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Molecular typing of aspergilli: Recent developments and outcomes

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Aspergillus spp. have been the subject of numerous epidemiological studies. The most useful typing techniques are DNA based methods including the random amplified polymorphic DNA technique, microsatellite length polymorphisms, restriction fragment length polymorphism (RFLP) analysis using retrotransposon-like sequences as probes, and multilocus sequence typing. The results of typing clinical isolates indicate that most of the invasive aspergillosis (IA) patients were infected by a single strain. Genetic analysis could not discriminate between clinical and environmental isolates of *Aspergillus fumigatus*, indicating that every strain present in the environment is a potential pathogen if it encounters the appropriate host. The source of infection can also be monitored by typing. Typing studies led to the discovery of a new pathogenic species, *A. lentulus*, and to the identification of several species not known previously to be pathogenic. Typing studies revealed the existence of two genetically isolated groups within a global *A. fumigatus* population. *Aspergillus fumigatus* was found to be the first example of a true cosmopolitan fungus. Additionally, the results obtained in several studies support the premise that recombination played an important role in *A. fumigatus* populations. The discovery of functional mating type genes in *A. fumigatus* indicates that past or recent sexual processes could be responsible for the observed recombining population structure.

Keywords *Aspergillus*, epidemiology, evolution, mating type, population structure

Introduction

Aspergillus spp. are common soil saprophytes which play an essential role in recycling carbon and nitrogen from organic debris [1]. On the other hand, several *Aspergillus* spp. are considered as opportunistic human and animal pathogens. Diseases caused by *Aspergillus* spp. are increasing in importance, especially among immunocompromised hosts. Clinical manifestations are variable, ranging from allergic to invasive disease, largely depending on the status of the host's immune system. *Aspergillus* spp. and may cause several diseases, such as allergic bronchopulmonary aspergillosis, aspergilloma, and invasive aspergillosis (IA), usually a fatal disease particularly in immunocompromised patients [2]. Invasive aspergillosis is a major cause of death at leukemia treatment centers and transplantation units. Today, *A. fumigatus* is responsible for more than 90% of IA cases in humans [3,4]. *Aspergillus fumigatus* is among the most ubiquitous fungi present worldwide on most surfaces and aerial environments. *Aspergillus fumigatus* has no known sexual cycle, although it is closely related to the homothallic *Neosartorya fischeri* [5,6]. The *A. fumigatus* life cycle is characterized by the abundant production of small (2–4 μm) asexual, haploid conidia. Given the ubiquitous presence of these conidia in ambient air, every human being is permanently exposed to them and hundreds of conidia are inhaled daily by every individual [7,8]. Besides *A. fumigatus*, other aspergilli like *A. niger*, *A. terreus*, *A. ustus* and *A. flavus* can also be the causative agents of various forms of infections including IA, sinusitis,

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osteomyelitis, keratitis, bronchiectasis, otomycosis and ophthalmitis [4].

Molecular strain typing of aspergilli can be used to reveal the genetic diversity of environmental and clinical isolates, to reveal the source of infection, and to clarify some aspects of the population genetics of aspergilli. In this review, we attempt to give an overview of the techniques used for typing aspergilli, and the results achieved by using these typing methods, with special emphasize on the *A. fumigatus* sp.

Molecular techniques used for typing aspergilli

Aspergillus fumigatus is generally regarded as a single homogeneous species, but strains may vary both in their phenotypic and genotypic characteristics. *Aspergillus fumigatus* has been the subject of numerous epidemiological studies due to the danger of nosocomial infection of immunocompromised patients. Among other approaches, secondary metabolite profiles and micromorphology of the strains were examined [9–11], DNA reassociation kinetics of some of the species were recorded [12], and isoenzyme and amplified DNA patterns of some species were compared [13]. However, only a limited number of these approaches were found to be useful for the intraspecific clustering or typing of *A. fumigatus* isolates (Table 1). The followings give a brief summary of these techniques. The techniques are dealt with in the order of their discriminatory power.

Multilocus enzyme electrophoresis (MLEE)

Before the advent of DNA-based techniques, electrophoretic analysis of isoenzymes has been the most widely used method for characterizing both prokaryotic and eukaryotic populations. The technique involves the separation of proteins by gel electrophoresis and the subsequent visualization of specific enzymes using appropriate staining reactions [14]. Any soluble protein that can be stained selectively can be extracted from a sample of the population and electrophoresed. If the protein bands are variable, they are considered to be alleles on the basis of mobility. Isolates with identical banding patterns are usually referred to as electrophoretic types. A drawback of MLEE is that it assays the genotype indirectly [15]. Several investigators attempted to use the analysis of isoenzyme patterns to reveal the intraspecific variability of *A. fumigatus* populations [13,16,17]. Although MLEE is not powerful enough to be used as a single tool for typing *A. fumigatus* populations, it is still widely used in combination with other methods for epidemiological purposes [16–22].

