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Molecular typing of *Malassezia furfur* and *Malassezia globosa* isolated from Tinea versicolor patients, based on Total protein extract and DNA finger print profiles.

Yehia A.-G.Mahmoud*, Metwally A.Metwally, Hanan H.Mubarak and Nesma El Zewawy

Tanta University, Faculty of Science, Botany Department, Mycology Research Lab., Tanta 31527, Egypt
*Current address: Albaha University, Faculty of Science, Biology Department, Albaha, Saudi Arabia
E-mail: Yehiamah@gmail.com

ABSTRACT:

The cellular proteins of the 40 studied isolates of *Malassezia furfur* were electrophoretically analyzed on SDS-PAGE. Analysis of the total cellular proteins from *M. furfur* isolates are grouped into two wide molecular weight range (11 - 112 kDa) . It is very interesting to notice that the band with molecular weight of 56 kDa may be specific to the type of tinea versicolor. The cluster analysis based on cellular proteins data of *M. globosa* separated the seven isolates into 2 groups according to the band with molecular weight 56 kDa, which is present in 6 cases (hyperpigmented) and absent in case (hypopigmented) lesions.

The polymorphism observed from RAPD markers revealed a high degree of genetic diversity in *M. furfur* and *M. globosa* isolates collected from different patients having tinea versicolor infection. Only primer E (5'-TGCCGAGCTG-3') generated a RAPD PCR banding pattern which allowed the identification of 6 different amplification groups within *M. furfur* and *M. globosa* that could be used as diagnostic tool for different isolates of *Malassezia*..

Key words: Tinea versicolor; Malassezia furfur; Malassezia globosa; RAPD PCR.

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INTRODUCTION

Malassezia is a dimorphic fungus has a pathogenic (filamentous) and opportunist forms (yeast). In both forms the fungus resides inside the stratum cornea where it is fed by free fatty acids, sebum triglycerides and keratinized epidermis. *Malassezia* is a lipophilic fungus which requires the addition of lipidic substances in the middle of culture like olive oil (1).

Although pityriasis versicolor (PV) is of worldwide distribution, its frequency is variable and depends on different climate, occupational and socioeconomic conditions (2). But it is particularly common in tropical (3) and subtropical countries. A high percentage, up to 50% of the populations in certain regions of Central and South America, as well as parts of Africa (4) were found to be highly affected. Singh and Nath (5) recorded that the incidence of tinea versicolor was about (14 %) among other skin disease in India. In Brazil, pityriasis versicolor represented 20 % cases of superficial mycosis (6). In Egypt, PV was found to be ranged in between (10-14%) among other skin diseases (7-8). However, El-Shanawany (9) found that tinea versicolor in Assiut Governorate was markedly increased about 40.8 % among other dermatomycosis and also he found the incidence of PV was 30 % in new valley governorate. Mubarak (10) showed that PV was found to be 35.7% among other dermatomycosis in El Gharbiah governorate. In contrast, only 0.5% of males and 0.3% of females were found to be suffered from tinea versicolor in the course of a population survey in Sweden (11) and 1.9% of factory workers examined in central Turkey presented with tinea versicolor lesions (12). In England, Nowiscki and Sadowska (13) found that PV was common (10 %) in temperate areas. But in other temperate countries, the incidence of the disease is much lower, representing up to 0.3 % patients seen by dermatologists during the summer months (14).

Several trials for the differentiation between different *Malassezia* species which cause various superficial disease including pityriasis versicolor and seborrhoeic dermatitis were carried out (15). The application of a PCR – based technique using restriction enzyme digestion for discrimination of different *Malassezia species* was employed by (16). Also, pulsed – field gel electrophoresis is a technique that can reliably differentiate between *Malassezia sp*. Guillot (17) used single PCR–restriction endonuclease analysis for rapid identification of *Malassezia sp.*

However, Makimura (18) identified Malassezia sp. using the DNA sequences of nuclear ribosomal internal transcribed spacer regions. Boekhout (19) used molecular typing to differentiate between Malassezia species with RAPD PCR technique.

Molecular typing could be particularly useful in solving epidemiological questions, for example revealing infective routes, common sources of infection and areas of dissemination, as well as determining whether the original isolate is responsible for reinfection or a new strain has been acquired (our work is in progress in these points). The aim of this study was focused on assessing the genetic variability between *Malassezia* species different isolates at the biochemical and molecular levels. Also, to assess the molecular epidemiology of *Malassezia* species using total cellular proteins and DNA finger print have been applied, each one demonstrating interesting aspects of the dispersion and ecology of this genus.

MATERIALS AND METHODS

A. Isolation and culture techniques:

Samples were gathered from patients clinically diagnosed to have tinea versicolor under aseptic conditions (dermatology department at Tanta University Hospital, Tanta, Egypt). The surface of the affected area was first cleaned with cotton swab moistened with 70 % ethyl alcohol prior to sampling. Sterile scalpel was used to collect skin scrapings on sterile glass slide (9). These were cultured on Sabouraud's dextrose agar (SDA) medium; each 1 liter of distilled water contained 30 g dextrose, 10 g peptone, 20 g agar, and 0.5 g from each chloramphenicol and cycloheximide were added to avoid bacterial contamination (20-21). Few drops of olive oil (100 µl) must be added to enhance yeast growth (22-23-24). The plates were incubated at 37 ˚C for 4 to 15 days.

B. Identification of yeasts

All cultured yeast plates were examined and identified by macroscopic and microscopic features. Also, physiological characteristics of yeast isolates (catalase test) and biochemical criteria (esculin and lipid assimilation tests) were carried out. The following references were used for the identification of the isolated yeasts (25-26-27-28-29).

C. Statistical analysis:

Statistical analysis was carried out to analyze the data of tinea versicolor infection through Anova – test (Oneway, twoway, multiway) and L.S.R calculations that were performed using SAS software for windows, version 6.12 © 1985.

D. Biochemical and molecular analysis:

A. Total cellular protein profile of *Malassezia* isolates by SDS polyacrylamide gel electrophoresis technique:

Polyacrylamide gel electrophoresis (PAGE) was used to determine quantitative and qualitative changes that occur in the soluble proteins of the isolates.

i. Extraction of yeast protein:

Protein was extracted from *Malassezia furfur* and *Malassezia globosa* previously grown on liquid YPD (1 % yeast extract, 2 % peptone, 2 % glucose; a according to Kushnirov (30). Yeast cells were harvested by centrifugation from liquid culture. These cells were resuspended in 100 ml distilled water, 100 ml of 0.2 M NaOH was added, incubated for 5 min at room temperature, pelleted, resuspended in 50 ml SDS sample buffer (0.06 M Tris HCl pH = 6.8, 5 % glycerol, 2 % SDS, 4 % β-mercaptoethanol, 0.0025 % bromophenol blue), boiled for 3 min and pelleted again. Protein content of supernatant was loaded into electrophoresis unit and the protein concentration was estimated according to the method of (Bradford) (31).

ii. Protein electrophoresis:

Preparation of gels (Hoefer SE 600) was made as described by Laemmli (32). Six µl of the crude protein solution was applied to the wells of the stacking gel. The samples covered with electrode buffer. Molecular weight protein (SIGMA) was used as a standard, then gels were stained and destained by conventional method used in this respect. Gel was scanned for molecular weight and quantity of bands was determined by using gel documentation system (AAB Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6c, Fullerton CA 92631). The different molecular weights of bands were determined using standard marker by UPGMA clustering using Systat software for windows, version 7.1 © 1997.

