Chapter 3

RESULTS

Optimization of the PCR Assays

In the development of an RAPD fingerprinting system for this study, seven oligonucleotide primers (Table 2) were tested to select the appropriate primer which yielded the greatest number of DNA bands. Determination of the optimal reaction conditions for RAPD was carried out, at first according to the reference methodology (Table 2) or alternatively, using adjustable genomic DNA, and changing the temperature profiles in the thermal cycles. The other parameter that was adjusted was the concentration of $MgCl_2$ (1, 1.5, 2, 2.5, 3, 3.5 mM).

The results showed that four of seven primers: primer 1, PA03, RSD11 and RSD12 gave DNA bands, whereas, the remaining three primers (M13, C1 and C3) never showed any bands despite adjusting the temperature profiles or concentrations of $MgCl_2$. The optimal concentration of $MgCl_2$ that yielded the most intense bands was 1.5 mM for the former group of four primers. Primer 1 gave only one band (Figure 4), primer PA03 gave 4 bands (Figure 5), and primers RSD11 and RSD12 gave several bands. However, RSD11 yielded more bands than RSD12 and the bands ranged from 0.5-2.0 kb while the bands generated through RSD12's were greater than 1.5 kb (Figure 6).

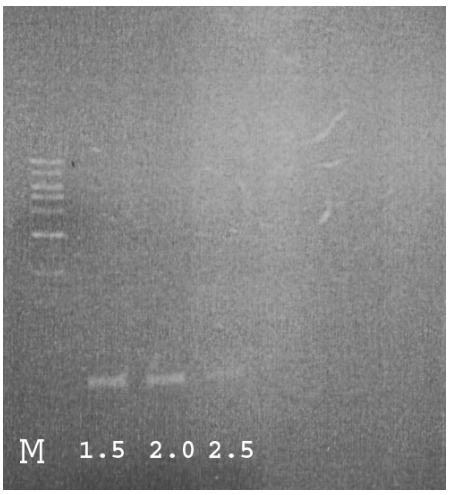

Figure 4 In optimization of PCR assays, only a single DNA band was generated from primer 1, despite adjustments of $MgCl_2$ concentrations (1.5 mM- lane 2, 2 mM- lane 3, 2.5 mM -lane 4). The 1.5 mM $MgCl_2$ exhibited the most intensity. (*C. albicans* used = A003/4A).

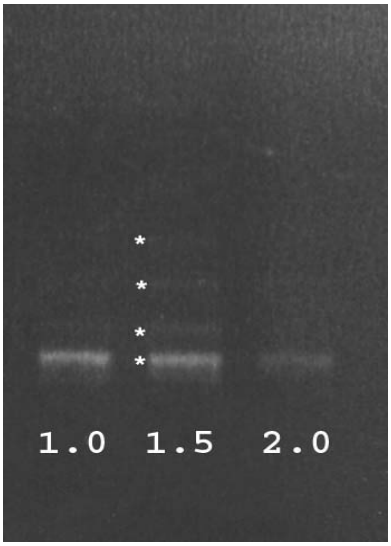

Figure 5 Four bands resulting from primer PA03 with various concentrations of $MgCl_2$ (1 mM - lane1, 1.5 mM – lane 2, 2 mM – lane 3) during optimization of PCR assays. (*C. albicans* used = N001/3).

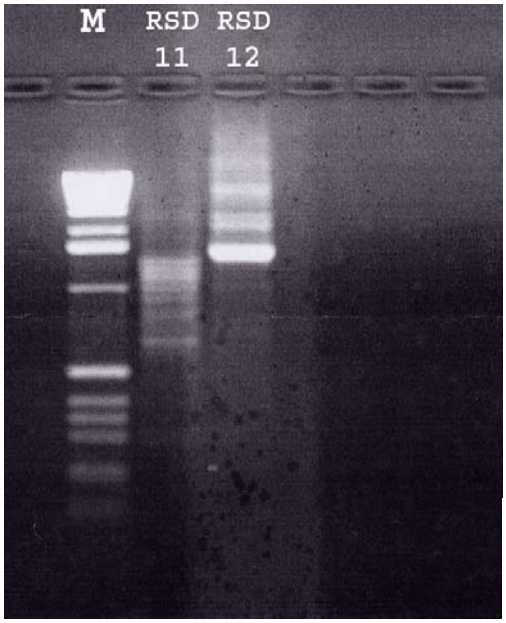

Figure 6 Band patterns generated using primer RSD11 (lane a) and RSD12 (lane b) during optimization of PCR assays. (*C. albicans* used = A025/4B).

