Molecular epidemiology of *Candida albicans* and *Candida glabrata* strains isolated from intensive care unit patients in Poland

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Over the last decades, Candida spp have been responsible for an increasing number of infections, especially in patients requiring intensive care. Knowledge of local epidemiology and analysis of the spread of these pathogens is important in understanding and controlling their transmission. The aim of this study was to evaluate the genetic diversity of 31 Candida albicans and 17 Candida glabrata isolates recovered from intensive care unit patients from the tertiary hospital in Krakow between 2011-2012. The strains were typed by random amplified polymorphic DNA (RAPD) polymerase chain reaction using five primers (CD16AS, HP1247, ERIC-2, OPE-3 and OPE-18). The results of the present investigation revealed a high degree of genetic diversity among the isolates. No clonal relationship was found among the C. albicans strains, whereas two C. glabrata isolates were identical. The source of Candida infection appeared to be mostly endogenous; however, the presence of two clonal C. glabrata strains suggested the possibility of cross-transmission of these pathogens. Our study confirmed the high discriminatory power of the RAPD technique in the molecular typing of Candida clinical isolates. This method may be applied to the evaluation of transmission routes of pathogenic fungi on a local level.

Key words: *Candida albicans - Candida glabrata* - genotyping - epidemiology - RAPD PCR

Over the last few decades, the incidence of fungal infections in humans has increased, especially in patients requiring intensive care. The Extended Prevalence of Infection in Intensive Care study demonstrated that *Candida* spp accounted for 18.5% of microorganisms isolated from intensive care unit (ICU) patients in both Western and Eastern Europe (Vincent et al. 2009). Invasive candidiasis and candidaemia, caused most commonly by *Candida albicans* and *Candida glabrata*, are of growing concern in the ICU (Morace & Borghi 2010, Pfaller et al. 2011, Rodrigues et al. 2013). Polish surveillance involving 94 units in 20 hospitals (including surgery, intensive care, neonatology, haematology and others) indicated that the highest degree of candidaemia was found in the ICU (30.8%) (Nawrot et al. 2013).

Furthermore, *C. albicans* and *C. glabrata* bloodstream infections have been associated with a high mortality rate (Weinberger et al. 2005). Risk factors for invasive fungal infection in ICU patients include the use of broad-spectrum antibacterial agents and immunosuppressive agents, parenteral nutrition, central venous catheters and mechanical ventilations. Immune status, comorbid diseases and the age of patients also contrib-

ute to the incidence of fungal diseases (Lass-Flörl 2009, Arendrup 2010). Although the most cases of *Candida* infections appear to originate from an endogenous source, there are cases of nosocomial transmission (Vrioni & Matsiota-Bernard 2001, Ben Abdeljelil et al. 2011). Identification of relatedness between *Candida* species is crucial for control measures to reduce the incidence of nosocomial candidiasis, but little is known of the epidemiology of these species in southern Poland. For this purpose, it is important to perform genotyping analysis that would allow evaluation of the spread of these pathogens. The most commonly used molecular typing methods are restriction endonuclease analysis, microsatellite analysis, multilocus enzyme electrophoresis, pulsed-field gel electrophoresis, multilocus sequence typing and random amplified polymorphic DNA polymerase chain reaction (RAPD PCR) (Krawczyk et al. 2009, Abbes et al. 2010, Karaman et al. 2013). The latter method was used in this study to evaluate the level of genetic diversity of *C. albicans* and *C. glabrata* strains isolated from ICU patients from the tertiary hospital in Krakow, Poland over a two-year period.

SUBJECTS, MATERIALS AND METHODS

Patients and isolates - A total of 136 and 145 yeast strains were isolated from patients hospitalised in the ICU of the Rydygier's Specialized Hospital in Krakow in 2011 and 2012, respectively. The species distribution is shown in Table I. The predominant species were *C. albicans* (162 strains) and *C. glabrata* (45 strains), obtained from endotracheal aspirates (ETA) and urine. Among the most frequently isolated species, subjected to further molecular studies, were 48 strains, including 31 *C. albicans* and 17 *C. glabrata* isolates cultured at concentrations of $\geq 10^4$ colony-forming unit (cfu)/mL. Of the study

group patients, 20 were female, 22 were male and the age range was 19-94 (mean 65) years. In 2011, there were 13 recovered *C. albicans* isolates and seven recovered *C. glabrata* isolates, whereas in 2012 there were 18 *C. albicans* and 10 *C. glabrata* isolated strains, respectively. Twenty nine strains of *C. albicans* and 17 strains of *C. glabrata* were isolated from ETA, while two *C. albicans* isolates were taken from urine. All strains were obtained once per patient, except for *C. albicans* CA11 and CA12 strains, which were isolated from the ETA and urine of the same patient. From five of the patients tested, both *C. albicans* and *C. glabrata* were cultured (Table II). Antifungal prophylaxis was not administered to the patients included in this study. This research was approved by the Ethical Committee of Jagiellonian University Medical College (protocol KBET/129/B/2011).

