

# First Characterization of *Candida albicans* by Random Amplified Polymorphic DNA Method in Nicaragua and Comparison of the Diagnosis Methods for Vaginal Candidiasis in Nicaraguan Women

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*A total of 106 women with vaginitis in Nicaragua were studied. The positive rate for the identification of Candida species was 41% (44 positive cultures out of 106 women with vaginitis). The sensitivity of microscopic examination of wet mount with the potassium hydroxide (KOH) was 61% and 70% with Gram's stain when using the culture of vaginal fluid as gold standard for diagnosis of candidiasis. Among the 44 positives cultures, isolated species of yeast from vaginal swabs were C. albicans (59%), C. tropicalis (23%), C. glabrata (14%) and C. krusei (4%). This study reports the first characterization of 26 C. albicans stocks from Nicaragua by the random amplified polymorphic DNA method. The genetic analysis in this small C. albicans population showed the existence of linkage disequilibrium, which is consistent with the hypothesis that C. albicans undergoes a clonal propagation.*

Key words: vulvovaginal candidiasis - diagnosis - *Candida albicans* - molecular typing - random amplified polymorphic DNA - population structure - Nicaragua

The genital candidiasis is one of the pathogenic demonstrations of yeast. *Candida albicans* is the most frequent species; it is usually isolated in 85 to 90% from the vaginal mycoses (Odds et al. 1988).

Vaginal candidiasis affects females at least once during their lifetime, at an estimated rate of 70 to 75%, of whom 40 to 50% will experience a recurrence (Sobel 1999).

In Nicaragua, we know very little about the prevalence and incidence of vaginal candidiasis and no study of the biology of *C. albicans* has been carried out. In this country, diagnosis of vaginal candidiasis is mainly based on the clinical presentation. Laboratories of the hospitals and health centres (peripheral laboratories) carry out only the microscopic diagnosis from the vaginal fluid. In the laboratory of the National Centre of Diagnosis and Reference of Nicaragua (CNDR), the yeast identification is based on the observation of the microscopic aspects, culture and biochemical tests.

Most of the genetic studies revealed that *C. albicans* is predominantly clonal (Pujol et al. 1993, Helstein et al. 1993, Lockhart et al. 1995, Xu et al. 1999). Some authors have proposed that clonal propagation with a remaining capacity of recombination, shape the population structure of *C. albicans* (Caugant & Sandven 1993, Gräser et al. 1996, Tibayrenc 1997).

Pujol et al. (1997) demonstrated that the fingerprinting of *C. albicans* random amplified polymorphic DNA (RAPD), multilocus enzyme electrophoresis (MLEE) and

Southern blot hybridization with the moderately repetitive DNA Ca3 probe, not only clustered moderately related isolates in a similar fashion but also afforded similar levels of resolution of microevolution within a clonal population.

The goal of this study was to use the RAPD method to examine the patterns of yeast genetic diversity among women with vaginitis from a single geographic area. We were specifically interested to know the frequency of yeast in vulvovaginal secretions. We also compared the conventional methods of yeast diagnosis from vaginal samples used in Nicaragua and yeast culture method.

## MATERIALS AND METHODS

The vaginal swabs were taken from 106 women exhibiting symptoms of vulvovaginitis, who were attended in the outpatient ward of the CNDR in Managua, Nicaragua, between June and August 1997. Swabs were processed by the method routinely used for the detection of germinated yeast pathogens: microscopic examination of wet mount, with a 10% potassium hydroxide (KOH) preparation, and the Gram's stain. Samples were inoculated into Sabouraud-glucose agar, supplemented with chloramphenicol, and were incubated at 37°C for 48 h. For identification of *C. albicans*, isolates were placed in foetal calf serum for 4 h to test for the production of germ tubes and were incubated on Rice-Agar-Tween (RAT®) BioMérieux Laboratories, France for 48 h to induce chlamydospores. All yeast isolates were preliminary identified to the species level according to the CHROMagar Albicans® Test (Mycoplasme International, Toulon, France). This medium contains a chromogene substrate for immediate identification of *C. albicans* (green), *C. tropicalis* (metallic blue), *C. glabrata* (pink) and *C. krusei* (pale pink). Yeast species were confirmed with ID 32C identification kits (bioMérieux, Marcy-l'Etoile, France). Growth at 45°C on

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Sabouraud glucose agar was used to distinguish between *C. albicans* and *C. dublinensis*.

