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Genetic relationships and isozyme profile of dermatophytes and *Candida* strains from Egypt and Libya

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Three molecular techniques random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR) and restriction fragment length polymorphism (RFLP) were employed for identification and to study the genetic relationship among six species of dermatophytes and three species of yeasts isolated from Egyptian and Libyan patients with skin mycosis. Each species was represented by two isolates, one from Egyptian patients and the second from Libyan. RAPD in which four random 10-mer primers and two ISSR primers were used to amplify the DNA fragments of target fungi and RFLP in which two universal primers (ITS1 and ITS4) were used to amplify the internal transcribed spacer (ITS) regions of the ribosomal (rRNA) gene in fungal isolates followed by digestion with *Hinf*I and *Hae*III endonucleases was carried out. Three molecular marker techniques showed considerable potential for identifying and discriminating dermatophytes and *Candida* species and the achieved results confirmed identification based on conventional morphological methods. Results of RAPD and ISSR markers revealed 78.7% genetic similarity (GS) between *Microsporum canis* and other tested fungi reflecting a relatively longer genetic distance from other isolates of dermatophytes and yeasts. *Candida krusei* and *Candida tropicalis* were closely related showing 93.3% GS. *C. albicans* showed 90.9% similarity with other species of *Candida. Epidermophyton floccosum* was easily separated from all *Trichophyton* species showing 87.3% similarity. Unique bands were displayed by certain fungi and can be taken as a positive marker for isolate identification and discrimination. RFLP technique revealed differences in the number (1 to 5) and size (8 to 378 base pairs) of DNA fragments depending on the fungal isolate and restriction enzyme used. Within each fungal species, different isolates of dermatophytes and *Candida* from Egypt and Libya showed close relationship. Seven isozyme systems namely esterase, peroxidase, malate dehydrogenase, acid phosphatase, glutamate-oxalo-acetate transaminase, Urease and protease were studied to detect the gene expression and genetic variability among the different isolates of dermatophytes and *Candida.*

Key words: Random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), restriction fragment length polymorphism (RFLP), dermatophytes, *Candida*, isozymes.

INTRODUCTION

Conventional methods for identification of fungi rely on macro and micro morphological features of the colonies on general and specific culture media and on some biochemical and physiological complementary test. However, outside factors such as temperature variation, medium and chemotherapy, can greatly influence the

phenotypic characteristic and consequently can make the identification more difficult (Weitzman and Summerbell, 1995; Faggi et al., 2001; Kanbe, 2008; De Baere et al., 2010). Recently, molecular marker approaches, such as nested-polymerase chain reaction (PCR) (Verrier et al., 2013), random amplified polymorphic DNA (RAPD)-PCR (Kim et al., 2001; Baeza et al., 2006; Leibner-Ciszak et al., 2010; Dobrowolska et al., 2011; Spesso et al., 2013), inter-simple sequence repeat (ISSR)-PCR (Cano et al., 2005; Khosrav et al., 2012), PCR- restriction fragment length polymorphism (RFLP) (Yang et al., 2008; Rezaei-Matehkolaei et al., 2012; Samuel et al., 2013), real time PCR (Bergmans et al., 2010) and multiplex PCR assay (Arabatzis et al., 2007; Kim et al., 2011) and others have been adapted for detection of dermatophytes from clinical specimens.

The RAPD approach which used to detect strain polymorphism depends on the application of short arbitrary primers that anneal to multiple genomic sites at appropriate temperature conditions and this technique does not depend on prior knowledge of species-specific sequence (Valério et al., 2006). RAPD methods have frequently been used for phylogenetic analysis and identification of dermatophytes. Spesso et al. (2013) used random amplified polymorphic DNA primers to recognise *Microsporum canis, Microsporum gypseum, Trichophyton rubrum* and *Trichophyton interdigitale*. For identification of *Trichophyton mentagrophytes* and *Trichophyton tonsurans,* Kim et al. (1999 and 2001) proposed a RAPD method, in which *T. mentagrophytes* was divided into subtypes on the basis of amplification patterns in RAPD using three primers.

The ISSR-PCR technique is highly reproducible and allows detection of interspecific and intraspecific DNA-polymorphisms. This method is based on PCR and uses primers containing microsatellite sequences and degenerate anchors at the 50 end (Zietkiewicz et al., 1994). Cano et al. (2005) and Khosrav et al. (2012) mentioned that ISSR-PCR is a simple and rapid method for identification of dermatophyte species and for differentiation. Therefore, it is better to use the RAPD and ISSR method in combination with other techniques such as gene-specific PCR-RFLP for fungal identification at the species level. Specific PCR products and RFLP patterns for MvaI, HinfI and HaeIII enzymes allowed the rapid identification and reliable differentiation of isolated dermatophytes at the genus or species level for 5 to10 day-old colonies (Mirzahoseini et al., 2009; Rezaei-Matehkolaei et al., 2012). Isozyme analysis is a powerful technique for taxonomic, genetic, and population studies, and has been successfully applied for identifying fungal species. Isozyme term means “the different molecular forms in which proteins may exist with the same enzymatic specificity” (Markert and Moller, 1959). Klaas (1998) reported that the isozyme markers can correctly identify several levels of taxa, accessions, and individuals, since the assumption of homology can be more accurate than for some genomic DNA markers. Siddiquee et al. (2010) used eight enzymes and single protein pattern systems to identify and study the genetic relationships among 27 isolates of *Trichoderma harzianum*, 10 isolates of *Trichophyton aureoviride*, and 10 isolates of *Trichophyton longibrachiatum* from Southern Peninsular Malaysia. They found that three isozyme and total protein patterns were useful for the detection of three *Trichoderma* species. The aim of this study was to identify and study the genetic relationship among six species of dermatophytes and three species of yeasts isolated from Egyptian and Libyan patients with skin mycosis using three molecular techniques (RAPD, ISSR and RFLP) and isozyme profiles.