B. DNA fingerprint of *Malassezia* isolates by RAPD-PCR technique:

i. DNA isolation and RAPD technique:

DNA isolated from 6 different isolates of Malassezia sp. (4 isolates of *M. furfur* and 2 isolates of *M. globosa*, one isolate from each group of the cluster analysis resulting from previous total cellular protein of all isolates) using Qiagen Kit (Clinilab, Cairo Egypt) for DNA extraction. The extracted DNA was dissolved in 100 µl of elution buffer. The concentration and purity of the obtained DNA was determined by "Gen quanta" system-pharmacia Biotech. The purity of the DNA for all samples was between 90-97% and the ratio between 1.7-1.8. Concentration was adjusted at 6 ng/µl for all samples using Tris base EDTA buffer (pH 8.0).

ii. Random amplified polymorphic DNA technique (RAPD):

Polymerase chain reaction (PCR) mixture was prepared with PCR beads tablet (manufactured by Amersham Pharmacia Biotech), which containing all of the necessary reagents except the DNA template and the primer, which added to the tablet. Thirty nanogram (ng) from the extracted fungal DNA were used for amplification reaction. 5µl of the random primer (15ng/ml) were added to a PCR bead tablets. The sequences of used primers were as follows: Primer A: 5'-ACCTGAACGG-3', Primer B: 5'-CCTTGACGCA-3', Primer C: 5'-GAGAGCCAAC-3', Primer D: 5'-AGCCAGCGAA-3' and Primer E: 5'-TGCCGAGCTG-3'

The total volume was completed to 25µl using sterile distilled water. The amplification protocol was carried out according to Williams, et al (33) using PCR unit II biometra: a- Denaturation was at 95°C for 5 min (one cycle), b- The following steps consist of 45 cycle each: 1-Denaturation was at 95°C for 1 min, 2-Annealing was at 36°C for 1 min, 3-Extension was at 72°C for 2 min, c-Final extension was at 72°C for 5min (one cycle) and d- Hold at 4°C. 7µl of 6x tracking buffer (manufactured by Qiagen Kit) were added to 25 µl of the amplification product.

iii. Amplification product analysis:

The amplified DNA samples were electrophoresed (15µl) using horizontal electrophoresis unit (WIDE mini-sub-cell GT bio-RAD) on 1% agarose gel containing Ethidium Bromide (0.5 µg/ml), at 75 constant volt, and determined with ultra violet transillminator, then gels were photographed. Electrophoresis buffer was TBE 10x pH=8.3.

iv. Gel analysis:

Gel was scanned, to determine the size of bands (bp) by using gel documentation system AAB (Advanced American Biotechnology 1166 E. Valencia Dr. unit 6C, Fullerton CA J2631). The different sizes of bands were determined using DNA standard (one kilo base marker) with molecular size 1300, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp by UPGMA clustering using Systat software for windows, version 7.1 © 1997.

RESULTS

Tinea versicolor was clinically diagnosed in 47 patients coming to the outpatient clinic of dermatology department at Tanta University Hospital, Tanta, Egypt during the year of study (January 2008 to December 2008) through one weekly visit, and were confirmed as *M. furfur*; 40 isolates and 7 isolates of M. globosa by positive culture results for yeast growth (Data to be presented elsewhere).

II. Biochemical and molecular analysis:

A. Total cellular protein profile of *Malassezia* isolates by SDS polyacrylamide gel electrophoresis technique:

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i. SDS-PAGE profile of 40 *M. furfur* isolates:

The cellular proteins of the 40 studied isolates of *M. furfur* were electrophoretically analyzed on SDS-PAGE. The extracted proteins were fractionated using one dimensional SDS-PAGE. The SDS-PAGE banding patterns and the survey for the cellular proteins bands of the 40 isolates are shown in fig (1) and table (1).

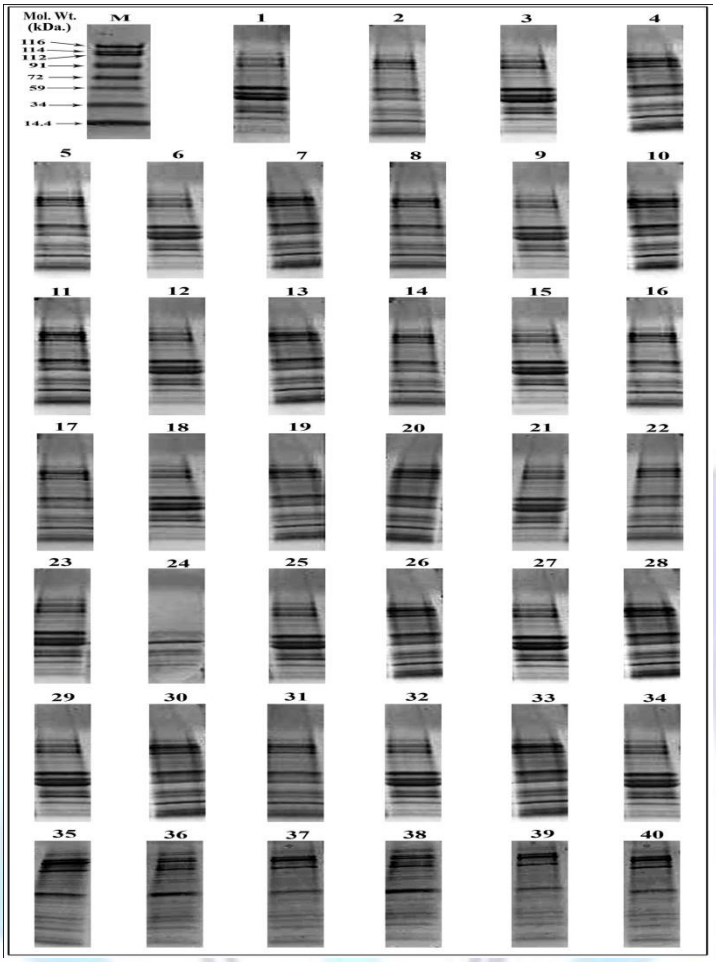

Figure1. SDS-PAGE band survey for the total cellular proteins extracted from each of the 40 isolates of *M. furfur* of tinea versicolor lesions (from 1 to 40) during the year of study (2008), and a standard protein markers mixture (M) with molecular weights of:116 kDa: β-galactosidase, 114 kDa: *E. coli* Hmp1 protein, 112 kDa: IgE seral immunoglobulin (against Mycoplasma antigen), 91 kDa: IgA linear immunoglobulin (against bullous disease antigen), 72 kDa: mammalian heat shock cognate protein (prp72), 59 kDa: eurofilament protein, 34 kDa: Soy bean seed oil-associated protein and 14.4 kDa: Lipoprotein lipase.

