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ARTICLE

Candida glabrata strain relatedness by new microsatellite markers

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Abstract We investigated six microsatellite markers to type 85 unrelated and 118 related isolates of *Candida glabrata* from 36 patients. Three new markers were selected from the complete sequence of CBS138 and three previously described markers, RPM2, MTI and ERG3 were used. We found a genetic diversity of 0.949 by combining four of them. By applying the new microsatellite markers GLM4, GLM5 and GLM6 we were able to discriminate 29 isolates, originally identified by the more established markers, RPM2, MTI and ERG3. When epidemiologically closely related isolates from 36 patients were typed, 25 patients (72%) exhibited identical or highly related multilocus genotypes. We noted a microvariation in 4 of the patients. This minor change of one locus could be explained by a single step mutation. Since one of these patients had not received antifungal treatment; thus, the relationship between genome variation and antifungal therapy remains controversial. We can conclude from our analysis of these new microsatellite markers that they are highly selective and therefore should be considered as a useful typing system for differentiating related and unrelated isolates of *C. glabrata,* as well as being able to detect microvariation.

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Introduction

Candida glabrata has recently emerged as a major pathogen, causing both mucosal and systemic infections [1, 2]. Several molecular methods have been used to differentiate *C. glabrata* strains. Most of them provide good discrimination power such as electrophoretic karyotyping, multilocus sequence typing (MLST), Southern blotting with probes and randomly amplified polymorphic DNA [3, 4]. Nowadays, microsatellite or simple tandem repeats (STR) polymorphism analyses are more commonly used for fungal typing [5, 6, 7]. In most cases, these techniques have satisfied length variations, thus making them sui for large-scale epidemiological studies of *C. glabrata*. Several polymorphic microsatellite loci have been identified in the *C. albicans* genome and provide good discriminatory power by combining up to three microsatellites used in multiplex PCR strategy [8, 9, 10]. For *C. glabrata*, only a limited number of authors have used microsatellite markers to investigate the delineation of clinical *C. glabrata* isolates [11, 12, 13]. The association of nine microsatellites provides better discrimination between *C. glabrata* isolates [11]. Genetic typing of *C. glabrata* can also provide information on strain variation, such as replacement, microvariation, and maintenance in the hosts during consecutive episodes of infection [14, 15]. The aim of our study was to assess genotype variation and its relationship to antifungal therapy between related isolates.

Materials and methods

Yeast isolates and identification

Eighty-five isolates from different anatomical sites of 85 patients were collected (40 blood cultures; 6 deep site localisation: 2 kidney abscesses, 2 lung abscesses, 1 spondylitis and 1 gastric biopsy; 36 urinary samples and 3 vaginal swabs). Of them, 36 patients were reviewed and sequential isolates were collected from each patient. One hundred and eighteen were from peripheral sites from consecutive episodes of urinary or vaginal infection. Isolates were collected both from inpatients on different wards (intensive units, infectious disease, renal unit or endocrinology wards) and from outpatients. Sequential samples were obtained and the interval time between the first and the last varied from 1 week to several months for each patient. Two *C. glabrata* reference strains were used in this study, CBS138 and ATCC 90020. For identification, isolates were plated onto Candiselect ID medium (Bio-rad, France) at 37°C for 48 h. *C. glabrata* was identified by its green colour and this identification was confirmed by ID32C (Biomérieux, France) assimilation test and Glabrata RTT (Fumouze, Diagnostic, France).

DNA extraction

Genomic DNA was prepared from cell pellets obtained from 5 ml of fresh overnight culture in YPG. Yeasts were digested with lytic enzyme followed by SDS/proteinase K. After proteinase K treatment, the DNA was purified with phenol/chloroform, and precipitated with ethanol, as described previously [16].

