Detection of food-relevant filamentous fungi by real time PCR

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Maria Landgraf

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Promotionsausschuss:

Vorsitzender: Prof. Dr. L.-W. Kroh

Berichter: Dr. K. Berghof-Jäger

Berichter: Prof. Dr. U. Stahl

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1 Summary

Due to serious incidences of food and feed poisoning in the 1960's caused by mycotoxins intensive research on the fungal metabolism began. Today about 400 different mycotoxins are known to cause food-borne diseases, allergic reaction and even cancer in humans (Müller *et. al* page 252 (1996)). Living filamentous fungi are also known to cause serious infection in immuno-compromised patients.

Food damage caused by fungi is often invisible and late detected by change of taste and odour. Early monitoring of fungal contamination during food production can help to avoid human health risks and economical losses. A PCR based method is one possible solution for monitoring and detection of filamentous fungi in food.

The aim of this work is to develop and establish a PCR based system for the detection of filamentous fungi in food by use of real time PCR in the LightCycler® instrument. Industrial demands were defined in advance of this work as the employment of non-toxic substances in the nucleic acid extraction and during PCR, high specificity and high sensitivity of the primer and probe system for detection of fungal nucleic acids as well as easier and faster data acquisition compared to conventional microbiological methods.

The fungal specific gene *EF-3*, elongation factor 3, is selected and evaluated for the usefulness as molecular marker in detection of filamentous fungi. As little sequence information is available in the public databases yet, for the first time sequence information of *EF-3* from about 101 different species of nearly all taxonomic divisions of filamentous fungi was generated in this thesis. The comparison of sequences shows a heterogeneous sequence nature in *Zygomycota* complicating the design of universal primers and probes for PCR. Nevertheless this problem was solved by inclusion of degenerated nucleotides into the sequence design. Different primer and probe systems were investigated. A real time consensus PCR system including detection by LightCycler® HybProbes was found as the best method to fulfil the industrial demands in terms of sensitivity and specificity. The specificity of the consensus PCR system is positively tested including about 165 different strains of filamentous fungi. The closed taxonomical relation between the ecological classifications yeasts and filamentous fungi implicate that yeasts are partially detected by the consensus PCR system for filamentous fungi. Indeed, exclusivity tests show around 68 % out of 56 yeast strains are cross amplified by primers and 10 % detected by probes derived from sequence information of filamentous fungi. As one application of the consensus PCR system is its use in sterility testing, it is chosen in this study to demonstrate the possibilities of PCR based methods in routine detection of viable fungi. For routine tests of viable fungi PCR and RT-PCR methods were compared and evaluated according to their practicability. PCR was confirmed as the more sensitive and robust method able to detect 3 – 30 copies of the target gene *EF-3*. Enrichment of filamentous fungi on nutrient pads was favoured in terms of adapting the preceding cultures to PCR based methods. The establishment of the consensus PCR system is shown by investigating fluid food samples demonstrating, that results from PCR are available before microbiological data. Differentiation between DNA from viable and non-viable cells is realized by excluding the DNA of dead cells from extraction and so finally from PCR. The main result of this work was an assay combining an efficient extraction method for DNA from filamentous fungi with a sensitive PCR system for specific amplification and detection of fungal DNA by real time PCR in the LightCycler® instrument.

1 Zusammenfassung

Mit der Entdeckung der Aflatoxine in den 1960-er Jahren begann eine intensive Erforschung des Stoffwechsels der Pilze. Seitdem wurden etwa 400 verschiedene Mykotoxine identifiziert, die unter anderem ernährungsbedingte Erkrankungen wie Infektionen, Allergien und sogar Krebs begünstigen und hervorrufen können (Müller *et. al* S.252 (1996)).
Die Nahrungsmittelkontamination durch Pilze wird häufig erst nach dem Transport zum Verbraucher sichtbar. Um gesundheitliche und finanzielle Risiken durch Verderb auszuschließen, werden Methoden für einen frühzeitigen Nachweis von lebenden filamentösen Pilzen in Nahrungsmitteln benötigt. Die Entwicklung eines derartigen Nachweises basierend auf molekularen Methoden wie zum Beispiel PCR oder RT-PCR war Aufgabe dieser Arbeit. PCR ist als Methode in der Lebensmittelanalytik weit verbreitet und wurden für den Nachweis von Pilzen in dieser Arbeit herangezogen. PCR und RT-PCR wurden mit Bezug auf die Anwendbarkeit in der Lebensmittel-Diagnostik verglichen. Da sich PCR sich dabei als die robustere Methode herausstellte war das Ziel dieser Arbeit die Entwicklung einer „real time" PCR basierten Untersuchungsmethode zum Nachweis von lebenden filamentösen Pilzen mit Hilfe des LightCycler® PCR-Gerätes. Für die Anwendung der PCR in der Routinediagnostik waren einige Vorgaben zu beachten, wie beispielsweise die Verwendung von nicht-toxischen Substanzen in der Nukleinsäureextraktion, die Bereitstellung von hoch spezifischen und sensitiven Primern und Sonden für den Nachweis von allen lebensmittel-relevanten Arten der Pilze und eine Verringerung der für die Durchführung benötigten Zeit gegenüber herkömmlichen Verfahren der Mikrobiologie.
Das pilzspezifische Gen *EF-3*, Elongationsfaktor 3, wurde für den Nachweis ausgewählt und bezüglich seiner Nutzbarkeit als molekularer Marker bewertet. Da zu Beginn dieser Arbeit kaum Sequenzinformationen von *EF-3* aus filamentösen Pilzen verfügbar waren, wurden in dieser Arbeit Sequenzen von 101 Arten aus fast allen taxonomischen Ebenen der Pilze generiert. Der Sequenzvergleich zeigte deutlich die besonders heterogene Natur der Sequenzen aus der Gruppe der *Zygomycota*. Jedoch konnte diese Variabilität der Sequenzen durch die Degenerierung der Primer und Sonden erfasst werden. Verschiedene Detektionssysteme wurden im Rahmen der Entwicklung einer „Konsensus"-PCR für die „real time" PCR getestet. Der Nachweis von spezifischen PCR Produkten mittels Sonden stellte sich als vorteilhaft heraus. Die Spezifität der ausgewählten Primer und Sonden dieser Konsensus-PCR wurde mit DNA von 165 verschiedenen Pilzstämmen positiv getestet. Die nahe verwandten Hefen wurden mit den vorgestellten Primern in 68 % und mit den Sonden in 10 % der getesteten Stämme nachgewiesen. Eine Erweiterung des Sondenspektrums zur Erfassung der Hefen in der Konsensus-PCR wird daher für eine Umsetzung in der Lebensmitteldiagnostik empfohlen.
Die Sterilitäts-Testung in Lebensmitteln als eine mögliche Anwendung der Konsensus-PCR wurde in flüssigen Lebensmitteln demonstriert. Es wurde gezeigt, dass durch die Anreicherung auf Nährkarton-Membranen in Kombination mit einer effizienten DNA Extraktion und der Konsensus-PCR ein Nachweis von Pilzen noch lange vor dem Sichtbarwerden von Kolonien möglich ist. Der Ausschluss von DNA toter Zellen wird während der DNA Extraktion erreicht. Lebende Zellen können auf diese Weise gezielt nachgewiesen werden, was die Möglichkeit der Umsetzung des hier vorgestellten Systems in der Routine eröffnet.

2 Introduction

2.1 Filamentous fungi and their importance in food and human health

By consuming food the human body is constantly exposed to microorganisms and their metabolic products. Some of these microorganisms are essential for processes like the digestion in the colon while others are contaminants forming toxic substances or even are pathogenic. Microorganisms like bacteria, yeasts and filamentous fungi are common contaminants leading to spoilage of food. Metabolic components of these organisms cause food borne diseases, allergic reaction and even cancer in humans (Mücke and Lemmen page 9 (1999)). Filamentous fungi are the slowest growing organisms of these groups. The presence of fungi in food or food sources may lead to the production of (and therefore contamination with) mycotoxins. Long storage under inconvenient conditions and late detection of fungi might be some reasons why food or food sources can become contaminated by mycotoxins. The consumer detects fungi and their metabolites as contaminants in food by occurrence of mycelia and changes in odour and taste. Many countries legislate for, or suggest, maximum permitted levels of mycotoxins in feeds and foods because of the potential safety and commercial implications of their presence (CBS (Centraalbureau voor Schimmelcultures) (2005), Official Journal of the European Communities (2003)).

In food industry rapid detection and identification of microorganisms or contamination sources is required to make decisions concerning the loss of raw material and production lots. This also minimizes the costs of unnecessary storage.

International transportation of commodities has increased the emergence of new pathogens, which even more complicates the rapid detection of that high diversity of pathogens. Both consumers and industry therefore demand the development of fast and reliable detection systems. High sensitivity and specificity of detection can be achieved by DNA based methods. The design and development of such a detection system for viable filamentous fungi in food is the major focus of this work.

2.1.1 Growth behaviour in food and adaptation to food preservation

To fulfil both the consumer's and industrial demands food is preserved for long-term storage and transportation. Several types of food are sterilized to exclude microbial growth, but some microorganisms including some fungal strains are resistant to preservation.

To avoid fungal damage of food and consequently enrichment of mycotoxins several methods of conserving food are practised in industrial food production. Different physical or chemical methods are applied like pasteurisation, sterilization, cold or frozen storage under modified atmosphere ($\uparrow CO_2$, $\uparrow N_2$), vacuum packing, canning, drying, irradiation and addition of chemical preservatives (such as diphenyl, thiabendazole, natamycin, sorbic acid, benzoic acid), respectively (Samson *et al.* page 289 (1995)). Most microorganisms should be killed by these methods, but some methods especially addition of chemical preservatives result in inhibition but not killing of microorganisms (Samson *et al.* page 289 (1995)). For example fungal hyphae often contain glycerol which enables some species to grow at temperatures as low as –8°C (Weidenbörner (1998); Reiß page 48-50 (1997)).

Additionally, fungi can adapt to physical treatments forming heat-resistant spores, which remain viable even through processes like pasteurisation (Beuchat and Pitt (2001), Yildiz *et al.* (2001)). An example is the duclauxin[1] producing *Talaromyces macrosporus,* a common contaminant in fruit juices, which forms dormant cells (ascospores) that need a rigorous external trigger for germination. Dijksterhuis *et al.* (2003) found, that extreme heat and high pressure can activate these cells to germinate. These data are supported by Merkulow (2001), who found that even a small fraction of fungal cells can survive extreme high pressure due to the fact, that it is impossible to define a limiting pressure for all fungal species. In this context one has to consider the ability of some fungi to produce chlamydospores, thick-walled hyphal fragments, which enables fungi to survive long-term storage of food within oxygen reduced atmosphere. Chlamydospores of *Paecilomyces variotii, Fusarium spp.* or sclerotia[2] of *Eupenicillium* can survive even heat shocks (Pitt *et al.* (1970)).
In general, sexual ascospores (teleomorph) of filamentous fungi are much more heat resistant than asexual (anamorph) mycelia and conidia (asexual spores) in *Ascomycota* and more resistant than yeast ascospores. The heat resistance proved to be dependent on the age of spores/conidia and on the physiological stage based on the composition of the growing and heating media at the time of the heat shock.
Mycelia and conidia are generally not very heat resistant. In contrast to ascospores, heating at 80°C for several minutes can be applied to achieve decimal reduction of germination (Samson *et al.* page 249 (1995), Vatilingom (1998), Beuchat and Pitt (2001), (Pitt *et al.* (1970), Shearer *et al.* (2002), Yildiz (2001)).

2.1.2 Fungal structures in food: a short introduction

Taxonomic relationships have to be considered in the development of a diagnostic test system aiming to detect all of the heterogeneous groups of filamentous fungi. Taxonomically, filamentous fungi are a heterogeneous group within the division of Eumycota (Berbee *et al.* (2001), Bowman *et al.* (1992), Hawksworth (1991), Prillinger (2000)). Most filamentous fungi are dimorphic organisms developing mycelia and yeast-like structures. *Ascomycota* and *Zygomycota* reproduce sexually forming meiospores and disperse asexually by the formation of mitospores. Food-relevant fungi can be divided into four main classes based on their reproduction modes: *Ascomycota* form ascospores, *Basidiomycota* form basidia (serve also in dispersal), *Zygomycota* form zygospores while the fourth class, *Deuteromycota* (*Fungi Imperfecti*), lacks a sexual reproduction mode and reproduces only asexually. *Deuteromycota* produce only asexual conidia and many species are found to belong to the *Ascomycota* forming the anamorphic stage of the fungi (Kuhls *et al.* (1996)). No sexual state is known for over 5000 species of fungi (Berbee (2001)). A fifth class *Oomycota* contains basically plant pathogens and is therefore considered to be non-important in food spoilage.
The knowledge of fungal morphology, physiology and taxonomy is of great importance for both understanding how fungi contaminate food and for developing a sophisticated detection assay.

[1] Duclauxin is a fungal secondary metabolite, which has cytotoxic and antitumoral potential.

[2] Sclerotia are specialized hyphal bodies involved in dormant survival. They germinate either by producing hyphae or a sexual fruiting body.

A selection of fungal structures that can be found in food is therefore given in Table 1. The biological function of the particular structure is explained.
Yeast and filamentous fungi are artificial ecological categories and contain species that belong to all of the different classes in the kingdom (Kurtzman (2000)). Species of both groups can be taxonomically closely related. However, significant differences in physiology exist. These differences are visible even on the molecular level and have to be considered in the development of DNA based detection systems. This is further demonstrated by the fact, that 31 % of the open reading frames found in *Aspergillus nidulans* were not detected in *Saccharomyces cerevisae* (Birren *et al.* (2002)).

Table 1 - Fungal structures. *Basidiomycota* (forming mainly meiospores for dispersal) are not considered.

	characteristic function	example of genus
Zygomycota		
mycelium growing from mitospores and meiospores		
mitospores:		
aplanospores	asexual reproduction by single celled sporangiospores	*Absidia*
merospores	row of spores in a braking cylindrical sporangium	*Syncephalastrum*
chlamydospores	thick walled resting cells of some species	*Mucor, Rhizopus*
oidia	thin walled spores of some species	*Mucor*
meiospores:		
zygospore	thick walled coloured spores of sexual reproduction resulting from fusion of two hyphal multinucleate gametangia	all *Zygomycota*
Ascomycota*, *Deuteromycotas (*Fungi Imperfecti* only anamorph state is known, no meiospores, using parasexuality for recombination)		
mycelium growing from mitospores and meiospores		
mitospores produced in anamorph (asexual reproductive) state:		
conidia	asexual reproduction state; coloured cells; single; in slime drops, in chains	all *Ascomycota* and Deuteromycetes
athroconidia	dark thick walled hyphae, falls apart into separate cells	*Auroebasdium, Geotrichum*
microspores	one-celled; manly developed in aerial mycelium, small cell	*Fusarium*
macrospores	septate sickle shaped large cells	*Fusarium*
ramoconidia	conidiophores fall apart into separate cells	*Cladosporium*
blastoconidia	multicelled conidia	*Epicoccum, Alternaria, Ulocladium*
chlamydospores	thick walled resting cells for long time surviving	*Byssochlamys, Monascus, Fusarium, Acremonium, Paecilomyces, Trichoderma, Moniliella, Phoma*
meiospores produces in teleomorphic (sexual reproductive) state:		
ascospores	formed in asci by sexual karyogamy of two gametangia (antheridium; ascogonium) often heat resistant	*Byssochlamys, Emericella, Eurotium, Monascus, Neosartorya, Talaromyces*
ascospores in fruit bodies	apothecia, cleistothecia, perithecia, pseudothecia	teleomorph of *Penicillium* and *Aspergillus*

Unlike yeast, most filamentous fungi are not able to change their metabolism to fermentation under anaerobic conditions, but to anaerobic respiration (*Neurospora crassa, Aspergillus nidulans*). Only a few fungal species are able to grow slowly under anaerobic conditions, such as *Mucor, Rhizopus, Absidia spinosa, Geotrichum candidum, Fusarium oxysporum, Fusarium solani* (Weidenbörner (1998)). In contrast to yeast, some species of *Mucor* and *Fusarium* have even been isolated as the source of alcoholic fermentation from fruit juices (Müller *et al.* page 114 (1996)).

2.1.3 Medical importance of fungal contaminated food

Especially young, old, pregnant and immunocompromised persons have a potential higher risk of food borne diseases. Therefore sterility control of food has important medical relevance for immunocompromised patients in oncology and transplantation surgery as well as diabetes and HIV-infected patients. Furthermore, today's general application of antibiotics results in increasing numbers of patients, which become temporarily immunocompromised. Infections of immunocompromised patients with *Aspergillus* and *Fusarium* species show a mortality of nearly 100 % (Turenne *et al.* (1999)). The filamentous fungus *Aspergillus fumigatus* produces very small conidia, which are able to reach the alveoli of the human lung. The fungus is able to grow at 37°C and produces pathogenic factors like the immunosuppressing gliotoxin and restrictotoxin, the latter inhibiting protein biosynthesis of human cells (Mücke and Lemmen page 27 (1999)). No effective antifungal drugs for those aggressive infections are known today. Fungi can cause different diseases such as opportunistic infections in immunocompromised patients, keratitis (fungal nail infection), pulmonary infections, food poisoning related diseases and even cancer. Other important fungi-related health risks are allergic reactions caused by inhalation of fungal particles, for example in the dust of the working environment. "Farmer's lung", "baker's lung", "organic dust toxic syndrome" and "exogene-allergic alveotitis" are only some today known occupational diseases caused mainly by spore producing fungi in the dust of offices, homes, various factories and farms.
A detailed insight in medically important fungi is given in http://www.doctorfungus.org, Mücke and Lemmen (1999) and Bridge *et al.* see page 267 – 288 (1998).

2.1.4 Mycotoxins

Acute food born diseases caused by fungal contamination are reactions to the toxins formed during spoilage. Acute mycotoxicosis is often diagnosed as aflatoxicosis, a food poisoning caused by the mycotoxin aflatoxin, which is in general the strongest known naturally occurring cancerogen. However, no data are available for general incidences of acute mycotoxicosis, while it has been suggested that long-term consumption of mycotoxin-contaminated food is associated with the development of liver cancer (Weidenbörner (1998)).
Most mycotoxins are low molecular substances, which are stable during food processing and can be highly toxic. Due to the occurrence of fungal growth and mycotoxin production in primary products, heat resistant mycotoxins can be detected in processed food, too (Mücke and Lemmen see page 89 (1999)). Not all strains of fungi produce mycotoxins in all stages of their life cycle, but the risk to contain mycotoxins is very high in mouldy food. Food and Agriculture Organization of the UN (FAO) estimates detectable amounts of mycotoxins in 25 % of all worldwide produced food (Mücke and Lemmen see page 86 (1999)).
In fungi as in all eukaryota essential metabolites are produced from intermediate metabolic pathways like glycolysis and the citric acid cycle. Secondary metabolism removes products from intermediate metabolic pathways when growth is temporarily restricted. According to this theory production of secondary metabolites only occurs if there is an oversupply of nutrient. Since fungal secondary metabolites like mycotoxins often have antibiotic properties, many of the today known antibiotics were derived from the large group of mycotoxins. Secondary metabolites such as mycotoxins are often specific for individual genera, species, or strains. In food production

however, mycotoxins are the most important secondary unwanted metabolites. Mycotoxins constitute a diverse range of compounds from different precursors and pathways that are grouped together based on their toxicity to higher animals and humans (Olsson (2000)).
Table 77 in the attachment gives an insight into the most common mycotoxins and their occurrence (Richard (2000), Reiß (1997), Mizakova (2002), Al-Gashgari (2002)).

2.2 Methods for the detection of filamentous fungi

The variability in fungal physiology and morphology complicates the establishment of a general method for the inhibition of fungal growth in food. Therefore viable fungal contaminants can be found in preserved food, too. Testing for absence of fungal growth is necessary especially for easily perishable food like non-pasteurised juices, instant food, ready-to-eat food and food products for medical diet, respectively. Industrial problems with filamentous fungi often become visible only after the product has been sent out to the costumer, in production machines by clotting nozzles or while testing food for mycotoxins. Amount and type of microorganisms in the final food product depend on the biological quality of the raw material and on the hygienic conditions during production and storage.
The introduction of food safety management tools has replaced the end point control of food products. Good Manufacturing Practice (GMP) and Good Hygiene Practice (GHP) describe the basic measures that have to be applied during production, processing, handling and distribution, storage, sale, preparation and use. (Brännback & von Blankenfeld-Enkvist (2002)). HACCP (Hazard Analysis and Critical Control Point) is a guideline to prevent hazards in food manufacturing lines. It includes the identification of hazards and the establishment of control systems based on GMP and GHP. Raw materials are controlled to exclude pathogens from the beginning of food production. Food processing is well defined to prevent the occurrence of harmful microorganisms (Mäntynen (1999)). In order to overcome the problems with fungal contaminants during food production a screening of fungal absence in the different production units is recommended. A control system during food production by monitoring the occurrence of fungi might be the best way to overcome quality loss and health problems caused by fungi in food.
Two different purposes of conventional food microbiology in industrial HACCP protocols are to test the absence or to enumerate the microorganisms using plate-counting or direct plating techniques followed by microscopical evaluation of the colonies on the solid plates. Plate count methods like the CFU (Colony Forming Unit) method are applied for estimating the quantity of spoilage organisms including fungi (ISO 7954 (1987)). However, the test of absence ‘sterility’ is not officially defined for filamentous fungi in food as opposed to pharmacological products. During the conventional incubation of pharmacological samples in fluid media, both absence of bacterial and fungal growth is tested in parallel. Spoilage is indicated by the fluid medium becoming cloudy after a simple incubation for 14 days at 25°C.
However, fungal growth requires aerobic conditions, which are not always given in fluids. Filamentous fungi grow slower than all other microorganisms found in food and they grow in a different way, usually on the surface of a fluid. As the fluid media routinely used are generally optimised for bacterial growth, the conditions for fungal development are not ideal. Lacking reliable protocols for testing absence of fungi, today mostly “in-house” protocols are carried out in the different manufactories. These protocols often combine culturing methods in fluids and on

solid plates due to the fact that filamentous fungi grow better and faster on solid agar plates than in fluids. Additionally, one can easier determine on plates whether the contaminating organism is a fungus, i.e. forming filamentous mycelia.
However, the success of detecting fungi from food by any culture-based method depends on appropriate media for enrichment, techniques for homogenizing of the particular food, filter systems for fluids and the strain composition of the food. Obviously a single media cannot provide conditions for growth of acidophilic, xerophilic, proteinophilic and osmophilic fungi. Conventional microbiological test systems are therefore limited in sensitivity and reproducibility. Particularly the group of spore-producing fungi is often insufficiently detectable in heat-processed food with common sterility test systems (Shearer *et al.* (2002)). Indeed, it has been estimated that less than 1 % of the microorganisms are cultivable (Mäntynen (1999)). As further applications in food diagnostics depend on the optimal enrichment careful optimisation of enrichment methods is important.

2.2.1 Conventional methods

The dilution plating or CFU method is the most commonly used technique for the examination of food and feedstuffs. Homogenising of the samples, preparing 10-fold dilutions and surface spreading on different agar plates are the characteristic steps of this method. The agar plates are incubated at 25°C for 5-14 days and the resulting colonies are counted and analysed (ISO 7954 (1987)). Obviously, the results of colony counting vary depending on the applied method for homogenising food, the sporulation and the choice of specific selective media (Olsson (2000)). Sensitivity and reproduction of results may be restricted in CFU methods caused by hydrophobic and microscopical surface structures of fungal spores. Homogenising of these spore clusters in solutions is problematic; homogenising of mycelium is impossible.
There are many differences in fungal demands concerning humidity (a_w-values), nutrient composition, temperature and pH, respectively. Exceptionally important for fungal growth in food is the amount of free available water, which is expressed as water activity (a_w) (Müller and Weber page 138 (1996)). The water activity can be calculated from the relative humidity of the air surrounding the sample when the air and the sample are at equilibrium. As the minimal necessary water activity for most filamentous fungi is 0.8-0.85 (Mücke and Lemmen see page 18 (1999)) it differs from that for most bacteria ($a_w \geq 0.91$) and yeasts ($a_w = 0.91$-0.87). Only xerophilic yeasts (for example *Debaryomyces, Hansenula, Pichia, Candida, Zygosaccharomyces*) share the same minimal water activity levels with filamentous fungi. In dry products ($a_w = 0.75$-0.65) only xerophilic slowly growing filamentous fungi are able to survive (for example *Eurotium, Aspergillus restrictus*) (Weidenbörner (1998)).
Therefore it is not surprising that the Centraalbureau voor Schimmelcultures (CBS) recommends no less than 37 different media for cultivating filamentous fungi to meet the different requirements. No universal medium exists and only a few media are widely accepted, among them DG18 (Hocking and Pitt (1980)) and DRBC (King *et al.*, 1979). To make it more difficult, a few microorganisms are only in community with other organisms able to grow and therefore hard to cultivate or non-cultivable at all; others suppress the growth of antagonists (Randhawa (2002), Bottone (1998)). According to Samson *et al.* see page 308-311 (1995) several widely accepted selective media for fungal isolation have been developed as shown in Table 2.

Table 2 - Selective media for fungal isolation

selection	name	species
moderately xerophilic fungi	DG 18 (Dichloran 18 % Glycerol Agar)	*Penicillium, Aspergillus, Wallemia, Eurotium*
extreme xerophilic fungi	MY50G (Malt Yeast 50 % Glucose Agar)	*Xeromyces, Eremascus, Chrysosporium*
acidophilic fungi	ADYS (Acetic Dichloran Yeast Extract Sucrose Agar)	*Penicillium roquefortii, Penicilliun carneum, Monascus rubber, Paecilomyces variotii*
proteinophilic fungi	CREAD (Dichloran Creatine Sucrose Bromocresole Agar)	*Penicillium commune, P. solitum, P. crustosum, P.expansum, Aspergillus clavatus, Asp. versicolor, Asp. sydowii, Asp. ustus*
Fusarium	CZID (Czapek Iprodione Dichloran agar)	/
Mucor	MYCK (Malt extract Yeast Chloramphenicol Ketoconalzol)	/
aflatoxigenic species	AFPA (Samson *et al.*page 308 (1995))	*Aspergillus flavus*

Conventional detection and identification of moulds are based on cultivable isolates and assessment of morphological features. Further identification of fungi based on morphological features requires work of experienced and well-educated mycologists. These methods are time consuming and labour-intensive (Wu (2003)). However, non-cultivable microorganisms can nowadays be detected e.g. by microscopic counting or by polymerase chain reaction (PCR) to amplify their DNA (Mäntynen (1999)).

2.2.2 Alternative methods for the detection of fungal specific metabolites

Today the evaluation of cultivation is done by a number of methods, traditionally by simple plate counting as described above. Conventional methods have some disadvantages, though. The occurrence of young fungal mycelia on the surface of agar plates remains difficult to distinguish from background material, especially if food samples are plated. Most colonies grow very fast and cannot be distinguished accurately during manual counting.

Antibody- as well as DNA-based methods as represented by the ELISA assay, PCR and PCR-ELISA, respectively, are the most widely used technologies in food diagnostics today. Also Immunomagnetic Separation (IMS) is applied in a number of commercially available kits and will become an even more important technology in the future (Brännback & von Blankenfeld-Enkvist (2002)). Advantages especially in PCR-based methods lie in the specific detection of small amounts of target organisms by amplifying their DNA in a considerable short timeframe. Since this method is highly relevant for the present work, a detailed description of DNA-based methods is given later.

The occurrence of mycotoxins at the end of the exponential growth phase is one reason for the importance of the detection of viable fungi in food. Growth factors, such as substrate composition, temperature, a_w, pH, atmosphere, redox potential, and microbial competition influence mycotoxin production. For many fungi the conditions that are required for mycotoxin production are more limiting than the range of condition allowing growth (Olsson (2000)). The methods to detect mycotoxins are complicate and expensive. Mycotoxins have to be extracted, cleaned up and concentrated before detection, quantification and identification. Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) are commonly used techniques for detection of the stable low molecular aromatic mycotoxins (Martins (2003)).

The identification is confirmed with a positive sample in high-resolution mass spectroscopy (MS). Some mycotoxins such as aflatoxin can emit the absorbed energy from UV-light as fluorescence light, which is used for detection. Additionally, ELISA techniques exist for mycotoxin detection in food.
Other methods in food diagnosis are based on the specific detection of the different volatile metabolites derived from primary and secondary metabolism that can indicate spoilage in food. Acetyl-CoA is the main precursor in the biosynthesis of volatile fungal metabolites. Most common fungal volatile compounds are 2-methyl-1-propanol, 3-methyl-butanol, 1-octene-3-ol, 3-octanone, ethyl acetate, 3-methylfuran, 2-methylisoborneol and geosmin. These components were already used to classify and identify microorganisms in food.
Larsen and Frisvad (1995) investigated the volatile compounds produced by 132 isolates of 25 *Penicillium* species for classification. The results of this analysis agree with previous classifications of *Penicillium*. Also, the production of mycotoxins in moulds correlates with the existence of volatile compounds. Unfortunately, bacteria and fungi are expected to produce similar volatile metabolites (Olsson (2000)). This makes the analysis of volatile compounds as specific marker for fungi less meaningful in food diagnosis.
Fungi including yeasts and filamentous fungi are able to utilise a variety of substrates (sugar, pectines, organic acids, proteins and lipids) and furthermore are relatively tolerant to low pH, low a_w, low temperatures and various preservatives. Therefore biotechnological cultivation of defined production strains has been and will continue to be an important technological method in food industry. For instance, yeasts are used to produce 60 million tons of beer, 30 million tons of wine and 600000 tons of baker's yeast annually. Special strains of filamentous fungi like *Aspergillus niger* are used to produce citric acid. *Aspergillus oryzae* is more important in asia and is used in production of soy sauce. By contamination with wildtype and by mutations production strains can lose their ability to efficiently produce metabolites. Using DNA based fingerprinting methods the quality of the production strain and/or the purity of the production lots is verified (Querol *et al.* (1992), Mäntynen (1999)). Different commonly used fingerprinting methods are available like DGGE (Denaturing Gradient Electrophoresis), TGGE (Temperature Gradient Gel Electrophoresis), SSCP (Single Strand Conformation Polymorphism), TRFLP (Terminal Restriction Fragment Polymorphism) and ARISA (Automated R-DNA Intergenic Spacer Analysis).
Other methods like flow cytometry are nowadays introduced into the food analysis. Especially FACS (Fluorescence Activated Cell Sorting) offers new possibilities in analysing the quality of liquid food. Cells are stained for analysis by nucleic acids stains, protein stains, antibodies, and dyes for membrane potential measurement and enzymatic activity, respectively. The specifically bound chromophor is stimulated and detected by light emission at a specific wavelength. Flow cytometry offers the discrimination between viable and dead cells in both a qualitative and a quantitative manner. But the technology is still very complex and cost intensive.
Also, impedance methods are used to investigate the growth of microorganisms in food samples as described by Huang *et al.* (2003) and Noble (1991), respectively. This technology is based on the discovery that microbiological metabolisation causes subtle changes in the ionic composition of the media. The uncharged or weakly charged substrates are modified by the microorganism into charged end products, which is measured as an increase in the conductance of the growth media. The main disadvantage is that impedance methods are non-specific.

Other methods are available for the detection of metabolic markers indicating fungal existence such as the cytoplasmic membrane compound ergosterol and the cell wall compounds chitin (ß-1,4-linked N-acetylglucoseamine), ß-1,2-/ß-1,6-glucan and cellulose (Müller and Weber page 71 (1996)). Ergosterol as an membrane integral part can be detected by HPLC and is relatively specific, whereas chitin as an cell wall compound can also be found in insects, nematodes, crustaceans, protozoans and diatoms, and is therefore a rather unspecific marker for detection of filamentous fungi in food.

2.2.3 Molecular methods: Introduction of PCR and real time PCR

Since DNA-based methods are highly relevant for the routine diagnostics in food production, they are discussed in detail in the present work. PCR (Polymerase Chain Reaction) is based on enzymatic amplification of a target DNA region defined by two oligonucleotides, called primers, which bind opposite to complementary DNA strands (see page 428 in Knippers (1997)). PCR has been developed in 1986 by Mullis and is widely applied in laboratories today. Originally, Taq polymerase has been isolated from the extremophil *Thermus aquaticus*. Today, heat resistant DNA polymerases are produced by recombinant techniques in *E. coli* enabling large-scale manufacturing. DNA polymerases extend a short primer at the free 3'-OH, which is hybridised to a single strand target DNA. According to the base pairing rules in the Watson-Crick-Helix desoxynucleotides are incorporated complementary into the new extending DNA strand. Cycle profiles like denaturing of target DNA, binding of primers and extension of new strands are repeated until a detection of amplification with suitable methods is possible. Assuming the PCR starts with 2 DNA target molecules, during exponential amplification (2^n) millions of molecules can be detected after 20-50 cycles.
For the detection of amplified molecules different formats are available today. One of the first formats was the examination of DNA separated by size in agarose gels using an intercalating dye, which is stimulated by UV light emitting a fluorescent signal. Today different fluorescent dyes, so-called fluorophores, with specific emission and excitation spectra are used in real time PCR. Fluorophores are bound to target-specific hybridising probes leading to highly sensitive detection systems. Real time PCR mixtures can for instance contain fluorescently labelled primers or fluorescent labelled probes or DNA intercalating dyes like SYBR Green I. A detection step is integrated into each PCR cycle and can be simultaneously monitored in real time and online. Amplification and detection of target DNA takes place simultaneously. This is a major advantage compared to conventional end point analysis of amplification products in agarose gels.

<u>Real time PCR in fungal diagnostics</u>

Fungi form a large eukaryotic kingdom comprising probably more than 100 000 species, including yeasts and moulds as well as mushrooms (Hawksworth (1991)). Based on the immense diversity of fungi, group or strain specific detection by selective primers is very difficult to realise. A detection strategy in Two-Steps as performed in real time PCR, where a specific amplification of target DNA by PCR is followed by detection using hybridising probes is much more specific and sensitive than a One-Step amplification by primers only. The principles of real time PCR are already applied in fungal diagnostics, for instance by detecting airborne filamentous fungi (Wu (2003)). Using primers for specific target regions in the DNA species-

specific question can be answered (Lantz *et al.* (2000)). This was successfully done by a PCR based method developed for detection of aflatoxigenic moulds in grains in 1996 by Sharpira *et al.* Targets were key enzymes and regulatory factors in aflatoxin biosynthesis: *ver-1*, *omt1* and *apa-2*, respectively. Other PCR based quality controls for toxigenic *Fusarium* spp. were developed by Mulfinger *et al.* (2000), Parry and Nicholson (1996) and Schilling *et al.* (1996). Voetz and Rath (2002) developed a real time PCR based assay for the identification and quantification of ochratoxin synthesizing fungi on cereals. Detection and Quantification of *Wallemia sebi* in aerosols by real-time PCR was performed by Zeng *et al.* (2004). Jimenez *et al.* (2000) established the use of PCR analysis for detecting low levels of bacteria and mould contamination in pharmaceutical samples. Real time PCR detection of the *tri5* gene in *Fusarium* species by LightCycler®-PCR using SYBR Green I for fluorescence monitoring was developed by Schnerr *et al.* (2001). Most of these assays aim to detect fungal species, which are related to a particular problem in the industry. A general method for the detection of a wide range of food relevant species does still not exist and is therefore highly demanded.

Additionally, PCR based methods are used in strain identification by other methods such as fingerprinting like RFLP, RAPD, direct sequencing or real time reverse transcription (RT)-PCR (Loeffler and Hebart (2002), Paffeti *et al.* (1995), Walsh *et al.* (1995), An *et al.* (2002), Reischl *et al.* (2001), Vaitilingom *et al.* (1998), Bleve *et al.* (2003), Turenne *et al.* (1999), Wu *et al.* (2003), Turin (2000)), the latter being one of the methods investigated in this work and therefore explained in detail later. An example may the development of reverse transcription (RT)-PCR and real time RT-PCR assays for rapid detection and quantification of viable yeasts and moulds contaminating yoghurts and pasteurised food products which was introduced by Bleve *et al.* (2003). Quantitative analysis of the relative transcript levels of ABC transporter *atr* genes in *Aspergillus nidulans* by real-time RT-PCR was performed by Semighini *et al.* (2002). Direct detection of viable bacteria, moulds and yeasts by RT-PCR in contaminated milk samples after heat treatment was performed by Vaitilingom *et al.* (1998).

Commonly target molecules in diagnostic PCR are 18S rDNA genes, internal transcribed spacer (ITS) regions of ribosomal genes, mitochondrial DNA and specific proteins (Fletcher *et al.* (1998), Kappe *et al.* (1998)). The properties of the target DNAs and the sequence variability have to be considered carefully aiming the detection of different taxonomic levels. The target gene sequences need to be conserved and universal and in some sections the sequence variability needs to allow species discrimination. Primers are designed based on the sequences in public databases or alternatively by sequencing the target gene. Taxon-specific markers generated by RAPD or other PCR fingerprinting methods can be cloned and sequenced, but these sequence-characterized amplified regions (SCARs) are then used to design specific primers for a particular detection assays (Bridge *et al.* (1998) see pages 12-13 and 294-295, Andrighetto *et al.* (2000)).

However, efficiency of DNA amplification depends on sample preparation since various components in food can inhibit the reaction (Al-Soud *et al.* (2000), Tichopad *et al.* (2004)). Several methods to extract nucleic acids from fungal mycelia have been reported demonstrating the difficulties in that field (Borges (2002), Müller (1998), Yeates *et al.* (1998), Van Burik *et al.* (1998), Rivas *et al.* (2001), Lecellier *et al.* (1994), Loeffler *et al.* (2002), Griffin *et al.* (2002), Manian *et al.* (2001), Nicolas (2002)). Most of the published methods for fungal DNA extraction have been developed using mycelium grown in liquid culture, which is far from the real fungal

environment (Olsson (2000)). Therefore DNA extraction has to be carefully validated for the various types of food.

2.2.4 Formats for detection of PCR products in real time PCR

The following paragraph will give a short summary of the available detection formats in real time PCR.

2.2.4.1 SYBR Green I format

The fluorophor SYBR Green I is able to bind to the minor groove of the DNA double helix. The stimulation of the dye/DNA complex at 530 nm leads to measurable light emission via fluorescence. In solution, the unbound dye emits very little fluorescent light. But the fluorescence is enhanced upon DNA-binding. This characteristic of SYBR Green I to intercalate into the DNA is used to measure the generation of new PCR products. During PCR the increase in SYBR Green I fluorescence is directly proportional to the amount of newly generated double-stranded DNA (Roche Applied Science LightCycler® DNA Master SYBR Green I (2005)). However, the detection of PCR products by intercalating dyes is unspecific. Unspecific signals, which often derive from primer dimers, can lead to wrong positive interpretations (McCartney *et al.* (2003)). Consequently a melting curve analysis has to be performed to prove that only the desired PCR product has been amplified.
The melting curve analysis immediately follows the PCR cycles. The temperature of the PCR mixture is increased continuously to 95°C, which causes melting of all double-stranded DNA and consequently a decrease of SYBR Green I fluorescence. The characteristic melting temperature (*T*m) of a particular DNA product depends on the length and the GC-content of that product. The melting temperature is monitored by the calculation of a particular melting peak for each PCR product. Consequently, primer dimers and specific fragments can be distinguished. The SYBR Green I format is additionally limited in the detection of small sequence variations as necessary for genotyping. As the sensitivity and accuracy of quantitative real-time PCR using SYBR Green I varies dependent on the cDNA synthesis conditions, especially the RT-PCR requires extraordinary developmental effort (Lekanne *et al.* (2002), Pfaffl (2003), Vandesompele (2001)).

2.2.4.2 Detection using specific probe formats

More specific detection is realised by including probes into the real time PCR. One simple format using single probes is the so-called Taqman assay (US-Patent 5.487.972).
Taqman assay, also known as 5'exonuclease assay or hydrolysing assay, benefits mainly from the 5' exonuclease activity of Taq polymerase and from quenching effects between two sterically close fluorophors. TaqMan PCR mixtures contain two target specific primers and one hybridising probe labelled on both ends with two fluorescent dyes. No fluorescence of the probe is detected in intact condition, where both dyes are in close contact. For example, the fluorescence of 6-carboxyfluorescein (6-FAM) is quenched by 6-carboxytetramethylrhodamine (TAMRA). During the extension of the new DNA strand the hybridised fluorescent TaqMan probe is hydrolysed by the Taq polymerases 5' exonuclease activity. The quenching is decreased as more and more fluorescent dyes become independent from each other during the PCR. The stimulation by light of specific absorption wavelength leads consequently to increasing emission

signals in each cycle. This assay was applied in different diagnostic areas by Chandler *et al.* (1998), Kalina *et al.* (1997), Nadkarni *et al.* (2002), Stults *et al.* (2001), Haugland (2004) and Knutsson *et al.* (2002), respectively.

The detection of PCR products including two fluorescently labelled hybridisation probes is mainly applied in the LightCycler® instrument (Roche Diagnostics Corporation). The fluorophors influence each other by Fluorescence Resonance Energy Transfer (FRET), if the probes are hybridised adjacent to each other. During FRET one dye is stimulated by light, absorbing light energy and transferring this energy to the nearby[3] dye, which reacts with detectable emission of fluorescent light of a longer wavelength. The absorption spectrum of the first dye and the emission spectrum of the second dye need to overlap for that purpose. A head-to-tail design of the hybridisation probes guarantees necessary close proximity (one to three base pairs distance) of the fluorescent dyes. FRET-based assays have been introduced by Reischl (2001); (2003) and applied to food diagnostics by Koo *et al.* (2003), O'Mahony *et al.* (2004) and Kiehne *et al.* (2005).

Other molecular probe formats are smart probes (also known as gene pins) and molecular bacons (Vogelstein (1999)). Smart probes were developed for fluorescent correlation spectroscopy in connection with confocal fluorescent microscopy. They consist of an end labelled fluorescent oligonucleotide, which is designed to form a hairpin structure. During the formation of the hairpin structure a special label, the fluorescent dye (oxazine dye JA242), comes in close proximity to a guanosine containing section in the complementary sequence of the oligonucleotide, which quenches the fluorescent signal of JA242. During hybridisation to the target sequence the probe undergoes a conformational change that forces the fluorescent dye and the guanosine residues apart and results in increasing fluorescence intensity.

A similar structure can be found in molecular bacons probes (Kramer and Tyagi (1996)), which contain a second quenching dye in the hairpin structure instead of guanosine.

Fluorescence increases in both systems by changing the conformation of the probe. Opening of the hairpin structure is mainly caused by the higher affinity of the probe to hybridise to a specific target DNA (Knemeyer (2000)).

Whitcombe *et al.* (1999) developed the Scorpions technology. Scorpions combine primer and Probes by linking them together forming hairpin structures. A scorpion primer carries a 5' extension comprising a probe element, a pair of self-complementary stem sequences, and a fluorophor/quencher pair. The probe extension is "protected" from copying by the inclusion of a blocking HEG (hexethylene glycol) monomer (Whitcombe (1999)). After one round of PCR the primer is extended and the newly synthesized target region is now attached to the same strand as the probe. During the next annealing phase, the probe sequence in the scorpion's tail curls back to hybridise to the target sequence in the newly formed PCR product (Whitcombe (1999)). The fluorophor/quencher pair is separated and fluorescent signals increase.

2.2.5 Target molecules for detection of filamentous fungi

Considering the industrial demands for the detection of viable filamentous fungi from food the use of PCR is a promising tool. Viable fungi contain both DNA and RNA. As DNA is often stable during food processing it is not a specific marker of viable cells (McKillip (1998)).

[3] The dyes are separated by 10–100 Å, which is a distance of approximately one to five base pairs.

However, this is true for rRNA, too. In *Escherichia coli* and *Staphylococcus aureus* it has been demonstrated that detection of 16S rRNA was not correlated to viable cells (McKillip (1998)). Only under extreme heat rRNA was destroyed experimentally. Using DNA or rRNA as target molecules for viable cells, nucleic acids from/or in dead cells in food have to be destroyed selectively by enzymatic digestion before running a PCR to avoid false positive signals.
As mRNA is found only in living organisms, it is a strong marker of viable cells and therefore the target molecule in several assays (Bustin (2000) and (2002), Sheridan (1998) and (1999)). A major disadvantage of mRNA is its instability. Compared to other RNA types in eukaryotic cells most mRNAs are up and down regulated as a reaction to environmental and endogenous conditions. Therefore a special mRNA pattern will be expected for different live stages. In general, stability of mRNA depends on the importance of the transmitted information coded in mRNA for overall cell physiology. Several stability signals are known in eukaryotic mRNA which are located in the 3'untranslated region, inside the coding region as well as in the 5' non-translated region (Drugeon (1997), Mugnier *et al.* (1999), McCarthy (1998)). As the mRNA of housekeeping genes are transcribed permanently those genes may serve as target molecules for reliable detection of viable cells (McKillip (1998), Pfaffl (2003)).
Surprisingly, also in spores of filamentous fungi mRNA detection has been described (Harper *et al.* (1980), Linz *et al.* (1982), Rhody *et al.* (2003), Smith (1979), Smith (1979)). Since a main task of this work was to proof whether the detection of mRNA in fungal spores and mycelia is of use in food diagnostics the next paragraph will explain the methods to detect mRNA in more detail.

2.2.5.1 Introduction of RT-PCR

RT-PCR (reverse transcription-polymerase chain reaction) is the most suitable technique for sensitive mRNA detection and quantification currently available. The reverse transcription of mRNA into cDNA is catalysed by the enzyme reverse transcriptase. RT enzymes such as Transcriptor Reverse Transcriptase and Avian-Myeloblastosis-Virus (AMV) Reverse Transcriptase combine the activity of reverse transcription with an additional RNase H activity, which removes the mRNA from the mRNA/cDNA hybrid molecule. The remaining cDNA serves as target molecule for amplification in PCR.
As positive and negative controls are essential parts of every experiment, they are important in the reverse transcription step and the PCR step of the RT-PCR as well (Pfaffl (2003)). RNA, which is free of DNA contamination, is a necessary demand to prevent false positive amplification during PCR. Consequently, the digestion of DNA in RNA preparations by DNase I is essential for the reliability of RT-PCR results. Unfortunately, only labour-intensive methods like ultra centrifugation and CsCl gradient purification deliver RNA totally free of DNA. However, these methods cannot be used routinely (Lion (2001)).
Choosing primers, that flank an intron in the target gene, DNA contamination can be monitored by the differences in the size of amplification products derived from RNA and DNA. For primer systems, which make no difference between RNA and DNA false positive amplifications have to be expected. Therefore, RT-PCR should be optimised by simultaneous amplification of a reference target as an internal control, which can be for instance a promoter region of the gene to be amplified (Mannhalter (2000)). As different specific stabilizing sequence regions can be found in eukaryotic mRNA, the stability of target and control genes can differ, which has to be

avoided in RT-PCR. This is especially important during transportation of samples over long distances.