Electrophoretic karyotype (EK) analysis

Chromosomal size variation (chromosome length polymorphisms, CLPs) is assayed via an electrophoretic technique, EK analysis, which uses electric fields of alternating orientation to move intact chromosomes through the agarose gel matrix. Since its discovery in 1984 [23], several variations of the theme have been developed including CHEF (contour clamped homogeneous electric field), FIGE (field-inversion gel electrophoresis), OFAGE (orthogonal field alternation gel electrophoresis), RFGE (rotating field gel electrophoresis), TAFE (transverse alternating field electrophoresis), and others. Besides separation of intact chromosomes, this technique has also been used to separate large restriction fragments cut with rare-cutting restriction endonucleases like *Sfi*I or *Not*I [24]. This technique has mostly been used for typing yeasts and other human pathogenic fungi including *Coccidioides immitis* and *Histoplasma capsulatum* [24–26]. The EK of a clinical *A. fumigatus* isolate has also been determined [27]. However, EK analysis has not been used for typing purposes in *A. fumigatus*. Besides, the technique is very labor-intensive and expensive, and the reproducibility of the results is low due to the fact that pulsed field gels are sensitive to electrophoretic parameters including the quality and concentration of agarose, temperature, buffer strength and other factors.

Table 1 Comparison of the performance of different techniques used for typing aspergilli (modified after [7,134,135])

Methodology	Ease of use	Ease of interpretation	Discriminatory power	Reproducibility	Cost	Availability
MLEE	Easy	Easy	Low/moderate	Moderate	Low	Moderate
PFGE	Difficult	Easy	Moderate	Good	High	Low
DNA fingerprinting	Difficult	Moderate	High	Good	High	Low
RAPD	Easy	Easy	High	Moderate	Low	High
SSDP	Easy	Easy	Low	Good	Low	High
MLP	Moderate	Moderate	High	Good	Moderate	Moderate
AFLP	Difficult	Moderate	High	Good	High	Low
Sequencing	Moderate	Easy	High	Good	High	Low

Additionally, the efficient removal of the cell wall and the number of protoplasts obtained also poses a bottleneck [28].

Random amplified polymorphic DNA (RAPD)

During RAPD analysis, a single 10-base oligonucleotide primer is used to amplify DNA fragments at low annealing temperatures [29,30]. A DNA amplification product is generated for each genomic region that is flanked by a pair of priming sites (in the appropriate orientation), which are within less than about 4–5000 bps from each other. Amplification products are usually analyzed by agarose gel electrophoresis. A particular DNA fragment generated for one individual but not for another represents a DNA polymorphism and can be used as a genetic marker. Random amplified polymorphic DNA analysis is technically simple and often detects variation among isolates that are shown to be invariant by MLEE or restriction fragment length polymorphism (RFLP) analysis. Although RAPD analysis was suggested to be prone to errors due to the low annealing temperatures [31], it is widely used for typing *A. fumigatus* populations [32–35]. Interlaboratory reproducibility of this technique is rather low, so external laboratories cannot always reproduce the conclusions obtained with this technique. The main advantage of RAPD is that no previous sequence information is needed in contrast with other techniques like RFLP or microsatellite typing. Another technique similar to RAPD uses primers specific to repetitive sequences within the genome. This technique, called rep-PCR (repetitive extragenic palindromic PCR) or interrepeat PCR [36] is mainly used for typing bacterial pathogens, but recently it has also been adopted to identify and type pathogenic aspergilli [37]. The discriminatory power of the technique is still quite low for intraspecific typing, although targeting more variable repetitive sequences could lead to the development of a useful typing technique.

Sequence specific DNA primer (SSDP) analysis is an extension of the RAPD technique. The method is based on the use of sequence specific primers (20-mers), designed on the basis of discriminating RAPD bands, in high stringency PCR reactions. The presence or absence of usually five SSDP fragments is scored. This method was first used for typing *A. fumigatus* isolates by Mondon *et al.* [38]. Although the SSDP technique overcomes the drawback of RAPD in terms of reproducibility, the method lacks discriminatory power, since the number of distinguishable genotypes for five biallelic loci is only 32 [16].

