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ORIGINAL ARTICLE

Multilocus microsatellite analysis of European and African *Candida glabrata* isolates

V. Chillemi[1] · C. L. Passo[1] · A. D. van Diepeningen[2] · S. Rharmitt[1] · D. Delfino[3] · A. Cascio[3] · N. E. Nnadi[4] · B. D. Cilo[5] · P. Sampaio[6] · H.-J. Tietz[7] · J. Pemán[8] · G. Criseo[1] · O. Romeo[1,9] · F. Scordino[9]

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Abstract This study aimed to elucidate the genetic relatedness and epidemiology of 127 clinical and environmental *Candida glabrata* isolates from Europe and Africa using multilocus microsatellite analysis. Each isolate was first identified using phenotypic and molecular methods and subsequently, six unlinked microsatellite loci were analyzed using automated fluorescent genotyping. Genetic relationships were estimated using the minimum-spanning tree (MStree) method. Microsatellite analyses revealed the existence of 47 different genotypes. The fungal population showed an irregular distribution owing to the over-representation of genetically different infectious haplotypes. The most common genotype was MG-9, which was frequently found in both European and African isolates. In conclusion, the data reported here emphasize the role of specific *C. glabrata* genotypes in human infections for at least some decades and highlight the widespread distribution of some isolates, which seem to be more able to cause disease than others.

Introduction

Candida glabrata is a haploid yeast historically believed to be harmless to humans and for a long time regarded as a non-pathogenic saprophyte of the normal flora of healthy individuals [1]. However, in recent decades, this view has been gradually weakened as a growing number of clinical reports have shown that this fungus is, on the contrary, an important pathogen [2–4].

The increase in patients with an impaired immune system, in addition to the widespread use of immunosuppressive therapies and broad-spectrum antibiotic drugs, has greatly contributed to rendering *C. glabrata* one of the most commonly isolated yeasts among all non-*C. albicans* species that infect humans [2–4]. However, although *Candida albicans* remains the most frequently isolated fungal pathogen in humans, *C. glabrata* has been reported to be an significant cause of oral infections, especially in elderly people [4, 5]. In addition,

✉ O. Romeo
oromeo@unime.it

[1] Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale F. Stagno d'Alcontres, 31, 98166 Messina, Italy

[2] CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

[3] Department of Human Pathology, University of Messina, Via Consolare Valeria, 98125 Messina, Italy

[4] Department of Microbiology, Plateau State University, P.M.B 2012, Bokkos, Plateau State, Nigeria

[5] Medical Microbiology Department, Faculty of Medicine, Uludag University, 16059 Bursa, Turkey

[6] Biology Department, Minho University, Campus de Gualtar, 4710-057 Braga, Portugal

[7] Institut für Pilzkrankheiten, Luisenstrasse 50, 10117 Berlin, Germany

[8] Servicio de Microbiología, Hospital Universitario y Politécnico La Fe, Valencia, España

[9] IRCCS Centro Neurolesi "Bonino-Pulejo", SS113, 98124 Messina, Italy

this species also shows a remarkable ability to infect other human body sites including the vagina and urinary tract [6, 7], and in recent years it has undoubtedly emerged as the second-leading cause of bloodstream infections (BSIs) in the USA and in Northern and Central European countries [3].

The reasons for the increased isolation rate of *C. glabrata* are multifactorial and difficult to pinpoint exactly. However, the issue of resistance to azole derivatives [3, 8] and the discovery and evolution of new strains [9, 10] are certainly partly responsible.

For population genetics studies in *C. glabrata*, microsatellites have increasingly become the markers of choice [11–14]. Most microsatellites are noncoding DNA, either in intergenic sequence regions or in introns. Thus, they can generally be assumed to evolve neutrally; thus, their level of polymorphism is proportional to the underlying mutation rate.

In this study, we report the genetic profiles, obtained through analysis of six microsatellite loci, of different clinical and environmental *C. glabrata* isolates from Europe and Africa. Our data showed a south–north and east–west distribution of closely related genotypes and highlight the widespread distribution of particular haplotypes, which seem to be more able to cause infections than others.

Materials and methods

A total of 127 *C. glabrata* isolates, of different clinical and geographical origin, were examined in this study (Table 1). All isolates were initially identified by the ID 32C system (bioMeriéux, France) and subsequently confirmed using a species-specific multiplex PCR-based method that allows *C. glabrata* to be discriminated from its closely related species *Candida bracarensis* and *Candida nivariensis* [15, 16].