Typing yeast isolates

The molecular typing of the *Candida glabrata* isolates obtained was performed using the multiplex PCR technique. Of the 6 microsatellite markers used for this technique, 3 were known markers [12] and 3 were our new ones. The latter were selected from the *C. glabrata* DNA sequences available in the GenBank database by using Tandem Repeats Finder software (http://tandem.bu.edu/trf/trf.html). Only tri microsatellite loci with perfect repeat sequences (having 100% identities between repeat units) with a copy number of >11 were selected. Imperfect repeats containing point mutation and/or insertion or deletion or having "mismatches" in their repeated units, were excluded. This software permit a flanking sequence for these selected microsatellites to be given. Primer sequences were designed with Primer3 software [17], and locations in the *C. glabrata* genome were determined using the Genolevures database (http://cbi.labri.fr/Genolevures/). The forward primers were labelled with a fluorophore (Applied Biosystems, Courtaboeuf, France; Table 1). PCR was conducted in a 20-µl reaction volume containing 2 µl of *C. glabrata* DNA, 1X STR* buffer (Promega, USA), 1.25 U of Ampli Taq gold (Applied Biosystems, Courtaboeuf, France), a reverse primer and a 5′-dye-labelled forward primer at 5 pM of RPM2, GLM4, GLM5 and GLM6, 10 pM for ERG3 and 20 pM of MTI marker (Table 1). After an initial step of 10 min at 95°C, the PCR included 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by an additional step of 5 min at 72°C. One microlitre of the PCR mixture was then added to 24 µl of formamide containing 0.5 µl of Genscan LIZ 500 marker (Applied Biosystems, Courtaboeuf, France) and denaturated for 2 min at 95°C. The PCR products were subjected to electrophoresis on an ABI 310 sequence analyser and the data were analysed with the Genescan software (Applied Biosystems, France).

Sequence analysis

To verify that the differences observed between the different alleles were due to the number of microsatellite

Table 1 Characteristics of the six microsatellite markers used for the analysis of polymorphism length

Marker	Repeats in CBS138	Primer sequences	Marker location
GLM4	$(TTA)_{11}$	FAM 5′ AGTGTTCATTGTCGCCTTC AATGCAGGCTCACCATTTTC	Chromosome C
GLM5	$(TCA)_{11}$	VIC5′ TGGGGATAGTGGGAACTCAA CGATGATTTCATGTCCGATG	Chromosome G
GLM6	$(CAA)_{16}$	FAM 5′GATGATTCTGCCCGTTAGGA CCTGAAGTAGGTGCCGAGAG	Chromosome I
RPM2	$(ACAAGC)_7$	FAM 5′ATCTCCCAACTTCTCGTAGCC ACTTGAACGACTTGAACGCC	Chromosome I
MTI	$(A)_{10}$….$(CAACAAA)_5$……$(CAAAAA)_1$ ……	FAM 5′CAGCAATAATAGCTTCTGACTATGAC GACAGGAGCAACCGTTAGGA	Chromosome D
ERG3	Irregular motif	VIC 5′ AGTGCGAGTGTATGTAAAGAATG CGTATACCTTATCTCCGTTCAA	Chromosome F

sequence repeats, a simple PCR was performed for each marker alone and then sequenced after amplification using unlabelled primers. The PCR products were purified using the Mini Elute purification kit (Qiagen, France) and subjected to bidirectional cycle sequencing with the Big Dye terminator V3.0 reaction kit. The sequencing reaction mixtures were analysed by an ABI 310 sequence analyser with Sequence Analysis software (Applied Biosystems, France).

Genetic parameter study

To determine all genetic parameters, we used Arlequin program software version 3.1. We calculated the allelic frequency, the discriminatory power by using Simpson's index of diversity [18] and molecular distance to group-unrelated isolates according to their genetic distance [19]. For the same patient, when isolates had the same genotype at all six loci, isolates were considered identical; a micro-variation was considered if two isolates differed by only one loci and isolates were noted to be different if two or more loci had a different size.

Statistical analysis

An UPGMA (unweighted pair group method with arithmetic mean) method was employed for phylogenetic analysis (http://pubmlst.org) and correlation analyses (Spearman's Rho) were performed using SPSS (Version 14.0).

Results

A total of 1,310 repeat unit motifs were obtained from the genomic DNA of *C. glabrata*. Three new microsatellites were selected and were used to type 85 unrelated *C. glabrata* isolates and 118 related isolates. This analysis revealed that all loci were polymorphic and each locus presented 4–9 alleles (Table 2).

