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Use of Amplified Fragment Length Polymorphism Analysis To Identify Medically Important *Candida* spp., Including *C. dubliniensis*

A. Borst,[1]* B. Theelen,[2] E. Reinders,[3] T. Boekhout,[2] A. C. Fluit,[1] and P. H. M. Savelkoul[3]

Eijkman-Winkler Center for Microbiology, Infectious Diseases and Inflammation, University Medical Center,[1] and Centraalbureau voor Schimmelcultures,[2] Utrecht, and Department of Medical Microbiology and Infection Control, VU University Medical Center, Amsterdam,[3] The Netherlands

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Non-*Candida albicans Candida* species are increasingly being isolated. These species show differences in levels of resistance to antimycotic agents and mortality. Therefore, it is important to be able to correctly identify the causative organism to the species level. Identification of *C. dubliniensis* in particular remains problematic due to the high degree of phenotypic similarity between this species and *C. albicans*. The use of amplified fragment length polymorphism (AFLP) analysis as an identification method for medically important *Candida* species was investigated. Our results show very clear differences among medically important *Candida* species. Furthermore, when screening a large collection of clinical isolates previously identified on CHROMagar as *C. albicans*, we found a misidentification rate of 6%. AFLP analysis is universally applicable, and the patterns can easily be stored in a general, accessible database. Therefore, AFLP might prove to be a reliable method for the identification of medically important *Candida* species.

In the past decade, the number of life-threatening forms of candidiasis increased dramatically (1). The attributable mortality of these infections is as high as 38% (34), whereas crude mortality rates exceed 50% (10, 27, 33). For a long time, *Candida albicans* was the main cause of invasive fungal infections. However, the number of infections by this species is declining whereas non-*albicans Candida* species like *C. glabrata*, *C. krusei*, and *C. parapsilosis* are increasingly being isolated. At present, non-*albicans Candida* species account for approximately 50% of all *Candida* infections (14).

In cases of candidiasis, it is important to be able to correctly identify the causative organism to the species level. Different species show differences in levels of resistance to antimycotic agents. *C. krusei* is innately resistant to fluconazole, and *C. glabrata* is able to acquire resistance to this drug rapidly. Furthermore, *C. glabrata* infections have been associated with a high mortality (11). A particular problem is formed by the recently recognized species *C. dubliniensis*. Like *C. glabrata*, this species is capable of acquiring stable fluconazole resistance rapidly (22, 23). Identification of *C. dubliniensis* remains difficult, due to the high degree of phenotypic similarity between this species and *C. albicans*. However, it is known that genotypically there is more variation between the two species (30). Therefore, molecular identification methods may be more reliable than identification methods based on phenotypic characteristics.

Amplified fragment length polymorphism (AFLP) analysis is a relatively new technique which has a discriminatory power that makes it suitable for identification as well as for strain typing (29, 32). In short, in AFLP analysis genomic DNA is digested with two restriction enzymes (e.g., *Eco*RI and *Mse*I) and double-stranded oligonucleotide adapters are ligated to the fragments. These adapters serve as targets for the primers during PCR amplification. To increase the specificity, it is possible to elongate the primers at their 3′ ends with one to three selective nucleotides. One of the primers is labeled with a fluorescent dye. The fragments are separated and analyzed using software packages like BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium). The advantage of AFLP analysis is that only a limited amount of DNA is needed since the fragments are PCR amplified. Furthermore, since stringent annealing temperatures are used during amplification, the technique is more reproducible and robust than other methods such as randomly amplified polymorphic DNA analysis (13, 29). This paper describes the use of AFLP as an identification method for medically important *Candida* species, including *C. dubliniensis*.

