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Phylogeny and molecular diagnosis of mycotoxigenic fungi

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Abstract

Phylogenetic studies of the fungi that produce the five major groups of mycotoxins are reviewed, with a focus on studies employing ribosomal and/or β-tubulin (*BenA*) gene sequences. The toxins aflatoxin and ochratoxin A are produced by several *Aspergillus* and *Penicillium* species classified in the *Trichocomaceae*, *Eurotiales*. The toxins fumonisin, deoxynivalenol and zearalenone are produced by several *Fusarium* species classified in the *Nectriaceae*, *Hypocreales*. Studies of ribosomal genes have revealed that the present generic concepts for *Aspergillus*, *Penicillium* and *Fusarium* will require some adjustment in order to conform to phylogenetic principles. Phylogenetic studies have resulted in generally narrower species concepts in all three genera but there is good correlation between these species and mycotoxin production. The development of molecular diagnostics for the critical mycotoxigenic species is considered, with particular emphasis on the development of DNA hybridization probes that can be used to detect and identify multiple species using species and/or clade specific oligonucleotides designed from one or more genes. As an illustration, a virtual array for identifying *Aspergillus* species and groups of species producing aflatoxin is presented, based on oligonucleotides selected and optimized from a database of internal transcribed spacer and partial β-tubulin sequences assembled from GenBank. It was possible to design acceptable oligos for all species and groups in the complex using the β-tubulin gene, but only for one species and the larger group using the less variable internal transcribed spacer of the ribosomal DNA.

Introduction

Because of their economic importance and impact on human and animal health, mycotoxigenic fungi have received abundant taxonomic and phylogenetic attention. Three anamorph genera, namely *Aspergillus*, *Fusarium* and *Penicillium*, are particularly important and are the focus of all the articles in this special issue of the European Journal of Plant Pathology. Species of these genera are responsible for producing the five major mycotoxins subject to regulation by most countries, namely aflatoxin, ochratoxin A, deoxynivalenol (=DON, or vomitoxin), zearalenone (ZEA) and fumonisins. Although the list of species thought to produce these toxins was once fairly short, changes in taxonomic concepts brought about by molecular phylogenetic studies, coupled with more sensitive chemical detection and identification technologies, has resulted in a longer list of species proven to produce these toxins (Table 1). The list of species *reported* to make these toxins is longer still, but misidentified strains and imprecise mycotoxin identifications cause some of this confusion. The question of whether other species make these toxins is secondary to the purpose of this article, which is to present a phylogenetic approach for developing comprehensive molecular detection and diagnostic tools for the major fungi producing these important five mycotoxins.

The preoccupation of taxonomic mycologists for the last decade has been the elaboration of molecular phylogenies, and in recent years the development of the phylogenetic species concepts (PSCs). A detailed discussion of the PSC is beyond the scope of this article, but the method defines the

Table 1. Species of *Aspergillus*, *Fusarium* and *Penicillium* producing the five major mycotoxins, according to modern phylogenetic species concepts

Species	Toxins	References
Aspergillus alliaceus	Ochratoxin A	Frisvad and Samson (2000)
A. bombycis	Aflatoxin	Ehrlich et al. (2003) Peterson et al. (2001)
A. carbonarius	Ochratoxin A	Heenan et al. (1998)
**A. flavus*	Aflatoxin B, G	
Group I	Aflatoxin B	Geiser et al. (2000)
Group II	Aflatoxin B, G	Geiser et al. (2000)
A. muricatus	Ochratoxin A	Frisvad and Samson (2000)
A. niger	Ochratoxin A (some strains)	Heenan et al. (1998)
**A. nomius*	Aflatoxin B, G	Kurtzman (1987)
**A. ochraceus*	Ochratoxin A	Frisvad and Samson (2000)
A. ochraceoroseus	Aflatoxin	Klich et al. (2000)
**A. parasiticus*	Aflatoxin	Klich and Pitt (1988a)
A. pseudotamarii	Aflatoxin	Ito et al. (2001)
Fusarium acutatum	Fumonisin B1, B2	Fotso et al. (2002)
F. andiyazi	Fumonisin B1	Rheeder et al. (2002)[a]
F. anthophilum	Fumonisin B1, B2	Rheeder et al. (2002)
F. begoniae	Fumonisin B1	Fotso et al. (2002)
F. brevicatenulatum	Fumonisin B1	Fotso et al. (2002)
**F. culmorum*	Deoxynivalenol	Ward et al. (2002)
F. dlamini	Fumonisin B1	Rheeder et al. (2002)
F. fujikuroi	Fumonisin B1	Rheeder et al. (2002)
F. globosum	Fumonisin B1, B2, B3	Rheeder et al. (2002)
**F. graminearum*	Deoxynivalenol, zearalenone	O'Donnell et al. (2000a) Ward et al. (2002)
lineages 1, 3, 4, 6, *7	Deoxynivalenol, zearalenone	O'Donnell et al. (2000a) Ward et al. (2002)
lineage 2	Zearalenone	O'Donnell et al. (2000a)
lineage 5	Deoxynivalenol	O'Donnell et al. (2000a) Ward et al. (2002)
Group 1 see *F. pseudograminearum*		
F. napiforme	Fumonisin B1	Rheeder et al. (2002)
F. nygamai	Fumonisin B1, B2, B3	Rheeder et al. (2002)
F. oxysporum	Fumonisin C (some strains)	Seo et al. (1996)
F. phyllophilum	Fumonisin B1	Fotso et al. (2002)
F. polyphialidicum	Fumonisin B1	Rheeder et al. (2002)
**F. proliferatum*	Fumonisin B1, B2, B3	Rheeder et al. (2002)
F. pseudocircinatum	Fumonisin B1, B2	Fotso et al. (2002)
**F. pseudograminearum*	Deoxynivalenol, zearalenone	Ichinoe et al. (1983) Sydenham et al. (1991)
F. pseudonygamai	Fumonisin B1, B2	Rheeder et al. (2002)
F. redolens	Fumonisin B1, B2, B3	Rheeder et al. (2002)
F. sacchari	Fumonisin B1	Rheeder et al. (2002)
F. subglutinans	Fumonisin B1	Rheeder et al. (2002)
F. thapsinum	Fumonisin B1, B2, B3	Rheeder et al. (2002)
**F. verticillioides*	Fumonisin B1, B2, B3	Rheeder et al. (2002)
**Penicillium nordicum*	Ochratoxin A	Larsen et al. (2001)
**Penicillium verrucosum*	Ochratoxin A	Lund and Frisvad (1994)

Species marked with * are considered significant producers of these toxins.
[a]This is a review article. The primary citations for toxin production by these species can be found there.

boundaries of a species by determining where the congruence stops between phylogenies derived from different genes for the same set of sample organisms (Taylor et al., 2000). As with most other phyla and kingdoms, the majority of DNA sequence data used for taxonomy of fungi comes from the nuclear ribosomal operon. Of the regions comprising the ribosomal operon, the DNA coding for the small (18S) and 5.8S subunits of eukaryotes are generally too conserved to be seriously considered for species boundaries delineation and diagnostics. Attempts have been made to utilize the more variable domains of the large subunit (26 or 28S) for species identification and

detection, particularly in yeasts, but in general this domain is also too conserved. This leaves the internal transcribed spacer of the ribosomal gene (ITS) as the region that has been generally considered suitable for species identification and detection. The ITS does not always provide species level resolution, but despite this, it is the only part of the transcribed ribosomal region that deserves serious consideration as a diagnostic marker for species. The high number of copies of the ITS per cell, in particular, makes it an attractive target for diagnostics and it can be detected with great sensitivity. In the past five years, sequences of intron-rich protein coding genes have been exploited for developing PSCs, and would be good targets for the development of species-specific molecular diagnostic tools. In this paper, we will focus on exons 3–6 of the β-tubulin gene (*BenA*) as a representative protein coding gene that has been applied to the three fungal genera of interest. Geiser et al. (this volume) consider the applications of another gene, elongation factor 1-α (*EF1*α), for identifying *Fusarium* species. For molecular diagnostics, there is a trade-off implicit in exploiting PSC databases for molecular detection because although single copy genes tend to be more variable and offer more resolution for species delimitation, they are less sensitive when used for direct detection assays because of their low copy number.

This paper is divided into two major sections. In the first, we review progress in phylogenetic studies on *Penicillium*, *Aspergillus* and *Fusarium*. Our focus is on the species responsible for the five groups of regulated toxins and the development of precise species concepts. Despite the relative stability of generic concepts (all of which are being revisited following molecular phylogenetic studies), the histories of the taxonomy of three mycotoxigenic mould genera are full of controversies over species concepts. Earnest discussions of taxonomic lumping and splitting dominated pre-molecular studies, when subjective interpretations of micromorphological, cultural and host or substrate-based characters were the basis for disagreements. Arguments that broad species concepts were necessary because they would allow even non-specialists to make acceptable identifications were gradually discounted. Against the hopes of many, molecular data suggest that narrow species boundaries are the norm in fungi (but not the rule). It is often difficult to target precisely the genetic boundaries of serological or phenotype-based taxa for diagnostic tests, but it is usually possible to target phylogenetic clusters at different hierarchical levels with DNA-based assays.

The development of molecular diagnostics has mostly been a focus for plant pathologists and medical mycologists. This is a pity, because much of the available molecular data has been under-exploited and there has been a lack of taxonomic rigour in some of the assays that have been developed. In the second section of this paper, we will describe an approach for developing molecular diagnostics based on the design of species (or group) specific oligonucleotides (=oligos). Such oligos can be used in a variety of assay platforms, including membrane-based macroarrays, glass slide-based DNA microarrays, or real-time PCR assays such as TaqMan^{TM}. Using existing data from public databases, we will demonstrate an approach to developing multiplex assays that could be used to individually or simultaneously detect and identify critical mycotoxin-producing species using oligos derived from the ITS and part of the DNA coding for the *BenA* gene.

Phylogeny: family, genus, infrageneric and species concepts

Penicillium *and* Aspergillus

Penicillium and *Aspergillus* are both anamorph genera classified in the *Trichocomaceae*, *Eurotiales*, one of the most intensely studied groups of ascomycetes. Many species are important in medicine, industrial microbiology, agriculture, biodeterioration and biotechnology. The taxonomic histories of *Aspergillus* and *Penicillium* were reviewed in the monographs of Raper and Fennel (1965) and Pitt (1979), and explored with considerable detail in three conference proceedings (Samson and Pitt, 1985, 1990, 2000). A 'List of Names in Current Use' was recently updated by Pitt et al. (2000), and includes 184 species of *Aspergillus*, 225 species of *Penicillium*, and 41 species of *Paecilomyces* (a genus not otherwise considered in this article). DNA sequence data, mostly ITS and large subunit rDNA, are now available for the majority of the species, allowing the monophyly of the genera and the infrageneric classifications proposed for them to be evaluated. These data, based primarily on the

work by Peterson (2000a, b), serve as the basis for the following phylogenetic discussion. Protein coding gene data is available for some groups of species, allowing a more resolved examination of species concepts. For this discussion of economically significant mycotoxins, we are primarily concerned with *Penicillium verrucosum*, *Aspergillus ochraceus*, *A. niger* and their closest relatives.

The morphological characters used to distinguish *Aspergillus* and *Penicillium* as genera mostly have held up to phylogenetic scrutiny. *Aspergillus* has traditionally been characterized by the production of unbranched, aseptate conidiophores with a swollen apex (the so-called vesicle), covered with a layer of sporulating cells (phialides), sometimes with an intermediate layer of supporting cells called metulae. *Aspergillus* was divided into 'groups' (Raper and Fennell, 1965), later formalized as subgenera and sections (Gams et al., 1985), based primarily on the colour of the conidia, the shape of the vesicles and the presence or absence of metulae on the vesicles. In contrast, most *Penicillium* species have branched, septate conidiophores, with the sporulating cells (also phialides) in clusters at the ends of up to four series of branches. Most *Penicillium* species have green conidia, so the division into subgenera has generally been based on the branching patterns of the conidiophores.

