Ph. D. Thesis

Comprehensive phylogenetical analysis of the genus *Aspergillus*; characterization of potential mycotoxin-producer and opportunistic pathogen black aspergilli

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INTRODUCTION

The genus *Aspergillus* is a diverse group of filamentous fungi. Some species of the genus have high economic and social impact because of their application in biotechnology and food industry. *Aspergillus* is one of the most difficult groups concerning classification and identification. Several closely related species cannot be reliably distinguished on the basis of morphological criteria alone. Molecular tools have been developed to solve this issue, with sequence-based methods being the most valuable for species delimitation. Partial sequences of the calmodulin or β-tubulin genes are suitable to discriminate species within the genus *Aspergillus*. *Aspergillus* has been the subject of a large number of taxonomic studies using DNA sequence data. Many of these studies focused on specific groups (species, sections, subgenera) within *Aspergillus* and the number of phylogenetic studies at the genus level and above are limited.

In order to recognize the phylogenetic relationships in the genus *Aspergillus*, a robust phylogenetic analysis was carried out, with the involvement of 93 species. The examined species represented the whole genus properly. Sequences of six genes were analysed: RPB1, RPB2 (subunits of the RNA polymerase gene), Tsr1 (putative ribosome biogenesis protein), Cct8 (putative chaperonin complex component TCP-1), Acl1 (ATP citrate lyase), MCM7 (minichromosome maintenance complex component 7). Combined sequence data were created and the phylogenetic analysis was carried out by Maximum Likelihood (ML) and Bayesian methods.

The black aspergilli (*Aspergillus* section *Nigri*) is an important group of species in food mycology, medical mycology and biotechnology. *A. niger* is used in the fermentation industry to produce hydrolytic enzymes, such as amylases or lipases, and organic acids, such as citric acid and gluconic acid. Besides their economic importance, black aspergilli are also important as opportunistic human pathogens and many species cause food spoilage and are potential producers of mycotoxins (fumonisins, ochratoxin A) which contaminate several agricultural products.

Fumonisins are carcinogenic mycotoxins that were originally identified in *Fusarium verticillioides*. Fumonisins can be divided into structurally distinct groups, four of which have been designated A, B, C and P fumonisins. Some reports indicate that fumonisins may be involved in esophageal cancer in South Africa, and have been shown to be involved in leukoencephalomalacia in horses, pulmonary edema in pigs, and cancer and neural tube defects

in experimental rodents. Black aspergilli can colonize numerous food produtcs thus these products can be contaminated with fumonisins.

In this study black *Aspergillus* isolates were collected from different agricultural products, and the isolates were identified at the species level based on calmodulin gene sequences. The fumonisin producing ability of the isolates and the fumonisin content of the examined products were tested. Raisin-, fig-, date- and onion sampes were investigated.

Black aspergilli are opportunistic human pathogens, but the importance of these diseases is growing with the spreading of immunosuppressive therapy. The disease usually occurs in the respiratory system, but they can also cause keratitis and otitis. In order to select the appropriate treatment, the correct identification of these clinical isolates is essential, as not all species have the same susceptibility patterns against several antifungal drugs. Clinical black aspergilli are usually identified as *A. niger* using conventional morphological methods. However, recent molecular analyses showed that, apart from *A. niger* other black aspergilli are also important as potential opportunistic pathogens in human infections.

We isolated black aspergilli from otomycosis cases in Iran and Hungary. All of the isolates were identified as *A. niger* based on morphological characters. Antifungal susceptibility tests have also been carried out against 5 antifungal drugs including amphotericin B, fluconazole, itraconazole, ketoconazole and terbinafine.

AIMS

- **Production of a robust genus-wide phylogeny to get insight into the evolutionary relationships of this economically important genus.** Ninety-three species and 6 genes sequences were involved. The analysis was carried out using Maximum Likelihood and Bayesian methods.

- **Analysis of the genetic variability of the two closely related black *Aspergillus* species *A. niger* and *A. welwitschiae*.** These two species cannot be distinguished from each other based on morphological characters, but their partial calmodulin gene sequences are obviously different. Our aim was to clarify, if this difference expands the whole genome. UP-PCR (Universally primed – Polymerase Chain Reaction) is suitable for examination of genomic variability between closely related species.

