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CHARACTERISATION OF MITOCHONDRIAL HAPLOTYPES OCCURRED IN A *CANDIDA ALBICANS* POPULATION

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The genetic background of mitochondrial DNA polymorphism in *Candida albicans* was studied by physical and functional mapping of four haplotypes identified recently in a hospital-population. The restriction patterns revealed considerable differences; however, the size of the mitochondrial DNA did not vary significantly. Sequence data demonstrated that size differences arose by short deletions, while restriction fragment length polymorphisms are caused by nucleotide substitutions in single sites. Gene rearrangement could not be detected; nevertheless, the coincidence of nucleotide substitution pattern in the inverted repeat region suggested the occurrence of homologue recombination.

Keywords: Candida albicans – mitochondrial DNA – sequences – PCR

INTRODUCTION

The diploid yeast *Candida albicans* exists commensally in the normal human microflora. At the same time, this species is still the most frequent pathogen isolated from human fungal infections [11, 13] despite the increasing number of cases connected to various non-albicans *Candida* species [15]. Infections usually develop in patients with disrupted balance of microflora or with natural or acquired immunodeficiency [16]. Owing to a number of virulence factors [2] expressed by *C. albicans*, it can cause superficial infections (i.e. cutaneous candidiasis, oropharyngeal candidiasis, and vulvovaginitis) and also invasive candidiasis; latter is associated with high morbidity and mortality rates [12].

The establishment of the source of the infection and to adapt prevention policies could be important from epidemiological point of view through the emergence of *C. albicans* strains resistant to antimycotic drugs [3]. Study of the mitochondrial genomes can be a useful tool to reveal the genetic relatedness of clinical isolates. Recently, the mitochondrial genome structure of *C. albicans* was revealed; numerous 40420-bp-long units of mtDNAs form a complex network of branched DNA mole-

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cule [5]. The 40.42 kb unit contains a large inverted duplication [17] where homologue recombination can occur [14]. Recent results evidenced the recombination events in the mitochondrial genome of this species [1, 5, 7].

In a population of clinical isolates, four different *C. albicans* haplotypes were detected on the bases of mitochondrial DNA polymorphism during an earlier study [4]. The RFLP patterns generated by *Bgl*II and *Eco*RV digestion differed markedly. In this study, the comparative mtDNA analysis of the haplotypes has been carried out to reveal the background of the variation.

MATERIALS AND METHODS

Strains and culture conditions

Candida albicans strains were grown in YPD media (1% peptone, 1% glucose, 0.5% yeast extract) for 24 hours at 30 °C with vigorous shaking. The strains used in the experiments are listed in Table 1.

Table 1
Candida albicans isolates

Strain number	Isolation source, location
C. albicans 10930 (Type I)	hemoculture, Hungary (Hun)
C. albicans 9132 (Type II)	hemoculture, Hungary (Hun)
C. albicans 5796 (Type III)	hemoculture, Hungary (Hun)
C. albicans 17471 (Type IV)	hemoculture, Hungary (Hun)
C. albicans SC5314	USA
C. albicans CBS 562	skin, Uruguay (Uru)
C. albicans CBS 2695	Puerto Rico (Pur)
C. albicans CBS 2706	nail, Argentina (Arg)
C. albicans CBS 2712	sputum, China (Chn)
C. albicans CBS 5736	vagina, Republic of South Africa (RSA)
C. albicans CBS 6589	vagina, United Kingdom (UK)
C. albicans CBS 6910	urine, New Zealand (NZL)
C. albicans CBS 8838	blood, USA
C. albicans CBS 9120	mouth, Japan (Jpn)
C. albicans ATCC 90028	blood, USA
C. albicans ATCC 10231	lung, unknown
C. albicans ATCC 10261	Brasil (Bra)

Nucleic acid isolation and analysis

Mitochondrial DNA was isolated by the method described previously [8]. Restriction digestion was carried out in 15 µl volume at 37 °C for 1 hour. *Eco*RI and *Eco*RV enzymes (Fermentas, Vilnius, Lithuania) were used for construction of the restriction map of the representative strains (haplotype I: 5796; II: 9132; III: 10930; IV: 17471). The *Eco*RI fragments of mtDNAs of the strains were cloned into pBluescript SK vector, and transformed into *E. coli*. The purified plasmids were digested with *Eco*RI-*Eco*RV and loaded onto agarose gel. After the separation of the bands, the fragments were compared to the single (*Eco*RI; *Eco*RV) and double digested (*Eco*RI-*Eco*RV) mtDNA [6].

