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Old and new concepts of species differentiation in *Aspergillus*

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The classification of the genus *Aspergillus* has been studied by many taxonomists. The most important monograph on which most taxonomies are derived from is strictly based on phenotypical characters. Later revisions of certain *Aspergillus* sections have been predominantly nomenclatural changes and primarily used morphological criteria. Many new taxa were added particularly in the genera *Emericella* and *Neosartorya*. Identification of the most common and often important species remains problematic due to the variability in the phenotypic characters. This has caused errors in the literature, especially concerning the links to mycotoxin formation. The new taxonomies are based on a polyphasic approach using phenotypical characters together with multigene DNA sequences. In a polyphasic approach micro- and macromorphology, physiology, metabolites produced and molecular data are all important, and in principle no particular method should be overemphasized. In particular extrolite profiles have proven to be specific for the taxa and this has contributed to a stable species concept, but DNA sequence data have also been very valuable in critical revisions of species and their taxonomy and phylogeny. Examples of new classifications for species in section *Circumdati, Flavi, Fumigati* and *Nigri* are presented. Although the polyphasic approach might reveal clear cut species, problems may arise for some species if they are to be separated based only on their microscopic features and few physiological features. Suggestions for new methods in order to carry out more fast and precise identifications will be discussed. Full genome sequencing and DNA arrays offers exciting new bases for identifying the Aspergilli, but recent methods based on image analysis of accurately fingerprinted phenotypes are also very promising. However both methods require a stable and well resolved taxonomy and nomenclature. Validated careful phenotypic classification (taxonomy) together with phylogenetic treatment of DNA sequence data is a prerequisite for reliable rapid identification methods and database formation. Concerning identification, DNA bar coding will be possible in the future, either based on molecular methods or certain phenotypic features.

Keywords *Aspergillus*, polyphasic taxonomy, extrolites, species concept, classification

Introduction

When Raper and Fennell [1] published a monograph of the genus *Aspergillus* they accepted 132 species subdivided into 18 groups. The generic and species concepts were well circumscribed and this monograph

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is still valuable and useful. However, many new species have been described and the relationships of *Aspergillus* with its teleomorphs have been elucidated. The species and varieties described since 1965 were critically reviewed [2–4]. In recent years several new taxa have been described [5] and a high number of new methods for classification and identification have been suggested and used. As *Aspergillus* is an extremely important genus concerning pathogenicity, mycotoxins, fundamental eukaryotic genetics and biotechnological exploration, several books have been dedicated to *Aspergillus* in general [6–10] and to the taxonomy and phylogeny of *Aspergillus* in particular [11–13].

Traditional *Aspergillus* taxonomy, based on phenotypical characters only, gives mostly a satisfactorily delimitation of the taxa. However, in several sections of the genus much morphological variation occurs, resulting in debatable taxonomic schemes. The taxonomy of *Aspergillus* and its teleomorphs has now recently been re-investigated by using a polyphasic approach in order to examine the variability within the species [14–17]. *Aspergillus* strains were studied by their macro-, and micro- morphology, growth characters, extrolite profiles and β-tubulin gene sequences. In section *Fumigati* also calmodulin and actin genes were sequenced, while Random Amplified Polymorphic DNA (RAPD) and other fingerprinting techniques have been used. In this paper we review the most recent taxonomies in *Aspergillus* sect. *Fumigati, Circumdati, Flavi* and *Nigri*, as these contain the most important human pathogenic Aspergilli and also the biotechnologically important filamentous fungi. The use of extrolites has become an important part of our investigations demonstrating that each *Aspergillus* taxon is characterized by a specific profile. Furthermore characterization of these chemical compounds also provides important information for the presence of mycotoxins. Extrolites were analysed by HPLC using alkylphenone retention indices and diode array UV-VIS detection [18,19].

Aspergillus Section *Fumigati*

A. fumigatus is by far the most important pathogenic *Aspergillus* species known [6,20,21] and until recently this was the only species that was considered pathogenic from *Aspergillus* section *Fumigati*. However, *Neosartorya pseudofischeri* was also reported as a potential human pathogen [22–24] and recently *A. lentulus* was described as a pathogen related to *A. fumigatus* [25].

A. lentulus is clearly separated based on multilocus sequence typing of five genes (the β-tubulin gene, the rodlet A gene, the salt-responsive gene, the mitochondrial cytochrome b gene, and the internal transcribed spacer regions [25]. In our studies [16] with many strains from soil we found that *A. lentulus* differs from *A. fumigatus* by thinner stipes, smaller and predominantly (sub)globose vesicles and growth at 10°C. In contrast with the original description we also noticed that the strains of *A. lentulus* do grow rapidly with similar growth rate as *A. fumigatus*. *A. lentulus* seems to have a wide geographical distribution and can be isolated from common places as soil and air, although it was only reported from clinical strains [25]. In our β-tubulin, calmodulin and actin analysis two groups, each with two strains were separated from *A. fumigatus* sensu stricto, *A. lentulus* and *N. fischeri*. These strains were proposed as new taxa [16]: *A. fumigatiaffinis* has comparatively small (sub)globose vesicles (16–24 μm) while *A. novofumigatus* has nearly flask shaped vesicles and comparatively large vesicle (15–30 μm). On the temperature growth regimes, the two species grew at 10°C and did not grow at 50°C. Also in their extrolite profiles these taxa differ from *A. fumigatus* sensu stricto and *A. lentulus*.

A. fumigatus is variable in macro- and micromorphology, and it is difficult to differentiate from the other closely related species only based on morphology. Fig. 1 illustrates the conidiophores and conidia of the four species showing that they are microscopically very similar. Nevertheless, *A. fumigatus* can be characterized by having usually subclavate vesicles while *A. lentulus, A. fumigatiaffinis* and *A. novofumigatus* usually have globose shaped vesicles. *A. fumigatus* grows at 50°C but do not at 10°C. Genetically *A. fumigatus* seems to be a very homogeneous taxon and unique and clearly separated from related species based on molecular characters including β-tubulin, actin and calmodulin gene sequences. Fig. 2 shows a combined tree with β-tubulin, calmodulin and actin gene sequences and the clear grouping of *A. fumigatus* and related anamorphic species. Therefore multilocus gene sequence could be critical determinants for the delineation of *A. fumigatus*.