- **Identification of black *Aspergillus* isolates from otomycosis cases, and investigation of their susceptibility against several antifungal drugs**. From otomycosis cases only *A. niger* strains were isolated, however other black *Aspergillus* species can also cause opportunistic mycosis. The closely related species cannot be distinguished from each other based on morphological characters. Our aim was to collect black *Aspergillus* isolates from otomycosis cases, and identify them at the species level based on calmodulin sequences. Furthermore, we intended to investigate the susceptibility of the isolates against several antifungal drugs using microdilution broth method.

- **Identification of black *Aspergillus* isolates from food samples, and analysis of their fumonisin producing ability and the fumonisin content of the investigated food samples**. Black aspergilli are present in numerous food commodities, and the potential of their fumonisin-production means an important health risk. Our aim was to collect black *Aspergillus* isolates from different food samples, and identify them at the species level using DNA based methods. We purposed to investigate the fumonisin producing ability of the isolates and the fumonisin content of the food materials by HPLC-MS method.

METHODS

Phylogenetic analysis

DNA isolation

Polimerase chain reaction (PCR)

Agarose gel electrophoresis

Capillary sequencing

Alignment of sequences

Creation of contigs

Maximum Likelihood analysis

Bayesian analysis

Investigation of the difference among two closely related species *A. niger* and *A. welwitschiae*

Universally primed (UP)-PCR

Neighbor joining analysis

Identification of black Aspergillus isolates originated from otomycosis cases

Breeding of pure cultures

Amplification of mycelia, DNA isolation

Molecular identification based on calmodulin gene sequences

Creation of phylogenetic tree using Maximum Parsimony

Microdilution antifungal susceptibility testing

Investigation of black aspergilli isolated from agricultural products

Isolation of fungal strains on Dichloran-rose bengal-chloramphenicol agar

Identification of isolates based on calmodulin gene sequences

Creation of phylogenetic tree using Maximum Parsimony

Extraction of fumonisins

Detection of fumonisin content using reverse phase HPLC/ESI-IT-MS method

RESULTS

1. Phylogenetic analysis of the genus *Aspergillus*

According to our data the genus *Aspergillus* can be divided into 6 subgenera and 22 sections. The *Aspergillus* (bootstrap value: 100/ posterior probability: 1), *Polypaecili* (100/1), *Cremei* (90/1), *Fumigati* (97/1) and *Nidulantes* (100/1) subgenera have high support values, however the subgenus *Circumdati* has lower bootstrap value (47/1). Except *Usti*, all the sections are monophyletic according to the ML analysis with moderate or high bootstrap values. The section *Usti* is divided into two groups, and *A. amylovorus* and *A. egypticus* represent a separate clade with high support values (92/1). Fungi belonging to *Polypaecilum* and *Phialosimplex* seem to be in relation with members of sections *Cremei* and *Aspergillus*, thus the former *Phialosimplex caninus*, *Phialosimplex clamydosporus*, *Phialosimplex sclerotiales*, *Polypaecilum insolitum* and *Polypaecilum pisci* species get the genus name *Aspergillus*. *Penicillium inflatum* is related to genus *Aspergillus*, consequently this species is transferred to genus *Aspergillus* under the name *A. inflatus*. According to our data, sections *Versicolores*, *Nidulantes*, *Aenei*, *Raperi*, *Usti*, *Bispori*, *Ochraceorosei* and *Sparsi* belong to one group. Species of *Versicolores* and *Nidulantes* are divided, but not at the section level. Section *Nigri* is the sister group of *Candidi* and *Terrei*, not *Flavi*. The sister group of *Flavi* is section *Circumdati*. Section *Cremei* is related to sections *Polypaecili*, *Restricti* and *Aspergillus*. *A. funiculosus* has been classified to section *Sparsi*, but this speies is related to *A. ochraceoroseus*, thus belongs to section *Ochraceorosei*.

These data contributed to decide the dispute about nomenclature of the genus *Aspergillus*. According to our data the genus *Aspergillus* is monophyletic, and the nomenclatural subdivision is unnecessary. Based on the decision of the Nomenclature Committee, the members of the genus can keep the name *Aspergillus*.

2. Intvestigation of the genetic variability of two closely related black *Aspergillus* species, *A. niger* and *A. welwitschiae*

The genetic variability of *A. niger* and *A. welwitschiae* was studied by the UP-PCR method. A Neighbour-joining tree was generated based on a binomial matrix which contains the data of 88 fragments. On this tree the two species appear in separated clades, thus these closely related species can be distinguished from each other with this method.

