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*review*

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# Evolutionary Relationships among *Aspergillus* Species Producing Economically Important Mycotoxins

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## Summary

Several mycotoxins are produced by aspergilli, which contaminate our foods and feeds. Economically the most important of these mycotoxins are aflatoxins, ochratoxins and patulin. Recent studies indicate that these compounds can be produced by a number of unrelated *Aspergillus* species. Evolutionary relationships among sections involving these mycotoxin producing species (*Aspergillus* sections *Circumdati*, *Flavi*, *Nigri*, *Fumigati* and *Clavati*) were evaluated using phylogenetic analysis of β-tubulin and ribosomal RNA gene sequences. Phylogenetically unrelated species were found to produce the same mycotoxins. For example, aflatoxins have been produced under laboratory conditions by species belonging to three different sections, while ochratoxin A (OA) and patulin have been produced by a variety of unrelated species. Based on this observation, mycotoxin-producing abilities of the isolates were lost (or gained) several times during the evolution of the genus. Previous studies revealed that biosynthetic genes of several mycotoxins are clustered in the genome, leading to the idea that these clusters could be horizontally transferred as a unit to unrelated species, leading to the biosynthesis of the same mycotoxins in phylogenetically unrelated fungi. Such a mechanism could also explain the widespread occurrence of ochratoxin and patulin production in aspergilli. The organisation of biosynthetic genes of patulin and ochratoxins is unknown. Research is in progress in our laboratory to clarify the genetic background of biosynthesis of these mycotoxins, and to develop biosynthetic gene based probes for the molecular detection of these mycotoxin-producing organisms.

*Key words: Aspergillus*, phylogeny, evolution, mycotoxin, aflatoxin, ochratoxin, patulin

## Introduction

Mycotoxins are fungal secondary metabolites that are harmful to animals or humans. Since the Middle Ages it has been known that fungal metabolites can cause animal or human diseases such as ergotism and St Anthony's fire *(1)*. However, mycotoxins first made headline news in the 1960's, when more than one hundred thousand turkeys died in England due to liver hemorrhages and necrosis. Extensive investigations revealed that the disease was caused by peanut meal heavily contaminated with *Aspergillus flavus (2)*. The toxin was na-

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med aflatoxin, after the producing organism. Several other *Aspergillus* mycotoxins which may contaminate our foods and feeds have also been identified. Economically the most important mycotoxins are aflatoxins, ochratoxins and patulin. These toxins were first identified in *A. flavus, A. ochraceus* and *A. clavatus*, respectively *(1)*. However, recent studies indicate that these compounds can be produced by a number of other *Aspergillus* species as well. Only a fraction of these mycotoxin producers can be treated as a health hazard, since they produce only traces of the given mycotoxin, and usually only under laboratory conditions (for example, aflatoxin production by *Eurotium rubrum*, or ochratoxin production by *A. wentiior* or *A. terreus*). Others are rarely encountered in food products (*e.g.* aflatoxin producing *A. ochraceoroseus*, *Emericella venezuelensis* and *E. acristata* isolates, or ochratoxin producing *A. auricomus* isolates) *(3)*. However, recent data indicate that some species recently discovered as mycotoxin producers can be treated as sources of mycotoxin contamination in various food products. For example, although ochratoxin producing abilities of black aspergilli have only recently been discovered, these fungi are now considered as major sources of ochratoxin contamination in wine, raisins and coffee *(3)*. Our aim was to examine the evolutionary relationships among these mycotoxin producing species.

Partly due to its economic importance, the genus *Aspergillus* has one of the better classifications among filamentous fungi. Raper and Fennell *(4)* described 18 species groups within this genus based mainly on cultural and morphological features. These were treated as sections belonging to 6 subgenera by Gams *et al. (5)*. Phylogenetic studies of ribosomal RNA gene sequences led to the acceptance of 3 subgenera with a total of 15 sections and the so-called »*Warcupiella* group«, a treatment currently accepted by most *Aspergillus* researchers (Table 1)*(6)*.