The various alleles observed at all loci were sequenced in order to determine the nature of the polymorphisms observed. The sequencing result confirmed that the GLM4, GLM5, and GLM6 alleles analysed were simple STR with one variable repetitive motif, and the differences in the molecular weights of the distinct alleles reflected the differences in the number of the repeated motifs.

The polymorphism study of 85 unrelated isolates with six microsatellite markers provided 37 different genotypes (Table 3). Some multilocus genotypes were more frequent than others and represented respectively 15.29%, 14.11%, 9.41% and 8.23%. For 25 isolates the multilocus genotype was unique and specific. Haplotypic distribution for the same isolate group was also calculated for only three previously described microsatellites, RPM2, MTI and ERG3. We found 19 multilocus genotypes (Fig. 1).

Discriminatory power

The diversity index of each locus was calculated using Simpson's formula as previously mentioned. The diversity index varied from 0.521 for RPM2 to 0.788 for GLM5 (Table 2). We found a discriminatory power of 0.941 by combining the six microsatellites. Moreover, a higher discriminatory power was obtained when combining four of them: MTI, ERG3, GLM4 and GLM5 (H=0.949).

Specificity and reproducibility

We were able to reproduce our results using the PCR technique with the sequencing size of the same DNA sample several times and the same result was found. Primer specificity was checked by analysing *C. glabrata* reference strains ATCC 90020 and CBS138 and non-*C. glabrata* reference strains. Primers failed to amplify the DNA of *Candida albicans* 3153A, *Candida parapsilosis* ATCC 22109, *Cryptococcus neoformans* wm179 and *Candida krusei* ATCC 6258.

Variation of genotype and susceptibility to fluconazole of isolates during sequential samples

The multiplex assay applied to differentiate multiple isolates from the same patient (36 patients), showed the same genotype in 61% of cases (22 patients) (Table 4). Among them, 4 patients who underwent fluconazole therapy acquired resistance to the drug (patients 12, 17, 20 and 25). Highly related multilocus genotype was detected in 11% of cases (patients 3, 7, 27 and 28), which differed by only one locus in ERG3 (patient 28), GLM4 (patient 7) and GLM5 (patients 3 and 27). Patients 3, 7 and 28 underwent fluconazole therapy. A microvariation and a new type of isolate occurred simultaneously in patient 3. On the other hand, 11 patients showed different genotypes. Among them,

Table 2 Diversity index for each microsatellite marker used

Markers	RPM2	MTI	ERG3	GLM4	GLM5	GLM6
Number of alleles	4	6	9	7	8	4
Size (bp)	121–139	227–247	181–260	261–288	259–307	280–325
Diversity index	0.521	0.714	0.757	0.751	0.788	0.545

Table 3 Haplotype distribution of 85 unrelated *C. glabrata* isolates

Haplotype	RPM2	MTI	ERG3	GLM4	GLM5	GLM6	Number of isolates (%)
1	121	246	190	267	262	295	3 (3.5)
2	127	227	181	276	265	325	1 (1.2)
3	127	227	227	267	259	325	1 (1.2)
4	127	227	181	273	301	295	1 (1.2)
5	127	237	197	273	259	298	4 (4.7)
6	127	237	197	273	301	295	1 (1.2)
7	127	237	197	273	295	295	1 (1.2)
8	127	238	197	273	304	295	2 (2.3)
9	127	238	197	288	271	295	7 (8.2)
10	127	238	197	273	298	295	12 (14.1)
11	127	238	197	267	262	295	3 (3.5)
12	127	238	197	270	298	295	1 (1.2)
13	127	238	197	273	301	295	1 (1.2)
14	127	238	197	270	271	289	1 (1.2)
15	127	238	197	273	259	295	1 (1.2)
16	127	238	197	276	262	298	1 (1.2)
17	127	238	198	273	298	295	3 (3.5)
18	127	238	198	273	262	295	2 (2.3)
19	127	238	227	273	259	298	1 (1.2)
20	127	239	198	273	298	295	1 (1.2)
21	127	239	227	267	259	325	1 (1.2)
22	127	239	260	276	259	325	8 (9.4)
23	127	239	260	276	262	298	1(1.2)
24	127	240	200	273	298	295	1 (1.2)
25	133	239	190	264	259	325	1 (1.2)
26	133	240	204	264	259	325	13 (15.2)
28	133	240	204	276	259	298	1 (1.2)
27	133	240	205	264	259	325	1 (1.2)
29	139	227	227	276	262	298	1 (1.2)
30	139	238	197	273	262	295	2 (2.3)
31	139	238	198	273	301	295	1 (1.2)
32	139	238	227	258	301	259	1 (1.2)
33	139	239	227	276	262	298	1 (1.2)
34	139	239	227	276	259	298	1 (1.2)
35	139	239	227	276	262	295	1 (1.2)
36	139	239	227	276	262	325	1 (1.2)
37	139	239	227	261	262	280	1 (1.2)