Multilocus analysis of polymorphic microsatellite markers was used to evaluate genetic relatedness among our *C. glabrata* isolates [11]. Six microsatellite markers were amplified by PCR using six pairs of primers, as described in Abbes et al. [11]. For automatic allele size determination the forward primer of each pair was 5′-fluorescently labelled with 6-carboxyfluorescein (FAM; GLM4-Fwd, GLM6-Fwd, RPM2-Fwd and MTI-Fwd primers), 6-carboxyhexafluorescein (HEX; GLM5-Fwd primer), and 6-carboxytetramethylrhodamine (TAMRA; ERG3-Fwd primer). For each isolate, six separate PCR amplifications were performed and amplicons were sent out to Eurofins-MWG/Operon (www.eurofinsdna.com) for fragment length analysis using an ABI 3130XL sequencing platform (Applied Biosystems) and GeneScan 500-ROX as the size standard. Allele binning was carried out using the program AutoBin [17]. Genetic relationships were estimated using the minimum-spanning tree (MStree) method, as implemented in BioNumerics, version 4.61 (Applied Maths, St.-Martens-Latem, Belgium).

The discriminatory power (*D*) of the microsatellite typing method used was calculated on-line (http://insilico.ehu.es/mini_tools/discriminatory_power/index.php) using Simpson's index of diversity [18].

For statistical analysis, all isolates were split according to their geographical origin: Africa (n = 22), Greece (n = 15), Italy (n = 30), Portugal (n = 15), Spain (n = 16), Turkey (n = 16) and Middle/Northern Europe (UK, the Netherlands, Austria, Finland, and Germany; n = 13). Haplotype frequencies and genetic diversity within each population in addition to inter-population analysis were computed using the software HAPLOTYPE ANALYSIS version 1.05 (www.uni-goettingen.de/en/134935.html). HAPLOTYPE ANALYSIS calculates genetic diversity within each population based on the number of different haplotypes (A) in that population, the number of unique or "private" haplotypes (P) specific to that population, the effective number of haplotypes (Ne) observed in the population, the estimated maximum haplotype richness or "allele richness" (Rh) defined as the number of alleles per locus and based on the rarefaction method, Nei's index of genetic diversity (He; the probability that two randomly chosen haplotypes are different) estimated without bias and the mean genetic distance between individuals (D_{sh}^2) as measures for genetic diversity.

Fisher's exact tests were used to test whether there were statistical differences in the proportion of the genotypes among the different countries. To better quantify the difference between the countries, we used the four clonal complexes (CCs) recognized in the 47 different microsatellite genotypes by the MStree in Fig. 1. These CCs were grouped into four classes based around the central genotypes.The classified *C. glabrata* populations were compared using Fisher's exact test giving us the probability that populations were similar (p values >0.05) or dissimilar (p values <0.05).

Results

All yeast isolates examined in this study were identified as *C. glabrata* isolates and no *C. nivariensis* or *C. bracarensis* were recovered. The multilocus microsatellite genotypes obtained are shown in Table 1 and Fig. 1.

A total of 84 different alleles were found for the six microsatellite loci analyzed (Table 2). Locus ERG3 showed the highest genotypic diversity with a *D* value of 0.85, whereas the lowest genotypic diversity was observed with the locus GLM5 (D = 0.62; Table 2).

When all six microsatellite markers were combined, a total of 47 diverse multilocus genotypes (MG) were obtained from 127 *C. glabrata* isolates (Fig. 1, Table 1). This led to an index of discrimination of 0.89 (Table 2).

One genotype (MG-9) was the most frequently observed (37 out of 127 isolates; 29 %) followed by genotypes MG-34

t1.1 **Table 1** Results obtained by microsatellite genotyping analysis of the European and African *C. glabrata* isolates examined in this study

