J. Med. Microbiol. — Vol. 49 (2000), 575–581

ISSN 0022-2615

MOLECULAR EPIDEMIOLOGY

Molecular genotyping of *Candida* species with special respect to *Candida* (*Torulopsis*) *glabrata* strains by arbitrarily primed PCR

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A set of 46 epidemiologically related or unrelated *Candida* (*Torulopsis*) *glabrata* isolates from four different medical centres in Germany and Hungary, and the type strain of this species, were genetically typed by arbitrarily primed PCR (AP-PCR). The resulting band patterns of *C. glabrata* strains were compared with those of other species of the genus *Candida* including *C. albicans*, *C. guilliermondii*, *C. kefyr*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*. After preliminary trials of various reaction parameters and control experiments to test the reproducibility of this method, it was found that consistently reproducible amplification patterns were obtained only when rigorously optimised and standardised reaction conditions were employed. Discriminatory abilities were studied with 29 generated 10-mer oligonucleotides of different G+C content. Typing of clinical isolates with the optimised AP-PCR protocol was then performed with the primer 50-1, with a G+C content of 50%. Sufficiently discriminatory polymorphisms were observed among the band patterns of the *Candida* species included. The gel electrophoresis patterns of each species showed an adequate similarity. Variations in minor bands were characteristic for comparison at the isolate level. Only three AP-PCR genotypes were identified among the clinical isolates of *C. glabrata* tested. Two of these genotypes were closely related and appeared to be widespread within German and Hungarian isolates. The third genotype of *C. glabrata* showed a distinct band pattern. With optimised, validated and standardised assay conditions, the feasibility, sensitivity and rapidity of AP-PCR may offer a discriminatory method for genotyping of yeasts in epidemiological studies, as well as in the control of nosocomial infections.

Introduction

In recent years, distinct shifts in the distribution of *Candida* species isolated from nosocomial infections have been reported. Although *C. albicans* remains the most frequent cause of fungaemia and haematogenously disseminated candidiasis, an increasing number of hospital-acquired infections due to *C.* (*Torulopsis*) *glabrata* and other 'non-*albicans*' *Candida* spp. is being observed [1–5]. *C. glabrata* currently ranks second or third in frequency among *Candida* spp., is associated with an equally high mortality rate and is innately resistant to antifungal agents, specifically the azoles [4, 6, 7].

Despite its increasing importance as an emerging nosocomial pathogen, little is known about the epidemiology and geographical distribution of *C. glabrata* compared to other *Candida* spp. [3–5, 8] In recent years, traditional methods of phenotyping have been mostly replaced by, or used in conjunction with, molecular methods [9]. AP-PCR [10, 11] belongs to a recent generation of genotyping methods in which short oligonucleotides with randomly chosen sequences are used in a modification of classical PCR protocols. Such arbitrary primers generate a set of amplicons of varying number and size, providing the basis for typing pathogen isolates. This technique is methodologically easier, less time-consuming and more cost-effective than the older genomic typing methods, particularly pulsed-field gel electrophoresis (PFGE). However, a limitation of AP-PCR has been the observation that the low-stringency conditions of this PCR-based method may result in poor reproducibility of typing results [12].

The objectives of the present study were to apply and

Received 12 July 1999; revised version received 27 Oct. 1999; accepted 18 Nov. 1999.
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optimise an AP-PCR protocol for reproducible and convenient typing and confirmation of the species identity of *C. glabrata* isolates, and also to employ these optimised conditions for molecular evaluation of *C. glabrata* clinical isolates from several European centres.