7 underwent antifungal therapy. In 2 patients (patients 3 and 11), a new genotype occurred, as well as a low susceptibility to fluconazole.

Phylogenetic analysis

Phylogenetic analysis was performed by the three previously described and by all six hyper-variable loci. Multilocus genotype distribution among unrelated isolates has been shown in Figs. 1 and 2 respectively. Isolates were collected from various anatomical sites; statistical analysis failed to show any correlation between body location of isolates and multilocus genotype data ($P>0.05$).

Discussion

In this study, we investigated 85 unrelated isolates of *C. glabrata* with 3 new microsatellite markers compared with 3 known and used markers. We found a genetic diversity (H) of 0.941. Foulet was the first to describe polymorphic microsatellite markers as a method of identifying and

Table 4 Genotypes of sequential isolates from recurrent infections of *Candida glabrata*

Patient	Number of episodes	Number of isolates	CMI FCZ (μg/ml)	Anatomical site	RPM2	MTI	ERG3	GLM4	GLM5	GLM6	Antifungal therapy
1	5	1	4	Urine	121	246	190	267	262	295	Fluconazole
2	3	1	0.125	Urine	127	238	197	273	304	295	Fluconazole
		2	2		133	240	204	264	259	325	
3	6	1	8	Urine	127	238	197	273	304	295	Fluconazole
		2	256		127	238	197	288	271	295	
		3	6		127	238	197	273	298	295	
4	3	1	2	Urine	127	238	197	273	298	295	Fluconazole
5	2	1	256	Vagina	133	240	204	264	259	325	Fluconazole
6	4	1	4	Urine	121	246	190	267	262	295	Fluconazole
7	9	1	6	Urine	127	238	197	288	271	295	Fluconazole
		2	0.016		127	238	197	276	271	295	
8	2	1	256	Urine	127	238	197	288	271	295	Fluconazole
		2	3								
9	2	1	256	Urine	127	238	197	273	298	295	No treatment
		2	256								
10	7	1	4	Urine	127	238	197	267	262	295	Fluconazole
		2	2		127	238	197	276	259	295	
		3	1.5		127	238	197	273	298	325	
11	5	1	1.5	Urine	127	238	197	273	298	295	Fluconazole
		2	32		133	240	197	264	259	325	
		3	256		139	238	197	289	271	295	
12	3	1	1.5	Urine	127	238	197	273	298	295	Fluconazole
		2	256		127	238	197	273	298	295	
13	3	1	0.125	Urine	127	239	197	273	298	295	No treatment
14	2	1	4	Urine	133	240	205	264	259	325	Fluconazole
15	2	1	0.125	Urine	127	240	200	273	298	295	Fluconazole
		2	2		127	238	228	273	298	295	
16	2	1	4	Urine	133	240	204	264	259	325	No treatment
17	2	1	15	Urine	139	227	227	276	262	298	Fluconazole
		2	256		139	227	227	276	262	298	
18	7	1	4	Urine	133	240	204	264	259	325	Fluconazole
19	2	1	1.25	Urine	127	238	197	273	298	295	Fluconazole
20	6	1	6	Vagina	133	240	204	264	259	325	Fluconazole
		2	8		127	238	197	289	271	295	
		3	256		127	238	197	289	271	295	
		4	0.25		127	238	197	273	298	295	
21	4	1	1	Vagina	127	238	198	273	298	295	Fluconazole
		2	3		133	240	204	273	307	295	
22	5	1	16	Urine	127	239	260	276	259	325	No treatment
		2	0.125								
23	3	1	1	Urine	127	238	197	267	262	295	No treatment
		2	32		133	240	204	264	259	325	
		3	0.5		127	238	197	273	301	295	
24	2	1	0.25	Urine	127	238	197	288	271	295	No treatment
		2	1		139	239	227	261	262	280	
25	2	1	4	Urine	127	238	197	270	271	289	No treatment
		2	256		127	238	197	270	271	289	
26	2	1	1.5	Urine	133	240	204	264	259	325	No treatment
27	2	1	1	Urine	127	238	197	273	259	295	No treatment
		2	1		127	238	197	273	271	295	
28	3	1	1.5	Urine	127	238	228	273	298	295	Fluconazole
		2	2		127	238	197	273	298	295	

