Investigation of Genetic Diversity and Relationships among the Clinical Candida species using Random Amplified Polymorphic DNA (RAPD) Analysis

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Abstract

This study included the isolation and diagnosis of twenty-one isolates belonging to the genus Candida which included nine of them: (C. albicans, C. glabrata, C. krusei, C. parapsilosis, C. kefyr, C. guilliermondii, C. famata, C. sphaerica and C. norvegensis. Microscopic and metabolic tests were performed to diagnose them, as well as molecular tests using the technique of RAPD-PCR using five random primers through which the diagnosis of isolates, where each initiator gave a number of the unique bands of each type of Candida which were characteristic of these species at certain molecular weights gave the primer OPM-20 highest number of unique bands nine unique bands followed by the primer OPE-16 gave six unique packages and then the primer OPL-05 gave five unique packages.

Each one was given a unique package One. There was a genetic hereditary difference between (7) and (8) with a genetic distance of 0.01709 and the highest genetic distance between specimens (13) and (19) with a genetic distance of 0.90944.

Keywords: Candida species, RAPD, DNA markers.

Introduction

DNA markers are defined as sequences that can be inferred from a specific location on the chromosome or genome and are used to study the genetic relationship among individuals as they reflect the differences in genetic information stored in them. These differences result either from the deletion, addition or rearrangement of the nucleotides in the studied individuals' The reason was as mutations for example, therefore, it has been adopted in the studies of molecular classification and evolutionary studies and in the construction of genetic maps and has become an important tool for the study of genetic diversity as it is an irreplaceable option in the development of appropriate conservation plans for species.[7]

DNA indicators have the ability to detect hundreds of sites and several alleles per location. Because these indicators reflect differences directly on the level of DNA, their preparation is great and is not affected by the environment and some of them give very quick results and reasonable cost. For these reasons, there are many areas of applications to many organisms including fungi where these indicators are used to study the genetic relationship between fungus species, especially disease, whether for humans, animals or plants to develop plans to take advantage of the results of these studies to resist these fungi.[5]

The diagnosis of fungus is one of the most important applications of DNA indicators because it is of great importance in terms of the development of plans for prevention and treatment of these fungal pathogens and since these indicators have the accuracy and speed in giving results, so it helps in the rapid identification of the fungus and thus the rapid diagnosis of appropriate treatment.[9]

One of the genetic indicators used to diagnose pathogenic fungi is the random multiple polymorphisms abbreviated as RAPD of the PCR-based DNA series where specific sites are multiplied on the DNA tape using short random sequences of about 10 nitrogen bases where these primers find complementary sites on the DNA tape and the result of the multiplication is a different length, number and the number of pieces resulting from the number of link locations as well as the distance between the location of a link and another.[19] Fungi eukaryotic organisms have about 50,000 species living in nature of which 80 species of yeast cause many diseases for humans and animals alike.[4]

Candida is a genus of yeasts, which includes many different species such as *C. albicans, C. glabrata, C. tropicalis, C. krusei, C. parapsilosis, C. kefyr, C. guilliermondii, C. famata, C. sphaerica* and *C. norvegensis* that are implicated in human disease (candidiasis).[15]

Candida is a normal flora in many healthy people, but in a few numbers as many studies indicate that colonies of *candida* are present in the oral cavity by 20-40% in healthy people in addition to their presence in vaginal and respiratory tract and others.[8]

Candida is transformed from a co-existing organism into a pathogen as opportunistic due to its ferocious factors such as adhesion to epithelial cell surfaces, production of lipid-digesting enzymes, proteins and formation of the germination tube. They take advantage of the vulnerability of the body to become a nurse called opportunistic fungi.[4] This study aims at identifying the best methods in the diagnosis of yeast.

Material and Methods

Collection of samples: Samples were collected from patients referred to the Yarmouk Teaching Hospital who were suspected of developing candidiasis as determined by the physician, aged between 15 and 50 years. Swabs were taken from the Thrush, Vagina and Upper respiratory tract.

Isolation and identification: After a transfer of the samples to the laboratory, culture media Sabouraud dextrose agar SDA and three replicates for each sample ensure that the fungal growth is not contaminated during the transplant and incubated the dishes grown at 37°C for 48 hrs.