3. Identification of black *Aspergillus* isolates from otomycosis cases, and their susceptibility to different antifungal drugs

Seven samples from Iran and 14 samples from Hungary were investigated. According to the clinical implication, all of them were identified as *A. niger* on the basis of conventional morphological methods. However, based on sequence analysis of a part of the calmodulin gene, our data showed that apart from *A. niger*, other *Aspergillus* species (*A. welwitschiae* and *A. tubingensis*) can also cause otomycosis. The susceptibilities of the isolates to commonly used antifungal agents (amphotericin B, itraconazole, fluconazole, ketoconazole and terbinafine) were tested *in vitro*. Based on the MIC_{100} values there were no significant differences among the susceptibilities of the different species. Terbinafine (MIC_{100}: 0.25-1 μg/ml) and itraconazole (MIC_{100}: 0.5-1 μg/ml) were more effective than ketoconazole (MIC_{100}: 8-16 μg/ml). Amphotericin B was more effective against the isolates from Hungary, than against the isolates from Iran.

4. Presence of black aspergilli on food samples and their ability to produce fumonisins and fumonisin content of food samples contaminated by black *Aspergillus* strains

Black *Aspergillus* strains were isolated from raisin, date, fig and onion samples. The isolates were identified based on partial calmodulin gene sequences. Among 32 black *Aspergillus* isolates from raisins, 16 were found to belong to *A. niger* and 16 to *A. welwitschiae*. Sixty-six percent of the isolates were found to be able to produce fumonisins. Several isomers of fumonisins were identified in the extracts of the isolates. The average production of fumonisins by the toxinogenic species was around 5 mg/kg, but two of the isolates produced fumonisins in a very high concentrations (15 and 17 mg/kg, respectively). The average fumonisin content of the raisin samples was around 7 mg/kg, but in the case of one sample we could detect 35 mg/kg (for comparison, the EU limit for unprocessed maize is 4 mg/kg). Regulations do not exist for the fumonisin content of raisins.

All of the 35 black *Aspergillus* strains isolated from dates were found to belong to *A. tubingensis* based on calmodulin sequence data. This species is not able to produce mycotoxins.

The majority of the isolates originated from figs were also belonging to *A. tubingensis*, but 6 *A. niger* isolates were also detected. One fig sample was contaminated by fumonisins at a low concentration (0.16 mg/kg).

All of the 35 black *Aspergillus* strains isolated from onion samples were found to belong to *A. welwitschiae* based on calmodulin sequence data. 15% of the isolates were able to produce fumonisins, while 17% produced ochratoxin A. Two of the onion samples were contaminated by fumonisins at low concentrations (0.32 and 0.33 mg/kg).

SUMMARY

Based on a genom wide phylogenetic analysis, we proved that:

- Section *Usti* represents two different clade
- Section *Versicolores* incorporates to the section *Nidulantes*
- Section *Nigri* is the sister group of *Candidi* and *Terrei*, not *Flavi*
- Section *Flavi* is the sister group of *Circumdati*
- *Polypaecilum* and *Phialosimplex* belong to genus *Aspergillus*
- *A. funiculosus* is a member of section *Ochraceorosei*, not *Sparsi*
- *Penicillium inflatum* belongs to *Aspergillus* section *Cremei*
- *A. clavatoflavus* and *A. zonatus* does not belong to the genus *Aspergillus*

Our data contributed to decide the dispute about nomenclature of the genus *Aspergillus*.

Genetic variability of two closely related species, *A. niger* and *A. welwitschiae* was proved by UP-PCR method.

Besides *A. niger* other black *Aspergillus* species (*A. tubingensis* and *A. welwitschiae*) can also cause otomycosis.

Fumonisin-producing black *Aspergillus* species are present in raisin, fig and onion samples.

Fumonisins produced by black *Aspergillus* species were detected in raisin, fig and onion sampels.

Production of FB_1, FB_3, 3-epi-FB_3, 3-epi-FB_4, izo-$FB_{2,3}$ isomers by *Aspergillus* species was detected for the fist time.

A. niger and *A. welwitschiae* species are responsible for fumonisin contamination of raisin worldwide.

The causative agent of black mold rot of onion is the species *A. welwitschiae* worldwide.

PUBLICATIONS

Publications in referred journals summarizing the results of this Ph. D. thesis

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