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Table 1. SDS-PAGE band survey for the total cellular proteins extracted from each of the 40 isolates of *M. furfur* of tinea versicolor lesions during the year of study (2008)

Isolate no.	Band number													
	1	2	3	4	5	6	7	8	9	10	11	12	13	**14**
	Band mol. wt. (kDa)													
	112	105	102	95	90	79	60	56	48	34	31	25	14	**11**
1	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
2	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
3	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
4	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
5	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
6	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
7	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
8	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
9	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
10	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
11	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
12	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
13	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
14	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
15	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
16	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
17	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
18	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
19	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
20	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
21	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
22	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
23	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
24	0	0	0	0	0	0	1	1	0	1	1	1	0	**0**
25	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
26	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
27	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
28	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
29	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
30	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
31	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
32	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
33	1	1	1	1	1	1	1	0	1	1	1	1	1	**1**
34	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
35	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
36	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
37	1	1	1	1	1	1	0	0	1	1	1	1	1	**1**
38	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
39	1	1	1	1	1	1	0	0	1	1	1	1	1	**1**
40	1	1	1	1	1	1	0	0	1	1	1	1	1	**1**
T	**39**	**39**	**39**	**39**	**39**	**39**	**37**	**18**	**39**	**40**	**40**	**40**	**39**	**39**

The isolates share a number of bands. These bands may be species specific bands. The banding patterns showed a total number of 14 bands with molecular weights range from 11 to 112 kDa. Data showed that the bands were monomorphic with range from 45% in some isolates to 100% in other isolates.

The frequency of each band was little different whereas the bands with molecular weight of (112, 105, 102, 95, 90, 79, 60, 48, 34, 31, 25, 14, 11 kDa) recorded the highest frequency between the 40 isolates. On the other hand, one band exhibited the lowest frequency (56 kDa) as shown in table (1).

The bands that show highest frequency with molecular weight (34, 31, 25 kDa) was found in 40 isolates and those with molecular weight (112, 105, 102, 95, 90, 79, 48, 11 kDa) was found in 39 isolates, while the band with molecular weight (60 kDa) was found in 37 isolates out of 40 isolates. On the other hand, the band that exhibited the lowest frequency (56 kDa) was found in 18 isolates.

A cluster analysis was performed using the UPGMA clustering as a means of visualizing relationships in the data. The cluster analysis based on cellular proteins data separated the isolates of M. furfur into 2 clusters as shown in figure (2). The first cluster included isolates no. (2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 26, 28, 30, 31, 33, 37, 39, 40). The second cluster included the isolates no. (1, 3, 6, 9, 12, 15, 18, 21, 23, 24, 25, 27, 29, 32, 34, 35, 36, 38). It is very interesting to notice that the band with molecular weight of 56 kDa may be specific to the type of tinea versicolor lesions as this band was found in isolates of hyperpigmented lesions (second cluster) and absent in isolates of hypopigmented one (first cluster) and this is the reason for classifying the isolates into 2 clusters. By drawing an arbitrary line at a genetic distance of 0.25 the isolates are classified in four groups as in figure (2).

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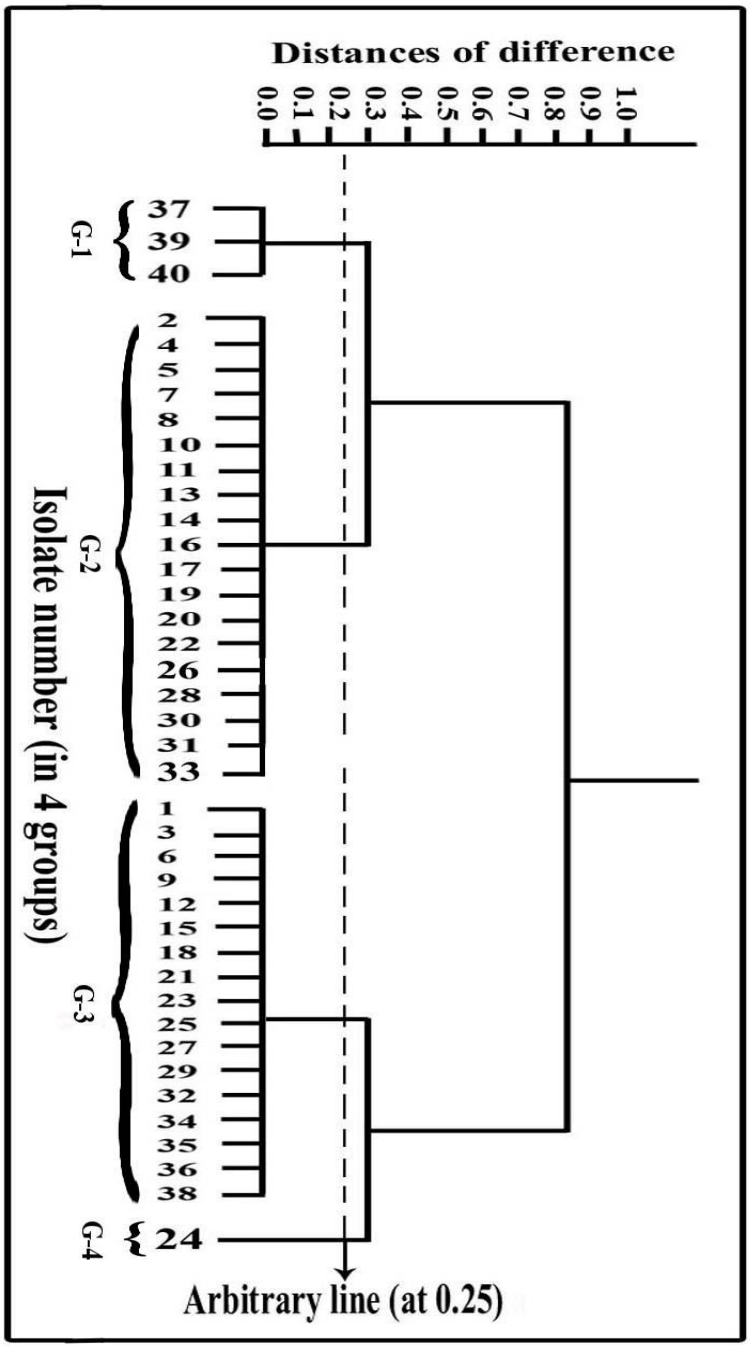

Figure. 2. Dendrogram derived from cluster analysis (UPGMA), showing relationship among the 40 isolates of M. furfur during the year of study (2008).

It was found according to patient history that isolates no. (37, 39, 40) in group no. 1 for patients in between 40 to 50 years old having hypo-pigmented tinea versicolor and isolate no. (24) in group no 4 were for only patient of 65 years old taking anti-depressive drugs having hyperpigmented tinea versicolor, while the rest of the isolates (group no. 2, 3) were for patients in range 6 - 40 years old having hypo and hyper-pigmented tinea versicolor, respectively.

This data proves that there may be a sort of relationship between phenotype produced by protein analysis of isolates, factors affecting human tinea versicolor infection and type of lesions.

ii. SDS – PAGE profile of 7 *M. globosa* isolates:

The cellular protein of 7 isolates of *M. globosa* analyzed on SDS – PAGE as in fig (3). The isolates share a number of bands. These bands may be species specific bands. The banding patterns showed a total number of 11 bands with molecular weight range from (14.4 – 112 kDa).