t1.2 Isolate	Origin	Year	Samples	Microsatellite loci						Genotype
t1.3				ERG3	RPM2	MTI	GLM4	GLM5	GLM6	
t1.4 CG125	Italy	2006	BPS	230	139	239	279	262	300	MG-1
t1.5 CG2018	Italy	2007	Sputum	200	125	233	284	258	295	MG-17
t1.6 CGES1	Italy	2012	Sputum	264	127	239	277	258	324	MG-4
t1.7 CGES2	Italy	2012	Sputum	207	133	239	265	258	324	MG-9
t1.8 CG2051	Italy	2007	BAL	201	127	238	275	298	295	MG-20
t1.9 CG3416	Italy	2008	BAL	207	133	239	265	258	324	MG-9
t1.10 CG4157	Italy	2009	BAL	207	133	239	265	258	324	MG-9
t1.11 CG3285L[a]	Italy	2008	BAL	230	139	239	279	262	300	MG-1
t1.12 CG3285S[a]	Italy	2008	Blood	230	139	239	279	262	300	MG-1
t1.13 CG3399	Italy	2008	Blood	207	133	239	265	258	324	MG-9
t1.14 CG157	Italy	2006	CVC	207	133	239	265	258	324	MG-9
t1.15 CG4795	Italy	2009	CVC	264	127	239	280	258	324	MG-5
t1.16 CG4768	Italy	2009	CVC	264	127	239	280	258	324	MG-5
t1.17 CG1626	Italy	2007	Feces	264	127	239	280	258	324	MG-5
t1.18 CG1724	Italy	2007	Feces	230	139	239	279	262	300	MG-1
t1.19 CG1828	Italy	2007	Feces	194	121	248	268	262	295	MG-25
t1.20 CG2081	Italy	2007	Feces	264	127	239	277	258	324	MG-4
t1.21 CG2086	Italy	2007	Feces	207	133	239	265	258	324	MG-9
t1.22 CG4836	Italy	2009	Feces	264	127	239	277	258	324	MG-4
t1.23 CG1580	Italy	2007	Urine	207	133	239	265	258	324	MG-9
t1.24 CG2108	Italy	2007	Urine	237	121	239	265	258	287	MG-12
t1.25 CG2453	Italy	2008	Urine	201	127	238	275	298	295	MG-20
t1.26 CG4819	Italy	2009	Urine	207	133	239	265	258	324	MG-9
t1.27 CGLA1	Italy	2011	Urine	207	133	239	265	258	324	MG-9
t1.28 CG1721	Italy	2010	Vaginal	230	127	239	268	258	324	MG-7
t1.29 CG1818	Italy	2007	Vaginal	207	133	239	265	258	324	MG-9
t1.30 CG2074	Italy	2007	Vaginal	276	127	233	280	264	310	MG-22
t1.31 CG2087	Italy	2007	Vaginal	237	133	248	280	270	305	MG-24
t1.32 CG4165	Italy	2009	Vaginal	207	133	239	265	258	324	MG-9
t1.33 CG4206	Italy	2012	Vaginal	207	133	239	265	258	324	MG-9
t1.34 NIG10	Nigeria	2011	Vaginal	264	127	239	277	258	324	MG-4
t1.35 NIG16p	Nigeria	2011	Vaginal	264	127	239	277	258	324	MG-4
t1.36 NIG19Bp	Nigeria	2011	Vaginal	237	133	238	268	258	295	MG-13
t1.37 NIG29p	Nigeria	2011	Vaginal	207	133	239	265	258	324	MG-9
t1.38 NIG64	Nigeria	2011	Vaginal	207	133	239	265	258	324	MG-9
t1.39 NIG71	Nigeria	2011	Vaginal	207	133	239	265	258	324	MG-9
t1.40 NIG108p	Nigeria	2011	Vaginal	207	133	239	265	258	324	MG-9
t1.41 NIG154	Nigeria	2011	Vaginal	207	133	239	265	258	324	MG-9
t1.42 NIG157p	Nigeria	2011	Vaginal	207	133	239	265	258	324	MG-9
t1.43 NIG162p	Nigeria	2011	Vaginal	264	127	246	277	258	324	MG-6
t1.44 NIG173p	Nigeria	2011	Vaginal	207	133	239	265	258	324	MG-9
t1.45 NIG176	Nigeria	2011	Vaginal	241	145	238	268	262	321	MG-32
t1.46 NIG190p	Nigeria	2011	Vaginal	264	127	239	277	258	324	MG-4
t1.47 NIG194	Nigeria	2011	Vaginal	237	133	238	268	258	295	MG-13
t1.48 NIG222p	Nigeria	2011	Vaginal	264	127	239	277	258	324	MG-4
t1.49 NIG223p	Nigeria	2011	Vaginal	237	133	238	280	258	295	MG-14
t1.50 NIG244	Nigeria	2011	Vaginal	207	133	239	265	258	324	MG-9

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t1.51 **Table 1** (continued)