The teleomorphs of *Aspergillus* and *Penicillium* complicate the picture (Table 2, Figure 1). *Aspergillus* anamorphs are associated with nine teleomorph genera. There is good correlation between some of the subgenera and sections of *Aspergillus* and these teleomorph genera. For example, subgenus *Aspergillus* section *Aspergillus* includes species associated with the teleomorph genus *Eurotium*. *Penicillium* is associated with two teleomorph genera, *Eupenicillium* and *Talaromyces*. The species of *Penicillium* associated with *Talaromyces* teleomorphs produce symmetrical conidiophores and a distinctive shape of sporulating cells, and are all classified in the *Penicillium* subgenus *Biverticillium*. The species of *Penicillium* associated with *Eupenicillium* teleomorphs are found in the other subgenera of *Penicillium*. Molecular studies have confirmed the morphological hypotheses (Malloch, 1985) that *Eupenicillium* and *Talaromyces* occur in distinct subfamilies within the *Trichocomaceae* (LoBuglio et al., 1993, 1994; Berbee et al., 1995). Considering the relatively distant relationships of these two groups of teleomorphs, it is not surprising that molecular phylogenies suggest that *Penicillium* is polyphyletic if subgenus *Biverticillium* is included. In addition to the distinctive morphological characters of subgenus *Biverticillium*, many additional characters such as ubiquinone patterns, isozyme analysis, secondary metabolite profiles and cell wall biochemistry suggest that it is distinct from the rest of *Penicillium* (see individual chapters in Frisvad et al., 1998). Removal of the subgenus *Biverticillium* would result in the rest of *Penicillium* being a monophyletic group. Early molecular phylogenetic papers on *Penicillium* showed that teleomorphs were phylogenetically intermixed with anamorphic species, indicating multiple losses of sexual states had occurred during evolution (LoBuglio et al., 1993); this phenomenon is also evident in Figure 1.

A second problem is the generic definition of *Aspergillus*. *Aspergillus* appears to be a phenotype that evolved once, associated with a group of teleomorph genera that appear to be individually and collectively monophyletic. In other words, *Aspergillus* is a useful name for a broad group of species, which includes several more narrowly defined groups with teleomorph-based names. In a phylogenetic classification, *Aspergillus* might have to be divided into multiple anamorph genera correlating with teleomorph genera or subjected to a combined re-evaluation of anamorph and teleomorph generic concepts to arrive at an intermediate solution.

The core group of economically important species of *Penicillium* is classified in subgenus *Penicillium*, and has been subject to a number of different morphological treatments. Raper and Thom (1949) put a heavy emphasis on cultural characters, including aspects of colony texture that were difficult to describe and illustrate effectively. Samson et al. (1976) emphasized micromorphology, in particular roughening of conidiophores and sizes of conidia, and recognized 13 species and eight varieties. Pitt (1979) emphasized colony growth rates on various media and conidial colours, and recognized 25 species. The classical morphological and cultural taxonomy of *Penicillium* was supplemented with secondary metabolite profiling (reviewed in Frisvad et al., 1998) and to a lesser extent isozymes patterns (Cruickshank and Pitt, 1987). Despite earlier reports to the contrary,

Table 2. Anamorph–teleomorph correlations, and phylogenetic evaluations of infrageneric classification of *Aspergillus*, *Penicillium* and *Fusarium*

Genus	Subgenus	Section	Associated teleomorphs	Monophyletic
Aspergillus	—	—	nine genera	No
	Aspergillus	*Aspergillus*	*Eurotium*	Yes
		Cremei	*Chaetosartorya*	Yes[a]
		**Flavi*	*Neopetromyces*	Yes[a]
		Flavipedes	*Fennellia*	Yes[a]
	Fumigatii	*Fumigatii*	*Neosartorya*	Yes
	**Circumdati*	—	four genera	No[b]
	Circumdati	**Circumdati*	*Petromyces*	Yes[a]
	Nidulantes	*Sparsi*	*Hemisartorya*	Yes[a]
		Nidulantes	*Emericella, Fennellia*	Yes
		Ornati	*Warcupiella*	
			Sclerocleista	
		Hemicarpentales	*Penicillium*	
Fusarium	—		four genera	No
	**Fusarium*		*Gibberella*	Yes
	Roseum		*Gibberella*	No
	Gibbosum		*Gibberella*	Untested
	**Liseola*		*Gibberella*	Yes
	Martiella		*Neocosmospora*	Yes
	Eupionnotes		*Cosmospora*	No?
	Spicarioides		*Albonectria*	Yes
Penicillium	—		two genera	No
	Biverticillium		*Talaromyces*, *Trichocoma*	Yes
	other subgenera		*Eupenicillium*	Yes
	**Penicillium*		*Eupenicillium*	Yes
	Furcatum		*Eupenicillium*	No
	Aspergilloides		*Eupenicillium*	No

Only infrageneric taxa with known anamorph-teleomorph connections are listed. Taxa marked with * included species that produce one or more of the five groups of regulated toxins. *Aspergillus* data extracted from Peterson (2000b); *Fusarium* data from Aoki and O'Donnell, (1999); O'Donnell et al., (1998a; 2000a, b); *Penicillium* data from Peterson (2000a).

[a]Indicates sections originally classified in subgenus *Circumdati*.

[b]Subgenus *Circumdati* as originally defined by Gams et al. (1985).

species were shown to have constant and reproducible profiles of secondary metabolites; each species produces signature metabolites. It was difficult to examine phylogenetic relationships among species using the metabolite profiles, but identification was possible. Early work on ochratoxin-A-producing *Penicillium* species attributed this toxin to strains of *P. viridicatum*. The initial chemotaxonomic work attributed OA-producing strains to a chemotype (Group III) of this species (Ciegler et al., 1981) which subsequently was identified as *P. verrucosum* (Lund and Frisvad, 1994). Until recently, this was the only species of *Penicillium* known to produce ochratoxin A. This species is relatively easy to recognize based on the combination of morphological (vertically compressed penicilli with short metulae and branches) and cultural characters (slow growth, green conidia lacking blue shades, and an inability to produce acid on creatine agar) (Samson et al., 2000). Recently, Larsen et al. (2001) added *P. nordicum*, a morphologically similar species to *P. verrucosum* that occurs primarily on meat, to the list of known OA-producing moulds.

Because of metabolite profiling, narrower species concepts were adopted in subgenus *Penicillium* before molecular data started to be generated in the late 1980s. Molecular studies showed that ITS sequences contained relatively little phylogenetic information that could be used to support species concepts based on mycotoxin profiles (Skouboe et al., 1999; Peterson, 2000a). Terminal clades of phylograms derived from *BenA* sequences, on the other hand, conformed perfectly with the myco-

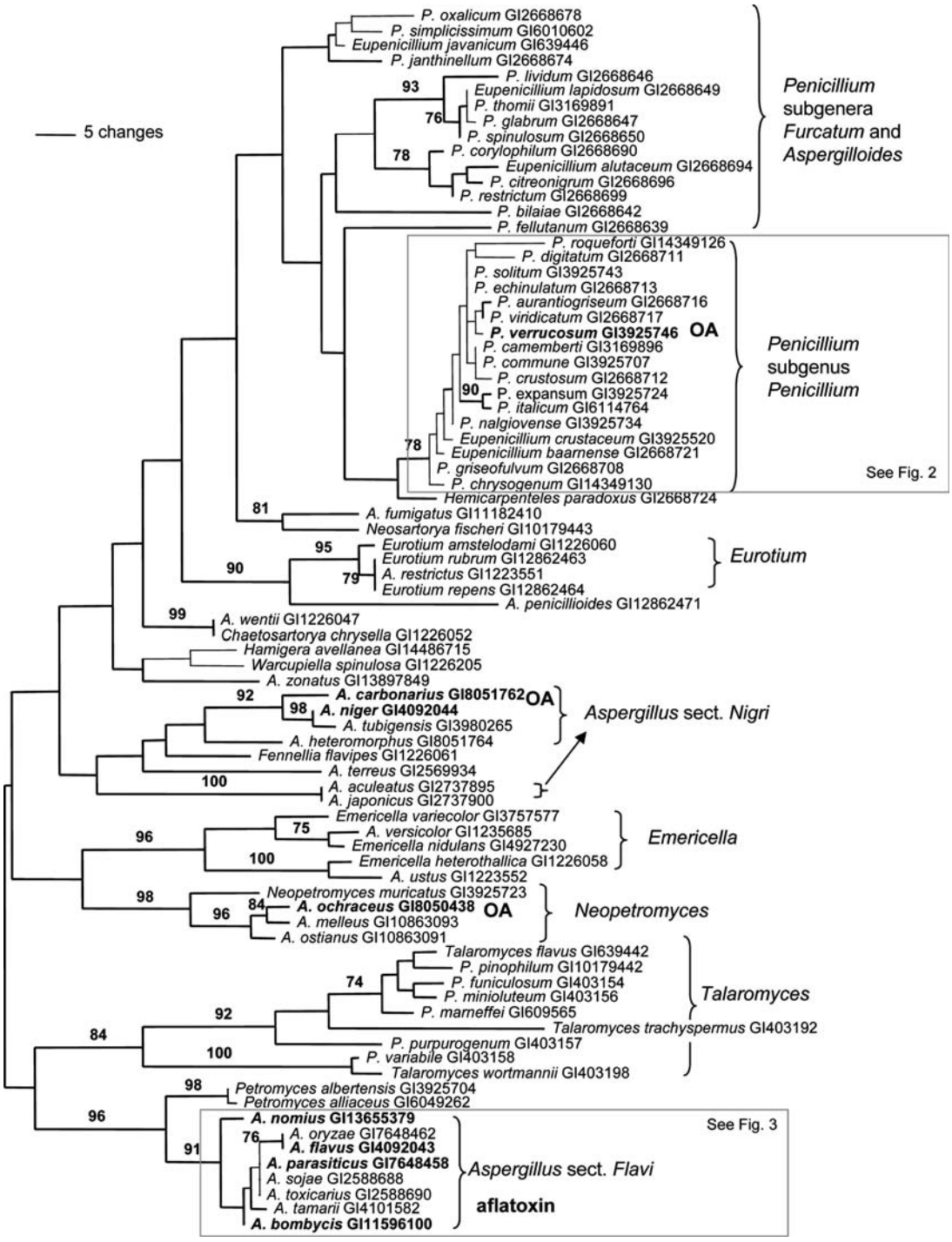

Figure 1. Phylogenetic relationships among selected species of the *Trichocomaceae*, *Eurotiales*, based on an heuristic search of aligned internal transcribed spacer (ITS) sequences, presented with midpoint rooting. Species producing the toxins of interest are in bold, with the toxin name in bold nearby (OA = ochratoxin A). One of 5000 equally parsimonious trees (incomplete search) 769 steps long based on 186 informative characters (CI 0.420, RI 0.781, RC 0.328, HI 0.580). The structure of the tree not represented by thinner lines was supported by the 100% consensus; bootstrap values > 70% based on 1000 replications using fast step-wise addition are placed on some branches. Teleomorph generic names are spelled out. *P.* = *Penicillium*.; *A.* = *Aspergillus*. Primarily based on the data of Peterson (2000a, b).

toxin-based species concepts (Seifert and Louis-Seize, 2000, see Figure 2). Continuations of *BenA* studies by Samson et al. (pers. comm.) suggest the existence of about 58 phylogenetic species in subgenus *Penicillium*, most of which can be recognized through combinations of micromorphology, cultural characters and secondary metabolite profiling.