A. fumisynnematus Horie *et al*. [26] was considered as a separate species because of its short conidiophores borne on funiculose aerial hyphae. It clustered separately from the other taxa in the section *Fumigati* because of its cytochrome b sequences [27]. However we are unable to confirm the taxonomy of this taxon because the type isolate is not available. Another *A. fumigatus*-like fungus from liver lesions of a dairy cow in Finland with a brown colony colour was described as *A. arvii* Aho *et al*. [28]. Although the description suggests that this taxon belongs in section *Fumigati* the type isolate is not available for study to determine it taxonomic position. Three

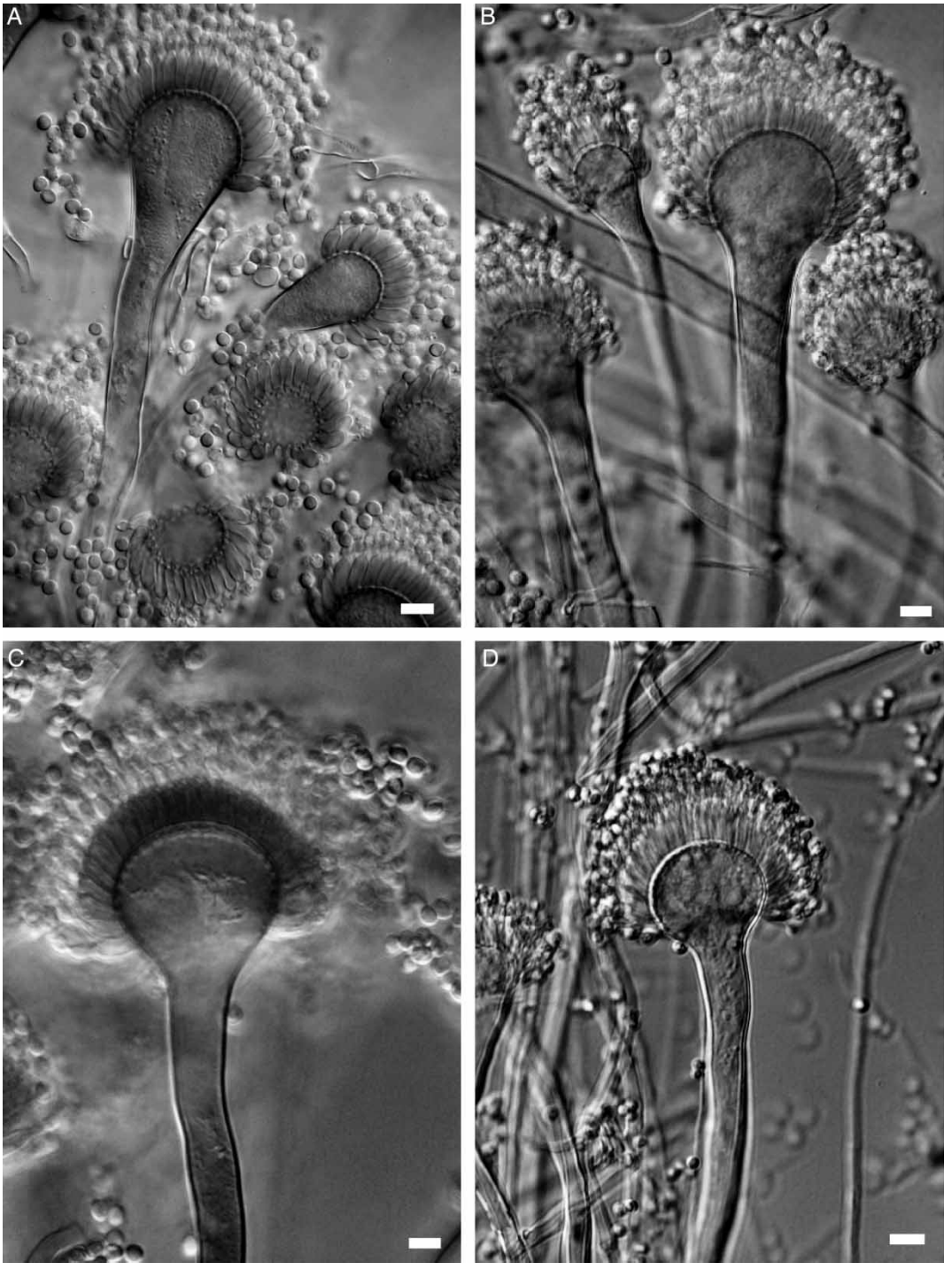

Fig. 1 Conidiophores of (A) *Aspergillus fumigatus*, (B) *Aspergillus lentulus*, (C) *Aspergillus novofumigatus* and (D) *Aspergillus fumigatiaffinis*.

new *Aspergillus* species from clinical sources as a causal agent of human aspergillosis in China were proposed [29]. The species are characterized by yellow to greyish green, olive yellow or dull green conidial suggesting *A. fumigatus* strains with abortive conidial structures. The strains are unavailable for comparison and are regarded as doubtful.

A. fumigatus strains produce a large number of extrolites. As for all filamentous fungi examined in detail, individual isolates produce a large number of

