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The current status of species recognition and identification in *Aspergillus*

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Abstract: The species recognition and identification of aspergilli and their teleomorphs is discussed. A historical overview of the taxonomic concepts starting with the monograph of Raper & Fennell (1965) is given. A list of taxa described since 2000 is provided. Physiological characters, particularly growth rates and the production of extrolites, often show differences that reflect phylogenetic species boundaries and greater emphasis should be placed on extrolite profiles and growth characteristics in species descriptions. Multilocus sequence-based phylogenetic analyses have emerged as the primary tool for inferring phylogenetic species boundaries and relationships within subgenera and sections. A four locus DNA sequence study covering all major lineages in *Aspergillus* using genealogical concordance theory resulted in a species recognition system that agrees in part with phenotypic studies and reveals the presence of many undescribed species not resolved by phenotype. The use of as much data from as many sources as possible in making taxonomic decisions is advocated. For species identification, DNA barcoding uses a short genetic marker in an organism"s DNA to quickly and easily identify it to a particular species. Partial cytochrome oxidase subunit 1 sequences, which are used for barcoding animal species, were found to have limited value for species identification among black aspergilli. The various possibilities are discussed and at present partial β-tubulin or calmodulin are the most promising loci for *Aspergillus* identification. For characterising *Aspergillus* species one application would be to produce a multilocus phylogeny, with the goal of having a firm understanding of the evolutionary relationships among species across the entire genus. DNA chip technologies are discussed as possibilities for an accurate multilocus barcoding tool for the genus *Aspergillus*.

Key words: multolocus phylogenetics, polyphasic taxonomy, species identification, species recognition

INTRODUCTION: Where things stand currently, and how we got here

The last complete monograph of *Aspergillus* was written in 1965 (Raper & Fennell 1965). They recognised 132 species and 18 varieties. This was a pragmatic treatise. Although they provided Latin descriptions for new species, they did not designate formal type specimens, instead using type strains. They described teleomorphs under the anamorph (*Aspergillus*) name. They divided the species into 18 informal "groups" based on the authors" opinions of probable relationships. The "groups" they established, which largely reflected groups defined in previous treatises (Thom & Church 1926; Thom & Raper 1945), have been amazingly stable through a great deal of morphological, physiological and molecular scrutiny over the subsequent 42 years. In an effort to bring the taxonomy of the genus in line with the International Code of Botanical Nomenclature, the species were typified (Samson & Gams 1985), the groups were revised and given formal taxonomic status as sections, and subgenera were added (Gams *et al.* 1985).

A number of changes have been made to the infrageneric taxa proposed by Gams *et al.* (1985). In his revision of the genus based on rDNA sequences, Peterson (2000) proposed eliminating three of the six subgenera established by Gams *et al.* (1985), retaining 12 of the 18 sections, modifying three of the sections and deleting the other three. Frisvad *et al.* (2005) proposed Section *Ochraceorosei* to accommodate the species *A. ochraceoroseus* and *A. rambellii.* The genus *Neopetromyces* was proposed by the same authors for the teleomorph of *A. muricatus* (Frisvad & Samson 2000).

Species named since the turn of the century are listed in Table 1. Raper and Fennell (1965) described 150 taxa in their monograph; the latest compilation of names in current use (Pitt *et al.* 2000) listed 182. Samson (2000) listed another 36 published between 1992 and 1999. More than 40 new species descriptions have been published since then (Table 1), bringing the total number to ~250. The number will continue to grow as we discover new species and refine species concepts.

Aspergillus is one of the most economically important genera of microfungi, so the rigor and stability of its taxonomy is of significant practical concern. We joke that everyone trying to break a patent is a "splitter", and everyone trying to retain a patent is a "lumper", but there is some truth in the joke. Some aspergilli produce metabolites toxic to animals that are highly regulated in many countries while other aspergilli cause allergies or mycoses. It is important that we carefully consider any taxonomic changes in order to keep the taxonomic system practical for economic and regulatory reasons. This has been accomplished through open discussion and consensus-building in meetings such as the *Aspergillus* Workshops, and in efforts such as the lists of accepted species and synonyms (Pitt *et al.* 2000).

Aspergillus taxonomists have a long tradition of an eclectic approach to our discipline. This is reflected in the methods used by many researchers to delineate new species (Table 1). In addition to morphological characters - the shapes and sizes of various structures, we have traditionally used physiological characters such as colony diam and production of coloured metabolites in our taxonomic systems. We now also use data on other metabolites as measured by chromatography and mass spectroscopy. As a rule, molecular data have supported relationships previously inferred based on morphological and physiological characters.

Table 1. Characters used in delineating new species of *Aspergillus* since 2000.