Materials and methods

Yeast strains and growth

In all, 47 clinical isolates of *C. glabrata* and the type strain of the species, ATCC 2001, were studied. Six of these isolates were from one patient with a putative *C. glabrata* endocarditis (four blood cultures and two isolates from abscess material) at the University Hospital, Münster. A further 40 clinical isolates (from blood cultures, catheter, urine or vaginal swabs) from individual patients were examined: 15 from the University Hospital, Münster, Germany; 10 from the University Hospital, Budapest, Hungary; 10 from the University Hospital, Würzburg, Germany; and 5 from the University Hospital (Charité), Berlin, Germany. The isolates were identified as *C. glabrata* by the API 32C identification system for yeasts (bioMérieux, Marcy-l'Etoile, France) and confirmed by standard taxonomic procedures [13, 14]. All strains were maintained on Kimmig agar plates. A total of 10 type or reference strains of *C. albicans* (ATCC 36801, ATCC 44374), *C. guilliermondii* (ATCC 90877), *C. kefyr* (Y0601), *C. parapsilosis* (ATCC 22019, Y0501), *C. tropicalis* (ATCC 90874, ATCC 28707) and *C. krusei* (ATCC 6258, ATCC 90878) was analysed to determine differences in the banding patterns of several yeast species. All *Candida* strains were cultivated and tested in their anamorphic form.

DNA extraction

Nucleic acid (NA) extracts were prepared from 10 ml of an 18-h culture in yeast extract-peptone-glucose (YPD) broth (yeast extract, Difco, 1%; polypeptone, Merck, Darmstadt, Germany, 2%; D-glucose 2%) with shaking at 37°C. Yeast cells were pelleted by centrifugation at 5000 *g* for 10 min, resuspended in 600 μl of sorbitol buffer (1 M sorbitol, 100 mM EDTA, 14 mM β-mercaptoethanol) with Lyticase (Sigma) 200 U, and incubated at 30°C for 0.5 h. Spheroplasts were centrifuged at 5000 *g* for 5 min and resuspended in 180 μl of ATL buffer (Qiagen, Hilden, Germany). Subsequently, NA preparations were extracted with the QIAamp® Tissue Kit (Qiagen) following the manufacturer's recommendations. NA samples were eluted with distilled water and adjusted to a final concentration of 1 μg/ml according to A_{260} values.

Optimisation of the PCR amplification

For primer evaluation, 29 arbitrary 10-mer oligonucleotides (Table 1) with a G+C content of 30–90%, obtained from MWG Biotech (Ebersberg, Germany) were tested. First, preliminary trials with different annealing temperatures (30, 32.5, 35, 37.5 and 40°C), cycle numbers (20, 25, 30, 35, 40), different units (1.0, 1.5, 2.0, 2.5, 3.0) and brands of DNA polymerases (*Taq* DNA Polymerase, Boehringer, Mannheim, Germany; PrimeZyme™, Biometra, Göttingen, Germany; *AmpliTaq*® and *AmpliTaq* Gold™ DNA Polymerase, Applied Biosystems, Foster City, CA, USA; BioTherm DNA Polymerase, GeneCraft, Münster, Germany) and with various concentrations of $MgCl_2$ (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mM) were performed to define the most suitable oligonucleotides. Prolonged ramping times between the annealing and the extension temperature (3, 5, 7, 9 min) were tested as described previously [15].

Table 1. Base sequences and G+C content of the arbitrary 10-mer oligonucleotide primers

Primer	Oligonucleotide sequence (5′–3′)	G+C content (%)
AP30-1	CAG ATA TGA T	30
AP30-2	CTG ATA TGA A	30
AP40-1	CCG ATA TGA T	40
AP40-2	TGG AAT TAC G	40
AP50-1	GAT TCA GAC C	50
AP50-2	ACG GGT TCA A	50
AP50-3	TCG CTA ATC C	50
AP50-4	GCC GTC AAA T	50
AP50-5	ACA TCG CAA C	50
AP50-6	CCT TAC GGA A	50
AP50-7	GAC GGA TTA G	50
AP50-8	GGA TTA CCT G	50
AP50-9	CAT GCA TTC G	50
AP50-10	AAA CGC TCA C	50
AP50-11	CCA AGG TTA C	50
AP50-12	GGG ATT CCT T	50
AP50-13	CGA TTA AGG G	50
AP50-14	GTA CCT GTC A	50
AP50-15	GCC CAA ATT G	50
AP50-16	CAA GCC ATT G	50
AP50-17	GCC ATT CAA G	50
AP60-1	GGT TCA CCA G	60
AP60-2	CCG GTC TAA G	60
AP70-1	CCG GTC TGA G	70
AP70-2	CCG GTC CAA G	70
AP80-1	CCG GTC TCG G	80
AP80-2	CCG CTC GGA C	80
AP90-1	CCG GCC TCG G	90
AP90-2	CCG GAC GCG C	90