Table 4 (continued)

Patient	Number of episodes	Number of isolates	CMI FCZ (μg/ml)	Anatomical site	RPM2	MTI	ERG3	GLM4	GLM5	GLM6	Antifungal therapy
29	3	1	2	Urine	139	239	228	276	262	298	No treatment
		2	0.125		133	239	228	264	259	325	
30	2	1	1	Urine	133	240	198	273	298	295	No treatment
		2	1		127	239	197	273	298	295	
31	2	1	0.125	Urine	133	240	204	264	259	325	No treatment
32	2	1	1.25	Urine	127	239	228	267	259	325	No treatment
33	3	1	16	Urine	139	239	228	276	262	298	No treatment
34	2	1	8	Urine	127	239	260	276	259	325	No treatment
35	5	1	2	Urine	139	239	227	276	262	295	Fluconazole
36	5	1	1	Urine	127	238	197	273	298	295	Fluconazole

delineating clinical *C. glabrata* isolates [12]. He found a discriminatory power of 0.84 by using a combination of the RPM2, MTI and ERG3 microsatellites. Grenouillet et al. described 6 new microsatellite markers, 4 of them provided a higher discriminatory power (ID=0.902) [13]. More recently, Brisse et al., reported 9 markers that provided a

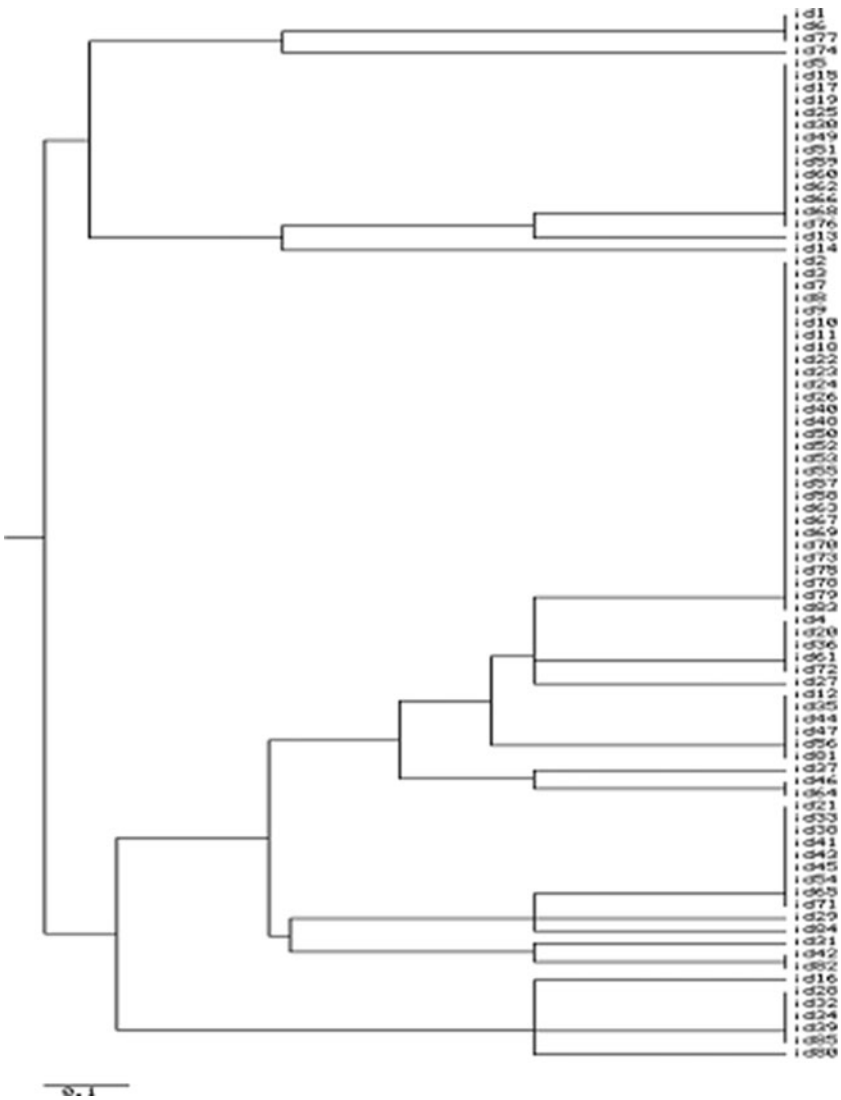

Fig. 1 Dendrogram generated by UPGMA (unweighted pair group method with arithmetic mean) methods showing the relationships among 85 unrelated *Candida glabrata* isolates by three microsatellite markers, RPM2, MTI and ERG3