There are three groups of *Aspergillus* that have particular relevance to the major mycotoxins. The *Aspergillus flavus* complex, also known as section *Flavi* of subgenus *Aspergillus*, includes several species that produce aflatoxins. This group of species is easily recognized by its yellowish-green masses of conidia; several of the species also form sclerotia. Only two teleomorph species are known in this group, both classified in *Petromyces*. Morphologically, the species of the *A. flavus* complex are difficult for inexperienced taxonomists to differentiate, but reliable, reproducible identifications based on critical examination of micromorphology are possible with experience (Klich and Pitt, 1988a; Klich 2002). Original molecular studies of this group of species emphasized the close genetic relationships among them based on DNA:DNA reassociation experiments (Kurtzman et al., 1986), which lead to the recognition of *A. nomius* (Kurtzman et al., 1987). The controversial assertion that the major aflatoxin-producing species were conspecific with 'domesticated' non-aflatoxigenic species used in oriental food fermentations, ie. *A. flavus* was the same as *A. oryzae* and *A. para-*

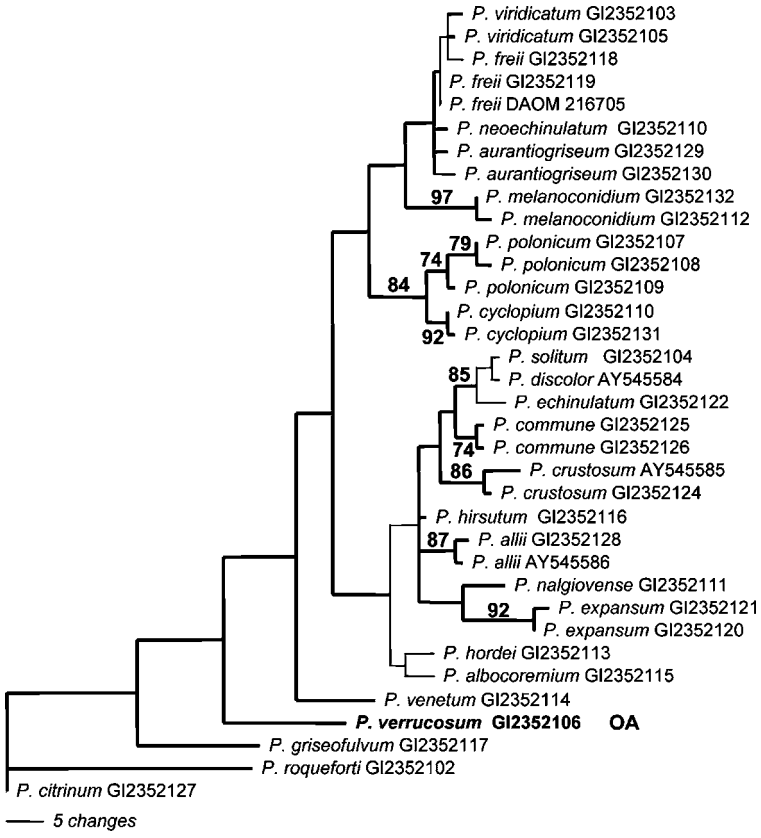

Figure 2. Phylogenetic relationships among the species of the *Penicillium aurantiogriseum* complex, based on an heuristic search of aligned *BenA* sequences, with *P. citrinum* used as an outgroup. Note the position of *P. verrucosum*, the only species of *Penicillium* conclusively proven to produce ochratoxin A. One of 5000 equally parsimonious trees (incomplete search) 214 steps long based on 86 informative characters (CI 0.589, RI 0.710, RC 0.418, HI 0.411). The structure of the tree represented by thicker lines was supported by the 100% consensus; bootstrap values >70% based on 1000 replications using fast step-wise addition are placed on some branches. Primarily based on Seifert and Louis-Seize (2000), in which the GenBank accession numbers were inadvertently printed incorrectly because of a sorting error during the preparation of camera ready copy; the correct numbers are reproduced here.

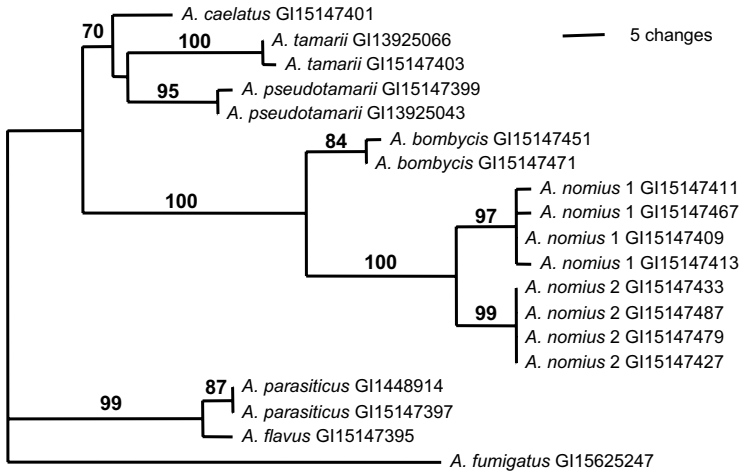

Figure 3. Phylogenetic relationships among the toxin-producing species of the *Aspergillus flavus* complex, based on an heuristic search of aligned *BenA* sequences, with *A. fumigatus* used as an outgroup. The domesticated species *A. sojae* and *A. oryzae* were not included in the analysis. Single most parsimonious tree 116 steps long based on 70 informative characters (CI 0.828, RI 0.929, RC 0.769, HI 0.172); bootstrap values >70% based on 1000 replications using full heuristic searches are placed on some branches. Primarily based on the data of Peterson et al. (2001) and Ito et al. (2001).

siticus was the same as *A. sojae* (Kurtzman et al., 1986), was supported by subsequent studies involving ITS and LSU rDNA sequencing (Peterson, 2000b; Rigo et al., 2002). Wang et al. (2001) presented a phylogeny of sect. *Flavi* based on sequences of the mitochondrial cytochrome *B* gene but found only seven variable sites in a 402 bp fragment among the 77 strains that they sequenced. Within *A. flavus*, some phenotypic and genetic variation has been noted that has yet to be formalized in the taxonomy. Cotty (1989), in part following observations by previous workers, divided *A. flavus* into two groups depending on whether they produced large (group L) or small (S) sclerotia. Geiser et al. (1998, 2000) used a multiple gene phylogeny to show the existence of two apparently isolated groups within *A. flavus*, with an imperfect correlation with the L and S phenotypic groups, although all Group II strains were of the S phenotype. Geiser et al. (2000) also noted that there was some evidence that *A. oryzae* was genetically distinct from *A. flavus*, but it is unclear whether the differences are entirely in the genes responsible for aflatoxin. Erlich et al. (2003) achieved similar results with phylogenetic analysis of two of the 23 genes involved in aflatoxin synthesis. The possibility that *A. nomius* could represent more than one species, first suggested on the basis of morphological and physiological characters (Feibelman et al., 1998), was also supported by the phylogenies of the aflatoxin genes (Ehrlich et al., 2003) and is evident from the *BenA* phylogeny as well (Figure 3). One species outside the *A. flavus* group, namely *A. ochraceoroseus*, was reported to produce high levels of aflatoxin (Klich et al., 2000), but this species is known only from a few strains and its significance as an aflatoxin producer in nature is unknown (R.A. Samson, pers. comm.).

Ochratoxin A (OA) was named because it was originally discovered in *A. ochraceus*. This species complex is relatively easily identified by its light brown or yellowish (i.e. ochre coloured) conidial masses, in combination with some clear micromorphological features. The one teleomorph species associated with this group of species is now assigned to *Neopetromyces* (Frisvad and Samson, 2000). The inconspicuous morphological distinctions between some of the species of this group, and the very similar LSU sequences, led Peterson (2000b) to suggest the synonymy of *A. mellus*, *A. ostianus* and *A. petrakii* with *A. ochraceus*. Frisvad and Samson (2000) maintained the species as distinct on the basis of differences in mycotoxin profiles, in addition to the originally described micromorphological differences, as did Varga et al. (2003) based on differences in ITS sequences. Furthermore, Varga et al. (2000) identified two

groups within *A. ochraceus* using ITS sequences, one of which apparently does not make OA.

The list of *Aspergillus* species reported to make OA is controversial, but it is now accepted that some strains of *A. niger* are able to produce this toxin, and that *A. carbonarius* is also a significant producer of OA in coffee and grapes in some parts of the world (Heenan et al., 1998; Abarca et al., 2003). The black aspergilli (or section *Nigri* of subgenus *Aspergillus*), of which *A. niger* is the quintessential representative, are ubiquitous, and their taxonomy has been problematic. Some species can be distinguished reliably on the basis of presence or absence of metulae and the shape and wall-roughening of the conidia. Distinctions based on minute differences in shades of dark brown or black have been difficult to apply. No teleomorphs are known for this group of species. The taxonomy developed by Al-Mussalam (1980), which accepted seven species, was adopted for the most part in the two most recent identification manuals (Klich and Pitt, 1988b; Klich, 2002) and has generally been supported by molecular studies. Kozakiewicz (1989) suggested a slightly higher number of species (11), based on differences in conidial ornamentation observed using SEM. Only the relationships between *A. tubingenensis*, *A. niger* and *A. foetidus*, suggested to represent either two or three species on the basis of various DNA fingerprinting techniques (e.g. Kusters van Someren et al., 1991; Parenicova et al., 1997, 2001), remain to be solved.

Fusarium

Fusarium is an anamorph genus classified in the *Nectriaceae*, *Hypocreales*. Species with teleomorphs attributed to other orders have now been moved to other genera, in particular the snow mould *Fusarium nivale* (now *Microdochium nivale*) and the common soil fungus and root pathogen *Fusarium tabacinum* (now *Plectosporium tabacinum*, the anamorph of *Plectosphaerella cucumerina*, *Phyllachorales*). The importance of some *Fusarium* species to agriculture and human health is outlined in Table 1. The current generic concept (Gams and Nirenberg, 1989), accepted by the majority of morphology-based taxonomists working on these fungi, is 'polythetic', accommodating the combinations of morphologically diverse synanamorphs (i.e. sporodochial macroconidia, various conidia produced in the aerial mycelium, chlamydospores) produced by the species in this genus. The teleomorphs of *Fusarium*, as they are currently understood, fall into four genera, with *Gibberella* being the core teleomorph genus for the main part of *Fusarium*, *Neocosmospora* (or *Haematonectria*) including the teleomorphs of the *F. solani* complex, and a smaller number of species with teleomorphs in *Cosmospora* and *Albonectria*. Most *Fusarium* taxonomists are comfortable with the current generic concept, but phylogenetic studies suggest that it might be paraphyletic, with several important genera of the *Nectriaceae* derived from within.

The modern taxonomy of *Fusarium* is based almost entirely on the study of pure cultures, which has made the interpretation of many species described before about 1950 problematic. The interpretation of the significance of various observed differences in micromorphology and cultural characters varied widely, with different 'schools' of taxonomy dominating prior to about 1985. The influential, but now mostly abandoned Snyder and Hansen system accepted only nine species (Snyder et al., 1956), with some variants within those species designated by a trinomial 'form' system. Wollenweber and Reinking (1935) recognized about 140 species or varieties, distinguished by critical micromorphological characters and variations in cultural characters. The photographic atlas by Gerlach and Nirenberg (1982), which accepted about 90 species or varieties, was a modern distillation and extension of the Wollenweber and Reinking (1935) work. The identification manual by Nelson et al. (1983) represented a break by one school of *Fusarium* taxonomists from the Snyder and Hansen system, accepting 30 common species and 15 less common species in the genus. It is the Gerlach and Nirenberg (1982), and Nelson et al. (1983) books that have been the springboards into the era of molecular phylogenetics. Presently, there are about 150 phylogenetically and/or morphologically distinct species well characterized and accepted by *Fusarium* taxonomists, but no comprehensive identification manual is available.