MATERIALS AND METHODS

Fungal strains

Eighteen (18) fungal strains were isolated from clinical cases in Assiut (Egypt) and Tripoli (Libya) and used in the present study (Table 1). Conventional morphological methods were employed for identification of these fungi where dermatophytes were cultured on Sabouraud`s Dextrose agar (SDA) with chloramphenicol and on bromocresol purple milk glucose agar (Ellis et al., 2007). *Candida* species were allowed to grow on SDA, followed by identification on Chromagar *Candida* medium (Pfaller et al., 1996). Mycological references including De-Hoog et al. (2000) and Ellis et al. (2007) were consulted for identification. All cultures were preserved in the culture collection of the Assiut University Mycological Centre (AUMC).

DNA extraction

Genomic DNA of each fungal strain was extracted using glass bead disruption (Yamada et al., 2002). Briefly, 300 mg of 0.5 mm diameter glass beads, 300 µl of lysis buffer (100 mM Tris-HCl pH 8, 10 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate (SDS), 2 % triton X-100) and 300 µl of phenol, chloroform-isoamyl, alcohol (25:24:1) were added to a fresh mycelium or cells in a 1.5 ml tube. Samples were shaken vigorously for 5 min, centrifuged for 10 min at 5000 rpm, and the supernatant was transferred to a fresh tube. The supernatant was extracted again with chloroform and DNA was precipitated by adding equal volume of cold isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.2). The solution was incubated for 10 min at -20°C and centrifuged for 15 min at 10000 rpm. The precipitant was washed with cold 70 % ethanol and dried in air. DNA was suspended in 80 µl free-nuclease-water and an aliquot was removed to determine nucleic acid purity and concentration by spectrophotometry. DNA was also checked by agarose (0.8 %) gel electrophoresis.

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Abbreviations: PCR, Polymerase chain reaction; **RAPD,** random amplified polymorphic DNA; **ISSR,** inter-simple sequence repeat; **RFLP,** restriction fragment length polymorphism; **SDA,** Sabouraud`s Dextrose agar; **SDS,** sodium dodecyl sulfate; **GS,** genetic similarity; **BP,** banding patterns; **ITS**, internal transcribed spacer; **ACP,** acid phosphatase; **MLEE,** multilocus enzyme electrophoresis.

Table 1. Source and code number of fungal isolates involved in the present study.

AUMC Number	Fungal species	Source	Country
7826	*Trchophyton mentagrophytes*	Onychomycosis	Libya
5505	*Trchophyton mentagrophytes*	Onychomycosis	Egypt
5479	*Trchophyton verrucosum*	Tinea cruris	Egypt
7460	*Trchophyton verrucosum*	tinea capitis (Kerion type)	Libya
5495	*Epidermophyton floccosum*	Tinea corporis	Egypt
7892	*Epidermophyton floccosum*	Tinea pedis	Libya
5453	*Microsporum canis*	Tinea capitis (Scaly ringworm)	Egypt
7893	*Microsporum canis*	Tinea capitis (Scaly ringworm)	Libya
5469	*Trchophyton rubrum*	Tinea capitis (Scaly ringworm)	Egypt
7808	*Trchophyton rubrum*	Onychomycosis	Libya
7980	*Candida albicans*	Onychomycosis	Libya
7981	*Candida albicans*	Onychomycosis	Egypt
5454	*T. violaceum*	Tinea corporis	Egypt
7895	*T. violaceum*	Tinea capitis (Black dot)	Libya
7985	*C. krusei*	Onychomycosis	Egypt
7984	*C. krusei*	Onychomycosis	Libya
7983	*C. tropicalis*	Onychomycosis	Egypt
7982	*C. tropicalis*	Onychomycosis	Libya

PCR-amplification for RAPD and ISSR

Four PCR-RAPD primers and two PCR-ISSR primers as shown in Table 2 were used to study the genetic differences and relationships among the 18 isolates of dermatophytes and Candida (Table 2). Each amplification reaction was performed in a final volume of 25 µl containing 1 µl of genomic DNA, 1.25U of *Taq* DNA polymerase, 0.3 mM of each four deoxynucleoside triphosphate, 1.5 mM of $MgCl_2$, 0.4 µM of each primer and 2.5 µl of 10X PCR buffer. PCR was carried out with the following program: 1 cycle of 5 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 34ºC and 2 min at 72°C and a final extension step at 72°C for 10 min. Amplified DNA fragments were run onto 1.4 % agarose gel electrophoresis in TBE buffer at 80 V for 2.5 h. The products were detected by staining with ethidium bromide and photographed. The molecular sizes of DNA fragments were determined in relation to molecular standards (100 to 1500 bp). The presence/absence of fragments from dermatophytes and *Candida* isolates was analyzed using the software package MVSP program of Nei and Li (1979) to estimate the genetic similarity (GS).