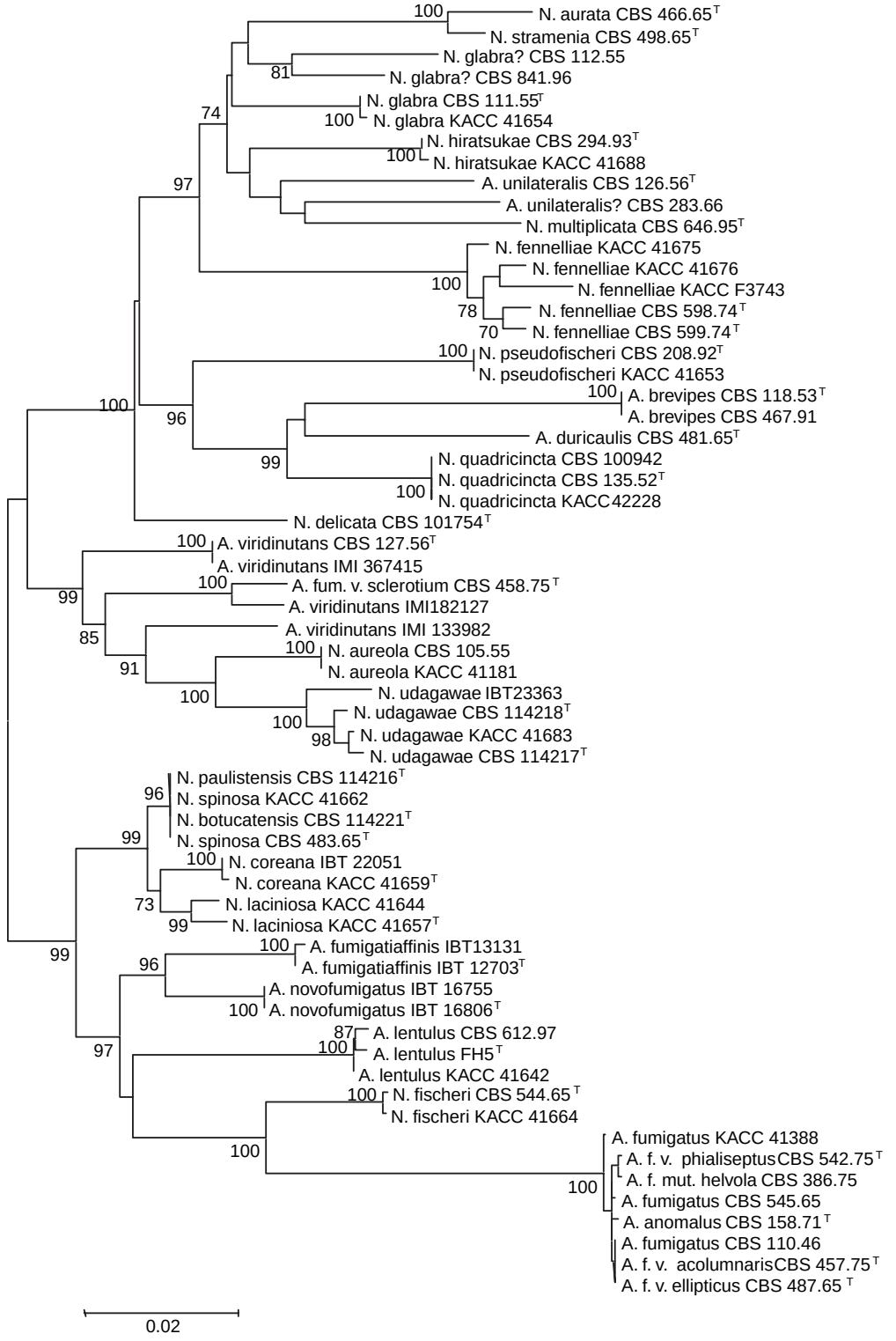

Fig. 2 Combined tree with β-tubulin, calmodulin and actin gene sequences of type and representative strains of *Aspergillus* section *Fumigati.* Three genes were concatenated and aligned by CLUSTAL W. Each output alignment was used for combined Neighbor-Joining Tree. The sequences were first analyzed using the Tamura-Nei parameter distance calculation model with gamma-distributed substitution rates, which was then used to construct the tree with MEGA version 3. Bootstrap indices, when higher than 70%, are indicated above or below each ramification node.

common species-specific extrolites in a qualitative sense [30]. An important metabolite is gliotoxin, which is one of the extrolites involved in pathogenesis [21,31,32], but other diffusible extrolites may also be important [21,33]. Further metabolites from *A. fumigatus* include fumagillin, helvolic acid, fumitremorgins and verruculogen, tryptoquivalins, fumigatin, pseurotins, pyripyropens, fumigaclavins, fumiquinazolins, trypacidin, and monomethylsulochrin [34–38].

In Table 1, the extrolite profiles of *A. fumigatus sensu stricto, A. lentulus* and related species are listed. Strains of *A. fumigatus sensu stricto* produced fumagillin, fumitremorgins, fumiquinazolins, gliotoxin, pseurotins, trypacidin and verruculogen, which are absent in *A. lentulus, A. fumigatiaffinis* and *A. novofumigatus*. Strains of *A. lentulus* produced cyclopiazonic acid and dimeric indoles showing its uniqueness in *Aspergillus* section *Fumigati*, while auranthine was shared with *A. fumigatiaffinis* and fiscalins with *A. novofumigatus*. Neosartorin was produced by *A. lentulus*, *A. fumigatiaffinis* and *A. novofumigatus*, but not by *A. fumigatus*. Pyripyropens, tryptoquivalins and tryptoquivalons are produced by *A. fumigatus*, *A. lentulus* and *A. novofumigatus*. *A. fumigatiaffinis* and *A. novofumigatus* shared an unknown compound with a characteristic UV spectrum and cycloechinuline.

Aszonalenin, fiscalins and neosartorin can also be found as extrolites in *Neosartorya fischeri* showing that these anamorph taxa are chemically related and indicating that in anamorph species teleomorph structures may be discovered in the future. The production of apolar indole alkaloids (of unknown structure) by *A. novofumigatus* further indicates that this taxon could have a capability to produce ascomata or sclerotia given the right conditions.

Ascospore ornamentation is an important morphological character in distinguishing species of the ascomycete genus *Neosartorya* [39] and on this basis several new taxa were described. In a study of isolates from Korean soils, it was demonstrated that *N. spinosa* showed low similarity with *N. glabra* but showed strong resemblance with two new taxa: *N. coreana* and *N. laciniosa* [16]. Based on a polyphasic study it was further concluded that *N. botucatensis* and *N. paulistensis* were identical with *N. spinosa*. Based on partial β-tubulin and calmodulin sequences and ascospore morphology two separate groups were distinguished and proposed as new species: *N. laciniosa* has

Table 1 Comparison of the profiles of extrolites produced by *Aspergillus fumigatus, Aspergillus lentulus* and related species [from 16].

Extrolites	*Aspergillus fumigatus*	*Aspergillus lentulus*	*Aspergillus fumigatiaffinis*	*Aspergillus novofumigatus*	*Neosartorya fischeri*
Apolar indolalkaloids*	–	–	–	+	–
Apolar unknown polyketide*	–	–	–	+	–
Aszonalenin*	–	–	–	+	+
Auranthine	–	+	+	–	–
Cycloechinuline	–	–	+	+	–
Cyclopiazonic acid	–	+	–	–	–
Dimeric indole*	–	+	–	–	–
Fiscalins*	–	+	–	+	+
Fischerin*	–	–	–	–	+
Fumagillin	+	–	–	–	–
Fumigaclavines	+	–	+?	–	–
Fumigatin	+/–	–	–	–	–
Fumitremorgins & verruculogen	+	–	–	–	+
Fumiquinazolins*	+	–	–	–	–
Gliotoxin	+	–	–	–	–
Helvolic acid	+	–	+	+	+
Neosartorin*	–	+	+	+	+
Palitantin	–	–	+	+	–
Pseurotins	+	–	–	–	–
Pyripyropens	+	+	+	–	+
Terrein	–	+	–	+	–
Territrem B	–	–	–	+/–	–
Trypacidin	+	–	–	–	+
Tryptoquivalins and tryptoquivalons	+	+	+	–	+
Unknown compound	–	–	+	+	–

*Based on UV spectrum evidence.

Table 2 Accepted species in *Aspergillus* section *Fumigati* and their synonyms.

