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Towards the Genotyping of Fungi: Methods, Benefits and Challenges (review)

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Abstract

The accessibility of comprehensive fungal genomes is fast increasing, providing a thorough and precise perspective on this "kingdom." A significant scientific achievement has been attained with the open access to over 1000 fungal genomes from various species, accompanied by the launch of new and engaging projects. The “1000 Fungal Genomes Project” constitutes one of the most enormous sequencing initiatives centered on fungal organisms, aiming to tackle deficiencies in fungal genomics. Currently, 329 fungal families possess at least one sequenced representative genome; nonetheless, other fungal families remain without any sequenced genomes. Furthermore, supplementary sequencing research contributed to the comprehension of genetic diversity among certain fungal species. The existence of many genomes per species aids in taxonomic classification, affords new views on fungal evolution in quick timeframes, clarifies regional distribution and dispersal patterns, and elucidates outbreaks and transmission routes, among other objectives. Genotyping strategies analyse solely a limited phase of an individual's genome and alternatively facilitate the rapid and inexpensive comparison of numerous isolates. The incorporation of comprehensive genomic approaches and enhanced genotyping panels focused on specific and pertinent single-nucleotide polymorphisms and/or repetitive areas can serve as efficient and pragmatic methods for investigating the local, regional, and global epidemiology of fungus.

Keywords: Filamentous, fungi, Genetic, transformation, Protoplast, Agrobacterium.

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Introduction

Fungal pathogens are responsible for a broad spectrum of disorders, from minor, superficial infections to serious, life-threatening infections. Studies have demonstrated the considerable growth in the medical significance of fungus, especially when immunocompromised patients are involved (1,2). There have been reports linking a few fungal infections to possible epidemics. Among the crucial fungi for medicine Candida, Cryptococcus, and other filamentous fungi like Aspergillus, Scedosporium, Penicillium, and Fusarium have all been linked to serious fungal infections (2). Despite the use of antifungal medications, many of these fungal infections had dismal outcomes. Preventative strategies and other early interventions are therefore necessary to enhance results. Precise species identification and genotyping are essential for epidemiological research aimed at comprehending the evolution and path of these organisms in medical settings (3).

Almost all environments in the kingdom are home to species of fungi. Fungi are multicellular and unicellular organisms with a variety of morphologies that can live freely and in symbiosis (4). Fungi can also develop in a range of habitats, infiltrate plant and animal cells, and take part in the cycling of vitamins in each terrestrial and aquatic ecosystem due to the fact of the genes encoded in their genomes. Both large-scale and individual efforts, like Génolevures, have produced the ever-growing databases of fungal genome sequences. (5). The Broad Institute's Fungal Genome Initiative and the 1000 fungal genomes project (http://1000.fungalgenomes.org). The result of these studies is a catalogue of the genomic structure and genes of fungi. By using the genomic databases, one can gain a deeper understanding of the ways in which fungi have adapted to their ecological niches and lifestyles. Large quantities of fungal transcriptomic and genomic data have made it easier to apply contemporary methods and improved the assessment of fungal evolution from the perspective of molecular sequences (6). Genome sequencing confirms that, in addition to microscopes, petri dishes, and field research, bioinformatics and comparative genomics are crucial instruments for studying fungal biology and evolution (4).

Typing refers to the investigation of isolates, either phenotypically or genetically, below the species level. This analysis is conducted to provide strain or clone-specific fingerprints or databases (7). Typing procedures might rely on phenotypic traits or involve analyzing changes in DNA sequences (genotyping). The utilization of phenotype-based approaches is diminishing due to their technical intricacy, limited repeatability, and/or inadequate Discriminatory capability. Importantly, a phenotype may not consistently reflect the genotype of a microbe, thus making it an unreliable and unstable epidemiological marker (8). The review aims to highlight their efficiency, limitations, and future applications in fungal biotechnology and industrial processes.

2- Fungal Genotyping

Fungal genotyping refers to the genetic examination of fungi, specifically below the species level, to identify strain-specific characteristics. Dactylograms. Genotyping generates molecular markers that facilitate the comparison of strains and the examination of their genetic relationships. In recent years, diverse molecular approaches have been utilized to classify different types of fungal infections (4). Diverse methodologies utilized for fungal genotyping embody multilocus enzyme electrophoresis (MLEE), random amplification of polymorphic DNA (RAPD), pulse discipline gel electrophoresis (PFGE), restriction fragment size polymorphism (RFLP) analysis, and amplified fragment size polymorphism (AFLP). Fragment size polymorphism (AFLP) analysis, variable range of tandem repeats (VNTR), and DNA sequence-based methodologies are frequently employed procedures. Multilocus sequence typing (MLST) (3,7).

Scientific mycology uses genotyping techniques to investigate fungal disease transmissibility and population dynamics. In settings that are both therapeutic and environmental. Numerous fungal infections that take advantage of opportunities are widely distributed in nature and have the potential to represent a considerable danger to vulnerable people. Thus, by utilizing data obtained from genotyping techniques, it is possible to analyze the connection between isolates from the environment and patients. This study facilitates the identification of the infection source inside the environment. In the event of a suspected outbreak of fungal diseases, it is crucial to employ an accurate genotyping method to verify or dismiss the epidemic status. Additionally, the examination of genotyping data makes it easier to determine which index patient is responsible for the epidemic and to identify patient-to-patient transmission. Exercise is essential in outbreak management, since the timely and efficient implementation of infection control measures is obligatory (1,2).

Genotyping facilitates a deeper comprehension of the behavior of a fungal infection inside a specific patient host. Several instances of isolations An organism can be present in a single host patient during a specific period of time. Often, the organism might exist either temporarily as transient colonization or for a longer period as persistent colonization, which can finally lead to a true invasive infection. Genotyping allows for the distinction of possible infection for these strains. Furthermore, there are contributing elements that lead to ongoing persistence. The assessment of colonization and infection is possible (1).