In terms of a fungi-specific PCR detection system, the primer region of the internal control has to be specific for fungi and conserved in all relevant species, too. Suitable control genes and target genes need to be transcribed with equal intensity and independent of cell cycle. Unfortunately, information about control genes with the mentioned features is very limited. Furthermore, the primer region should prevent amplification of potential pseudogenes[4] to avoid false positive results. Therefore primers for RNA should be tested with DNA for pseudogene amplification. Some genes identified in the human genome are known to have pseudogenes, which have influenced the results in RT-PCR assays, such as aldolase, beta-actin (M55014), dihydropholate reductase (DHFR), glyceraldehyd phosphate dehydrogenase (GAPDH), histone 3.3 (H 3.3), and hypoxanthine phosphoribosyltransferase (HPRT), respectively (Lion (2001)). However, for most genes the existence of pseudogenes is unknown; nevertheless it is important to test for homologue pseudogenes especially in systems where no control of the reverse transcription step is done.

As mentioned earlier, the amount of DNA in RNA preparations can be decreased by DNase I treatment, but due to varying amounts of DNA in samples total removal of DNA is difficult in routine testing. Additionally, DNase I treatment can influence the stability of mRNA, which often limits the sensitivity in RT-PCR applications. Matrix inhibition[5] can decrease sensitivity, too. Diluting of samples and addition of non-target carrier DNA may compensate the effects of matrix inhibitors in detecting small amounts of mRNA. Additional to internal controls, external positive controls, which monitor the quality of the RT-PCR reaction mix can be added artificially (Lion T. (2001)).

At this point targeting rRNA is considered again, because many authors consider rRNA as a target for PCR applications (Chen and Eisner (2000), Chen *et al.* (1991), Dlauchy *et al.* (1999), Gargas *et al.* (1995), James *et al.* (2000), Jamet-Vierny *et al.* (1997), Kurtzman *et al.* (1998), Turenne *et al.* (1999), Wu (2003)). If one ignored the problems emerging with the stability of rRNA for the distinction of viable and dead organisms as mentioned earlier, rDNA gene clusters seem to combine many features of a promising target for RT-PCR applications. Up to 100 copies of 18S rDNA genes are found in genomic DNA promising high sensitivity in RT-PCR (Wu *et al.* (2003)). The sequences of rRNA genes contain well-conserved regions for the development of a consensus PCR or RT-PCR system. Consequently, the 18S rDNA gene is considered to be an excellent target for highly sensitive applications. In fact, the detection of airborne fungi by PCR (using this gene) showed an amplification limit of 2-4 spores (Wu *et al.* (2003)).

However, it was found that the amount of rRNA gene copies varies between the species of fungi in a range of 20-200 (Berbee (2001), Krutzman (1994)). The nucleolar organizer regions which harbour tandemly repeated rRNA gene copies are dynamic, and the number of rRNA genes may change even during the lifespan of a single cell (Pawlowska and Taylor (2005)). Considering filamentous fungi as a very heterogeneous group one has to expect an unequal amplification of fungi harbouring a lower amount of gene copies in a potential consensus PCR assay. From that

[4] Pseudogenes are genomic DNA sequences similar to normal genes but non-functional; they are regarded as non-functional relatives of functional genes (www. pseudogene.org).

[5] Matrix inhibition is an inhibition effect caused by components of the investigated food sample (matrix).

point of view the consensus PCR for all fungi would be difficult to optimise in terms of sensitivity and specificity targeting the rRNA gene cluster. Therefore, this target cannot be supported in this work for detection of viable fungi by RT-PCR.
To focus on the mRNA of housekeeping genes might not only be the more direct way to detect viable organisms but also to target a molecule in RT-PCR which is more or less equally present in the cells of all filamentous fungi (MarLleo *et al.* (2000), Scheridan *et al.* (1998)). Therefore, in the beginning of this work the mRNA of a housekeeping gene was defined as target molecule for the detection of viable filamentous fungi.

2.2.6 Specific detection of filamentous fungi using a single copy target gene

Most eukaryotic organisms contain the two essential elongation factors EF-1α and EF-2 in their ribosomes. EF-1α catalyses the binding of the aminoacyl-tRNA to the ribosomal A-site while EF-2 mediates the translocation of the peptidyl-tRNA from the A site to the P site of the ribosome (Sandbaken M.G. *et al.* (1990)). In addition to EF-1α and EF-2, a third essential factor, elongation factor 3 (*EF-3*) has been identified in a cell free translation system of *Saccharomyces cerevisae* for the synthesis of polyphenylalanin (Uritani M. (1996)). Subsequently, *EF-3* sequence evaluation, determination of protein function in several yeasts strains, assessment of its expression level, sequence uniformity and fungal specificity have been extensively characterised in Chakraburtty (1999), (1998), (2001), Colthrust *et al.* (1992), (2000), Kovalchuke *et al.* (1994), (1995).
The investigation of lethal mutations led to the assumption, that *EF-3* is an essential protein of the translation machinery in fungi (Colthrust *et al.* (1991), Yang (1996), Belfield *et al.* (1993)). The functional uniformity of *EF-3* in different species was shown by compensation of the lethal *EF-3* mutation in *S. cerevisae* by an *EF-3* copy of *Candida albicans.* It has been suggested that *EF-3* is responsible for the exact translation of the information from the mRNA by stimulating the selective binding of the correct aa-tRNA in the A site of ribosomes. Additionally, polypeptide chain elongation in fungi requires *EF-3* dependent ATP hydrolysis to release deacetylated tRNA from the ribosomal E-site (Triana *et al.* (1995)). Additionally *EF-3* contains an ATP dependent GTPase activity, which is essential for elongation (Uritani *et al.* (1988), Miyazaki *et al.* (1988)).
Since EF-3 was not found in higher eukaryota, the existence of the additional elongation factor 3 is a unique characteristic of yeast and fungi (Dasmahaptra et al. (1981)). As one promising molecular marker for specific detection of fungi, EF-3 has been extracted from a multitude of different possible target genes such as *Hex-1* (Lim (2001), Tenney et al. (2001)), *brlA* (Han et al. (1993) Prade (1993), Boylan et al. (1987)), chitin synthase (Bowen (1992)), actin gene (Cox (1995)), translation elongation factor 1 *alpha* (Negrutskii et al. (1998), Roger (1999)) and mitochondrial cytochrome B gene (Yokoyama et al. (2000)), respectively.
Uritani *et al.* (1999), Blakely *et al.* (2001), Colthurst *et al.* (1992) and Nakayama *et al.* (1998) designed primers for sequencing of the gene *EF-3* from a selection of yeast strains. Sequences of several yeasts strains are therefore available from public databases. However, for filamentous fungi only one *EF-3* sequence from *Aspergillus fumigatus* was published in the beginning of this work. Some more sequence information of filamentous fungi was obtained during this work generated by ongoing genome projects in Whitehead Institute Centre for Genome Research.

Mäntynen *et al.* (1999) suggested the *EF-3* gene as a target for a fungal specific diagnostic assays in food.

There are several different evolutionary conserved domains in *EF-3* (El'Skaya *et al.* (1995), Kamath *et al.* (1986), Ladror *et al.* (1998)). Significant sequence similarity to membrane associated proteins of the ATP-binding cassette (ABC) family, especially in the highly conserved ATP/GTP binding motifs walker A and walker B was determined. The only protein factor with significant sequence homology to *EF-3* is found in the Chlorella virus CVK2 (McCarthy *et al.* (1998)). A similar gene in *E.coli,* RbbA, shares a number of biochemical properties with *EF-3* and cross reacts with antibodies specific for *EF-3*.

Initially, *EF-3* was identified as a single locus gene on chromosome XII of *Saccharomyces cerevisae* by Qin *et al.* (1976), Belfield *et al.* (1993). The location of the gene in filamentous fungi differs from chromosome XII in the yeast strains as the amount of chromosomes in filamentous fungi is often less than XII (*Aspergillus parasiticus* V-VII (40 Mb), *Aspergillus flavus* VI-VIII (33-36 Mb); Arora (2003) page 7). The *EF-3* gene contains one single open reading frame with 3132 nucleotides in yeast. The protein consists of 1044 amino acids of 115860 Dalton. No intron could be identified. The hydrophobic part of the *EF-3* protein shows 82 % identity to S5 protein from *E. coli*, but no functional analogue was found in any other organism for the whole protein. S5 in *E. coli* is partly responsible for proofreading activity of aa-tRNA in the ribosomal A site. There is another part of *EF-3* with homology to S5 that seems to interact with rRNA (Ross-Smith *et al.* (1995)).

The carboxyterminal part of *EF-3* is mainly exposed and charged by lysine blocks; it is considered to react with negatively charged mRNA (Kambampati *et al.* (1997)). *EF-3* can interact with all three major types of RNA: rRNA, tRNA and mRNA (Gontarek *et al.* (1998), Rodnina *et al.* (1994)).

As discussed earlier, the target gene in a potential RT-PCR based assay for the detection of viable filamentous fungi needs to be expressed permanently. Swoboda (1994) describes that *EF-3* mRNA levels were not significantly affected by heat-shock but were tightly regulated during batch growth of the yeast form[6], reaching maximal levels in exponential phase. However, in spores of filamentous fungi detection of *EF-3* mRNA has not been described yet. It is presumed that there is little *EF-3* mRNA in spores of filamentous fungi and that it is up-regulated during spore germination reaching similar levels as in the exponential growth phase of yeasts. According to Swoboda *et al.* (1994) the expression of *EF-3* correlates to growth rates which make it a promising target for the detection of viable fungi by RT-PCR.

Sequence analysis of yeast *EF-3* protein shows a unique region of 106 amino acids with functional importance for the protein in fungi (Uritani (1999)). This region is considered to serve as a target region for fungal specific detection of nucleic acids. The *EF-3* unique sequence region has been described as more species specific since it does not contain the conserved domains known in other members of the ABC family. This region was of interest in this work for the development of a fungal specific detection system based on methods like PCR or RT-PCR.

[6] On solid growth media with limiting nitrogen source, diploid budding-yeast cells differentiate from the yeast form to a filamentous, adhesive, and invasive form (Prinz S. *et al.* (2004)).

3 Aims

For the detection of fungal contaminants in food molecular methods are not adequately implemented yet, because only a few suitable molecular markers have been described so far. Previous studies suggested the gene *EF-3*, elongation factor 3 as a promising fungal-specific marker for the detection in food. Therefore, the aim of this study was to detect viable fungi in food based on molecular methods targeting the gene *EF-3*. Several industrial specifications for the system have been defined in advance of this work, as there are the implementations of PCR or RT-PCR techniques. Both methods were supposed to be tested for their application in routine use.

The development of a consensus PCR system based on a suitable target gene for simultaneous and sensitive detection of all food relevant filamentous fungi including the more rare species was therefore a major goal of this work. Since there was hardly any sequence information available at the beginning of this work, it was a key requirement to generate new sequence information for the design of suitable primers and probes for the amplification of fungal *EF-3*.

The development of a sensitive extraction protocol for nucleic acids from pure cultures and from food was one important basic demand for the application of PCR and RT-PCR in this work. Simple handling and the use of non-toxic substances were desired for this extraction method. Optimisation of the mechanical disruption process for fungal mycelia was additionally necessary.

The consensus PCR or RT-PCR system had to be adaptable to real time PCR platforms in particular to the LightCycler® instrument. Different available formats in the real time PCR had to be compared to find the most suitable for the detection of the *EF-3* target gene sequence. Adapting the features of primers, probes as well as the reaction mixtures (i.e. buffer composition) and the PCR profile was one part of the work. The design and development of an internal positive control for monitoring potential matrix inhibition and an external positive control to provide evidence of reaction mix quality was another part included into the optimisation of the consensus PCR.

4 Materials and methods

All chemical compounds, solvents and biochemicals used in this work are summarized in the attached Table 79. Primer and oligonucleotide probes were obtained from the MWG-BIOTECH AG (Ebersberg), the Metabion GmbH (Planegg-Martinsried) and the Thermo ELECTRON CORPORATION. The fungal reference strains used in this work are summarized in chapter 9.4.

4.1 Culturing

4.1.1 Culturing filamentous fungi for nucleic acid extraction

Table 3 - Growth media

organism	name of medium	composition
fungi pH 7.2	DSM 310; V-8 Juice Agar	200 ml/l V-8 juice 3 g/l calciumcarbonate ($CaCO_3$) 15 g/l agar
fungi pH 5.6	DSM 90; Malt extract Tryptone Agar (M)	30 g/l malt extract 3 g/l tryptone 15 g/l agar
fungi pH 5.4 – 5.6	Potato Dextrose Agar (PDA)	80 g/l potato mash (Pfanni) 20 g/l dextrose 15 g/l agar
fungi n.d.	DSM 187 Osmophile medium for fungi (M40 Y)	400 g/l sucrose 20 g/l malt extract 5 g/l yeast extract 15 g/l agar
fungi pH 6.5	DSM 393; YPD; YEPD	20 g/l peptone 10 g/l yeast extract 75 ml glucose (26.67 %) autoclaved separately 15 g/l agar
fungi pH 7.2	DSM 189; oat flake medium (OM)	30 g/l oat flake 15 g/l agar
fungi pH 5.6	Sabouraud	5 g/l peptone fromcasein 5 g/l peptone from meat 40 g/l glucose 15 g/l agar
bacteria; fungi pH 7.2	Caso (Soya-bean casein digest medium)	17 g/l pancreatic digest of casein 3 g/l papaic digest of soya-bean meal 5 g/l sodium chloride 2.5 g/l dipotassium hydrogen phosphate 2.5 g/l glucose monohydrate 15 g/l agar

Fungi are more tolerant to low pH than bacteria. Consequently, the media for the selective cultivation of filamentous fungi are characterised by a low pH. The fungal specific media contained the antibiotic Chloramphenicol (50 mg/ml). The herein applied media are summarized in Table 3. The particular strains cultured in this work are summarized in the attached Table 75 and Table 76. Additionally, the culturing media are assigned to the strains cultured in this work in Table 75 and Table 76.

Plate cultures

Agar plates totally covered with mycelia were prepared according to the following protocol. By dilution plating of spores mycelia were grown from one CFU. Using a sterile cork borer equal circles of mycelia were cut from the agar plates. The pieces of mycelia were transferred to 2 ml tubes containing 10 Zirconium beads/ml saline (0.9 % NaCl). The solution was homogenized in the MagNA Lyser (Roche) for 1 min at 6500 rpm. A 1 ml pipette tip was cut and 100 µl of the solution evenly spread on the agar plates. The plates were incubated at 25°C for 2-14 days. Again defined circles were cut with a sterile tool for nucleic acid extractions.

4.1.2 Germination of fungal spores for food investigation

In a biological safety cabinet spores from agar plate cultures were transferred into 1ml of spore resuspension buffer (0.1 % Tween 80, 20 % Glycerol) and shaken vigorously. The sedimented lower part of the suspension was resuspended in the MagNA Lyser (1 min at 6500 rpm). Subsequently the lower part was transferred into a new tube and diluted 1:100 for quantification. Quantification of spores was performed in a Neubauer chamber using a Novex microscope K-range Holland (400x) according to the manufacturers protocol. The total spore count was calculated according to the following equation using the spore count of 4 large squares. Based on the equation media and food were spiked with defined amounts of spores.

$$\mathbf{CFU/ml = MV * 10^4 * DF}$$

CFU (Colony forming units)
MV (Mean value of 4 large squares)
DF (Dilution factor)

The germination of spores from pure cultures was performed in 2 ml tubes containing fluid media at 20-25°C for at least 16 hours. Germination of spores was observed using a Novex microscope K-range Holland. The duration of spore germination depended on the composition of the media, the type of fungal strain, the amount of spores, the maturity and age of the spores, the type of spores and the temperature, respectively. Therefore, the duration of germination was carefully determined for each model strain used in the experiments.
The optimal time point for nucleic acid extraction from germinated spores in fluid media was determined by performing a quality control of DNA in gel electrophoresis. DNA was extracted from 0, 4, 8 and 16 hours old spore cultures. After 8 hours detectable amounts of DNA were extracted for the first time, but an over night incubation (16 hours) resulted in larger amounts of DNA leading to reliable results in PCR (data not shown).
To meet the requirements of PCR the enrichment of filamentous fungi was carried out as described in the following part. As it is too difficult to take out equal homogenous amounts of fungi, the culturing in fluid media was not practicable. But agar cultures offered no alternative as well, because the fungal hyphae are clotted together with agar. Agar is inhibiting PCR and therefore needs to be avoided in the DNA samples. A high reproducibility of PCR results can only be achieved by DNA extraction from equal amounts of samples. Consequently the enrichment of filamentous fungi was performed on the surface of membrane filters covering nutrient pads or agar plates. Nutrient pads are favourable as they are already validated for culturing in industry and accepted by AOAC (Association Of Analytical Communities). The whole membrane filter was disrupted during nucleic acid extraction. The filter membranes from Pall (0.45 µm) were tested and do not influence PCR (data not shown). The chances for detecting fungi by PCR are maximized using this type of enrichment as all viable fungi in the particular sample are transferred to the DNA extraction.

4.2 Nucleic acid preparation

Although chloroform extraction generally improves the purity of the extracted nucleic acid it is toxic and not suitable in routine handling. The industry demands to use alternative methods. Silica binding and anion exchange resins are the most commonly used alternative nucleic acid extraction methods. Both principles were applied in this work.

The disruption of fungal cell walls is a requirement and a major challenge during nucleic acid extraction. Problems during the extraction of nucleic acids from filamentous fungi are basically caused by the compact cell wall structures from chitin, cellulose, ß-1-3-glucan, chitosan, and mannan. Usually grinding with mortar and pestle in liquid nitrogen is the most efficient method for disruption of the rigid fungal cell walls of hyphae and conidia (Karakousis (2005)). Unfortunately this handling was not suitable for this study, especially if one considers the dust containing living pathogens that may derive from grinding. Consequently one has to consider grinding down the hyphae in a closed compartment for a routine use. This was realised in this study by disruption of cells in the FastPrep^{TM} FP120 BIO101 Thermo Savant or MagNA Lyser (Roche) using 2 ml tubes with Zirconium beads (1.2 mm) for 1–2 min at 6000-7000 rpm. The shear forces in the tubes improved the cell disruption and nucleic acid extraction immensely. The quality of DNA and RNA was verified by agarose gel electrophoresis. The protocols applied herein for RNA/DNA extraction from fungi are based on silica membranes and ion exchanger Chelex 100. DNA and RNA can simultaneously be extracted by splitting the samples and substituting the DNA digestion with RNase treatment. Nucleic acids were separated electrophoretically on 1 % or 2 % horizontal agarose gels (120V, TBE buffer final concentration 0.5x). The DNA was loaded onto the gel in presence of loading buffer and the size was determined by comparison with molecular size standard VI (Roche) run on the same agarose gel. The nucleic acids were stained with the intercalating dye ethidiumbromide and visualized by UV light. Photographs were taken under UV illumination using gel documentation system (Herolab) by means of the software Easywin 32. The quality of RNA was controlled in 2 % agarose gels.

Protocol 1

The Protocol of GMO Sample Prep Kit (Roche) was used for extraction of RNA from fungal hyphae with the changes outlined below.

1. Young fungal mycelia were grown on agar plates until the whole surface was covered with fungal mycelia. Samples of 2 cm were cut out of the mycelia by using a cork borer.
2. The fungal surface was removed from agar and transferred into 2 ml tubes containing Zirconium beads (1.2 mm).
3. 400 µl Lysis Buffer and 400 µl Binding Buffer were added as prepared in the GMO Sample Prep Kit.
4. Samples were crushed 2 x 60 sec at 6500 rpm in the MagNA Lyser instrument (Roche).
5. A centrifugation step for 1 min at 12000 rpm followed. The supernatant was transferred into a new tube.
6. Before the samples were transferred into the silica columns of the Kit. 200µl isopropanol were added. The columns were centrifuged for 1min at 12000 rpm.
7. The columns were washed by addition of 450 µl Wash Buffer twice as prepared in the GMO Sample Prep Kit and subsequent centrifugation for 1 min 12000 rpm followed.
8. Samples were dried by centrifugation at maximal speed for 2 min.
9. DNA and RNA were eluted with 65 µl Elution Buffer as prepared in the GMO Sample Prep Kit.
10. Sigma DNase I (6.5 µl 10 x Reaction Buffer + 1 µl AMP D1 DNase I) was added.
11. DNA was digested for 30 min at room temperature. The DNase I was inactivated for 10 min at 70°C. The RNA was stored at –20°C.

Protocol 2

Protocol for extraction of RNA from fungal spores, mycelia and membrane filters using ion exchanger Chelex 100 (pH Chelex > 10). 1.2 mm Zirconium beads and MagNA Lyser instrument (Roche).

1. 2 ml tubes with screw-type cap containing 15 beads were placed on ice.
2. Spores, mycelia or membrane filters were added to the tubes. Additionally, 0.2–0.5ml Chelex 100 (10 %) were added and the tubes were placed on ice.
3. The disruption was carried out in MagNA Lyser for 2 x 60 sec at 6500 rpm and was followed by incubating the samples for 2 min at 95°C and 3 min on ice. The samples were centrifuged for 3 min at 12000 rpm and the supernatant was transferred into a new tube (0.5 ml).
4. 1 µl Protector RNase Inhibitor was added. The samples were centrifuged and the clear supernatant was transferred to a new tube.
5. DNase I (Roche) 0.8 µl /20 µl was added to the supernatant and the tubes were incubated at 37°C for 30 min. EDTA was added to a final concentration of 5 mM in the supernatant or alternatively the supernatant was added to a tube containing dried Chelex 100. The DNase I was heat inactivated for 10 min at 70°C.
6. Quality was checked by agarose gel electrophoresis.
7. Samples were centrifuged before adding them to the RT-PCR master mix.
8. RNA was checked in a PCR to exclude amplification of contaminating DNA in RT-PCR applications.

Protocol 3

Protocol for extraction of DNA from viable fungi and membrane filters including non- gelatinised food samples using ion exchanger Chelex 100 (pH Chelex > 10). 1.2 mm Zirconium beads and MagNA Lyser instrument (Roche).

1. 2 ml tubes with screw-type cap containing 15 beads were placed on ice.
2. Spores, mycelia or membrane filters including food samples were added into the tubes. Filter membranes were folded before transferring them.
3. 0.5 – 0.86 ml sterile water and 40 µl proteinase K were added and incubated for 20 min at 65°C.
4. Proteinase K was inactivated for 5 min at 95°C and cooled down to 4°C.
5. 5 µl (50U) of DNase I (Roche) and 50–100 µl 10x buffer (1M NaAc; 100 mM $MnCl_2$) were added and incubated for 30 min at 37°C and mixed several times.
6. Samples were centrifuged for 1 min at 13200 rpm and the supernatant was discarded.
7. 250 µl Chelex 100 (20 %) was added and the samples were incubated for 5 min at 96°C.
8. MagNA Lyser was applied for 2 min at 6500 rpm. Afterwards, the samples were immediately cooled down to 4°C.
9. Samples were centrifuged at 13200 rpm for 3 min. The supernatant contained DNA. The RNA was digested by adding RNase and incubating for 30 min at 37°C.

This method resulted in the digestion of the background contaminating DNA found in food samples before disrupting the viable cells in MagNA Lyser.

DNA from heat gelatinised food

To extract DNA from heat gelatinised food protocol 3 was changed in the following way. Chelex 100 (20 %) (500 µl) was added to the filter membranes and Zirconium beads contained in 2 ml MagNA Lyser tubes that were then incubated in the MagNA Lyser instrument for 60 sec at 6500 rpm. The samples were immediately cooled down and the MagNA Lyser disruption of the samples was repeated. The samples were cooled down for 3 min at 4°C and centrifuged for 1min at 13000 rpm. The supernatant contained the DNA for downstream applications like PCR.
According to the manufacturer's instructions nucleic acids were quantified by measuring the absorbance at 260 nm in a spectrophotometer and by applying Quant-iT™ PicoGreen® dsDNA Assay Kit in the LightCycler® instrument.

4.2.1 Primer used for sequence generation

Table 4 - Primer used during amplification for sequence generation. The region SP1 – SP 10 see in Figure 3.

region	name	function	Sequence 5'→ 3'	author
SP 1	HS3	not suitable for sequencing	GAATTYTCTYTKGCWTAYGG	Uritani *et al.* (1999)
SP 2	ML1 ABC	sequencing of *Zygomycota*	GGTGGTTGGAAGATGAAG	this work
SP 3	mix 10, mix 20 fw, mix 20 rev	primer for sequencing of *Zygomycota* and *Ascomycota*	this work; Table 5	this work
SP 3	HS10	primer for sequencing	ATGGMGITWCCANWCYGGTGA	BCD
SP 4	primer from region 155	primer for sequencing of *Zygomycota*	this work mix 157 rev; Table 5	this work
SP 5	ML1f	test of clones	TCAGTTCCGTCCCCTGGTCCG	this work
SP 6	HS9	not improving sequencing	GAGATTGAGGCCCTCTG	BCD
SP 7	HSabc3	alternative primer for sequencing	CIAIIACAIIYTTIACYTTYTGRCCACCNG	BCD
SP 7	primer from region 400	primer for sequencing of *Zygomycota*	this work 437ML12r; Table 5	this work
SP 8	ML2r	test of clones	GCGGCAGGACGACCTTGACA	this work
SP 9	HS4	primer for sequencing	ACTGARGAAGTYTGG	Uritani *et al.* (1999)
SP 10	HSwb1	alternative primer for sequencing	ITIITIITIGAYGARCCIACYAACCATYTNG	BCD

4.2.2 Primer for amplification of filamentous fungi

Table 5 - PCR primer for amplification of *Ascomycota*, *Deuteromycota*, *Basidiomycota*, *Zygomycota* and *Oomycota*. The primers were designed based on the sequence alignments in the attachment and some were used in the consensus PCR assay. Some of the primers were additionally helpful during the sequencing of *Zygomycota*.

	name	Sequence 5'→ 3'	degeneration[7]
mix 10:	10ML29fA	GGCGGTTCCAAACTGGTGAAGATCGTG	0
	10ML29fB	GGCGGTACCAGACCGGTGAGGATCGTG	0
	10ML30f	GCGGTACCAGTCTGGTGAAGATAAGG	0
mix 140:	140ML31r	GCCTTGCGACGTCCATAGAGATCC	0
	140ML32r	TTCATATTGGAAAGTCTTCTTGAGTTTAGTACG	0
	140ML33r	GTATTCGTAGGATTGCTTGAGCTTACGG	0
	140ML45r	CATATTGAAATGTCTTCTTGAGCTTAGTACG	0
	140ML46r	CTCGTAGGTGTTCTTGAACTTACGACG	0
	140ML62rev	TCGTATTC(AG)TAAGATTG(CT)TTGAGCTTTC	4
	140ML63rev	TC(AG)TA(CT)TC(AG)TAAGATTGCTT(AG)AGCTT	16
	140ML64rev	CGTACTCGTACGACTGCTT(GC)AGCTT	2
	140ML65rev	GTACTCGAAAGAGCGCTTGGCCTT	0
mix 157 rev	157ML13r	CTCGTACTCGTACGAGTTCTTGAACTT	0
	157ML14r	CTCGTACTCGTAAGAGTTCTTGAACTT	0
	157ML15r	CTCGTACTCGTAAGTGTTCTTGAACTT	0
	157ML16r	CTCGTACTCGTAGCTGTTCTTGAACTT	0
	157ML8r	CTCGTACTCGTAGGAGTTCTTGAACTT	0

[7] Degree of degeneration of oligonucleotides = product of possible base substitutions in the oligonucleotide and the wildtypes.

	157ML9r	CTCGTACTCGTAGGTGTTCTTGAACTT	0
mix 20 fw:	20ML5f	GGTGAGGAYCGYGARACYATGGATCG	16
	20ML6f	GGTGAGGAYCGYGARACYATGGACCG	16
	20ML7f	GGTGAAGATCGYGAAACYATGGACAG	4
mix 20 rev:	20MLF34r	GTGAGGACCGTGAGACTATGGACCG	0
	20MLF35r	GTGAGGATCGTGAGACCATGGATCG	0
	20MLF36r	GTGAGGACCGAGAAACCATGACCG	0
	20MLF37r	TGGTGAAGATCGTGAAACCATGGACAG	0
	20MLF38r	CTGGTGAAGATCGTGAAACTATGACAG	0
	20MLF39r	TGAGGATCGTGAGACCATGGACCG	0
	20MLF40r	GAGGATCGTGAGGCCATGGATCG	0
	20MLF41r	GAGGATCGCGAGACTATGGACCG	0
	20MLF42r	GGTGAGGATCGTGAGACTATGGATCG	0
	20MLF43r	GGTGAGGATCGTGAGACTATGGATCG	0
mix –30 f:	-30ML47F	GACAAGACTCCCTCTGA(AG)TA(CT)AT(ACT)CA(AG)TGG	24
	-30ML48F	GACAAGACCCCGTCCGA(AG)TA(CT)AT(ACT)CA(AG)TGG	36
mix 400_52 r:	400ZML51r	G(CT)TTGAC(CT)TTTTG(AG)CCACC(AG)GA(AC)A(AG)A(GC)C	128
	400ZML52r	GAGGACGAG(CT)TTGAC(CT)TTTTG(AG)CCACC	8
	437ML12r	GRACRACCTTRACACGYTGTCCACC	16
mix –60 f:	-60ML49F	CAACATGCTTTCGC(AGCT)CA(CT)AT(ACT)GA(CT)AA(CT)C	96
	-60ML50F	AGCACGCTTTCGC(AGCT)CA(CT)AT(ACT)GA(CT)AA(CT)C	96
	-60ML51F	T(CT)GC(CT)C(AG)ACACGCTTTCCACCACA	8
	-60ML52F	C(CT)CAGCACGCTTTCCACCA(CT)ATTG	4
	-60ML53F	CACGCTTTCGCTCACAT(CT)GATAACC	2
	-60ML54F	CACGCCTTCGCGCACATCG	0
	-60ML55F	CACGCTTTCCACCACAT(CT)GA(AG)AAGC	4
	-60ML56F	CGC(CT)TTCGC(GT)CACAT(CT)GA(CT)CACC	16
	-60ML57F	ACGC(CT)TTCGC(GT)CA(CT)AT(CT)GA(CT)CACC	32
RT reaction	OligodT35	(GCT)(AGC)(AGCT)CT(GCT)AAGA$(T)_{35}$	108

4.2.3 Probes for detection of filamentous fungi

Table 6 - TaqMan probes.

name	5’ exonuclease assay
S1ML-RV	5’-Fam-CTTGTTGGCRCGRTCCATGGTYTCRCG-Tamra-3’
S1ML	5’-Fam-CGYGARACCATGGAYCGYGCCAACAAG -Tamra-3’

Table 7 – HybProbes. The following degenerated probes[8] were designed as HybProbes for detection of significant fungal species in Table 75 and Table 76.

name	bp	sequence	degeneration
A FML12rev	24	5’ CTTGTTGGCNCGRTCCATRGTCTC Fluo 3’	16
A FML14rev	30	5’ GACRATCTTGTTGGCTCTGTCCATRGTTTC Fluo 3’	4
A FML15rev	26	5’ ATTCGGTTGGCTCGGTCCATGGTTTC Fluo 3’	0
Z FML11rev	31	5’ CRTTCTTRTCRAGTTCTCACGATCTTCACC Fluo 3’	8
Z FML12rev	30	5’ TACCTTKTCSARYTCYTCWCGRTCTTCACC Fluo 3’	128
A LCRed640ML4rev	24	5’ Red640 CGRTCCTCACCRGTNTGGAACCGC Pho 3’	16
Z LCRed640ML5rev	35	5’ Red640 TAGCATAACGCCATTGRATRTAYTCRTTRGGAGT Pho 3’	32
Z LCRed640ML6rev	26	5’ Red640 GTMGCRTAWCGCCACTGGATGTACTC Pho 3’	8
A LCRed640ML7rev	24	5’ Red640 GCRTCCTCACCRGANTGGTACCGC Pho 3’	16
A LCRed640ML10rev	26	5’ Red640 CGATCTTCACAGAYTGGWACCKCCA Pho 3’	8

[8] R = G or A, Y = C or T, K = G or T, W = A or T, S = C or G, M = A or C, B = C, G or T, D = A, G or T, H = A, C or T, V = A, C or G, N = A, C, G or T

4.3 Generation of sequence information

4.3.1 PCR amplification of fungal genomic DNA

The following chapter describes characteristics of fungi, which were taken into consideration before extraction of new *EF-3* sequences. Particularly one has to consider, that mycelia grown from one CFU are typically not grown from one spore. Caused by hydrophobic surface structures, spores can clot together during plating. That means that even though filamentous fungi are haploid the mycelia grown from spores can contain a mixture of fungi with different alleles. Haploid conidia are consequently formed from different genotypes and the extracted DNA contains the whole genetic variability. This has to be considered in the use of this DNA for cloning and sequencing. Due to the variability of PCR products amplified with degenerated primers direct sequencing of the PCR products can result in distortion of obtained sequences. Consequently, separation of different alleles by ligation into a vector system and cloning was performed as described later. The general PCR protocol is summarized in
Table 8.

Table 8 - PCR profile and PCR master mix used in amplifying fungal DNA for sequencing. The HTB-mixture consisted of Taq (HBT) polymerase 0.1 U/µl. 1/8 of final volume of ClAK (antibody) and buffer at final 1 x (Clontech).

components	final conc. in 25 µl
$MgCl_2$	2 mM
each dNTP	each 0.4 mM
10 x Buffer	1 x
Primer 10 pmol	0.4 pmol/µl
HTB-mixture	0.25 µl HTB-mixture
template DNA	<0.25 ng

temperature	time	cycles
95°C	5 min	1
95°C	30 sec	30
60°C	30 sec	
72°C	1 min	
72°C	10 min	1

To avoid unspecific amplification during PCR hot start enzymes were used in the generation of PCR products for sequencing. Antibody (CIAK) that binds to the polymerase nucleotide binding domain was used to render the polymerase inactive. Upon heating, the compound dissociated from the polymerase, restoring enzyme activity (Miller (1996)).

4.3.1.1 Generating sequences for forward and reverse primer design of *Zygomycota*

Amplification of products for cloning and sequencing of the region for forward primer design of *Zygomycota* was performed with the two PCR protocols in Table 9.
In order to generate sequences for the specific design of reverse primers of *Zygomycota* the experimental procedure in Table 9 was repeated with the PCR profiles of PCR 1 and PCR 2 using the forward primer ABC ML1 and the reverse primers 400-51 and 400-52 in PCR 1 (derived from artefact alignment A in Figure 29). The PCR 2 mixture combined forward primers -60ML55, -60ML56 and -60ML57 and reverse primers Rv 400-51 and 400-52 resulting in a 531 bp product. The specific 531 bp product was separated from other unspecific products by gel electrophoresis, extracted from the gel by Qiaquick Gel Extraction Kit, cloned and sequenced as described in 4.3.2. The alignment C (see Figure 29) was generated from the resulting sequences.

Table 9 a and b - PCR protocols for sequencing *Zygomycota.* Products of the first PCR were separated by agarose gel electrophoresis, fragments of the expected size were cut from the gel and transferred to the second PCR.

PCR 1

Forward primer ABC ML1 derived from SP2 was combined with the reverse primers 140ML31, 140ML32, 140ML33, 140ML45, 140ML46, 157ML8r, 157ML9r, 157ML13r, 157ML14r, 157ML15r and 157ML16r derived from SP 4. The primers 140ML31, 140ML32, 140ML33, 140ML45 and 140ML46 were derived from the artefact alignment from region SP4. Apart from the expected specific 780 bp product additional products were amplified with these primers in the PCR.

PCR 1	time	temperature	
	10 min	95°C	1x
	1 min	45°C	1x
	2 min	72°C	1x
	20 sec	95°C	35x
	20 sec	50°C	
	45 sec	72°C	
	7 min	72°C	1x

PCR 2

Forward primer ABC ML1 was combined with reverse primers 10ML29Arev, 10ML29Brev and 10ML30rev derived from the artefact alignment A of *Zygomycota* sequences in region SP3. Apart from the expected specific 650 bp product additional products were amplified with these primers in the PCR.
Products of this PCR were separated by agarose gel electrophoresis; fragments of the expected size 650 bp were extracted from the gel, cloned and sequenced as described later.

PCR 2	time	temperature	
	10 min	95°C	1x
	20 sec	95°C	30x
	20 sec	52°C	
	45 sec	72°C	
	7 min	72°C	1x

4.3.2 Cloning and sequencing

Some problems while cloning PCR products into Vector pGEM®T were solved by adding additional adenine nucleotides to the PCR products using the following protocol. Amplicons generated with Taq DNA Polymerase typically have A overhangs, which led to the method referred to as T-vector cloning. The plasmid cloning vector is engineered to contain 3'-T overhangs that match the 3'-A overhang of the amplicon (Promega). In 'A tailing' the *Taq* DNA Polymerase adds a nontemplate-dependent A residue to the 3´-end of probably blunt-ended PCR products, which were than successfully ligated.

Table 10 – Protocol for „ A tailing" which was used to increase ligation efficiency.

component	conc.
PCR product extracted from gel	7 µl
10 x buffer	1 x
$MgCl_2$ (50 mM)	2 mM
dATP (10 mM)	0.4 mM
FastStart-Taq	5 U

time	temperature	cycle
10 min	95°C	1
30 min	70°C	1

Products of this reaction were ligated into the vector pGEM®-T (Promega) according to the manufacturer's protocol. Briefly, the ligation mixture contained 5 µl 2 x Buffer, 2.5 – 3.8 µl Insert, x µl sterile aqua bidest, 0.4 µl Vector pGEM T (50ng/µl) and 0.8 µl ligase (3U/µl). The components of the ligation mixture were carefully mixed in a final volume of 10 µl. Incubation was done for 16 h at 4°C or alternatively for 1-2 hours at room temperature followed by 1-2 hours at 4°C.
PCR products using "sequencing" primers Table 11 were separated on agarose gels, fragments of expected sizes were cut from the gels and purified according to the manual of Quiaquick Gel Extraction kit (Qiagen Hilden). The results were controlled by gel electrophoresis. The ligation of fragments into the vector pGEM®-T (Promega) was done according to the manufacturers protocol. Competent cells were produced by standard $CaCl_2$ method (Maniatis *et al.*1982). The resulting ligation products (plasmids) were transferred to competent cells (*E.coli* XL-1 blue) as follows. Frozen aliquots of cells were thawed and plasmids added. The mixture was incubated for 20 min on ice, followed by 45 sec in 42°C and 2 min on ice. SOC[9] medium (900 µl) was added to the tubes and cells were incubated at 37°C for 1 ½ h with shaking. For the selection of positive clones the mixture was plated on LB/ampicillin/IPTG/X-Gal agar[10] plates and incubated over night at 37°C. Selection was performed using the 'blue white method' according to the manual from Promega (pGEM-T Vector System). White colonies on LB/ampicillin/IPTG/X-Gal agar plates contained *EF-3* positive clones and were transferred into fluid selective LB medium including ampicillin. Clones were grown at 37°C overnight.
The clones were tested for *EF-3* by a PCR using primer ML1f and ML2r or standard M13 primers. The plasmids of *EF-3* positive clones were extracted from cells by Qiagen Plasmid Mini Kit (Qiagen Hilden) according to the manufacturer's protocol.

Table 11 – Standard sequencing primers.

contained	name	function	sequence	origin
in Vector pGEM® T	M13 FP	Primer for sequencing from Vector pGEM® T	TGTAAAACGACGGCCAGT	Standard primer
in Vector pGEM® T	M13 RP	Primer for sequencing from Vector pGEM® T	CAGGAAACAGCTATGACC	Standard primer

The plasmid sizes were compared to a negative control (uncut vector pGEM®-T) by gel electrophoresis. Sequencing was performed by GATC BIOTECH AG (Konstanz). Depending on the size of the fragment either both standard primers M13 Fw and M13 Rev or only one of these primers was used.

4.3.3 Compilation of sequence alignment and oligonucleotide design

Some important considerations concerning primer and probe design will be described in the following part. Primer design and probe design should be done by using mathematical algorithm for equalising of annealing temperatures. Specific melting temperatures have to be the same in forward and reverse primers. To achieve high specificity the melting temperature has to be as

[9] SOC medium consited of 2.0 g peptone, 0.5 g yeast extract, 1 ml (1 M) NaCl, 0.25 ml (1M) KCl, 1 ml (2 M) Mg^{2+} (1M $MgCL_2$ x 6 H_2O/ 1M $MgSO_4$ x 7 H_2O) and 1 ml (2 M) glucose.
[10] LB Agar was autoclaved, cooled down to 50°C and ampicillin (final conc.: 100 µg/ml), IPTG (final conc.: 0.5 mM) and X-Gal (final conc.: 80 µg/ml solvable in Formamid!) were added.

high as possible. This is especially important in the design of primers for the RT-PCR because of the occurrence of secondary structures in the RNA (Kuo (1997)). The software used in sequence analysis is listed in Table 12.

Table 12 – Software used in sequence evaluation.

name	function
Vector NTI 7.1 (Feb.4, 2002) 1994-2002 InforMax, Inc.	Including AlignX generating alignments using ClustalW algorithm
GeneDoc Version 2.6.002 1997, Nicholas, Karl B., Nicholas, Hugh B.Jr.	Multiple Sequence Alignment Editor & Shading Utility
Chromas Version 1.45 Conor McCarthy School of Health Science Griffith, Australia	Sequencing control program
MeltCalc Professional Edition Version2.x for commercial use	Thermodynamic DNA stability calculations and automatic design of hybridisation probes

By evaluating the sequences obtained from GATC BIOTECH AG with the mentioned software primer and probe design was prepared. The sequences were aligned by the Vector NTI software applying the ClustalW algorithm in AlignX. Sequence trees were obtained by applying the neighbour joining analysis method of the software. Additionally, the nucleotide sequences were translated into protein sequences and aligned forming protein alignments. In general, some sequences were aligned and edited using the alignment editor GeneDoc software (freeware).

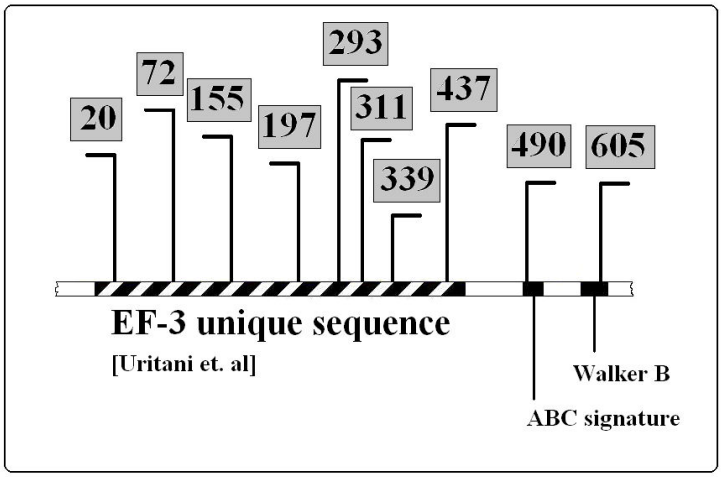


Figure 1 - Potential primer or probe hybridisation sites in the generated *EF-3* sequences. As shown in the figure several promising regions have been identified for a potential primer or probe hybridisation.

The *EF-3* unique region (Uritani *et al.* (1999)) was chosen for primer and probe design. The protein alignment of this region was searched for areas of 8-15 amino acids, which include little taxonomical variation. Comparison of these protein regions with the sequence alignment of nucleotides was done. Regions were selected which showed conserved amino acid triplets within all or a large group of fungal sequen-ces. The nucleotide sequences of the 10 selected regions were ordered into groups by extracting all occurring sequence variants. In this order the nucleotide 1 in the triplet code of the corresponding amino acid was weighted stronger as a feature than nucleotide 2 and 3, because the variation in the first base of the codon triplets are directly transmitted to the protein level and therefore not accidentally enriched during evolution. The variations in nucleotide 2 and 3 of the amino acid code were included into the primer design by degenerating the primers as recommended in Berbee (2001), Compton (1990). Degeneration can therefore decrease a large amount of single primer variants. Degeneration of primers was done within the 5'-end of the primer sequence mainly in the third position of the amino acid code.

4.4 Consensus PCR (protocol A)

Table 13 - Master mix of consensus PCR including HybProbes The enzyme-mixture contained UNG and Fast Start Taq Polymerase (0.35 µl Fast Start Taq (2 U); 0.04 µl UNG (2U/µl); 0.61 µl storage buffer for fast start).

component	final concentration in 20 µl
genomic DNA or dilutions	1 pg -100 ng
Enzyme-mixture including UNG; Fast Start Taq Polymerase	each enzyme 2U
10x buffer	1x
BSA 30 mg/ml	0.03 mg/ml
$MgCl_2$ 100 mM	4 mM
each dNTP 10 mM	0.4 mM each dGTP, dATP, dCTP; 0.3 mM dUTP; 0.1 mM dTTP
A Primer Fwd Mix 10 pmol/µl	0.2 pmol/µl
A Primer Rev Mix 10 pmol/µl	0.2 pmol/µl
Z Primer Fwd Mix 10 pmol/µl	0.4 pmol/µl
Z Primer Rev Mix 10 pmol/µl	0.4 pmol/µl
each Fluorescein labelled probe: 10 pmol/µl	0.075 pmol/µl
each LC-Red labelled probe: 10 pmol/µl	0.15 pmol/µl
PCR water	x

Table 14 - PCR profile of consensus PCR

temperature	time	cycles
50°C	2 min	1
95°C	10 min	1
95°C	0 sec	20
50°C single measuring	0 sec	
55°C	5 sec	
72°C	10 sec	
95°C	0 sec	45
50°C single measuring	0 sec	
55°C	30 sec	
72°C	40 sec	

Table 15 – HybProbes used in consensus PCR

	Fluorescein probes	LC Red probes
Asco.	F-AML14rev	A-LCRed640ML4rev
	F-AML15rev	A-LCRed640ML7rev
	F-AML12rev	A-LCRed640ML10rev
Zygo.	F-ZML12rev	Z-LCRed640ML5rev
	F-ZML11rev	Z-LCRed640ML6rev

Table 16 - Primer mixture of consensus PCR

A Primer Fwd Mix 1:1	-60ML49F; -60ML50F
A Primer Rev Mix 1:1:1:1:1:1	157ML8r;157ML9r; 157ML13r;157ML14r; 157ML15r; 157ML16r;
Z Primer Fwd Mix 1:1:1:1:1:1:1	-60ML55, -60ML56, -60ML57, -60ML51,-60ML52,-60ML53,-60ML54
Z Primer Rev Mix 1:1:1:1:1:1:1:1:1	140ML31, 140ML32, 140ML33, 140ML45, 140Ml46, 140ML62rev, 140ML63rev, 140ML64rev, 140Ml65rev

Reference strains of filamentous fungi summarized in Table 75 and in Table 76 were included in the experiments concerning PCR and RT-PCR.