In this review, we wish to give a general overview of the taxonomic relationships among economically important mycotoxin producing *Aspergillus* species based mainly on our recent studies. The techniques applied included PCR based methods, RFLP techniques, and phylogenetic analysis of β-tubulin and ribosomal RNA gene sequences in comparison with morphological and physiological features.

## *Aspergillus* section *Circumdati*

*Aspergillus* section *Circumdati* historically includes species with biseriate conidial heads in shades from yellow to ochre. Species of *Aspergillus* section *Circumdati* are economically important as ochratoxin A (OA) producing spoilage organisms. They are also useful for biotransformation of steroids and alkaloids, while sclerotia of several species contain antiinsectan compounds. The genetic variability of the most well-known species of the section, *Aspergillus ochraceus*, was examined by using genotypic methods *(7)*. Based on mitochondrial DNA restriction profiles, PCR based techniques and ITS sequences, most isolates formed two distinct groups (data not shown). None of the isolates in group 2 produce OA, so these isolates could be used safely in steroid bioconversions.

Table 1. Classification of *Aspergillus* species based on rDNA sequences (the sections examined are set in bold type)

| Sections | Assignment to subgenus | | Associated teleomorph |
|---|---|---|---|
| | Gams *et al. (5)* | Peterson *(6)* | |
| *Aspergillus* | *Aspergillus* | *Aspergillus* | *Eurotium* |
| *Restricti* | *Aspergillus* | *Aspergillus* | – |
| *Cervini* | *Fumigati* | *Aspergillus* | – |
| *Terrei* | *Nidulantes* | *Aspergillus* | *Fennellia* |
| *Flavipedes* | *Nidulantes* | *Aspergillus* | *Fennellia* |
| ***Nigri*** | ***Circumdati*** | ***Aspergillus*** | **–** |
| ***Circumdati*** | ***Circumdati*** | ***Aspergillus*** | ***Neopetromyces*** |
| ***Flavi*** | ***Circumdati*** | ***Aspergillus*** | ***Petromyces*** |
| *Cremei* | *Circumdati* | *Aspergillus* | *Chaetosartorya* |
| *Candidi* | *Circumdati* | *Aspergillus* | – |
| *Wentii* | *Circumdati* | – | – |
| ***Fumigati*** | ***Fumigati*** | ***Fumigati*** | ***Neosartorya*** |
| ***Clavati*** | ***Clavati*** | ***Fumigati*** | ***Neocarpenteles*** |
| *Nidulantes* | *Nidulantes* | *Nidulantes* | *Emericella* |
| *Versicolores* | *Nidulantes* | – | – |
| *Usti* | *Nidulantes* | – | – |
| *Sparsi* | *Circumdati* | *Nidulantes* | – |
| *Ornati* | *Ornati* | *Nidulantes* | *Sclerocleista* |
| *Warcupiella* group | *Ornati* | *Warcupiella* group | *Warcupiella* |

Interspecific variability of species assigned to this section was also examined using phenotypic features and sequences of the intergenic transcribed spacer regions (ITS region) and the 5.8 S rRNA gene (Fig. 1). Phylogenetic analysis of sequence data indicated that *Aspergillus campestris*, *A. lanosus* and *A. dimorphicus* with *A. sepultus* belong to *Aspergillus* sections *Candidi*, *Flavi* and *Cremei*, respectively (Figs. 1 and 2)*(8)*. Two teleomorphic species previously assigned to this section, *Petromyces alliaceus* and *P. albertensis*, together with the asexual *A. lanosus* were found to belong to *Aspergillus* section *Flavi (9)*. These results were also supported by phenotypic data, and by the main ubiquinones observed in these species. Species of the revised *Aspergillus* section *Circumdati* formed two main clades, which could also be distinguished based on phenotypic methods. A sexually reproducing ochratoxin producing species, *Neopetromyces muricatus* was also found to belong to this section *(8,10)*. All these species are characterised by Q-10($H_2$) ubiquinone system. *A. auricomus* and *A. elegans* were found not to belong to any of these clades. Ochratoxin producing abilities of the isolates examined did not correlate with their taxonomic relationships based on ITS sequence data. However, a clade found not to produce ochratoxins during an earlier study of genetic variability of *A. ochraceus* could be identified in this study *(7)*. This clade also includes an ochratoxin non-producing *A. melleus* isolate and an *A. petrakii* strain together with a number of *A. ochraceus* isolates (Fig. 1).

