Lehrstuhl für Technische Mikrobiologie

Classification, Identification and Detection of Toxigenic Moulds

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Doctoral Thesis

Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt,

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I. Introduction

1. Fungi in food production

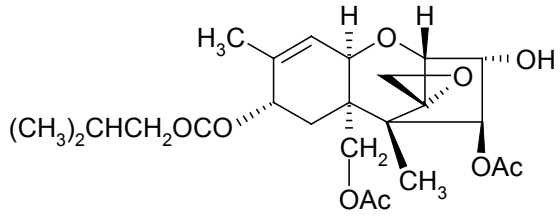
Fungi are widely used for fermentation of food and beverages, improving sensory and dietary quality of raw products. Beer, wine and other alcoholic beverages result from the fermentation of sugar containing liquids (i. e. wort and grape juice) with yeasts. Sake is made in a complex production procedure with *Aspergillus oryzae* as the most important organism involved in the process. Kefir and Kumiss are drinks made from milk fermented with lactobacilli and different yeasts. Apart from various well known cheese products like Stilton, Gorgonzola, Roquefort, Camembert etc., many traditional foods are made with the use of fungi. Examples are Tempeh and Sufu in Asia, Ogi in Nigeria and sierra rice in Ecuador (Nout, 2000, Weidenbörner, 1998). In addition to that fungal biomass itself serves as human nutrition. Apart from macromycetal fruiting bodies, e. g. mushrooms, until now the only industrial produced foodstuff is “Quorn” (Marlow Foods ltd.), consisting of processed hyphae of a non-toxigenic strain of *Fusarium venenatum* (O´Donnell et al., 1998, Yoder et al., 1998). On the other hand, uncontrolled fungal growth often leads to considerable decrease in sensory and nutritional quality of affected commodities. Moreover, chemical very diverse secondary metabolites produced by fungi cannot be overestimated. They are mainly produced during the ideophase of their growth. Many of these substances are harmful to animals and humans and some can enter the food chain via feedstuff or be carried over via meat or milk. These mycotoxins (Greek: “mykes” meaning fungus and Latin “toxicum”: poison or toxin) thus have significant impact on human health and in the current debate on food safety have gained much attention.

2. Mycotoxins and mycotoxicoses

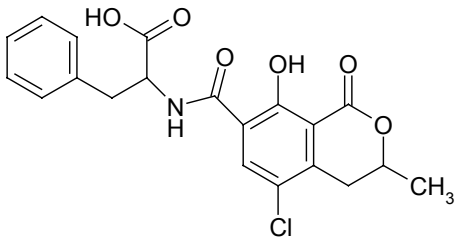
Filamentous food spoiling fungi have major impact on human health since ancient times. When mycotoxins are ingested via food the various resulting clinical pictures are called mycotoxicoses. Ergotism, referred to as “holy fire” (sacer ignis) caused by alkaloides produced by *Claviceps purpurea* and closely related species is believed to have killed hundreds of thousands of people during the Middle ages. For thousands of thousands of Russians the outbreak of alimentary toxic aleukia in the late 40s of the last century was fatal. Other acute diseases that are likely linked to fungal metabolites are acute cardiac beriberi (yellowed rice syndrome), though nowadays of less importance. Onyalai, an acute disease endemic especially in Southern Sahara regions, is characterized by haemorrhaging lesions in the mouth and can be traced back to *Phoma sorghina*. A phototoxic dermatitis is linked to the exposure of celery infected with *Sclerotinia sclerotiorum*, the causative agent of the pink rot disease of celery. For references see Angsubhakaron (1991), Pitt (1991), Pitt (2000), Pitt et al. (2000) and Rabie et al. (1975). Mycotoxicoses are of great economic importance when domesticated animals are affected. An acute epidemic of stachybotryotoxicosis killed tens of thousands of horses in the 1930s in the USSR . The turkey-X-disease dispatched 100 000 young turkeys in the UK in 1960 (Weidenbörner, 2001). This outbreak lead to the discovery of the mycotoxin, referred to as aflatoxin in 1963 by Asao et al. (1963).

Apart from the described acute cases of intoxication, the long term influence of mycotoxin intake is of higher significance to human health since the effects can accumulate. Among more than 400 mycotoxins identified (Weidenbörner, 1998) today about 20 are considered to be relevant to public health (Geisen, 1998). Aflatoxins, ochratoxin A (OTA), fumonisins, trichothecenes and zearalenone are regarded as the most important compounds produced by fungi relevant to food safety (Pitt et al., 2000). Undoubtedly, aflatoxins have by far the highest impact on food and feed, where aflatoxin B1 is regarded as the most potent

hepatocarcinogen and the strongest natural known carcinogen to humans (Riley, 1998). Fumonisins are 20-carbon aliphatic diesters resembling sphingosin which also explain their mode of action. They inhibit sphingolipid biosynthesis (WHO, 2002) and were therefore made responsible for equine leukoencephalomalacia. Fumonisins have hepatotoxic and hepatocarcinogenic effects in rats and are linked to an increased risk of oesophageal cancer in humans. Zearalenone is sometimes referred to as a mycooestrogen rather than as mycotoxin indicating that its acute toxiticity is quite low though the risks are still not yet assessed (Riley, 1998).



T-2 Toxin



Ochratoxin A

Figure 1: Chemical structures of T-2 toxin and ochratoxin A

Trichothecenes are a chemically very diverse group of tricyclic sesquiterpenes and are produced by various *Fusarium* species (Marasas et al., 1984). Among the different groups of trichothecenes the ones of type A are the most potent regularly detected in cereal samples (Torp and Langseth, 1999). This group includes neosolaniol, diacetoxyscirpenol, and T-2 toxin as the most frequently encountered. T-2 toxin (figure 1) and its deacetylated form HT-2 toxin are highly toxic as they strongly inhibit protein biosynthesis and induce DNA fragmentation typical of apoptosis (WHO, 2002). The biosynthetic pathway of the toxin production is partially understood and some genes encoding for the enzymes involved have been characterized (Edwards et al., 2002, Sweeny and Dobson, 1998).

Ochratoxin A is a chlorinated isocumarine derivative linked to phenylalanine via a peptide like bond. Figure 1 shows its chemical structure. The pentaketide mycotoxin was first isolated in 1965 by van der Merwe et al. (1965). Huff and Hamilton (1979) proposed a pathway of

ochratoxin biosynthesis, but Harris and Mantle (2001) disproved this hypothesis leaving the real mechanism to be clarified. In conclusion, little is known neither on the enzymatic nor on the genetic level concerning biosynthesis of ochratoxins.

The mycotoxin is considered to be nephrotoxic, carcinogenic, teratogenic and immunosuppressive. Though several proposals for its mode of action have been made the mechanism remains unclear (Höhler, 1998, WHO, 2002). It has been linked to Balkan Endemic Nephropathie (BEN), but it remains uncertain whether it is the causative agent of that disease (WHO, 2002, Abouzied et al., 2002). Petzinger and Weidenbach (2002) summarized the origin and the proportion of OTA intake in Germany. Apart from cereal derived products like bread, rolls and beer which contribute to approximately half of the total daily OTA intake, coffee and fruit juices have a significant toxin burden attributing to the total daily intake of OTA with 14.2-14.5 and 6.6-7.7 %, respectively. A less significant part of OTA intake is due to the consumption of meat and sausages (especially blood and liver-type sausages) products of swine and poultry. Because of the established decomposition of OTA in the rumen the contribution of beef is of little significance. In 2002 the European authorities have set up maximum levels for OTA in certain food commodities (European Commission, 2002).

3. Toxigenic fungi

The organisms responsible for production of the mentioned mycotoxins mainly belong to three anamorph genera of fungi: *Fusarium*, *Penicillium* and *Aspergillus*. Fumonisins, zearalenone and trichothecenes are produced by various *Fusarium* species. The genus comprises more than 90 species and varieties linked to several teleomorphs in the ascomycete family *Nectriaceae* within the *Hypocreales* (Gerlach and Nirenberg, 1982). The species of major importance regarding trichothecene production are *Fusarium graminearum* and *F. culmorum* (WHO, 2002). T-2 toxin production in Europe has long been assigned to *Fusarium* species within the sections *Gibbosum* and *Sporotrichiella*. Important toxigenic species

from this group are *F. equiseti* and the recently described *F. armeniacum*, while *F. acuminatum* does not produce type A trichothecenes (Altomare et al., 1996, Burgess and Summerbell, 2000, WHO 2002). In the *Fusarium* section *Sporotrichiella*, *F. sporotrichioides* and *F. poae* have been reported to produce T-2 toxin. However, only in cultures of *Fusarium* isolates having an intermediate phenotype between *Fusarium sporotrichioides* and *F. poae* T-2 and HT-2 toxin were detected in substantial quantities. According to the appearance of the colonies on CZID agar these strains were called "powdery *F. poae*" (Torp and Langseth, 1999). None of the "true" *F. poae* isolates in that study was able to produce T-2 toxin. The authors point out that "powdery *F. poae*" is one of the major sources for T-2 toxin contamination of cereals in countries of Northern Europe.

While *Fusarium* species typically infect crops on the field and thus are responsible for preharvest contamination of the crops with mycotoxins, postharvest contamination with mycotoxins is mainly due to the growth of fungi during storage under favourable conditions, i. e. high moisture and temperature. *Aspergillus* and *Penicillium* species are typical storage fungi causing safety problems in a wide variety of foods (Pitt, 2000, Samson et al., 2000). These two genera are associated to the ascomycete family *Trichocomaceae* within the *Eurotiales* (Berbee and Taylor, 1999).

According to Pitt et al. (2000) the genus *Aspergillus* comprises more than 180 accepted species. Aflatoxins are produced by members of the section *Flavii* within the *Aspergillus* subgenus *Circumdati* (Gams et al., 1985). The known aflatoxinogenic species are *Aspergillus bombycis*, *A. pseudotamarii*, *A. nominus*, *A. parasiticus* and *A. flavus* (Ito et al., 2001, Peterson et al., 2000) with only the latter two being of importance in food commodities like peanuts, almonds, figs and spices (Geisen, 1998). In contrast to aflatoxinogenic aspergilli ochratoxin A production is not linked to a restricted taxonomic group of fungi. Ochratoxinogenic aspergilli are scattered throughout different sections. Apart from *Aspergillus ochraceus*

ochratoxin A production has been reported for a lot of other *Aspergillus* species. Table 1 gives an overview on the known ochratoxin A producing fungi.

However, only *Aspergillus alliaceus*, *A. niger*, *A. carbonarius* and *A. ochraceus* are considered to be of major relevance for human health (Baymann et al., 2002 Cabañes et. al., 2002, Taniwaki et al., 2003). *A. alliaceus* has been linked to the OTA load of dried figs (Baymann et al., 2002). *A. carbonarius* and *A. niger* are suspected to be the responsible for the contamination of grape derived foods with OTA (Abarca et al., 2003, Cabañes et al., 2002, Torp et al., 2002). However, all these authors point out that *A. niger* seems to be of much less importance compared with *A. carbonarius.* In coffee, the latter two species have some impact on the OTA load, but in this commodity *A. ochraceus* accouts for the major of ochratoxin A contamination (Bucheli and Taniwaki, 2002, Joosten et al., 2001, Mantle and Chow 2000, Taniwaki et al., 2003). Batista et al. (2003) recently studied toxigenic fungi associated with coffee. Unlike others, the authors did not find any *A. cabonarius* strains and assigned ochratoxin production mainly to *A. sulphureus*. However, *A. ochraceus* and *A. sulphurueus* are not easily distinguished by morphological characters (Samson et al., 2000).

Table 1: Known ochratoxinogenic *Aspergillus* species

Section[1)]	Anamorph	Teleomorph	Reference
Aspergillus	*A. glaucus*	*Eurotium herbariorum*	Chelkowski et al., 1987
Fumigati	*A. fumigatus*	unknown	Abarca et al., 2001
Nigri	*A. carbonarius*	unknown	Schuster et al., 2002
	A. niger	unknown	Schuster et al., 2002
Terrei	*A. terreus*	unknown	Abarca et al., 2001
Usti	*A. ustus*	unknown	Abarca et al., 2001
Versicolores	*A. sydowii*	unknown	Abarca et al., 2001
	A. versicolor	unknown	Abarca et al., 2001
Wentii	*A. wentii*	unknown	Varga et al., 1996
Flavi	*A. alliaceus*	*Petromyces alliaceus*	Varga et al., 1996
	(syn. *A. albertensis*)	(syn. *P. albertensis*)	Frisvad and Samson, 2000
Circumdati	*A. auricomus*	unknown	Varga et al., 1996
	A. melleus	unknown	Abarca et al., 2001
	A. muricatus	*Neopetromyces muricatus*	Frisvad and Samson, 2000
	A. ochraceus	unknown	van der Merwe et al., 1965
	A. ostinatus	unknown	Abarca et al., 2001
	A. petrakii	unknown	Abarca et al., 2001
	A. sclerotiorum	unknown	Abarca et al., 2001
	A. sulphureus (syn. *A. fresenii*)	unknown	Abarca et al., 2001

[1)] according to Gams et al., 1985

While in tropical and subtropical regions ochratoxin A production is attributed to *Aspergillus* species, *Penicillium* species are responsible for ochratoxin A contamination of food and feed commodities in temperate climates (Larsen et al., 2001). The anamorph genus comprises more than 220 accepted species (Pitt et al., 2000). Though it was believed that a number of *Penicillium* were able to synthesise ochratoxin A, it was recognized that *P. verrucosum* was the only species within this genus to produce the toxin (Frisvad and Filtenborg, 1983, Pitt, 1987). These authors recognized two different chemotypes and it was subsequently concluded to raise *P verrucosum* and "*P nordicum*", which are to date the only known ochratoxinogenic penicillia (Larsen et al., 2001). Apart from differences in the secondary metabolites produced *P. verrucosum* is mainly isolated from plant associated material, while "*P. nordicum*" was isolated from meat-derived products. Furthermore, it grows slower and produces more OTA under laboratory conditions. So, in contrast to what is known in *Aspergillus*, OTA production seems to be restricted to a rather small and well defined taxonomic group in the genus *Penicillium*.

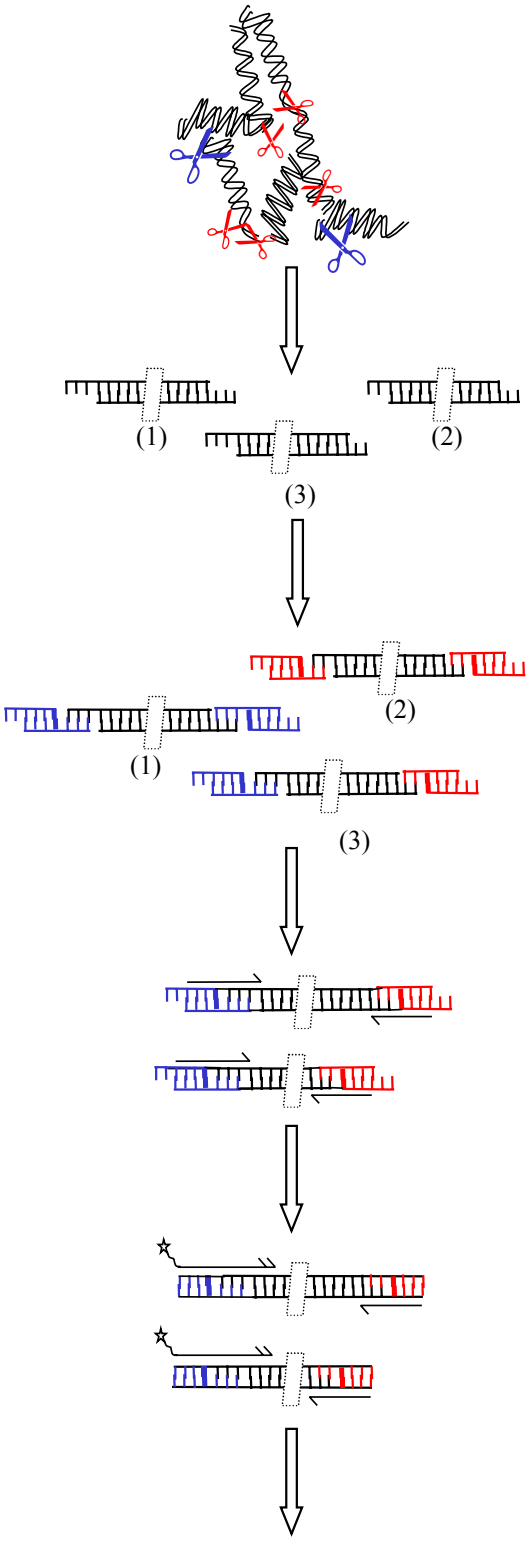
4. Problems in fungal classification

Classification of fungi still relies mainly on morphological and cultural characters. The system used to date in *Fusarium* by Gerlach and Nirenberg (1982) is completely founded on morphological observation. The same holds true for the monographs of aspergilli and penicillia (Raper and Fennell, 1965), and the subgeneric system for aspergilli (Gams et al., 1985), which is however pretty well supported by molecular techniques (Peterson, 2000). Additional tools for the classification of fungi are substrate utilization patterns. Seifert et al. (2000) and Kiil and Sasa (2000) described the use of the BIOLOG system for classifying and identifying *Penicillium* species. Chromatographic analysis of secondary metabolites has been demonstrated to be useful for identification and classification of a wide variety of fungi (Filtenborg and Frisvad, 1989, Fischer et al., 2000) Recently, FTIR has been demonstrated to be a valu-

able taxonomic tool for the identification of yeasts (Wenning et al., 2002) and penicillia (Fischer et al., 2002).

Isozym profiling and the comparison of protein sequences have been used for the classification of fungi but these techniques have been overcome by methods relying on DNA sequences rather than on proteins. Today, comparison of DNA sequences for taxonomic studies is the approach most widely used. Berbee and Taylor (2001) give an overview on fungal molecular evolution and useful genes. However, DNA sequencing is still rather expensive. This excludes the technique from high throughput screening and therefore alternative methods for DNA base comparison have been developed (Scott and Strauss, 2000).

DNA fingerprinting is comparably cheap and allows discrimination of fungal strains from the genus down to clone level (Chulze et al., 2000, Louws et al., 1999). Fingerprinting techniques have been suggested to function as a core technique in a polyphasic taxonomic system (Savelkoul et al., 1999). RFLP was the first DNA fingerprinting technique used in microbial taxonomy. This technique has the drawback that it requires vast quantities of high quality DNA. RFLP was applied for example to examine the taxonomic position of the species in the *Aspergillus* section *Nigri* (Varga et al., 2000b, Parenicova et al., 2000). RAPD or AP-PCR is a PCR-based fingerprinting technique relying on the random amplification of DNA fragments. This technique is straightforward and has been applied e. g. for the characterization of *Penicillium roqueforti* strains (Geisen et al., 2001).



Digestion of total genomic DNA with two restriction enzymes, one rare cutter (big blue scissors) and one frequent cutter (small red scissors).

Restriction fragments of different size are generated resulting in three classes of fragments: (1) sticky ends created only by rare cutter, (2) sticky ends created only by frequent cutter, and (3) with different sticky ends.

Two different adapters are ligated to the restriction fragments.The adapters are designed in a way that the ligation destroys the recognition site of the coresponding restriction enzyme (indicated by the bold „base pair“). Thus both, restriction and ligation can be performed in the same tube.

After dilution of the restriction/ligation mix this solution is used as a template in a PCR. This pre-amplification leads to selective enrichment of the fragments of class (3).

In the selective PCR additional selective bases are used to reduce the number of fragments.The application of a labelled primer (indicated by the star) allows easy detection. The “rare“ primer is used in tenfold lower concentration than the “frequent”. The PCR thus stops when this primer is exhausted.This makes the method relatively independent from variation in template concentrations.

Electrophoresis and resolution of variable length fragments followed by computer assisted pattern analysis.

Figure 2: Schematic representation of the different steps in an AFLP protocol.

Amplified fragment length polymorphism (AFLP) is a technique first described by Vos et al. (1995) and resembles a hybrid between RFLP an RAPD. The advantages are its robustness, high reproducibility and the fact that it can be applied with only minor modifications to any DNA without prior knowledge of the composition of the genome (Blears et al., 1998, Louws et al., 1999, Savelkoul et al., 1999). Figure 2 shows the principle of the AFLP technique. With cDNA as a starting point a slight variation of the AFLP protocol, called DD-AFLP or cDNA AFLP has been successfully used for expression studies (Gellatly et al., 2001).

Coupled with an automated DNA sequencer and computer software for pattern analysis the set up allows fingerprinting of DNA samples at high throughput (Aarts and Keijer; 1999; Kristensen et al., 2001). Such a set up for AFLP typing of fungi is outlined in figure 3.

For toxigenic fungi the technique was first applied by Leissner et al. (1997) who described the identification of *Fusarium graminearum* isolates. Later, the technique has been applied for a wide variety of *Fusarium* species (e. g. Baayen et al., 2000, Leslie et al., 2000) and penicillia (Castella et al., 2000, Kure et al., 2003). In addition to that AFLP can be applied for the detection of specific marker sequences (Behura et al., 2000).

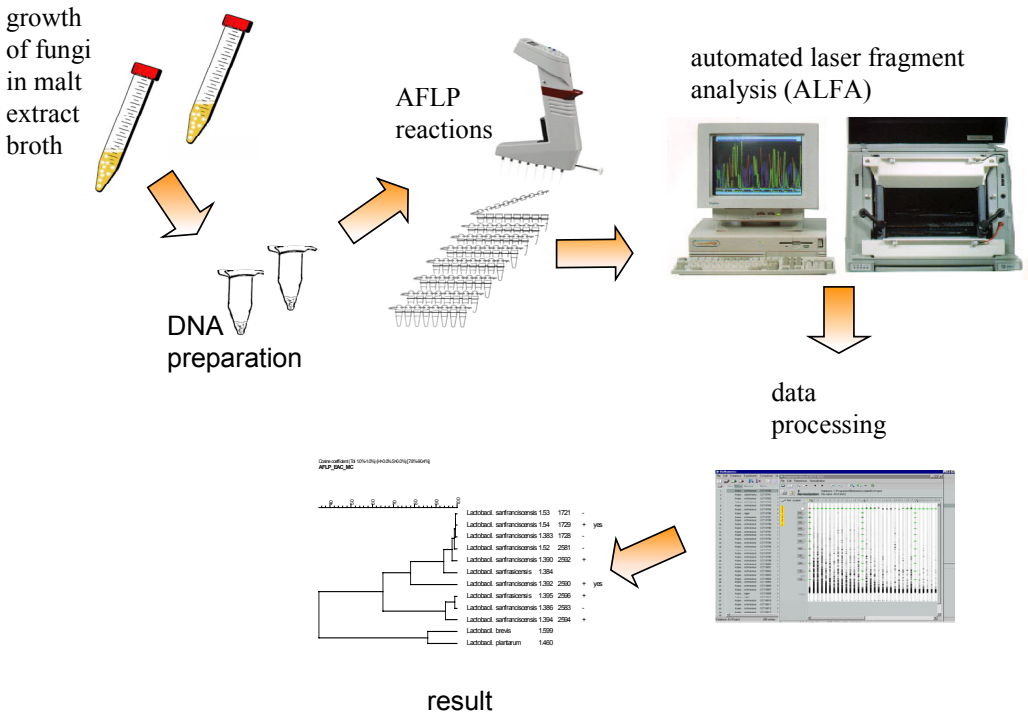


Figure 3: Outline of a set up for HTS AFLP typing of fungi.

5. PCR based detection of toxigenic fungi

The polymerase chain reaction first described by Saki et al. (1985) requires the presence of specific target sequences. When genes involved in the biosynthetic pathway are known, they represent a valuable target for the specific detection of toxigenic strains. One of the first to use this approach for the detection of toxigenic fungi were Geisen et al. (1996) and Shapiro et al. (1996) who described a diagnostic PCR directed against DNA sequences in the aflatoxin biosynthetic gene cluster. However, when the genes responsible for mycotoxin production are unknown other sequences can function as target. Examples are rDNA sequences, genes or anonymous DNA marker sequences. Geisen (1998) and Edwards et al. (2002) reviewed available diagnostic PCRs for mycotoxigenic fungi. The advantages of the PCR based approach for the detection of toxigenic fungi compared to classical mycological or chemical analysis is mainly the time aspect. For the chemical analysis of mycotoxins in food elaborated protocols for sample preparation and expensive laboratory equipment are necessary. Classical myco-

logical analysis requires isolation and cultivation of the fungi on different media and at least one week of growth for their reliable identification. In addition to that much expertise is required to recognize the species especially for the main genera of toxigenic fungi *Fusarium*, *Penicillium* and *Aspergillus*. DNA extraction from food samples and food raw materials can be performed in a few minutes (Knoll et al., 2002 a). Also the use of modern thermocyclers can reduce analysis time to less than one hour (Knoll et al., 2002 b). The same authors demonstrated the use of detection strips for the rapid analysis of PCR products. Recently, quantitative PCRs were applied for enumeration of copy numbers of genes involved in mycotoxin biosynthesis. Schnerr et al. (2001) correlated the copy number of the *tri5* gene and the DON content of cereal samples. Using the *nor-1* gene as target, Mayer et al. (2003a) were able to demonstrate the correlation of the copy numbers of the gene and colony forming units (cfu) in pepper, paprika and maize. Using a RT-PCR approach the expression of that gene was quantified by real time PCR during growth of *A. flavus* in wheat (Mayer et al., 2003b). These examples show that PCR with the decrease of costs in equipment and reagents is a promising alternative to other methods to asses the risk of contamination of food with toxigenic fungi.

6. Objectives of this study

The knowledge of systematic relationship is the basis for the detection of a specific group of organisms. The aim of a collaborative study was to clarify the taxonomy of "powdery *Fusarium poae*". An other aim of the study was to elucidate whether a relation between the ability to produce OTA and the taxonomic position of the fungus at a infraspecific level exists. AFLP functioned as a core technique due to the outlined advantages of the technique. These results could be taken as basis for the detection of specific marker sequences based on which PCRs for the identification of the organisms of interest could be developed. Further on the applicability of these PCRs for the detection of the target fungi in coffee were to be tested.

II. Materials and methods

1. Organisms

Fusarium isolates belonging to the section *Sporotrichiella* were obtained from different laboratories from all over Europe. Information on the *Fusarium* isolates is given by Torp and Adler (2003).

BFE strains of *Penicillium*: kindly provided by R. Geisen (Bundesforschungsanstalt für Ernährung, Karlsruhe, Germany).

M-strains of *Aspergillus carbonarius*: kindly provided by H. Joosten (Nestlé Research Center, Lausanne, Switzerland).

CECT-strains: Colección Española de Cultivos Tipo (University of Valencia, Spain).

CCT and ITAL strains: kindly provided by M. Taniwaki (Instituto de Tecnologia de Alimentos, Campinas-SP, Brazil).

TMW-strains: Lehrstuhl für Technische Mikrobiologie (TU München, Freising, Germany)

DSM-strains: Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany.

Aspergillus ochraceus KA103: kindly provided by J. Chelkowski (Institute for Plant Genetics, Polish Academy of Sciences, Poznan, Poland).

Aspergillus ochraceus A8: kindly provided J. Cabañes (Dept. Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona, Bellaterrra, Spain).

For further details on the fungal strains the reader is referred to the appendix.

2. Maintenance of fungal cultures

Routinely, fungi were cultivated on malt extract agar (MEA, 20 g malt extract, 1 g peptone from soybeans, 20 g glucose, 20 g agar, Gams et al., 1998) at room temperature to check purity and identity of the culture.

For long time preservation fungi were cultivated for 7 to 10 days in ME broth containing little porous clay granules of approximately 0.5 cm in diameter used normally for the cultivation of plants (Niessen and Vogel, 1997). With a sterile spoon, part of the mycelium and the stones were transferred into a sterile 1.8 ml tube (Nunc GmbH & Co., Wiesbaden, Germany) containing 1ml 80 % glycerol. These tubes were stored at –80 °C.

3. DNA extraction from pure fungal cltures (Möller et al., 1992)

For DNA preparation fungal isolates were grown in 15 ml plastic tubes (Sarstedt, Nümbrecht, Germany) containing 3 ml 2 % malt extract broth (Gams et al., 1998) for 7 to 10 days at room temperature on a rotary shaker (140 rpm). Mycelia were harvested by centrifugation (15 min, 4500 x g), washed once with sterile distilled water and lyophilised. One spoon of heat sterilized sea sand (Merck, Darmstadt, Germany) was added to each sample and mycelia were grinded together with a total of 500 µl of TES (100 mM Tris, pH 8.0, 10 mM EDTA, 2 % SDS) containing 1 % proteinase K solution. Following incubation at 60 °C for 1 hour 150 µl 5M NaCl and 65µl (ca. 1/10 of the total volume) of previously heated CTAB solution were added to the lysis reaction. Samples were incubated for 10 min at 65 °C and an equal volume of CIA (chloroform : isoamylalcohol 24:1, about 700 µl) was added. After mixing the samples tubes were put on ice for 30 min.

Samples were centrifuged at 5000 rpm for 10 min at 4 °C, obtaining a pellet and two phases with an interphase of proteins. The upper aqueous phase, containing DNA, was transferred to a sterile 1.5 ml test tube. 225 µl of ammonium acetate were added and the tubes were put in ice for 30 min. To pellet the remaining proteins and cell debris the samples were centrifuged at 14000 rpm for 10 min at 4 °C in a bench top centrifuge 1K15 (Sigma Laborzentrifugen, Osterade, Germany). Supernatant was transferred to new 1.5 ml tubes and mixed with 0.55 volumes (about 400 µl) of isopropanol. DNA was precipitated at room temperature for at least two hours and pelleted in a bench top centrifuge at 14000 rpm for 15 min. The supernatant

was discarded and the pellet was washed with 200 µl ethanol 70 % (-20°C). The pellet was dried at room temperature and resuspended in 30 to 50 µl of TE (10 mM Tris, pH 8.0, 1 mM EDTA).

4. DNA concentration measurement

DNA concentration was measured using a SpectraFluor (TECAN Deutschland GmbH, Crailsheim, Germany), with SYBR green I (Molecular Probes, Europe BV, Leiden, The Netherlands) as dye at 485 nm excitation and 520 nm emission. Calf thymus or λ DNA of a concentration of 1ng/µl was used as standard. Alternatively, DNA concentration was determined photometrically by measuring the absorption at 260 nm (Sambrook et al., 1989).

5. DNA preparation from green coffee samples (Knoll et al., 2002b)

Green coffee samples (50 g) and data on their ochratoxin A content were kindly provided by commercial laboratories and coffee companies. Ochratoxin A concentration was determined by HPLC. For extraction of DNA, 5 g of green coffee beans were ground with a commercial coffee grinder. Isolation of DNA was performed using ultrasonification and a commercial extraction kit as described by Knoll et al. (2002b) for wheat. Briefly, 5 g of ground coffee were mixed with 7 ml lysis buffer (20 mM EDTA, 10 mM Tris-base, 1% Triton-X, 500 mM guanidin-HCl, 250 mM NaCl) containing 6 % polyvenyl propylene and ultrasonified for 1 min with a UP 200S processor (Dr. Hieschler GmbH, Germany) equipped with a 14 mm diameter steel sonotrode (model S14, energy density = 105 W cm^{-2}). Coffee particles were allowed to settle and 0.8 ml supernatant were mixed with 0.4 ml ethanol (-20°C). This solution was further used for DNA preparation with the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturers recommendations.

6. Oligonucleotides for AFLP

6.1 Adapter preparation

E1: 5′-CTCGTAGACTGCGTACC-3′

E2: 5′-AATTGGTACGCAGTCTAC-3′

M1: 5′-GACGATGAGTCCTGAG-3′

M2: 5′-TACTCAGGAGTCAT-3′

For the preparation of the adapter molecules the oliconucleotides were resuspended in distilled water and mixed in equimolar amounts, heated to 94 °C and slowly cooled down. For simplification of the protocol the concentration of the EcoRI adapter solution was ten times less than that of the MseI adapter solution, i. e. 0.5 µl E1 and E2 (each 100 pMol/µl) and 99 µl water formed the EcoRI adapter and 5 µl M1 and M2 (each 100 pMol/µl) and 90 µl water formed the MseI/BfaI adapter.

6.2 Primer sequences

EcoRI: 5'-GACTGCGTACC**AATTC***NNN*-3'

BfaI: 5'-GATGAGTCCTGAG**TAG***NNN*-3'

MseI: 5'-GATGAGTCCTGAG**TAA***NNN*-3'

The primers used in the PCRs during the AFLP protocol consisted of a core sequence (NORMAL letters), an enzyme specific sequence (**BOLD** letters) and the selective nucleotides (in *ITALICS*). For the pre-amplification primers had no or one, for selective amplification one to three selective bases.

7. AFLP (Vos et al., 1995, Aarts and Keijer, 1999)

7.1 Template preparation

For template preparation all reaction mixtures contained 10 mM Tris-HAc pH 7.5, 10 mM $MgAc_2$, 50 mM KAc, 5 mM DTT and 50 ng/µl BSA. 200 ng of isolated genomic DNA were digested in a total volume of 15 µl at 37 °C for 3 h with 2U of EcoRI (TaKaRa Shuzo Co., Ltd., Japan) and 2U of MseI or BfaI (New England Biolabs, Frankfurt, Germany). Following incubation, 5 µl of a solution containing 2 pMol EcoRI adaptor, 20 pMol MseI adaptor, 0.4U T4 DNA ligase (MBI Fermentas, St. Leon-Rot, France) and 1 mM ATP were added. Ligation of adaptors was performed for 12 h at room temperature in a total volume of 20 µl. This mixture was diluted tenfold with 10 mM Tris-HCl pH 8.0..

7.2 Pre-amplification

Five microliters of the diluted restriction/ligation mixture were added to a 15 µl mastermix I resulting in the following concentration: $MgCl_2$ (1,5 mM), KCl (50 mM), dNTPs (0.2 mM), PreE-primer, 0.3 mM, PreM- or PreB-primer, 0.3 mM and Taq-polymerase, 0.02 U/µl. The PCR had the following temperature profile:
Denaturation at 94 °C for 1 min, 94 °C for 30 s, annealing at 56 °C for 1 min, extension at 72 °C for 1 min, 20 cycles; final extension at 72 °C for 3 min.

7.3 Selective Amplification

The pre-amplification was diluted tenfold with 10 mM Tris-HCl, pH 8.0 and 5 µl of this dilution were added to 15 µl mastermix II. The reaction thus had the following composition: $MgCl_2$ (1.5 mM), KCl (50 mM), dNTPs (0.2 mM), E*-primer (0.05 mM), M-primer (0.3 mM) and Taq-polymerase (Promega, Mannheim, Germany, 0.02 U/µl). The touch down PCR used the following temperature profile:

Denaturation at 94 °C for 1 min; 13 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, extension at 72 °C for 1 min in each cycle the annealing temperature was reduced by 0.7 °C ; followed by 23 cycle of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 1 min. Again, a final elongation of 72 °C for 3 min was applied.

7.4 Automated laser fluorescence analysis (ALFA)

8.5 µl of formamide containing 5 mg/ml dextran blue, and a 35 bp and 650 bp internal standard were added to the 20 µl PCR product resulting from the selective amplification. The mix was denatured by heating to 94°C for 3 min and the tubes immediately transferred to ice. Analysis of the denatured Cy5-labelled fragments was performed on an ALFexpress sequencer (Amersham Pharmacia, Freiburg, Germany). Five µl sample were loaded on a 0.3 mm denaturing 5 % PAA gel. An external standard was run every 12 lanes. Electrophoresis was carried out at 45 W, 60 mA and 50 °C for 6 h.

8. AFLP for the recovery of marker sequences

8.1 Modifications of the AFLP protocol

Recovery of AFLP fragments from silver stained PAA gels for sequencing was carried out according to Chalhoub et al. (1996) with some modifications. Pre-amplification was conducted as described above. The selective PCR (a total of 40 µl) was carried out using both the MseI/BfaI primer and the EcoRI primer in equal concentrations of 0.3 mM. All other components had the same concentration as described above for the selective PCR. A total of four to five selective bases was used in this second PCR. After the PCR reaction the products were evaporated to dryness and resuspended in 5 µl of water.

8.2 Electrophoresis on polyacrylamide gels and silver staining

AFLP fragments for marker detection were separated on a native CleanGel Long-10 polyacrylamide gel using the DNA LongRun Buffer (ETC-Elektrophorese-Technik, Kirchentellinsfurt, Germany) on a Multiphor II electrophoresis system (Amersham Pharmacia, Freiburg, Germany). Electrophoresis was carried out as described by the manufactures protocol with the exception that only 1 µl of sample buffer was added to 5 µl concentrated AFLP product. After loading the gel a delay of approximately five minutes before applying power was carried out. After electrophoresis, fragments were silver stained according to Bassam et al. (1991). After fixation gels were washed for 10 min in distilled water and the banding pattern was observed.

8.3 DNA recovery, cloning, sequencing and PCR conditions

Bands considered to be characteristic were cut out from the gel. DNA was eluted in 20 µl water at 4 °C overnight. 5 µl of this eluate were used in the subsequent reaction. For reamplification the same primers as for the selective PCR were used. The temperature profile was as follows: 94 °C for 1 min, 40 cycles: 30 s at 94 °C, 60 s at 56 °C, 72 °C 1 min and finally 3 min at 72 °C. The resulting fragments were separated on a 2 % agarose gel. After staining with ethidium bromide bands were cut out from the gel under UV. DNA was extracted using a DNA extraction kit (Genomed, Bad Oeyenhausen, Germany). Purified DNA fragments were ligated into the pGEM®-T Easy Vector (Promega, Madison, USA) according to manufactures instructions. The vector was transformed into *E. coli* XL1 blue (Stratagene, Amsterdam, The Netherlands) by electroporation (Sambrook et al., 1989). Plasmides were prepared using either the boiling method (Holmes and Quigley, 1981) or a plasmid midi kit (Qiagene, Hilden, Germany). Sequencing of the inserts was done with the ABI Prism Dye Terminator Cycle Sequencing Kit (Perkin Elmer, Boston, USA) on a ABI 373 stretch sequencing system by a commercial service (SequiServe, Vaterstetten, Germany)

9. Standard polymerase chain reactions (PCRs)

The standard PCR reactions for specificity testing or the detection of fungal DNA was carried out in a total volume of 25 µl and had the following composition: 10 x PCR buffer (Roche Diagnostics, Penzberg, Germany) 5 µM each forward and reverse primer, 0.25 µl Taq Polymerase (Promega, Heidelberg, Germany, 5 U/µl), dNTPs (10 mM each) 0.5 µl, 1 µl of template DNA, and water to a final volume of 25 µl. PCR was performed in a Mastercycler Gradient (Eppendorf, Hamburg, Germany) with the following temperature profile. Initial denaturation at 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and final elongation for 2 min at 72 °C. PCR products were separated electophoretically on a 2 % agarose gels and visualized by ethidium bromide staining (Sambrook et al., 1989)

10. Quantitative PCR

Quantitative real time PCR was carried out using a LightCycler™ (Roche Diagnostics, Penzberg Germany). The reaction mixture was prepared using the QuantiTect™ SYBR® Green PCR kit (Qiagene, Hilden, Germany). Reaction mix composition was as follows: 10 µl 2x mastermix; 1 µl forward primer OCA V (5'-ATA CCA CCG GGT CTA ATG CA-3', 50 pMol/ µl,) and 1 µl reverse primer OCA R (5´-TGC CGA CAG ACC GAG TGG ATT-3´, 50 pMol/ µl), 0.5 µl uracil-N-glycosylase (1 U/ µl, MBI Fermentas, St. Leon-Rot, Germany), 2 µl of template DNA and water to a final volume of 20 µl. Temperature profile in the LightCycler instrument were as follows: 2 min at 50 °C, 15 min at 95 °C, 50 cycles of 94 °C for 15 s, 55 °C for 25 s, 72 °C for 30 s and 77 °C for 20 s in which the fluorescence was acquired. After the final PCR cycle, melting curve analysis of the PCR products was performed by heating from 40 to 94 °C and continuous measurement of the fluorescence to verify the PCR product.

After calibrating the instrument with dilution series of purified *A. ochraceus* DNA a standard curve was set up. Quantification was done using the 2nd derivative maximum method. Statisti-

cal analysis of data was performed either with the LightCycler software version 3.5 (Roche Diagnostics, Penzberg, Germany) or Sigma Plot 8.0 (SPSS Inc.).