Fungal genotyping techniques vary in their capacity to distinguish between distinct fungal strains, which in turn affects their usefulness in distinguishing between different types of fungi. There are two strains. Methods utilizing gels, such as PFGE, RAPD, and AFLP, have been commonly employed.

While these methods provide a considerable level of selective capability, their interpretations and analyses are typically challenging and susceptible to individual variations, which are influenced by the operator.

VNTR and MLST, in contrast, offer simpler interpretation since they produce data that is unambiguous, allowing for reliable comparison between different laboratories. Nevertheless, these methods typically incur higher expenses compared to the previous methods (1,2). Hence, every medical laboratory must assess its requirements and capacities before selecting the most suitable genotyping techniques for clinical application (1).

Fungal classification is significantly challenging due to the restricted morphological traits and the intricate evolutionary level of fungi. Classifying fungal species and determining their relatives based only on exterior morphology, internal structure, and physiological and biochemical indications of fungus is a challenging task. Significant advancements in fungal categorization have been made through the utilization of molecular biology, bioinformatics, genomics, and comparative genomics technologies. Lifeasible can integrate gene sequencing and bioinformatics technology to offer genotyping services specifically for plant and crop fungus (Figure 1) (9).

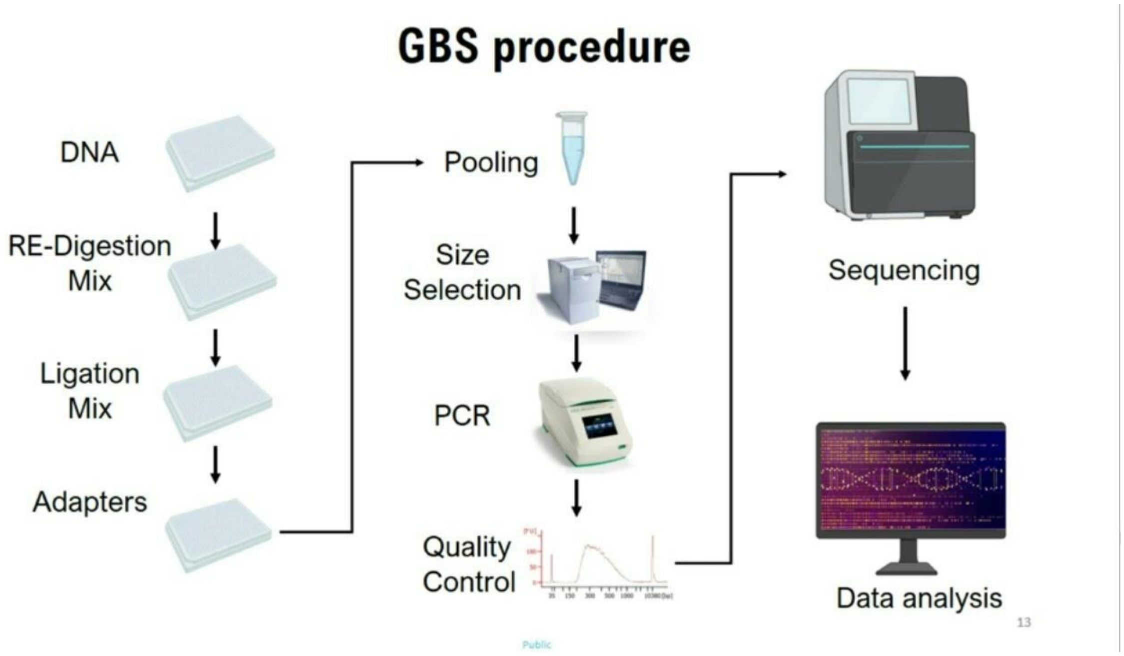

Figure 1. Genotype by sequencing (GBS) protocol for fungal genomes (10)

3- Identifying sequence variation

Though there are small variances among individuals within a species, each biological species is identified by a unique set of common features. Within the human species, physical characteristics like hair or eye color are easily distinguishable between individuals. Certain individual differences also exist in other species, even in those that are thought to be non-living, like viruses, but they might be more modest. Those members of a species that do differ significantly from one another, but not enough to be considered a separate species, are known as variations. The word variation refers to the trait that varies among individuals in a population or within a single species. One common fungal infection, for instance, is resistant to particular populations of soybean plants (9). Variation denotes the characteristic that differentiates individuals within a group or a specific species. Certain populations of soybean plants demonstrate resistance to the common fungal infection, Phytophthora root rot, whereas other populations are susceptible to the same pathogen. Fungal resistance might be characterized as a type of diversity (9). The population of plants resistant to phytophthora root rot possesses a unique characteristic that allows them to protect themselves from the fungus. If the ability to withstand fungal infections is inherited by future generations of plants, this characteristic is probably encoded in the plant's genome. Both environmental and genetic factors contribute to the development of these observable or phenotypic characteristics. Given that environmental alterations are not passed down between generations, the focus of most academics lies in investigating genetic variances that lead to these physical abnormalities. Genetic diversity can be inherited by the following generation and has the potential to either enhance or diminish the adaptability of a particular species (9).

4- Fungal Reproductive Mechanisms and the Creation of Genetic Variation

Fungi can exhibit sexual reproduction, asexual reproduction, or a combination of both in their natural environment. Approximately 20% of fungus have traditionally been believed to be asexual or clonal. However, fresh evidence has emerged to support the notion that even these fungi may possess sexual reproduction capabilities. Species have the potential to undergo recombination (11,12). Authors suggest that really asexual organisms are rare in nature, with only a limited number of rotifers identified as entirely asexual (13). Certain fungal species exhibit distinct sexual and asexual cycles, such as *Aspergillus nidulans*, whereas others have infrequent or nonexistent sexual reproduction, as seen in *Penicillium marneffei* (14). Asexuality is a common occurrence among fungi due to its energy efficiency, it does not contribute to long-term reproductive success. The limited number of offspring resulting from sexual reproduction, the continual requirement to find a mate (with limited opportunities for genetic recombination), and the potential conflicts between individuals with diverse genetic backgrounds are sufficient reasons for microbial eukaryotes to engage in asexual reproduction (15).