Identification and antifungal susceptibility testing of Candida strains - All analysed clinical specimens, from ETA (46) and urine (2), were cultured by quantitative technique on Sabouraud dextrose agar (bioMérieux, Poland) and incubated at 37ºC for 48 h. Fungal concentrations between 10^4 cfu/mL and 10^6 cfu/mL for ETA and 10^5 cfu/mL for urine were observed after 48 h of incubation (Müller et al. 1986).

Preliminary fungal strain identification was based on colony morphology on CHROMAgar *Candida* (bioMérieux), while the identification to the species level was confirmed by the Vitek 2 Compact automatic system (bioMérieux). Susceptibility of strains to amphotericin B, fluconazole, 5-fluorocytosine and voriconazole was determined by using the Vitek 2 Compact with AST-YS01 cards and by interpreting results according to the Clinical and Laboratory Standards Institute criteria (CLSI 2008).

TABLE I

Yeast species isolated from the intensive care unit patients of the Rydygier's Specialized Hospital in Krakow, Poland, between 2011-2012

| Yeast species | Year of isolation | |
| | 2011 | 2012 |
	n (%)	n (%)
Candida albicans	76 (55.9)	86 (59.3)
Candida glabrata	23 (16.9)	22 (15.2)
Candida tropicalis	11 (8.1)	8 (5.5)
Candida krusei	5 (3.7)	8 (5.5)
Candida kefyr	4 (2.9)	6 (4.1)
Candida dubliniensis	3 (2.2)	2 (1.4)
Candida parapsilosis	3 (2.2)	6 (4.1)
Other[a]	11 (8.1)	7 (4.8)
Total	136 (100)	145 (100)

a: *Candida lusitaniae*, *Candida lipolytica*, *Candida guilliermondii*, *Candida inconspicua*, *Saccharomyces cerevisiae*, *Geotrichum* sp.

DNA extraction - Fungal genomic DNA was extracted with a Genomic Mini AX YEAST isolation kit (A&A Biotechnology, Poland) according to the manufacturer's instructions. Evaluation of the DNA concentration and purity was performed by spectrophotometry at 260 nm and the ratio of the absorbance at 260 nm and 280 nm, respectively. The total genomic DNA was stored at -20ºC for further molecular studies.

RAPD reaction - Five arbitrary primers were used for RAPD analysis of *C. albicans* and *C. glabrata* strains: CD16AS (5'-CTCTTGAAACTGGGGGAGACTTGA-3'), HP1247 (5'-AAGAGCCCGT-3'), ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') (Trojanowska et al. 2010), OPE-3 (5'-CCAGATGCAC-3') and OPE-18 (5'-GGACTGCAGA-3') (Marol & Yücesoy 2008) (Blirt, Poland). The RAPD reactions were performed with 25 ng of *Candida* genomic DNA in a final volume of 25 µL containing 100 pmol of primer (Blirt, Poland), 100 µM of each nucleotide (Fermentas, Lithuania), 0.75 mM $MgCl_2$, 0.625 U of GoTaq DNA Polymerase in 5 µL and 5x colourless GoTaq Flexi Buffer (pH 8.5) provided by the manufacturer (Promega, USA). Amplification was carried out in a thermocycler T personal (Biometra, Germany) with an initial denaturation at 95ºC for 2 min followed by 40 cycles of denaturation at 95ºC for 1 min, annealing at 47ºC for 1 min, elongation at 72ºC for 2 min and a final extension at 72ºC for 5 min. The DNA fragments were then separated by 2% agarose gel electrophoresis and visualised by ethidium bromide staining (Sigma-Aldrich, Germany). The PCR product size was determined by comparison with a molecular weight standard (O'Gene Ruler 100 bp DNA Ladder Plus; Thermo Scientific, USA).