11. Computing and analysis of biological relationships

11.1 Fingerprints

ALFexpress data files were converted to TIFF images using the ALF2TIFF software provided by the manufacturer using the following settings: resample 10, gutter 5, lane width 15 and inversion off. These images were imported into the BioNumerics version 2.5 software package (Applied Maths, Sint-Martens-Latem, Belgium) and further processed. For cluster analysis usually fragments between 70 bp and 650 bp were considered. Similarity coefficients were calculated using the Pearson correlation, a method that calculates similarities and standardizes the fingerprints according to the relative intensity of the signals. Thus, similarities calculated were based on the shape of the densiometric curve of the fingerprints rather than on the appearance of a single band. Dendrograms were calculated using the Ward algorithm or the UPGMA method. For a extensive description and discussion of these methods, see the book of Sneath and Sokal (1973). For each node the cophenetic correlation was calculated and in UPGMA dendrograms the error flags, i. e. the standard deviation within a cluster, was calculated.

11.2 Composite data sets

11.2.1 Sequence data

An alignment of the DNA sequences was calculated according to the algorithms provided by the BioNumerics software. For alignment and similarity calculation no conversion costs were used and no gap penalty was assigned. The alignment was checked manually and the similarity between the sequences was calculated using the correction of Jukes and Cantor (1969).

11.2.2 Chromatographic data

Chromatographic data on secondary metabolites were obtained and processed as described by Thrane et al. (2003). The calculated similarity matrix was directly imported into the BioNumerics software.

11.2.3 Phenotypical data

Morphological and phenotypical data were provided by M. Torp (National Veterinary Institute, Oslo, Norway) and coded in a binary character table. The resemblance was determined using the Dice coefficient (Dice, 1945).

11.2.4 Consensus matrix

The consensus matrix was calculated using the values from the similarities of the individual datasets using the arithmetic average. No weights were assigned to the individual experiments. To visualize the obtained consensus matrix a dendrogram was calculated by the unweighted pair-group method using arithmetic averages (UPGMA, Sokal and Michener, 1958). From the same matrix a non-metric multidiminsional scaling (MDS, Shepard 1962) was computed. To check the robustness of the dendrogram and groups the cophenetic correlation and error flags for each node of the dendrogram (standard deviation) were determined.

11.2.5 Comparison of datasets

Pair wise resemblance between data matrices was computed using the Pearson correlation (Pearson, 1926). All calculations were carried out on complete datasets using the BioNumerics version 2.5 software (Applied Maths, Sint-Martens-Latems, Belgium).

III. Results

1. AFLP typing of *Fusarium poae*, *F. sporotrichioides* and *F. langsethiae* Torp and Nirenberg ined.

For the typing of *Fusarium* species complexities of AFLP fingerprints obtained was low when the combination of *Eco*RI and *Mse*I and a total of five selective ends (E-ACT or E-AAC together with M-CA) was applied (data not shown). A total of four selective bases (E-AC, M-CA) turned out to provide a sufficiently complex pattern for fragment analysis. Figure 4 shows AFLP profiles of some representative strains. Within these runs, fragments were present throughout the range from 70 to 650 bp. Besides several very prominent peaks a number of less intense signals were recognizable. At high molecular weight peaks were generally smaller and less frequent compared to lower molecular weights. In summary, a total of approximately 20 different peaks within the range of fragment sizes from 70 to 650 bp contributed to cluster analysis.

Among the fragments analysed some were found to appear in fingerprints of a specific group of isolates (see figure 4). For example, fragment A (85 bp) appeared only in fingerprints of *Fusarium poae* whereas the 290 bp fragment C was detected exclusively in fingerprints of *F. sporotrichioides* strains. Fragment B of approximately 220 bp was present in both *F. sporotrichioides* and *F. langsethiae*. In strain IBT 9945 identified as *F. sporotrichioides* only fragment B could be found resembling AFLP profiles of *F. langsethiae*. Fingerprints of strains IBT 9958 and IBT 9951 (data not shown) clearly differed from fingerprints of all of the species mentioned.

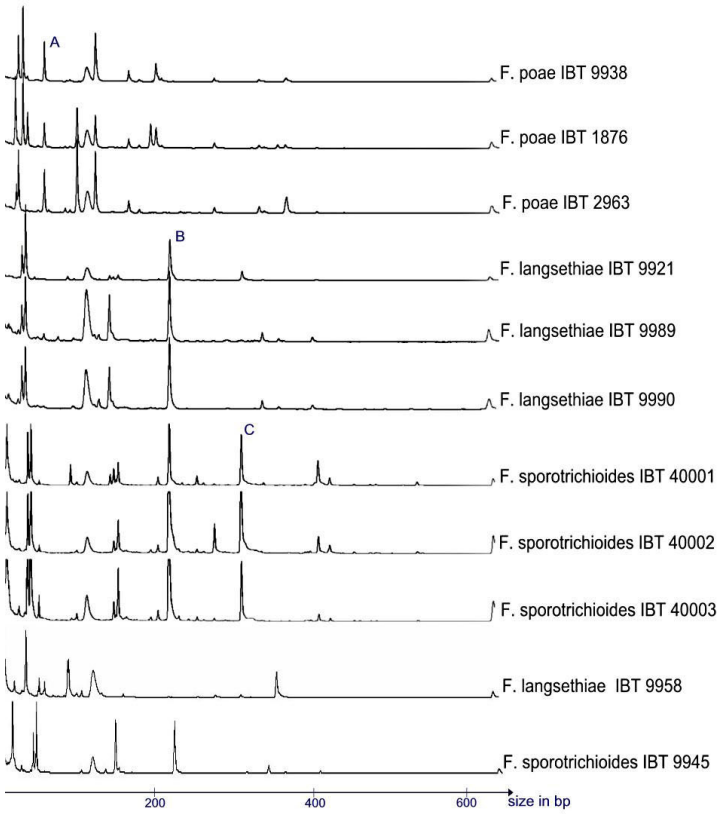


Figure 4: AFLP fingerprints of representative *Fusarium* strains examined in this study. Sizes of nucleotides on the x-axis, the y-axis shows the relative intensity of the signal in the electropherogram. Primers M-CA and E-AC were used to generate the pattern. A indicates an anonymous marker present only in *F. poae* isolates, C a marker present only in *F. sporotrichioides*. Fragment B is present in both *F. sporotrichioides* and *F. langsethiae.*

AFLP profiles were compared using the Pearson correlation. The Ward dendrogram calculated from this similarity matrix is presented in figure 5. In this graph, organisms were clustered according to the shape of their AFLP profiles in a dichotomous way. In this cluster analysis two major groups, I and II, appeared which were clearly separated from another and well supported by a high overall cophenetic correlation of 81 % for the complete dendrogram. These two clusters were further divided into subgroups. *Fusarium sporotrichoides* strains were exclusively found in group I. Within this group all *F. sporotrichoides* strains studied formed a distinct subcluster (IA) with one exception (IBT 9945). In the other subcluster (IB), grouping of all strains assigned to *F. langsethiae* was well supported by a cophenetic correla-

tion of 68 %. In this group, *F. sporotrichoides* IBT 9945 was the only outlier among the majority of the *F. langsethiae* isolates examined. Cluster II comprised all *F. poae* isolates and was rather heterogeneous compared to I. Two strains of *F. langsethiae* and the analysed strain of *F. kyushuense* could also be found in this group. The latter three strains formed rather segregated branches within the *F. poae* strains, indicating a rather distant relationship of these strains to the *F. poae* strains within cluster I.

Figure 5

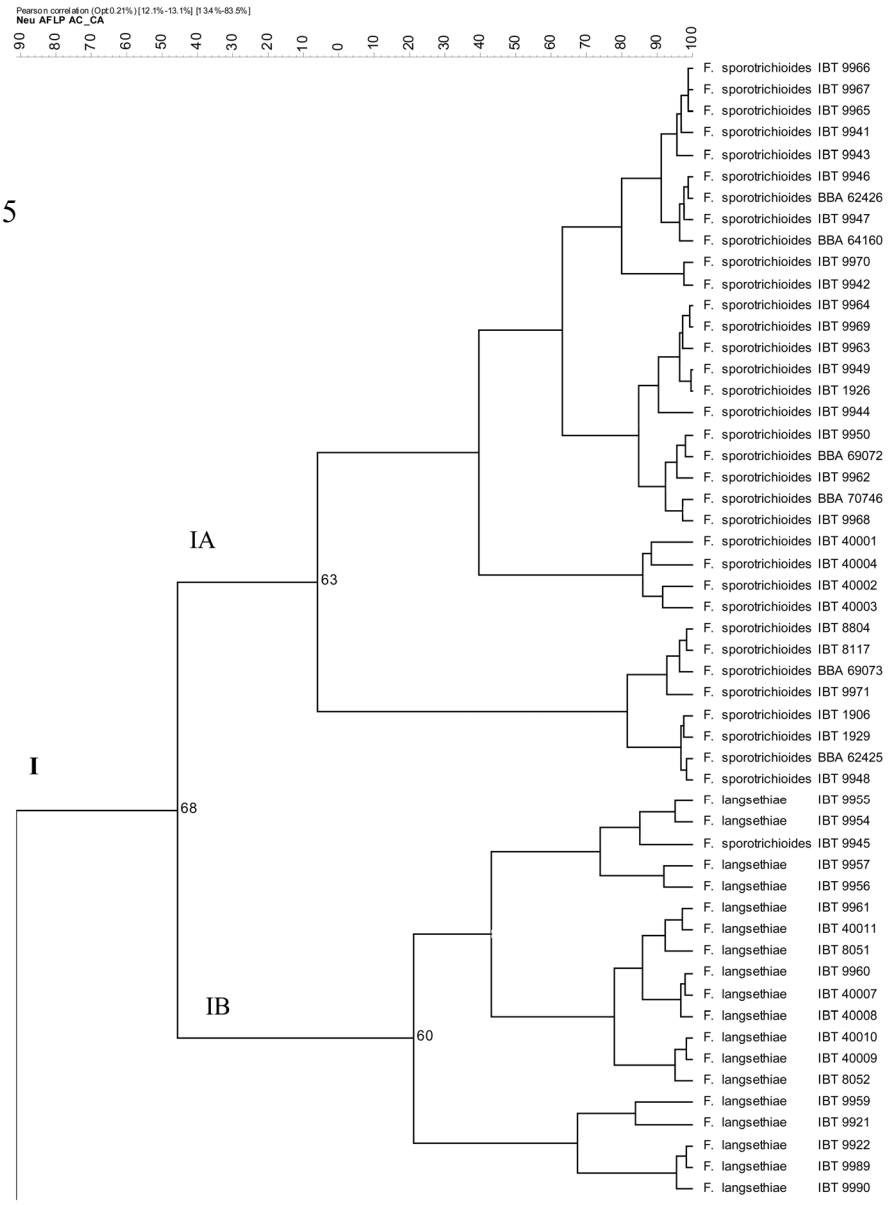


Figure 5 continued

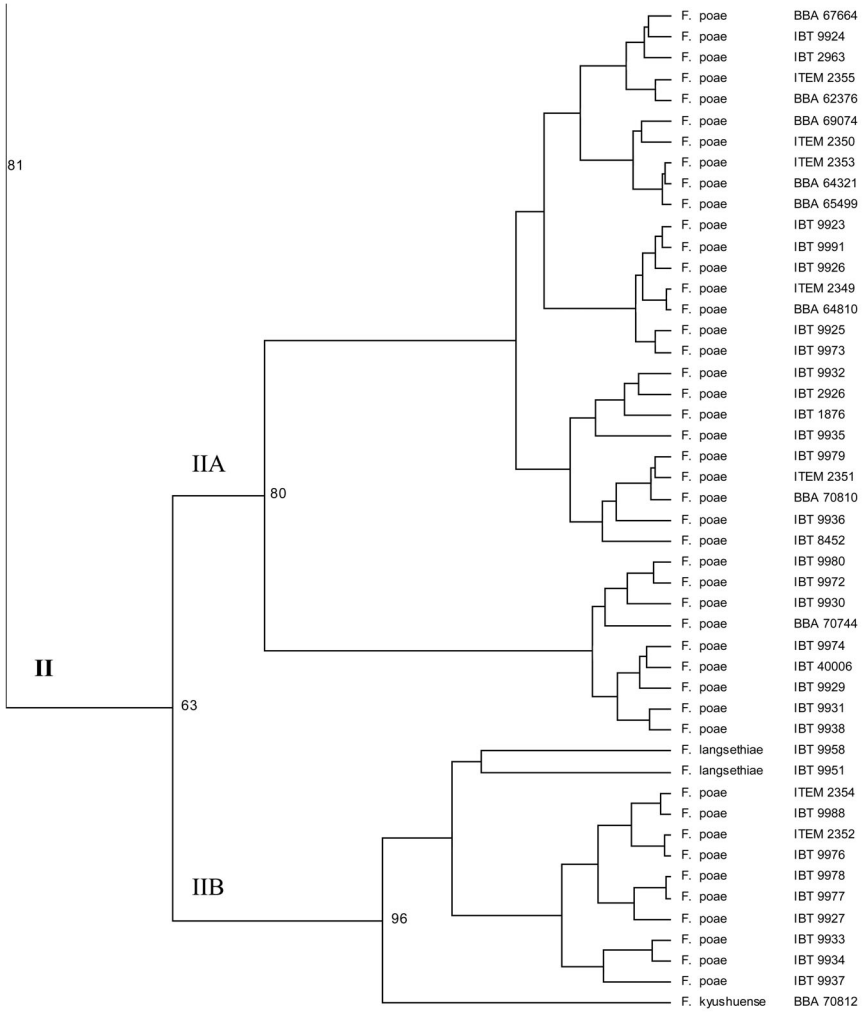


Figure 5: Ward dendrogram calculated from the comparison of AFLP fingerprints of *Fusarium* strains examined in this study. Similarities were determined using the Pearson correlation. Fragments from 70 bp to 650 bp were taken into account. The cophenetic correlation at the major branching points is given in %. Notice that cluster I comprises only *Fusarium sporotrichioides* and *F. langsethiae* isolates. All *F. poae* isolates studied are found in cluster II. With the exceptions of IBT 9958 and IBT 9951 all *F. langsethiae* strain are found in subcluster IB.

2. Cluster analysis of composite datasets of *Fusarium* section *Sporotrichiella* isolates

A number of different data were available for the different *Fusarium* strains and a panel of strains was chosen for which a complete character set was available. From these data a consensus matrix was calculated and transformed into a UPGMA dendrogram (figure 6). This dendrogram showed a clear separation of the three *Fusarium* species. The main groups of the phenogram were well supported by a rather high cophenetic correlation. However, the standard deviations, which indicate the homogeneity of a group, were quite high at the basal nodes. The error flags at the basal parental nodes did not overlap with those of the daughter nodes for any of the three main groups, pointing to the conclusion that the three main clusters represent distinct entities. It should be stressed that the *F. langsethiae* strain IBT 9959 formed a rather separate branch within the *F. langsethiae* cluster. From the analysis of these data, it was visible that *F. sporotrichioides* was the most homogeneous group of the three taxa.

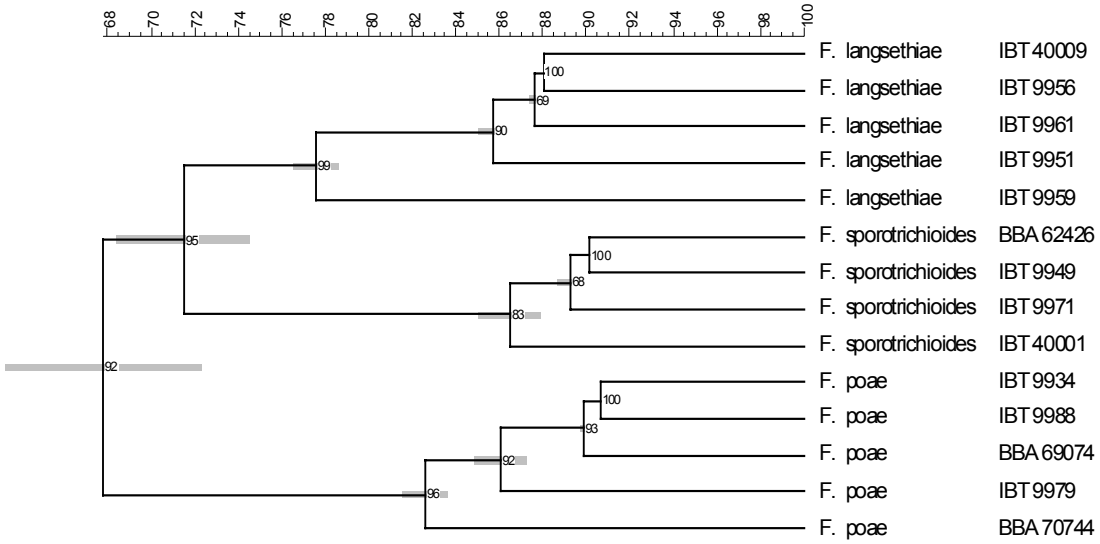


Figure 6: UPGMA dendrogram calculated from the combined similarity matrix of IGS, ITS as well as partial DNA sequences of the EF-1-α and β-tubulin genes, chromatographic data, AFLPs and phenotypical data. Numbers at the nodes give the cophenetic correlation. Bars indicate the standard deviation for the corresponding cluster. The scale at the top indicates the percentage of similarity.

Multidimensional scaling (MDS) is a nonhierarchical grouping technique, and was used to compute a three dimensional graphic image of the relationships between individual strains from the composite similarity matrix of the selected strains (i.e. excluding the EF-1α data, figure 7). This ordination method allows to interpret the similarity data leading to the separation of groups as well as the analysis of homogeneity within a group. The three main groups which correspond to the three *Fusarium* species studied were clearly separated. However, within the *F. langsethiae* group strain IBT 9959 was clearly separated from all other *F. langsethiae* strains.

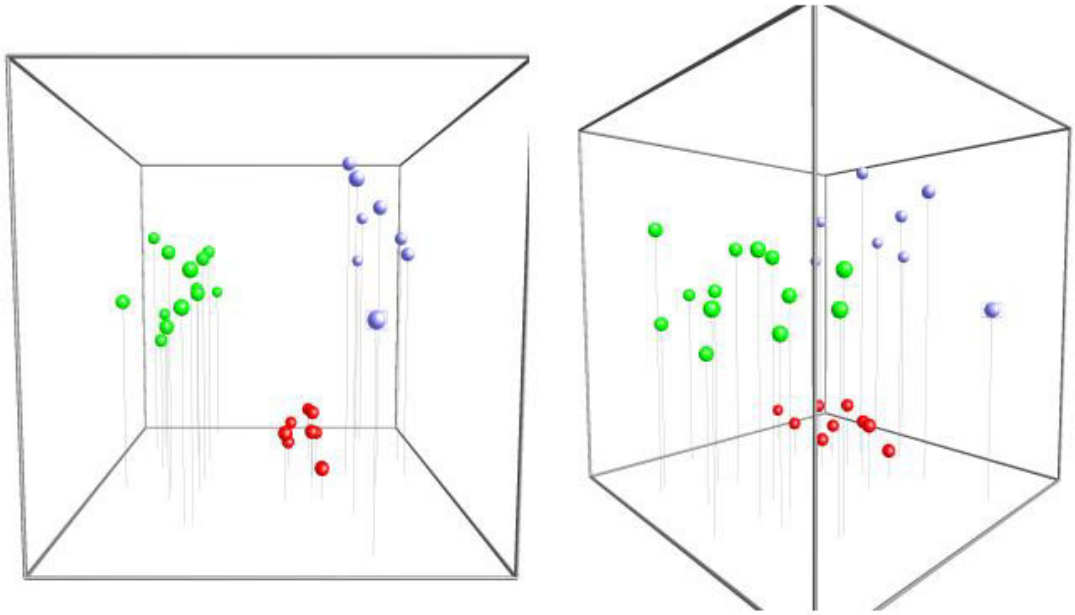


Figure 7: Two views of the multideminsional scaling (MDS) calculated from the similarity matrix combined from the matrices of IGS, ITS as well as partial β-tubulin DNA sequences, chromatographic data, AFLPs and phenotypical data. ● : *F. poae*-strains, ● : *F. sporotrichiodes*-strains, ● : *F. langsethiae*-strains. The box indicates *F. langsethiae*-strain IBT 9959.

The result of comparative congruence analysis of the individual datasets is presented in figure 8. Comparison of the similarity matrices of two and two datasets shows that the highest concordance between two individual datasets was found between the similarity matrices of the partial EF-1-α and β-tubulin gene sequences. The lowest concordance came from the comparison of ITS and phenotypically derived similarity matrices. It is interesting to note that the highest consilience of the composite matrix with an individual matrix is found with the one derived from the AFLP experiments.

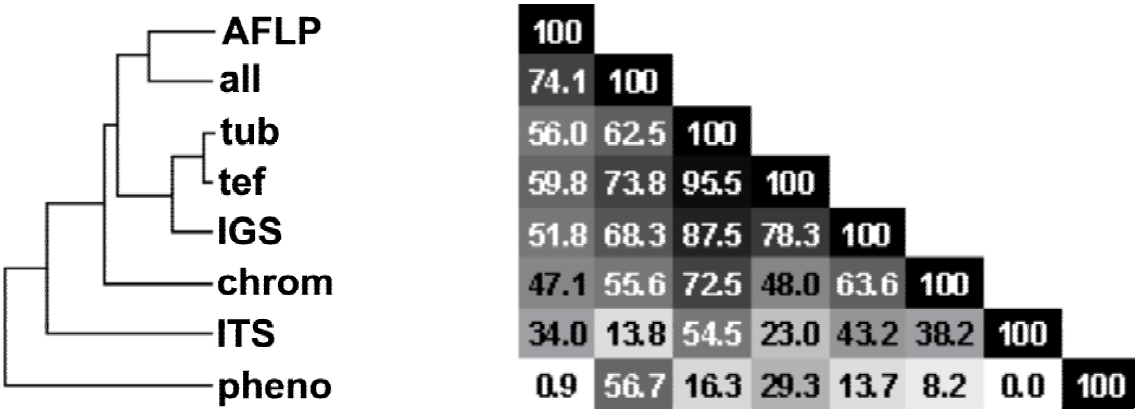


Figure 8: Congruence between the experiments leading to the clustering in fig. 6. The similarity matrix derived from the pairwise comparison of the individual experiments (right side of the figure) was transformed to the UPGMA dendrogram at the left side. AFLP: data derived from the AFLP experiments, all: composite dataset, chrom: chromatographic data, IGS: DNA sequences of the IGS region, ITS: DNA sequences of the ITS region, pheno: similarity data derived from the coding of phenotypical data, tef: partial DNA sequences of the EF-1α-gene, tub: partial DNA sequences of the EF-1α-gene.

3. AFLP typing of ochratoxinogenic *Penicillium* strains

For AFLP typing of *Penicillium* strains the combination of the restriction enzymes *Eco*RI in combination with *Bfa*I was used. *Bfa*I creates the same sticky ends as *Mse*I and for this reason the *Mse*I adapters originally described can be used. A total of three selective bases used in the second PCR resulted in a complex, yet clearly resolved banding pattern. The Ward dendrogram calculated from the AFLP fingerprints obtained from the different *Penicillium* strains resulted in two well separated groups (figure 9). These two groups were in good agreement with those found by Castella et al. (2002). Group I contained non-citirinin-producing strains that synthesized high amounts of OTA. In contrast to this all citrinin-producing strains were found in group II. Members of this group also produced relatively low amounts of OTA.

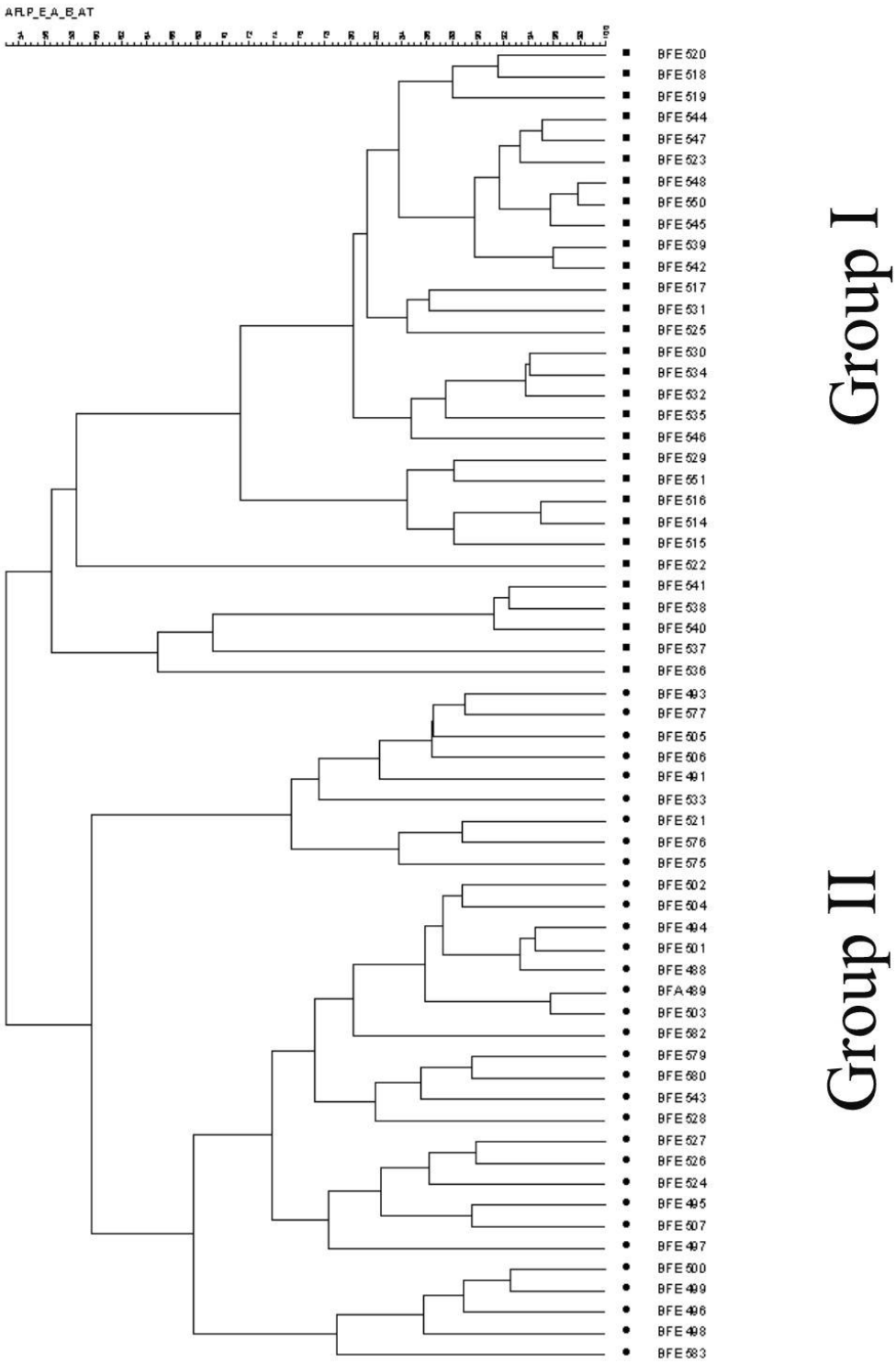


Figure 9: Ward dendrogram generated by cluster analysis of AFLP patterns obtained from *Penicillium verrucosom* and "*P. nordicum*" strains with the restriction enzymes *Bfa*I and *Eco*RI using a total of three selective nucleotides in the selective PCR (E-A/B-AT). Group I: strains producing high amounts of OTA. Group II: strains producing low amounts of OTA.

4. AFLP typing of *Aspergillus niger* and *A. carbonarius* and construction of SCAR primers

The AFLP applied for automatic laser fluorescence analysis (ALFA) resulted in a sufficiently complex, yet clearly resolved banding pattern of approximately 20 bands within the range of 70 to 650 bp. For the creation of fingerprints the combination of the restriction enzymes *Eco*RI and *Mse*I together with a total of four selective nucleotides in the selective PCR was used. This fingerprint turned out to be well suited for cluster analysis (figure 10). The dendrogram calculated from the similarity matrix obtained from the comparison of the fingerprints (figure 10) showed a clear separation of the *A. niger* / *A. carbonarius* strains from the rest of the fungal species analysed. The *A. carbonarius* strains formed a well separated group in which the three non-toxigenic isolates studied were rather dispersed. Interestingly, the CBS strain of *Aspergillus niger var. niger* clustered close to the *A. carbonarius* strains while all other strains belonging to the *A. niger* aggregate like *A. awamorii*, *A. usamii* var. *shiro-usamii*, and *A. foetidus*, formed a separate unit, which was less homogeneous when compared with the *A. carbonarius* strains. *A. niger* strains ITAL 638 and ITAL 630 were segregated from the rest of the *A. niger* strains. *A. fotidus* CBS 114.49 and *A. niger* CECT 2090 apparently did not belong to the *A. niger* "core group", whereas *A. awamorii* CBS 101704 and *A. usamii* var. *shiro-usamii* CBS 101700 were close to the genotype of the majority of the *A. niger* strains examined. As for *A. carbonarius*, the strains described as toxigenic and non-toxigenic in this group were rather scattered within this part of the cluster.

Based on these findings it was decided to look for a species specific marker for *Aspergillus carbonarius* since the incident of ochratoxin A producing strains is much higher than in *Aspergillus niger*. In order to identify marker fragments, AFLPs were separated on polyacrylamide gels and subsequently silver stained. Several fragments characteristic for *A. carbonarius* strains were detected. The bands considered to be species specific were cut out

from the gel, and after elution of DNA reamplified with the AFLP primers. Two of these DNA fragments, assigned A and C, were cloned and subsequently sequenced. The obtained sequences were searched against Genebank (http://ncbi.nlm.nih.gov/BLAST) and submitted to the EMBL nucleotide database (http://ebi.ac.uk/embl) accession numbers AJ516957 and AJ516956, respectively. Comparison of the sequence of fragment A resulted in no significant hits while the sequence of fragment C showed high similarity to a protein of unknown function "related to ahmp1" in *Neurospora crassa* (EMBL accession AL355928.2) using the blastx algorithm (Altschul et al. 1997).

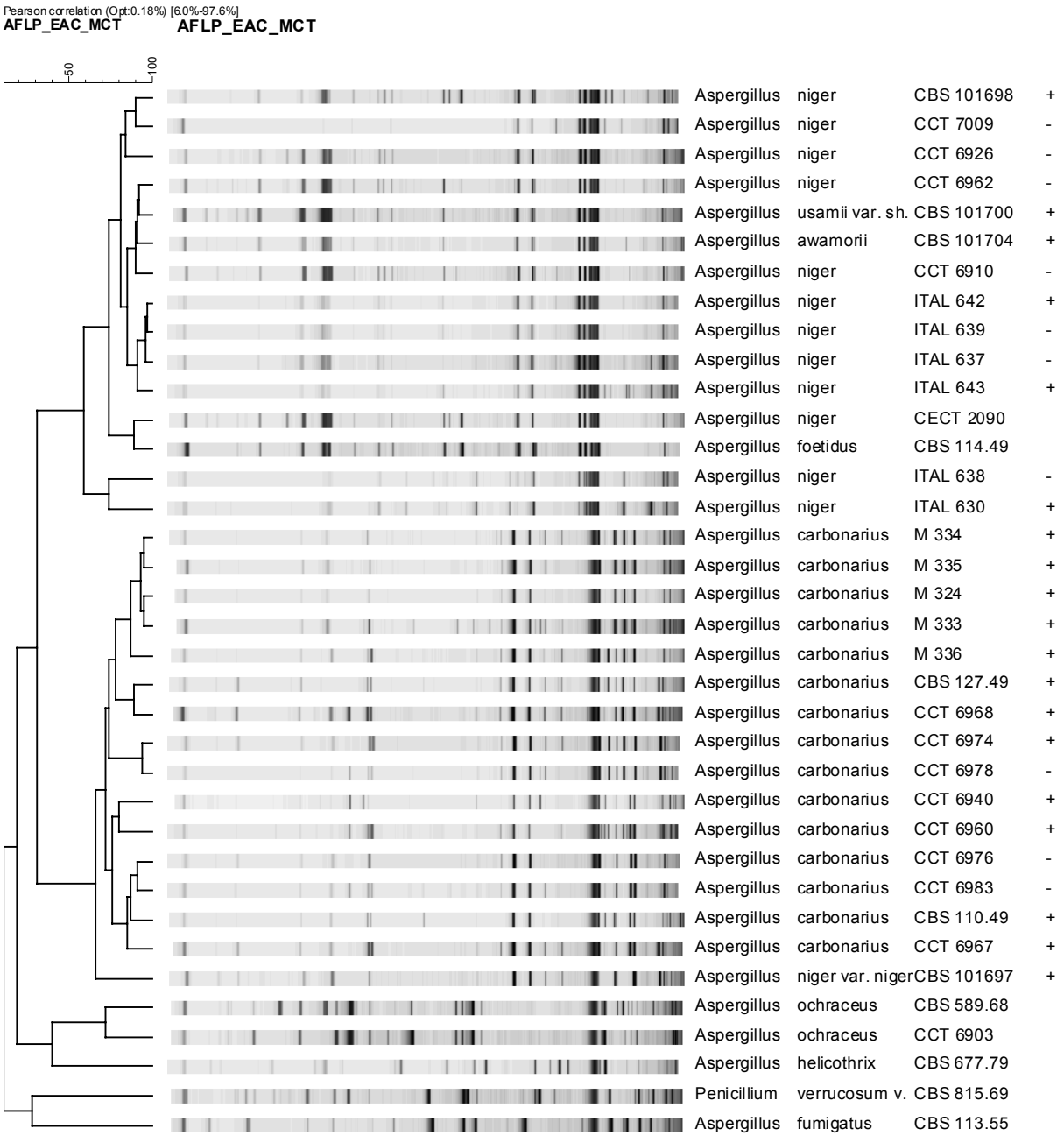


Figure 10: UPGMA dendrogram calculated from comparison of AFLP fingerprints generated with a total of four selective bases (E-AT/M-CT). Fragments between 70 and 650 bp are shown. For similarity calculation the Pearson correlation was used. + and – indicate the ability of the isolate to produce ochratoxin A, where no + or - is displayed the production of the toxin was not determined. The cophenetic correlation of the dendrogram is 0.95.

Based on these sequences, two primer pairs (A1B_fw/ A1B_rv and C1B_fw/ C1B_rv) were designed. PCR was optimized and primers were tested for specificity against a panel of 20 fungal strains belonging to 17 different *Aspergillus* and *Penicillium* species (figure 11).

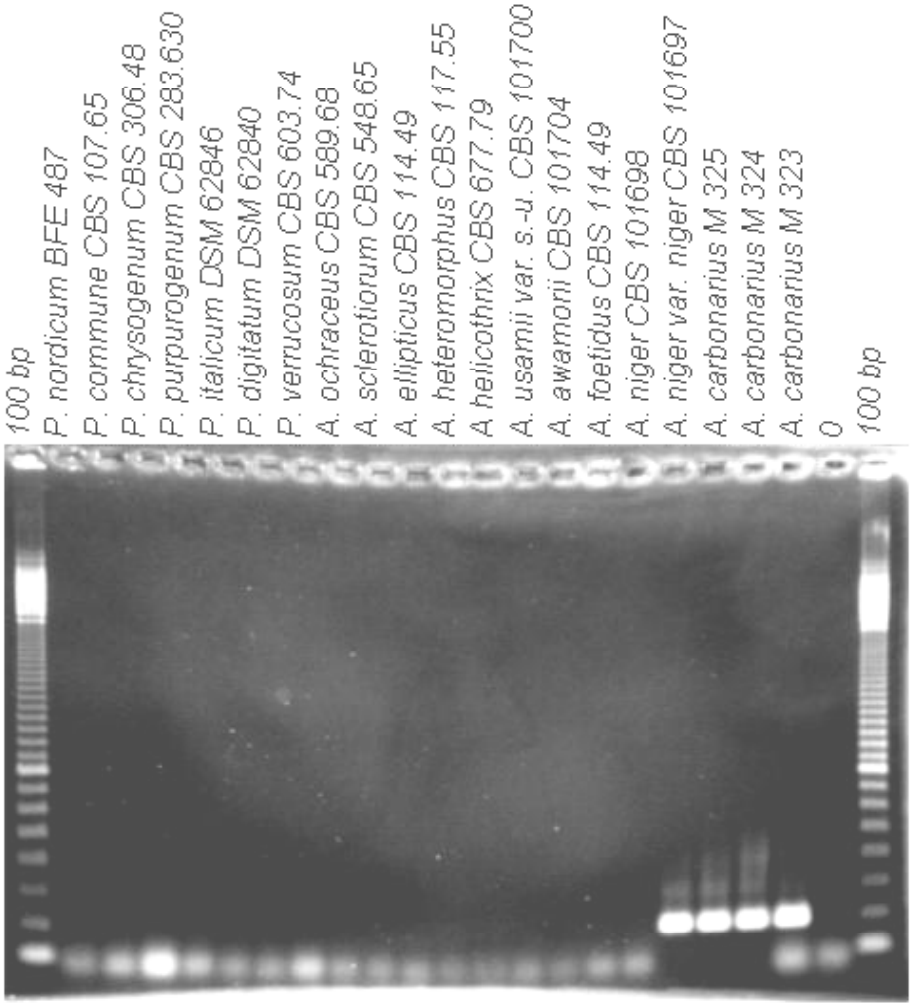


Figure 11: PCR reaction carried out with the primer pair A1B_fw/A1B_rv under the conditions summarized in table 2. Notice that only when DNA isolated from *A. carbonarius* and *A. niger var. niger* CBS 101697 was used the a 189 bp product resulted. 100 bp: 100 base pair ladder, 0: negative control.

PCR conditions and primer sequences are summarized in table 2. Both primer pairs specifically yielded products of 189 bp and 351 bp (not shown) with DNA from *A. carbonarius* strains and *A. niger var. niger* CBS 101697 using primers A1B_fw/ A1B_rv and C1B_fw/ C1B_rv, respectively.

Table 2: Primer sequences and PCR conditions used for the specific detection of *Aspergillus carbonarius*.

Marker	Primers (5`to 3`)	Amplification buffer mix (10x)	Cycling conditions
A (EMBL accession number: ACA516957)	A1B_fw GAA TTC ACC ACA CAT CAT AGC A1B_rv: TTA ACT AGG ATT TGG CAT TGA AC	500 mM KCl 100 mM Tris-HCl 15 mM $MgCl_2$ pH 8.6	94 °C, 4 min initial denaturation followed by 35 cycles 94 °C, 30 s; 64 °C, 30 s; 72 °C 30 s, followed by 5 min final elongation at 72 °C. Temperature of heated lid was set to 100 °C.
C (EMBL accession number: ACA516956)	C1B_fw GAA TTC ACG GTG CTC GAC CC C1B_rv TTA ACT GCT GGC GGA AGA GGC	500 mM KCl 100 mM Tris-HCl 15 mM $MgCl_2$ pH 8.6	94 °C, 4 min initial denaturation followed by 35 cycles 94 °C, 30 s; 64 °C, 60 s; 72 °C 60 s, followed by 5 min final elongation at 72 °C. Temperature of heated lid was set to 100 °C.

5. Typing of *A. ochraceus* and construction of SCAR primers

For typing of strains the restriction enzymes *Bfa*I in combination with *EcoR*I were chosen. The use of a total of three selective ends (E-A/ B-AT) turned out to produce a complex yet well resolved fingerprint pattern. The region from approximately 70 to 650 bp was considered for similarity calculation and subsequent graphical visualisation. The UPGMA dendrogram constructed from this matrix is shown in figure 12 for all taxa treated. All *Aspergillus ochraceus* strains examined displayed a high degree of similarity. There was no clear correlation between the genetic similarity and the potential of the strain to produce OTA. OTA-producers and non-producers were scattered throughout the dendrogram. All other *Aspergillus* species considered formed distinct groups.

