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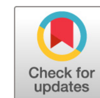

Investigating Clinical Issues by Genotyping of Medically Important Fungi: Why and How?

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Published 10 May 2017

Citation Alanio A, Desnos-Ollivier M, Garcia-Hermoso D, Bretagne S. 2017. Investigating clinical issues by genotyping of medically important fungi: why and how? Clin Microbiol Rev 30:671–707. https://doi.org/10.1128/CMR.00043-16.

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SUMMARY Genotyping studies of medically important fungi have addressed elucidation of outbreaks, nosocomial transmissions, infection routes, and genotype-phenotype correlations, of which secondary resistance has been most intensively investigated. Two methods have emerged because of their high discriminatory power and reproducibility: multilocus sequence typing (MLST) and microsatellite length polymorphism (MLP) using short tandem repeat (STR) markers. MLST relies on single-nucleotide polymorphisms within the coding regions of housekeeping genes. STR polymorphisms are based on the number of repeats of short DNA fragments, mostly outside coding regions, and thus are expected to be more polymorphic and

more rapidly evolving than MLST markers. There is no consensus on a universal typing system. Either one or both of these approaches are now available for *Candida* spp., *Aspergillus* spp., *Fusarium* spp., *Scedosporium* spp., *Cryptococcus neoformans*, *Pneumocystis jirovecii*, and endemic mycoses. The choice of the method and the number of loci to be tested depend on the clinical question being addressed. Next-generation sequencing is becoming the most appropriate method for fungi with no MLP or MLST typing available. Whatever the molecular tool used, collection of clinical data (e.g., time of hospitalization and sharing of similar rooms) is mandatory for investigating outbreaks and nosocomial transmission.

KEYWORDS genotyping, short tandem repeat, multilocus sequence typing, molds, yeasts, *Aspergillus* spp., *Fusarium* spp, *Scedosporium* spp., *Candida* spp., *Cryptococcus neoformans*, *Pneumocystis jirovecii*, endemic mycoses, microsatellite length polymorphism

INTRODUCTION

More and more fungal species are being associated with human diseases, either as allergen or toxin producers or as infectious agents causing invasive and systemic infections (1). The increased incidence of invasive fungal diseases (IFDs) has been linked to the growing number of patients receiving immunosuppressive treatment in hematology and solid organ transplant units. Moreover, many patients in intensive care units are also considered immunocompromised, while immunosuppressive drugs (e.g., steroids or anti-tumor necrosis factor [anti-TNF]drugs) are increasingly being used in more medical fields. Given the multiplicity of ways to develop IFDs, it will be important to explain the physiopathology and transmission of fungal diseases, and fungal genotyping will be an important part of strategies to achieve this.

Genotyping of infectious agents can help with understanding the evolution, geographical distribution, and spread of disease, providing insights into genomic recombination, linkage, and mode of reproduction. Beyond population genetics, genotyping can be used to study local disease epidemiology (outbreaks, nosocomial acquisition, and patient-to-patient transmission), route of transmission (airborne, waterborne, foodborne, or through contaminated intravenous or contact lens solutions), specific clinical questions (infection due to patient isolates or from environmental or animal isolates and distinction between reoccurrence and new infection), and genotype-phenotype correlations (acquisition and spread of antifungal resistance and delineation of genotypes with higher virulence), and this list is not exhaustive.

Here we focus on fungi of medical interest for which genotyping is available and can impact patient care, such as prevention of infection or cross-contamination. Therefore, the genetics of the fungi will not be discussed as long as there is no direct implication reported for patient care, acknowledging that the distinction between the different uses and studies using genotyping is artificial. Similarly, when typing studies are focused mainly on deciphering the number of species in a given fungal group for taxonomic reasons, these studies will not be considered here. This is the case for diverse species, such as *Aspergillus niger* (2), *Sporothrix schenkii* (3), *Malassezia* spp. (4), or *Trichosporon* spp. (5), for which the current studies are more focused on taxonomy.

DNA-BASED METHODS DEVELOPED DURING THE 1980s AND 1990s

Several methods developed during the 1980s have now been practically abandoned. Pulsed-field gel electrophoresis approaches require spheroplast preparation for chromosome preparation and require several days to perform, and the results are not polymorphic enough for genotyping purposes. Restriction fragment length polymorphism (RFLP) based on DNA digestion, Southern blotting, and probe hybridization was widely used during the 1990s. However, this approach requires demanding DNA preparation steps to obtain long DNA fragments and homogenous digestion by restriction enzymes, gel electrophoresis, ethidium bromide staining, Southern blot transfer, and probe (usually radiolabeled) hybridization. Although these methods could be discriminating, depending on the probe used, and despite some possibility of digitizing the

results after scanning and imaging manipulations (6), they were hardly reproducible between laboratories, and most of them have been abandoned for genotyping. The PCR-based methods developed in the 1990s were less labor-intensive and included randomly amplified polymorphic DNA analysis (RAPD), single-strand conformation polymorphism analysis (SSCP), and amplified fragment length polymorphism analysis (AFLP) approaches.

RAPD was very popular because of its simplicity and low cost. No previous knowledge of the DNA sequences of the fungal species is needed for RAPD, which depends on amplification using short primers (<10 bp long), amplification under low-stringency conditions, and migration in an agar or acrylamide gel. However, RAPD's simplicity also has drawbacks. Because of its low-stringency conditions and use of random primers, the reproducibility of RAPD is low, even within a single laboratory. RAPD is also dependent on DNA quality, does not detect accidental mixtures, and is not amenable to digitization and storage in a database for subsequent comparisons. Moreover, the nature of the RAPD polymorphism is unknown, hampering reliable taxonomic analyses, and the ploidy of fungi cannot be assessed. The use of RAPD should be restricted to initial assessment of the degree of polymorphism before applying more reliable methods. For instance, the commercial repetitive sequence-based PCR (DiversiLab; bioMérieux) was tested on 99 *Candida parapsilosis* isolates, and all were shown to have identical profiles, which could have led to the conclusion of cross-contamination. However, microsatellite length polymorphism (MLP) typing identified 56 different genotypes among this collection of 99 isolates, with a completely different conclusion (7).

SSCP is based on conformational differences of single-stranded nucleotide sequences of identical length. After denaturation of double-strand DNA amplicons, single-stranded DNA folds in 3 dimensions, assuming unique conformational states based on DNA sequence. If the amplified DNA sequences are different, they migrate differently on a denaturing electrophoresis gel, despite having equal nucleotide lengths. SSCP shares several limitations with RAPD, including low reproducibility, lack of portability, impossibility of detecting accidental mixtures, and homoplasia. SSCP was developed in human genetics for the detection of heterozygosity and has since been superseded by sequencing approaches.

AFLP is based on double enzymatic digestion (e.g., by EcoR1 with rare cuts and MseI with frequent cuts), ligation of adaptors, amplification with one labeled primer, and migration in denaturing gels. Although very discriminating and based on stringent PCR conditions, this method is subject to many variables (e.g., ligation yield and DNA quality) and has been restricted to a few laboratories.

CURRENT PCR-BASED METHODS

One of the main disadvantages of the methods presented above is the impossibility of detecting species identification errors. Because genotyping addresses differences between isolates, it is mandatory to start with pure colonies, which is not always possible when multiple colonies are obtained or when working directly with the clinical sample. In designing species primers, microsatellite length polymorphism (MLP) typing and multilocus sequence typing (MLST) circumvent these pitfalls. If no amplification is obtained, the first explanation is an incorrect identification of the colony instead of technical reasons.

MLP Typing

Microsatellite length polymorphism (MLP) typing is based on the amplification of short tandem DNA repeats (STRs) located at numerous loci in eukaryotic genomes. The diversity of STRs and their mutation rates is high, although the variation rate depends on the species (8). The term microsatellite was coined after the name "satellite" used to characterize the layer separated from the bulk DNA upon centrifugation in cesium chloride. Since this "satellite" layer was shown to correspond to long repeated DNA fragments, the word minisatellite was used to name short DNA repeats. Today, the term microsatellite is often replaced by STR, which is more understandable. The term

variable-number tandem repeat (VNTR) is sometimes used as a synonym of STR, but it usually refers to repeats longer than 5 to 6 bp. The STRs reported for typing the different fungal species described below are usually made from di- to pentanucleotides and are repeated between 5 and 50 times.

STRs located outside coding regions are preferred for genotyping applications because of the lack of adaptation pressure and hence an expected higher variability than for STRs in coding regions. When located inside the gene or in the regulatory flanking regions, STR variations can lead to phenotypic modifications, such as those described in human triplet extension diseases with low polymorphism (9). Changes in repeat numbers arise from replication slippage, with successions of slippages at a single repeat unit over several generations producing alleles of various lengths. The polymorphism can then be easily identified by digitization after migration in capillary electrophoresis as used for sequencing applications. Slippage events depend on the length and the repeat number of each STR, as well as across different species (10).

The initial step in developing a practical microsatellite typing strategy is to identify STRs within the genome of the investigated species (11), for which several types of software are available (e.g., Tandem Repeats Finder [12] and Websat [13] [http://omictools.com/microsatellite-detection-category]). Next, primers are designed in the flanking regions of the STR, assuming that these regions are identical for all isolates of a given species. However, because only the length of the PCR product is used for analysis, there is a risk of wrongly concluding that an STR marker is identical between isolates, whereas sequence variations are present in the flanking regions. For example, a high-resolution DNA melting analysis of a single STR marker of *Candida albicans* showed different melting curves, which were shown to be due a single-nucleotide polymorphism (SNP) in the flanking region using a SNaPshot minisequencing approach (14). Furthermore, the absence of an amplification product suggests an error in the species identification, which represents an internal quality control (see above).

After PCR with one of the two primers labeled, the size of amplicons is calculated according to their mobility in capillary electrophoresis. Since only the size of the PCR product is considered, perfect STRs (i.e., with only one type of nucleotide repeat) are preferable to distinguish different alleles (e.g., different mixtures of di-tri- or tetranucleotide repeats on the same DNA strand can lead to the same size). Several loci can be tested simultaneously using primers with different dyes, allowing multiplexing. As the amplification product lengths are calculated automatically, according to standards, decimal values can be obtained. Including several reference strains with a known number of repeats allows these decimal values to be converted into base pair numbers (15). For diploid organisms, heterozygotes can be easily identified, since MLP typing detects different alleles at a given locus. Therefore, the MLP method not only can detect misidentification, because no amplification is observed due to the species-specific nature of the primers, but also can detect mixtures of different isolates when an inappropriate number of alleles is obtained for a species known to be haploid or diploid (15, 16).