The *Eco*RI fragments of haplotype I, II and IV cloned into pBluescript SK vector were sequenced with T3 and M13F universal primers (Table 2) by Macrogen (Seoul, Republic of Korea).

Partial genetic map was also constructed with the following *C. albicans* homologue hybridization probes: *cox1, cox2, cox3, cob, nad1, nad2, nad4, nad5* and *atp6*. Digoxigenin Labeling and Detection Kit (Roche, Mannheim, Germany) was used for hybridization experiments.

Table 2
Genbank accession number of the mtDNA fragments of *C. albicans* strains

NCBI Genbank Accession number	Sequence details
GU395655	strain 5796 mtDNA *Pvu*II fragment A
GU395656	strain 9132 mtDNA *Pvu*II fragment A
GU395657	strain 5796 mtDNA *Pvu*II fragment B
GU395658	strain 9132 mtDNA *Pvu*II fragment B
HQ721189	strain 10930 mtDNA *Pvu*II fragment A
HQ721191	strain 17471 mtDNA *Pvu*II fragment A
HQ721190	strain 10930 mtDNA *Pvu*II fragment B
HQ721192	strain 17471 mtDNA *Pvu*II fragment B
GU395659	strain 10930 partial mitochondrial sequence containing cox2
GU395660	strain 9132 partial mitochondrial sequence containing cox2
GU395661	strain 17471 partial mitochondrial sequence containing cox2
GU395662	strain 10930 partial intergenic sequence of cox3a and atp6
GU395663	strain 9132 partial intergenic sequence of cox3a and atp6
GU395664	strain 17471 partial intergenic sequence of cox3a and atp6
GU395665	strain 10930 partial intergenic sequence of cox3b and nad4
GU395666	strain 9132 partial intergenic sequence of cox3b and nad4
GU395667	strain 17471 partial intergenic sequence of cox3b and nad4

The total DNA was extracted with the MasterPure™ Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) according to the instructions of the manufacturer. DNA pellet was re-suspended in 50 µl distilled water and treated with 100 µg/ml RNase for 30 min at 37 °C.

PCR

CapvuA frw - CapvuA rev (5'- CGG ATC GGG TGT AGT GGA GG -3' and 5'- CGT GGA CTT AAC CAA GGA -3', respectively), and CapvuB frw - CapvuB rev (5'- GGA GAT CAT ACT TTA GCA G -3', and 5'- GGG TGT AGT GGA GGT TTA TC -3', respectively) primers were designed to amplify the smallest *Pvu*II fragments (*Pvu*IIA and *Pvu*IIB) of the isolates. The amplification was carried out in a MJ Mini, Personal Thermal Cycler (Bio-Rad, Hercules, CA, USA) using the following protocol: 95 °C for 2 min followed by 30 cycles at 95 °C for 1 min, 60 °C for 30 sec and 72 °C for 2 min, with a final extension at 72 °C for 5 min.

The PCR products were sequenced by Macrogen (Seoul, Republic of Korea) using the PCR primers.

RESULTS AND DISCUSSION

The genetic diversity of 44 *C. albicans* strains was investigated in a previous study [4]. The strains could be clustered into four groups that are four haplotypes, based on the restriction fragment length polymorphism (RFLP) of their mtDNA. Further on the haplotypes will be represented by specific strains; haplotype I: strain No. 10930, haplotype II: strain No. 9132, haplotype III: strain No. 5796, haplotype IV: strain No. 17471).

In this study the physical map of the mtDNA of the representative strains belonging to the four haplotypes was constructed on the basis of the pattern generated by *Eco*RI and *Eco*RV restriction endonucleases (Fig. 1). The *Eco*RI and *Eco*RV-pattern of *C. albicans* strain SC 5314 was generated *in silico* and it is proved identical to haplotype III.