Strictly anamorph species

Aspergillus brevipes G. Sm.
Aspergillus duricaulis Raper & Fennell
Aspergillus fumigatus Fresen.
=*A. anomalus* Pidoplichko & Kirilenko
=*A. fumigatus* var. acolumnaris Rai et al.
=*A. fumigatus* var. *ellipticus* Raper & Fennell
=*A. fumigatus* mut. *helvola* Rai et al.
=*A. phialiseptus* Kwon–Chung
=*A. neoellipticus* Kozak.
Aspergillus fumigatiaffinis S.B. Hong, Frisvad & Samson
Aspergillus lentulus S.A. Balajee & K.A. Marr
Aspergillus novofumigatus S.B. Hong, Frisvad & Samson
Aspergillus unilateralis Thrower
=*A. brevipes* var. *unilateralis* (Thrower) Kozakiewicz
Aspergillus viridinutans Ducker & Thrower
=*A. fumigatus* var. *sclerotium*

Doubtful species and considered to be invalid

? *Aspergillus arvii* Aho et al.
? *Aspergillus beijingensis* D.–M. Li, Horie, Y.-Z. Wang R.-Y Li
? *Aspergillus fumisynnematus* Y. Horie, Miyaji, Nishim., Taguchi & Udagawa
? *Aspergillus qizutongii* D.-M. Li, Horie, Y.-Z. Wang R.-Y Li
? *Aspergillus wangduanlii* D.-M. Li, Horie, Y., Y.-Z. Wang R.-Y Li

Neosartorya

Neosartorya aurata (Warcup) Malloch & Cain [anamorph: A. igneus Kozak.]
Neosartorya aureola (Fennell & Raper) Malloch & Cain [anamorph: A. aureoluteus Samson & Gams]
Neosartorya coreana S.B. Hong, Frisvad & Samson, [anamorph: *A. coreanus* S.B. Hong et al.]
Neosartorya fennelliae Kwon-Chung & S.J. Kim [anamorph: *A. fennelliae* Kwon-Chung & S.J. Kim]
?=*Neosartorya otanii* Takada, Y. Horie & Abliz [anamorph: *A. otanii* Takada et al.]
Neosartorya fischeri (Wehmer) Malloch & Cain [anamorph: *A. fischeranus* Kozak.]
Neosartorya glabra (Fennell & Raper) Kozak. [anamorph: *A. neoglaber* Kozak.]
Neosartorya laciniosa S.B. Hong, Frisvad & Samson, [anamorph: *A. laciniosus* S.B. Hong et al.]
Neosartorya spinosa (Raper & Fennell) Kozak. [anamorph: *A. spinosus* Kozak.]
= *Aspergillus fischeri* var. *spinosus* Raper & Fennell 1965 (basionym)
= *Sartorya fumigata* var. *verrucosa* Udagawa & Kawasaki
= *Neosartorya botucatensis* Y. Horie, Miyaji & Nishim. [anamorph: *A. botucatensis* Y. Horie et al.]
= *Neosartorya paulistensis* Y. Horie, Miyaji & Nishim. [anamorph: *A. paulistensis* Y. Horie et al.]
? = *Neosartorya takakii* Horie, Abliz & K. Fukush. [anamorph: *A. takakii* Horie et al.]
Neosartorya quadricincta (J.L. Yuill) Malloch & Cain [anamorph: *A. quadricingens* Kozak.]
=?*Neosartorya primulina* Udagawa, Toyaz. & Tsub. [anamorph: *A. primulinus* Udagawa et al.]
Neosartorya stramenia (R.O. Novak & Raper) Malloch & Cain [anamorph: *A. paleaceus* Samson & Gams]
Neosartorya spathulata Takada & Udagawa [anamorph: *A. spathulatus* Takada & Udagawa]
Neosartorya hiratsukae Udagawa, Tsub. & Y. Horie [anamorph: *A. hiratsukae* Udagawa et al.]
Neosartorya pseudofischeri S.W. Peterson [anamorph: *A. thermomutatus* (Paden) Petersen]
Neosartorya tatenoi Horie, Miyaji, Koji Yokoy., Udagawa & Camp.-Takagi [anamorph: *A. tatenoi* Horie et al.]
=?*Neosartorya delicata* H.Z. Kong [anamorph: A. delicatus H.Z. Kong]
Neosartorya multiplicata Yaguchi, Someya & Udagawa [anamorph: *A. muliplicatus* Yaguchi et al.]
Neosartorya udagawae Horie, Miyaji & Nishim. [anamorph: *A. udagawae* Horie et al.]
Neosartorya sublevispora Someya, Yaguchi & Udagawa [anamorph: *A. sublevisporus* Someya et al.]

Doubtful species and considered to be invalid

?*Neosartorya nishimurae* Takada, Y. Horie & Abliz [anamorph: *A. nishimurae* Takada et al.]
?*Neosartorya indohii* Y. Horie [anamorph: *A. indohii* Y. Horie]
?*Neosartorya tsurutae* Y. Horie [anamorph: *A. tsurutae* Y. Horie]

microtuberculate ascospores with two bent crests and two distinct equatorial rings of small projections. *N. coreana* has rugose to weak reticulate ascospores with two often bent crests but without the equatorial rings of small projections [16]. The separation of these two new taxa was also supported by their specific extrolite profile. In Fig. 2 the phylogeny of *Neosartorya* and related taxa in section *Fumigati* is shown in a

Table 3 Species in *Neopetromyces* and *Aspergillus* subgenus *Circumdati* section *Circumdati* and their mycotoxin and mellein production [from 14].

Species	Ochratoxins	Penicillic acid	Xanthomegnins	Mellein
(Aspergillus robustus)	–	–	–	–
Aspergillus auricomus	–	+	+	+
Aspergillus bridgeri	–	+	+	–
Aspergillus cretensis	+	+	–	+
Aspergillus elegans	–	–	+	–
Aspergillus flocculosus	+	+	+	+
Aspergillus insulicola	–	+	+	+
Aspergillus melleus	–	+	+	+
Aspergillus neobridgeri	–	+	+	–
Aspergillus ochraceus	+/–	+	+	+
Aspergillus ostianus	–	+	+	+
Aspergillus persii	–	+	+	–
Aspergillus petrakii	–	+	+	+
Aspergillus pseudoelegans	+	+	–	+
Aspergillus roseoglobulosus	+	+	+	–
Aspergillus sclerotiorum	+/–	+	+	–
Aspergillus steynii	+	–	+	+
Aspergillus sulphureus	+	+	+	–
Aspergillus westerdijkiae	+	+	+	+
Neopetromyces muricatus	+	+	+	–

combined tree with partial β-tubulin, calmodulin and actin tree showing a clear separation of the accepted taxa. In combination with characters of morphology, physiology and extrolites a list of accepted species with synonyms is presented in Table 2.

