Genotyping of Scedosporium species: a review of molecular approaches

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Scedosporium species are increasingly encountered opportunistic fungal pathogens not only in immunocompromised patients but are also significant primary pathogens in immunocompetent individuals. The environmental reservoir of these fungi is uncertain and the epidemiology and mode of transmission are not well-defined. Conventional phenotypic methods are of limited use for epidemiological purposes since they are insensitive and inadequately discriminatory. Molecular techniques not only enable accurate phylogenetic delineation of species but also provide the means for rapid, reliable genotyping of strains for epidemiological and population genetic studies. This review discusses the methods that have been applied for genotyping of these increasingly important pathogens.

Keywords Scedosporium, genotyping, RAPD, RFLP, PCR fingerprinting, MLST

Introduction

Infections caused by Scedosporium species are increasingly encountered in seriously ill and immunocompromised patients [1,2]. Traditionally, the genus Scedosporium comprised two species of clinical interest: Scedosporium apiospermum (teleomorph Pseudallescheria boydii) and Scedosporium prolificans (teleomorph unknown), which is phylogenetically distant from Scedosporium apiospermum [3]. Recently, multilocus phylogenetic studies have shown that S. apiospermum/P. boydii is a complex of at least eight phylogenetic species and that P. boydii and S. apiospermum are sufficiently distinct to be considered as two separate species [3,4]. The most commonly encountered species of this complex among clinical isolates are P. boydii, S. apiospermum and the newly-described Scedosporium aurantiacum [3,5]. Given the morbidity and poor clinical outcomes associated with Scedosporium infections, and that invasive scedosporiosis is often refractory to treatment, early preventive and/or therapeutic strategies are of paramount importance [2]. The successful implementation of such strategies requires good understanding of the epidemiology and mode of transmission of infection.

Over the years, many molecular techniques have been applied to genotype Scedosporium isolates with the objectives of discriminating between strains and to identify possible sources of Scedosporium infection. Methods include: multilocus enzyme electrophoresis (MLEE) [6], random amplification of polymorphic DNA (RAPD) [6–9], PCR fingerprinting [5,8,10]/inter-simple-sequence-repeat PCR (ISSR-PCR) [11], intergenic spacer region PCR (IGS-PCR) [12], restriction fragment length polymorphism (RFLP) analysis [5,10,13], and amplified fragment-length polymorphism (AFLP) analysis [5]. In addition to these methods, more recent research has focused on the development of a robust multilocus Sequence Typing (MLST) system to provide a reliable and reproducible method of strain differentiation (see below).

This review aims to describe the molecular tools that have been used, or are under development for the genotyping of Scedosporium species.
Molecular typing methods

Multilocus enzyme electrophoresis (MLEE)

MLEE is a technique that evaluates the polymorphisms of selected housekeeping fungal enzymes. In this method, the enzymes of interest are extracted from the fungal cells, electrophoretically separated on a gel and then visualized by specific enzyme-staining procedures. Most of these enzymes display a limited number of isotypes. Isotypes that are present are mainly a result of homologous amino acid substitutions which are transferred from parental strains, thus enabling the establishment of phylogenetic relatedness by comparing adequate numbers of selected enzymes [14]. If a panel of carefully selected enzymes is used, the information derived will enable the detection of microevolution within strains [15]. Typing of several fungi including Candida and Aspergillus by MLEE has been reported [14,16–18].

Random amplification of polymorphic DNA (RAPD) analysis

The technique of RAPD analysis is based on the amplification of random DNA fragments using several arbitrary, short primers [19]. This procedure allows the primers to bind randomly along the template DNA; thus no knowledge of the DNA sequence for the target gene is required. The primers detect polymorphisms which represent genetic markers enabling construction of genetic maps [19]. RAPD has been found to be useful in genotyping of several fungi including Candida spp. and Aspergillus fumigatus [16,20,21].