Genetic Analysis

As the molecular profile resolution was great with primer RSD11, the latter was used in all subsequent RAPD assays with all 189 strains. They were oropharyngeal *C. albicans* strains from 41 HIV-infected patients and, 17 strains from 6 healthy subjects with no HIV infection (see Appendix I).

1. Clonal variability of *C. albicans*

RAPD analysis was performed to determine the clonal distinction. Of 41 HIV-infected patients studied, 8 patients possessed one strain each while the remaining 33 patients had 2-15 strains of *C. albicans* (see Appendix I). All isolates from each patient showed distinct RAPD patterns. Most of the HIV-infected individuals exhibited varying and disparate clonal patterns either within the same individual or between them. The gel images indicated different

genetic patterns from different individuals as shown in the example of 12 patients in Figure 7.

Using dendrogram data clonal distinction of *C. albicans* strains was assessed of thirty-three patients who yielded more than one strain. Almost all patients, accounting for 96.6% (32/33) had more than one distinct clonal type of *C. albicans*. On further analyses it was evident that a single patient could be colonized by up to 15 clonal types (see Appendix I). Only one patient 3.4% (1/33) had one genotype for two identical strains. One half of these individuals (17/33) had multiple strains positioned in the same cluster implying the close similarity in genetic pattern of yeasts derived from the same individual. Further, with regard to strains from HIV-uninfected subjects, only one person yielded several strains while others possessed only one genotype each; genetic variety in this group was noted in 10 different clones from 12 strains.

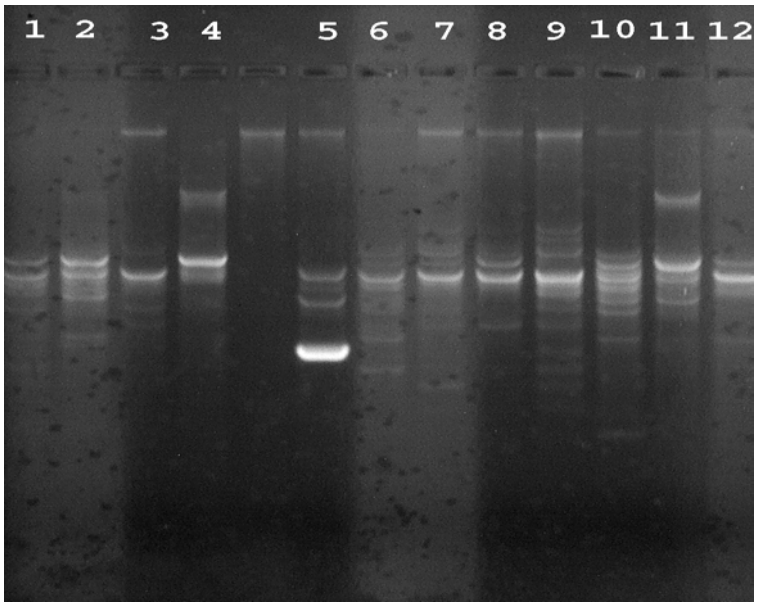

Figure 7 Twelve different genotypes of *C. albicans* (lane 1-12) derived from twelve HIV-infected individuals. All of the 12 lanes showed the dissimilarity of band pattern.

2. Genetic relatedness

2.1 Genetic relatedness of *C. albicans* in HIV-infected individuals

The dendrogram data was evaluated to determine strain relatedness. The S_{AB}s ranges from 0.00 to 1.00. A threshold of S_{AB} 0.80 has been suggested to be the arbitrary limit for clusters of moderately related isolates. [41,96] The moderately to highly related strains were noted from S_{AB}s of 0.80 upwards. The remaining, distantly related or unrelated strains were noted at the other end of the scale with S_{AB}s < 0.80.

The dendrograms based on S_{AB} values generated for 189 strains of 41 HIV-infected individuals with the primer RSD11 is shown in Figure 8, and the 17 strains of 6 HIV-uninfected subjects are shown in Figure 9.