Occasionally cultures ex type of newly described taxa of *Aspergillus, Emericella* and *Neosartorya* are difficult to obtain. For example the sequences of *Neosartorya otanii* Takada *et al*. [40] when blasted in GenBank showed 100% homology with the type of *N. fennelliae* and the description of the species is not different from *N. fennelliae*. This indicates that the species is synonymous with *N. fennelliae*, but mating experiments, requiring a living culture, should be carried out to confirm this. Analysis of extrolites will also require a living culture.

The speciation in *A. fumigatus* and related taxa has been subject for more detailed molecular studies. Pringle *et al*. [41] found cryptic speciation in *A. fumigatus* with two phylogenetic taxa with a global distribution, while Katz *et al*. [42] demonstrated multiple genetically distinct groups among clinical isolated of *A. fumigatus*. Now *A. fumigatus* has been full genome sequenced [43], this will allow for detailed studies of the physiology and differentiation of this species [44].

Aspergillus Section *Circumdati*

Members of *Aspergillus* section *Circumdati* (= *A. ochraceus* group) are important because of their production of several mycotoxins including ochratoxin A [45–48], penicillic acid [48], xanthomegnin, viomellein and vioxanthin [49,50]. Some of the species are also used in the bioindustry, especially for biotransformations [51,52]. The group has been taxonomically revised by Christensen & Raper [53,54]. Later Varga *et al*. [55–58] presented molecular analysis of the genetic variability in species of this section. A few new species have been described *A. sepultus* Tuthill & Christensen [59], *Neopetromyces muricatus* [37,60], and *A. persii* Zotti & Montemartini Corte [61].

Based on rDNA sequence data Peterson [62] indicated that *Petromyces alliaceus*, *P. albertensis*, and *A. lanosus* belong to section *Flavi,* which was supported by phenotypic data [37]. Another species originally placed in section *Circumdati* was *A. campestris*. This species was transferred to section *Candidi* [62–64]. Finally *A. dimorphicus* [65] and *A. sepultus* were transferred to section *Wentii* [37,62], leaving a homogeneous series of very closely related fungi in section *Circumdati* with only *A. robustus* being different from the remaining species in the section. The number of species in section *Circumdati* is currently 19 in addition to *A. robustus* [66].

This group of organisms is especially well known for its production of ochratoxin A, named after the producer *A. ochraceus* CBS 263.67 [45,46] and has attracted much interest by its role in the ochratoxin contamination of coffee [66–79]. Interestingly CBS 263.67 = NRRL 3174 has now been re-identified as *A. westerdijkiae* [14], so many of the species reported

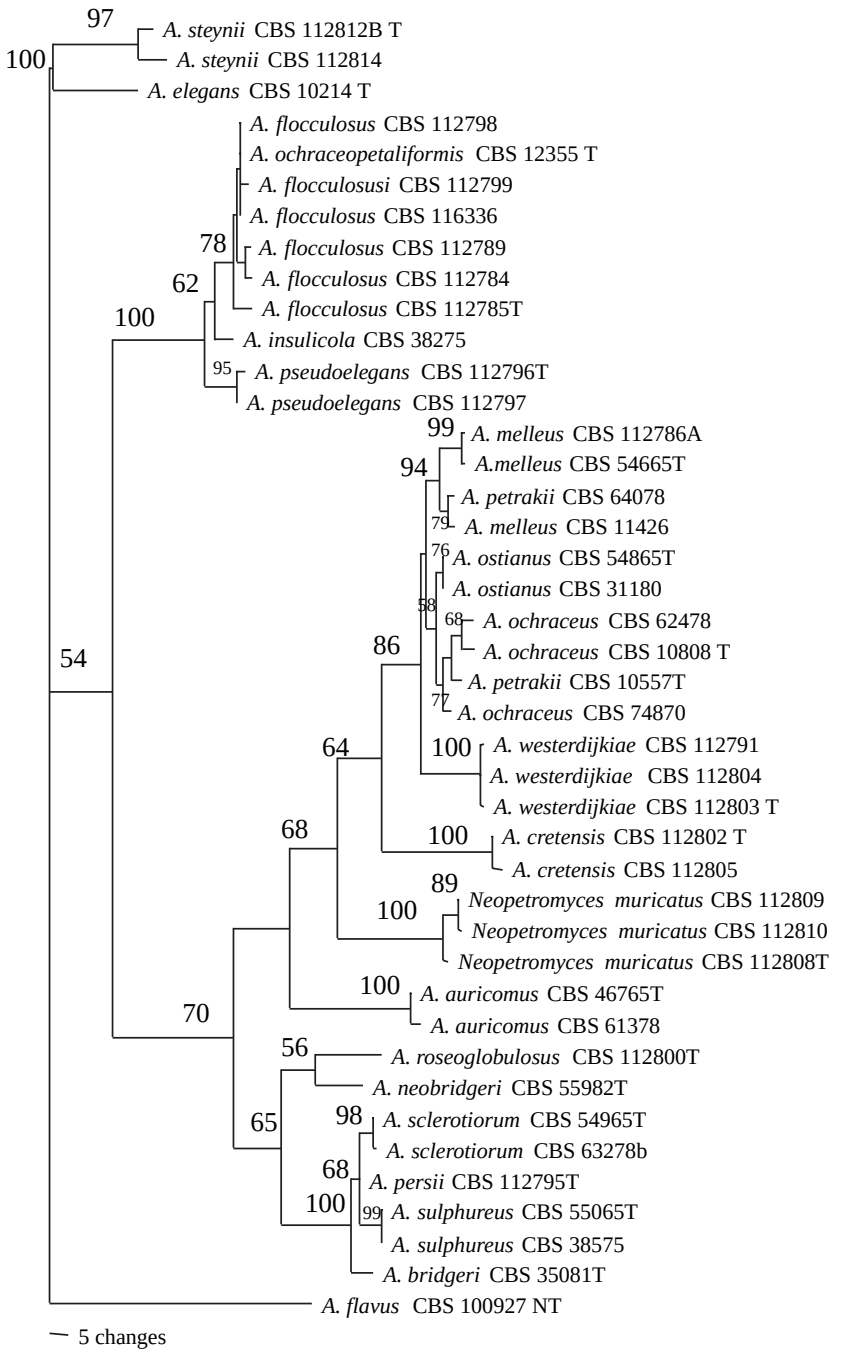

Fig. 3 One of the 5000 equally MPT of 517 steps based on heuristic search partial beta-tubulin sequences of type and representative strains of *Aspergillus* section *Circumdati* with *Aspergillus flavus* as an outgroup The branches in bold are 100% in the 70% majority-rule consensus of equally parsimonious trees. The numbers represent bootstrap percentages >50% (Ci = 0,654, Ri = 0,884 Rc = 0,578, Hi = 0,346). From [14].