Section	Species	Morphology	Physiology	Molecular	Reference
Circumdati	*A. persii*	i	x		Zotti & Corti (2002)
	A. cretensis	i	x	*BenA*	Frisvad *et al.* (2004)
	A. flocculosus	i		*BenA*	Frisvad *et al.* (2004)
	A. neobridgeri	i	g	*BenA*	Frisvad *et al.* (2004)
	A. pseudoelegans	i	x	*BenA*	Frisvad *et al.* (2004)
	A. roseoglobosus	i	x	*BenA*	Frisvad *et al.* (2004)
	A. steynii	i	g x	*BenA*	Frisvad *et al.* (2004)
	A. westerdijkiae	i	x	*BenA*	Frisvad *et al.* (2004)
Nigri	*A. costaricaensis*	i	g x	*BenA*	Samson *et al.* (2004)
	A. homomorphus	i	g x	*BenA*	Samson *et al.* (2004)
	A. lactocoffeatus	i	g x	*BenA*	Samson *et al.* (2004)
	A. piperis	i	g x	*BenA*	Samson *et al.* (2004)
	A. sclerotioniger	i	g x	*BenA*	Samson *et al.* (2004)
	A. vadensis	i	g x	multi	Vries *et al.* (2005)
	A. ibericus	i	g x	multi	Serra *et al.* (2006)
	A. brasiliensis	i	g x	multi	Varga *et al.* (2007)
	A. uvarum	i	g x	multi	Perrone *et al.* (2007)
	A. aculeatinus	i	g x	multi	Noonim *et al.* (2007)
	A. sclerotiicarbonarius	i	g x	multi	Noonim *et al.* (2007)
Flavi	*A. bombysis*	i	g x	multi	Peterson *et al.* (2001)
	A. pseudotamarii	i	g x	multi	Ito *et al.* (2001)
	A. arachidicola	i	g x	multi	Pildain *et al.* (2007)
	A. minisclerotigenes	i	g x	multi	Pildain *et al.* (2007)
	A. parvisclerotigenus	i	gx	*BenA*	Frisvad *et al.* (2005)
Ochraceorosei	*A. rambellii*	i	g x	ITS, RAPD	Frisvad *et al.* (2005)
Fumigati	*A. lentulus*	i		multi	Balajee *et al.* (2005)
	A. fumigatiaffinis	i	x	multi	Hong,*et al.* (2005)
	A. novofumigatus	i	x	multi	Hong *et al.* (2005)
	A. turcosus	i	g x	multi	Hong *et al.* (2007)
Neosartorya	*N. nishimurae*	a i	g		Takada *et al.* (2001)
	N. otanii	a I	g		Takada *et al.* (2001)
	N. takakii	a			Horie *et al.* (2001)
	N. indohii	a			Horie *et al.* (2003)
	N. tsurutae	A			Horie *et al.* (2003)
	N. coreana	a		*BenA*/calm	Hong *et al.* (2006)
	N. laciniosa	a		*BenA*/calm	Hong *et al.* (2006)
	N. assulata	a i	g x	multi	Hong *et al.* (2007)
	N. denticulata	a i	g x	multi	Hong *et al.* (2007)
	N. galapagensis	a i	g x	multi	Hong *et al.* (2007)
	N. australensis	a i	g x	multi	Samson *et al.* (2007)
	N. ferenczii	a i	g x	multi	Samson *et al.* (2007)
	N. papuensis	a i	g x	multi	Samson *et al.* (2007)
	N. warcupii	a i	g x	multi	Samson *et al.* (2007)
Emericella	*Em. qinqixianii*	a i			Horie *et al.* (2000)
	Em. venezuelensis	a	x	*BenA*	Frisvad & Samson (2004)
Eurotium	*Eu. taklimakanense*	a i	g		Abliz & Horie (2001)

Abbreviations: a = ascospore characters; I = anamorph characters; g = Growth rate; x = Extrolites; BenA = β-tubulin; Calm = calmodulin; multi = three or more molecular probes.

There is no one method (morphological, physiological or molecular) that works flawlessly in recognising species. Perhaps this is why *Aspergillus* taxonomists have so readily embraced the eclectic approach. Morphological characters can vary. For instance, sclerotia which are "characteristic" of some species are not always present in all isolates of a species, and their production can vary among cultures of the same isolate. Currently we are beginning to question the past emphasis on ascospore wall characteristics in systematics. A case in point is the *Emericella nidulans* variants that possess rough-walled ascospores normally characteristic of *Em. rugulosa* (Klich *et al.* 2001). Physiological characters may vary, or in the case of metabolites, be absent altogether in some isolates. Multilocus DNA sequence data are extremely useful for recognising species boundaries, but we do not have strict criteria as to where to draw the line between phylogenetic species and well-differentiated populations that are potentially capable of interbreeding, at the same time integrating them into a coherent species concept. Therefore, we advocate the use of morphological, physiological and molecular data in circumscribing *Aspergillus* species wherever possible.