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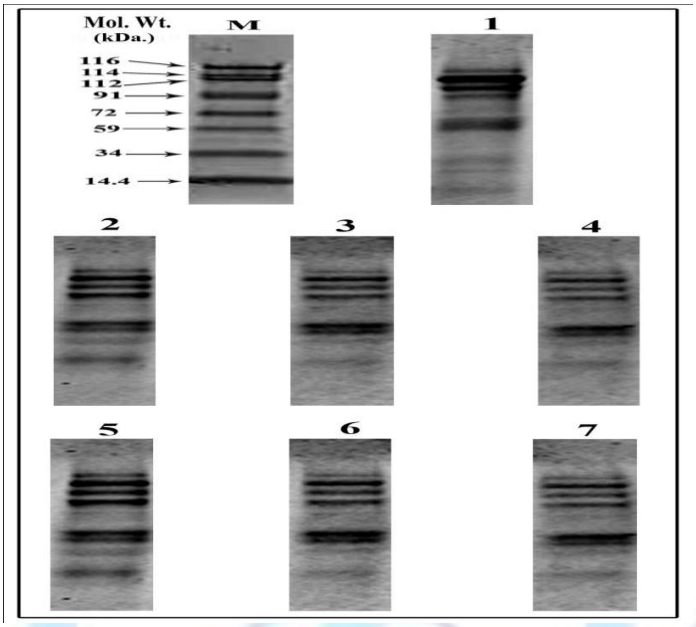

Figure 3. SDS-PAGE band survey for the total cellular proteins extracted from each of the 7 isolates of M. globosa of tinea versicolor lesions (from 1 to 7) during the year of study (2008), and a standard protein markers mixture (M) with molecular weights of: 116 kDa: β-galactosidase, 114 kDa: E. coli Hmp1 protein, 112 kDa: IgE seral immunoglobulin (against Mycoplasma antigen), 91 kDa: IgA linear immunoglobulin (against bullous disease antigen), 72 kDa: mammalian heat shock cognate protein (prp72), 59 kDa: eurofilament protein, 34 kDa: Soy bean seed oil-associated protein, 14.4 kDa: Lipoprotein lipase.

The frequency of each band was little different. Whereas the bands with molecular weight of (112, 95, 91, 87, 78, 66, 59, 34) showed the highest frequency that were found in 7 isolates, and that with molecular weight (56 kDa) was found in 6 isolates (isolates no. 2, 3, 4, 5, 6, 7), while the bands with molecular weight (29 and 4.4 kDa) was found in 1 isolate (isolate no. 1) as shown in table (2).

Table 2. SDS-PAGE band survey for the total cellular proteins extracted from each of the 7 isolates of *M. globosa* of tinea versicolor lesions during the year of study (2008)

Isolate no.	**Band number**										
	1	2	3	4	5	6	7	8	9	10	**11**
	Band mol. wt. (kDa)										
	112	95	91	87	78	66	59	56	34	29	**4.4**
1	1	1	1	1	1	1	1	0	1	1	**1**
2	1	1	1	1	1	1	1	1	1	0	**0**
3	1	1	1	1	1	1	1	1	1	0	**0**
4	1	1	1	1	1	1	1	1	1	0	**0**
5	1	1	1	1	1	1	1	1	1	0	**0**
6	1	1	1	1	1	1	1	1	1	0	**0**
7	1	1	1	1	1	1	1	1	1	0	**0**
T	7	7	7	7	7	7	7	6	7	1	**1**

The cluster analysis based on cellular proteins data separated the isolates of *M. globosa* into 2 clusters. The first cluster included isolate no.1. The second cluster included the rest of the isolates. By drawing an arbitrary line at genetic distance of 0.35 the isolates are classified into 2 groups as in figure (4).

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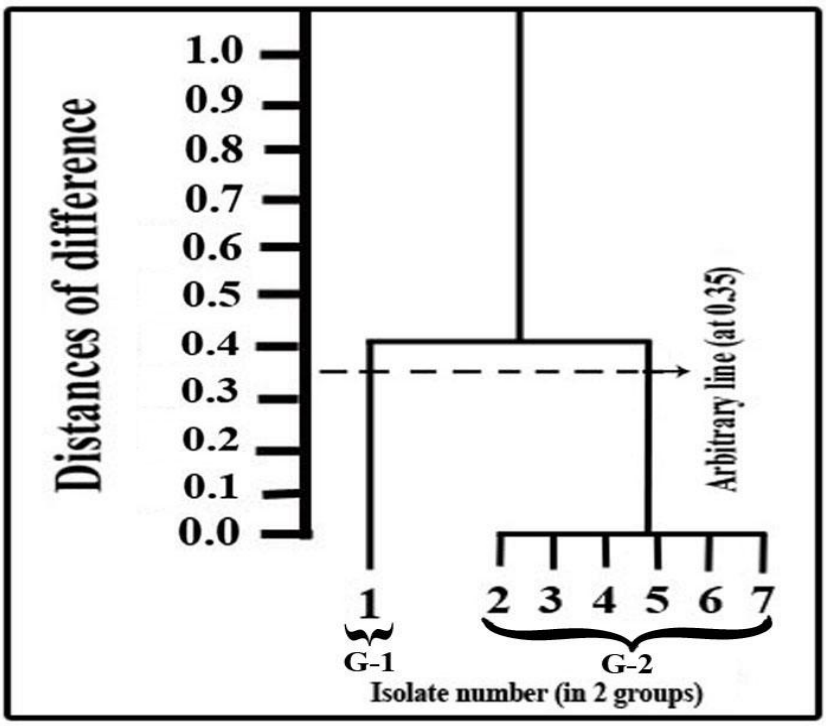

Figure 4.Dendrogram derived from cluster analysis (UPGMA), showing relationship among the 7 isolates of *M. globosa* during the year of study (2008).

It is very interesting also to notice the band with molecular weight of 56kDa may be specific to type of tinea versicolor lesion as this band was found in hyperpigmented lesion (as in isolates no. 2, 3, 4, 5, 6, 7) and absent in hypopigmented one (as in isolate no. 1). Interestingly, we found that isolate no. 1 was a pregnant woman versus the rest of isolates that were men and this may be the cause for the presence of bands with molecular weight (29 and 4.4 kDa) in this isolate and absent in other isolates. So, this data also proves that there is a sort of relationship between the phenotypes of isolates and type of lesions.

B. DNA fingerprint of *Malassezia* isolates by RAPD-PCR technique:

Genomic DNA was extracted from a subset of 6 isolates (4 isolates of *M. furfur* no. 40, 16, 18, 24) and 2 isolates (no.1 and 5) of *M. globosa*. DNA amplification was performed using five random primers of 10 nucleotides. One primer resulted in appearance of PCR products with varied numbers of DNA fragments and the other four primers didn't give any variations. The resulted amplified fragments are shown in figs (5) and (6).