However, engaging in sexual reproduction can also provide significant benefits by expediting the process of adapting to challenging surroundings and eliminating harmful traits. Adverse mutations can threaten a species' survival; nevertheless, diploid states substantially alleviate the effects of deleterious mutations, enabling the persistence of organisms. Fungi exhibit two reproductive strategies: heterothallism, requiring two compatible partners for mating, and homothallism, permitting self-fertilization by a solitary organism. Yeast and molds have unique pheromones and receptors that allow them to identify and distinguish between different mating kinds. Recombination and chromosomal rearrangements frequently occur under stress, resulting in the formation of opportunistic infections such as *Candida albicans* (11,16). The sexual reproductive mode has lately been validated in numerous Aspergillus and Penicillium species, imparting new insights into fungi that had been earlier labeled as asexual for many years. Uncertainties persist regarding the influence of sexual reproduction on the development of strains exhibiting reduced antifungal susceptibility and/or heightened pathogenicity; yet, the variety generated by this process is substantial and necessitates quantification (17).

5- Molecular genotyping methods

Historically, the traits of bacteria and fungi were determined through the morphology of their colonies on particular medium, biochemical assays, serological methods, susceptibility to bacteriocins, and resistance typing (18). Nonetheless, these systems exhibited insufficient consistency, were sometimes excessively complicated, and, crucially, possessed inadequate differentiation capabilities. A variety of typing methodologies, including Amplified Fragment Length Polymorphism (AFLP), Multilocus Sequence Typing (MLST), Pulsed-Field Gel Electrophoresis (PFGE), Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Retrotransposon Insertion-Site Context (RISC), PCR-Restriction Fragment Length Polymorphism (PCR-RFLP), Repetitive-Sequence-Based polymerase chain reaction (rep-PCR), Sequence-Specific DNA Primer (SSDP), and Variable Number of Tandem Repeats (VNTR) typing, have been employed for fungal genotyping (19).

5.1 DNA-fingerprinting tools

Facilitated the comparative analysis of the strains' genomes. The foundation of fingerprinting techniques is the idea that information about the strains' whole genomes may be inferred from examinations of specific genomic regions, known as genetic markers. This occurs because clonal organisms exhibit low or no chromosomal recombination and meiosis. Chromosomes are passed from the progenitor organism to its offspring unchanged, with genetic alterations, including point mutations and the insertion or deletion of DNA segments, serving as the primary source of variation. As the modifications are passed on to succeeding generations as a unit, comparing a sufficient number of genetic markers enables the detection of genetic relatedness and facilitates the creation of dendrograms (Figure 2) (19). However, in the case of a sexually reproducing organism, the exchange of genetic markers between chromosomes makes it impossible to determine genetic distances by comparing only a limited number of genetic markers. (Figure 3) (19).

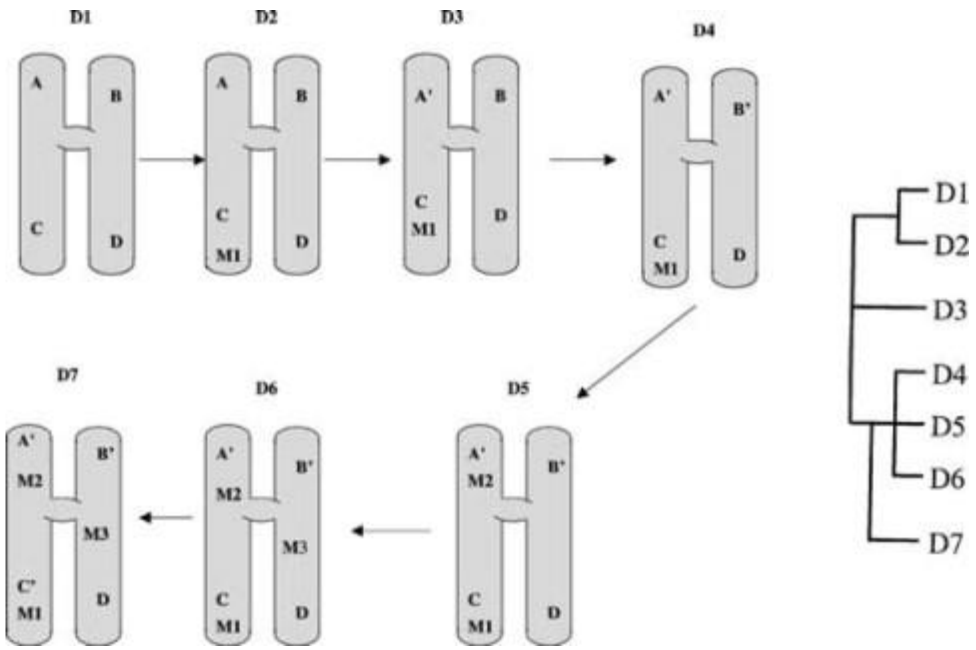

Figure 2: Demonstrates the evolution of genetic material in an organism that undergoes asexual reproduction. To ensure clarity, only one chromosome is illustrated. D1–D7 illustrate strains that have been systematically cultivated. A, B, C, and D exemplify polymorphism genetic markers. M1-M3: Mutations not detected by the chosen genetic markers. Genetic relatedness can be assessed by analyzing the quantity of shared genetic markers among different strains. This data is utilised to create the dendrogram illustrated in the picture. D1 and D2 share the same set of markers: A, B, C, and D. Consequently, they are deemed closely connected and categorized under a cluster. D4, D5, and D6 possess identical A, B′, C, and D, and are arranged in a cluster. D3 exhibits shared components B, C, and D with D1 and D2, as well as A′, C, and D with D4–D6, signifying a moderate degree of association with both clusters. D7 shares shared elements A′, B′, and D with the D4-D7 cluster, suggesting a modest degree of relatedness. Nonetheless, D7 is less strongly associated with D1 and D2 (19).