Although the majority of fungal systematists now utilise molecular data to some extent, researchers give varying levels of weight to molecular versus other sources of information in making taxonomic decisions. Indeed, *Aspergillus* systematists are no exception, and a rather large diversity of opinions on this matter exists even among the authors of this paper! Rather than dwelling on these disagreements, we emphasise the considerable common ground among us, which is leading to a taxonomic system that reflects morphological, physiological, ecological and phylogenetic relationships. Dobzhansky (1951) noted that the discontinuous distribution of variation among organisms is a matter of observation consistent with species being real entities, and that discontinuity is as evident in the genus *Aspergillus* as it is anywhere. While there are inherent problems of logic with placing these discontinuous units into species using consistent rules (Hey 2001) we have to do the best job we can, and the approaches employed over recent decades appear to have served us well. We advocate the use of as much data from as many sources as possible in making taxonomic decisions (Samson *et al.* 2006).

The roles of molecular, morphological and physiological characters in species recognition

It is important to consider carefully the roles of different kinds of characters in drawing boundaries between species. Variable DNA sequence characters provide the best means for inferring relationships among organisms, simply because it is possible to sample very large numbers of variable characters, and for the most part, those characters vary because they are under little or very weak selection. Within and between sister species, the genealogies inferred from single genes do not reflect the underlying organismal genealogies, because of the stochastic effects of the segregation of variation during speciation, and recombination. However, the point where different gene genealogies become concordant is a useful place to assign a species boundary, as it is likely to reflect a lack of historical recombination among species (Dykhuizen & Green 1991; Koufopanou *et al.* 1997; Geiser *et al.* 2000; Taylor *et al.* 2000). In practice, this has proven to be a powerful tool in the fungi and in the genus *Aspergillus*, as discussed below.

An inference of a species boundary based on multilocus data provides an objectively determined line of demarcation, but as a rule in fungi, it appears that reproductive isolation precedes morphological differentiation in speciation (Taylor *et al.* 2006). This can lead to some unsettling observations. On the one hand, it is comforting that there is usually a very strong correlation between biological species in fungi defined based on laboratory mating tests and those defined based on genealogical concordance principles (O"Donnell *et al.* 1998; O"Donnell 2000). In some cases, closely related phylogenetic species show some degree of cross-fertility (Dettman *et al.* 2006), allowing a window into the forces of hybridisation that affect fungal evolution as much as they do animals and plants, yet have gone mostly unobserved because fungal morphological species concepts tend to be too broad to allow hybridisation to be considered (O"Donnell *et al.* 2004). What is less satisfying about the genealogical concordance approach is that it tends to yield species that are morphologically and otherwise biologically cryptic (Koufopanou *et al.* 1997; Geiser *et al.* 1998; O"Donnell *et al.* 2004; Pringle *et al.* 2005; Balajee *et al.* 2005), in some cases leaving authors with little choice but to describe species using nucleotide characters as the primary descriptors (Fisher *et al.* 2002; O"Donnell *et al.* 2004). However, this may be a simple matter of not looking hard enough. Indeed, initial observations of the morphology and physiology of the cryptic human pathogenic species *Coccidioides immitis* and *C. posadasii* uncovered no morphological or physiological differences other than growth rates on media with high salt concentration (Fisher *et al.* 2002), but molecular evolutionary studies of a proline-rich antigen in these species showed different patterns of positive selection that need to be considered in vaccine design (Johannesson *et al.* 2004). While these species may be cryptic to the human eye, perhaps they can be distinguished by the human immune system, and we are served well by recognising them as separate species.

Most *Aspergillus* species were defined based on morphology, with the additional consideration of molecular and extrolite data used in recent years (Hong *et al.* 2005). Molecular phylogenetics has uncovered cryptic speciation in a number of taxa (Geiser *et al.* 1998; Pringle *et al.* 2005; Balajee *et al.* 2005), suggesting that morphological characters provide a very broad species concept that does not reflect the true extent of evolutionary divergence and reproductive isolation, as appears to be the rule in fungi (Geiser 2004). However, physiological characters, including growth rates and the production of extrolites, often show differences that reflect phylogenetic species boundaries (Geiser *et al.* 2000). Considering this, greater emphasis should be placed on extrolite profiles and growth characteristics in species descriptions.

Molecular characters provide the greatest number of variable characters for fungal taxonomy, they can be generated using a widely available technology, that technology comes with an extremely well-developed bioinformatic infrastructure that allows worldwide communication and comparison of results, and they produce results that generally correlate with reproductive barriers and physiological differences. This utility ensures that molecular characters will have a primary role in recognising fungal taxa. However, good taxonomy does not end with the recognition of a species and a Latin binomial. Species descriptions should include data from as many sources as possible, comprising morphology, physiology and molecular data, which can be used not only as tools for identifying an isolate, but understanding its biology.