The identification of the genus *Candida* and its dependent species are based on a series of tests which include the following:

Cultural characteristics: The shape, colour and texture of the *Candida* spp colonies developing on the media Sabouraud dextrose agar are examined.[3]

Microscopic test: The shape of the yeast cells was examined microscopically after dyeing by gram stain and then examined under the force 40X depending on to detect their response to stain shapes of their arrangement and yeast budding form.[12]

Urea hydrolysis test: This test was used to investigate the yeast's ability to produce Urease.

Growth on ChromAgar Culture: The ChromAgar containing dishes were vaccinated with yeast colonies isolated by a sterile loop in a streaking and then incubated at 37°C for 48 h after which we observed the growth and colour of the colonies.[10]

Germ tube production test: In this test, small clean test tubes containing 0.5 ml of human serum are vaccinated by the yeast to be tested. The tubes were incubated at 37°C for 3-4 hours, followed by a drop of a mixture and placed on a clean glass slide and then microscopically covered and tested at 40X to detect the germination tube.[14]

Vitek2 test: The pipes for the device were prepared and placed in a special mould called Cassete. These tubes were filled with 3 ml of the saline solution of the device and then placed in a fraction of yeast colonies to be diagnosed at the age of 1 - 2 days. After a transfer of the colonies to the tubes by sterile needle, the tubes were well shaken by the vibrator to absorb the suspension. Density is not to exceed the prescribed limits of the device since the density of yeast is 1.8-2.2, then the serial number data of the card was entered by a Barcode and then the laser beam was directed to the serial number of each card and inserted into the computer with a special list for each template which contains only ten isolates to hide; after that, a number was written for each isolation and the data was saved. The template was then entered into the filler door which is empty of air. The door is closed and the button is locked. The process continues for 70 seconds. After completion of three and a half minutes, the operations are completed within this section where the device cuts heat to the capillary tube connected to each card called the Sealing process and then the device raises the cards for the purpose of working on it inside the machine. In a process called loading it then indicates the machine word. Remove device is required to open the door and take out the mould and then the samples inside the device is incubated to the next day where the completion of the results is displayed in the form of a report.[2]

DNA extraction: DNA was prepared and purified according to the genomic isolation kit provided by Geneaid Company/Taiwan (Cultured Cell Protocol Procedure Ver.11.21.13). Liquid nitrogen (-170°C) was used with the commercial kit since the very low temperature helped to prevent DNase activation.[1] The Nanodrop system (BioDrop/UK) was used for the measurement of the concentration and purity of the DNA according to Sambrook and Russell[16] using 2 µl of each DNA sample.

The amplification reaction programmes for RAPD-PCR: PCR amplification was performed in a final volume of 20 µl, the reaction consisted of 5 µl of the PCR ready mix (Bioneer/Korea), 11 µl of double distilled water (ddH2O), 2 µl of template DNA and 2 µl of each primer. 7 µl of PCR amplified products were electrophoresed on 1.5% agarose gel (2 hr 5 V/cm, 1X Tris-borate buffer). The DNA bands were visualized under UV light and photographed after staining the agarose gels with ethidium bromide (5 µg/ml).

Results and Discussion

A total of 30 specimens of yeast-like isolates were obtained from patients between 15 and 50 years old. These specimens included 7 thrush samples with 13 vaginal and 10 upper respiratory tract swabs. At the time of the tests, 21 samples were positive for *Candida spp.* and the other 9 samples were negative. The developing colonies appeared on Sabouraud dextrose agar SDA in the form of white colonies to the cream of colour, smooth and in circular colonies where this result is consistent with what Singh and his group reported that *Candida spp.* colonies appear to have a creamy when planted on the said medium to provide conditions for growth. The isolated species gave positive results to interact with the chromium dye where the cells appeared oval to spherical or oval to longitudinal or cylindrical shape and this result was identical with Boon and his group.