We compared the microscopy and KOH test with the culture (chosen as gold standard), which is at present considered as the most sensitive method (Sobel 1999).

Sensitivity and specificity of each technique were estimated with the culture results with the following formulas:

Sensitivity = True positives/True positives + False negatives  
Specificity = True negatives/True negatives + False positives

The identified *C. albicans* stocks were used to study the genetic structure of this population. The stocks were stored in 1.5 ml of a stationary phase culture (until reaching a density of  $2 \times 10^8$  cells/ml), mixed with 0.1 ml of dimethyl sulfoxide and frozen at  $-70^\circ\text{C}$  until use. Four reference *C. albicans* strains of the American Type Culture Collection (ATCC) were used as control strains: 90028, 64548, 64550 and 64551. Six stocks belonging to other species from the strains collection of the Parasitology Laboratory of the Purpan Hospital of Toulouse, France, were used as outgroups for phylogenetic analysis (*C. krusei*, *C. tropicalis*, *C. glabrata*, *C. parapsilopsis*, *Saccharomyces cerevisiae* and *Geotrichum candidum*).

**DNA extraction** - A modification of procedure previously applied to *S. cerevisiae* was used (Philippsen et al. 1991). Briefly, the yeast colonies were taken from each agar plate and were inoculated in 15 ml of YPD (2% yeast extract, 1% peptone, 2% dextrose). Yeast were grown overnight at  $37^\circ\text{C}$  to a cell density of approximately  $2 \times 10^8$  cells/ml (early stationary phase). The yeast were collected by centrifugation at 3200 g for 5 min, washed in 15 ml of water at 1700 g for 5 min and suspended once again in 1.5 ml of a solution (pH 7.5) of 0.9 M sorbitol, 0.1 M EDTA, 50 mM dithiothreitol plus 0.5 mg of Zymolias 20T (ICN, biomedical inc, UK). After 1 h of incubation at  $37^\circ\text{C}$ , the cells were centrifuged at 1700 g for 5 min and suspended in 0.5 ml of lysis buffer: 50 mM Tris HCl pH 8.0, 10 mM EDTA, 1% SDS (W/V), plus 5  $\mu\text{l}$  of proteinase K 20  $\mu\text{g}/\text{ml}$ . The mixture was incubated at  $56^\circ\text{C}$  for 2 h. Eight  $\mu\text{l}$  of RNase A (100  $\mu\text{g}/\text{ml}$ ) were then added, followed by mixing and incubation at  $37^\circ\text{C}$  for 30 min. DNA purification was carried out by classical phenol-chloroform extraction (Sambrook et al. 1989). The DNA was precipitated with cold ethanol at  $-70^\circ\text{C}$  for 1 h and centrifuged at 5000 g for 30 min. The pellet was washed twice with 70% ethanol. The precipitate was dried and suspended in 100  $\mu\text{l}$  of purified water.

**RAPD** - Conditions for the RAPD have been previously described (Williams et al. 1990). Briefly, a polymerase chain reaction was performed in 0.5 ml microcentrifuge tubes in a final reaction mixture (60  $\mu\text{l}$ ) containing 20 ng of *C. albicans* DNA, 6  $\mu\text{l}$  of 10X buffer for Taq DNA polymerase, 0.9 U of Taq DNA polymerase (Boehringer Mannheim, Meylan, France), 100  $\mu\text{M}$  each dATP, dCTP, dGTP and dTTP (Boehringer Mannheim), and 200 nM of the primers (Operon Technologies, Alameda, USA). The decameric primers used were A2 (5' CAGGCCCTTC 3'); A3 (5' AGTCAGCCAC 3'); A5 (5' AGGGGTCTTG 3'); A9 (5' GGGTAACGCC 3'); A12 (5' TCGGCGATAG 3'); A15 (5' TTCCGAACCC 3'); A18 (5' AGGTGACCGT 3') and A20 (5' GTTGCATCC 3'). Amplifications were performed

in a thermal cycler PTC-100 (MJ Research Inc., USA) programmed for 45 cycles of 1 min at  $94^\circ\text{C}$  (denaturation), 1 min at  $36^\circ\text{C}$  (annealing) and extension at  $72^\circ\text{C}$  for 2 min, with a final 10 min extension at  $72^\circ\text{C}$  for the last cycle. Amplified DNA fragments were analyzed by electrophoresis on 1.6 % agarose gels stained with ethidium bromide.