Fusarium was divided into several taxonomic sections by Wollenweber and Reinking (1935), and this system of organizing species within the genus was followed, with some variations, by most subsequent authors. Some sections correlate with

teleomorph genera, but those working with molecular phylogeny have discounted this formal nomenclatural rank in favour of the cladistic-friendly concept of species complexes. Molecular phylogenies suggest the existence of several major clades within the *Gibberella* clade of *Fusarium* (Figure 4). O'Donnell et al. (1998a) and other others have heavily sampled the *G. fujikuroi* complex (section *Liseola*), which now includes more than 40 species and is discussed in more detail below. This clade is a sister group to the *F. oxysporum* complex (section *Elegans*). No teleomorphs are known in the latter complex, but its phylogenetic relationship to *Gibberella* is unequivocal. The number of species has yet to be elucidated, but it is clear that *F. oxysporum* in the commonly understood sense will eventually be divided into several phylogenetic species. *Fusarium* sect. *Fusarium* (more widely known as sect. *Discolor*) seems to be divided into two main clades, one producing type A trichothecenes and the other producing type B trichothecenes.

The principal type B trichothecene-producing species are *F. graminearum* and *F. culmorum*. Distinguishing between these morphospecies is relatively simple because of the rather broad macroconidia of *F. culmorum*, but designing primers to distinguish them in PCR reactions using ITS sequences was not possible (Schilling et al., 1996). The concept of *F. graminearum* has changed significantly in recent years. In morphologically-based taxonomic treatments, the species was recognized by the production of straight, moderately robust macroconidia produced in almost colourless sporodochia, an absence of microconidia in the aerial mycelium, and the eventual production of chlamydospores in the vegetative mycelium and older macroconidia. *Fusarium graminearum* first was divided into two groups based on differences in ecology and the ability to produce perithecia in culture (Burgess et al., 1975; Francis and Burgess, 1977). The common producer of deoxynivalenol and zearalenone, which readily produced perithecia in agar culture, was designated as Group II, and caused spikelet diseases in wheat and cob rot in maize. Group I occurred in arid areas, caused crown rot of wheat, and rarely produced perithecia in agar culture; it was subsequently named *F. pseudograminearum* (Aoki and O'Donnell, 1999). Although a member of the same clade as *F. graminearum*, it is somewhat distantly related. However, it also produces both deoxynivalenol and zearalenone (Ichinoe et al., 1983; Syndenham et al., 1991). O'Donnell et al. (1998a) presented a six gene phylogeny for *F. graminearum*, showing that the species could be divided into seven phylogenetic lineages (or phylogenetic species) with distinct geographic origins. The principal grain and maize pathogen, and main producer of deoxynivalenol and zearalenone in North America and northern Europe, is lineage 7. Deoxynivalenol was detected in all but lineage 2, and zearalenone in all but lineage 5. Miller et al. (1991) recognized three chemotypes within the broader concept of *F. graminearum*. (i) the NIV chemotype producing only nivalenol and acetylated derivatives, (ii) the 15ADON chemotype producing deoxynivalenol and 15-acetyldeoxynivalenol and (iii) the 3ADON chemotype producing deoxynivalenol and 3-acetyldeoxynivalenol. Oddly, phylogenies derived from eight toxin coding genes (from a single gene cluster) do not correlate with the phylogenies suggested by other genes, suggesting that the acquisition of the toxin genes predated speciation, and that a distinct genetic mechanism unrelated to recombination has been responsible for the maintenance of chemotypes across the phylogenetic species within the morphospecies *F. graminearum* (Ward et al., 2002).

The prevailing concept of morphological species in the *Gibberella fujikuroi* complex was that of Nirenberg (1976), who recognized four species. Species in this complex produce abundant microconidia, sometimes in chains, otherwise in slimy heads, from monophialides or polyphialides, and relatively uniform, straight, narrow macroconidia from sparsely produced sporodochia. Biological species concepts, based on mating of compatible strains on carrot agar, started to extend the number of species (Leslie, 1995). The definition of *Fusarium* section *Liseola* (the section including the *G. fujikuroi* complex, and the majority of fumonisin producing species) has been confusing, because the description excluded species with chlamydospores (Nirenberg, 1976). Later workers found species that otherwise would fit in section *Liseola*, except that they produced cells that looked very similar to chlamydospores. This group was later segregated in section *Dlaminia* (Kwasna et al., 1991). O'Donnell et al. (1998a) showed that species attributed to sections *Liseola* and *Dlaminia* were phylogenetically intermingled, and that nei-

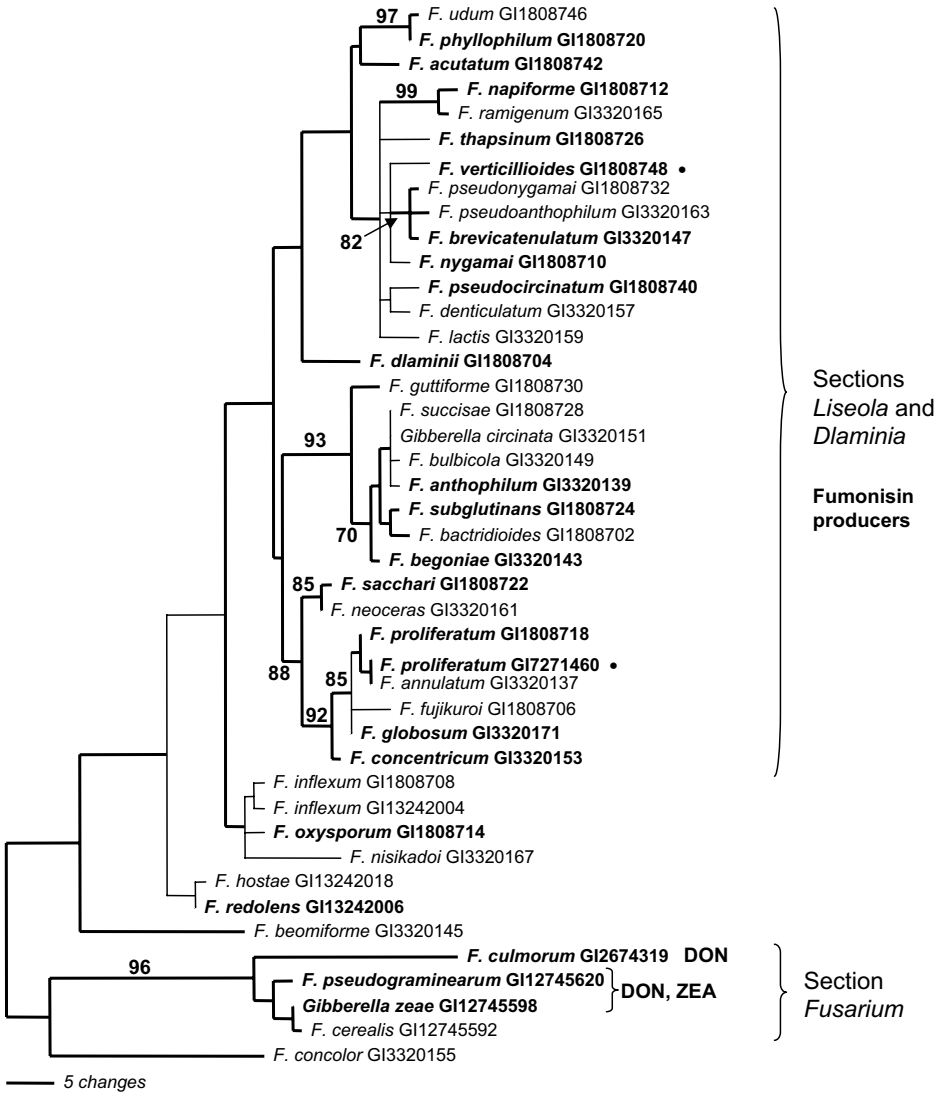

Figure 4. Phylogenetic relationships among selected species of *Fusarium* classified in sections *Fusarium*, *Liseola* and *Dlaminia*, based on an heuristic search of aligned *BenA* sequences, with species of section *Fusarium* used as the outgroup Fumonisin-producing species are in the upper clade of the tree in bold type, with the particularly significant species indicated with a bullet. Deoxynivalenol (DON) and zearalenone (ZEA) producers are in the basal clade of the tree. One of 540 equally parsimonious trees 245 steps long based on 112 informative characters (CI 0.629, RI 0.828, RC 0.521, HI 0.371). The structure of the tree represented by thicker lines was supported by the 100% consensus; bootstrap values >70% based on 1000 replications using fast step-wise addition are placed on some branches. Primarily based on the data of O'Donnell et al. (1998a, b; 2000a, b).

ther section was monophyletic. Clearly, the concept for section *Liseola* should be emended to include species with chlamydospores and the result will be a monophyletic group, sister to *F. oxysporum* clade (i.e. section *Elegans*).

The discovery of fumonisins in *F. verticillioides* (Gelderblom et al., 1988) led to intense taxonomic consideration of this species and its relatives. Nirenberg and O'Donnell (1998) and O'Donnell et al. (1998a) revised the *Gibberella fujikuroi* complex, and showed a correlation between previously overlooked micromorphological characters and a molecular phylogeny based on LSU, ITS, *BenA* and mtSSU sequences. The result was the recog-

nition of about 35 named species, with several unnamed species recognized (Figure 4, Nirenberg and O'Donnell, 1998; Marasas et al., 2001; Britz et al., 2002). O'Donnell et al. (2000b) supplemented the original data set with sequences of calmodulin and *EF1α*; the latter is now considered the optimal gene for identifying unknown *Fusarium* spp. using DNA sequencing (Geiser et al., this volume) but some common species are not yet represented in GenBank for this gene. Many of these newly recognized *Liseola* species have been tested for fumonisin production. Rheeder et al. (2002) confirmed that *F. proliferatum* and *F. verticillioides* are the most prolific producers of fumonisins B1, B2 and B3, with *F. nygamai* producing lower but still significant amounts of all three toxins. Fotso et al. (2002) demonstrated the production of fumonisins by five of the newly recognized species, but significant amounts of fumonisin B1 were produced only by *F. phyllophylum*. As can be seen in Figure 4, fumonisin production seems to be a synapomorphic character in the *G. fujikuroi* complex that has been lost in some species.

Molecular diagnostics: PCR-based diagnostics using species-specific oligonucleotides derived from phylogenetic studies

Single species assays

Until now, most molecular detection protocols were designed to detect single species. In conventional PCR-based assays, specific primer pairs produce a single band when the target species is present. The specificity can be incorporated in one or both primers. Most assays rely on specific primers that amplify either an anonymous fragment shown to be specific to the target species, or parts of known gene regions that have been shown to be diagnostic. Many examples are reviewed by the other authors in this issue, and a comprehensive review of PCR methods for detection of plant pathogens was given by Martin et al. (2000).