RFLP analysis

Restriction endonucleases recognize specific DNA sequences, usually four to six nucleotides in length, and cut the DNA in or near the recognition site. The alteration of the recognition sequence by nucleotide substitution, insertion or deletion or for some restriction enzymes by methylation of nucleotides can prevent the restriction endonuclease from acting, and can change the fragment pattern. Insertions and deletions in the region between restriction sites can also change the pattern. Any region of the DNA (locus) can be used for RFLP analysis, if the variation (alleles) can be visualized, either directly, due to multiple copies (mitochondrial DNA, ribosomal RNA repeat unit; also called REA, restriction enzyme analysis), or indirectly, usually by hybridization to probe DNA. One of the main drawbacks of RFLP analysis is the use of radioactively labeled probes, but this limitation can be overcome by using non-radioactive labels. Restriction fragment length polymorphism analysis of the mitochondrial DNA of *A. fumigatus* isolates could not be used for strain typing, as most isolates exhibited the same mitochondrial DNA pattern using several different restriction enzymes [13]. Restriction analysis of total genomic DNA using the restriction enzymes *Xba*I, *Sal*I, or *Xho*I, exhibited a limited degree of discrimination among strains [39,40]. Southern blot patterns obtained with non-species specific probe sequences such as intergenic spacer of ribosomal RNA (rRNA) of *A. nidulans* [41], telomeric sequences of *Fusarium oxysporum* [42] or the whole M13 genome [32] did not reveal enough variability between strains.

The application of repeated sequences as probes, however, provides an excellent tool for strain typing. For RFLP fingerprinting, the probe is a moderately repeated DNA sequence that hybridizes to restriction fragments containing the repeated DNA. Owing to the large number of fragments and the mutability of repeated DNA sequences, RFLP fingerprints are quite variable and provide superior discrimination among individual fungal isolates. This method was used successfully for typing *A. fumigatus* populations [8,43]. The repeat sequence *Afut1*, used in most studies as probe, was found to represent a defective retrotransposon element with a sequence and organization characteristic of the *gypsy* family of retrotransposons [3,44]. At least 10 copies of *Afut1* were found in the genome of *A. fumigatus*. The multiple stop codons found in the putative coding domains indicate that *Afut1* (and *Afut2*) are defective retroelements [45]. The stability of the *Afut1* banding patterns within a single strain over many generations suggests that the *Afut1*

sequence is not evolving as rapidly as, for example, the *ca*3 repeated sequence of *Candida albicans* [15]. Southern blots patterns obtained with *Afut1* are amenable to computer-assisted analysis of the band position and intensity in Southern blot hybridization patterns, enabling highly reproducible and reliable comparison among large number of strains [8,43].

Other defective retrotransposons, including *Afut2* [45], *Af4*, *Af4A* [46], and *Taf1* [47] could also be used successfully for strain typing in *A. fumigatus*. In the recent paper on sequencing of the *A. fumigatus* genome, several retrotransposons have been identified, about half of them belonging to the *gypsy* family of retrotransposons with long terminal repeats [48]. The application of these transposon-like sequences as probes could lead to an even higher discriminatory power of this technique.

Amplified fragment length polymorphisms (AFLPs)

Amplified fragment length polymorphism fingerprinting is a generic fingerprinting method with intrinsic superior reproducibility and a high degree of discriminatory power compared to other random amplification methods like RAPD [49]. This genetic marker system basically uses restriction fragment analysis, but a polymerase chain reaction (PCR) approach is used to visualize fragments instead of hybridization with a probe. Initially, DNA is cut with two different restriction enzymes to produce well defined restriction fragments with sticky ends. Synthetic double stranded linkers with matching sticky ends are ligated on all the restriction fragments, and the ligated fragments are subsequently amplified in PCR in a two-step amplification procedure. First, part of the total number of restriction fragments are amplified during 'preamplification' with primers containing one extra 'selective' nucleotide at their 3′ end. This selective nucleotide will allow amplification of those restriction fragments which have a matching nucleotide next to the linker. With one selective nucleotide on both primers only 1/16th of all restriction fragments in the mixture will amplify during preamplification. During the subsequent 'selective' amplification, one or two additional selective nucleotides on each primer will further reduce the number of fragments amplified. Amplified fragment length polymorphism fingerprinting was applied for determining the epidemiological relatedness between *A. fumigatus* isolates [50].

Microsatellite length polymorphisms (MLPs)

Microsatellites (also called short tandem repeats, STRs, or simple sequence repeats, SSRs) are short repetitive sequences distributed across eukaryotic genomes. Repeat units vary from one to five nucleotides in length at different loci [51]. Because of the great variability of the repeat number at most loci, microsatellites are widely used for genetic mapping and strain typing purposes. Microsatellites are short enough to be analyzed by PCR, combined with gel electrophoresis, avoiding the need for hybridization. The use of labeled primers can improve reproducibility, allow the analysis of several microsatellite loci in one PCR reaction, and provide computerizable data. The method is rapid, highly reproducible and, in contrast to RAPD, uses unique primers and specific sequences flanking microsatellite loci. This technique was used successfully for strain typing in *A. fumigatus* [52–54]. Comparing three typing techniques – RAPD, RFLP fingerprinting and MLP – Bart-Delabesse *et al.* [54] found that RFLP fingerprinting is the most powerful technique giving mostly concordant results with MLP data, while RAPD was found to be inferior to the other two techniques in view of its discriminatory power. Taking into consideration the advantages and drawbacks of the different typing techniques, microsatellite-based approaches are the most promising tools for typing *A. fumigatus* isolates, since these techniques are relatively simple, inexpensive, has high disriminatory power compared to for example multilocus sequence typing (MLST), and the drawback of potentially ambiguous interpretation (stutter peaks) can be overcome by appropriate data analysis. Recently, de Valk *et al.* [55] developed a panel of nine short tandem repeats for fingerprinting *A. fumigatus* isolates. The discriminatory power of this technique was found to be extremely high: 96 of the 99 examined isolates could be distinguished.