4.4.1 Carry-over prevention using UNG during PCR

The Uracil-DNA Glycosylase (UNG) is suitable for preventing carry-over contamination. The incorporation of deoxyuridine triphosphate (dUTP) into the newly synthesized DNA is necessary to cleave the carry-over PCR products by UNG at any UTP-site. During the denaturation step in the PCR these fragmented DNA residues hydrolyse and cannot serve as target DNA any longer. Native DNA on the other hand is still available for PCR, because it does not contain uracil. UNG

was also included into the consensus PCR master mix used in this work. The dTTP in the dNTP mixture was partly substituted by dUTP, resulting in a mix containing 25 % dTTP and 75 % dUTP. The final concentration of UNG in the PCR master mix was 0.08 U/reaction.

4.4.2 Hot Start PCR using FastStart Taq DNA polymerase

FastStart Taq DNA Polymerase is a thermostable, chemically modified variant of recombinant Taq DNA Polymerase, which is used in the herein described experiments. It is a highly processive 5'-3' DNA polymerase that lacks 3'-5' exonuclease activity.
The polymerase only becomes active at temperatures above 75°C when the DNA is completely denatured, which is called 'Hot Start'. The reaction starts only in case of specific primers bound to the target DNA.

4.4.3 Color Compensation

In a multicolour experiment as performed herein one has to apply a colour compensation to avoid unspecific signals. Unspecific signals result from fluorescent cross talk of different channels. The consensus PCR system is a dual colour experiment including both LightCycler® - Red 640 and LightCycler® - Red 705 labelled hybridisation probes in a single capillary. For unambiguous results in this system use of a colour compensation file in the LightCycler® instrument is necessary. The generation of such a colour compensation file is achieved with the LightCycler® Color Compensation Set (Cat. No. 02 158 850 001).

4.4.4 Monitoring matrix inhibition

The internal positive control (IPC), also called internal amplification control or internal control, is included in the PCR test system to monitor false-negative results in a particular DNA sample (Siebert and Larrick (1993), Pallen *et al.* (1992)).

Table 17 - Development of the IPC molecule. In PCR 1 a short fragment of 62 bp (+ 26 bp artificially overhanging sequence) was amplified from DNA of *Neurospora crassa* DSM 1129. In PCR 2 a longer fragment of 187 bp (+ 26 bp overhanging sequence) was amplified from DNA of *Botrytis spp*. B 2674 A2/1. The products of PCR1 and PCR2 were pooled, hybridised as shown below and amplified in PCR3. The resulting product is equivalent to the IPC molecule. The HybProbe designed for the detection of the IPC molecule was labelled by the dye LC-RED705. The IPC was therefore detected in a separate channel in the LightCycler® instrument at 705 nm. The donor probe AML14rev was labelled by Fluorescein.

	PCR 1	PCR 2	PCR 3
target DNA	*Neurospora crassa* DSM 1129	*Botrytis spp*. B 2674 A2/1	product PCR1 + product PCR2
product	short fragment 62 bp + 26 bp overlapping sequence	long fragment 187 bp + 26 bp overlapping sequence	including *EF-3* primers; 275 bp IPC
primer	-60ML50FW, IPC primer1rev	IPC primer2 fw, 157ML14RV	-60ML50FW, 157ML14RV

Bold letters indicate the artificial sequence in the overlapping hybridisation in PCR 3.

3'AGACTCATGTAGGTCACC **TGAAGTAGACGAATGGAAGGGCGCAC** 5' IPC primer1rev

IPC primer2 fw 5' **ACTTCATCTGCTTACCTTCCCGCGTG** TGAAACTATGGACAGAGCCAAC 3'

In the fungal specific PCR system matrix inhibition control was achieved by inclusion of a plasmid consisting of the pGEM®T vector and the target *EF-3* sequence in each reaction, which was artificially changed at the binding position of one HybProbe. More precisely, the sequence of the HybProbe LCRed640ML10rev was substituted by an artificially designed sequence using PCR mutagenesis. The primers were the same in the amplification of IPC and wild type target DNA. The amount of IPC DNA in the PCR mixture was optimised by careful titration. Thereby quenching effects due to inclusion of large amounts of IPC DNA in the PCR mixture were avoided achieving a high sensitive PCR. The principle of the IPC plasmid construction by PCR mutagenesis applied in this work is summarized in Table 17. The PCR protocol in Table 18 was applied to construct the IPC molecule by PCR mutagenesis. The products of PCR 1 and PCR 2 (see Table 17) were separated on a 2 % agarose gel. Specific fragments were purified and the PCR products of PCR 1 and PCR 2 were pooled before adding them to PCR 3. The forward primer -60ML50F and the reverse primer 157ML14r were included in PCR 3 to amplify the IPC molecule from the pooled products (PCR 1 and PCR 2). The resulting products of PCR 3 were separated by electrophoresis. Specific products were purified by Qiaquick Gel Extraction Kit (Qiagen). Cloning and sequencing of the products was performed according to the protocol described in 4.3.2.

Table 18 – PCR protocol used in IPC development. The PCR protocol shown in this table was applied in PCR 1, PCR 2 and PCR 3. The primer specifically designed for the PCR mutagenesis were primer1rev and primer 2 fw as shown in Table 17.

master mix	final conc. in 20 µl
$MgCl_2$	4 mM
each dNTP	each 0.2 mM
10 x Puffer	1 x
Primer forward	0.5 pmol/µl
Primer reverse	0.5 pmol/µl
Fast Start Taq Polymerase	2U
Casein	100 mg/ml in HSTE
DNA	<0.25 ng/ 25 µl

time	temperature	cycles
10 min	95°C	1x
10 sec	95°C	35x
20 sec	50°C	
45 sec	72°C	

A positive control is included as a quality control of the entire PCR master mix in each PCR run and is amplified in a separate external capillary. The external positive control was amplified from *Botrytis cinerea* DSM 4709 DNA as described for the IPC in PCR3. The positive control was detected by HybProbes FAML14rev and ALCRed640ML10rev. Both IPC and positive control were sequenced (see Figure 2).

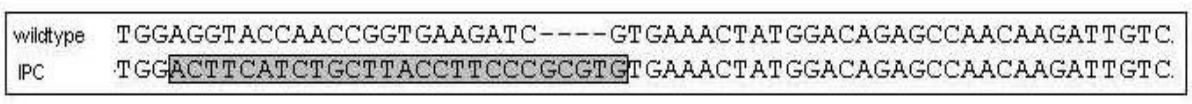


Figure 2 – IPC probe binding site. The sequences of wild type positive control *Botrytis spp.* (BCD 3776) and IPC in the region of the HybProbe binding are shown. The sequence of the specific IPC artificial HybProbe is labelled.

The lengths of the sequences of wild type and IPC differ in four nucleotides as shown in Figure 2. The plasmids of IPC and positive control (PC) were linearised by restriction endonuclease *Sac* I according to manufacturer's instructions, before addition to the PCR.

5 Results

The first part of this chapter will focus on the herein generated specific sequences of primers and probes. The second and third sections describe the experimental results of the implementation of RT-PCR based methods. As the RT-PCR was found to be too complex in routine detection of viable fungi, the fourth and fifth sections include experimental results concerning the specificity and sensitivity of PCR based methods. The adaptation of PCR based methods to relevant food matrices is demonstrated in the sixth section.

5.1 Sequences and Oligonucleotides

One of the major parts of this work focused on the generation of sequences from a specific part of the elongation factor 3 gene that included the so-called "*EF-3* unique sequence" of *EF-3*. With the aim to design primers and probes for detection of all food-relevant viable filamentous fungi, 103 sequences from a wide range of different species were obtained by cloning and sequencing of PCR fragments. The sequence reactions were performed twice for each sample to avoid sequencing errors. The consensus sequence of the two obtained sequences was used in the computation of the alignments. The sequences belong to 101 different species originated from *Ascomycota*, *Basidiomycota*, *Deuteromycota*, *Zygomycota* and *Oomycota*. All species, which were successfully sequenced, are summarized in the attached Table 75. The sequences differed significantly from each other resulting in difficulties to create a complete alignment including all relevant strains. Additionally, the computation of a complete alignment was limited by the algorithm in the program Vector NTI. Consequently, different alignments for *Ascomycota* (including *Basidiomycota* and *Deuteromycota*) and for *Zygomycota* were created from the obtained sequences.

5.1.1 Alignment of *Ascomycota*, *Basidiomycota* and *Deuteromycota*

The strategy to amplify PCR products for sequencing of *EF-3* is described in this section. Sequences of the oligonucleotides derived from the regions in Figure 3 are summarized in the paragraph results in 4.2.2 and in 4.2.3. In order to obtain specific PCR fragments for sequencing of different filamentous fungi, 'nested' PCR was performed combining primers derived from the regions SP1 to SP10 in Figure 3. In the beginning of this work primers SP1 (HS3) and SP9 (HS4) were included in a first PCR amplification and primer combinations SP3 (HS10) and SP9 (HS4) or SP3 (HS10) and SP6 (HS9) , which both span the '*EF-3* unique sequence' (see Figure 3 (Uritani *et al.* (1999)) were included in a second PCR. The obtained PCR products were cloned and sequenced as described in 4.3.2. The sequences were evaluated and compared to existing *EF-3* sequences from yeasts. Queries in databases showed, that unfortunately not all sequences were derived from the *EF-3* target gene. In order to obtain specific *EF-3* sequences from all fungal strains the available degenerated primers SP6 (HS9), SP3 (HS10), SP1 (HS3), SP9 (HS4), SP5 (ML1f) and SP8 (ML2r) have been combined in all possible variants for amplification of DNA from filamentous fungi. The majority of the finally sequenced PCR products have been amplified using the primers SP9 (HS4) and SP3 (HS10), resulting in fragments of up to 620 bp in length (see Figure 30).

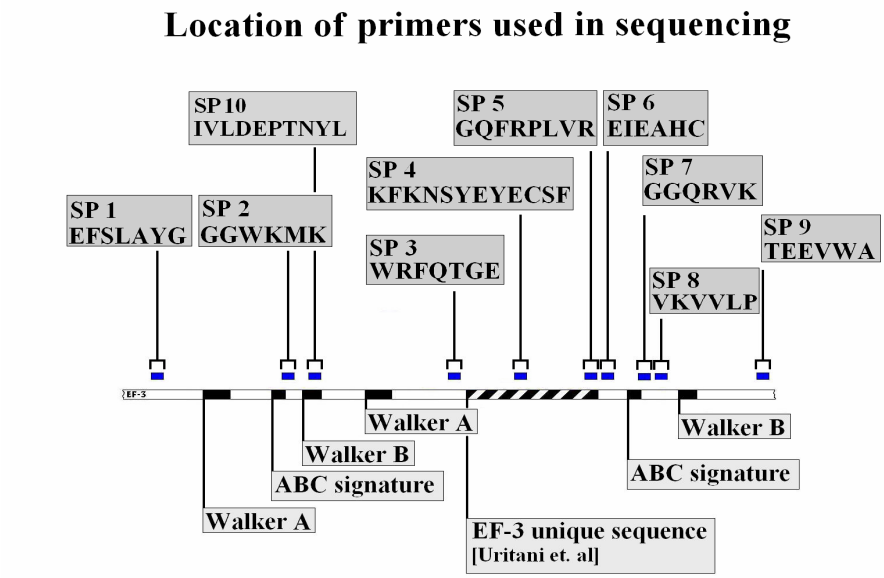


Figure 3 - Location of the primers used in sequencing of *EF-3* in relation to functional structures. The main amino acid sequences in the primer-binding region are specified to demonstrate the potential variability of the chosen region at the nucleotide level. A higher variability and therefore a need to degenerate the primers for the DNA amplification of all different fungal strains was expected in regions containing the amino acids Leucin (L), Arginin (R) and Serin (S), respectively. Consequently, it was tried to avoid the inclusion of these amino acids in the regions chosen for the primer design.

Primers SP1 (HS3) and SP6 (HS9) were found to have a low affinity for DNA from filamentous fungi. However, a control PCR using only one primer in the PCR was demonstrating, that the primers SP3 (HS10) and SP9 (HS4) bind to more than one existing binding sites in the fungal genome (see Figure 4), which resulted in unspecific sequencing in some strains**.** As a consequence, a screening system for specific *EF-3* PCR products in clones prepared for sequencing was developed including the primers SP5 (ML1f) and SP8 (ML2r). In part these primers served also in cloning and sequencing of *Cladosporium herbarum* BCD 388*, Absidia glauca* CBS 100.48*, Actinomucor elegans* BCD 486*,* and *Eurotium rubrum* BCD 2172*.*

Some PCR products remained difficult to clone. For optimisation of the cloning process the so-called 'A-tailing' was performed as described in Table 10 (Peist (2002)). However, generation of PCR products was still observed, which did not contain *EF-3* sequences. For some strains it was therefore necessary to perform another nested PCR using the outer primers SP7 (HSabc3) and SP10 (HSwb1) in a first PCR and in the second PCR the unspecific products were amplified with the more specific primers SP3 (HS10) and SP9 (HS4) resulting in a 620 bp. The resulting alignment is attached to this document (see Figure 30)

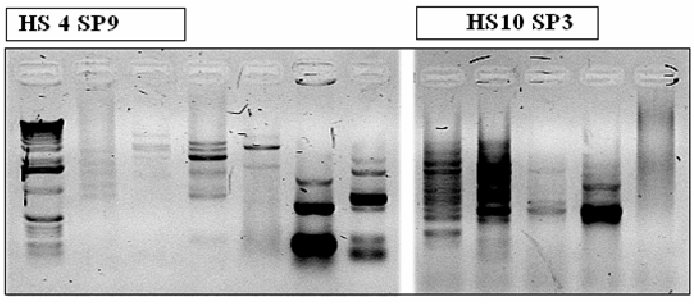


Figure 4 - Control of PCR specificity using only one primer. The occurrence of unspecific PCR products demonstrated, that primers SP3 (HS10) and SP9 (HS4) bind to more than one existing binding sites in fungal DNA.

5.1.2 Alignments of *Zygomycota*

Not all strains of the *Zygomycota* could be sequenced correctly using the primers mentioned in 5.1.1. Some inhomogeneous sequences were still obtained, leading to the assumption of artefact amplification (see Figure 6). The attached alignments indicated by A in Figure 29 and in Figure 30 contain the artefact sequences. As no other sequence information was available, the oligonucleotides derived from the artefact alignment served as primers in the further sequencing of *Zygomycota*. Additionally, for the sequence generation of *Zygomycota* a new short primer was designed derived from the 6 conserved amino acids GGWKMK close to the first ABC signature. The PCR in 4.3.1.1 was performed as a 'PCR walking' between the first ABC signature (SP 2) and primers in region SP 3 (see Figure 3). As shown in 4.3.1.1 the first 'PCR walking' experiment was performed upstream of the *EF-3* unique sequence resulting the corresponding alignment B in Figure 29. Forward primers were designed for *Zygomycota* using the sequence information of alignment B. The second 'PCR walking' experiment aimed to amplify partly the *EF-3* unique sequence. Alignment C in Figure 29 was the result of this sequencing. Reverse primers for *Zygomycota* detection were derived from alignment C. The primers and probes designed with help of alignment B and C were combined resulting in specific primer and probes hybridisation during PCR amplification (see Figure 5).

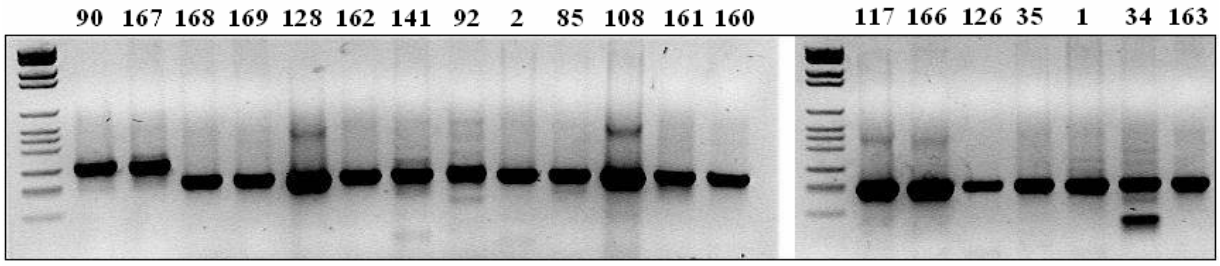


Figure 5 – Specific amplification. The combination of primers created in this work delivers specific amplification products of *Zygomycota*. The probes hybridised specifically, too. Amplification of the strains 90 and 167 (lab-code in Table 75 and Table 76) of the *Zygomycota* results in bigger fragments. The sequencing of these fragments shows an insertion in the *EF-3* unique sequence as can be seen in the attached alignment in Figure 31.

Compared to other sequences generated in this work, the sequences derived from *Zygomycota* contained exceptionally many insertions and deletions. For example, an insertion was found in strain *Syncephalastrum racemosum* BCD 162, which belongs to the *Zygomycota*. Probes or primers in the consensus PCR system were consequently not located at this position.

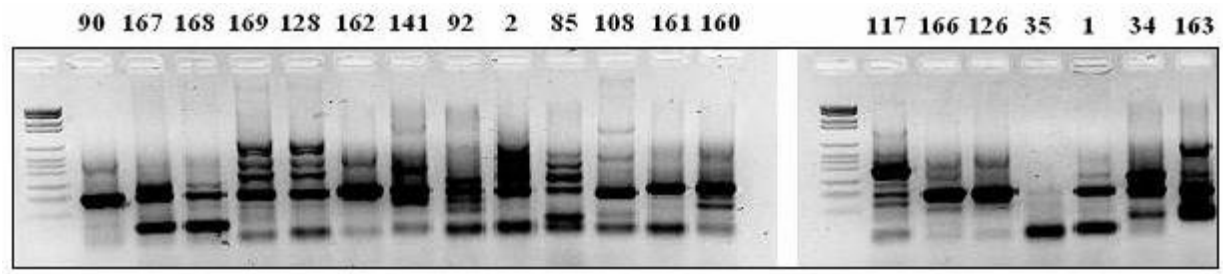


Figure 6 – Unspecific amplification with primers derived from inhomogeneous sequences. Non-specific amplification products resulted from PCR including primers derived from the artefact alignment of *Zygomycota*.

5.1.3 Detection of fungal specific amplification

In the following part the possibilities of detecting the *EF-3* PCR amplification from filamentous fungi in real time PCR are described. Different principles in the design of primers and probes have been tested for their ability to specifically detect filamentous fungi. These principles included detection of PCR products by unspecific binding of SYBR Green I and specific binding of probes, such as TaqMan and HybProbes. Based on the sequence information given in the attached alignments the most efficient combination of primers was chosen for each probe format.

5.1.3.1 Primer design and SYBR Green I

Using the SYBR Green I format the specificity of potential primer combinations was tested in the beginning of this work. The application of SYBR Green I in food diagnosis was found to be not suitable in a diagnostic assay, because of its disadvantages as described in 2.2.4.1. In the development and optimisation of specific amplification products the detection by SYBR Green I was helpful and is therefore mentioned here.

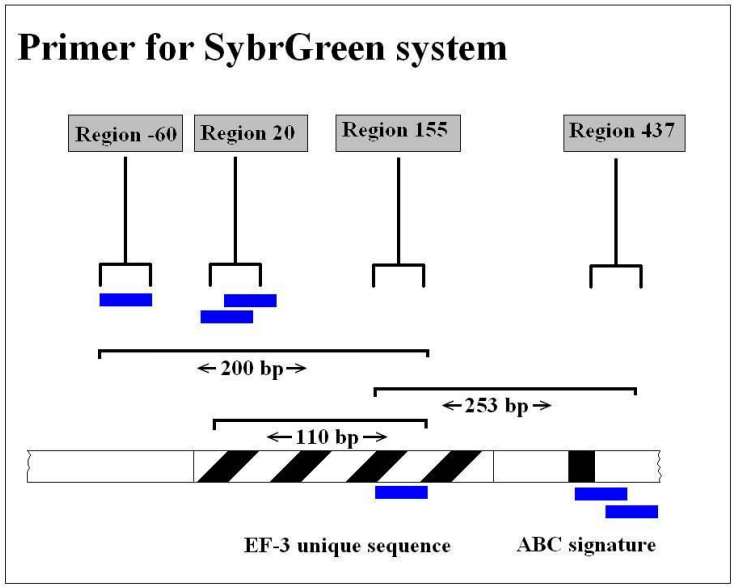


Figure 7 - Potential primer hybridisation sites for a PCR system including SYBR Green I.

The combinations of primer derived from different regions (see Figure 3) was optimised using SYBR Green I. This resulted in the amplification of *EF-3* specific PCR products as summarized in Figure 7. Oligonucleotides derived from region –60 and region 20 served as potential forward primers and sequences from region 155 and region 437 as potential reverse primers. The combination of primers derived from region 20 and primers derived from region 437 resulted in some non-specific amplification products and were therefore not suitable for further applications. The amplification of the 200 bp product[11] and the 110 bp product was in general more specific than the amplification of the 253 bp product. The 200 bp product was favoured for the final detection in 4.4 including probe hybridisation in the region 20.

[11] Product size does not include primers.

5.1.3.2 Primer design and TaqMan probes

Screening the sequence of *EF-3* led to the identification of potential probe binding areas with respect to the application of the TaqMan probe format.

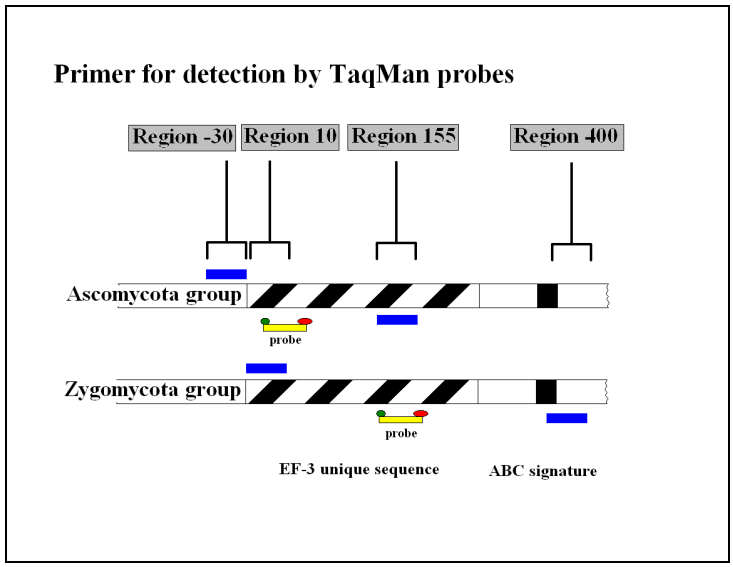


Figure 8 - Potential PCR system including TaqMan probes.

TaqMan probes are comparatively small and can therefore be placed in different regions within the *EF-3* sequence. Figure 8 shows a possible consensus primer system using TaqMan probes. The system was realised resulting in co amplification of two different PCR fragments, one for the group of *Zygomycota* another for the group of *Ascomycota* including *Deuteromycota* and *Basidiomycota***.** The amplification resulted in specific detection in real time PCR as demonstrated in 5.2.3. As an representative example the primer system was positively tested including the TaqMan probe S1ML in RT-PCR.

5.1.3.3 Primer design and HybProbes

The consensus PCR system including HybProbes consisted of forward primers derived from one region (-60) upstream of the *EF-3* unique sequence combined with reverse primers derived from the region (155) within the *EF-3* unique sequence. This resulted in a PCR product of about 200 bp (see Figure 7). Within this PCR product one region (20) was chosen for detection by HybProbes. To use this region for detection with TaqMan probes might also be possible but was not realised herein.

The conserved regions within the *EF-3* unique sequence and the 200 bp product were not long enough to place the two HybProbes next to each other in one highly conserved region. Hence both probes were degenerated. The Fluorescein labelled probes were degenerated at the 3'-end of the oligonucleotide and the probes labelled at the 3'-end with LCRed640 were degenerated at the 5'-end. As a result of degeneration the probes melt in a temperature range of 50°C – 67°C.

Based on the alignments DNA sequences of the potential probe binding areas were divided into groups. One specific probe was designed for each group. The probability of cross hybridisation with DNA from other sequence groups of the alignment was high, because of the probe degeneration. Functional domains were excluded from probe design to achieve fungal specific hybridisation. The minimal amount of necessary probes for detecting all strains was determined

empirically. This empirical testing resulted in the design of 5 Fluorescein labelled donor probes and 5 LCRed640 labelled acceptor probes (see Table 7). By combining all probes the PCR system was able to detect the DNA samples of heterogeneous groups such as *Basidiomycota*, *Ascomycota* (including *Deuteromycota*) and *Zygomycota*. The hybridisation region of the HybProbes was located on the reverse strand close to the forward primer region. It was located as far away as possible from the reverse primer binding position. An insertion was found in some *Zygomycota* making it impossible to place primers and HybProbes within the region 20 in the *EF-3* unique sequence (see *Zygomycota* alignment in 9.3).

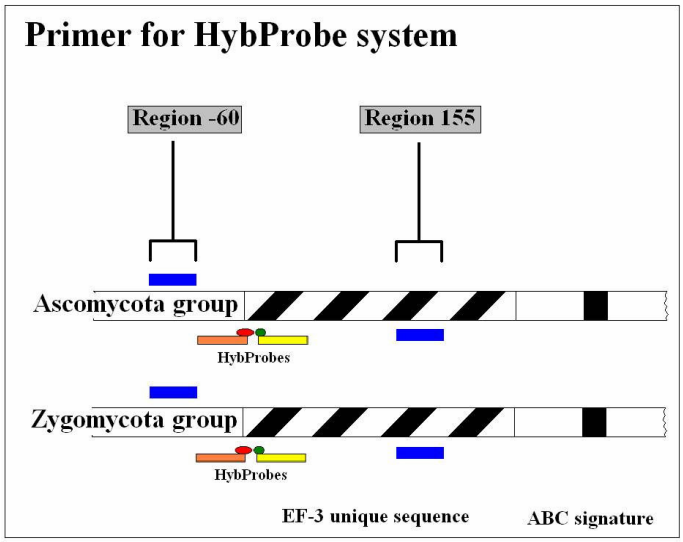


Figure 9 - Schematic overview of the consensus PCR system in protocol A including HybProbes.

The forward primers are therefore located outside of the *EF-3* unique sequence as demonstrated in Figure 9. The sequences of primers and probes designed for this consensus PCR are summarised in the paragraph 4.2.2. The resulting consensus PCR system was optimised for the investigation of food samples. The corresponding PCR protocol is called 'PCR protocol A' (see 4.4) and was applied in the experiments with emphasise on specificity, sensitivity and food testing.

5.2 Detection of viable filamentous fungi by RT-PCR based methods

5.2.1 Preliminary considerations in RT-PCR design

Two-Step versus One-Step RT-PCR

It was necessary to determine whether a Two-Step or a One-Step RT-PCR meets the requirement of a particular application. In a Two-Step RT-PCR the product of the reverse transcription reaction (cDNA) is transferred into the PCR mixture. The enzymes (Reverse Transcriptase and Taq Polymerase, respectively) act separately in two different compartments and do therefore not interact. In contrast, in the One-Step RT-PCR the different enzymes are present in the same master mix and the reactions take place successively in the same compartment. Two-Step RT-PCR is the more sensitive procedure and offers the chance to repeat the PCR step. As the interaction of the different components in the One-Step RT-PCR reaction mixture is complex, One-Step RT-PCR is less sensitive and was therefore suggested to be applied for samples containing abundant RNA. On the other hand the One-Step RT-PCR offers convenient handling and the chance to avoid cross-contamination.

Oligo (dT) primers versus specific primers

Oligo (dT) primers bind to the poly-A tail of mRNA and are therefore able to transcribe the whole mRNA spectrum in the cell (Nam *et al.* (2002)) including the *EF-3* target mRNA. To avoid random binding of the oligo (dT) primer at the poly A tail, anchored oligo (dT) primers were designed, which contained a G; C or A at the 3'-end of the primers. Specific reverse PCR primers are homologue to the mRNA. Since those primers are already present in the potential One-Step RT-PCR mixture which decreases the chances of cross-contamination, they are favoured in this work.

mRNA extraction versus total RNA extraction

Both mRNA and total RNA require different strategies and equipment during the nucleic acid preparation with the extraction of mRNA being the more complicated method. During mRNA extraction the poly-A tail of the mRNA binds to an artificial poly-T tail, which is linked to a specific matrix, for example magnetic beads or membrane filters. Commercial kits are available for mRNA extraction. The amount of extracted mRNA depends on the concentration of the specific target molecule in the RNA pool. The amount of mRNA copies depends on the regulation of the gene expression and the half-life time of the specific mRNA. The extraction of total RNA is less complicate and commercial kits are available as well. One disadvantage in the extraction of total RNA is, that different types of RNA are present in the sample, which can influence the hybridising during the RT reaction.

These preliminary considerations influenced the experimental procedure concerning RT-PCR, which is explained in this chapter. Industrial laboratories tend to prefer the most time and cost saving procedures in diagnostic systems. The One-Step RT-PCR offers the option of a timesaving single step mechanism for detection of viable organisms and prevention of cross contamination.

These considerable advantages led to the idea to prove whether a One-Step RT-PCR can be established for the detection of all food-relevant viable fungi using the LightCycler® instrument. The Two-Step RT-PCR is used in preliminary investigations of the RT-PCR master mix components. Therefore initial RT reactions were performed in Two-Step in a block cycling machine. Later the ability of the different RT-PCR components to act in the same reaction mixture was investigated. The reaction mixture was optimised according to the different demands of One-Step RT-PCR versus Two-Step RT-PCR. The next step was the performance of a Two-Step RT-PCR in reaction tubes made of different materials. For instance, RT reaction was performed in plastic tubes in the block cycling machine and inside the glass capillaries in the LightCycler® instrument. Consequently the chances to establish a One-Step RT-PCR in the block cycling machine and inside the glass capillaries of the LightCycler® instrument were investigated. Additionally, the blocking of the glass surfaces in the LightCycler® capillaries was optimised to perform a One-Step RT-PCR. Different primers and detection formats were applied during the experiments in order to find the most robust and sensitive combination. Table 19 summarizes the results concerning One-Step and Two-Step RT-PCR establishment.

Table 19 - Systems of One-Step and Two-Step RT-PCR. The details of the different protocols including the primer combinations are described in next chapters.

detection format	Two-Step RT-PCR	One-Step RT-PCR	products
SYBR Green I	yes	yes	one specific product
TaqMan	yes	-	two specific products
HybProbes	yes	yes	one specific product

5.2.2 RT-PCR including SYBR Green I

5.2.2.1 Two-Step RT-PCR including SYBR Green I

The following experiment demonstrates the ability to detect EF-3 mRNA in a Two-Step RT-PCR system with SYBR Green I. The RNA was extracted from mycelia (circles of 2 cm in diameter) of filamentous fungi as described in 4.2 applying protocol 1. Quality control of RNA was checked in 2 % agarose gels. The RNA was controlled for contaminating DNA by PCR and found to be free of any contaminating DNA. The following table describes the master mix for a Two-Step RT-PCR including and excluding specific primers from RT reaction.

Table 20 – Reverse transcription master mix A 1. Specific primers simulating the primer conditions in One-Step RT-PCR were included in the transcription reaction. Components included in the reaction mixture were hold at 4°C until the reaction was started.

component final volume 13 µl	final concentration
spec. primer mix 10 :10ML29f, 10ML30f	0.3 pmol/µl
spec. primer -30ML47F, -30ML48F	0.3 pmol/µl
spec. primer 157ML8r, 157ML9r, 157ML13r, 157ML14r; 157ML15r, 157ML16r	0.3 pmol/µl
spec. primer mix: 400_52	0.3 pmol/µl
RNA	50 pg - 1µg
water (DEPC treated)	x

Table 21 - Reverse transcription master mix A 2. As a ‘no primer’ control the transcription mixture did not include any primers.

component (final volume 13 µl)	final concentration
RNA	50 pg - 1µg
water (DEPC treated)	x

Table 22 - Master mix B was added into the two different variations of Master mix A 1 and A2. RT reaction was carried out in a Block cycler in plastic tubes for 30 min at 53°C.

component: final volume 20 µl	final concentration
dNTP 10 mM	0.4 mM each
Protector RNase Inhibitor	20 U
10 x buffer	1 x
Transcriptor Reverse Transcriptase	4U
$MgCl_2$	4 mM
water (DEPC treated)	x

Table 23 - PCR mixture with SYBR Green I. The PCR was performed by transferring 5µl cDNA from RT reaction to PCR.

PCR components	final conc.
dNTP Mix with dTTP (10 mM)	0.4 mM
Fast Start Taq DNA Polymerase	2 U
10x buffer	1 x
BSA (30 mg/ml)	3 mg/ml
$MgCl_2$ 50 mM	4 mM
SYBR Green I 10x dilution buffer	0.4x/µl
2x Primer Fwd Mix 10 pmol/µl Mix 10: 10ML29f, 10ML30f, Mix –30: -30ML47F, -30ML48F	0.4 pmol/µl
2x Primer Rev Mix 10 pmol/µl 400_52; Mix 157:157ML8r, 157ML9r, 157ML13r, 157ML14r; 157ML15r, 157ML16r	0.4 pmol/µl
water (DEPC treated)	x

Table 24 - PCR profile.

temperature	time	cycles
95°C	10 min	1
95°C	10 sec	45
55°C	3 sec	
72°C	20 sec	

SYBR Green I fluorescence was detected at 530 nm at 72°C. Specific results were obtained by melting curve analysis using stepwise increase of temperature (0.1°C/s). PCR products of 110 bp melt around 85-91°C. The RNA samples were free of any detectable DNA contamination, which was confirmed in a PCR including the RNA sample only (data not shown).

Table 25 - Comparison of results with and without specific primers from the RT step in Two-Step RT-PCR. +/- is equivalent to weak amplification results, + corresponds to positive results and – to negative results. For lab-code numbers see Table 75 and Table 76.

lab-code	master mix A1: RT reaction with specific primers	master mix A2: RT reaction without any primer
126	-	-
146	-	+/-
54	+	+
26	+/-	+
0.3	+/-	+
145	-	+/-

The *EF-3* mRNA from several filamentous fungi was successfully transcribed and amplified by specific primers in RT reaction and the PCR with SYBR Green I detection. Surprisingly, the RT reaction without any primer resulted in better amplification of the 110 bp products. However, the amount of fungal mycelia used for RNA extraction was much more than the amount generally expected in food samples, even after enrichment. Moreover, the resulting RT-PCR fragments were still weak in some species.

5.2.2.2 One-Step RT-PCR including SYBR Green I

The following experiment aimed to establish a One-Step RT-PCR including the detection by SYBR Green I.

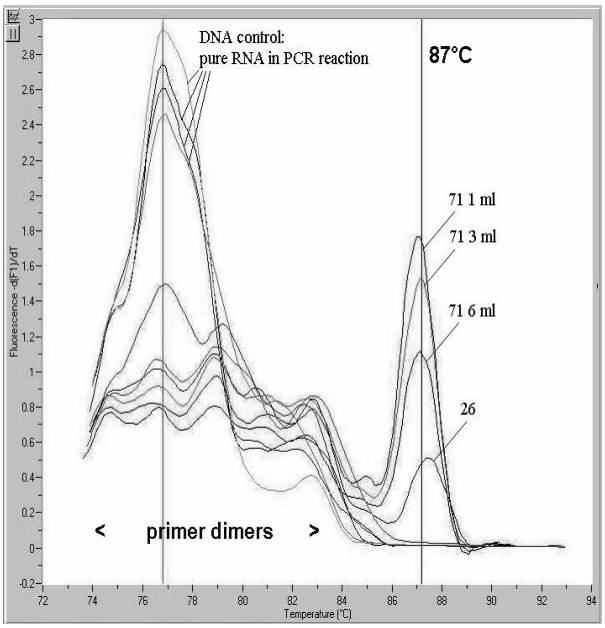


Figure 10 - SYBR Green I detection One-Step RT-PCR. Melting curve plot of fluorescence versus temperature generated in the LightCycler®. Specific amplification products are detected at 87°C and are labelled by the lab-code numbers 71 (1 ml), 71 (3 ml), 71 (6 ml) and 26 of the strains summarized in Table 75 and in Table 76. Unspecific amplification signal derive from primer dimers were detected at 77-82°C. RNA was extracted according to protocol 2 in 4.2 and free of any detectable contaminating DNA (see DNA control samples).

The One-Step RT-PCR was performed using RNA of two different fungal strains. The RNA of three parallel cultivations of *Aspergillus niger* DSM 872 (lab-code 71) in 1 ml , 3 ml, 6 ml media was compared to demonstrate the reproducibility of the assay. RNA was extracted according to protocol 2 in 4.2 and free of any detectable contaminating DNA. The amplification of specific products of the One-Step RT-PCR is shown in the melting curve analysis in Figure 10.

Table 26 - PCR mixture The One-Step RT-PCR was performed in the LightCycler® instrument (Roche) in glass capillaries according to the following protocol.

components in 20 µl	final conc.
Oligo dT35	0.4 pmol/µl
10 Fwd Mix 10 pmol/µl	0.4 pmol/µl
140 Mix Rev Mix 10 pmol/µl	0.4 pmol/µl
157 Mix Rev Mix 10 pmol/µl	0.4 pmol/µl
20 Fwd Mix 10 pmol/µl	0.4 pmol/µl
dNTP 10 mM	0.8 mM each
Protector RNase Inhibitor	20 U
10 x Puffer	1 x
Transcriptor Reverse Transcriptase	4U
$MgCl_2$	8 mM
Faststart Taq DNA Polymerase	2 U
SYBR Green I 10x Dilution	1x
Casein (0.01 g/ml HSTE)	0.6 mg/ml

Table 27 - PCR profile. All reagents were hold at 4°C until the reaction was started, running the indicated profile on LightCycler® instrument.

temperature	time	cycles
53°C	30 min	1
95°C	10 min	1
95°C	10 sec	45
58°C	3 sec	
72°C	20 sec	
50°C	30 sec	1

5.2.3 RT-PCR including TaqMan probes

TaqMan probes as one possible type of detection format were investigated concerning the inclusion in the consensus RT-PCR. The ability of the TaqMan probes to detect the products in the Two-Step RT-PCR is demonstrated in the experiment in Figure 11.

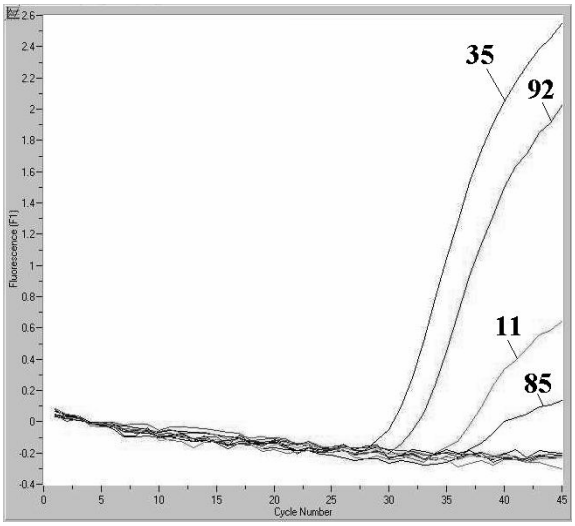


Figure 11 - TaqMan Two-Step RT-PCR curves. Fluorescence is plotted versus cycle number. DNA control = RNA proved in PCR. DNA controls were not amplified, curves remained flat. The specific amplification curves from cDNA are labelled by lab-code numbers, which correspond to Table 28.

Fungi were grown on the surface of liquid YEPD in Petri dishes for 3 days. Circles of 2 cm in diameter were cut from the cultures. RNA was extracted according to protocol 1 in 4.2 using the GMO sample Prep Kit (Roche). The RNA was free of any detectable contaminating DNA as shown in Figure 11. The reverse transcription (RT) reaction, was performed as described in the experiment 5.2.2.1 including *EF-3* specific primers. The PCR protocol is described in Table 35. Figure 11 shows a plot of the fluorescence of the TaqMan probes versus the cycle number measured during the amplification of 4 representative strains in Table 28. The mentioned crossing point (CP value) in Table 28 is defined as the fluorescence signal, which first exceeds the level of background noise in that plot. It can be correlated to the original amount of DNA.

Table 28 - Summary of the results of Two-Step RT-PCR with TaqMan probes.
+ corresponds to positive results, – corresponds to negative results.

lab-code	nucleic acid	check in 2 % agarose gel	PCR with TaqMan probe S1ML, CP values
85	cDNA	+	35.24
11	cDNA	+	34.79
92	cDNA	+	32.36
35	cDNA	+	30.56
NC RT	no	-	-
85	RNA	-	-
11	RNA	-	-
92	RNA	-	-
35	RNA	-	-
NC PCR	no	-	-

5.2.4 RT-PCR including HybProbes

5.2.4.1 Two-Step RT-PCR including HybProbes

The general feasibility to detect *EF-3* derived cDNA by HybProbes is demonstrated in the LightCycler® instrument. Conidia of *Aspergillus niger* DSM 872 (10^6 spores) were incubated 16 hours at 25°C in fluid YEPD. The germination of spores was controlled visually. According to protocol 2 (in 4.2) the RNA was extracted and stabilised by addition of 50 mM EDTA and on Chelex 100 before heat inactivation of the DNase I. The RT reaction was performed as in the protocol used in the experiment 5.2.2.1. The RNA was reverse transcribed using specific primers. The PCR was performed according to the protocol outlined in Table 29.

Table 29 - PCR mixture of Two-Step RT-PCR with HybProbes.

The transcribed cDNA was amplified in a PCR with primers –60ML49F; –60Ml50F and 157ML8r, 157ML9r, 157ML13r, 157ML14r, 157ML15r and 157ML16r. Two specific fluorescently labelled probes (LightCycler® HybProbes) A F12MLrev and A LCRed640ML7rev were added to the PCR.

components in 20 µl	final conc.
cDNA from RT reaction or RNA 1:25	5 µl
10 x buffer	1x
each dNTP (10 mM)	0.4 mM
forward primer (10 mM)	0.6 mM
reverse primer (10 mM)	0.6 mM
$MgCl_2$ (50 mM)	4 mM
Fast Start *Taq*-Polymerase	2 U
Fluorescein probe (donor) 10 µM	0.075 µM
LC Red probe (acceptor) 10 µM	0.15 µM
Casein in HSTE (100 mg/ml)	0.1 mg

Table 30 – Corresponding PCR profile. A single reading of fluorescence at 640 nm was included at 50°C.

temperature	time	cycles
95°C	10 min	1
95°C	0 sec	35
50°C	0 sec	
55°C	30 sec	
72°C	40 sec	

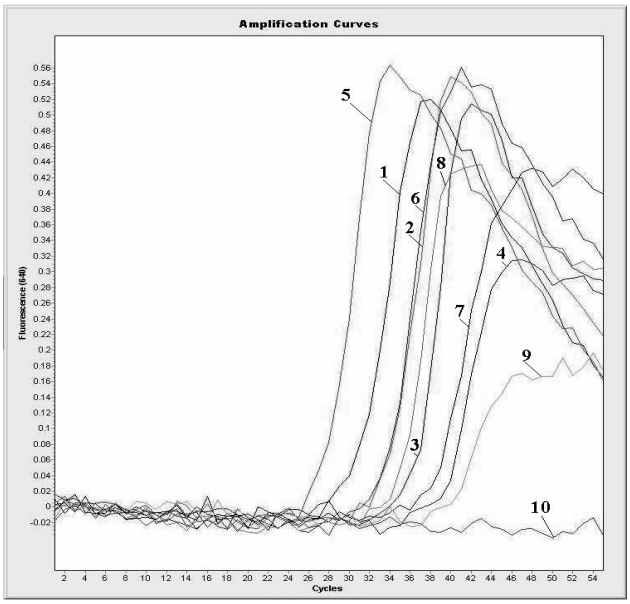


Figure 12 – Example for amplification curves using HybProbes in RT-PCR. Fluorescence is plotted versus cycle number. CP values are summarized in Table 31.

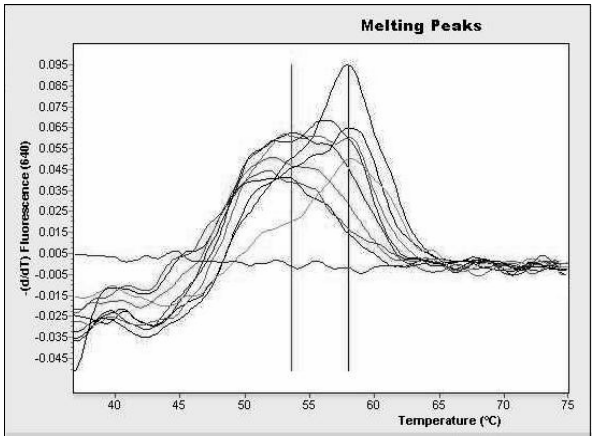


Figure 13 – Example for melting curve of HybProbes. Fluorescence is plotted versus temperature. Finally melting curves were determined by continuous increase of temperature in steps of 0.1° C/sec. The HybProbes were melted at about 50°-67°C from the target cDNA in the LightCycler® instrument.