Some insights on *Aspergillus* species recognition based on multilocus phylogenetics

In recent years, molecular tools such as RFLP"s, RAPD"s, AFLP, MLEE, ribosomal RNA sequences, and protein-coding gene sequences have been applied to taxonomic questions in the genus. Multilocus DNA sequence studies of some anamorphic species have shown that the patterns of polymorphisms in different genes are consistent with recombination in these asexual species (Geiser *et al.* 1998). Clonal lineages accumulate large numbers of deleterious mutations over time but perhaps aspergilli survive because they are not as clonal as we once thought. The discovery of MAT idiomorphs in the complete genome sequence of *A. fumigatus* and the subsequent discovery of both MAT idiomorphs in populations of *A. fumigatus* (Paoletti *et al.* 2005; Dyer & Paoletti 2005; Nierman *et al.* 2006; Rydholm *et al.* 2007) strongly indicate that this species is heterothallic, consistent with population genetic patterns suggesting some level of recombination (Pringle *et al.* 2005). These indications that putatively asexual *Aspergillus* species are actually recombining allow the use of genealogical concordance methods for delimiting species (Taylor *et al.* 2000).

Single locus DNA sequence studies have been conducted in *Aspergillus* using different loci, and there are extensive databases available for nuclear ribosomal RNA genes (large subunit, internal transcribed spacers) and β-tubulin. Any of these gene regions alone may serve as an effective tool for identifying well-defined species, but a weakness of the single locus approach is that not all species can be identified from DNA polymorphisms therein.

In order to resolve the central question of species boundaries in *Aspergillus* and detection of those species using methodologies based on DNA sequences, a four locus DNA sequence study was undertaken covering all major lineages in *Aspergillus* and have included most of the known and accepted species. Interpretation of the results using genealogical concordance theory results in a species recognition system that agrees in part with phenotypic studies and reveals the presence of many undescribed species not resolved by phenotype.

A primary question of this study is whether the *Aspergillus* anamorph is a reliable marker for species that belong in the *Aspergillus* clade. The species of *Sclerocleista*, *S. ornata* and *S. thaxteri,* are vastly different from most *Aspergillus* species and form a distinct group distantly related to the main *Aspergillus* clade. *A. zonatus*, *A. clavatoflavus* (section *Flavi*) and *Warcupiella spinosa* (section *Ornati*), like the *Sclerocleista* species are distant from the main body of species with *Aspergillus* anamorphs and should be placed in their own groups too. *Hemicarpenteles paradoxus*, *A. malodoratus* and *A. crystallinus* are phylogenetically outside of all other *Aspergillus* species (Stevenson, Samson & Varga, unpubl. data).

In examining close relationships, it was found that *Aspergillus niveus* ex type is not the same species as *Fennellia nivea* ex type; *Chaetosartorya stromatoides* ex type is not the same species as *Aspergillus stromatoides* ex type (Peterson 1995) and *Fennellia flavipes* ex type is not the same species as *A. flavipes* ex type. This raises a nomenclatural challenge, because multilocus DNA analysis showed that the assignments of anamorph-teleomorph connections were incorrect. Additionally, among isolates identified as *A. flavipes* there are at least three new species.

In the *Eurotium* lineage, a teleomorphic state was identified that belongs with *A. proliferans*. This species was placed in the *Eurotium* clade even though it was anamorphic (Raper & Fennell, 1965). The isolates identified as teleomorphs of *A. proliferans* had previously been described by Raper & Fennell (1965) as a colonial variant of *A. ruber* (NRRL 71) or a transitional strain somewhere between *A. ruber* and *A. mangini* in taxonomic terms (NRRL 114). Because these isolates are conspecific with *A. proliferans* they should have a distinct *Eurotium* name and description. Separately, about 80 isolates of *A. restrictus* were sequenced at a single locus. Three of those isolates were identical with known *Eurotium* species at that locus. Subsequent sequencing of the other three loci conclusively showed that the anamorphs of *E. intermedium, E. repens* and *E. amstelodami* can be found in nature apart from their teleomorphic state.

Aspergillus terreus isolates contain a greater amount of intraspecific variation than in many other species, and additionally, among the many *A. terreus* isolates examined were several new species. In this species, protein coding genes seem to provide a good set of loci for strain typing. It remains to be seen whether the typing possible in this group with these loci will have relevance for medical treatments.

The internet provides a means for the rapid distribution of data, but not every site on the web contains data of equal quality. GenBank contains sequences of questionable quality and probable errors in the identity of source organisms. Proposals from the MLST community would create curated sites with a more narrow scope than GenBank. At such a site, researchers with a putative new sequence type submit their DNA sequence along with corroborating information, such as the tracings from the DNA sequencer, to the curator of the site. The curator verifies the quality of the data and either asks for more information from the submitting scientist or posts the new sequence type to the web site. In this way, data that has gone through a third party quality check quickly appears at a central site accessible from all over the globe.