Anonymous fragments are DNA sequences of unknown function that serve as specific markers for a group of interest. Their development is analogous to the approach used for antibody-based species-specific tests because a unique binding molecule must be detected in a randomly generated set, and this process must be repeated for each species added at a later date. In the early 1990s, random amplification of polymorphic DNA (RAPD) was one of the most common methods for developing genetic species and population markers in fungi. Nowadays, this has been largely replaced by a more robust method, amplification fragment length polymorphism (AFLP). Unique anonymous fragments can be sequenced and new PCR primers designed that may or may not maintain the desired specificity, a technique known as sequence characterized amplified region (SCAR). For mycotoxigenic fungi, Ouellet and Seifert (1993) employed SCAR primers, as well as RAPDs, to track strains of *Fusarium graminearum* in field experiments. Nicholson et al. (1998) developed species-specific primers for *F. graminearum* and *F. culmorum* using SCAR. Similarly, Yoder and Christianson (1998) developed species-specific primers for *F. cerealis*, *F. sambucinum*, *F. torulosum* and the Quorn fungus, *F. venenatum*. In the latter example, two primer pairs intended to be specific for *F. culmorum* and *F. cerealis* did not give species-specific results, but precise identification of these species was nevertheless possible when all six primer pairs were used in combination. As shown by some of these examples, unique anonymous fragments can be used successfully for species-specific detection, but are amenable only to a certain level of multiplexing.

Species specific assays for a wide range of organisms have been developed from phylogenetic databases of ubiquitous gene regions such as the rDNA. An alternative strategy that pertains more specifically to mycotoxigenic fungi is to derive primers or probes from the genes from the 'toxin clusters', i.e. the actual genes responsible for mycotoxin production (Edwards et al., 2002). This approach has the advantage of detecting the fungi using the very genes that make them of importance, but so far has been less fruitful than expected. This is partly because the phylogeny of the toxin gene clusters themselves is sometimes different from the phylogeny of the species (Ward et al., 2002), and partly because non-functional toxin genes are sometimes present in fungi that do not produce the toxins. For example, homologue genes for parts of the ochratoxin and aflatoxin pathways exist in toxin-free species (Edwards et al., 2002). A detection approach based on both toxin-related and toxin-independent markers might be interesting for complex environmental

samples, especially if non-toxigenic strains of a given species exist, as is the case with *Aspergillus flavus* (Egel et al., 1994). Reverse transcriptase PCR (RT-PCR), in which a PCR product is generated from active RNA in the cell, is one way to restrict the assays to expressed genes.

Detection of groups of species producing toxins, rather than specific species, has also been attempted using primers developed from sequences of toxin cluster genes. Niessen and Vogel (1998) used one of the genes from the trichodiene synthase family (*Tox5*) to detect a group of trichothecene-producing *Fusarium* spp. from environmental samples. They used these primers to detect DNA of *F. graminearum* and *F. culmorum* in cereal samples, showing some correlation with DON levels (Knoll et al., 2002a, b). Ward et al. (2002) also designed chemotype specific PCR primers for the various chemotypes present in the *F. graminearum* complex using *Tri3* and *Tri12* genes. Bluhm et al. (2002) developed PCR primers with different levels of specificity for the detection of fumonisin- and trichothecene-producing species of *Fusarium*. Primers at the *Fusarium* genus level were based on the ITS region whereas primers for detecting the fumonisin and trichothecene producing groups of species were based on *Fum5* and *Tri6* gene sequences, respectively. Attempts to develop PCR-based assays for aflatoxin-producing species based on gene sequences from the aflatoxin biosynthetic gene cluster had difficulty distinguishing *A. flavus* and *A. parasiticus* (Shapiro et al., 1996; Farber et al., 1997; Shapiro and Mullins, 1997).

Multiplex PCR: detecting multiple species with multiple markers

PCR assays intended to detect a number or species, often using multiple genes or multiple markers, are known as multiplex assays. In multiplex PCR assays based only on electrophoresis of amplicons, the risk of misinterpretation and false positives increases with the increasing number of bands from different species that can be distinguished only by amplicon size. At some point, multiplexing must be accompanied with techniques that verify the identity of the amplicons. Sequencing of bands is definitive confirmation but this approach is impractical for routine testing. There are three possible approaches:

(i) DNA hybridization during or after PCR amplification to ensure that an amplicon from an unidentified sample is homologous to the targeted gene of an identified taxon or taxonomic group. Amplicons can be immobilized on a membrane after size fractionation on an electrophoretic gel (Southern blot) or by direct spotting of the PCR products (dot blot). The uncharacterized amplicons on the membranes are then hybridized with a series of probes comprising either species-specific oligonucleotides from the amplified region if a universal gene is used, or entire amplicons from reference strains if unique anonymous fragments are used (Figure 5A). This approach is cumbersome if the presence or absence of several species must be verified, because the single amplicon must be hybridized in series with homologous fragments for all possible species. Replicate dot blot membranes can be made from 96- or 384-well plates for PCR in roboticized high-throughput systems, which can be hybridized at once with a corresponding number of different probes.

(ii) Alternatively, the same diagnostic hybridization oligonucleotides can be immobilized on a solid surface to create a DNA array (reverse dot blot), on nylon membranes (macroarray) for smaller numbers or on glass slides (microarray) for larger numbers, to which the PCR products from the unknown are hybridized (Figure 5B). This approach allows detection of multiple species from an environmental sample with a single PCR reaction and is amenable to detection using multiple genes. The number of probes that can be immobilized on arrays continues to increase with improved technologies. Hybridization of the PCR product from a sample to bound oligonucleotide(s) results in a positive dot, or pattern of dots, that represents the species present (single or multiple) in the sample. Optimally, multiple oligonucleotides would be present for each target species, preferably from two or more genes. For example, Wilson et al. (2002) developed a DNA microarray for 18 potential biowarfare agents, mainly bacteria and viruses but including *F. sporotrichoides*, with 3–10 diagnostic regions amplified by PCR for each organism. The microarray included over 50,000 probes, giving extensive oligonucleotide-probe coverage for all genes and species in the assay. The amplicons were from virulence genes when known, but mostly from shotgun sequencing of uncharacterized clones.

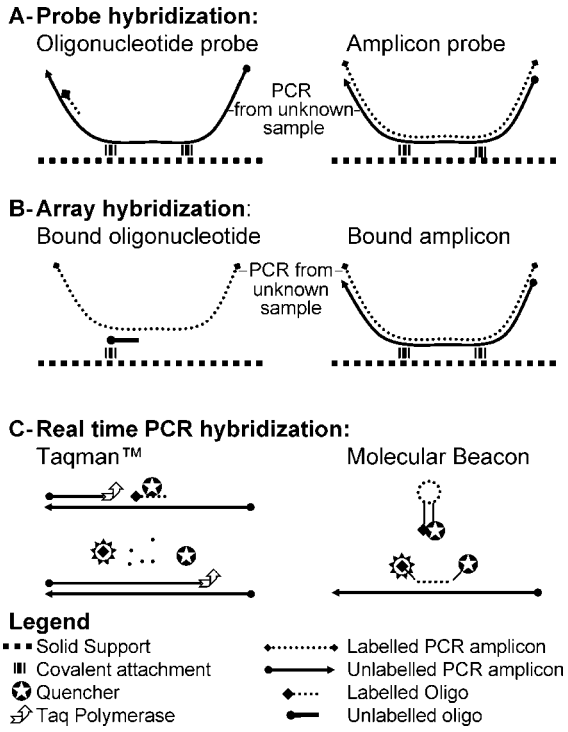

Figure 5. Different hybridization reactions that can be used to characterize PCR amplicons. Probe and array hybridizations have one of the complementary strands bound to a solid support, whereas in real-time PCR the hybridizations occur in the aqueous phase.

(iii) Real-time quantitative PCR with Molecular Beacons or TaqMan™ is another approach to verify that the amplicons are indeed homologous with the intended target (Schaad and Frederick, 2002). Specific oligonucleotide probes nested within the targeted PCR amplicon hybridize during PCR (Figure 5C). Because hybridization results in fluorescence, quantitative estimates of the amount of amplicon can be obtained at each cycle of the PCR. Measurement of the signal after amplification requires less expensive equipment but is less quantitative. By using different fluorescent dyes, the amplification of multiple targets can be followed in a single well. For example, a PCR-based system for identification of fungi and bacteria occurring in indoor environments was patented by Haugland and Vesper (2002), based on the real-time TaqMan™ assay. The procedure allows detection of individual species or simultaneous detection of up to 40 species or species groups based on primers to parts of the ITS, and specific, oligonucleotide fluorescent probes that hybridize to the PCR products. Although the patent does not report specific detection of any of the mycotoxigenic species considered in this article, two primers and a probe are noted that are relevant for the detection of a group of species related to *P. verrucosum*. A multiplex assay based on a single gene region should be more amenable to quantification because there is a greater likelihood of similar copy number across species for a given gene than for different genes or random fragments.

The design and properties of oligonucleotides for standard hybridization (Southern and dot blot), for DNA arrays and for TaqMan™ are very similar and will be the focus of the rest of this paper. For ecological studies, arrays offer the ability to detect and identify microorganisms from all taxonomic groups, eukaryote and prokaryote, with a single set of techniques. Arrays can be easily customized for particular commodities or niches by selecting a set of relevant oligonucleotides from a large library (Wilson et al., 2002; Fessehaie et al., 2003; Lievens et al., 2003). It is also possible to continue adding oligonucleotides as new species are discovered or new genes are studied, increasing the comprehensiveness and taxonomic rigour of the resulting system. The development of a multiplex approach with oligonucleotides designed in a hierarchical fashion for phylogenetically relevant groups, clades and species allows the detection of unknown species in environmental samples if the broader phylogenetic group they belong to is represented. Membranes used for macroarrays can be erased and re-used (Fessehaie et al., 2003), a significant cost saving over microarrays.

One of the challenges in designing species-specific PCR primers or oligos has been the limitations of the human brain to process the large volume of data necessary to discover the 'signature oligonucleotides' that can be used to detect individual species or groups of species. It has been necessary to align sequences, group them according to similarities, and examine the matrix by eye to locate specific sequence regions. For an alignment of any significant size, this process takes weeks of eye-straining labour. Also, the alignment context tends to introduce a cladistic bias to the process. Because these cladistic concepts downplay the value of gaps and insertions, and emphasize shared characters, it is easy to overlook areas

in the alignment that actually contain unique characters (nucleotides or deletions) for specific oligonucleotides. The design of group-specific oligonucleotides is even more difficult because conserved regions must be considered while keeping the overall alignment in perspective.

We have designed a program called 'Signature Oligo' to facilitate the selection of species or clade specific oligos and have automated the steps required for the design of specific oligonucleotides. This will be described in detail elsewhere by Zahariev et al. (unpublished), and we will only summarize the process here. This approach could be used to design PCR primers, but we will focus our demonstration on oligonucleotides that could be used for the different oligonucleotide hybridization techniques (Figure 5). Although this is a virtual experiment and the results have not been tested with DNA samples, we believe that this will demonstrate the potential power of this bioinformatics approach and provide putative specific oligos for further assay development. For the purposes of this exercise, we will focus on internal transcribed spacer (ITS) and *BenA* sequences for the toxin producing species of *Aspergillus*, *Penicillium* and *Fusarium* that were the focus of the first part of this article. ITS sequences for approximately 600 species of ascomycetes, including all the ITS and *BenA* sequences for species of the *Trichocomaceae* and *Nectriaceae* available in October–November 2000, were downloaded from GenBank. The sequences were trimmed to common 5′ and 3′ points. For ITS, any segments of large or small subunit rDNA were removed (except for the CATTA at the end of the small subunit, which served as a useful signature at the start of the sequences) and sequences that were either only ITS1 or ITS2 were removed. A large FASTA format file was generated for each gene region following this editing and trimming. For *BenA*, we focussed on the fragment amplified by primers Bt2a and Bt2b (Glass and Donaldson, 1995). For most of the fungi considered here, this region contains either only very short introns, or no introns at all; however, this is the part of the *BenA* gene with the largest number of representative sequences in GenBank. Both data sets were supplemented with our own in-house sequences, most of them for *Penicillium*, but these are unlikely to have influenced the results in any significant way for this exercise. The *BenA* analysis was repeated in August 2003 with additional sequences released in GenBank in the intervening time. The final *BenA* analysis included approximately 200 species.