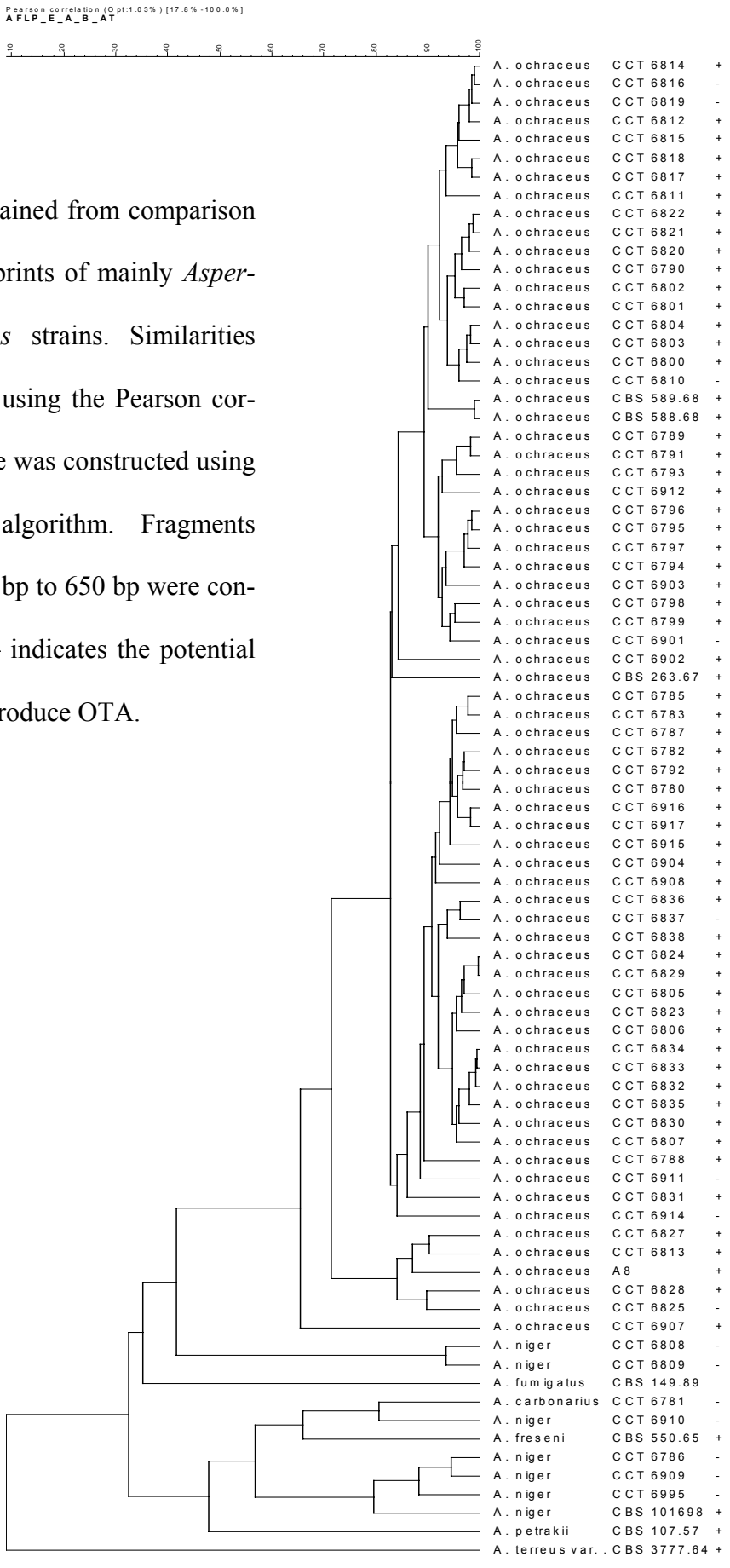


Figure 12:

Dendrogram obtained from comparison of AFLP fingerprints of mainly *Aspergillus ochraceus* strains. Similarities were calculated using the Pearson correlation. The tree was constructed using the UPGMA algorithm. Fragments from approx. 70 bp to 650 bp were considered. + and – indicates the potential of the strain to produce OTA.

Based on these results the three CBS strains of *A. ochraceus* were selected to serve as references for further studies aiming at detection of specific marker sequences. For this purpose a number of different combinations of *Eco*RI with *Mse*I or *Bfa*I were used in the subsequent AFLP reactions. In the selective PCR reactions of these AFLPs the number of selective bases was increased to 4 to reduce the number of fragments. AFLP fragments could be clearly separated on polyacrylamide gels and visualised by silver staining. By this means a number of bands considered to be unique for *Aspergillus ochraceus* were detected. Figure 13 shows an example of a silver stained AFLP separated on a polyacrylamide gel. Some of the bands were cut out from the stained gel and DNA could be reamplified following elution. Subsequently, the fragments could be successfully utilized for ligation into the pGEM®-T Easy vector which was then transformed into *E. coli* to allow sequencing. Using this approach a number of sequences could be obtained which were searched against Genebank (http://ncbi.nlm.nih.gov/BLAST). Neither fragment sequenced showed a significant similarity to other sequences in the database. DNA sequences of fragment H10, A and D were utilized for the design of primers and have been submitted to the EMBL nucleotide database (http://ebi.ac.uk/embl), accession number AJ511647, AJ511648, and AJ511647 respectively. PCR with the primer pairs designed according to the obtained sequences were optimized for sensitivity and specificity prior to testing against DNA isolated from a panel of representative strains. Table 3 summarizes information on markers, sequences of derived primers and PCR conditions. Figure 14 shows an example of a PCR performed with DNA of a selected test panel of strains representing distantly related fungal species and a set of closely related yellow aspergilli with the PCR conditions employed as displayed in table 3.

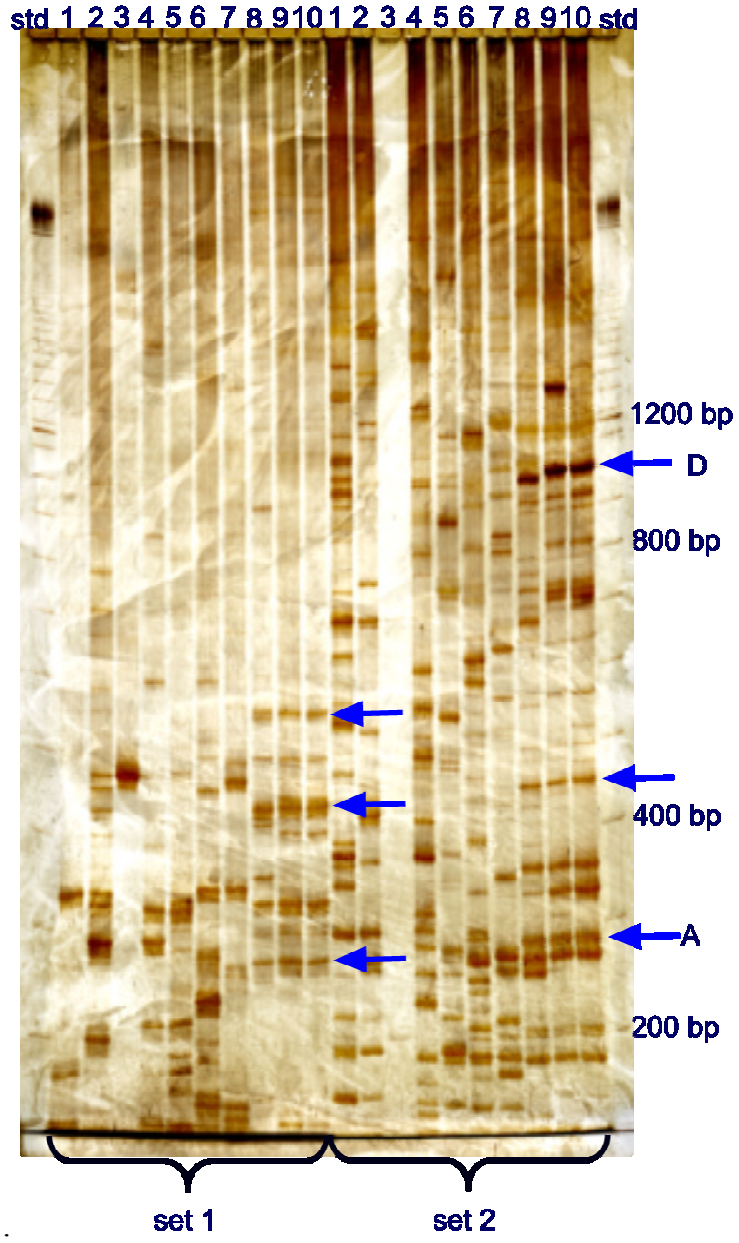


Figure 13: Silver stained AFLP fragments created by the primer combination E-AC/M-CT (set 1) and E-A/M-CAG (set 2) separated on a native polyacrylamide gel. std: 100 bp ladder, 1: *Stachybotris chartarum* TMW 4.0654, 2: *Penicillium verrucosum* CBS 603.74, 3: *Aspergillus auricomus* CBS 467.65, 4: *A. sulphureus* CBS 550.65, 5: *A. bridgeri*: CBS 350.81, 6: *A. petrakii* CBS 101.57, 7: *A. spec.* A 91, 8: *A. ochraceus* CBS 263.67, 9: *A. ochraceus* CBS 589.68, 9: *A. ochraceus* CBS 588.68. Arrows indicate some characteristic fragments common in all *A. ochraceus* strains and absent in the other strains. A and D are fragments that lead to the corresponding marker sequences mentioned in the text and table 3.

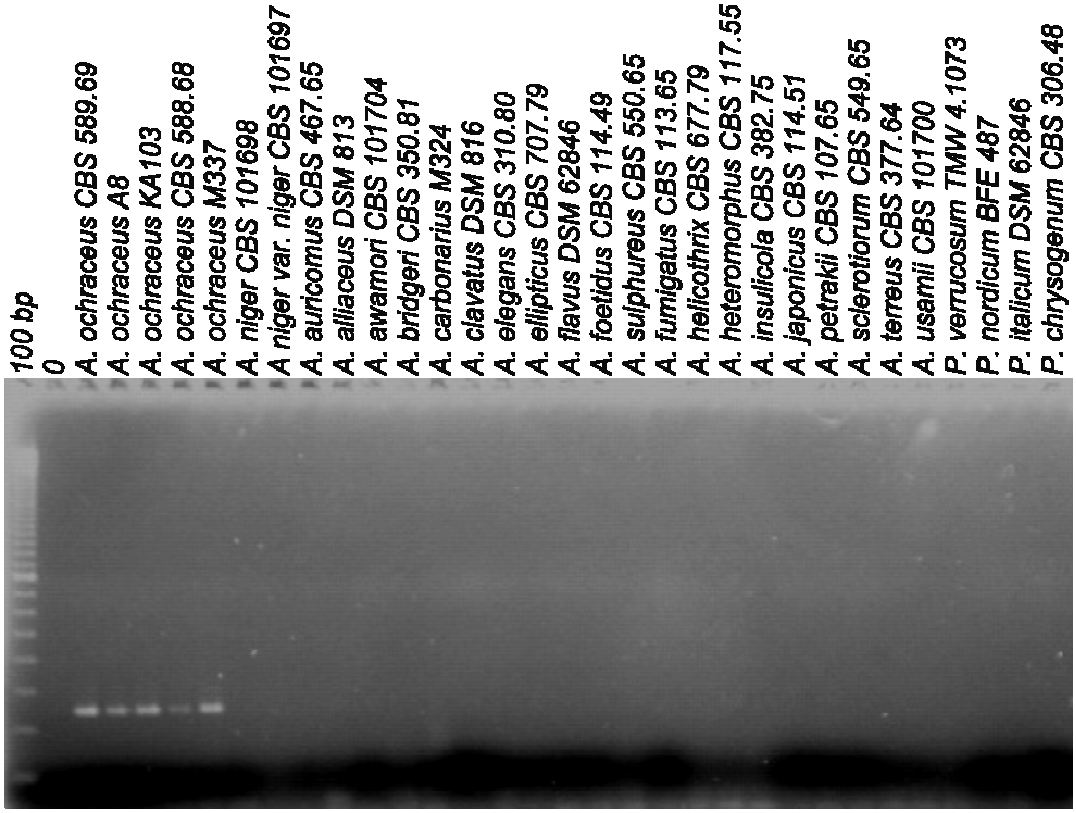


Figure 14: Reactivity of the primer pair OCA-V/OCA-R against *Aspergillus and Penicillium* spec. 100bp:100 bp ladder, 0: negative control. Notice that only DNA from *Aspergillus ochraceus* strains result in the amplification of a 260 bp product. For PCR conditions see table 3.

Table 3: PCR conditions and primer sequences used for the specific detection of marker sequences in *A. ochraceus*. The numbers in the brackets indicate the EMBL accession number.

Marker	Primers (5`to 3`)	Amplification buffer mix	Cycling conditions
H10 (AJ511647)	OCA-V: ATA CCA CCG GGT CTA ATG CA OCA-R: TGC CGA CAG ACC GAG TGG ATT	500 mM KCl 100 mM Tris-HCl 15 mM $MgCl_2$ 60 % (v/v) formamide 50 mM $(NH_4)_2 SO_4$ pH 8.6	94 °C, 4 min initial denaturation followed by 35 cycles 94 °C, 30 s; 55 °C, 30 s; 72 °C 30 s, followed by 5 min final elongation at 72 °C. Temperature of heated lid was set to 100 °C.
A (AJ511648)	Ochra pA-F: TTA ACA GGG AAC AAT CCA TAT AG Ochra pA-R GAA TTC ACC CAG AAC TTG CCG	500 mM KCl 100 mM Tris-HCl 15 mM $MgCl_2$ 30 % (v/v) formamide 50 mM $(NH_4)_2 SO_4$ 0.1 % gelatine pH 8.6	94 °C, 4 min initial denaturation followed by 35 cycles 94 °C, 30 s; 55 °C, 30 s; 72 °C 30 s, followed by 5 min final elongation at 72 °C. Temperature of heated lid was set to 100 °C.
D (AJ511649)	Ochra pD-F: TTA ACA GGC ACC GGA GAC ATA GTT AG Ochra pD-R: TTA ACA GAT TTG AGA ACC CCA TTC	500 mM KCl 100 mM Tris-HCl 15 mM $MgCl_2$ 30 % (v/v) formamide 0.3 % gelatine pH 8.6	94 °C, 4 min initial denaturation followed by 35 cycles 94 °C, 30 s; 57.5 °C, 30 s; 72 °C 30 s, followed by 5 min final elongation at 72 °C. Temperature of heated lid was set to 100 °C.

6. Detection of *Aspergillus ochraceus* in coffee samples

As a first step towards the detection of the fungus in coffee samples, green coffee artificially infected with *A. ochraceus* KA103 was blended with uninfected, sterilized green coffee. When this mixture was used for DNA preparation it was possible to clearly identify the fungus in the sample by the PCR described. A clearly visible band of a PCR product of the expected size (260 bp) was still present at a portion of 0.1 % (w/w) infected in uninfected coffee (data not shown). Neither in the uninfected control sample, nor in the no target control a band was visible. The same was true for coffee spiked with purified *Aspergillus ochraceus* DNA. As shown in figure 15 the reaction resulted in one single clearly detectable PCR product of the expected size of approximately 260 bp. A minimum of 1 µl of a concentration of 1.9 µg/µl per 5 g coffee could be detected. This corresponded to approximately 0.4 ng *A. ochraceus* DNA per reaction if 100 % recovery during DNA preparation is assumed.

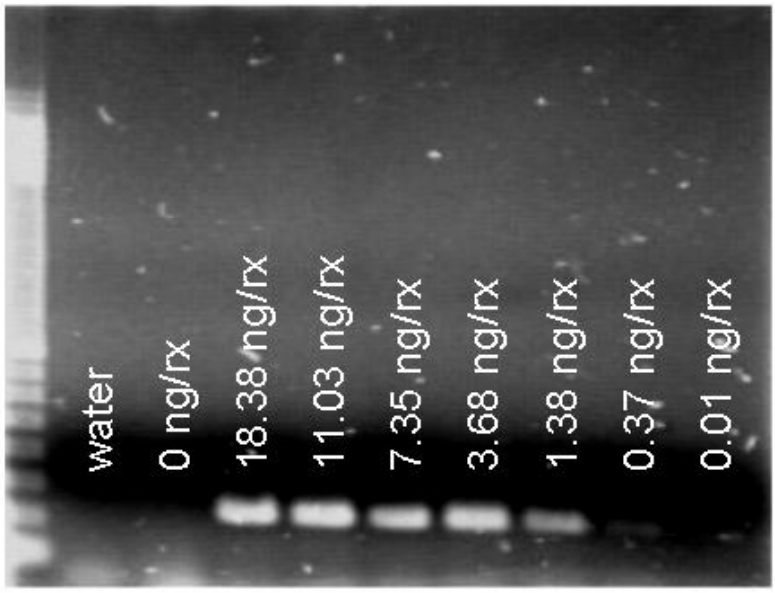


Figure 15: Agarose gel showing the PCR reaction with DNA prepared from green coffee spiked with different amounts of purified *A. ochraceus*-DNA. Water means water control, the ng/rx ng of DNA expected to be present with an recovery rate of 100 % assumed in the reaction (=rx).

7. Quantification of *A. ochraceus* DNA in coffee

When starting PCR optimization it turned out, that the annealing temperature used was appropriate for the amplification of pure fungal DNA. However, when only small amounts of template DNA were present, the melting curve analysis displayed, that primer dimers occurred. As a consequence, the fluorescence data were acquired at 77 °C to avoid false positive measurements. Figure 16 shows a melting curve analysis of the PCR products formed during the reaction with different amounts of template DNA.

The reproducibility of the system was tested by comparing the different standards used for calibration. The sensitivity of the system was higher compared to that of the convential PCR. 4.7 pg template DNA per reaction were determined as the lower detection limit. The reproducibility was as follows. At a DNA concentration of 5950 pg/rx the crossing point was calculated to be 24.2 cycles with a standard deviation of 0.2, at 372 pg/rx 28.9 ± 0.4 cycles, at 93 pg/rx 30.8 ± 0.6 cycles and at 23 pg/rx 28.9 ± 0.5 cycles with n=5. The recovery of DNA from spiked green coffee was found to be in the average 77 % ± 38 %.

Quantification of *A. ochraceus* DNA in all 30 samples was done in triplicate. Plotting DNA content against the ochratoxin A content of the samples resulted in the graph shown in figure 16. The standard deviations of the determined DNA content in each sample was calculated. For the two parameters a linear regression was calculated with a coefficient of correlation r=0.55. A t-test was performed to analyse the quality of correlation found. A positive correlation between the two parameters was secure at the 99 % level of significance. The 95 % interval of confidence was calculated and 3 out of the 30 data points clearly did not lie within this area.

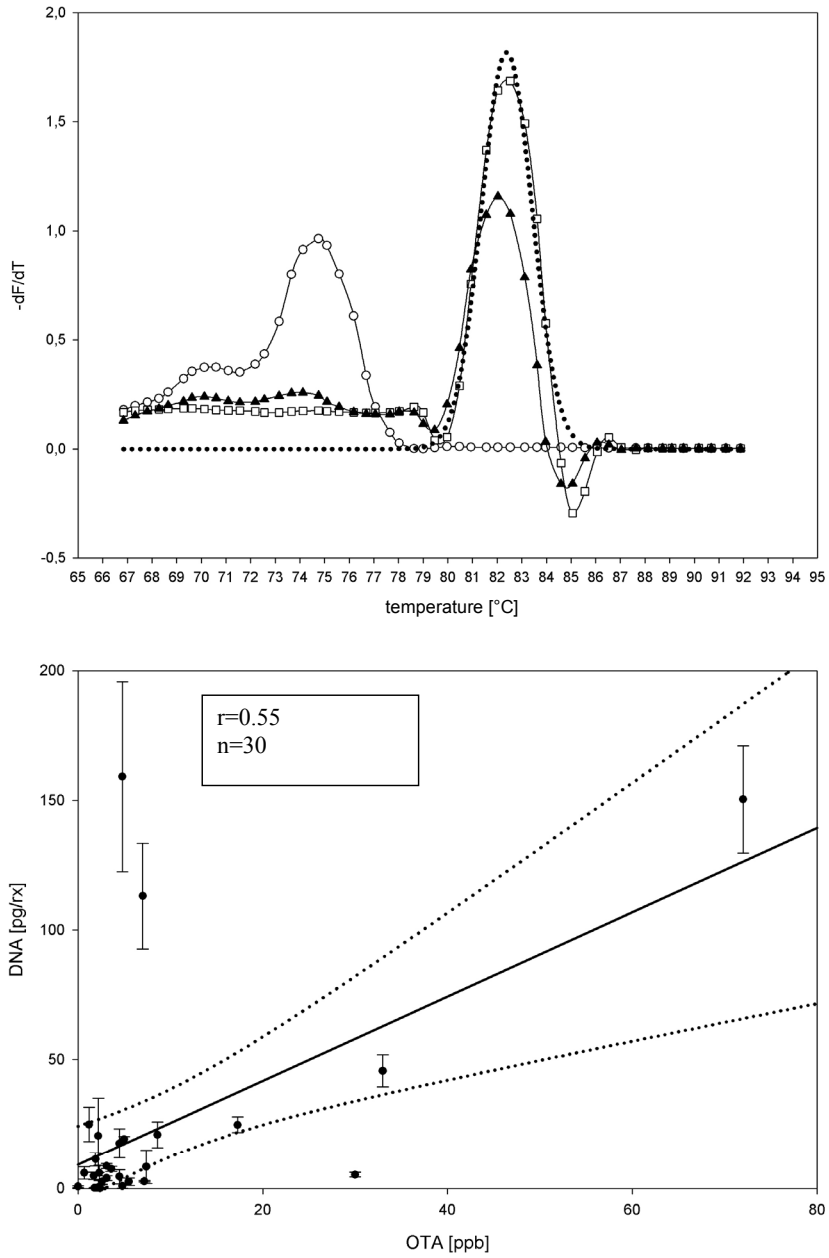


Figure 16: **A**: Melting curve analysis of different PCR resulting from different templates. ○: green coffee with no *A. ochraceus*-DNA detectable; ▲: green coffee with *A. ochraceus*-DNA detectable; □: DNA prepared from *A. ochraceus*; •••: Gaussian regression calculated from □.

B: Plot of *A. ochraceus*-DNA content of the 30 green coffee samples against ochratoxin A content. The error bars are the standard deviations. The solid line is the regression calculated, the dotted line is the 95 % interval of confidence. The regression coefficient was calculated to be r=0.55.

IV. Discussion

1. Methods

One of the core techniques in this thesis was AFLP (amplified fragment length polymorphism) a DNA fingerprinting technique universally applicable to any genome without prior knowledge on its composition (Savelkoul et al., 1999, Vos et al., 1995). Since its first application for distinction and identification of fungi (Majer et al., 1996), lots of different fungal isolates have been studied using the AFLP technique (e.g. Mueller et. al., 1996). In *Fusarium* taxonomy AFLP was first applied to discriminate between different strains of *Fusarium graminearum* (Leissner et al., 1997). In this paper the method was used for the comparison of different *Fusarium* species and applied for *Pencillium* and *Aspergillus* isolates. The comparison of different methods for the taxonomy of *Fusarium poae*, *F. langsethiae* and *F. sporotrichioides* displayed that the similarity matrix obtained from a composite dataset consisting of two coding (tef-1, beta tubulin) and two non coding DNA sequences (IGS, ITS), chromatographic and phenotypic data and AFLP fingerprints themselves is best resembled by AFLP as an individual experiment. This strengthens the statement of Savelkoul et al. (1999) that DNA fingerprinting techniques can function as a core technique in a polyphasic taxonomic approach. Baayen et. al. (2000) stress the higher resolution of AFLP analyses at a refined taxonomic level compared with EF-1α and mtSSU rDNA sequences in *F. oxysporum.* However, for analysis of relationships above the genus level, DNA fingerprinting techniques are not applicable, since incidental similarities may occur. For phylogenetic studies, i. e. the use of character based algorithms as most parsimony or maximum likelihood fingerprints are always problematical since the homology of anonymous DNA fragments is not always given. As a consequence for phylogenetic analysis DNA sequences seem to be suited better. On the other hand DNA fingerprinting techniques sample polymorphisms from throughout the whole

genome and thus comparison of the organisms is not only based on a few DNA sequences. For understanding fungal evolution sequencing and comparison of whole genomes would be the best approach. Unfortunately, until now *Aspergillus niger*, *Schizosaccheromyces pombe* and *Saccheromyces cervisiae* are the only complete fungal genome sequences available and those of *Aspergillus nidulans*, *A. fumigatus*, *Neurospora crassa*, *Ustilago mayidis*, *Cryptococcus neoformans* and *Candida albicans* are being sequenced (http://www.tigr.org). However, costs for DNA sequencing exclude this for large scale screening at the moment (Myburg et al., 2001).

The detected polymorphisms may serve as a source of target sequences for the specific identification or detection of an organisms. The possibility to recover DNA sequences from silver stained AFLP polyacrylamide gels was first described by Behura et al. (2000). Anonymous marker sequences for identification of fungi has been demonstrated by Schilling et al. (1996). Using DNA markers for the detection of toxigenic aspergilli and penicillia in food has proven to be a valuable tool for the rapid and easy detection of these moulds (e. g. Boysen et al., 2000, Geisen et al., 1996, Färber et al., 1997).

2. *Fusarium poae*, *F. sporotrichioides* and *F. langsethiae* Torp and Nirenberg ined.

As pointed out already, in some instances comparison of rather distantly related organisms has lead to misinterpretation of dendrograms when these organisms eventually cluster together (Louws et al., 1999, Savenkoul et al., 1999). Maybe this is the case for the two *F. langsethiae* isolates IBT 9958 and IBT 9951 as they do not seem to be well fitting into group II of the dendrogram presented in figure 5. Strain IBT 9945 of *F. sporotrichioides*, which clustered perfectly within the *F. langsethiae* group indicates the close relationship between the two species and may be misidentified based on morphological characters. When taking into account the dendrogram calculated from the AFLP fingerprints of the rest of the examined *F. langsethiae* isolates, they can be considered as a group well separated from

F. sporotrichioides. The relatively high cophenetic correlation (68 %) at the basal node of group I further supports the division of the two taxa. Together with the homogeneity of the cluster obtained by AFLP fingerprinting, this justifies the creation of a separate taxon.

This is also supported from the analysis of the similarity matrix of the combined datasets. However, in most cases the grouping of operational taxonomic units (e.g. single strains) coincide, and it has been pointed out that neither approach may truly reflect natural classification (Sneath and Sokal, 1973). If one assumes that all the data used to construct the similarity matrix are ultrametric and evolve at the same speed, UPGMA would be suited for phylogentic analysis (Hillis et al., 1996). Such an assumption would not be valid for the data analysed. A phenetic approach may be an indicator of phylogenetic relationship, but it is not necessarily congruent with the latter. However, in most cases the grouping of operational taxonomic units (e.g. single strains) coincide, and it has been pointed out that neither approach may truly reflect natural classification. Considering this, the dendrogram shown in figure 5 most likely gives a picture of the natural relationships. The appearance of characteristic fragments in the electropherograms of *Fusarium* AFLPs (figure 4) point towards the possibility of the specific detection of *F. poae* and *F. sporotrichioides*. However, *F. langsethiae* would only be distinguishable from *F. sporotrichioides* by the combination of two independent PCRs. Using the *tri*5 gene as target, Niessen et al. (2003) also found, that only the combination of two PCRs would allow the discrimination of the two species. For practical purposes the exact differentiation of the two species is however not to relevant, since both produce neosolaniol, T2- and HT-2 toxin (Thrane et al., 2003, Torp and Langseth 1999) and thus are of major concern for food safety.

3. *Penicillium verrucosum* and "*P. nordicum*"

Ochratoxin A producing *Penicilium* isolates have been assigned to lots of different species. Pitt (1987) stated that ochratoxin A production can only be assigned to *P. verrucosum*. Fris-

vad et al. (2000) agreed with this though members of the species are isolated from two completely different habitats, i. e. cereals from temperate zones and salted meat products and cheese from Northern and Southern Europe. In addition to that both authors retained one species, though the isolates belonged to two different chemotypes. Larsen et. al. (2001) separated two chemotypes by a combination of chromatographic methods and cluster analysis of the metabolite data. The largest group of isolates produced citrinin and relative low amounts of ochratoxin A. This group contained the type strain of *Penicillium verrucosum* and was isolated mainly from plant sources. The other group contained the former type culture of *P. nordicum* and members of this group were not able to produce citrinin. These strains were, however, generally strong ochratoxin A producers and were mainly isolated from cheese and meat products. Studies on molecular relationships using ITS sequence data, RAPD and AFLP fingerprints were further carried out (Castella et al., 2002). A difference of only two single nucleotides was found between the ITS1-5.8S-ITS2 sequences of the two groups. However, both AFLP and RAPD analysis clearly separated the two groups indicating that the isolates belong to two different, albeit closely related species (Castella et al., 2002). The concordance between these two methods illustrates the value of DNA fingerprinting as a quick, economic and effective tool for taxonomic and identification purposes.

4. *Aspergillus carbonarius* and *A. niger*

Black aspergilli have been studied extensively using both phenotypical and genotypical data (e. g. Varga et al., 1993, Parenicová et al., 1997, Hamari et al., 1999, Parenicová et al., 2000, Varga et al., 2000, Parenicová et al., 2001). Schuster et al. (2002) summarize the accepted species within the section. It is now generally accepted that in this group ochratoxin producing strains are only found in *A. niger s. s.* (comprising the formerly separated *A. awamorii*, *A. usamii*, *A. phoenicus* etc.) and *A. carbonarius* (Varga et al., 2000b). While *A. carbonarius* can easily be recognized by the size of its echinuate conidia the differentiation

of the other black aspergilli based on morphological characters is more difficult. On the molecular level the two species can be easily recognized. The AFLP analysis clearly distinguishes the two species (figure 10). The studied strain *of A. niger var. niger* groups close to *Aspergillus carbonarius*. This may be either due to hybrid formation or misidentification by the depositors. The conidia of this *A. niger var. niger* strain strongly resembled that of *A. carbonarius* and this together with the fact that the fungus produces vast amounts of ochratoxin A leads to our conclusion that it should be referred to as *A. carbonarius* rather than *A. niger*.

The developed SCAR primers A1B_fw/ A1B_rv and C1B_fw/ C1B_rv specifically react with *A. carbonarius* (and *A.niger var. niger* CBS 101697) but not with the other black aspergilli examined (*A. ellipticus*, *A. heteromorpus*, *A. helicotrix* and the strains belonging to *A. niger* including *A. foetidus*). The fungus is regarded as the main causative agent of ochratoxin A in grape derived products (Abarca et al., 2003, Cabañes et al., 2002, Torp et al., 2002). It significantly contributes to the contamination of raw coffee with this mycotoxin (Taniwaki et al., 2003). For both reasons, the early detection and identification of this fungus are of major concern to food safety. The SCAR-PCR primers designed during the current study allow the reliable and rapid identification of *A. carbonarius* and may serve as a basis for a rapid culture independent detection system for the fungus in present different food samples and raw material and for the identification of pure cultures.

5. *Aspergillus ochraceus*

The *Aspergillus ochraceus* strains studied seem to form a quite homogeneous group with a high degree of similarity among the isolates (figure 12). The grouping in figure 12 is based on complex AFLP patterns, which allow a distinction of very closely related strains but since incidental similarities among the fingerprints of distantly related organisms could occur the dendrogram may not reflect true phylogenetic relationships. In contrast to Varga et al. (2000a)

this work could not demonstrate any correlation of OTA-production to the genotype for *A. ochraceus*. Varga et al. (2000a) state that several lineages of *A. ochraceus* have lost their ability to synthesise OTA as revealed by cluster analysis of DNA fingerprints. However, when ITS sequence based phylogenetic analysis of members of the section *Circumdati* or even different *A. ochraceus* isolates was performed, no clear correlation between the taxonomic position and the OTA production could be detected (Varga et al., 2000a , Varga et al., 2000c, Varga et al., 2000d). Though the algorithms employed for cluster analysis do not necessarily reflect true evolution (Hillis et al., 1996) our AFLP work confirmed these findings. For aflatoxinogenic aspergilli both RAPD analysis (Tran-Dinh et al., 2000) and ITS sequence comparison (Peterson, 2000) showed no homogeneous distribution of toxigenic and nontoxigenic strains or species. It is also known that the potential for aflatoxin synthesis is easily lost in the course of ongoing sub cultivation (Horn and Dorner, 2001). Analogous observations have been made for ochratoxin A (Harwig, 1974, Varga et al., 1996). Experience in our laboratory also shows that the ability of a strain to produce the toxin is highly variable (unpublished data). Thus looking at ochratoxin production alone in culture without taking into account other characters is probably not very helpful for classifying yellow aspergilli and the comparison of DNA fingerprints may not be a reasonable approach for the detection of specific marker genes involved in ochratoxin biosythesis.

Aspergillus ochraceus is regularly isolated from different food and feed, especially coffee but also from cereals (Wilson et al., 2002). While the source of ochratoxin A in different commodities has been assigned to other aspergilli and penicillia (Wilson et al., 2002) in coffee infection of coffee beans with *A. ochraceus* – partly mediated by insects - is responsible for OTA formation (Taniwaki et al., 2003). Therefore, prevention of the growth of this fungus during storage and its rapid and early detection during processing are of importance in coffee manufacturing (Bucheli and Taniwaki, 2002).

The developed primer pair OCA-R/OCA-V was also tested in a ring study involving seven European laboratories in the course of the EU-Project DeTox Fungi. In this test, cycling conditions, template DNA and the primers were supplied. Participants used different thermocyclers and Taq-DNA-polymerases. Tests were performed in triplicate. When pure fungal DNA was used as template all laboratories involved were able to clearly identify the fungus without any cross reactions. This demonstrates the robustness of this PCR for diagnostic purposes. Preliminary results with pure fungal cultures and a simplified DNA extraction protocol points towards similar findings. However, in this set up DNA extraction an the age of the fungal culture seems to be of major importance.

The use of PCR as a tool for the rapid detection of toxigenic fungi in food has been demonstrated recently (Färber et al., 1997, Knoll et al., 2002a). The target sequence has immanent influence on the quality an sensitivity of the PCR. ITS and anonymous DNA markers have been shown to perform well in different *Fusarium* species (Schilling et al., 1996; Yoder et al., 1998). However, one of the drawbacks of these markers is that the number of copies in a genome is not exactly known. The use of genes involved in the biosynthesis of a mycotoxin is of course much more valuable when one intents to find a correlation between DNA concentration and the amount of toxin. The correlation between DNA content and mycotoxin load or the presence of toxigenic fungi has been demonstrated for *Fusarium* and *Aspergillus* species (Schnerr et al., 2002; Mayer et al., 2003a). Mayer et al. (2003b) also used a real time RT-PCR approach to monitor the correlation of gene expression and aflatoxin biosynthesis in wheat.

Edwards et al. (2002) described the presence of a specific polyketide synthase of *Aspegillus ochraceus*. However, the authors suggest that this gene is not involved in the biosynthesis of ochratoxin A. As no gene is known in the ochratoxin biosynthetic pathway and the pathway itself is rather unclear (Mantle and Chow, 2000) the detection of the microorganisms capable of producing the toxin is one approach. *A. ochraceus* is reagarded as the main causative agent

of ochratoxin A in green coffee (e. g. Urbano et al., 2001). However, not all isolates of the fungus are capable of producing the toxin under in vitro conditions and other ochratoxigenic fungi have also been isolated from green coffee, e. g. *A. sulphureus* and *A. carbonarius* (Batista et al., 2003, Joosten et al., 2001,). Moreover, *A. ochraceus* is only one among many *Aspergillius* species that have the potential to produce ochratoxin A (Frisvad and Samson, 2000, Abarca et al., 2001). Recently, Batista et al. (2003) assigned ochratoxin A production in green coffee mainly to *A. sulphureus*, a species which is closely related to *A. ochraceus* but not detected by the primer pair used for DNA-quantification during the current study. One should keep in mind these facts when interpreting the data regarding the relation of DNA content of *A.ochraceus* and ochratoxin A load presented in the current study. Detection as well as quantification of fungal DNA by PCR in coffee could was possible, but the correlation of data with ochratoxin A concentrations was low, with several data points being clear outliers. However, a positive correlation between the presence of the fungus and ochratoxin A could be established. This is not surprising since occurence of moulds is the prerequisite for the production of the metabolite. One of the biases in this study may be the rather limited set of samples which were analysed. Among the 30 samples only 10 had an ochratoxin A content exceeding 5 ppb. Also with classical methods it has been demonstrated that the presence of the fungus does not necessarily mean ochratoxin A contamination (e. g. Taniwaki et al., 2003). In addition to that, sampling is a crucial point for mycotoxin analysis. Even in ground coffee there can be inhomogeneities in OTA content (Scott, 2002).

Nevertheless, this work offers the tools necessary for a rapid detection of the presence of *Aspergillus ochraceus* and *A. carbonarius* for further detailed investigations about distribution and occurrence without the need for time consuming sub cultivation and identification.

V. Summary

In this work mycotoxigenic fungi belonging to the three genera of major importance for food *Fusarium*, *Penicillium* and *Aspergillus* have been investigated. Comparison of DNA-fingerprints of *Fusarium poae*, *F. sporotrichioides* and *F. langsethiae* (ined.) revealed several markers specific for each group of isolates. In addition to this an integrated systematic study was carried out using a composite dataset. This set consisted of coding and non codingDNA sequences, AFLP fingerprints, chromatographic data on secondary metabolites and morphology. From these combined data a consensus matrix was calculated which was used as the basis for the construction of an UPGMA dendrogram and a multidimensional scaling, both of which revealed a clear separation of the three taxa. IGS, partial EF-1α and β-tubulin sequence- as well as chromatography- and AFLP-derived similarities turned out to be comparably consistent, while ITS sequence- and morphology-derived similarity matrices were rather divergent. The data support *F. langsethiae* as a new *Fusarium* species, for the time being in the section *Sporotrichiella*.

The comparison of ochratoxinogenic *Penicillium* isolates by AFLP revealed two groups of strains which were also concordant to the groups found by RAPD analysis. These two groups corresponded to the described chemotypes *Penicillum verrucosum* and "*P. nordicum*" supporting the later as a separate species. Toxigenic and non-toxigenic black aspergilli belonging to the *Aspergillus niger* aggregate and *A. carbonarius* isolated from Brazilian coffee related sources, were characterized by DNA fingerprinting and compared with other strains. AFLP fingerprints showed a clear separation of *A. niger* from *A. carbonarius*. However, no clear correlation between the genetic similarity of the strains and the potential to produce ochratoxin A could be found. Based on AFLP, marker sequences were selected and used for the construction of SCAR-PCR primers. Using these primers PCR assays were developed and optimised for sensitivity and specificity for the detection of *A. carbonarius*, the fungus con-

sidered to be one of the main causative agents for ochratoxin A in coffee and grape derived products. A similar approach was used for *A. ochraceus* an other fungus of major importance considering ochratoxin A contamination of coffee. Cluster analysis of *Aspergillus ochraceus* strains mainly isolated from Brazilian coffee related sources revealed a very close genetic relationship among most of the strains. A set of three species specific SCAR PCR-primer pairs was constructed. One of this primer pairs was used for PCR and Real-Time PCR detection of *A. ochraceus* in green coffee. The *Aspergillus ochraceus*-DNA content of 30 naturally contaminated green coffee samples was determined and compared to the ochratoxin A concentrations of respective samples. *A. ochraceus* could be rapidly and specifically detected and quantified in green coffee by Real-Time PCR. A positive correlation between the ochratoxin A content and the DNA quantity was established.

VI. Zusammenfassung

In dieser Arbeit wurden mykotoxinbildende Pilze der Gattungen *Fusarium*, *Penicillium* und *Aspergillus*, die für Lebensmittel von größter Bedeutung sind, untersucht. Der Vergleich von DNA-Fingerprints von *Fusraium poae*, *F. sporotrichioides* und *F. langsethiae* ließ einige Marker erkennen, die für eine Gruppe von Isolaten spezifisch waren. Zusätzlich wurde eine integrierte systematische Studie durchgeführt, die einen aus mehreren DNA-Sequenzen, AFLP-Fingerprints, Daten über Sekundärmetabolite und Morphologie zusammengesetzten Datensatz nutzte. Aus diesen kombinierten Daten wurde eine Konsensus-Matrix errechnet, auf deren Basis ein UPGMA-Dendrogram und ein „Multi-Dimensional-Scaling“ konstruiert wurden, die beide eine klare Trennung der drei Taxa offenbarten. IGS-, partielle EF-1α– und β-Tubulinsequenzen, wie auch auf Chromatographie und AFLP basierende Ähnlichkeiten zeigten sich als vergleichsweise konsistent, während auf ITS- und Morphologie basierende Ähnlichkeitsmatrizen sich als relativ divergent erwiesen. Die Daten stützen *F. langsethiae* als neue *Fusarium*-Spezies in der im Umbau begriffenen Sektion *Sporotrichiella*.