In order for this approach to be sufficiently precise, the selected genetic markers must meet many criteria. Initially, they should undergo mutation at a moderate frequency. If the mutation frequency is too low, it becomes difficult to distinguish variations among different strains. Conversely, if the frequency is excessively high, the majority of isolates will exhibit significant differences, making it impossible to form clusters. Furthermore, they must not be influenced by evolutionary pressure, as this would introduce a bias in genetic relatedness. Furthermore, the likelihood of a mutation reverting in these organisms should be extremely minimal (for instance, a transposable element would serve as an erroneous indicator)

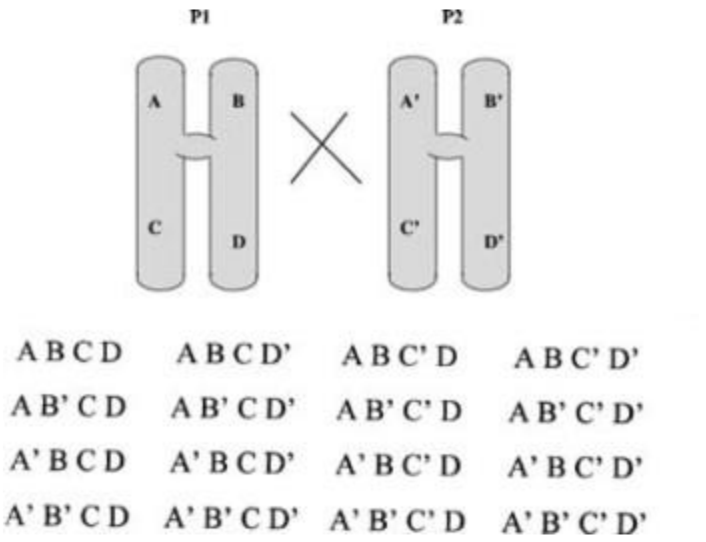

Figure 3: Exhibits the modifications in the genetic material of a sexually reproducing organism over time. To provide clarity, only one chromosome from each parental strain is depicted. A, B, C, and D serve as exemplars of polymorphic genetic markers. Post-recombination, 16 potential combinations of the genetic markers emerge. All these pairings demonstrate equivalent genetic relatedness in the initial generation, making the creation of a dendrogram impractical (19).

Ultimately, the likelihood of the identical mutation arising in two separate isolates should be exceedingly minimal to prevent the occurrence of homoplasy, which refers to shared traits that lack a shared ancestor (19). Multiple criteria have been suggested for evaluating the ability of a fingerprinting approach to accurately determine genetic relatedness. The authors suggest that an effective technique should be able to withstand changes in the environment and frequent rearrangements in the genome. Techniques that rely on minichromosomal or DNA sequences that undergo frequent and potentially irreversible rearrangements will not be considered valid. Furthermore, the created fingerprints must remain consistent over time. Therefore, the selected sequences must have minimal or no recombination. Furthermore, the approach must be able to withstand homoplasy. Moreover, it is essential that the data produced is capable of being replicated; the identical strains should provide consistent outcomes in all instances examined, both within the same research facility and across several laboratories and time periods. Ultimately, a genetic fingerprinting approach must possess the ability to evaluate genetic relatedness with the required level of precision to address the given inquiries. The approach should have the capability to distinguish between unrelated strains, potentially related strains, strains that are likely to have undergone microevolutionary changes, and strains that are highly likely to have a recent common ancestor based on their molecular type (18).

By employing this approach, it is possible to calculate a similarity coefficient for every pair of isolates, which may then be utilized to create dendrograms. The effectiveness of the strategy in grouping the lines at different phases of relatedness requires validation by an assessment with an unrelated method. Considering this problem is essential when evaluating the epidemiology of different yeasts that experience microevolution or "substrain shuffling." There may be slight variations in banding patterns across epidemiologically related isolates in this situation when analysed using different fingerprinting methods. This raises a question regarding the degree of divergence in fingerprint patterns required for two isolates to be securely classified as distinct strains. In order to facilitate retrospective analysis and comparisons across several laboratories, the statistics produced by this method should be appropriate for computer-assisted assessment and archiving (19). The techniques must be efficient, economically viable, and compatible with the technological capabilities of the majority of medical microbiological laboratories.

5.2 Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is a technique utilized to distinguish species by analyzing the patterns produced from the enzymatic breakdown of their deoxyribonucleic acid (DNA) using restriction enzymes. If two species exhibit variation in the spatial distribution of cleavage sites due to a specific endonuclease, the resulting deoxyribonucleic acid (DNA) fragments will vary in size after digestion with that enzyme. The variations in the produced patterns can be employed to distinguish species and subtypes. The process begins with the extraction and purification of DNA from tissue. Subsequently, a particular gene sequence, such as mitochondrial 12S ribosomal RNA (mt12S rRNA), is amplified via polymerase chain reaction (PCR). Thereafter, the amplicon undergoes digestion with one or more restriction endonucleases, and the ensuing fragments are separated with the aid of agarose gel electrophoresis (AGE) and visualized with ethidium bromide (EB). Species identification with PCR-RFLP basically relies on the desire of the goal sequence, the utility of limit endonucleases, and the dimensions of the resultant fragment. PCR-RFLP is regularly employed through researchers to decide meat species. However, it may be inappropriate for evaluating foods that have undergone detrimental DNA processing. Enzymatic restriction requires substantial DNA fragments, whereas thermal breakdown of DNA may impede the amplification process (Wang et al. 2010). Restriction fragment length polymorphism (RFLP) is a technique utilized to distinguish species by analyzing the patterns produced from the enzymatic breakdown of their DNA using restriction enzymes. If two species exhibit variation in the spatial distribution of cleavage sites due to a specific endonuclease, the resulting DNA fragments will vary in size after digestion with that enzyme. T-RFLP can affect the quantification of endophytes because of the differing amount of fragments generated. Thus, it is more advantageous as it requires only one or two specific primers.