The Dendron database formed 20 clusters containing 4 small clusters (cluster 1,2,3,4) and 16 larger clusters (cluster 5-20) at the S_{AB} 0.02 which was extremely loosely connected within this yeast population (Figure 8). Only 46 of 189 strains (24%) were grouped into clusters at S_{AB}s of ≥ 0.80. Within these 46 strains, 13 sets of identical strains (37 strains) or called “isotypes” were noted (Table 4). The details of these moderately to highly related strains are shown in Table 5. Most strains accounting to 76% (143/189) of the cohort were distantly related. Commonly, strains from the same patient were fitted into the same cluster (Figure 8).

In the relatedness among clusters, the small cluster 1, 2, 3 were absolutely unrelated to any other cluster since they were connected at S_{AB}s of 0.00 where as the larger number of clusters (cluster 4 to 20) were connected at S_{AB}s of 0.01. Within each of the eleven clusters, cluster1, 2, 3, 4, 7, 9, 10,11, 13, 19 and 20, were internally distantly connected. From the threshold of 0.80 which has been suggested to be the cut of point for determining whether the strains were distantly related, 143 strains (76%) were distantly related as shown in the dendrogram. There were six strains that were neither related nor grouped into any cluster, i.e. strain A 059/1 B, A 016/2 B, A 016/3 B, A 016/4 B, A

018/4 A and A 018/1 B. It is therefore noted that all the clusters were distantly connected with diverse genotypes in and between persons.

On further analysis of inter-cluster relationships, five major clusters were noted. The first major cluster consisted of three clusters, where the clusters 4, 5, 6 were connected at S_{AB} of 0.01 (Figure 8). The second major cluster consisted of cluster 8, 9, 10, 11, 12 and 13. Cluster 9 and 10 were firstly connected at S_{AB} of 0.05 and then connected to cluster 11 at S_{AB} of 0.02. The cluster 12 and 13 were firstly connected at S_{AB} of 0.04 and all were connected at S_{AB} of 0.01. The third major cluster consisted of clusters 14 and 15 connected to each other at S_{AB} of 0.02. The fourth major cluster consisted of clusters 16, 17 and 18. Clusters 17 and 18 were firstly connected at S_{AB} of 0.07 and then connected to cluster 16 at S_{AB} of 0.06. The last major cluster consisted of cluster 19 and 20 which were connected to each other at S_{AB} of 0.02.

In conclusion, this dendrogram generated through RAPD data revealed random distribution of *C. albicans* strains and random cluster formation, implying that the strains from HIV-infected individuals does not represent a genetically distinct clone.

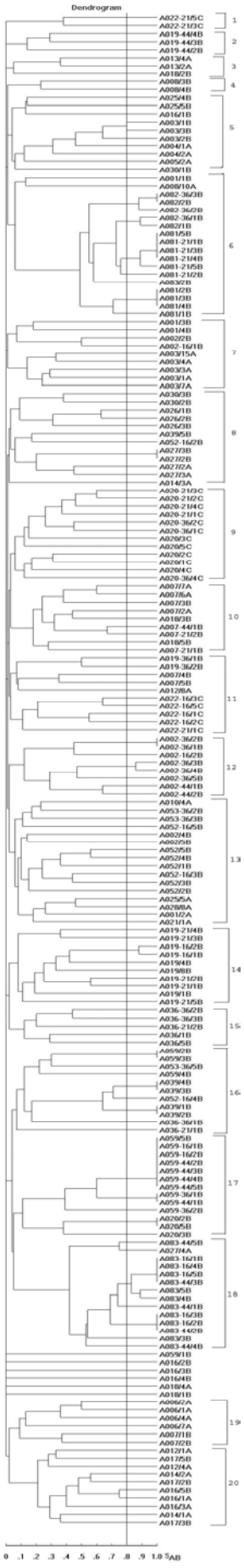

Figure 8 Dendrogram generated by cluster analysis of 189 *C. albicans* strains.