RNA was free of any detectable DNA contamination (data not shown). Specific PCR products

were detected by HybProbes and the specific size of about 210 bp was confirmed by agarose gel electrophoresis. RNA samples of 10^6 germinated conidia were diluted as summarized in Table 31 to simulate lower RNA amounts. In the experiment no significant differences between the RNA samples stabilized by EDTA and samples stabilized on dried Chelex 100 were detected.
The Two-Step RT-PCR including HybProbes was able to detect RNA from dilutions of 1:10000 which correlates to 10^2 germinated spores. However, this is probably not sensitive enough for a system suitable for food diagnostics.

Table 31 - Results of Two-Step RT-PCR including HybProbes using different RNA stabilizers. Numbers correlate to labels in Figure 13. RNA samples extracted of 10^6 over night germinated conidia were diluted.

numbers	strain	RNA dilution	stabilizing RNA	specific amplification	CP values
1	*Aspergillus niger* DSM 1988	no dilution	EDTA in RNA	+	29.74
2	*Aspergillus niger* DSM 1988	1:100	EDTA in RNA	+	32.53
3	*Aspergillus niger* DSM 1988	1:1000	EDTA in RNA	+	34.81
4	*Aspergillus niger* DSM 1988	1:10000	EDTA in RNA	+	37.96
5	*Aspergillus niger* DSM 1988	no dilution	RNA on Chelex 100	+	26.22
8	*Aspergillus niger* DSM 1988	1:100	RNA on Chelex 100	+	33.28
6	*Aspergillus niger* DSM 1988	1:1000	RNA on Chelex 100	+	32.28
7	*Aspergillus niger* DSM 1988	1:10000	RNA on Chelex 100	+	37.21
9	*Aspergillus niger* DSM 1988	1:10000	RNA on Chelex 100	+	38.20
10	PCR NC (negative control)	-		-	-

5.2.4.2 One-Step RT-PCR including HybProbes

RNA from Two-Step RT-PCR experiments (5.2.4.1.) was used in One-Step RT-PCR experiments in the LightCycler® instrument. The following reaction mixture was applied in the One-Step RT-PCR.

Table 32 - One-Step RT-PCR mixture including HybProbes.

component in 20 µl	final conc.
Sample volume	50 pg - 1µg
Fast Start Taq Polymerase	2 U
10x Buffer	1 x
Casein (100 mg/ml HSTE)	0.15 mg
$MgCl_2$ 100 mM	10 mM
each dNTP 10 mM	1 mM
primer Mix –30 fw 10 pmol/µl	0.4 pmol/µl
primer Mix 157 rev 10 pmol/µl	0.8 pmol/µl
Fluorescein probe A-F-12MLrev: 5 pmol/µl	0.075 pmol/µl
LC Red 640 probe A-R-7MLrev: 5 pmol/µl	0.15 pmol/µl
Protector RNase Inhibitor	20 U
Transcriptor Reverse Transcriptase	4 U
water (DEPC treated)	x

Table 33 - One-Step RT-PCR profile.

temperature	time	cycle
53°C	30 min	1
95°C	10 min	1
95°C	5 sec	45
50°C	30 sec	
72°C	20 sec	

The resulting product/probe binding was monitored in a melting curve analysis by continuous increase of the temperature by steps of 0.1° C/sec. Specific probes melt at around 50°C – 67°C.
The results demonstrate, that the One-Step RT-PCR is generally functioning. By including HybProbes in the system specifically amplified PCR products were detectable. However, sensitivity seemed to be not as high as in the Two-Step RT-PCR. The One-Step RT-PCR is the more complex system compared to the Two Step RT-PCR, because the enzymes compete for the

basic components like primers and dNTPs, respectively. Increasing complexity of the system may decrease the sensitivity of the One-Step RT-PCR system.

Table 34 - Results of detecting different amounts of RNA by One-Step RT-PCR with HybProbes.
RNA extracted of 10^6 over night germinated conidia was diluted.

strain	RNA dilution	EDTA stabilized	specific amplification	CP values
Aspergillus niger DSM 1988	1:10	RNA	+	15.76
Aspergillus niger DSM 1988	1:100	RNA	+	19.02
Aspergillus niger DSM 1988	1:1000	RNA	-	-
Aspergillus niger DSM 1988	1:10000	RNA	-	-
Aspergillus niger DSM 1988	n.d.	cDNA PC	+	17.00
n.d.	n.d.	water	-	-

5.3 Experiments for optimisation of RT-PCR

5.3.1 Experiments with 'no-primer' controls in the RT step

Since the complex One-Step RT-PCR master mix contains specific *EF-3* primers the influence of the specific primers during the RT step was investigated including TaqMan probes for the detection. In the following experiment different test strains were included in the Two-Step RT-PCR experiments investigating the phenomenon of positive products in 'no primer' controls.
Mycelia were grown for 10 days until the whole agar plate was covered. The RNA of different species was extracted as described before by protocol 1 in 4.2. see Table 37. The reverse transcription (RT) reaction was performed according to the protocol in the experiment 5.2.2.1 with and without specific primers. The PCR protocol is described in Table 35.
The experiment shows differences between products of the RT reaction with and without specific primers. RT reactions seemed to be more specific if no primers were included. The results are summarized in Table 37.

Table 35 - PCR mixture including TaqMan probes.

PCR components in final vol. 20	final conc.
Fast Start Taq DNA Polymerase including 10 x buffer (12.5 µl enzyme solution + 60 µl 10x buffer)	2 U
$MgCl_2$ 50 mM	4 mM
TaqMan Probe S1ML 5 pmol/rct.	0.4x/µl
dNTP Mix (10 mM)	0.4 mM
2x Primer Fwd Mix 10 pmol/µl Mix 10:	0.4 pmol/µl
2x Primer Rev Mix 10 pmol/µl 400_52;	0.4 pmol/µl
water (DEPC treated)	x

Table 36 – Corresponding PCR profile.

temperature	time	cycles
95°C	10 min	1
95°C	10 sec	45
55°C	5 sec	
72°C	20 sec	

The RNA of samples 126 and 145 were not detected by Two-Step RT-PCR. Further optimisation of primers and TaqMan probes would be necessary to detect all relevant species. The range of species detected from different divisions by RT-PCR was broader without any primer in the RT reaction step than including specific primers. The agarose gel in Figure 14 shows additionally a more distinct band pattern (without a smear) for the samples without primers in the RT reaction. The formation of primer dimers and artefacts was stronger for the samples including specific primers in the RT reaction.

Table 37 - Reproducibility of results with and without specific primers in the RT step of Two-Step RT-PCR. Specific products were detected by TaqMan probes as well as in gel electrophoresis shown in Figure 14.

lab-code	division	RT reaction including	check in 2 % agarose gel	CP values
126	*Zygomycota*	spec. primers	-	-
146	*Zygomycota*	spec. primers	-	-
54	*Ascomycota*	spec. primers	+	21.28
26	*Ascomycota*	spec. primers	-	-
0.3	*Basidiomycota*	spec. primers	-	-
145	*Basidiomycota*	spec. primers	-	-
54	*Ascomycota*	spec. primers	+	31.47
126	*Zygomycota*	excluding primers	-	-
146	*Zygomycota*	excluding primers	+	27.59
54	*Ascomycota*	excluding primers	+	23.50
26	*Ascomycota*	excluding primers	+	26.70
0.3	*Basidiomycota*	excluding primers	+	25.83
145	*Basidiomycota*	excluding primers	+/-	39.66
54	*Ascomycota*	excluding primers	+	25.15

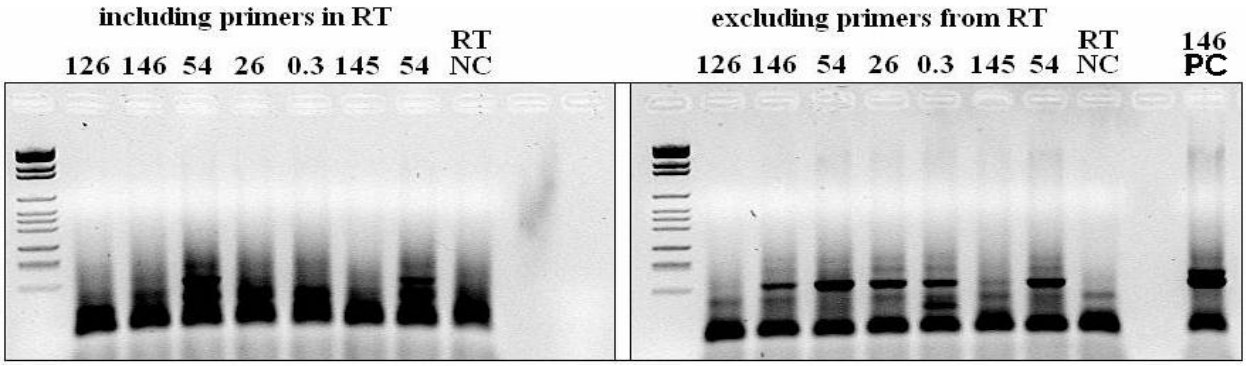


Figure 14 – Comparison of amplification with and without applying primers in the RT step of Two-Step RT-PCR in agarose gel electrophoresis. Primer dimer formation is stronger in the RT reaction including specific primers, leading to less amounts of specifically amplified cDNA. The RNA was free of any detectable contaminating DNA (data not shown).

As there were no positive signals expected in the RT-PCR without any primers, these results were surprising. The results demonstrate the complexity of the RT-PCR mechanisms. As the reverse transcriptase does need some primers in the beginning of the transcription, one can assume that other unspecific oligonucleotides were present in the RNA. If one suspects the DNase I digestion not being complete, small undigested DNA fragments might be present in the RT reaction. The small DNA fragments might be able to serve as primers for the enzyme Transcriptor Reverse Transcriptase during the transcription of total RNA into cDNA. Theoretically, these small DNA or RNA oligonucleotides can hybridise to the specific primers forming unspecific fragments and primer dimers in the RT-PCR. This would explain why a stronger primer dimer formation is observed by agarose gel electrophoresis in the samples including specific primers in the RT reaction (see Figure 14). However, the background of these results is difficult to explain, because even small RNA fragments can serve as primers in the RT reaction. The experiment led to the idea of the formation of different compartments for the RT and PCR components during the RT step, which is realized in a experiment 5.3.2.

5.3.2 Formation of compartments in RT-PCR

The experiments excluding specific primers in the RT reaction resulted in better amplification products as shown in Figure 14 by agarose gel electrophoresis and by TaqMan probe hybridisation (see Table 37). The specific PCR primers in the RT-PCR mixture might be the main problem in One-Step RT-PCR assays. The next set of experiments was therefore designed to exclude the specific primers from the RT reaction in a One-Step RT-PCR format.
Conidia of filamentous fungi were grown for three days in fluid media (YEPD). Mycelia of 2 cm in diameter were cut by a cork borer. RNA extraction was performed according to protocol 1 in 4.2. The experimental procedure can be described as a Two-Step RT-PCR, where all necessary RT-PCR components are contained in a single tube. The handling is yet too complicated to perform those experiments routinely. However, the experiment was designed to demonstrate the possibility to perform a Two-Step RT-PCR in a single LightCycler® capillary in a single run, 'One Tube Two-Step'. Specific primers were separated from the RT reaction by a layer made up of PCR wax[12] (Ampli Wax PCR Chem 50 Perkin Elmer). The wax was melted during the activation step of the Fast Start Taq Polymerase at 95°C for 10 min. Simultaneously, the RT enzyme was inactivated and the RT reaction stopped.

The preparation of the LightCycler® capillary. The specific PCR primer mixture was centrifuged into a LightCycler® capillary. One piece of wax was added to the capillary and the capillary was closed by a lid. The capillary was heated in a 1.5 ml tube containing water. Still warm, the capillary was centrifuged and cooled down, resulting in a layer of wax on top of the PCR primer mixture. The lid was opened again and the RT-PCR master mix excluding any specific primer was added. RNA was added to the capillary and the lid was closed again. The PCR wax was a stabile barrier between the primers and the master mix during the RT reaction (30 min at 53°C).

The experiment offers an idea how to separate PCR primers from the rest of the RT-PCR master mix to avoid primer dimer formation and increase sensitivity during the RT reaction in a single tube assay. Including a PCR wax barrier in the capillary a separation of antagonistic RT-PCR components is possible. This demonstrates a possibility to perform the more sensitive Two-Step RT-PCR assay in a single tube in a single experiment (run) in the LightCycler® instrument.

Table 38 – RT-PCR mixture added to the capillary on top of PCR wax. The specific PCR primers were separated by wax and are summarized in PCR protocol A (see 4.4).

RT-PCR master mix 20 µl	final conc.
RNA	50 pg - 1µg
oligo dT35 (10 pmol/µl)	0.16 pmol/µl
each dNTP 10 mM	0.8 mM each
Protector RNase Inhibitor	20 U
10 x Buffer	1 x
Transcriptor Reverse Transcriptase	4U
$MgCl_2$	8 mM
Fast Start Taq Polymerase	2 U
SYBR Green I 10x dilution	0.32 x/µl
Casein (100 mg/ml)	0.15 mg
water (DEPC treated)	x

Table 39 – Corresponding One Step RT-PCR profile.

temperature	time	cycles
53°C	30 min	1
95°C	10 min	1
95°C	10 sec	45
65°C-58°C each step 0.5°C	3 sec	
72°C	20 sec	
50°C	30 sec	1

[12] Similar ideas are already realised in products for RT-PCR in block cycling machines from MBP Molecular BioProducts (San Diego, CA 92121) for example.

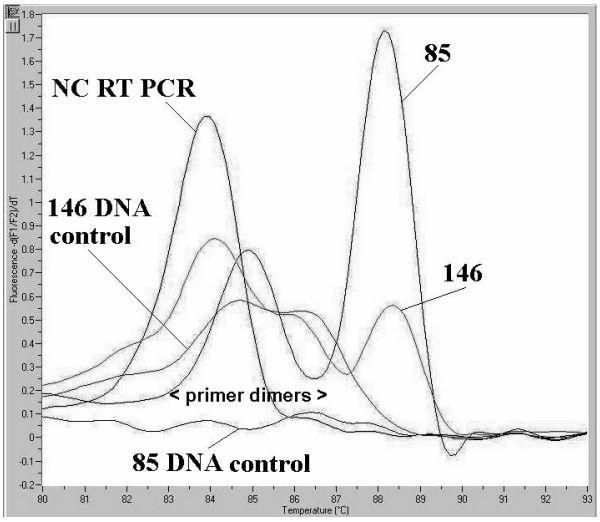


Figure 15 – Corresponding melting curve with SYBR Green I to Table 40. Fluorescence is plotted versus temperature. Labelled specific amplification corresponds to the lab-code numbers in Table 75.

The resulting RT-PCR products were detected by SYBR Green I as shown in Figure 15. Additionally, the experiment indicates the potential of encapsulation experiments in RT-PCR applications. However the handling is still too complicate for routine application.

Table 40 - Summary of RT-PCR results using PCR wax to separate specific primers from RT reaction.

lab-code	strain	specific product in One-Step RT-PCR with SYBR Green I including wax in the capillaries	specific product in Two-Step RT-PCR including detection by TaqMan probes
85	*Mucor hiemalis* BCD 3498	yes	yes
146	*Neurospora crassa* DSM 1129	yes	yes
85	*Mucor hiemalis* BCD 3498	no (DNA control)	no (DNA control)
146	*Neurospora crassa* DSM 1129	no (DNA control)	no (DNA control)

5.3.3 Reproducibility of DNA digestion

As contaminating DNA was a problem in the reproducibility of the RT-PCR, the following experiments were performed to optimise this step. It was demonstrated that the DNA digestion was not always complete depending on the amount of initial fungal cells. Different amounts of young spores (10^2, 10^3, 10^4, 10^5 and 10^6) from three different species (i.e. *Aspergillus niger* DSM 872, *Aspergillus parasiticus* DSM 2028, and *Penicillium expansum* DSM 1994) were germinated for 16 hours in fluid YEPD. Germination of the spores was microscopically observed. DNA and RNA were extracted from young hyphae changing the protocol 2 in 4.2. as follows. The addition of Chelex 100 (10 %) was followed by 10 min incubation at 95°C. The MagNA Lyser was applied twice followed again by 10 min incubation at 95°C and 3 min centrifugation at 12000 rpm. The supernatant containing DNA and RNA was divided. One aliquot was digested by DNase I (Sigma).

Aim of this experiment was to check whether some residual DNA is present in the samples after digestion with DNase I. For a better accuracy the RNA was not stabilized during heating of the sample by EDTA or dried Chelex 100. Consequently, the RNA hydrolysis during this steps was achieved without adding RNases before determining the rest of DNA from DNase I digestion by

PCR. Also, this experiment was performed to proof the sensitivity of the extraction protocol for DNA from germinated spores with the results shown in Table 41. The PCR reproducibly detected 10^3 germinated spores from 16 hours old pure cultures. The threshold value using this PCR system was determined with 10^2 germinated spores. The amount of DNA varied according to the amount of spores originally germinated.

Table 41 - Results of PCR checking the reliability of DNA digestion in RNA. PCR was performed by the PCR system including the following primers 10ML29f; 10ML30f; 400_52; Mix 157; Mix –30. The detection was performed by SYBR Green I in the LightCycler® instrument and visually in 2 % agarose gels as shown in Figure 16.

nucleic acid	lab-code	strain	16 hours germinated spores				
			10^2	10^3	10^4	10^5	10^6
DNA	11	*Aspergillus parasiticus* DSM 2028	+	+	+	+	+
RNA	11	*Aspergillus parasiticus* DSM 2028	-	-	-	-	+
DNA	71	*Aspergillus niger* DSM 872	-	+	+	+	+
RNA	71	*Aspergillus niger* DSM 872	-	-	-	-	+
DNA	106	*Penicillium expansum* DSM 1994	+	+	+	+	+
RNA	106	*Penicillium expansum* DSM 1994	-	-	-	-	-

No PCR amplification was expected after DNA digestion in the RNA samples. The data indicate that a complete DNA digestion depends on the originally contained amount of DNA in the sample. This result was reproducible and implicates wrong positive results in RT-PCR in case of high background values of DNA. In samples with low DNA background contamination the primer hybridisation can be influenced by short DNA fragments as shown before. DNA digestion by DNase I becomes even more complicate in food. Consequently DNA digestion remains one unsolved problem in the application of RT-PCR in routine testing of living food contaminants.

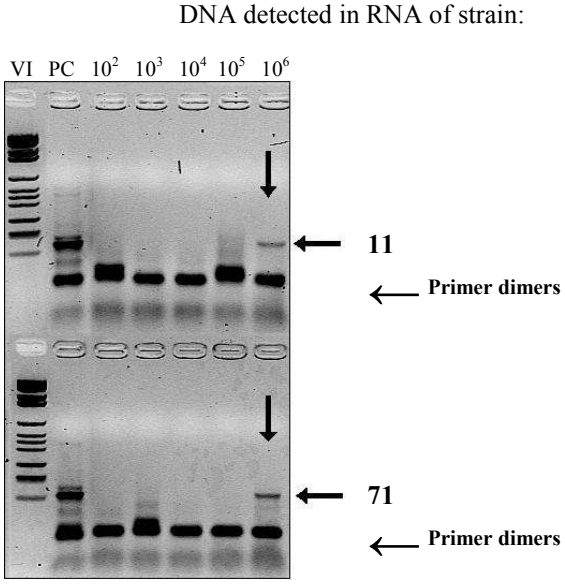


Figure 16 - Gel electrophoresis of non-digested DNA in RNA samples.

Another experiment further demonstrates the occurrence of false positive amplification. Different amounts of RNA were transcribed by the One-Step RT-PCR protocol and detected by SYBR Green I as described in Table 26. Two different representative strains of filamentous fungi were included in this experiment. The melting curves of the strains are shown in Figure 18

and Figure 17 while the results are summarized in Table 42. In the strain 26 the One-Step RT-PCR was positive. The strain 0.3 failed, because RNA of sample 0.3 (lab-code) was found to contain contaminating DNA, which led to wrong positive results in the RT-PCR see Figure 18. By diluting the RNA samples this DNA contamination disappeared.

Table 42 - Results of One-Step RT-PCR decreasing the RNA amount. DNA control = RNA checked in PCR.

lab-code	RNA ng/µl in RT	RT- PCR including Reverse Transcriptase?	signal in melting curve
0.3	25	yes	+
0.3	10	yes	+
0.3	5	yes	+
0.3	25	no (DNA control)	+/-
0.3	10	no (DNA control)	+/-
0.3	5	no (DNA control)	-
26	20	yes	+
26	8	yes	+
26	4	yes	+
26	20	no (DNA control)	-
26	8	no (DNA control)	-
26	4	no (DNA control)	-

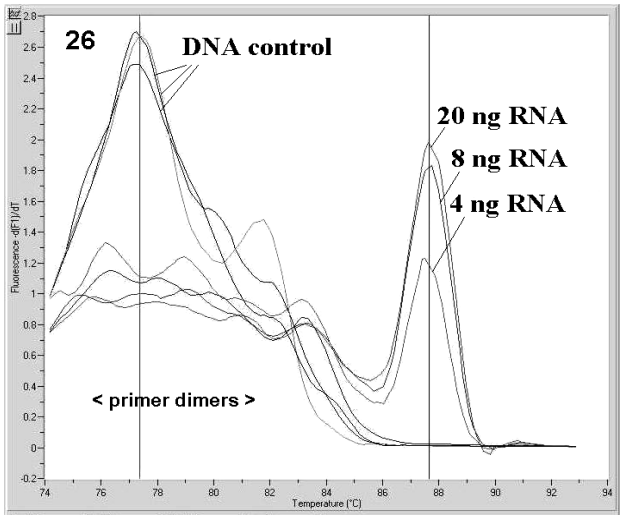


Figure 17 - Melting curve sample 26. RNA was free of any contaminating DNA. 4 ng RNA in sample 26 gives still a significant peak.

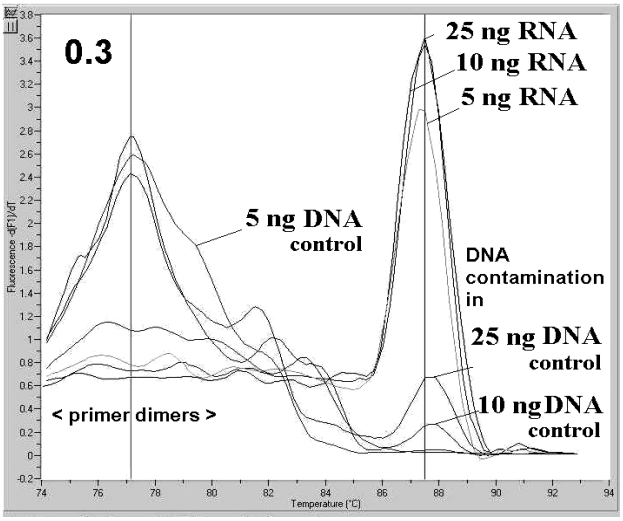


Figure 18 - Melting curve of sample 0.3. Significant DNA contamination was detected in 25 ng and 10 ng RNA. Fluorescence is plotted versus temperature.

5.3.4 Stabilizing of RNA by adding chelators

Heat inactivation of DNase I and other enzymes added during RNA extraction procedure is necessary for further application like RT-PCR. However, heating increases RNA hydrolysis in presence of bivalent cations (Zagorowska (1998)). Some RNA targets form secondary structures, which are able to inhibit the reaction of the reverse transcriptase (Kuo (1997)). By heating in presence of chelating agents secondary structures can be denatured and RNA can be stabilized. RT-PCR applications require sensitive extraction of RNA, which than needs to be stabilized. Addition of 50 mM EDTA can stabilise RNA. However, this is not always suitable, because of dilution affects in samples containing small amounts of RNA. Additionally EDTA cannot be easily removed from the RNA solution and will be transferred into downstream applications.

From that point of view an alternative-chelating agent (insoluble chelating resin Chelex 100) avoiding the above mentioned problems was induced in the RNA extraction protocol. RNA samples containing DNase I enzyme and cations were added directly to the dried[13] Chelex 100 and were heat inactivated at 95°C for 2 min. Using Chelex 100 RNA was successfully stabilized and amplified in the Two-Step RT-PCR as can be seen in Table 31.

5.3.5 Sensitivity in RT-PCR

5.3.5.1 Detection limit of DNA contaminating RNA samples of non-germinated conidia

Table 43 - Sensitivity of DNA extraction and DNA digestion tested in PCR with SYBR Green I.

lab-code	conidia/ml	nucleic acid	specific product in melting curve
11	10^2	DNA	-
	10^3	DNA	-
	10^4	DNA	+
	10^5	DNA	+
	10^6	DNA	+
	10^2	RNA	-
	10^3	RNA	-
	10^4	RNA	-
	10^5	RNA	+ weak
	10^6	RNA	-
71	10^2	DNA	-
	10^3	DNA	+ weak
	10^4	DNA	+
	10^5	DNA	+
	10^6	DNA	+
	10^2	RNA	-
	10^3	RNA	-
	10^4	RNA	-
	10^5	RNA	-
	10^6	RNA	-
106	10^2	DNA	+ weak
	10^3	DNA	+ weak
	10^4	DNA	+
	10^5	DNA	+
	10^6	DNA	+
	10^2	RNA	-
	10^3	RNA	-
	10^4	RNA	-
	10^5	RNA	-
	10^6	RNA	-

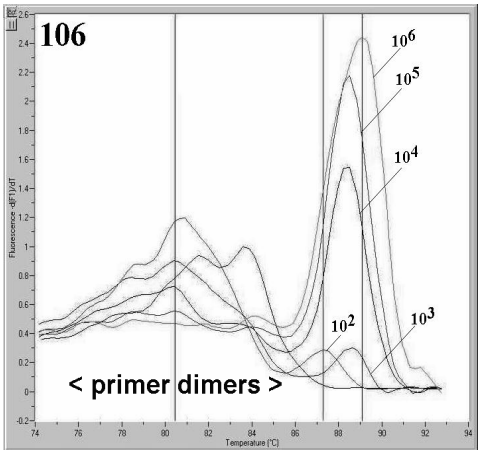


Figure 19 – Demonstration of weak detection of DNA of 10^2 non-germinated conidia by PCR in non-germinated conidia. The enrichment of DNA by germinating spores can increase detection. SYBR Green I fluorescence is plotted versus temperature in a melting curve. DNA of strain 106 in Table 43 was investigated.

To show that DNA was extracted from non-germinated conidia and to define the PCR detection limit of DNA contaminating RNA samples of non-germinated spores was one aim of this section. Another aim was to monitor the reproducibility of the DNA digestion in the RNA samples. The sensitivity of DNA extraction from non-germinated conidia was shown for DNA including three different fungal strains (lab-code 11, 71 and 106). Mycelia of these strains were cultured until spore formation was observed. Conidia were separated from the mycelia by several washing steps in saline (0.9 % NaCl) and controlled microscopically to avoid hyphal contamination. DNA and RNA were extracted simultaneously from defined amounts of conidia

[13] Chelex 100 (10 %) was aliquoted, centrifuged at 12000 rpm from 3 min, and the pellet was dried at about 95°C for 10 min.

using protocol 2 (see 4.2). The PCR system was the same as used in the experiment 5.3.5.1. DNA of 10^4 conidia was repeatedly detected and limited at about 10^3 conidia partly at 10^2 conidia. The DNA digestion in the RNA samples remained to be not complete and not reproducibly. The detection of food-relevant amounts of spores in a range of 10-100 spores/g food (Samson *et al.* see page 277 (1995)) was not achieved reproducibly. The amplification of different DNA amounts (10^2-10^6 conidia) of strain 106 is shown in Figure 19 as a representative example for weak signals in DNA samples of 10^2 and 10^3 conidia. The sensitivity of the given assay including nucleic acids extraction and PCR is not as high as necessary to detect food-relevant amounts of DNA in RNA. Since DNA is more stabile than RNA and the DNA detection seems to be already limited in food-relevant amounts of non-germinated conidia, mRNA detection in 10^2 or 10^3 non-germinated conidia was not expected.

5.3.5.2 Is EF-3 mRNA from non-germinated conidia detectable in RT-PCR?

Conidia of *Aspergillus niger* DSM 1988 were separated from mycelia by centrifugation and several washing steps in saline (0.9 % NaCl). Repeated microscopical controls showed that no mycelia were present in the spore suspension. The spores (10^{10} spores/ml) were counted in a Neubauer chamber. The RNA was extracted from 10^{10} conidia by applying the protocol 2 in 4.2. DNA was digested by DNase I (Roche) and samples were inactivated on dried Chelex 100 at 95°C for 2 min. Two-Step RT-PCR was performed according to the protocol in experiment 5.2.2.1 using PCR primers –30ML47F, -30ML48F and Mix 157. The amplification was detected by SYBR Green I as shown in Figure 20. It was found, that the spores indeed contained *EF-3* mRNA, although in a very low amount. The *EF-3* mRNA was detected by RT-PCR including 10 µl of the extracted RNA from 10^{10} conidia, but the specific signal was weak. Using only half of the RNA amount in the RT reaction of the Two-Step RT-PCR resulted in a fail.

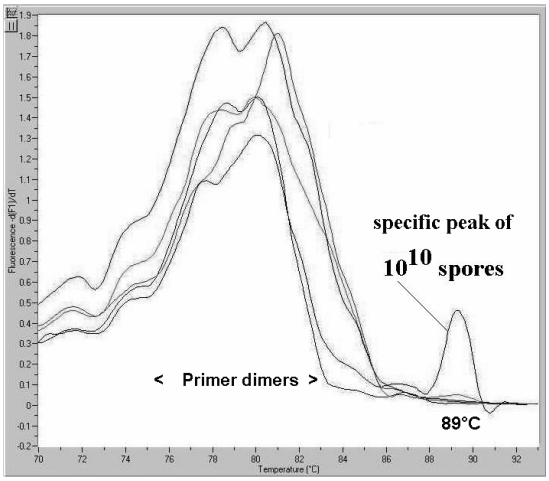


Figure 20 - Detection of EF-3 mRNA in non-germinated conidia. The melting curve of specific amplified cDNA detected with SYBR Green I is shown. Fluorescence is plotted versus temperature. The RNA was free of any detectable DNA contamination. EF-3 mRNA was detected in 10^{10} non-germinated conidia of Aspergillus niger DSM 1988, indicated by a specific amplification product which melted at 89°C.

The amount of spores included in the RNA extraction was much more than usually contained in food. It was impossible to detect *EF-3* mRNA from spores in a range of 10-100 spores, which would be relevant in food testing. The RT-PCR of the single copy gene *EF-3* from non-germinated conidia remains to be impossible for routine applications. Consequently an enrichment step needs to be done for the detection of viable spores by RT-PCR.

5.3.5.3 Sensitivity of the Two-Step RT-PCR

The aim of this section was to compare the sensitivity of the Two-Step RT-PCR in the detection of different species. Conidia of two representative strains *Aspergillus niger* DSM 872 and *Aspergillus parasiticus* DSM 2038 were germinated in YEPD at 25°C for 16 hours. RNA was extracted as described before in protocol 2 in 4.2. DNase I was inactivated on dried Chelex 100 (10 %). The RNA was free of any detectable contaminating DNA. The data in Table 44 result from Two-Step RT-PCR experiments.

Table 44 - Differences in sensitivity of the RT-PCR between two strains of *Aspergillus*.

strains	germinated conidia	RNA in PCR	RNA in Two-Step RT-PCR
Aspergillus niger DSM 872	10^6	-	+
	10^3	-	+
	10^2	-	+
Aspergillus parasiticus DSM 2038	10^6	-	+
	10^3	-	-
	10^2	-	-

The sensitivity in the Two-Step RT-PCR was different between the two examined strains. This implicates, that it is not advisable just to transfer the sensitivity results from one strain to another in RT-PCR. As the *EF-3* mRNA might be expressed in a strain-dependent way, the optimisation of the RT-PCR for routine use becomes a challenge.

5.3.6 Detection of viable fungi in juice by RT-PCR

5.3.6.1 Two-Step RT-PCR including HybProbes

Table 45 – Composition of RT reaction mixture. RT reaction was performed for 30 min at 50°C. RT products, cDNA was transferred to the PCR mixture. Primer mixture and PCR mixture were composed as described in PCR protocol A in 4.4.

component	final conc.
Rev Primer Z	0.3 pmol/µl
Rev Primer A	0.3 pmol/µl
RNA	50 pg - 1µg
each dNTP 10 mM	0.4 mM each
Protector RNase Inhibitor	20 U
10 x Puffer	1 x
Transcriptor Reverse Transcriptase	4U
$MgCl_2$ 50 mM	4 mM

The next section demonstrates the principle possibility to detect 10^2 conidia/ml juice by Two-Step RT-PCR after two days enrichment. Conidia of *Aspergillus niger* DSM 1988 were added into orange juice (FS10) to a final amount of 10^2 spores/ml juice. The juice samples were enriched on membrane filters as described in chapter 4.1.2. The samples were simultaneously cultured on nutrient pads (Schaufus Pottinger; sample 1 and 2) and agar plates (Caso + chloramphenicol final conc. 50 mg/l; sample 3 and 4) for 48 hours at 25°C. The control enrichment (growth control) was done on membrane filters without adding juice samples. As the colonies were forced to grow slowly by adding them on Caso agar, they were hardly visible after 48 hours enrichment in juice samples at the time of RNA extraction. The RNA extraction was performed according to protocol 2 in 4.2 including the whole membrane filter in the disruption step. RNA was stabilised by EDTA addition and free of any detectable DNA contamination (data not shown). The composition of the RT reaction mixture is summarized in Table 45.

Table 46 – Results of Two-Step RT-PCR including juice samples. Conidia were grown on membrane filter. FS 10 = food sample orange juice

in FS	conidia added to juice	media	specific amplification	CP value
FS 10	100	nutrient pad	+	32.11
without FS	100	nutrient pad	+	28.57
FS 10	100	Caso agar	-	-
without FS	100	Caso agar	+	20.59
-	- (NC RT)		-	-
-	- (PC 32C)		+	13.30

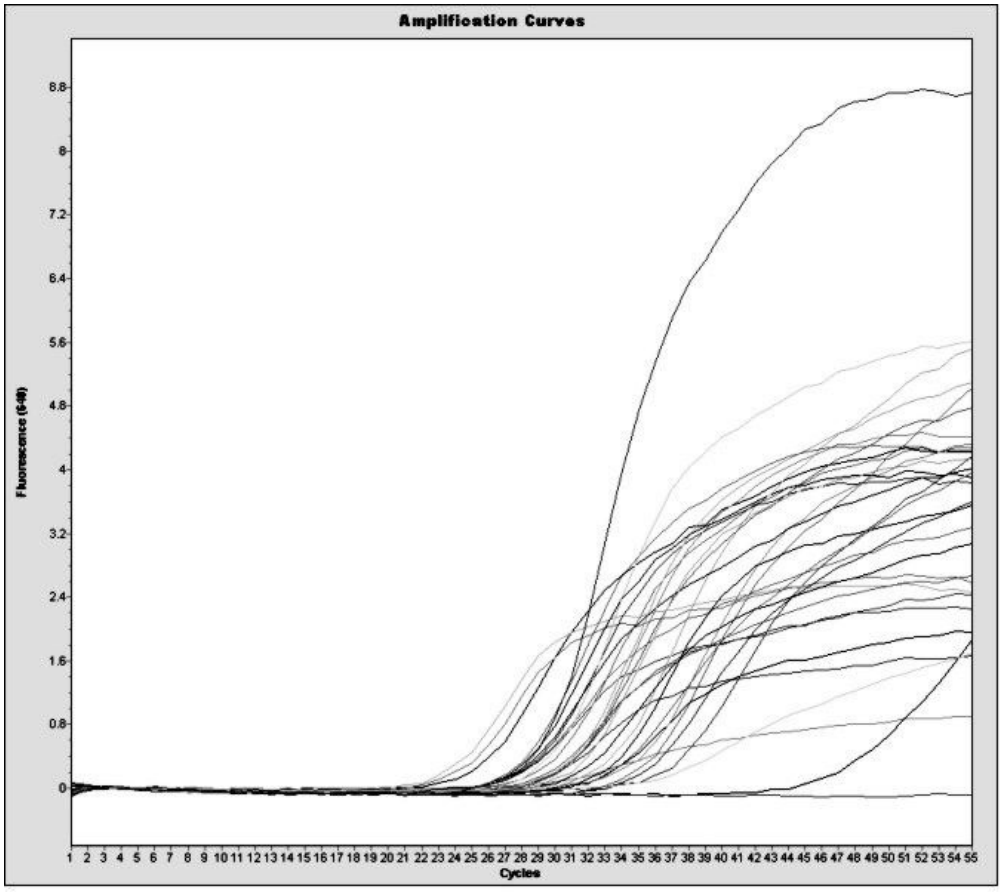
The PCR was performed according to PCR protocol A as mentioned earlier in the chapter 4.4. Two-Step RT-PCR detected 100 conidia after two days enrichment in orange juice. At least one RNA sample failed to amplify in this experiment (see Table 46) showing again the problems arising in optimising and validating RT-PCR. The experiment was repeated in a One-Step RT-PCR format and showed a positive detection of fungal *EF-3* mRNA in spiked juice samples, which were enriched on nutrient pads. However, One-Step RT-PCR was again less sensitive than the Two-Step RT-PCR.

5.3.7 Summary of RT-PCR experiments

Two-Step RT-PCR and One-Step RT-PCR assays were established on the LightCycler® instrument using the Transcriptor Reverse Transcriptase in the RT reaction and the Fast Start Taq Polymerase in the PCR. Different detection formats were evaluated according to their potential to specifically detect fungal nucleic acids. The probe hybridisation was found to serve as the most reliable detection format. As a result of this work a SYBR Green I assay is additionally available. However, the use of probe formats to specifically detect *EF-3* PCR products, mainly the HybProbes format, was selected for further specific detection in this work.
The *EF-3* mRNA was detected in non-germinated conidia by RT-PCR. However, the possibility to detect viable conidia in food without any enrichment was not practicable, because the expression level of *EF-3* mRNA in the conidia was unfortunately too small to be detectable in food relevant amounts of spores.
Consequently, enrichment of fungal nucleic acids by germinating the conidia was suggested. In a model experiment including young hyphae (16 hours old) *EF-3* mRNA was detected by Two-Step RT-PCR in a range of 10-100 spores, which is approximately the amount in food. As the conditions in this model experiments were far from the situation in food, the basic possibility of RT-PCR including RNA samples from food (in orange juice) was demonstrated.
The sensitivity and reproducibility of RT-PCR experiments apparently depended on a large complex of different parameters. Especially the situation in the One-Step RT-PCR mixture is too complicate for justifiable trouble shooting in routine use. From that point of view it might be better to abandon the optimisation of RT-PCR experiments for routine applications in food.

5.4 Specificity of the consensus PCR (protocol A)

5.4.1 Detection of food-relevant filamentous fungi by PCR



lab-code	CP value
47	24.68
151	29.48
16	28.43
120	44.52
48	27.12
9	36.30
15	31.70
6	34.21
29	22.63
18	28.77
129	23.17
124	26.82
-5	27.17
137	28.91
111	32.58
121	27.98
110	30.62
113	27.04
132	32.69
127	30.15
114	30.21
136	28.27
115	30.26
83	27.03
75	34.46
131	34.54
102	30.68
96	29.00
148	35.52
7	32.35
27	28.64
NC	-

Figure 21 - Specific amplification of genomic DNA from pure cultures of filamentous fungi using the consensus PCR system including HybProbes. The strain numbers are included in the attached Table 75 and Table 76 (lab-code). Fluorescence is plotted versus cycle number.

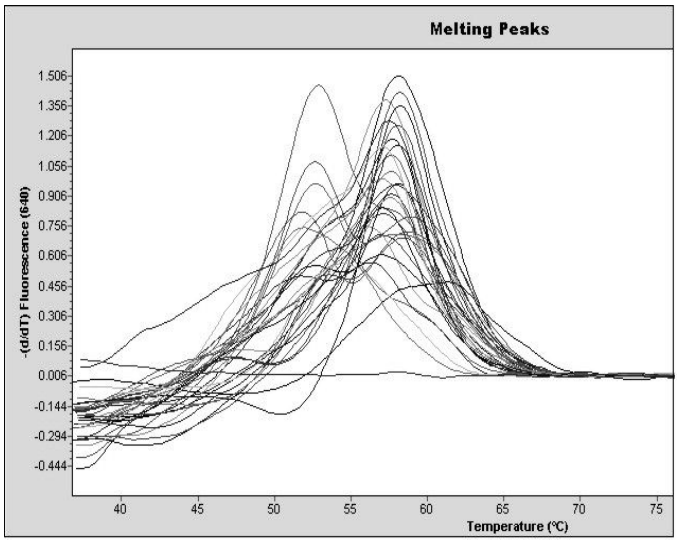


Figure 22 - Melting curves of the consensus PCR HybProbes. Shown is a plot of fluorescence versus temperature using the PCR products shown in Figure 21.

Figure 21 shows the amplification curves generated by the consensus PCR system using protocol A and fungal specific HybProbes for a selection of 31 strains. Specific PCR products from a total of 163 fungal isolates summarised in Table 75 and Table 76 could be detected by applying the consensus PCR system using HybProbes in protocol A. Figure 22 shows the melting peaks of HybProbes used in the detection of the mentioned fungal DNA samples. They were generated from the decrease in the fluorescent signal during the melting curve. Caused by the degenerated nature of the HybProbes the melting peaks were hetero-

geneous and located within a range of 47°C and 60°C. The specificity of the consensus PCR was additional tested in experiments (see Table 47) including environmental food-related strains.

Table 47 - Detection of environmental food-related fungal strains by consensus PCR.

place of air collector installation	manufacturer of	results in PCR with HybProbes	CP values	results in PCR without HybProbes
air directly at bottler	soft drinks	+	12.78	+
air directly at bottler	soft drinks	+	13.59	+
air directly at bottler	soft drinks	+	13.09	+
indoor air in production facility	pharmacy plant product	-	-	+/- weak
indoor air in production facility	pharmacy plant product	+	14.63	+
indoor air in production facility	pharmacy plant product	+	19.92	+
indoor air in production facility	pharmacy plant product	-	-	+/- weak
indoor air in production facility	pharmacy plant product	+	11.75	+
indoor air in production facility	pharmacy plant product	+	23.44	+
indoor air in production facility	pharmacy plant product	+	16.31	+
indoor air in production facility	soft drinks	+	9.75	+
indoor air in production facility	soft drinks	+	11.71	+
indoor air in production facility	soft drinks	+	12.86	+
indoor air in production facility	soft drinks	+	12.14	+
indoor air in production facility	soft drinks	-	-	+ /-weak

The selected strains were isolated from air collections in food production facilities. The air samplers were installed directly in the food production facilities of different beverage manufacturers. Some air samples were taken directly at the bottler of the facility. DNA was extracted from 15 airborne isolates according to protocol 3 in 4.2 and applied to the consensus PCR. Specific PCR products were amplified for all 15 fungal isolates. Unfortunately, three samples were only weakly amplified and therefore not detected with the HybProbes as can be seen in the summarized results in Table 47. The fungal isolates have not been identified.

Table 48 - Detection of pure cultures of unidentified fungal strains extracted from food by PCR. Inhibition of PCR occurred in sample 3, 10 and 12.

No.	food sample	visual description of strain	PCR without HybProbes	PCR including detection by HybProbes	CP value
1	parsley	white mycelia	+	+	8.40
2	parsley	black mycelia	+	+	12.16
3	salami	mycelia with yellow brown spores	-	-	-
4	salami	mycelia with grey spores	+	+	13.73
5	skin cream	white mycelia	+	+	12.31
6	breadcrumbs	mycelia with green spores	+	+	9.60
7	wheat whole meal	mycelia with grey spores	+	+	9.52
8	wheat whole meal	mycelia with grey brown spores	+	+	7.63
9	corn meal	mycelia with green spores	+	+	8.89
10	corn meal	mycelia with brown spores	+/-	-	-
11	fruit tee	white air mycelia with black spores	+/-	+	14.84
12	fruit tee	mycelia with black spores	+/-	-	-
13	almonds grounded	grey air mycelia	+/-	+	12.17
14	almonds grounded	mycelia with green spores	+	+	10.66
15	maize grit	white mycelia	+	+	10.89
16	maize grit	mycelia with green spores	+	+	10.41

Additionally, filamentous fungi from different food samples listed in Table 48 were isolated and DNA was extracted. DNA from these samples was amplified in the consensus PCR and detected by HybProbes as well. The fungal isolates have not been identified. Other results further extending the specificity testing should be mentioned here. For instance the fungal background DNA contamination was detected by the consensus PCR (see Table 63), if no DNase I treatment was performed during DNA extraction from juices. Positive amplification represented non-cultivable and non-identifiable fungi contaminating the raw products.

5.4.2 Exclusion of bacterial DNA from detection

During the fungal enrichment of non-sterile food bacterial growth is generally inhibited by antibiotics but cannot be prevented in all samples. As a large spectrum of different bacterial species can be present in various food samples before the enrichment of fungi, some species can be resistant against the employed antibiotics. Additionally, isolated bacterial DNA can be found in large amounts in some food samples (see http://www.bacteriamuseum.org).