The principle limitations of the MLP method from the technical point of view are errors in the amplification products caused by difficulties of the DNA polymerases in correctly amplifying repeated elements. It is therefore common to observe stutter peaks (17). Some polymerases are also prone to adding a supplementary adenine, changing the estimated amplicon size. All these artifacts depend on the STR and the experimental conditions, which need to be controlled (17). For heterozygous fungi, the amplification process strongly favors the shorter alleles, so that longer alleles can be overlooked, underlying the need to optimize the PCR to detect low-intensity peaks in each run. For these technical reasons, MLP typing results are portable (i.e., suitable for interlaboratory comparisons) only if results are expressed relative to an allelic ladder. An allelic ladder can be built with a well-defined mixture of alleles with a known number of repeats, at best defined after Sanger sequencing, and used in the same run as the samples to create reference positions (18, 19) (Fig. 1). As a consequence, no public databases are currently available to our knowledge.

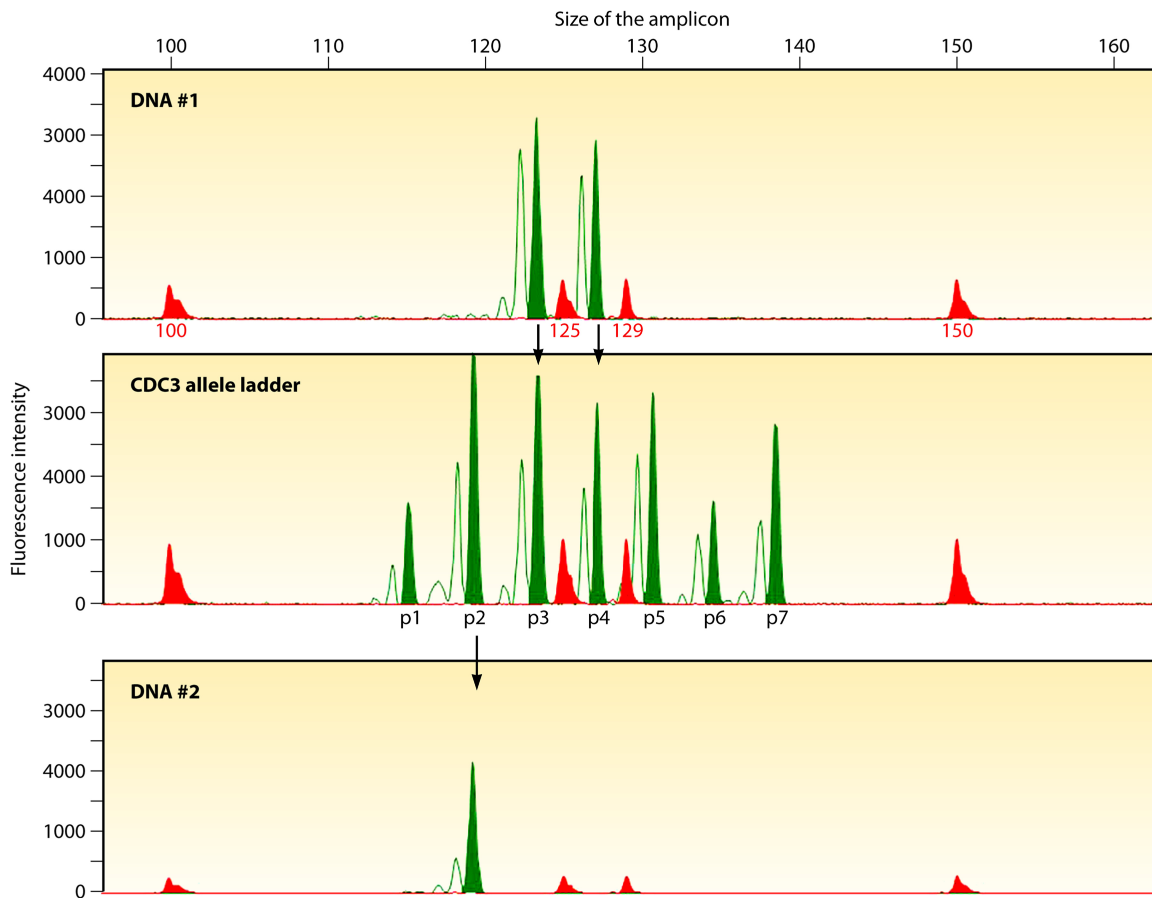

FIG 1 Allele assignment using the CDC3 allelic ladder for DNA 1 and DNA 2. Peaks (p1 to p7) are the different alleles in the ladder (in green). The red peaks represent the internal size standard GeneFLo 625, with sizes in bp below each peak. Isolate 1 is p3-p4 heterozygous, and isolate 2 is p2 homozygous (18).

MLST

Multilocus sequence typing (MLST) relies on the sequencing of multiple loci and was developed primarily by bacteriologists (20). MLST makes use of SNPs within selected regions. The retained loci are usually housekeeping genes to ensure that every isolate of a given species is correctly amplified, although some evolutionary constraints are expected to limit mutations in such essential genes. Nevertheless, the rate of nucleotide change in housekeeping genes is expected to be sufficient to discriminate between isolates. A preliminary step in MLST is to design primers outside the tested locus in flanking nonvariable regions. After PCR amplification and Sanger sequencing, the point mutations define sequence type (ST) or diploid sequence type (DST) for haploid or diploid microorganisms, respectively. These STs and DSTs correspond to numbers assigned to each unique combination of genotypes. The method is technically robust, providing the sequences are of adequate quality. MLST is then unambiguous and easily portable. As a consequence, numerous websites are presently available for asking whether the ST or DST has been already reported (Table 1). One important issue in MLST is the selection of adequate target genes (locus) and the number of loci needed for discriminating isolates (see below). Another limitation of MLST for diploid organisms is the impossibility of assigning a haplotype to a given isolate when heterozygosity is observed, because the loci are sequenced independently (Table 2).

There are no technical limitations of MLST when dealing with pure colonies obtained in culture. For genotyping directly from clinical samples, MLST typing is limited by the low detection rate for mixtures due to Sanger sequencing, which cannot detect minority alleles below 20 to 30% (21).

TABLE 1 Fungi of medical importance, with MLP and/or MLST typing when available

Fungal species	MLP	MLST	MLST website(s)
Molds			
Aspergillus fumigatus	Yes	Yes	http://pubmlst.org/ (no updating)
Aspergillus flavus	Yes	No	
Aspergillus terreus	Yes	No	
Aspergillus niger	No	No	
Fusarium spp.	No	Yes	http://www.cbs.knaw.nl/fusarium/, http://isolate.fusariumdb.org/blast.php
Pseudallescheria/Scedosporium species complex	No	Yes	http://mlst.mycologylab.org/
Yeasts			
Candida albicans	Yes	Yes	http://pubmlst.org/calbicans/
Candida dubliniensis	No	Yes	
Candida glabrata	Yes	Yes	http://pubmlst.org/cglabrata/
Candida tropicalis	Yes	Yes	http://pubmlst.org/ctropicalis/
Candida parapsilosis	Yes	No	
Pichia kudriavzevii (*Candida krusei*)	No	Yes	http://pubmlst.org/ckrusei/
Cryptococcus neoformans	Yes	Yes	http://mlst.mycologylab.org/
Cryptococcus gattii	No	Yes	http://mlst.mycologylab.org/
Pneumocystis jirovecii	Yes	Yes	http://mlst.mycologylab.org/
Endemic mycoses			
Talaromyces marneffei	Yes	Yes	
Histoplasma capsulatum	Yes	Yes	
Blastomyces dermatitidis	Yes	No	
Paracoccidioides spp.	Yes	No	
Coccidioides spp.	Yes	No	
Dermatophytes	No	No	

Comparison of MLP and MLST Methods

For the medical applications listed in the introduction, the main feature of a genotyping method is its discriminatory power (DP) (the average probability that two unrelated specimens randomly chosen will be assigned to different types by the method), expressed as follows:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1)$$

where N is the number of isolates, s is the number of groups, and x_j is the number of isolates falling into the jth group (22). Indeed, the principal issue being addressed is whether isolates are similar to each other; the nature of the polymorphism and the genetic distance are secondary considerations. The higher the DP, the more discriminant the genotyping method is. Confidence in the typing results is assumed for DP values greater than 0.9 (22). As a consequence, the number of markers to be tested before concluding identity between isolates depends on the DP of the genotyping method used.

When MLP and MLST methods have been compared, the relatedness of isolates has been similar (18, 23, 24), suggesting that either method can be used (15). However, the MLP method was shown to be more polymorphic with a higher DP. When testing 100 isolates of *Aspergillus fumigatus*, nine STR markers provided 96 different genotypes with a DP of 0.9994 (25), whereas seven MLST markers had a DP of only 0.93 (26). Similarly, only three STR markers performed as well as seven MLST markers for typing *C. albicans* (27). For *Candida glabrata*, MLST was found insufficient for genotyping compared to a system of nine STR markers (28). These differences can be explained by a higher instability and mutation rate of STRs than of SNPs located in housekeeping genes. Therefore, MLST could rather be used to assess broad subpopulations of a given species, whereas MLP could be used for tracing strains (28). The main differences between MLP and MLST typing are summarized in Table 2.

TABLE 2 Comparison of MLP and MLST for typing medically important fungi

Parameter	MLP	MLST
Previous knowledge on DNA sequences	Yes	Yes
Marker selection	Free software available for selecting STRs	Analyze of housekeeping genes through sequencing
Species specificity	Yes	Yes
Analytical result interpretation	Skill needed	Simple
Risk of assignment of two different PCR products to a given allele	Possible[a]	No
Heterozygosity detection	Simple	No haplotype assignment possible for diploid organisms
Minority allele detection (mixture)	At least 10%[b]	Above 40%[c]
Discriminatory power	High	Moderate
Reproducibility	High	High
Digitization	Yes	Yes
Portability	Currently limited	Excellent
Data bank available	No	Yes
Ease of use[d]	High	High
Cost	Moderate	High
Labor-intensive	No	Yes

[a]When the fragment length is the only result considered, there is a possibility of sequence differences in the STR flanking regions.
[b]Threshold not formally defined for every species.
[c]Sensitivity threshold of Sanger sequencing.
[d]Assuming that sequencing equipment is available.

The MLP method offers better options for speed, throughput, workload, and, as a consequence, cost. However, the workload depends on the number of markers to be tested for a given question. For example, when aiming to rapidly test the similarity of isolates at the beginning of an epidemic, testing a few loci with either method can rapidly provide answers regarding the clonal or nonclonal hypothesis for the epidemic. Thus, the choice between the two methods depends on the goal of the genotyping, the equipment available and the skills of each team.

If DP is the main feature for distinguishing between isolates, it is also important to group isolates even for medical applications, e.g., for zoonoses to know whether an animal can serve as a reservoir for human contamination. The most commonly used method to compare or group genotyping data is the unweighted pair group method with arithmetic mean (UPGMA). The UPGMA tree is built based on a distance matrix and uses agglomerative hierarchical clustering based on the average linkage method (29). This method allows visualization of clusters of isolates (Fig. 2). However, no definite threshold in terms of distance could be assigned to describe what is a cluster and how distant are different isolates or clusters.