The analyses of the genetic relatedness of the *Candida* isolates were performed by Bio 1D++ software (Vilber-Lormat, France) via unweighted pair-group method with arithmetic averages using the Dice coefficient. When the similarity value was 100%, the strains were determined to be identical, while isolates with values ranging between 80-99% were considered related and those with values under 80% were considered unrelated. The experiments were carried out in duplicate and there were no differences in the RAPD profiles.

RESULTS

The minimal inhibitory concentration (MIC) values for amphotericin B, fluconazole, 5-fluorocytosine and voriconazole for all tested isolates are shown in the Table II. Forty-seven *Candida* strains were susceptible to the antifungal agents tested in this study, except for one *C. glabrata* strain (CG7) that had intermediate susceptibility to fluconazole (MIC 32 mg/L).

Genetic diversity of 31 *C. albicans* and 17 *C. glabrata* isolates were examined in this study. *C. albicans* isolates displayed from one to nine; two-nine, four-nine, four-10 and three-13 bands for OPE-18, HP1247, OPE-3, CD16AS and ERIC-2 primers, respectively. The RAPD patterns obtained for *C. glabrata* were composed of one-five, one-eight, two-10, six-11 and three-12 DNA fragments for HP1247, CD16AS, ERIC-2, OPE-3 and OPE-18

TABLE II

Description of 31 *Candida albicans* and 17 *Candida glabrata* isolates comprising gender and age of patients,
date of isolation, clinical sample, antifungal agents minimal inhibitory concentration (MIC) values and results
of random amplified polymorphic DNA polymerase chain reaction (RAPD) analysis