Restriction fragment length polymorphyism (RFLP) analysis

PCR amplification for rRNA gene

Fungal-specific universal primer pairs were used to amplify internal transcribed spacer 1 (ITS1) 5.8S -ITS2 regions of rDNA in the tested fungi. The amplification reaction was performed in a final volume of 25 µl containing 1 µl of extracted genomic DNA (about 20 ng), 1.25 U of *Taq* DNA polymerase, 0.3 mM of each deoxy-nucleoside triphosphate mix (dATP, dTTP, dGTP, dCTP), 0.4 µM of each of forward ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and reverse ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers, 1.5 mM of $MgCl_2$, and 2.5 µl of 10X PCR buffer. PCR was carried out in a thermal cycler with the following temperature profile: 1 cycle of 5 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 56°C and 1 min at 72ºC and a final extension step at 72°C for 10 min. PCR products were analyzed in 2% agarose gel with 0.5 X Tris-Borate-EDTA buffer, stained with ethidium bromide and visualized in UV light.

Digestion with restriction enzymes (endonucleases)

10 µl of each PCR product were separately digested with 10 U of *Hinf*I and *Hae*III restriction enzymes at 37°C overnight. Restriction fragments were analyzed in 2.5% agarose gel electrophoresis in 0.5 X Tris-Borate-EDTA buffer for about 2.5 h at 80 V and visualized by staining with 0.5 µg/ml of ethidium bromide.

Data analysis

Agarose gel photos were scanned by the Gene Profiler 4.03 computer software program that uses automatic lane and peak finding for detecting the presence of banding patterns, and calibrating them for size and intensity. A binary data matrix recording the presence (1) or the absence (0) of bands was made. The software package Multi-Variant Statistical Package (MVSP) was used and genetic similarities were computed using the Dice coefficient of similarity of (Nei and Li, 1979). Cluster analysis was carried out on similarity estimates using the pair-group method with arithmetic average (UPGMA) software.

Isozyme profiles of fungal isolates

Polyacrylamide gel electrophoresis

The electrophoresis was carried out in vertical polyacrylamide gels, using the slab gel apparatus " SE 600, vertical slab gel", according to Laemmli (1970) with 7.5 % acrylamide.

Preparation of samples

Enzymes were extracted by crushing 1.0 g of fungal hyphae in 1.0 ml extraction buffer (0.1 M Tris-HCl + 2 mM EDTA, pH 7.8). To avoid denaturation of the enzymes by overheating, the samples were cooled with ice during crushing and preparation. Then, the samples were centrifuged for 25 min at 10.000 rpm at 4°C. 100 µl of the supernatant was mixed with 100 µl of sample application buffer which was composed of 2.5 mM Tris-HCl (pH 6.8), 10.0 % glycerol, 4.0% SDS, 0.02% bromophenol blue and 10% mercaptoethanol.

Table 2. Nucleotide sequences of primers used for PCR-RAPD and PCR-ISSR analysis.

Primer	Nucleotide sequence
PCR-RAPD primer	
OPA06	(5'-GGTCCCTGAC-3')
OPA09	(5'-GGGTAACGCC-3')
OPW15	(5'-ACACCGGAAC-3')
OPA13	(5'-CAGCACCCAC-3')
PCR-ISSR primer	
844A	(5'-CTCTCTCTCCTCTGC-3')
HB15	(5'-GTGGTGGTGGC-3')

Samples were then loaded directly in the electrophoresis apparatus for isozymes analysis.

Staining of gel and detection of enzymes

The gels were stained for seven enzyme systems. The staining protocols were according to Guidkema and Sherman (1980) for peroxidase (E.C.1.11.1.7); for esterase (E.C.3.1.1.2), glutamate-oxaloacetate-transaminase (E.C.2.6.1.1), malate dehydrogenase (E.C1.1.1.37) and acid phosphatase (E.C.3.1.3.2), staining protocols were according to Tanksley and Orton (1986), staining protocol for urease (E.C.3.5.1.5) was according to Fishbein (1969) and protease (PROT E.C.3.4.2.3) staining gel was according to Sarath et al. (1999).

Data collection and analysis

Stained gels were placed in a light box to determine their isozyme banding patterns (BP). The number of bands was recorded and their relative mobility (Rf) was obtained using the formula:

$$\mathrm{RF} = \frac{\text{Distance travelled by the band}}{\text{distance traveled by the tracking dye}}$$

Multivariate analysis of the isozyme profiles was done and clustering was based on the results of unweighted pair group method using averages (UPGMA) cluster analysis performed on the (Nei and Li, 1979) similarity coefficient matrices. Dendrogram presenting the genetic relationships of the different isolates were constructed using the numerical taxonomy and multivariate analysis system (NTSYS).