Der Vergleich ochratoxinogener *Penicillium*-Isolate ergab zwei Gruppen von Stämmen, die mit der durch RAPD gefundenen Gruppierung übereinstimmten. Diese beiden Gruppen entsprachen den beschriebenen Chemotypen *P. verrucosum* und „*P. nordicum*“ und unterstützen letzteren als eigene Art. Toxigene und nicht toxigene schwarze Aspergillen, die dem *Aspergillus niger* Aggregat und *A. carbonarius* angehörten und von brasilianischem Kaffee isoliert wurden, wurden mittels DNA-Fingerprinting miteinander und mit Stämmen anderer Arten verglichen. Die AFLP-Fingerprints zeigten eine klare Abgrenzung von *A. niger* und *A. carbonarius*. Es konnte jedoch keine klare Korrelation zwischen genetischer Ähnlichkeit der Stämme und dem Potential Ochratoxin A zu produzieren gefunden werden. Basierend auf AFLP wurden Marker-Sequenzen ausgewählt und für die Konstruktion von SCAR-PCR Primern genutzt. Mit Hilfe dieser Primer wurden PCRs entwickelt und

hinsichtlich Empfindlichkeit und Spezifität für die Detektion von *A. carbonarius*, dem Pilz der als einer der Hauptverursacher von Ochratoxin A in Kaffee und in aus Weintrauben erzeugten Produkten gilt, optimiert. Ein ähnlicher Ansatz wurde für *A. ochraceus* verwendet, ein weiterer Pilz von Interesse bezüglich Ochratoxin A Kontamination von Kaffee. Die Cluster Analyse von *A. ochraceus* Isolaten, hauptsächlich von brasilianischem Kaffee, zeigte eine sehr enge genetische Verwandtschaft der meisten Stämme. Drei artspezifische SCAR-PCR Primerpaare wurden entwickelt und eines davon für die PCR- und Real-Time-PCR-basierende Detektion von *A. ochraceus* in grünem Kaffee genutzt. Der Gehalt an *A. ochraceus*-DNA in grünem Kaffee wurde bestimmt und mit den Ochratoxin A Konzentrationen verglichen. *A. ochraceus* konnte schnell und spezifisch in grünem Kaffee gefunden und quantifiziert werden. Es zeigte sich eine positive Korrelation zwischen DNA-Menge und Ochratoxin A-Gehalt.

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VIII. Appendix

List of publications resulting from this thesis.

H. SCHMIDT, M. EHRMANN, R. F. VOGEL, M. H. TANIWAKI AND L. NIESSEN: Molecular typing of *Aspergillus ochraceus* and construction of species specific SCAR-primers based on AFLP. Sys. Appl. Microbiol. **26,** 138-147, **2003.**

H. Schmidt wrote the paper and carried out the experiments. M.H. Taniwaki provided the fungal strains and data on ochratoxin A production. M. Ehrmann helped with cloning and sequencing of DNA fragments.

G. CASTELLA, T. O.LARSEN, F. J. CABAÑES, **H. SCHMIDT**, A. ALBORESI, L. NIESSEN, P. FÄRBER, AND R. GEISEN: Molecular characterization of ochratoxin A producing strains of the genus *Penicillium*. System. Appl. Microbiol. **25,** 74-83, **2002**.

AFLP typing of *Penicillium* strains was carried out by H. Schmidt.

H. SCHMIDT, L. NIESSEN, AND R. F.VOGEL: AFLP analysis of *Fusarium* species in the section *Sporotrichiella* – evidence for *F. langsethiae* as a new species. Int. J. Food Microbiol. *in press.*

H. Schmidt wrote the paper and carried out the experiments.

H. SCHMIDT, A.HOLST-JENSEN , S. KLEMSDAL, C. M. KULLNIG-GRADINGER, C.P. KUBICEK, R. MACH, L. NIESSEN, H. NIRENBERG, U. THRANE, M. TORP, T. YLI-MATTILA AND, R. F. VOGEL: An integrated taxonomic study of *Fusarium langsethiae*, *F. poae* and *F. sporotrichioides* based on the use of composite datasets. *in press.*

H. Schmidt wrote the paper, collected and organized the data from the co-authors and performed the calculations of the composite datasets.

H. SCHMIDT, R. F. VOGEL M. H. TANIWAKI AND L. NIESSEN: PCR based characterisation, distinction and detection of toxigenic and non-toxigenic strains of *Aspergillus niger* and *Aspergillus carbonarius*. s*ubmitted*

H. Schmidt wrote the paper and carried out the experiments. M.H. Taniwaki provided the fungal strains and data on ochratoxin A production.

H. SCHMIDT, M.BANIER, L. NIESSEN AND R. F.VOGEL: Detection and quantification of *Aspergillus ochraceus* in green coffee by PCR. *submitted*

H. Schmidt wrote the paper and carried out the experiments. M. Banier helped with DNA isolation and qPCR.

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SYSTEMATIC AND APPLIED MICROBIOLOGY

Molecular Typing of *Aspergillus ochraceus* and Construction of Species Specific SCAR-Primers Based on AFLP

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Summary

70 strains of *Aspergillus ochraceus* mainly isolated from Brazilian coffee related sources were investigated for genetic relatedness using automated laser fluorescence analysis of AFLP fragments. Cluster analysis of fingerprints revealed a very close relationship among most of the strains. Based on these results, a sub-set of characteristic *A. ochraceus* strains was chosen for the detection of marker sequences. These sequences were obtained from silver stained AFLPs separated on polyacrylamide gels. A number of bands characteristic for *A. ochraceus* were detected and cut out from the gels. DNA was reamplified, cloned and fragments were sequenced. Based on these sequences a set of SCAR PCR-primers was constructed. PCRs were optimised for specificity and subsequently tested against a panel of *Aspergillus* species. Using this approach a PCR specific for *Aspergillus ochraceus* was developed.

Key words: *Aspergillus ochraceus* – AFLP – SCAR – PCR – detection – coffee – taxonomy

Introduction

Ochratoxin A (OTA) is a chlorinated isocumarine derivative linked to phenylalanine via a peptide like bond. Although OTA was first isolated from cultures of *Aspergillus ochraceus* in 1965 by van der Merwe et al. [42], little information is available concerning the biosynthesis of this compound [19]. OTA production has been reported for other fungi as *Penicillium verrucosum*, *P. nordicum* [26] and a wide variety of *Aspergillus* species belonging to different sections [2]. The mycotoxin is considered to be nephrotoxic, carcinogenic, teratogenic and immunosuppressive. It has been linked to Balkan Endemic Nephropathie (BEN), but it remains unclear whether it is the causative agent of that disease [3, 50]. Recently, the European Commission has set up maximum levels for the toxin in certain food products [14a]. While *Penicillium* species are considered to be the main organisms responsible for OTA contamination of food and feed in temperate climates, *Aspergillus* species are regarded as the main spoilage organisms producing OTA in warmer climates [50]. *A. alliaceus* has been reported to be responsible for OTA contamination of figs [6], *A. niger* and *A. carbonarius* are suspected to be the major source of OTA in grape-derived products [11]. The latter two species are also regarded as contaminating agents in green coffee, but *Aspergillus ochraceus* is considered to be the most important causative agent for OTA load in that commodity [38]. Though *Aspergillus ochraceus* is a rather common species in different habitats the taxonomy of this and related species is confusing. The species was originally described by Wilhelm [51]. Later the yellow aspergilli were treated by different authors leading to a certain confusion within this group [13, 33, 39]. Sequence analysis of the ITS region and observation of the secondary metabolites helped to clarify the situation towards a stable taxonomic system. [17, 31]. One aim of the current study was to examine the genetic relatedness of OTA producing and non-producing *A. ochraceus* isolates in order to elucidate whether OTA production might be linked to a certain population of *A. ochraceus* strains. Such DNA studies and derived sequences could serve as a basis for the construction of specific primers to allow easy and quick PCR-based identification of the organisms of interest [41]. This is of special importance in aspergilli where the correct identification of species requires much exper-

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tise and time using classical methods. Further on, such primers could serve as the basis for a culture independent detection system of the fungus without the need of labour-intensive isolation and sub culturing. AFLP, first described by Vos et al. [49], has proven to be a powerful taxonomic tool especially at low taxonomic ranks [8, 14, 35]. The advantages of this technique are its high reproducibility, robustness and that it can be easily automated [1]. In addition to this, AFLP allows to differentiate a large number of polymorphisms with only little variation of the protocol. Thus, AFLP is well suited for the detection of markers and the technique has been applied successfully for the discovery of specific marker sequences. Such sequences obtained from AFLP fragments could serve as basis for the construction of SCAR primers which allow identification and detection of the organism of interest [7].

Materials and Methods

Mycological analysis and isolation of strains

Samples of coffee cherries or beans were surface disinfected with 0.4% chlorine solution for 1 min [32], and then a total of 50 cherries or beans were plated directly (10 particles per plate) onto Dichloran 18% Glycerol agar [21]. The plates were incubated at 25 °C for 5 to 7 days, then inspected for colony growth visually and with the aid of a stereomicroscope. Representative colonies of *Aspergillus* species with the potential to produce OTA were isolated in pure culture.

Identification of fungi

Aspergillus isolates were grown on standard identification media, Czapek yeast extract agar and malt extract agar, and identified according to Klich and Pitt [25]. Isolates identified as *A. ochraceus* or closely related species, *A. carbonarius* and *A. niger* were tested for OTA production.

Test for OTA production by isolated fungi

The isolates identified as *A. ochraceus* or closely related species, *A. niger* and *A. carbonarius* were

Table 1. Strains isolated and used in this study. OTA: Potential of the isolated strain to produce ochratoxin A is indicated by + or –.

Aspergillus strains isolated from Brasilian coffe related sources					
CCT 6781	*Aspergillus*	*carbonarius*	Storage	São Paulo	–
CCT 6786	*Aspergillus*	*niger*	Drying area	São Paulo	–
CCT 6808	*Aspergillus*	*niger*	Drying area	São Paulo	–
CCT 6809	*Aspergillus*	*niger*	Drying area	São Paulo	–
CCT 6895	*Aspergillus*	*niger*	Storage	São Paulo	–
CCT 6909	*Aspergillus*	*niger*	Storage	Minas Gerais	–
CCT 6910	*Aspergillus*	*niger*	Drying yard	Minas Gerais	–
CCT 6780	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6782	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6783	*Aspergillus*	*ochraceus*	Drying area	São Paulo	+
CCT 6785	*Aspergillus*	*ochraceus*	Drying area	São Paulo	+
CCT 6787	*Aspergillus*	*ochraceus*	Coffee raisins	São Paulo	+
CCT 6788	*Aspergillus*	*ochraceus*	Coffee raisins	São Paulo	+
CCT 6789	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6790	*Aspergillus*	*ochraceus*	Coffee raisins	São Paulo	+
CCT 6791	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6792	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6793	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6794	*Aspergillus*	*ochraceus*	Coffee cherries	São Paulo	+
CCT 6795	*Aspergillus*	*ochraceus*	Drying yard	São Paulo	+
CCT 6796	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6797	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6798	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6799	*Aspergillus*	*ochraceus*	Coffee raisins	São Paulo	+
CCT 6800	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6801	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6802	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6803	*Aspergillus*	*ochraceus*	Coffee raisins	São Paulo	+
CCT 6804	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6805	*Aspergillus*	*ochraceus*	Coffee cherries	São Paulo	+
CCT 6806	*Aspergillus*	*ochraceus*	Drying area	São Paulo	+
CCT 6807	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6810	*Aspergillus*	*ochraceus*	Coffee raisins	São Paulo	–
CCT 6811	*Aspergillus*	*ochraceus*	Coffee raisins	São Paulo	+
CCT 6812	*Aspergillus*	*ochraceus*	Drying area	São Paulo	+
CCT 6813	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6814	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6815	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6816	*Aspergillus*	*ochraceus*	Coffee raisins	São Paulo	–
CCT 6817	*Aspergillus*	*ochraceus*	Drying area	São Paulo	+
CCT 6818	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6819	*Aspergillus*	*ochraceus*	Drying area	São Paulo	–
CCT 6820	*Aspergillus*	*ochraceus*	Drying area	São Paulo	+
CCT 6821	*Aspergillus*	*ochraceus*	Drying area	São Paulo	+
CCT 6822	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6823	*Aspergillus*	*ochraceus*	Coffee raisins	São Paulo	+
CCT 6824	*Aspergillus*	*ochraceus*	Drying area	São Paulo	+
CCT 6825	*Aspergillus*	*ochraceus*	Drying aerea	São Paulo	–
CCT 6827	*Aspergillus*	*ochraceus*	Drying aerea	São Paulo	+
CCT 6828	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6829	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6830	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
CCT 6831	*Aspergillus*	*ochraceus*	Storage	São Paulo	+
Reference strains used in this study					
DSM 813	*Aspergillus*	*alliaceus*			
CBS 467.65	*Aspergillus*	*auricomus*			
CBS 10704	*Aspergillus*	*awamori*			
CBS 350.81	*Aspergillus*	*bridgeri*			
M324	*Aspergillus*	*carbonarius*			
CBS 513.45	*Aspergillus*	*clavatus*			

Table 1. continued.

CBS 310.80	*Aspergillus*	*elegans*
CBS 707.79	*Aspergillus*	*ellipticus*
CBS 113.32	*Aspergillus*	*flavus*
CBS 114.49	*Aspergillus*	*foetidus*
CBS 550.65	*Aspergillus*	*sulphureus*
CBS 113.55	*Aspergillus*	*fumigatus*
CBS 149.89	*Aspergillus*	*fumigatus*
CBS 677.79	*Aspergillus*	*helicothrix*
CBS 117.55	*Aspergillus*	*heteromorphus*
CBS 382.75	*Aspergillus*	*insulicola*
CBS 114.51	*Aspergillus*	*japonicus*
CBS 101698	*Aspergillus*	*niger*
CBS 101697	*Aspergillus*	*niger var. niger*
A8	*Aspergillus*	*ochraceus*
CBS 263.67	*Aspergillus*	*ochraceus*
CBS 588.68	*Aspergillus*	*ochraceus*
CBS 589.68	*Aspergillus*	*ochraceus*
KA103	*Aspergillus*	*ochraceus*
M 337	*Aspergillus*	*ochraceus*
CBS107.57	*Aspergillus*	*petrakii*
CBS 549.65	*Aspergillus*	*sclerotiorum*
A 91	*Aspergillus*	*spec.*
CBS 550.65	*Aspergillus*	*sulphureus*
CBS 377.64	*Aspergillus*	*terreus var. terreus*
CBS 101700	*Aspergillus*	*usamii var. shiro-usamii*
CBS 306.48	*Penicillium*	*chrysogenum*
DSM 62846	*Penicillium*	*italicum*
BFE 487	*Penicillium*	*nordicum*
CBS 603.74	*Penicillium*	*verrucosum*
TMW 4.0654	*Stachybotris*	*chartarum*

grown on yeast extract 15% sucrose agar at 25 °C for 7 days and evaluated for the production of OTA by the agar plug technique, which tests small samples from Petri dishes by thin layer chromatography (TLC) [16]. Larger samples of isolates which tested negative for OTA production by this technique were then analysed by TLC as well. For this analysis, the colony and yeast extract sucrose medium from a Petri dish were extracted with chloroform (50 ml) in a Stomacher for 3 min [37]. Extracts were filtered and concentrated in a water bath at 60 °C to near dryness and then dried under a stream of N_2. Residues were resuspended in chloroform and spotted on TLC plates which were developed in toluene/ethyl acetate/formic acid (5 : 4 : 1) and visualised under UV light at 365 nm. An OTA standard (Sigma Chemical Co., St Louis, USA) was used for comparison. Information on the isolates and other strains employed in this study is summarised in Table 1.

DNA preparation

Fungal strains were cultivated for 12 to 14 days at ambient temperature in 15 ml sterile plastic tubes (Sarstedt, Nümbrecht, Germany) containing 3 ml ME broth (20 g malt extract, 1 g peptone, 20 g glucose, distilled water ad 1000 ml). Mycelia were harvested by centrifugation, washed twice with 5 ml sterile distilled water and freeze dried. DNA was prepared according to the CTAB protocol provided by Möller et al. [27]. DNA concentration was determined using a SpectraFluor (Tecan, Crailsheim, Germany) measuring fluorescense with SYBR®green I (Molecular Probes, Leiden, The Netherlands) using lamda-DNA (Promega, Madison, USA) as standard for calibration.

Template preparation and AFLP reactions

AFLP was carried out as described by Vos et al. [49] and modified by Aarts and Keijer [1] with minor changes. For template preparation all reaction mixtures contained 10 mM Tris-HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT and 50 ng/µl BSA. 200 ng of isolated genomic DNA were digested in a total volume of 15 µl at 37 °C for 3 h with 2 U of EcoRI (TaKaRa Shuzo Co., Ltd., Japan) and 2U of BfaI or Mse I (New England Biolabs, Frankfurt, Germany). BfaI (recognition site 5′-C↓TAG-3′) creates the same sticky ends as MseI and the adaptors originally described can be used with this enzyme combination. Following digestion 5 µl of a solution containing 2 pMol EcoRI adaptor, 20 pMol MseI/BfaI adaptor, 0.4U T4 DNA ligase (MBI Fermentas, St. Leon-Rot, France) and 1 mM ATP was added. Ligation was performed for 12 h at room temperature in a total volume of 20 µl. This mix was diluted 1:10 with 10 mM Tris-HCl pH 8.0. Five microliters of this dilution were used as template for the pre-amplification. Primers used in this PCR reaction were those described by Vos et al. [49] and had a total of one or two selective bases (indicated by bold letters). The pre-EcoRI-primer used the sequence 5′-GAC TGC GTA CCA ATT CA-3′, the pre-MseI primer 5′-GAT GAG TCC TGA GTA AC -3′ and the pre-BfaI primer 5′-GAT GAG TCC TGA GTA G-3′.

Automated laser fluorescense analyses (ALFA) and data processing

For ALFA the combination of EcoRI and BfaI was chosen. For selective amplification, carried out as described by Vos et al. [49] in a total volume of 20 µl, the EcoRI primer was labelled with Cy5 to allow fluorescence detection of fragments. The EcoRI primer had A and the BfaI primer AT as selective end. 8.5 µl of formamide containing 5 mg/ml dextran blue, a 35 bp and a 650 bp internal standard were added to the selective amplification. The mix was denatured by heating to 94 °C for 3 min and immediately transferring the tubes to ice. Analysis of the denatured Cy5-labelled fragments was performed on an ALFexpress sequencer (Amersham Pharmacia, Freiburg, Germany). 5µl sample were loaded on a 0.3 mm denaturing 5% PAA gel. An external standard was run every 12 lanes. Files were converted to TIFF images and these were examined with the BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium).

For clustering, fragments between 70 bp and 650 bp were analysed. The Pearson correlation [29] was used for the curve based pair wise similarity calculation. The dendrogram was calculated using the UPGMA algorithm [36].

Silver staining of AFLP-fragments

Recovery of AFLP fragments from silver stained PAA gels for sequencing, was carried out according to Chalhoub et al. [12] with some modifications. Pre-amplification was conducted as

described above. The selective PCR was carried out using both the MseI/BfaI primer and the EcoRI primer in equal concentrations as described by Chalhoub et al. [12]. A total of four selective bases was used in this second PCR (total of 40 µl). After the PCR reaction the products were evaporated to dryness and resuspended in 5 µl of water.

AFLP fragments were separated on a native CleanGel Long-10 polyacrylamide gel using the DNA LongRun Buffer (ETC-Elektrophorese-Technik, Kirchentellinsfurt, Germany) on a Multiphor II electrophoresis system (Amersham Pharmacia, Freiburg, Germany). Electrophoresis was carried out as described by the manufacturers protocol with the exception that only 1 µl of sample buffer was added to 5 µl concentrated AFLP product. After loading the gel a delay of approximately 5 minutes before applying power was carried out.

Fragments were silver stained according to Bassam et al. [5]. After fixation gels were washed for 10 min in distilled water and the banding pattern was observed.

DNA recovery, cloning, sequencing and PCR conditions

Bands considered to be characteristic were cut out from the gel. DNA was eluted in 20 µl water at 4 °C overnight. 5 µl of this eluate were used in the subsequent reaction. For reamplification the same primers as for the selective PCR were used. The temperature profile was as follows: 94° C for 1 min, 40 cycles: 30 s at 94 °C, 60 s at 56 °C, 72 °C 1 min and finally 3 min at 72 °C. The resulting fragments were electrophorised on a 2% agarose gel. After staining with ethidiumbromide bands were cut out from the gel under UV. DNA was extracted using a DNA extraction kit (Genomed, Bad Oeyenhausen, Germany). Purified DNA fragments were ligated in the pGEM®-T Easy Vector (Promega, Madison, USA) according to manufacturers instructions. The vector was transformed into *E. coli* XL1 blue (Stratagene, Amsterdam, The Netherlands) by electroporation [34]. Plasmides were prepared using either the boiling method [22] or a plasmid midi kit (Qiagene, Hilden, Germany). Sequencing of the inserts was done with the ABI Prism Dye Terminator Cycle Sequencing Kit (Perkin Elmer, Boston, USA) on ABI 373 stretch sequencing system by a commercial service (SequiServe, Vaterstetten, Germany).

In order to optimise PCRs for sensitivity and specificity the annealing temperature for cycling conditions, adjustment of the pH, addition of $MgCl_2$, formamide, gelatine and $(NH4)_2SO_4$ was tested. PCR was carried out in total volume of 25 µl using 1.25 U Taq polymerase (Promega, Madison, USA) per reaction. Table 2 summarises the PCR conditions for the different primer pairs, buffers and cycling conditions.

Results

For typing of the strains the restriction enzymes BfaI in combination with EcoRI were chosen, since the recognition site of BfaI is less AT-rich than that of MseI which should result in statistically bigger fragments, as eucaryotic DNA is rich in AT. The use of a total of three selective ends (E-A/B-AT) turned out to produce a complex yet well resolved fingerprint pattern. Figure 1 shows representative AFLP profiles of treated strains. In profiles of *A. ochraceus* more than 40 peaks were detected. The region shown (approx. 70 to 650 bp) was considered for similarity calculation and subsequent graphical visualisation. The UPGMA dendrogram constructed from this matrix is shown in Figure 2 for all taxa treated. All *Aspergillus ochraceus* strains examined displayed a high degree of similarity. There was no clear correlation between the genetic similarity and the potential of the strain to produce OTA. OTA-producers and non-producers were scat-

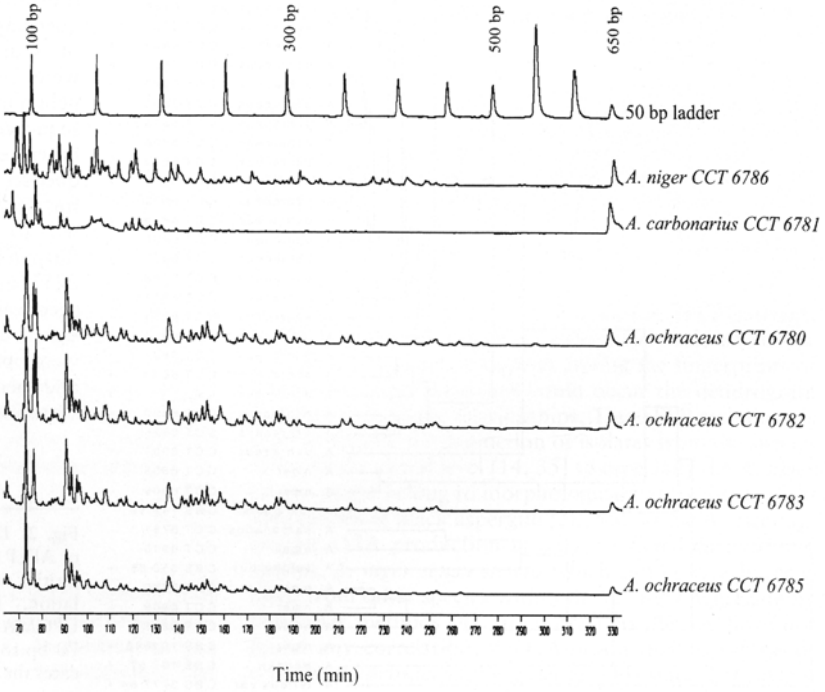
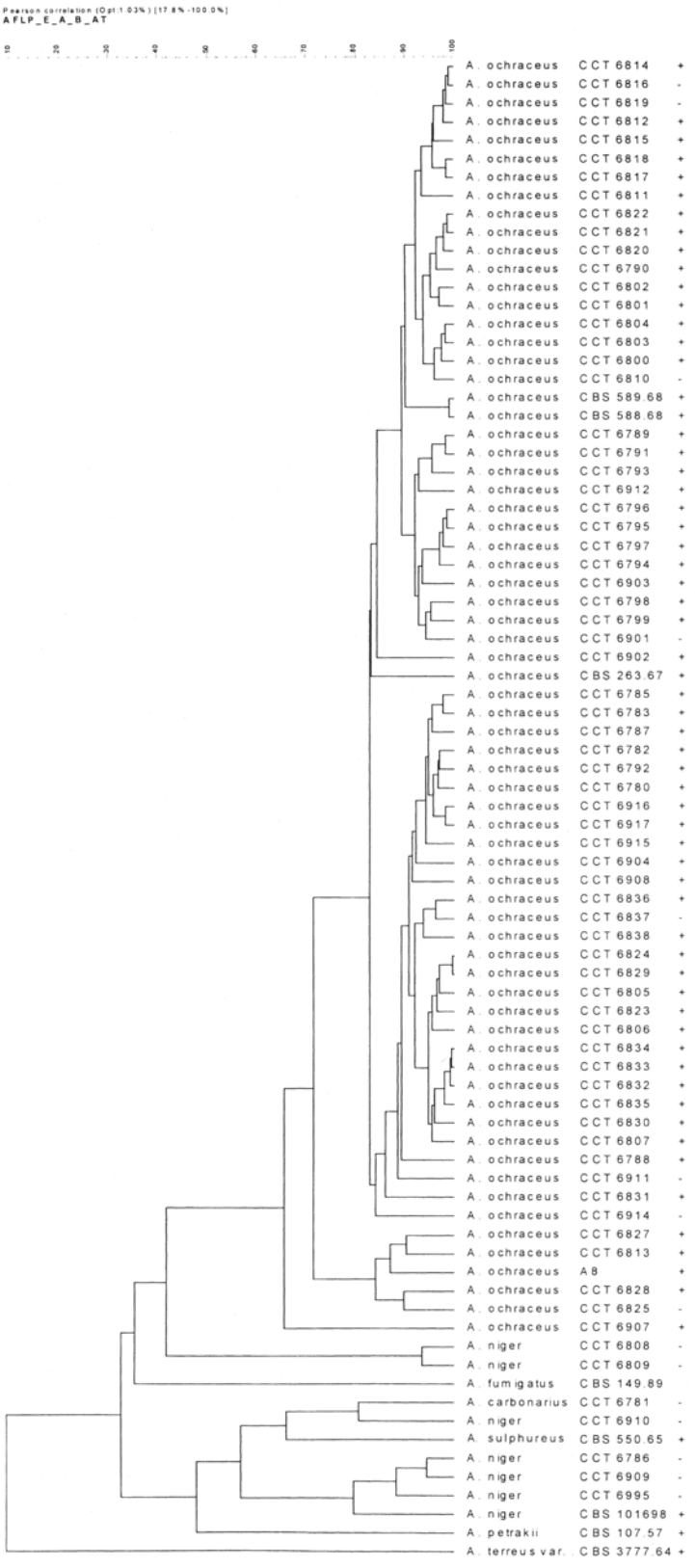


Fig. 1. AFLP fingerprints of representative *Aspergillus* strains examined in this study. The X-axis indicates the time, on the y-axis the relative intensity of the signal in the electropherogram obtained from fluorescence analysis of the fragments on an ALFexpress sequencer are shown. Primers B-AT and E-A were used to generate the pattern.



tered throughout the dendrogram. All other *Aspergillus* species considered form distinct groups. Multidimensional scaling (MDS), a non-hierarchical grouping technique calculated from the obtained similarity matrix which shows entries as „clouds" in a three dimensional space is shown in Figure 3. The MDS revealed a rather close grouping of the *Aspergillus ochraceus* strains, whereas the other strains (e. g. *A. niger*) examined are rather dispersed throughout the plot.

Based on these results the three CBS strains of *A. ochraceus* were selected to serve as references for further studies aiming at detection of specific marker sequences. For this purpose a number of different combinations of EcoRI with MseI or BfaI were used in the subsequent AFLP reactions. In the selective PCR reactions of these AFLPs the number of selective bases was increased to 4 to reduce the number of fragments. AFLP fragments could be clearly separated on polyacrylamide gels and visualised by silver staining. By this means a number of bands considered to be unique to *Aspergillus ochraceus* could be detected. Figure 4 shows an example of a silver stained AFLP separated on a polyacrylamide gel. Some of the bands were cut out from the stained gel and after elution DNA could be reamplified. Subsequently, the fragments could be successfully utilised for ligation into the pGEM®-T Easy vector which was then transformed into *E. coli* to allow sequencing. Using this approach a number of sequences could be obtained which were searched against Genebank (http://ncbi.nlm.nih.gov/BLAST). Neither fragment sequenced showed a significant similarity to other sequences in the database. DNA sequences of fragment H10, A and D used for the design of primers have been submitted to The EMBL nucleotide database (http://ebi.ac.uk/embl), accession number AJ511647, AJ511648, and AJ511647, respectively. PCR with the primer pairs designed according to the obtained sequences were optimised for sensitivity and specificity and then tested against a test panel of representative strains. Table 2 summarises infor-

Fig. 2. Dendrogram obtained from comparison of AFLP fingerprints of *Aspergillus* strains. Similarities were calculated using the Pearson correlation. The tree was constructed using the UPGMA algorithm. Fragments from approx. 70 bp to 650 bp were considered. + and – indicates the potential of the strain to produce OTA.

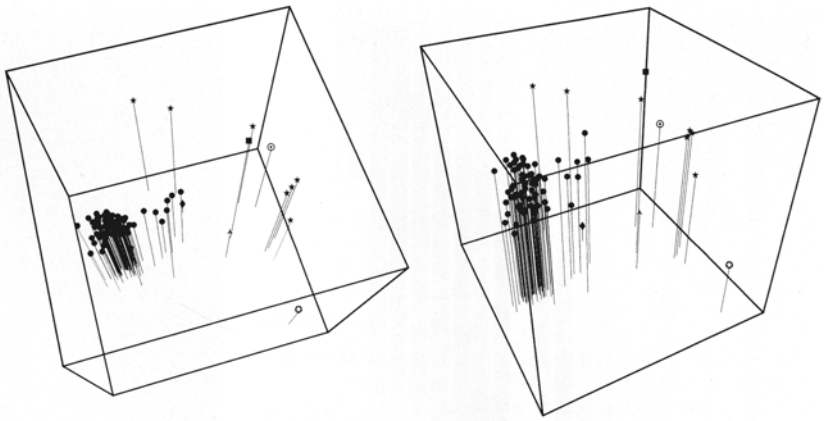


Fig. 3. Non-metric multidimensional scaling (MDS) calculated from the similarity matrix leading to the dendrogram in Figure 2.

⊙: *A. carbonarius*,
■: *A sulphureus*,
◆: *A. fumigatus*,
★: *A niger*,
●: *A. ochraceus*,
⅄: *A. petrakii*,
✪: *A. terreus var. terreus*.

Table 2. PCR conditions and primer sequences used for the specific detection of marker sequences in *A. ochraceus*. The numbers in the brackets indicate the EMBL accession number.

Marker	Primers (5′to 3′)	10 × Amplification buffer mix	Cycling conditions
H10 (AJ511647)	OCA-V: ATA CCA CCG GGT CTA ATG CA OCA-R: TGC CGA CAG ACC GAG TGG ATT	500 mM KCl 100 mM Tris-HCl 15 mM $MgCl_2$ 60% (v/v) formamide 50 mM $(NH_4)_2$ SO_4 pH 8.6	94 °C, 4 min initial denaturation followed by 35 cycles 94 °C, 30 s; 55 °C, 30 s; 72 °C 30 s, followed by 5 min final elongation at 72 °C. Tempterature of heated lid was set to 100 °C.
A (AJ511648)	Ochra pA-F: TTA ACA GGG AAC AAT CCA TAT AG Ochra pA-R GAA TTC ACC CAG AAC TTG CCG	500 mM KCl 100 mM Tris-HCl 15 mM $MgCl_2$ 30% (v/v) formamide 50 mM $(NH_4)_2$ SO_4 0.1% gelatine pH 8.6	94 °C, 4 min initial denaturation followed by 35 cycles 94 °C, 30 s; 55 °C, 30 s; 72 °C 30 s, followed by 5 min final elongation at 72 °C. Tempterature of heated lid was set to 100 °C.
D (AJ511649)	Ochra pD-F: TTA ACA GGC ACC GGA GAC ATA GTT AG Ochra pD-R: TTA ACA GAT TTG AGA ACC CCA TTC	500 mM KCl 100 mM Tris-HCl 15 mM $MgCl_2$ 30% (v/v) formamide 0.3% gelatine pH 8.6	94 °C, 4 min initial denaturation followed by 35 cycles 94 °C, 30 s; 57.5 °C, 30 s; 72 °C 30 s, followed by 5 min final elongation at 72 °C. Tempterature of heated lid was set to 100 °C.

mation on markers, sequences of primers derived and PCR conditions. PCR conditions were optimised for sensitivity and specificity of detection of *A. ochraceus* strains. Figure 5 shows an example of a PCR performed against a selected test panel of strains representing distantly related fungal species and a set of closely related yellow aspergilli with the PCR conditions employed as displayed in Table 2.

Discussion

The *Aspergillus ochraceus* strains studied seem to form a quite homogenous group with a high degree of similarity among the isolates (Figs. 2 and 3). In contrast to this the *A. niger* strains seem to be less homogenous. The grouping in Figure 2 is based on complex AFLP patterns, which allow a distinction of very closely related strains but since incidental similarities among the fingerprints of distantly related organisms could occur the dendrogram may not reflect true relationships. The AFLP technique is only applicable for distinction of isolates from the species down to the clonal level [14, 35] so especially the *A. niger* strains might belong to morphological hardly distinguishable species of black aspergilli [28, 46]. In the *A. niger* aggregate OTA production is only restricted to a certain species, *A. niger sensu stricto* which can be clearly separated from other species within the *A. niger* aggregate by molecular methods [4]. In contrast to this we have not found any correlation of OTA-production to the genotype for *A. ochraceus*. Varga et al. [44] state that several

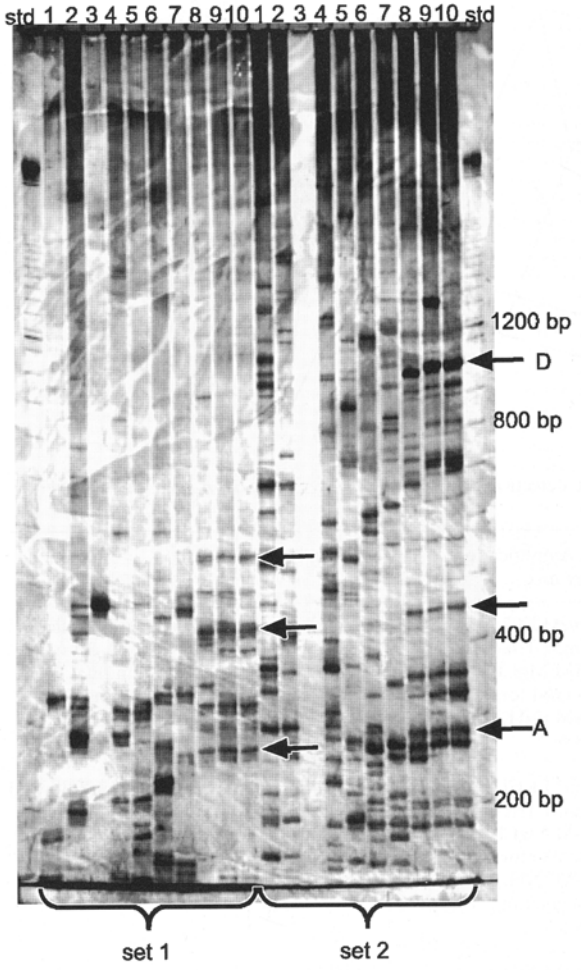


Fig. 4. Silver stained AFLP fragments created by the primer combination E-AC/M-CT (set 1) and E-A/M-CAG (set 2) separated on a native polyacrylamide gel. std: 100 bp ladder, 1: *Stachybotris chartarum* TMW 4.0654, 2: *Penicillium verrucosum* CBS 603.74, 3: *Aspergillus auricomus* CBS 467.65, 4: *A. sulphureus* CBS 550.65, 5: *A. bridgeri*: CBS 350.81, 6: *A. petrakii* CBS 101.57, 7: *A. spec.* A 91, 8: *A. ochraceus* CBS 263.67, 9: *A. ochraceus* CBS 589.68, 9: *A. ochraceus* CBS 588.68. Arrows indicate some characteristic fragments common in all *A. ochraceus* strains and absent in the other strains. A and D are fragments that lead to the corresponding sequences mentioned in Table 2.

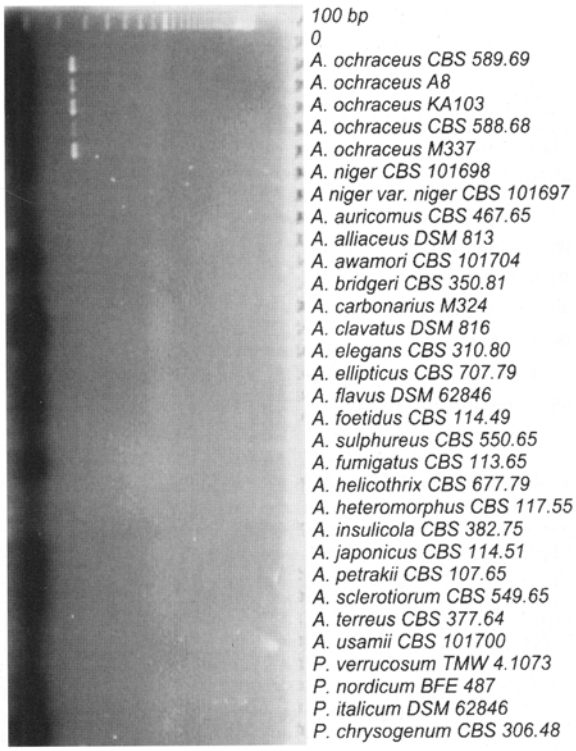


Fig. 5. Reactivity of the primer pair OCA-V/OCA-R against *Aspergillus* and *Penicillium spec.* 100bp:100 bp ladder, 0: negative control. Notice that only *Apergillus ochraceus* strains result in the amplification of a 260 bp product. For PCR conditions see Table 2.

lineages of *A. ochraceus* have lost their ability to synthesise OTA as revealed by cluster analysis of DNA fingerprints. However, when ITS sequence based phylogenetic analysis of members of the section *Circumdati* or even different *A. ochraceus* isolates was performed no clear correlation between the taxonomic position and the OTA production could be detected [44, 45, 47]. Though the algorithms employed for cluster analysis and the MDS do not necessarily reflect true evolution [23] our AFLP work confirmed these findings. For aflatoxinogenic aspergilli both RAPD analysis [40] and ITS sequence comparison [30] showed no homogenous distribution of toxigenic and nontoxigenic strains or species. It is also known that the potential for aflatoxin synthesis is easily lost in the course of ongoing sub cultivation [24]. Analogous observations have been made for ochratoxin A [20, 46]. Our experience also shows that the ability of a strain to produce the toxin is highly variable (unpublished results). Thus looking at ochratoxin production alone in culture without taking into account other characters is probably not very helpful for classifying yellow aspergilli.

Aspergillus ochraceus is regularly isolated from different food and feed, especially coffee but also from cereals [52]. While the source of ochratoxin A in different commodities has been assigned to other aspergilli and penicillia [52] in coffee infection of coffee beans with *A. ochraceus* – partly mediated by insects – is responsible for OTA formation [38]. Therefore, prevention of the growth of this fungus and its rapid and early detection are of importance in coffee manufacturing [10]. Using DNA markers for the detection of toxigenic aspergilli and penicillia in food has proven to be a valuable tool for the rapid and easy detection of these moulds [9, 15, 18].