This method provides advantages of speed, simplicity, and cost-efficiency. Nonetheless, RFLP exhibits certain methodological limitations. The intricacy of the fungal genome obscures the clarity of bands denoting distinct sequences because of the presence of several bands. Furthermore, similar to all eukaryotic genomes, fungal genomes possess repetitive rRNA genes arranged in cistrons. These genes exhibit very similar sequences and are separated by intergenic regions. Consequently, rDNA sequences, along with mitochondrial DNA sequences to a lesser degree, constitute the predominant strong bands in an RFLP pattern. However, these fragments lack sufficient information to determine the similarity of closely related isolates (20).

5.3 Random Amplified Polymorphic DNA (RAPD)

Randomly primed PCR analysis uses a single, randomly selected primer with roughly ten nucleotides. It is technically simple, carefully constructed, and often detects differences between isolates that do not change in RFLP analysis, even when Southern blot hybridization is present (17). Although a single primer frequently yields one to three different bands that may also differ among isolates, it can also provide a complex sample that differs among isolates. In these situations, it is crucial to use a large number of primers, evaluate each one independently, and then combine the findings (17). Using a single primer and polymerase chain reaction (PCR), a Random Amplified Polymorphic DNA (RAPD) evaluation is a molecular marker method that can yield a complicated profile that differs between isolates. It shows that the amplified band samples, which are also known as Mendelian genetic markers, vary among genetically varied individuals. Under specific PCR conditions, the method uses a single 10-mer primer to allow for random amplification. The distribution and size of annealing sites throughout the genome determine the quantity of amplified fragments (21). Only when primers attach to certain sites on both strands that are no more than three to four kilobases apart does amplification take place. On an agarose gel, the PCR product is easily visible, and the band pattern that results is the DNA fingerprint. By changing the base composition of the used short DNA segment, RAPD provides a more stochastic depiction of the genome than other genetic markers and can detect an infinite number of distinct loci in the DNA sequence. The dominant expression of alleles, which makes evaluating multilocus patterns more difficult, is the main drawback of RAPD markers. Some have expressed concerns about the accuracy of amplification. Taxonomic identification, hybridization, reproductive behaviour, and population genetic structure studies have all made extensive use of RAPDs. This method has been used to track genes that are very important financially. Amplification of unknown DNA sequences with short, random oligonucleotide primers produces RAPD markers. This technique no longer depends on knowing the DNA sequence beforehand (Figure 4) (22).

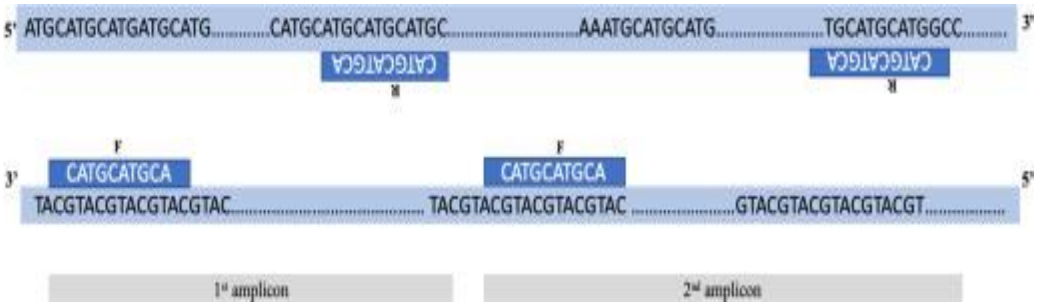

Figure 4. Random Amplified Polymorphic DNA (RAPD) analysis: A single primer functions as both forward (F) and reverse (R), binding to two sites on the target double-stranded DNA (dsDNA) sequence, resulting in amplicons of varying lengths. Amplicon 1 and amplicon 2 differ in both base pair quantity and sequence composition. The number of amplicons generated by RAPD for each sample is contingent upon the specific DNA sequence targeted (22).

5.4 Analysis of Single and Multiple Gene Sequencing

To differentiate strains, gene sequencing analysis is useful. Although internal transcriber spacer regions 1 and 2 are commonly used for fungal identification, there are a number of differences in these regions that can help differentiate between different strains of the fungus. Alternative genomic regions may be sporadically employed; however, the importance of a singular gene is limited, leading to reduced discriminatory capacity. Consequently, multilocus sequence analysis (MLSA) and multilocus (23). MLSA and MLST are endorsed as efficient solutions for the genotyping of fungal species. These techniques, grounded in genomic research of several genes, serve as robust instruments for examining population structure and molecular epidemiology. MLST is a prevalent approach that yields reliable and unambiguous data, facilitating the global interchange of information among laboratories via a common database (http://www.mlst.net/) (12,23). Genetic variety metrics have been assessed by using Multi-locus Sequence Typing (MLST) evaluation in *C. albicans*, different Candida species, and A. fumigatus. The acquired genotyping profiles.

The MLST databases, accessible online, embody international studies. Currently, there are 2,217 profiles for *C. albicans*, 152 profiles for *C. krusei*, 389 profiles for *C. tropicalis*, and forty-three profiles for *A. fumigatus*. The software of MLSA (Multi-Locus Sequence Analysis) and MLST (Multi-Locus Sequence Typing) strategies can be luxurious and labor-intensive when assessing massive collections of medical and/or environmental isolates. Alternative methodologies using single nucleotide polymorphisms (SNPs) have been proposed to enhance approaches concentrated on many genes with applicable facts (24,23).