MATERIALS AND METHODS

Yeast strains. The yeast strains and isolates used are listed in Table 1. Reference strains were obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, and the American Type Culture Collection, Manassas, Va. Clinical isolates were obtained from the European SENTRY collection (Eijkman-Winkler Center, Utrecht, The Netherlands) (26) and from the hematology ward of the VU University Medical Center (VUMC), Amsterdam, The Netherlands. Upon receipt of the SENTRY isolates in the central laboratory (Eijkman-Winkler Center), CHROMagar (CHROMagar, Paris, France) was used to distinguish between *C. albicans* and non-*C. albicans* species. Non-*C. albicans* species were further identified by using the API *Candida* system (bioMérieux, Marcy-l'Etoile, France). In addition, Vitek YBC cards (bioMérieux) were used when the results obtained by the API *Candida* system were inconclusive or differed from the identification made by the center where the *Candida* species was isolated. The isolates from the VUMC were identified using the germ-tube test. The isolates that were negative in this assay were further identified by using Vitek YBC (bioMérieux).

Extraction of DNA. DNA was extracted from approximately 10^7 CFU using a DNeasy tissue kit (Qiagen, West Sussex, England) according to the manufacturer's instructions (protocol for isolation of genomic DNA from yeasts). DNA was eluted in 100 μl of elution buffer (buffer AE of the kit) and stored at −20°C.

AFLP. (i) Restriction and ligation of adapters. The sequences of the adapters and primers used for AFLP analysis are given in Table 2. DNA was extracted

* Corresponding author. Present address: Division of Bacterial and Mycotic Diseases, Centers for Disease Control and Prevention, Mailstop G-11, 1600 Clifton Rd., Atlanta, GA 30333. Phone: (404) 639-3547. Fax: (404) 639-3546. E-mail: aoz0@cdc.gov.

TABLE 1. Reference strains and clinical isolates used in this study

Species[a]	Strain or isolate (collection)	Origin	Source
C. albicans	CBS 562	Uruguay	Skin of man with interdigital mycosis
	CBS 1905	Unknown	Man
	CBS 1912	Norway	Sputum of asthma patient
	ATCC 90028	Iowa, United States	Blood
	ATCC 90029	Iowa, United States	Blood
	04A080 (SENTRY)	Paris, France	Blood
	06A309 (SENTRY)	Lille, France	Blood
	07C069 (SENTRY)	Freiburg, Germany	Pneumonia
	08E058 (SENTRY)	Dusseldorf, Germany	Wound, skin, or soft tissue
	10A173 (SENTRY)	Genoa, Italy	Blood
	10C007 (SENTRY)	Genoa, Italy	Pneumonia
	11A134 (SENTRY)	Rome, Italy	Blood
	11C034 (SENTRY)	Rome, Italy	Pneumonia
	12E033 (SENTRY)	Utrecht, The Netherlands	Wound, skin, or soft tissue
	15A020 (SENTRY)	Coimbra, Portugal	Blood
	15A206 (SENTRY)	Coimbra, Portugal	Blood
	15A561 (SENTRY)	Coimbra, Portugal	Blood
	16A232 (SENTRY)	Seville, Spain	Blood
	16A438 (SENTRY)	Seville, Spain	Blood
	16C088 (SENTRY)	Seville, Spain	Pneumonia
	17A381 (SENTRY)	Madrid, Spain	Blood
	19A164 (SENTRY)	Lausanne, Switzerland	Blood
	19A519 (SENTRY)	Lausanne, Switzerland	Blood
	19A567 (SENTRY)	Lausanne, Switzerland	Blood
	19A568 (SENTRY)	Lausanne, Switzerland	Blood
	23D045 (SENTRY)	Ankara, Turkey	Urinary tract
	TY727 (VUMC)	Amsterdam, The Netherlands	Oral cavity
	TY728 (VUMC)	Amsterdam, The Netherlands	Oral cavity
	TY729 (VUMC)	Amsterdam, The Netherlands	Oral cavity
	TY732 (VUMC)	Amsterdam, The Netherlands	Feces (human)
C. dubliniensis	CBS 7987	Dublin, Ireland	Oral cavity of HIV-infected patient
	CBS 7988	Melbourne, Australia	Oral cavity of HIV-infected patient
	CBS 8500	Nijmegen, The Netherlands	Blood of 38-year-old woman with chronic myelogenous leukemia
	CBS 8501	Nijmegen, The Netherlands	Child with neutropeny induced by chemotherapy
	02A038 (SENTRY)	Brussels, Belgium	Blood
	05C118 (SENTRY)	Lyon, France	Pneumonia
	05C121 (SENTRY)	Lyon, France	Pneumonia
	18A221 (SENTRY)	Barcelona, Spain	Blood
	20C149 (SENTRY)	London, United Kingdom	Pneumonia
	23A137 (SENTRY)	Ankara, Turkey	Blood
C. glabrata	CBS 138	Unknown	Feces (human)
	ATCC 90030	Iowa, United States	Blood
	TY714 (VUMC)	Amsterdam, The Netherlands	Oral cavity
	TY715 (VUMC)	Amsterdam, The Netherlands	Feces (human)
	TY716 (VUMC)	Amsterdam, The Netherlands	Oral cavity
	TY717 (VUMC)	Amsterdam, The Netherlands	Oral cavity
	TY718 (VUMC)	Amsterdam, The Netherlands	Oral cavity
	TY719 (VUMC)	Amsterdam, The Netherlands	Oral cavity
	TY731 (VUMC)	Amsterdam, The Netherlands	Oral cavity
C. guilliermondii	CBS 566	Unknown	Sputum (human)
	CBS 2024	Berlin, Germany	Ulcer on horse
	14A097 (SENTRY)	Cracow, Poland	Blood
C. krusei	CBS 573	Colombo, Sri Lanka	Sputum of bronchitic convict
	TY722 (VUMC)	Amsterdam, The Netherlands	Oral cavity
	TY723 (VUMC)	Amsterdam, The Netherlands	Oral cavity
	TY726 (VUMC)	Amsterdam, The Netherlands	Feces (human)
C. lusitaniae	CBS 4413	Portugal	Cecum of pig
C. parapsilosis	CBS 604	Puerto Rico	Case of sprue (human)
	CBS 2195	Austria	Infected nail of 11-year-old boy