Table 49 - Non-specific primer binding to bacterial DNA. No specific signals with HybProbes were found.

strain	DSM, if no other collection mentioned.	control of PCR products in agarose gel	PCR using detection by HybProbes
Burkholderia cepacia	7288	-	-
Campylobacter jejunii	33560	-	-
Enterobacter sakazakii	4485	+ non-specific	-
Shigella baydii	7532	-	-
Shewanella putrafaciens	6067	+ non-specific	-
Yersinia enterocolitica	4780	+ non-specific	-
Zymomonas mobilis	424	+ non-specific	-
Lactobacillus lindneri	20690	+ non-specific	-
Listeria monocytogenes	19118 ATCC	+ non-specific	-
Staphylococcus aureus	20231	-	-
Bacillus alcalophilus	485	-	n.d.
Bacillus cereus	10980	-	n.d.
Bacillus psychrophilus	3	-	n.d.
Cedecea davisae	4568	+	-
Citrobacter koseri	4595	+	-
Clostridium perfringens	12709	-	n.d.
Erwinia carotovora	30168	+ non-specific	-
Escherichia coli	NCTC 12790	+	-
Hafnia alvei	30163	+ non-specific	n.d.
Klebsiella planticola	4617	+	-
Klyvera cryocrescens	4588	+	-
Leclercia adecarboxylata	5077	+	-
Leuconostoc mesenteroides	20241	-	n.d.
Moellerella wisconsensis	5076	+ non-specific	-
Pantoea agglomerans	0202	+ non-specific	-
Plesiomonas shigelloides	8224	-	n.d.
Proteus rettgeri	1131	+ non-specific	-
Serratia marcescens	1636	+	-
Yersinia pseudotuberculosis	8992	+ non-specific	-
Yokenella regensburgei	5079	+ non-specific	-

It was found, that the PCR primers used in fungal consensus PCR bind to bacterial DNA and form unspecific products as shown in Table 49. The fungal specific HybProbes did not bind to bacterial DNA, but competitive PCR amplifying unspecific bacterial DNA and specific fungal

target DNA might influence the sensitivity of the fungal specific consensus PCR. Table 49 shows the results from testing bacterial DNA with the fungal primer and HybProbe system.

5.4.3 Exclusion of eukaryotic DNA from detection

5.4.3.1 Exclusion of DNA from higher eukaryota

Apart from bacterial contamination the DNA extracted from food generally contains a mixture of DNA from different organisms. Therefore fungal specific primers need to avoid non-specific binding to other eukaryotic DNA in food. The DNA derived from different eukaryotic organisms was extracted by High Pure PCR Template Preparation Kit (Roche) as shown in Table 50.

Table 50 - Results testing DNA of higher eukaryota in the consensus PCR according to protocol A. The quality of the eukaryotic DNA was determined applying specific PCR systems.

DNA of	specific PCR systems	fungal PCR
meat from turkey	+ RAPD M13fw	-
chicken	+ RAPD M13fw	-
kidney (pig)	+ RAPD M13fw	-
heart (chicken)	+ RAPD M13fw	-
mixed mincemeat (pig/ cow)	+ RAPD M13fw	-
human (Novagen)	+ ß-catenin primer	-
broccoli	+ plant specific primer GMO screening Kit (Roche)	-
green algae (Baltic Sea)	+ plant specific primer GMO screening Kit (Roche)	-

The fungal specific consensus PCR system did not amplify the DNA from the selected samples listed in Table 50. The quality of the DNA was determined by applying the DNA to different specific PCR systems. Plant DNA was amplified by specific primers from the GMO screening Kit (Roche). Human DNA (Novagen) amplification was shown by PCR including primers for ß-catenin. DNA from different meat samples was tested by RAPD including M13fw primers. Based on these data it was concluded, that a parallel amplification of DNA from other eukaryota than fungi in food is impossible using the fungal specific consensus primers.

5.4.3.2 Exclusion of yeast DNA from detection

The potential to amplify and detect yeast DNA using the consensus PCR designed for the detection of filamentous fungi was estimated including 56 yeast strains. The yeast strains, which were detected by the fungal specific consensus PCR are shown in the attached Table 78. The PCR protocol A was performed including 50 pg yeast DNA. In a first step the PCR was performed without HybProbes. In the second step the positive samples were reapplied in a PCR including HybProbes. The results of both PCR experiments were confirmed by agarose gel electrophoresis. Different yeast strains were investigated. By PCR without HybProbes 38 out of 56 strains were positively amplified, while the inclusion of HybProbes into the PCR resulted in the specific detection of 15 strains out of the 38 being positively amplified. Six yeast strains out of these 15 positive strains showed a strong probe binding. The specific amplification curves of five of these strongly detected yeast strains *Citeromyces matritensis* BCD 1061, *Yarrowia lipolytica* BCD 2556, *Endomycopsis burtonii* DSM 3505, *Candida versatilis* DSM 6956 and *Metschnikowia pulcherrima* DSM 70321 are shown in Figure 23, whereas the sixth strain *Candida rugosa* BCD 814 was included in another experiment and is not shown.

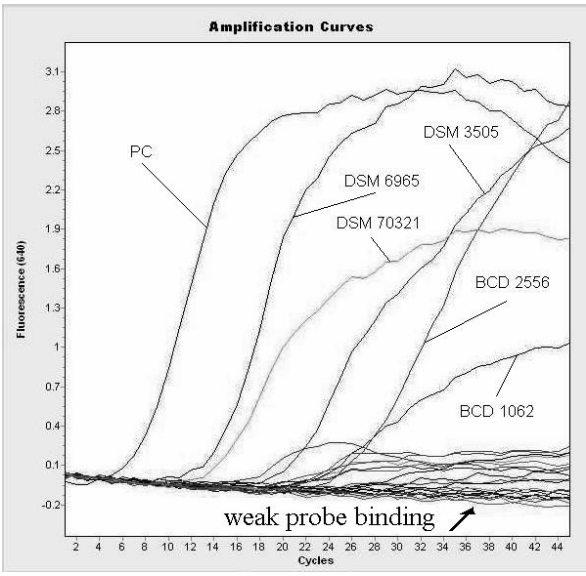


Figure 23 - HybProbe hybridisation to yeast DNA.
The amplification curves are shown in a plot of fluorescence versus cycle number.

The weak binding to HybProbes is indicated in the Figure 23 by an arrow and mentioned in attached Table 78. The experiments were repeated and the results were reproduced. The summarised results of all tested yeast DNA are given in Table 51.

Table 51 - Summary of PCR specificity experiments including yeast DNA.

	positive tested strains out of 56	%
binding of primers to yeast DNA samples	38	67.9
binding of HybProbes to specific amplified PCR products of yeast DNA	15	26.9
Strong binding of HybProbes to specific amplified PCR products of yeast DNA	6	10.7

Detection of unknown yeast isolates

Table 52 - Detection of non-identified yeast isolates from food by consensus PCR. The data show, that yeasts and filamentous fungi share similar environments.

food sample	PCR without HybProbes	PCR with detection by HybProbes	microbiological determination of	
			yeast	filamentous fungi
corn	+	+	+	+
pepper	+	-	+	+
pepper	+	-	+	+
pepper	+	+	+	+
garden beans	+	-	+	+
brussels sprout	+	-	+	+
chocolate cream puff	+	-	+	+
brittle tart	+	+	+	+

The possibility to detect yeasts isolated from real food samples using the consensus PCR system is demonstrated in the following experiment. Yeasts and filamentous fungi were separated by dilution plating and single colonies were transferred to Sabouraud agar plates. The DNA was extracted applying protocol 3 in 4.2. Both filamentous fungi and yeast were isolated from the food samples listed in Table 52. PCR amplification was performed according to protocol A. All

yeast DNAs were included in the PCR using the consensus PCR primers. Three yeast strains were detected including HybProbes in the PCR. The question arises how yeasts might influence the results of the consensus PCR designed for filamentous fungi in case of cross contamination in food.

Cross contamination - yeasts and filamentous fungi

As shown earlier many types of yeast were amplified by primers derived from alignments of filamentous fungi sequences. This was not surprising, because yeast and filamentous fungi are taxonomically closely related. Yeast and filamentous fungi are defined as ecological categories as opposed to phylogenetical different groups. However, the differences in morphology, growth behaviour and physiology between yeast and filamentous fungi cause specific problems for each group during manufacturing of food. Yeasts cause immense problems by unwanted fermentation of food while filamentous fungi produce mycotoxins during long storage and transportation. As yeasts can grow rapidly during the enrichment of filamentous fungi, the question arises how this influences the detection of filamentous fungi using the consensus PCR system in 4.4. The detection of filamentous fungi from a mixed culture of yeast and filamentous fungi is demonstrated in the following experiment.

Table 53 - Simultaneous enrichment of yeasts and filamentous fungi. *Schizzosaccharomyces maliclevorans* DSM 70572 was detectable by fungal specific primers in the consensus PCR but not by hybridisation of HybProbes.

yeast cells	name of yeast	conidia	name of filamentous fungi	PCR HybProbe CP values	mycelia growth	yeast growth
0	n.d.	10^2	*Aspergillus niger* DSM 1988	18.71	22 CFU	n.d.
10^2	*Schizzosaccharomyces maliclevorans* DSM 70844	10^6		5.77	laminar cover	not visible
10^3		10^6		5.32	laminar cover	not visible
10^6		10^2		15.84	11 CFU	weak film
10^6		10^3		15.57	80 CFU	weak film
10^2		10^2		16.61	10 CFU	weak film
10^3		10^3		14.81	58 CFU	weak film
10^9		10^2		24.49	not visible	strong film
10^9		10^2		26.26	not visible	strong film

The yeast strain selected for this experiment (*Schizzosaccharomyces maliclevorans* DSM 70572) was detectable by fungal specific primers in the PCR but not by hybridisation of HybProbes developed for the detection of filamentous fungi. The enrichment was performed on membrane filters on top of agar plates containing chloramphenicol for 48 hours. Food samples were not included. The conidia of filamentous fungi and yeast were quantified in the Neubauer chamber before they were mixed together and transferred to the filter membranes. The DNA was extracted from mixed cultures of *Schizzosaccharomyces maliclevorans* DSM 70572 and *Aspergillus niger* DSM 1988 applying protocol 3 in 4.2, and PCR was performed according to protocol A.

DNA from a mixture of 10^2 young hyphae of *Aspergillus niger* and $>10^9$ CFU of *Schizzosaccharomyces maliclevorans* was extracted and compared in the consensus PCR to DNA from a pure culture of *Aspergillus niger* (10^2 young hyphae). Only DNA from *Aspergillus niger* was detected using HybProbes although yeast cells were included in the mixture. The data show that the CP values of *Aspergillus niger* from a mixed culture were significantly increased by 6.6 CP compared to pure culture.

The shift is probably caused by co-amplification of DNA from both the yeast and the filamentous fungi. The mixture of the DNA might lead to a statistical decrease of the primers available to bind to the fungal DNA compared to pure culture. Consequently it was concluded, that a quantification of DNA from filamentous fungi in the presence of large amounts of yeast DNA in real time PCR is not applicable. Importantly, the quenching effect of the yeast DNA on the PCR amplification of DNA from filamentous fungi were demonstrated to show the demand for careful validation of the PCR system.

Cross contamination - yeasts and filamentous fungi in juice

The experiment including mixed cultures of yeast and filamentous fungi was extended by the addition of a food sample. *Rhodutorula* was contained in a juice. The DNA of *Rhodutorula* was amplified by primers developed from the alignment of filamentous fungi but could not be detected by the HybProbes. *Aspergillus niger* DSM 1988 conidia were artificially added to the juice samples. 1 ml of the mixture was enriched on membrane filters on Sabouraud agar plates for 43 hours at 25°C. The whole filter membrane was disrupted during the DNA extraction as mentioned in protocol 3.

Table 54 - Cross contamination of yeast and filamentous fungi. *Rhodutorula* was contained in juice 10 as a contamination, which was not detectable by HybProbes. Conidia of *Aspergillus niger* DSM 1988 were artificially added.

			microbiological result in CFU counted after 72 hour incubation	
juice	conidia *Asp.nig.*	CP values	*Aspergillus niger* DSM 1988	*Rhodutorula*
10	0	-	0	membrane filter covered
10	10	33.37	5	membrane filter covered
10	100	34.31	43	membrane filter covered
10	1000	31.24	not countable	membrane filter covered
10	10000	30.34	not countable	membrane filter covered

The membrane filters were completely covered by *Rhodutorula* after 43 hours. The cells of *Aspergillus niger* were visible by yellow circles after 72 hours of enrichment. The PCR including HybProbes did not detect the sample containing only *Rhodutorula* cells. The experiment confirms the detection of 10 and 100 spores of *Aspergillus niger* DSM 1988 in the presence of yeast cells (*Rhodutorula*) after enrichment in a juice sample for 43 hours.

5.5 Sensitivity of the consensus PCR system

The amplification of genomic DNAs and IPC molecules are introduced in this chapter in the context of sensitivity of the PCR assay. The inclusion of too many molecules of the IPC plasmid in the PCR can inhibit the amplification of low amounts of specific target DNA. To avoid those quenching effects a careful titration of the number of IPC plasmids in the PCR was performed as shown in the following experiments.

5.5.1 Sensitivity of the PCR demonstrated in genomic DNA and IPC plasmid DNA

Genomic DNA was extracted by protocol 1 in 4.2 and the concentration of the DNA was determined by measuring the optical density at 260 nm. The sensitivity of the consensus PCR was estimated by inclusion of serial dilutions of genomic DNA in the consensus PCR protocol A. The detection limit was determined at 1 pg genomic DNA in representative strains of *Zygomycota* and *Ascomycota* (see for example Table 55 *Aspergillus flavus* ATCC 9643).

Table 55 - Sensitivity of the PCR including fungal specific DNA and IPC. The detection limit of genomic DNA was estimated with 1 pg, which corresponds to 30 or 60 genome equivalents if one underlies a mean genome size of 15 –30 Mb in the presented filamentous fungi. Results of titration IPC and estimation of PCR sensitivity including DNA of *Aspergillus flavus* and IPC are summarized. The specific target DNA was detected in the 640 nm channel whereas the IPC (in general 0,1 fg) was detected at 705 nm. Increasing fluorescence at 705 nm indicates the exclusion of matrix inhibition in negative samples.

strain	amount of genomic DNA	amount of IPC 146 C	IPC copies?	PCR result HybProbe (640 nm)	CP value (640 nm)	PCR IPC HybProbe (705 nm)	CP value (705 nm)
Paecilomyces variotii CBS 62866	100 pg	n.d.	n.d.	+	35.87	n.d.	n.d.
	10 pg	n.d.	n.d.	+	35.30	n.d.	n.d.
	1 pg	n.d.	n.d.	+	42.19	n.d.	n.d.
Aspergillus fumigatus DSM 819	100 pg	n.d.	n.d.	+	31.01	n.d.	n.d.
	10 pg	n.d.	n.d.	+	35.06	n.d.	n.d.
	1 pg	n.d.	n.d.	+	39.00	n.d.	n.d.
Rhizopus nigricans BCD 3506	100 pg	n.d.	n.d.	+	34.51	n.d.	n.d.
	10 pg	n.d.	n.d.	+	37.94	n.d.	n.d.
	1 pg	n.d.	n.d.	-	-	n.d.	n.d.
Actinomucor elegans BCD 3486	100 pg	n.d.	n.d.	+	29.15	n.d.	n.d.
	10 pg	n.d.	n.d.	+	32.83	n.d.	n.d.
	1 pg	n.d.	n.d.	+	33.16	n.d.	n.d.
Aspergillus flavus ATCC 9643	1 ng	n.d.	n.d.	+	20.28	n.d.	n.d.
	100 pg	n.d.	n.d.	+	24.61	n.d.	n.d.
	1 pg	n.d.	n.d.	+	32.66	n.d.	n.d.
	0.1 pg	n.d.	n.d.	-	-	n.d.	n.d.
	0.01 pg	n.d.	n.d.	-	-	n.d.	n.d.
	1 fg	n.d.	n.d.	-	-	n.d.	n.d.
	1 ng	0.1 fg	30	+	18.40	-	-
	0.1 ng	0.1 fg	30	+	24.96	+/-	30.31
	1 pg	0.1 fg	30	+	30.04	+	29.99
	0.1 pg	0.1 fg	30	-	-	+	30.70
	1 ng	0.01 fg	3	+	19.72	-	-
	0.1 ng	0.01 fg	3	+	23.11	-	-
	1 pg	0.01 fg	3	+	31.35	+	31.95
	0.1 pg	0.01 fg	3	-	-	+	33.48
	0.01 pg	0.01 fg	3	-	-	+	35.19

If one underlies a GC content of 50 % in genomic DNA of fungi and a mean genome size of 15-40 Mb in the presented filamentous fungi, the detection limit of 1 pg genomic DNA corresponds to the detection of 25-65 genome equivalents. The amplification of genomic DNA from pure fungal cultures showed an efficiency[14] of the amplification in a range between 70 % and 100 %. This range was probably caused by errors during DNA quantification, dilution and additionally by the strain-dependent differences in the primer and probe hybridisation. In complex primer and target DNA systems (such as consensus PCR) small errors in sample preparation multiply, as can be concluded from changes in the efficiency. As IPC molecules were included into each PCR capillary of consensus PCR experiments (as described in 4.4.4), quenching effects caused by competing for PCR compounds between the IPC and specific target DNA might occur. To avoid this situation careful titration was performed. Besides the sensitivity for IPC plasmid DNA of the consensus PCR was estimated by correlating the amount of IPC DNA to the copy number of the IPC plasmid. The detection of IPC DNA was demanded if no specific target DNA occurred in the sample. The Table 55 shows a representative result of the titration with genomic target DNA and IPC. The DNA was amplified by the consensus primer system for filamentous fungi applying the PCR protocol A. The amplification of the IPC is quenched by specific target DNA at 0.1 ng and more. In contrast the amplification of specific target DNA is not quenched by the IPC. In the consensus PCR described in protocol A in 4.4 finally 0.1 fg IPC plasmid DNA were included in each amplification.

5.5.2 Detection limit of IPC and PC

To avoid quenching effects of the IPC in the PCR an exact determination of the amount of IPC plasmid copies in the DNA sample was necessary. To determine the amount of gene copies in a IPC plasmid DNA, it was necessary to quantify and calculate the molecular mass of the plasmid DNA including the target IPC sequence. The quantification of IPC plasmid DNA was performed using two different methods. One method was the application of the Quant-iT™ PicoGreen® dsDNA Assay Kit, the other method was measuring the DNA amount in an Ultraspec 4000 UV/visible Spectrophotometer. Both methods gave similar results for the IPC and the positive control (PC) plasmid.

The molecular weight (Da) of one plasmid was calculated from the content of the particular nucleotide bases and the molecular weight of the corresponding nucleotides. As the molar mass is the product of the molecular weight of one plasmid, the Avogadro number and the mass of hydrogen, one could calculate the mass of one double strand plasmid copy (1 gene copy). These data were correlated to the quantified plasmid DNA samples enabling the inclusion of defined amounts of IPC plasmids in the consensus PCR. Based on defined plasmid amounts the threshold values of the IPC and the PC were determined in a PCR including pure IPC and PC DNA (see Table 56). The PCR was performed according to PCR protocol A including HybProbes for the detection of specific amplification products. Serial dilutions were performed in HSTE[15] buffer in steps of 1:10 until 0.001 fg/µl was achieved. Different channels in the LightCycler® instrument served for reading the absorption of positive control (at 640 nm) and internal control (at 705 nm).

[14] See 5.5.3 for the principle of efficiency calculations.
[15] HSTE contained Tris HCl (Sigma) 0.01M; EDTA 0.5 mM; Salmon Sperm DNA (Sigma No. D-1626) 0.01 mg/ml.

As described in material and methods the color compensation was applied to distinguish the signals. Due to dilution errors, the detection of 3 copies was not reproducible. The threshold value of the IPC and PC was around 30 gene copies.

Table 56 - Detection limits of the consensus PCR system with defined amounts of pure IPC and PC plasmids. The absorption of positive control (at 640 nm) and internal control (at 705 nm) was measured in different channels in the LightCycler® instrument.

amount of PC (32 C) plasmid in PCR	amount of IPC (146 C) plasmid in PCR	content of plasmids copies in PCR	PCR result measured at 640 nm	CP value at 640 nm	PCR result measured at 705 nm	CP value at 705 nm
n.d.	1 fg	300	-		+	20.10
n.d.	0.1 fg	30	-		+	24.57
n.d.	0.1 fg	30	-		+	24.54
n.d.	0.01 fg	3	-		+	28.98
n.d.	0.01 fg	3	-		-	
n.d.	0.001 fg	0.3	-		-	
n.d.	0.001 fg	0.3	-		-	
1 fg	n.d.	300	+	22.53	-	
0.1 fg	n.d.	30	+	27.83	-	
0.1 fg	n.d.	30	+	26.08	-	
0.01 fg	n.d.	3	+	24.44	-	
0.01 fg	n.d.	3	+	27.4	-	
0.001 fg	n.d.	0.3	-	/	-	
0.001 fg	n.d.	0.3	-	/	-	

5.5.3 Efficiency of IPC detection in the consensus PCR

The PCR profile originally included 35-45 cycles, which led to limitations of assay sensitivity in food. Therefore the PCR profile was optimised by the addition of 20 short preceding PCR cycles as included in the general PCR protocol A (see 4.4).

Table 57 - Comparison of two different PCR profiles to increase PCR sensitivity.

PCR profile with regular 35-45 cycles.	
IPC 146 C in PCR sample	CP value
100 fg	23.81
100 fg	22.53
10 fg	26.63
10 fg	26.24
10 fg	27.55
1 fg	29.65
1 fg	31.27
0.1 fg	34.83
0.1 fg	34.90

PCR profile with 20 preceding cycles plus 35 regular cycles	
IPC 146 C in PCR sample	CP value
1 fg	27.02
1 fg	26.14
1 fg	26.70
1 fg	26.47
1 fg	26.52
0.1 fg	29.77
0.1 fg	29.89
0.1 fg	30.46
0.1 fg	30.59

This resulted in a shift of the CP values by up to 4 CP values compared to the profile including regular 35-45 cycles. For example, the IPC amount for 0.1fg DNA was detected at CP 30 instead of CP 34 (see Table 57).

The efficiency of the PCR amplification control was determined for the data in Table 57 by 81.7 ± 5.9 % with regular 35-45 cycles and by 90.04 ± 8.2 % including preceding cycles.

In fact, the efficiency is considered 100 % if the CP value decreases by one cycle with each replication of X in (1) (Tichopad (2002)). The efficiency of the PCR reaction directly reflected the quality of the sample preparation. The efficiency of the IPC amplification was calculated using the following equation (Deprez (2002)):

(1) $Y = X (1+E)^n$

Y (amount of end product)
X (amount of starting target DNA copies)
E (efficiency of the reaction)
n (number of PCR cycles 'CP value')

The efficiency of an optimised PCR assay should be in a range of 90-100 %. However, this is only a theoretical assumption. Normally multiplex PCR systems are expected to result in lower efficiency values largely due to their complex primer and probe composition. Influences like the unpredictable hybridisation behaviour of degenerated probes, primer dimer formation or differences in the sample matrices can change the efficiency of the PCR.
In Figure 24 the data of Table 57 achieved without preceding cycles were compared to the data achieved including preceding cycles. Figure 24 shows a plot of these data, CP values are plotted versus the log(X). The slope (a) was calculated from the linear fit of the data. The efficiency was finally calculated from the slope a = (-1/log1 + E) of the linear plot (y = ax+b).

(2) $E = 100 \cdot (10^{-1/a} - 1)$

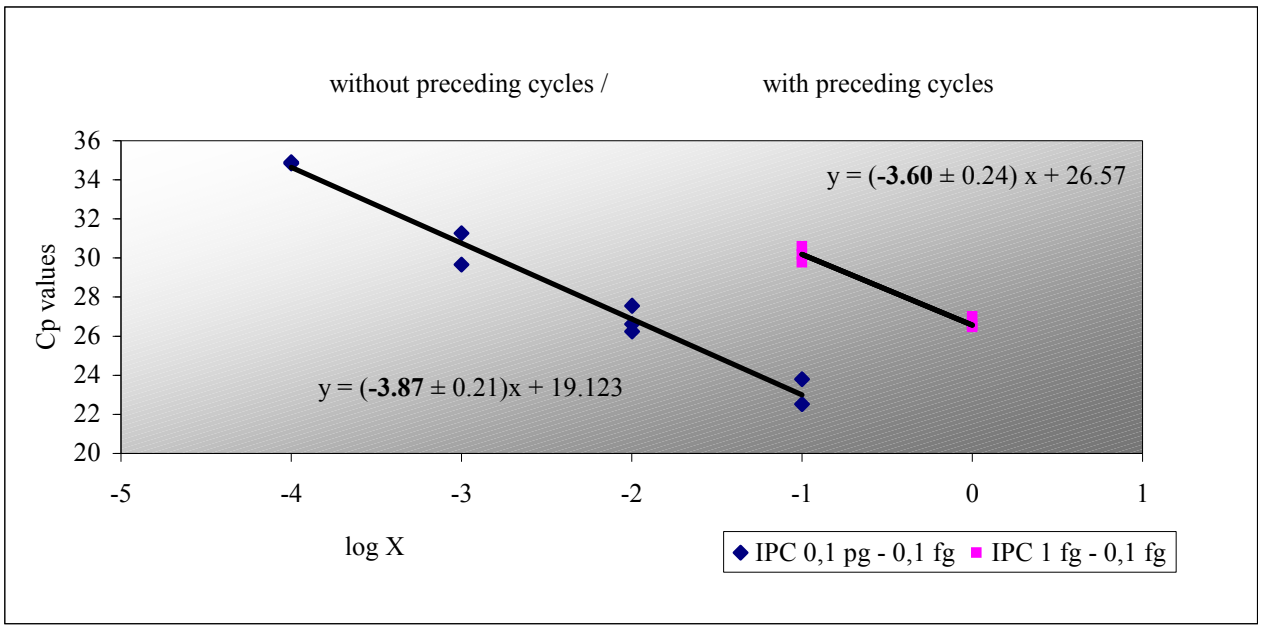


Figure 24 – Efficiency comparison of PCR profiles including and excluding preceding cycles. Corresponding data are shown in Table 57**.** The ideal slope =-3.3 reflects 100 % efficiency.

5.6 Detection of viable filamentous fungi by PCR based methods

The main difference in the detection of viable fungi by PCR compared to RT-PCR is that the target molecule, DNA is not necessarily derived from viable fungi. The fraction of DNA in a food sample after enrichment may contain DNA from both living and dead cells. To distinguish between DNA from living and dead cells one has to selectively exclude DNA from dead cells before PCR amplification as shown in the following chapter.

5.6.1 Investigating the phenomenon of background DNA contamination

5.6.1.1 Isolation of fungal strains from food

Fungal contamination in food was determined by plating food samples on different solid media. Several fungal strains were isolated from the food samples listed in Table 58. Some of these strains were later used to spike food samples. Fungal growth was already visible after 2 days incubation at 20°C-25°C. The fungal isolates were not identified.
Even though samples were incubated for 14 days, no fungi were isolated from tomato and orange juice in Table 58. A strain isolated from another orange juice was used as an additive strain in the following experiments. This orange juice isolate was microscopically evaluated and probably belongs to the genus *Cephalosporium*. As mentioned earlier the amount of viable fungi in food is quite small and depends on the method of preservation. To detect even small amounts of contaminants by PCR and to distinguish these from background DNA one needs to enrich the viable particles until they reach a sufficient amount that is detectable by PCR. The method to enrich fungi from food samples has been described in 4.1.2.

Table 58 - Overview of the food samples used for fungal isolation. 0.1 g of food samples or pellet concentrated by centrifugation of 10 ml juice from samples was applied.

	food sample	Fungal growth on solid media?			
		PDA	**M**	**OM**	**YEPD**
1	maize grit	yes	yes	no	yes
2	corn meal	yes	yes	yes	bacteria
3	wheat whole meal	yes	yes	yes	bacteria
4	parsley	yes	bacteria	yes	bacteria
5	currant	no	no	no	no
6	breadcrumbs	n.d.	bacteria	yes	bacteria
7	fruit tee	n.d.	yes	yes	yes
8	almonds grounded	n.d.	yes	yes	yes
9	tomato juice	n.d.	no	no	no
10	orange juice	n.d.	no	no	no

5.6.1.2 Digestion of background DNA contamination in juice

In order to investigate the special problems arising in fruit juice production food sample 10 (orange juice) was chosen for the following experiment. No living fungi could be isolated from this food sample not even by plating statistical large amounts of juice in different media for more than 14 days. However, fungal DNA was detected by consensus PCR in food sample 10 (see Figure 25 lane 1 – 4).

Table 59 - Digestion of background DNA in non-enriched food sample 10. In sample 7 and 8 mycelia of 10^6 germinated spores of *Aspergillus niger* DSM 1988 were included. The digestion of the background DNA contamination was performed before disrupting the intact living cells in the MagNA Lyser. Intact living cells were grown from spores overnight and some juice samples were spiked with these spores prior to DNA extraction. The results were monitored by PCR. The PCR was performed according to protocol A in 4.4 without inclusion of HybProbes. See corresponding Figure 25, the amplification was observed by agarose gel electrophoresis. The extraction protocol included proteinase K and/or DNase I (Roche) treatment (protocol 3) in 4.2, which were applied to digest the background DNA contamination in samples 5, 6, 9 and 10.

gel lane	medium	in food sample	proteinase K	DNase I	*Aspergillus niger* DSM 1988	PCR without HybProbes
1	YEPD	10	-	-	-	+
2	M	10	-	-	-	+
3	YEPD	10	+	-	-	+
4	M	10	+	-	-	+
5	YEPD	10	+	+	-	-
6	M	10	+	+	-	-
7	YEPD	10	+	+	+	+
8	M	10	+	+	+	+
9	YEPD	10	-	+	-	-
10	M	10	-	+	-	-

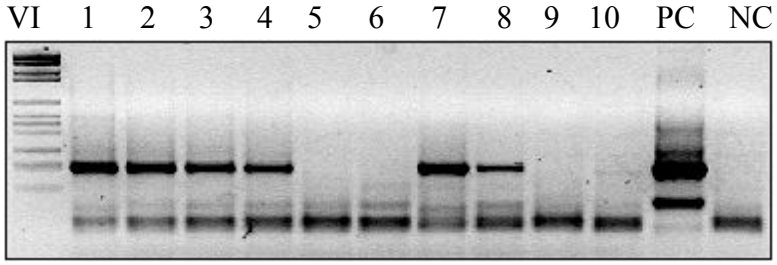


Figure 25 – Agarose gel demonstrates digestion of background DNA in lane 5, 6, 9 and 10. Numbers correlate to Table 59.

This DNA was referred to as background DNA contamination in juice and might represent free DNA in the solution of fruit juices. To avoid wrong positive signals in PCR one has to get rid of this background DNA by digesting it. Table 59 shows PCR results of an experiment with and without digestion of the background DNA contamination during DNA extraction. It was shown, that the background DNA contamination could be digested either by DNase I alone or in combination with Proteinase K[16] in advance to the disruption of living cells in the MagNA Lyser. The digestion of background DNA and the disruption of intact living cells in the MagNA Lyser were performed in the same tube. The living cells were added to the samples in order to demonstrate that the digestion of the background contaminating DNA did not affect the DNA extraction from those cells (see sample 7 and 8 in Figure 25). Indeed, it was shown, that the DNase I treatment did not influence the DNA extraction from living cells spiked in the juice as shown later. The application of this method for the extraction of DNA from food samples avoids wrong positive results from background DNA.

[16] Proteinase K can improve DNA digestion by destroying DNA binding proteins before DNase I treatment.

5.6.1.3 Effect of background DNA digestion for the detection of small amounts of viable cells

This experiment demonstrates the influence of the digestion of the background DNA contamination on small amounts of viable cells. Spores from isolates of food sample 2 and 7 in Table 58 (CFU 10^2 to 10^6) were germinated overnight and added to the media and/or fruit juice sample. DNA extraction was repeated as in the experiment in Figure 25 with the digestion of background DNA contamination before disruption of viable cells. PCR was performed according to PCR protocol A in 4.4 using SYBR Green I instead of HybProbes. The existence of background DNA contamination was demonstrated by performing the DNA extraction protocol without proteinase K or DNase I treatment. The digestion of background DNA was demonstrated in non-spiked food samples grown in YEPD. As shown in Table 60, it is possible to detect up to 10^2 germinated spores by this method. The digestion of background contaminating DNA did not influence the sensitivity of DNA extraction from small amounts of living cells.

Table 60 – Testing the influence of DNase I treatment on different amounts of viable fungi. Fungi were isolated from food samples in Table 58 and added to food samples 2 and 7 of Table 62.

food sample/ media	fungi from food sample	proteinase K	DNase I	germinated spores from food-isolates	PCR (SYBR Green I) detection
2	2	+	+	10^6	+
2	2	+	+	10^5	+
2	2	+	+	10^4	+
2	2	+	+	10^3	-
2	2	+	+	10^2	-
YEPD	2	+	+	10^6	+
YEPD	2	+	+	10^5	+
YEPD	2	+	+	10^4	-
YEPD	2	+	+	10^3	+
YEPD	2	+	+	10^2	+
2	-	+	+	-	-
2	-	-	-	-	+
7	7	+	+	10^6	+
7	7	+	+	10^5	+
7	7	+	+	10^4	+
7	7	+	+	10^3	+
7	7	+	+	10^2	+/-
YEPD	7	+	+	10^6	+
YEPD	7	+	+	10^5	+
YEPD	7	+	+	10^4	+
YEPD	7	+	+	10^3	+
YEPD	7	+	+	10^2	+
7	-	+	+	-	-
7	-	-	-	-	+/ -

5.6.2 Enrichment of viable filamentous fungi

The enrichment of viable fungi in media without addition of antibiotics can end up in bacterial contamination, which can inhibit fungal growth. Additionally, bacterial DNA might be able to inhibit fungal-specific PCR. Therefore antibiotics had to be included in fungal cultivation. Fungal culturing is often performed in bottles containing fluid media. This is not suitable for spore germination as Deacon (1997 see page 165) demonstrated in germination experiments

concerning brake of dormancy and fungistasis[17]. Since fungi are aerobe microorganisms, they grow on the surface of fluids. Inside a fluid media fungi usually do not grow homogenously but form nests on particles. This makes it difficult to take out a homogenous aliquot for the PCR. However, reliable reproduction of PCR results can only be achieved using homogenous samples in DNA extraction. Homogenizing of the fluid samples is impossible for fungi because mycelia are disrupted and DNA will be lost. Considering these observations and including the fact that equal sample amounts have to be taken from the culture, the enrichment in fluid media was not suitable. As an alternative cultivation method, the enrichment on membrane filters, which were covering nutrient pads or agar plates, was used in this study.
Nutrient pads, such as OGY, Orange serum, Sabouraud, Schaufus Pottinger and Würze have been successfully used in combination with the membrane filter method for more than 20 years. The food samples were prepared according to their consistency (diluted or concentrated) and spread on top of the membrane filter or filtered through the membranes, if fluid. Nutrient pads differed from agar plates in that they were sucking the dilution fluid of the homogenized food sample like a sponge, which results in growth conditions similar as in food.

Table 61 - Enrichment of *Aspergillus niger* DSM 1988 conidia on membranes on top of nutrient pads and comparatively on top of agar plates for the detection by PCR including HybProbes. Fungal conidia of *Aspergillus niger* strain DSM 1988 were spiked into the solution produced from orange juice pellet in YEPD as well as in pure YEPD as a control containing chloramphenicol. The enrichment was performed on the surface of nutrient pads 'Schaufus Pottinger' plus filter membrane (Pall 0.45 µm) as well as on the surface of filter membrane (Pall 0.45 µm) on top of agar plates (Caso agar). To demonstrate a positive enrichment the CP difference of DNA extracts from the 1st day and the 2nd day of enrichment was calculated.

PCR CP value[18]	CP difference between 1st day and 2nd day	enrichment	nutrient pad + membrane + spiked spores	day	media	food sample
24.85	5.77	**positive**	50 CFU	1st	YEPD	10
19.08			50 CFU	2nd	YEPD	10
23.26	4.13	**positive**	50 CFU	1st	YEPD	-
19.13			50 CFU	2nd	YEPD	-
PCR CP value	CP difference between 1st day and 2nd day	enrichment	Caso agar + membrane + spiked spores	day	media	food sample
24.37	4.8	**positive**	50 CFU	1st	YEPD	10
19.57			50 CFU	2nd	YEPD	10
23.10	2.78	**positive**	50 CFU	1st	YEPD	-
20.32			50 CFU	2nd	YEPD	-

The cultivation of fungi on nutrient pads allows to take out equal amounts of the food samples by just transferring the whole membrane filter into a tube prepared for DNA extraction. The membrane of the nutrient pad can be destroyed by disruption using Zirconium beads (1.2 mm) in a MagNA Lyser (Roche). This principle enables one to extract DNA from the entire amount of

[17] Spores are held in a dormant state by some form of feedback inhibition which signals that the environment is unsuitable. Spore germination is inhibited on 'nutrient-leaching' systems as on flowing and shaken fluids containing little nutrients Deacon (1997).
[18] The CP values in a real time PCR experiment are correlated to the amount of target DNA originally contained in the sample. As the amount of target DNA in a real time PCR sample is inversly proportional to the CP values, smaller CP value in a particular sample represent more detectable target DNA.

fungi grown in the food sample. In this work nutrient pads were partly substituted by agar plates containing chloramphenicol (final conc. 50 mg/l) on which a sterile filter membrane was put on top.
The experiments in Table 61 demonstrate the fungal enrichment on membrane filters after 1-2 days. DNA was extracted by protocol 3 in 4.4 using 860 µl VE water and 100 µl 10x buffer and excluding the DNase I treatment. On the 1st and on the 2nd day filter membranes were washed in sterile plastic bags by 3 ml of sterile water. The resulting solutions were transferred into tubes containing Zirconium beads. Extracted DNA was applied to the fungal specific consensus PCR protocol A including HybProbes. The PCR signals in this experiment derived from DNA of dead and living cells. To monitor the occurrence of viable cells the samples from the 1st and the 2nd day (after enrichment) were compared by real time PCR. The CP values show a significant difference, which was interpreted as enrichment of DNA during growth of the cells. The resulting CP values are listed in Table 61. In this experiment it was investigated to wash the membrane filters and extract DNA from the washing solution. Unfortunately, microscopically examination revealed that most of the fungi were still stuck to the filter membranes after washing and were not included into the nucleic acid extraction. Therefore it was decided to disrupt the whole membrane filters by Zirconium beads in the MagNA Lyser to isolate the fungal DNA from the entire mycelia grown during the enrichment. This procedure increased the CP difference significantly, which consequently improved the reliability of this method considerably.
Due to the occurrence of background contaminating DNA, a potential test of absence of viable cells requires the comparison of two samples before and after enrichment. The handling would be more suitable and the assay cheaper, if one aimed to develop a single sample test system, which can be achieved by digesting the background contaminating DNA.

5.6.3 Testing preserved food: fruit juice matrix

Heat and pressure stable structures of filamentous fungi are mainly introduced into juice production by processing contaminated mouldy fruits. Therefore screening of viable fungi in raw materials of fruit juices is recommended. Considering the special problems associated with fungi in juice production, juices were chosen from the large spectrum of food samples as a matrix to model fluids. The juices in Table 62 have been investigated. Interestingly, no living fungi could be isolated by any method from these juices samples.

Table 62 - Overview of fruit juice matrices.

number	type of juice	percentage of pulp according to the producer
1	peach juice	50 %
2	carrot juice	not specified
3	multi vitamin juice 11 fruits	100 %
4	sherry juice	50 %
5	multi vitamin juice 12 fruits	55 %
6	A+C+E+ fibre vitamin drink	60 %
7	orange juice 1 l package	100 %
8	tomato juice	made from concentrate
9	orange juice 0.2 l package	100 %
10	pineapple juice	100 %
11	apple juice natural	100 %

The samples were pre-treated by sterile transfer of 10 ml of the whole juice into a Falcon tube and centrifugation at 5000 rpm for 5 min at 4°C. The supernatant was discarded and the pellet was diluted in 10 ml autoclaved DI water. This step enables one to adjust a neutral pH in the solution. This is particularly important in strong acidic juices like pineapple and orange juice. The DNA was extracted according to protocol 3 in 4.2. Potential contaminating fungi were determined on Sabouraud agar plates containing chloramphenicol by direct plating.

5.6.3.1 DNase I digestion of background DNA contamination in juices

To develop a single sample assay the background DNA contamination in juices needs to be digested before living cell structures are destroyed during DNA extraction as shown earlier in 5.6.1.2. In the following experiment 10 juices were investigated for the occurrence of background DNA contamination and were digested using DNase I (Roche) according to protocol 3 4.2 without proteinase K.

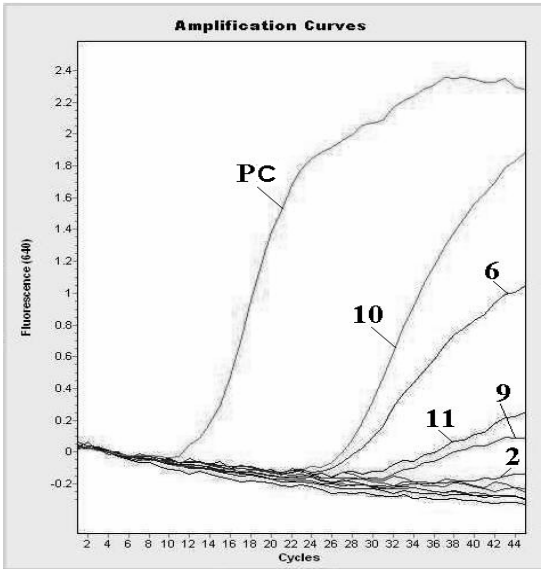


Figure 26- Amplification curves show background DNA detection in five juice samples out of 10 before DNase I treatment. Fluorescence is plotted versus cycle number. PC = positive control

The results were compared and are shown in Table 63. The PCR was performed according to protocol A with and without detection by HybProbes, to monitor yeast amplification. The background DNA contamination was detected by PCR including HybProbes in 5 of the 10 juice samples as shown in Figure 26. In order to detect amplified DNA, which was not detected by HybProbes, samples were run on 2 % agarose gels to identify the PCR products by size. Background DNA contamination could not be digested in all samples and was still detected in sample 6, 9, 10, 11. However, these DNAs were not detectable by HybProbes. One may assume that the detection of the background DNA contamination in a PCR including HybProbes is not as sensitive as the detection in agarose gels. However, background DNA contamination detected in agarose gels can originate from yeast DNA amplification. As shown earlier yeast DNA is only partly detectable by the given HybProbes.

Table 63 - Detection of background DNA contamination in fruit juices. 10 ml of the juice samples were centrifuged and the supernatant was discarded. The pellet was homogenized in 3 ml of water to neutralize the pH. 1 ml of this solution was loaded on membrane filters and incubated at 20°C-25°C to dry the solution. DNA from two membranes with or without DNase I digestion was compared. In samples with DNase I digestion, the whole filter was treated in a solution containing DNase I. Matrix inhibition was monitored by addition of the IPC in the PCR master mix and was found in juice sample 1. To monitor yeast amplification PCR was performed with and without detection by HybProbes. - = negative result in amplification; + = positive amplification; +/- = weak amplification

	PCR with HybProbes		PCR without probes, detection in agarose gel		
juice sample	treatment with DNase I	no DNase I treatment	no DNase I treatment no filter membrane	treatment with DNase I	no DNase I treatment
	sample	sample	sample	sample	sample
1	-	-	-	-	-
2	-	+/-	+	-	+
3	-	-	+	+	+
5	-	-	+/-	-	+
6	-	+	+	-	+
7	-	-	+/-	-	+
8	-	-	+/-	-	+
9	-	+	+/-	+	+
10	-	+	+	-	+
11	-	+	+	-	+

Reproducibility of the background DNA digestion and detection of viable fungi

To demonstrate the reproducibility of the background DNA digestion by DNase I and the detection of a defined number (10 and 100) of young hyphae in juice samples the following experiment was performed. Young conidia of *Aspergillus niger* DSM 1988 were added to juice samples and incubated for two days on membrane filters on Sabouraud agar plates containing chloramphenicol (final 50 mg/l) at 20°C-25°C. It was expected to detect fungal growth after two days by the consensus PCR without HybProbes. Samples with or without enrichment of *Aspergillus niger* spores were compared. As shown in Figure 29 some background DNA contamination could still be detected. Samples 9 and 10 showed significant amplification of the background DNA contamination which would lead to wrong positive results in a single sample assay. However repeated culturing experiments demonstrated, that contamination with yeast was the reasons of the positive amplification in these samples.

Table 64 - Results of PCR detected in agarose gels in samples spiked with conidia before enrichment. Background DNA detected in samples after DNase I treatment derived from yeasts, as was shown in repeated cultivations. These results demonstrate the difficulties with co-amplification of yeast DNA, which was not digested as background DNA was. Unfortunately, no selective agents are known today to separate filamentous fungi from yeasts during cultivation on different selective media. (see corresponding Figure 27)

	Aspergillus niger DSM 1988		
juice sample	0 CFU	10 CFU	100 CFU
1	-	-	+
2	+/-	+	+
3	-	+	+
5	+/-	+	+
6	-	+	+
7	-	+	+
8	-	+/-	+
9	+	+	+
10	+	+	+
11	+/-	+	+

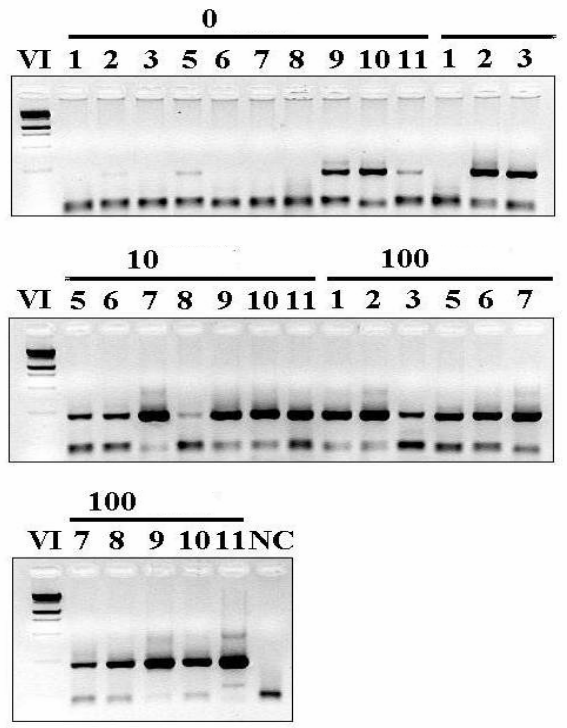


Figure 27 - Gel electrophoresis of PCR performed without HybProbes. In spiked juice samples (10 and 100 conidia) fungal DNA was detected after 48 hours enrichment. Background DNA contamination, which was correlated with growth of yeasts in those samples by additional plating, is significantly amplified in sample 9 an 10 after 48 h incubation.