PCR fingerprinting/inter-simple-sequence-repeat PCR (ISSR-PCR)

PCR fingerprinting is based on the amplification of DNA sequences, which are flanked by hypervariable repeat units. The technique uses a single primer specific to microsatellite or minisatellite DNA repeats, which were originally designed as hybridization probes used in DNA fingerprinting [22]. Following PCR amplification, the amplified interrepeat DNA sequences are separated by agarose gel electrophoresis. The banding patterns can be analysed visually or by using commercially available software programs. The primers used in PCR fingerprinting include the minisatellite-specific core sequence of the wild-type phage M13 (5’ GAGGGTGCCGTTCT 3’), and the microsatellite specific primers (GTG)_5 and (GACA)_4 [22]. The application of PCR fingerprinting using these primers has been demonstrated in numerous studies of clinical and environmental isolates of the Cryptococcus species complex (Cryptococcus neoformans and Cryptococcus gattii), where fingerprint patterns were highly reproducible and able to detect sufficient genetic variability to differentiate among individual strains [22–24]. In addition this approach has allowed the accurate species identification and strain typing of a large number of fungal species including Candida spp. [25], Aspergillus spp. [26] and Penicillium marneffei [27].

ISSR-PCR is a method similar to PCR fingerprinting. It also involves amplification of DNA regions located between closely adjacent microsatellites to produce a unique fingerprint. The ISSR-PCR method has been used for population genetic, epidemiological and ecological studies of several fungi [28].

Intergenic spacer region PCR (IGS-PCR)

The IGS is the most variable part of the rDNA complex and has also been applied as a target for fungal strain typing [12,29]. The procedure includes the amplification of the IGS region, followed by agarose gel electrophoresis of the amplicons. Using a low annealing temperature multiple banding patterns are generated [29]. Although multiple banding patterns usually infer inherent genetic variability, these may also be the result of random amplification due to the low annealing temperatures being used.

Restriction fragment-length polymorphism (RFLP)

RFLP is utilized for the identification of species by comparing DNA restriction patterns. The method is simple to use and a large number of restriction enzymes are commercially available. Digestion of template DNA with these enzymes results in a set of fragments of predetermined and welldefined length. These restriction fragments are then separated according to their length by agarose gel electrophoresis. As the distance between the locations cut by restriction enzymes varies between individual isolates, the fragment lengths also vary, creating polymorphisms. The PCR-RFLP technique has been well established and used for molecular typing of many fungi [30–35]. For instance, PCR-RFLP of the internal transcribed spacer (ITS) region of the rDNA gene cluster has been demonstrated to be useful in assessing the diversity of fungal species among soil fungi [30]. ITS-RFLP has also been used to differentiate between closely related species such as Candida dubliniensis and Candida albicans [35] whilst genetic characterization of Cryptococcus neoformans [33] and Cryptococcus gattii [34] has been successfully conducted by RFLP of the URA5 gene.
**Amplified fragment-length polymorphism (AFLP) analysis**

AFLP analysis is a highly discriminatory method to differentiate microorganisms at intra-species level. In AFLP analysis, fragments are amplified from random localizations throughout the genome of an organism in a highly reproducible manner [36]. The AFLP technique involves digestion of the DNA template with restriction enzymes and ligation of specific adapters to selected restriction sites. Typically, this is followed by two consecutive PCR reactions, which are performed on the restricted template, using specifically designed primers that allow only a subset of the restriction fragments to be amplified. The products are then separated by polyacrylamide gel electrophoresis and the analysis of the banding patterns can be performed using a number of different software programs. This technique has been successfully applied for molecular epidemiological studies of fungal pathogens such as *Candida* spp. [37] and *Aspergillus fumigatus* and to investigate the mode of reproduction in natural populations of *C. gattii* [38].