RESULTS

Genotypic relationship among dermatophytes and *Candida* species

RAPD and ISSR analysis

A total of 58 DNA bands ranging from 119 (HB15) to 863 bp (OPW15) were generated by the six primers. The number of bands per one primer ranged from 12 bands in the case of OPA06 RAPD primer to eight bands with 844A ISSR primer. *C. krusei* displayed the highest number of total DNA fragments (43 bands/six primers), whereas *T. rubrum* yielded the lowest (35 bands) as shown in Figure 1 and Table 3. These variations are mainly due to variations in primer structure and number of annealing sites in the genomic DNA of fungal strains (Kernodle et al., 1993). Polymorphic bands were detected with all tested primers in all isolates of dermatophytes and *Candida*. Out of 58 DNA-bands, 26 were conserved among all tested isolates while 32 (55.2 %) were polymorphic (Table 3).

The monomorphic bands are constant bands and cannot be used to study the diversity, while polymorphic bands revealed differences and could be used to establish a systematic relationship among the fungal genotypes (Hadrys et al., 1992). The presence of a unique band for a given isolates is taken as a positive marker while the absence of a unique band is referred as a negative marker. Such bands could be used as DNA markers for isolate identification and discrimination. The 844A ISSR primer showed one specific band for *T. mentagrophytes* at molecular weight of 377 bp and three unique bands for *M. canis,* two of which are expressed as positive bands (261 and 221 bp) and the 3rd was negative band (287 bp). One DNA fragment at molecular weight of 212 bp generated by OPA09 primer could be used as positive marker for *C. krusei*.

The OPA13 RAPD primer produced specific bands for *M. canis* and *E. floccosum* with molecular weights of 485 and 552 bp, respectively. Cluster dendrogram (Figure 2) based on similarity matrix obtained with unweight pair group method using arithmetic means (UPGMA) showed that the genetic similarity among the 18 isolates of dermatophytes and *Candida* was high, ranging from 78.7 to 93.3%. The dendrogram also showed that *M. canis* isolates were separated in a single branch with 78.7% genetic similarity, reflecting a relatively longer genetic distance from other isolates. *C. krusei and C. tropicalis* are closely related (93.3% genetic similarity). *Candida albicans* showed 90.9% similarity with both *C. krusei* and *C. tropicalis. E. floccosum* was easily separated from all *Trichophyton* species showing 87.3% similarity.

RFLP analysis

Two consecutive PCR steps were done. Firstly, the internal transcribed spacer (ITS) region of rDNA gene was amplified using the universal primers ITS1 and ITS4. Amplicons produced by *T. mentagrophytes, Trichophyton verrucosum* and *T. rubrum* were about 680 bp in length. Those from *Trichophyton violaceum, M. canis* and *E. floccosum,* were larger, 690, 720 and 780 bp, respectively. The ITS region amplified from *C. krusei, C. tropicalis* and *C. albicans,* were 510, 524 and 550 bp, respectively (Figure 3a and Table 4). Secondly, PCR products were subjected to RFLP analysis by digestion with the restriction enzymes *Hinf*I and *Hae*III in order to generate species-specific patterns for fungal identification. Digestion with *Hinf*I endonuclease produced different fragment patterns varying in number and size. *T.*