as *A. ochraceus* may indeed be *A. westerdijkiae*. *A. ochraceus* and ochratoxin A have also been detected in rice [80,81]. On the other hand *A. ochraceus* and allied species are less common on wheat and corn [82–84]. Mycotoxins from *A. ochraceus* can cause mycotoxicoses in mice and swine when grown on rice [85]. Furthermore members of the section have been found on salted or dried fish [46]. Other members of section *Circumdati sensu stricto* have been reported to produce ochratoxin A: *A. auricomus, A. elegans*, *A. insulicola*, *A. melleus*, *A. ostianus*, *A. petrakii*, *A. sclerotiorum*, *A. sulphureus*, and *Neopetromyces muricatus* [37,45,48, 71,77,86]. However, there is much confusion in the literature about the production of mycotoxins by other members of the section, indicating that many strains have earlier been regarded as intermediate forms between known species [46,47]. We believe that such intermediate forms between species do not exist, but

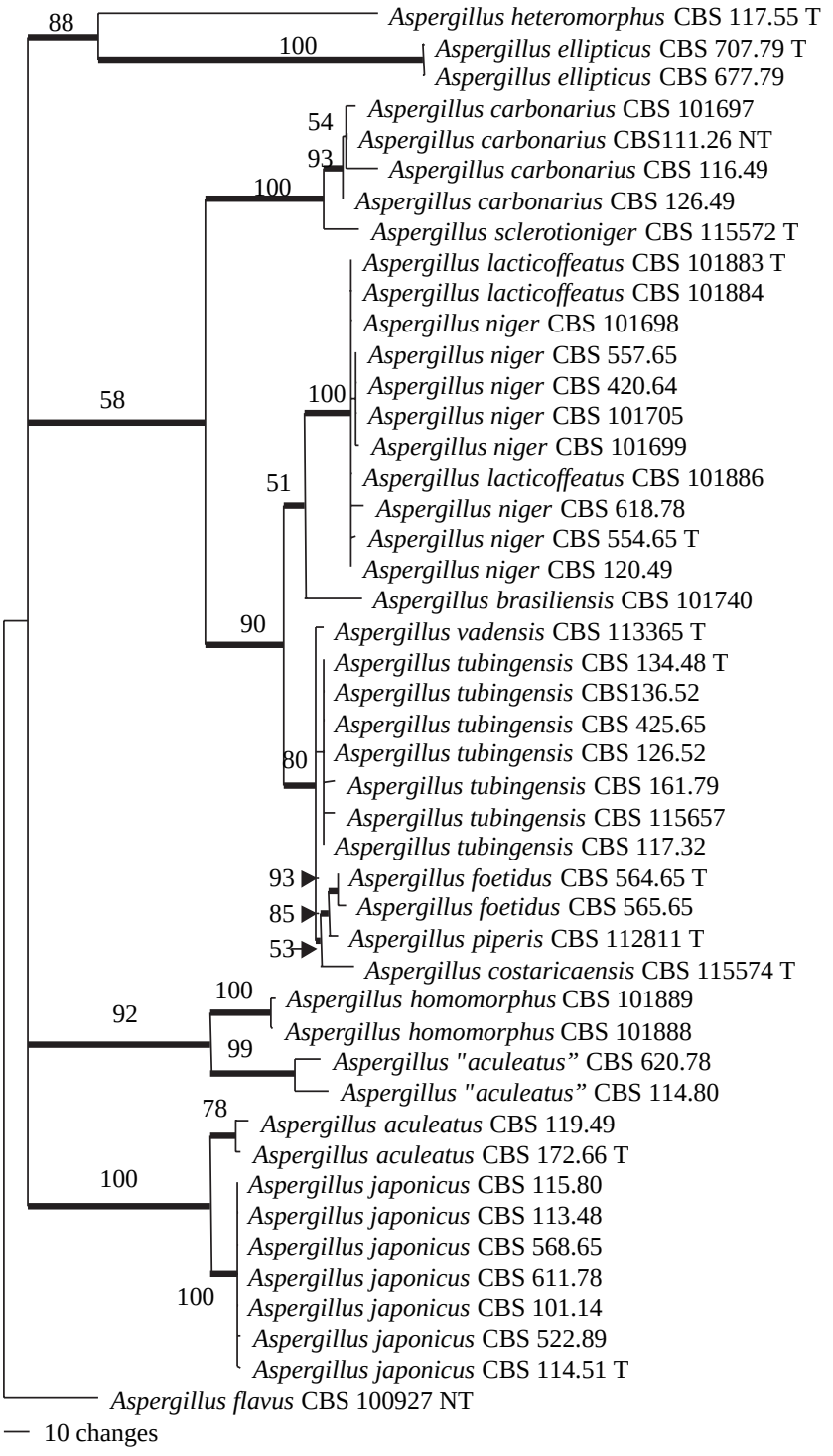

Fig. 4 One of the 5000 equally MPT of 517 steps based on heuristic search partial beta-tubulin sequences of type and representative strains of *Aspergillus* section *Nigri* with *Aspergillus flavus* as an outgroup The branches in bold are 100% in the 70% majority-rule consensus of equally parsimonious trees The numbers represent bootstrap percentages >50% (Ci = 0,654, Ri = 0,884 Rc = 0,578, Hi = 0,346). From [15].

that the use of only one kind of feature, such as morphology, may render a species look superficially like an intermediate form. During our mycological investigations of various substrates we have collected several new taxa of the section, including some which have formerly been identified as *A. ochraceus*. Furthermore an overview of the mycotoxins produced by species assigned to this section has been published [14].

Table 4 Production of sclerotia, ochratoxin A and other extrolites by species in *Aspergillus* section *Nigri* [from 15].

Species	Ochratoxin A	Pyranonigrin	N-γ-P[1]	Asp[2]	SeD[3]	Ant[4]	Afl[5]	Cor[6]	Kot[7]
Aspergillus aculeatus	–	–	–	–	+	–	–	–	–
Aspergillus brasiliensis	–	–	+	–	–	–	–	–	–
Aspergillus carbonarius	+	+	+	–	–	–	–	–	–
Aspergillus costaricaensis	–	–	+	–	–	–	+	+	–
Aspergillus ellipticus	–	–	–	–	–	–	–	–	–
Aspergillus foetidus	–	+	+	+	–	+	–	–	–
Aspergillus heteromorphus	–	–	–	–	–	–	–	–	–
Aspergillus homomorphus	–	–	–	–	+	–	–	–	–
Aspergillus japonicus	–	–	–	–	–	–	–	–	–
Aspergillus lacticoffeatus	+	+	–	–	–	–	–	–	+
Aspergillus niger	+/–	+	+	–	–	–	–	–	+/–
Aspergillus piperis	–	+	+	–	–	–	+	–	–
Aspergillus sclerotioniger	+	+	+	–	–	–	–	+	–
Aspergillus tubingensis	+/–	+	+	+	–	–	–	–	–
Aspergillus vadensis	–	–	+	+	–	–	–	–	–

[1]N-γ-P: Naphtho-γ-pyrones; [2]Asp = asperazine; [3]SeD = secalonic acid D; [4]Ant = antafumicin; [5]Afl = aflavinines; [6]Cor = Corymbiferan lactones; [7]Kot = Kotanins (kotanin, desmethylkotanin, orlandin).