Acknowledgments

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SYSTEMATIC AND APPLIED MICROBIOLOGY

Molecular Characterization of Ochratoxin A Producing Strains of the Genus *Penicillium*

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Summary

Sixty-six strains classified as *P. verrucosum* based on morphological criteria were characterized by molecular methods like RAPD, AFLP and ITS sequencing. Two groups could be identified by RAPD and AFLP analyses. The two RAPD as well as the two AFLP groups were completely coincidental. Strains in the two groups differed in their ability to produce ochratoxin A, with group I containing mainly high producing strains, and group II containing moderate to non-producing strains. The strains from group I originate from foods, such as cheeses and meat products, while the strains from group II originate from plants. The ribosomal ITS1–5.8S-ITS2 sequences were similar, except for two single nucleotide exchanges in several strains of each group.

A chemotaxonomical analysis of some of the strains identified differences between the groups in secondary metabolite production. Strains from group I possessed the chemotype of *P. nordicum* and strains from group II that of *P. verrucosum*. The differences at the RAPD and AFLP level, which parallel the chemotypic differences, are consistent with the recent reclassification of ochratoxin A producing penicillia to be either *P. verrucosum* or *P. nordicum*. The homolgy between the ITS sequences however indicates phylogenetic relationship between the two species.

Key words: ochratoxin – *Penicillium verrucosum* – *Penicillium nordicum* – RAPD typing – AFLP typing – ITS sequences – chemotyping

Introduction

Penicillium verrucosum is the most important ochratoxin A producing fungus in regions with a moderate climate. It has been quite frequently isolated from cereal crops, meat products and cheeses (PITT and HOCKING, 1997). Ochratoxin A is a nephrotoxic and carcinogenic secondary metabolite (HÖHLER, 1998) and has been detected in food commodities such as cereals and cereal products (WOLFF, 2000), beverages, such as coffee, tea, beer, wine and fruit juices (BRESCH et al., 2000, OTTENEDER and MAJERUS, 2000), meats and meat products (GAREIS and SCHEUER, 2000), and dairy products (ENGEL, 2000). The blood serum of 98.1% of a group of volunteers contained ochratoxin A in a concentration above the detection limit of 0.06 ng/ml (ROSNER et al., 2000) This situation points out the importance of ochratoxinogenic fungi to human and animal health.

The species concept of ochratoxin A producing Penicillia has been a matter of discussion (LARSEN et al., 2001). *P. verrucosum* was taxonomically grouped in relation to *P. viridicatum* (RAPER et al., 1949, FRISVAD and FILTENBORG, 1989;). PITT (1987) reclassified ochratoxin A producing *P. viridicatum* strains as *P. verrucosum* and specified that *P. verrusosum* was the only ochratoxin A producing species within the genus *Penicillium*. LARSEN et al. (2001) analyzed different ochratoxin A producing penicillia and found two distinct groups based on chemotaxonomic data. Both groups produced a different set of secondary metabolites. One group included the ex-type culture of *P. nordicum*. For this reason ochratoxin A producing strains, whith a secondary metabolite profile similar to that of *P. nordicum* were shifted into this species, whereas the other group was classified as *P. verrucosum*.

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Molecular methods may be used to differentiate between fungal strains with similar morphology. The RAPD technique (randomly amplified polymorphic DNA) monitors ample differences between strains at the genomic level (WILLIAMS et al., 1990; SYMOENS et al., 2000). The RAPD approach usually has a discriminatory power at the species or subspecies level, depending on the primer used (GUTHRIE et al., 1992). The AFLP technique (amplified fragment length polymorphism) has a similar resolution than the RAPD technique however the reproducibility is higher (WOLFENBERGER, 1999). The sequence comparison of variable DNA regions on the other hand can reveal differences at the nucleotid sequence level. Most commonly, the ribosomal ITS sequences are used for phylogenetic purposes (BRYAN et al., 1995; BOYSEN et al., 1996), however sequences of other genes also have been compared (GEISER et al., 1998). As these approaches detect differences at different phylogenetic levels, the obtained results can supplement each other.

Our objective in this study was to use molecular techniques to confirm that ochratoxin A producing *Penicillium* strains belong to two closely related, but clearly different taxa, which correspond to the two groups identified with the chemotaxonomic data (LARSEN et al., 2001).

Materials and Methods

Strains and culture conditions

The strains used in this study (Table 1) were routinely grown in malt extract broth (Merck, Darmstadt, Germany) or on malt extract agar plates, both media were supplemented with glucose (5 g/l). For ochratoxin A production, the strains were grown on malt extract, CYA and YES agar plates (SAMSON and van REENEN-HOEKSTRA, 2000) at 25 °C for 5 days.

Determination of Ochratoxin A

Thin layer chromatography (TLC) was used for semiquantitative determination of ochratoxin A. For this analysis 0.1 g of mycelium (dry weight) was harvested and extracted by intensive shaking in 1 ml chloroform for 30 min in a microreaction tube. The mycelium was discarded and the chloroform was evaporated to dryness in a speed-vac concentrator. The residue was redissolved in 20 µl chloroform and applied to a silica gel plate (Silica gel 60, Merck, Darmstadt, Germany). The TLC plate was developed in a chloroform-methanol-acetic acid (90:5:5) system. The plates were documented under UV light in a FlourS-Image analyzer (Biorad, Gaitherburg, USA) and the signals were semiquantified by measuring signal intensity.

For quantitative determination ochratoxin A production strains were grown either on Yeast Extract Sucrose (YES) medium (with additional 0.5 g/l $MgSO_4$) or on Czapek Yeast Extract (CYA) agar for 7 days at 25 °C. With a steril corer (diameter 6 mm), three agar plugs from each colony were removed and extracted in 0.5 ml methanol for 60 min. The extracts were filtered and used for HPLC analysis. HPLC analysis was performed essentially as described by BRAGULAT et al. (2001). All experiments were carried out in duplicate.

Determination of citrinin

Citrinin production was assessed by a TLC method. Samples were prepared as described above, and than applied to a silica gel plate (Silica gel 60, Merck, Darmstadt, Germany) The plate was developed in toluol-ethylacetate-chloroform-acetic acid (70:50:50:20) and the spots were subsequently visualized as described above.

Isolation of fungal DNA

DNA was isolated with a method modified from that of YELTON et al. (1984). 72 to 96 h old mycelia were harvested from a submerged culture by filtration. The mycelium was transferred to a mortar and frozen in liquid nitrogen. The frozen mycelium was ground and resuspended in lysis buffer (50 mM EDTA; 0.2% SDS; pH 8.5). This suspension was heated to 68 °C for 15 min and centrifuged for 15 min at 15.000 × g. After centrifugation 7 ml of the supernatant was transferred to a new centrifuge tube and 1 ml of 4 M sodium acetate added. This solution was placed on ice for 1 h and centrifuged for 15 min at 15000 × g. After centrifugation 6 ml of the supernatant were transferred to a fresh tube. The solution was phenol extracted and the isolated DNA was precipitated by the addition of 2.5 volumes of ethanol. The isolated DNA was checked on an agarose gel and the concentrations were determined spectrophotometrically as described by SAMBROOK et al. (1989).

RAPD analysis

The isolated chromosomal DNA was diluted to 2 µg/ml and used as template DNA for RAPD-PCR reactions. The PCR reaction mixture contained: 5.0 µl Taq DNA polymerase buffer (10×, Pharmacia, Uppsala, Sweden), 8 µl nucleotide mixture (dATP, dCTP, dTTP, dGTP); 2.5 mM each, (Boehringer, Mannheim)) 5.0 µl $MgCl_2$ (25 mM), 1.25 µl primer (120 pmol μl^{-1}), 0.1 µl Taq polymerase (5 U/µl (Pharmacia, Uppsala, Sweden)), 5.0 µl template DNA (2 µg/ml) and 25.0 µl H_2O. Polymerase chain reactions were performed in 44 cycles (Eppendorf Mastercycler 5330, Eppendorf, Hamburg, Germany): 1 min at 95 °C, 1 min at 36 °C, 4 min at 72 °C. The sequence of the random primer ari1 was 5′TGC TTG GCA CAG TTG GCT TC3′. This primer is 21 nucleotides in lenght, instead of the usual 10 (Guthrie et al., 1992) resulting in higher reproducibility of the PCR results. The RAPD-PCR products were separated on an 0.8% agarose gel and the band patterns were analysed with the UPGMA algorithm of the BIORAD fingerprinting software (version 1) from the Molecular Analyst series.

AFLP analysis

AFLP was carried out as described by VOS et al. (1995) and modified by AARTS and KEIJER (1999) with minor changes. For template preparation all reaction mixtures contained 10 mM Tris-acetate pH 7.5, 10 mM Mg acetate, 50 mM K acetate, 5 mM dithiothreitol and 50 ng/µl bovin serum albumine. At first, 200 ng of genomic DNA were digested in a total volume of 15 µl at 37 °C for 3 h with 2U of *Eco*RI (TaKaRa Shuzo Co., Ltd., Japan) and 2U of *Bfa*I (New England Biolabs, Frankfurt). *Bfa*I (recognition site 5′-C↓TAG-3′) creates the same sticky ends as *Mse*I and the adaptors originally described can be used with this enzyme combination. Second, 5 µl of a solution containing 2 pMol *Eco*RI adaptor, 20 pMol *MseI*/*Bfa*I adaptor, 0.4U T4 DNA ligase (MBI Fermentas, St. Leon-Rot, Germany) and 1 mM ATP was added. Ligation was performed for 12 h at room temperature in a total volume of 20 µl. This mix was diluted 1:10 with 10 mM Tris-HCl pH 8.0. Five µl of this dilution were used as template for the first PCR amplification.

PCR was performed as described by VOS et al. (1995) in a total volume of 20 µl with the following modifications. For the pre-amplification the *Eco*RI-primer had the sequence 5′GAC TGC GTA CCA ATT CA3′ and the BfaI primer 5′GAT GAG TCC TGA GTA G3′.

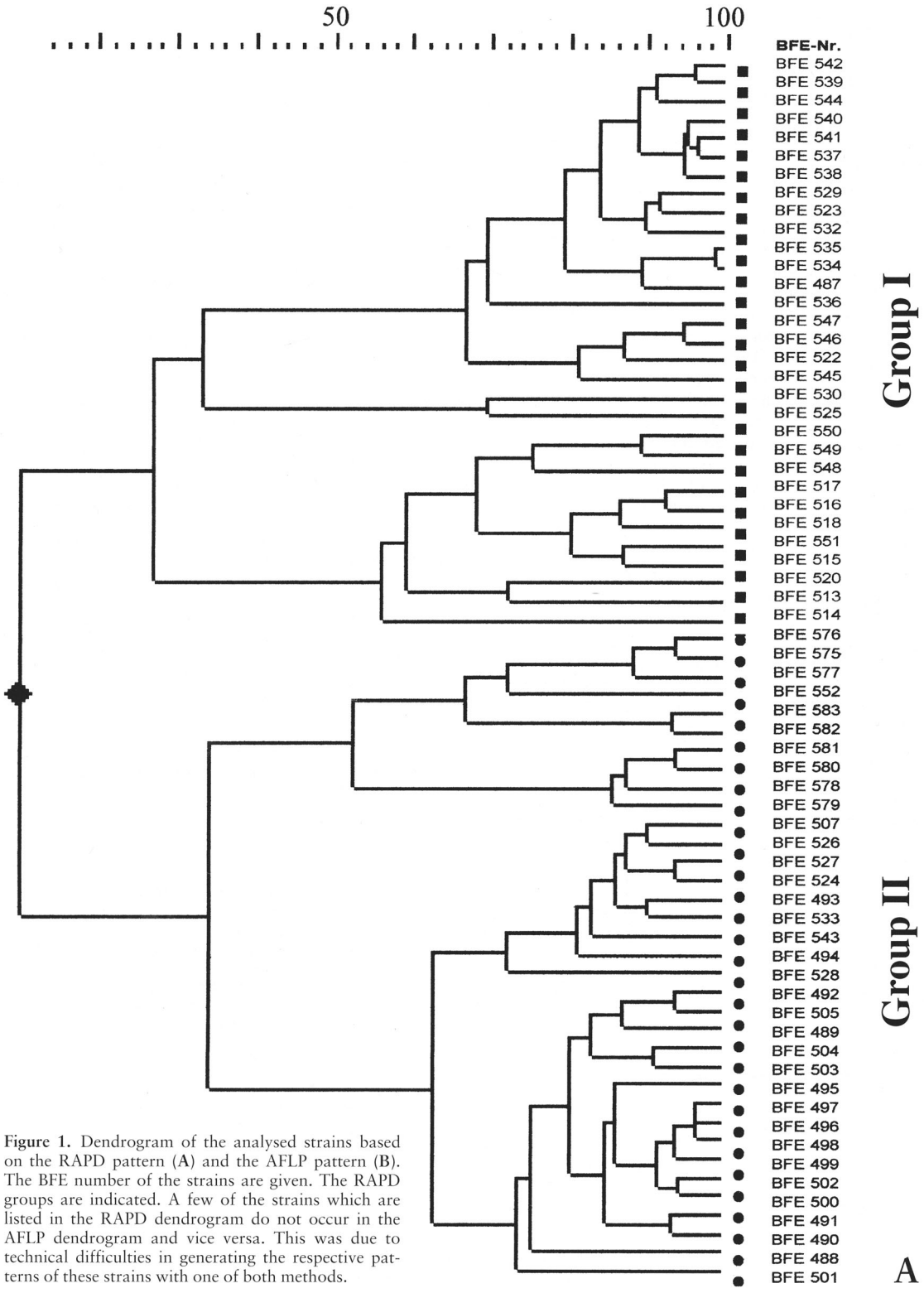
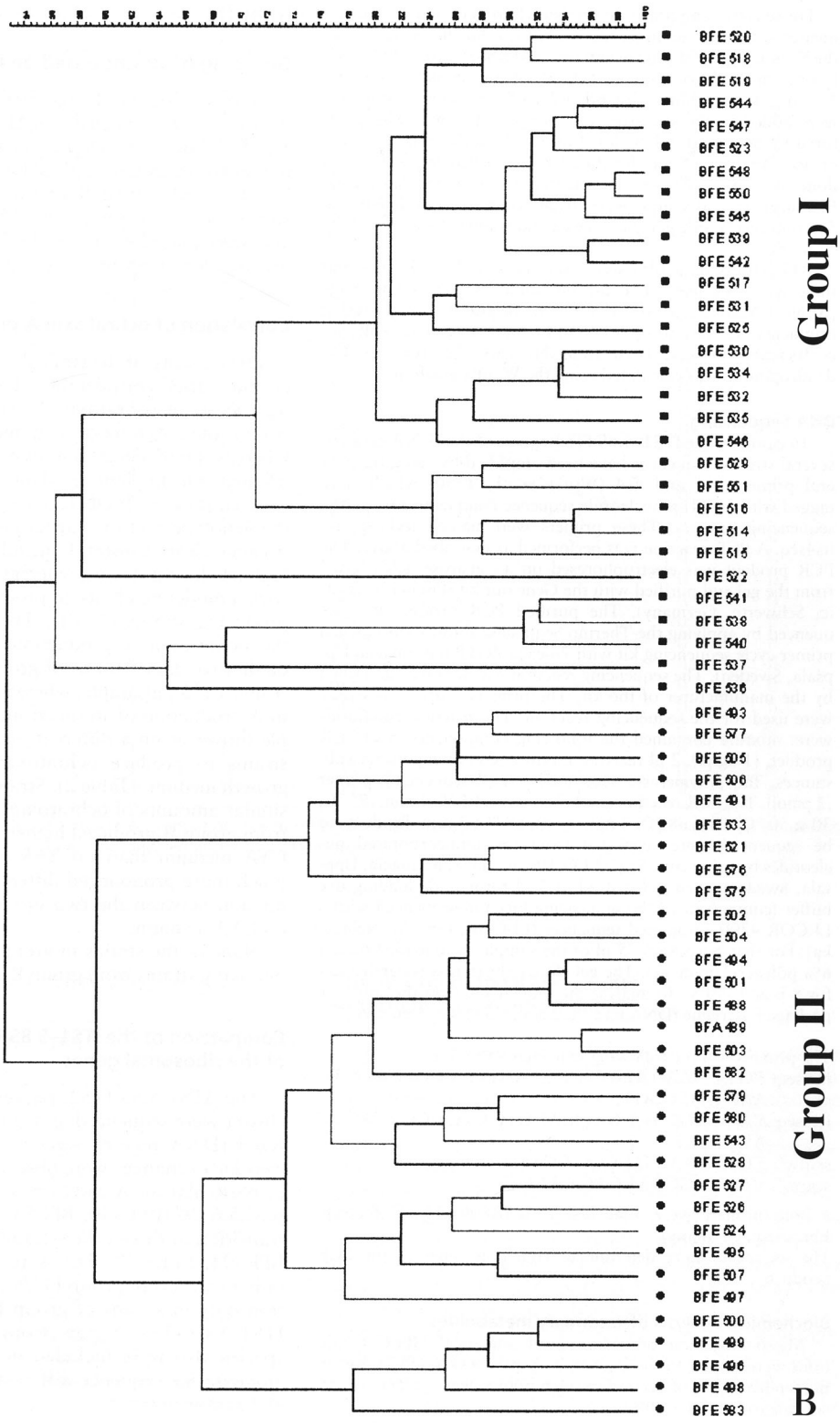


Figure 1. Dendrogram of the analysed strains based on the RAPD pattern (**A**) and the AFLP pattern (**B**). The BFE number of the strains are given. The RAPD groups are indicated. A few of the strains which are listed in the RAPD dendrogram do not occur in the AFLP dendrogram and vice versa. This was due to technical difficulties in generating the respective patterns of these strains with one of both methods.


BFE 520
BFE 518
BFE 519
BFE 544
BFE 547
BFE 523
BFE 548
BFE 550
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BFE 539
BFE 542
BFE 517
BFE 531
BFE 525
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BFE 501
BFE 488
BFA 489
BFE 503
BFE 582
BFE 579
BFE 580
BFE 543
BFE 528
BFE 527
BFE 526
BFE 524
BFE 495
BFE 507
BFE 497
BFE 500
BFE 499
BFE 496
BFE 498
BFE 583
Group I
Group II
B

For selective amplification the *Eco*RI primer had the same sequence as for the pre-amplification but was labelled with Cy5 at the 5′ end. The *Bfa*I primer had two additional bases (AT) as selective ends. A volume of 8.5 µl of formamid containing 5 mg/ml dextran blue, a 35 bp and a 650 bp internal standard were added to the selective amplification. The mix was denatured by heating to 94 °C for 3 min and transferring the tubes on ice. Analysis of the denatured Cy5-labelled fragments was done on an ALFexpress sequencer (Amersham Pharmacia, Freiburg). Five µl sample were loaded on a 0.3 mm denaturing 5% polyacrylamide gel gel. An external standard was run every 12 lanes.

ALFexpress data files were converted to TIFF images and these were examined with the BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium). For clustering fragments between 50 bp and 650 bp were analysed. Similarity coefficients were calculated using the Pearson correlation. The dendrogramm was calculated using the Ward algorithm.

DNA Sequencing

To compare the ITS1–5.8S-ITS2 region of the rDNA genes of several strains, sequences have been amplified by using the general primers its1 and its4 (WHITE et al., 1990) which were tagged with an 11bp nucleotide sequence complementary to the sequencing primers. These primers were named its1seq and its4seq. A PCR reaction was performed as described above. The PCR product was electrophoresed on an agarose gel, excised from the gel and purified with the Gene out kit (Fischer Scientific, Schwerte, Germany). The purified PCR product was sequenced by applying the Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Pharmacia, Uppsala, Sweden). The sequencing reaction was set up as described by the manufacturer of the kit. The primers seqfw and seqrev were used for the sequencing reaction. The reaction conditions were: mixture contained the following components: 5 µl PCR product, (1 ng/µl), 2 µl reaction mixture (containing buffer substances, Taq polymerase, nucleotides), 1 µl sequencing primer (2 pmol). The PCR reaction conditions were: 95 °C 4 min, (95 °C, 30 s; 50 °C, 40 s; 68 °C, 3 min) × 30, 68 °C, 3 min. Products to be sequenced were separated from the unincorporated nucleotides by using AutoSeq TM G-50 columns (Pharmacia, Uppsala, Sweden), then redissolved in 3 µl formamide loading dye buffer (component of the sequencing kit) and sequenced with a LI-COR 4200 automated sequencer (LI-COR, Lincoln, Nebraska). For that purpose 0.75 µl of the sample were loaded onto a 6% polyacrylamide gel. The gel electrophoresis was carried out for 8 h at 1300 V. To analyse the sequencing data the software package Lasergene (DNAStar, Madison, USA) has been used.

The primers had the following sequences (5′to 3′):
its1seq: 5′GTA AAA CGA CGG CCA GTT CCG TAG GTG AAC CTG CGG3′
its4seq: 5′AAC AGC TAT GAC CAT GTC CTC CGC TTA TTG ATA TGC3′
seqfw#: 5′GTA AAA CGA CGG CCA GT3′
segrev#: 5′AAC AGC TAT GAC CAT G3′

both primers were 5′labelled with IR800 (MWG Biotech, Ebersberg, Germany)
The sequences were determined from both sides of the PCR product.

Biochemical analysis of secondary metabolites

Micro-extraction procedures and analytical HPLC conditions were similar to those given by SMEDSGAARD (1997). Retention indices (RI) of secondary metabolites were calculated according to FRISVAD (1989).

Results

Grouping of strains based on RAPD and AFLP data

Sixty-six strains of potential ochratoxin A producing Penicillia were subjected to RAPD and AFLP analysis (Fig 1.). With both methods very similar results were obtained in that strains could be placed in one of two large clusters based on either their RAPD or AFLP profile. All of the strains placed in group I by AFLP analysis for example also were placed in group I by RAPD analysis. The same was true for the strains in group II.

Correlation of ochratoxin A production to genotype

The capacity of the strains to produce ochratoxin A or citrinin varied considerably (Table 1). Strains producing high levels of ochratoxin A usually had been recovered from protein rich foods, e. g. meat products and cheeses, whereas the moderate and non-producing strains usually originated from plants or plant products.

According to Table 1, a very clear tendency between the genotype and the capacity to produce ochratoxin A became clear. Cluster I includes mainly ochratoxin A high producing strains, whereas cluster II includes strains with a moderate ability to produce ochratoxin A or non-producing strains (Tab.1). The quantitative analysis of the ochratoxin A production was consistent with the qualitative data. Strains of group I produced ochratoxin A up to 115 µg/sample, whereas the maximum ochratoxin A production of strains from group II was 34 µg/sample (however on a different medium). The ability of the strains to produce ochratoxin A was altered by the growth medium (Table 2). Strains from group I produced similar amounts of ochratoxin A on both media. Strains from group II produced higher levels of ochratoxin A on CYA medium than on YES medium. Thus there is a much more pronounced difference in ochratoxin A production between the two groups on YES medium than on CYA medium.

None of the strains in group I could produce citrinin, but many strains from group II could.

Comparison of the ITS1–5.8S-ITS2 region of the ribosomal genes

The ITS1-5.8S-ITS2 regions of strains from each cluster were sequenced and compared (Fig 2.). The analyzed rDNA regions were very similar. Only two nucleotide exchanges were observed between some strains, in particular an A to C transversion in three strains at position 20 (BFE530, BFE537, BFE541) and a A to G transition in three other strains at position 70 (BFE489, BFE504, BFE577). The A to C transversion occurred only in strains of group I (3/6), whereas the other mutation only in strains of group II (3/7). As a control the ITS1–5.8S-ITS2 region from two other *Penicillium* species also were included in the analysis. For *P. nalgiovense* the sequence differs at 6 and for *P. roqueforti* at 16 positions.

Table 1. The capacity of the strains to produce ochratoxin A, citrinin and their classification into genotype groups.

Strain No	Ochratoxin	Citrinin	RAPD group	AFLP group	Strain No	Ochratoxin	Citrinin	RAPD group	AFLP group
BFE532	+[1]	–	I	I	BFE521	–	–	II	II
BFE487	+++++	–	I	I	BFE527	+	+	II	II
BFE513	++++	–	I	I	BFE524	+	+	II	II
BFE529	–	–	I	I	BFE543	+	–	II	II
BFE520	–	–	I	I	BFE488	–	–	II	II
BFE535	+	–	I	I	BFE505	–	+	II	II
BFE534	+	–	I	I	BFE492	++	+	II	II
BFE523	+	–	I	I	BFE489	++	+	II	II
BFE539	+	–	I	I	BFE533	–	–	II	II
BFE540	++++	–	I	I	BFE503	+	+	II	II
BFE537	++++	–	I	I	BFE497	+	+	II	II
BFE541	++++	–	I	I	BFE498	+	+	II	II
BFE544	+++	–	I	I	BFE495	++	–	II	II
BFE538	++++	–	I	I	BFE494	+	–	II	II
BFE551	++	–	I	I	BFE491	–	+	II	II
BFE542	++++	–	I	I	BFE499	–	–	II	II
BFE518	+	–	I	I	BFE493	++	–	II	II
BFE515	++	–	I	I	BFE490	–	–	II	II
BFE517	+++	–	I	I	BFE504	–	–	II	II
BFE516	+	–	I	I	BFE502	++	–	II	II
BFE514	+	–	I	I	BFE507	++	–	II	II
BFE547	+++	–	I	I	BFE500	+	–	II	II
BFE546	+++	–	I	I	BFE501	–	–	II	II
BFE548	++	–	I	I	BFE582	+	–	II	II
BFE550	+++	–	I	I	BFE577	–	+	II	II
BFE549	++++	–	I	I	BFE583	+	+	II	II
BFE545	+++	–	I	I	BFE578	–	+	II	II
BFE522	+	–	I	I	BFE576	–	+	II	II
BFE536	+	–	I	I	BFE581	–	+	II	II
BFE525	+++	–	I	I	BFE580	–	+	II	II
BFE530	+	–	I	I	BFE579	–	+	II	II
BFE528	++	–	II	II	BFE552	–	+	II	II
BFE526	+	–	II	II	BFE575	–	+	II	II

[1] semiquantitive analysis of the ochratoxin A production, the number of + indicates the estimated amount of ochratoxin A produced, – indicates that ochratoxin A could not be detected under the conditions used

Table 2. Correlation of the genotype groups to quantitative ochratoxin A production.

Strain No	AFLP/RAPD group	HPLC[a] (CYA)	HPLC[a] (YES)
BFE487	I	84.2	115.1
BFE540	I	29	39
BFE537	I	40.6	35.5
BFE538	I	36.8	76.4
BFE542	I	38.8	38.6
BFE549	I	84.7	26.3
BFE514	I	19.3	4.9
BFE522	I	3.7	7.1
BFE520	I	1.9	3.2
BFE526	II	30.4	0.9
BFE527	II	15.7	0.2
BFE524	II	15.4	2.1
BFE493	II	0.5	0.1

[a] – The values are given in µg/sample (see Materials and Methods)

Comparison of the RAPD results with chemotaxonomical data

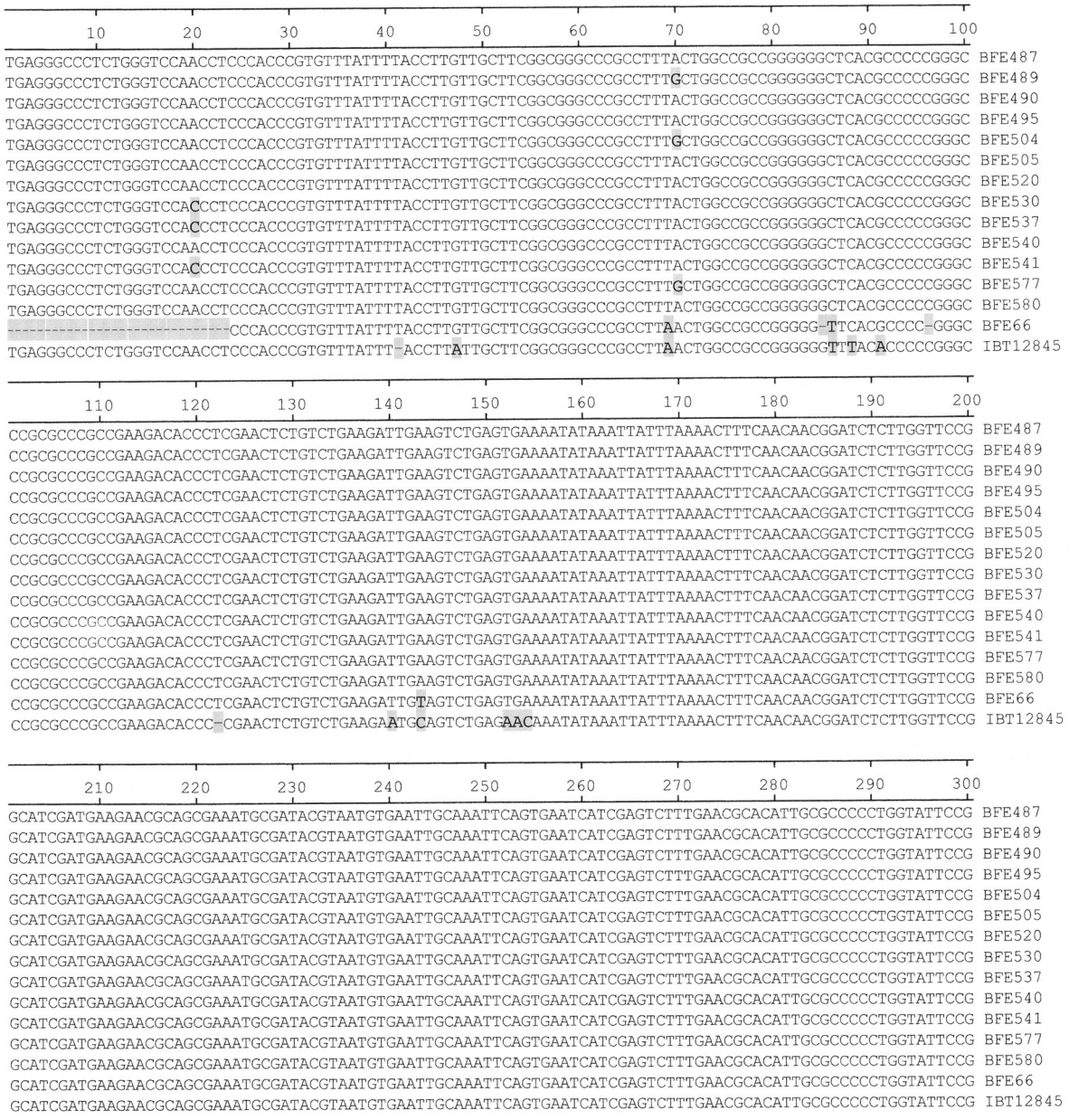
Fifteen representative isolates were further analyzed by microextraction followed by HPLC coupled to diode array detection. All isolates tested produced verrucolone and related α-pyrones (Table 3), metabolites very consistently produced by ochratoxin A producing penicillia. Nine of the fifteen isolates produced either anacines or sclerotigenin or both often in combination with production of high levels of ochratoxin A and low levels of ochratoxin B. Altogether a typical profile of secondary metabolites for *P. nordicum*. None of the six remaining isolates (BFE: 493, 524, 526, 527, 581, 583) produced anacines or sclerotigenin, but instead all produced verrucines (LARSEN et al., 1999), which are compounds commonly produced by *P. verrucosum*. Three of these six isolates produced ochratoxin A in relatively small amounts and three isolates produced citrinin. Thus the

15 isolates examined can be divided into two groups – one group of nine that is similar to *P. nordicum* and another group of six that is similar to *P. verrucosum*.

The results show, that the observed differences at the genotypic level correlate very well with the differences observed at the level of secondary metabolism. The strains from cluster I had the same secondary metabolite profile as *P. nordicum* (LARSEN et al., 2001). Strains from cluster II are coincidental with *P. verrucosum* according to their secondary metabolite profile.

Discussion

The molecular results described here strongly support the reclassification of ochratoxin A producing penicillia into two species (LARSEN et al., 2001), *P. verrucosum* and *P. nordicum*. Both are very similar at the morphological level. They show however one phenotypic difference when grown on YES medium. *P. verrucosum* produces a characteristic dark brown colour on the reverse, whereas *P. nordicum* does not.



The RAPD and AFLP analysis clearly divided the analyzed strains into two genetically different groups. Group I is able to produce high amounts of ochratoxin A. None of these strains were able to produce citrinin, instead they produced anacines and/or sclerotigenin. Almost all of these strains were derived from proteinacous foods like cheeses and meats. According to the chemotaxonomic analyses these strains belong to *P. nordicum* (LARSEN et al., 2001).

Strains from groups II originate from plants and are moderate ochratoxin A producers to non producers at the conditions used here. Many of these strains were able to synthezise citrinin and in addition verrucines, which are typical for *P. verrucosum*.

It is discussed by LARSEN et al. (2001) that the production of these additional secondary metabolites maybe the reason for the reduced capacity of *P. verrucosum* strains to produce ochratoxin A compared to *P. nordicum*. In the

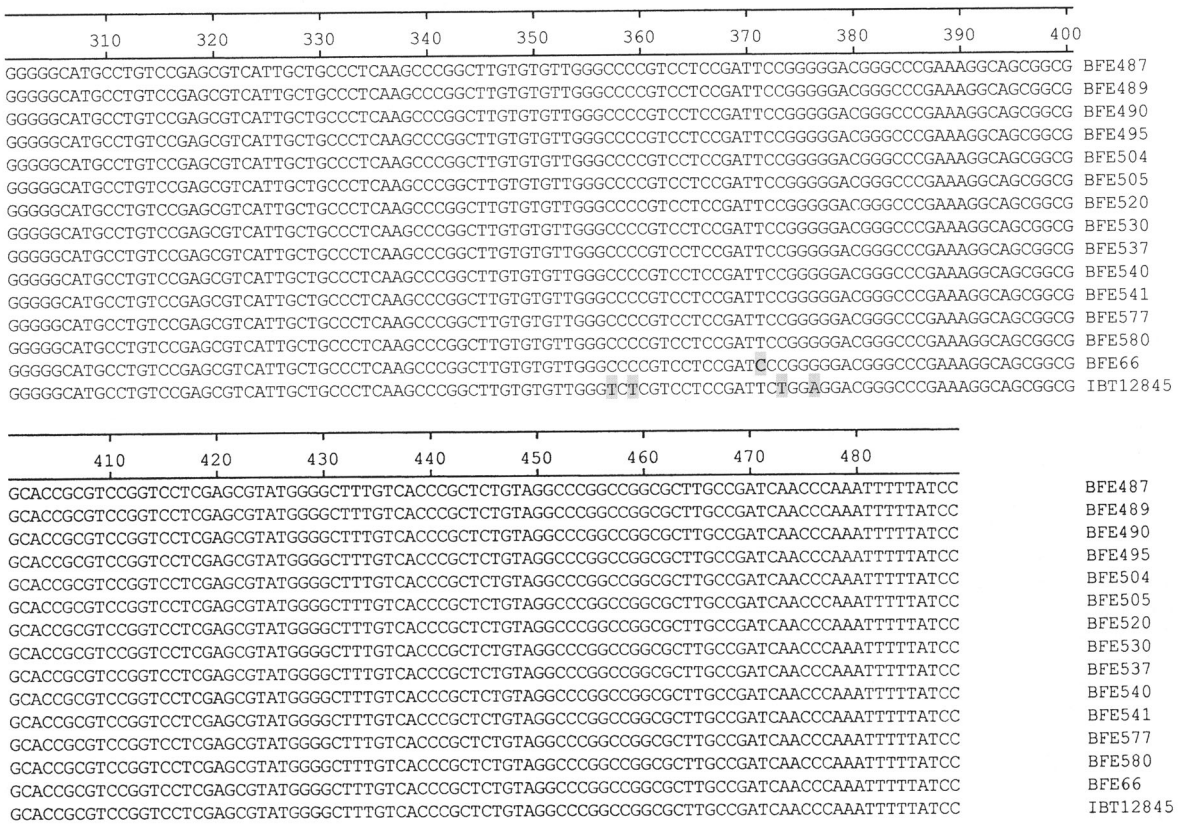


Figure 2. Sequences of the ITS1–5.8S-ITS2 region of representative strains of each genotypic group. Differences between the sequences are marked with a box. Nucleotide 1 is the first (except strain *P. nalgiovense* BFE66) and nucleotide 498 the last common nucleotide for all samples. Strains BFE487, BFE541, BFE520, BFE530, BFE537 and BFE540 belong to group I; strains BFE490, BFE495, BFE504, BFE505, BFE489, BFE577 and BFE580 to group II. BFE66 is a strain of *P. nalgiovense* and IBT12845 a strain of *P. roqueforti*. The sequence of *P. roqueforti* IBT12845 (accession number AJ005677) was taken from GenBank (www.ncbi.nlm.nih.gov)

Table 3. Correlation of phenotypic data to genotypic data.

Strain No	verrucolones	OTA	OTB	sclerotigenin	anacines	verrucines	citrinin	Species[a]	AFLP/RAPD group[b]
BFE487	×	×	×	×				*P. nordicum*	I
BFE514	×	×		×				*P. nordicum*	I
BFE520	×			×	×			*P. nordicum*	I
BFE522	×	×		×				*P. nordicum*	I
BFE537	×	×	×	×	×			*P. nordicum*	I
BFE538	×	×	×	×	×			*P. nordicum*	I
BFE540	×	×	×	×	×			*P. nordicum*	I
BFE542	×	×	×	×	×			*P. nordicum*	I
BFE549	×	×	×		×			*P. nordicum*	I
BFE493	×	×				×		*P. verrucosum*	II
BFE524	×	×				×	×	*P. verrucosum*	II
BFE526	×	×				×		*P. verrucosum*	II
BFE527	×	×				×	×	*P. verrucosum*	II
BFE581	×					×	×	*P. verrucosum*	II
BFE583	×					×	×	*P. verrucosum*	II

[a] – according to Larsen *et al.* (2001); [b] – according this report

biosynthesis of both secondary metabolites the same precursors as for the biosynthesis of ochratoxin A are utilized, phenylalanine in the case of the verrucines and the isocoumarin moiety in the case of citrinin. The production of ochratoxin A by the two species seems to be differentially regulated on the two media tested. The production of ochratoxin A is much less influenced by the composition of the growth medium in the case of *P. nordicum* than of *P. verrucosum*. *P. verrucosum* produces much higher amounts of ochratoxin A on CYA than on YES medium. The citrinin production by *P. verrucosum* on the other hand is much higher on YES medium than on CYA medium (data not shown), which supports the hypothesis of LARSEN et al. (2001) that the production of citrinin is in expense of the amount of ochratoxin A produced.

The fact that *P. verrucosum* is able to produce citrinin in addition to ochratoxin, explains the observation that both mycotoxins can be isolated from cereal products (SCUDAMORE et al., 1993; VRABCHEVA et al., 2000). In contrast to that *P. nordicum* is obviously not well adapted to plant products like cereals. Under the aspect of food safety this fact can be regarded as an advantage, because this species is able to produce ochratoxin A in very high amounts. If *P. nordicum* is able to produce ochratoxin on proteinaceous foods, it may constitute a serious health hazard for these commodities.

The analysis of ITS1-5.8S-ITS2 sequences gives evidence that both species are related. The analyzed DNA region is nearly identical for all ochratoxin A producing strains, indicating a close phylogenetic relationship. Non ochratoxin A producing *Penicillium* species showed a much higher sequence divergence. It has to be kept in mind however, that also the generally homologous 5.8S region was included in the analyzed region. High sequence similarity of this DNA region between related species was also demonstated by other authors. ACCENSI et al. (1999) described nearly identical sequences for *Aspergillus niger* and *A. tubingensis*. BOYSEN et al. (1996) reclassified *P. roqueforti* strains according to their differences in the ITS1-5.8S-ITS2 regions into three species, namely *P. roqueforti*, *P. carneum* and *P. paneum*. *P. carneum* and *P. roqueforti* differed in only two positions. PAŘENIKOVÁ et al. (2001) also analyzed the ITS1-5.8S-ITS2 region of the two closely related *Aspergillus japonicus* and *A. aculeatus* species. They also found nucleotide exchanges at only three positions. However if these sequences were compared to the ones of other species of the black Aspergilli they identified up to 19% dissimilarity. In combination with other molecular and biochemical methods these authors could clearly separate both morphologically nearly identical species.