Single nucleotide polymorphisms (SNPs)

Single-nucleotide polymorphisms are single-base variations at unique loci. A variety of techniques is available for SNP identification [56]. For examining single-strand conformation polymorphisms (SSCPs), DNA regions with potential polymorphisms are amplified by PCR, and the products are denatured by formamide and heat, generating single-stranded DNAs which adopt a tertiary structure that depends on the sequence. A difference in one base generally forms a different conformer and migrates differently on a non-denaturing polyacrylamide gel [57]. Single-strand conformation polymorphisms analysis of parts of the rRNA gene cluster has been used to identify a wide variety of fungi, but has not been assessed on more polymorphic genes to fingerprint fungal strains of the same species [58,59]. Another technique used to detect SNPs is

heteroduplex mobility assay. After PCR amplification of DNA regions of interest, the DNA is denatured by heat and allowed to anneal, forming homoduplexes when the sequences are identical, and heteroduplexes when there is a difference in one or more bases. A single-base mismatch can produce conformational changes in the double helix that cause the differential migration of homoduplexes and heteroduplexes during gel electrophoresis [57]. A heteroduplex mobility assay with a panel of reference fragments has been used to assess intraspecific variation in a group of aflatoxin-producing *Aspergillus* isolates [60].

Single nucleotide pollymorphism can also be assessed by MLST analysis. This technique was originally developed for characterizing isolates of bacterial species using the sequences of internal fragments of six to ten house-keeping genes. Approximately 450–500 bp internal fragments of each gene are used [50]. For each house-keeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each locus define the allelic profile or sequence type. Each isolate of a species is therefore unambiguously characterized by a series of six to ten integers which correspond to the alleles at the six to ten house-keeping loci. The great advantage of MLST is that sequence data are unambiguous and the allelic profiles of isolates can easily be compared to those in a central database (www.mlst.net).

However, the MLST technique revealed insufficient genetic variation among isolates of some pathogenic fungi including *Coccidioides* sp. and *A. fumigatus* [61–63]. In a preliminary study, Platt *et al.* [63] screened 28 loci of different *A. fumigatus* isolates for polymorphic sites. Nucleotide sequence variation was observed in eight loci, from which only the sequences of a catalase (*catB*) and a polyketide synthase (*pksP*) gene fragment showed some nucleotide variation in all isolates. This phenomenon could possibly be caused either by recent speciation or selective pressure. In these cases, sequencing of numerous, more variable microsatellite loci (the so-called multilocus microsatellite typing, MLMT system) was found to provide sufficient variability for population genetic studies. This typing system provides data similar to those obtained by MLST, although in the MLMT system length polymorphisms are searched for. The technique was successfully used to examine the population structure of *Coccidioides* sp. and *A. fumigatus* [64,65].

In conclusion, microsatellite based and MLST analyses are the most powerful tools for strain typing of *Aspergillus* isolates (Table 1). The strain-typing data obtained by the methods detailed above can be used not only for analyzing genotypic diversity among fungal isolates, but also for revealing genetic differentiation or isolation and the mode of reproduction of *Aspergillus* populations.

Outcomes of molecular typing studies in aspergilli

Clinical aspects

Aspergillus conidia are permanently present in the air. Chalazet *et al.* [8] detected that the concentration of *A. fumigatus* conidia in the air of a hospital varied between 0–3 m^{-3}, while Hospenthal *et al.* [66] could detect 11.6 conidia m^{-3} in an oncology unit of a hospital (1.83 conidia m^{-3} on average). The extent of genetic diversity of environmental and clinical isolates has been demonstrated in an *Afut1*-RFLP-based study including 879 clinical and environmental isolates from different geographical locations [43]. Upon analysis of the 424 distinct unique genotypes obtained, only 14 identities between strains of different geographical origins could be found. Correlation was not observed between the genotypes and geographical origin of the isolates [43]. In another study, 700 isolates came from the hospital environment and patients were examined [8]. The authors detected 85% of the genotypes only once, while 15% of the genotypes were detected several times and were found to persist in the hospital environment for months. Patients hospitalized in different parts of the same hospital can be infected with the same strain since every patient might inhale the same spore population. Isolates came from the environment or from patients could not be distinguished based on their RFLP profiles [7]. Other genetic methods were also unable to discriminate between clinical and environmental isolates of *A. fumigatus*, indicating that every strain present in the environment is a potential pathogen if it encounters the appropriate host. The ability of any strain of *A. fumigatus* to become pathogenic also provides evidence for the absence of host specificity [7].