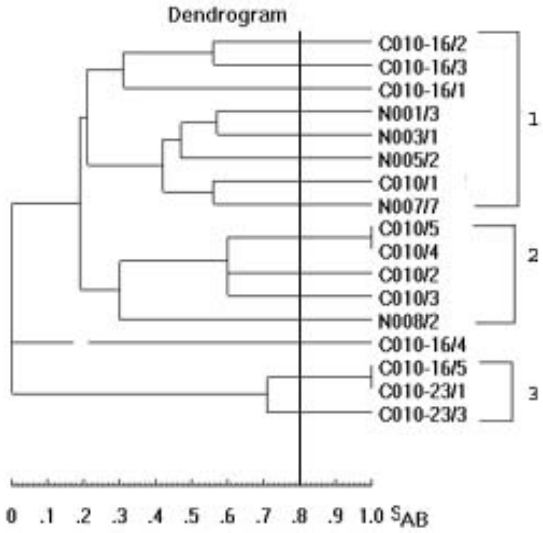

Figure 9 The dendrogram of 17 *C. albicans* strains obtained from non-HIV subjects

Table 4 Isotypes from 189 strains situated in the clusters shown in the dendrogram

Cluster No.	Strain name
6	A 082 - 36/3 B , A 082/2B A 081/2 B, A 081-21/1B, A081-21/3B, A081-21/4B A 081/2 B, A 081/3B, A081/4 B
8	A 027/3 B, A 027/2 B
12	A 002-36/2 B, A 002-36/1B
16	A 059/2 B, A059/3 B A 039/4 B, A 039/3 B A 039/1 B, A 039/2 B
17	A 059/5 B, A 059-16/1 B, A 059-16/2 B, A 059-44/2 B, A 059-44/3 B A 059-44/4B, A 059-44/5 B A 059-36/1 B, A059-44/1B A 020/2 B, A 020/5 B
18	A 083-16/1 B, A 083-16/ 4 B, A 083-16/5 B, A 083-44/3 B A 083-16/3 B, A 083-16/2 B, A 083-44/2 B

Table 5 Details of *C. albicans* strains tightly related at S_{AB}s of 0.80 or greater

Cluster	Number of strains	Patient	S_{AB} connected
5	2	A 003 B	0.80
6	3	A 082 B	0.89
	2	A 082 B	0.90
	5	A 081 B	0.90
8	2	A 027 B	1.00
12	2	A 002 B	1.00
	2	A 002 B	0.87
14	2	A 019 B	0.89
16	2	A 059 B	1.00
	2	A 039 B	1.00
	2	A 039 B	1.00
17	7	A 059 B	1.00
	2	A 059 B	1.00
	2	A 020 B	1.00
18	6	A 083 B	Subgroup*a*:1.00 Subgroup **b***:0.90 "a" connected to b***:0.84
	3	A 083 B	1.00

* Subgroup a : S_{AB} at subgroup "a" connection consisted of A 083-16/1 B, A 083-16/4 B, A083-16/5B, A083-44/3B

**subgroup b : S_{AB} at subgroup "b" connection consisted of A 083/5 B, A083/4 B

*** "a" connected "b" : S_{AB} at the connection of subgroups "a" and "b"

2.2 Genetic relatedness in HIV-uninfected subjects

The 17 *C. albicans* strains from HIV-free subjects showed a diverse genetic profile, as exemplified in Figure 9. Of the 17 strains analyzed by dendrogram three clusters and, one unrelated strain were categorized at S_{AB} = 0.02 (Figure 9). Four isotypes were situated in cluster 2 and 3, namely C010/5 and C010/4, C010-16/5 and C010-23/1. Besides these isotypes which were highly related, the other 13 strains (76.5%) were all distantly related situated at

S_{AB}s of less than 0.80. The clusters 1 and 2 were connected at S_{AB} of 0.19, and then connected to cluster 3 at S_{AB} of 0.00.

To conclude then, both dendrograms from HIV-positive individuals and normal subjects demonstrated similar random distribution of *C. albicans* strains. Thus 20 clusters were derived from 189 strains of HIV-infected individuals and 3 clusters from 17 strains of healthy individuals. Most of them were distantly related : 143 of 189 strains (76%) and 13 of 17 strains (76.5%) in diseased and healthy individuals, respectively. From this it may be postulated that the genetic patterns of *C. albicans* isolates of both of HIV-infected patients as well as healthy subjects are diverse.

Phenotypic Analysis

The two phenotypic systems included in this study were biotyping and antifungal susceptibility testing

1. Biotyping

One hundred and six strains were biotyped based on the report of Williamson et al.[131] Briefly, it comprises two commercially available kits, API ZYM for enzyme profile assessment and API 20C AUX for sugar assimilation, and one boric acid sensitivity test. The nomenclature is designated by two letters and one central figure : the first letter is from API ZYM, the central figure is from API AUX 20C and the last letter is referred to “sensitive” or “resistant” to boric acid. According to this method fifty-seven biotypes were generated when 106 strains of *C albicans* were analysed (Table 6). The A1S biotype was the most predominant, found in 30.4 % (32/106). From the data of figure 10 and Appendix III, the categorisation of the identical phenotype into different genotypic clusters are summarized in Table 7.