AP-PCR

Amplification reactions were performed in 100 μl of a PCR mixture containing 100 μM (each) dATP, dCTP, dGTP and dTTP. The master mix contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 50 pmol primer and 2.5 U DNA polymerase. The amplification was performed in an automated thermocycler with a hot bonnet (Hybaid, Teddington). The thermal cycling conditions were 30 cycles of denaturation at 94°C for 1 min (2 min for the first cycle), annealing at 35°C and polymerisation at 72°C for 2 min. Amplified products (10 μl) were resolved by agarose 2% gel electrophoresis at 150 V for 1.5 h. The

gel was stained with ethidium bromide, exposed to UV light (254 nm) to visualise the amplified products and photographed (665 P/N, Polaroid, Cambridge, MA, USA).

The following working definitions were used for interpretation of the gel electrophoresis patterns. A major band was present if the amplicon produced a clearly visible and reproducible band that could be used for genotyping. In contrast, minor bands were diffuse, blurred and sometimes unreproducible bands of inferior quality and application for typing purposes. An AP-PCR genotype was deduced from the gel electrophoresis pattern of major bands observed in more than one isolate. AP-PCR subtypes were closely similar to this genotype, but differed in size or intensity in one or more minor bands.

Results

Choice of the primer

First, the discriminatory power of 29 random primers (Table 1) of different G+C content was assessed by studying a limited set of epidemiologically unrelated isolates from four different medical centres and the type strain of *C. glabrata*. The presence of *c*. 10 or more separate, reproducible bands of sufficient intensity was used as the major criterion for primer selection and for determination of optimum conditions. It appeared that primers with a G+C content of 40–50% provided acceptable discrimination. Among these, the AP primer 50-1 showed the maximum discriminatory power. When the lengths of the DNA fragments and the number of bands were investigated, this particular primer was observed to generate more than eight amplicons ranging from 300 bp to *c*. 3000–4000 bp. In contrast, the majority of other primers generated fewer amplicons or smaller size values, or both. Subsequently, all 46 clinical isolates and the reference strains of the different *Candida* spp. were typed with 50-1 primer from the same batch, as in preliminary tests different primer batches resulted in differences in amplification patterns. All strains were typable by AP-PCR.

Optimisation of AP-PCR

As test parameters such as the concentration of $MgCl_2$, DNA concentration, primer concentration, polymerase type or thermal cycler profile are critical factors in producing informative patterns, the optimum conditions for the AP-PCR were determined. On the basis of these preliminary experiments (data not shown), the optimised and standardised amplification protocol employed $MgCl_2$ at a concentration of 1.5 mM. Lower concentrations reduced the numbers of bands that could be discriminated. Optimal results were found with 50 pM primer and 2.5 U DNA polymerase (Boehringer, Mannheim). The use of alternative DNA polymerases showed no advantages in this AP-PCR, yet changes of batches affected the banding patterns, resulting in minor differences in the number and size of amplified bands. As it is known that *Taq* polymerases are frequently contaminated with bacterial DNA [16, 17], this could be the reason for this effect. To study this phenomenon, several batches and brands were tested in control reaction tubes without *C. glabrata* DNA template. Sometimes, minor bands were still observed.