The potential sources of air-borne *A. fumigatus* conidia are potted plants, construction work, aerosols (e.g. showers), personal and medical materials (e.g. pillows), etc. [67–69]. The importance of alternative sources of infection, with special emphasize on water is increasing [67,70]. Recently, environmental isolates recovered from either air or water could have been distinguished by AFLP analysis [50]. The source of infection (community acquired vs. nosocomial) can also be monitored by typing. The isolation of identical strains from a patient and from the hospital environment of that patient indicates that the infection was

nosocomially acquired, although the huge genetic diversity of *A. fumigatus* isolates makes it difficult to ascertain a hospital source of the infection [71]. Chalazet *et al.* [8] detected isolates with identical genotypes from two patients or from a patient and the patient's environment in the same hospital for 30 of 73 patients (41%). These genotypes accounted for 17 of the 80 clinical genotypes (21%) studied, whereas only 21 (4.8%) common genotypes were identified among 433 unique environmental genotypes encountered in the hospitals. Comparison of these data suggested that infection was nosocomial for these 30 patients [8]. Bart-Delabesse *et al.* [51] observed that among 12 patients with IA in a hematological unit, five (42%) had hospital-acquired IA based on MLP genotyping of 27 and 62 *A. fumigatus* isolates came from the patients and the environment, respectively. The nosocomial origin of *A. fumigatus* infections has also been suspected based on typing of environmental and clinical isolates in other studies. The techniques used include random microsatellite typing [72], RAPD [73,74], RAPD combined with SSDP analysis [75], or with SSDP and MLEE [19]. Lair-Fullinger *et al.* [76] examined the epidemiology of avian aspergillosis in breeding turkeys by MLP. The authors detected 10 genotypes occurring both in animals and in air samples. Whereas older animals harbored several combinations of genotypes, one-day-old chicks carried a unique genotype, which was frequently recovered in air samples [76].

Aspergilloses caused by other aspergilli could also be identified as nosocomial infections. In these cases, the genetic variability is less pronounced, so identification of the source is an easier task. For *A. flavus* infections, the most frequently used technique is RAPD analysis [34,77–79]. James *et al.* [80] used hybridization with a repetitive DNA sequence, pAF28 to identify the source of *A. flavus* infections in a neonatal unit. The cutaneous infection of the infants was possibly caused by an adhesive tape contaminated by an *A. flavus* isolate exhibiting the same genotype as the clinical isolates [80]. For *A. terreus*, RAPD analysis was used to identify the source of infection (in-hospital plants) in a hematological unit [81].

The results of typing clinical isolates indicate that most of the IA patients were infected by a one to two strains, although mixed infections with more strains of *A. fumigatus* can also occur [7,19]. In contrast, several genotypes were isolated from airways of patients suffering from cystic fibrosis. For example, five to ten different types were found in six cystic fibrosis patients with bronchial colonization by using *Afut1*-RFLP analysis [82,83]. The same genotypes were found repeatedly over time, demonstrating that cystic fibrosis patients are colonized permanently by the same strains of *A. fumigatus* without causing pathological changes. Cimon *et al.* [75] observed that most recently colonized patients carry numerous genotypes, while chronically colonized patients usually harbor a limited number of genotypes with the occurrence of a dominant one. The authors suggested that selection for some strains of *A. fumigatus* during the prolonged colonization of the airways could cause this phenomenon. On the other hand, isolates collected from a cystic fibrosis patient with aspergilloma displayed the same genotype, suggesting that the infection was due to a single strain [75].

Besides *A. fumigatus*, other aspergilli including *A. terreus*, *A. niger*, *A. flavus* and several *Neosartorya* spp. have also been implicated in human diseases. In a recent publication, Lionakis *et al.* [84] observed *Aspergillus* isolates other than *A. fumigatus (A. terreus*, *A. niger*, *A. flavus*) at an increased frequency in cancer patients predisposed to antifungal drugs. *Aspergillus ustus* infections have also been found in stem cell transplant patients treated either with votriconazole or caspofungin [85]. Among *Neosartorya* spp., *N. pseudofischeri*, *N. fischeri* and *N. spinosa* have long been known to be able to cause different forms of aspergillosis [86–92]. Typing studies led to the identification of species not known previously to be pathogenic, including *Neosartorya hiratsukae* [93], *N. udagawae* [S.A. Balajee, personal communication] and several unnamed aspergilli related to *A. fumigatus*, *A. viridinutans* and *N. spinosa* [94]. Other isolates have also been described as distinct from most *A. fumigatus* strains on the basis of molecular data [95,96]. More recently, the anamorph of *Neosartorya pseudofischeri*, *A. thermomutatus* could not be distinguished from *A. fumigatus* isolates without using molecular analysis [97].