Whatever the method retained for genotyping, several major points have to be underlined. The first one is the quality of the sampling for calculating the DPs of the methods. The isolates and reference strains used must be independent, which is difficult when collecting isolates from a given ward in a given hospital (22). Isolates should come from different hospitals or from different individuals from different populations. The second point is the quality of the clinical data associated with the isolates, which is very demanding when implementing prospective collections. The third point, more specific to outbreak investigations, is the chronological history, which can support the possible transmission according to what is known from the natural behavior of the fungus investigated.

ASPERGILLUS MOLDS

Aspergillus fumigatus

Aspergillus fumigatus is the most common species responsible for aspergillosis, including noninvasive diseases (30, 31) and invasive diseases (32). Typing methods were developed initially to understand the pathophysiology of human aspergillosis and route of contamination, then to investigate population genetics of *A. fumigatus* (which is not reviewed here), and finally to understand the origin of azole-resistant isolates recovered from naive or azole-preexposed patients. An MLST scheme using seven or

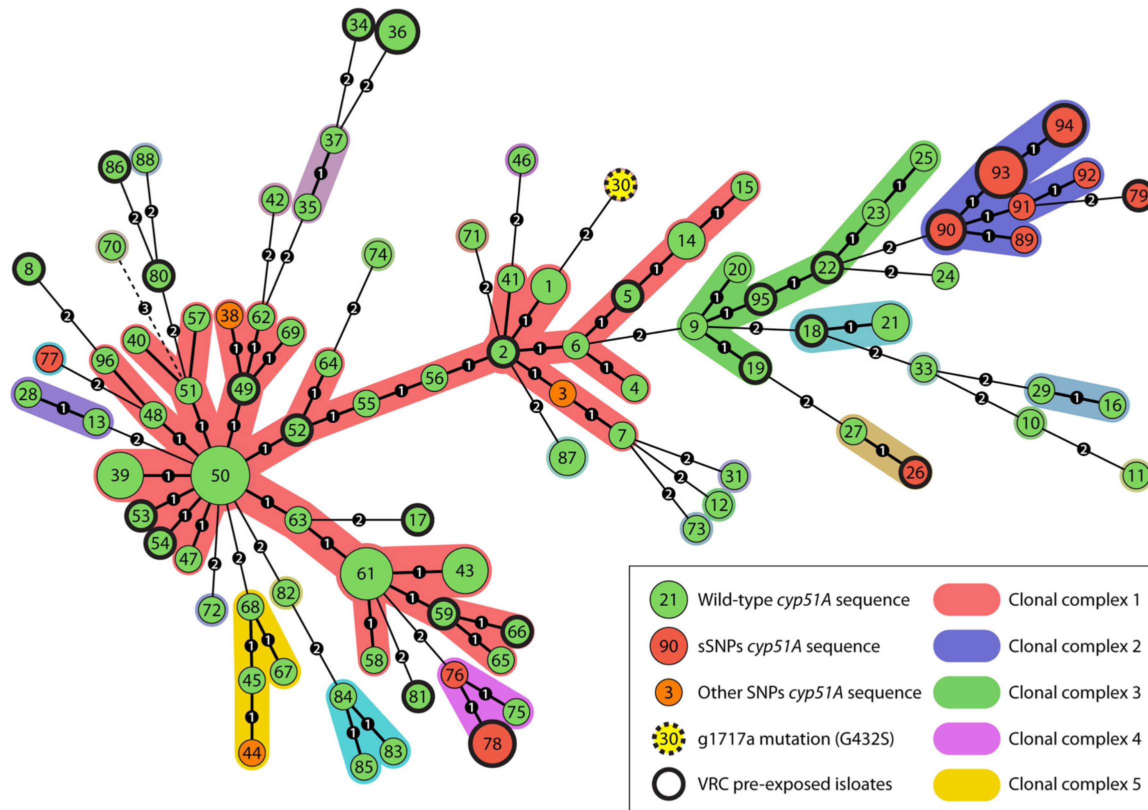

FIG 2 Minimum spanning tree analysis of an MLP typing study of 114 *A. fumigatus* isolates from patients with or without voriconazole preexposure (62). The distances were calculated after the number of allelic mismatches among the MLP profiles. Each genotype (Gt) corresponds to one cycle, with the number of isolates of the same Gt indicated inside. The smallest circles contain one isolate, and the size increases logarithmically with the number of isolates. The higher the number of different markers between linked genotypes, the thicker the connecting bars. The numbers of allelic mismatches between genotypes are indicated in black circles on the connecting bars. A clonal complex (CC) is defined as Gts having a single allelic mismatch with at least one other Gt of the complex and appears as colored zones surrounding some groups of circles. The only azole-resistant isolate sampled has a unique Gt (Gt30) (dotted black circle). The red circles indicate the isolates that harbor serial SNPs in *cyp51A* sequence compared to the wild type (green circles). Note that all but 3 isolates harboring SNPs are grouped in CC2 (blue) and CC4 (pink). The comparison of allelic profiles provides the relatedness between the different GTs using the minimum spanning tree (MStree) method (BioNumerics software v6.5; Applied Maths, Sint Maartens-Latem, Belgium). MLP data were treated as multistate categories, assuming that all changes are equally probable. A maximal allelic divergence of one marker to group genotypes into CCs was used (368). The genotypes not grouped in CCs had at least two allelic mismatches with any other Gt and were considered singletons.

eight housekeeping genes has been shown to have a DP of 0.93, which was considered insufficient (26), and MLP methods have since been preferred.

A first MLP typing, based on four dinucleotide repeats of *A. fumigatus* and with a DP of 0.994, was published in 1998 (33). This set of four markers compared favorably to the previously reported RFLP and RAPD typing methods (34). More recently, a set of nine STRs with a DP of 0.9994 has been developed (25), and an allelic ladder was proposed for homogenizing the results between laboratories (19). Comparison of these nine STR markers and AFLP-based data showed very few discrepancies (35), and comparison with MLST (26) showed that MLP typing and MLST had the highest and lowest DPs, respectively (36). A simpler method, cell surface protein (CSP) typing, has been developed based on the sequencing of one gene containing repeated motifs. CSP typing identified 18 and 19 different genotypes in collections of 209 (37) and 164 (38) isolates, respectively. CSP typing was thus positioned between MLST and MLP typing in terms of DP (37, 39). CSP typing is simple to perform and has good interlaboratory reproducibility (40).

Pathophysiology of aspergillosis. The first relevant studies that aimed at understanding the pathophysiology of invasive aspergillosis (IA) were conducted using RFLP and a probe containing the repeated sequence *Afut* after Southern blot hybridization

(41, 42). Debeaupuis et al. genotyped 849 isolates that showed no geographical clustering and no clustering between environmental and clinical isolates (41). A study of 700 environmental and clinical isolates from various hospitals in France found that 85% of genotypes were unique and that some identical genotypes could be isolated from different hospital sites and could persist over time. In addition, the same genotypes could be recovered from one specific environment and in patients exposed to that environment (42). In parallel, an MLP genotyping method using four markers was developed and, in similar studies, also failed to detect clustering between environmental and clinical isolates (33, 43). Similar observations have also been reported in other settings using various methods (44–46). Using MLP typing, avian isolates were shown to harbor genotypes also observed in human IA, suggesting that either environmental or animal genotypes could infect immunocompromised human individuals (47).

The second issue addressed by genotyping was whether the patient can be infected by one or several genotypes. Several studies have reported the recovery of identical clinical isolates from different deep tissue sites after dissemination (35, 43, 48, 49). Although a multiplicity of genotypes in iterative samples over the course of IA was described during the 1990s, this was deemed rare (43, 50). However, more recent publications have demonstrated that several different genotypes can contribute to IA (35, 48, 49). It is possible that earlier typing methods were not sensitive enough to detect initial mixed genotypes. Alternatively, this genotype multiplicity could be explained by a current more chronic evolution of invasive IA compared to that in the 1990s (32, 51), allowing contamination by several genotypically different isolates. Additionally, a more chronic course of IA could enable microevolution to occur, as previously proposed (48). Thus, although IA typically arises from a single contaminating genotype, several genotypes may be encountered, especially when the risk factors for IA persist.

In cystic fibrosis patients with chronic *Aspergillus* colonization, respiratory samples can harbor various colonization patterns (e.g., multiple unique genotypes, a predominant genotype, or genotypes succeeding each other) (52). Interestingly, some genotypes could be recovered consistently over long periods, suggesting the possibility of prolonged colonization or a better adaptation to the human respiratory epithelium (53).

MLP typing has also being used to investigate several outbreaks of IA (54–56). However, because of the possible recovery of the same genotype in different places and at different times (41–43), it is impossible to say when and where the patients were infected when relying only on genotyping. Given the high DPs of the MLP typing methods, when a patient is contaminated by the same genotype as that found in the environment, there is a high likelihood that the isolates are identical (57). However, the time and site of patient contamination cannot be ascertained (43). Thus, the risk of nosocomial acquisition is real, and all sources of potential contamination should be investigated for the benefit of the immunocompromised patients. The chronology (i.e., contamination in a theater or in an incubator) is suggestive of a common source whatever the genotyping results. Even the identification of several genotypes cannot exclude that the patients were infected at the same time and the same place. CSP typing can be a first approach to avoid additional genotyping investigations when the CSP results are already different (56). Moreover, when dealing with cutaneous aspergillosis, the patients can be the source of the environmental contamination and not the other way around (54). Therefore, when investigating *A. fumigatus* outbreaks, the stress should be put on the clinical and environmental investigation.

Azole-resistant *Aspergillus fumigatus*. Genotyping can be particularly useful for understanding azole-resistant acquisition of *A. fumigatus* due to mutations in the CYP51A protein, the main protein responsible for azole susceptibility. Two hypotheses were proposed: either the acquisition of resistance in the patient under the pressure of azole therapy or the inhalation of azole-resistant isolates with subsequent development of IA. Both possibilities were shown to occur.

Several publications have well documented the occurrence of resistance in patients

treated with azole. In a patient treated with itraconazole over two periods of 6 months and 4 to 7 months for aspergilloma, Chen et al. showed using RAPD that azole-susceptible and azole-resistant isolates harboring the M220I mutation shared the same genotype (58). Such evolution after prolonged azole treatment has been described with other mutations (G448S, G54E, P216L, and F219I) in the CYP51A protein and other mechanisms of resistance (59–61). The impact of azole therapy in patients could also be less obvious than the occurrence of *in vitro* resistance of *A. fumigatus* isolates. For instance, the itraconazole MICs of *A. fumigatus* isolates recovered from patients under voriconazole prophylaxis were increased, although without reaching the threshold defined for resistance (62). These isolates carried serial polymorphisms (F46Y, M172V, N248T, D255E, or E427K) and were associated with two clonal complexes (CCs) (Fig. 2). These results suggested that azole even at a prophylactic dosage could select specific genotypes associated with *cyp51A* polymorphisms and higher itraconazole MICs (62).