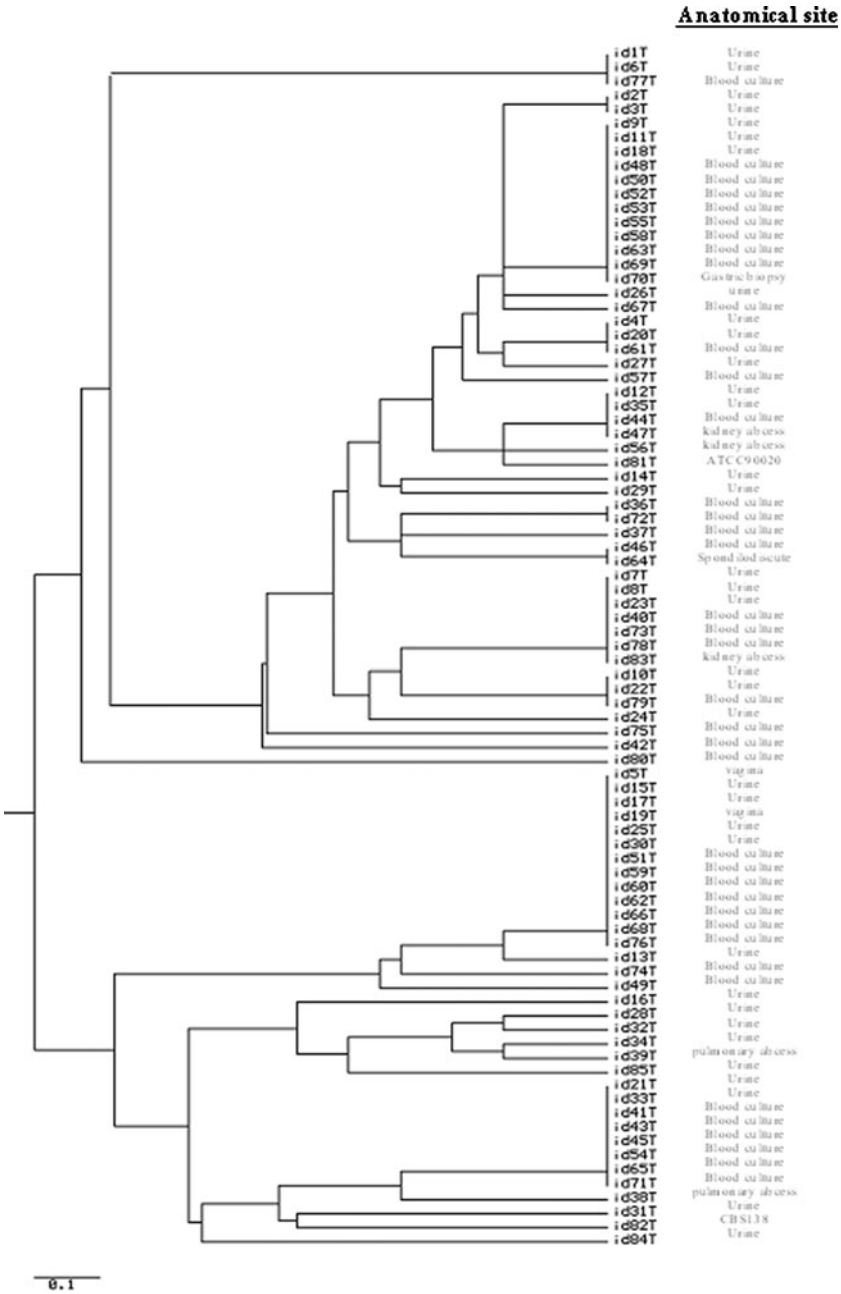

Fig. 2 Dendrogram generated by UPGMA (unweighted pair group method with arithmetic mean) methods showing the relationships among 85 unrelated *Candida glabrata* isolates by the six microsatellite markers

discriminatory power (ID=0.96) [11]. In our study, by combining 4 microsatellite markers, we achieved a high discriminatory power (ID=0.949), making this method suitable and less expensive for epidemiological studies and routine use in clinical laboratory.

Our research was based on random distribution of alleles and revealed that several alleles were dominant, since 4 multilocus genotypes represented 47% of the isolates studied. Our sample collection of isolates was obtained from a restricted geographical area that could partially explain the predominance of some of the genotypes [12, 20]. Indeed, Dodgson AR et al. analysed 103 isolates from diverse geographic origin by MLST and identified 5 major clades [21]. Three of them exhibited significant geographical bias. Other investigators assigned some genotypes a selective ecological advantage [12, 13].

The use of the three new microsatellite markers in assessing genetic ties of unrelated samples disclosed 18 additional multilocus genotypes that had not been identified by RPM2, MTI and ERG3 markers and allowed the discrimination of 29 isolates that were confirmed as being identical by RPM2, MTI and ERG3. The use of GLM4, GLM5 and GLM6 allowed the subdivision of the most common mutilocus genotype (127-238-197) into 9 separate groups. Also, the 3 new markers confirmed the identity of 12 additional isolates.

Lin and al., when analysing 80 isolates, identified 15 sequence types and 54 genotypes by MLST and PFGE respectively [22]. In our setting, 85 isolates yielded 37 multilocus genotypes. Microsatellite markers seem to have a better discriminatory power than MLST.