An increase in cycle numbers (>30) did not substantially improve the discrimination power. In contrast, higher cycle numbers could result in a background of non-specific and non-reproducible minor bands. Reducing the number of cycles to below 30 yielded results varying in number and intensity of amplicons. Annealing temperatures ranging from 30 to 40°C were tested, and an intermediate temperature of 35°C was chosen as the optimum temperature. Higher temperatures resulted in increased differences in band intensities and decreasing numbers of reproducible and utilisable bands. In contrast, lower annealing temperatures generated more multiple, diffuse bands ('background'). For ramping times, 7-min ramps enhanced the number and yield of the amplified bands [15].

Reproducibility of AP-PCR

Several control experiments were done to test the reproducibility of the AP-PCR method. When duplicate cultures were grown and extracted DNA was used simultaneously for AP-PCR, the patterns from both cultures of the same isolate were indistinguishable. Also, the patterns obtained were not affected by testing the same DNA extraction at different positions in the thermal cycler. When prolonged ramp times were used, the same samples placed on different thermal cyclers resulted in the same band pattern. When the same DNA preparation from a given isolate was used on different days, the amplification bands yielded the same pattern, but sometimes different intensities.

AP-PCR patterns

After optimisation of AP-PCR procedures, the AP-PCR generated distinct patterns for the seven yeast species tested (Fig. 1). The banding patterns were much more similar for the profiles derived from strains of the same species than were the banding patterns from other yeast species.

Three distinct major profiles were observed for the 47 clinical isolates and the type strain of *C. glabrata*; these were designated genotypes A, B and C. Fig. 2 shows some representative AP-PCR banding patterns of *C. glabrata*. The profiles of the genotypes A and B appeared to be closely related. Both these genotypes shared similar major (2.0, 0.3 kb) and minor (2.7, 2.4, 1.7, 1.1, 0.9, 0.5 kb) bands, but also differed significantly and reproducibly in a few bands (1.4, 0.45,