Table 6 The API biotype profiles and their percentage distribution of 106 *C. albicans* strains from HIV-infected patients.

Biotypes	Number of isolates	Percentage	Biotypes	Number of isolates	Percentage
A 1 S	32	30.4	B 6 S	1	0.95
A 1 R	1	0.95	B 15 S	1	0.95
A 4 S	1	0.95	B 16 S	1	0.95
A 6 S	1	0.95	B 19 S	1	0.95
A 6 R	1	0.95	B 20 S	1	0.95
A 7 S	1	0.95	B 21 R	1	0.95
A 8 S	3	2.85	B 22 S	1	0.95
A 13 S	3	2.85	B 24 S	1	0.95
A 13 R	1	0.95	C 1 S	2	1.90
A 14 S	3	2.85	C 1 R	1	0.95
A 14 R	1	0.95	C 2 S	2	1.90
A 15 S	1	0.95	C 13 S	2	1.90
A 16 S	1	0.95	C 22 R	1	0.95
A 17 S	1	0.95	C 26 S	1	0.95
A 17 R	1	0.95	C 29 S	1	0.95
A 18 S	1	0.95	D 1 S	3	2.85
A 19 S	1	0.95	D 1 R	1	0.95
A 21 S	1	0.95	D 8 S	2	1.90
A 22 S	1	0.95	D 14 S	1	0.95
A 22 R	1	0.95	E 2 R	1	0.95
A 23 S	1	0.95	E 14 R	1	0.95
A 23 R	1	0.95	E 21 S	1	0.95
A 24 S	1	0.95	E 29 R	1	0.95
A 25 S	1	0.95	F 4 S	1	0.95
A 26 S	1	0.95	G 1 S	1	0.95
A 27 R	1	0.95	G 2 S	3	2.85
A 30 S	1	0.95	G 5 R	1	0.95
B 1 S	3	2.85	G 6 R	1	0.95
B 4 S	3	2.85			

Table 7 Identical biotypes of *C. albicans*, but with varying RAPD genotypes, found in the same cluster.

Cluster No.	Diffent genotypes	Biotype	Cluster No.	Diffent genotypes	Biotype
4	A 020/5 B	A 1 S	5	A 026/1 B	A 1 S
	A 020/3 B	A 1 S		A 026/2 B	A 1 S
12	A 059/2 B	G 2 S	7	A 007/5 B	A 1 S
	A 059/ 4 B	G 2 S		A 007/4 B	A 1 S
13	A 081/1 B	A 1 S	6	A 007/2 A	A 1 S
	A 081/5 B	A 1 S		A 007/7 A	A 1 S
	A 082/2 B	A 1 S		A 020/3 C	A 1 S
	A 082/1 B	A 1 S		A 020/5 C	A 1 S
8	A 003/4 A	A 1 S		A 020/2 C	A 1 S
	A 003/15 A	A 1 S		A 020/1 C	A 1 S
	A 003/3 A	A 1 S		A 020/4 C	A 1 S
	A 003/7 A	A 1 S			

2. Ketoconazole susceptibility test

The antifungal susceptibility test for ketoconazole was performed with 94 *C. albicans* isolated from HIV-infected patients. The distribution of MICs of the yeasts are shown in Table 8. The MICs ranged from 0.0075 to ≥ 4.0 μg/ml and the median of MICs was 0.031 μg/ml. The susceptibility values were then used to sub-divide the *C. albicans* strains evaluated into two groups as "high" and "low" susceptible strains. The MICs ≤ 0.125 μg/ml were defined as "high susceptible" while MICs > 0.125 μg/ml were classified as "low susceptible". Three quarters of the strains tested belonged to high susceptible group. When these results were matched with dendrogram findings, the strains were widely distributed in several clusters (Appendix IV and Figure 10). In addition, it was observed that each patient had multiple strains with different genotypes that had similar MIC values.

As the biotyping data were also recorded concomitantly with genotype and ketoconazole susceptibility (Figure 10). The A1S biotype or other minor biotypes were distributed in several clusters and, randomly distributed. No specific biotypes could be categorized as showing either high or low sensitivity to the antifungal drug.