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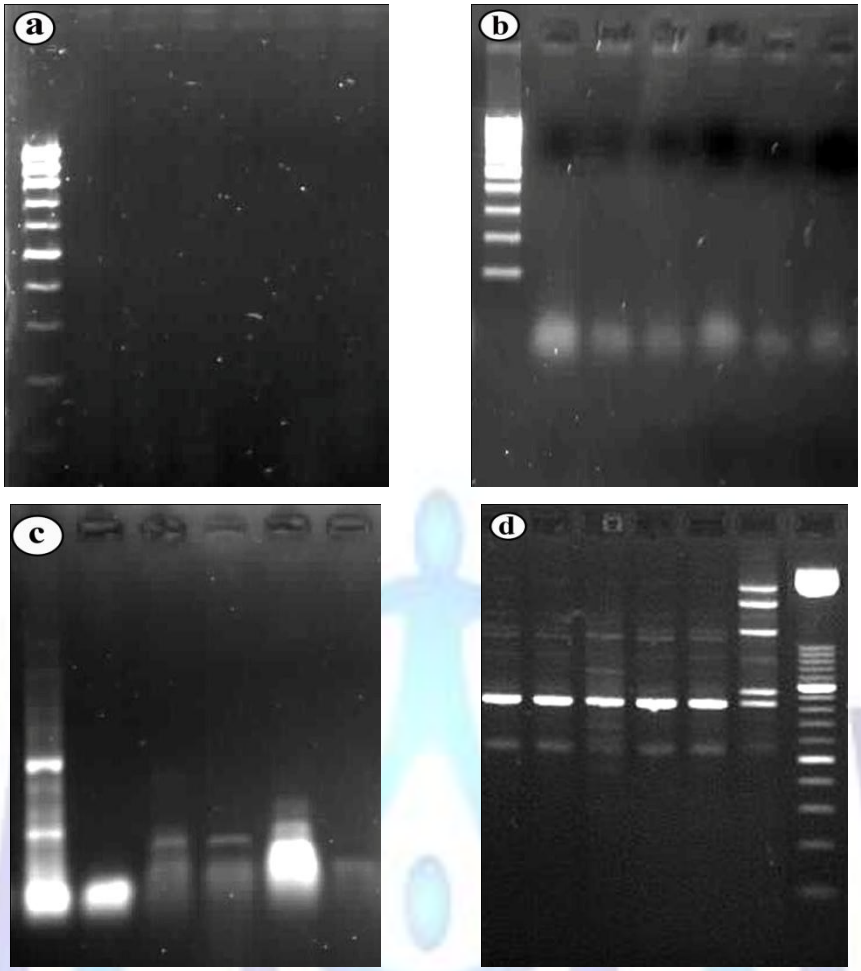

Figure 5. RAPD-PCR band survey for the DNA finger prints of representative samples for *M. furfur* (isolates no. 40, 16, 18, 24) and *M. globosa* (isolates no. 1, 5), isolated from tinea versicolor lesions during the year of study (2008), using different random primers.Primer (a)= 5'-ACCTGAACGG-3', Primer (b)= 5'-CCTTGACGCA-3', Primer (c)= 5'-GAGAGCCAAC-3, Primer (d)= 5'-AGCCAGCGAA-3'.

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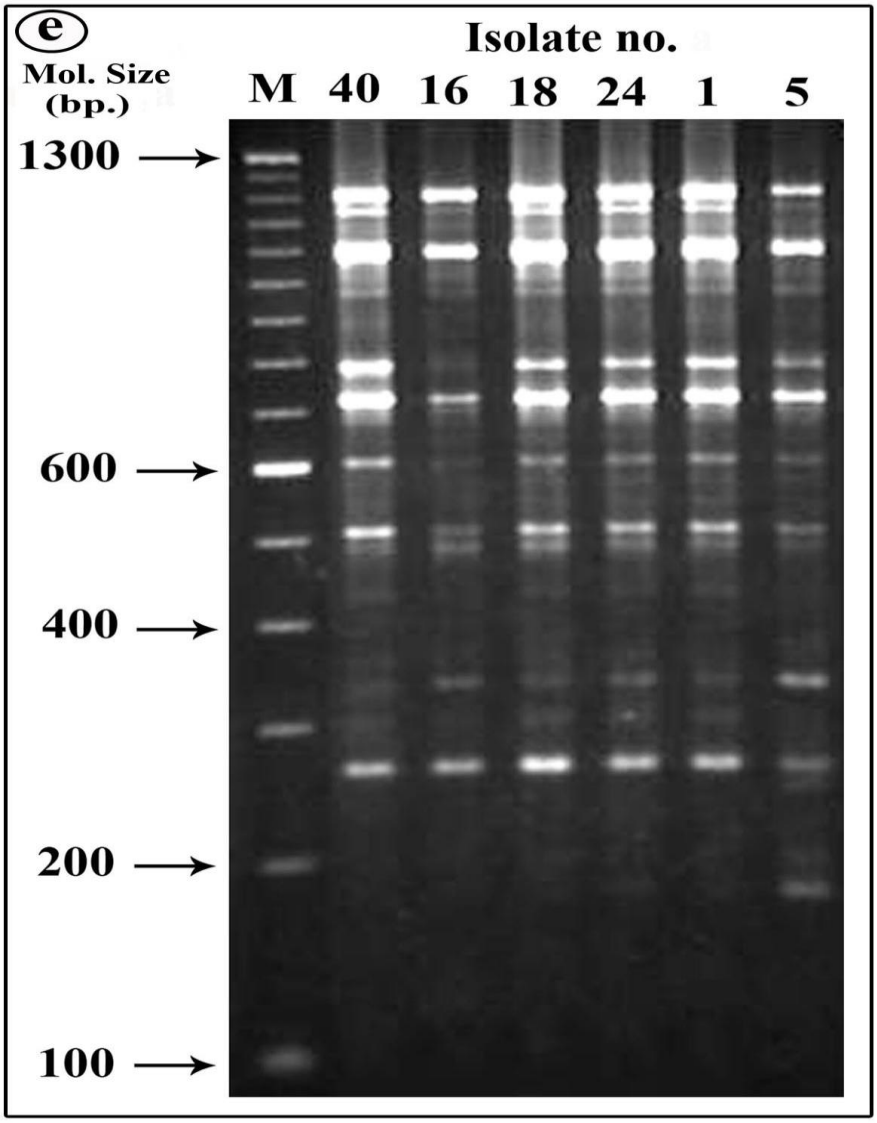

Figure 6. RAPD-PCR band survey for the DNA finger prints of representative samples for *M. furfur* (isolates no. 40, 16, 18, 24) and *M. globosa* (isolates no. 1, 5), isolated from tinea versicolor lesions during the year of study (2008), using primer (e).

M= one kilo base DNA marker mixture.

Primer (e)= 5'-TGCCGAGCTG-3'.

The number of amplified DNA fragments was differed due to different primers and different isolates. The highest total number of bands identified by the five primers used was 14, for isolates no. 18, 24 of M. furfur and no. 5 of M. globosa; and the lowest number was 9, for isolate no. 16 of *M. furfur*.

Genomic DNA was extracted from a subset of 6 isolates (4 isolates of M. furfur no. 40, 16, 18, 24) and 2 isolates (no.1 and 5) of *M. globosa*. DNA amplification was performed using five random primers of 10 nucleotides. One primer resulted in appearance of PCR products with varied numbers of DNA fragments and the other four primers didn't give any variations. The number of amplified DNA fragments was differed due to different primers and different isolates. The highest total number of bands identified by the five primers used was 14, for isolates no. 18, 24 of *M. furfur* and no. 5 of *M. globosa*; and the lowest number was 9, for isolate no. 16 of M. furfur.

Furthermore, primer E (5'-TGCCGAGCTG-3') produced the highest number of bands among the 5 primers, with a total of 18 bands as in table (3).