Continued on following page

TABLE 1—*Continued*

Species[a]	Strain or isolate (collection)	Origin	Source
	ATCC 90018	Virginia, United States	Blood
	07A212 (SENTRY)	Freiburg, Germany	Blood
	10A120 (SENTRY)	Genoa, Italy	Blood
	10A311 (SENTRY)	Genoa, Italy	Blood
	14A161 (SENTRY)	Cracow, Poland	Blood
	TY735 (VUMC)	Amsterdam, The Netherlands	Oral cavity
	TY736 (VUMC)	Amsterdam, The Netherlands	Unknown
C. pseudotropicalis	CBS 607	Sri Lanka	Bronchitic patient
C. tropicalis	CBS 94	Unknown	Bronchitic patient
	CBS 2310	Unknown	Unknown
	11D028 (SENTRY)	Rome, Italy	Urinary tract
	TY737 (VUMC)	Amsterdam, The Netherlands	Oral cavity
	TY739 (VUMC)	Amsterdam, The Netherlands	Oral cavity

[a] Identification of SENTRY isolates based on AFLP patterns.

from approximately 10^7 CFU of *C. albicans* as described above. Five microliters of the DNA samples was added to 5 microliters of restriction-ligation reaction mixture (1× T_4 DNA ligase buffer, 0.05 M NaCl, 0.5 μg of bovine serum albumin, 2 pmol of the *Eco*RI adapter, 20 pmol of the *Mse*I adapter, 80 U of T_4 DNA ligase, 1 U of *Eco*RI, 1 U of *Mse*I) and incubated overnight at 37°C. All enzymes were obtained from New England Biolabs (Beverly, Mass.). The mixture was diluted 1:5 with 0.1× TE (5 mM Tris-HCl [pH 7.5], 1 mM EDTA).