5.5 Single Nucleotide Polymorphisms (SNPs)

The SNaPshot mini-sequencing test is a rapid, sensitive, and reliable method employing extension primers in conjunction with fluorescently labeled dideoxynucleotides (25). Targeting small PCR products (even partially broken DNA), removing stutter products, multiplexing flexibility, and adding or changing markers are some of the features this test offers. The restricted quantity of markers and alleles produced by these tests may constrain specific genomic investigations. Proposed SNP assays with restrictions have been suggested for fungal genotyping. The diversity of Pandora neoaphidis was assessed using 13 SNPs across six genomic areas, resulting in the identification of 15 profiles among 23 isolates, thereby creating an effective genotyping methodology (12). A take a look at consisting of a hundred SNPs was once developed for *Cryptococcus gattii*, meant to characterize molecular subtypes VGIIa, VGIIb, and VGIIc, and to analyse genetic range (29) To detect, identify, and genotype Aspergillus fumigatus in clinical specimens, the single nucleotide polymorphism multiplex assay (SNaPAfu assay) uses a panel of 20 SNPs found in MLST genes (26). Twenty-seven SNP markers have been suggested for the identification and genotyping of Podosphaera plantaginis, highlighting that co-infections significantly influence pathogen evolution. Recent PCR melting analyses conducted in yeast have yielded significant discriminatory results (26). However, these methods offer an indirect assessment of the sequencing data, rendering it more prone to errors. Single nucleotide polymorphisms (SNPs) serve as valuable markers for assessing recombination, linkage, and aneuploidy, with their significance recently confirmed in *Saccharomyces cerevisiae* (12).

5.6 Microsatellite or Short Tandem Repeat (STR) Analyses

Because of its high discriminatory strength (up to 0.995 in positive panels), convenience of data processing, affordability, and laboratory reliability, microsatellite typing is frequently considered the best technique for studying local epidemiology (27). Tri-nucleotide, tetra-nucleotide, and pentanucleotide sequences are optimal for reducing typing ambiguities associated with dinucleotide repeat motifs, which are particularly susceptible to DNA polymerase slippage during amplification (27). Because it is easy to include markers in multiplex systems, microsatellite analyses can be both efficient and economical (28). The observed inconsistency in microsatellite results from various laboratories, attributable to sequencing instrument configurations, can be effectively mitigated and rectified by the use of allelic ladders (12). However, a comprehensive database on relevant fungi is lacking. Microsatellites' reduced capacity to identify recombination within populations presents a subsequent challenge. Numerous microsatellite panels, such as those for *Candida albicans*, *Candida parapsilosis*, *Candida glabrata*, *Candida fumigatus*, *Candida flavus*, *Candida niger*, and *Candida terreus*, and a number of additional environmental moulds, have been characterised for the most clinically important fungus (29). Microsatellites can occasionally aid in the identification of closely related species (Figure 5) (29).

5.7 Multilocus enzyme electrophoresis (MLEE)

Multilocus enzyme electrophoresis (MLEE) was among the initial techniques employed for genetic and epidemiological research, utilizing protein (enzyme) polymorphism and gel electrophoresis rather than DNA. Different mobility banding patterns could also be a sign of changes in the amino acid sequence. However, a number of nucleotide substitutions did not cause changes to amino acids (silent mutations). Consequently, MLEE exhibits little capacity to account for microevolutionary changes (30). Even though it consistently distinguishes between various residues of *C. albicans* and *Cryptococcus sp*. Limitation fragment length polymorphism (RFLP), which uses restriction enzymes to digest DNA and analyses the resultant fragments based on their varying lengths assessed by gel electrophoresis, was the first method for DNA profiling. The variations in size originate from mutations or deletions of restricted site sequences, as well as DNA deletions and insertions occurring between the recognition sites (30). This method is simple, quick, and cost-effective, demonstrating favorable discriminatory findings for local epidemiology; yet, it suffers from a lack of reproducibility (12). Consequently, this technology is now regarded as obsolete for genotyping.

5.8 Multilocus sequence typing (MLST)

The initial application of MLST in fungus was for species identification. Conventional techniques for identifying fungal species, mostly through phenotypic assessment or mating experiments when feasible, are being replaced by phylogenetic approaches that utilize nucleotide sequences from several gene lineages. Identifying species as clades of genetically separate individuals in nature has revealed novel, cryptic species within *Candida albicans* (31).

Although MLST is founded on the ideas of MLEE, alleles are found using nucleotide sequencing as opposed to examining the related enzymes' electrophoretic mobilities. An allele count is assigned to each locus after internal sections of around seven housekeeping genes, spanning 450–500 base pairs, are sequenced. The combination of this data results in a conclusive generic allelic profile or sequence type that is accessible via open databases (e.g., http://pubmlst.org) (32). MLST has obtained significance by using without delay sampling the full-size variety of nucleotide sequences, enabling every new discovery to make use of and increase all previously gathered data. Consequently, the dataset for every species is always up to date and handy to all fascinated events by means of an online platform. MLST has supplanted antiquated strategies that dim variation, such as MLEE (multilocus enzyme electrophoresis), as well as these necessitating an escalating variety of comparisons amongst installed genotypes with every new addition, like DNA–DNA hybridisation, and techniques inclined to minor fluctuations in laboratory conditions, such as electrophoretic karyotyping or randomly amplified polymorphic DNA (RAPDs) (31). MLST offers an advantage over single nucleotide polymorphism (SNP) analysis by identifying and integrating unique polymorphic nucleotide locations within any gene fragment sequence into the database. This feature allows for the incorporation of individuals from diverse geographic regions into the study without the risk of polymorphic changes detected in the initial sample becoming monomorphic in the newly included populations, as can occur with SNPs (31).

5.9 Variable number tandem repeat (VNTR) typing

Multi-Locus sequence typing (MLST) has gained importance by promptly sampling a comprehensive array of nucleotide sequences, allowing each new study to utilize and expand upon every piece of previously gathered information. Consequently, each species' dataset is continuously up to date and available to all interested parties through an internet platform. MLST has taken the place of antiquated techniques like MLEE (multilocus enzyme electrophoresis) that mask variation, as well as those requiring an increasing number of comparisons among established genotypes with each new addition, like DNA–DNA hybridization, and techniques susceptible to minor fluctuations in laboratory conditions, such as electrophoretic karyotyping or randomly amplified polymorphic DNA (RAPDs). This allows for the extraction of VNTR blocks via restriction enzymes, analysis of restriction fragment length polymorphism, amplification via polymerase chain reaction (PCR), and size comparison by gel electrophoresis. The markers' adaptation and utility in genotyping and diversification studies were assessed in the laboratory, demonstrating significant advantages in their adaptability. They are readily measurable and may be analyzed utilizing agarose gels, rendering them a valuable asset, particularly for less sophisticated laboratories (33,32).