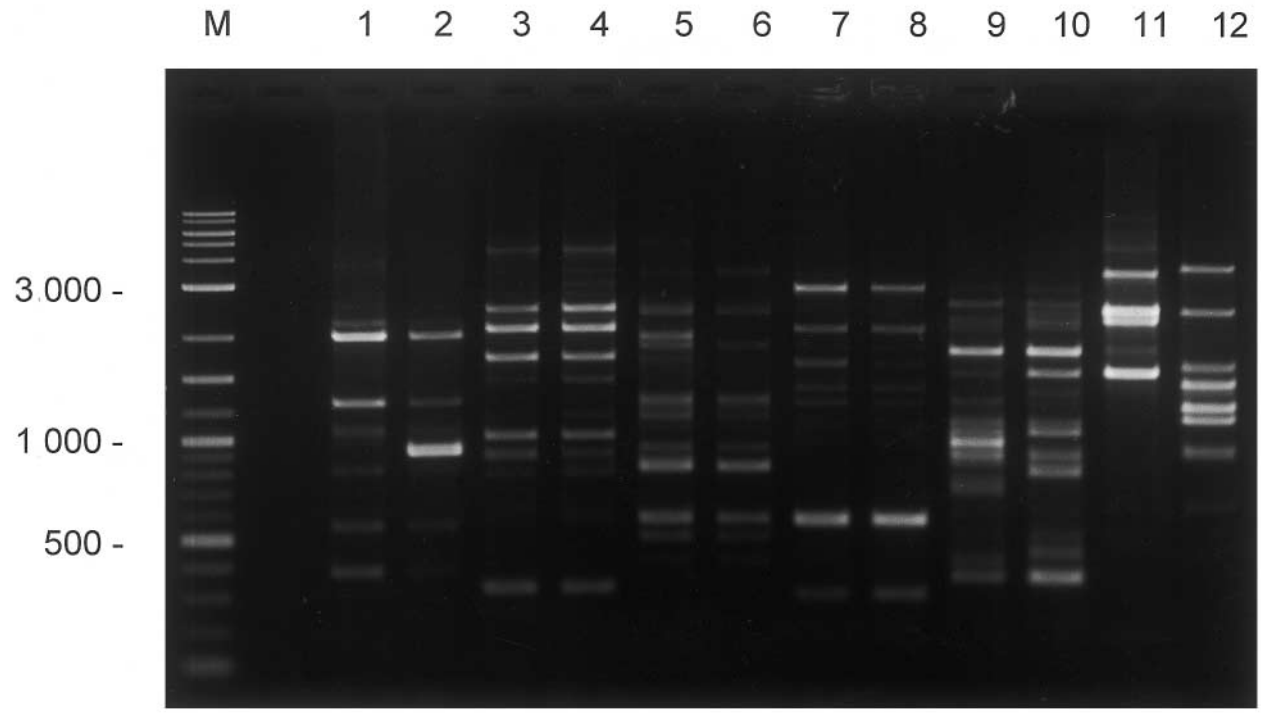

Fig. 1. Agarose gel electrophoresis of AP-PCR of *Candida* reference strains. Lane **M**, DNA molecular size marker, combined 100-bp/1-kb DNA ladder (New England Biolabs, Beverly, MA, USA); **1** and **2**, *C. albicans*; **3** and **4**, *C. parapsilosis*; **5** and **6**, *C. tropicalis*; **7** and **8**, *C. krusei*; **9** and **10**, *C. glabrata*; **11**, *C. guilliermondii*; **12**, *C. kefyr.*

0.4 kb) (Fig. 3). Furthermore, subtypes slightly different in the number, size, or intensity of a few amplification bands were observed. Genotype C showed a more obvious distinct banding pattern (similar to differences found at species level). This genotype shared only two major bands (2.0, 0.3 kb) and a 3.0-kb minor band with strains of genotypes A and B, respectively. Overall, these bands may represent the *C. glabrata* species-specific banding pattern.

Genotypes A and B with their subtypes were observed in more than one medical centre. Genotype A occurred in one isolate from a Hungarian patient as well as in isolates from Würzburg and Münster. The second widespread AP-PCR pattern, genotype B, was found in all the German centres studied. Representatives of both genotypes A and B were detected in the Hungarian isolates. The results of the geographically widespread patterns A and B were confirmed by the use of other random primers that produced good results (AP 40-1, AP 50-4). The more distinct genotype C was identified only in two isolates from Würzburg (Table 2).

The *C. glabrata* type strain ATCC 90030 showed a banding pattern that belonged to genotype B. The amplification patterns for the six isolates from the patient with endocarditis were identical and belonged to genotype A. Intervals between recovery of the first isolate and recovery of the subsequent isolates from this patient were 2 and 5 months, respectively.

Discussion

This report describes the successful application of AP-PCR to the genomic typing of *C. glabrata*. Technical aspects of feasibility and rapidity in assay performance have made AP-PCR an increasingly popular tool for the epidemiological evaluation of infections. AP-PCR has been used in various epidemiological studies for genomic fingerprinting of several bacterial pathogens and to a limited extent to the analysis of pathogenic yeasts, including *C. glabrata* [18–21]. However, the applicability of AP-PCR is hampered by the facts that variations in amplicon patterns between isolates may be caused by assay conditions such as stringency, and under non-optimal conditions amplicon pattern similarity may be due to a small number of amplified PCR products [22, 23]. Therefore, the strategies used in the present study for assay optimisation have focused on reproducibility and high discriminatory power in investigating *C. glabrata* isolates.