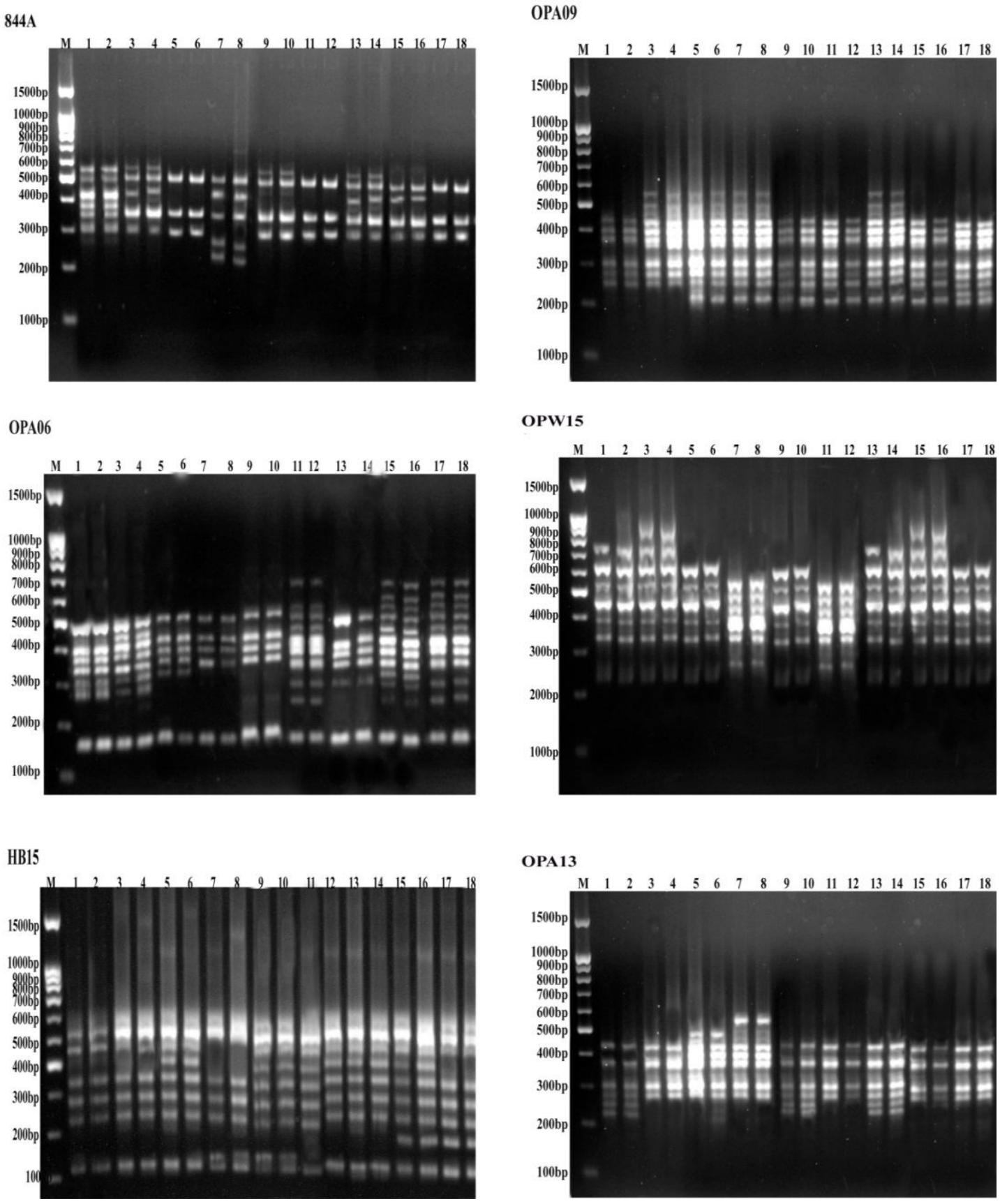

Figure 1. Agarose gel electrophoresis of fungal DNA fragments amplified with the RAPD and ISSR primers.

Table 3. Number of DNA fragments amplified by the 6 primers of RAPD and ISSR analysis of fungal isolates.

Primer	Number of amplified bands																		Total amplified bands	Number of polymorphic bands	% of polymorphic bands
	T. mentagrophytes (L)	*T. mentagrophytes* (E)	*T. verrucosum* (L)	*T. verrucosum* (E)	*E. floccosum* (L)	*E. floccosum* (E)	*M. canis* (L)	*M. canis* (E)	*T. rubrum* (L)	*T. rubrum* (E)	*C. albicans* (L)	*C. albicans* (E)	*T. violaceum* (L)	*T. violaceum* (E)	*C. krusei* (L)	*C. krusei* (E)	*C. tropicalis* (L)	*C. tropicalis* (E)			
844A	6	6	5	5	3	3	5	5	4	4	3	3	5	5	4	4	3	3	8	6	75%
OPA09	6	6	9	9	10	10	10	10	7	7	7	7	10	10	8	8	7	7	11	5	45.5%
OPA06	7	7	8	8	6	6	5	5	5	5	10	10	6	6	12	12	11	11	12	7	58.3%
OPW15	6	6	7	7	5	5	6	6	5	5	6	6	6	6	7	7	5	5	9	5	55.6%
OPA13	6	6	4	4	6	6	6	6	6	6	4	4	6	6	4	4	4	4	9	5	55.6%
HB15	6	6	6	6	7	7	6	6	8	8	7	7	7	7	8	8	7	7	9	4	44.4%
Total bands	37	37	39	39	37	37	38	38	35	35	37	37	40	40	43	43	37	37	58	32	55.2%