t1.52	Isolate	Origin	Year	Samples	Microsatellite loci						Genotype
					ERG3	RPM2	MTI	GLM4	GLM5	GLM6	
t1.53	CBS12544	Zimbabwe	2011	Fruit pulp	237	127	232	268	258	289	MG-16
t1.54	CBS2175	UK	1955	Vaginal	207	133	239	268	258	324	MG-10
t1.55	CBS2192	South Africa	1955	Malt	207	133	239	265	258	324	MG-9
t1.56	CBS2498	Netherlands	1956	Medium ph2	264	127	239	277	258	324	MG-4
t1.57	CBS4692	Austria	–	GM	207	133	239	265	258	324	MG-9
t1.58	CBS5040	South Africa	–	Insect larvae	207	133	239	265	258	324	MG-9
t1.59	CBS6144	Finland	–	Vaginal	230	127	239	268	258	324	MG-7
t1.60	CBS860	Netherlands	1935	Urine	207	133	239	265	258	324	MG-9
t1.61	CBS861	Netherlands	1942	Mouth	207	133	239	280	258	324	MG-11
t1.62	CBS862	Netherlands	1948	Mouth	230	127	239	268	258	324	MG-7
t1.63	CGS-65	Spain	2011	Blood	207	133	239	265	258	324	MG-9
t1.64	CGS-66	Spain	2011	Blood	207	133	239	265	258	324	MG-9
t1.65	CGS-67	Spain	2011	Blood	207	133	239	265	258	324	MG-9
t1.66	CGS-68	Spain	2011	Blood	264	127	239	277	258	324	MG-4
t1.67	CGS-75	Spain	2012	Blood	207	133	239	265	258	324	MG-9
t1.68	CGS-77	Spain	2013	Blood	230	127	239	268	258	324	MG-7
t1.69	CGS-78	Spain	2013	Blood	230	127	239	268	258	324	MG-7
t1.70	CGS-79	Spain	2013	Blood	207	133	239	265	258	324	MG-9
t1.71	CGS-80	Spain	2013	Blood	207	133	239	265	258	324	MG-9
t1.72	CGS-81	Spain	2013	Blood	216	122	242	268	264	307	MG-29
t1.73	CGS-82	Spain	2013	Blood	194	121	247	268	260	295	MG-26
t1.74	CGS-86	Spain	2014	Blood	207	133	239	265	258	324	MG-9
t1.75	CGS-87	Spain	2014	Blood	230	127	239	268	258	324	MG-7
t1.76	CGS-88	Spain	2014	Blood	276	127	232	280	264	310	MG-23
t1.77	CGS-89	Spain	2014	Blood	215	127	225	268	282	321	MG-44
t1.78	CGS-90	Spain	2014	Blood	202	121	225	277	273	307	MG-30
t1.79	GER1	Germany	2015	Vaginal	230	139	239	277	260	298	MG-2
t1.80	GER2	Germany	2015	Vaginal	230	139	239	268	258	280	MG-3
t1.81	GER3	Germany	2015	Vaginal	216	133	238	271	258	324	MG-15
t1.82	GER4	Germany	2015	Vaginal	230	127	239	265	258	324	MG-8
t1.83	GER5	Germany	2015	Vaginal	207	133	239	265	258	324	MG-9
t1.84	GER6	Germany	2015	Vaginal	207	133	239	265	258	324	MG-9
t1.85	GRE1	Greece	2010	Vaginal	237	127	232	268	258	289	MG-16
t1.86	GRE2	Greece	2010	Vaginal	230	139	239	277	260	298	MG-2
t1.87	GRE3	Greece	2010	Vaginal	201	125	238	268	270	290	MG-21
t1.88	GRE5	Greece	2010	Vaginal	194	121	247	268	260	283	MG-27
t1.89	GRE6	Greece	2010	Vaginal	194	127	247	268	260	310	MG-28
t1.90	GRE8	Greece	2010	Vaginal	230	139	239	277	260	298	MG-2
t1.91	GRE9	Greece	2010	Vaginal	207	133	239	265	258	324	MG-9
t1.92	GRE10	Greece	2010	Vaginal	207	133	239	265	258	324	MG-9
t1.93	GRE11	Greece	2010	Vaginal	207	133	239	265	258	324	MG-9
t1.94	GRE12	Greece	2010	Vaginal	264	127	239	277	258	324	MG-4
t1.95	GRE13	Greece	2010	Vaginal	230	139	239	277	260	298	MG-2
t1.96	GRE16	Greece	2010	Vaginal	207	133	239	265	258	324	MG-9
t1.97	GRE17	Greece	2010	Vaginal	230	127	239	268	258	324	MG-7
t1.98	GRE18	Greece	2010	Vaginal	264	127	239	277	258	324	MG-4
t1.99	GRE19	Greece	2010	Vaginal	215	127	225	265	279	321	MG-45

t1.100 **Table 1** (continued)