As summarised previously [22], modifications of $MgCl_2$ concentration, DNA concentration, primer concentration, number of cycles, polymerase type, annealing temperature, or batch-to-batch variations of assay components such as polymerases changed the amplification pattern in this AP-PCR assay and had to be optimised. An increase in the number of cycles, as well as elevation of the annealing temperatures had a direct effect on the discriminatory power and reprodu-

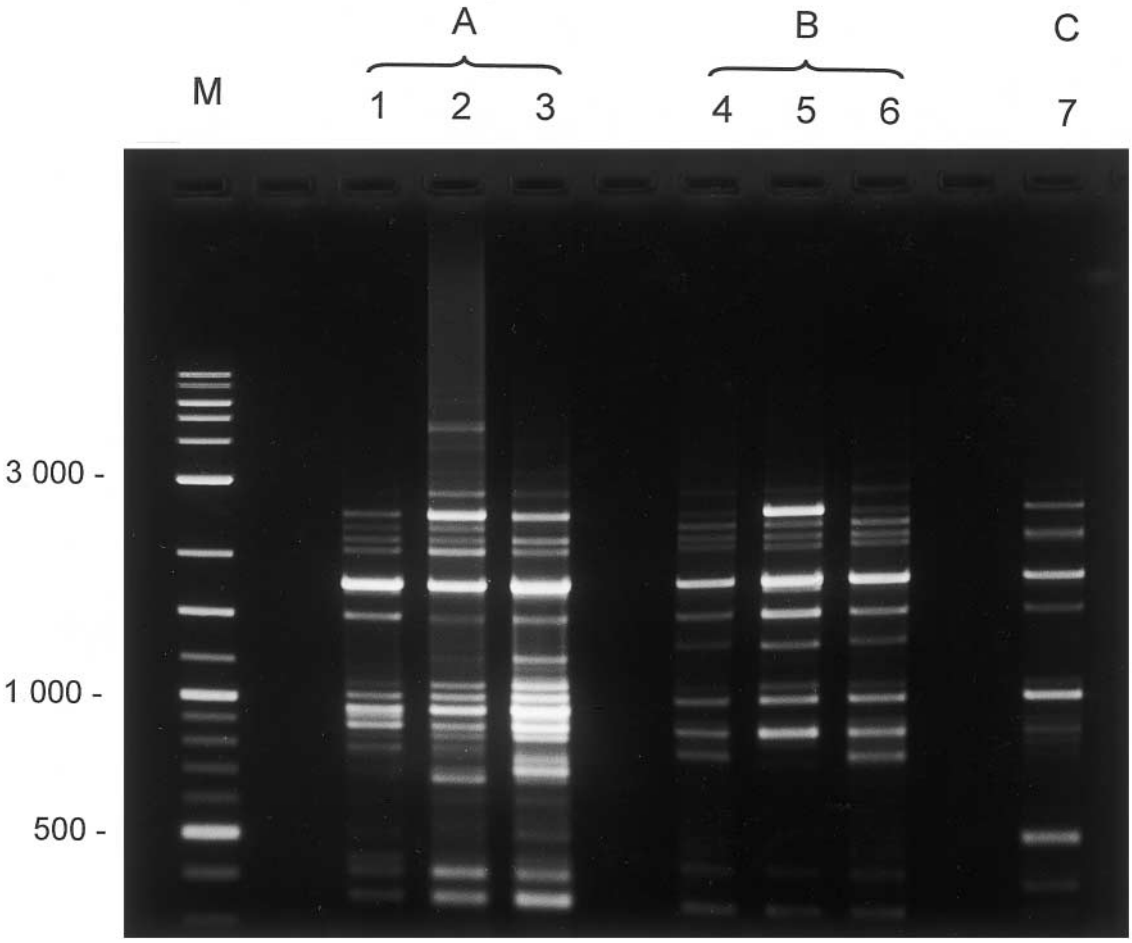

Fig. 2. Agarose gel electrophoresis patterns demonstrating the three different genotypes (A, B, C) and examples of their subtypes of AP-PCR-amplified products of clinical *C. glabrata* strains. Lane **M**, DNA molecular size marker, combined 100-bp/1-kb DNA ladder (New England Biolabs); **1**–**3**, amplicons of genotype A; **4**–**6**, amplicons of genotype B; **7**, amplicon of genotype C.