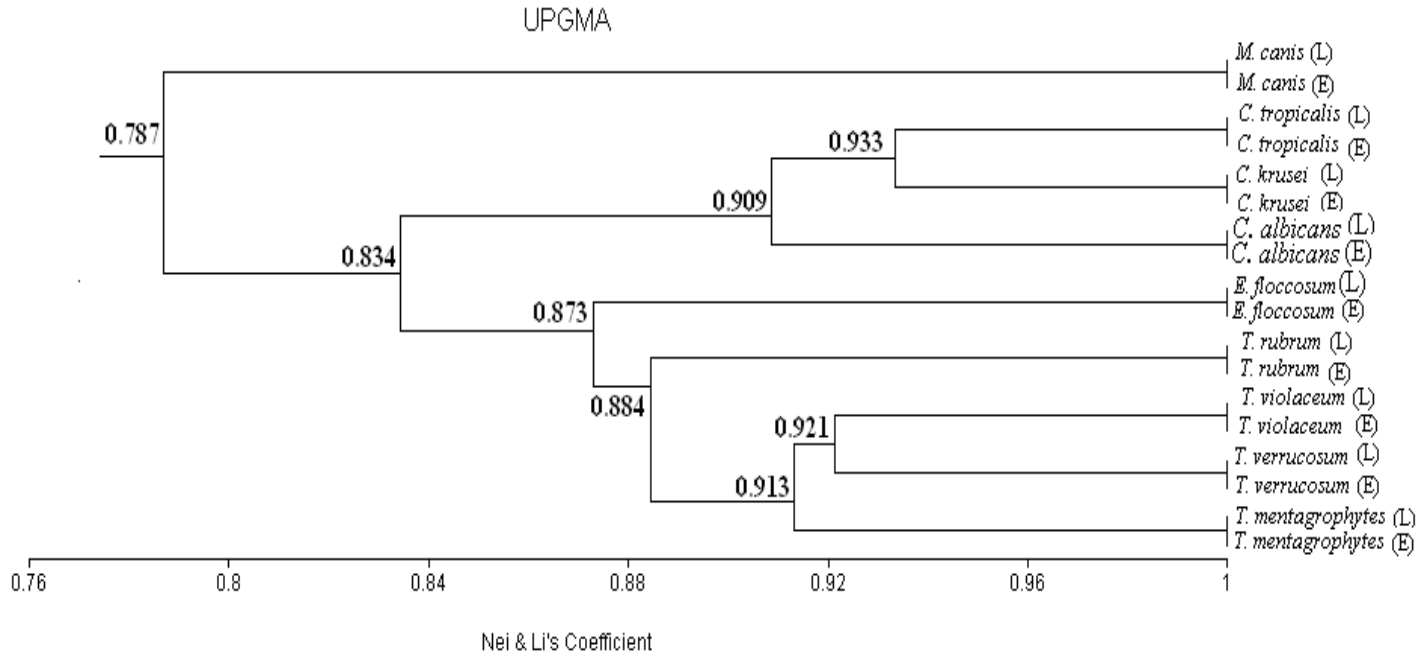

Figure 2. Dendrogram developed from PCR- RAPD and PCR-ISSR primers using UPGMA analysis. The scale is based on Nei and Li coefficients of similarity.

mentagrophytes, T. verrucosum and *E. floccosum* created five fragments, *T. rubrum, T. violaceum* and *C. tropicalis* showed four fragments and three fragments for *C. krusei* and *C. albicans.* Restriction fragments showed marked variations in size ranging from 8 to 378 bp. It was interestingly to note that in case of *M. canis*, *Hin*fI was not able to produce any obvious cutting pattern (Table 4 and Figure 3b). The endonuclease *Hae*III was able to digest the ITS region of rDNA gene producing 2 to 3 fragments of 40 to 450 bp depending on the fungal isolate. Great similarity was observed among fragments of *C. albicans* and *C. tropicalis* (420 and 90 bp for each). *C. krusei* yielded three fragments of different sizes (370, 90 and 40 bp). Similarly, dermatophytes produced three fragments one of which (100 bp) was shared by all isolates of *Trichophyton* and *Microsporum* but not by *Epidermophyton*. However, these dermatophytes showed marked variations in the size of other fragments as seen in Figure 3c