Isolate code	Patient code	Gender	Age	Date of isolation	Clinical sample	MIC values (mg/L)				RAPD profile	RAPD cluster
						AMB	FLC	5FC	VRC		
CA1	SE	F	78	12 Jan 2011	ETA	1	4	≤ 1	≤ 0.12	A	A
CA2	DM	M	25	16 Mar 2011	ETA	1	≤ 1	≤ 1	≤ 0.12	U1	U
CA3	WM	M	80	30 May 2011	ETA	0.5	≤ 1	≤ 1	≤ 0.12	R	R
CA4	BL	M	53	07 Jun 2011	ETA	0.5	≤ 1	≤ 1	≤ 0.12	D	D
CA5	PA	M	50	18 Aug 2011	ETA	0.5	≤ 1	≤ 1	≤ 0.12	N	N
CA6	LP	M	28	05 Sep 2011	ETA	0.5	≤ 1	≤ 1	≤ 0.12	K1	K
CA7	KL	M	28	21 Sep 2011	Urine	0.5	≤ 1	≤ 1	≤ 0.12	O1	O
CA8	PB	F	43	22 Sep 2011	ETA	0.5	≤ 1	≤ 1	≤ 0.12	O2	O
CA9	SR	M	38	05 Oct 2011	ETA	0.5	≤ 1	≤ 1	≤ 0.12	K2	K
CA10	SL	M	90	07 Oct 2011	ETA	0.5	≤ 1	≤ 1	≤ 0.12	L	L
CA11	BM	F	66	15 Nov 2011	ETA	1	≤ 1	≤ 1	≤ 0.12	P	P
CA12	BM	F	66	16 Nov 2011	Urine	1	≤ 1	≤ 1	≤ 0.12	M1	M
CA13	LF	M	90	29 Dec 2011	ETA	0.5	≤ 1	≤ 1	≤ 0.12	M2	M
CA14	WH	M	34	03 Jan 2012	ETA	0.5	≤ 1	≤ 1	≤ 0.12	C	C
CA15	BuM	F	53	12 Jan 2012	ETA	1	≤ 1	≤ 1	≤ 0.12	E1	E
CA16	PZ	M	87	09 Mar 2012	ETA	1	≤ 1	≤ 1	≤ 0.12	T1	T
CA17	ZW	M	83	04 Apr 2012	ETA	0.5	≤ 1	≤ 1	≤ 0.12	E2	E
CA18	KT	M	61	04 Apr 2012	ETA	0.5	≤ 1	≤ 1	≤ 0.12	F	F
CA19	NA	F	61	16 Apr 2012	ETA	0.5	≤ 1	≤ 1	≤ 0.12	E4	E
CA20	ZJ	F	68	08 Oct 2012	ETA	0.5	≤ 1	≤ 1	≤ 0.12	J1	J
CA21	OJ	M	69	08 Oct 2012	ETA	0.5	≤ 1	≤ 1	≤ 0.12	T2	T
CA22	PP	M	19	10 Oct 2012	ETA	0.5	≤ 1	≤ 1	≤ 0.12	E3	E
CA23	NN	F	41	12 Oct 2012	ETA	0.5	≤ 1	≤ 1	≤ 0.12	J2	J
CA24	OA	F	45	17 Oct 2012	ETA	≤ 0.25	≤ 1	≤ 1	≤ 0.12	G	G
CA25	MR	M	86	12 Nov 2012	ETA	≤ 0.25	4	≤ 1	≤ 0.12	U2	U
CA26	TP	M	57	26 Nov 2012	ETA	0.5	≤ 1	≤ 1	≤ 0.12	H1	H
CA27	ZaJ	F	79	28 Nov 2012	ETA	0.5	≤ 1	≤ 1	≤ 0.12	H2	H
CA28	ZS	F	94	28 Nov 2012	ETA	0.5	≤ 1	≤ 1	≤ 0.12	S	S
CA29	KM	F	76	18 Dec 2012	ETA	0.5	≤ 1	≤ 1	≤ 0.12	I1	I
CA30	CM	F	69	24 Dec 2012	ETA	≤ 0.25	≤ 1	≤ 1	≤ 0.12	B	B
CA31	KH	F	62	28 Dec 2012	ETA	0.5	≤ 1	≤ 1	≤ 0.12	I2	I
CG1	SZ	F	80	28 Apr 2011	ETA	≤ 0.25	8	≤ 1	≤ 0.12	a	a
CG2	WM	M	80	30 May 2011	ETA	0.5	4	≤ 1	0.25	f1	f
CG3	PJ	M	80	15 Jun 2011	ETA	1	8	≤ 1	≤ 0.12	g1	g
CG4	WJ	F	47	29 Jun 2011	ETA	0.5	8	≤ 1	≤ 0.12	h	h
CG5	CA	M	57	16 Sep 2011	ETA	1	8	≤ 1	≤ 0.12	g2	g
CG6	SL	M	90	07 Oct 2011	ETA	1	4	≤ 1	≤ 0.12	i	i
CG7	SJ	M	70	28 Oct 2011	ETA	0.5	32	≤ 1	≤ 0.12	f2	f
CG8	WE	F	37	31 Jan 2012	ETA	1	8	≤ 1	1	e2	e
CG9	PZ	M	87	04 Apr 2012	ETA	0.5	4	≤ 1	0.25	d1	d
CG10	LG	F	72	18 Apr 2012	ETA	1	4	≤ 1	≤ 0.12	d1	d
CG11	JJ	M	57	07 Aug 2012	ETA	0.5	4	≤ 1	≤ 0.12	e1	e
CG12	BG	F	77	08 Aug 2012	ETA	0.5	8	≤ 1	≤ 0.12	c	c
CG13	ZJ	F	68	04 Oct 2012	ETA	0.5	2	≤ 1	≤ 0.12	d3	d
CG14	OJ	M	69	05 Oct 2012	ETA	1	2	≤ 1	≤ 0.12	d2	d
CG15	GG	F	78	25 Oct 2012	ETA	≤ 0.25	2	≤ 1	≤ 0.12	d4	d
CG16	GH	F	87	18 Dec 2012	ETA	0.5	2	≤ 1	≤ 0.12	j	j
CG17	CM	F	69	24 Dec 2012	ETA	0.5	8	≤ 1	≤ 0.12	b	b

AMB: amphotericin B; CA1-CA31: *C. albicans* strains; CG1-CG17: *C. glabrata* strains; ETA: endotracheal aspirate; F: female;
FLC: fluconazole; M: male; VRC: voriconazole; 5FC: 5-fluorocytosine.

oligonucleotides, respectively. The results of the RAPD reactions obtained with five primers were used for the construction of dendrograms.

Among 31 *C. albicans* strains, 31 RAPD profiles were distinguished. Twenty *C. albicans* isolates were allocated into nine clusters (E, H-K, M, O, T, U) with homology levels higher than 80%, whereas the remaining 11 genotypes (A-D, F, G, L, N, P, R, S) were represented by single isolates only. Four isolates belonged to cluster E, while clusters H-K, M, O, T and U consisted of two strains each (Fig. 1).