*Eco*RI digestion generated identical patterns in all the four haplotypes while considerable differences were detected in the *Eco*RV digestion patterns. Nucleotide sequence analysis of the *Eco*RI fragments terminals in haplotypes I, II and IV revealed that single nucleotide polymorphisms (SNPs), resulting in distinctive *Eco*RV patterns were responsible for the polymorphism.

The background of the detected size differences [4] was analyzed by nucleotide sequencing. The size variation (about 100 bp) could be attributed to the smallest *Pvu*II fragment (1305 bp in strain SC 5314). As this fragment was located within the inverted repeat (IR) region, therefore, it was duplicated (*Pvu*IIA and *Pvu*IIB). The length and the nucleotide sequence (AF285261) of the two fragments were the same in strain SC 5314. Primers were designed to the neighboring regions (CapvuA frw

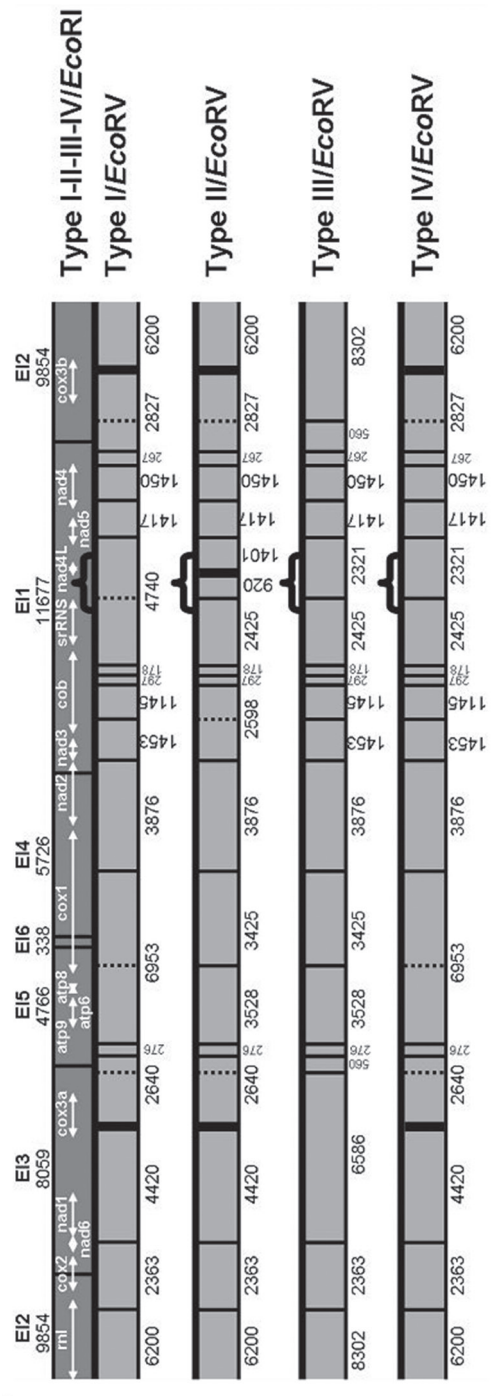

Fig. 1. Restriction and partial genetic maps of the mtDNA of the representative strains belonging to the four *C. albicans* haplotypes. The brackets designated the CALDIF region. Thick lines: new *Eco*RV digestion sites, dotted lines: ceased *Eco*RV digestion sites

and CapvuA rev, CapvuB frw and CapvuB rev), and both fragments were amplified and sequenced (Table 2.).

The nucleotide sequence of *Pvu*IIA and *Pvu*IIB fragments of haplotype III showed 100% similarity to the corresponding region of strain SC 5314. Results revealed two regions indicated S50 and S55 (Fig. 2). The lack of the S55 region resulted in 55-bp-long deletions in the position 36217–36271 bp and 9689–9743 bp in haplotypes I and II. Fifty-bp-long deletions were detected in the position 9143–9192 bp and 36768–36817 bp as the consequence of the absence of the S50 region in haplotype IV. Therefore, the mitochodrial genome of haplotypes I, II and IV was smaller compared to haplotype III. The size of the mtDNA proved 40310 bp in haplotypes I and II, while it was 40320 bp in haplotype IV.