From species recognition to species identification

DNA barcoding is a taxonomic method which uses a short genetic marker in an organism"s DNA to quickly and easily identify it as belonging to a particular species. A DNA sequence should meet several criteria to be used successfully for species identification. DNA sequences should be orthologous in the examined organisms, and variable enough to allow species identification, with low levels of intraspecific variation (Hebert *et al.* 2003). A DNA barcode should be easily accessible (universally amplified/sequenced by standardised primers from a wide set of organisms), relatively short (≤ ~ 500–600 bp), simple to sequence, easily alignable [although this problem can be overcome by using Composition Vector Tree analysis (Chu *et al.* 2006), or other non-alignment based algorithms developed recently (Little & Stevenson 2007)], with no recombination. An ~ 600 bp region of the 5" end of the mitochondrial cytochrome oxidase subunit 1 (*cox1*, usually referred to as CO1 in barcoding studies) was proposed to be a good candidate for barcoding animal species including birds (Hebert *et al*. 2004b), fishes (Ward *et al*. 2005), and Lepidopteran insects (Hebert *et al*. 2004a; Hajibabaei *et al*. 2006). The *cox1* region was also used successfully to develop DNA barcodes for red algae (Saunders 2005). However, recent studies have indicated that mtDNA based barcoding region on its own is not suitable for species identification in several cases. Factors such as interspecific hybridisation (Hurst & Jiggins 2005; Bachtrog *et al.* 2006), presence of mtDNA derived genes in the nuclear genome (Thalmann *et al.* 2004; Bensasson *et al.* 2001), and infection by maternally transmitted endosymbionts

such as *Wolbachia* are known to cause flow of mitochondrial genes between biological species, so species groupings created using mtDNA can differ from the true species groupings (Hurst & Jiggins 2005; Withworth *et al.* 2007). Infra-individual heterogeneity of mitochondrial sequences can also cause problems (Sword *et al.* 2007). Besides, data presented by Wiemers & Fiedler (2007) indicate that the "barcoding gap" (i.e. the difference between inter- and intraspecific variability) is an artifact of insufficient sampling, and suggested that other characters should be examined to identify new species. Hickerson *et al.* (2006) claimed that single-locus mtDNA based barcodes can only consistently discover new species if their populations have been isolated for more than 4 million generations. Other authors also suggested that mtDNA-based barcodes should be supplemented with nuclear barcodes (Moritz & Cicero 2004; Dashamapatra & Mallet 2006). Attempts have been made to evaluate various regions of the nuclear ribosomal RNA gene cluster for barcoding animals (Sonnenberg *et al.* 2007; Schill & Steinbrück 2007; Vences *et al.* 2005a).

For some animal groups, other (usually nuclear) genomic regions have been proposed to be used for species identification. In nematodes, a small region of the 18S rRNA gene was proposed as a DNA barcode (Bhadury *et al.* 2006). For dinoflagellates and sponges the intergenic transcribed spacer (ITS) region was found to be the most promising for species identification (Park *et al.* 2007; Litaker *et al.* 2007), while for Cephalopoda rRNA sequences were suggested to be used as DNA barcodes (Strugnell & Lindgren 2007). Regarding plants, the *cox1* region was found to be inappropriate for species identification because of a much slower rate of evolution of *cox1* (and other mitochondrial genes) in higher plants than in animals (Kress *et al.* 2005; Chase *et al.* 2005). A variety of loci have been suggested as DNA barcodes for plants, including coding genes and non-coding spacers in the nuclear and plastid genomes. In flowering plants, the nuclear ITS region and the plastid *trnH-psbA* intergenic spacer are two of the leading candidates (Kress *et al*. 2005). More recently, the non-coding *trnH-psbA* spacer region coupled with the coding *rbcL* gene have been suggested as a two-locus global barcode for land plants (Kress & Erickson 2007). In protists, Scicluna *et al.* (2006) used successfully part of the ssu rRNA gene for species identification, while spliced leader RNA sequences have been used as barcodes in Trypanosomatidae (Maslov *et al.* 2007).

Regarding fungi, ITS and translation elongation factor 1-alpha (*tef1*) based DNA barcodes have been developed recently for identification of *Trichoderma* and *Hypocrea* species (Druzhinina *et al.* 2005). The ITS region was also found to be useful for species identification in other fungal groups including Zygomycetes (Schwarz *et al.* 2006), dematiaceous fungi (Desnos-Ollivier *et al.* 2006) and *Trichophyton* species (Summerbell *et al.* 2007). For the identification of *Fusarium* species, Geiser *et al.* (2004) developed FU.S.A.RIUM-ID v.1.0, a publicly available sequence database of partial *tef1* sequences. Recently, the applicability of the *cox1* region for species identification in *Penicillium* subgenus *Penicillium* has been examined by Seifert *et al.* (2007). They found that representatives from 38 of 58 species could be distinguished from each other using *cox1* sequences. Work is in progress in several laboratories to develop DNA barcodes for various fungal groups.