5.6.3.2 Enrichment of conidia in juice samples

In order to compare the PCR results received after 48h enrichment with microbiological data the following experiments were performed. The enrichment of conidia (*Aspergillus niger* DSM 1988) and spores originated from an orange juice isolate was performed in two juice samples for about 72 hours at 20°C-25°C. According to microscopical examination the isolate from the original orange juice was probably *Cephalosporium*. The conidia were of different age. The general ability of the pure conidia to germinate and grow was determined on Sabouraud agar plates and in the juice matrix as shown in Table 65 and Table 66. The enrichment was detected by PCR and these data were compared to results from conventional visual evaluation of agar plates as shown in Table 67.

The microbiological counting of fungal colonies was performed in two juice samples on membrane filters on top of Sabouraud agar plates containing chloramphenicol. The same enrichment procedure was performed for the PCR.

Table 65 - Pure cultures. Determination of fungal growth on Sabouraud agar plates containing chloramphenicol. Colonies were counted.

	isolate from orange juice			*Aspergillus niger* DSM 1988			
CFU	0	10	100	0	10	100	after
plate 1	0	0	0	0	0	0	**24 h**
plate 2	0	0	0	0	0	0	**24 h**
plate 1	0	0	0	0	**43**	**388**	**48 h**
plate 2	0	0	**4**	0	**48**	**>500**	**48 h**
plate 1	0	0	**21**	0	n.d.	n.d.	**72 h**
plate 2	0	**1**	**19**	0	n.d.	n.d.	**72 h**
plate 1	0	0	n.d.	0	n.d.	n.d.	**96 h**
plate 2	0	**1**	n.d.	0	n.d.	n.d.	**96 h**
plate 1	0	0	n.d.	0	n.d.	n.d.	**144 h**
plate 2	0	**1**	n.d.	0	n.d.	n.d.	**144 h**

Table 66 - Cultures in juice samples. Determination of fungal growth in juice samples 5 and 7 (see Table 62) on Sabouraud agar plates containing chloramphenicol. Colonies were counted.

	isolate from orange juice			*Aspergillus niger* DSM 1988			
CFU	0	10	100	0	10	100	after
juice 5	0	0	0	0	0	0	**24 h**
juice 7	0	0	0	0	0	0	**24 h**
juice 5	0	0	0	0	0	0	**48 h**
juice 7	0	0	0	0	0	0	**48 h**
juice 5	0	0	**1**	0	**8**	**78**	**72 h**
juice 7	0	0	0	0	**10**	**82**	**72 h**
juice 5	0	0	**2**	0	n.d.	n.d.	**96 h**
juice 7	0	0	**4**	0	n.d.	n.d.	**96 h**
juice 5	0	0	n.d.	0	n.d.	n.d.	**144 h**
juice 7	0	0	n.d.	0	n.d.	n.d.	**144 h**

Conidia (2 - 3 days old) from *Aspergillus niger* DSM 1988 were found to germinate in juice samples on membrane filters after about 72 h. The DNA extraction using whole filters for PCR was performed according to the protocol 3 including proteinase K and DNase I treatment in a single sample assay.

Table 67 - Comparison of PCR and microbiological results from culturing for two different strains. Duplicate plates have been investigated. The juice samples are described in Table 62.

		fungal isolate (orange juice)			*Aspergillus niger* DSM 1988		
CFU		0	10	100	0	10 (=10 spores)	100 (=40 spores)
plate	juice	PCR determination (in CP values)					
1	5	-	-	-	n.d.	-	-
2	7	-	-	-	n.d.	-	-
1	5	-	-	-	n.d.	**weak 35**	**25.19**
2	7	-	-	**>60**	n.d.	**weak 35**	**22.94**
1	5	-	-	-	n.d.	n.d.	n.d.
2	7	-	-	**>35**	n.d.	n.d.	n.d.
1	5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

fungal isolate (orange juice)				*Aspergillus niger* DSM 1988		
0	10	100	0	10 CFU (9 spores)	100 (41.4 spores)	after
microbiological determination (in colonies)						
0	0	0	0	0	0	**24h**
0	0	0	0	0	0	**24h**
0	0	0	0	0	0	**48h**
0	0	0	0	0	0	**48h**
0	0	0	0	**12**	**47**	**72h**
0	0	0	0	**6**	**36**	**72h**
0	0	0	0	n.d.	n.d.	**96h**
0	0	0	0	n.d.	n.d.	**96h**

The enrichment of the strain DSM 1988 was successfully detected by PCR at around 48 hours, at a time, where microbiological counting of the fungi was not possible. The detection of fungal growth in juice at around 24 hours was impossible with both conventional and PCR based methods. The growth of the original fruit juice isolate (100 conidia) was evidently inhibited in the juice matrix and was hardly detectable by conventional visual methods after 96 hours (see Table 66). Apparently, the germination of the conidia or other structures in the sample limits the speed of the whole test.

Furthermore the data demonstrate differences in the ability of the two strains to grow on membrane filters in juice samples. The experiment gives an idea about the influence of the age and the particular fungal strain at the time of enrichment, as the fungal spores isolated from orange juice were older than conidia from *Aspergillus niger* DSM 1988. Older spores seemed to

loose or reduce their germination potential. The same differences in growth potential can be expected for fungal spores, which underwent processing during food production.
In general, the germination time of spores depends on different factors such as age of spores, type of strain, type of spores, amount of spores, growth media, a_w, temperature, food matrix, inhibitors, pH, genetically determined growth rates etc. Therefore it is extremely difficult to find the optimal time point to detect all viable fungi from only one sample.

5.6.3.3 Comparison of PCR data with fungal growth on plates

Table 68 - Comparison of PCR and microbiological data concerning the necessary time frame of the assay. *Aspergillus niger* DSM 1988 conidia were spiked into juices (see Table 62) on membrane filters on top of agar plates and incubated at 25°C for 48 h. The DNA was extracted by protocol 3 in 4.2 and amplified by consensus PCR (protocol A) resulting in the given CP values. Simultaneously, reference plates were incubated to count the visible colonies at the time of DNA extraction and later. The juice samples are described in Table 62. Detection of germinated conidia by PCR was possible after 48 hours and by microbiological counts after 67 hours enrichment. Background DNA was not detectable by PCR after DNase I treatment in this experiment.

spiked conidia	juice matrix	results of micro-biological counts	time	detection of PCR products in agarose gel	PCR detection by HybProbes: CP values	
0	3	0	48 h	-	n.d.	n.d.
0	3	0	48 h	-	n.d.	n.d.
0	9	0	48 h	-	n.d.	n.d.
0	9	0	48 h	-	n.d.	n.d.
0	10	0	48 h	-	n.d.	n.d.
0	10	0	48 h	-	n.d.	n.d.
10	3	0	48 h	+	+	24.90
10	3	0	48 h	+	+	25.22
10	9	0	48 h	+	+	22.06
10	9	0	48 h	+	+	25.12
10	10	0	48 h	+	+	23.86
10	10	0	48 h	+	+	24.73
100	3	Hardly visible, colony growth was not countable.	48 h	+	+	19.58
100	3		48 h	+	+	19.90
100	9		48 h	+	+	21.86
100	9		48 h	+	+	19.38
100	10		48 h	+	+	19.40
100	10		48 h	+	+	18.93
only microbiological counts, no PCR above 48 h:						
10	3	6 CFU	67 h	n.d.		
100	3	53 CFU	67 h			
10	9	7 CFU	67 h			
100	9	67 CFU	67 h			
10	10	8 CFU	67 h			
100	10	55 CFU	67 h			
10	no	3 CFU	42 h			
100	no	59 CFU	42 h			

The next experiment demonstrated the potential of the consensus PCR to detect food-relevant amounts of filamentous fungi in juice long before any growth was visible on agar plates. For that purpose enrichment of *Aspergillus niger* DSM 1988 conidia on membrane filters was performed for 48 hours at 20°C-25°C as described before. At about 48 hours the DNA was extracted and the samples containing 100 conidia already showed visible growth. These samples were included in the PCR to serve as positive controls. The detection of food-relevant amounts of 10 young

hyphae[19] in juice was possible at that time by PCR. The DNA extraction was performed according to protocol 3 in 4.2 including treatment with proteinase K and DNase I. The results of the PCR are summarized in Table 68. The experiment shows a clear detection of fungal growth (*Aspergillus niger* DSM 1988) by consensus PCR within 48 h of enrichment on membrane filters. Fungal growth in a range of 10 young hyphae was detected, which simulates closely the naturally occurring amount of fungi in food. The young hyphae were hardly visible within the two days of enrichment in juices.
The above experiments are based on the knowledge of the particular properties of the investigated model strain (i.e. *Aspergillus niger*) in food. As the properties of the various food relevant filamentous fungi differ immensely, the enrichment time remains an open question. It might be possible to determine the growth data for the most interesting food relevant key strains (marker strains) to validate the consensus PCR in food.

5.6.3.4 Exclusion of fungal spores from detection

In the experiments above DNA was extracted from germinated conidia and young mycelia of fungi, respectively. As described earlier, several other fungal structures can occur in food such as ascospores, asci, conidia and athrospores. The ability of the DNA extraction protocol to yield DNA from those usually dead but still intact structures was tested in the experiment described below. Since those structures were probably not destroyed during proteinase K and DNase I treatment, they might be destroyed during the disruption of the young hyphae in the MagNA Lyser. Applying this DNA to the consensus PCR no distinction between DNA from viable mycelia or dead conidia was possible.

Table 69 - Exclusion of dead spores from disruption and finally from PCR. Different spores were added to juices on top of filter membranes. The membrane filters were disrupted during the DNA extraction from juices. Simultaneously, spores were added to juices and from these mixtures DNA was extracted as described in protocol 3 in 4.2 without inclusion of the filter membrane during the cell disruption. Both samples were compared in the PCR. Fortunately, DNA from dead spores including the filter membrane in the cell disruption was not detectable by PCR. Only whole asci in large amounts gave a weak positive signal.

juice	strain; collection number	type of spores	amount of spores	PCR including membrane in disruption	PCR without membrane in disruption
1	*Byssochlamys fulva* BCD 955	conidia	10^6	-	+
1	*Byssochlamys fulva* BCD 955	conidia	10^3	-	+
1	*Byssochlamys fulva* BCD 955	conidia	10^2	-	+
2	*Aspergillus niger* DSM 1988	conidia (autoclaved)	10^6	-	+
2	*Aspergillus niger* DSM 1988	conidia (autoclaved)	10^3	-	+
2	*Aspergillus niger* DSM 1988	conidia (autoclaved)	10^2	-	+
3	*Chaetomium globosum* ATCC 62005	ascospores	10^6	-	+
3	*Chaetomium globosum* ATCC 62005	ascospores	10^3	-	+
3	*Chaetomium globosum* ATCC 62005	ascospores	10^2	-	+
4	*Byssochlamys nivea* BCD 956	whole asci	10^6	+/-	+
4	*Byssochlamys nivea* BCD 956	whole asci	10^3	-	+
4	*Byssochlamys nivea* BCD 956	whole asci	10^2	-	+

[19] Young hyphae are equivalent to germinated spores.

The experiment should answer the question, whether DNA from dead and living spores added into juices could be detected if the whole membrane filters were used in the extraction protocol. Control experiments excluding the membrane filter from the DNA isolation protocol were performed to show, that DNA from other fungal structures than mycelia can be extracted by bead crushing. Different types of spores were included in the test as summarized in Table 69.
These different spore types were added to the juices and loaded onto the filter membranes on Sabouraud agar plates. The plates were dried until the filter could be removed from the plates. The DNA was extracted from the filters in presence of proteinase K and DNase I applying protocol 3. As an additional control, the conidia of *Aspergillus niger* were autoclaved before spiking the juices. The experimental data show that no spores in the relevant amount (10-100 spores) were detectable by PCR, using the outlined extraction protocol including the whole membrane filters during the disruption in the MagNA Lyser.
Performing the DNA extraction in the same way but without the membrane filters spores can be detected by PCR (data not shown). It was therefore concluded, that the DNA extraction protocol could indeed be optimised to avoid disruption and therefore exclude the detection of non-germinated dead spores. This might be achieved by combining the filter membrane and the Zirconium beads of the adequate size during the crushing process in the MagNA Lyser.

5.6.4 Testing of non-preserved food samples

The enrichment of non-preserved (i.e. non-sterile) food samples on membrane filters on top of Sabouraud agar was performed as described in Table 70. As DNA could not be properly extracted from heat gelatinised food samples, protocol 3 in 4.2 was adapted to the properties of agents like starch as described in the chapter 4.2. DNA from non-gelatinised samples was extracted according to protocol 3 in 4.2 including proteinase K and DNase I digestion. The background DNA contamination was not digested in food samples containing gelatinised agents, because necessary heating steps for the inactivation of proteinase K and DNase I could not be performed for heat-gelatinised food samples. Therefore DNA samples taken before and after enrichment were compared in this experiment. Since no specific PCR products were detected in any food sample in Table 70 before enrichment, the data are not shown.

Table 70 - Results of PCR with representative non-preserved food samples. In food sample 1, 2, 4 and 7 fungal growth was detected within 48 hours. Food samples are described in Table 58. Due to matrix inhibition effects, two samples (3 and 8) give no results in PCR. Bacterial growth in sample 3 seems to inhibit fungal specific detection. fungi = filamentous fungi

food sample	results of PCR with HybProbes	CP value	results of microbiological control at time of DNA extraction	Results of micro-biological control after enrichment	heat application in extraction
1	+	20.4	48 hours no growth	fungi at 72 hours	-
2	+	26.94	48 hours growth	fungi at 72 hours	-
3	-	-	48 hours bacterial growth	fungi, bacteria at 72 hours	-
4	+	41.74	72 hours yeast like organisms	fungi at 96 hours	+
7	+	16.61	72 hours yeast like organisms	fungi at 96 hours	+
8	-	-	48 hours fungal growth	fungi at 48 hours	-

The resulting CP values in Table 70 represent the viable fungi after the indicated time of enrichment. Heat-gelatinised food samples (1, 2 and 3, see Table 58) were compared to non-gelatinised food samples (4, 7 and 8, see Table 58). The data show, that enrichment of fungi was detected by PCR in food samples both in the presence as well as in the absence of gelatinised agents. In contrast to juices, food containing gelatinising agents, requires the comparison of samples before and after enrichment.

5.6.5 Strategies to detect fungal enrichment

The PCR technology offers several advantages over traditional culturing methods. For example, fungal contamination was detected by PCR before reliable microbiological data were available without any mycological expertise.

The different types of food used in this study require two basically different DNA extraction protocols. Consequently two different principles for determination of the amount of fungal enrichment in food by PCR can be derived from the presented experiments.

- In some food samples heat treatment resulted in the formation of jelly, which caused fixation of DNA. Consequently, proteinase K and DNase I treatment could not be applied, because heat treatment needed to be performed to inactivate these enzymes. Therefore background DNA contamination found typically in food could not be disposed and could consequently lead to wrong positive results if one investigates only food samples after enrichment. For those types of food always two different samples, i.e. before and after enrichment, have to be compared in the PCR. As a result the difference between the CP values of the DNA samples before and after enrichment has to be calculated.
- In other food samples such as juices, tea and spices, heat treatment was possible without formation of jelly. Here the background DNA contamination was digested by proteinase K and DNase I followed by heat inactivation of these enzymes. As a consequence, only a single food sample after enrichment of viable fungi was examined. No comparison needed to be done between samples before and after enrichment.
- As shown in the experiment above, it was possible to design the DNA extraction in a way that DNA of dead fungal spores was not extracted from food and consequently not detectable by PCR.

6 Discussion

6.1 General proceedings in the consensus PCR assay

The quality and consistency of food must be reliably evaluated in manufacturing food. Due to the adaptation of filamentous fungi to a wide spectrum of nutrients in foodstuffs, their ability to tolerate low pH, a_w, extreme temperatures, chemical preservation and other special environmental conditions they are an important group of contaminants in food products. Formation of heat and pressure resistant spores of some filamentous fungi are further aspects influencing storage, preservation and transportation of food products. Therefore consumers and industry demand fast and reliable diagnostic systems for the selective detection of viable filamentous fungi in food.

Today molecular methods are more and more introduced into food diagnostics replacing conventional labour-intensive and time consuming culturing methods. PCR and RT-PCR have been suggested to serve as most promising analytical tools in detecting fungal contaminants because of their sensitivity and specificity (Vaitilingom (1998), Bleve (2003)).

The aim of this work was to develop a consensus PCR system for the specific detection and monitoring of viable filamentous fungi in food. A universal primer and probe system was designed based on the fungal specific gene *EF-3* (elongation factor 3). Additional to real time PCR, real time RT-PCR was investigated for its practicability in routine food testing. After all the DNA based consensus real time PCR was favoured as the more suitable method for the detection of filamentous fungi in routine compared to real time RT-PCR. The first part of this paragraph will focus on the DNA based assay, whereas in the second part critical points in DNA and RNA based methods are discussed.

6.1.1 Implementation of the consensus PCR system in food testing

As the specificity of the detection of viable fungi by PCR depended on the germination of spores or growth of mycelia, the accuracy of the whole assay is determined by the enrichment and DNA extraction procedure. The PCR system was therefore combined with a special enrichment procedure on membrane filters and a corresponding DNA extraction from the filters to detect selectively viable filamentous fungi.

As important perception it was found, that food can contain a background DNA derived from non-cultivable or dead organisms. It was demonstrated in this work that DNA from cells killed by heat or other treatments was able to serve as template for PCR, which led to false positive signals. This background contaminating DNA was not correlated to any fungal growth in the investigated food matrices. To overcome this problem contaminating DNA in food was digested by DNase I during the nucleic acid extraction prior to mechanical disruption of living mycelia in the MagNA Lyser instrument. The selectivity for viable fungi is therefore determined by the accuracy of the DNase I digestion during the DNA extraction in a single sample assay. It was shown in fruit juices, that contaminating DNA could be removed from the samples without destroying the DNA from viable cells.

The selective DNA extraction could not be reproduced in all types of food tested, because food is composed of various chemical substances, which require different solutions in the sample preparation before enrichment and during the nucleic acid extraction. It was found, for instance, that in heat gelatinising solid food (flour) the extraction protocol needed to be performed under low temperature conditions. The contaminating DNA originated from dead organisms could not be digested in those samples, because the required heating steps (to inactivate DNase I) gelatinised the sample resulting in loss of the DNA.
Consequently two different strategies for the determination of fungal enrichment in food were introduced. The first strategy aimed to detect viable fungi by including two different samples into the whole assay. One sample was taken before the enrichment and DNA was extracted. The CP value of this sample was compared in the consensus PCR to the CP value of a sample taken after the enrichment period. The enrichment was defined to be positive in case of smaller CP value of the samples taken after enrichment. Because of the calculation of a ΔCP the digestion of contaminating DNA by DNase I could be ignored and difficult samples (for instance from heat gelatinising solid food) could be investigated. However, this strategy had to be optimised and validated for several food types separately, because difficulties can result from high background levels of contaminating DNA. As the difference between the samples can become to small to be distinguishable from a strong background signal, the background contaminating DNA preferably has to be digested.
The second strategy involved the removal of contaminating DNA by DNase I digestion as described above. Consequently, only one sample had to be amplified in the PCR, which lowers the costs of the assay. However, the reproducibility of the DNA digestion was dependent on the special chemical composition of the particular food sample and might fail for some reasons. Therefore a careful validation of this step needed to be done before applying the assay to other food samples than the one being tested. Moreover, the phenomenon of incomplete DNA digestion is less important, because one can switch to the first strategy and measure the ΔCP.

6.1.2 The principles of DNA enrichment during cultivation

DNA enrichment was based on the successful cultivation of viable fungi from food samples under favourable conditions with the effect of replication of the fungal DNA, which then served as target DNA for detection, by the consensus PCR.
Before adding the samples to the enrichment media samples were pre-treated according to their aggregate state and other characteristics. For instance the samples needed to be neutralized by NaOH if acidic (Samson, Hoekstra (2004)). The viable particles in fluid food samples including fungal spores needed to be concentrated before transfer to the enrichment media. This could be done by filtration through membrane filters of clear fluids like water or by centrifugation and filtration of fluids like juices. Food samples like flour needed to be diluted in media containing antibiotics. In order to detect ascospores or other heat resistant fungal structures the food products need to be heated at 75°C for 30 min according to Pitt *et al.* (1992).
The relatively small amount of target organisms in food mentioned in the literature demonstrates the importance of preceding enrichment steps like filtration and centrifugation to concentrate viable fungal spores before the enrichment process. Engel (1991), for example, described

Byssochlamys nivea ascospores, which were found in raw and pasteurised milk in a range of 1 and 100 CFU/l. Other species like *Monascus ruber* could only be detected at a level of 2.5 CFU/l. Therefore it seems to be necessary to include statistically relevant food amounts and replicates into the diagnostic experiments.
Due to surface structures of the fungal spores, filamentous fungi are growing in nest-like structures, which are inhomogeneously distributed in the whole food sample. Therefore it is not possible to take out equal sample amounts for reproducible investigation by PCR from a fluid enrichment media. Consequently the enrichment methods were adapted to the molecular methods in this work. The enrichment of food samples on membrane filters on top of nutrient pads was successfully established in this work. The whole food sample was then included in the DNA extraction by transferring the whole membrane filters to the disruption process. This enabled to extract DNA from a defined amount of food sample and to allow reproducible results in the PCR. The procedure is relatively simple, because nutrient pads are ready-to-use products and have been widely accepted and validated. Quality changes from lot to lot as opposed to conventional agar plates are not expected.

6.1.3 Efficiency of fungal cell wall disruption

A sensitive extraction protocol for nucleic acids from pure cultures and from food samples enriched on nutrient pads has been developed in this study. The handling was relatively simple and no toxic substances were included. It was shown, that the use of this method allowed to selectively extract DNA from living mycelia growing on the membrane filters (see 5.6.2). The method was developed on the basis, that the cell walls of young mycelia are thinner (about 50 nm) than of older mycelia (about 125 nm) especially in the area of the apex (Deacon see page 29 (1997), Müller *et al.* (1995)). Based on this fact the disruption of fungal hyphae was suggested to be performed directly after germination of the spores.
Due to the diversity of fungal cell wall components an enzymatic digestion of the cell walls was impossible. In this work the mechanical disruption of the fungal cell walls was combined with the disruption of the filters on which they were enriched. The disruption was performed in a MagNA Lyser instrument (Roche) by Zirconium beads in a closed tube to avoid cross contamination. Enzymatic digestion of contaminating DNA was done before the mechanical disruption of living cells as described earlier.
It is known that only parts of the spores formed in the fungal sporulation process become mature and some need a rigorous external trigger to overcome fungistasis (Deacon see page 166 (1997). During food processing only a part of the mature spores remain capable of germinating. That means that the fraction of potential viable spores is decreased immensely during food processing and the major parts of the spores contained in food are dead. It was an important demand to exclude these spores from detection in PCR. This was realized in this work by optimising the mechanical disruption in a way that only mycelia were destroyed (see 5.6.3.4). DNA from non-germinated or dead spores was not extracted and consequently not detected by PCR.

6.1.4 Evaluation of the target gene *EF-3*

The consensus PCR system used in this study was based on sequences of the fungal specific gene *EF-3*. Prior to the design of consensus primers and probes sequence information from the whole spectrum of food-relevant strains was generated as shown in the attached alignments. In this work as in Mäntynen *et al.* (1999), *EF-3* was found to be able to serve as a fungal specific marker at higher taxonomic levels like divisions. The *EF-3* sequence in *Pneumocystis carinii* was found to have 57 % identity to *EF-3* from *Saccharomyces cerevisae* (YPMA-Wong (1992), Jackson (1991)), which makes it a potential target for species identification in yeast. The homology of EF-3 sequences found in yeasts with 77.5-76.6 % emphasised the chances of identification (Mita *et al.* (1997), Myers (1992)). However *EF-3* sequences generated in this work from filamentous fungi were in general not suitable to distinguish fungal genera or species by the design of species-specific probes. Only in the two genus *Emericella* and *Phytophthora* regions were identified to distinguish these from the other fungi by the inclusion of TaqMan probes for instance (see Table 71).

Table 71 Potential regions for identifying the two genus *Emericella* and *Phytophthora* from other fungi.

name	sequence
Emericella rugulosa DSM 945, *Emericella nidulans* BCD 3554	gcccaggaggctctcaagagcggtaaca
Phytophthora infestans MUCL 43045	tactggtagcgccagcggatgtactc

Sequences of *Ascomycota* and *Zygomycota* were significantly different, which consequently led to the design of different primers and probes for the two groups. The complexity of the consensus PCR was especially increased by addition of primers and probes for the detection of *Zygomycota*.

As expected, the diversity of fungal sequences within the *EF-3* unique area was greater than in the regions containing the functional domains. Therefore one of the primers originated from the *EF-3* unique area making the PCR system even more specific for fungi. The primers were designed to include a highly specific part at the 3' end of the oligonucleotide, because the DNA polymerase needs a correct base pairing in this area. The region at the 5' end of the primers was variable to enable a hybridisation to different phylogenetic groups. The sequence of *EF-3* contained just a few small conserved regions for the hybridisation of HybProbes. It was therefore necessary to degenerate the HybProbes (Compton (1990)), which resulted in difficulties while optimising the probe annealing temperature in the PCR profile due to a melting range (see Figure 22.

As can be seen from the protein alignments (see Figure 29) members of the group of the *Zygomycota* differ significantly from the group of *Ascomycota*, *Basidiomycota* and *Deuteromycota* by the deletion of the region characterized mainly by the amino acid sequence ALGENVGMKNERW (see Figure 29). Whether this is a potential spot to distinguish *Zygomycota* from the other groups or is a characteristic of a potential pseudogene might be a future research interest and requires generation of more sequences from *Zygomycota*.

Table 72 Insertion in *Zygomycota.*

lab-code	sequence of insertion
90	AG**AAAAAAA**GAAAGGGCGAGGGTGG**TTTTTTT**CGTGTTAGCAAGGTATTGATTGTTTATTCTTGTGCTCTAGGTCG
167	CGC**AAAAAAA**CTATCTGCTTATATCATGCACCAACACAACCAACGATCTTACTAGGTG

A significant insertion was found in two different strains (lab-code 90, 167) of *Syncephalastrum racemosum* (*Zygomycota)*, which was mainly characterized by a 75 base pair insertion in strain 90 and a 57 base pair insertion in strain 167 (see Figure 29). The insertion in strain 90 contains a potential hairpin structure (bold letters), which is only partly conserved in strain 167 (Table 72). The insertions might represent intron relics, but this still remains to be proved experimentally. As mentioned earlier it was difficult to align the sequences derived from *Ascomycota* and *Zygomycota* in a common alignment. The most different sequences derived from *Ascomycota* strains *Hyalodendron lignicola* DSM 1877, *Humicola fuscoatra* BCD 3590, *Aspergillus penicillioides* DSM 1623 and *Byssochlamys fulva* BCD 955 and showed more similarity to the *Zygomycota* than to the other *Ascomycota* in the investigated *EF-3* unique region. *Zygomycota* were mainly aligned on the basis of these four sequences of *Ascomycota* in the protein alignment in Figure 29.

6.1.5 The specificity and sensitivity of the consensus PCR system

Considering the immense diversity of fungal strains in food the selection of around 160 species from 82 genera tested in this work is far from giving a complete insight. The specificity of the primer and probe system was shown by the positive amplification results including all food relevant fungal strains from the attached Table 75 and Table 76. The primers and probes were designed for universal detection of fungal DNA. The probe design was more specific and therefore inclusion of more than the given probes might be necessary for detection of exotic species not tested in this work. As the specificity testing was performed for only a limited selection of the 50 000 species of filamentous fungi known today (Mücke *et al.* page 14 (1999)), it is more than likely that some species are not detected by the consensus PCR system. Other limitations of the consensus PCR can be caused by different matrix inhibition, low DNA concentration, inhibition of the reaction by fungal compounds like dyes from secondary metabolism, problems in primer binding etc. (Chandler *et al.* (1998), Kreader *et al.* (1996) and Trichopad *et al.* (2004)). Some strains might require much stronger cell wall disruption during DNA extraction while for other strains one might have to dilute the DNA sample to overcome inhibition from secondary metabolites like dyes. However, a wide spectrum of food-borne fungi is detectable by the given PCR system, but the immense diversity of fungi could not be investigated here.

The mean genome size of filamentous fungi was estimated in a range of 15-40 Mb (Cervelatti *et al.* (2004)). The detection limit of the consensus PCR was estimated in fungal genomic DNA derived from different divisions such as *Ascomycota* and *Zygomycota* at about 1 pg (see Table 55). Because the genomes size in filamentous fungi was estimated within a range of 15-40 Mb the detection of 1 pg fungal genomic DNA in PCR is concordant with the detection of 25-65 genome equivalents. Caused by the diversity of the published data concerning fungal

genome sizes more detailed data could not be estimated. Certainly, not all fungal genomes sizes are known today, which complicates the situation. However, by detecting 10 genome equivalents similar sensitivity was achieved for instance for the single copy gene *EF-1* alpha in *Candida spec*. in blood samples Trépanier (2001) indicating, that the consensus PCR system demonstrated in this work achieves sufficient sensitivity.

The whole PCR assay including enrichment and nucleic acid extraction was optimised to detect 10-100 pure germinated spores of *Aspergillus niger* DSM 1988 after 16 hours enrichment at 25°C on membrane filter. However the sensitivity can change depending on different germination and nuclei replication velocity of the fungal strains with respect to different food types. Further applications of the assay require careful validation and optimisation for the particular application.

It was not possible to estimate the amount of fungal nuclei in the different germinated spores. Therefore, the correlation between the detection limit of germinated spores and the detection limit at 25- 65 Mb was only a theoretical calculation. Knowing the duration of the cell cycle of the particular fungal strain under defined conditions those theoretical assumptions are possible.

For instance, *Aspergillus nidulans* is well suited for cell cycle analyses and mitosis, since it has a short cell cycle of about 90 to 120 min (Bergen *et al.* (1983), Kobayashi *et al.* (1998)). That means that the nucleus of one spore from *Aspergillus nidulans* may replicate 24 times within 48 hours of enrichment. The detection limit of the whole assay for 3 days old conidia of *Aspergillus niger* DSM 1988 in juices after 48 hours enrichment was 10 germinated spores. This would correlate to a detection limit of 240 nuclei (240 GE in a single copy gene) assuming the same replication rate as published for *Aspergillus nidulans*.

However, the time of the first division is found to be a function of the age of the spores and the temperature during germination (Kobayashi *et al.* (1998)). For another fungus, *Neurospora crassa,* the duration of the cell cycle has been estimated as approximately 100 min (Martegani (1980)). In *Colleotrichum gloeosporioides* two rounds of mitosis take place during germination and appressorium formation within 90 min (Kuo (1999)). Other fungal nuclei might replicate much slower as one can expect from slow growth rates like 2.5 mm in 7 days (*Xeromyces bisporus, Aspergillus restrictus, Basipetospora halophila*). Additionally, many fungal spores exhibit a crowding effect in which the spores contain a pre-packaged self-inhibitor that prevents germination under crowded, high cell density conditions (Hornby *et al.* (2004)). For fungi with different replication rates after the enrichment different amounts of gene copies will be found; an exact quantification of the amount of viable fungi is therefore difficult even than measuring the DNA amount.

In this work the consensus PCR was performed on the LightCycler® instrument. Since the inclusion of 20 short preceding cycles into the PCR profile resulted in a better sensitivity, the final PCR profile consisted of three major steps. The first was the denaturing of the target DNA and activation of the 'hot start' polymerase; the second step included 20 short preceding cycles and the third step the amplification and measurement of the ongoing PCR process. It was demonstrated, that the consensus PCR is easily adaptable to other real time PCR platforms using for example the TaqMan probe format (see 5.1.3.2). The most suitable detection format in the LightCycler® instrument was estimated as the HybProbe format.

Other detection formats were not suitable because of several disadvantages. For example the specificity and sensitivity of PCR including SYBR Green I was not satisfying due to the formation of primer dimers. Stringent PCR conditions have to be applied in SYBR Green I assays to prevent primer dimer formation and detection. However, this was limited by the degeneracy of the primers in the consensus PCR. The alternative method to prevent wrong positive signals in SYBR Green I assays by measuring the amplification signals above the melting temperatures of primer dimers (Ball *et al.* (2003)) requires a detectable difference in the melting temperature of specific target and primer dimers. This was not suitable for short *EF-3* amplification products. As shown in the melting curve Figure 28 the primer dimer peak was overlapping with the specific PCR peak in the SYBR Green I assay for the detection of the different filamentous fungi. The detection of specific amplification products by HybProbes was favoured as in the longer double probe hybridisation region (compared to single probe assays) more sequence information is included. Acting on the assumption that the consensus PCR is more specifically in this way.

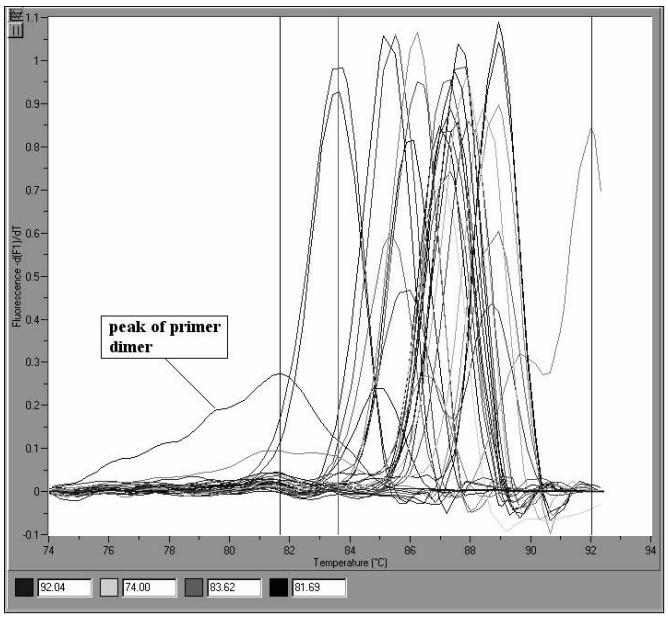


Figure 28- Melting curve of different fungal strains. Fluorescence of SYBR Green I detected samples versus temperature is plotted. Peak of primer dimers is too closed to the specific peaks to exclude it from detection by measuring fluorescence of specific products above 82°C.

6.1.6 Monitoring matrix inhibition and PCR quality

As food samples are highly complex chemical mixtures, they can contain several components, which inhibit PCR (Konietzny *et al.* (2003)). Due to these matrix inhibition effects, the detection of false-negative results is important to obtain reliable results in investigating food. Reliable controls monitoring the cause of a potential fail were included into the consensus PCR system in this work. Some food types are known to inhibit PCR amplification, for example spices, milk powder and cacao, respectively (Biochemica No.4 (2002)) Inhibition of PCR can further occur

in some strains containing PCR inhibiting secondary metabolites such as fat, polysaccharide, polyphenols and dyes. This was for instance found for the black dye of *Stachybotrys chartarum* DSM 2144 (data not shown) or for unknown fungi in Table 48. This matrix inhibition cannot be totally avoided if food samples are investigated (Siebert and Larrick (1993), Pallen *et al.* (1992)). Matrix inhibition was monitored by the inclusion of an internal positive control into each PCR reaction. The internal positive control consisted of a plasmid constructed by PCR mutagenesis carrying a copy of the target region. The sequence of a particular fungal specific probe inside of the target region was replaced by an artificially designed intercept, which was detected in the real time PCR by a specific probe labelled for detection in a separate channel. The inclusion of an internal positive control required careful titration of the DNA amount to avoid quenching effects and loss of sensitivity. Quenching effects between target DNA and IPC arise when both compete for PCR components like primers or dNTPs. By detecting 3-30 gene copies of the IPC the sensitivity of the PCR system to amplify and specifically detect IPC is comparable to other systems (Levett *et al.* (2004)).
The general quality of a PCR mixture was monitored by the inclusion of an additional sample containing a positive control. This external control was amplified from a plasmid carrying one original target sequence from the same size as the expected PCR product from the fungal samples. The amplification of the external positive control monitors the ability of the PCR mixture to amplify DNA in case of matrix inhibition. Carry-over contamination was generally avoided by inclusion of the Uracil-N-DNA Glycosylase (Longo *et al.* (1990)) as explained earlier.

6.2 Critical reconsideration of the consensus PCR assay

The major critical points of the consensus PCR assay are discussed as follows.

6.2.1 The duration of the enrichment

The detection of viable fungi by PCR depends on the cultivation of the fungi, the germination time frame of fungal spores and the ability of the culturing methods to break dormancy and senescence. The enrichment by special culturing methods limited the whole assay. The moment for DNA preparation and PCR applications still needed to be validated carefully for a spectrum of fungi, which are expected from a particular food type. It was shown that the detection of viable fungi was faster by PCR than by conventional microbiological methods. But this advantage is based upon the knowledge of the germination time frame of the strains in the experiments. Therefore the estimation of the time frame for germination and growth of marker strains in a particular food product would be helpful during large-scale validation. Finally, results of those validation experiments would enable a standardization of the system and allow quality control of PCR as described by Burkardt (2000).
In contrast to test at only one special time point, the samples should rather be taken at several time points to inspect the progress of the enrichment within a time frame. In correlation to these findings the European Pharmacopoeia instruct the investigator to do sterility testing for bacterial and fungi over a time period of 14 days. However, it might be difficult to determine the time

frame for the enrichment, but the experiments basically show the ability of PCR to detect fungi before microbiological methods can do so.

6.2.2 Evaluating the sequencing of the *Zygomycota*

The development of the consensus PCR was based on the generation of new sequence information by cloning and sequencing of the target region from a limited number of fungal strains. A critical point in this work was the specific amplification of *EF-3* from strains of the heterogeneous group of *Zygomycota*. These difficulties during sequencing and developing a primer system for amplification of *Zygomycota* have to be considered on the background of the polyphyly of the group. The primers used initially to amplify fragments for sequencing have been designed from multiple existing regions in *EF-3*. Those PCR systems have been called multitemplate systems. Exact sequencing of those regions and genes provided many difficulties, because of artefact amplifications in PCR (Karnati (2003), Gasch (1992)). Genes that do duplicate to form gene families require extra sequencing effort (Berbee (2001)). One may speculate about the existence of *EF-3* gene copies based on the difficulties during sequencing the *Zygomycota*. In fact, unspecific amplification has been explained from the technical point of view by the slow change of temperature during annealing in block PCR machines (Kurata (2004)). PCR products were separated in agarose gels from unspecific targets in this work. However, artefacts of the same size as the target DNA can be extracted by this method, too (Gasch (1992)). Cloning and sequencing of those artefacts resulted in sequences similar to the target gene, which did not exist in cells (artificial gene diversity). This might be another reason for diffculties in sequencing *Zygomycota*. However, the reason of the formation of artefacts during *Zygomycota* sequencing remains unclear.
Due to the formation of PCR artefacts the sequence alignments were difficult to interpret during the primer and probe design. On the basis of the 'artefact' alignment (A) primers and probes were designed, but amplification remained still unspecific and probes did not detect the original fungal genomic DNA. Sequences generated by a Two-Step 'PCR walking' method differed considerably from the sequences in the 'artefact' alignment but comparably little among one another. The primers and probes designed on the basis of the new alignment resulted in a specific amplification of PCR products and a specific detection of the *Zygomycota* strains.

6.2.3 Additional information from public databases

The partial sequences of *EF-3* generated in this work were compared with the information available from public sequence databases. Some sequences listed as predicted mRNA were significantly similar to *EF-3* sequences of the filamentous fungi generated from filamentous *Ascomycota* in this project. The sequences of XM 328627 *Neurospora crassa*, XP 664304 *Aspergillus nidulans,* XM 364494 *Magnaporthe grisea* found in the public databases (at NCBI, EMBL) are defined as predicted mRNA or hypothetical protein. They show significant similarity to *EF-3* sequences generated in this work. These sequences might represent *EF-3*.
In yeast *EF-3* was identified on chromosome XII. In filamentous fungi only 5-9 chromosomes exist (Arora and Khachatourians (2003) page 7). The sequence of *Magnaporthe grisea* XM

364494 is derived from chromosome IV. Therefore one can speculate that *EF-3* in other filamentous fungi may be located on chromosome IV, too.
Other similar sequences were found on different chromosomes, supporting the idea of existing gene copies of *EF-3*. Considering that, the existence of pseudogenes cannot absolutely be excluded for *EF-3* in the genome of filamentous fungi. Pseudogenes are characterized by a high sequence homology to the functional gene copy. The existence of pseudogenes or alleles need to be considered during primer and probe design to avoid unspecific hybridisation. Conventional pseudogenes contain insertions, deletions or stop codons in the exons, whereas processed pseudogenes do not contain any promoter region. Processed pseudogenes probably descended from inclusion of reverse transcribed mRNA (Hagemann (1999)). Indeed, in the yeast *Saccharomyces cerevisae* two different gene copies of *EF-3* have been identified, YEF3A and YEF3B (Uritani (1999)). YEF3A was the first identified *EF-3* gene and is shown to be essential while YEF3B is neither expressed nor essential (Sarthy (1998)). YEF3B seems to be a pseudogene-like copy of YEF3A. In yeast there are many duplicated chromosomal regions, which led to the proposal of whole genome duplications one hundred million years ago (Arora (2003) page 216). Analysis suggests about 55 major regions of clustered gene duplications (Arora (2003) page 216). But this observation has not been confirmed in filamentous fungi yet. Little sequence information about *EF-3* in filamentous fungi existed at the beginning of this work. To enlarge the sequence information for the primer and probe design a database search was done. The search showed the existence of homologue sequences in the databases, which might be closed members within the gene family or even pseudogenes. However the 4 sequences in Table 73 show more homology to each other than to *EF-3* and cannot be identified as *EF-3*.

Table 73 - *EF-3* sequences below were found in databases. They are similar to *EF-3* but show significant differences to the *EF-3* sequences generated in this work.

accession number	organism	family
XM_388708	*Giberella zeae* PH-1 chromosome 2 strain PH-1; NRRL 31084	*Ascomycota*
XM_329650	*Neurospora crassa* strain OR74A	*Ascomycota*
XM_410788	*Aspergillus nidulans* FGSC A4, AN6651.2 predicted mRNA	*Ascomycota*
XM_366496	*Magnaporthe grisea* 70-5 chromosome VII	*Ascomycota*

The appearance of the gene copy YEF3B in yeast supports the general possibility of the existence of *EF-3* pseudogenes in filamentous fungi. However, this remains to be proved. In contrast the relative high detection limit of the consensus PCR at 25-65 genome equivalents estimated in this work shows at least, that probably no multiple copies of *EF-3* are detected. However, using multicopy genes as a basis to design universal primers one need to make sure that the copies are identical, and only the interesting gene copy is specifically amplified and detected. The situation is especially complicated, as different alleles of *EF-3* have been identified in yeast (Dalle *et al.* (2000)). In this work the sequences in Table 73 were aligned to the newly generated sequences of *EF-3*. The chance of the universal primers to hybridise to the sequences given in Table 73 was minimized during primer and probe design.
As pseudogenes can originate from the activities of viruses and transposable elements one has to consider the existence of gene copies in that context. The Chlorella Virus CVK2 carries a virus specific homologue to *EF-3* (Yamada *et al.* (1993), Reischl (2001)).

Table 74 - Demonstration of the existence of functional domains in *EF-3* in different sequences. The bold letters show the previous defined domains of *EF-3* in yeast (Uritani *et al.* 1999).

organism	Walker A1	ABC 1 signature	Walker B1	Walker A 2	ABC 2 signature	Walker B 2
XP 664304	**GPNGSGKTTL**	**LSGG**WKMKLAL	**ILLLD**EPTNHL	**GPNGAGKSTL**	**LSGG**QR	V**IVLD**
XM 328627	**GPNGSGKSTL**	**LSGG**WKMKLAL	**ILLLD**EPTNHL	**GPNGAGKSTL**	**LSGG**QR	L**IVLD**
XM 388708	**GRNGAGKSTL**	**LSGG**WKMKLAL	VLLLDEPTNHL	**GPNGAGKSTL**	**LSGG**QK	LLVLD
XM 410788	**GRNGAGKSTL**	**LSGG**WKMKLAL	VLLLDEPTNHL	GGNGAGKSTF	**LSGG**QK	LLVLD
XM 329650	**GRNGAGKSTL**	**LSGG**WKMKLAL	VLLLDEPTNHL	GPNGAGKSTL	**LSGG**QK	MLVLD
XM 366496	**GRNGAGKSTL**	**LSGG**WKMKLAL	VLLLDEPTNHL	GANGAGKSTL	**LSGG**QK	LLVLD
Chlorella virus CVK2 D16505	**GPNGAGKSTL**	**LSGG**WKMKLAL	LLLLDEPTNHL	GENGAGKSTL	**LSGG**QK	V**IVLD**

The *EF-3* homologue cloned from the *Chlorella* virus shows a low identity (36 %) to the yeast *EF-3* and the functional significance of the latter gene has not been established (Blakely (2001)). Whether the *EF-3* like gene in the *Chlorella* virus is a relict of a potential horizontal gene transfer remains unclear. Due to the existence of group I introns[20] found in protein encoding genes in fungi, algae and *Chlorella* virus, horizontal gene transfer is assumed between algae and fungi (Yamada (1993)). The theory of horizontal gene transfer is also supported by the existence of plasmids found in mitochondria and the nucleus of fungi. Viruses and virus like particles (VLPs) in fungi are common and have been identified as double stranded RNA or DNA including a capsid and often occur as large crystals in the cytoplasm in old hyphal regions (Deacon see page 139-141 (1997)). During the design of universal primers and probes it was tried to avoid hybridisation of primers and probes to *EF-3* similar sequences and the *Chlorella* virus sequence.

The functional domains in *EF-3* can be found in several other members of the ABC family in bacteria and humans as well as in fungi (Ganoza and Kiel (2001), Belfield and Bauer *et al.* (1995)). The sequence areas of domains have therefore been excluded from the design of primers and probes. It was shown experimentally in this work, that DNA from higher eukaryota was not amplified using the fungal specific consensus PCR system.