In this work the combination of chemotaxonomical and various molecular data clearly demonstrates that ochratoxin A producing penicillia belong to two different, albeit related species, thereby confirming the data of LARSEN et al. (2001) which were mainly based on the different chemotypes of the species.

Acknowledgments

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AFLP analysis of *Fusarium* species in the section *Sporotrichiella* – evidence for *F. langsethiae* as a new species

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Abstract

Amplified fragment length polymorphism (AFLP) was applied to compare 100 isolates of *Fusarium poae*, *F. sporotrichioides* and *F. langsethiae*. Comparison of fingerprints revealed several markers specific for each group of isolates. Cluster analysis showed a clear separation of *F. poae* from *F. sporotrichioides* and *F. langsethiae. F. langsethiae* isolates formed a rather homogeneous group separated from *F. sporotrichioides*. The AFLP data obtained support *F. langsethiae* as a new *Fusarium* species, for the time being in the section *Sporotrichiella.*

Keywords: *Fusarium*, fungi, AFLP, fingerprinting, DNA, cluster analysis

1. Introduction

Most molecular systematic approaches rely on the comparison of one or more distinct DNA sequences. Many different kinds of data have been obtained and are used for classifying organisms. Taxonomists dealing with fungi favor IGS, ITS, actin, β-tubulin, mtDNA and other sequences for differentiating their isolates (Berbee and Taylor, 2001). However, in choosing a certain sequence type one defines the discriminatory power of his experimental design and, strictly speaking, uses only a small subset of the information present in an organism´s genome. As a consequence the use of multiple sequences has become the most frequently applied approach when studying taxonomical relationships (e. g. O´Donell et al., 1998). The best way to characterize the genotype would of course be to determine the complete sequence of the genome and compare it to other genomes. Yet, *Saccharomyces cervisiae* and *Schizosaccharomyces pombe* are the only fungi up to now for which the entire genome sequence is available (Goffeau et al., 1996; Wood et al., 2002). Only a few genomes of fungi are on their way to be completed (http://www.tigr.org). The tremendous amount of money, time and the very limited throughput exclude this approach from taxonomic studies for the time being.

To overcome these problems, different genotyping techniques like restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) have been developed and extensively used over the last few years. For comprehensive information see the reviews by Louws et al. (1999) and Soll (2000). These fingerprinting techniques take into account information distributed over the whole genome of an organism and generally permit discrimination at species to strain level. It has been suggested that fingerprinting techniques can function as a core technique in a polyphasic taxonomic system (Savelkoul et al., 1999).

Among these techniques AFLP is famous for its robustness, reproducibility and high discriminatory power (Vos et al., 1995). No previous information on the genome is required as the AFLP protocol is universally applicable, needing only minor adaptation to the group of organisms studied. The modifications depend on the size and composition of the examined genome and affects the choice of restriction enzymes as well as the kind and number of selective bases used in the PCRs. Once having determined these conditions AFLP procedures can easily be automated and standardized when using fluorescently labelled primers and automated DNA sequencers. Coupled with modern data evaluation software this set up allows typing, clustering and identification of organisms with high throughput (Blears et al., 1998; Myburg et al., 2001). Due to this advantages AFLP has been widely applied in fungal taxonomy since described by Vos et al. (1995), initially being used in fungal taxonomy by Majer et al. (1996) who studied genetic variations in the plant pathogenic species *Cladosporium fulvum* and *Pyrenopeziza brassicae*. Soon thereafter other phytopathogenic and toxigenic fungi, mainly of the genus *Fusarium* were studied using AFLP (Leissner et al., 1997).

F. sporotrichioides has been recognized as the main source for the highly potent mycotoxins T-2 and HT-2 toxin (Marasas et al., 1984). Recently a group of T-2 toxin producing isolates has been identified which are morphologically distinct from *F. poae* and *F. sporotrichioides* (Torp and Langseth, 1999; Torp and Nirenberg, 2003). The aim of the current study was to evaluate relationships among the T-2 toxin and HT-2 toxin producing taxa *F. sporotrichioides* and *F. langsethiae* (Torp and Nirenberg, 2003) and to compare them with *F. poae* by the use of AFLP as a tool for analysing relationships at low taxonomic levels.

2. Material and methods

2.1 Strains, culture conditions and DNA preparation

Information of the strains examined in this study is given in the table of the introduction to this issue (Torp and Adler, 2003. For DNA preparation fungal isolates were grown in 15 ml plastic tubes (Sarstedt, Nümbrecht, Germany) containing 3 ml 2 % malt extract broth (Gams et al., 1998) for 7 to 10 days at room temperature on a rotary shaker (140 rpm). Mycelia were harvested by centrifugation (15 min, 4500 x g), washed once with sterile distilled water and lyophilised. DNA used for AFLP experiments was extracted according to the CTAB protocol by Möller et al. (1992).

2.2 Template preparation and AFLP reactions

AFLP was performed as described by Vos et al. (1995) and modified by Aarts and Keijer (1999) with minor changes to the protocol. For template preparation all reaction mixtures contained 10 mM Tris-HAc pH 7.5, 10 mM $MgAc_2$, 50 mM KAc, 5 mM DTT and 50 ng/µl BSA. In a first step, 200 ng of isolated genomic DNA were digested in a total volume of 15 µl at 37 °C for 3 h with 2U of EcoRI (TaKaRa Shuzo Co., Ltd., Japan) and 2U of MseI (New England Biolabs, Frankfurt, Germany). Secondly, 5 µl of a solution containing 2 pMol EcoRI adaptor, 20 pMol MseI adaptor (Vos et al., 1995), 0.4U T4 DNA ligase (MBI Fermentas, St. Leon-Rot, France) and 1 mM ATP were added. Ligation of adaptors was performed for 12 h at room temperature in a total volume of 20 µl. This mixture was diluted tenfold with 10 mM Tris-HCl pH 8.0. Five microliters of this dilution were used as template for the first PCR amplification.

PCRs were performed as described by Vos et al. (1995) in a total volume of 20 µl with the following modifications. For pre-amplification the EcoRI-primer had an adenin (A) and the MseI primer a cytosin (C) as an additional base at the 3´-end, respectively. The selective amplification

was carried out using Cy5-labeled EcoRI primers to allow automated laser fluorescence analysis (ALFA, Grundmann et al., 1995). Different numbers and kinds of selective bases were tested. EcoRI primers had ACT, AAC or AC as selective ends. The MseI primer had either CT or CA as selective ends.

2.3 Fragment Analysis and data processing

For fragment analysis 8.5 µl of formamid containing 5 mg/ml dextran blue and a 650 bp Cy5-labelled DNA fragment as internal standard, was added to the selective amplification. The mix was denatured by heating to 94 °C for 3 min and the tubes immediately transferred to ice. Analysis of the denatured Cy5-labelled fragments was done on an ALFexpress sequencer (Amersham Pharmacia, Freiburg, Germany). Five µl sample were loaded on a 0.3 mm denaturing 5 % PAA gel. An external standard was run every 12th lane. Electrophoresis was carried out at 45 W, 60 mA and 50 °C for 6 h.

ALFexpress data files were converted to TIFF images using the ALF2TIFF software provided by the manufacturer. These images were imported into the BioNumerics version 2.5 software package (Applied Maths, Sint-Martens-Latem, Belgium) and further processed. For cluster analysis fragments between 70 bp and 650 bp were considered. Similarity coefficients were calculated using the Pearson correlation, a method that calculates similarities and standarizes the fingerprints according to the relative intensity of the signals. Thus, similarities calculated were based on the shape of the densiometric curve of the fingerprints rather than on the appearance of a single band. The dendrogram was calculated using the Ward algorithm. For each node the cophenetic correlation was calculated. For a extensive description and discussion of these methods, see the book of Sneath and Sokal (1973).

3. Results

Complexity of AFLP fingerprints obtained was low when the combination of *Eco*RI and *Mse*I and a total of five selective ends (E-ACT or E-AAC together with M-CA) was applied (data not shown). A total of four selective bases (E-AC, M-CA) we found to provide a sufficiently complex pattern for fragment analysis. Fig. 1 shows AFLP profiles of some representative strains. Within these runs, fragments are present throughout the range from 70 to 650 bp. Among several very prominent peaks a number of less intense signals are recognizable. At high molecular weights peaks were generally smaller and less frequent. In summary, a total of approximately 20 different peaks within the range of fragment sizes from 70 to 650 bp contributed to cluster analysis.

AFLP profiles were compared using the Pearson correlation. The Ward dendrogram calculated from this similarity matrix is presented in Fig. 2. In this graph, organisms were clustered according to the shape of their AFLP profiles in a dichotomous way. In this cluster analysis two major groups, I and II, appeared which were clearly separated from one another and well supported by a high overall cophenetic correlation of 81 % for the complete dendrogram. These two clusters were further divided into subgroups. *Fusarium sporotrichoides* strains were exclusively found in group I. Within this group all *F. sporotrichoides* strains studied formed a distinct subcluster (IA) with one exception (IBT 9945). In the other subcluster (IB), grouping of all strains asigned to *F. langsethiae* was well supported by a cophenetic correlation of 68 %. In this group, *F. sporotrichoides* IBT 9945 was the only outlier among the majority of the *F. langsethiae* isolates examined. Cluster II comprised all *F. poae* isolates and was rather heterogeneous compared to I. Two strains of *F langsethiae* and the analysed strain of *F. kyushuense* could also be found in this group. The latter three strains formed rather segregated

127 branches within the *F. poae* strains, indicating a rather distant relationship of these strains to the
128 *F. poae* strains within cluster I.
129 Among the fragments analysed some were found to appear in fingerprints of a specific group of
130 isolates (see Fig. 1). For example, fragment A (85 bp) appeared only in fingerprints of *Fusarium*
131 *poae* whereas the 290 bp fragment C was detected exclusively in fingerprints of
132 *F. sporotrichioides* strains. Fragment B of approximately 220 bp was present in both
133 *F. sporotrichioides* and *F. langsethiae*. In strain IBT 9945 identified as *F. sporotrichioides* only
134 fragment B could be found resembling AFLP profiles of *F langsethiae*. Fingerprints of strains
135 IBT 9958 and IBT 9951 (data not shown) clearly differed from fingerprints of all of the species
136 mentioned.

137 **4. Discussion**

138 Since its first application for distiction and identification of fungi (Majer et al., 1996), lots of
139 different fungal isolates have been studied using the AFLP technique (Mueller et al., 1996). In
140 *Fusarium* taxonomy AFLP was first applied to discriminate between different strains of
141 *Fusarium graminearum* (Leissner et al., 1997). Up to now many questions concerning mating
142 types, populations and *formae speciales* in the genus *Fusarium* have been resolved using this
143 technique (Baayen et al., 2000; Bao et al., 2002). AFLP is well suited for distinguishing closely
144 related organisms at the species to strain level. Bakkeren et al. (2000) pointed out that the
145 phylogenetic trees obtained from AFLP markers are quite similar to those obtained by ITS
146 sequences in *Ustilago* species, but generally permit distinction of closely related isolates which
147 can not be resolved by ITS sequence comparison. Baayen et al. (2000) stress the higher resolution
148 of AFLP analyses at a refined taxonomic level compared with EF-1α and mtSSU rDNA
149 sequences in *F. oxysporum*. Recently, the high discriminatory power of AFLP was also

demonstrated by Chulze et al. (2000) who traced isolates of *F. verticillioides* and *F. proliferatum* down to the clonal level.

On the one hand, AFLP generally resolves taxa at a high resolution but on the other hand AFLP is not suited for distinguishing organisms at a level ranking above the species. The cophenetic correlation (81 %) - a parameter for the consistency of a cluster (Sneath and Sokal, 1973) - indicates that the similarity matrix is reflected rather well by the dendrogram. Thus the overall grouping of the entries can be regarded as rather robust. However, in some instances comparison of rather distantly related organisms has lead to misinterpretation of dendrograms when these organisms eventually cluster together (Louws et al., 1999, Savenkoul et al., 1999). Maybe this is the case for the two *F. langsethiae* isolates IBT 9958 and IBT 9951 as they do not seem to fit well into group II of the dendrogram. Strain IBT 9945 of *F. sporotrichioides*, which clustered perfectly within the *F. langsethiae* group indicates the close relationship between the two species and may be misidentified based on morphological characters. When taking into account the dendrogram calculated from the AFLP fingerprints of the rest of the *F. langsethiae* isolates examined, they can be considered as a group well separated from *F. sporotrichioides.* The relatively high cophenetic correlation (68 %) at the basal node of group I further supports the division of the two taxa. Together with the homogeneity of the cluster obtained by AFLP fingerprinting, this justifies the creation of a separate taxon, at the rank of species or sub-species, remains to be elucidated by other techniques.

Acknowledgments

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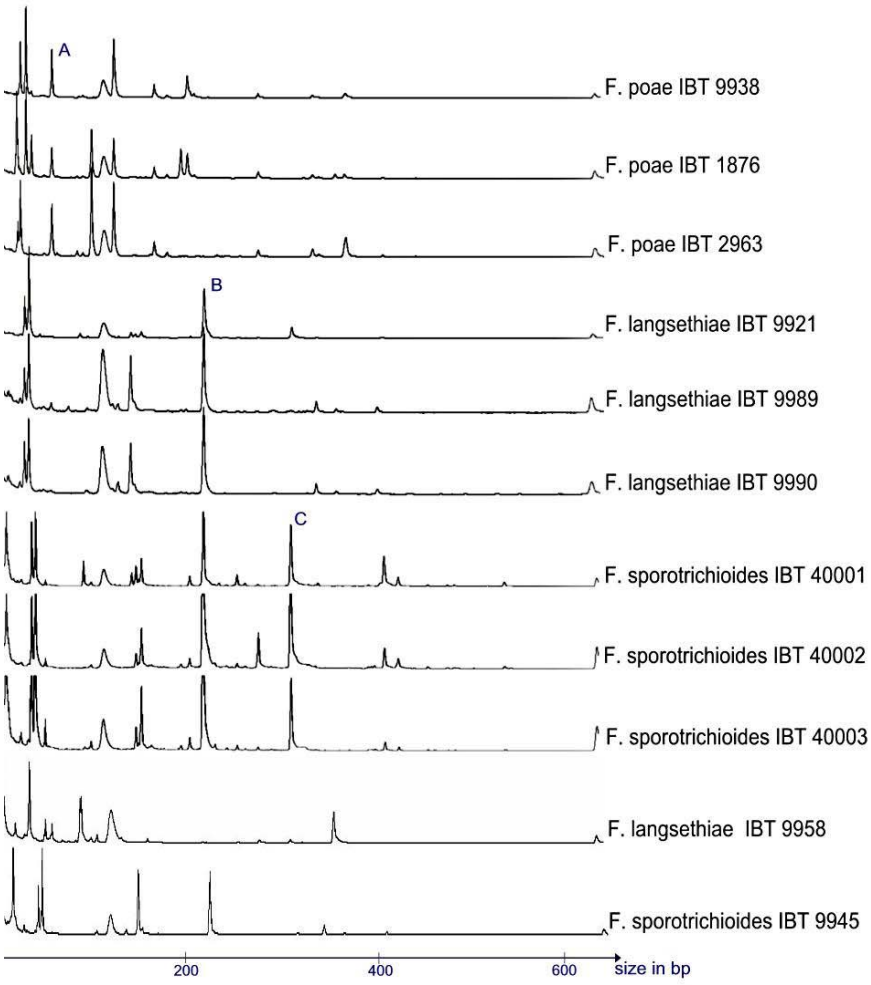
Legends to figures:

Figure 1: AFLP fingerprints of representative *Fusarium* strains examined in this study. Sizes of nucleotides on the x-axis, on the y-axis the relative intensity of the signal in the electropherogram is shown. Primers M-CA and E-AC were used to generate the pattern. A indicates an anonymous marker present only in *F. poae* isolates, C a marker present only in *F. sporotrichioides*. Fragment B is present in both *F. sporotrichioides* and *F. langsethiae.*

Figure 2: Ward dendrogram calculated from the comparison of AFLP fingerprints of all strains examined in this study. Similarities were determined using the Pearson correlation. Fragments from 70 bp to 650 bp were taken into account. The cophenetic correlation at the major branching points is given in %. Notice that cluster I comprises only *Fusarium sporotrichioides* and *F. langsethiae* isolates. All *F. poae* isolates studied are found in cluster II. With the exceptions of IBT 9958 and IBT 9951 all *F. langsethiae* strain are found in subcluster IB.


A
B
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F. sporotrichioides IBT 40001
F. sporotrichioides IBT 40002
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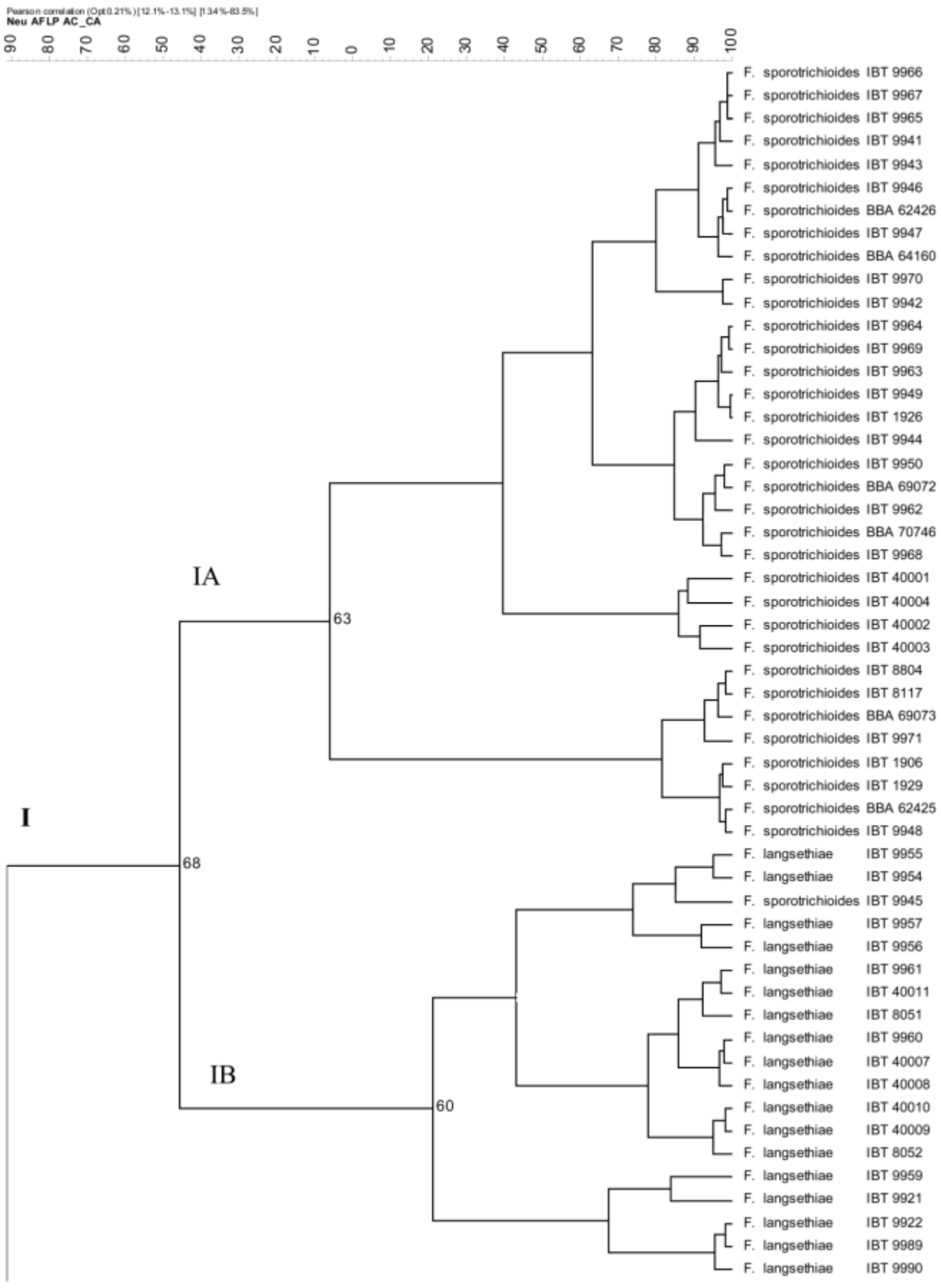
Pearson correlation (Opt:0.21%) [12.1%-13.1%] [13.4%-83.5%]
Neu AFLP AC_CA
90 80 70 60 50 40 30 20 10 0 10 20 30 40 50 60 70 80 90 100
F. sporotrichioides IBT 9966
F. sporotrichioides IBT 9967
F. sporotrichioides IBT 9965
F. sporotrichioides IBT 9941
F. sporotrichioides IBT 9943
F. sporotrichioides IBT 9946
F. sporotrichioides BBA 62426
F. sporotrichioides IBT 9947
F. sporotrichioides BBA 64160
F. sporotrichioides IBT 9970
F. sporotrichioides IBT 9942
F. sporotrichioides IBT 9964
F. sporotrichioides IBT 9969
F. sporotrichioides IBT 9963
F. sporotrichioides IBT 9949
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F. sporotrichioides IBT 9944
F. sporotrichioides IBT 9950
F. sporotrichioides BBA 69072
F. sporotrichioides IBT 9962
F. sporotrichioides BBA 70746
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IA
F. sporotrichioides IBT 40001
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F. sporotrichioides IBT 40002
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F. langsethiae IBT 9961
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F. langsethiae IBT 8051
F. langsethiae IBT 9960
F. langsethiae IBT 40007
F. langsethiae IBT 40008
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F. langsethiae IBT 40010
F. langsethiae IBT 40009
F. langsethiae IBT 8052
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F. langsethiae IBT 9959
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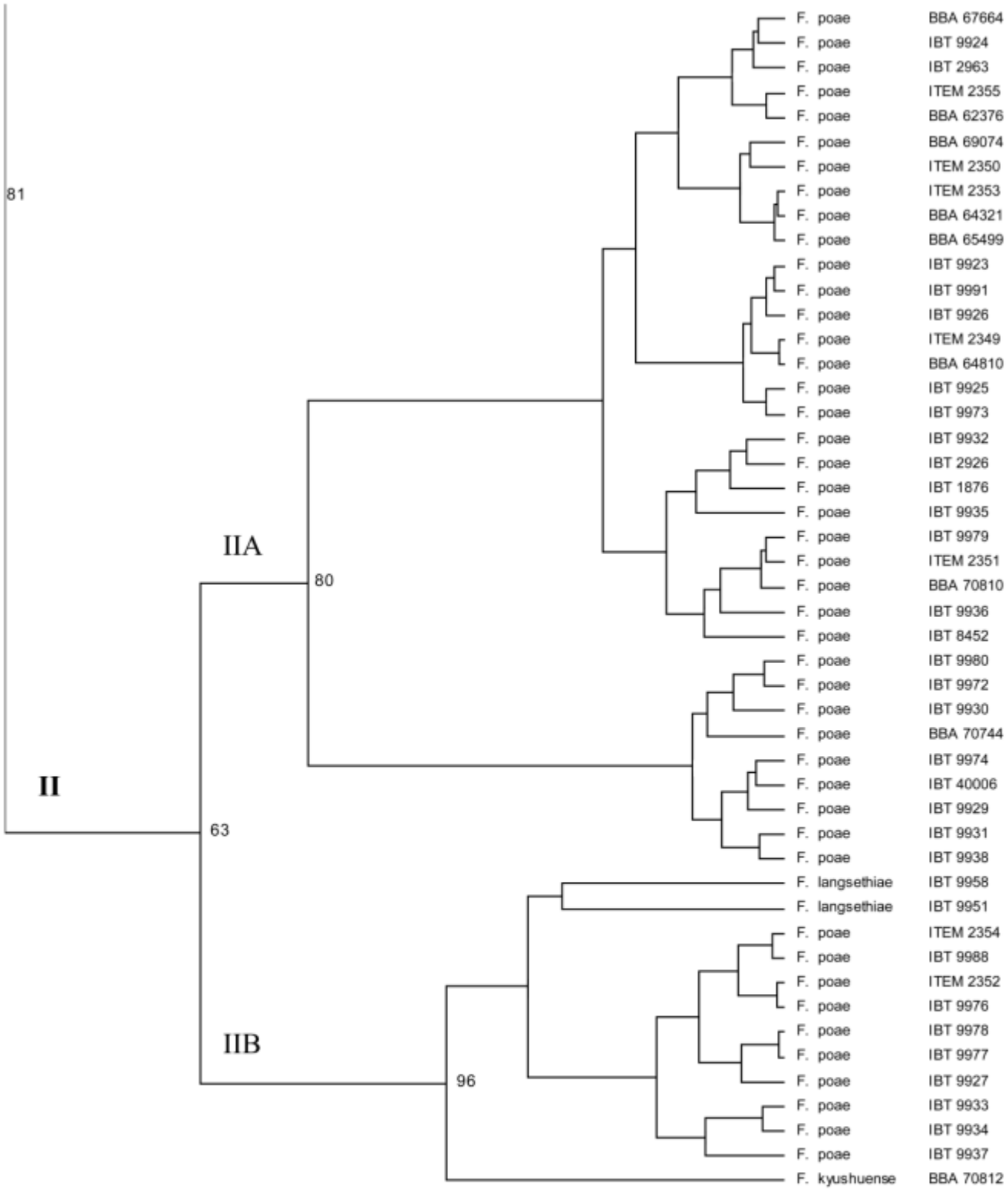
F. langsethiae IBT 9989
F. langsethiae IBT 9990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IIA
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F. poae BBA 67664
F. poae IBT 9924
F. poae IBT 2963
F. poae ITEM 2355
F. poae BBA 62376
F. poae BBA 69074
F. poae ITEM 2350
F. poae ITEM 2353
F. poae BBA 64321
F. poae BBA 65499
F. poae IBT 9923
F. poae IBT 9991
F. poae IBT 9926
F. poae ITEM 2349
F. poae BBA 64810
F. poae IBT 9925
F. poae IBT 9973
F. poae IBT 9932
F. poae IBT 2926
F. poae IBT 1876
F. poae IBT 9935
F. poae IBT 9979
F. poae ITEM 2351
F. poae BBA 70810
F. poae IBT 9936
F. poae IBT 8452
F. poae IBT 9980
F. poae IBT 9972
F. poae IBT 9930
F. poae BBA 70744
F. poae IBT 9974
F. poae IBT 40006
F. poae IBT 9929
F. poae IBT 9931
F. poae IBT 9938
F. langsethiae IBT 9958
F. langsethiae IBT 9951
F. poae ITEM 2354
F. poae IBT 9988
F. poae ITEM 2352
F. poae IBT 9976
F. poae IBT 9978
F. poae IBT 9977
F. poae IBT 9927
F. poae IBT 9933
F. poae IBT 9934
F. poae IBT 9937
F. kyushuense BBA 70812

An integrated taxonomic study of *Fusarium langsethia*, *F. poae* and *F. sporotrichioides* based on the use of composite datasets

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Abstract

An integrated systematic study was carried out to clarify the taxonomical position and relationship of *Fusarium langsethiae* (Torp and Nirenberg, 2002, this issue) within the *Fusarium* section *Sporotrichiella*. Different isolates of this species were compared with strains of the closely related species *F. poae* and *F. sporotrichioides* using a composite dataset. This set consisted of DNA sequences derived from the intergenetic spacer region (IGS), the internal transcribed spacer region (ITS); partial sequences of the β−tubulin and the elongation factor-1 alpha (EF-1α) gene, AFLP fingerprints, chromatographic data on secondary metabolites and morphology. From these combined data a consensus matrix was calculated. This was used as the basis for the construction of an UPGMA dendrogram and a multidimensional scaling both of which revealed a clear separation of the three taxa. IGS, partial EF-1-α and the β-tubulin sequence-derived similarities as well as chromatographic and AFLP-derived turned out to be comparably consistent while ITS and morphological derived similarity matrices resulted in rather different similarity matrices. Based on the comparison of the discriminatory power of the different approaches *Fusarium langsethiae* is considerd to be a close sister taxon to *F. sporotrichioides*

Keywords: Fusarium, fungi, polyphasic, taxonomy, numerical, composite dataset

Introduction

For the elucidation of taxonomic problems mycologists (are) used to rely on the examination of a (more or less) limited set of characters. On the one hand, morphological and phenotypical observations are still essential for the valid description of a fungal species, whereas, on the other hand molecular data such as DNA sequences and fingerprints are about to dominate fungal systematics. However, neither approach

45 can reflect the true relationship amongst different fungal groups and multidisciplinary studies are
46 demanded. It has been discussed how mycologists could effectively combine these different data to
47 produce reliable classifications and identification regimes (Seifert et al., 2000). Due to the rapid
48 development in computer technology and software engineering and the availability of diverse datasets,
49 the prerequisites for this task are now met.

50 Because of their importance in agriculture and human health, *Fusarium* species belong to the best
51 studied group of fungal organisms. The impact of these organisms is mainly based on their ability to
52 cause a number of plant diseases and to synthesise a variety of mycotoxins which can contaminate food
53 and feed. The trichothecens are considered to be the most hazardous compounds produced by
54 *Fusarium* species. Type A trichothecens, especially T2- and HT2-toxin, are the most dangerous
55 substances regularly detected in cereal samples (Marasa et al., 1984). Recently, *F. langsethiae* Torp
56 and Nirenberg, was described as a new species that produces these two toxins in vast amounts. This
57 species resembles *F. poae* in several morphological features, but the latter species does not produce
58 type A trichothecenes. Thus *F. langsethiae* has some affinity to *F. sporotrichioides* (Torp and
59 Langseth, 1999, Torp and Nirenberg, 2002, this issue). Indeed, when toxin patterns are compared the
60 species seems to be more similar to *F. sporotrichioides* than to *F. poae* (Thrane et al., 2002, this
61 issue). Logrieco et al. (2002, this issue) discriminated *F. poae* and *F. sporotrichioides* from
62 *F. langsethiae* by their ability to produce beauvericin. Phylogenetic analyses of combined ITS, IGS and
63 partial sequences of the β−tubulin (Yli-Mattila et al., 2002, this issue) and of the elongation factor-
64 1 alpha (EF-1α) gene (Knutsen et al., 2002 this issue) as well as cluster analysis of AFLP fingerprints
65 (Schmidt et al., 2002 this issue) strongly suggest that *F. sporotrichioides* and *F. langsethiae* form a
66 group separated from *F. poae*. PCR-based methods for the rapid detection and distinction of the
67 species have been developed (Konstatinova and Yli-Mattila, 2002, this issue, Mach et al., 2002, this
68 issue, Niessen et al., 2002, this issue).

Even though lots of polyphasic studies especially in bacterial an yeast systematics exist, only a few combine multiple data to produce one consensus model of the taxonomic relationships among the organisms studied (e. g. O´Donell et al. 1998a). Therefore there is an immanent demand for polyphasic taxonomic studies (Petrini and Petrini, 1996, Seifert et al., 2000).

Here we present a collaborative study aimed at the clarification of the taxonomic position of *F. langsethiae* within the *Fusarium* section *Sportrichiella*. This work intends to consider all available data, such as morphological and phenotypic as well as information on the genotype like fingerprints and nucleotide sequence data. By choosing this strategy we hope to contribute to the understanding of the true connections between the different species and deliver an image of the whole fungus (Kendrick, 1979).

Material and methods

Fungal strains

Information on the examined strains can be found in table XXX in Torp and Nirenberg (2002, this issue). Strains for the different data analysis were chosen according to the availability of data.

Sequence data

The DNA sequences of the intergenetic spacer region (IGS), the internal transcribed spacer region (ITS) and partial sequences of the β−tubulin gene used are those described by Yli-Mattila et al. (2002, this issue) the partial sequences of of the elongation factor-1 alpha (EF-1α) of the examined strains are those determined by Knutsen et al. (2002, this issue). An alignment of the sequences was calculated according to the algorithms provided by the BioNumerics software. For alignment and similarity calculation no conversion costs were used and no gap penalty was assigned. The alignment was checkt

manually and the similarity between the sequences was calculated using the correction of Jukes and Cantor (1969).

Chromatographic data

Chromatographic data on secondary metabolites were obtained and processed as described by Thrane et al. (2002, this issue). The calculated similarity matrix was directly imported into the BioNumerics software.

AFLP

AFLP data were obtained and processed as described by Schmidt et al. (2002, this issue). The resemblance between the fingerprints was calculated using the Pearson correlation (Pearson, 1926).

Phenotypical data

Morphological and phenotypical data were recorded as described by Torp and Nirenberg (2002, this issue) and coded in a binary character table (0;1). The following characters were used: 1. macroconidia present, 2. microconidial shape globose/subglobose/pyriform only, 3. only monophialides present, 4. chlamydospores present, 5. powdery appearance on Czapek-dox iprodione dichloran agar (CZID), 6. colony diameter on potato sucrose agar (PSA) after 6 days exceeding 85 mm, 7. height of aerial mycelium on PSA exceeding 3 mm, 8. fruity odour present in culture. The resemblance was determined using the Dice coefficient (Dice, 1945).

Composite dataset, congruence of characters and software

The consensus matrix was calculated using the values from the similarities of the individual datasets. No weights were assigned to the individual experiments. To visualise the obtained consensus matrix a

dendrogram was calculated by the unweighted pair-group method using arithmetic averages (UPGMA, Sokal and Michener, 1958). From the same matrix a non-metric multidiminsional scaling (MDS, Shepard 1962) was computed. To check the robustness of the dendrogram and groups the cophenetic correlation and error flags for each node of the dendrogram (standard deviation) were determined. Pairwise resemblance between the data matrices was computed using the Pearson correlation (Pearson, 1926). All calculations were carried out on complete datasets using the BioNumerics Version 2.50 software (Applied Maths, Sint-Martens-Latems, Belgium).

Results

According to the availability of data, a set of strains was chosen for which all data of DNA sequences of the intergenetic spacer region (IGS), the internal transcribed spacer region (ITS), partial sequences of the elongation factor-1 alpha (EF-1α) and the β–tubulin gene as well as AFLPs, chromatographic and morphological data were available. From these data a consensus matrix was calculated and transformed into an UPGMA dendrogram (fig. 1). This dendrogram showed a clear separation of the three *Fusarium* species. The main groups of the phenogram were well supported by a rather high cophenetic correlation. However, the standard deviations, which indicate the homogeneity of a group, were quite high at the basal nodes. But the none-overlapping of the error flags at the basal parental nodes with those of the daughter nodes of neither of the three main groups supported the three main clusters. It should be stressed that the *F. langsethiae* strain IBT 9959 formed a rather separate branch within the *F. langsethiae* cluster. From the analysis of this data, it was visible that *F. sporotichioides* was the most homogenous group of the three taxa.

Calculation of the congruence of the experiments is represented in fig. 2. From this paire wise comparison of the similarity matrices resulting from the data of two experiments it can be recognised that

the highest concordance of two experiments was found between the similarity matrices derived from the comparison of the partial EF-1-α and the β-tubulin sequences. The lowest concordance is to be found when ITS and phenotypically derived similarity matrices are compared. It is interesting to note that the highest consilience of the composite matrix with an individual matrix is with the one derived from the AFLP experiments. Though the similarity values between the phenotype-derived matrix and the rest of the experiments are rather low, the highest similarity value is found with the composite data.

As the set of strains for which a complete dataset was available turned out to be rather limited, the partial gene sequences of the elongation factor-1 α was omitted from the calculations. The intention was to have a higher number of samples as the information implemented in this sequence seemed to be rather redundant compared with the β-tubulin genes. This new set of organisms consisted of 30 strains of *Fusarium poae*, *F. langsethiae* and *F. sporotichioides*. From the new calculated datamatrix, a new UPGMA dendrogram was computed. Basically, the same statements concerning the grouping of the strains could be concluded from these data as from the combined dataset including the EF-1α sequences. The three main clusters were well separated and showed a high consitency. The branching pattern found with the increased number of datasets was at least at the very basic nodes somehow doutful. The standard deviation within the clusters was rather high and the error flags overlap at the basal nodes.

The multidimensional scaling (MDS), a nonhierachical grouping technique calculated from composite similarity matrix of the selected strains is shown in fig. 4. This ordination method allows to interpret the similarity data leading to the separation of a group as well as the homogeneity of the group. Figure 4 clearly shows the separation of the three main groups corresponding to the three *Fusarium* species studied. However, within the *F. langsethiae* group strain IBT 9959 was clearly separated from all other *F. langsethiae* strains.

Comparison of the congruence of the individual experiments is displayed in fig. 5. The highest

159 concordance of two individual experiments could be found between IGS- and partial β-tubulin gene
160 sequence comparison. On the other hand the lowest value was discovered between the phenotypical
161 data and the ITS sequence derived similarities. The highest resemblance between the composite data
162 matrix and an individual experiment is achieved again with AFLP.

163 Discussion

164 Independently of the of choice of datasets and algorithms the three species *Fusarium poae*,
165 *F. sporotrichioides* and *F. langsethiae* could clearly separated by the compination of different
166 datasets. These groups are well supported by statistical means. All data have been analysed using
167 algorithms that measure the overall similarity between the individual strains and these matrices were
168 transformed into graphs. The reason for the choice of this approach is to be found in the different kinds
169 and formats of the available data. Neither the AFLPs nor the chromatographic data could be processed
170 with purely phylogenetic algorithms as no distinct characters and character states (e. g. band matching
171 table) were available.
172 The MDS calculated shows the three species as distinct clouds in spaces with the exception of strain
173 IBT 9959 which is an outlier from the *Fusarium langsethiae*-group. The exceptional position of strain
174 IBT 9959 was found by other workers, too(Yli-Mattila et al., 2002, Knutsen et al., 2002). This can
175 also be seen in the calculated UPGMA from the combined datasets. In fig. 1 and 3 this strain also forms
176 a rather separate branch within the *Fusarium langsethiae* cluster. Generally, the UPGMA algorithm is
177 not suited for creating reliable phylogenies unless all characters are ultrametric and evolve at the same
178 speed (Hills, et al., 1996). This cannot be assumed for the data analysed. A phenetic approach may be
179 an indicator of cladistic relationship, but it is not necessarily congruent with the latter. However, in most
180 cases the classifications are coincident and it is stressed that neither approach may truly reflect natural
181 classification. (Sneath and Sokal, 1973). The differences in the branching patterns in fig. 1 and 4 can be

attributed to the different weights the individual experiments have. In fig. 1 morphological and ITS sequence derived similarities contribute relatively less to the overall similarity compared to fig. 3. In each composite dataset the data matrices of the latter two experiments seem to be the most inhomogeous compared to the others. This results in the different branching patterns observed. IGS, partial EF-1-α and the β-tubulin sequence-derived similarities as well as chromatographic and AFLP-derived similarity matrices are comparably consistent. ITS and morphological derived similarity matrices form rather distinct branches. The value of different DNA sequences and DNA-fingerprinting methods for *Fusarium* taxonomy has been been extensively discussed (Yli-Mattila et al., 2002, Knutsen et al., 2002). Also the usefulness of chemotyping for the identification and classification of fungal species has been reported (O´Donell et al. 1998a, Thrane et al., 2002 this issue). While ITS sequence comparison has proven to be rather helpful for taxonomical purposes in many fungal species (Burns et al., 1991) this approach seems to be rather unsuited for the genus *Fusarium* (O´Donell et al., 1998b) even though Tapani et al.(2002) discusses the concordance of the ITS derived gene trees with that from other sequences. The difficulties in choosing and coding morphological data to create numerical classification systems is immanent and requires much expertise. Analyses of the similarity matrix of the combined data with that obtained from morphological inspection show that the coding and grouping used is helpful for the morphological recognition of the three species. Apart from the microscopic observation the described characters, molecular methods for the early detection the of *Fusarium langsethiae* are available faciliating the recognition of the species (Mach et al., 2002; Konstatinova and Yli-Mattila, 2002, this issue)

Phylogenetic analysis of nucleotide sequences found *Fusarium langsethiae* to be a sister group of *F. sprotrichioides* (Yli-Mattila et al., 2002, Knutsen et al., 2002). The results from the combined datasets lead to similar findings. In fig 1 the account congruent phylogenetic markers is relatively higher

than in figure 3. As a consequence the dendrogram shown in figure 1 should represent a better picture of the natural relationships than that in figure 3.

While EF-1α sequences as well as tubulin sequence analysis generally resolve higher taxonomic levels, AFLP is a method for distinguishing fungal species down to the level of clones (Baayen et al., 2000). The high concordnace of the overall similarities of the data with AFLP suggests *F. langsethiae* to be a sister species closely related to *F. sporotrichioides*.