**Multilocus sequence typing (MLST)**

Multilocus sequence typing (MLST) is a method to characterize organisms using partial sequence analysis of a defined set of 7–10 housekeeping genes [39–41]. For each locus studied, different genetic sequences present in a species are assigned as distinct alleles. The combination of the identified alleles at each of the loci defines the allelic profile or sequence type for each isolate. The data generated can be used to determine whether the fungal isolates are clonal or have undergone recombination. As MLST is a sequence based technique, inter-laboratory differences can be minimized by standardization of primers and PCR protocols, resulting in unambiguous, reproducible results that can be compared and made publicly available via electronic networking. MLST has been successfully used to characterize bacterial pathogens including *Neisseria meningitides*, where it has demonstrated a high degree of discrimination [41]. The method has also been useful for the characterization of a number of fungal pathogens including *Candida albicans* [42], *Candida tropicalis* [43], *Candida glabrata* [44], Cryptococcus neoformans [45], and *Fusarium oxysporum* [46].

**Application of molecular methods to type *Scedosporium* isolates**

**MLEE**

Earlier studies have investigated the use of MLEE as a typing tool for *Scedosporium apiospermum* strains. One study employed 14 different enzymes to reveal a high degree of polymorphism with 27 polymorphic loci within this species [6]. Strain differentiation was successfully achieved by combining the data obtained for different enzyme patterns. Polymorphisms were most frequently seen in profiles representing the superoxide dismutase, carboxyl esterase and malate dehydrogenase enzymes [6].

**RAPD**

RAPD has been performed by a number of authors to differentiate between *Scedosporium* isolates. San Millan *et al.* studied 17 *Scedosporium prolificans* isolates using a panel of 12 primers [7]. These authors studied the discriminatory ability of each primer as well as that of combining patterns of each primer into a single RAPD profile. Results obtained by visual inspection of the gels were compared to those generated by computer analysis using the program GelCompar ver. 4 (Applied Maths, Kortrijk, Belgium). Both approaches enabled the discrimination of all isolates. The combination of RAPD patterns of three primers (UBC 701, AB1.08 and AB1.11 or UBC 701, AB1.08 and UBC 707) allowed for the discrimination of all isolates. Multiple isolates originating from the same patient generated identical profiles [7]. Similar findings were observed in a study by Ruiz-Diez *et al.*, where a combination of three primers (GC70, GC80, M13) was also noted to enable adequate discrimination between *S. prolificans* isolates recovered from four immunocompromised patients [8].

RAPD analysis has also been used to investigate the genetic diversity among epidemiologically unrelated strains of *S. apiospermum* and its former teleomorph, *Pseudallescheria boydii*, from different areas in Europe [6]. In this study, 20 primers were used and none of them individually allowed for the discrimination of all studied strains. Three primers, GC70, UBC-701 and UBC-703, gave the most discriminating results [6]. The same primer set was also used in a study of *S. apiospermum* isolates from cystic fibrosis patients in France [9], where 129 sequential and multiple isolates of *S. apiospermum* from nine patients were analyzed. In this report, the combined results obtained by this primer set identified 16 genotypes among the studied *S. apiospermum* isolates. There was no predominant genotype amongst the patients and there was no clustering of patterns according to the geographic origin of the isolates [9].

**PCR fingerprinting/ISSR-PCR**

Ruiz-Diez *et al.* performed PCR fingerprinting on four *S. prolificans* isolates implicated in a nosocomial
outbreak together with 10 other epidemiologically unrelated strains [8]. Using the primer M13, these authors found different banding patterns among the isolates implicated in the case cluster. The isolates from distinct geographic localities also demonstrated different patterns [8].

This technique has also been applied to investigate the molecular variability of P. boydii. In one study, highly heterogeneous profiles within 25 P. boydii isolates were observed [10]. Strains from the same patients were noted to be identical. Five S. prolificans strains were included in this analysis. With exception of one isolate, these S. prolificans isolates were found to have identical banding pattern.