6.2.4 Non-specific amplification using the consensus primer system

Another critical point, which was complicated the consensus PCR assay was the cross detection of DNA from yeasts and filamentous fungi by the consensus primer and/or probe system. This was expected due to the closed taxonomical proximity of yeast and filamentous fungi. The yeasts detected by HybProbes in the consensus PCR system are defined as additional detected contaminants found in cheese and sausages (*Yarrowia lipolytica* BCD 2556), fermenting brine (*Candida versatilis* DSM 6956), cherries (*Metschnikowia pulcherrima* DSM 70321), milk and fruit syrup (*Citeromyces matritensis* BCD 1062), respectively. As demonstrated experimentally the co-amplification of yeast DNA, which was not detectable by the specific HybProbes, and DNA from filamentous fungi could lead to a switch of the CP values of the filamentous fungi.

[20] Group I introns have been demonstrated to self-splice and to propagate themselves in the genome. The intronic ORF encodes functions for splicing (RNA maturase) and mobility (‘homing endonuclease'). Host-encoded machinery is recruited for splicing.

This might be caused by a competition for the PCR components. In food containing high levels of yeast DNA, the sensitivity to detect filamentous fungi is therefore unpredictably decreased.
Some differences between filamentous fungi and yeasts are found in the sequence of *EF-3* but the conservation of differences is low. Consequently the total exclusion of all yeasts from hybridising to primers and probes is impossible. A solution of this problem would be the addition of probes for specifical detection of the yeast species amplified by the consensus primer. As long as these yeast specific probes are not included in the consensus PCR system, the whole assay has to be performed in a two-step mechanism investigating samples before and after enrichment.

6.2.5 Background contaminating fungal DNA in food samples

The detection of fungal DNA by PCR in food commodities indicates the occurrence of living fungi in the raw material. Theoretically the total amount of the genomic DNA point out if and how much fungal growth occurred or will potentially occur in the raw material of the particular food product.
Basic fungal contamination in raw material of food cannot be avoided because fungi occur ubiquitously. Probably one can set threshold values for the distinction of basic fungal DNA levels from increased levels by PCR. The amount of fungi and consequently the amount of DNA should be limited in the raw products. Since PCR can be performed quantitatively in real time PCR by comparison to a standard DNA one can probably indicate the degree of contamination by measuring the amount of fungal DNA in a PCR assay based on a single copy gene as *EF-3* is supposed to be.
However, careful validation is necessary to correlate these PCR results to the amount of viable fungal organisms and consequently the occurrence of mycotoxins. This is complicated by the fact that the amount of DNA cannot be correlated to the microbiologically estimated CFU thresholds values, because fungi can contain several nuclei in a single cytoplasm. Due to this multinucleate character of mycelia quantitative questions aiming a result in CFU may not be answered by PCR. Correlating the amount of DNA with the amount of mycotoxins is difficult as well, since not all strains always produce mycotoxins during growth. Often mycotoxins were determined not until fungal sporulation was completed (Samson (1995)).

6.2.6 Quantitative determination of filamentous fungi

A quantitative fungal specific real time PCR assay targeting the amplification of 18S rRNA genes was introduced by (Loeffler *et al.* (2002)). Loeffler *et. al* correlated the amount of target DNA to the amount of medically relevant spores (conidia) in blood. For the determination of infection levels it was assumed in the real time PCR that one spore contains one rRNA gene cluster. However, quantification of conidia does not reflect the true situation in food. The filamentous growth type and the ability of fungi to survive hyphae divisions are the main problems in the quantification of fungi by PCR in food. The principles of quantifying bacteria, yeasts or spores, where one can usually assume that one CFU is equivalent to one single haploid organism carrying one single copy of a particular target gene sequence, cannot be applied to filamentous fungi. Fungal mycelia contain the potential to form several single haploid organisms

by random division during the handling of the food. Consequently, mycelia are able to divide and grow separately as new independent organisms, which randomly changes the CFU results.
Therefore it is difficult to determine the amount of target gene copies in fungal hyphae and to correlate this to microbiological data based on CFU.
The amount of gene copies of a single locus gene can be determined by a correlation to standard curves in a quantitative real time PCR assay. This is difficult within the group of food relevant filamentous fungi considering the differences in growth rates, which indicate that the replication rates also vary. From that point of view it seems not possible to optimise a standard curve for the quantification of filamentous fungi. The implementation of a general quantification assay for all food relevant fungi is therefore not advisable. Another point indicating the difficulties in the quantification of viable filamentous fungi by PCR is shown in this work by the cross contamination of the investigated food samples with yeast cells. If one tried to correlate those data to an external standard curve to determine the gene copies, one would end up in false-negative or low-level results. It is not possible to quantify filamentous fungi in the presence of yeasts using the assay developed in this work.
One question in diagnostics is to know the amount of spores. One spore as a potential viable structure contains exactly one gene copy of *EF-3*, if a single nucleus is contained. Therefore the amount of initial gene copies amplified in the consensus PCR estimated by a correlation to an external standard curve can be defined as the amount of spores. This might be of interest for the quantitative detection of airborne fungal spores in the working environment of food manufactories. However those considerations are restricted to spores containing a single nucleus and not for *Fusarium* spores containing up to 8 nuclei in one spore for example.

6.2.7 Implementation of RT-PCR in food testing

The ability to detect viable fungal cells by RT-PCR based on the primers and probes derived from the single copy gene *EF-3* was demonstrated in this work. Experiments were done with orange juice samples including conidia, which were enriched on nutrient pads. Viable fungal mycelia were detected using the real time Two-Step RT-PCR system including the HybProbe format. RT-PCR as a PCR based method was tested for implementation in routine use. It was found that the error proneness of the demonstrated method was too high for routine use and the handling was still to complicate. The susceptibility to interferences and the unsatisfactory sensitivity of the mentioned RT-PCR assays (especially of the One-Step RT-PCR) were the major reasons to reject the method RT-PCR for the detection of viable fungi in food. The following arguments and summarised experiments emphasise this decision.

6.2.7.1 Stability of mRNA

A major problem in applying RT-PCR to food diagnostics is the instability of the mRNA target molecule during the nucleic extraction, which leads to loss in sensitivity compared to PCR methods. Naturally mRNA is not stabilized outside the intact cell and degrades when heated in the presence of bivalent cations. RNases are difficult to inactivate and are found ubiquitously. Inactivation of RNases in the laboratory was achieved by treating the equipment in extreme heat and with toxic DEPC. RNase activity is also a problem during transportation, storage and

extraction of investigated samples (Lion (2001)). Nevertheless it was possible in this work to extract stable RNA from enriched food samples. This was achieved by the inclusion of different RNA stabilizing agent during the RNA extraction and the RT-PCR. As stabilizing agents Protector RNase Inhibitor (Biochemica 4 (2002)), EDTA and Chelex 100 were favoured. Altogether stabilizing of RNA is one of the most fragile steps in the application of RT-PCR assays for routine use.

It is known that, specific extraction of poly (A) RNA (mRNA) might result in higher yields of cDNA than could be transcribed from total RNA (Lion (2001)). On the other hand, poly (A) RNA (mRNA) extraction methods are bound to the application of commercial kits, which base for instance on complicate methods including hybridisation to magnetic beads. These methods are not advantageous compared to Chelex 100 based methods, as both require DNase I digestion. Finally Chelex 100 based methods were favoured due to low costs and better chance of implementation into the food analysis.

A sensitive detection of small-scale m-RNA depends mainly on the m-RNA stabilisation during the RNA extraction. A sensitive detection of non-germinated viable conidia from fungi by RT-PCR targeting *EF-3* was not possible. In fact, *EF-3* mRNA was detected by RT-PCR in fungal conidia, but the amount of mRNA in the non-germinated conidia seemed to be too small to be detectable in food. Consequently, enrichment of mRNA by germinating the spores has to be performed for sensitive detection by RT-PCR.

Another point certainly causing the limitation of the RT-PCR sensitivity is given by the fact, that *EF-3* is described as a single copy gene Qin *et al.* (1976), Belfield *et al.* (1993). From the theoretical point of view a single copy gene might be weaker expressed than a multicopy gene. In dormant conidia (10^{10} spores) a low amount of *EF-3* mRNA was detected by RT-PCR. After germination of spores the *EF-3* mRNA from at least 10-100 young hyphae (originally conidia) was detected by RT-PCR. The expression of the *EF-3* mRNA was apparently up regulated during the germination. However, due to the fact, that mRNA is derived from a single copy (*EF-3*) the RT-PCR assay must be highly sensitive. Unfortunately the sensitivity to detect 10-100 young hyphae was difficult to reproduce in different strains. The data shown led to the assumption of differential expression patterns within the heterogeneous groups of fungi and during fungal development. Data from yeasts show a significant transcription of the *EF-3* target mRNA (Swoboda *et al.* (1994)). However, equal expression of the *EF-3* mRNA in filamentous fungi was not experimentally proved. As filamentous fungi are highly diverse and differ in some points from yeasts one cannot assume equal transcription levels of the *EF-3* mRNA for all food relevant fungi. Additional data on the transcription of *EF-3* in filamentous fungi would be necessary to complete the development of an RT-PCR based assay. Optimisation of RT-PCR is certainly difficult to achieve with respect to the required sensitivity.

6.2.7.2 Complexity of the One-Step RT-PCR

The experiments including ‘no primer’ controls in the RT reaction (see 5.3.1) demonstrated the complexity of the One-Step RT-PCR and may explain why the One-Step RT-PCR was less

sensitive than the Two-Step RT-PCR. As mentioned before, randomly produced oligonucleotides in the RNA sample may serve as additional primers for the Transcriptor Reverse Transcriptase leading to aberrant result in the 'no primer' controls. This may block the hybridisation of the specific RT primers. This additional so-called endogenous priming might end up with formation of artefacts and primer dimers and is lowering the sensitivity of the One-Step RT-PCR. This was also found for cardiac MHC RNA by Haddad F. and Baldwin and is described in Sambrook (1989). They have supposed that short antisense RNA molecules prime the reverse transcription reaction to create a background cDNA pool that works as a template for the PCR amplification (Lavorgna (2004)). However, based on this observation Haddad *et al.* (2005) decided to run a 'no primer' RT reaction for all RT-PCR reactions and treated the obtained signals as a background to be subtracted from the specifically primed RT-PCR reaction signal to avoid an overestimation of transcription results. Consequently one had to include a second control into the RT-PCR assay, which is probably not suitable for a routine use. This demonstrates that RT-PCR results can be confusing and the optimisation of RT-PCR for industrial applications is a highly complex task, which opens still new questions.

An additional problem in the practical application of RT-PCR is the complicate troubleshooting in RT-PCR systems, which needs to be performed in quality control checks during the production of a potential RT-PCR Kit. This makes the system extremely expensive. Before the RNA samples are applied to the established RT-PCR assays, one need to control the samples in a PCR to monitor contaminating DNA. Otherwise this would lead to false positive results from even trace amounts of residual DNA. The amount of DNA varies in real food samples according to the amount of contaminating organisms. It was shown that large amounts of DNA were difficult to digest by DNase I treatment, therefore the optimisation of this step is still an unsolved problem. Unfortunately the PCR target gene *EF-3* does not contain introns. It was not possible to select primers that span an intron in the PCR to monitor the DNA contamination. To distinguish large PCR products derived from DNA containing the intron from small PCR product derived from cDNA was therefore impossible. Consequently the Two-Step RT-PCR needed to include a control, which monitored DNA contaminations. This led to the investigation of two samples, which makes the assay more time consuming. Another critical point is the development and inclusion of reliable internal matrix inhibition and external positive controls into the RT and the PCR steps, respectively. As positive controls for the RT reaction one can consider artificially produced RNA using transcription vectors including the *EF-3* gene sequence (Schwab (1997), Gross (1998). However, this was not performed here, because the error-proneness and complexity of the assays rejected the further optimisation of RT-PCR.

The RT-PCR system is generally much more complex than conventional (genomic) PCR assays, and the costs of the system increase immensely by the inclusion of necessary controls. One control has to be performed in the PCR step for each sample to show the absence of contaminating DNA. The DNA digestion in the RNA samples is difficult to optimise and was shown to depend on the amount of DNA. As the amount of DNA varies in the various samples

the optimisation of this step seemed to be too difficult for routine use. The contaminating DNA would lead to false positive results in the RT-PCR.

6.2.7.3 A short outlook

The implementation of RT-PCR into food testing might be applicable in a combined test. The strategy, which includes two samples taken before and after enrichment can be carried out for RT-PCR. However, one would have to investigate 4 samples simultaneously, two RNA samples and two samples controlling the absence of DNA. The detection of viable fungi would be confirmed twice in this assay. Firstly by the comparison of samples before and after enrichment and secondly by the *EF-3* mRNA target molecule in RT-PCR. By performing this complicate experimental procedure the detection of viable fungi is certainly improved. However, experiments like this would be too expensive and too complicate for routine use.

Finally it has to be mentioned that mRNA editing enzymes were found to occur in fungi, too (Dance (2001), Kobayashi *et al.* (1998), Xie (2004)). The probes shown in this work were designed using sequence information derived from DNA. Theoretically one cannot exclude RNA editing in the *EF-3* mRNA of filamentous fungi. Therefore differences in the sequences on the DNA and the RNA level may exist influencing the primer and probe hybridisation to the cDNA in the RT-PCR based assay. Regarding the complete development of an RT-PCR based assay one had to proof the efficiency of the primer and probe hybridisation including mRNA from all food relevant fungi.

However, the main consequence from the mentioned error-proneness is that RT-PCR cannot meet the requirements of routine analysis for detection of food borne fungi yet. DNA seems to be the more robust target nucleic acid. Additionally, PCR is less complicate and the more robust method, too.

7 Conclusions

The herein presented consensus PCR system could detect specifically and sensitively DNA of a wide range of taxonomically different filamentous fungi relevant in food spoilage. In combination with methods for the enrichment of fungi from food samples the detection of viable filamentous fungi using this PCR system was demonstrated. A PCR system was established, which now remains to be tested in large spectrum of food samples.

The methods for the enrichment and DNA extraction from food could be optimised in a way to detect only DNA from viable fungal hyphae excluding the detection of dead fungal spores. The determination of the necessary time frame for the enrichment still needs to be validated carefully with respect to the expected fungal strain spectrum in a particular food product. Referring to the diversity of fungi, the definition of the time frame for the enrichment remains one of the major critical points. The sensitivity of the assay needs to be further optimised and validated in order to correlate data from fungal germination and PCR detection limits.

As certain yeast DNAs are co-amplified and detected in the consensus PCR, the system still needs to be extended by inclusion of yeast specific probes for a routine use. By careful validation and optimisation of the assay it might be possible to use the system to determine roughly the amount of fungal DNA in raw food, which can for instance be a parameter for quality testing concerning determination of mycotoxin production before its appearance during storage. Additionally, the assay can be applied to monitor the working environment and the air quality in food production facilities as this becomes more and more a major focus of hygiene control in food industry.

The group of filamentous fungi contains not only important food contaminants. The vast majority of fungal species in related to the environment decomposing organic material and can be naturally found in soil and air. Many industrial products contain organic substances, which serve as ideal medium for fungal growth. This poses a problem during storage of those materials in humid environments especially in the tropical areas. Therefore, monitoring of fungal growth in products made of paper, cotton, leather, rubber, wood, wool, cooling lubricants or even of inorganic materials like aluminium might be helpful in sophisticated industrial production control (Reiß pages 232-251 (1997)). In that context for instance the packaging industry, which needs to deliver sterilized products for the pharmaceutical and food industry, is one of the target groups of the herein presented assay (according to Müller (1995)).

8 Outlook

Nowadays, PCR based methods obtain increasing acceptance for testing the microbiological safety of food, because of their high sensitivity and specificity. Despite the apparent problems of fungal contamination in food and the demand of reliable test systems, today, only a few general obligations exist for fungal testing in food. The non-existence of guidelines for fungal specific detection and the lack of internationally validated standard protocols, reagents and equipments hamper the implementation of sensitive and cost-effective molecular methods in routine diagnostic.

Compared to the immense diversity in food, the experiments presented in this work investigated only a small number of available fungal strains and relevant food matrices. This implicates that a validation of the fungal specific PCR assay remains to be done. For this purpose validated and standardised use of the polymerase chain reaction (PCR) can facilitate the implementation of fungal diagnostic PCR in routine. The main properties of the assay, such as sensitivity and specificity, still need to be validated with respect to the key food-contaminants in a particular food type. Therefore, the establishment of collections containing certified DNA samples and the development of databases with information about food related fungal key strains are highly demanded. Further investigations should include the validation of pre-PCR sample treatment methods for enrichment of contaminants and their DNA. Certainly, those validation experiments can monitor the contamination of raw materials and give a more detailed insight in the relation of fungal contamination with food-borne diseases.

In the establishment of quantitative PCR based methods, especially in the detection of filamentous fungi, the measure of food contamination should be reconsidered.

As filamentous fungi often contain several nuclei in one CFU and the amount of the nuclei changes with the type of the fungal strain, PCR results cannot be correlated to conventionally achieved CFU results. It was shown in this work, that DNA of dead fungal cells could be ignored in that context, because it is removed during the DNA extraction process. The intended quantitative or semi-quantitative PCR based assays detecting filamentous fungi have to present the results in units of DNA mass.

As other PCR based assays (Loeffler (2002)), the one presented in this work implicates the possibility of automation, too. Since new PCR platforms like the LightCycler 480 Real-Time PCR System (Roche) can investigate 96 or 384 PCR samples simultaneously, the technical requirements for automation are already available.

Moreover, the probe format is not necessarily defined. Furthermore, it implicates the adaptation of the probe system to other platforms for instance based on the exonuclease assay using TaqMan probes instead of HybProbes.

9 Appendix

9.1 Literature

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9.2 Abbreviations

– no amplification, +/- weak amplification, + positive amplification

6-FAM	6-carboxyfluorescein
A	Adenosine
AOAC	Association Of Analytical Communities
ARISA	Automated rDNA Intergenic Spacer Analysis
ATP	Adenosine triphosphate
a_w	water activity
BCD	BIOTECON Diagnostics
C	Cytidin
CBS	Centraalbureau voor Schimmelcultures
CFU	Colony Forming Unit
conc.	concentration
DGGE	Denaturing Gradient Electrophoresis
DSM, DSMZ	German National Resource Centre for Biological Material
EDTA	Ethylenediaminetetraacetic acid
EF-3	Elongation factor 3
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence activated cell sorting
FAO	Food and Agriculture Organization of the UN
FRET	Fluorescence Resonance Energy Transfer
G	Guanosin
GHP	Good Hygiene Practice
GMP	Good Manufacturing Practice
GTP	Guanosine triphosphate
HACCP	Hazard Analysis and Critical Control Point
HEG	hexethylene glycol
HPLC	High Pressure Liquid Chromatography
HSTE	salmonsperm DNA plus Tris-EDTA buffer
IMS	Immunomagnetic separation
IPC	Internal Positive Control
IPTG	Isopropyl-β-D-Thiogalactopyranoside
ITS	Internal Transcribed Spacer
Mb	Mega bases
mRNA	messenger RNA
n.d.	not determined
NC	Negative Control
PC	Positive Control
PCR	Polymerase Chain Reaction
q.v.	quod vide
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
rRNA	ribosomal RNA
RT-PCR	Reverse Transcription PCR
SCAR	Sequence-Characterized Amplified Regions
SSCP	Single Strand Conformation Polymorphism
T	Thymidin
TAMRA	6-carboxytetramethylrhodamine
TBE	Tetrabromoethane
TGGE	Temperature Gradient Gel Electrophoresis
TLC	Chromatography
TRFLP	Terminal Restriction Fragment Polymorphism
tRNA	transfer RNA
U	Uracil
UNG	Uracil-DNA Glycosylase
UV	Ultraviolet Radiation
X-Gal	5-brom-4-chloro-3-indolyl-beta-D-galactopyranoside

9.3 Alignments

Figure 29 - Overview of the three different alignments received from Zygomycota DNA. The alignments A, B and C are attached to this document. The location of insertions and deletions compared to sequences from *Ascomycota* is indicated. The hybridisation sites of primers and probes indicated by bars demonstrate the situation in the consensus PCR, which included the detection by HybProbes.

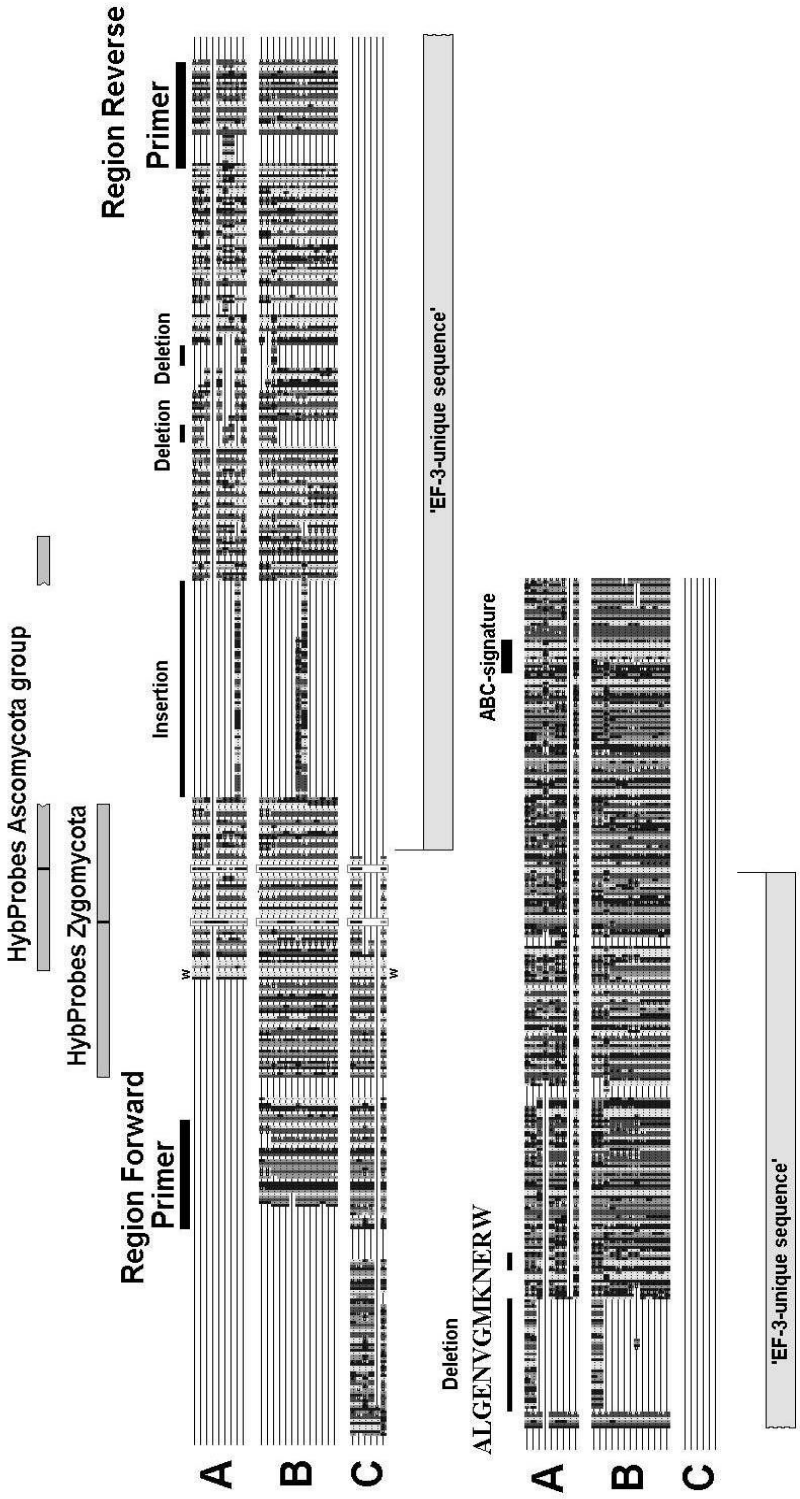
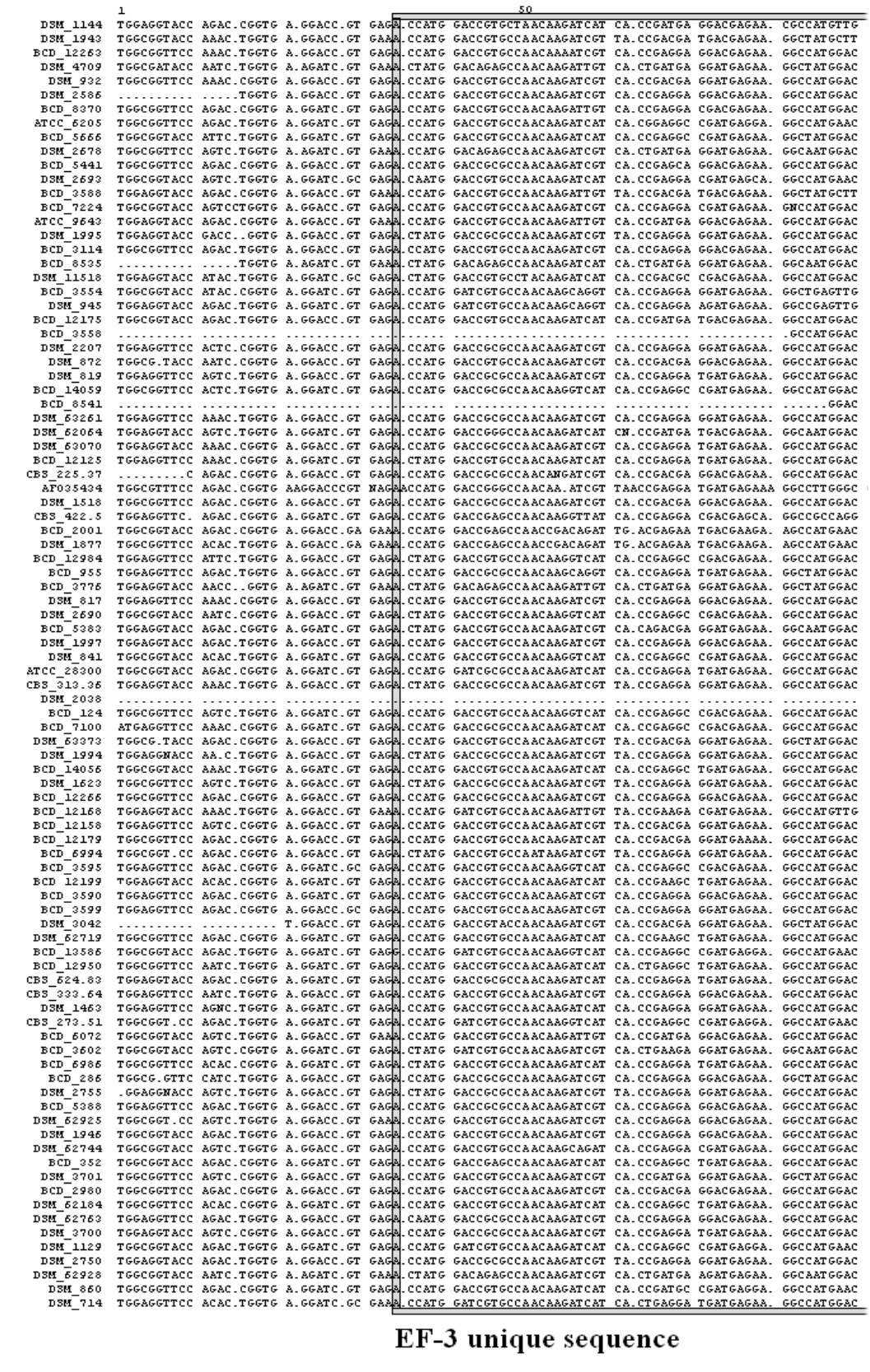
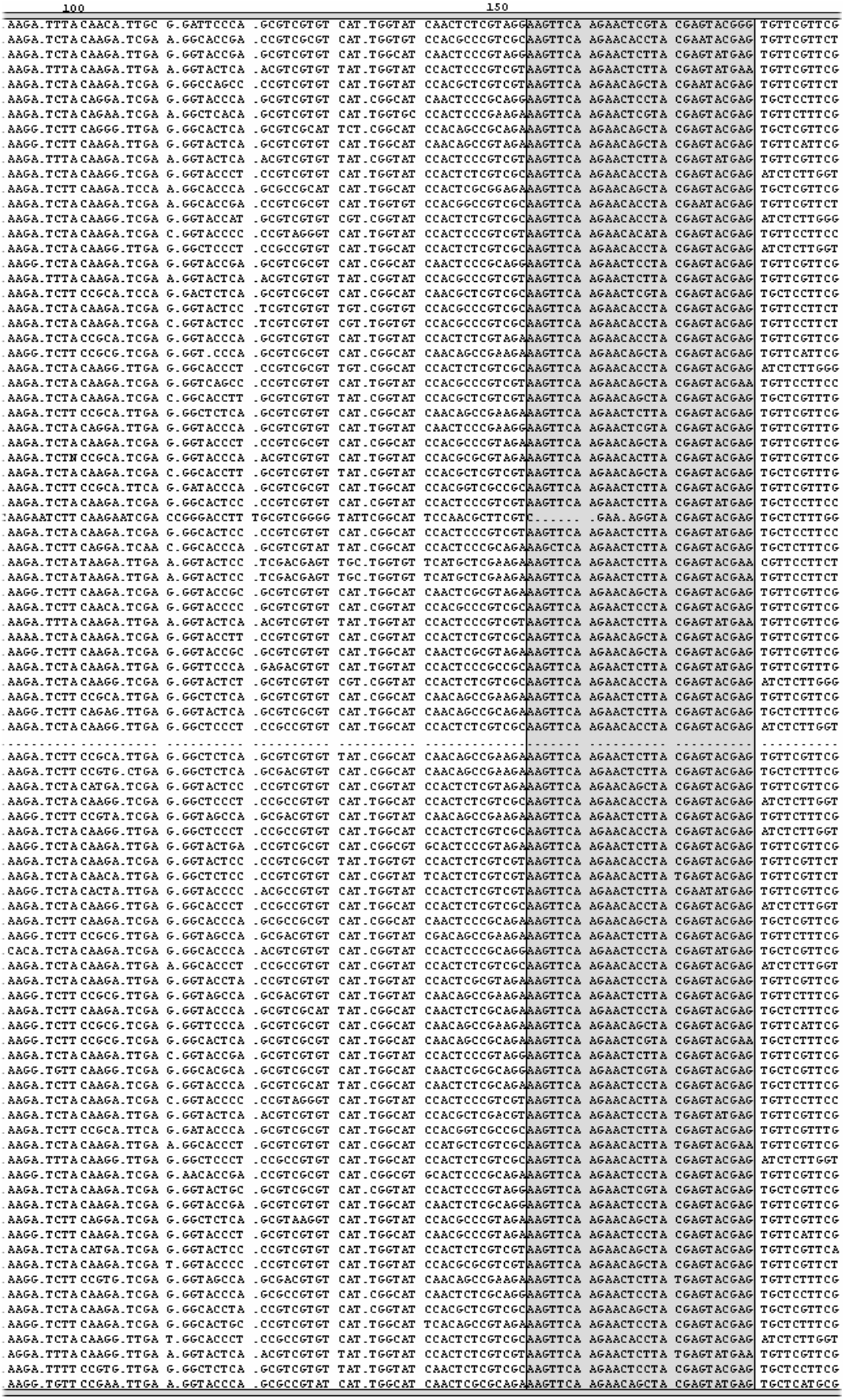
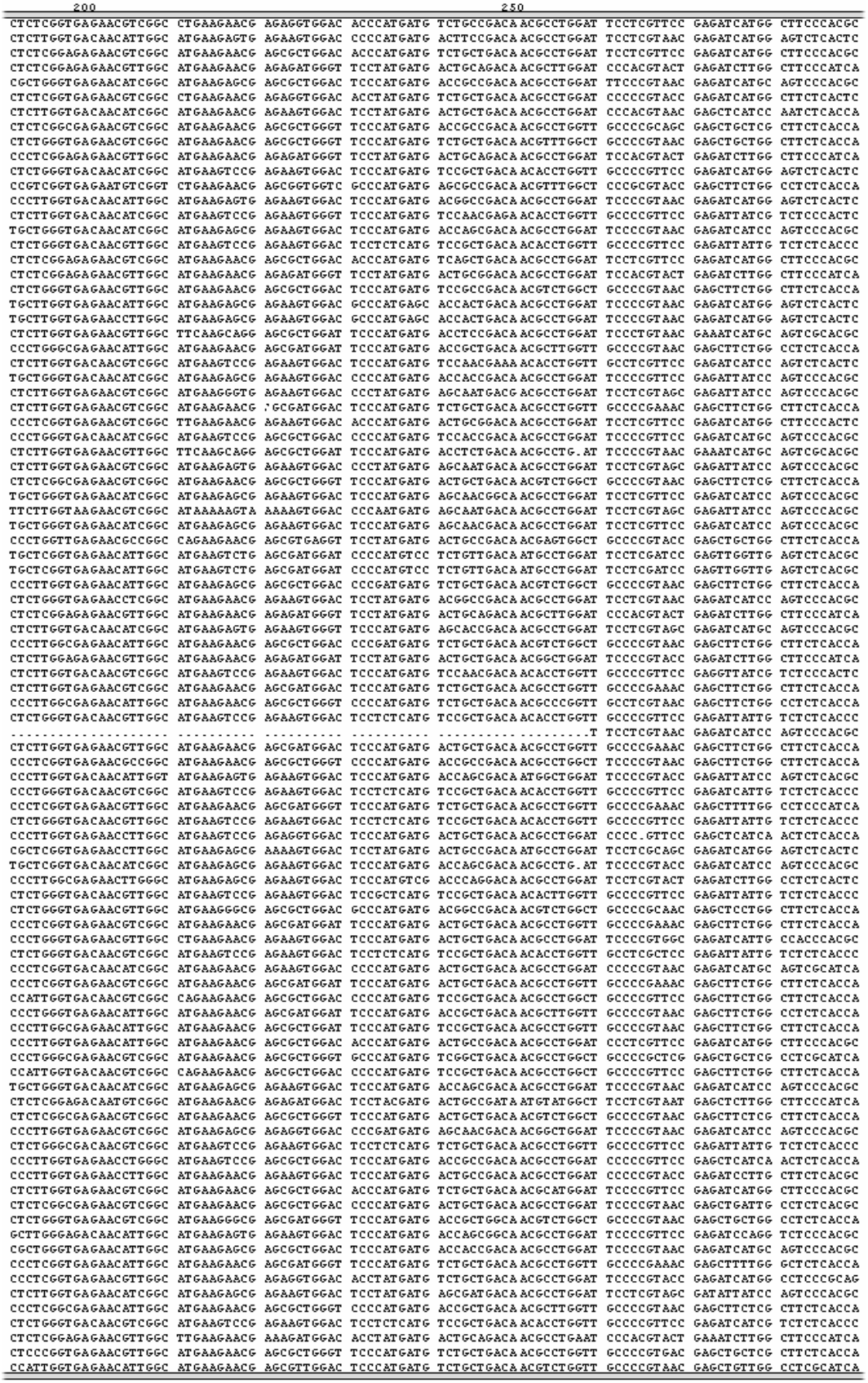


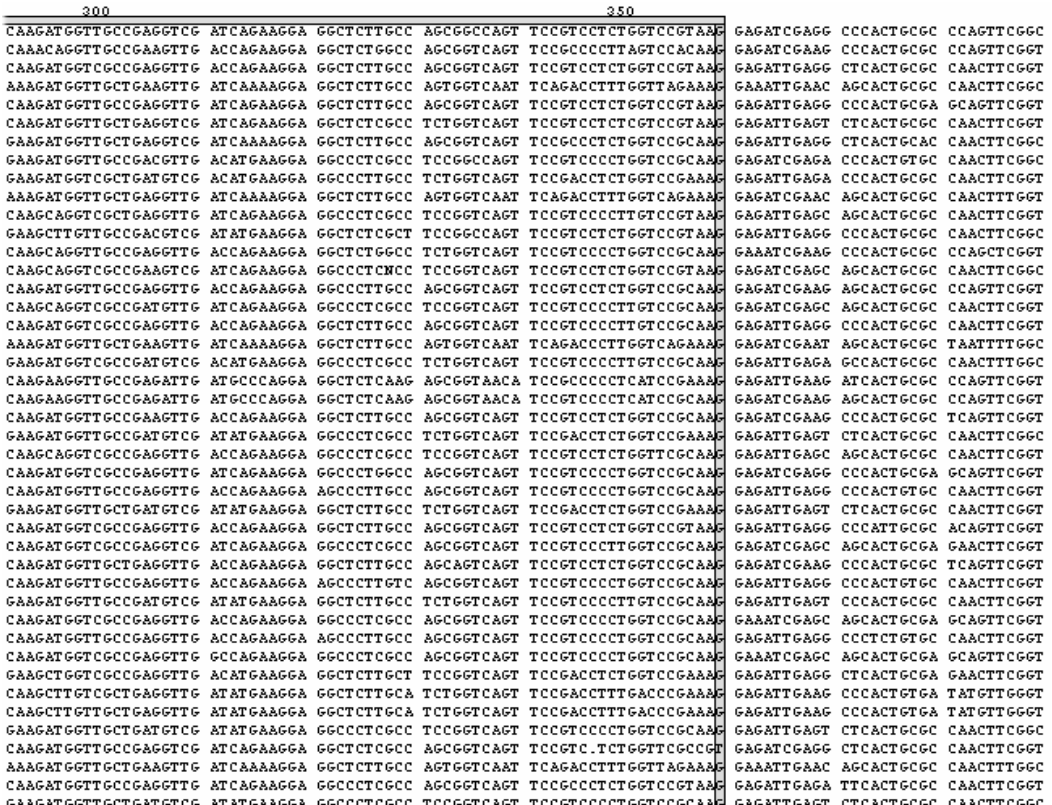
Figure 30 Alignment including sequences of *Ascomycota, Deuteromycota and Basidiomycota*.





Primer region 155

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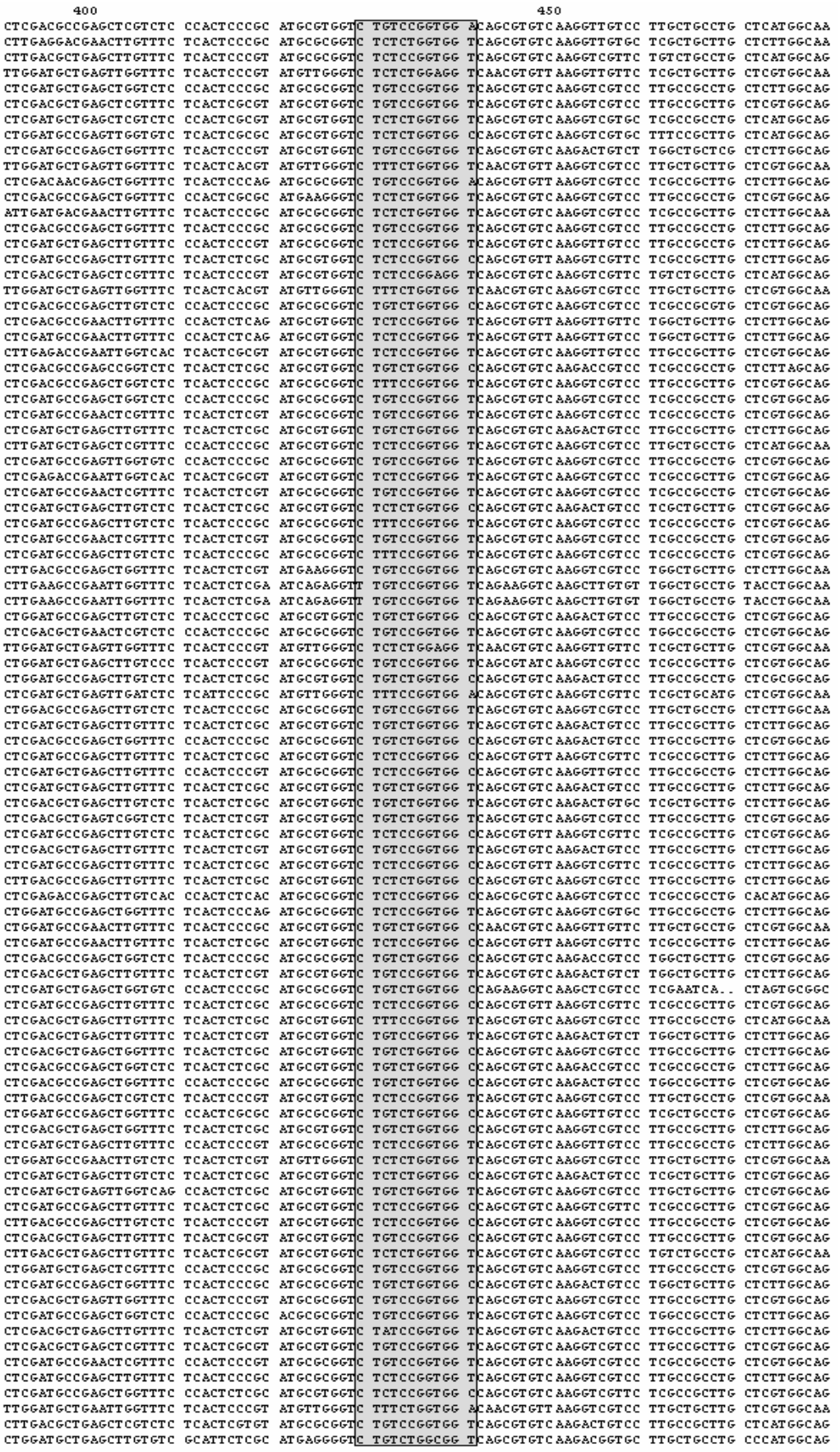
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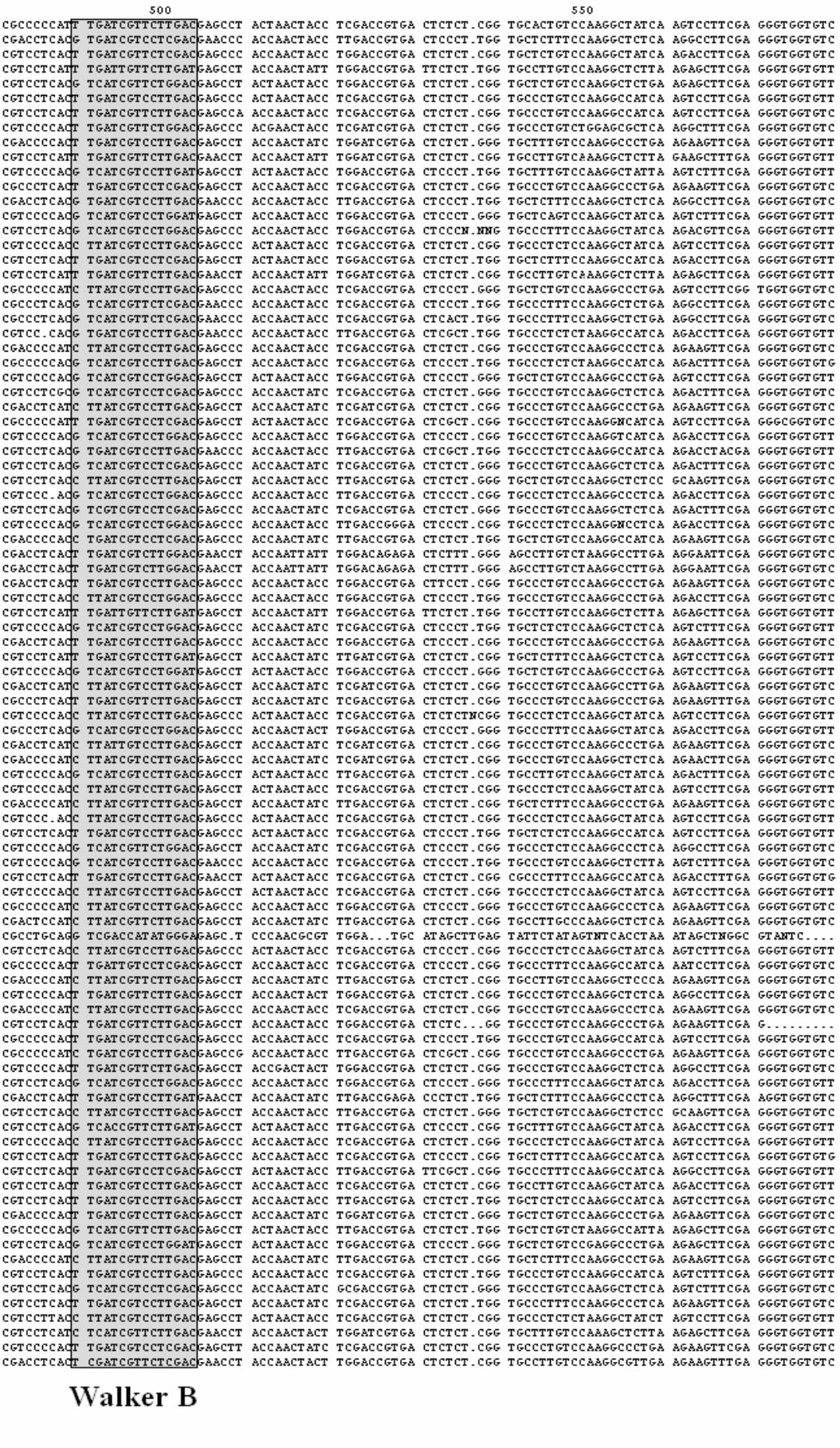
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Walker B