*A. robustus* and *A. ochraceoroseus* were found not to be related to any of the species examined. *A. ochraceoro-*

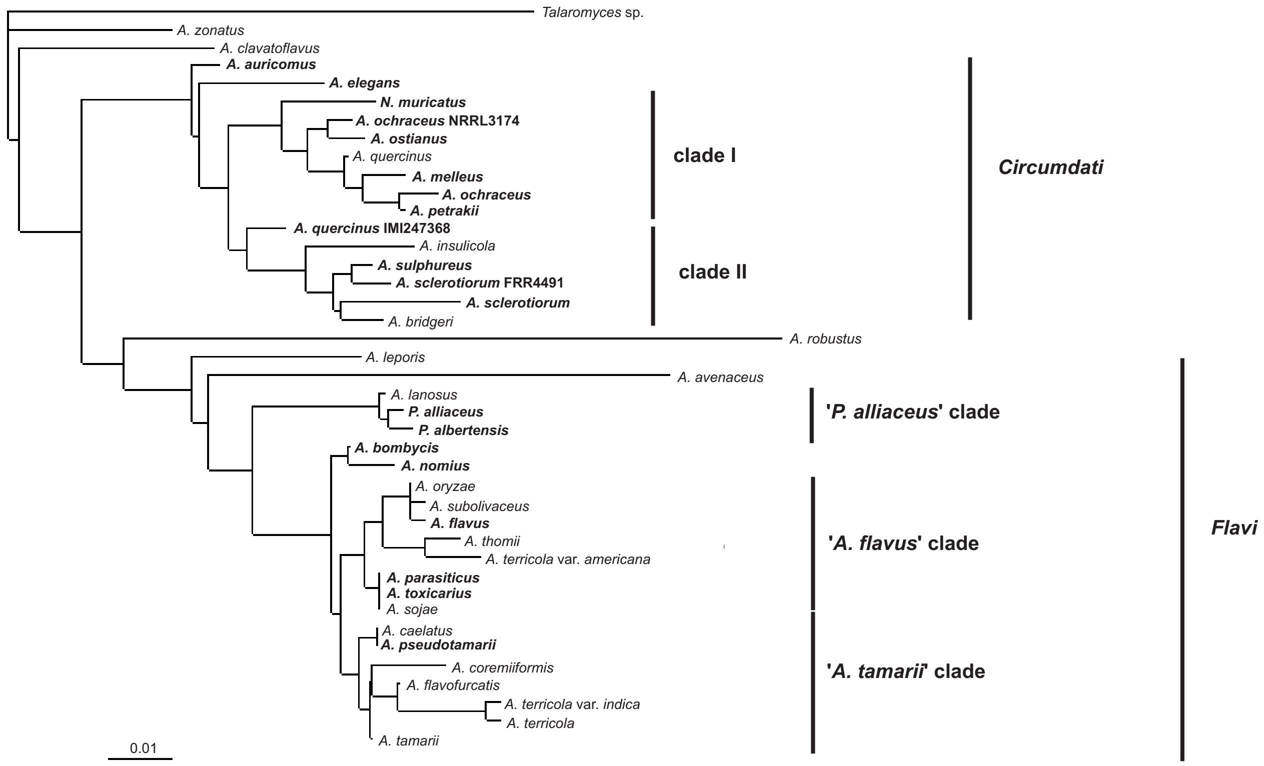

**Fig. 1. Neighbour-joining tree of ITS sequences of species assigned to *Aspergillus* sections *Circumdati* and *Flavi*.** Ochratoxin producing isolates are set in bold type, while aflatoxin producing isolates are in bold small capitals. Species names alone indicate the positions of type strains.