(ii) Preselective and selective PCRs. Preselective PCR was performed using the core sequences, i.e., primers without extensions. The AFLP primers, core mix, and internal size standard were supplied by Applied Biosystems (Nieuwerkerk aan den IJssel, The Netherlands). Four microliters of diluted restriction-ligation product was added to 15 μl of AFLP amplification core mix, 0.5 μl of the *Eco*RI core sequence, and 0.5 μl of the *Mse*I core sequence. The mixture was amplified in a GeneAmp PCR System 9700 machine under the following conditions: 2 min at 72°C, followed by 20 cycles of 20 s at 94°C, 30 s at 56°C, and 2 min at 72°C. The PCR product was diluted by adding 25 μl of sterile double-distilled water. In a second PCR more-selective primers were used: *Eco*RI-AC (labeled with 6-carboxyfluorescein) and *Mse*I-C. The conditions were 2 min at 94°C, followed by 10 cycles consisting of 20 s at 94°C and 30 s at 66°C (with this temperature decreasing 1°C with each succeeding cycle), and a final extension of 2 min at 72°C. This sequence was followed by 25 cycles consisting of 20 s at 94°C, 30 s at 56°C, and 2 min at 72°C and a final incubation of 30 min at 60°C.

(iii) Capillary electrophoresis and data analysis. The samples were prepared for capillary electrophoresis by adding 2 μl of the selective PCR product to 24 μl of deionized formamide and 1 μl of GeneScan-500 (6-carboxy-X-rhodamine [ROX] labeled) as an internal size standard. They were run on an ABI 310 genetic analyzer for 30 min each. Data were analyzed with the BioNumerics software package, version 2.5 (Applied Maths) by using the Pearson correlation as a similarity coefficient in combination with unweighted pair group method with arithmatic mean cluster analysis. The statistical reliability of the clusters was investigated by using the cophenetic values, which calculate the correlation between the calculated similarities and the dendrogram-derived similarities.

TABLE 2. Adapter and primer sequences used for AFLP

Adapter or primers	Sequence[a]
Adapters	
*Eco*RI	5′-CTCGTAGACTGCGTACC-3′ 3′-CATCTGACGCATGGTTAA-5′
*Mse*I	5′-GACGATGAGTCCTGAG-3′ 3′-CTACTCAGGACTCAT-5′
Primers	
*Eco*RI	5′-GACTGCGTACCAATTC**AC**-3′
*Mse*I	5′-GATGAGTCCTGAGTAA**C**-3′

[a] The bold type represents selective nucleotides (added to the core sequence and used only in the second PCR).

RESULTS AND DISCUSSION

A dendrogram representing all reference strains and clinical isolates is depicted in Fig. 1. The AFLP patterns of the reference strains clearly show that each species forms a distinct cluster. The cophenetic values were 78 for *C. albicans*, 92 for *C. dubliniensis*, 99 for *C. glabrata*, 84 for *C. krusei*, 98 for *C. pseudotropicalis*, 85 for *C. tropicalis*, 91 for *C. parapsilosis*, 98 for *C. lusitaniae*, and 94 for *C. guilliermondii*. These results were highly reproducible.

The *C. albicans* isolates show two main clusters. One cluster contains clinical isolates from the VUMC and the SENTRY collection as well as reference strains from the CBS. The other cluster contains only isolates from the SENTRY collection. There is no clear relation between these clusters and the geographical origins or sources of the isolates. North American *C. albicans* isolates show a three-part division by several typing methods, such as randomly amplified polymorphic DNA analysis, multilocus enzyme electrophoresis, and Southern blot hybridization with the moderately repetitive *C. albicans*-specific Ca3 probe. In South Africa, an additional cluster besides these three clusters has been found (4, 18, 28). It will be interesting to investigate whether the two AFLP clusters of *C. albicans* correspond with the North American or South African clusters.