5.10 Repetitive-sequence-based PCR (rep-PCR)

Numerous interconnected repetitive sequences that are present in intergenic areas across the genome make up bacterial chromosomes. Oligonucleotide probes use noncoding, repeating sequence blocks as genetic targets to create unique DNA profiles or fingerprints for particular bacterial strains. DNA fingerprinting is the process of separating different-sized DNA fragments from chromosomal or plasmid DNA using restriction endonuclease digestion and/or DNA amplification to produce a band profile that acts as a distinct identification. Without evaluating gene expression or enzyme activity, these distinct "bar codes" or DNA fingerprints identify every bacterial chromosome. The effectiveness of genotypic or molecular methods in classifying bacterial species or strains into discrete gender-based groups varies (34).

5.11 Pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) is a band-based molecular subtyping technique that excludes PCR and instead employs macro restriction of whole bacterial DNA (including chromosomal and plasmid) utilizing a rare-cutting restriction enzyme. Large DNA fragments generated during restriction digestion are prone to mechanical shearing; hence, to avert DNA fragmentation, entire bacterial cells are encapsulated within an agarose plug. After all the cells are embedded, they undergo lysis using detergents or enzymes and are thoroughly washed to eliminate any residual cellular or chemical impurities. The agarose plug containing genomic DNA is subsequently digested with a restriction enzyme classified as a 'rare cutter.' After digestion, a segment of the digested plug is introduced into an agarose gel, similar to PCR; however, it is then separated using a specialized electrophoresis device known as the contour-clamped homogeneous electric field electrophoresis system. This technique employs 24 electrodes uniformly distributed in a hexagonal configuration, in contrast to post-PCR gel electrophoresis. This electrophoresis technique generates uniform and homogenous electric fields across the gel by continuously switching current at 120° angles, facilitating the even migration of long DNA segments through the gel and preventing distorted bands at the bottom of the lanes (8, 35).

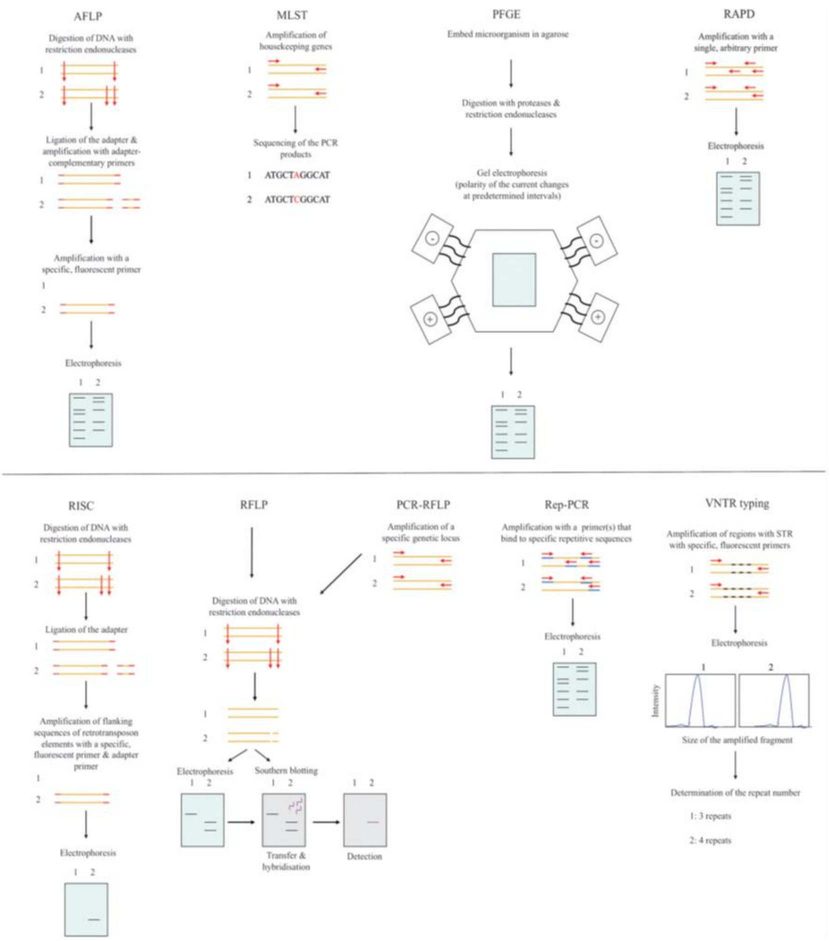

Figure 5: Summary of the many stages involved in commonly employed genotyping techniques for fungus (8 and 35)

6. Advantages of Typing Systems for Clinical Studies

Typing technologies can ascertain the genetic identification of clinical isolates, facilitating the genetic characterization and differentiation of strains. This comprehension is especially vital during clinical outbreaks due to the transfer of strains across individuals (12). Genotyping technologies facilitate the identification of identical fungal footprints across several anatomical sites or the evaluation of whether patients have distinct, unrelated strains. Genotyping-based mycological range and identification encounter significant global hurdles, including the lack of efficient, cost-effective, and rapid identification methods, accessible molecular data, and the ability to trace the origins and biogeography of strains. In challenging circumstances, many molecular techniques, including RAPD, MLSA, MLSD, SNP, CGH, and MALDI-TOF MS, can be employed for strain-level differentiation. SSR markers are advantageous due to their high repeatability, superior discriminative capacity, compatibility with multiplexing, and relative cost-effectiveness for screening many samples (36,37). The same strain of *Candida albicans* may cause recurrent infections throughout several episodes, or distinct strains may be responsible. It is possible to use yeast genetic information to identify the infection's origin and confirm that antifungal treatment techniques are working for patients (12). Patients can simultaneously experience many manifestations of the same fungus species. The implications of this presence and co-evolution are not yet fully understood; nonetheless, they may contribute to the creation of more virulent or drug-resistant forms. Furthermore, the simultaneous presence of numerous lines can obscure the recognition of perilous lines amid less hazardous variants. Typing approaches have been employed to examine fungal evolution in individuals with cystic fibrosis (CF) and in patients without CF who are persistently colonized by molds (38).