The inhalation of azole-resistant isolates is the second mechanism for developing azole-resistant IA. Azole-resistant isolates with a promoter duplication (TR_{34}) and point mutation (L98H) in the CYP51A, which have been well described in The Netherlands, clustered together using MLP typing in a clade separated from azole-susceptible isolates (25, 63). In a genotyping study comparing environmental and clinical azole-resistant isolates, all itraconazole-resistant isolates tested harbored unique genotypes, whereas the environmental TR_{34}/L98H isolates clustered with clinical TR_{34}/L98H isolates (64). These findings highly suggested the environmental origin of the azole-resistant TR_{34}/L98H isolates recovered from humans. Overall, these TR_{34}/L98H isolates have been found in several countries all over the world (65). Using CSP markers, European TR_{34}/L98H isolates showed less diversity than resistant isolates harboring other mutations or wild-type isolates, suggesting that the acquisition of this genetic event was recent (66). This phenomenon was also observed for TR_{46}/Y121F/T289A isolates first reported in The Netherlands (67) and later shown to cluster with TR_{46}/Y121F/T289A isolates from India, whereas German isolates with the same mutation did not (68). Thus, the wide spread of azole-resistant isolates might not result from uniform mechanisms. Finally, the TR_{34}/L98H isolates were shown to be able to outcross with wild-type isolates of different origins, allowing propagation of the genetic abnormality to various wild-type genetic backgrounds (66). However, another report has suggested that Dutch azole-resistant isolates reproduced and disseminated mainly asexually (69).

Given the diversity of the mechanisms of acquisition of azole resistance, several laboratories suggest regular screening, collecting, and reporting of azole resistance of *A. fumigatus* isolates from immunocompromised patients (70, 71). Such collections could be useful not only to estimate the prevalence of azole resistance for deciphering the best antifungal strategy (65) but also for further genotyping analyses.

Aspergillus flavus

Although several studies using RAPD, RFLP, AFLP, SSCP, or MLST have been described, most of these studies aimed to identify new species within the section Flavi (72). Only a few of these studies have focused on genotyping *A. flavus sensu stricto* isolates. *A. flavus* IA is much less common than *A. fumigatus* IA, accounting for $<$10% all cases (32, 73, 74).

Genotyping has been used to resolve nosocomial *A. flavus* infections (32). Using the RAPD technique in the context of postsurgical infection, a clinical isolate was shown to be identical to two environmental isolates found in the operating room, suggesting a nosocomial infection, with the limitation that only eight unrelated control strains were tested (75). Six cases of *A. flavus* stomatitis in leukemia patients were investigated, and RAPD profiles suggested a common source of infection (76). A repetitive DNA probe was used to genotype two geographically and temporally related clinical isolates responsible for cutaneous infection in neonates (77). A common profile between clinical isolates and isolates recovered from an ambulance was observed, suggesting contamination from material or fomites from the ambulance. MLP typing has also been applied to *A. flavus* (78). Using MLP typing to test *A. flavus* infections in hematology

wards, investigators found identical genotypes in the environment, suggesting nosocomial transmission (79, 80).

Genotyping has also been used to decipher the various clinical presentations of *A. flavus* infections. Using nine STR markers, Rudramurthy et al. analyzed 162 clinical isolates recovered from 162 Indian patients over a 2-year period. Thirteen isolates were shown to be mixtures of different genotypes, showing the advantage of MLP typing over methods unable to detect mixtures of isolates. The remaining 149 pure isolates were distributed in 26 clusters (81). No correlation between genotypes and clinical presentation was observed (81). This high diversity of the isolates was also observed when using six markers (82). These observations were consistent with what had been previously reported about *A. fumigatus* infections (see above). However, Hadrich et al. reported mixtures of genotypes in IA but unique genotypes in noninvasive disease (82), which is contrary to what was observed with *A. fumigatus*.

For veterinary medicine, Hadrich et al. described identical genotypes between avian isolates recovered from lung biopsy specimens and the environment of the birds using VNTR markers (83). The same authors also reported a high genetic diversity between human isolates and environmental and avian isolates (84), which is different from what was reported for *A. fumigatus*, where differences between the genotypes of avian and human isolates were not detected using STR markers (47).

Aspergillus terreus

Aspergillus terreus is increasingly reported as an agent of IA, especially in Austria (85) and the United States (86), although it accounts for fewer than 10% of IA cases (2% in France [32] and 4.4% [87] or 7.4% [88] in the United States), and represents 1.9 to 6.2% of the colonizing molds in cystic fibrosis patients (89). In the late 1990s, typing of *A. terreus* was performed, based mainly on RFLP or RAPD, with better discrimination using RAPD (90). RAPD allowed the discrimination of unrelated European isolates, whereas during follow-up of patients with cystic fibrosis or IA, isolates were genetically identical using RAPD (91). Comparison between isolates from patients with hematological malignancies and isolates collected from plants in the same hospital suggested these in-hospital plants as a potential source of infection (92).

MLP typing has also been developed using four markers (93). Typing of 113 isolates (from the eastern and western United States, France, Belgium, and Italy) revealed 111 different genotypes, thus confirming the great genetic variability of this organism (93). A study based on 243 clinical and environmental isolates from the United States, Austria, and other European countries suggested that three major genetic complexes could be delineated using eight newly described STR markers, including either clinical or environmental isolates. The authors suggest that the high incidence of IA due to *A. terreus* could be related to a specific environmental exposure in the Inn valley (Tyrol, Austria) (94).

The high diversity of this species was also observed using AFLP and MLP typing with nine markers in India, with 75 distinct genotypes delineated from 101 isolates and 38 genotypes from 47 widely distributed isolates (95). This diversity was also observed in five cystic fibrosis patients (89). Analysis of 115 isolates (15 to 39 isolates per patient) in 45 respiratory samples revealed 17 distinct genotypes and three colonization patterns (patients with one repeated dominant genotype, patients with repeated mixtures of genotypes, and patients with transiently present genetically diverse isolates) (89).

FUSARIUM SPECIES

Fusarium spp. are ascomycetous molds with a worldwide distribution in nature, as soil saprophytes or facultative plant pathogens. At least 20 different species complexes of the genus *Fusarium* have been described (96). In recent years, several molecular approaches (RFLP, RAPD, AFLP, MLST, and STR) have been applied to *Fusarium* isolates, aimed at determining the genetic diversity of the genus. Most of these analyses have been performed on environmental isolates (reviewed in reference 97). A multilocus species/haplotype nomenclature system has been established (98–100) and is available

at http://www.cbs.knaw.nl/fusarium/ (101) and http://isolate.fusariumdb.org/blast.php (Table 1). The *Fusarium solani* species complex is the complex most frequently associated with human infection (60%), followed by the *Fusarium oxysporum* (10%), *Fusarium fujikuroi* (10%), and *Fusarium dimerum* (5%) species complexes (100, 102).

As part of a keratitis investigation, 191 corneal and patient environment isolates were analyzed by MLST. One haplotype of each of the three most common complexes (*F. solani* species complexes 1-a and 2-d and *F. oxysporum* species complex 3-a) accounted for more than 50% of the environmental and clinical isolates, leading to the conclusion that the corneal isolates came from multiple environmental sources (103). An epidemic of *Fusarium solani* keratitis involving 66 patients was also investigated in Singapore using AFLP and RAPD (104). The authors observed a high polymorphism of the infecting isolates, also making the hypothesis of a common infecting source unlikely (104).

More recently, the genetic relatedness between *F. solani* species complex isolates recovered from hematology and dermatology patients, as well as from environmental isolates, mainly from water hospital sources, was studied (105). MLST analysis of 166 isolates demonstrated that *F. solani* species 2 (subtype 2-d) predominated in both invasive and superficial isolates from patients, whereas *F. oxysporum* species complex-33 accounted for more than 50% of environmental isolates (105). However, the limited environmental sampling prevented any conclusion on the environmental isolates as the cause of the disease (105).

Another MLST study of isolates from plumbing systems in the United States ($n = 297$) and patients ($n = 717$) found that the isolates mainly belonged to the *F. solani* species complex (around 60%), both in the environment and in patients (106). STR markers have been described for *F. oxysporum* and *F. verticillioides* (107–109) but have yet to be applied to clinical studies.

SCEDOSPORIUM SPECIES

The species of the *Pseudallescheria*/*Scedosporium* complex (*S. apiospermum*, *S. aurantiacum*, *S. boydii*, *S. dehoogii*, and *S. minutisporum*) or relatives (*Lomentospora prolificans*) (110) have been implicated in lung colonization and severe infections, ranging from skin to brain lesions, especially in immunocompromised patients (111). The genetic relatedness of *Scedosporium* species has been explored using various techniques, including multilocus enzyme electrophoresis, RAPD, inter-simple-sequence-repeat PCR, RFLP, and AFLP (reviewed in reference 112). Most of these studies have been limited to the analysis of a small number of isolates from cystic fibrosis patients. A study using MLST of 34 clinical *S. apiospermum* and *S. boydii* isolates from different parts of Germany (113) confirmed the persistence of unique genotypes over time, as previously reported using other methodologies (114, 115).

Two different MLST databases are currently available at the same site, one for *S. apiospermum* and *S. boydii* and one for *S. aurantiacum* (Table 1). The *Pseudallescheria*/*Scedosporium* species complex was also found to have a high degree of genetic variation (114, 116–118), whereas *L. prolificans* seems to have low to no intraspecies genetic heterogeneity (119).

CANDIDA YEASTS

Candida albicans

Candida albicans is the main yeast responsible for opportunistic fungal diseases. Despite its diploidy and clonal reproduction, the yeast has a genomic plasticity capable of generating high genetic diversity through various mechanisms, such as recombination (120) or chromosomal polymorphisms or gene replacement (121, 122), which contribute to the genomic microvariation reported for multiple isolates from single patients (123–126). Genotyping of *Candida albicans* using several methodologies has been reported, including electrophoretic karyotyping, RFLP, Southern blot hybridization, and RAPD, which have been reviewed elsewhere (6, 127, 128). However, MLP typing and MLST have since superseded these methods.

MLP typing was first developed for *C. albicans* in the late 1990s (129), with three noncoding region loci (EF3, CDC3, and HIS3) on different chromosomes able to achieve a DP of 0.97 (130). An allelic CDC3 ladder was developed to promote data portability of *C. albicans* MLP genotyping. This CDC3 ladder can be used as an internal standard for accurate allele assignment (18). Recently, a comprehensive protocol for *C. albicans* genotyping using five markers amplified in duplex (loci CDC3 and EF3) or singly (loci HIS3, CDR1, and STPK) has been reported (15). Others authors have used different combinations of STR loci (131–135).

In 2003, a highly discriminant consensus MLST scheme was proposed (136), relying on SNPs within seven housekeeping genes (137, 138) and comparison of the DSTs obtained to those available in the MLST database (Table 1). Other authors have proposed an SNP array typing system for *C. albicans*, which is based on a 79-SNP set across 19 loci of the seven genes originally used for MLST analyses (139). Medical investigations of invasive candidiasis, mainly candidemia, typically use either MLP or MLST genotyping approaches. The main source of invasive *C. albicans* was confirmed to originate from the endogenous mycobiota (6, 130, 140, 141). Indeed, identity between isolates from blood and colonized anatomical sites was almost always observed, leading to the conclusion that nosocomial transmission between patients is likely a rare event (141, 142), even if an outbreak is suspected (143), although some authors have reported nosocomial transmission in hospital environments (144–148). MLP typing has also been used to track the origin of graft site candidiasis after kidney transplantation (149). The results have shown that the contaminating genotype originated from the donor and that the contamination occurred during organ recovery (149).