In this study, we wished to evaluate the usefulness of *cox1* for species identification in the *Aspergillus* genus. Our studies were focused on *Aspergillus* section *Nigri*. Our previous work on mitochondrial genetics of aspergilli indicated that the *cox1* gene, or more generally any genes located on the mtDNA of aspergilli does not meet all criteria needed for a DNA barcode. Regarding intraspecific variability, several studies found high levels of intraspecific variability among black aspergilli (Varga *et al.* 1993, 1994; Hamari *et al.* 1997; Kevei *et al.* 1996), which is manifested not only in the presence or absence of intronic sequences, but also in exonic regions (Hamari *et al.* 2001; Juhász *et al.* 2007). Both inter- and intraspecific recombination has been detected in aspergilli even under non-selective conditions (Earl *et al.* 1981; Tóth *et al.* 1998; Kevei *et al.* 1997; Hamari *et al.* 2003; Juhász *et al.* 2004). Additionally, the *cox1* gene of *Aspergillus* species examined so far carries numerous introns which could make further work cumbersome (Hamari *et al.* 2003). Several studies also indicated that phylogenetic trees based on mitochondrial and nuclear sequence data are incongruent (Geiser *et al.* 1996; Wang *et al.* 2000). Although DNA barcodes are not meant to be used for phylogenetic analyses, the standard short barcode sequences (*ca.* 600 bp) were found to be unsuitable for inferring accurate phylogenetic relationships among fungi (Min & Hickey 2007). The authors proposed to extend the barcoding region from 600 to 1200 bp to be able to distinguish between these closely related species; however, longer sequences are impractical for use in high-throughput screening programs.

During this study, we concentrated on the *A. niger* species complex, which includes eight species: *A. niger, A. tubingensis, A. foetidus, A. piperis, A. brasiliensis, A. vadensis, A. costaricensis* and *A. lacticoffeatus* (Samson *et al.* 2004). According to our previous studies, all these species can be distinguished from each other using calmodulin sequence data, and all except one could be distinguished using β-tubulin sequence data (*A. lacticoffeatus* had identical β-tubulin sequences to some *A. niger* isolates; Samson *et al.* 2004; Varga *et al.* 2007). The ITS data set delimited 4 groups: 1. *A. niger* and *A. lacticoffeatus* isolates; 2. *A. brasiliensis*; 3. *A. costaricaensis*; 4. *A. tubingensis, A. foetidus, A. vadensis* and *A. piperis* (Varga *et al.* 2007). We also examined the applicability of the IGS (intergenic spacer region) for species identification; our data indicate that this region exhibits too high intraspecific variability to be useful for DNA barcoding. Other genomic regions examined by other research groups could also distinguish at least 2–5 species in the *A. niger* species complex, including pyruvate kinase, pectin lyase, polygalacturonase, arabinoxylan-arabinofuranohydrolase and several other genes (Gielkens *et al.* 1997; de Vries *et al.* 2005; Parenicova *et al.* 2001), translation intitiation factor 2, pyruvate carboxylase, 70 kD heat shock protein, chaperonin complex component (TCP-1), ATPase (Witiak *et al.* 2007), and translation elongation factor 1-α, RNA polymerase II and actin gene sequences (S.W. Peterson, pers. comm.).

To evaluate the usefulness of the *cox1* gene for DNA barcoding, we amplified and sequenced part of the *cox1* gene from about 45 isolates of the *A. niger* species complex using the primer pairs developed by Seifert *et al.* (2007), and examined their properties for species delimitation. Sequence alignments and phylogenetic analysis were made by the MEGA 3 software package (Kumar *et al. 2004).* For parsimony analysis, the PAUP v. 4.0 software was used (Swofford 2003). Alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option with 100 random taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1000 bootstrap replications (Hillis & Bull 1993). Thirty-seven variable sites were found in the 501 bp region, ten of which were parsimony informative (excluding *A. ellipticus*,