7. Current Challenges for Fungal Genetic Diversity

The volume of data generated by these emerging devices is substantial, and it is indisputable that sophisticated and inventive bioinformatics tools are required. Thus, there is a pressing need for comprehensive and centralized databases that are user-friendly for storing, retrieving, and exchanging data, facilitating collaboration among researchers. Single researchers or groups working with large-scale genomic data may face challenges due to the necessity for shared databases. This is particularly important for association and population genetics studies, since it allows for the depositing and amplification of the untapped potential of such data among the scientific community. The success of MLST in microbiology can be partially attributed to the convenience of depositing and sharing data among laboratories (12). However, it is important not to overlook the data and tools that were previously accessible. The exchange and sharing of genetic information among disparate databases should be enhanced. MLST@SNaP is a newly developed software that enables the translation of MLST sequences to SNP data and vice versa (42). These tools are essential for optimizing technology application, addressing global challenges, and fostering collaboration among researchers and diverse genetic platforms (12).

It is crucial to comprehend the interplay of microorganisms, whether they belong to the same species or not, as well as their communities and the transmission of microbes that have heightened pathogenicity or resistance to antifungal treatments. Research conducted in controlled clinical settings is essential for the development of strategies aimed at enhancing the health and well-being of patients, particularly those undergoing immunosuppressive therapies and extended hospital admissions. Continuous efforts should be made to produce and provide clinical controls, recommendations, and guidelines for public access. These publications ought to emphasise community protection and prevention while offering particular details about medical services (39,40).

More practical and cost-effective. The democratization of advanced sequencing technologies may significantly transform molecular diagnostics and alter our understanding of microbial evolution. These applied sciences will provide the concurrent detection, identification, and molecular characterization of complete populations within biological materials. This reality facilitates a fuller understanding of our anatomies and the habitats we occupy, and has the potential to reveal significant evolutionary dynamics of microbial partnerships. Exciting prospects exist in the field of molecular mycology in the near future (12, 41). With the use of second- and third-generation large-scale sequencing analysis in molecular mycology, a new era is dawning (Table 1) (12).

Table 1 Challenges of molecular mycology (12).

Challenges of fungal typing for next years
- A comprehensive method for recording the diversity of microbes, Relationships among gene characterization, genomic evolution, pathogen biology, epidemiology, and structural analysis - Real-time monitoring makes it possible to assess the efficacy of antifungal treatments and determine the best time to administer azole antifungals. - Creation of harmful or antibiotic-resistant variations determining the origins of the fungal agents, including a description of endemic, exotic, and/or regional strains. - Fungal strain biogeography and models of adaptive mechanisms - Next-generation sequencing that is affordable for routine clinical use - Easily accessible online archives of next-generation sequencing data - Bioinformatics tools for sharing genetic information across numerous databases

Conclusion

Recent developments in DNA and RNA sequencing technologies allow for the thorough investigation of fungal communities, including the analysis of their taxonomic profiles and ecological and functional traits. These data make it possible to interpret communities in a more complex way and to ask questions about how ecosystems function. Further investigation into the pathogenicity, epidemiology, and transmissibility of fungal species is necessary due to their emergence as opportunistic infections. The purpose of fungal genotyping has been to explore the genetic linkages within the organism in order to improve patient care and answer epidemiological questions (e.g., outbreak confirmation). There are several fungal genotyping methods that can be chosen according to the desired use and laboratory capacity.

Conflicts of interest

The authors declare that there is no conflict of interest.

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نحو التنميط الجيني للفطريات: الأساليب والفوائد والتحديات (مقالة)

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الخلاصة

تتزايد إمكانية الوصول إلى الجينات الفطرية الشاملة بسرعة، مما يوفر منظورا شاملا ودقيقا لهذه "المملكة". تم تحقيق إنجاز علمي مهم من خلال الوصول المفتوح إلى أكثر من 1000 جينوم فطري من مختلف الأنواع، مصحوبا بإطلاق مشاريع جديدة وجذابة. يشكل "مشروع 1000 جينوم فطري" واحدة من أكثر مبادرات التسلسل ضخامة التي تركز على الكائنات الفطرية، بهدف معالجة أوجه القصور في علم الجينوم الفطري. حاليا، تمتلك 329 عائلة فطرية جينوم تمثيلي متسلسل واحد على الأقل. ومع ذلك، لا تزال العائلات الفطرية الأخرى بدون أي جينومات متسلسلة. علاوة على ذلك، ساهمت أبحاث التسلسل التكميلي في فهم التنوع الجيني بين بعض الأنواع الفطرية. يساعد وجود العديد من الجينات لكل نوع في التصنيف التصنيفي، ويوفر وجهات نظر جديدة حول تطور الفطريات في أطر زمنية سريعة، ويوضح أنماط التوزيع والتشتت الإقليمية، ويوضح تفشي المرض وطرق الانتقال، من بين أهداف أخرى. تحلل استراتيجيات التنميط الجيني مرحلة محدودة فقط من جينوم الفرد وبدلا من ذلك تسهل التباين السريع وغير الباهظ الثمن للعديد من العزلات. يمكن أن يكون دمج الأساليب الجينومية الشاملة ولجان التنميط الجيني المحسنة التي تركز على تعدد الأشكال و / أو المناطق المتكررة المحددة وذات الصلة بمثابة طرق فعالة وعملية للتحقيق في علم الأوبئة المحلي والإقليمي والعالمي للفطريات.

الكلمات الافتتاحية: فطريات خيطية، تحويل وراثي، بـروتوبلاست، أكروباكتيريوم.