The appearance of *Candida* cells is dyed blue due to its containment of the peptidoglycan layer in its cell wall.[18] In the development of the *Candida spp.* isolated on the culture media ChromAgar for 24-48 hours at 37°C, the results showed that each species has a colour of its own where six samples appeared grew in green colour dating back to *C.albicans* and three samples have a pink colour back to *C.krusei.*

The remaining isolates were purple for other yeast species and this result was identical to the study of Manikandan and Amsath[10] on the isolation and rapid diagnosis of *Candida species* and as one of the most important areas used in the diagnosis of fungi as it depends on the diagnosis of colouring in the media.[11] All the isolates of type *C.albicans* had formed the tube of germination at incubation at 37°C and for 3-4 hours in 0.5 ml of human serum but all other species did not have a tube of germs under the same conditions and these results came similar to Boon and his group. *C. albicans* only has the ability to form the tube of the germs in the serum and that the tube of the germs plays role in the process of penetrating the layer of epithelial cells lining the body and tissues and access to the bloodstream as well as it is necessary to feed the yeast so this test is to diagnose the genus *C.albicans* on the rest of the species.[18]

All ovarian isolates gave a negative result to the production of urease enzyme because the genus of candida is weak to analyse urea and this result is agreeable to Abd and Abbas.[1] As for the Vitek2 test, the results of the diagnosis of specimens (1,2,3,4,5) showed that they belong to the species *C.albicans* while the sixth sample was not accurate, either *C.albicans* or *C.famata* samples (7,8,9,10,11) showed that they back to *C.sphaerica* while the twelfth sample was not accurate, either *C.sphaerica* or *C.Kefyr* samples (13,14,15) showed that they back to *C.krusei* when the sixteenth sample is *C.famata*, samples (17,18) they were not accurate, either *C.guilliermondii* or *C. famata*, the nineteenth sample was *C.parapsilosis* while samples (20,21) were *C.glabrata* and *C.norvegensis* respectively .

Note from the results of the table 3 convergence in the results of the current study with previous studies taking into account the difference in the number of samples and differences in the age groups and the study period and place of injury. *C.albicans* was the most isolated isolate with six isolates; the reason is that they have fermenting factors such as their ability to stick to epithelial membranes in a high degree compared with other species[4] followed by *C.sphaerica*, five out of 21 isolates. This result is consistent with most studies including the study of Abd and Abbas.[1]

In addition, the most samples were isolated from the vagina due to the fact that this area is exposed to conditions of humidity and temperature in addition to the continuous coverage of this region as well as the immune state of the patient. The treatment of antibiotics and long periods and the first to be exposed is the mouth will weaken the immunity and thus cause the infection of Candidiasis as opportunistic fungi.[4]

Genetic diagnosis: The genomic DNA was extracted efficiently from *Candida spp.* isolates using a genomic DNA extraction kit to yield intact DNA with a good-quality and high purity for use in PCR techniques. The concentration of the extracted DNA ranged between 50–200 ng/µl with a purity of 1.6-2 was obtained according to Saiki et al[17], adequate to ensure a good yield of the desired PCR products.

In the current study, the RAPD-PCR technique was used to detect DNA polymorphism of different Candida spp. in order to search for the source of differences that could be used as a DNA marker specific for each Candida spp. as well as the knowledge of the efficiency of molecular tests compared to the tests of biochemical accuracy in the diagnosis of the *Candida spp.* A randomized secretion of *Candida spp.* DNA was performed using five random primers under optimal conditions in terms of thermocycler device programming and reagent concentration to produce accurate results.[20]

The table 4 shows five random primers each consisting of 10 nitrogen bases used to diagnose 21 isolates belonging to the *Candida spp*. The OPI-06 primer produced 144 bands samples of *Candida* 21 and its molecular weights were between 2700-100bp and gave one unique band to *C. parapsilosis* at molecular weight 2700 bp and 24 polymorphic bands in addition, gave a monomorphic band with molecular weight 100bp that distinguished *C. guelliermondii* from other *Candida spp* which gave the highest percentage of polymorphism as 96% while OPL-05 primer produced 139 bands samples of *Candida* 21 and its molecular weights were between (2900-130)bp and gave five unique bands.

C. albicans produced two unique bands whose molecular weights are 240bp and 950bp, while the *C. krusei* gave a single unique band at the molecular weight of 1060bp in addition to a monomorphic band with molecular weight 100bp while the *C. famata* produced two unique bands of molecular weight 520bp and 2300 bp and 26 polymorphic bands.