These isolates were analysed for their genetic variability, but no correlation was found between genotype and anatomical site (superficial or deep location) as suggested by some authors [21, 23].

When 118 epidemiologically related isolates were taken from 36 patients, 26 (73%) exhibited identical or highly related multilocus genotypes (4 cases of microvariation). The highly selective power of these new microsatellite markers revealed the presence of three basic figures: isolates with the same genotype, isolates showing microvariation and isolates with different types. Of the 4 patients whose isolates revealed microvariation, 1 had not received antifungal treatment; thus, the relationship between genetic variation and antifungal therapy remains controversial. Several investigators analysed genotypic variability, among *C. glabrata*-related bloodstream isolates without antifungal therapy, by electrophoretic karyotyping, and showed that fine karyotypic changes can be developed rapidly [24, 25].

Furthermore, isolates from 11 patients (28%) displayed different multilocus genotypes, suggesting strain replacement. The apparition of isolates with different genotypes in recurrent infection was documented by some authors [15, 20] and two hypotheses could be evoked. The first was that these patients were initially colonised by various isolates and a selection of one isolate occurred under some factors, such as antifungal treatment, as observed in patients 3 and 11. The second hypothesis was a reinfection with a new isolate from another anatomical site or environmental source.

In conclusion, the analysis with the new microsatellite markers provided high discriminatory power, allowing a useful typing system to differentiate unrelated and related isolates and to detect microvariations.

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