t1.101 Isolate	Origin	Year	Samples	Microsatellite loci						Genotype
				ERG3	RPM2	MTI	GLM4	GLM5	GLM6	
t1.102 SAN1B	Morocco	2015	Vaginal	216	134	238	271	260	298	MG-31
t1.103 SAN2	Morocco	2015	Vaginal	235	125	230	263	256	287	MG-37
t1.104 HSJ118	Portugal	2005	Blood	205	131	238	280	256	321	MG-33
t1.105 8B	Portugal	2004	Urine	205	131	238	263	256	321	MG-34
t1.106 CIPO55	Portugal	2005	Feces	205	131	238	263	256	321	MG-34
t1.107 CIPO102	Portugal	2005	GI tract	235	125	230	265	256	321	MG-36
t1.108 HSJ42	Portugal	2005	Blood	205	131	238	263	256	321	MG-34
t1.109 CIPO88	Portugal	2005	Vaginal	205	131	238	263	262	318	MG-35
t1.110 HSJ177	Portugal	2005	Blood	205	131	238	263	256	321	MG-34
t1.111 HSJ117	Portugal	2005	Blood	205	131	238	263	256	321	MG-34
t1.112 CIPO103	Portugal	2005	RF	228	125	237	265	256	321	MG-38
t1.113 HSJ96	Portugal	2005	Blood	262	125	327	275	256	321	MG-42
t1.114 30C	Portugal	2004	Vaginal	205	131	238	263	256	321	MG-34
t1.115 H38	Portugal	2004	Vaginal	205	131	238	263	256	321	MG-34
t1.116 HSJ54	Portugal	2005	Blood	205	131	238	263	256	321	MG-34
t1.117 HSJ55	Portugal	2005	Blood	205	131	238	263	256	321	MG-34
t1.118 CIPO44	Portugal	2005	Feces	205	131	238	263	256	321	MG-34
t1.119 TUR1	Turkey	2014	Blood	198	125	236	272	295	292	MG-46
t1.120 TUR2	Turkey	2013	Blood	205	131	238	263	256	321	MG-34
t1.121 TUR3	Turkey	2014	Urine	228	137	237	275	258	295	MG-18
t1.122 TUR4	Turkey	2014	Urine	228	125	231	265	270	295	MG-43
t1.123 TUR5	Turkey	2013	Urine	228	125	237	265	256	321	MG-38
t1.124 TUR6	Turkey	2014	Urine	262	130	231	275	258	295	MG-19
t1.125 TUR7	Turkey	2013	Urine	205	131	238	263	256	321	MG-34
t1.126 TUR8	Turkey	2014	Urine	198	124	236	265	268	287	MG-47
t1.127 TUR9	Turkey	2014	Urine	205	131	238	263	256	321	MG-34
t1.128 TUR10	Turkey	2014	CSF	228	125	237	265	256	321	MG-38
t1.129 TUR11	Turkey	2013	Blood	228	125	237	265	255	321	MG-39
t1.130 TUR12	Turkey	2015	Urine	198	125	236	272	295	292	MG-46
t1.131 TUR13	Turkey	2014	Blood	198	125	236	272	295	292	MG-46
t1.132 TUR14	Turkey	2015	Urine	234	125	237	278	258	321	MG-41
t1.133 TUR16	Turkey	2014	Urine	243	125	237	268	256	321	MG-40
t1.134 TUR20	Turkey	2014	Blood	228	125	237	265	256	321	MG-38

BPS biopsy of the paravertebral space, *BAL* bronchoalveolar lavage, *CVC* central venous catheter, *GM* case of generalized mycosis, *GI* gastrointestinal, *RF* respiratory fluid, *CSF* cerebrospinal fluid

[a] Same patient

(13 out of 127; 10 %), MG-4 (11 out of 127; ~9 %) and MG-7 (7 out of 127; 5 %). The remaining 43 genotypes were found with an incidence of below 5 % (Table 1). In total, we analyzed 51 vaginal samples, 30 blood samples, and 17 from urine as the three most common sample types, whereas the remaining 29 were from a variety of other specimens. However, statistical analysis showed no significant association between the microsatellite genotypes and the origin or date of isolation of our isolates.

The predominant genotype MG-9 was found in 35 clinical samples from different countries and in two environmental samples (insect larvae and malt) in South Africa. Most of the MG-9 isolates were found in Italian (12 out of 37; 32 %) and African (10 out of 37; 27 %) specimens, whereas the remaining isolates were spread over Spain, Greece, and the Middle/Northern European countries (Fig. 1). Interestingly, another closely related genotype (MG-4), was also frequently encountered in Italy (3 out of 11; 27 %) and Africa (4 out of 11; 36 %;