Most recently, PCR fingerprinting was undertaken to examine the genetic variation among 146 Scedosporium isolates from Australia [5]. Notable findings from this survey include the separation of Scedosporium isolates into three major clusters which in turn, corresponded to the three major species, i.e., S. apiospermum, S. aurantiacum and S. prolificans. The results also showed considerable genetic polymorphisms within each species [5] with S. apiospermum demonstrating the highest intra-species variation, followed by S. prolificans and then S. aurantiacum [5]. The technique has also been applied to discriminate between additional novel species of Scedosporium following their description. Fig. 1 shows the resulting banding patterns seen with S. prolificans and the other species in the Pseudallescheria species complex.

Sole et al. employed ISSR-PCR to examine the genetic variation among 84 clinical and environmental S. prolificans isolates [11]. In their study, 35 unique patterns were observed, which were stable and reproducible [11]. Isolates from the United States were found to be genetically distant from the ones obtained from Spain and Australia.

IGS-PCR

IGS-PCR was used by Williamson et al. to examine the variability in Scedosporium spp. Twenty distinct banding patterns were noted among the 52 S. apiospermum isolates recovered form sputum of patients with cystic fibrosis and bronchiectasis [12].

RFLP analysis

The use of RFLP for genotyping of Scedosporium spp. has also been reported [5,10,13]. In a study of the molecular variability of P. boydii, Rainer et al. performed a 18S rDNA amplification, followed by digestion with the restriction endonucleases HaeIII, Hinfl, DdeI, RsaI, TaqI, MspI, and HhaI [10]. This group found considerable intra-specific variability within P. boydii, reflecting the unresolved phylogeny of this species complex [10].

RFLP analysis of the ITS region of the rDNA gene cluster was recently used in a study of Australian Scedosporium isolates [5]. In this study, the ITS amplicons of 146 isolates of S. apiospermum, S. aurantiacum and S. prolificans were double-digested with two restriction endonucleases Sau96I and HhaI. The resulting banding patterns revealed a unique pattern for each of the three species. The results enabled the identification of a substantial number of isolates originally identified as “S. apiospermum”, as the newly described species, S. aurantiacum [5]. Fig. 2 demonstrates the differences in the ITS-RFLP patterns.
obtained from isolates of S. prolificans, S. apiospermum and S. aurantiacum, confirming the ITS-RFLP patterns described by Delhaes et al. [5].

In another study, RFLP analysis of the IGS region of the rDNA gene cluster was employed to examine intra-specific diversity within the P. boydii complex [13]. HaeIII and MboI were used to digest the IGS amplicons of 22 strains, resulting in seven and five distinct patterns, respectively. The combination of electrophoretic patterns obtained with both enzymes resulted in a total of eight distinguishable patterns [13].

AFLP analysis

AFLP analysis was applied by Delhaes et al. to study the genetic variation of 12 Australian S. prolificans isolates recovered from patients involved in two presumptive case clusters [5] and 23 epidemiologically unrelated isolates. Thirty-two AFLP profiles were obtained with identical profiles noted in isolates recovered from the same patient. No clustering of the isolates was identified. The results of the AFLP analysis had good agreement (100%) with those obtained by M13 PCR fingerprinting performed in the same study [5].

MLST

A MLST system to genotype Scedosporium species has not yet been developed. Given the premise of the utility of MLST in the study of genetic variation in other fungi, such a system for Scedosporium species is under development. Previous molecular studies have pointed towards the use of several candidate gene loci for MLST analysis [3,13]. Wedde et al. utilized partial sequences of the internal transcribed spacer regions (ITS1 and ITS2) including 5.8S rDNA to study 16 isolates of Pseudallescheria and Scedosporium species [47], in which a low genetic diversity of the ITS sequences was observed. The ITS region has also been sequenced to study the molecular variability of P. boydii [10]. In contrast to the study by Wedde et al. [47], this study observed a high variability in the ITS1 and ITS2 regions [10]. Others, using sequences of the ITS regions and partial sequences of the large subunit (LSU) of the rRNA gene, have also observed a considerably high genetic variability among the P. boydii cluster, reflecting the unresolved phylogeny of this species complex [48]. S. prolificans, demonstrated relatively low intra-specific variability, in agreement with a previous report [10].