Typing studies also contributed to the identification of previously undescribed clades in pathogenic aspergilli, especially within the *A. fumigatus* 'species'. An '*A. fumigatus*' isolate (FRR 1266) found to be distinct from typical *A. fumigatus* isolates using isoenzyme and RAPD analyses [13] was later assigned to the *A. viridinutans* sp. on the basis of the sequence analysis of the β-tubulin gene [6,98]. Two other isolates, which were morphologically similar to *A. fumigatus*, but could be distinguished from it by molecular techniques (sequence analysis, mitochondrial DNA RFLPs) have also been suggested to represent a new species [6,98]. Later, sequence typing of clinical isolates led to the description of a new pathogenic species, *A. lentulus* [99]. This newly described species also includes the two isolates mentioned above [6,98], and several Korean

isolates [100]. *A. lentulus* isolates have so far been found in several parts of the United States (California, Florida, New York, Seattle, Texas), in Korea, Australia, Africa and in The Netherlands [98–101].

Population structure of A. fumigatus

Pringle *et al.* [65] carried out multilocus microsatellite sequence typing of five microsatellite loci of 63 *A. fumigatus* isolates collected from five continents. The data obtained revealed the existence of two genetically isolated groups or phylogenetic species, the 'fumigatus' and the 'occultum' clade within the global *A. fumigatus* population [65]. The authors could not find any correlation between genotypes and geographical origin of the isolates, and concluded that *A. fumigatus* is the first fungal species which was shown by population genetic evidence to have a global population structure. All other fungi postulated to be cosmopolitan previously were found to be more narrowly distributed based on population genetic analyses [65].

Aspergillus fumigatus isolates produce conidia (mitospores) abundantly, so asexual or clonal reproduction is a given capability of this species. The question is whether the fungus can also undergo recombination, either through a sexual or a parasexual cycle. Although numerous papers were published on the application of several typing techniques to *A. fumigatus* isolates, only some authors attempted to examine the reproductive mode of *A. fumigatus* populations. The extremely high degree of polymorphism within *A. fumigatus* populations observed by Chalazet *et al.* [8] and Depeaupuis *et al.* [43] can be due to past or recent actions of retroelements, or can also be viewed as an indicator of recombination. There are some other data supporting the view that recombination plays (or played) a role in *A. fumigatus* populations. Most investigations using isoenzyme data found some overrepresented genotypes, this was indicative of clonal reproduction [102]. For example, Rinyu *et al.* [13] observed that from 60 tested isolates 19 exhibited the same β-arylesterase and acid phosphatase profiles. Similarly, Cimon *et al.* [75] observed identical SSDP profiles in 63 of 109 examined isolates. On the other hand, the presence of all six possible two-locus genotypes of the three β-arylesterase and two phosphatase alleles among the strains tested by Rinyu *et al.* [13] was an indicator of recombination. The observation that 28 of the 32 possible combinations of the five biallelic SSDP loci were recovered in the studies carried out in the late 90s also indicated that some recombination took place in the examined *A. fumigatus* populations [18–20,38,75,103]. Parsimony analysis of both the isoenzyme and RAPD datasets of Rinyu *et al.* [13] resulted in trees with lack of substructuring and low bootstrap values [104]. The lack of clearly identifiable 'discrete typing units' was treated as an indicator of a recombining or 'nonstructured' population by Tibayrenc [105]. The absence of a strict correlation between trees based on different datasets, e.g. on the isoenzyme and RAPD data [13], or on the SSDP, RAPD and microsatellite data [18,19] also indicated that recombination took place in the examined populations [105]. Further support for ongoing or recent sexual reproduction in *A. fumigatus* comes from the presence of a defective transposable element, *Afut1*, within the genome. The propagation of this element must once have relied on sexual reproduction, and it appears to bear traces of repeat induced point mutation, which is specifically induced at meiosis in other fungal taxa [65,106]. The genome of *A. fumigatus* also contains other presumably active transposable elements [42], suggested to be another characteristic of sexual species by several authors [107–109].

Recently, several methods have been described for distinguishing recombination from clonality in fungal populations [15,110]. Most genetic methods used to distinguish between these two modes of reproduction are based on the assumption that under clonality different regions of the genome are inherited as a unit, while in the case of recombination they may have different evolutionary histories. In bacteria, the mosaic structure of certain genes was treated as evidence for recombination [111]. Another possibility is the examination of multilocus genotypes. Two methods frequently used for distinguishing between clonal and recombining reproduction are the index of association test, and the parsimony tree length permutation test (PTLPT) [15].