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```

Figure 31 Artefact alignment A of *Zygomycota*

```
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 BCD_12179 TGGCGGTTCC AGACCGGTGA GGACCGTGAG ACCATGGACC GTGCC.....
  BCD_6069 TGGAGGTACC AGTCTGGTGA AGATCGTGAA GAATTGGACA AGGTT.....
MUCL_43045 .......... .......... .......... .......... ..........
CBS_100.48 TGGCGGTTCC AAACTGGTGA AGATCGTGAA GAATTGGAAA AGGTC.....
  BCD_3498 TGGCGGTACC AGTCTGGTGA AGATAAGGAA GTTGCTGAAA AGGAA.....
   DSM_960 TGGCGGTACC ATTCCGGTGA AGATAAGGAA GTAGCTGAGA AGGAA.....
   BCD_162 TGGAGGTACC AAACCGGTGA AGATCGTGAG GAGCTCGACA AGGTAAGAAA
  BCD_3494 TGGCGGTACC AGACCGGTGA GGATCGTGAG GCCTACAACA AGGCC.....

           51                                                           100
  BCD_2001 .......... .......... .......... .......... ..........
 BCD_12179 .......... .......... .......... .......... ..........
  BCD_6069 .......... .......... .......... .......... ..........
MUCL_43045 .......... .......... .......... .......... ..........
CBS_100.48 .......... .......... .......... .......... ..........
  BCD_3498 .......... .......... .......... .......... ..........
   DSM_960 .......... .......... .......... .......... ..........
   BCD_162 AAAGAAAGGG CGAGGGTGGT TTTTTTCGTG TTAGCAAGGT ATTGATTGTT
  BCD_3494 .......... .......... .......... .......... ..........

           101                                                          150
  BCD_2001 .......... .......... ..AACCGACA GATTGACGAG AATGACGAAG
 BCD_12179 .......... .......... ..AACAAGAT CATCACCGAC GAGGATGAAA
  BCD_6069 .......... .......... ..GACCGTAT CATCACCGAA GACGAAGAAA
MUCL_43045 .......... .......... .......... .......... ..........
CBS_100.48 .......... .......... ..GACCGTAT CATCACTGAA GAAGAAGAAA
  BCD_3498 .......... .......... ..ACTCGTGC TTGGAGTGAA GATGAAATTG
   DSM_960 .......... .......... ..ACTCGTGT CTGGTCTGAT GAAGAAAAGG
   BCD_162 TATTCTTGTG CTCTAGGTCG ACCGTGTCAT CACC...GAG GAGGAGGCCA
  BCD_3494 .......... .......... ..ACTCNCCA GATCTCGCCC GAGGAGGAGG

           151                                                          200
  BCD_2001 AAGCCATGAA CAAGATCTAT AAG....... ......ATTG AAGGT...AC
 BCD_12179 AGGCCATGGA CAAGGTCTAC ACTA...... .......TTG AGGGT...AC
  BCD_6069 AGCAAATGAA CCAAGCTTTC GTC....... ......ATCG ATGGT...GA
MUCL_43045 .......... .......... .......... .......... ..........
CBS_100.48 AGCAAATGAA CCAAGCCTTT GTC....... ......ATTG AAGGT...GA
  BCD_3498 CTCAAATGAA CAA....... .......... .........G ATGGTTCCTG
   DSM_960 CTCAAATGGA AAAAAT.... .......... .........G ATCTC...TG
   BCD_162 AGCAGATGCA GCAGCACTTC GTC....... ......ATCG AGGGT...GA
  BCD_3494 CCCAGATGAA GAAGTTCATC CAGTGGGAG. ATTAACGAGA AGATT...GA

           201                                                          250
  BCD_2001 TCCTCGACGA GTTGCTGGTG TTCATGCTCG AAGA...... ....AAGTTC
 BCD_12179 CCCACGCCGT GTCATTGGTA TCCACTCTCG TCGT...... ....AAGTTC
  BCD_6069 AAAGCGTATC GTTGAAGACA TTGTTGGTCG TCGT...... ....AAGCTC
MUCL_43045 .......... .......... .......... .......... ..........
CBS_100.48 AAAGCGCGTC GTCGATGAAC TTGTTGGTCG CCGT...... ....AAGCTC
  BCD_3498 TTAACGGTGA AGAACGTCAA ATTGAAACTC TTTTAGGTCG TACTAAACTC
   DSM_960 TCAGCGGTGA AGAACGTCAA ATTGAGGCTT TGTTGGGTCG TACTAAGCTC
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  BCD_3494 GAAGGTCCAG ATCGAGGATC TCTATGGACG TCGC...... ....AAGGCC

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  BCD_6069 AAGCAATCCT ACGAATACGA A......... .......... ..........
MUCL_43045 .......... .......... .......... .......... ..........
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  BCD_3498 AAGAAGACTT TCCAATATGA A......... .......... ..........
   DSM_960 AAGAAGACAT TTCAATATGA A......... .......... ..........
   BCD_162 AAGAACAGCT ACGAGTACGA G......... .......... ..........
  BCD_3494 AAGCGCTCTT TCGAGTACGA G......... .......... ..........
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 BCD_12179  CATGAAGAGC GAGAAGTGGA CTCCCATGTC GACCCAGGAC AACGCCTGGA
  BCD_6069  .......... ATTTCATGGA AGGGCAAGTC CTCCGTTGAA AACACTTTCA
MUCL_43045  .......... .......... .......... .......... ..........
CBS_100.48  .......... GTCACCTGGG TCGGTCGCTC TTCCGTTGAC AACCTCTGGA
  BCD_3498  .......... ATTAAGTGGA AGAACAAGCT TCACAAGTTC AACGGTTGGT
   DSM_960  .......... ATTAAGTGGA AGAACAAGCT TCACAAGTTT AACAGCTGGT
   BCD_162  .......... A......... .......... .......... ..........
  BCD_3494  .......... GTTCAGTTTG TTGGCCGCAC CTATGAGGAC AACGCCTGGA

            351                                                       400
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 BCD_12179  TTCCTCGTAC TGAGATCTTG GCCTCTCAC. .....TCAAA GATGGTTGCC
  BCD_6069  TCTCTCGTCA ACGTCTTGAA CAAATGGGTT TC...AGCAA GAAGATTGCT
MUCL_43045  .......... .......... .......... .......TAT GGCGGTACCA
CBS_100.48  TCTCCCGCCA AAAGCTCGAA AACATGGGTT TC...TCCAA GAAGATTGCC
  BCD_3498  TCTCTCGTGA AAAGTTGCTC GAACTTGGCT TC...CAAAA GCTTGTTCAA
   DSM_960  TCTCTCGTGA AAAGTTGCTT GAATTGGGTT TC...CAGAA GTTGGTCCAA
   BCD_162  .......... .......... .......... .......... ..........
  BCD_3494  TTGCTCGTGA GAAGCTCGAG GAGTGGGGTT TCGAGAAGGT CCTCCAGGCT

            401                                                       450
  BCD_2001  GAG.GTTGAT ATGAAGGAGG CTCTTGCATC TGGT...CAG TTCCGACCTT
 BCD_12179  GAA.GTTGAT CAGAAGGAAG CTCTCGCCAG CGGT...CAA TTCCGTCCTC
  BCD_6069  GAA.GTTGAT GCTCAAGAAG CTGCCAAGAT GGGT...CTT AACCGTCCTT
MUCL_43045  AACCGGTGAG GACAAGGAA. CTTCTGGCTA AGGA.....G ACCCGTAAGC
CBS_100.48  GAA.GTCGAT GCCCTCGAAG CCGCCAAGTT GGGT...CTT AACCGTCCTT
  BCD_3498  CAA.TTTGAT GATAAAGAAG CTTCTCGTGA AGGTTTGCTT TACCGTGAAT
   DSM_960  CAA.TTCGAT GACAAGGAAG CTTCTCGCGA AGGTCTCTTG TACCGTGAAC
   BCD_162  .......... .......... .......... .......... ..........
  BCD_3494  TTC.GATGAC AAGGAGGCTG CCAAGGCTGG TGCC...TGG ACCCGCTCCT

            451                                                       500
  BCD_2001  TGACCCGAAA GGAGATTGAA GCCCACTGTG ATATGTTGGG TCTTGAAGCC
 BCD_12179  TGGTCCGTCG TGAGATTGAA GTTCACTGCT CCAACTTTGG TCTGGATGCC
  BCD_6069  TGACCTCCAA GGAAATTGAA AAGCACTTGA ACGAAGTTGG TTTGGAACCC
MUCL_43045  TGTCCAAGGA GGAGAAGGAG CACTACAAGA AGCCGGTGAA CTGGGAGGGC
CBS_100.48  TGACCTCCAA GGAAATCGAA AAGCACTTGA CCGAAGTTGG TTTGGAACCT
  BCD_3498  TAAGTATTCC TCATATTCGT CAACATTTCA ATGATATTGG TCTTGATCCT
   DSM_960  TAAGCATGCC CCACATTCGT CAACACTTTG CCGATATTGG TCTTGATCCT
   BCD_162  .......... .......... .......... .......... ..........
  BCD_3494  TGACCGCTGT TGAGGTCGAG AAGCACTTGG GTGATCTCGG TCTCGACCCC

            501                                                       550
  BCD_2001  GAATTGGTTT CTCACTCTCG AATCAGAGGT TTGTC.CGGT GGTCAGAAGG
 BCD_12179  GAACTTGTTT CTCACTCCCG CATGCGTGGT CTGTC.TGGT GGCCAACGTG
  BCD_6069  GAATTCGCCA CTCACTCCCG TATCCGTGGT TTGTC.CGGT GGCCAAAAAG
MUCL_43045  GAG..AAGCG CGTTCTCGAG GAGATTGTGT CTCGTCGTAA GTTCAAGAAC
CBS_100.48  GAATTCGCCA CTCACTCTCG CATCCGTGGT TTGTC.CGGT GGTCAAAAGG
  BCD_3498  GATATTGCTC AATACACCAA GATGAGTGCT CTTTC.TGGT GGTCAGAAGG
   DSM_960  GATATTGCTC AATACACTAA GATGGGTGCT CTTTC.TGGT GGTCAAAAGG
   BCD_162  .......... .......... .......... .......... ..........
  BCD_3494  GAG.TTCTCC ACTCACTCCC GTATTAAGGG TCTTTCTGGT GGCCAAAAAG

            551       566
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 BCD_12179  TCAAGGTTGT TCTTGC
  BCD_6069  TCAAACTCGT CCTCGA
MUCL_43045  ACCTACGAGT ACGAGA
CBS_100.48  TCAAACTCGT CCTCGA
  BCD_3498  TCAAGCTCGT CCTCGA
   DSM_960  AATCACTAGT GCGGCC
   BCD_162  .......... ......
  BCD_3494  TCAAGCTCGT CCTCGA
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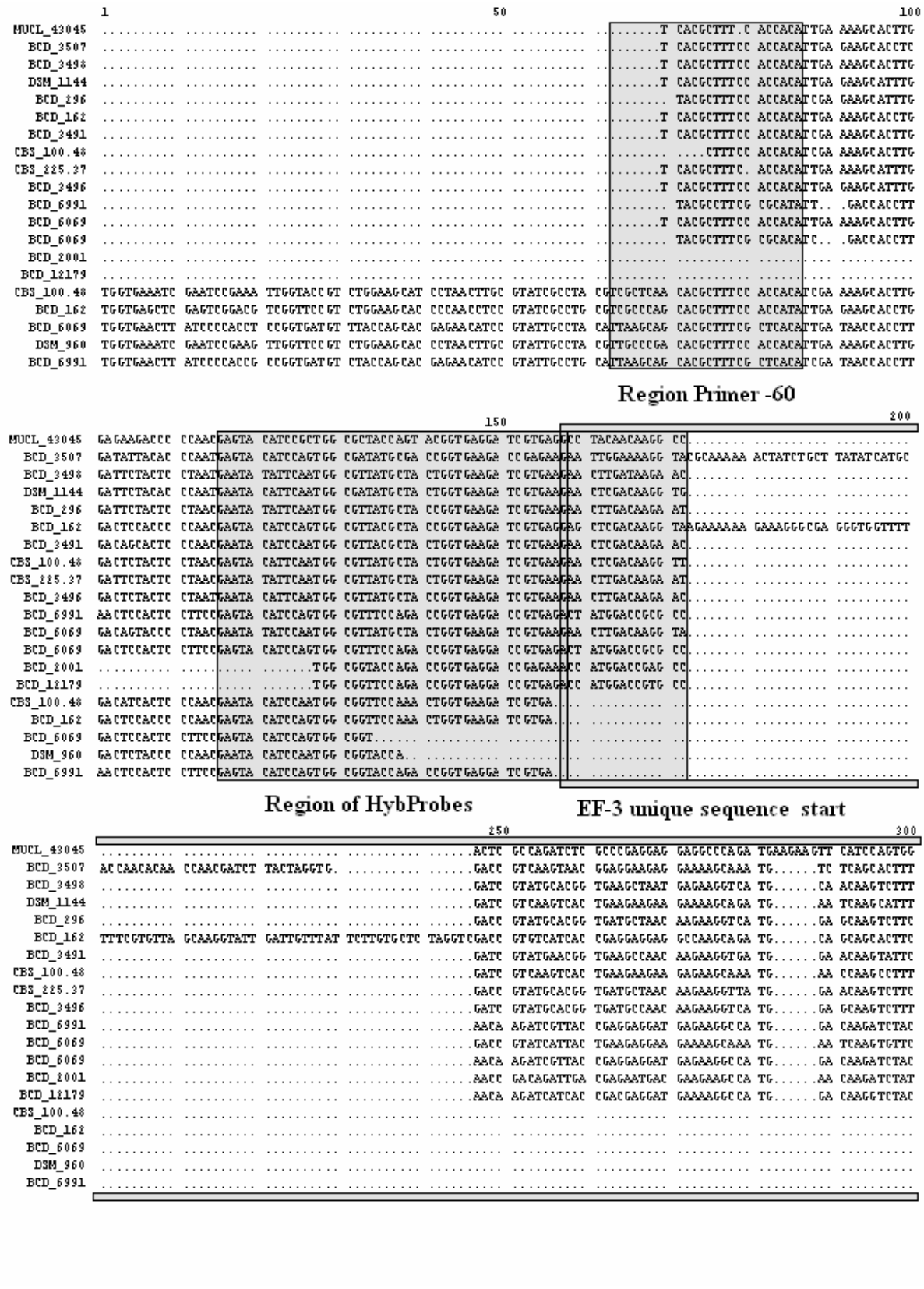
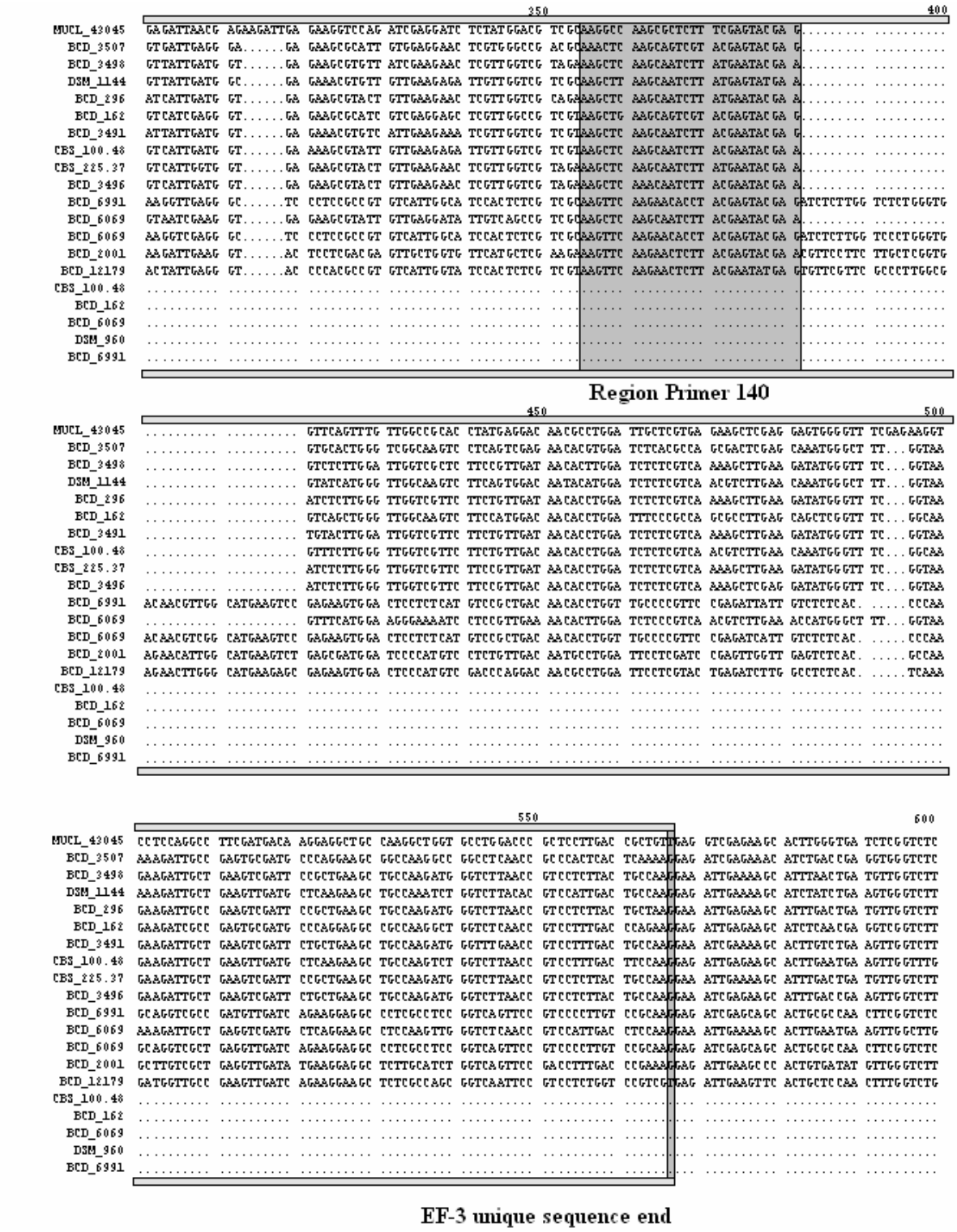
Figure 32 Alignment B and C of *Zygomycota*

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EF-3 unique sequence end

9.4 Reference strains

Table 75 - Summarizes the strains, which have been sequenced in this project and are detectable by the consensus PCR system including HybProbes (protocol A).

BCD-code	Lab-code	*genus*	*species*	collection number	division	growth media
14478	1	*Absidia*	*corymbifera*	DSM 1144	*Zygomycota*	M
3481	34; 165	*Absidia*	*glauca +*	CBS 100.48	*Zygomycota*	PDA
6986	2	*Acremonium*	*strictum*	HN 1006 1.11.P2(8)	*Ascomycota*	OM
12263	36	*Alternaria*	*alternata*		*Ascomycota*	PDA
8541	118	*Ascochyta*		HN 1134	*Ascomycota*	OM
14480	0.1	*Aspergillus*	*carneus*	DSM 1518	*Ascomycota*	PDA
10160	37	*Aspergillus*	*clavatus*	DSM 817	*Ascomycota*	PDA
14476	4	*Aspergillus*	*ficuum*	DSM 932	*Ascomycota*	PDA
5440	28	*Aspergillus*	*flavus*	ATCC 9643	*Ascomycota*	PDA
14474	38	*Aspergillus*	*fumigatus*	DSM 819	*Ascomycota*	PDA
10164	71	*Aspergillus*	*niger*	DSM 872	*Ascomycota*	PDA
12158	23	*Aspergillus*	*ochraceus*		*Ascomycota*	M
6072	10	*Aspergillus*	*oryzae*	2001	*Ascomycota*	PDA
918	11	*Aspergillus*	*parasiticus*	DSM 2038	*Ascomycota*	PDA
	70	*Aspergillus*	*penicillioides*	DSM 1623	*Ascomycota*	M
14488	74	*Aspergillus*	*sydowi*	DSM 63373	*Ascomycota*	M40Y
5441	39	*Aspergillus*	*tamarii flavus*		*Ascomycota*	PDA
12168	24	*Aspergillus*	*ustus*		*Ascomycota*	PDA
917	19	*Aspergillus*	*versicolor*	DSM 1943	*Ascomycota*	PDA
14485	64	*Aspergillus*	*wentii*	DSM 3701	*Ascomycota*	PDA
124	125	*Aspergillus*	*glaucus*	1005	*Ascomycota*	PDA
14484	40	*Aureobasidium*	*pullulans*	DSM 3042	*Ascomycota*	PDA
12199	42	*Botryotrichum*	*cf.piluliferum*		*Fungi Imperfecti*	OM
14486	32	*Botrytis*	*cinerea*	DSM 4709	*Ascomycota*	M
3776	17	*Botrytis*	*spp.*	B 2674 A2/1	*Ascomycota*	M
955	42	*Byssochlamys*	*fulva*	220201	*Ascomycota*	PDA
1288	122	*Cephalosporium*	*aphidicola*	ATCC 28300	*Ascomycota*	OM
2980	109	*Cephalosporium*	*roseum*		*Ascomycota*	OM
5383	119	*Ceratocystis*	*pilifera*	WFPL 55a	*Ascomycota*	OM
8370	116	*Cercospora*		HN 1063	*Ascomycota*	PDA
5387	44	*Chaetomium*	*globosum*	ATCC 6205	*Ascomycota*	PDA
14449	126	*Choanephora*	*infundibulivera var.cucurbitarum*	DSM 960	*Ascomycota*	YEPD
2143	73	*Chrysosporium*	*farinicola*	CBS 624.83	*Zygomycota*	M
12266	68	*Cladosporium*	*cladosporioides*		*Ascomycota*	PDA
5388	47	*Cladosporium*	*herbarum*	SP 44	*Ascomycota*	M
	151	*Claviceps*	*purpurea*	DSM 714	*Ascomycota*	YEPD
7988	21	*Colletotrichum*	*gloeosporoides*	DSM 11518	*Ascomycota*	M
3491	166	*Cunninghamella*	*elegans*		*Ascomycota*	OM
	139	*Curvularia*	*geniculata*	CBS 333.64	*Zygomycota*	OM
12950	112	*Cylindrocarpon*		HN 1179	*Ascomycota*	OM
3554	48	*Emericella*	*nidulans*		*Ascomycota*	PDA
14477	12	*Emericella*	*rugulosa*	DSM 945	*Ascomycota*	PDA
14482	9	*Epicoccum*	*nigrum*	DSM 2586	*Ascomycota*	OM
12125	15	*Eremascus*	*albulus*	ATCC	*Ascomycota*	M40Y
14481	29	*Eupenicillium*	*abidyesnum*	DSM 2207	*Ascomycota*	M
12175	18	*Eurotium*	*amstelodami*	SBUG M	*Ascomycota*	M40Y
14487	0.2	*Eurotium*	*chevalieri*	DSM 62064	*Ascomycota*	M40Y
14059	5	*Fusarium*	*oxysporum*	MA 1244	*Ascomycota*	PDA

BCD-code	Lab-code	*genus*	*species*	collection number	division	growth media
7100	111	*Fusarium*	*solani*	3532	*Ascomycota*	PDA
10162	121	*Fusarium*	*oxysporum sp. Vasinfectum*	DSM 841	*Ascomycota*	PDA
14056	110	*Fusarium*	*graminearum*	MA 1241	*Ascomycota*	PDA
94	75	*Fusarium*	*culmorum*	DSM 62184	*Ascomycota*	PDA
	148	*Gaeumannomyces*	*graminis*	DSM 1463	*Ascomycota*	PDA
14451	91	*Giberella*	*cyanea (Fusarium heterosporum)*	DSM 62719	*Ascomycota*	PDA
	140	*Gloeosporium*	*pedemontanum*	CBS 273.51	*Ascomycota*	OM
3588	50	*Helminthosporium*	*spp.*			YEPD
5394	51	*Humicola*	*grisea*	DSM 2690	*Ascomycota*	PDA
3590	76	*Humicola*	*fuscoatra*		*Ascomycota*	PDA
14453	103	*Hyalodendron*	*lignicola*	DSM 1877	*Ascomycota*	M
286	65	*Monascus*	*purpureus*		*Basidiomycota*	YEPD
8535	67	*Monilia*	*spec.*	HN 1128	*Ascomycota*	M
	153	*Monilia*	*fructigena*	DSM 2678	*Fungi Imperfecti*	PDA
3499	2	*Mucor*	*racemosus*	CBS 225.37	*Zygomycota*	M
3498	85	*Mucor*	*hiemalis*		*Zygomycota*	M
6069	108	*Mucor*	*circinelloides*	0101	*Zygomycota*	PDA
6069	161	*Mucor*	*circinelloides*	101		
3496	160	*Mucor*	*genevensis*		*Zygomycota*	PDA
	154	*Mycosphaerella*	*pinodes*	DSM 62763	*Zygomycota*	M
12984	77	*Myrothecium*		HN 64	*Ascomycota*	V8
14456	104	*Neosartorya*	*fischeri*	DSM 3700	*Ascomycota*	PDA
3558	4	*Neurospora*	*spp.*		*Ascomycota*	PDA
	146	*Neurospora*	*crassa*	DSM 1129	*Ascomycota*	PDA
1089	5	*Nigrospora*	*clurus*	CBS 313.36	*Ascomycota*	PDA
3595	98	*Paecilomyces*	*lilacinus*		*Ascomycota*	PDA
10123	54	*Penicillium*	*camembertii*	DSM 1995	*Ascomycota*	PDA
10124	25	*Penicillium*	*citrinum*	DSM 1997	*Ascomycota*	M
	149	*Penicillium*	*digitatum*	DSM 2750	*Ascomycota*	PDA
886	106	*Penicillium*	*expansum*	DSM 1994	*Ascomycota*	PDA
3599	3;7	*Penicillium*	*roquefortii*		*Ascomycota*	PDA
2001	6	*Penicillium*	*thomii*	B 513	*Ascomycota*	PDA
6994	93	*Penicillium*	*crustosum*	HN 1014 1.11.P2(1)	*Ascomycota*	PDA
10127	105	*Penicillium*	*italicum*	DSM 2755	*Ascomycota*	PDA
7224	0.3	*Rhizoctonia*	*solani*	N 17	*Ascomycota*	YEPD
	144	*Pithomyces*	*chartarum*	DSM 62925	*Basidiomycota*	PDA
5422	26	*Philaphora*	*hoffmanii*	DSM 2693	*Ascomycota*	PDA
3114	56	*Phoma*	*spec.*	B-2532 A167/2	*Ascomycota*	PDA
1155	152	*Phomopsis*	*phaseoli*	CBS 422.50	*Ascomycota*	PDA
	156	*Pleospora*	*herbarum*	DSM 62928	*Ascomycota*	PDA
	141	*Phytophthora*	*infestans*		*Oomycota*	PDA
6991	128	*Rhizopus*	*cf oryzae*	HN 1011 1.11.P2(3)	*Ascomycota*	V8
14460	155	*Sclerotinia*	*sclerotiorum*	DSM 1946	*Zygomycota*	PDA
3602	59	*Scytalidium*	*lignicola*		*Ascomycota*	M
162	90	*Syncephalastrum*	*racemosum*	0102	*Zygomycota*	M
3507	167	*Syncephalastrum*	*racemosum*		*Zygomycota*	M
296	168	*Syncephalastrum*	*racemosum*	0101	*Zygomycota*	M
480	169	*Syncephalastrum*	*racemosum*	89101	*Zygomycota*	M
12179	72	*Talaromyces*	*flavus*	FSU Weimar	*Zygomycota*	YEPD
	145; 147	*Tiasporella*	*phaseolina*	DSM 62744	*Ascomycota*	YEPD

5666	69	*Trichoderma*	*spp.*		*Basidiomycota*	YEPD
352	138	*Trichoderma*	*viride*	CSM	*Ascomycota*	M
945	61	*Trichothecium*	*roseum*	DSM 860	*Ascomycota*	M
5436	62	*Ulocladium*	*chartarum*	DSM 63070	*Ascomycota*	OM
13586	20	*Verticillium*		HN 1234	*Ascomycota*	PDA

Table 76 - Summarizes additional strains, which are detectable by the consensus PCR system including HybProbes (protocol A).

BCD-code	Lab-code	*genus*	*species*	collection number	division	growth media
3485	163	*Absidia*	*spinosa*		*Zygomycota*	PDA
3486	35	*Actinomucor*	*elegans*		*Zygomycota*	YEPD
525	14	*Alternaria*	*citri*	CBS 106.27	*Ascomycota*	PDA
482	123	*Aspergillus*	*ruber*	2302	*Ascomycota*	PDA
10159	30	*Aspergillus*	*aculeatus*	DSM 63261	*Ascomycota*	PDA
12139	22	*Aspergillus*	*candidus*		*Ascomycota*	M
10161	31	*Aspergillus*	*terreus*	DSM 826	*Ascomycota*	PDA
	142	*Basipetospora*	*halophila*	MUCL 28807	*Fungi Imperfecti*	M
5381	133	*Beauveria*	*bassiana*	DSM 875	*Ascomycota*	PDA
956	130	*Byssochlamys*	*nivea*	220102	*Ascomycota*	PDA
	134	*Ceratocystis*	*paradoxa*	MUCL1869	*Ascomycota*	OM
164	117	*Cunninghamella*	*elegans*	0201	*Zygomycota*	OM
3491	166	*Cunninghamella*	*elegans*		*Zygomycota*	OM
14479	16	*Diaporthe*	*citri*	DSM 1159	*Ascomycota*	OM
7979	120	*Drechslera*	*sp.*	HN 1060	*Ascomycota*	OM
12172	129	*Eurotium*	*rubrum*		*Ascomycota*	M40Y
3557	124	*Eurotium*	*amstelodami*		*Ascomycota*	M40Y
7005	137	*Fusarium*	*acuminatum*	70234 HN 1033	*Ascomycota*	PDA
14062	113	*Fusarium*	*sambucinum*	MA 1253	*Ascomycota*	PDA
14058	132	*Fusarium*	*cerealis*	MA 1243	*Ascomycota*	OM
100	127	*Fusarium*	*sporotrichoides*	DSM 62429	*Ascomycota*	PDA
119	114	*Fusarium*	*avenaceum*	FU 0501	*Ascomycota*	PDA
110	136	*Fusarium*	*equiseti*	FU 0514	*Ascomycota*	PDA
120	115	*Fusarium*	*moniliforme*	FU 1402	*Ascomycota*	PDA
114	83	*Fusarium*	*moniliforme var. subglutinans*	FU 1401	*Ascomycota*	PDA
149	131	*Fusarium*	*poae*	1002	*Ascomycota*	PDA
253	102	*Fusarium*	*proliferatum*	1501	*Ascomycota*	PDA
133	96	*Fusarium*	*bulbigenum*	0301	*Ascomycota*	PDA
14483	7	*Geomyces*	*pannorum*	DSM 2689	*Ascomycota*	OM
985	27	*Geotrichum*	*candidum*	41-0201/0201	*Ascomycota*	M
4093	84	*Gliocladium*	*spp.*	B-2773 A6	*Ascomycota*	Sabouraud
14445	97	*Moniliella*	*suaveolens*	DSM 2400	*Fungi Imperfecti*	M
3494	92	*Mortierella*	*macrocystis*		*Fungi Imperfecti*	OM
6069	161	*Mucor*	*circinelloides*	101	*Zygomycota*	M
3496	160	*Mucor*	*genevensis*		*Zygomycota*	M
5397	53	*Paecilomyces*	*variotii*	CBS 62866	*Ascomycota*	PDA
10168	86	*Penicillium*	*griseofulvum*	DSM 896	*Ascomycota*	M
10172	99	*Penicillium*	*simplicissimum*	DSM 1097	*Ascomycota*	M
10170	94	*Penicillium*	*oxalicum*	DSM 898	*Ascomycota*	M
451	87	*Penicillium*	*viridicatum*	DSM 62878	*Ascomycota*	M
460	79	*Penicillium*	*islandicum*	ATCC 36062	*Ascomycota*	M
1180	100	*Penicillium*	*funiculosum*	DSM 1960	*Ascomycota*	M
3006	95	*Penicillium*	*purpurogenum*	0401	*Ascomycota*	M
5233	88	*Penicillium*	*chrysogenum*	DSM1075	*Ascomycota*	PDA
12165	135	*Penicillium*	*aurantiogriseum*		*Ascomycota*	M
12182	80	*Penicillium*	*corylophilum*	SBGU-M	*Ascomycota*	M
10169	107	*Penicillium*	*nalgiovense*	DSM 897	*Ascomycota*	M

BCD-code	Lab-code	*genus*	*species*	collection number	division	growth media
519	101	*Penicillium*	*verrucosum*	DSM 1838	*Ascomycota*	PDA
	141	*Phytophthora*	*infestans*	MUCL 43045	*Oomycota*	OM
3506	162	*Rhizopus*	*nigricans*	BCD	*Zygomycota*	PDA
	150	*Stachybotrys*	*chartarum*	DSM 2144	*Ascomycota*	OM
162	90	*Syncephalastrum*	*racemosum*	0102	*Zygomycota*	YEPD
7384	66	*Thielaviopsis*	*spec.*	HN 1035/70364	*Ascomycota*	YEPD
6990	89	*Trichoderma*	*hamatum*	HN 1010 1.11P2(10)	*Ascomycota*	OM
6061	81	*Trichophyton*	*mentagrophyton*	0201a	*Ascomycota*	V8
	159	*Verticimonosporium*	*ellipticum*			PDA
14463	82	*Wallemia*	*sebi*	DSM 5329	*Ascomycota*	M40Y
	158	*Xeromyces*	*bisporus*	CBS 347.94	*Ascomycota*	M40Y

Table 77 - Summary of major food-relevant mycotoxins.

mycotoxin	genus	species	effect	food
aflatoxin M1	*non-specified*		carcinogenic, hepatotoxic	milk
aflatoxin (B1,B2,G1, G2, M1)	*Aspergillus*	*flavus*	carcinogenic, hepatotoxic	grain, peanuts
aflatoxin (B1,B2,G1, G2, M1)	*Aspergillus*	*parasiticus*	carcinogenic, hepatotoxic	grain, peanuts
altertoxin I	*Alternaria*	*alternata*	mutagenic	carrots, tomato
citrinin	*Penicillium*	*citrinum*	nephrotoxic	grain
citrinin	*Aspergillus*	*sp.*	nephrotoxic	grain
cyclopiaconic acid	*Penicillium*	*cyclopium*	toxic	grain, peanuts
deoxynivalenol, vomitoxin (DON)	*Fusarium*	*graminearum*	vomiting, immunotoxic	grain
deoxynivalenol, vomitoxin (DON)	*Fusarium*	*culmorum*	vomiting, immunotoxic	grain
ergot, ergotamine, ergovaline	*Claviceps*	*purpurea*	ergotism	grain
ergot, ergotamine, ergovaline	*Claviceps*	*fusiformis*	ergotism	grain
ergot, ergotamine, ergovaline	*Sphacelia*	*sorghi*	ergotism	grain
fumonisin	*Fusarium*	*moniliforme*	carcinogenic	grain
fumonisin	*Fusarium*	*proliferatum*	carcinogenic	grain
gliotoxin	*Aspergillus*	*fumigatus*	immunotoxic	hay
gliotoxin	*Penicillium*	*sp.*	immunotoxic	hay
gliotoxin	*Gliocladium*	*sp.*	immunotoxic	hay
moniliformin	*Fusarium*	*moniliforme*	toxic	grain
moniliformin	*Fusarium*	*proliferatum*	toxic	grain
nivalenol	*Fusarium*	*nivale*	not investigated	
ochratoxin	*Aspergillums*	*ochraceous*	nephrotoxic	soya, coffee, grain
ochratoxin	*Penicillium*	*verrucosum*	nephrotoxic	soya, coffee, grain
ochratoxin	*Aspergillus*	*niger*	nephrotoxic	soya, coffee, grain
oosporein	*Oospora*	*colorans*	nephrotoxic	grain, poultry
oosporein	*Acremonium*	*sp.*	nephrotoxic	grain, poultry, meat
oosporein	*Chaetomium*	*sp.*	nephrotoxic	grain, poultry
oosporein	*Penicillium*	*sp.*	nephrotoxic	grain, poultry
oosporein	*Beauveria*	*sp.*	nephrotoxic	grain, poultry
patulin	*Penicillium*	*patulinum*	carcinogenic	fruits (apple, grapes)
patulin	*Aspergillus*	*sp.*	carcinogenic	fruits (apple, grapes)
stachybotrys toxin	*Stachybotrys*	*chartarum*	necrotic	cellulose
sterigmatocystin	*Aspergillus*	*nidulans*	carcinogenic	barley, coffee, rise
T2-toxin (trichothecene)	*Fusarium*	*sporotrichoides*	inhibition protein, DNA, RNA synthesis	feed, poultry
tremorgens	*Penicillium*	*crustosum*	neurotoxic	grain
tremorgens	*Aspergillus*	*spp.*	neurotoxic	grain
tremorgens	*Claviceps*	*paspali*	neurotoxic	grain
zearalenone	*Fusarium*	*graminearum*	binds to estrogene receptor,	grain

			changing hormones	
zearalenone	*Fusarium*	*roseum*	changing hormones	grain

Table 78 - Test including yeast DNA.

name	collection	PCR without HybProbes	PCR detection by HybProbes	CP value	fluorescence level
Bullera dendrophila	DSM 70745	+	-	n.d.	n.d.
Candida albicans	BCD 517	-	n.d.	n.d.	n.d.
Candida boidinii	BCD 794	-	n.d.	n.d.	n.d.
Candida catenulata	DSM 70136	+	+	15.26	weak probe binding
Candida glabrata	BCD 8932	+/-	-	n.d.	n.d.
Candida inconspicua	BCD 14590	+	-	n.d.	n.d.
Candida intermedia	BCD 685	+	+	16.07	weak probe binding
Candida kefyr	BCD 5186	+	+	23.3	weak probe binding
Candida melinii	BCD 807	+	-	n.d.	n.d.
Candida rugosa	BCD 814	+	+	12.08	strong probe binding
Candida sake	BCD 1054	+	-	n.d.	n.d.
Candida tenuis	BCD 819	+	-	n.d.	n.d.
Candida tropicalis	BCD 1173	+	-	n.d.	n.d.
Candida versatilis	DSM 6956	+	+	13.56	strong probe binding
Candida zeylanoides	BCD 690	+	-	n.d.	n.d.
Citeromyces matritensis	BCD 1062	+	+	21.92	strong probe binding
Clavispora lusitaniae	DSM 70102	+	+	15.8	weak probe binding
Cryptococcus albidus	DSM 70197	-	n.d.	n.d.	n.d.
Cryptococcus laurentii	BCD 824	+/-	-	n.d.	n.d.
Debaromyces hansenii	BCD 3512	+	+	17.39	weak probe binding
Debaromyces marama	BCD 825	+/-	-	n.d.	n.d.
Dekkera custersiana	DSM 70736	-	n.d.	n.d.	n.d.
Dekkera intermedia (bruxellensis)	DSM 3429	-	n.d.	n.d.	n.d.
Endomycopsis (Hyphopichia) burtonii	DSM 3505	+	+	19.78	strong probe binding
Endomycospis fibuliger	DSM 70554	-	n.d.	n.d.	n.d.
Issatchenikia orientalis	BCD 843	+	-	n.d.	n.d.
Kloeckera africana	BCD 662	-	n.d.	n.d.	n.d.
Kloeckera apiculata	BCD 3526	+	+	20.8	weak probe binding
Klyveromyces vanudenii	BCD 849	+	-	n.d.	n.d.
Lodderomyces elongisporus	BCD 854	+	-	n.d.	n.d.
Metschnikowia pulcherrima	DSM 70321	+	+	13.32	strong probe binding
Pichia anomala	DSM 70783	-	n.d.	n.d.	n.d.
Pichia carsonii	DSM 70392	+	-	n.d.	n.d.
Pichia etchellsii	BCD 860	+		n.d.	n.d.
Pichia fermentans	BCD 711	-	n.d.	n.d.	n.d.
Pichia membranaefaciens	DSM 70178	+	-	n.d.	n.d.
Rhodotorula minuta	DSM 70408	+	-	n.d.	n.d.
Rhodotorula rubra	BCD 4081	+	+	14.77	weak probe binding
Saccharomyces bayanus	DSM 70412	-	n.d.	n.d.	n.d.
Saccharomyces carlsbergensis	BCD 728	-	n.d.	n.d.	n.d.
Saccharomyces cerevisae (diastaticus)	DSM 70487	-	n.d.	n.d.	n.d.
Saccharomyces diastaticus	BCD 1257	-	n.d.	n.d.	n.d.
Saccharomyces elegansbombax	BCD 1424	+	+	19.62	weak probe binding
Saccharomyces exiguus	BCD 1491	-	n.d.	n.d.	n.d.
Saccharomyces exiguus	BCD 14594	+	-	n.d.	n.d.
Saccharomyces pastorianus	DSM 6580	-	n.d.	n.d.	n.d.
Saccharomyces pastorianus	DSM 6580	+	-	n.d.	n.d.
Saccharomyces unisporus	BCD 877	-	n.d.	n.d.	n.d.
Schizzosaccharomyces octosporus	DSM 70573	-	n.d.	n.d.	n.d.

name	collection	PCR without HybProbes	PCR detection by HybProbes	CP value	fluorescence level
Schizzosaccharomyces pombe	BCD 773	+	-	n.d.	n.d.
Schizzosaccharomyces maliclevorans	DSM 70572	+	-	n.d.	n.d.
Torulaspora delbrueckii	BCD 780	+	-	n.d.	n.d.
Trichosporon cutaneum	DSM 70684	-	n.d.	n.d.	n.d.
Yarrowia lipolytica	BCD 2556	+	+	25.99	strong probe binding
Zygosaccharomyces bailii	BCD 14592	+	+	19.28	weak probe binding
Zygosaccharomyces rouxii	BCD 2141	+	-	n.d.	n.d.

9.5 Chemicals

Table 79 – Chemicals.

chemicals	manufacturer
DNA loading buffer agarose gels: 100 mg Xylene Cyanole FF (2000 bp); 100 mg Bromphenolblue (200 bp); 40 ml Glycerine (125 g/100ml);100 ml H_2O	
5 x TBE	Merck 1.06177
Agar Agar	Carl Roth GmbH 5210.2
Agarose	Biozyme
Ampicillin	Carl Roth GmbH K028.1
Bromphenolblue	Sigma B-5525
$CaCl_2$	Sigma cat: C4901
Casein	Sigma
Caso boullion	Merck 1.05459
Chelex 100 Resin	Bio Rad 1422832
Chloramphenicol (final 50 mg/l)	Carl Roth GmbH
D(+)-Glucose-Monohydrat	Merck 1.08342
Dimethylformamid DMF	SAF Riedel de Haen 15440
dNTP Set	Roche Diagnostics GmbH
EDTA	Sigma E-7889
Ethidiumbromide	Merck cat: 111615
Glycerine	Merck 3783.1
IPTG	Carl Roth GmbH
Isopropanol	Carl Roth GmbH 6752.1
Mashed potatoes	Pfanni
KCl 3M	Merck
Malt extract	Merck 1.05391
$MgCl_2$ solution	Sigma M-1026
$MgSO_4$	Merck 1.05886
$MnCl_2$	Merck
NaAc	Fluka 71188
NaCl	Merck 1.06404
PCR water	Roche Diagnostics GmbH
Peptone	Merck 1.02239
Sac I + 10x NEB buffer	New England BioLabs
Saccharose	Merck 1.07651
Schmelzflocken	Kölln
Tris HCL	Sigma T2788
Tween 80	Carl Roth GmbH 9139.1
x-Gal	Carl Roth GmbH 2315.2
Xylene Cyanole FF	Sigma X-4126
Yeast Extract	Merck 1.03753

Kits, biochemicals and lab equipment:	
10 x PCR Buffer	Roche Diagnostics
dNTP	Roche Diagnostics
RNase	Sigma
DNase I	Sigma
DNase I	Roche Diagnostics GmbH
Transcriptor Reverse Transcriptase	Roche Diagnostics GmbH
Protector RNase Inhibitor	Roche Diagnostics GmbH
SYBR Green I	Roche Diagnostics GmbH
HSTE: Tris HCl (Sigma) 0.01M; EDTA 0.5 mM; Salmon Sperm DNA (Sigma No. D-1626) 0.01 mg/ml	
Qiaquick gel extraction kit	Qiagen Hilden
Qiagen Mini Prep Plasmid Kit	Qiagen Hilden
chemagen mRNA T Kit	chemagen Biopolymer-Technologie AG
High Pure GMO Sample Preparation Kit	Roche Diagnostics GmbH
Quant-iT™ PicoGreen® dsDNA Assay Kit	Invitrogen
SYBR Green I Nucleic Acid Gel Stain	Roche Molecular Biochemicals, Mannheim
SUPER TAQ	HT BIOTECHNOLGY LTD, England
TaqStart™ Antibody	Clontech Laboratories, Inc.
DNA Molecular Weight Marker 0.15-2.1 kbp	Roche Diagnostics GmbH, Mannheim
pGEM-T Vector System	Promega Corporation, Madison USA
FastStart Taq Polymerase	Roche Diagnostics GmbH
High Pure PCR Template Preparation Kit	Roche Diagnostics GmbH
Herolab Geldokumentationssystem	Herolab Molekulare Trenntechnik, Wiesloch
MagNA Lyser	Roche Diagnostics GmbH
capillaries	Roche Diagnostics GmbH
LightCycler®	Roche Diagnostics GmbH
Ultraspec 4000 UV/visible Spectrophotometer	Pharmacia Biotec
Spore resuspension buffer 20 ml: 0.1 % Tween 80 (20 µl), 20 % glycerol (4 ml 88 %), rest water	

10 Curriculum vitae

Name:	Maria Landgraf
Geburtsdatum:	07.04.1974
Geburtsort:	Berlin
Staatsangehörigkeit:	deutsch

Ausbildung

1994 –1999	Studium der Biologie an der 'Humboldt-Universität zu Berlin' Abschluss: Diplombiologin Diplomarbeitsthema: 'Phylogenetic relationships among species of the genus Buxus inferred from *mat* K sequences'
1992 - 1994	Berufsausbildung zur Gärtnerin im Zierpflanzenbau; Bezirksgärtnerei Berlin-Charlottenburg
1992	Abitur an der „Max-Planck-Oberschule" 1. Gymnasium Berlin-Mitte

Berufserfahrung

2002 - 2005	Anstellung als wissenschaftliche Mitarbeiterin in der Firma: BIOTECON Diagnostics GmbH Promotionsprojekt: „Detection of food-relevant filamentous fungi by real time PCR" Patent Nr.102005058431.4-44: „Verfahren und Nukleinssäuremoleküle zum Nachweis von Hefen und filamentösen Pilzen"
2001- 2002	Anstellung als wissenschaftliche Mitarbeiterin in der Firma: 8sens biognostic AG, Berlin Deutschland
2001 - 2001	Anstellung als wissenschaftliche Mitarbeiterin in der Firma: Biodetection ltd., Hongkong, SAR
2000 - 2001	Forschungsaufenthalt an der Hongkong University of Science and Technology Hongkong SAR im Rahmen der Wissenschaftsförderung des DAAD im Department of Biology bei Prof. Dr. Maria Li Lung
1999 - 2000	Anstellung als Diplombiologin in der Firma: Invitek Gesellschaft für Biotechnik & Biodesign mbH