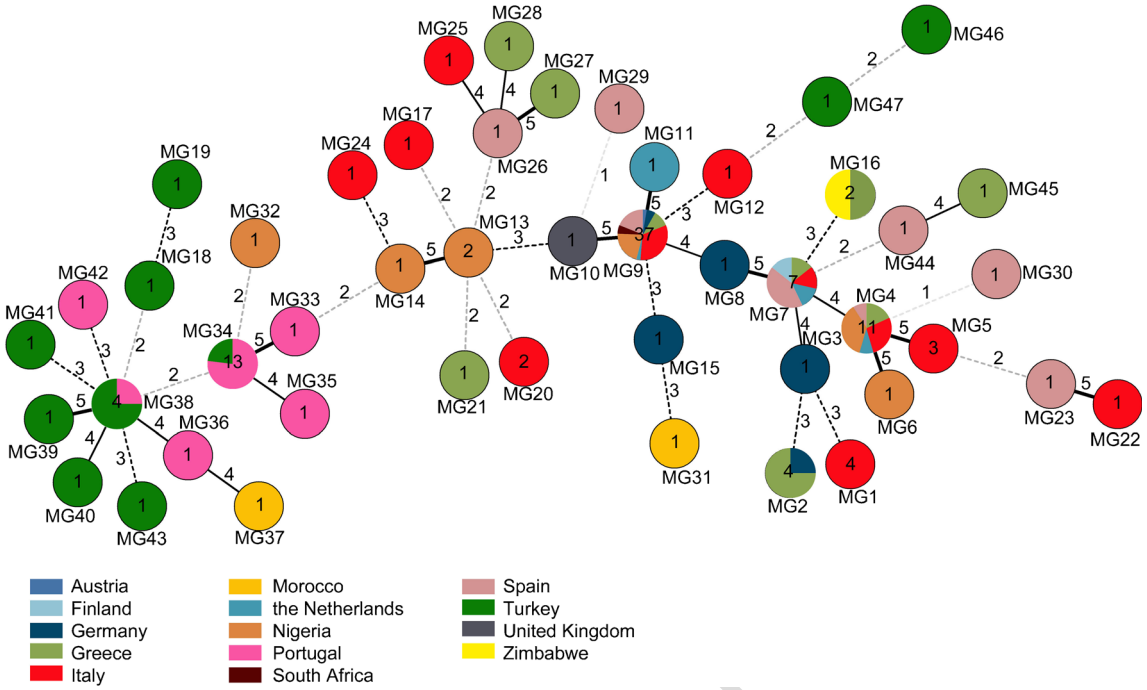

Fig. 1 Minimum spanning tree showing the differences based on a categorical analysis between the genotypes. Each *circle* represents a unique haplotype with the number of isolates of that genotype inside subdivided by country of origin based on the colors. Numbers at the connecting lines correspond to the number of identical microsatellites between the isolates. Similarities between genotypes were visualized using BioNumerics version 4.61 treating the data as categorical information

Fig. 1, Table 1). Conversely, the second most common genotype (MG-34) found in this study was exclusively recovered from Portuguese and Turkish clinical samples. This clone, together with the MG-38 genotype and a number of closely related genetic variants, form a particular cluster of isolates evolutionarily distinct from other European isolates (Fig. 1).

All our observed microsatellite genotypes form a large network in the MStree analysis with no outliers (Fig. 1). However, several related CCs or microsatellite classes can be defined based on the central, dominant genotypes. For our analysis, we defined four clonal clusters (CC1, CC2, CC3, and CC4) with the following central types MG-34, MG-13, MG-9, and MG-7 respectively (Fig. 1). The CC1, with MG-34 as the central type, contains the genotypes MG-18, MG-19, and MG-32 to MG-43; CC2, with MG-13 as the central type, contains MGs 14, 17, 20, 21, and 24–28; CC3, with MG-9 as the central type, includes MGs from 8 to 12, 15, 29, 31, 46, and 47, while the remainder (MGs 1–7, 16, 22, 23, 30, 44, and 45) fall into CC4, with MG-7 as the central type (Fig. 1).

According to their geographical origin, our isolates can be subdivided into seven different populations (Table 3). Even though the populations differed in their sample sizes, the number of haplotypes detected in each population and the numbers of private haplotypes specific to a given population did not differ very much. Portugal and Spain were the two countries with the lowest haplotypic richness and diversity, whereas the highest were in Middle/Northern Europe and Turkey (Table 3). However, a normalized pairwise population matrix of Nei's genetic distance showed that Portugal and Turkey in particular were more distant from the other countries.

The populations of Africa, Middle/Northern Europe, Italy, Greece, and Spain did not differ significantly from one another (p values >0.05) whereas the Portuguese and Turkish

Table 2 Number of alleles and index of diversity for each microsatellite marker examined in this study

	Microsatellite loci						Total
	ERG3	RPM2	MTI	GLM4	GLM5	GLM6	
Number of alleles	19	12	14	11	13	15	84
Range size (bp)	181–260	121–139	227–247	261–288	259–307	280–325	–
Diversity index	0.85	0.78	0.64	0.79	0.62	0.70	0.89

Table 3 Population analysis based on the microsatellite haplotypes

Population	*n*	A	P	Ne	Rh	He	D_{sh}^2
Africa	22	9	6	3,841	5,372	0775	627,636
Greece	15	9	4	6,429	7,190	0905	822,749
Italy	30	11	8	4,787	5,865	0818	884,636
Middle/Northern Europe	13	9	5	6,259	8,000	0910	361,863
Portugal	15	6	4	2,143	4,333	0571	643,863
Spain	16	8	5	4,000	5,873	0800	657,771
Turkey	16	10	8	7,529	7,682	0925	860,621
Mean ± SD	18 ± 5.9	9 ± 1.6	5.7 ± 1.7	5.0 ± 1.9	6.3 ± 1.3	0.81 ± 0.12	694 ± 182

N sample size in each population, *A* number of haplotypes detected in each population, *P* number of private haplotypes, *Ne* effective number of haplotypes, *Rh* haplotypic richness, *He* genetic diversity, D_{sh}^2 mean genetic distance between individuals, *SD* standard deviation

populations differed significantly from all others (*p* values of 6.7e-4 and smaller), but not between themselves (*p* value = 0.330).