Since the endogenous mycobiota is the source of invasive infections, some authors have investigated whether this flora is stable over time. The persistence of the same strain type at different site locations or over long periods of time seems to be the most common scenario (131). However, if an adaptive response to a particular environment is needed, the strain may undergo microevolution (132, 141, 150–153). These genetic variations have been widely observed among closely related strains and are mainly associated with loss of heterozygosity (120, 124, 125, 144). Only a few studies have reported strain replacement with a completely different type of *C. albicans* (131, 132).

Genotyping is also widely used to assess the occurrence of antifungal resistance in patients. When resistance appears within isolates of a given species, it is important to determine whether the patients acquired a new genotype or whether resistance occurred in the same genotype (154).

Several studies have aimed at identifying an association between a genotype and some virulence traits. Clade-specific associations with different properties of *C. albicans* isolates have been explored (128, 155). Odds et al. found one clade associated with superficial infections and other clades associated with commensal carriage (156). In contrast, using 11 STR markers with 147 isolates, L'Ollivier et al. failed to demonstrate an association between a given genotype and its clinical or commensal origin, supporting the hypothesis that isolates share the same overall pathogenicity whatever their origin (157).

Candida dubliniensis

The ascomycetous diploid yeast *Candida dubliniensis* is phylogenetically closely related to *C. albicans*. *C. dubliniensis* is an opportunistic human pathogen isolated worldwide, mainly associated with mucous candidiasis in HIV-infected patients. *C. dubliniensis* is also part of the commensal microbiota of the oral cavities (158). McManus et al. used a combination of 10 MLST loci, previously validated for *C. albicans*, to investigate the *C. dubliniensis* population structure and proposed a combination of eight loci with a DP of 0.909 (159).

C. dubliniensis has also been recovered from seabird excrement, indicating a nonhuman habitat (160, 161). Using MLST, a *CDR1* gene polymorphism, and mating-type analysis to study the genetic relatedness of avian and human isolates, McManus et al.

concluded that the majority of avian isolates represent a distinct subgroup in the C1 clade but that some genetic profiles are shared between human and bird isolates, suggesting a possible interspecies transmission (161).

Candida glabrata

Candida glabrata is a haploid ascomycetous yeast belonging to *Saccharomycetaceae*. This yeast is a commensal of the human gut (162) and has a decreased susceptibility to azoles and especially to fluconazole (163). After *C. albicans*, *C. glabrata* is the second leading yeast species responsible for human bloodstream infection in Europe and the United States (164–167). *C. glabrata* has a mainly clonal mode of reproduction, with no sexual reproduction yet reported, although recombination events do seem to occur in some subpopulations (168–170).

In 2003, Dodgson et al. described an MLST scheme for *C. glabrata*, consisting of six single-copy housekeeping genes located on six separate chromosomes (171). From 107 clinical isolates, Dodgson et al. defined 30 STs grouped into five major clades and identified 81 polymorphic sites among the 3,345 nucleotides sequenced (171). No correlation was detected between STs and fluconazole susceptibility, although a geographical specificity was identified (171). An online database containing molecular information on 209 isolates from Japan, Taiwan, Europe, South America, and North America, corresponding to 68 STs, has been made available (Table 1). Geographical clade specificity has been frequently observed using MLST typing but without evidence of association between genotypes and clinical data (e.g., site of isolation and underlying disease) or antifungal susceptibility (168, 172, 173).

STR markers have also been proposed for genotyping *C. glabrata* isolates (174–177). In 2005, Foulet et al. described three STR markers using 138 independent clinical isolates and reference strains with a low DP of 0.84 (174). The markers were shown to be stable after 25 subcultures, and 21 allelic associations could be identified (174). The authors failed to find any correlation between genetic profiles and clinical data (174). Nevertheless, as already mentioned for results obtained using MLST (171), a skewed distribution of the *C. glabrata* population has been described, with two genotypes accounting for almost 50% of all isolates (174). Foulet et al. also tested several isolates from different anatomical sites of the same patients and found that these isolates had similar genotypes, suggesting that patients were infected with their own colonizing genotype (174). These three STR markers (174) were complemented by three additional markers with trinucleotide repeats to obtain a DP of 0.949 (177). In genotyping epidemiologically closely related isolates from 36 patients, Abbes et al. observed identical or highly related genotypes for 25 patients and a microvariation in four of these patients (177), which had already been reported using Southern blot hybridization (178). In 2007, six other STRs were evaluated using 127 *C. glabrata* isolates: 37 genotypes were identified, leading to a DP of 0.902, with three genotypes representing 52% of the isolates (175).

More recently, Brisse et al. described eight new STRs located in coding and intergenic regions, with a DP of 0.96 (24). By testing 198 isolates, 90 genotypes were identified, and the clustering of isolates was congruent with that obtained using the housekeeping gene *NMT1* (171). These authors also compared MLP typing and MLST and concluded that they are complementary but at distinct evolutionary time scales, with MLP typing being a tool for fine-scale population genetic studies (24). In 2010, Enache-Angoulvant et al. used these eight STRs to genotype 180 strains, including blood isolates and isolates from the digestive tracts of nonhospitalized European patients (28). The digestive tract isolates differed from the blood isolates because of a higher genotypic diversity (28). They also demonstrated microevolution of isolates from the digestive tract, confirming that *C. glabrata* is a component of the human microbiota.

A commercial sequence-based service for typing *C. glabrata* has recently been proposed (179), which associates two STR markers, one of which is similar to one already described by Grenouillet et al. (175). The primers used for this commercial kit

were not provided because of an ongoing patent. Correct amplification was observed for 102 of the 104 isolates tested, with a DP of 0.95 (179). The size and sequences of the STR amplification were analyzed not using capillary electrophoresis but using Sanger sequencing. Four out of the five patients sampled from one hospital studied shared rare alleles, which suggested nosocomial transmission. Since fluconazole resistance developed in these isolates but with different mechanisms, the authors hypothesized that resistance was independently acquired after the interpatient transmission (179).

Recently, Dellière et al. genotyped 268 *C. glabrata* isolates using 10 STR markers (180). The authors found that acquisition of resistance was associated with drug exposure and not with mutations in the mismatch repair gene *MSH2*. The *MSH2* sequence type was then associated with the MLP genotype (180), suggesting that *MSH2* mutations would not directly be the cause of acquisition of resistance, at least for fluconazole (180), in contrast to what has been suggested by other authors (181).

Using the same complete set of nine STR markers proposed by Brisse et al. (24), Al-Yasiri et al. investigated whether gulls could be a reservoir for humans (182). In analyzing 190 *C. glabrata* isolates from hospitalized patients and from gull droppings in breeding areas along the Mediterranean seashore, the authors suggested that gulls are a reservoir of *C. glabrata* with possible transmission to humans through environmental contamination. The authors also suggested that other vertebrate hosts might be reservoirs as well.

Candida tropicalis

Candida tropicalis is one of the four major *Candida* species responsible for candidemia worldwide, accounting for 4 to 20% of all candidemia cases (167, 183, 184) and even the first species in India (185). *C. tropicalis* is an ascomycetous diploid yeast belonging to the CTG clade (species in which CTG codes for serine instead of leucine) of the *Saccharomycetales* (186, 187).

In the 1990s, the epidemiology and origin of fungal outbreaks due to *C. tropicalis*, especially in neonatal intensive care units, were investigated using RFLP and pulsed-field gel electrophoresis (188–192). The conclusion was that *C. tropicalis* frequently originated from the patient's own endogenous microbiota.

In 2005, Tavanti et al. described MLST using six loci and identified 88 DSTs, with a DP of 0.994 (193). The authors identified three clades and one additional putative clade containing flucytosine-resistant isolates (193). To date, 533 DSTs for 620 isolates are available in the online database (Table 1). The conclusion of the MLST studies was that *C. tropicalis* has clonal expansion, although recombination events could select one successful clade associated with humans (194). Jacobsen et al. also found that multiple isolates from a given patient shared the same MLST profile or showed evidence of microevolution, such as loss of heterozygosity (194). Magri et al. studied the genetic diversity of 61 isolates from 43 patients hospitalized in Brazil (195), reporting 39 DSTs. Among the 14 patients with multiple isolates, seven had microvariation in a single gene from sequential isolates and three had microvariation in six gene fragments. Finally, they identified three isolates resistant to fluconazole but did not find any correlation between this resistance and the isolate clustering (195).

To explore the origin of lower susceptibility to flucytosine, 130 *C. tropicalis* isolates from positive blood cultures collected in the Paris (France) area were studied by combining four MLST loci, SNPs on internal transcribed spacer (ITS) regions, and two STR markers (196). A cluster of flucytosine-resistant isolates was linked to hematological malignancies. Surprisingly, the patients infected with isolates of this cluster had a better prognosis than patients infected with flucytosine-resistant isolates not belonging to this cluster (196). The use of two STR makers and *URA3* sequencing was proposed to track this specific clone (196). Fluconazole-resistant isolates belonging to an MLST cluster have also been identified in Taiwan (197).

Different studies have suggested an endogenous origin for infecting strains of *C. tropicalis* and a mainly clonal mode of reproduction, even though parasexuality has been recently described (198). MLST and MLP typing are useful for determining the

possible origin of an outbreak, for example, in cases of contamination of grafts and/or preservation solutions (199) or cases of acquired antifungal resistance due to therapeutic pressure (200). More recently, Wu et al. described six STRs with DPs varying from 0.70 to 0.95 in testing 65 clinical isolates. Although the global DP of the six STRs combined was not provided, the authors concluded that their typing method was similar to MLST for discriminating *C. tropicalis* isolates (201). No clinical data were presented with this new set of STRs (201). A new set of eight STRs with a DP of 0.99 has recently been reported (202).

Candida parapsilosis

Candida parapsilosis is a frequent colonizer of skin and a component of the human microbiota. *Candida parapsilosis* belongs to the *Lodderomyces-Spathaspora* clade in the family *Debaryomycetaceae* (203). Like *C. tropicalis*, *C. parapsilosis* belongs to the CTG clade (186, 187). *C. parapsilosis* is frequently involved in invasive infection worldwide, with a specific link with use of intravascular catheters, probably due to its ability to form biofilms (204). This species is also frequently involved in neonates with nosocomial outbreaks of infections involving manual transmission from health care workers, which stimulates genotyping studies (205–208).