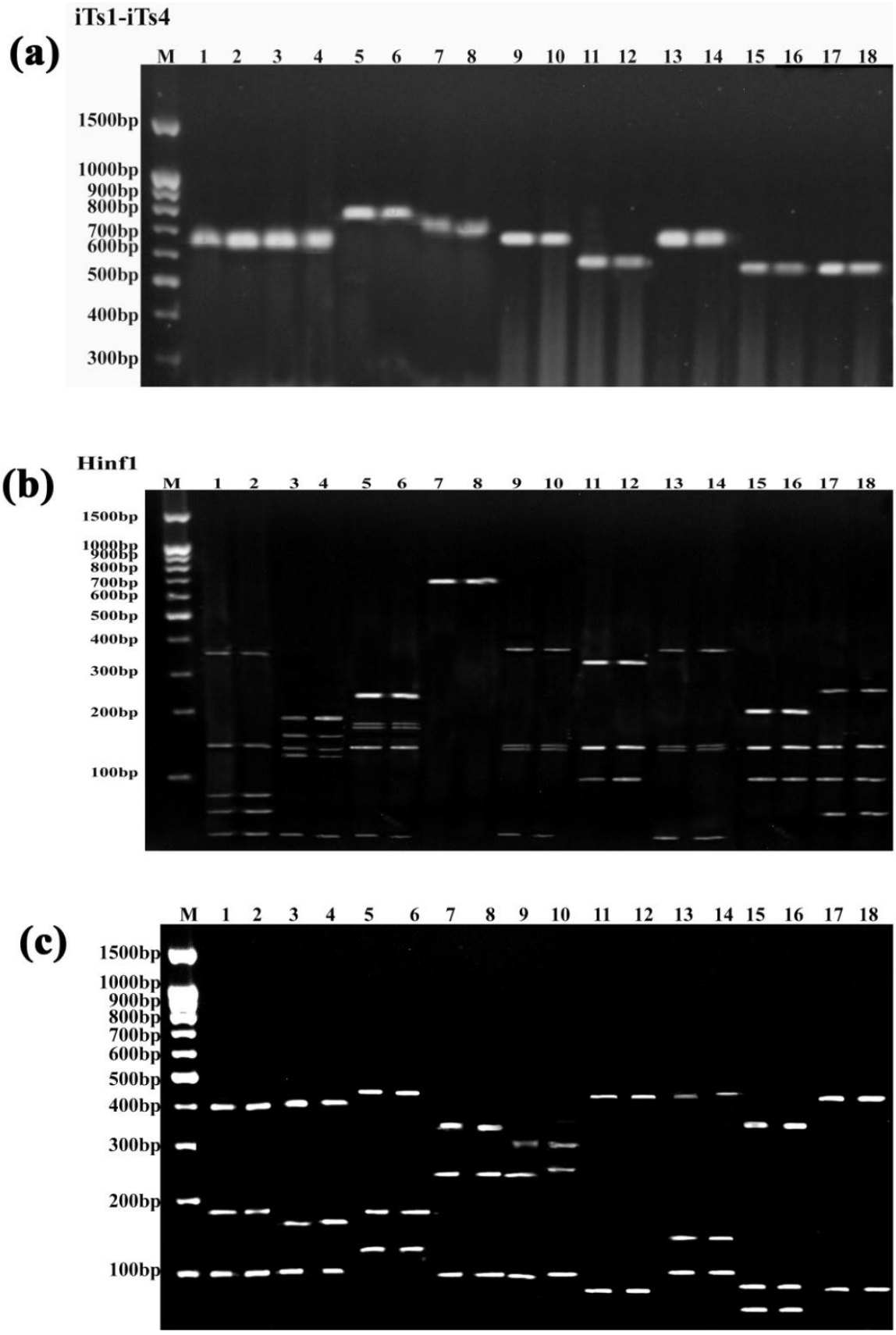

Figure 3. Agarose gel electrophoresis of fungal isolates. **a,** ITS-PCR product; **b,** ITS-PCR after digestion with *Hinf*1 endonuclease; **c,** ITS-PCR after digestion with *Haelll* endonuclease.

Table 4. RFLP analysis of ITS region of rDNA after digestion by *Hinf*I and *Hae*III endonucleases.

Fungal specie	Size of ITS products (bp)	Size of products of *Hinf*I (bp)	Size of products of *Hae*III (bp)
T. mentagrophytes	680	375, 158, 81, 65, 8	400,180,100
T. verrucosum	682	200, 163, 154, 151, 8	405, 175, 100
T. rubrum	680	378, 154, 152, 8	320, 260, 100
T. violaceum	690	378, 158, 154, 8	428, 162, 100
M. canis	720	-	370, 250, 100
E. floccosum	780	255, 182, 180, 155, 8	450, 190, 140
C. albicans	550	331,150,100	420,90
C. krusei	510	230,150, 135	370, 90, 40
C. tropicalis	524	351,150,100,50	420,90

and Table 4.

Isozyme profiles of dermatophytes and *Candida* isolates

Esterase (EST, E.C.3.1.1.2)

Three loci of isozyme activity (EST1, EST2 and EST3) were observed for esterase (Figure 4a). EST1 locus was polyomorphic and produced one band shown in *T. mentagrophytes, T. verrucosum, T. rubrum* and *T. violaceum* isolates, but was not detected in other fungi. All tested fungi were heterozygous for EST2 and EST3 loci. The tested fungi displayed three bands of EST3 at Rf values of 0.69, 0.73 and 0.76. Although at EST2 loci (Rf = 0.38, 0.43 and 0.48), a number of isolates including *E. floccosum, C. albicans, C. tropicalis* and *C. krusei,* showed only two out of the three bands (Table 5 and Figure 4a). The missing of such bands in the heterozygous state may be due to the least expression of such isozyme band or lower activity.

Peroxidase (PRX, E.C.1.11.1.7)

Six peroxidase isozyme loci (Prx-1 to PRX-6) were detected in the tested fungi. PRX-3, 4, 5 and 6 isozyme bands were monomorphic, and all fungi displayed these bands. PRX-1 and PRX-2 were polymorphic in which PRX-1 was detected in all fungi, except *C. albicans*, *C. krusei* and *C. tropicalis*. Prx-2 showed lower polymorphism (2/18) than PRX-2 (16/18) which was detected in all tested isolates, except *E. floccosum* (Table 5 and Figure 4b).

Protease (PROT, E.C.3.4.2.3)

Fungal protease was shown to be controlled by two loci PROT1 and PROT2 (Table 5 and Figure 4c). PROT1 was monomorphic and showed one band in all isolates at Rf value of 0.62. On the other hand, PROT2 locus showed heterozygosity in all tested fungi, except *C. albicans, C. tropicalis* and *C. krusei* which were homozygous for PROT2a allele which produced one band at this locus.

Urease (URA, E.C.3.5.1.5)

Urease electrophoretic pattern showed five different enzymatic bands: URA1-URA6 in the tested isolates (Figure 4d and Table 5). URA1 and URA4 were monomorphic bands which were expressed in all isolates while the other bands were polymormorphic. URA2 and URA3 were detected in all isolates except *T. rubrum*, *C. albicans, C. tropicalis* and *C. krusei*. The URA5 enzymatic band was only expressed in *T. mentagrophytes* (Libya), *T. verrucosum* (Libya) and *T. violaceum* (Libya). It is worthy to mention that the similarity in urease isozyme profile among *T. rubrum* and all *Candida* isolates is in harmony with the biochemical test for urease production by these fungal species which appeared to be urease negative as shown in Table 5.