Table 8 Details of ketoconazole susceptibility testing in 94 strains.

Classified susceptibility	MIC value	No. of strains	Percentage	
High	0.0075	4	4.25	
	0.015	24	25.53	
	0.025	1	1.06	73%
	0.03, 0.031	25	26.6	
	0.06, 0.062	11	11.7	
	0.125	4	4.25	
Low	**0.25**	**7**	**7.45**	
	0.5	**3**	**3.19**	
	1.0	**5**	**5.32**	27%
	2.0	**1**	**1.06**	
	≥ 4.0	**9**	**9.57**	

Relationship between Genotype and Biotype, Antifungal Susceptibility

1. Genetic relatedness of *C. albicans* for the assessment of genotype-phenotype relationship

The 112 *C. albicans* strains that were phenotyped were evaluated by dendrogram generation and mapped, concurrently for biotyping and antifungal

susceptibility relationships (Figure 10). The dendrogram generated had 14 clusters consisting of 5 small clusters (cluster 3, 8, 9, 10, 14) and nine larger clusters (cluster 1, 2, 4, 5, 6, 7, 11, 12, 13). The relatedness of clusters and genotypes were similar to the bigger pool of 189 strains. There were 4 unrelated clusters and the remaining 10 clusters were distantly related (see Appendix III). Ninety-nine of 112 strains (85%) were distantly related. The details of the 106 strains that were biotyped and 94 strains that were tested for their antifungal susceptibility, are provided according to their clusters and genetic strains in Appendix III and figure 10.

2. Genotype and biotype

When the information related to dendrogram finding, and the biotypes, were compared, the major biotype (A1S) and other minor biotypes (56 biotypes) were generally distributed in several clusters (see Appendix IV). For example 32 strains belonging to A1S biotype were distributed amongst 9 clusters and, a single unclustered strain (Figure 11). Since the A1S strains were non-specifically distributed amongst several culsters which contained many different genotypes, it could be concluded that no relationship existed between the derived for RAPD analysis and the API biotype.

3. Genotype and ketoconazole susceptibility

When the high and low ketoconazole susceptible strains were evaluated for the relationship between genotype and ketoconazole susceptibility, the organisms were distributed amongst several clusters representing different genotypes as shown in Figure 12.

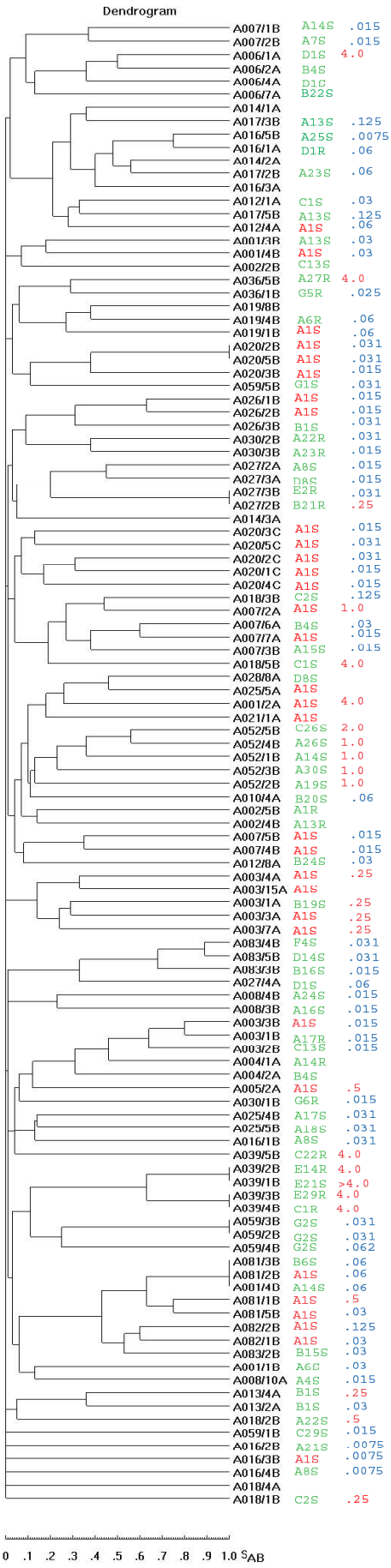

Figure 10 The details of genotyping by dendrogram, biotyping and MICs.