Aspergillus section *Circumdati* contains species with yellow to ochre conidia and non-black sclerotia that produce at least one of the following extrolites: ochratoxins, penicillic acids, xanthomegnins or melleins. The exception to this is *A. robustus*, which produces black sclerotia, phototropic conidiophores and none of the extrolites listed above. Based on a polyphasic approach using morphological characters, extrolites and partial β-tubulin sequences (Fig. 3), 20 species can be distinguished, that, except for *A. robustus*, are phylogenetically and phenotypically strongly related. Seven new species were described, *A. cretensis*, *A. flocculosus*, *A. neobridgeri*, *A. pseudoelegans*, *A. roseoglobulosus*, *A. steynii*, and *A. westerdijkiae* [14]. Twelve species of section *Circumdati* produce mellein, 17 produce penicillic acid and 17 produce xanthomegnins. Eight species consistently produce large amounts of ochratoxin A: *A. cretensis*, *A. flocculosus*, *A. pseudoelegans*, *A. roseoglobulosus*, *A. westerdijkiae*, *A. sulphureus*, and *Neopetromyces muricatus*. Two species produce large or small amounts of ochratoxin A, but less consistently: *A. ochraceus* and *A. sclerotiorum*. Ochratoxin production in these species has been confirmed using HPLC with diode array detection and comparison to authentic standards. Four further species produce ochratoxin A inconsistently and in trace amounts according to the literature: *A. melleus*, *A. ostianus, A. petrakii*, and *A. persii*. In Table 3 the accepted species of section *Circumdati* are listed with their extrolites production. The most important species regarding potential ochratoxin A production in coffee, rice, beverages and other foodstuffs are *A. ochraceus, A. westerdijkiae* and *A. steynii*.

Aspergillus Section *Nigri*

The black aspergilli are among the most common fungi causing food spoilage and biodeterioration of other materials. They have also been extensively used for various biotechnological purposes, including production of enzymes and organic acids [87]. The taxonomy of *Aspergillus* section *Nigri* has been studied by many taxonomists and was recently reviewed [88]. Mosseray [89] described 35 species black aspergilli, while Raper and Fennell [1] reduced the number of species accepted within their *A. niger* group to 12. Al-Musallam [90] revised the taxonomy of the *A. niger* group, primarily based on morphological features. She recognized seven species (*A. japonicus*, *A. carbonarius*, *A. ellipticus*, *A. helicothrix*, *A. heteromorphus*, *A. foetidus*, *A. niger*), and described *A. niger* as an aggregate consisting of seven varieties and two formae. Kozakiewicz [38] distinguished *A. ellipticus*, *A. heteromorphus*, *A. japonicus*, *A. helicothrix*, *A. atroviolaceus* and *A. carbonarius* as species exhibiting echinulate conidial ornamentations distinct from the remaining black *Aspergillus* taxa, which produce verrucose conidia. Within the verrucose category, *A. fonsecaeus*, *A. acidus, A. niger* var. *niger*, *A. niger* var. *phoenicis*, *A. niger* var. *ficuum*, *A. niger* var. *tubingensis*, *A. niger* var. *pulverulentus*, *A. niger* var. *awamori*, *A. citricus* (*A. foetidus*) and *A. citricus* var. *pallidus* were recognized. Currently 14 species are accepted [15] but several taxa are under study and will be described in the near future.

A. niger is the most frequently reported species in this section, and has often been included in biotechnological processes that are Generally Regarded as Safe (GRAS). However, species concepts are uncertain in

Table 5 Production of aflatoxins and other extrolites by species in *Aspergillus* section *Flavi*.

	Kojic acid	Aflatoxin B type	Aflatoxin G type	Cyclopiazonic acid	Aspergillic acid	Ochratoxin A	Chrysogine	Parasi-ticolide
Aspergillus arachidicola	+	+	+	−	+	−	+	+
Aspergillus avenaceus	−	−	−	−	−	−	−	−
Aspergillus bombycis	+	+	+	−	+/−	−	−/+	−
Aspergillus caelatus	+	−	−	+	−	−	−	−
Aspergillus flavus	+	+/−	−	+	+	−	−	−
Aspergilus lanosus	+	−	−	−	−	−	−	−
Aspergillus leporis	+	−	−	−	−	−	−	−
Aspergillus minisclerotium	+	+	+	+	+	−	−	−
Aspergillus nomius	+	+	+	−	+	−	−/+	−
Aspergillus oryzae (domesticated from *A. flavus*)	+	−	−	+	−	−	−	−
Aspergillus parasiticus	+	+	+	−	+	−	−	+
Aspergillus parvisclerotigenus	+	+	+	+	+	−	−	−
Aspergillus pseudotamarii	+	+	−	+	−	−	−	−
Aspergillus sojae (domesticated from *A. parasiticus*)	+	−	−	−	+	−	−	−
Aspergillus tamarii	+	−	−	+/−	−	−	−	−
Aspergillus toxicarius	+	+	+	−	+	−	−	+
Petromyces albertensis	+	−	−	−	−	+	−	−
Petromyces alliaceus	+	−	−	−	−	+	−	−

this complex and occasionally the name *A. niger* has been used for any member of the section. Taxonomic studies using molecular methods have divided the *A. niger* complex into two species, *A. niger* and *A. tubingensis* [for overview see 88]. Some further species have been described but not considered in revisions or reviews. *A. ellipsoideus* was described as a new species with ellipsoidal greyish black conidia [91]. Recently, a new species *A. vadensis,* with a different extrolite profile, colony characters and unusually low citric acid production, was proposed [92].

Ueno *et al*. [93] were the first to report on ochratoxin A (OA) production by a black *Aspergillus* species, *A. foetidus,* even though OA production by this particular species has not been confirmed later: The *A. foetidus* reported to produce ochratoxin later [94,95] was actually an *Aspergillus niger*. Abarca *et al*. [96] reported that two strains of *A. niger* produced OA, which was confirmed in numerous studies [75,94,97–107]. Horie [108] reported OA in *A. carbonarius*, which was later confirmed [75,94,104,105,109–119] about 160 black *Aspergillus* strains from collections and from field isolates for OA production were tested using an immunochemical method and thin layer chromatography [110]. The strains examined included 12 *A. carbonarius* and 45 *A. japonicus* strains from culture collections and field isolates from all over the world, including about 100 strains belonging to the *A. niger* species complex.