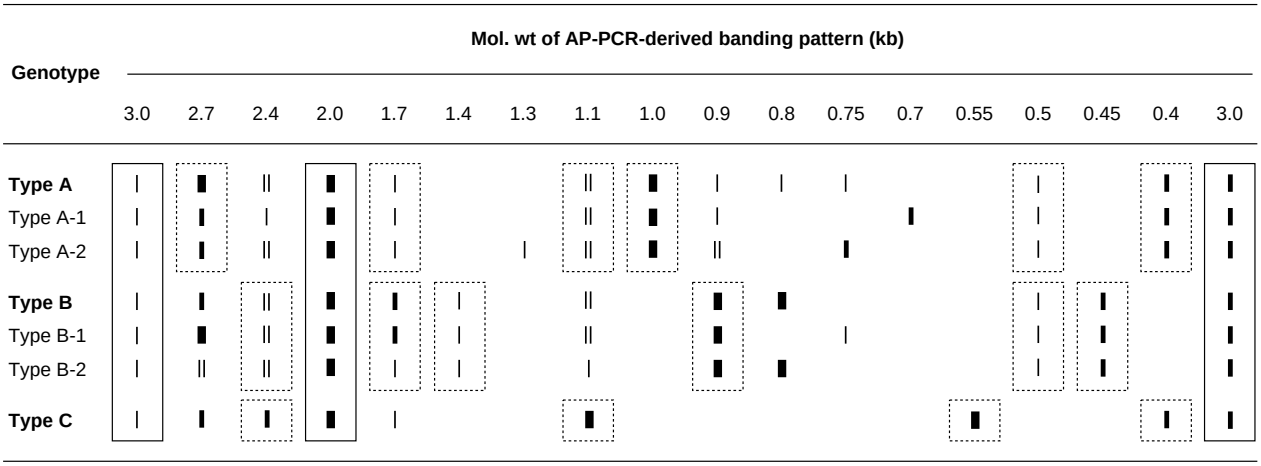

Fig. 3. Schematic representation of *C. glabrata* banding pattern of the three genotypes with examples of their subtypes. For a better graphical view, the lanes were arranged by counterclockwise rotation of 90°. ▌, ▊, major bands; |, minor bands; ||, double bands; ☐ *C. glabrata* species-specific banding pattern; ⬚ *C. glabrata* type-specific banding pattern.

cibility in this test system. Inter-assay variability of pipetting volume, enzyme activity or temperature profile fluctuations may all result in amplification pattern variation in low-stringency AP-PCR protocols. Thus, constancy of all assay parameters, particularly of batch-to-batch differences, is pertinent to obtain reproducible results. In the present study, the use of prolonged ramp time intervals extended the PCR procedure, but is a simple and practical way to improve the typing efficiency.

Table 2. Distribution of *C. glabrata* genotypes in German and Hungarian medical centres

	Number of isolates of genotype		
Medical centre	A	B	C
Berlin, Germany	–	5	–
Budapest, Hungary	1	9	–
Münster, Germany	11*	11	–
Würzburg, Germany	5	3	2
Total	17	28	2

*Number includes the six clonal identical isolates from a patient with endocarditis.

As observed in diagnostic amplification with highly conserved regions as primer target, e.g., 16S rDNA, commercially available brands of *Taq* polymerases are contaminated with bacterial DNA [16, 17]. In the case of PCR with arbitrary primers, this exogenous DNA contamination may represent a serious problem. This was demonstrated in the control experiments in the absence of *C. glabrata* DNA template, when minor bands were observed. An excess of target DNA over contaminating DNA and the use of DNA inactivating agents for assay reagents may ensure that the target DNA will be amplified preferentially [24].