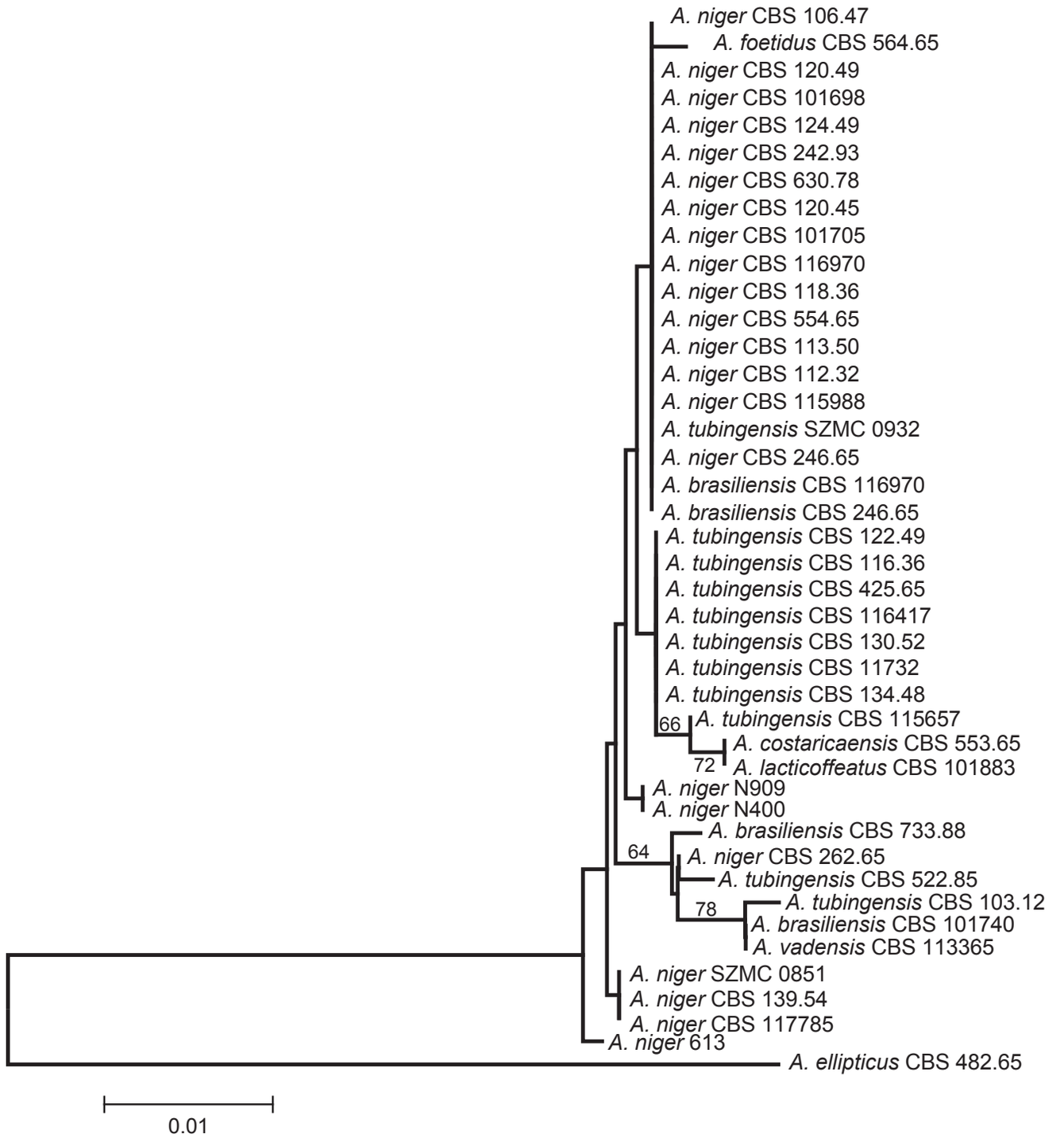

Fig. 1. Neighbour-joining tree based on cox1 sequences of the *A. niger aggregate. Aspergillus ellipticus CBS 482.65 was used as outgroup.* Bootstrap values above 50 % are shown on the branches. Partial *cox1* sequences of the examined isolates have been deposited in the GenBank database under accession numbers EU021012–EU021046.

which was used as outgroup, 8 of the 12 variable characters were parsimony informative). The topology of the Neighbour-joining tree shown in Fig. 1 is the same as one of the 76 maximum parsimony trees (length: 56 steps, CI = 0.9038, RI = 0.8936, RC = 0.8077). The intraspecific genetic distances calculated using Kimura"s 2-parameter model (Kimura 1980) were 0.003 both for *A. niger* and *A. tubingensis*, 0.007 for *A. brasiliensis*, and 0.002 for *A. foetidus*. The interspecific diversities were in the same range: 0.003 between *A. niger* and *A. tubingensis* or *A. foetidus*, 0.004 between *A. tubingensis* and *A. foetidus*, and 0.005–0.006 between *A. brasiliensis* and the other species examined. The genetic distance from *A. ellipticus* varied between 0.084–0.090 for the different species of the *A. niger* aggregate. Although most isolates of either *A. tubingensis* or *A. niger* were found to have identical *cox1* sequences, several isolates of these species did not fit into these clades. We could not identify a single site which could be used without ambiguity for distinguishing *A. niger* from *A. tubingensis* using a character-based approach as suggested by DeSalle *et al.* (2005). Although at position 328 all *A. tubingensis* isolates contained an A nucleotide and most *A. niger* isolates contained C, some *A. niger* isolates also had A in this position (data not shown). This nonsynonymous substitution resulted in an amino acid change from leucine to isoleucine. Regarding other amino acid sequences, altogether 5 variable sites have been found, 3 of which

were parsimony informative. All these amino acid substitutions represented intraspecific variability within the species (data not shown).