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Table 3. RAPD-PCR band survey for the DNA finger prints of representative samples for *Malassezia* sp., isolated from tinea versicolor lesions during the year of study (2008), using primer (E)

		40	16	18	24	1	5	
		Cluster no.						
		G:f-1	G:f-2	G:f-3	G:f-4	G:g-1	G:g-2	
	1200	1	1	1	1	1	1	
	1165	1	0	1	1	1	0	
	1050	1	1	1	1	1	1	
	980	1	1	1	1	1	1	
	960	1	1	1	1	1	1	
	900	1	1	1	1	1	1	
	790	1	0	0	0	0	0	
	720	1	1	1	1	1	1	6
7	600	1	0	1	1	1	1	5
10	500	1	1	1	1	1	1	6
11	510	1	1	1	1	1	1	
12	370	0	0	0	1	0	0	
13	350	0	1	1	1	1	1	
14	320	0	0	1	1	1	0	
15	290	1	1	1	1	1	1	
16	280	0	0	0	0	0	1	1
17	200	0	0	1	0	0	1	2
18	180	0	0	0	0	0	1	**1**

G: f-1= *M. furfur* isolate no. 40, G: f-2= *M. furfur* isolate no. 16,

G: f-3= *M. furfur* isolate no. 18, G: f-4= *M. furfur* isolate no. 24;

G: g-1= *M. globosa* isolate no. 1, G: g-2= *M. globosa* isolate no. 5.

The amplification profiles of all isolates were polymorphic with primer E as in fig (6). Each isolate gave different DNA profile. The cluster analysis of the DNA fragments produced by primer E is shown in figure (7).

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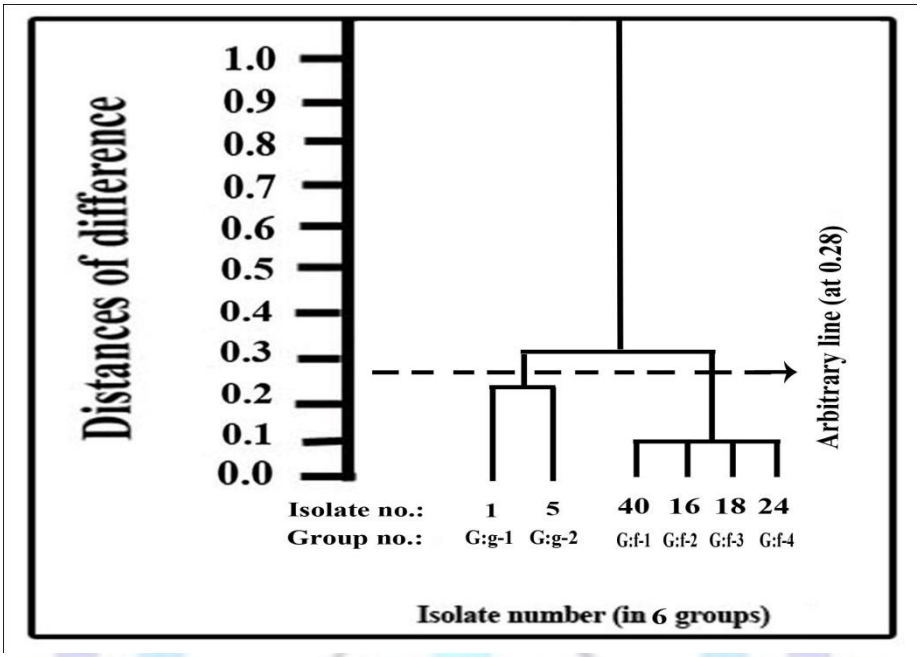

Figure 7. Dendrogram derived from cluster analysis (UPGMA), showing relationship among the DNA finger prints of representative samples for *Malassezia sp*., isolated from tinea versicolor lesions during the year of study (2008), using primer: E (5'-TGCCGAGCTG-3').

The dendogram classified the isolates into 2 clusters. By drawing an arbitrary line at a distance of 0.28, the RAPD primer E identified the isolates into six genetic groups but the results generated by the other primers did not separate the genetic group that defined by this primer, as G:f-1 refers to patients in between 40 to 50 years old had hypopigmented lesions of tinea versicolor caused by M. furfur, G:f-2 refers to patients in range 6-40 years old had hypopigmented lesions of tinea versicolor caused by M. furfur, G:f-3 refers to patients in range 6-40 years old had hyperpigmented lesions of tinea versicolor caused by M. furfur, G:f-4 refers to patient of 65 years old taking anti-depressive drugs had hyperpigmented lesions of tinea versicolor caused by M. furfur, G:g-1 refers to patient had hypopigmented lesions of tinea versicolor caused by M. globosa, and G:g-2 refers to patient had hyper pigmented lesions of tinea versicolor caused by M. globosa.

By comparing the total cellular protein of isolates with RAPD genotypes, an association was found between them especially in primer E as it gave the same six groups of those obtained from total protein by SDS- PAGE.

So, the present work provide evidence for the association between RAPD bands, factors affecting human tinea versicolor infection and type of lesions, it is possible that the variant bands of the amplification products of the isolates may be linked to the factors affecting tinea versicolor that may be expressed by genes.

DISCUSSION

The electrophoretic separation of protein is a useful tool for differentiating fungal taxa, also can be used to evaluate levels of genetic diversity and systematic relationships (34). In our present study, the electrophoretic analysis of the total cellular proteins of the 40 examined isolates of M. furfur demonstrated that all isolates showed a similarity in electrophoretic pattern with range from 45 % in some isolates to 100 % in other isolates. Almost similar data was obtained from study carried on different isolates of *Malassezia pachydermatis* (35). In the present study the cellular protein from M. furfur isolates are grouped within a relatively wide molecular weight range (11 - 112 kDa) compared with the molecular weight range (16 - 141 kDa) which reported by.35 This contradiction in data could be attributed to the variation in the temperature conditions, time of incubation for each fungus; these parameters were found to have a great efficacy on the electrophoretic patterns of microorganisms (36). Our recent work represents that in case of M. furfur, many of protein bands exhibited high frequency but the protein band that exhibited the lowest frequency was the band with molecular weight of 56 kDa. This band was present in hyperpigmented lesions in 18 isolates and absent in 22 isolates which are hypopigmented. This data proved that this band of molecular weight 56 kDa may be related to the action of some enzymes that secreted by *Malassezia* affecting melanocytes and causing cosmetic disrupting leading to the appearance of hypo and hyperpigmented lesion. Moreover, we noticed that the band with molecular weight 60 kDa was present only in 3 isolates of patients in between 40 to 50 years old (isolate no.37, 39, 40). Also, we found that the bands with molecular weight (112, 105, 102, 95, 90, 79, 48, 14, 11 kDa) was absent in isolate no. 24 of patient of 65 years old taking anti-depressive drugs. So it could be concluded that these bands may be correlated to the factors affecting patients of tinea versicolor.

Also, the electrophoretic analysis of the total cellular proteins of the seven examined isolates of *M. globosa* demonstrated that the frequency of protein bands was little different in some isolates and similar in others with molecular weight range from (14.4 - 112 kDa).