The sequences were aligned and subjected to parsimony analyses (e.g. Figures 1–4) in order to identify species and groups of species that were targets for oligonucleotide design. In some genera, particularly with ITS sequences in *Fusarium* and *Penicillium*, multiple species have identical sequences. For the purpose of the analysis, these were considered 'sequence species', because any attempt to find oligonucleotides to distinguish the actual species would be futile. The alignment and analysis exercise is also necessary to detect and correct problems arising from misidentified strains.

The two main programs employed utilize unaligned sequences in the FASTA format, with one sequence per file. In order to begin the analysis, all sequences in the single large FASTA file were converted to this format using the utility PUMA (Parsing Utility with Many Applications, N. Tinker unpublished), and sorted into folders containing the sequences for which we wanted specific oligos. For the analysis of each gene set, a single hierarchy level of folders was created, each containing all the sequences of particular target taxa. The largest analysis included species folders, with subsequent analyses including sequences sorted into larger clades (species complexes, subgenera, genera, etc.). Once Signature Oligo encounters a folder within the database, it searches for a unique oligonucleotide conserved for all the sequences within that folder and yet different from any other sequences in the database. Although the ITS and *BenA* analyses were conducted separately here, this was not necessary. All sequences that were downloaded for comparative purposes, but that did not represent actual target species or groups, were placed in a folder labelled 'other'.

Signature Oligo allows the user to designate the length of the 'word' that will be the basis for the search, i.e. a number of bases that would correspond to a hypothetical diagnostic oligonucleotide. The software essentially searches for unique short sequences in the data. In a data set of highly divergent sequences, a search for longer 'words' (e.g. 24 bases) is often successful. Once the data is organized into higher taxonomic categories (e.g. folders representing genera instead of species or clusters), it is more difficult to find

long oligonucleotides conserved in all sequences within each folder, and it helps to set a shorter oligonucleotide length. The algorithm scans each sequence starting at base 1 for oligonucleotides of length *n*, with the restriction that if sequences are in a folder instead of alone, the unique oligonucleotide must be present in all sequences within that folder. The analyses we have done to date are completed in a few seconds. This speed allows experimentation in the length of 'word', so that an acceptable number of oligonucleotides can be identified. One output format is a list of all the unique oligonucleotides per species. Another output is a text file, with each row denoting a target sequence (the representative one when there are many sequences in a folder), and tab delimited numbers representing each base number in the sequence where a unique oligonucleotide is centered.

The tab delimited output format of Signature Oligo is suitable for input to Array Designer 1.1 (Premier Biosoft International, Palo Alto, CA), which we use for optimizing hybridization oligonucleotides. This software analyzes each location identified by Signature Oligo to select oligonucleotides of a given melting temperature (55 °C at 6 × SSC), without dimers or hairpins. In Array Designer, the user has some flexibility in the minimum and maximum length of the oligonucleotides and their location with respect to the input file (e.g. ±5 bases from the site identified by Signature Oligo). This allows optimization of the oligonucleotides for their intended use. Generally, this process results in the rejection of 1/4–1/3 of the oligos for reasons related to secondary structure or Tm. Shifting of the oligo sequence during optimization away from the originally different central locations of adjacent oligos often results in some identical oligos for one diagnostic sequence section.

Finally, Array Designer 1.1 creates a batch file to automate a BLAST search of all putative oligonucleotides through the Internet. This begins the validation for the oligonucleotides, allowing their actual specificity to be assessed against the entire GenBank database. It is surprising how often 18–22 base oligonucleotides have no BLAST matches apart from their own sequences. Usually, unexpected perfect matches are from various genomic projects that are not homologous with the gene being amplified, and are of no consequence in the design of arrays based on the amplification of these genes. This process often takes several hours, and sometimes several days, depending on the speed of the Internet connection and traffic load at GenBank. The end result is a spreadsheet with each row representing a potential oligonucleotide, with its location as originally identified by Signature Oligo, the columns representing the parameters for each oligonucleotides, and a hyperlink to the stored BLAST search. Depending on the relative comprehensiveness of the GenBank holdings compared to internal databases, an internal BLAST search of in-house sequences can also be a useful means of evaluating the oligonucleotides. We have performed such in-house BLAST searches for our *BenA* database and have created a second column of hyperlinks to access these results rapidly for each oligonucleotide.

Evaluation of the oligos resulting from this process involves careful examination of the BLAST results. Many oligos can be rejected quickly because the optimization process has destroyed their specificity. Consideration of the mismatches that the proposed oligo has with closest neighbours is critical. Because differences in resulting Tm would interfere with hybridization with imperfect matches in the relatively stringent conditions used for arrays or macroarrays, mismatches of more than 2–3 bases are certain to prevent non-target hybridization. Central mismatches in the oligo are preferred, in contrast to the design of PCR primers, where especially 3′ mismatches will interfere with successful priming. Central mismatches of C with A, for example, are virtually certain to result in poor hybridization because of the highly unstable nature of C–A mismatches, whereas G–T mismatches are more stable and thus less favourable.

The results for this process for the *A. flavus* complex are presented in Table 3 and Figure 6, based on publicly available ITS and *BenA* sequences. Unfortunately, not all species of the complex were represented in the *BenA* database; in particular, homologous sequences for the domesticated species *A. sojae* and *A. oryzae* were not available. It is noteworthy that in general, the internal transcribed spacer yields fewer species-specific oligos than does the more variable *BenA*. For the ITS, in general we would like to be able to choose at least two oligonucleotides, one from the ITS1 and one from the ITS2, but as this example

Table 3. Oligonucleotides designed for 55 °C and 6 × SSC hybridization to distinguish mycotoxigenic species of *Aspergillus, Fusarium* and *Penicillium* producing the five major mycotoxins

Species or Group	ITS (position) oligo sequence	Notes	β-Tubulin (position) oligo sequence	Notes
Aflatoxin-producing species				
A. bombycis	One oligo rejected	–	(24) TATGCTTTGGACCCAGGACC	F
			(36) CCAGGACCTCAGCAGAAACA	F
A. flavus	Four oligos rejected	–	(1) ATGTCTCAATGCCTTCGAGTTAG	C
			(63) GGATGTGTCCTGTTATATCTGCC	C
A. flavus incl. *A. oryzae*	(485) GCCGAACGCAAATCAATCTTTT	C (t/a), B1	Not tested	
A. nomius	(130) ACCACGAACTCTGAACGATCT	G	(41) GCCTCAGCAGAAACATGAGC	
			(306) TTTTCAGCCGCTATAAAAACGC	C (g/t)
A. nomius 1	Not tested	–	(169) ACACCTCGAATGAACGACGA	F
			(198) ATTGGAAGAGTTTTGGATGGGTC	C
A. nomius 2	Not tested	–	(13) CCCCGAGTCAATATGCTTTGG	A2
			(148) GGTGTGTAAGTACAACCCGTG	B2
			(209) TTTCGGATGGGTCTGACAGG	A4, B3
A. parasiticus	One oligo rejected	–	(8) GATACCTTCGAGTTAATATGCTTTGG	A4
			(65) ATGTGTCCTATTATATCTGCCACG	B1, C(a/g)
A. parasiticus/flavus	None found	–	(50) AAAGCATGATCTCGGATGTGTC	E
			(304) TTCAGCATCTATGAAAACGCTTTG	C
A. pseudotamarii	Not tested	–	(25) ATGCTTTGGACCAAGGAACTTC	B2
			(66) TGTGCCCTATTGCGTTTGC	F
			(72) CTATTGCGTTTGCTAACATCCTTG	F
subgenus *Flavi*	(125) GGAGACACCACGAACTCTGT	F	(326) CTGACCGCTTCTCCAGGC	
Deoxynivalenol- and zearalenone-producing species				
F. culmorum	None found	–	(27) GTGTTTACAACGGCACCTCC	A1
			(95) GTCACTCCTGCTACGAAAAACA	C(t/c)
			(105) CTACGAAAAACACAAGCTCACG	B2
F. graminearum	None found	–	(80) AACGAGGTTTGTTCAGTCACTAC [a]	B2
F. pseudograminearum	None found	–	(196) CGCCGTTCGTGCTGGT	A1, B4
subgenus *Fusarium*	None found	–	None found	-
Fumonisin-producing species				
F. acutatum	None found	–	(109) GTGGCCGTGTTGCTATGAAG [b]	F
F. anthophilum	(413) TCGTTACTGGTACTCGTCGC	C (c/a)	(7) GCTGCTTCAGACTTCCGTAAC	C (a/t)
			(66) TCTGATTTTGCACATGAAGATTATTTG	C (a/g)
F. begoniae	One oligo rejected	–	(64) ATTCTGATGTAGCACATGGAGATTAT	2B, C (a/t)
F. dlamini	One oligo rejected	–	(182) CCTGGTACTATGGACGCCG	C (c/t), F
F. nygamai	(18) CGGTAAAACGGAACGGCC	C (a/g)	One oligo rejected	
F. proliferatum	None found	–	(114) CCACAAGCTCACACAACTAGG[c]	A2
F. pseudocircinatum	None found	–	(65) ATGAGTGTTTATTTCAACGAGGTATG	C (t/c)
F. sacchari	(345) GAGTCAAATCCGTTCCCCAAAT	C	(96) ACAGTCAATGCCAATAATTCCCA4[d]	C (t/g)

Table 3. (Continued)

Species or Group	ITS (position) oligo sequence	Notes	β-Tubulin (position) oligo sequence	Notes
			(411) CTCGCAACTGGTAATCGTCG	F
F. thapsinum	None found	–	(83) GAGGTATGTATTAGCAGTCTATGTCA	F
F. verticillioides	None found	–	(109) AGAGTTCACACGCTCACACA[e]	F
fukikuroi/*oxysporum* complex	None found	–	(31) CGCTACCTGACCTGCTCG	
Ochratoxin-producing species				
A. carbonarius	(96) GCATCTCTGCCCCTCGG	F		No data
	(145) CTGTCTGAAATCGTGAAGTCTGA	F		
	(386) CCCTGTCTGGGGGGACG	F		
	(486) TGCCGACAACTCCAACCTT	F		
A. ochraceus complex	(121) GGAGACACCAACGTGAACAC	F		No data
			(128) CCAACGTGAACACTGTCTGAA	F
P. verrucosum	None found	–	(165) GTTCCAGTCGTTGAACTCACAT	A, C(a/g)
			(171) GTCGTTGAACTCACATGGTTAAAG	B4, C(a/g)
			(259) GCAATCCACCAGAAACCAACA	A3, B4
			(269) AGAAACCAACATCACCATTAAAACTTA	A1, C(a/c)
Penicillium subgenus, *Penicillium*	None found	–		

Rejected oligos are those that were initially identified as species-specific, but were not suitable for use as hybridization probes. No species-specific oligos were detected for *Aspergillus alliaceus*, *A. muricatus*, *A. niger*, *A. ochraceus*, *Fusarium andyazi*, *F. brevicatenulatum*, *F. fujikuroi*, *F. globosum*, *F. napiforme*, *F. polyphialidicum*, *F. phylophyllum*, *F. pseudonygamae*, *F. subglutinans* and the lineages of *F. graminearum*. Codes for notes: A – 5′ mismatch(es) with closest relatives; B – 3′ mismatch(es) with closest relatives; C – central mismatch with closest relatives, for single mismatches with the corresponding mismatched bases in parentheses in lower case; D – matches only target species, but not all strains; E – no homologies with homologous sequences closest relatives; F – similar to but different from only non-homologous genomic sequences; G – no similar sequences. Numbers following letter codes A, B, C indicate the number of mismatched bases.

[a] Also reacts with *F. flocciferum*.