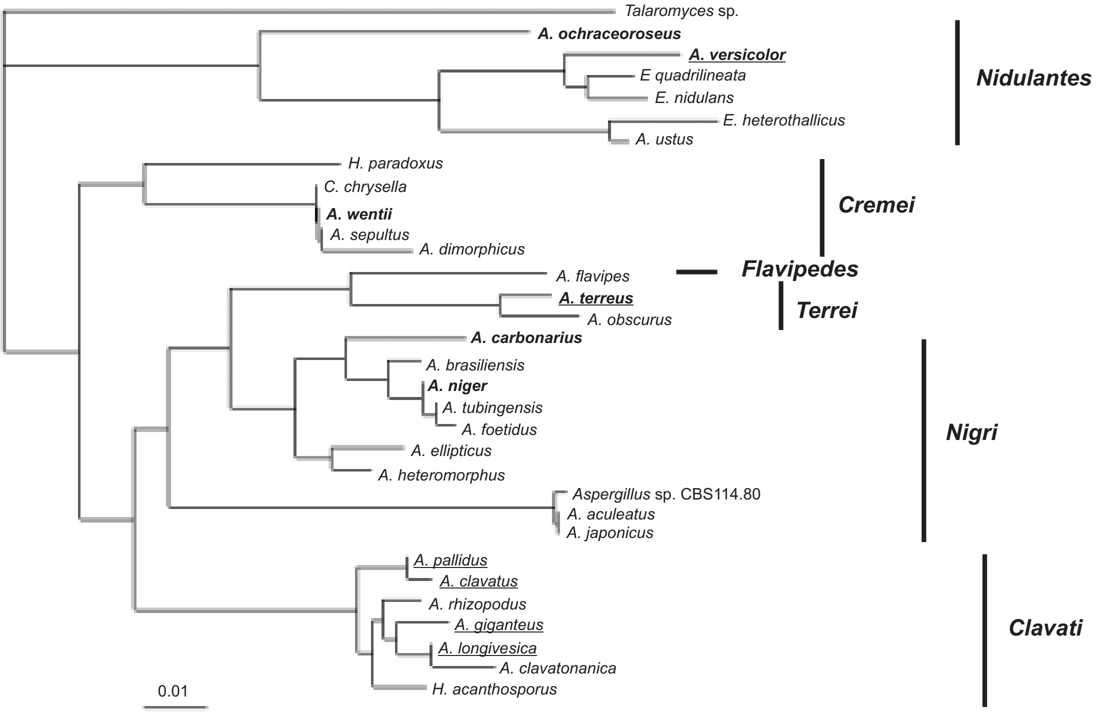

**Fig. 2. Neighbour-joining tree of ITS sequences of species assigned to *Aspergillus* sections *Clavati*, *Nigri*, *Cremei*, *Terrei* and *Nidulantes*.** Ochratoxin producing isolates are set in bold type, while aflatoxin producing isolates are in bold small capitals, and patulin producing isolates are underlined. Species names alone indicate the positions of type strains. Note that evolutionary relationships of these sections were analysed together only for convenience, not to indicate the relationships between these sections (*e.g.* section *Clavati* is most closely related to section *Fumigati*, not shown on this figure).

*seus* is an aflatoxin producing member of the genus *(11)*. This is the only species known to accumulate aflatoxin $B_1$ and sterigmatocystin simultaneously. This species has previously been assigned either to *Aspergillus* sections *Wentii*, *Cremei* or *Circumdati*, respectively *(12–14)*. However, ITS and 26 S rDNA data indicate that this species is closely related to section *Nidulantes* (Fig. 2). Its distant relationship to *Aspergillus* section *Flavi* is supported by the observation that *A. ochraceoroseus* DNA hybridised only weakly if at all to the *A. flavus* and *A. parasiticus* aflatoxin biosynthetic gene probes *(11)*. Additionally, the order of genes of the aflatoxin biosynthetic gene cluster of *A. ochraceoroseus* was more similar to that of the sterigmatocystin gene cluster of *A. nidulans* than to that of *A. parasiticus (11)*. These data indicate that the aflatoxin and sterigmatocystin biosynthetic pathway genes in *A. ochraceoroseus* are different from known pathway genes. Additionally, aflatoxin production has also been observed recently in *Emericella venezuelense* and *E. acristata* isolates, which are also closely related to *A. nidulans* (J. C. Frisvad and R. A. Samson, personal communication).