A specific region named EO3 was identified within the inverted sequences of *C. albicans* mtDNA [9]. This region showed length and restriction site polymorphism among 154 *C. albicans* strains. Earlier studies revealed the variable presence of two ~50 bp-sequences within this region [10]. The *Pvu*IIA fragment overlapped with the previously described EO3 region. The EO3 region exhibited DNA-size polymorphism, yielding three fragment types: L, M (I and II), and S [10]. We managed to correlate the identified haplotypes to the clusters established by Miyakawa and co-workers [10]: haplotype III and SC 5314 belonged to group L, haplotypes II and I matched M-II, while haplotype IV fitted to MI, group S did not have representatives in the present population of *C. albicans*. Moreover, the exact size of the two small sequence regions was determined during this work; one proved 50 bp and the other one 55 bp.

Nucleotide sequence analysis revealed 27 polymorphic positions within the *Pvu*IIA fragment of the four haplotypes. All these differences restricted to the same positions both in the aligned sequences of *Pvu*IIA and *Pvu*IIB fragments within one strain. As it was mentioned before, both fragments were located within the inverted repeat (IR) of the mtDNA. Gerhold et al. [5] identified putative replication origin within these regions and they demonstrated that recombination driven replication (RDR) frequently initiated at the end of the IRs. Our results are in accordance with their findings as the nucleotide changes at the same positions of the *Pvu*IIA and *Pvu*IIB fragments could be explained as the result of recombination within one mtDNA molecule rather than independent events, because in the latter case more

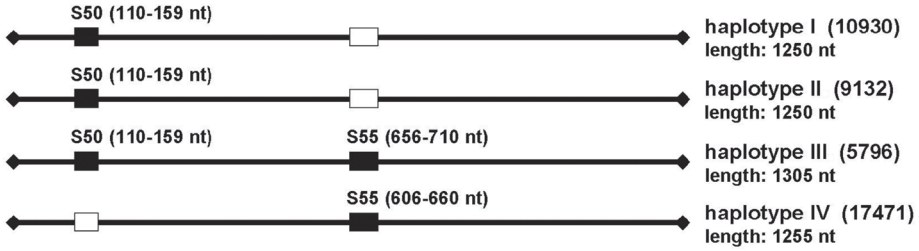

Fig. 2. Schematic drawing of the structure of the *Pvu*IIA fragments of the analyzed strains. Black box S50: 50-bp-long segment, black box S55: the 55-bp-long segment. Empty box: place of the deletion