Discussions and conclusions

In recent years, important changes in the epidemiology of candidiasis have been observed worldwide, highlighting a significant increase in non-*C. albicans* species with different degrees of virulence and pathogenicity [3, 15]. Among these species, *C. glabrata* has emerged as an important cause of human disease, proving to possess an extraordinary ability to infect different body sites [3–7, 19]. However, based on our results, it seems that not all *C. glabrata* isolates are equally as likely to cause human infections and some of them, especially those with the MG-9 haplotype, appear to be clinically most relevant and cause disease significantly more often than others (Fig. 1). This remarkable capacity of some isolates to infect different host tissues may be the result of their unique genetic background (Table 1). This observation agrees with previous studies [12, 14] and suggests that the identification of specific genotypes could represent an important step for future studies on the virulence of *C. glabrata*.

In this study, the combined analysis of the six microsatellite markers reached a *D* value of 0.89 and the 127 *C. glabrata* isolates were divided into 47 different genotypes. Our *D* value was not consistent with that reported by Abbes et al. [11], and it is below the desirable value of 0.95 for an "ideal" typing system [20]. Therefore, our results suggest that the present markers should be used with caution if utilized in an attempt to recognize outbreaks of infections, but they can be used for some population studies.

In this study, we were able to recognize at least four CCs. Looking at the distribution of these clonal complexes over the different geographical regions sampled, we observed an expansion of closely related genotypes (MG-9, MG-7, and MG-4) that were prevalent in southern countries (Italy and Spain) and in Africa and were probably spread by the huge flow of migrants that occurs between these two continents. Conversely, two closely related genotypes, MG-34 and MG-38, were restricted only to Turkey and Portugal, even if they showed distantly genetic relationships with the African types MG-37, MG-32, and MG-14. This latter, together with the other Nigerian MG-13, correlates more directly with the main type, MG-9.

The MG-9 type was the most frequently observed genotype spreading between Africa and European countries for long periods of time. In fact, infections caused by this genotype date back to more than 80 years ago (1935) as demonstrated by some *C. glabrata* strains in the CBS culture collection that were isolated during the two decades between 1935 and 1955 (Table 1).

All other clinical isolates were recovered during or after 2004 and the MG-9 genotype was still present in recent clinical samples by demonstrating its exceptional, strong adaptation to specific geographical areas. Hence, the MG-9 seems to be the main European type, while its branching out to other main genotypes via the German MG-8 and the UK sample MG-10 suggests that these branches might have been crucial to the expansion of *C. glabrata* in Europe.

Even though in part, the same set of microsatellite loci was used in other *C. glabrata* population studies, it proves difficult to directly compare the results, as small size differences can be observed in the dominant types. These could be due to actual size differences or to errors in the measurements. To avoid such problems in the future we advocate the use of a standard set of reference strains, available from public sources. For this purpose we deposited a representative panel of strains (MGs 7, 9, 13, and 34 in this study) to the CBS culture collection (www.cbs.knaw.nl) in Utrecht, the Netherlands, and the strains are also available from the corresponding author.

In conclusion, the data reported here emphasize the role of specific *C. glabrata* genotypes in human infections for at least

a few decades. We recommend further genetic studies that may detect a different reality than that currently known with regard to the population structure, epidemiology, and spread of this fungal pathogen.

Compliance with ethical standards

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Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study formal consent is not required.