Besides the rDNA genes, there are other gene targets that have been used for species delineation and which may also serve as potential markers for molecular typing. Gilgado et al. have shown variability in the β-tubulin and calmodulin genes in their phylogenetic study of the P. boydii species complex [3]. These loci are good candidate genes to be used in MLST studies as they are known to demonstrate substantial genetic variation. Most recently, Zeng et al. have further demonstrated genetic variability within the elongation factor 1α gene of P. boydii and related species [13]. Further studies of all those candidate genes in the context of the development of an MLST typing scheme for Scedosporium spp. is warranted.

To evaluate the usefulness of several candidate genes to be used in a MLST typing scheme for Scedosporium spp. a preliminary study has been performed. Isolates chosen in this study include genetically closely related and unrelated isolates of the two phylogenetically well characterized species, S. prolificans and S. aurantiacum. Several gene loci have been examined, which were selected from previous studies or from studies of genetic variation in other filamentous fungi. These
loci include manganese superoxide dismutase (SOD2) [49], elongation factor 1z (EF1z) [13], two loci of β-tubulin (BT2, TUB) [3,50], calmodulin (CAL) [3], ATPase subunit 6 (ATP6) [51], ITS region of rDNA complex [52], mitochondrial small rRNA (mt SSU rRNA) [53] and chitin synthase (CHS) [54] (Table 1). The EF1z locus, the first β-tubulin locus (BT2), the second β-tubulin locus (TUB), the SOD2 locus, the chitin synthase (CHS) locus and the calmodulin (CAL) locus showed polymorphic nucleotides for both studied species, whereas the ITS, mt SSU rRNA and ATP6 loci did not show considerable polymorphism in both Scedosporium species (Table 1). Based on these early findings, EF1z, SOD2, β-tubulin, CHS and CAL have demonstrated good potential for inclusion in an MLST scheme for Scedosporium spp.

**Technical issues**

All molecular methods used for genotyping fungi have their strengths and limitations. With regard to MLEE, despite its ability to differentiate between strains, this method is not adequately discriminatory for the use as a single tool for strain typing. Since the organism is not being examined directly, variation at the nucleotide level may be missed. Nucleotide substitutions do not necessarily result in alteration in the amino acid composition and subsequent changes in the electrophoretic profile may not be visualized. As a consequence, alleles which appear homologous from a different individual may represent different gene alleles. Moreover, MLEE is time-consuming and requires evaluation of at least 10 enzymes to give adequate discriminatory results [14,15].

RAPD has been widely used, as it has the advantage of being rapid and simple to use. However, even though it is highly discriminatory, its widespread adaptation into practice is limited by the absence of standardized methodologies, inadequate reproducibility and the requirement for a strict quality control.

Data reported from various studies involving PCR fingerprinting did show that this method enables strains differentiation. However, despite the highly discriminatory ability of this approach, inter-laboratory variation may occur due to varying experimental conditions and the use of different laboratory reagents. Consequently, PCR fingerprinting profiles may not be reproducible when undertaken in different laboratories rendering this technique unsuitable for multi-institutional typing studies.

Inter-laboratory reproducibility is also an issue for the IGS-PCR due to the use of low annealing temperatures.

Similar problems are found with the AFLP analysis. An additional drawback of AFLP is that variation of banding patterns may be artefactual, reflecting the result of incomplete digestion before the specific amplification of the subset of DNA fragments being detected. As a result, performing amplification in duplicate is necessary.