Varga and Tóth [103] examined the reproductive mode of *A. fumigatus* populations on the basis of isoenzyme and SSDP data using statistical tests. Both the index of association tests and PTLPT tests supported the premise that recombination played an important role in *A. fumigatus* populations. The analyses indicated the presence of an epidemic structure in most examined *A. fumigatus* populations. The prevalence of a single or some related electrophoretic types in local *A. fumigatus* populations could be due to selection. Although any environmental strain of *A. fumigatus* can become pathogenic, the severity of the disease caused can be different for individual isolates since the virulence of a given strain can be influenced by several factors. In experimental models of IA, it was demonstrated that *A. fumigatus* isolates differed in virulence, with clinical isolates being more virulent than environmental strains, suggesting that pathogenicity in

this case depended not only on the immune status of the host, but also on the fungal isolate involved [112]. Indeed, recently correlation was observed between elastase activities and invasiveness of *A. fumigatus* isolates [113]. Similarly, *A. fumigatus* isolates varied in their abilities to inhibit phagocytosis by macrophages [114]. In a recent publication, correlation has also been detected between *in vitro* growth rate and virulence of *A. fumigatus* isolates [115]. It has also been suggested that gliotoxin producing abilities of the isolates also influence the virulence of the isolates [116]. Genetically distinct *A. fumigatus* isolates might respond to environmental changes differently, resulting in the outbursts of some electrophoretic types. Network methods have also been used successfully to reveal the recombining population structures of *A. fumigatus* populations [103].

In a recent publication, Pringle *et al.* [65] also observed that *A. fumigatus* 'fumigatus' appears to recombine in addition to clonal reproduction when MLMT datasets were examined. Evidence of recombination was found only when clone-corrected datasets were examined by the index of association test. However, clone correction was irrelevant when two other tests, the PTLPT and the incongruence length difference/partition homogeneity test were applied; both analyses rejected the null hypothesis of clonality for the examined dataset.

Paoletti *et al.* [117] identified and sequenced three intergenic regions from 106 *A. fumigatus* isolates, and examined the dataset by the index of association test. The association index of the observed dataset was found to be 0.24, whereas the association index of 1000 artificially recombining datasets ranged from −20 to 0.29, with a mean value of zero. These results present clear evidence that recombination had occurred within the test samples.

Population structures of other *Aspergillus* spp. have also been examined recently. In *A. flavus*, Geiser *et al.* [118] compared genealogies of five partial gene sequences, and observed two cryptic species. In one of these species, lack of concordance of gene genealogies was treated as an evidence for recombination. Van Diepeningen [119] examined the population structure of black aspergilli using partial sequences of three genes coding for non-essential extracellular enzymes. Comparing the genealogies of these genes, she found evidence for a very low level of nuclear recombination in *A. niger*. Using a similar approach by comparing phylogenies of five nuclear genes, Peterson *et al.* [120] observed recombining population structure in an *A. nomius* population, but could not find evidence for recombination in *A. bombycis*.

The applied techniques did not enable the researchers to distinguish between the alternative hypotheses of whether past meiotic exchanges, parasexuality or a cryptic sexual stage were responsible for the recombining population structure of *A. fumigatus*. For past or present sexual recombination, functional MAT genes are necessary in fungi [121]. In the followings, the results of hunting for genes needed for sexual recombination in aspergilli will be presented.

Genes necessary for sexual reproduction in aspergilli

Mating processes in fungi are governed by mating type genes. Most (if not all) heterothallic filamentous ascomycetes have a dimictic mating system with two alleles (called idiomorphs as they do not share any significant sequence similarity) located in a single locus [121]. One idiomorph (*MAT1-2*) contains a single open reading frame (ORF) encoding a regulatory protein with a DNA-binding domain of the high mobility group (HMG) type, while the other (*MAT1-1*) contains an ORF encoding a protein with a motif called alpha box also present in the *MATα1* protein of *Saccharomyces cerevisiae* [121]. The presence of a mating type gene (*MAT1-2*) in *A. fumigatus* was first reported by Poggeler [122] and Varga [123]. Recently, Paoletti *et al.* [117] reported the presence of both MAT idiomorphs in a global *A. fumigatus* population. The proportion of isolates carrying either the *MAT1-1* or *MAT1-2* homologs was about the same (43% and 57%, respectively). Interestingly, the flanking region of the *MAT1-1* idiomorph carries part of the *MAT1-2* idiomorph, a feature not seen before among fungi. Regarding the evolution of MAT loci among aspergilli, the presence of a partial *MAT1-2* sequence within the idiomorph-flanking region is consistent with the proposal that species such as *A. fumigatus* arose from a homothallic ancestor in which *MAT1-1* and *MAT1-2* genes originally were adjacent, but the genes then became separated as a result of a translocating break or aberrant segregation [117]. This hypothesis is also supported by the extensive conserved synteny observed at the MAT loci of *A. nidulans*, *A. fumigatus* and *A. oryzae* [124]. However, it remains possible that homothallic aspergilli arose from heterothallic ancestors as suggested for *Neurospora* and *Cochliobolus* spp. [117]. This alternative scenario is supported by the observation that several relatives of *A. fumigatus* are heterothallic, including *Neosartorya fennelliae*, *N. spathulata*, *N. udagawae*, *N. nishimurae* and *N. otanii* [125]. However, previous studies clarified that at least three of these heterothallic species form separate clades not closely related to each other [5,6].