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Legends

Figure 1:

UPGMA dendrogram calculated from the combined similarity matrix of IGS, ITS as well as partial DNA sequences of the EF-1-α and β-tubulin genes, chromatographic data, AFLPs and phenotypical data. Numbers at the nodes give the cophenetic correlation. Bars indicate the standard deviation for the corresponding cluster. The scale at the top indicates the percentage of similarity.

Figure 2:

Congruence between the experiments leading to the clustering in fig. 1. The similarity matrix derived from the pairwise comparison of the individual experiments (right side of the figure) was transformed to the UPGMA dendrogram at the left side. AFLP: data derived from the AFLP experiments, all: composite dataset, chrom: chromatographic data, IGS: DNA sequences of the IGS region, ITS: DNA sequences of the ITS region, pheno: similarity data derived from the coding of phenotypical data, tef: partial DNA sequences of the EF-1α-gene, tub: partial DNA sequences of the EF-1α-gene.

Figure: 3

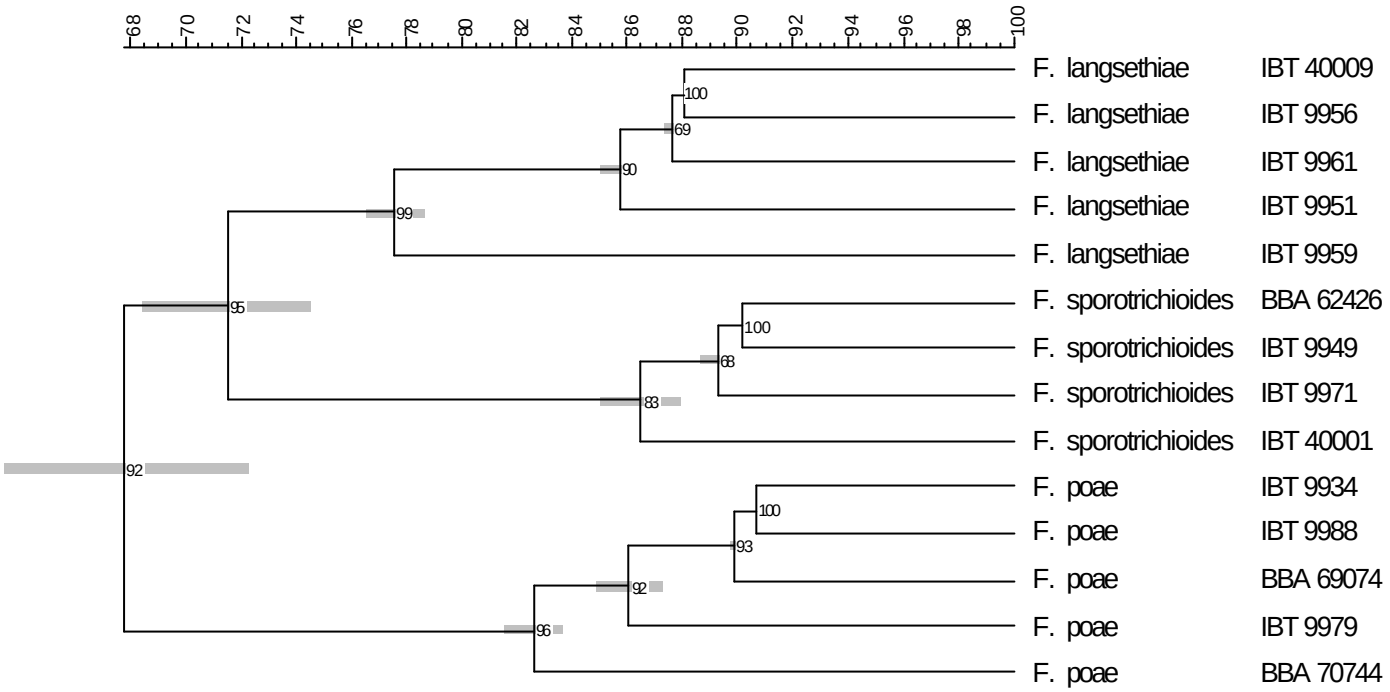
UPGMA dendrogram calculated from the combined similarity matrix of IGS, ITS as well as partial β-tubulin DNA sequences, chromatographic data, AFLPs and phenotypical data. Numbers at the nodes give the cophenetic correlation and the bars indicate the standard deviation for the corresponding cluster. The scale at the top indicats the percentage of similarity.

Figure 4:

Two views of the multideminsional scaling (MDS) calculated from the similarity matrix leading to the dendrogram in fig. 3. ●: *F. poae*-strains, ★: *F. sporotrichiodes*-strains, ◆: *F. langsethiae*-strains. The circle indicates *F. langsethiaea*-strain IBT 9959.

Figure 5:

Congruence between experiments leading to the clustering in fig. 3 and the MDS in fig 4. Structure of figure and abbriviations are in analogy to fig. 2.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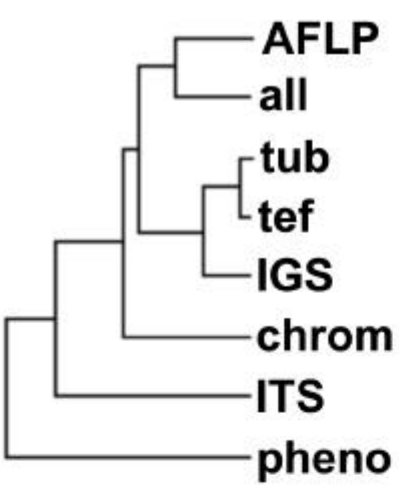

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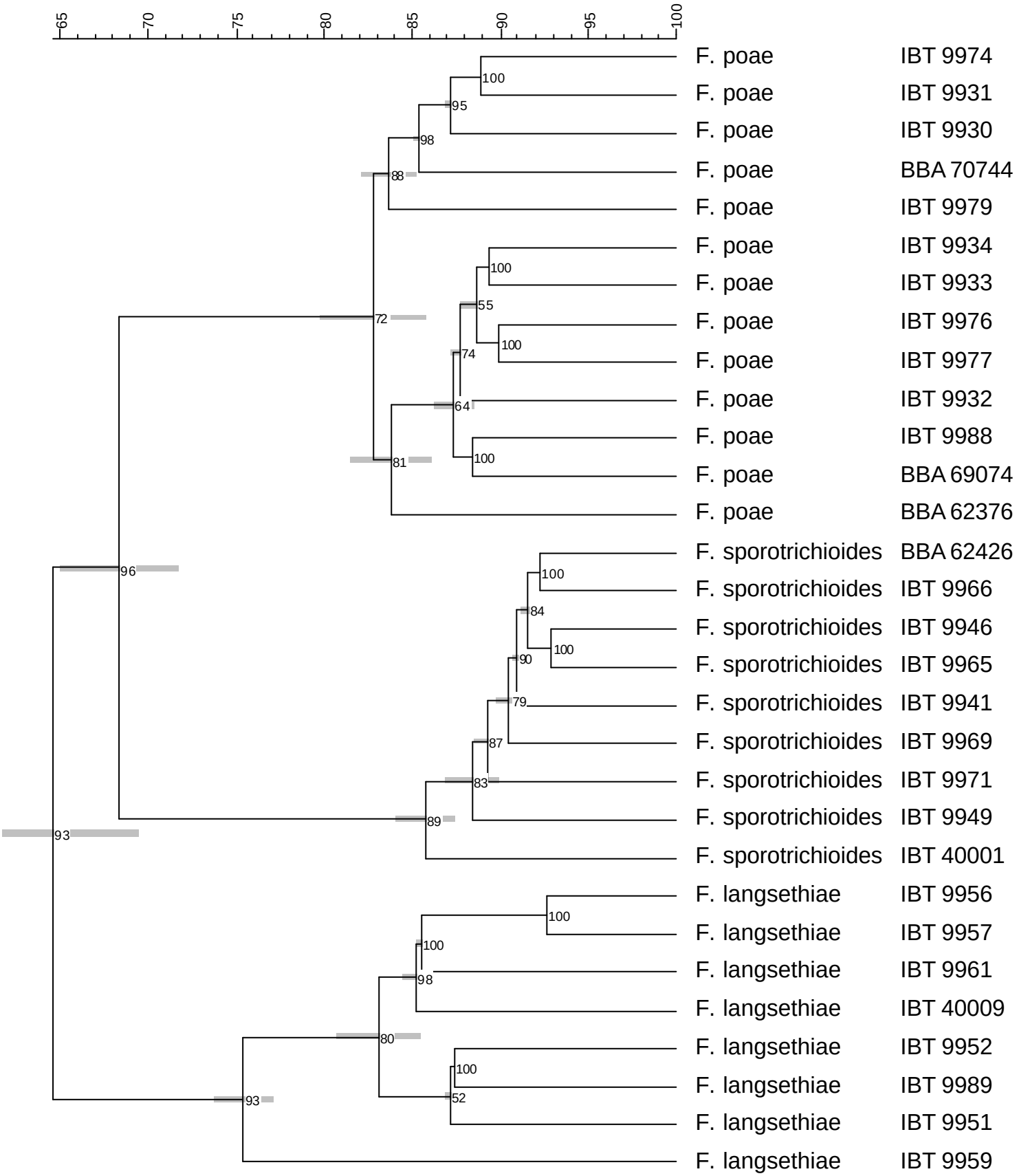
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F. poae IBT 9934
F. poae IBT 9933
F. poae IBT 9976
F. poae IBT 9977
F. poae IBT 9932
F. poae IBT 9988
F. poae BBA 69074
F. poae BBA 62376
F. sporotrichioides BBA 62426
F. sporotrichioides IBT 9966
F. sporotrichioides IBT 9946
F. sporotrichioides IBT 9965
F. sporotrichioides IBT 9941
F. sporotrichioides IBT 9969
F. sporotrichioides IBT 9971
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F. sporotrichioides IBT 40001
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F. langsethiae IBT 9957
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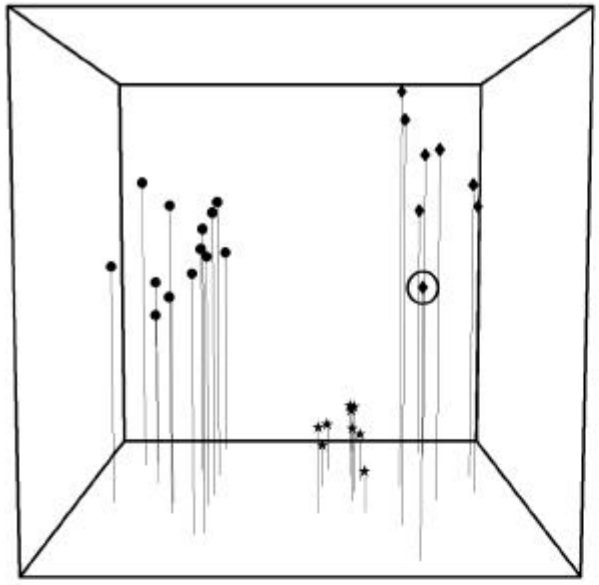

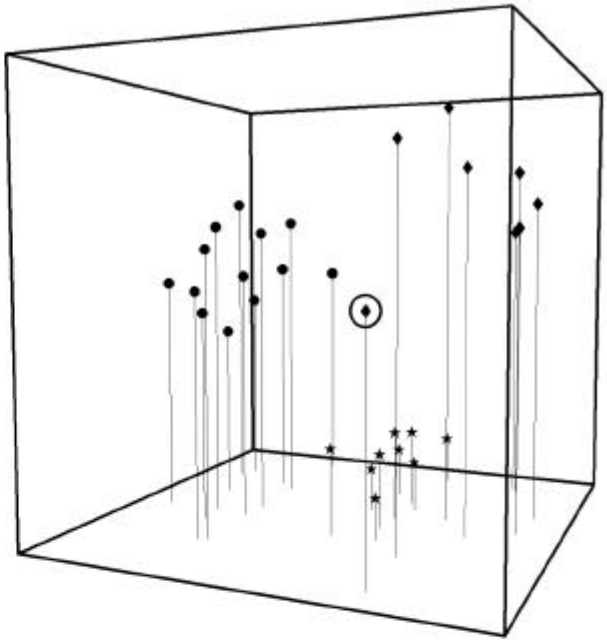

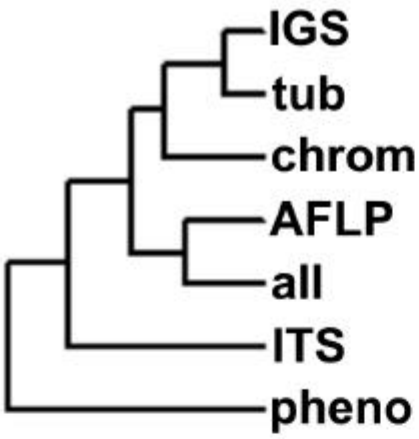

100						
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68.3	62.5	55.6	74.1	100		
43.2	54.5	38.2	34.0	13.8	100	
13.7	16.3	8.2	0.9	56.7	0.0	100

PCR based characterisation, distinction and identification of *Aspergillus niger* and *Aspergillus carbonarius*

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Running Titel: PCR based identification of *A. carbonarius*

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Summary

Aims: The objective of this work was to test whether ochratoxin A production of *Aspergillus niger* and *A. carbonarius* is linked to a certain genotype and to identify marker sequences useful for the identification of *A. carbonarius*, a fungus of major concern regarding ochratoxin A production.

Methods and Results: *Aspergillus niger* and *A. carbonarius* were isolated mainly from Brazilian coffee related sources and their ability to produce ochratoxin A was tested by TLC. The strains were genetically characterized by AFLP and compared with each other and with reference strains. Cluster analysis of fingerprints showed clear separation of *A. niger* from *A. carbonarius* strains. To obtain marker sequences, AFLP fragments of were separated on polyacrylamide gels, silver stained, cloned and sequenced. The sequences obtained were used to develop species specific SCAR-PCR primers for the identification of *A. carbonarius*.

Conclusions: No clear correlation between the genetic similarity of the strains studied and their potential to produce ochratoxin A could be found. Primers designed from AFLP markers are a useful and specific tool for the identification of *A. carbonarius.*

Significance and Impact of Study: The developed PCRs allow the reliable identification of *A. carbonarius*, the fungus considered to be one of the major causative agents for ochratoxin A in coffee and grape derived products and these PCRs may serve as a basis for a culture independent detection system.

Keywords: ochratoxin A, AFLP, DNA, detection, fungi, mycotoxins

Introduction

Black aspergilli, i. e. the section *Nigri* within the *Aspergillus* subgenus *Circumdati* Gams *et al.* (1985*)* are of immanent importance for the biotechnological industry. Especially *Aspergillus niger* is exploited in production of enzymes, biotransformations of organic compounds. Moreover this organism is used since the early 20s of the last century for the production of citric, gluconic and fumaric acid (Deacon 1997, Archer 1999). However, the black aspergilli are hard to identify and the classification of these organisms is still matter of intense research and debate especially since the production of ochratoxin A has been reported for strains of *Aspergillus niger* Arbaca *et al.* (1994), which is generally regarded as safe (GRAS). A recent review by Schuster *et al*.(2002) summarized the accepted species within this group. Though Yakoama *et al*. (2001) consider *A. japonicus* and *A. aculeatus* as identical based on cytochrome c gene sequences, several other authors (Parenicová *et al.* 2001, Parenicová *et al.* 2001, Varga *et al,* 2000) agree that the section comprises *Aspergillus aculeatus*, *A. carbonarius*, *A. ellipticus*, *A. heteromorphus, A. japonicus* and *A. niger*. The latter species also contains the well known morphospecies *A. foetidus* and *A. citricus* which are by some authors considered to be separate species, but are hard to distinguish by molecular methods (Parenicová *et al.* 2001, Varga *et al.,* 2000). On the other hand, the name "*A. tubingense*" has been assigned to a not yet validly described group of strains being morphological similar to *A. niger* (Varga *et al.,* 2000, Pitt *et al.* 2000). In addition to the genetic variation, none of the strains belonging to "*A. tubingense*" was able to produce ochratoxin A, while all strains producing ochratoxin A were found to be *A. niger sensu stricto* (Varga *et al.,* 2000, Accensi *et al.* 2001). However, only a small percentage of *Aspergillus niger* strains (3 %) were described to produce ochratoxin A under laboratory conditions (Taniwaki *et al.* 2003, Bucheli and Taniwaki 2002). In contrast, the incidence of ochratoxin A producing isolates among strains of the closely related species *A. carbonarius* is quite high

(77 %) (Taniwaki *et al.* 2003, Bucheli and Taniwaki 2002). This species is frequently encountered in food commodities in tropical regions. It has been isolated from green coffee and is thus believed to contribute to a significant part to the ochratoxin A load of this commodity. In wine and other grape derived products, this organism is considered to be the main causative agent for this mycotoxin (Cabañes *et al*. 2002, Torp *et al.* 2002). Ochratoxin A is of major concern for food safety and human health since it may cause renal tumours and is immunosupressive. In addition, ochratoxin A has genotoxic and teratogenic effects as it can cross the placenta (WHO, 2002). For these reasons the prevention of ochratoxin A from entering the food chain is of immanent importance and the European Commission has recently set up limits for the toxin in certain feeds and foods (European Comission, 2002). This indicates the requirement for a rapid and reliable identification and detection of this fungus in raw materials used in food production. Culture dependent identification and isolation requires much time and expertise and is rather labour intensive. Culture independent systems for the detection of mycotoxigenic fungi based on PCR have been published and proven to be a reliable tool for the quick detection of the organism of interest (Edwards *et al.* 2002) .

The aim of this study was to check whether ochratoxin A production could be linked to a certain genotype of either *Aspergillus niger* or *Aspergillus carbonarius* using AFLP, a technique which can resolve organisms down to the level of clones (Chulze *et al.* 2000). Furthermore, AFLP can serve as a basis for the detection of DNA markers for a certain population of organisms (Behura *et al.* 2000) and provide a starting point for the design of SCAR primers which can be used for a PCR based identification or detection system. PCR based systems valuable for the screening and quantification of toxigenic fungi have been described recently (Edwards *et al.* 2002). Methods using genes from the mycotoxin biosynthetic pathways as target sequences in combination with real-time PCR turned out to a valuable tool for studying the quantity of toxins or toxigenic fungi themselves. Schnerr *et al*.

(2002) correlated DNA quantity with the concentration of the trichothecene deoxynivalenol (DON) in wheat and Mayer *et al*.(2002) described a real-time PCR based method for the determination of *Aspergillus flavus* in food.

Materials and methods

Isolation of strains and determination of ochratoxin A production.

Isolation of strains and determination of ochratoxin A production was carried out as described by Taniwaki *et al*.(2003) Briefly, strains were isolated from surface disinfected coffee cherries or beans (by treatment with 0.4% chlorine solution for 1 min) which were plated on Dichloran 18% Glycerol agar (DG18, Samson *et al.* 2000) and incubated for 5 to 7 days at 25°C. Isolates were identified according to Klich and Pitt (1988). In order to test strains for their ability to produce ochratoxin A isolates were grown on yeast extract 15% sucrose agar (YES, Samson *et al.* 2000) at 25ºC for 7 days. Analysis for ochratoxin A was performed by the agar plug technique (Hocking and Pitt, 1997). Isolates which were found to be non-producers by this technique were checked again. For this purpose colony and medium from a whole Petri dish was extracted wit 50 ml chloroform. The extracts were evaporated to dryness and the residues resuspended in cholroform and spotted on TLC plates. Plates were developed in toluene/ethyl acetate/formic acid (5:4:1) and visualized under UV light (365 nm). Isolation of the *A. carbonarius* strains M 323, M 324, M 325, M 333, M 334, M 335, CBS 110.49 and CBS 127.49 was performed by Joosten *et al.* (2001) and these strains were kindly provided by H. Joosten. Data on the strains are summarized in table 1.

DNA isolation, AFLP and computing

After cultivation of the strains for 12 to 14 days in malt extract broth (20 g malt extract, 1 g peptone, 20 g glucose, dest. water ad 1000 ml, pH. 5.6) mycelia were harvested by centrifugation, washed with distilled water and freeze-dried. DNA was prepared according to

the CTAB protocol by Möller *et al*.(1992). AFLP was carried out as described by Vos *et al*. (1995), and modified by Aarts and Keijer (1999). For template preparation all reaction mixtures contained 10 mM Tris-HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT and 50 ng/µl BSA. Two hundred nanograms of isolated genomic DNA were digested in a total volume of 15 µl at 37°C for 3 h with 2U of EcoRI (TaKaRa Shuzo Co., Ltd., Japan) and 2U Mse I (New England Biolabs, Frankfurt, Germany). Following digestion 5 µl of a solution containing 2 pMol EcoRI adaptor, 20 pMol MseI adaptor, 0.4U T4 DNA ligase (MBI Fermentas, St. Leon-Rot, France) and 1 mM ATP was added. Ligation was performed for 12 h at room temperature in a total volume of 20 µl. This mixture was diluted 1:10 with 10 mM Tris-HCl pH 8.0. Five microliters of this dilution were used as template for the pre-amplification. Primers used in this PCR reaction were those described by Vos *et al*. (1995) and had a total of two selective bases (indicated by bold letters). The pre-EcoRI-primer used had the sequence 5´-GAC TGC GTA CCA ATT C**A**-3'and the pre-MseI primer 5'-GAT GAG TCC TGA GTA A**C** -3´. The selective PCR was carried out using a total of 4 selective bases (E-AT/M-CT). The EcoRI primer was Cy5-labelled to allow analysis of DNA-fragments on an ALFexpress automated DNA sequencer (Amersham Pharmacia, Freiburg, Germany). After the second PCR 8.5 µl sample buffer (5 mg dextrane blue in 1 ml formamide) were added, the samples denatured and 5 µl of the mixture were loaded onto a 0.3 mm denaturing polyacryl amide gel. The ALF-files were converted into TIFF-images and imported into the BioNumerics Version 3.0 software package (Applied Maths, Sint-Martens-Latem, Belgium). After normalisation the similarity between the fingerprints was calculated using the Pearson correlation (Pearson, 1926). For this purpose fragments between 70 and 650 bp were considered. The dendrogram was constructed using the UPGMA algorithm (Sokal and Michner 1958).

Silver staining of AFLP fragments for marker detection

For silver-staining AFLP was modified as described by Chaloub *et al*. (1997).The selective PCR was carried out in a volume of 40 µl with a total of four selective bases using both the MseI primer and the EcoRI primer in equal concentrations. PCR products were evaporated until dryness and resuspended in 5 µl distilled water. Electrophoresis was performed with a Multiphor II electrophoresis system (Amersham Pharmacia, Freiburg, Germany) using a CleanGel Long-10 polyacryl amide gel in combination with the DNA LongRun Buffer (ETC-Elektrophorese-Technik, Kirchentellinsfurt, Germany). One microliter of sample buffer was added to the concentrated PCR product and loaded onto the gel. Electrophoresis was carried out as recomended by the manufacturer. Silver staining was performed according to Bassam *et al.* (1991).

DNA recovery, cloning and sequencing

DNA was eluted from the bands cut out of the silver stained polyacryl amide gel with 20 µl TE buffer. Ten microliters of the eluate were used for re-amplification. The composition of the PCR reaction was similar to that used for pre-amplification, but the total volume was 40 µl. The temperature profile was as follows: initial denaturation at 94°C (60 s), 40 cycles of 94°C (30s), 56°C (60s), 72°C (60s), followed by final elongation for 3 min at 72°C. The whole PCR reaction was loaded on an 1,5% agarose gel, elctrophorised and stained with ethidium bromide. Fragments were visualised under UV and cut out from the gel. DNA was extracted from the agarose gel with the E.Z.N.A.® Gel Extraction Kit (PEQLAB, Erlangen, Germany) according to the manufacturer's instructions. Seven microliters of the extracted DNA were used for the ligation of the product into the pSTBlue-1 vector (Novagene, Darmstadt, Germany) which was carried out in a total volume of 10 µl according to the manufacture´s instruction. The plasmids were transformed into *E. coli* strain DH5α using the heat shock method (Sambrock *et al.* 1989). After transfer, the clones were checked for the

presence of the insert by colony PCR. Plasmids were prepared using the Plasmid Miniprep Kit 1 (PEQLAB, Erlangen, Germany). Sequencing of the inserts was done with the ABI Prism Dye Terminator Cycle Sequencing Kit (Perkin Elmer, Boston, USA) on ABI 373 stretch sequencing system by a commercial service (SequiServe, Vaterstetten, Germany).

Primer construction, PCR optimisation and test for specifity

The primers were designed based on the obtained sequences and tested for optimal PCR conditions. Primers were tested for specificity against a set of fungal strains. PCRs were carried out in a total volume of 25 µl using a standard PCR buffer (Roche Diagnostics, Penzberg, Germany) and 1.25 U Taq Polymerase (Promerga, Mannheim, Germany) in an Mastercycler gradient (Eppendorf, Hamburg, Germany) thermocycler. For cycling conditions and primer details see table 2.

Results

The AFLP applied for automatic laser fluorescence analysis (ALFA) resulted in a sufficiently complex, yet clearly resolved banding pattern of approximately 20 bands within the range of 70 to 650 bp. This fingerprint turned out to be well suited for cluster analysis (fig. 1). The dendrogram calculated from the similarity matrix obtained from the comparison of the fingerprints (fig.1) shows a clear separation of the *A. niger* / *A. carbonarius* strains from the rest of the fungal analysed. The *A. carbonarius* strains formed a well separated group in which the three non-toxigenic isolates studied were rather dispersed. Interestingly, the CBS strain of *Aspergillus niger var. niger* clustered close to the *A. carbonarius* strains while all other strains belonging to the *A. niger* aggregate like *A. awamorii*, *A. usamii* var. *shiro-usamii*, and *A. foetidus*, formed a separate unit, which was less homogenous when compared with the *A. carbonarius* strains. *A. niger* strains ITAL 638 and ITAL 630 were segregated from the rest of the *A. niger* strains. *A. fotidus* CBS 114.49 and *A. niger* CECT 2090 apparently did not belong to the *A. niger* "core group", whereas *A. awamorii* CBS 101704 and

188 *A. usamii* var. *shiro-usamii* CBS 101700 were close to the genotype of the majority of the *A.*
189 *niger* strains examined. As for *A. carbonarius*, the toxigenic and non-toxigenic members of
190 this group were rather scattered within this part of the cluster.
191 Based on these findings it was decided to look for a species specific marker for *Aspergillus*
192 *carbonarius* since the incident of ochratoxin A producing strains was much higher than in
193 *Aspergillus niger.* In order to identify marker fragments AFLP were separated on polyacryl
194 amide gels and subsequently silver stained. Figure 2 shows an example of these AFLP
195 derived from different *Aspergillus* spp.. Several fragments characteristic for *A. carbonarius*
196 strains were detected. The bands considered to be species specific were cut out from the gel,
197 and after elution of DNA reamplified with the AFLP primers. Two of these DNA fragments,
198 assigned A and C, were cloned and subsequently sequenced. The obtained sequences were
199 searched against Genebank (http://ncbi.nlm.nih.gov/BLAST) and submitted to the EMBL
200 nucleotide database (http://ebi.ac.uk/embl) accession numbers AJ516957 and AJ516956,
201 respectively. Comparison of the sequence of fragment A resulted in no significant hits while
202 the sequence of fragment C showed high similarity to a protein of unknown function “related
203 to ahmp1” in *Neurospora crassa* (EMBL accession AL355928.2) using the blastx algorithm
204 (Altschul *et al.* 1997). Based on these sequences, two primer pairs (A1B_fw/ A1B_rv and
205 C1B_fw/ C1B_rv) were cdesigned. PCR was optimised and primers were tested for
206 specificity against a panel of 20 fungal strains belonging to 17 different *Aspergillus* and
207 *Penicillium* (figure 3). PCR conditions and primer sequences are summarized in table 2. Both
208 primer pairs specifically yielded products of 189 bp and 351 bp with DNA from *A.*
209 *carbonarius* strains and *A. niger var. niger* CBS 101697 using primers A1B_fw/ A1B_rv and
210 C1B_fw/ C1B_rv, respectively (figure 2).

Discussion

The taxonomy of black aspergilli has been studied extensively using phenotypical and genotypical data (Varga *et al*. 1993, Parenicová *et al.* 1997, Hamari *et al.* 1999, Parenicová *et al.* 2000, Varga *et al.,* 2000, Parenicová *et al.* 2001, Schuster *et al.* 2002). Recently, Varga *et al*. (2000) divided the *A. niger* aggregate into two groups, *A. niger* and "*A. tubingense*" regarding *A. foetidus* as a synonym to *A. niger* which comprises also well known names such as as *A. usami* and *A. awamorii*. Parenicová *et al.*(2000) agreed with the separation of the morphologically similar "*A. tubingens* " from *A. niger*, but separated *A. foetidus* from the other taxa. Within these taxa, ochratoxin A production has only been described for *A. niger* while "*A. tubingense*" and *A. foetidus* are not believed to produce ochratoxin A (Accensi *et al.* 2001). The current AFLP analysis of the *A. niger* strains isolated from coffee related sources indicate a close relationship of the strains with ochratoxin A producing isolates being dispersed throughout the whole cluster of *A. niger* strains. Although strains ITAL 638 and ITAL 630 are somehow separated from the "core group" of the rest of the *Aspergillus niger* cluster the ability of ITAL 630 to synthesise ochratoxin A suggests that this two isolates also belong to *A. niger*. The studied strain of *A. foetidus* is genotypically similar to the rest of the *A. niger* strains indicating that it does not belong to the *A. foetidus* genotype described by Parenicová *et al.* (1997). Clustering of *A. usamii* and *A. awamorii* within *A. niger* is in good agreement to the work of other authors (Varga *et al.* 2000) encouraging the assumption that only strains of *A. niger sensu stricto* are able to produce ochratoxin A amongst the taxa in the *A. niger* species complex. The other ochratoxinogenic taxon in the *Aspergillus* section *Nigri*, *Aspergillus carbonarius,* can be distinguished form *A. niger* by the size and the shape of its big echinulate conidia (Raper and Fenell 1965). On the molecular level this species can be straightforwardly recognized (Hamari *et* al. 1999, Parenicová *et al.* 2000, Varga *et al,* 2000). Varga et al. (2000) described a high degree of genotypic similarity among *A. carbonarius* strains with the exception of one isolate based on which they propose "*A. carbonarius var.*

237 *indicus*" as a new taxa. The *A. carbonarius* strains examined in the current study form a group
238 of high genetic similarity with toxigenic and non-toxigenic isolates distributed across the
239 cluster. The studied strain of *A. niger var. niger* groups close to *Aspergillus carbonarius.* This
240 may be either due to hybrid formation or misidentification by the depositors. The conidia of
241 this *A. niger var niger* strain strongly resembled that of *A. carbonarius* and this together with
242 the fact that the fungus produces vast amounts of ochratoxin A leads to our conclusion that it
243 should be referred to as *A. carbonarius* rather than *A. niger*. This is also supported by the
244 positive reaction of its DNA with both primer pairs A1B_fw/ A1B_rv and C1B_fw/ C1B_rv.
245 *A. carbonarius* is regarded as the main causative agent of ochratoxin A in grape derived
246 products (Accensi *et al.* 2001). It significantly contributes to the contamination of raw coffee
247 with this mycotoxin (Taniwaki *et al.* 2003). For both reasons, the early detection and
248 identification of this fungus is of major concern to food safety. The SCAR-PCR primers
249 designed during the current stud allow the reliable and quick identification of *A. carbonarius*
250 and may serve as a basis for a rapid culture independent detection system for the fungus
251 present different in food samples and raw material.

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Legends to Tables

Table 1

Strains used in this study. + and – indicates the ability of an isolate to produce ochratoxin A, n. d.: the production of the toxin was not determined.

Table 2

Primer sequences and PCR conditions used for the specific detection of *Aspergillus carbonarius.*

Legends to Figures

Fig. 1. UPGMA dendrogram calculated from the comparison of AFLP fingerprints generated with a total of four selective bases (E-AT/M-CT). Fragments between 70 and 650 bp are shown. For similarity calculation the Pearson correlation was used. + and – indicate the ability of an isolate to produce ochratoxin A, where no + or - is displayed the production of the toxin was not determined. The cophenetic correlation of the dendrogram is 0.95.

Fig. 2. Silver stained AFLP (created with the primer pair E-AC/M-CT) separated on a polyacryl amide gel. The arrows indicate representative bands characteristic for *Aspergillus carbonarius* mentioned in the text. Numbers at the right margin indicate the approximate size of the fragments. 100 bp: 100 base pair ladder.

Fig . 3.

Fig. 3a.

PCR reaction carried out with the primer pair C1B_fw/C1B_rv under the conditions summarized in table 2.

Fig. 3b

PCR reaction carried out with the primer pair C1B_fw/C1B_rv under the conditions summarized in table 2.

Notice that only when DNA isolated from *A. carbonarius* and *A. niger var. niger* CBS 101697 was used as template a 189 bp (Fig 3a) and a 351 bp (Fig 3b) product resulted. 100 bp: 100 base pair ladder, 0: no template control.

392 Tabl2 1

Species			Reference	Strain	OTA
Aspergillus	*awamorii*			CBS 101704	+
Aspergillus	*carbonarius*	Coffee	Joosten *et al.* (2001)	CBS 110.49	+
Aspergillus	*carbonarius*	Air	Joosten *et al.* (2001)	CBS 127.49	+
Aspergillus	*carbonarius*	Coffee sample from drying area, São Paulo, Brazil		CCT 6940	+
Aspergillus	*carbonarius*	Coffee sample from drying area, São Paulo, Brazil		CCT 6960	+
Aspergillus	*carbonarius*	Coffee sample from drying area, São Paulo, Brazil		CCT 6967	+
Aspergillus	*carbonarius*	Coffee sample from drying area, São Paulo, Brazil		CCT 6968	+
Aspergillus	*carbonarius*	Coffee sample from drying area, São Paulo, Brazil		CCT 6974	+
Aspergillus	*carbonarius*	Coffee sample from drying area, São Paulo, Brazil		CCT 6976	-
Aspergillus	*carbonarius*	Coffee sample from drying area, São Paulo, Brazil		CCT 6978	-
Aspergillus	*carbonarius*	Coffee sample from drying area, São Paulo, Brazil		CCT 6983	-
Aspergillus	*carbonarius*	Coffee, Thailand	Joosten *et al.* (2001)	M 323	+
Aspergillus	*carbonarius*	Coffee, Thailand	Joosten *et al.* (2001)	M 324	+
Aspergillus	*carbonarius*	Apples	Joosten *et al.* (2001)	M 325	+
Aspergillus	*carbonarius*	Coffee, Thailand	Joosten *et al.* (2001)	M 333	+
Aspergillus	*carbonarius*	Coffee, Thailand	Joosten *et al.* (2001)	M 334	+
Aspergillus	*carbonarius*	Coffee, Thailand	Joosten *et al.* (2001)	M 335	+
Aspergillus	*carbonarius*	Coffee, Thailand	Joosten *et al.* (2001)	M 336	+
Aspergillus	*ellipticus*			CBS 707.79	n. d.
Aspergillus	*foetidus*			CBS 114.49	n. d.
Aspergillus	*fumigatus*			CBS 113.55	n. d.
Aspergillus	*helicothrix*			CBS 677.79	n. d.
Aspergillus	*heteromorphus*			CBS 117.55	n. d.
Aspergillus	*insulicola*			CBS 382.75	n. d.
Aspergillus	*japonicus*			CBS 114.51	n. d.
Aspergillus	*niger*			CBS 101698	n. d.
Aspergillus	*niger*	Coffee sample from drying yard, Minas Gerais, Brazil		CCT 6910	-
Aspergillus	*niger*	Coffee sample from storage, Minas Gerais, Brazil		CCT 6926	-

Aspergillus	*niger*	Coffee sample from storage, São Paulo, Brazil	CCT 6962	-
Aspergillus	*niger*	Coffee sample from drying yard, São Paulo, Brazil	CCT 7009	-
Aspergillus	*niger*		CECT 2090	n. d.
Aspergillus	*niger*	Coffee raisins, São Paulo, Brazil	ITAL 630	+
Aspergillus	*niger*	Coffee raisins, São Paulo, Brazil	ITAL 637	-
Aspergillus	*niger*	Coffee raisins, São Paulo, Brazil	ITAL 638	-
Aspergillus	*niger*	Coffee raisins, São Paulo, Brazil	ITAL 639	-
Aspergillus	*niger*	Coffee raisins, São Paulo, Brazil	ITAL 642	+
Aspergillus	*niger*	Coffee raisins, São Paulo, Brazil	ITAL 643	+
Aspergillus	*niger var. niger*		CBS 101697	+
Aspergillus	*ochraceus*		CBS 589.68	n. d.
Aspergillus	*ochraceus*		CCT 6903	n. d.
Aspergillus	*sclerotiorum*		CBS 549.65	n. d.
Aspergillus	*usamii var. shiro-usamii*		CBS 101700	n. d.
Penicillium	*chrysogenum*		CBS 306.48	n. d.
Penicillium	*commune*		CBS 107.11	n. d.
Penicillium	*digitatum*		DSM 62840	n. d.
Penicillium	*italicum*		DSM 62846	n. d.
Penicillium	*nordicum*		BFE 487	n. d.
Penicillium	*purpurogenum*		CBS 283.630	n. d.
Penicillium	*verrucosum*		CBS 603.74	n. d.
Penicillium	*verrucosum var. verrucosum*		CBS 815.69	n. d.

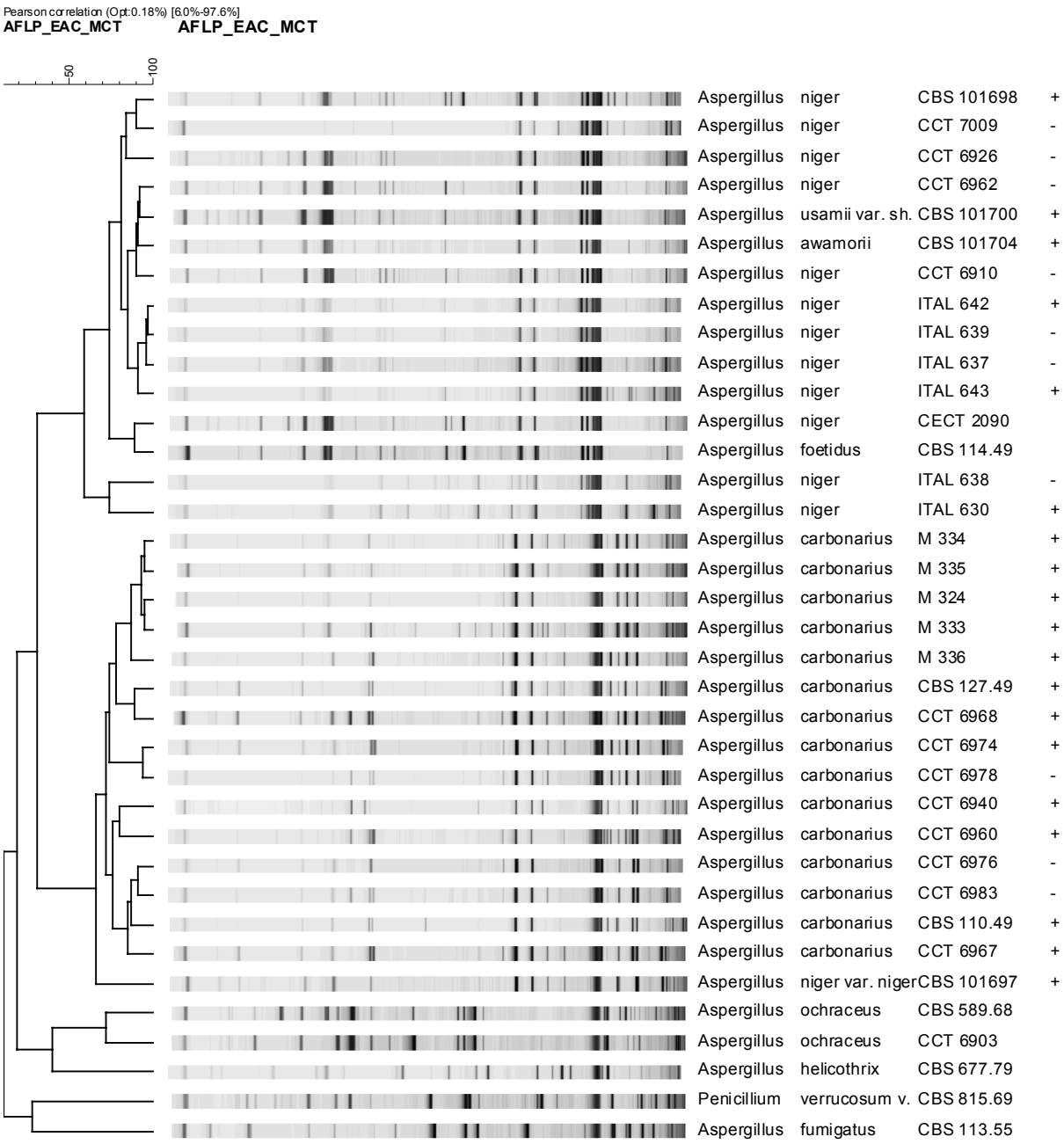
393 BFE: Bundesforschungsanstalt für Ernährung, Karlsruhe, Germany
394 CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands
395 CECT: Colección Española de Cultivos Tipo, Valencia, Spain
396 DSM: Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany
397

397 Table 2

Marker	Primers (5`to 3`)	Amplification buffer mix (10x)	Cycling conditions
A (EMBL accession number: ACA516957)	A1B_fw GAA TTC ACC ACA CAT CAT AGC A1B_rv: TTA ACT AGG ATT TGG CAT TGA AC	500 mM KCl 100 mM Tris-HCl 15 mM $MgCl_2$ pH 8.6	94 °C, 4 min initial denaturation followed by 35 cycles 94 °C, 30 s; 64 °C, 30 s; 72 °C 30 s, followed by 5 min final elongation at 72 °C. Tempterature of heated lid was set to 100 °C.
C (EMBL accession number: ACA516956)	C1B_fw GAA TTC ACG GTG CTC GAC CC C1B_rv TTA ACT GCT GGC GGA AGA GGC	500 mM KCl 100 mM Tris-HCl 15 mM $MgCl_2$ pH 8.6	94 °C, 4 min initial denaturation followed by 35 cycles 94 °C, 30 s; 64 °C, 60 s; 72 °C 60 s, followed by 5 min final elongation at 72 °C. Tempterature of heated lid was set to 100 °C.