The strains CA11 and CA12, cultured from ETA and urine of the same patient, were unrelated with homology levels under 80%. Two pairs of isolates, CA17-CA22 (cluster E) and CA29-CA31 (cluster I) showed the highest degree of similarity with 94% and 90% homology, respectively. None of the *C. albicans* isolates revealed the same RAPD profile when results from all primers were combined to generate the dendrogram (Fig. 1, Table II).

Sixteen patterns were observed in the RAPD analysis of the 17 *C. glabrata* strains. When 80% homology

was used as a cut-off point, the *C. glabrata* strains were allocated into four genetic similarity groups (d-g) and six unique profiles were distinguished (a-c, h-j) (Fig. 2). The cluster d comprised of five isolates that presented four different patterns, whereas the remaining four clusters, e-g, contained two isolates each. Among the *C. glabrata* strains, only two isolates, CG9 and CG10 (d1), were identical. Both of them were collected in April 2012 from two patients hospitalised in the ICU. Despite 100% homology, they presented different MIC values to the antifungal agents amphotericin B (MIC 0.5 and 1 mg/L) and voriconazole (MIC 0.25 and ≤ 0.12 mg/L) (Table II). The analysis of the dendrogram revealed three groups of isolates: CG8 and CG11 (cluster e), CG9, CG10 and CG14 (cluster d), CG13 and CG15 (cluster d), with 97%, 91% and 90% homology, respectively.

The discriminatory power (DP) of the RAPD method was calculated for each species according to the Simpson index (Hunter & Gaston 1988). When all tested oligonucleotides were combined, the DP value was 1 for *C. albicans* and 0.99 for *C. glabrata*.
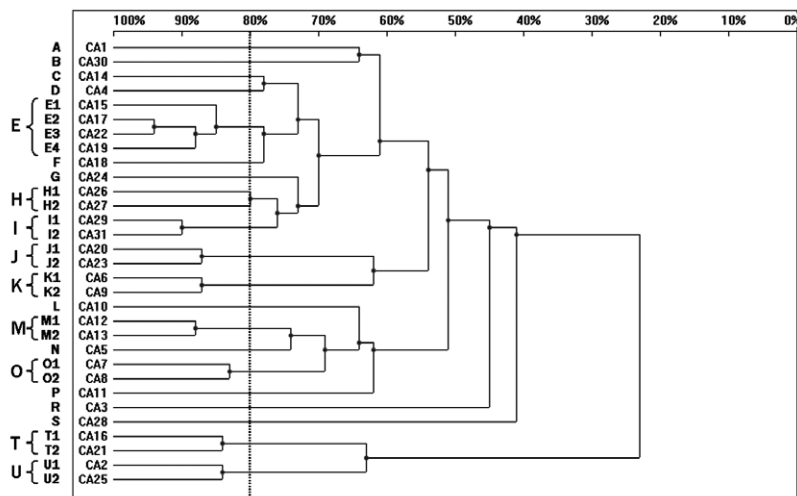

Fig. 1: dendrogram presenting the genetic relatedness of 31 *Candida albicans* strains from intensive care unit patients generated by random amplified polymorphic DNA polymerase chain reaction, using CD16AS, HP1247, ERIC-2, OPE-3 and OPE-18 primers. The vertical line divides the strains according to the level of genetic similarity into related and unrelated.
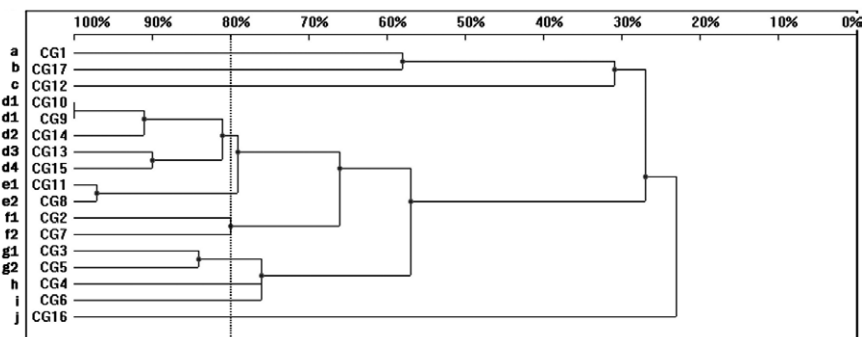

Fig. 2: dendrogram presenting the genetic similarity among 17 *Candida glabrata* strains generated by random amplified polymorphic DNA polymerase chain reaction, using CD16AS, HP1247, ERIC-2, OPE-3 and OPE-18 primers. The vertical line divides the strains according to the level of genetic similarity into related and unrelated.