Homologs of other genes necessary for mating in yeasts have also been identified in *A. fumigatus* and other aspergilli [122,123]. In a recent publication on sequencing the *A. nidulans* genome, homologs of more than 200 genes taking part in sexual processes in other fungi have been identified in the genomes of *A. nidulans*, *A. oryzae* and *A. fumigatus* [124]. These homologs include genes taking part in the mating process, pheromone response, fruit body development, karyogamy and meiosis. In all cases, if a gene was present in the *A. nidulans* gemone, it was also present in the genomes of *A. oryzae* and *A. fumigatus* [124]. Homologs of the a-factor like pheromone precursor gene (*ppgB*) could not be identified unambiguously in any aspergilli [124].

Paoletti *et al.* [117] investigated whether putative genes involved in mating processes were expressed at the RNA level during mycelial growth of *A. fumigatus.* The authors observed that both *MAT1-1* and *MAT1-2* were expressed in a mating-type specific manner. The pheromone-receptor genes *preA* and *preB* were also expressed, with no clear differences evident between the *MAT-1* and *MAT-2* isolates. In contrast, the expression level of the α-pheromone precursor gene was higher in the *MAT1-1* than the *MAT1-2* isolate. A similar expression pattern has been observed in *Neurospora crassa*, a heterothallic ascomycete, in which pheromone-receptor gene expression is constitutive, whereas pheromone-precursor gene expression is mating-type dependent [126].

Mating type gene homologs have also been identified recently in the homothallic *A. nidulans* [127], and in the asexual *A. flavus*, *A. sojae*, *A. parasiticus*, *A. oryzae* [121,128,129] and *A. niger* spp. [J. Varga, unpublished results]. In asexual species, the mating type genes were distributed in a 'heterothallic' manner, and the ratio of isolates carrying either *MAT1-1* or *MAT1-2* was found to be similar in most species [124]. On the contrary, most (more than 80%) of the black *Aspergillus* isolates tested were found to carry the *MAT1-1* idiomorph, indicating that these species lost their ability to sexual recombination long time ago, and reproduce clonally [J. Varga, unpublished data]. Experiments are in progress to cross *A. fumigatus* and *A. oryzae* isolates in order to induce a sexual cycle in these species [P. Dyer, personal communication]. The observation of abortive cleistothecia in interspecific pairings between some *A. fumigatus* isolates and an 'A' mating-type strain of *N. fennelliae* indicate that such crosses might lead to the discovery of the sexual cycle in these fungi [130].

Conclusions

Several molecular techniques have been developed recently for typing *Aspergillus* spp. Among them, RFLP fingerprinting, MLPs and MLST have the best discriminatory power and reliability. Hopefully, the completion of the genome sequencing of *A. fumigatus* will speed up the identification of new markers, including microsatellite loci as happened in other aspergilli [131–133]. Molecular strain typing techniques have been used to reveal the genetic diversity of environmental and clinical isolates, to reveal the source of infection, and to address several other questions related to the epidemiology of aspergilloses. Data obtained by molecular typing also help to address questions related to the population genetics of aspergilli, including the presence of cryptic species, and the role of recombination in *Aspergillus* populations. The identification of mating type genes in *A. fumigatus* populations indicates that either recent or ongoing sexual processes could be responsible for the observed recombining population structure of this human pathogen. Further studies should be pursued to reveal differences between the two phylogenetic species of *A. fumigatus* [65]. It would be interesting to see whether the two clusters corresponding to air-derived and water-borne *A. fumigatus* isolates observed by Warris *et al.* [49] by AFLP analysis correspond to the two phylogenetic species recognized in the global *A. fumigatus* population by Pringle *et al.* [65]. Additionally, a thorough study of the genetic diversity of *A. fumigatus* may provide some insight in the polygenic nature of virulence and improve our understanding about the variation of the pathogenicity of natural isolates.

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