In 2005, differences in four gene sequences resulted in the identification of three distinct species. The former *C. parapsilosis* groups I, II, and III were then named *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*, respectively (209). The three groups were physiologically and morphologically indistinguishable and were initially identified on the basis of molecular data (209). Ultimately, the four MLST markers listed above revealed a very low degree of variability for *C. parapsilosis* isolates (210). In 2010, Tavanti et al. used AFLP to genotype *C. parapsilosis* isolates and found limited DNA sequence variability, in line with previous DNA sequencing data, as well as a lower genetic variability for *C. parapsilosis* than for *C. orthopsilosis* or *C. metapsilosis*, supporting the hypothesis of a clonal expansion mode of reproduction of *C. parapsilosis* (211). Furthermore, recent genomic analysis suggests that, in contrast to the case for the *C. parapsilosis* population, events of recombination and hybridization between type I and type II were observed in *C. orthopsilosis* isolates, resulting in novel subspecies (212).

Since the identification of *Candida parapsilosis sensu stricto*, 11 STR markers with various sets of primers have been described. First, Lasker et al. used seven STRs (six dinucleotide repeat markers [A to F] and one trinucleotide repeat marker [G]) to genotype 42 isolates recovered from the United States (213). They obtained 30 genotypes, a DP of 0.971, and a good concordance with Cp3-13 DNA hybridization (213). The authors concluded that five of their markers (A, B, C, E, and G) were chromosomally linked (206) and proposed to combine two of their markers, D and F, with three (CP1, CP4, and CP6) of the four markers described by Sabino et al. in 2010 (214). Sabino et al. described four STRs (CP1, CP4, CP6, and B5) and genotyped 236 clinical and environmental isolates, recovered mainly from Portugal but also from France, Spain, the United States, and Peru (214). Their results demonstrated that these STRs were suitable for outbreak investigations and confirmed a likely clonal expansion mode of reproduction (214).

Since 2006, STR markers have been frequently used to study outbreaks of *C. parapsilosis*, especially in neonates. Studies based on STRs, together with other tools such as Cp3-13 DNA probes, suggested persistence of genotypes during recurrent infection, horizontal transmission in intensive care units, and microevolution of *C. parapsilosis* (7, 206, 208, 214–218).

Pichia kudriavzevii (*Candida krusei*)

Pichia kudriavzevii (synonym, *Issatchenkia orientalis* or *Candida krusei*) is an ascomycetous yeast belonging to the *Saccharomycetaceae* that is responsible for almost 2% of fungemia worldwide and frequently associated with solid tumor and solid organ transplantation (167). This species is resistant to fluconazole and is associated with a higher mortality in humans than *C. albicans* (167).

In 1994, Carlotti et al. described the first RFLP method for genotyping *P. kudriavzevii* (219). This study allowed the delineation of 12 clusters for the 48 distinct types identified among the 58 isolates. Similar genotypes were found among multiple isolates recovered from different anatomic sites of the same patients at different times, suggesting an endogenous origin of the infecting isolates. In 2001, Shemer et al. described a polymorphic degenerate STR (i.e., with intercaling base pair substitutions) and suggested clonal reproduction using 50 clinical isolates and six reference strains (220).

In 2007, using 129 isolates of different geographical origins, Jacobsen et al. (221) described an MLST method to genotype *P. kudriavzevii* isolates based on the sequencing of six loci (Table 1). They identified 94 DSTs clustered into four subgroups by a UPGMA dendrogram and found heterozygous sequences for some alleles, confirming that *P. kudriavzevii* is a diploid organism for at least part of its genome. The authors did not find evidence of geographical associations among the subtypes. A total of 60 SNPs were identified, 30 of which were synonymous and 30 nonsynonymous, and among the latter, 16 changes were found to be nontrivial (i.e., with side chains changing from acidic to basic or from aliphatic to aromatic). By analysis of pairs of isolates from different times or different sites recovered from seven patients, the authors confirmed the hypothesis of an endogenous origin of the infecting strains.

The same MLST method was used in 2015 by Tavernier et al. to determine the genotypes of successive *C. krusei* clinical isolates recovered from bone marrow transplant patients (222). The authors observed microvariation of allelic profiles within a single patient and demonstrated that genetically linked isolates acquired resistance to echinocandins following exposure to micafungin and caspofungin.

CRYPTOCOCCUS NEOFORMANS/CRYPTOCOCCUS GATTII

Cryptococcus neoformans is a human fungal yeast causing life-threatening meningoencephalitis, mainly in patients with AIDS or other cellular immune defects (223). Two varieties of *C. neoformans* have been described: *C. neoformans* var. *grubii* (serotype A), recovered worldwide, and *C. neoformans* var. *neoformans* (serotype D), found mainly in Europe (224, 225). *C. neoformans* and *C. gattii*, initially identified as two varieties, are now considered two distinct species (226). Delineation of these two species and their varieties is of the utmost importance, not only for the epidemiology of the yeast but also for their medical impact, with *C. gattii* justifying a more intensive therapeutic approach (227). Recently, seven species have been proposed within the *Cryptococcus neoformans/Cryptococcus gattii* species complex (228). However, this classification is not accepted by the whole community (229).

Since the 1990s, various methods have been tested to understand the *C. neoformans* and *C. gattii* population structures (230). Multiple hypotheses on the biology, pathophysiology, and epidemiology of cryptococcosis have been proposed, such as infection by a unique strain, dormancy of *C. neoformans* isolates (231), geographic distribution of the serotypes (232, 233), environmental origins (234) and descriptions of interspecies and intervariety hybrids (235–237). Until recently, PCR amplification using M13 primers, AFLP, and RAPD were principally used for typing *C. neoformans/C. gattii* isolates. These methods described 10 major molecular types (236, 238–242) (Table 3). The VGII genotype accounted for more than 97% of the isolates recovered during the Vancouver Island outbreak, which could be further divided in two populations (VGIIa and a minor VGIIb population) (243). This Vancouver Island outbreak started in 1999 (244), and in August 2001, 12 cases of animal cryptococcosis were diagnosed, with a concomitant increase in human cases within the same geographic area (245). Forty-five animal cases from different mammals and 59 human cases, mainly among immunocompetent individuals, were identified at the end of 2002 (245). Furthermore, *C. gattii* was isolated from trees and soil.

In 2005, Fraser et al. genotyped *C. gattii* isolates involved in the Vancouver Island outbreak using eight MLST unlinked loci (246). This MLST differentiated all four major molecular types (VGI to VGIV) and also distinguished both subgenotypes, VGIIa and

TABLE 3 Different molecular types of *Cryptococcus neoformans*/*Cryptococcus gattii* species[a]

AFLP type	RAPD type(s)	Serotype	Actual nomenclature	Proposed nomenclature
AFLP1	VNI	A	*C. neoformans* var. *grubii*	*C. neoformans*
AFLP1A	VNII			
AFLP1B	VNB			
AFLP3	VNIII	AD	AD hybrids	AD hybrids
AFLP2	VNIV	D	*C. neoformans* var. *neoformans*	*C. deneoformans*
AFLP4	VGI	B	*C. gattii*	*C. gattii*
AFLP6	VGIIa, VGIIb			*C. deuterogattii*
AFLP10	VGIV, VGIIIc			*C. decagattii*
AFLP5	VGIII	C		C. *bacillisporus*
AFLP7	VGIV			*C. tetragattii*
AFLP8		BD	BD hybrids	BD hybrids

[a]See references 228, 230, 231, 232, 233, and 234.

VGIIb. Fraser et al. also demonstrated that the major genotype was hypervirulent and that the minor genotype was severely attenuated. Another study, using four STR markers, found that strains genetically similar to those from the Vancouver Island outbreak could be isolated in other parts of the world (247). In 2006, Litvintseva et al. described new MLST markers and combined them with previously described markers to propose a set of 12 MLST markers, dispersed across nine chromosomes (248). To harmonize *Cryptococcus* genotyping, an international working group was formed in 2007, and a consensus set of seven markers was proposed (238). These markers are unlinked and not under any selective pressure. A database has been established, including data for serotypes A and D and *C. gattii* isolates (Table 1) (238).

In 2005, an abundance of cryptococcal STRs was reported once the *C. neoformans* var. *neoformans* genome had been sequenced (249). In 2008, Hanafy et al. used three STR loci to study serotype A isolates from 12 countries (250). The method yielded 30 different genotypes, with a DP of 0.992. Karaoglu et al. used seven STR markers with a DP of 0.99 to study both varieties and an AD hybrid isolate of *C. neoformans* (249). The genetic diversity of the VNI and VNII *C. neoformans* var. *grubii* molecular types was found to be similar.

In 2010, Illnait-Zaragozi et al. described nine STRs to genotype 190 serotype A isolates from Cuba, 122 from humans and 68 from pigeon guano (251). The authors found 104 genotypes and achieved a DP of 0.993. The authors identified 11 microsatellite complexes (MCs) corresponding to genotypes differing in up to two STR markers. More than 70% of isolates from pigeon guano were clonally related, with the absence of temporal and spatial variability. On the other hand, several MCs did not contain environmental isolates, which suggests unidentified additional niches of *C. neoformans* var. *grubii* possibly responsible for human infection. This also suggests that not all the environmental isolates are equally pathogenic for humans. This hypothesis is supported by evidence that pigeon isolates were less pathogenic than human isolates in a mouse model (252).

Hagen et al. genotyped Dutch clinical isolates collected between 1977 and 2007 by AFLP and MLP analyses (253). The AFLP typing provided three groups: AFLP1 (81.7%), AFLP2 (12%), and AFLP3 (4.7%). The authors used the nine STRs previously described for serotype A (251) and designed seven new STRs for serotype D. For 259 serotype A isolates, the authors obtained 196 genotypes grouped into 11 MCs, including two novel clusters, MC13 and MC14, with a DP of 0.994. Thirty-two genotypes were identified in 53 isolates of serotype D or hybrids (36 serotype D, 14 AD hybrid, and 3 BD hybrid), with a DP of 0.966. The authors described some mixed infections ($n = 7$) due to isolates with different genotypes. MC and genotype could not be associated with a geographical origin or clinical data. However, some AFLP groups were associated with different susceptibility to antifungal drugs: the AFLP1 group with lower susceptibility to amphotericin B and AFLP2 isolates with lower susceptibility to flucytosine and higher susceptibility to azoles (251).

Pathophysiology of *Cryptococcus* Species

Based on PCR fingerprinting profiles, Igreja et al. found that the majority of infections in Brazil were caused by a single strain (254). However, Desnos-Ollivier et al., using an MLST method, showed that almost 20% of patients having cryptococcosis diagnosed in France were infected by multiple isolates (with different genotypes and/or different serotypes) (255, 256). Similarly, Wiesner et al. found that 17% of Ugandan patients were infected with multiple genotypes (257). Whatever the heterogeneity of the infecting strains was, a major clinical point is that the patients had acquired the infecting strain long before the onset of the cryptococcal meningitis (231). As determined using two methods to genotype strains recovered from patients who developed cryptococcosis in France, the isolates from patients originating from Africa and having left Africa for some time (median, 110 months) differed from those from patients originating from Europe (231). This epidemiological finding has been recently corroborated by biological evidence of dormancy during host interaction (258).