## *Aspergillus* section *Flavi*

*Aspergillus* section *Flavi* historically includes species with conidial heads in shades from yellow-green to brown, and dark sclerotia. Several species of section *Flavi* produce aflatoxins; *A. flavus* and *A. parasiticus* are pathogenic on peanuts, corn and cotton *(1)*, while isolates of other so-called domesticated species, such as *A. oryzae* and *A. sojae* as well as *A. tamarii*, are used in oriental food fermentation processes, and as hosts for heterologous gene expression *(15)*. ITS sequences of type strains or representative isolates of the species and subspecies currently assigned to this section were analysed. Phylogenetic analysis of sequence data indicated that species of *Aspergillus* section *Flavi* form distinct clades (Fig. 1) *(16)*. The three main clades identified based on sequence data could also be distinguished based on colony colour and their ubiquinone systems. The »*A. flavus*« clade includes species characterised with Q-10($H_2$) as their main ubiquinone, conidial colours in shades of green and dark sclerotia. Studies on the genetic variability of *A. flavus* indicated that the name is currently applied to a paraphyletic group of isolates that may produce aflatoxins B or G, and have large or small sclerotia *(17)*. It was suggested that isolates with small sclerotia, able to produce both aflatoxins B and G (group II), deserve recognition as a new species *(17)*. The other group (group I) includes isolates producing only aflatoxin B and having large or small sclerotia (Table 2). This group also includes isolates of *A. oryzae*, which has previously been described as having a recombining population structure *(17)*. Although several lines of evidence suggest that *A. oryzae* and *A. sojae* are morphological variants of *A. flavus* and *A. parasiticus*, respectively, it was suggested that these taxa should be retained as separate species due to the regulatory confusion that conspecificity might generate in the food industry. The »*A. tamarii*« clade involves species with ubiquinone system Q-10($H_2$), and conidia in shades from olive to brown, while the »*A. alliaceus*« clade consists of species with Q-10 ubiquinone system, and conidia in shades of ochre. Two species of this clade, *P. alliaceus* and *P. albertensis*, produce high amounts of OA (50–300 mg/mL), and are considered to be responsible for OA contamination of figs *(18,19)*. The synnematous species *A. coremiiformis* was closely related to species in the »*A. tamarii*« clade. The recently described aflatoxin producing species *A. pseudotamarii* and *A. bombycis* are closely related to *A. caelatus* and *A. nomius*, respectively *(20,21)*. Physiological properties and mycotoxin producing abilities of these taxa justify their treatment as separate species (Table 2)*(20,21)*. While no evidence of genetic recombination was found in *A. bombycis*, cryptic genetic recombination was observed in an *A. nomius* population *(20)*. Two other species, *A. avenaceus* and *A. leporis,* were found to form separate lineages not closely related to any of the main clades identified. It is suggested that *A. clavatoflavus* and *A. zonatus* be excluded from *Aspergillus* section *Flavi*, a suggestion previously made by Kozakiewicz, based on her scanning electron microscopic studies of the group *(14)*. Phylogenetic analysis of partial 26 S rRNA gene sequences supported our findings.

Table 2. Mycotoxin producing abilities of the economically important species of *Aspergillus* section *Flavi* (compiled from refs. *17,20,21*)

| Species | Aflatoxins | | CPA* | Kojic acid | Nominine |
|---|---|---|---|---|---|
| | B | G | | | |
| *A. flavus* group I | + | – | ± | + | – |
| *A. flavus* group II | + | ± | + | + | – |
| *A. oryzae* | – | – | ± | + | – |
| *A. parasiticus* | + | + | – | + | – |
| *A. sojae* | – | – | – | + | – |
| *A. nomius* | + | + | – | + | + |
| *A. bombycis* | + | + | – | + | ? |
| *A. tamarii* | – | – | + | + | – |
| *A. caelatus* | – | – | – | + | – |
| *A. pseudotamarii* | + | – | + | + | – |

*CPA, cyclopiazonic acid

Aflatoxin producing species (*A. flavus*, *A. parasiticus*, *A. nomius*, *A. bombycis* and *A. pseudotamarii*) were scattered throughout the dendrogram indicating that aflatoxin producing ability was lost (or gained) several times during evolution.