The *C. dubliniensis* isolates also show two clusters whose isolates have remarkably high similarities (91 and 98%). One cluster contains all reference strains used and one SENTRY clinical isolate; the other cluster is composed of SENTRY isolates only. Using the *C. dubliniensis*-specific fingerprinting probe Cd25 on a panel of 98 isolates, Gee et al. also recognized two different clusters, one of which contained mainly isolates derived from human immunodeficiency virus (HIV)-infected individuals, while the other cluster contained mainly isolates derived from HIV-negative individuals (9). Strains CBS 7987 and CBS 7988, both part of the same AFLP cluster, were isolated from an HIV-infected individual. However, data on the HIV status of the patients from which the other isolates (CBS 8500, CBS 8501, and SENTRY isolates) were obtained are lacking. Further investigations are necessary to examine whether the AFLP clusters correspond with the Cd25 clusters.

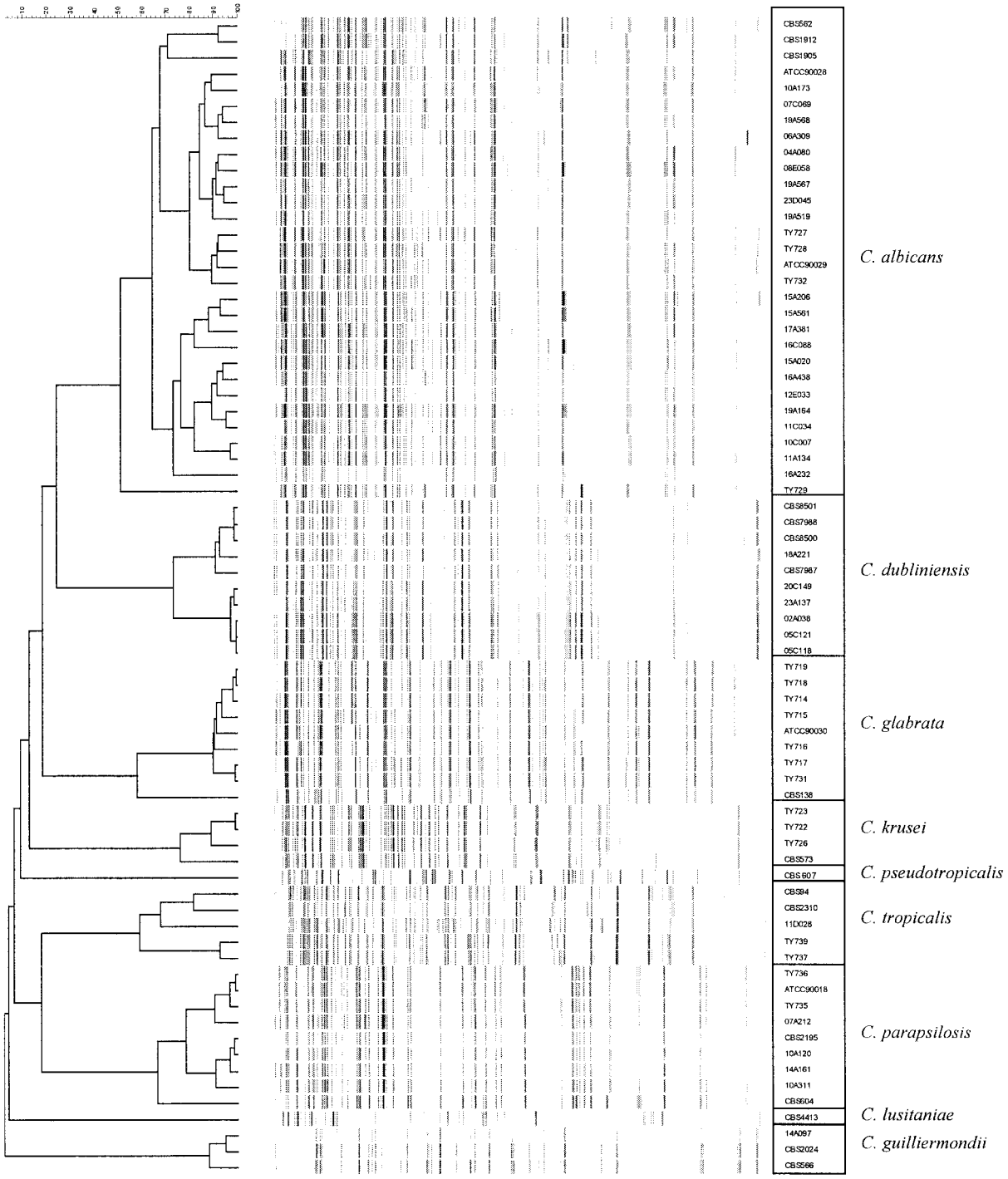

FIG. 1. Dendrogram representing all reference strains and clinical isolates (see also Table 1).

Another noteworthy finding is that all of the AFLP patterns for the *C. glabrata* isolates are very similar (90% similarity) except for that of the CBS reference strain (58% similarity). This reference strain (CBS 138) was isolated from human feces and was first described in 1917. The fact that all of the other isolates studied were clinical isolates which were isolated fairly recently may account for this difference.

The AFLP patterns of the 18 isolates from the VUMC all corresponded with the results of the phenotypic identification (obtained by using the germ-tube test and Vitek YBC cards). The clinical isolates from the European SENTRY collection were all originally identified on CHROMagar as being *C. albicans*. However, based on the AFLP patterns shown in Fig. 1, some of these strains were presumably misidentified and belong to different species. When the total collection of isolates previously identified as *C. albicans* ($n = 213$) was screened by

AFLP analysis, a misidentification rate of 6% was observed. Six strains are now identified as *C. dubliniensis*, four are identified as *C. parapsilosis*, one is identified as *C. tropicalis*, and one is identified as *C. guilliermondii* (results are partly shown in Fig. 1).