Genotyping has also suggested an association between *C. neoformans* genotypes and virulence during human infection, although some reports do not confirm such associations. In 2012, Wiesner et al., using eight MLST markers, analyzed 111 isolates from cerebrospinal fluid (CSF) samples (107 of serotype A and 8 AD hybrids) from Ugandan AIDS patients before receiving antiretroviral therapy (257). The mortality was different according to the MLST groups, which suggested that cryptococcal strain variation could play an important role in human immune responses and, as a consequence, in mortality. Although based on a limited number of cases, the Ugandan hybrid strains were associated with an increased mortality (257), which is inconsistent with other reports (255). Other authors genotyped *C. neoformans* var. *grubii* isolates from CSF samples from 230 South African HIV-infected patients between 2005 and 2010 (259). The authors also identified genotypes associated with phenotypic features. The VNII group isolates had a significantly better CSF survival and a higher laccase activity than VNI and VNB isolates. However, a study of 54 clinical isolates (serotype A, mating type alpha) from France did not show any correlation between DSTs and clinical data or *in vitro* data (i.e., phagocytosis index and intracellular proliferation) (260, 261).

Other studies focused on the correlation between the immune status of patients and the infecting genotype. Choi et al. genotyped 78 isolates from patients hospitalized for cryptococcosis in different provinces of the Republic of Korea between 1990 and 2008 using MLST and PCR fingerprinting typing (262). The HIV-positive patients were all infected with genotype VNI, whereas the remaining patients were infected mainly with genotype VNIc, previously identified as the major genotype in China (263). Most of the VNIc strains were also associated with HIV-negative patients in Korea (262), similar to a subcluster (VNIgamma) identified in HIV-negative patients in Vietnam (264). Similarly, Illnait-Zaragozi et al. reported an association between a specific genetic cluster and HIV-negative patients in Cuba (251). Therefore, it may be possible that some *Cryptococcus* genotypes could be better adapted to patients with specific underlying immunodepression or that these specific groups of patients could have been exposed more preferentially to these genotypes.

From a medical point of view, the genetic analyses discussed above have allowed a better delineation of the eight major molecular types of *C. gattii* and *C. neoformans* which are now validated. Their identification, which is important for medical management, can be achieved using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), currently routinely used in laboratories for fungal species identification (228, 265).

PNEUMOCYSTIS JIROVECII

Pneumocystis species belong to Taphrinomycotina, one of three subdivisions of ascomycetous fungi that can infect various mammalian hosts. *Pneumocystis jirovecii* is the species specifically associated with humans and could be considered a commensal (266). Almost all individuals are exposed to *P. jirovecii* before reaching the age of two (267–271). Whether pneumocystis pneumonia (PCP) in immunocompromised individ-

uals comes from recently acquired or from dormant organisms, or both, is still debated. Indeed, *Pneumocystis* DNA can be detected in patients without PCP, leading to the concept of carriage (272). Moreover, the genotypes from PCP patients and carriers did not differ, and carriers can harbor *P. jirovecii* for long periods (273). Thus, all carriers, and probably all individuals, can potentially act as a reservoir and transmit the fungus to immunocompromised hosts, as extrapolated from transmission experiments in mice (274, 275). All these studies on the pathophysiology of *P. jirovecii* depend on genotyping, which must be performed directly on clinical specimens because of the absence of simple culture procedures (276). For carriers with low fungal loads, the amount of DNA can be too low for correct amplification for typing, introducing potential biases if low fungal loads are associated with some genotypes (21).

Since airborne transmission between humans is the currently accepted hypothesis (277) with ascus (previously called cyst) as the most probable infecting agent, as demonstrated in mice (278), genotyping of clinical respiratory specimens has been used to investigate potential nosocomial PCP outbreaks. The first studies were based on PCR-SSCP of nuclear and mitochondrial loci (279–281). PCR-SSCP was then replaced by direct DNA sequencing using an MLST scheme (282). MLST confirmed the presence of identical genotypes recovered from different patients during outbreaks (283–285). An optimized MLST scheme restricted to three loci (nuclear and mitochondrial genes) achieved a DP of 0.987 (286). Similarly, studies using ITS sequencing with subcloning of the PCR products (287) and multitarget SSCP (288) suggested that epidemic cases were mostly due to a single genotype. These results were obtained with highly discriminant methods, since the number of genotypes was estimated at 43 using the SSCP method (289) and as many as 60 using ITS sequencing methods (290). A common nomenclature for the various alleles of these MLST markers has led to the creation of a website allowing identification of alleles to harmonize the results (Table 1).

Besides investigation of clustered cases, genotyping has been used to characterize the organisms present in a given individual, and all the typing methods have reported the presence of mixtures of two or more genotypes in single patients. The range of mixture detection varied according to the method used, from a few percent using Sanger DNA sequencing to about 70% using SSCP (21, 281, 291–296). When focusing on mitochondrial polymorphisms using SNaPshot minisequencing, a method based on one-nucleotide extension of primers developed for the analysis of SNPs, mixed mitochondrial genotypes were associated with the highest fungal loads observed, suggesting either coinfections with several genotypes or accumulation of mutations due to a high replication rate of the microorganism (21). This minisequencing method also showed that the minority *P. jirovecii* populations below 20% are not detected when using Sanger sequencing, a method not sensitive enough to detect minority alleles (21).

A specific clinical issue has emerged with the use of trimethoprim-sulfamethoxazole (co-trimoxazole) prophylaxis of patients with PCP and AIDS. Although dihydropteroate synthase (DHPS) mutations have been reported in PCP treatment failure, suggesting selection by drug pressure, the same mutations have also been detected in PCP patients not receiving co-trimoxazole (297). Therefore, rather than selection pressure by sulfa prophylaxis, the presence of DHPS mutations could be explained by incidental interhuman transmission and may serve as an epidemiological marker rather than a marker of resistance (298, 299).

Molecular methods for *P. jirovecii* genotyping are continually evolving. Recently, implementation of MLP methods (300, 301) and next-generation sequencing (NGS) (302) has allowed easy detection of low abundances of coinfecting types, with ratios of about 1:50 and 1:1,000, respectively.

DERMATOPHYTES

Dermatophytes are very common fungi responsible for hair (tinea capitis), skin (tinea corporis), and nail (tinea unguium) diseases, the taxonomy of which has recently been widely modified (303). Concern has been raised about the high propagation rate of *Trichophyton tonsurans* (304), with outbreaks in pediatric care centers (305, 306) and

among wrestlers (307). Transmission can occur through hairdressing (308) or direct contact with an infected individual.

An epidemic involving 21 individuals, including health staff members corresponding to repeated admissions of an infected child, was investigated using PCR-RFLP of three different loci (ribosomal DNA nontranscribed spacer and the enzymes alkaline protease and leucine aminopeptidase). The authors concluded that the infected child was the source of infections for the other people, although a formal DP of the typing system was not established (306). The typing system was completed using sequence variation in 10 additional gene loci using PCR-RFLP and testing of 198 isolates from 14 countries (309). This complete set of markers was used to study transmission of *T. tonsurans* among wrestlers (307). The authors assessed the clonal nature of the *T. tonsurans* strain infecting 14 of the 29 wrestlers tested (307). The same PCR-RFLP markers were used to study single and mixed infections in children (310). Colonies obtained from 252 children were genotyped, and 57 children had two distinguishable strains, underlining the possible complexity of typing when mixed genotypes are simultaneously present in clinical samples (310). Similarly, an outbreak of *Microsporum canis*, a zoophilic species, affecting 42 children was investigated using RAPD, amplification of ribosomal DNA nontranscribed spacer, and ITS sequencing. The authors concluded that there was a common source of the tinea capitis among the children, although the typing method was not evaluated with independent isolates (311). Another PCR-based method using primers consisting of trinucleotide repeats has been used to determine whether the *M. canis* isolates were shared between patients and their cat (312).

ENDEMIC MYCOSES

Talaromyces marneffei

The thermally dimorphic fungus *Talaromyces marneffei* (formerly *Penicillium marneffei*) is a member of the subgenus *Biverticillium* and an emergent pathogen affecting humans and animals (313). Its endemicity in tropical areas of Asia (Thailand, northeastern India, China, Hong Kong, Vietnam, and Taiwan) combined with the emergence of HIV/AIDS changed the frequency of this disease, making it a significant health problem in those areas.

Initial studies using an MLST approach were designed to distinguish between clinical and environmental isolates from Thailand, China, and Hong Kong (314). Haploid genotypes were assigned using sequences of five polymorphic loci with a high DP of 0.949. The MLST analysis of 24 isolates found a geographical separation between the samples from Thailand or China, but with some mixed geographical clusters (314). In parallel, an MLP typing system identified 23 genetic loci amplified among 23 clinical isolates (315). The typeability and reproducibility of the typing system were high (99.6% and 100%, respectively). The analysis of 21 independent isolates resulted in 19 unique genotypes with a DP of 0.995. The observation of a single allele for a given locus for each strain suggested that *T. marneffei* is haploid. In addition, the extensive multilocus linkage disequilibria shown by the high index of association (3.414; $P < 0.01$) suggested a clonal mode of reproduction of the fungus (315).

T. marneffei is thought to be transmitted primarily by inhalation. In the absence of a known environmental reservoir, Huang et al. and Cao et al. aimed to determine whether the human disease was acquired principally from rodents (e.g., bamboo rats of the genera *Rhizomys* and *Cannomys*) or whether it was directly acquired from the environment, independently of animals (316, 317). Six STRs were chosen from the panel described by Fisher et al. (318) to study 43 rodent isolates and 40 human isolates (317). Human *T. marneffei* isolates were found to be similar to those infecting rats. However, in the absence of environmental isolates sampled, the authors could not discriminate between human contamination directly from the environment or following amplification of infectious dispersal stages by rats (317).

Histoplasma capsulatum

Histoplasma capsulatum is a haploid ascomycetous fungus with a worldwide distri-

bution (319) that causes systemic mycosis in mammals, including humans. This thermo-dimorphic pathogen is found in its mycelial phase in rich soil, particularly soils associated with bird and bat guano. In humans, *H. capsulatum* proliferates as an intracellular yeast in macrophages and monocytes (320, 321). Historically, three varieties were described based on morphological and pathogenic characteristics: *H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii*, and *H. capsulatum* var. *farciminosum* (321).

Several genotyping assays performed since the 1980s have revealed considerable polymorphisms among isolates from particular geographical locations (322). An MLST study showed that the *H. capsulatum* complex was not monophyletic and consisted of seven phylogenetic species, with an African clade including *H. capsulatum* var. *duboisii* but also isolates morphologically identified as *H. capsulatum* var. *capsulatum* (323). There is, however, no consensus genotyping method for this fungus. Although outbreaks of histoplasmosis are regularly reported (324), the current genotyping studies are more focused on the genetic variation and environmental dispersion of *H. capsulatum* (325).

Blastomyces dermatitidis

Blastomyces dermatitidis is a haploid thermo-dimorphic fungus responsible for pulmonary and disseminated infections in humans and other mammals (dogs, bats, and sea lions). Blastomycosis is endemic to regions of North America (the Ohio and Mississippi River valleys, the Great Lakes, and the St. Lawrence River) and has also been described in Africa, India, and South America. The infection occurs after inhalation of airborne conidia disrupted from wet soil or organic material (326). The ecological niche of *B. dermatitidis* remains poorly defined, although the disease appears to be more frequent along waterways (327, 328).