**Genetic analysis** - Genetic relationships among the stocks were estimated by the Jaccard's genetic distance (Jaccard 1908). Each RAPD band was coded with a number, starting with 1 for the slowest band. The distance was estimated based on the following formula:

$$D = 1 - [a/(a + b + c)]$$

a = number of bands that are common to the two compared genotypes

b = number of bands present in the 1st genotype and absent in the 2nd

c = number of bands absent in the 1st genotype and present in the 2nd

The UPGMA method [Unweighted Pair-Group Method with Arithmetic Averages, Sneath and Sokal (1973)] was used to cluster the genotypes together according to their Jaccard's distances.

Population genetic analysis was based on linkage disequilibrium statistics, with random mating as null hypothesis. The following four probability values proposed by Tibayrenc et al. (1990) were used: *d1*: the combinatorial probability of sampling the most frequent genotype as often as or more often than actually observed if there were random recombination; *d2*: the probability of observing any genotype as often as or more often than the most common genotype actually observed if there were random recombination; *e*: the probability of observing as few or fewer genotypes than actually observed if there were random recombination; and *f*: it gives the probability of observing linkage disequilibrium as high, or higher than actually observed in the sample if there were random recombination. If a probability is non significant ( $p > 5 \times 10^{-2}$ ), random recombination can not be rejected, but it is significant ( $p < 5 \times 10^{-2}$ ), it supports the nonrandom association of loci. The *f* test is based on Montecarlo simulations with  $10^4$  iterations. A level of significance  $< 10^{-4}$  means that no case was observed out of  $10^4$  iterations (Tibayrenc et al. 1991).

## RESULTS

**Frequency of candidiasis** - Yeast were isolated in 44 out of 106 women (42%), while no trace of yeast was found in 62 women (58%). *C. albicans* was the most frequently isolated species accounting for 26 (59%), followed by the next most frequent yeast species, *C. tropicalis*, which was isolated from 10 (23%) of the women. Other species of *Candida* were also cultured: *C. glabrata* from 6 (14%) and *C. krusei* from 2 (4%).

**Laboratory methods** - The microscopic examination gave 31 positive results and 13 negative (Table I). Among the 44 positive in culture, the sensitivity of the microscopic examination by the Gram's stain was 70%. The KOH test was positive in 27 cases (61%). The specificity of both techniques was 100% (all negative results in microscopic examination and KOH test were culture negative).

The filamentation test was positive for all the *C. albicans* stocks and negative for the other species. The RAT confirmed the presence of chlamyospore of *C. albicans*, whereas for the other species, we observed only the presence of yeast.

**RAPD** - The 26 *C. albicans* stocks collected in this study were analyzed with 8 individual primers. These primers were selected from 20 primers tested for their capacities to discriminate variability and reproducibility. Eight primers for the *C. albicans* stocks generated 37 bands. From the total samples (*C. albicans* and the others species) the number of bands was 78. RAPD profiles were close to the four reference stocks. The RAPD profiles obtained with the A3 and A20 primers were better for differentiating the yeast stocks (Fig. 1).

**Genetic diversity** - All primers tested showed the polymorphic bands. The level of RAPD resolution was high: among 26 stocks, 23 different rapdemes were observed (genotype diversity = 0.88). All the primers exhibit variability.

**Phylogenetic clustering** - The UPGMA tree derived from RAPD data showed that all *C. albicans* fell into a single cluster, in which Jaccard's genetic distances were fairly low, while the other non-*C. albicans* stocks fell apart

in a sharply distinct cluster (Fig. 2). The most important genetic distance among the stocks of our sample was 0.49, with an average of  $0.26 \pm 0.1$ , theoretical maximum is 1.0. The level of polymorphism was 0.75 and the mean genetic diversity was 0.45.