## *Aspergillus* section *Nigri*

*Aspergillus* section *Nigri* are industrially one of the most important taxa of filamentous fungi *(15)*. Several strains belonging to this section are used in the fermentation industry for the production of different organic acids and hydrolytic enzymes. The observation that black aspergilli including *A. niger (22–25)* and *A. carbonarius (26,27)* are able to produce ochratoxins is economically important since *A. niger* is extensively used in the food industry. Several morphologically similar »species« were included in an *A. niger* species complex by Al-Musallam *(28)*. Taxonomic evaluation of this species complex was carried out using different methods. Among the genotypic approaches, nuclear and mtDNA polymorphisms and PCR based techniques led to the recognition of four species within this species complex (*A. niger, A. tubingensis, A. brasiliensis, A. foetidus*)*(29–36)*. Several well-known species names such as *A. awamorii, A. usamii, A. phoenicis* and *A. ficuum* have been reduced to synonymy.

Regarding other black *Aspergillus* species, phylogenetic analysis of ITS sequence data indicates that at least 9 species belong to this section (*A. heteromorphus, A. ellipticus, A. carbonarius, A. japonicus, A. aculeatus, A. niger, A. tubingensis, A. foetidus* and *A. brasiliensis*; Fig. 2)*(32,36)*. The uniseriate species *A. japonicus, A. aculeatus* and isolate CBS 114.80, which is considered to represent a new species, form one well-defined clade, while the biseriate species are on a separate branch (Fig. 2). Ochratoxin production has been observed only in *A. niger* and *A. carbonarius* isolates *(24,37)*. These species are now considered as major sources of ochratoxin contamination in tropical and subtropical foods including dried vine fruits, wines and coffee *(3,37,38)*.

## *Aspergillus* section *Fumigati*

This section involves the anamorphs of at least 20 sexual *Neosartorya* species, and 5 asexual aspergilli. The most important species among them is *Aspergillus fumigatus*, which is an ubiquitous filamentous fungus in the environment, and also an important human pathogen *(4)*. Several *Neosartorya* species have also been described as causative agents of human diseases including invasive aspergillosis, osteomyelitis, endocarditis and mycotic keratitis. Many of the *Neosartorya* species examined produce heat-resistant ascospores that are frequently encountered in different food products, particularly heat pasteurised foods. In addition, several mycotoxins are produced by these species, many of which may cause serious health hazards *(4)*. Phylogenetic analyses of sequences of part of the β-tubulin gene have recently been carried out on this group (Fig. 3)*(39–41)*. The recently described *Neosartorya pseudofischeri* was found to be basal to all other species of section *Fumigati (39,42)*. Phylogenetic analyses supported the hypothesis that heterothallism is a derived character, and that sexuality was lost several times during the evolution of *Aspergillus* section *Fumigati (39–41)*. The heterothallic *N. fennelliae* and *N. udagawae* strains were closely related to the homothallic *Neosartorya* sp. NRRL 4179 and *N. aureola*, respectively. Strain FRR 1266, which was earlier classified as a highly divergent *A. fumigatus* isolate, was found to belong to the *A. viridinutans* species *(40,43)*. The ochratoxin A producing *A. viridinutans* strain IMI 306135 was most closely related to an ochratoxin producing asexual strain isolated from cocoa beans (JV3, Fig. 3). These two latter strains were more closely related to *A. fumigatus* and *N. fischeri* than to any *A. viridinutans* strains, and possibly represent a new species in *Aspergillus* section *Fumigati*. These conclusions were also supported by carbon source utilisation data and restriction analysis of the mitochondrial and nuclear DNA of the strains (data not shown). The results indicate that the presence or absence of nodding conidial heads is not an unequivocal morphological