Outbreaks of blastomycosis have been associated with work-related or leisure activities and with exposure to moist soil enriched with decaying vegetation (326). An RFLP study using various rRNA probes of 59 isolates of *B. dermatitidis* collected from 15 regions (in the United States, India, Africa, and Canada) found high genetic similarity among isolates, with the definition of only three major groups (329). With this RFLP typing method, the soil isolates could not be deemed responsible for the majority of cases during an outbreak in Eagle River, WI (329). An additional RFLP study of 116 isolates explored the polymorphism in the promoter region of the *BAD-1* gene (330) and described two new genetic groups in addition to those previously identified (329).

Using an MLP typing scheme (328), the relationship between genetic groups and clinical symptoms in 227 clinical isolates from the outbreak in Eagle River, WI, was evaluated (331). In univariate analysis, pulmonary-only infections and fever were more likely associated with group 1 isolates, while disseminated disease, older patient age, and comorbidities were more likely associated with group 2 isolates characterized by a high allelic diversity (331). However, in multivariate analysis, only disease onset to diagnosis of more than 1 month, older age at diagnosis, and smoking status remained predictors for group 2 infections (331). Although this study revealed clinical differences between the two genetic groups, the molecular basis of these differences was not resolved (331).

Paracoccidioides Species

The genus *Paracoccidioides* is responsible for the endemic systemic fungal infection paracoccidioidomycosis. According to multilocus genealogy, this genus consists of two haploid thermo-dimorphic species: *P. brasiliensis* (332, 333) and the recently defined species *P. lutzii* (334, 335). Paracocciodiomycosis is restricted to Latin America, from Mexico to Argentina, with the highest prevalence in Brazil, Colombia, Venezuela, and Argentina (336, 337). Autochthonous human paracoccidioidomycosis has been reported in some countries (e.g., Chile, Guyana, Surinam, French Guyana, Belize, and Nicaragua) (338). Paracoccidioidomycosis can be acquired by the inhalation of environmental infectious propagules, with the lung as the portal of entry, from where the fungus can disseminate as a yeast-like parasitic form (339). Few reports have been

made about the isolation of *Paracoccidioides* spp. in the environment in zones of endemicity. These species have been repeatedly recovered from armadillos (*Dasypus novemcinctus* and *Cabassus centralis*), which are considered natural reservoirs (340, 341). The identification of the environmental habitat of *Paracoccidioides* species is still under study. Although STR markers (333) are available for this species, they were used for phylogeny, and to our knowledge no report deals with a specific clinical question.

Coccidioides Species

Coccidioides immitis and *Coccidioides posadasii* are the two thermo-dimorphic fungi responsible for the animal or human mycosis coccidioidomycosis. Recent studies have evidenced that genetic exchange between these species is possible (342). *C. immitis* is endemic in central and southern California, whereas *C. posadasii* is present mainly in Arizona and extends to Texas and New Mexico (343). *C. posadasii* can be found outside the United States in parts of Mexico and Central and South America (343). The habitat of *Coccidioides* spp. is mainly the warm, arid, and desert regions with annual rainfalls not exceeding 60 cm and with very hot summers (344). The species have been recovered from soil and are frequently associated with rodents and Amerindian burial places in desert zones of the southwest of the United States (345). The major route of infection leading to a chronic pulmonary disease is the inhalation of wind-borne arthroconidia, with subsequent spherule transformation once inside the host. Coccidioidomycosis may disseminate and become fatal in those cases (346).

The first typing approaches to explore the intraspecific relationships of *C. immitis* were performed in the 1990s using RFLP (347, 348) and genealogies of five nuclear genes (349). The authors thus distinguished Californian isolates from non-California isolates (349). Later, Fisher et al. corroborated this clustering using nine STR loci among 167 clinical isolates from all known regions of endemicity, including Venezuela, Mexico, and Brazil (350). The authors formally recognized that the monotypic genus consisted of two closely related species and therefore named the new species *C. posadasii* (350). The same nine-STR scheme was applied to 129 clinical isolates of *Coccidioides* spp. to address the presence of a hypervirulent strain due to an increased rate of coccidioidomycosis in southern Arizona (351). The majority of isolates (92%) were identified as *C. posadasii*. The high level of STR variation among these isolates and the absence of a particular genotype pattern did not support the hypothesis of contamination with a hypervirulent strain as previously suspected (351). In 2014, Luna-Isaac et al. also applied the same nine-STR scheme to establish the predominant *Coccidioides* species in Mexico, to delineate the current geographical locations of both *Coccidioides* species, and to identify a possible correlation between clinical symptoms and a specific genotype (352). One hundred sixty isolates (155 clinical, 4 environmental, and 1 animal) were recovered in Mexico between 1957 and 2010. *C. posadasii* was the predominant species (82%), but no significant correlation of genotypic groups with patients' characteristics was found.

PERSPECTIVES ON NGS

The development of genotyping methods can be time-consuming and demand a minimum of DNA data on the investigated genomes. Moreover, MLST or MLP focuses on the diversity of a restricted number of loci. Consequently, if alleles of those markers are different between two isolates, it is easy to exclude identity between these isolates. In contrast, even with a very discriminant method, there is always the risk of concluding that there is similarity between individuals just because the informative loci are not included in the panel tested, and only whole-genome comparison has the potential for answering this question of identity between two isolates. The current trend is therefore to move to next-generation sequencing (NGS) to pursue genetic analyses at the individual level, which eventually can be achieved at lower costs than to search for new MLST or STR markers and to validate each of them for genotyping. NGS is also the most rapid way to identify SNPs in the genomes of species not already investigated. These new methods are diverse but are currently dominated by two

options for medical applications: (i) PCR amplification of marker sequences with subsequent high-throughput sequencing of the constructed amplicon library and (ii) whole-genome sequencing (WGS) of isolates.

High-throughput sequencing of amplicons provides the possibility to upgrade MLST to a new-generation MLST. This was applied to *C. neoformans* and allowed sequencing of 96 isolates in one run, decreasing costs and time (353). A recent report describes the polymorphism in portions of the mitochondrial large-subunit rRNA gene, ITS2 region, and DHFR gene of the *P. jirovecii* genome using ultradeep pyrosequencing and shows that the polymorphism rate is higher than that previously described using less-sensitive methods (302).

WGS typing allows analysis of the entire collection of polymorphisms within the genomes of each studied isolate. This has been used to understand the diversity of *C. gattii* isolates in the Pacific Northwest. Results of the analysis were comparable to those obtained by MLST (354) but with a greater resolution among isolates, with detection of up to 1,512 SNPs along the genome in isolates harboring identical MLST sequences (354). Comparative genomics of *C. gattii* isolates allowed recognition of four lineages (VGI, VGII, VGIII, and VGIV), with the identification of VGII as the ancestral *C. gattii* population (355, 356).

There are now an increasing number of publications reporting WGS data to understand nosocomial transmission and to investigate outbreaks (357). WGS was applied in a case of organ donor-transmitted coccidioidomycosis. The results showed a high genetic similarity between the three genomes studied with only three SNPs identified among them, suggesting that the organ donor was the source of the three *C. immitis* isolates recovered from the three transplant recipients (358). WGS has been used to confirm genetic identity between environmental and infecting isolates of *C. immitis* in Washington State (359). WGS determined that a single strain of *Exserohilum rostratum* was responsible for the contamination of methylprednisolone batches that caused human infections in the United States (360), with only 8 SNPs detected among the outbreak isolates within a genome of 33.8 Mb. WGS demonstrated the diversity of strains responsible for a tornado-associated cluster of wound mucormycosis due to *Apophysomyces trapeziformis* (361). An outbreak of *Saprochaete clavata* in French leukemic patients with a high mortality rate was investigated using WGS (362). A single clone was responsible for the outbreak (362). A *Mucor circinelloides* var. *circinelloides* outbreak of invasive wound infections in a burn unit was investigated using WGS (363). Analysis of the whole sequences of 23 isolates and 1 reference strain suggested that the patients were infected by different genotypes, supporting that the contaminating source(s), which unfortunately was not identified, would contain several different strains (363). Another *Mucor circinelloides* outbreak, originating from contaminated yogurt products and responsible for digestive symptoms in more than 200 consumers, was reported, and one isolate of this outbreak was analyzed (364). WGS confirmed that the studied isolate was close to the *M. circinelloides* var. *circinelloides* subgroup, which is more often involved in human diseases than the other *M. circinelloides* subgroups (364).

WGS has also been used to investigate azole resistance not due to the *cyp51A* mutations, leading to the identification of the putatively involved P88L mutation in the *hapE* gene (365). For the azole-resistant TR_{34}/L98H *A. fumigatus* isolates, WGS was used to determine their genetic diversity. WGS revealed that country and continental genetic diversities were of a similar scale, with the exception of India, where highly related genotypes were observed. This study confirmed that TR_{34}/L98H isolates recombined and that the TR_{34}/L98H allele was able to segregate in various genetic backgrounds (366).

WGS was used for investigating serial clinical isolates of *C. neoformans* from a patient with AIDS with relapsing episodes of meningoencephalitis. These isolates presented phenotypic differences, despite having highly conserved genomes. WGS highlighted in the second isolate the loss of a predicted transcriptional regulator gene involved in melanin and capsule formation, carbon source use, and dissemination in the host,

which was consistent with the modifications of the phenotype between the two isolates (367).

CONCLUSION

The genotyping of medically important fungi is currently done almost exclusively using MLP and MLST approaches. These methods are technically robust (15), with differences between them (Table 2), and several schemes are available for various fungal species (Table 1). The choice between these methods depends on the available equipment, the skill of each team, the species being investigated, and the clinical question. When investigating a disease outbreak, it can be sufficient to know that two isolates are different, thereby excluding cross-contamination or a common source, whereas multiple loci should be investigated when aiming to prove that two isolates have the same genotype. For molds, MLP is more discriminant than MLST and is now preferred for epidemiological studies. For yeasts, MLP seems, at present, to be the most robust genotyping method to discriminate isolates in cases of outbreak or nosocomial infection, whereas MLST is more effective for the determination of long-term genetic relatedness or population structure (24, 211, 213). Moreover, data banks are available for MLST but not for STR markers (Table 1).

Further typing studies could clarify the correlation between strain genotypes and geographical location and body site of isolation, colonization, or infection. The collection and identification of environmental isolates from patients' surroundings will be useful for exploration of infection sources. In this context, the use of NGS technologies has great potential for the investigation of new species and genetic variability among isolates. WGS and comparison of isolates of rare fungal organisms are nowadays an easy and quick method when adequate bioinformatics pipelines are available and validated. However, for rare and usually poorly known fungal organisms, the analysis of genomes of isolates involved in an outbreak still requires knowledge about the plasticity of the genomes and the rate of mutation acquisition in the environment and upon interaction with host in noninvasive or invasive infections.

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