**Genetic population tests** - The linkage disequilibrium tests (*dI*, *e*, and *f*) were significant within the *C. albicans* group with all stocks, but when repeated genotypes were removed, the *f* test only showed a borderline p value (0.06).

**DISCUSSION**

**Laboratory diagnosis** - When we used the culture as gold standard for detecting *Candida* sp. the sensitivity of microscopic examination by Gram's stain was more effective than the KOH test. These results are similar to a study made by Geiger et al. (1995). A positive culture does not necessarily indicate that the yeast is responsible for the vaginal symptoms. The diagnosis of *Candida* vaginitis requires a correlation between clinical conditions, and laboratory results.

Studies indicate that *Candida* sp. may be isolated from lower genital tract of approximately 20% (occasional studies set the upper limit at 55%) of asymptomatic healthy

TABLE I  
Results of diagnosis of *Candida* sp. by microscopy and potassium hydroxide (KOH) test on 44 culture positive vaginal fluid samples from Nicaraguan vaginitis patients

Isolates <sup>a</sup>	Microscopy				KOH test				Total	
	Positive		Negative		Positive		Negative		N	%
	N	%	N	%	N	%	N	%		
<i>C. albicans</i>	19	73	7	27	16	61	10	39	26	59
<i>C. tropicalis</i>	6	60	4	40	5	50	5	50	10	23
<i>C. glabrata</i>	4	67	2	33	4	67	2	33	6	14
<i>C. krusei</i>	2	100	0	0	2	100	2	100	2	4
Total	31	70	13	30	27	61	17	39	44	100

a: isolates were typed based on the chromagar test and confirmed with ID 32C identification kits; N: number of corresponding samples

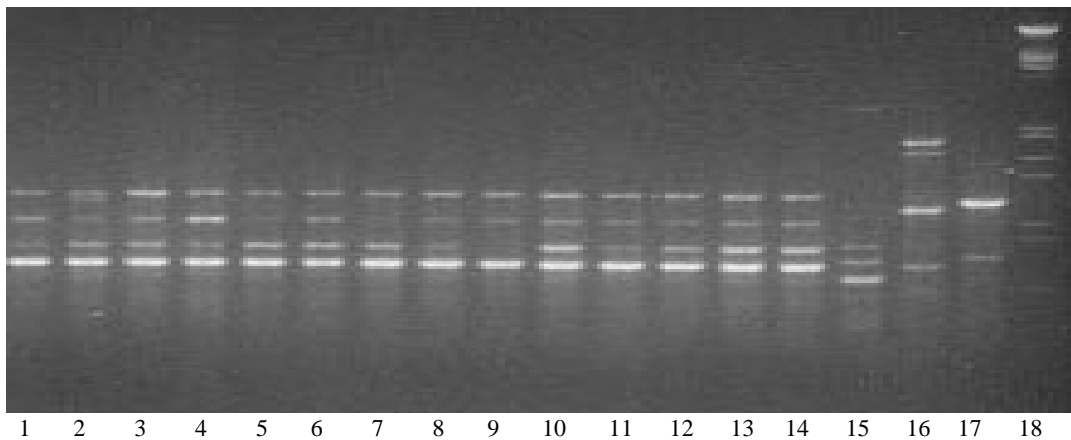


Fig. 1: random amplified polymorphic DNA patterns obtained with the primer A3 for the *Candida albicans* stocks from Nicaragua: 1. CANO1; 2. CANO2; 3. CANO3; 4. CANO4; 5. CAN14; 6. ATCC 64550; 7. CAN20; 8. CAN07; 9. ATCC 90028; 10. CANO9; 11. CAN10; 12. CAN11; 13. CAN12; 14. CAN13; 15. *Geotrichum candidum*; 16. *Sacharomyces cerevisiae*; 17. *C. krusei*; 18. Momcular weight marker.

women (Drake & Maibach 1973). Our results in symptomatic women showed that yeast were present in 41% of the vaginal specimens. This study was based on the isolation of agent from vaginal fluid in culture, which does not allow to differentiate pathogenic from saprophytic *Candida* sp. As these microorganisms are common colonisers of the female genital tract, it would be useful to have a gold standard for identification of *Candida* sp. able to distinguish pathogenic forms from saprophytic ones.