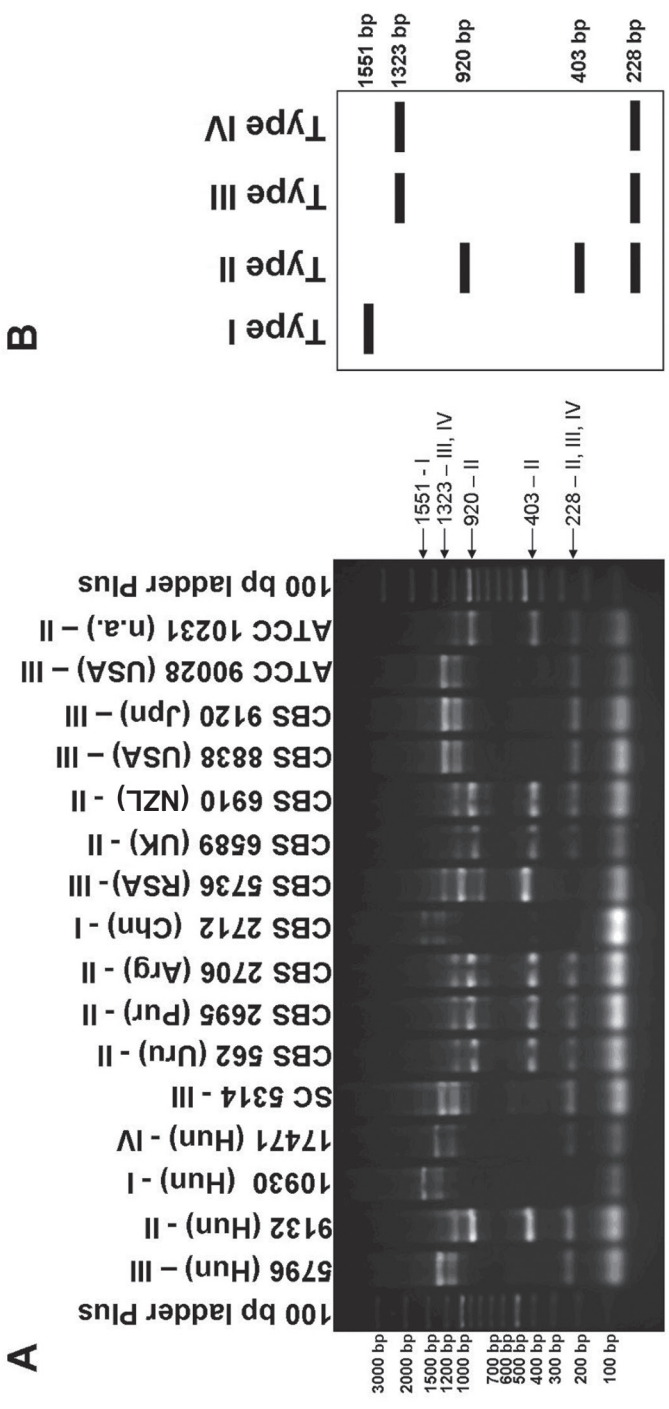

Fig. 3. A) The PCR RFLP pattern of the CALDIF region of *C. albicans* strains. B) Schematic drawing of PCR-RFLP pattern of the CALDIF region generated by *Eco*RV digestion

variation would be expected both in distribution of sites affected by mutation and within the sites where different states might be fixed.

Nevertheless, gene rearrangements in consequence of recombination could not be detected. The partial genetic map constructed with *C. albicans* homologue gene probes (*cox1, cox2, cox3, cob, nad1, nad2, nad4, nad5* and *atp6*) revealed the same gene order in the four types (Fig. 1).

Nucleotide sequence analysis detected 100% sequence similarity in the *Pvu*IIA and *Pvu*IIB fragments of haplotyp I and II containing the majority of the strains [23 and 11 strains, respectively]. Therefore, a region which differed remarkably corresponding to the *Eco*RV sites was chosen (CALDIF: Candida albicans different) on the basis of the restriction maps. This region (the end sequence of *srRNA*, an intergenic sequence and the whole *nad4l* gene [28321–29888 bp]) was amplified by specific (caldif frw and rev) primers and digested with *Eco*RV. The PCR RFLP patterns (Fig. 3) were distinct among haplotypes I, II, and III/IV, while they were identical inside the groups (haplotype I: no *Eco*RV site, 1551 bp; haplotype II: two *Eco*RV sites, 920 bp, 403 bp, 228 bp; haplotype III: one *Eco*RV site, 1323 bp, 228 bp; haplotype IV: same as haplotype III). To differentiate between haplotype III and IV, the PCR products were digested with *Bgl*II, which resulted different patterns (data not shown).

The PCR-RFLP of the CALDIF region of several strains from different geographic origin (Table 1) was analyzed to validate the results. All of them could be classified in the four haplotypes (Fig. 3). The most prevalent Hungarian haplotype I was represented only by a Chinese isolate, the isolates from South America, UK and New Zealand belonged to haplotype II, isolates from the USA and Japan proved haplotype III. The PCR RFLP pattern of the South African isolate could not be classified into the groups we created upon the *Eco*RV PCR-RFPL, however, using *Bgl*II it was similar to haplotype III. The most infrequent haplotype IV was not represented by any of the foreign isolates. Based on these results, we could conclude that PCR-RFLP analysis of the CALDIF region would be a good epidemiological and typing tool for *C. albicans:* it could refine the results of the abovementioned EO3 region analysis or substitute that approach.

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