RFLP on the other hand is relatively easy to use and is more reproducible. Nevertheless, this technique is of

<table>
<thead>
<tr>
<th>Species</th>
<th># of strains tested/haplotype</th>
<th>Gene locus</th>
<th># of polymorphic nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aurantiacum</td>
<td>12/4</td>
<td>Manganese superoxide dismutase (SOD2)</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>12/8</td>
<td>Elongation factor 1z (EF1z)</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>12/5</td>
<td>β-tubulin (BT2)</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>12/7</td>
<td>β-tubulin (TUB)</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>12/1</td>
<td>Internal transcribed spacer region (ITS)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12/1</td>
<td>ATPase subunit 6 (ATP6)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12/5</td>
<td>Calmodulin (CAL)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>12/2</td>
<td>Mitochondrial SSU rRNA (mtSSU)</td>
<td>1</td>
</tr>
<tr>
<td>S. prolificans</td>
<td>12/1</td>
<td>Manganese superoxide dismutase (SOD2)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12/3</td>
<td>Elongation factor 1z (EF1z)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>12/1</td>
<td>Internal transcribed spacer region (ITS)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>12/3</td>
<td>β-tubulin (BT2)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>12/4</td>
<td>β-tubulin (TUB)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>12/1</td>
<td>ATPase subunit 6 (ATP6)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12/6</td>
<td>Chitin synthase (CHS)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>12/1</td>
<td>Mitochondrial SSU rRNA (mtSSU)</td>
<td>0</td>
</tr>
</tbody>
</table>
limited use for studies in which cluster analyses of related isolates is necessary, since it only allows identification to the species level.

Inter-laboratory variation can be eliminated in MLST typing, with the standardization of the PCR condition and sequencing protocols. However, the ability of the MLST analysis to give high strain discrimination relies on the choice of genes or loci selected in each study. Accurate sequence determination and comparison is crucial as a single base difference denotes a different haplotype.

Conclusion and recommendations

Scedosporium species are important emerging fungal pathogens. Despite antifungal therapy, mortality remains high [2]. Given the need to improve outcomes, early interventions including preventative measures are urgently required. Epidemiological investigation of Scedosporium infections requires accurate species identification and genotyping. Various molecular techniques have been applied, aiming at accurate and reliable strain typing. These molecular approaches differ in their discriminatory power and hence, their applicability in differentiating between Scedosporium strains. A comparison of the major features of the methods is summarized in Table 2.

Table 2 Comparison of different methods used for genotyping of Scedosporium species

<table>
<thead>
<tr>
<th>Method</th>
<th>Turn around time</th>
<th>Discriminatory power</th>
<th>Inter-laboratory reproducibility</th>
<th>Interpretation</th>
<th>Costs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLEE</td>
<td>1-2 weeks</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Easy</td>
<td>Low</td>
<td>[6]</td>
</tr>
<tr>
<td>RAPD</td>
<td>1-2 days</td>
<td>High</td>
<td>Poor</td>
<td>Difficult</td>
<td>Low</td>
<td>[6-9]</td>
</tr>
<tr>
<td>PCR fingerprinting</td>
<td>1-2 days</td>
<td>High</td>
<td>Poor</td>
<td>Difficult</td>
<td>Low</td>
<td>[5,8,10]</td>
</tr>
<tr>
<td>RFLP</td>
<td>2 days</td>
<td>Low</td>
<td>Good</td>
<td>Easy</td>
<td>Moderate</td>
<td>[5,10,13]</td>
</tr>
<tr>
<td>AFLP</td>
<td>2-3 days</td>
<td>High</td>
<td>Moderate</td>
<td>Moderate</td>
<td>High</td>
<td>[5]</td>
</tr>
<tr>
<td>MLST</td>
<td>1-2 weeks</td>
<td>High</td>
<td>Excellent</td>
<td>Easy</td>
<td>High</td>
<td>[under development]</td>
</tr>
</tbody>
</table>

Previous studies indicate that genetic polymorphisms within the rDNA gene cluster are valuable markers for species delineation although may not be adequately discriminatory in differentiating between individual strains. In comparison to the rDNA gene cluster other gene targets, such as β-tubulin [3,4], calmodulin [3] and EF1α [13] genes have exhibited considerable genetic variability to open up a promising future for their use in the development of an MLST typing scheme for Scedosporium species. The usefulness of these gene loci have been demonstrated in a preliminary study involving selected strains of S. prolificans and S. aurantiacum isolates.

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