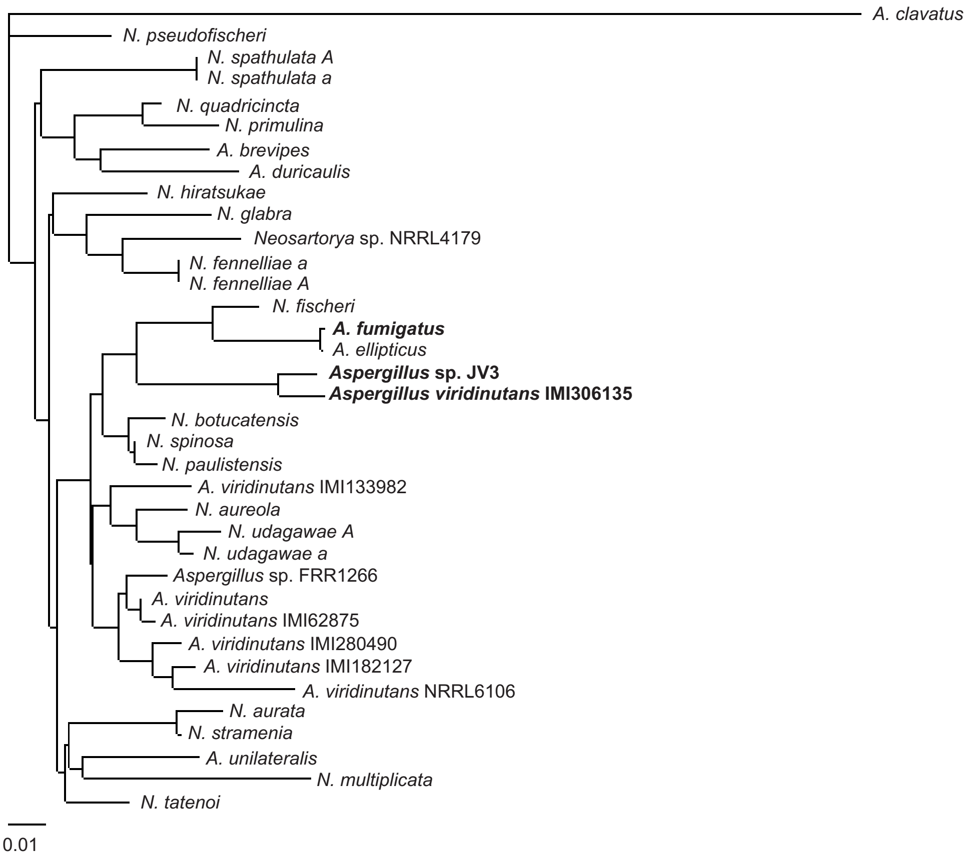

**Fig. 3. Neighbour-joining tree of β-tubulin gene sequences of isolates assigned to *Aspergillus* section *Fumigati*.** Ochratoxin producing isolates are set in bold type. Species names alone indicate the positions of type strains.

character for the identification of species within *Aspergillus* section *Fumigati*.

## *Aspergillus* section *Clavati*

Species belonging to *Aspergillus* section *Clavati* are characterised by clavate shaped vesicles and large blue-green conidial heads. The most economically important species of the section, *A. clavatus* can be isolated mainly from soil and dung, but also occurs on stored products (mainly cereals) with high moisture content *(44)*. *A. clavatus* was found to be responsible for an extrinsic allergic alveolitis known as malt worker's lung. *A. clavatus* and its relatives produce a number of mycotoxins including patulin, kojic acid, cytochalasins, and tremorgenic mycotoxins. The carcinogenic mycotoxin patulin is receiving world-wide attention due to its frequent occurrence in apple juices. *A. clavatus* and *A. giganteus* isolates also produce ribotoxins, which are promising tools for immunotherapy of cancer *(45)*. Mycotoxin producing abilities and phylogenetic relationships among isolates representing the six species currently assigned to this section were examined *(46)*. *A. longivesica* was found for the first time to produce patulin. A phylogenetic analysis of intergenic transcribed spacer sequence data indicated that most isolates belong to two main clades, which have been identified previously using 26 S rDNA sequence data (Fig. 2)*(6)*. *A. pallidus* isolates clustered together with *A. clavatus* strains. The anamorph of *Hemicarpenteles acanthosporus* was also found to belong to this section *(6,46)*. A new genus, *Neocarpenteles* was proposed to accommodate this species as *Neocarpenteles acanthosporum (47)*. Correlation was not observed between patulin production and the taxonomic position of the isolates tested, indicating that patulin producing abilities were lost several times during the evolution of *Aspergillus* section *Clavati*.