As previously reported, electrophoretic patterns of the cellular proteins of *M. furfur* isolates covered a relatively wide molecular weight range extended from (11 -112 kDa) and from (14.4 - 112 kDa) in *M. globosa*. However, the range of the cellular proteins in fungi distinctly varied from species to another and even between the isolates at the same species, as it covers a range of molecular weight varying from14.4 to 200 kDa in four isolates of species of Candida sp.36 and 18 – 66 kDa in Candida albicans.35 This may be partly attributed to genetics of the examined microorganisms and partly to the culture conditions as well as other growing agents

The cluster analysis based on cellular proteins data of *M. globosa* separated the seven isolates into 2 groups according to the band with molecular weight 56 kDa, which is present in 6 cases (hyperpigmented) and absent in case (hypopigmented). Also, we found that the bands with molecular weight (29, 4.4 kDA) was present in isolate no. 1 of pregnant woman and absent in the rest men isolates (no. 2, 3, 4, 5, 6, 7). Combining all these data together showed that there may be a relationship between phenotype produced by protein analysis of isolates, type of lesions, and factors affecting human tinea versicolor infection.

The analysis of the electrophoretic protein profiles combining computer static program enabled the identification, classification and even the reclassification of a number of strains, species and genera of bacteria and yeast in taxonomic and epidemiological studies (37-38-39-40-41-42).

The advent of DNA–based molecular methods provided useful tools with which to study the phylogeny of microorganisms and to differentiate species, formae speciales, races and strains. RAPD markers have been previously used to study intra-specific variation *of Malassezia* sp (43-44-45-46-47).

The polymorphism observed from RAPD markers revealed a high degree of genetic diversity in *M. furfur* and *M. globosa* isolates collected from different patients having tinea versicolor infection. This was consistent with the considerable genetic variation among the isolates of M. furfur from patients of pityriasis versicolor and seborrhoeic dermatitis as reported by (48). Additionally, Durate and Hamdan (49) have analysed the DNA profile of *Malassezia furfur*, *Malassezia slooffiae* and *Malassezia pachydermatis* by random amplified polymorphic DNA (RAPD)–PCR to compare the genetic diversity between isolates from the external ears of cattle, dogs and humans. The analysis showed genetic heterogeneity between RAPD profiles of *Malassezia furfur* and *Malassezia slooffiae* isolates from humans and cattle and between *Malassezia pachydermatis* isolates from dogs and cattle. Intra-species variations in DNA pattern of *Malassezia* isolates and the presence of specific genetic types in cattle, dogs or humans were observed. A review of genetic heterogeneity of these yeast in veterinary and human medicine studies is given considering a possible transmission animal to human or human to animal. Phenetic characteristics often do not allow the identification or delineation of closely related *Malassezia* spp., such that molecular tools need to be used to assist in fundamental studies of the epidemiology and ecology of *Malassezia* as well as aspects of the pathogenesis and disease caused by members of this genus (50). They reviewed the morphological and biochemical methods commonly used for the identification of *Malassezia* as well as DNA technological methods that have been established for the specific identification of members of this genus and the diagnosis of their infections. New avenues for the development of improved molecular-diagnostic methods to overcome diagnostic limitations and to underpin fundamental investigations development of improved molecular-diagnostic methods to overcome diagnostic limitations and to underpin fundamental investigations of this interesting group of yeasts are proposed.

Only primer E (5'-TGCCGAGCTG-3') generated a RAPD banding pattern which allowed the identification of 6 different amplification groups within *M. furfur* and *M. globosa*.This primer can be used for the development of a PCR-based molecular diagnostic tool for different isolates of *M. furfur* and *M. globosa*, as it is used as a probe in molecular hybridization analysis. By comparing the total cellular protein of isolates with RAPD genotypes, an association was found between them especially in primer E as it gave the same six groups of those obtained from total protein by SDS- PAGE.

The current data provide evidence for the association between RAPD bands, factors affecting human tinea versicolor infection and type of lesions, it is possible that the variant bands of the amplification products of the isolates may be linked to the factors affecting tinea versicolor that may be expressed by genes. Sugita et al. (51) compared the genotypes of *M. globosa* colonizing the skin surface of 32 atopic dermatitis (AD) patients and 20 healthy individuals for polymorphism of the intergenic spacer (IGS) 1 region of the rRNA gene. Sequence analysis demonstrated that *M. globosa* was divided into four major groups, which corresponded to the sources of the samples, on the phylogenetic tree. Of the four groups, two were from AD patients and one was from healthy subjects. These findings suggest that a specific genotype of M. globosa may play a significant role in AD, although *M. globosa* commonly colonizes both AD patients and healthy subjects.

There was a correlation between RAPD patterns and factors affected the studied isolates of tinea versicolor patients. The results from RAPD analysis and total cellular protein analysis were similar and suggest a considerable genetic diversity among isolates of *M. furfur* and *M. globosa*. From the results of these two methods of analysis, we noticed that there was a relationship between genotype and phenotype with the factors affecting human tinea versicolor infection and type of lesions.

REFERENCES

[1] Faergemann, J.M.D. 1985.Lipophilic yeasts in skin disease. Senin Dermatology 4: 173 – 184.

[2] Sunenshine, P.J., Schwartz, R.A. and Janniger, C.K. 1998.Review. Tinea versicolor. International Journal of Dermatology 37: 648-655.

[3] Savin, R. 1996. Diagnosis and treatment of tinea versicolor. J. Fam. Pract., 43: 127-132.

[4] Assis, T.L., Aguira, E.J., Nery, J.A., Gurmaraes, A.A. and Azulay, R.D. 1999. Data and comments on 4060 mycological tests on skin scales, hairs and nails. Medicina Cutanea Iberolantino – Americana 13: 209 – 218.

[5] El-Hefnawi, H.; El-Gothamy, Z. and Refai, M. 1971. Studies on pityriasis versicolor in Egypt. Mycoses, 14: 225 – 231

[6] El-Gendy, Z.K.A. 1988. Studies on dermatophytes in Minia Governorate. M.Sc. Thesis, Botany Department, Faculty of Science, Minia Univ., Egypt.

[7] El–Shanawany, A.A. 1993. Human dermatophytes in Assiut and new Valley Governorates. Ph.D. Thesis, Bot. Dept ., Fac. Sci., Assiut Univ ., Egypt.

[8] Mubarak, H.M. 1996. Studies on some dermatophytic fungi. M.Sc. Thesis, Botany department, Faculty of Science, Tanta University, Egypt

[9] Hellgren, L. and Vincent, J. 1983. The incidence of tinea versicolor in central Sweden. Journal of Medical Microbiology 16: 501-502.

[10]Celik, E., Ilkit, M. and Tanir, F. 2003. Prevalence and causative agents of superficial mycoses in a textile factory in Adana, Turkey. Mycoses 46: 329-333.

[11] Nowiscki, R. and Sadowska, E. 1993. Mycotic infections in the Gdansk area. Przegl Dermatology 80: 245 – 250.

[12]Faergemann, J.M.D. 1995. Tinea versicolor (pityriasis versicolor). Clinical Dermatology. Philadelphia: Lippincott-Raven, 20: 1 – 11.

[13]Gupta, A.K., Kholiand, Y. and Summerbell, R.C. 2000. Molecular differentiation of seven Malassezia species. Journal of Clinical Microbiology, 38: 1869-1875.