The OPM-20 primer produced 107 bands of *Candida spp.* 21 and its molecular weights were between (3000-160)bp and gave 9 unique bands of *C. albicans* that are isolated from thrush, produced single unique band whose molecular weights are 1300bp and *C. albicans* that are isolated from upper respiratory tract produced single unique band whose molecular weights are 1350bp while the *C. kefyr* gave a single unique band at the molecular weight of 950bp while the *C. guelliermondii* produced single unique band of molecular weight 565bp while *C. parapsilosis* gave four unique bands whose molecular weights are 700bp, 1770bp, 2150bp and 3000bp while *C. norvegensis* gave single unique band of molecular weight 1750bp in addition, gave three packs of monomorphic two which came back to *C. guelliermondii* with a molecular weight of 450 and 650bp and the third belonged to *C. albicans* with a molecular weight of 750bp and 20 polymorphic bands which gave the lowest percentage of polymorphic as 69%.

The OPE-16 primer produced 153 bands of *Candida spp.* 21 and its molecular weights were between (2520-192)bp and

gave 6 unique bands as *C. guelliermondii* produced single unique band whose molecular weight is 2520bp while the *C. parapsilosis* gave a single unique band at the molecular weight of 1200bp while *C. glabrata* gave four unique bands whose molecular weights are 192bp, 524bp, 1340bp and 1600bp and 19 polymorphic bands.

The OPQ-01 primer produced 95 bands of *Candida spp.* 21 and its molecular weights were between (2100-230)bp and gave one unique band to *C. glabrata* at molecular weight 1700 bp and 13 polymorphic bands. In addition, it gave a monomorphic band with molecular weight 320bp that distinguished *C. krusei* from other *Candida spp.*

The presence or absence of associate amplified RAPD band (DNA polymorphism) resulted from rearrangements or mutations either at or between the priming sites. Many studies have supported the construct that composition variations among *C. albicans* isolates may well be correlative with their invasive environments or completely different body sites.[6]

The table 5 shows the accuracy of the PCR technique in the diagnosis of types of the genus *Candida* compared to the Vitek2 system which was not accurate in the diagnosis of four isolates (6) *C. albicans*, (12) *C. kefyr*, (17), (18) *C. guilliermondii*, while they were accurately diagnosed by PCR technique.

The genetic distance between the twenty-one samples of the Candida species was estimated using the genetic program (NTSYS-PC. Version 2.02i) which is based on the existence of bundles between each pair of yeasts based on Nei and Li where using the primers, the genetic dimension and the similarity between the studied species can be found. If the genetic material matches between two studied species, this indicates that the genetic dimension between them should be equal to zero. The genetic similarity ratio shall be equal to one (100%).

The table (6) shows the degree of genetic dimension between isolates 21 of *Candida* where it appears that less distance between isolates (7) and (8) *C. sphaerica* was isolated from thrush where the genetic distance between them (0.01709) shows how similar the two samples are. They also shared the highest number of packages and the highest genetic distance between the sample (13) *C. krusei* and the sample (21) *C. norvegensis* with genetic dimension (0.90944) which means that there is the least similarity in the genetic material between these two types that is, they have subscribed to the lowest number of packages. The genetic relationship between the genotypes of the Candida genus was determined based on the genetic dimension values as in fig. 1, where the studied species were divided into major and subgroups.

First major group: This group included specimens (1,2,3,4,5,6) that belong to *C. albicans* where these samples were separated by themselves in the tree diagram in figure 1. These samples were characterized by a number of phenotypic, microscopic and metabolic characteristics as compared to the rest of the other samples, although these samples were similar in their effect to the other *Candida species* but differed in green colour in the test of ChromAgar culture as well as in the formation of the germ tube. This group included three subgroups where the first group included samples (5) and (6) where they were genetically closer to the rest of the samples of this group (0.14700), where they were isolated from the same source upper respiratory tract. The second subgroup included samples (3) and (4) genetic distance (0.21304) and the third subset included sample (1) which was farthest from the rest of the group but closer to the sample (3) and sample (2) although it belongs to the same the source of thermal insulation showed that farm, microscopic and metabolic tests were identical in their results. However, molecular tests using RAPD-PCR technique using random primers showed the difference between the samples of this group which is due to the mutation, deletion or rearrangement of DNA nucleotides of these samples.