As previously observed in others studies (Tietz et al. 1995, Mendoza et al. 1999),

*C. albicans* was the most frequent specie of yeast isolated from these vaginal samples from Nicaragua, with a total of 59%.

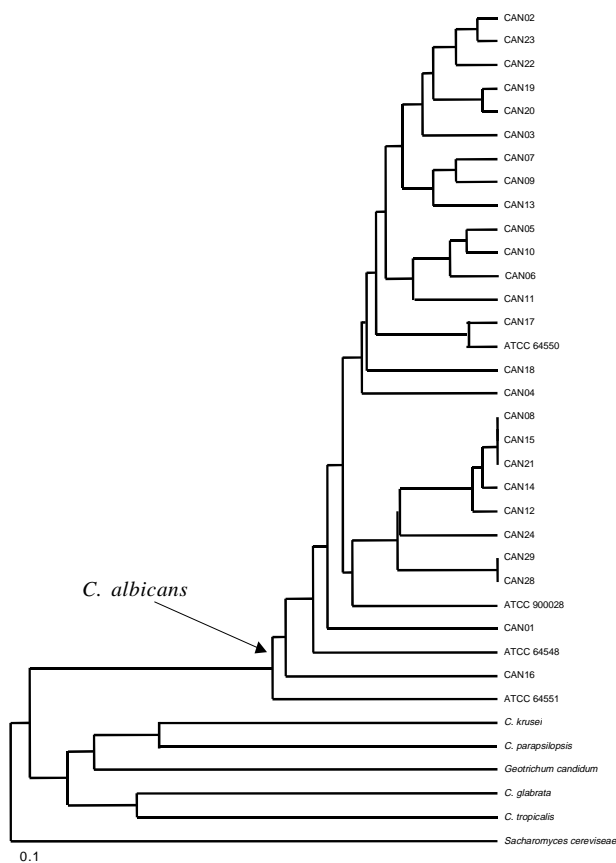


Fig. 2: UPGMA dendrogram derived from Jaccard's distances after random amplified polymorphic DNA data of the 26 *Candida albicans* isolates from Nicaragua and reference stocks.

When we compared ours results with the data from others tropical countries (Venezuela, Angola, and Madagascar), no statistical difference in the *C. albicans*/non-*C. albicans* distribution was observed ( $P = 0.75$ ). However, compared to results from Dublin (Al-Rawi et al. 1999), a statistical difference was observed ( $P < 0.002$ ), with a higher frequency of *C. albicans* in Dublin (Table II). Recruitment strategy of each study could have modified these results, but they suggest the possibility of differences linked to climate or to socio-economic development, further studies on this thematic are necessary.

**Genetic and phylogenetic diversity of *C. albicans*** - The present study confirmed early results and demonstrated that stocks attributed to *C. albicans* by classical morphological and biological criteria display a genetic and phylogenetic diversity. The results recorded here confirm a tendency, already noted by Schmid et al. (1993), in a population of *C. albicans* strains from vaginal samples using the DNA fingerprints with the moderately repetitive sequence Ca3, observing that the majority of the stocks of *C. albicans* are in a relatively homogeneous group. A high genetic similarity was also reported, using the same Ca3 probe, in strains isolated from women with vaginal candidiasis, from a same geographical region (Schmid et al. 1999).

**Population genetic analysis** - Like many other pathogens, the question of the population structure of *C. albicans* has been the subject of intense debates (Tibayrenc 1997, Vilgalys et al. 1997). Early studies addressing this question (Tibayrenc et al. 1991, Caugant & Sandven 1993) have recorded low levels of linkage disequilibrium (nonrandom association of genotypes occurring at different loci), by comparison with other pathogens such as *Trypanosoma* or *Leishmania* (Tibayrenc et al. 1990). By contrast, other authors (Hellstein et al. 1993, Pujol et al. 1993, Xu et al. 1999, Arnavielhe et al. 2000) have found considerable levels of linkage in different *C. albicans* populations and have concluded that these populations propagate clonally. Tibayrenc (1997) has proposed that the relevant boundary is not between clonal and sexual species, but rather between species that are structured into stable evolutionary units (DTUs), and species in which genetic exchange is frequent enough to render impossible that maintaining of such stable subdivisions.