References

1. Bolotin-Fukuhara M, Fairhead C (2014) *Candida glabrata*: a deadly companion? Yeast 31:279–288. doi:10.1002/yea.3019
2. Pfaller MA, Andes DR, Diekema DJ, Horn DL, Reboli AC, Rotstein C, Franks B, Azie NE (2014) Epidemiology and outcomes of invasive candidiasis due to non-*albicans* species of *Candida* in 2,496 patients: data from the Prospective Antifungal Therapy (PATH) registry 2004–2008. PLoS One 9, e101510. doi:10.1371/journal.pone.0101510
3. Quindós G (2014) Epidemiology of candidaemia and invasive candidiasis. A changing face. Rev Iberoam Micol 31:42–48. doi:10.1016/j.riam.2013.10.001
4. Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J (2012) *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. FEMS Microbiol Rev 36:288–305. doi:10.1111/j.1574-6976.2011.00278.x
5. Li L, Redding S, Dongari-Bagtzoglou A (2007) *Candida glabrata*: an emerging oral opportunistic pathogen. J Dent Res 86:204–215. doi:10.1177/154405910708600304
6. Tomczak H, Szałek E, Grześkowiak E (2014) The problems of urinary tract infections with *Candida* spp. aetiology in women. Postepy Hig Med Dosw (Online) 68:1036–1039. doi:10.5604/17322693.1118989
7. Sardi JC, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini MJ (2013) *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J Med Microbiol 62:10–24. doi:10.1099/jmm.0.045054-0
8. Pfaller MA, Castanheira M, Messer SA, Moet GJ, Jones RN (2010) Variation in *Candida* spp. distribution and antifungal resistance rates among bloodstream infection isolates by patient age: report from the SENTRY Antimicrobial Surveillance Program (2008–2009). Diagn Microbiol Infect Dis 68:278–283. doi:10.1016/j.diagmicrobio.2010.06.015
9. Turner SA, Butler G (2014) The *Candida* pathogenic species complex. Cold Spring Harb Perspect Med 4:a019778. doi:10.1101/cshperspect.a019778
10. Chapeland-Leclerc F, Hennequin C, Papon N, Noël T, Girard A, Socié G, Ribaud P, Lacroix C (2010) Acquisition of flucytosine, azole, and caspofungin resistance in *Candida glabrata* bloodstream isolates serially obtained from a hematopoietic stem cell transplant recipient. Antimicrob Agents Chemother 54:1360–1362. doi:10.1128/AAC.01138-09
11. Abbes S, Sellami H, Sellami A, Hadrich I, Amouri I, Mahfoudh N, Neji S, Makni F, Makni H, Ayadi A (2012) *Candida glabrata* strain relatedness by new microsatellite markers. Eur J Clin Microbiol Infect Dis 31:83–91. doi:10.1007/s10096-011-1280-4
12. Amouri I, Sellami H, Abbes S, Hadrich I, Mahfoudh N, Makni H, Ayadi A (2012) Microsatellite analysis of *Candida* isolates from recurrent vulvovaginal candidiasis. J Med Microbiol 61:1091–1096. doi:10.1099/jmm.0.043992-0
13. Brisse S, Pannier C, Angoulvant A, de Meeus T, Diancourt L, Faure O, Muller H, Peman J, Viviani MA, Grillot R, Dujon B, Fairhead C, Hennequin C (2009) Uneven distribution of mating types among genotypes of *Candida glabrata* isolates from clinical samples. Eukaryot Cell 8:287–295. doi:10.1128/EC.00215-08
14. Foulet F, Nicolas N, Eloy O, Botterel F, Gantier JC, Costa JM, Bretagne S (2005) Microsatellite marker analysis as a typing system for *Candida glabrata*. J Clin Microbiol 43:4574–4579. doi:10.1128/JCM.43.9.4574-4579.2005
15. Criseo G, Scordino F, Romeo O (2015) Current methods for identifying clinically important cryptic *Candida* species. J Microbiol Methods 111:50–56. doi:10.1016/j.mimet.2015.02.004
16. Romeo O, Scordino F, Pernice I, Lo Passo C, Criseo G (2009) A multiplex PCR protocol for rapid identification of *Candida glabrata* and its phylogenetically related species *Candida nivariensis* and *Candida bracarensis*. J Microbiol Methods 79:117–120. doi:10.1016/j.mimet.2009.07.016
17. Guichoux E, Lagache L, Wagner S, Chaumeil P, Léger P, Lepais O, Lepoittevin C, Malausa T, Revardel E, Salin F, Petit RJ (2011) Current trends in microsatellite genotyping. Mol Ecol Resour 11:591–611. doi:10.1111/j.1755-0998.2011.03014.x
18. Hunter PR, Gaston MA (1998) Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol 26:2465–2466
19. Esposto MC, Prigitano A, Romeo O, Criseo G, Trovato L, Tullio V, Fadda ME, Tortorano AM; FIMUA Working Group (2013) Looking for *Candida nivariensis* and *C. bracarensis* among a large Italian collection of *C. glabrata* isolates: results of the FIMUA working group. Mycoses 56:394–396. doi:10.1111/myc.12026
20. Van Belkum A, Tassios PT, Dijkshoorn L, Haeggman S, Cookson B, Fry NK, Fussing V, Green J, Feil E, Gerner-Smidt P, Brisse S, Struelens M (2007) Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clin Microbiol Infect 13:1–46. doi:10.1111/j.1469-0691.2007.01786.x