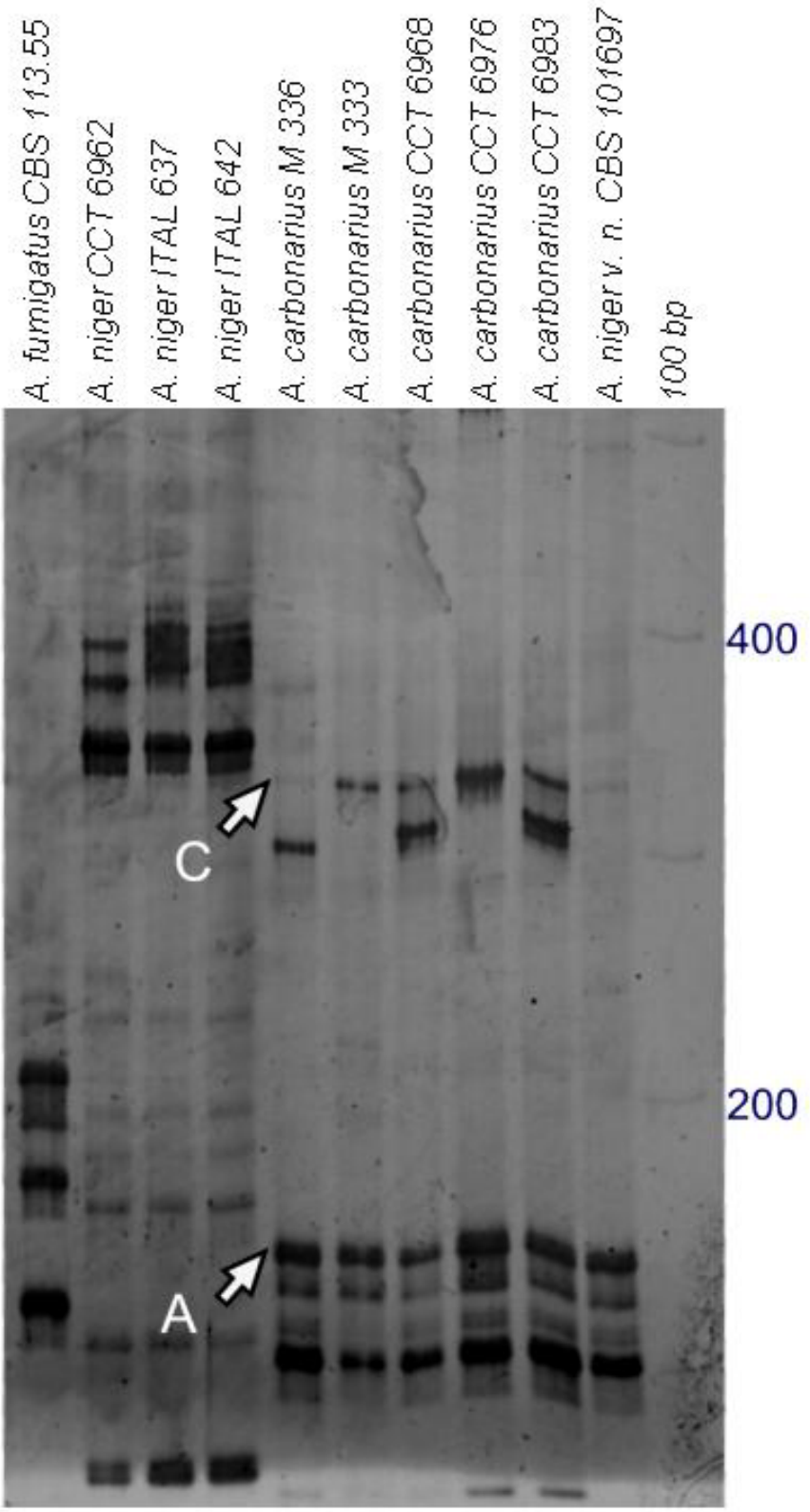
398

398 Figure 1



399
400

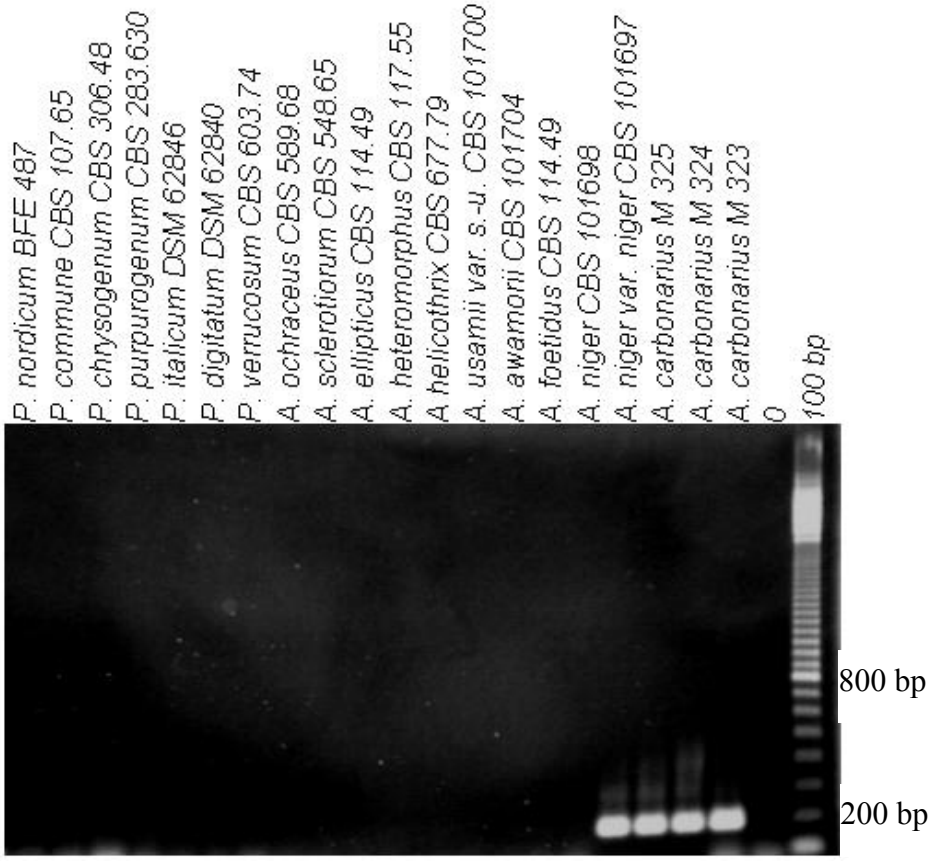
400 Figure 2



401

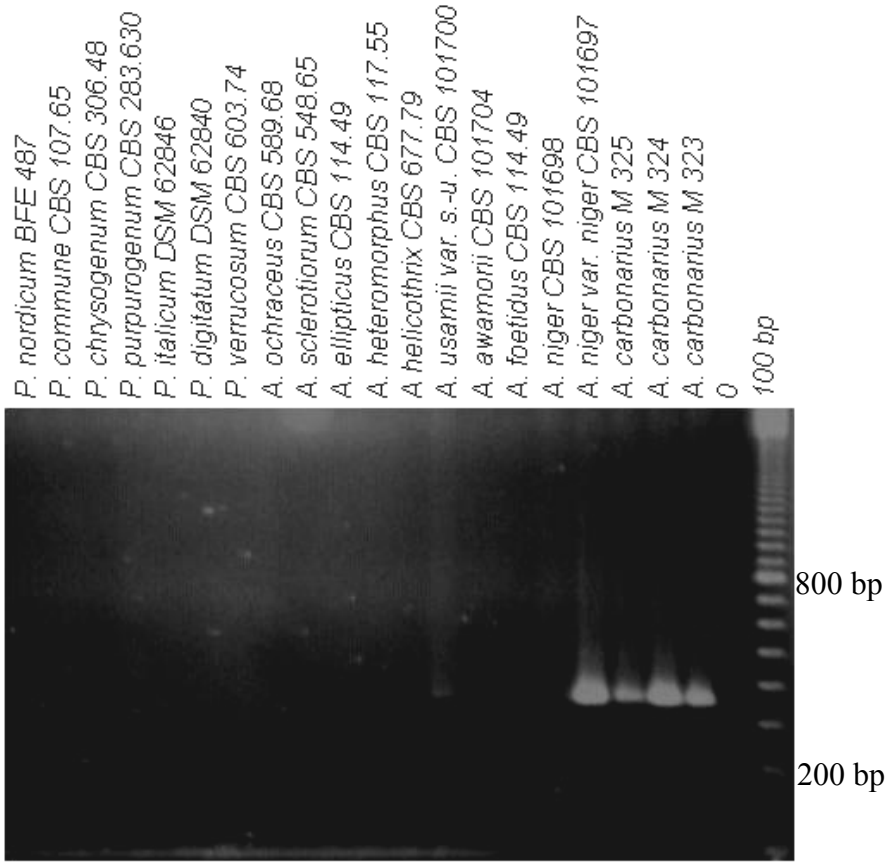
402

402 Figure 3a



403

404 Figure 3b



405

Detection and quantification of *Aspergillus ochraceus* in green coffee by PCR

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Summary

Aims: The aim of this study was to the detect and quantify DNA of the ochratoxinogenic fungus *Aspergillus ochraceus* in green coffee and to compare the results with the ochratoxin A content of naturally contaminated samples.

Methods and Results: A DNA extraction protocol based on a combination of ultrasonification and a commercial kit was tested for the recovery of fungal DNA. PCR and Real-Time PCR protocols were established for the detection of *A. ochraceus*. Sensitivity of the PCR was checked by the addition of inoculated green coffee and pure fungal DNA to uncontaminated green coffee samples. The *Aspergillus ochraceus*-DNA content of 30 naturally contaminated green coffee samples determined and compared to the ochratoxin A concentrations.

Conclusions: *A. ochraceus* can be rapidly and specifically detected in green coffee by PCR. A positive correlation between the ochratoxin A content and the DNA quantity was established.

Significance and Impact of the Study: This work offers a quick alternative to the conventional mycological detection and quantification of *A.ochraceus* in green coffee.

Keywords: Real-Time PCR, detection, ochratoxin A, DNA, fungi

Introduction

Ochratoxin A is a mycotoxin produced by *Penicillium verrucosum*, *P. nordicum* (Larsen et al., 2001; Castella et al., 2002) and a number of *Aspergillus* species (Frisvad and Samson, 2000; Abarca et al., 2001). The toxin is considered to be nephrotoxic, carcinogenic and since it can cross the placenta, also embryotoxic and teratogenic (WHO, 2002). The European Comission has established limits for ochratoxin A in certain foods (European Comission, 2002). Coffee contributes to a significant level to the total daily intake of ochratoxin A. In Germany Petzinger and Weidebach (2002) assigned a percentage of 14.2-14.5 % of the total daily intake of OTA to coffee consumption. Recently, *A. sulphureus* has been suspected to play an important role in this commodity (Batista et al., 2003). However, the production of the mycotoxin is mainly assigned to the growth of *Aspergillus ochraceus*, *A.carbonarius*, *A. niger* with *A. ochraceus* being the most important species (Urbano et al., 2001; Bucheli and Taniwaki, 2002; Taniwaki et al., 2003). Good manufacturing practice and correct storage can significantly contribute to the prevention of fungal growth and thus ochratoxin A production, but quality assurance of the raw product is still a major concern in coffee industry (Taniwaki et al., 2003).

Chemical analysis of mycotoxins is a rather elaborate and requires expensive equipment for sample preparation and analysis (Scott, 2002). Classical mycological analysis on the other hand is time consuming and the correct identification and quantification of the fungus of interest requires much expertise. With the decrease of costs for equipment and reagents PCR is becoming an alternative to the culture dependent analysis of food samples. Applications of PCR for the detection of mycotoxin producers have been reviewed e. g. by Geisen (1998) and Edwards et al. (2002) With the help of a RapidCycler device in combination with DNA detection strips Knoll et al. (2002a) could detect the toxigenic *Fusarium graminearum* in cereal samples within 20 minutes using the *gaoA* gene as target sequence. Schnerr et al. (2002) used the *tri5* gene sequence

in a quantitative real-time PCR to correlate the amount of target DNA with deoxynivalenol contents in wheat samples. Mayer et al. (2003a) demonstrated the correlation of the copy number of the *nor*-1 gene and the cfu and aflatoxinogenic *Aspergillus* species. Recently, Schmidt et al. (2003) developed several different PCRs specific for the detection of pure fungal DNA of *Aspergillus ochraceus* based on SCAR. The objective of this study was to check whether PCR was applicable for the detection and quantification of the fungus in naturally contaminated green coffee and to check if the presence of template DNA correlates with the amount of ochratoxin A present in this commodity.

Materials and methods

Fungal strains, cultivation, DNA extraction and concentration measurement

For DNA preparation, fungal strains were cultivated for 12 to 14 days at ambient temperature in 15 ml sterile plastic tubes (Sarstedt, Nümbrecht, Germany) containing 3 ml ME broth (20 g malt extract, 1 g peptone, 20 g glucose, distilled water ad 1000 ml). Mycelia were harvested by centrifugation, washed twice with 5 ml sterile distilled water and freeze dried. DNA was prepared according to the CTAB protocol provided by Möller et al. (1992). DNA concentration was measured photometrically according to Sambrook et al. (1989). Reference strain were *Aspergillus ochraceus* CBS 589.68 and *A. ochraceus* KA103, kindly provided by J. Chelkowski and identified by molecular techniques.

Green coffee samples and DNA extraction from coffee beans

Green coffee samples (50 g) and data on ochratoxin A content of the samples were kindly provided by commercial laboratories and coffee companies. Ochratoxin A concentration was determined by HPLC. For extraction of DNA, 5 g of green coffee beans were ground with a

commercial coffee mill. Isolation of DNA was performed using ultrasonification and a commercial extraction kit as described by Knoll et al. (2002b) for wheat.

PCR detection of *A. ochraceus* in green coffee

Non-quantitative

Approximately 200 g of uninfected green coffee beans were autoclaved and inoculated with 10 ml spore suspension of *A. ochraceus* prepared from an overgrown malt extract agar plate. Samples were incubated for 7 days at room temperature to allow growth of the fungus and ground as described above. Uninfected ground green coffee was blended with the infected samples at different mixing ratios. DNA from these mixtures was extracted as described above. Alternatively, 5 g of ground coffee were spiked with different amounts of genomic *A. ochraceus* DNA. The PCR reaction for the detection of the fungus was carried out in a total volume of 25 µl and had the following composition: 10xPCR buffer (Roche Diagnostics, Penzberg, Germany) 2,5 µl; forward primer OCA V (5'-ATA CCA CCG GGT CTA ATG CA-3', 50 pMol/ µl,) 0,25 µl; reverse primer OCA R (5´-TGC CGA CAG ACC GAG TGG ATT-3´, 50 pMol/ µl) 0,25 µl; Taq Polymerase (Promega, Heidelberg, Germany, 5 U/µl) 0,25 µl; dNTPs (10 mM each) 0,5 µl; 1 µl of template DNA; and water to a final volume of 25 µl. PCR was performed in a Mastercycler Gradient (Eppendorf, Hamburg, Germany) with the following temperature profile. Initial denaturation at 94 °C for 2 min, 35 cylces of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec, and final elongation for 2 min at 72 °C. PCR products were separated electophoretically on a 2 % agarose gel and visualised by ethidium bromide staining (Sambrook et al., 1989).

Quantitative PCR

Quantitative real time PCR was carried out using a LightCycler (Roche Diagnostics, Penzberg Germany). The reaction mixture was prepared using the QuantiTect™ SYBR® Green PCR kit

(Qiagene, Hilden, Germany). Reaction mix composition was as follows: 10 µl 2x mastermix; 1 µl forward primer OCA V and 1 µl reverse primer OCA R (concentrations and sequences see above); 0,5 µl uracil-N-glycosylase (1 U/ µl, MBI Fermentas, St. Leon-Rot, Germany), 2 µl of template DNA and water to a final volume of 20 µl. Temperature profile in the LighCycler instrument were as follows: 2 min at 50 °C; 15 min at 95 °C; 50 cycles of 94 °C for 15 sec, 55 °C for 25 sec, 72 °C for 30 sec and 77 °C for 20 sec in which the fluorescence was measured. After the final PCR cycle melting curve analysis of the PCR products was performed by heating 40 to 94°C and continuous measurement of the fluorescence to verify the PCR product.

After calibrating the instrument with dilution series of purified *A. ochraceus* DNA of standard curve was created. Quantification was done using the ^{2nd} derivative maximum method. Statistical analysis of data was performed either with the LightCycler software version 3.5 (Roche Diagnostics, Penzberg, Germany) or Sigma Plot 8.0 (SPSS Inc.,).

Results

Detection of *Aspergillus ochraceus* in coffee samples

As a first step towards the detection of the fungus in coffee samples, coffee artificially infected with *A. ochraceus* was blended with green coffee. When this mixture was used for DNA preparation it was possible to clearly identify the fungus in the sample by the PCR described. A clearly visual band of a PCR product of the expected size (260 bp) was still at portion of 0.1 % (w/w) infected in uninfected coffee (data not shown). Neither in the uninfected control sample, nor in the no target (ntc) control a band was visible. The same was true for coffee spiked with *Aspergillus ochraceus* DNA. As shown in figure 1 the reaction resulted in one single clearly detectable PCR product of the expected size of approximately 260 bp. Down to 1 µl of a

concentration of 1.9 µg/µl per 5 g coffee could be detected. This corresponds to approximately 0.4 ng *A. ochraceus* DNA per reaction if 100 % recovery during DNA preparation is assumed.

Quantification of *A. ochraceus* DNA in coffee

When starting PCR optimization it turned out, that the annealing temperature used was appropriate for the amplification of pure fungal DNA. However, when only small amounts of template DNA were present, the melting curve analysis displayed, that primer dimers occurred. As a consequence, the fluorescence data were acquired at 77 °C to avoid false positive measurements. Figure 2 shows a melting curve analysis of the PCR products formed during the reaction with different amounts of template DNA.

The reproducibility of the system was tested by comparing the different standards used for calibration. Figure 3 shows real-time PCR kinetics of a serial dilution of *A. ochraceus* DNA used as external standards and a calibration curve calculated from these data. The sensitivity of the system was higher compared to that of the convential PCR. 4.7 pg template DNA per reaction were determined as the lower detection limit. The reproducibility was as follows. At a DNA concentration of 5950 pg/rx the crossing point was calculated to be 24.2 cycles with a standard deviation of 0.2, at 372 pg/rx 28.9 ± 0.4 cycles, at 93 pg/rx 30.8 ± 0.6 cycles and at 23 pg/rx 28.9 ± 0.5 cycles with n=5. The recovery of DNA from spiked green coffee was found to be in the average 77 % ± 38 %.

Quantification of *A. ochraceus* DNA in all 30 samples was done in triplicate. Table 1 summarizes the measured values. Plotting DNA content against the ochratoxin A content of the samples resulted in the graph shown in figure 4. The standard deviations of the determined DNA content in each sample was calculated. For the two parameters a linear regression was calculated with a coefficient of correlation r=0.55. A t-test was performed to analyse the quality of correlation found. A positive correlation between the two parameters was secure at the 99 % level of

146 significance.. The 95 % interval of confidence was calculated and 3 out of the 30 data points
147 clearly did not lie within this area.

148 Discussion

149 The use of PCR as a tool for the rapid detection of toxigenic fungi in food has been demonstrated
150 recently (Färber et al., 1997; Knoll et al., 2002a). The target sequence has immanent influence on
151 the quality an sensitivity of the PCR. ITS and anonymous DNA markers have been shown to
152 perform well in different *Fusarium* species (Schilling et al., 1996; Yoderet al., 1998). However,
153 one of the drawbacks of these markers is that the number of copies in a genome is not exactly
154 known. The use of genes involved in the biosynthesis of a mycotoxin is of course much more
155 valuable when one intents to find a correlation between DNA concentration and the amount of
156 toxin. The correlation between DNA content and mycotoxin load or the presence of toxigenic
157 fungi has been demonstrated for *Fusarium* and *Aspergillus* species (Schnerr et al., 2002; Mayer et
158 al., 2003a). Mayer et al. (2003b) also used a real time RT-PCR approach to monitor the
159 correlation of gene expression and aflatoxin biosynthesis in wheat.
160 While Edwards et al. (2002) have described the presence of a specific polyketide synthase of
161 *Aspergillus ochraceus*. However, the authors suggest that this gene is not involved involved in
162 the biosynthesis of ochratoxin A. As no gene is known in the ochratoxin biosynthetic pathway
163 and the pathway itself is rather unclear (Mantel and Chow, 2000) the detection of the
164 microorganisms capable of producing the toxin is one approach. *A. ochraceus* is reagarded as the
165 main causative agent of ochratoxin A in green coffee (e. g. Urbano et al., 2001). However, not all
166 isolates of the fungus are capable of producing the toxin under in vitro conditions and other
167 ochratoxigenic fungi have also been isolated from green coffee (Joosten et al., 2001; Batista et
168 al., 2003). Moreover, *A. ochraceus* is only one among many *Aspergillius* species that have the
169 potential to produce ochratoxin A (Frisvad and Samson, 2000, Abarca et al., 2001). One should

keep in mind these two facts when interpreting the data between the relation of DNA content of *A.ochraceus* and ochratoxin A load presented in the current study. Detection as well as quantification of fungal DNA by PCR in coffee could be performed, but the correlation of data with ochratoxin A concentrations was low, with several data points being clear outliers. However, a positive correlation between the presence of the fungus and ochratoxin A could be established. This is not surprising since occurence of moulds is the prerequisite for the production of the metabolite. One of the biases in this study may be the rather limited set of samples which were studied. Among the 30 samples only 10 had an ochratoxin A content exceeding 5 ppb. Also with classical methods it has been demonstrated that the presence of the fungus does not necessarily mean ochratoxin A contamination (e. g. Taniwaki et al., 2003). In addition to that sampling is a crucial point for mycotoxin analysis. Even in ground coffee there can be inhomogeneities in OTA content (Scott, 2002). Nevertheless, this study offers the necessary tools for a rapid detection of the presence of *Aspergillus ochraceus* and for further detailed investigations about *Aspegillus ochraceus* distribution without the need for time consuming cultivation and identification.

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251

Legend to table

Table 1: Coffee samples, their ochratoxin A load and the determined *A. ochraceus*-DNA concentration. STD is the calculated standard deviation.

Legend figures

Figure 1: Agarose gel showing the PCR reaction with DNA prepared from green coffee spiked with different amounts of purified *A. ochraceus*-DNA. Water means water control, the ng/rx means ng of DNA expected to be present with an recovery rate of 100% assumed in the reaction (=rx).

Figure 2: Melting curve analysis of different PCR resulting from different templates. ○: green coffee with no *A. ochraceus*-DNA detectable; ▲: green coffee with *A. ochraceus*-DNA detectable; □: DNA prepared from *A. ochraceus*; •••: gausian regression calculated from □.

Figure 3: Calibration of the LightCycler™ instrument. B: Regression line calculated. The regression coefficient r was calculated to be 1.00. B: Fluorescence kineticsof PCR with different DNA concentrations used for the creation of the calibration curve. ng/rx or pg/rx means ng or pg DNA per reaction.

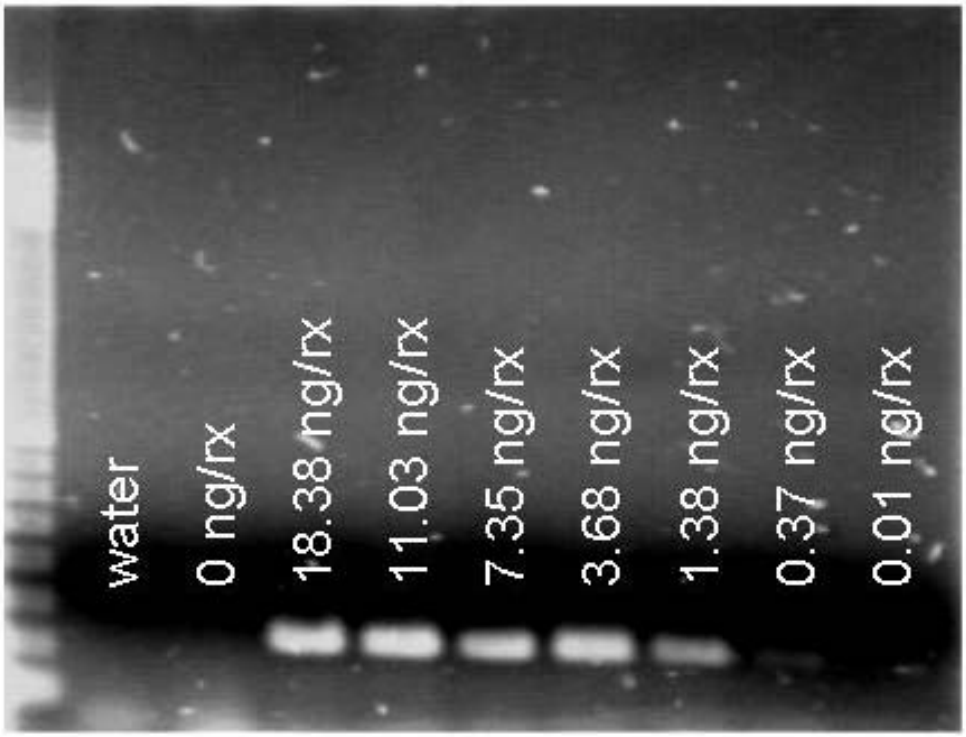
Figure 4: Plot of *A. ochraceus*-DNA content of the 30 green coffee samples against ochratoxin A content. The error bars are the standard deviations. The solid line is the regression calculated, the dotted line is the 95 % interval of confidence. The regression coefficient was calculated to be r=0.55.

271 Table 1

Sample and Origin	OTA [ppb]	DNA [pg/rx]	STD
1 India	72.0	150.3	20.7
2 Burundi	33.0	45.7	6.2
3 Brazil	17.3	24.8	3.0
4 Costa Rica	7.4	8.4	6.5
5 Costa Rica	7.2	2.7	0.3
6 Vietnam	5.5	2.6	1.4
7 Indonesia	5.0	19.3	1.0
8 Indonesia	4.8	0.9	0.8
9 Costa Rica	30.0	5.3	0.9
10 Burundi	8.6	20.9	5.1
11 Brazil	3.1	8.7	1.0
12 Costa Rica	2.4	0.1	0.0
13 Burundi	2.2	20.6	14.5
14 Vietnam	2.0	0.3	0.2
15 Burundi	1.9	11.3	2.6
16 Vietnam	1.8	0.2	0.1
17 Burundi	4.5	17.5	5.6
18 India	4.8	159.1	36.6
19 Costa Rica	4.5	4.5	2.8
20 Vietnam	3.1	3.9	0.9
21 Vietnam	2.6	2.5	0.9
22 Indonesia	2.3	5.9	3.0
23 Vietnam	2.3	0.6	0.6
24 Vietnam	2.2	0.2	0.2
25 unknown	7.0	113.0	20.4
26 unknown	3.6	7.6	1.0
27 unknown	0.7	6.0	2.4
28 unknown	1.2	25.0	6.6
29 unknown	1.7	4.8	1.4
30 unknown	0.0	0.7	0.4

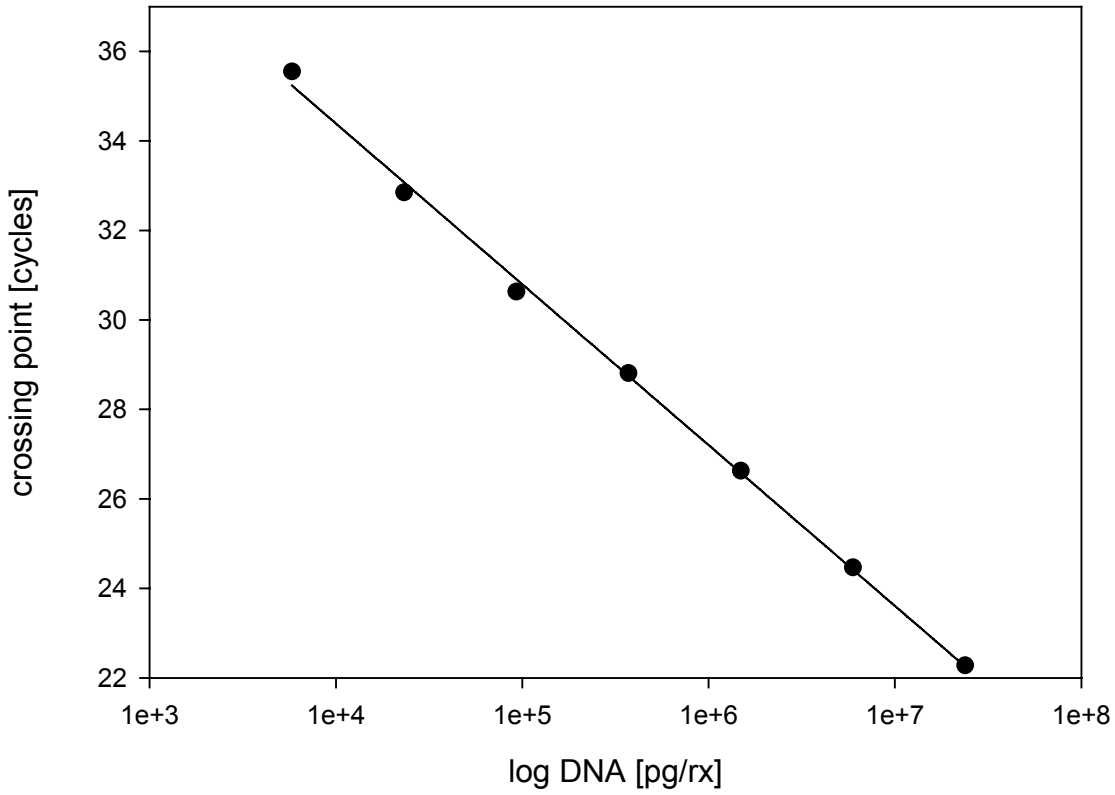
272

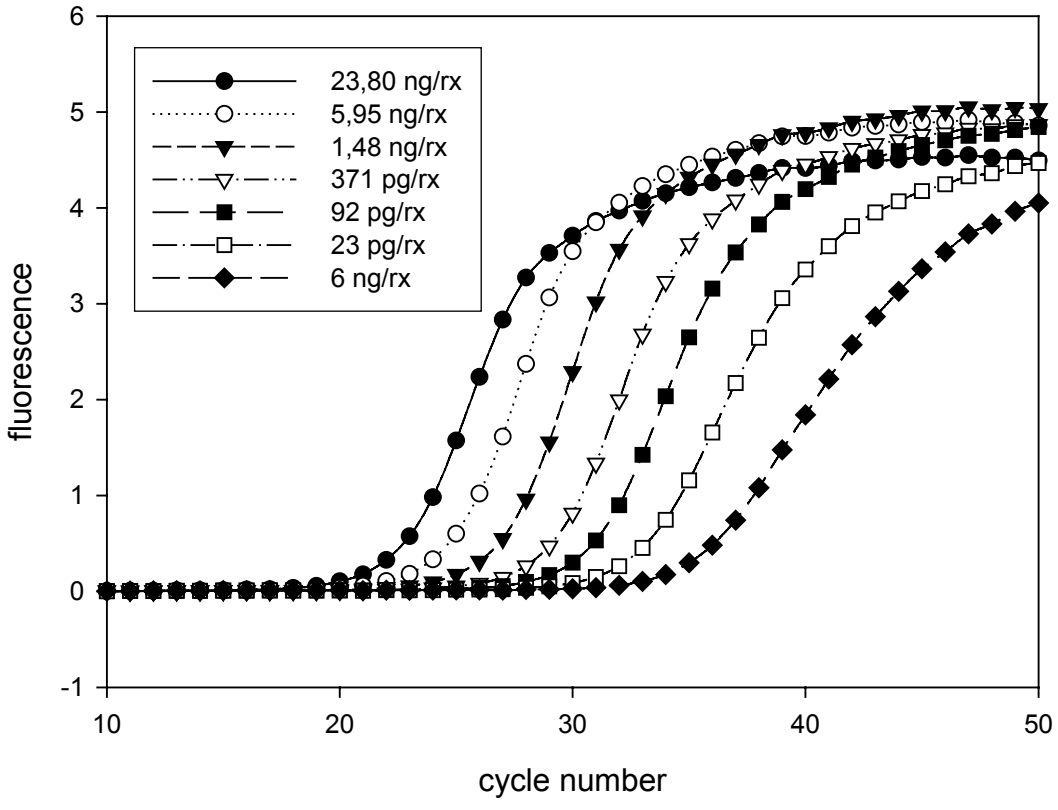
272 Figure 1



273

274 Figure 2





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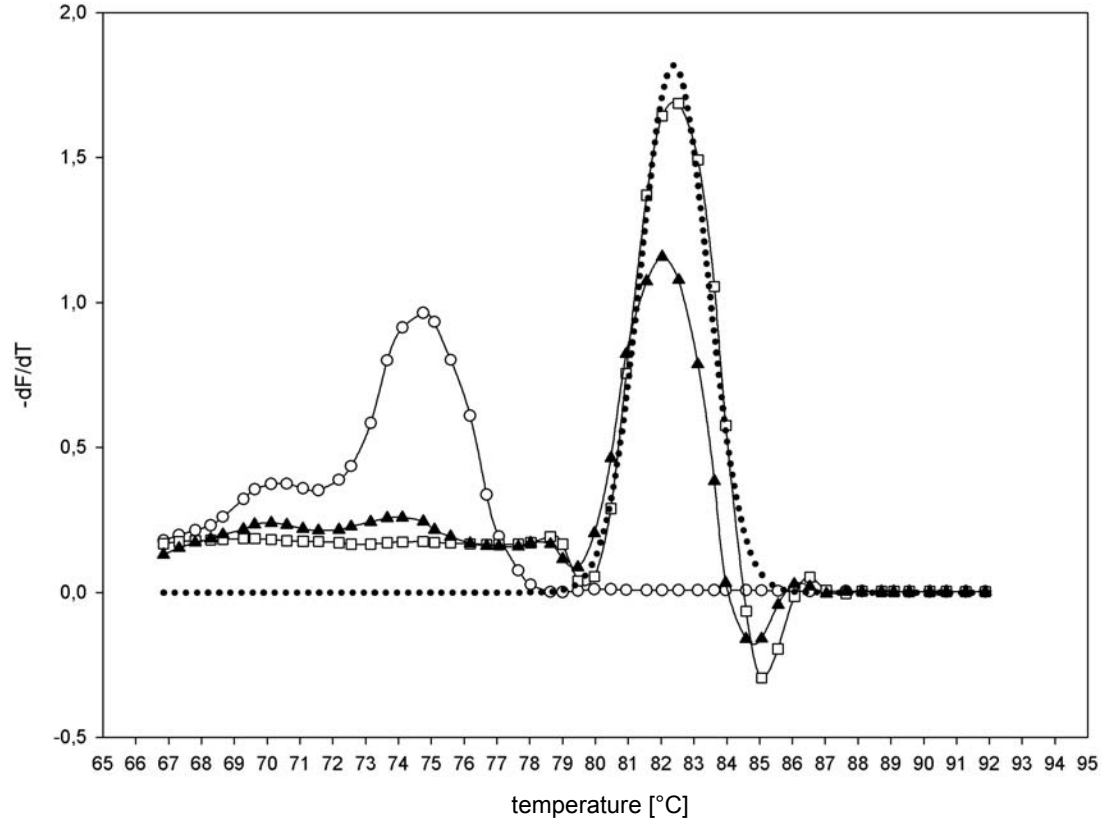
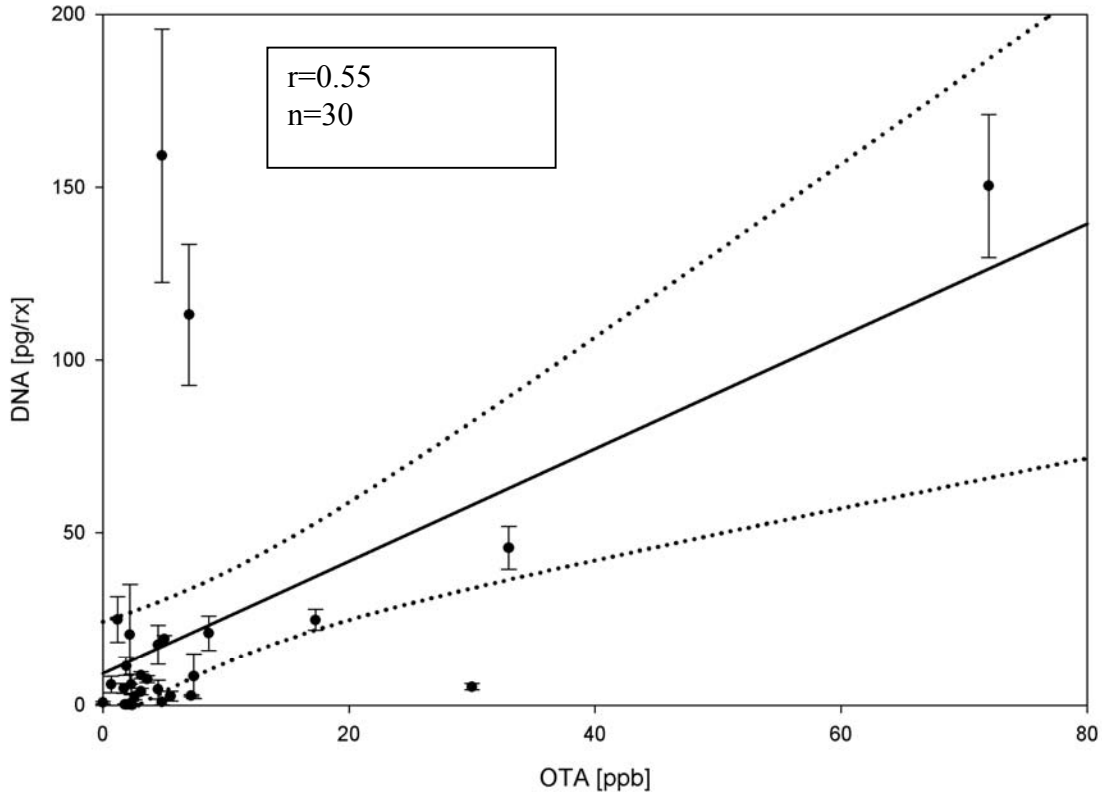
276 Figure 3





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