Our data indicate that *cox1* is not appropriate to be used as DNA barcode in black aspergilli since none of the eight species of the *A. niger* species complex could be identified unequivocally (Fig. 1). The *cox1* gene sequences of the *A. niger* species complex do not obey either of the two most frequently used methods for species delimitation, reciprocal monophyly as proposed by Wiens & Penkroft (2002), or the "10× rule", the observation of 10 times greater average pairwise genetic difference between the *n*1 individuals of the candidate species and the reference species than the average within-species pairwise differences found in the particular taxonomic group (Hebert *et al.* 2004). The phylogenetic tree constructed based on the *cox1* sequences shows an overlap between intra- and interspecific variation possibly due to past mitochondrial DNA recombination events as suggested earlier (Tóth *et al.* 1998). Although a high degree of heterokaryon incompatibility was observed among isolates of the *A. niger* species complex (van Diepeningen *et al.* 1997), mtDNA transfers occur readily even between incompatible isolates (Tóth *et al.* 1998). Our data are in agreement with those of Min & Hickey (2007), who recently analyzed the *cox1* sequences of *A. niger* and *A. tubingensis* isolates available from the GenBank database. The intra- and interspecific genetic diversity was found to be in the same range, and the amino acid sequences of the *cox1* barcoding region of isolates of the two species were the same. In the barcoding region, 4 variant sites have been found, of which 2 represent intraspecific variability within *A. niger*, one substitution was present in an *A. niger* and an *A. tubingensis* isolate, and one substitution represented interspecific variability between *A. niger* and *A. tubingensis*. This very low level of interspecific variability compared to the similar level of intraspecific diversity in our view is unsatisfactory for a DNA barcode to be used successfully for species identification.

Recent attempts to use the *cox1* gene for species identification in other fungal groups including *Fusarium* species and basidiomycetes have also met with limited success (K. Seifert, pers. comm.). Comparing the phylogenies based on *cox1*, ITS, β-tubulin and calmodulin sequences, either β-tubulin and calmodulin could serve as a suitable region for species identification among black aspergilli. Recently, participants of the All Fungi DNA Barcoding Planning Workshop (Smithsonian Conservation and Research Center, Front Royal, Virginia, 13–15 May, 2007) accepted the ITS region as the first choice for DNA barcoding of the Fungal Kingdom (www.allfungi.org). However, ITS does not always resolve very closely related phylogenetic species (Bruns 2001), whereas intron-rich protein coding genes generally do much better (Geiser 2004). In *Fusarium*, partial translation elongation factor 1-alpha sequences have emerged as the most useful single-locus identification tool (Geiser *et al.* 2004). In the case of aspergilli, our opinion is to use either β-tubulin or calmodulin sequences for accurate species identification because of their prevalence in public databases universality of application, and relative resolving power. However, studies in progress indicate that other regions of the genome might serve better both for species identification and for phylogenetic studies (V. Roberts, pers. comm.). Further studies are in progress to evaluate the usefulness of *cox1* for DNA barcoding in another *Aspergillus* section, and work is in progress to examine the applicability of other loci for species identification.

Looking ahead: genomics, many-locus phylogenies, and multilocus sequence typing

With the complete genome sequences of eight *Aspergillus* species (*Em. nidulans*, *A. oryzae, A. fumigatus, N. fischeri*, *A. terreus, A. clavatus, A. niger, A. flavus*) in advanced states of release, opportunities are upon us to generate data from far more loci in characterising *Aspergillus* species. One application would be to produce a many-locus phylogeny, with the goal of having a firm understanding of the evolutionary relationships among species across the entire genus. Previous phylogenetic studies across the genus have utlised one or a few markers, and unsurprisingly, do not resolve backbone relationships among sections and subgenera (Berbee *et al.* 1995; Geiser *et al.* 1996; Ogawa & Sugiyama, 2000; Peterson, 2000). Rokas *et al.* (2003) showed that strongly inferred nodes could be inferred consistently in the genus *Saccharomyces*, when approximately 20 genes were used, or about 8 000 orthologous nucleotide sites. With the goal of producing a well-supported phylogeny for the genus, we are using the available complete genome sequences to design new sequence markers that can be applied across the genus. Primers were designed to amplify 500–1 300 bp regions of protein coding genes, using conserved stretches of amino acids as primer sites. Our goal is to generate data from up to 20 genes across the entire genus. Initial results based on ten genes and 36 taxa indicate that backbone node support remains elusive across the genus, but excellent bootstrap support is generally obtained for relationships among species within sections and subgenera (Witiak & Geiser, unpubl.). Poor backbone support may be due to the overwhelming amount of sequence diversity in the genus, leading to saturation of third codon positions and long branch attraction artifacts. These problems may be averted by selecting genes exhibiting appropriate levels of resolving power (Townsend 2007) or perhaps with full taxon sampling breaking up long branches. Regardless of any inability to resolve early evolutionary events in the evolution of the genus, these data will provide the basis for multilocus sequence typing schemes that will allow for the precise identification of unknowns and the discovery of new taxa. With advances in the utility of DNA chip technologies accompanied by lower cost, we should be looking toward the application of an affordable and accurate multilocus barcoding chip for the genus *Aspergillus* in the foreseeable future.

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