The significant tests of linkage disequilibrium observed here in the population of 26 *C. albicans* allow us to reject the hypothesis of a panmictic structure within these *C. albicans* stocks from Nicaragua. However, when

TABLE II

Distribution of *Candida albicans* and non-*C. albicans* species in vaginal samples from patients with vaginitis in five countries

Species	Nicaragua		Angola <sup>a</sup>		Madagascar <sup>a</sup>		Venezuela <sup>b</sup>		Dublin <sup>c</sup>	
	N	%	N	%	N	%	N	%	N	%
<i>C. albicans</i>	26	59	35	63	53	64	12	52	26	93
Non- <i>C. albicans</i>	18	41	19	36	32	36	11	48	2	7
Total	44	100	54	100	85	100	23	100	28	100

a: Tietz et al. (1995); b: Mendoza et al. (1999); c: Al-Rawi et al. (1999)

the repeated genotypes were removed, only a limited, non-significant, linkage disequilibrium was found. A statistical type II error (lack of power of the test used in our small sample) could explain this fact. This population genetic analysis indicates that the two fundamental consequences of sexual reproduction (segregation and recombination) are apparently absent or rare in this *C. albicans* population. Now, it is impossible to identify clear-cut subdivisions within the *C. albicans* cluster (Fig. 2). It is worth noting that for another pathogen, *T. cruzi*, the agent of Chagas disease, clear subdivisions could be individualized by both isoenzyme electrophoresis and RAPD typing with an even lower set of primers (Tibayrenc et al. 1993). This result has been fully confirmed by another study involving a broader range of primers (Brisse et al. 1998). The results obtained here: significant linkage disequilibrium with apparent lack of stable and clear-cut subdivisions, are consistent with the proposition that the propagation of *C. albicans* is mainly clonal.

Nevertheless, complementary studies with other molecular markers and with other *C. albicans* populations from Nicaragua are necessary to ascertain the *C. albicans* population structure in this country. In any case, confrontation of clinical and therapeutic data with set of molecular data, such as RAPD, would be useful for a better understanding of the epidemiological aspects of the candidiasis infections.