Second major group: This group included the samples (7,8,9,10,11) belonging to the *C. sphaerica*, This group included three subgroups where the first group included samples (7) and (8), which are considered as the genetically closest among the studied samples with genetic distance (0.01709) because of their participation in the highest number of packages and their compatibility in the results of the biochemical tests and isolate them from the same site. For the rest of the samples, the sample (2) of *C. albicans* has the same genetic distance from samples (7) and (8) (0.69300) and the samples (5,6) of *C. albicans* have the same dimension from samples (7) and (8) (0.62631 and 0.54865) respectively while the sample (10), which is of the same type, has the same distance samples (7) and (8) (0.02594).

Also, for the sample (16), *C. famata* has the same genetic distance from samples (7) and (8) (0.56500), while samples (17) and (18) *C. guilliermondii* have both the same genetic dimension as (7) and (8) (0.75091 and 0.57061) respectively indicating the genetic convergence between samples (7) and (8). The second subgroup included samples (9) and (10) genetic distance between them 0.01755, which were isolated from the vagina while the third group included the sample (11), which was far from the rest of the samples of the group but closer to the samples (9) and (10) of samples (7) and (8) being isolated from the vagina.

The third major group: It included the samples (13,14,15) belonging to *C. krusei* separated from the rest of the samples. This was observed in the biochemical tests in pink colour in the test of ChromAgar culture. Samples (14) and (15) were genetically closer to the sample of the group with a genetic distance of 0.09946 and the sample (13) was closer to the sample (14) than the sample (15). The rest of the samples were fairly close.

Table 1
RAPD primers and their sequences.

Primer Name	Sequence (5́ - 3′)
OPI_06	AAGGCGGCAG
OPL_05	ACGCAGGCAC
OPM_20	AGGTCTTGGG
OPE_16	GGTGACTGTT
OPQ_01	GGGACGATGG

Table 2
RAPD-PCR program

Steps	Temperature	Time(min.)
Initial denaturation	94	5
Denaturation	94	1
Annealing	36	2
Extension	72	1
Final extension	72	10

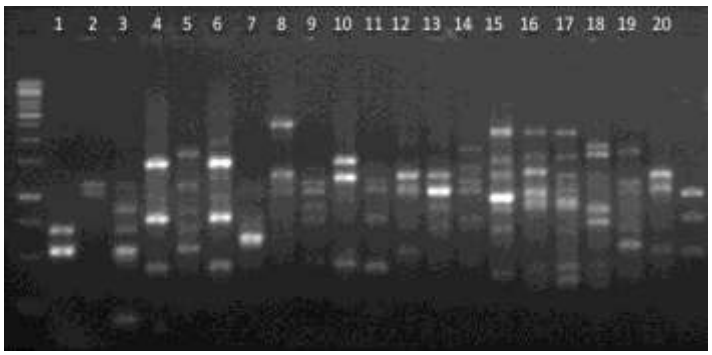

Image 1: Electrophoresis of the primer OPI-06 (packs) by RAPD-PCR of different *candida* spp. Lanes 1, 2, 3, 4, 5 and 6 for *C. albicans.* Lanes 7, 8, 9, 10 and 11 for *C. sphaerica.* Lane 12 for *C. kefyr.* Lanes 13, 14 and 15 for *C. krusei*. Lane 16 for *C. famata.* Lanes 17 and 18 for *C. guelliermondii.* Lane 19 for *C. parapsilosis*. Lane 20 for *C. glabrata.* Lane 21 for *C. norvegensis*

Table 3
The types of *Candida spp.*, isolation and isolation source and the number of isolates for each species as well as the results of the biochemical tests