[14]Theelen, B.; Silvestri, M.; Gueho, E.; Belkum, V. and Bekhout, T. (2001): Identification and typing of Malassezia yeasts using amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and denaturing gradient gel electrophoresis (DGGE). FEMS Yeast Research 1: 79-86.

[15]Guillot, J., Deville, M., Berthelemy, M. and Gueho, E.A 2000. Single PCR-restriction endonulease analysis for rapid identification of Malassezia species. Letter of Applied Microbiology 31: 400-403.

[16]Makimura, K., Tamura, Y., Kudo, M., Uchida, K.; Saito, H. and Yamaguchi, H. 2000. Species identification and strain typing of Malassezia species stock strains and clinical isolates based on the DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. Journal of Medical Microbiology 49: 29–35.

[17]Boekhout, T., Kamp, M. and Guého, E. 1998. Molecular typing of Malassezia species with PFGE and RAPD. Medical Mycology 36: 365-372.

[18]Al – Doory, Y. 1980. Laboratory medical mycology . Lea & Febiger Philadilphia Kimpton publishers, London.

[19]Khosravi A.R., Eidi S., Ziglari T. and Bayat, M. 2008. Isolation and Differentiation of Malassezia Species Isolatedfrom Healthy and Affected Small Animals, Ear and Skin. World Journal of Zoology 3 (2): 77-80, 2008

[20]Aste, N., Biggio, P. and Pau, M. 1987. Pityriasis versicolor. Journal of Micologia Dermatologica, 1: 121 – 131.

[21]Yi-Ming Fan, M.D., Wen-Ming Huang, Shun-Fan Li, Guo-Feng Wu, M.M., Kuan Lai, M.M. and Rong-Yi 2006. Granulomatous Skin Infection Caused by Malassezia pachydermatis in a Dog Owner. Archive Dermatology 142: 1181-1184

[22]Chaudhary R., Singh, S., Banerjee T. and Tilak R. 2010. Prevalence of different Malassezia species in pityriasis versicolor in central India. Indian Journal of Dermatology 76 (2):159-164

[23]Roberts, S.O.B. 1996. Pityriasis versicolor: a clinical and mycological investigation. British Journal of Dermatology 81: 315 – 326.

[24]Gueho, E., Midgley, G. and Guillot, J. 1996. The genus Malassezia with description of four new species. Antonie van Leeuwenhock, 69: 337-355.

[25]Scmidt, A. 1997. Malassezia furfur : A fungus belonging to the physiological skin flora and it's relevance in skin disorders. Cutis 59: 21-24.

[26]Nakabayashi, A., Sei, Y. and Guillot, J. 2000. Identification of Malassezia species isolated from patients with seborrhoeic dermatitis, atopic dermatitis, pityriasis versicolor and normal subjects. Medical Mycology 38: 337–341.

[27]Nell, S.A., James, C.J., Bond, H. and Herrtage, M.E. 2002. Identification and distribution of novel Malassezia species yeast on normal equine skin. Veterinary Record 150: 395 – 398.

[28]Kushnirov V.V. 2000. Rapid and reliable protein extraction from yeast. Yeast, 16: 857–860.

[29]Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

[30]Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 277: 680-685.

[31]Williams, J.G.K., Kubelic, A.R. , Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Research 18: 6531-6535.

[32]Tucker, W.D.L. and Noble, W.C. 1991. Polyacrylamide gel electrophoresis patterns of some Microsporum species. Mycoses, 34: 303-307.

[33]Coutinho, S.D., Souza, T. and Paula, C.R. 1997. Protein profiles of Malassezia pachydermatis isolated from dogs. Mycopathologia 139: 129-35.

[34]Hofling, J.F., Moreira, D., Spolidorio, D., Rodrigues, M. and Rosa, E. 2001. Biotypes in the oral cavity of school children from different socioeconomic categories in Piracicaba, Brazil. Pesquisa Odontológica Brasileira 15: 187 – 195.

[35]Kersters, K. 1985. Numerical methods in the classification of bacteria by protein electrophorsis. Computer Assisted Systematic, Acadimic Press, England, 20: 330 - 337

[36]Tanner, A.C.R; Listgarten, M.A; Ebersole, J.L. and Strzempko, M.N. 1986. Bacteroides forsythus from the human oral cavity. International journal of systematic bacteriology 36: 213 – 221.

[37]Coastas, M.; Holmes, B. and Wood, A.1989. Numerical analysis of electrophoretic patterns of Providencia rettgeri from human faeces , urine and other specimens. Journal of Applied Bacteriology 67: 441 – 452.

[38]Maiden , M.F. and Tanner, A.1991. Identification of oral yeast by polyacrylamide gel electrophoresis. Oral microbiology and immunology 6: 187 – 190 .

[39]Vancanneyt, M.; Pot. B.; Hennebert, G. and Kersters, K. 1991. Differentiation of yeast species based on electrophoretic whole cell protein patterns. System Applied Microbiology 14: 22 – 23.

[40]Vancanneyt, M;. Lerberge, E. ; Bernny, J. and Kersters, K. 1992. The application of whole cell protein electrophoresis for the classification and identification of basidiomyceous yeast species. Antonien van leeuwenhoec, 61: 69 – 78

[41]Boekhout, T.; Kamp, M. and Guého, E. 1998. Molecular typing of Malassezia species with PFGE and RAPD. Medical Mycology 36: 365-372

[42]Soll, D.R. 2000. The ins and outs of DNA fingerprinting the infectious fungi. Journal of Clinical Microbiology 13: 332–370.

[43]Theelen, B.; Silvestri, M.; Gueho, E.; Belkum, V. and Bekhout, T. 2001. Identification and typing of Malassezia yeasts using amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and denaturing gradient gel electrophoresis (DGGE). FEMS Yeast Research 1: 79-86.

[44]Cabanes, F.J.; Theelen, B.; Castella, G. and Boekhout, T. 2007. Two new lipid-dependent Malassezia species from domestic animals. FEMS Yeast Research 7: 1064–1076.

[45]Duarte, E.R. and Hamdan, J.S. 2008. Molecular characterization of Malassezia sympodialis and Malassezia furfur from cattle with and without otitis. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 60: 779-785.

[46]Gandra, R.F., Replace Rinaldo, F. and Rita, C.G. 2006. Genotyping by RAPD-PCR analyses of Malassezia furfur strains from pityriasis versicolor and seborrhoeic dermatitis patients. Mycopathologia, 162: 273–280.

[47]Duarte, E.R. and Hamdan, J.S 2010. RAPD differentiation of Malassezia spp. from cattle, dogs and humans. Mycoses 53(1):48-56.

[48]Cafarchia, C., Gasser, R.B., Figueredo, L.A., Latrofa, M.S. and Otranto, D. 2011. Advances in the identification of Malassezia. Molecular Cell Probes 25(1):1-7

[49]Sugita, T., Kodama, M., Saito, M., Ito T., Kato, Y., Tsuboi, R. and Nishikawa A. 2003. Sequence Diversity of the Intergenic Spacer Region of the rRNA Gene of Malassezia globosa Colonizing the Skin of Patients with Atopic Dermatitis and Healthy Individuals. Journal of Clinical Microbiology 41 (7): 3022-3027