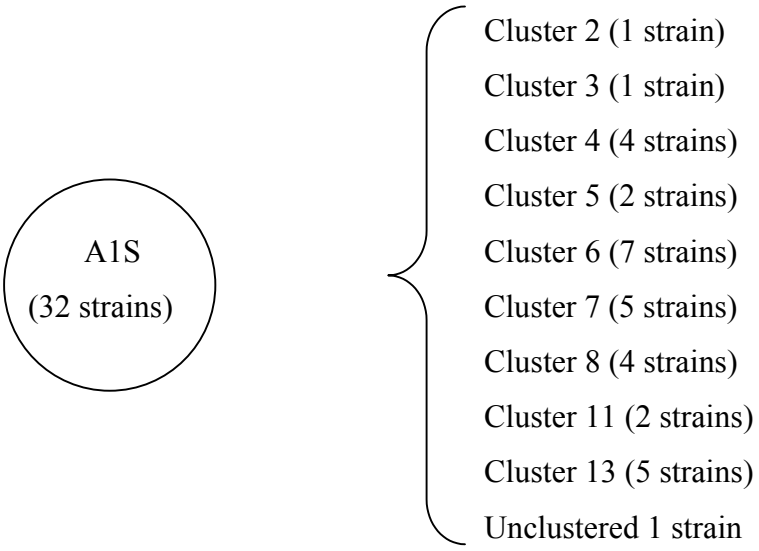

Figure 11 Thirty-two A1S strains situated in 9 clusters and one unclustered strain.

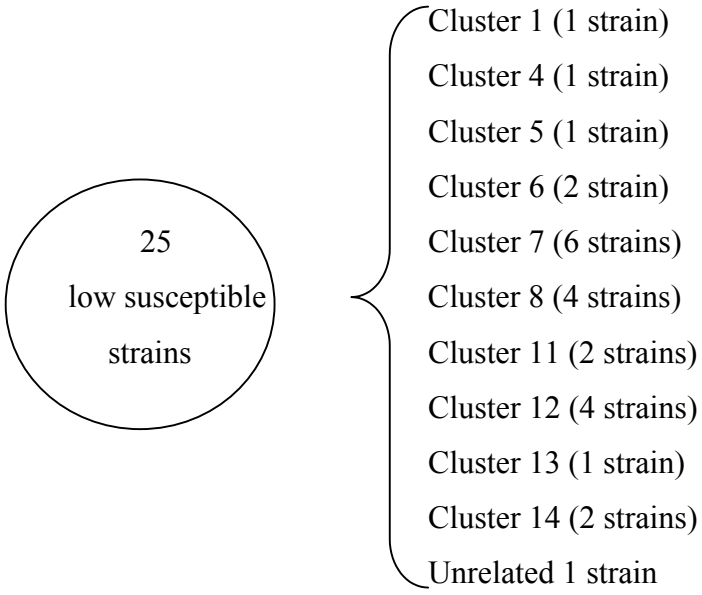

Figure 12 The distribution of low MIC *C. albicans* strains in 10 clusters and one unrelated strain comprising many genotypes.

4. Statistical analysis of association between *C. albicans* genotype, biotype, and ketoconazole susceptibility

There was no statistically significant association between clusters (representing different genotypes) and biotypes (major-A1S and minor biotypes) when evaluated using Chi-square test. Similarly, there was no association between genotype and ketoconazole susceptibility when analyzing high and low sensitive clusters (Table 9). However, both biotype and ketoconazole susceptibility were associated with the hosts or patients at p=0.001 and p=0.000respectively (Table 9). Furthermore, it was found that the biotype was not associated with ketoconazole susceptibility when evaluated by Chi-square test. Either the major or minor biotypes were not restricted to groups of high or low susceptibility to ketoconazole. This was confirmed by Odds Ratio analysis (Table 10).

Table 9 Statistical analysis by Chi-square test

	N. of valid cases	**Asymp. Sig. (2-sided)**
Host - biotype	106	.001
Host – ketoconazole	94	.000
Biotype - ketoconazole	94	.264

Table 10 Odds ratio analysis in biotypes and ketoconazole susceptibility of 94 *C. albicans* strains isolated from HIV-infected individuals.

	value	**95%Confidence interval**	
		Lower	**Upper**
Odds ratio for major/minor	1.204	.439	3.302
For cohort: high susceptible	1.049	.213	1.353
For cohort: low susceptible	.872	.410	1.255
No. of valid cases	94		