[b] Also similar to *Fusarium* spp. NRRL 26756, 26757.

[c] Also reacts with *F. annulatum*.

[d] Also reacts with *F. neoceras*.

[e] Also reacts with *G. fujikuroi*.

Figure 6. Locations of species- and group-specific oligonucleotides for aflatoxin-producing members of the *Aspergillus flavus* complex in portions of an alignment for the Bt2a-b region of the *BenA* gene. Oligonucleotides that are specific to a cluster or subgenus are boxed. Overlapping oligonucleotides are underlined with their intercepts italicized. The bases that impart specificity are in bold. A combination of bases is often required for the overall specificity.

shows, this is not always possible. The *BenA* gene was a much more productive region for oligonucleotides in this case, and the reason for this is obvious when the cladogram (Figure 3) is considered. All of the species and groups are well defined and separated by enough genetic distance to allow the consideration of several possible sites for oligos for each species or group. We were able to design at least two oligonucleotides for all the aflatoxin-producing species in this cluster, as well as oligos that recognize two infraspecific groups within *A. nomius*, the *A. flavus*/*A. parasiticus* species complex, and the entire *A. flavus* complex. These oligos could be incorporated into an array that would detect hitherto unknown species of the *A. flavus* complex (and thus potential producers of aflatoxin) and individual aflatoxin-producing species. In general, in order to use the forward and reverse strands of each labelled amplicon, we would use reverse complement oligos for detecting group oligos, and sense oligos for detecting species. The same oligos could be used in standard hybridization, TaqMan™ or Molecular Beacon assays, although the addition of complementary bases at each end of the oligonucleotide will be necessary to make a stem for the beacon.

In the case of the *Aspergillus flavus* complex, the data for the ITS and *BenA* is relatively complete within the group, but there are only scattered *BenA* data for species of *Aspergillus* outside the complex. This is unlikely to cause problems with the species-specific oligonucleotides because a good sampling of *BenA* haplotypes for each species is available, although it is always possible that newly discovered haplotypes might have mismatches. It is possible, however, that the proposed group oligos may have a broader hybridization activity than we are able to predict at this time. We have not attempted to distinguish the subgroups of *A. flavus* detected by Geiser et al. (1998, 2000), which appear to be very similar in their *BenA* sequences.

In *Fusarium*, the phylogenetic tree derived from *BenA* species (Figure 4) shows that there is a very small genetic distance between several of the species that produce fumonisin. It is perhaps not surprising that we were unable to detect species-specific oligos for many of these species using either the ITS or *BenA* data. Similarly, no specific oligos were detected for the phylogenetic lineages of *F. graminearum* (O'Donnell et al., 2000a) using either ITS or *BenA*. One oddity is the discovery of one potentially diagnostic oligo for *F. nygamai* in the ITS, but the absence of any diagnostic oligos for this species based on *BenA*.

Four potentially diagnostic oligos for the best known OA-producing species of *Penicillium*,

P. verrucosum, were detected in the relatively complete *BenA* database assembled for the subgenus *Penicillium*. However, at the time of writing, no ITS or *BenA* data was published for the closely related *P. nordicum*. Similarly, *BenA* sequences for OA-producing *Aspergillus* species are currently unavailable. Because of its comparative genetic distance from the other black *Aspergillus* species, *A. carbonarius* can be distinguished easily by two oligos in each of the ITS1 and ITS2 regions. However, no species-specific oligos were found in the ITS for *A. niger* or *A. ochraceus*.

Concluding remarks

Species concepts for many mycotoxigenic fungi have fluctuated over time. Phylogenetic databases and studies have provided new insights to resolve many of these taxonomic issues and are likely to result in a more stable taxonomic framework for identification of these important fungi. Better taxonomic keys and sequence databases will make identification of pure cultures faster and more accurate. Species specific DNA-based assays have been designed for several mycotoxigenic fungi, enabling direct detection from environmental samples without the isolation and purification of cultures. The exercise described above demonstrates how libraries of species-specific oligos can be identified from phylogenetic DNA databases. The use of the new software 'Signature Oligo' in combination with the existing oligo optimization software 'Array Designer' greatly facilitates the oligo design process. These oligos do not have unacceptable dimers or hairpins and are adjusted for identical hybridization conditions. However, we emphasize that the oligos identified by this 'electronic exercise' still need to be validated and tested using DNA from properly identified samples before being used in the 'real world'. For example, we have not tested whether these oligonucleotides are located in a DNA region with strong secondary structures, thus making the PCR amplicon unavailable for hybridization at the oligo site. We feel that the combination of ITS and *BenA* is a promising first step towards the development of multigene, multispecies detection and identification arrays. With more comprehensive databases, it should be technically feasible to have a single assay that would detect all known mycotoxigenic species. The ability to monitor hundreds of microorganisms simultaneously in a particular niche or commodity will revolutionize our understanding of how microorganisms interact in nature. We hope that the mycotoxigenic fungi will be among the first moulds to be included in such arrays.

References

Abarca ML, Accensi F, Bragulat MR, Castella G and Cabanes FJ (2003) *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. Journal of Food Protection 66: 504–506.

Al-Mussalam A (1980) Revision of the black *Aspergillus* species. PhD, Rijkuniversiteit Utrecht, Utrecht, the Netherlands.

Aoki T and O'Donnell K (1999) Morphological and molecular characterization of *Fusarium pseudograminearum* sp. nov., formerly recognized as the Group 1 population of *F. graminearum*. Mycologia 91: 597–609.

Berbee ML, Yoshimura A, Sugiyama J and Taylor JW (1995) Is *Penicillium* monophyletic? An evaluation of phylogeny in the family Trichocomaceae from 18S, 5.8S and ITS ribosomal DNA sequence data. Mycologia 87: 210–222.

Bluhm BH, Flaherty JE, Cousin MA and Woloshuk CP (2002) Multiplex polymerase chain reaction assay for the differential detection of trichothecene- and fumonisin-producing species of *Fusarium* in cornmeal. Journal of Food Protection 65: 1955–1961.

Britz H, Steenkamp ET, Coutinho TA, Wingfield BD, Marasas WFO and Wingfield BD (2002) Two new species of *Fusarium* section *Liseola* associated with mango malformation. Mycologia 94: 722–730.

Burgess LW, Wearing AH and Toussoun TA (1975) Surveys of fusaria associated with crown rot of wheat in eastern Australia. Australian Journal of Agricultural Research 26: 791–799.

Ciegler A, Lee LS and Dunn JJ (1981) Production of naphthoquinone mycotoxins and taxonomy of *Penicillium viridicatum*. Applied and Environmental Microbiology 42: 446–449.

Cotty PJ (1989) Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79: 808–814.

Cruickshank RH and Pitt JI (1987) Identification of species in *Penicillium* subgenus *Penicillium* by enzyme electrophoresis. Mycologia 79: 614–620.

Edwards SG, O'Callaghan J and Dobson ADW (2002) PCR-based detection and quantification of mycotoxigenic fungi. Mycological Research 106: 1005–1025.

Egel DS, Cotty PJ and Bhatnagar D (1994) Variability among atoxigenic *Aspergillus flavus* strains in ability to prevent aflatoxin contamination and production of aflatoxin biosynthetic pathway enzymes. Applied and Environmental Microbiology 60: 2248–2251.

Ehrlich KC, Montalbano BG and Cotty PJ (2003) Sequence comparison of aflR from different *Aspergillus* species provides evidence for variability in regulation of aflatoxin production. Fungal Genetics and Biology 38: 63–74.

Farber P, Geisen R and Holzapfel HW (1997) Detection of aflatoxigenic fungi in figs by a PCR reaction. International Journal of Food Microbiology 36: 215–220.

Feibelman TP, Cotty PJ, Doster MA and Michailides TJ (1998) A morphologically distinct strain of *Aspergillus nomius*. Mycologia 90: 618–623.

Fessehaie A, De Boer SH and Lévesque CA (2003) An oligonucleotide array for the identification and differentiation of bacteria pathogenic on potato. Phytopathology 93: 262–269.

Fotso J, Leslie JF and Smith JS (2002) Production of beauvericin, moniliformin, fusaproliferin, and fumonisins B1, B2, and B3 by fifteen ex-type strains of *Fusarium* species. Applied and Environmental Microbiology 68: 5195–5197.

Francis RG and Burgess LW (1977) Characteristics of two populations of *Fusarium roseum* 'Graminearum' in eastern Australia. Transactions of the British Mycological Society 68: 421–427.

Frisvad JC and Samson RA (2000) *Neopetromyces* gen. nov and an overview of teleomorphs of *Aspergillus* subgenus *Circumdati*. Studies in Mycology: 201–207.

Frisvad JC, Bridge PD and Arora DK (eds) (1998) Chemical Fungal Taxonomy 398 pp. Marcel Dekker, New York, USA.

Gams W and Nirenberg H (1989) A contribution to the generic delimitation of *Fusarium*. Mycotaxon 35: 407–416.

Gams W, Christensen M, Onions AHS, Pitt JI and Samson RA (1985) Infrageneric taxa of *Aspergillus*. In: Pitt JI (ed), *Advances in Penicillium and Aspergillus Systematics* (pp. 55–62) Plenum Press, New York, USA.

Geiser DM, Pitt JI and Taylor JW (1998) Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. Proceedings of the National Academy of Sciences of the United States of America 95: 388–393.

Geiser DM, Dorner JW, Horn BW and Taylor JW (2000) The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus oryzae*. Fungal Genetics and Biology 31: 169–179.

Gelderblom WCA, Jaskiewicz K, Marasas WFO, Thiel PG, Horak RM, Vleggaar R and Kriek NPJ (1988) Fumonisins – Novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. Applied and Environmental Microbiology 54: 1806–1811.

Gerlach W and Nirenberg H (1982) The genus *Fusarium* – a pictorial atlas. Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin-Dahlem 209: 1–406.

Glass NL and Donaldson GC (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied and Environmental Microbiology 61: 1323–1330.

Haugland R and Vesper S (2002) Method of identifying and quantifying specific fungi and bacteria. US 6,387,652 B1. United States Environmental Protection Agency, USA.

Heenan CN, Shaw KJ and Pitt JI (1998) Ochratoxin A production by *Aspergillus carbonarius* and *A. niger* isolates and detection using coconut cream agar. Journal of Food Mycology 1: 67–72.

Ichinoe M, Kurata H, Sugiura Y and Ueno Y (1983) Chemotaxonomy of *Gibberella zeae* with special reference to production of trichothecenes and zearalenone. Applied and Environmental Microbiology 46: 1364–1368.

Ito Y, Peterson SW, Wicklow DT and Goto T (2001) *Aspergillus pseudotamarii*, a new aflatoxin producing species in *Aspergillus* section *Flavi*. Mycological Research 105: 233–239.

Klich MA (2002) Identification of common *Aspergillus* species. Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands.

Klich MA and Pitt JI (1988a) Differentiation of *Aspergillus flavus* from *A. parasiticus* and other closely related species. Transactions of the British Mycological Society 91: 99–108.

Klich MA and Pitt JI (1988b) A Laboratory Guide to Common *Aspergillus* Species and their Teleomorphs. CSIRO Division of Food Processing, North Ryde, Australia.

Klich MA, Mullaney EJ, Daly CB and Cary JW (2000) Molecular and physiological aspects of aflatoxin and sterigmatocystin biosynthesis by *Aspergillus tamarii* and *A. ochraceoroseus*. Applied Microbiology and Biotechnology 53: 605–609.

Knoll S, Mulfinger S, Niessen L and Vogel RF (2002a) Rapid preparation of *Fusarium* DNA from cereals for diagnostic PCR using sonification and an extraction kit. Plant Pathology 51: 728–734.