***Candida* spp.**	**Origin**	**Nu. of isolates**	**Colour colonies to distinguish between *Candida* spp.**		**Germ tube test**	**Urea hydrolysis test**
			SDA culture	**ChromAgar culture**		
C.albicans	Thrush, Vaginitis, Upper respiratory tract	6	White to cream	Green	+	-
C.sphaerica	Thrush, Vaginitis	5	cream	purple	-	-
C.Kefyr	Vaginitis	1	cream	purple	-	-
C.krusei	Thrush	3	cream	Pink	-	-
C.famata	Vaginitis	1	cream	purple	-	-
C.guilliermondii	Vaginitis	2	cream	purple	-	-
C.parapsilosis	Vaginitis	1	cream	purple	-	-
C.glabrata	Upper respiratory tract	1	cream	purple	-	-
C.norvegensis	Upper respiratory tract	1	cream	purple	-	-

Table 4
The random primers used to amplify the DNA of each type of candida and the number of beams produced by each primer

Primer name	Sequences (5`- 3`)	Size range (bp)	Nu. of bands amplified in different *Candida* spp.			Primer efficiency (%)	Polymorphism (%)	Discrimination power (%)
			Total	Unique	Polymorphic			
OPI-06	AAGGCGGCAG	2700-100	144	1	24	22.57	96	26.08
OPL-05	ACGCAGGCAC	2900-130	139	5	26	21.78	84	18.26
OPM-20	AGGTCTTGGG	3000-160	107	9	20	16.77	69	21.73
OPE-16	GGTGACTGTT	2520-192	153	6	19	23.98	76	20.65
OPQ-01	GGGACGATGG	2100-230	95	1	13	14.89	93	14.13
Total			638	22	92			

Table 5
Comparison between automated (Vitek2 YST card) and the RAPD- PCR identification of *Candida spp.*

Isolates	Origin	Vitek2	RAPD-PCR
1-2-3	Thrush	*C. albicans*	*C. albicans*
4	Vaginitis	*C. albicans*	*C. albicans*
5	Upper respiratory tract	*C. albicans*	*C. albicans*
6	Upper respiratory tract	Low discrimination *C. famata* / *C. albicans*	*C. albicans*
7-8	Thrush	*C. sphaerica*	*C. sphaerica*
9-10-11	Vaginitis	*C. sphaerica*	*C. sphaerica*
12	Vaginitis	Low discrimination *C. kefyr* / *C. sphaerica*	*C. kefyr*
13-14-15	Thrush	*C. krusei*	*C. krusei*
16	Vaginitis	*C. famata*	*C. famata*
17-18	Vaginitis	Low discrimination *C. guilliermondii* / *C. famata*	*C. guilliermondii*
19	Vaginitis	*C. parapsilosis*	*C. parapsilosis*
20	Upper respiratory tract	*C. glabrata*	*C. glabrata*
21	Upper respiratory tract	*C. norvegensis*	*C. norvegensis*