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#### REFERENCES

- Al-Rawi N, Kavanagh K 1999. Characterisation of yeasts implicated in vulvovaginal candidosis in Irish women. *Br J Biomed Sci* 56: 99-104.
- Arnavielhe S, De Meeus T, Blancard A, Mallie M, Renaud F, Bastide JM 2000. Multicentric genetic study of *Candida albicans* isolates from non-neutropenic patients using MLEE typing: population structure and mode of reproduction. *Mycoses* 43: 109-117.
- Brisse S, Barnabé C, Tibayrenc M 1998. *Trypanosoma cruzi*: how many relevant phylogenetic subdivisions are there? *Parasitol Today* 14: 178-179.
- Caugant DA, Sandven P 1993. Epidemiological analysis of *Candida albicans* strains by multilocus enzyme electrophoresis. *J Clin Microbiol* 31: 215-220.
- Drake TE, Maibach HI 1973. *Candida* and candidiasis. 1. Cultural conditions, epidemiology and pathogenesis. *Postgrad Med J* 53: 83-87.
- Geiger AM, Foxman B, Sobel JD 1995. Chronic vulvovaginal candidiasis: characteristics of women with *Candida albicans*, *C. glabrata* and no *Candida*. *Genitourin Med* 71: 304-307.
- Gräser YM, Volovsek J, Arrington G, Schöniat W, Presber TG, Mitchell Vilgalys R 1996. Molecular markers reveal that population structure of the human pathogen *Candida albicans* exhibits both clonality and recombination. *Proc Natl Acad Sci USA* 93: 12473-12477.
- Hellstein J, Vawter-Hugart H, Fotos P, Schmid J, Soll DR 1993. Genetic similarity and phenotypic diversity of commensal and pathogenic strains of *Candida albicans* isolated from the oral cavity. *J Clin Microbiol* 31: 3190-3199.
- Jaccard P 1908. Nouvelles recherches sur la distribution florale. *Bull Soc Vaudoise Sci Nat* 44: 223-270.
- Lockhart S, Fritch J, Meier A, Schröppel K, Srikantha T, Galask R, Soll D 1995. Colonizing populations of *Candida albicans* are clonal in origin but undergo microevolution through C1 fragment reorganization as demonstrated by DNA fingerprinting and C1 sequencing. *J Clin Microbiol* 33: 1501-1509.
- Mendoza M, Gonzalez I, Bellorin EJ, Salazar W, Mendoza L, Zambrano EA, De Albornoz MC 1999. Isolation, identification and serotyping of yeasts obtained from the vaginal fluid in patients with clinical vaginitis. *Invest Clin* 40: 25-36.
- Odds FC 1988. *Ecology of Candida and Epidemiology of Candidosis*, Bailliere Tindall, London, p. 124-125.
- Philippen P, Stotz A, Scherf C 1991. DNA of *Saccharomyces Cerevisiae*. *Meth Enzymol* 194: 169-173.
- Pujol C, Renaud F, Mallie M, de Meeus T, Bastide JM 1997. Atypical strains of *Candida albicans* recovered from AIDS patients. *J Med Vet Mycol* 35: 115-121.
- Pujol C, Reynes J, Renaud F, Raymond M, Tibayrenc M, Ayala FJ, Janbon F, Mallie M, Bastide JM 1993. The yeast *Candida albicans* has a clonal mode of reproduction in a population of infected human immunodeficiency virus-positive patients. *Proc Natl Acad Sci USA* 90: 9456-9459.
- Sambrook J, Fritsch EF, Maniatis T 1989. *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, NY.
- Schmid J, Rotman M, Reed B, Pierson CL, Soll DR 1993. Genetic similarity of *Candida albicans* strains from vaginitis patients and their partners. *J Clin Microbiol* 31: 39-46.
- Schmid J, Scott H, Hunter PR, Cannon RD, Salleh Y, Samad S, Carr M, Parr D, McKinney W, Schousboe M 1999. Evidence for a general-purpose genotype in *Candida albicans*, highly prevalent in multiple geographical regions, patients types and types of infection. *Microbiology* 145: 2405-2413.
- Sneath PHA, Sokal RR 1973. Numerical taxonomy. In DAP Kennedy, *The Principle and Practice of Numerical Classification*, Freeman, San Francisco, 537 pp.
- Sobel J 1999. Vulvovaginal candidiasis. In KK Holmes, P-A Mardh, PF Sparling (eds), *Sexually Transmitted Diseases*, Mc Graw Hill, UK, p. 629-639.
- Tibayrenc M 1997. Are *Candida albicans* natural populations subdivided? *Trends Microbiol* 5: 253-257.
- Tibayrenc M, Kjellberg F, Arnaud J, Oury B, Brenière SF, Dardé ML, Ayala FJ 1991. Are eukaryotic microorganisms clonal or sexual? A population genetics advantage. *Proc Natl Acad Sci USA* 88: 5129-5133.
- Tibayrenc M, Kjellberg F, Ayala FJ 1990. A clonal theory of parasitic protozoa: the population structure of *Entamoeba*, *Giardia*, *Leishmania*, *Naegleria*, *Plasmodium*, *Trichomonas*, and *Trypanosoma* and their medical and taxonomical consequences. *Proc Natl Acad Sci USA* 87: 2414-2418.
- Tietz H-JA, Kussner M, Thanos M, Pinto de Andrade M, Presber W, Shonian G 1995. Phenotypic and genotypic characterization of unusual vaginal isolates of *Candida albicans* from Africa. *J Clin Microbiol* 33: 2462-2465.
- Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV 1990. DNA polymorphisms by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 18: 6531-6535.
- Xu J, Boyd CM, Livingston E, Meyer W, Madden JF, Mitchell TG 1999. Species and genotypic diversities and similarities of pathogenic yeasts colonizing women. *J Clin Microbiol* 37: 3835-3843.