Knoll S, Vogel RF and Niessen L (2002b) Identification of *Fusarium graminearum* in cereal samples by DNA Detection Test Stipes™. Letters in Applied Microbiology 34: 144–148.

Kozakiewicz Z (1989) *Aspergillus* species on stored products. Mycological Papers 161: 1–188.

Kurtzman CP, Smiley MJ, Robnett CJ and Wicklow DT (1986) DNA relatedness among wild and domesticated species in the *Aspergillus flavus* group. Mycologia 78: 955–959.

Kurtzman CP, Horn BW and Hesseltine CW (1987) *Aspergillus nomius*, a new aflatoxin-producing species related to *Aspergillus flavus* and *Aspergillus tamarii*. Antonie Van Leeuwenhoek 53: 147–158.

Kusters van Someren MA, Samson RA and Visser J (1991) The use of RFLP analysis in classification of the black *Aspergilli* – Reinterpretation of the *Aspergillus niger* aggregate. Current Genetics 19: 21–26.

Kwasna H, Chelkowski J and Zajkowski P (1991) Grzyby (Mycota), tom. XXII. Siepik (*Fusarium*). Polska Akademia Nauk, Flora Polska, Warszawa-Krakow, Poland.

Larsen TO, Svendsen A and Smedsgaard J (2001) Biochemical characterization of Ochratoxin A-producing strains of the genus *Penicillium*. Applied and Environmental Microbiology 67: 3630–3635.

Leslie JF (1995) *Gibberella fujikuroi*: available populations and variable traits. Canadian Journal of Botany 73: S282–S291.

Lévesque CA (2001) Molecular methods for detection of plant pathogens – What is the future? Canadian Journal of Plant Pathology 24: 333–336.

Lievens B, Brouwer M, Vanachter A, Lévesque CA, Cammue B and Thomma B (2003) Design and development of a DNA array for rapid detection and identification of multiple tomato vascular wilt pathogens. FEMS Microbiology Letters 223: 113–122.

LoBuglio KF, Pitt JI and Taylor JW (1993) Phylogenetic analysis of two ribosomal DNA regions indicates multiple independent losses of a sexual *Talaromyces* state among asexual *Penicillium* species in subgenus *Biverticillium*. Mycologia 85: 592–604.

LoBuglio KF, Pitt JI and Taylor JW (1994) Independent origins of the synnematous *Penicillium* species, *P. duclauxii*, *P. clavigerum* and *P. vulpinum*, as assessed by two ribosomal DNA regions. Mycological Research 98: 250–256.

Lund F and Frisvad JC (1994) Chemotaxonomy of *Penicillium aurantiogriseum* and related species. Mycological Research 98: 481–492.

Malloch D (1985) Taxonomy of the Trichocomaceae. In: Arai T (ed) Filamentous Microorganisms, Biomedical Aspects (pp. 37–45) Japan Scientific Societies Press, Tokyo, Japan.

Marasas WFO, Rheeder JP, Lamprecht SC, Zeller KA and Leslie JF (2001) *Fusarium andiyazi* sp. nov., a new species from sorghum. Mycologia 93: 1203–1210.

Martin RR, James D and Lévesque CA (2000) Impacts of molecular diagnostic technologies on plant disease management. Annual Review of Phytopathology 38: 207–239.

Miller JD, Greenhalgh R, Wang YZ and Lu M (1991) Trichothecene chemotypes of three *Fusarium* species. Mycologia 83: 121–130.

Nelson PE, Toussoun TA and Marasas WFO (1983) *Fusarium* Species: An Illustrated Manual for Identification. Pennsylvania State University, University Park, PA, USA.

Nicholson P, Simpson DR, Weston G, Rezanoor HN, Lees AK, Parry DW and Joyce D (1998) Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays. Physiological and Molecular Plant Pathology 53: 17–37.

Niessen ML and Vogel RF (1998) Group specific PCR-detection of potential trichothecene-producing *Fusarium* species in pure cultures and cereal samples. Systematic and Applied Microbiology 21: 618–631.

Nirenberg H (1976) Ubersuchungen über die morphologische und biologische Differenzierung in der *Fusarium*-Sektion *Liseola*. Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin-Dahlem 169: 1–117.

Nirenberg HI and O'Donnell K (1998) New *Fusarium* species and combinations within the *Gibberella fujikuroi* species complex. Mycologia (USA) 90: 434–458.

O'Donnell K, Cigelnik E and Nirenberg HI (1998a) Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90: 465–493.

O'Donnell K, Kistler HC, Cigelink E and Ploetz RC (1998b) Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proceedings of the National Academy of Sciences 95: 2044–2049.

O'Donnell K, Kistler HC, Tacke BK and Casper HH (2000a) Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proceedings of the National Academy of Sciences 97: 7905–7910.

O'Donnell K, Nirenberg HI, Aoki T and Cigelnik E (2000b) A multigene phylogeny of the *Gibberella fujikuroi* species complex: Detection of additional phylogenetically distinct species. Mycoscience 41: 61–78.

Ouellet T and Seifert KA (1993) Genetic characterization of *Fusarium graminearum* strains using RAPD and PCR amplification. Phytopathology 83: 1003–1007.

Parenicova L, Benen JAE, Samson RA and Visser J (1997) Evaluation of RFLP analysis of the classification of selected black aspergilli. Mycological Research 101: 810–814.

Parenicova L, Skouboe P, Frisvad J, Samson RA, Rossen L, ten Hoor-Suykerbuyk M and Visser J (2001) Combined molecular and biochemical approach identifies *Aspergillus japonicus* and *Aspergillus aculeatus* as two species. Applied and Environmental Microbiology 67: 521–527.

Peterson SW (2000a) Phylogenetic analysis of *Penicillium* species based on ITS and LSU-rDNA nucleotide sequences. In: Pitt JI (ed) Integration of Modern Taxonomic Methods for *Penicillium* and *Aspergillus* (pp. 163–178). Harwood Academic Publishers, Amsterdam, the Netherlands.

Peterson SW (2000b) Phylogenetic relationships in *Aspergillus* based on rDNA sequence analysis. In: Pitt JI (ed). Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* (pp. 323–355) Harwood Academic Publishers, Amsterdam, the Netherlands.

Peterson SW, Ito Y, Horn BW and Goto T (2001) *Aspergillus bombycis*, a new aflatoxigenic species and genetic variation in its sibling species, *A. nomius*. Mycologia 93: 689–703.

Pitt JI (1979) The Genus *Penicillium* and its Teleomorphic States *Eupenicillium* and *Talaromyces*. Academic Press, London.

Pitt JI, Samson RA and Frisvad JC (2000) List of accepted species and their synonyms in the family *Trichocomaceae*. In: Pitt JI (ed). Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* (pp. 9–49) Harwood Academic Publishers, Amsterdam, the Netherlands.

Raper KB and Fennell DI (1965) The Genus *Aspergillus*. Williams and Wilkins, Baltimore, MD, USA.

Raper KB and Thom C (1949) A Manual of the Penicillia. Williams and Wilkins, Baltimore, MD, USA.

Rheeder JP, Marasas WFO and Vismer HF (2002) Production of fumonisin analogs by *Fusarium* species. Applied and Environmental Microbiology 68: 2101–2105.

Rigo K, Varga J, Toth B, Teren J, Mesterhazy A and Kozakiewicz Z (2002) Evolutionary relationships within *Aspergillus* section *Flavi* based on sequences of the intergenic transcribed spacer regions and the 5.8S rRNA gene. Journal of General and Applied Microbiology 48: 9–16.

Samson RA and Pitt JI (eds) (1985) Advances in *Penicillium* and *Aspergillus* Systematics. Plenum Press, New York, USA.

Samson RA and Pitt JI (eds) (1990) Modern Concepts in *Penicillium* and *Aspergillus* Classification. Plenum Press, New York, USA.

Samson RA, Stolk AC and Hadlok R (1976) Revision of the subsection *Fasciculata* of *Penicillium* and some allied species. Studies in Mycology 11: 1–47.

Samson RA and Pitt JI (eds) (2000) Integration of Modern Methods for *Penicillium* and *Aspergillus*, 510 pp. Harwood Academic Publishers, Amsterdam, the Netherlands.

Samson RA, Hoekstra ES, Frisvad JC and Filtenborg O (2000) Introduction to Food and Airborne Fungi, 6th edn. Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands.

Schaad NW and Frederick RD (2002) Real-time PCR and its application for rapid plant disease diagnostics. Canadian Journal of Plant Pathology 24: 250–258.

Schilling AG, Moller EM and Geiger HH (1996) Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*. Phytopathology 36: 515–522.

Seifert KA and Louis-Seize G (2000). Phylogeny and species concepts in the *Penicillium aurantiogriseum* complex as

inferred from partial beta-tubulin-gene DNA sequences. In: Pitt JI (ed). Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* (pp. 189–198) Harwood Academic Publishers, Amsterdam, the Netherlands.

Seo J-A, Kim J-C and Lee Y-W (1996) Isolation and characterization of two new type C fumonisins produced by *Fusarium oxysporum*. Journal of Natural Products 59: 1003–1005.

Shapiro A and Mullins JT (1997) Localization of cytoplasmic water-soluble reserve (1–3)-beta-glucans in *Achlya* with immunostaining. Mycologia 89: 89–91.

Shapira R, Paster N, Eyal O, Menasherov M, Mett A and Salomon R (1996) Detection of aflatoxigenic molds in grains by PCR. Applied and Environmental Microbiology 62: 3370–3273.

Skouboe P, Frisvad JC, Taylor JW, Lauritsen D, Boysen M and Rossen L (1999) Phylogenetic analysis of nucleotide sequences from the ITS region of terverticillate *Penicillium* species. Mycological Research 103: 873–881.

Snyder WC, Hansen HN and Oswald JW (1956) Cultivars of the fungus, *Fusarium*. Journal of Madras University B 27: 185–192.

Syndenham EW, Marasas WFO, Thiel PG, Shephard GS and Niewenhuis JJ (1991) Production of mycotoxins by selected *Fusarium graminearum* and *F. crookwellense* isolates. Food Additives and Contaminants 8: 31–41.

Taylor JW, Jacobson DJ, Kroken S, Takao K, Geiser DM, Hibbett DS and Fischer MC (2000) Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology 31: 21–32.

Varga J, Kevei E, Toth B, Kozakiewicz Z and Hoekstra RF (2000) Molecular analysis of variability within the toxigenic *Aspergillus ochraceus* species. Canadian Journal of Microbiology 46: 593–599.

Varga J, Rigo K, Toth B, Teren J and Kozakiewicz Z (2003) Evolutionary relationships among *Aspergillus* species producing economically important mycotoxins. Food Technology and Biotechnology 41: 29–36.

Wang L, Yokoyama K, Takahasi H, Kase N, Hanya Y, Yashiro K, Miyaji M and Nishimura K (2001) Identification of species in *Aspergillus* section *Flavi* based on sequencing of the mitochondrial cytochrome b gene. International Journal of Food Microbiology 71: 75–86.

Ward TJ, Bielawski JP, Kistler HC, Sullivan E and O'Donnell K (2002) Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic *Fusarium*. Proceedings of the National Academy of Sciences 99: 9278–9283.

Wilson WJ, Strout CL, DeSantis TZ, Stilwell JL, Carrano AV and Andersen GL (2002) Sequence-specific identification of 18 pathogenic microorganisms using microarray technology. Molecular and Cellular Probes 16: 119–127.

Wollenweber HW and Reinking OA (1935) Die Fusarien, ihre Beschreibung, Schadwirkung und Bekämpfung. Paul Parey, Berlin, Germany.

Yoder WT and Christianson LM (1998) Species-specific primers resolve members of *Fusarium* section *Fusarium*. Fungal Genetics and Biology 23: 68–80.