CHROMagar identification of *Candida* species is based on differences in colony color. It has been shown that the reliability of this method depends on the incubation time and temperature used (2, 24, 35). However, even when optimum conditions are used, the method is not ideal and the differentiation between *C. albicans* and *C. dubliniensis* is especially problematic. Kurzai et al. reported that only 81% of their *C. dubliniensis* isolates showed the dark-green color on CHROMagar, which is considered indicative of *C. dubliniensis* (17). Furthermore, 15.9% of their *C. albicans* isolates also showed a dark-green coloration instead of the usual lighter green. Tintelnot et al. (31) reported an even lower number, 57%, of *C. dubliniensis* isolates that showed the dark-green coloration on CHROMagar, and only 48% of the isolates of Kirkpatrick et al. (15) showing the dark-green coloration turned out to be *C. dubliniensis*.

Other commercial tests that allow (presumptive) identification of *C. albicans* as well as non-*albicans Candida* species usually show high sensitivities and specificities for *C. albicans* but are less reliable or need further testing for the identification of other, less common species (3, 5, 8, 12). *C. dubliniensis*-specific PCR assays as well as generic PCR assays in combination with species-specific probes have been developed (6, 7, 16, 19, 25). The advantage of AFLP analysis, however, is that this method is based on the ligation of known sequences (adapters) to restriction fragments, which function as targets for the PCR primers. Therefore, the technique is universally applicable. In the present assay we made use of two subsequent amplifications, but similar results were obtained when only the second amplification was used (unpublished observations). The use of an internal size standard with every sample for normalization purposes greatly enhances the reproducibility between tests. Storing all patterns, including those of the reference strains, in a general, accessible database will provide a screening library for the identification of *Candida* species.

Two other universally applicable methods for the identification of *Candida* species have been described: PCR fingerprinting and reference strand-mediated conformational analysis (20, 21). However, whereas PCR fingerprinting uses mini- and microsatellite sequences as targets for the primers and reference strand-mediated conformational analysis is based on 18S rRNA sequences, AFLP patterns are a representation of the whole genome. Our results show very clear differences among medically important *Candida* species. Therefore, AFLP analysis might prove to be a reliable method for the identification of medically important *Candida* species, including *C. dubliniensis*.

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