DISCUSSION

C. albicans is an important cause of nosocomial infections; however, in the last decade, non-*albicans* species were responsible for an increasing number of infections (Bacelo et al. 2010, Nawrot et al. 2013). Yeasts are members of the normal microflora of the skin, mucous membranes and gastrointestinal tract in healthy individuals. However, as opportunistic pathogens, they may also cause endogenous infections in immunocompromised patients. Exogenous *Candida* infections are observed less frequently (Bacelo et al. 2010, Bonfim-Mendonça et al. 2013). Knowing the source of infection and detection of possible routes of transmission can help with the prevention of clonal spread of pathogenic fungi. RAPD is one of the most frequently used genotyping methods for epidemiological investigations of *Candida* infections worldwide (Lian et al. 2004, Marol & Yücesoy 2008, Muthig et al. 2010, Karaman et al. 2013). This technique is easy to perform as well as rapid, cost-effective and suitable for the typing of a large number of strains (Noumi et al. 2009). RAPD may result in low interlaboratory reproducibility, nevertheless this technique can be applied for the investigation of *Candida* epidemiology on a local level (Saghrouni et al. 2013).

In our study, we performed RAPD reactions to evaluate the genetic relatedness of *C. albicans* and *C. glabrata* isolates from southern Poland. We noted a high DP of the RAPD technique. This fact has also been supported by other investigators who reported that RAPD was able to clearly distinguish the genomic variability within the *Candida* isolates (Robert et al. 1995, Pinto et al. 2004).

In the molecular typing of *C. albicans*, there were no strains that generated identical RAPD profiles, which may indicate an endogenous source of infection. These findings were in accordance with results obtained by da Costa et al. (2012) who studied the genetic homology of 15 *C. albicans* strains using five primers. Similar results were found by Marol and Yücesoy (2008) who used four primers for typing 42 *C. albicans* strains recovered from ICU patients. Furthermore, four *C. albicans* isolates cultured from various specimens from the same patients were determined to be different (Marol & Yücesoy 2008). In our study, the strains CA11 and CA12, isolated from the respiratory tract and urinary system of a single patient, were also unrelated. However, in some studies, *C. albicans* strains cultured from two sources from the same patient were genotypically identical. Brillowska-Dabrowska et al. (2010) typed 34 *C. albicans* strains that revealed the same RAPD patterns in blood samples and colonisation specimens collected from a single patient. Bonfim-Mendonça et al. (2013) also observed that *C. albicans* strains recovered from different sites from a single patient were identical. Such results indicate the migration of yeasts from colonisation sites and confirm that previous colonisation is an important factor predisposing for systemic infection.

Molecular typing of 17 *C. glabrata* isolates revealed 16 different genotypes. We observed two strains generating identical RAPD profiles. Another study analysing the diversity of 19 *C. glabrata* isolates originating from oral and vaginal specimens from Tunisia hospitals, revealed an even higher degree of genetic diversity among strains (Noumi et al. 2009). Much lower genotypic variability of *C. glabrata* isolates, with two primers tested, was shown in a recent study from Krasnodębska-Szponder et al. (2005). The investigation performed by Szweda et al. (2013) revealed 26 genotypes among 54 *C. glabrata* isolates, but it is noteworthy that the authors used only one primer in the study. Additionally, Ergon and Gülay (2005) observed a lower degree of genetic diversity among *C. glabrata* strains. The authors suggested that it is possible that primers used in the study were not appropriate for the typing of non-albicans species (Ergon & Gülay 2005). Some authors highlighted that the strains classified as identical by one primer are not always classified as belonging to the same cluster when analysed by another primer (Bonfim-Mendonça et al. 2013). Therefore, the data obtained with a number of oligonucleotides should be combined to generate higher DP (Soll 2000).

In the present study, we noticed a few groups of *C. albicans* and *C. glabrata* strains with similarity values between 80-99%, which represented related, but not identical clinical isolates. These findings may suggest microevolutionary changes of a single strain occurring during adaptation to environmental conditions (Soll 2000, Bonfim-Mendonça et al. 2013). Furthermore, there was the possibility that the unique strains presented in the dendrograms belonged to other species, such as *Candida africana* or *Candida bracarensis*, which are closely related to *C. albicans* and *C. glabrata*. However, these species can be distinguished only with molecular methods, including sequencing of the D1/D2 region of 26S rDNA (Taverna et al. 2013).

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