Ochratoxin A production was detected in about 6% of the strains from the *A. niger* species complex [94,95]. Of the 13 *A. carbonarius* strains tested, six produced both OA and ochratoxins B [94,109]. *A. ellipticus*, *A. heteromorphus*, *A. japonicus* and *A. tubingensis* strains did not produce detectable amounts of ochratoxins. However, *A. japonicus* was later claimed to produce OA [103,114], a claim that has later been questioned [102,115,116]. Recently *Aspergillus tubingensis* has also been claimed to produce ochratoxin A by at least three different sources [115–117].

Aspergillus section *Nigri* includes some of the most important species for biotechnology and its species are of widespread occurrence. In addition, based on a polyphasic approach using traditional characters, extrolites and β-tubulin sequencing, a provisional revision and synoptic key of section *Nigri* was proposed (Fig. 4) [15]. Fifteen species were accepted, four of these produce ochratoxin A. Ochratoxin A producing species of section *Nigri* occurring on grapes, raisins and in wine include *A. carbonarius* and to a lesser extent *A. niger*. Four species recovered from coffee, viz. *A. carbonarius*, *A. niger*, *A. lacticoffeatus* and *A. sclerotioniger*, all produce ochratoxin A, but other species of *Nigri* also occur on this substrate, including *A. japonicus* and *A. tubingensis*. The 10 species not producing ochratoxin A are especially interesting for biotechnological exploration, as many other extrolites are produced by these species.

Aspergillus Section *Flavi*

Raper and Fennell [1] described the *Aspergillus flavus* group with nine species and two varieties, mainly based on the colour of the conidial heads and ornamentation of the conidia. Various studies have been done [for review see 13]. Apart from the three species with yellow to ochre conidia, *Petromyces alliaceus*, *P. albertensis* and *A. lanosus* [37], the green to brown coloured species in section *Flavi* now include *Aspergillus flavus*, *A. parasiticus*, *A. oryzae, A. sojae, A. tamarii*, *A. leporis*, *A. caelatus*, *A. nomius* [120], *A. pseudotamarii* [121], *A. bombycis* [66,122], *A. toxicarius* [66,123] and *A. parvisclerotigenus* [66,124]. *A. avenaceus* seems to be an outgroup to all these species [62] and other species such as *A. zonatus* and *A. clavatoflavus* first included in section *Flavi* [1,125] were later shown to be quite distantly related to this section [62]. *Aspergillus coremiiformis* and the closely related fungus *A. togoensis* (*Stilbothamnium togoensis*) appear to be closely related to section *Flavi* however [66,126,127]. *A. flavus* and *A. parasiticus* are by far the most common and most important aflatoxins producers in foods and feeds, but there have been indications that these two species may contain more than one species each [128,129]. Using a polyphasic approach with strains isolated from Argentina, two new taxa, *A. minisclerotiorum* and *A. arachidicola* will be proposed (Pildain *et al*., unpublished data). The section *Flavi* currently contains 18 accepted species of which two are domesticated forms. In Table 5 an overview is given of the extrolite production.

One species, *A. oryzae*, has been fully genome sequenced [130] and therefore studies of this species can be made very focused, specifically using DNA arrays and comparison with other Aspergilli [44]. *A. flavus* NRRL 3357 will also soon be fully genome sequenced.

Modern methods for *Aspergillus* identification

Several new phenotypic and genotypic methods are available for classifying, cladifying, identifying and fingerprinting *Aspergillus* species [30]. Among the phenotypic methods are the accurately photo documented image analysis of fungal colonies (colours and texture) [131]. By feature extraction, accurate colours and textures can be recorded for taxonomic purposes [132]. Extrolites, especially secondary metabolites, have been tested for consistency on a large number of strains and can often be used for identification of fungi, at least in pure culture [30]. Profiles of secondary metabolites are very characteristic for each species [see Table 1,3–5] and mostly very consistent from isolate to isolate. On the other hand, all secondary metabolites from the profile known from pure cultures are not necessarily produced in lung tissue, so new and sensitive methods have to be developed for accurate identification in each case. Selective ion monitoring MS may be a good method in such cases [31,32]. Gliotoxin has been detected in mouse lungs this way, but *A. fumigatus* is known to produce several other mycotoxins (Table 1).

Systems such as pyrolysis gas chromatography mass spectrometry (Py-GC-MS) and fatty acid methyl ester (FAME) analysis have been used for fungal identification [see for example 132], but these methods have not been validated on a sufficient number of isolates and species to be of any use in genus or species identification yet.

FTIR, NIR and related methods may also be used for identification and this has been proposed for the identification of some *Aspergillus* and *Penicillium* species [133]. Modern chemometric methods may be used for the evaluation of such data including 2D gel electrophoretic patterns and NMR spectra of extracts of the fungi or plants to be identified [134]. Again these methods should be validated on a large number of isolates in each relevant species for the problem at hand.

Immunological methods are not straightforward, as several cross reactions will occur [135], for example between the closely related species in *Aspergillus* section *Fumigati*. Immunological methods may work if the fungus causing disease is *A. fumigatus* with high probability, but the other species in section *Fumigati*, such as *A. lentulus* and *N. pseudofischeri* may cause cross-reactions and thus accurate identification of the cause of aspergillosis may not be achievable.

Several molecular methods are available for identification and molecular typing of *Aspergillus* species [136–139]. For example the ITS1 and ITS2 region of rDNA, which will not always give a sufficient number of DNA sequence differences, was anyway useful for differentiation of different *Aspergillus* species. In one case the amplicons from the PCR reaction of the ITS regions of *A. fumigatus* and other fungi were detected in a simple, colorimetric enzyme immunoassay system [140]. However, as is often the case, closely related and relevant pathogenic species, such as *A. lentulus* and *N. pseudofischeri* were not tested. Such systems need to be tested using several isolates of all relevant fungi for the problem at hand, as seen from a taxonomic point of view. Furthermore it is often necessary to use sequences from more than one gene [137,139] to get sufficient specificity.

Bar coding methods will be used more frequently in the future and with the publication of several *Aspergillus* genomes [43,44,129], simpler methods based on ca. 25 bp sequences may be selected for fast identification [140,141]. Microarrays, reusable nylon-based macroarrays and colour-coded DNA detection beads analysed by flow cytometry may all be used for practical identification [142].

The mycological literature is full of misidentifications and wrong *Aspergillus* names. This is not only the case in the medical mycology literature but also in numerous publications on the mycotoxinogenic fungi associated with foods and feeds [143]. It is hoped that all these new techniques will be contribute to a stable taxonomy and nomenclature.

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