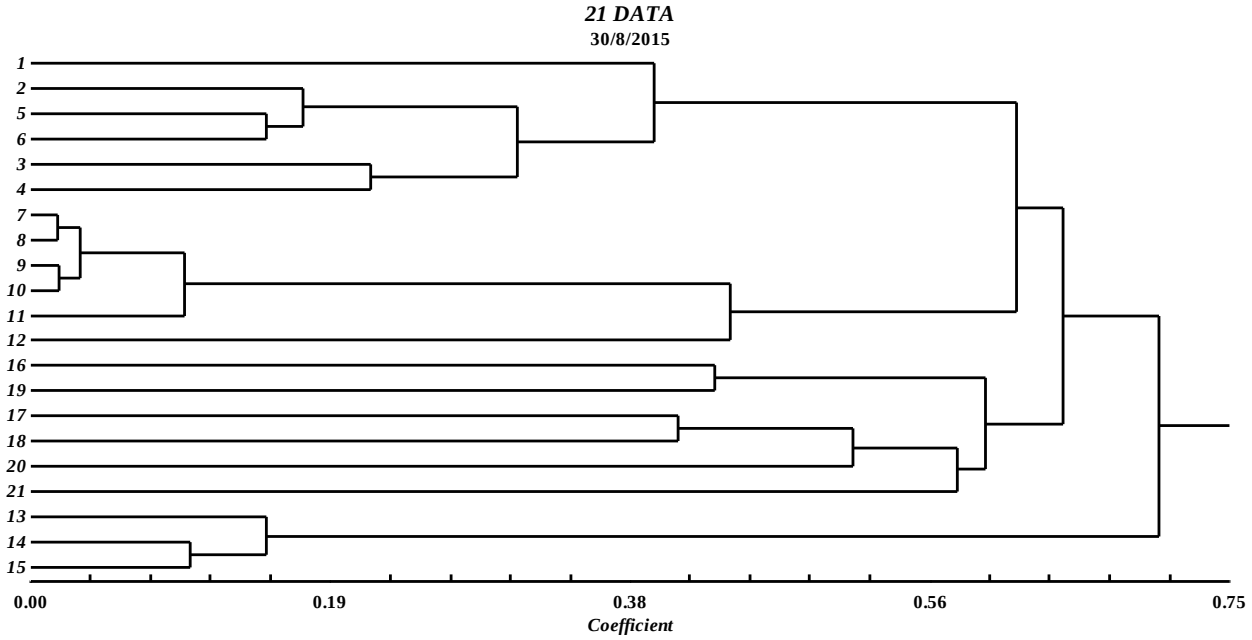

Figure 1: The genetic relations between the types of the genus *Candida* according the indicators of RAPD.

Table 6
The genetic dimensional values depending on indicators of RAPD

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	0.00000																				
2	0.36287	0.00000																			
3	0.31760	0.20127	0.00000																		
4	0.37934	0.34532	0.21304	0.00000																	
5	0.44083	0.17185	0.24295	0.39662	0.00000																
6	0.45050	0.16851	0.26300	0.37738	0.14700	0.00000															
7	0.54263	0.69300	0.70914	0.79335	0.62631	0.54865	0.00000														
8	0.57248	0.69300	0.74304	0.83257	0.62631	0.54865	0.01709	0.00000													
9	0.54639	0.66691	0.71695	0.80648	0.63300	0.52256	0.04411	0.02609	0.00000												
10	0.53408	0.68445	0.70059	0.78480	0.61776	0.54011	0.02594	0.02594	0.01755	0.00000											
11	0.51654	0.56847	0.58774	0.65806	0.56847	0.49179	0.11962	0.10020	0.07411	0.09165	0.00000										
12	0.44083	0.54654	0.50957	0.45068	0.60906	0.56316	0.42271	0.44938	0.45068	0.44083	0.42328	0.00000									
13	0.63606	0.65814	0.73993	0.61851	0.72267	0.67677	0.64461	0.67538	0.68103	0.66683	0.74773	0.54035	0.00000								
14	0.62811	0.61941	0.69931	0.58158	0.77848	0.73257	0.72903	0.76182	0.76963	0.75327	0.80472	0.56225	0.11572	0.00000							
15	0.66683	0.62736	0.64755	0.50410	0.72267	0.71067	0.84528	0.88302	0.85693	0.83673	0.85693	0.56852	0.17934	0.09946	0.00000						
16	0.73878	0.63477	0.68481	0.75513	0.63477	0.61964	0.56500	0.56500	0.56708	0.58463	0.53891	0.60492	0.65675	0.57044	0.54854	0.00000					
17	0.61316	0.54562	0.46213	0.59561	0.51745	0.52870	0.75091	0.75091	0.79628	0.77746	0.69092	0.66698	0.88595	0.80249	0.71605	0.52563	0.00000				
18	0.65168	0.55337	0.52117	0.60336	0.52520	0.50747	0.57061	0.57061	0.57351	0.53390	0.51635	0.64298	0.72380	0.68508	0.66318	0.58603	0.40544	0.00000			
19	0.77746	0.69977	0.65450	0.79628	0.57461	0.58933	0.62170	0.65247	0.62638	0.61316	0.65813	0.76876	0.68528	0.70718	0.74780	0.42800	0.57536	0.58312	0.00000		
20	0.69106	0.74906	0.58288	0.61100	0.74906	0.73824	0.66786	0.69961	0.70631	0.65931	0.64177	0.68237	0.73144	0.69271	0.64183	0.56700	0.51071	0.51846	0.64791	0.00000	
21	0.71953	0.71083	0.72451	0.73973	0.71083	0.74189	0.69171	0.65662	0.63053	0.64807	0.66562	0.67447	0.90944	0.81356	0.79166	0.59849	0.63060	0.46142	0.69729	0.64599	0.00000

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