The selection of an appropriate random primer is of great importance for optimisation of the discriminatory power of AP-PCR analysis and, optimally, primer composition is individualised for each species [25]. Not every arbitrary oligonucleotide produced a useful banding pattern. The present study tested several 10-mer primers with G+C contents varying from 30 to 90%. Oligonucleotides containing a G+C content of 50% were found to be optimal in defining differences among *C. glabrata* strains. It was suggested that the discriminatory power of AP-PCR methods could be increased by using more than one primer [26]. In preliminary experiments (data not shown) it was observed that only a little additional information (one or two minor bands, partly inconstant) was gained when two primers were used.

The AP-PCR protocol specifically adapted and optimised for *C. glabrata* strains was used to evaluate a representative set of 47 clinical strains from four Central European centres. So far, the rare studies applying molecular methods to the typing of *C. glabrata* isolates have all been limited to outbreaks. Lee *et al*. [27] used a restriction enzyme analysis (REA) to investigate a hospital outbreak. Khattak *et al*. [28] examined PFGE for this purpose and Arif *et al*. [21] compared various genotyping methods.

In the present study, only three genotypes were identified in clinical *C. glabrata* isolates. Surprisingly, strains representing two of these genotypes appear to be widespread in Germany and Hungary. Schönian *et al*. reported a predominant pattern of *C. glabrata* among patients from several departments of a hospital and from different geographic origins [29]. Recently, Xu *et al*. showed a lack of genetic differentiation between two geographically diverse samples of *C. albicans* from the USA and Brazil [30]. Another recent search for genetic isolation failed to detect differences among Asian, North American and European isolates of *C. albicans* [31]. The reason for these concordant observations of widespread prevalent genotypes among epidemiologically unrelated yeast strains is still unknown. Redkar *et al*. [32] described a type of *C. rugosa* isolates to be predominant in burn patients, consistent with the theory that pathogenic isolates of this yeast species have emerged and persisted because of extensive use of topical nystatin [33]. It is known that the prophylactic use of ketoconazole in neutropenic patients may result in the emergence of a higher prevalence of *C. glabrata* among surveillance cultures [5]. Despite some published contradictory results, the increase of *C. glabrata*, especially in neutropenic patients prophylactically treated with fluconazole, has been widely recognised and attributed to selection in immunocompromised patients [3, 34–36]. Moreover, some *C. glabrata* clones may have a slower evolution rate, resulting in a reduced diversity among isolates.

In contrast to the relatively similar AP-PCR pattern of the *C. glabrata* genotypes A and B, an almost completely distinct genotype C – sharing only two common major bands with the other *C. glabrata* genotypes – was observed in the present study. Phenotypically similar but genetically distinct organisms that may be masquerading as one species have been described among *C. parapsilosis* and *C.* (*T.*) *haemulonii* isolates [37]. Further epidemiological investigations to study the micro-evolution of this species by (i) comparing the AP-PCR results with other molecular methods of typing, (ii) determination of the sequences of AP-PCR-generated products and (iii) including additional isolates from multiple centres are necessary to examine these phenomenona.

In summary, discriminative and reproducible AP-PCR band patterns were obtained from *C. glabrata* strains with a strictly standardised test protocol that included prolonged ramp times. This AP-PCR method appears to be a simple and reliable technique for typing large numbers of yeast isolates and may provide a substitute for more expensive molecular typing methods such as PFGE for comparing *C. glabrata* isolates in global epidemiological studies, as well as for typing purposes in infection control. This tool may also contribute to the understanding of intra-species diversity and clonal predominances.

We are indebted to D. Harmsen (Würzburg), H. Schulze (Münster), H-J. Tietz (Berlin) and I. Török (Budapest) for providing *C. glabrata* strains and M. Schulte and B. Schuhen for expert technical assistance. We are grateful to M. Herrmann for critical reading of the manuscript.

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