## Conclusions and Future Prospects

Phylogenetic analysis of sequences of the ribosomal RNA gene cluster and the β-tubulin gene were found to be very useful for clarifying taxonomic relationships among toxigenic aspergilli. Phylogenetically unrelated species were found to produce the same mycotoxins: aflatoxins are produced by species belonging to three different sections, while OA and patulin are produced by species assigned to 7 and 8 different sections, respectively. In addition, ochratoxins are produced by some penicillia *(48–50)*, while patulin is produced by a variety of other fungal genera *(51)*. Based on this observation, mycotoxin producing abilities of the isolates were lost (or gained) several times during the evolution of the genus. Our data indicate that it is obviously not possible to develop rDNA based gene probes for the detection of OA or patulin producing fungi, an approach applied successfully for fumonisin producing fusaria. Such an approach is further hampered by the fact that in the cases of OA and patulin, different species have been found to be responsible for mycotoxin contamination in different plant products. For example, OA contamination in figs is caused by *P. alliaceus (18)*, that in grapes and wine by *A. carbonarius* and *A. niger (38)*, that of cereals by *P. verrucosum (3)*, while in cheese and meat products *P. nordicum* is responsible for OA contamination *(52)*. Instead, biosynthetic gene based probes are necessary for molecular detection of these mycotoxin producing organisms.

Genes of several pathways described as having »dispensable« metabolic functions, as well as biosynthetic genes of several mycotoxins including aflatoxins, trichothecenes, fumonisins and ergot alkaloids are also clustered in the genome *(53)*. This observation led to the speculation that these clusters could be horizontally transferred as a unit to unrelated species, leading to the biosynthesis of the same mycotoxins in phylogenetically unrelated fungi *(54)*. The organisation of biosynthetic genes of patulin and ochratoxins is unknown, although experiments are in progress in several laboratories to clarify the genetic background of biosynthesis of these mycotoxins. Such knowledge is essential for clarifying the evolution of mycotoxin biosynthesis in aspergilli.

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# Evolucijski odnos između vrsta *Aspergillus* koje proizvode ekonomski važne mikotoksine

## Sažetak

Aspergili proizvode nekoliko mikotoksina koji onečišćuju hranu i krmiva. Ekonomski najvažniji mikotoksini su aflatoksini, ohratoksini i patulin. Novija istraživanja pokazuju da ove spojeve proizvodi određeni broj nesrodnih vrsta *Aspergillus.* Evolucijski odnosi između sekcija, obuhvaćajući vrste koje proizvode mikotoksine (*Aspergillus* sekcije *Circumdati, Flavi, Nigri, Fumigati* i *Clavati*), utvrđeni su filogenetskom analizom β-tubulina i sekvencijom ribosomskog RNA gena. Nađeno je da filogenetski nesrodne vrste proizvode iste mikotoksine. Npr. u laboratorijskim je uvjetima aflatoksin proizveden od vrsta koje pripadaju trima različitim sekcijama, dok su ohratoksin i patulin bili proizvedeni od raznolikih nesrodnih vrsta. Na osnovi tih spoznaja gubila se (ili dobivala) sposobnost proizvodnje mikotoksina pojedinih izolata nekoliko puta tijekom evolucije genusa. Prethodna su ispitivanja otkrila da biosintetski geni nekoliko mikotoksina čine nakupine u genomu, pa se može zaključiti da se te nakupine horizontalno prenose kao cjelina na nesrodne vrste,

omogućujući time biosintezu istih mikotoksina u filogenetski nesrodnim fungima. Takav mehanizam može također objasniti učestalost proizvodnje ohratoksina i patulina u aspergilima. Nepoznat je način organizacije biosintetskih gena patulina i ohratoksina. U našem su laboratoriju u tijeku radovi kojima želimo objasniti genetičku osnovu biosinteze tih mikotoksina te razviti sonde temeljene na biosintetskom genu za molekularnu detekciju organizama proizvođača mikotoksina.