Glutamate – oxaloacetate - transaminase (GOT, E.C.2.6.1.1)

Two bands (GOT1, GOT2) were detected in the zymogram of glutamate oxaloacetate transaminase. GOT1 was monomorphic which appeared in all tested isolates. Meanwhile, GOT2 was detected in all isolates, except *C. albicans, C. tropicalis* and *C. krusei* (Table 5 and Figure 4e).

Malate dehydrogenase (MDH, E.C. 1.1.1.37)

The electrophoretic analysis of MDH reveled two enzymatic bands; one of them (MDH1) was monomorphic which appeared in all tested isolates. The other band (MDH2) was polymorphic and was detected in all isolates, except *C. albicans, C. tropicalis* and *C. krusei* (Figure 5a and Table 5).

Acid phosphatase (ACP, E.C. 3.1.3.2)

No differences were observed among the tested isolates of dermatophytes and *Candida* in the isozyme pattern of ACP in which only one band was detected for such enzyme (Figure 5b and Table 5).

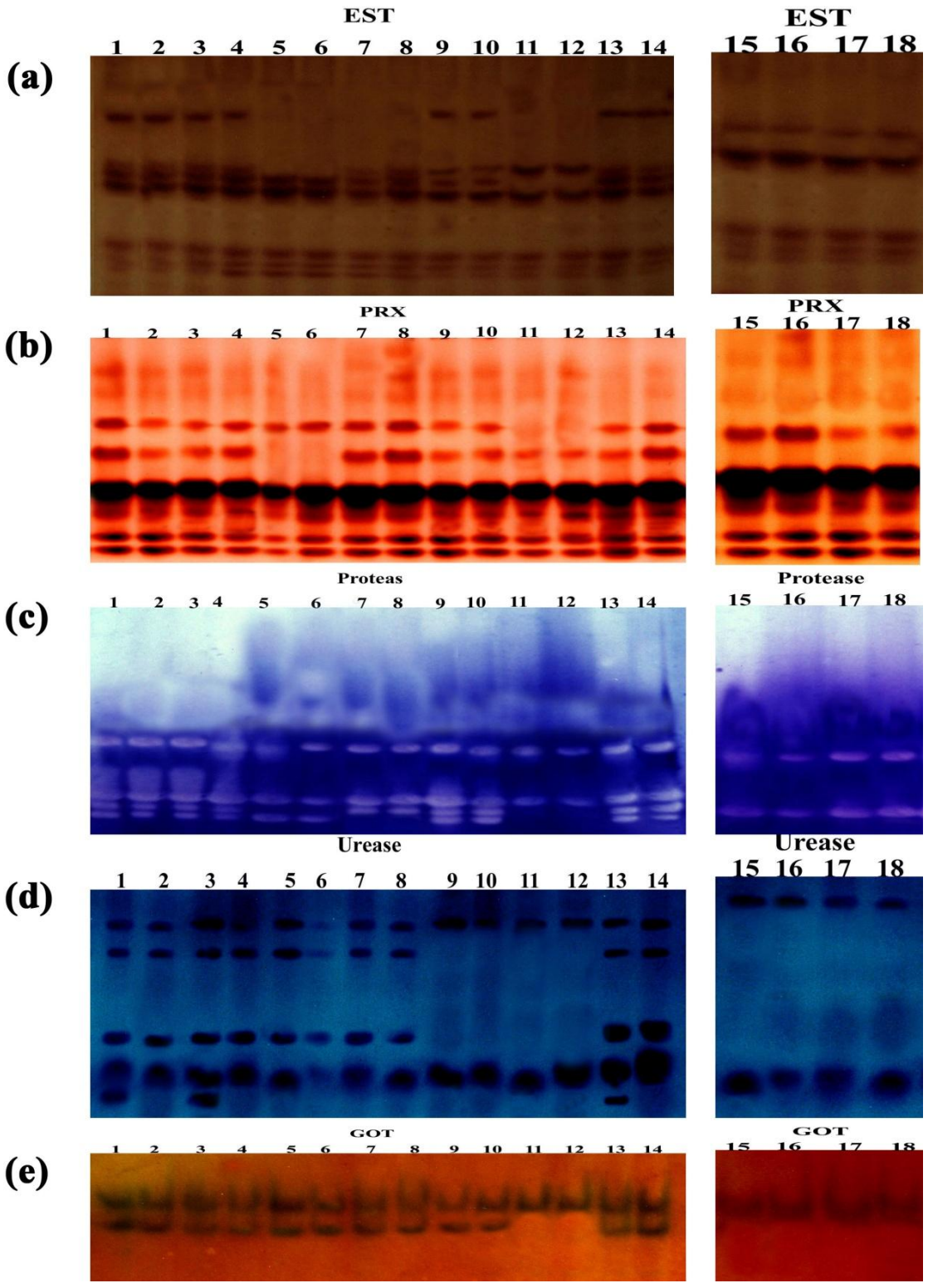

Figure 4. Electrophoretic isozyme patterns detected in all tested fungal isolates. a, Esterase (EST); b, peroxidase (PRX); c, protease (PROT); d, urease (URA); e, glutamate oxaloacetate transaminase (GOT).

Cluster analyses of isozyme profiles

Cluster analysis based on the combined data of all tested isozymes was more valuable for determining relationships among the isolates than if each isozyme was to be analyzed separately. Genetic distances were calculated

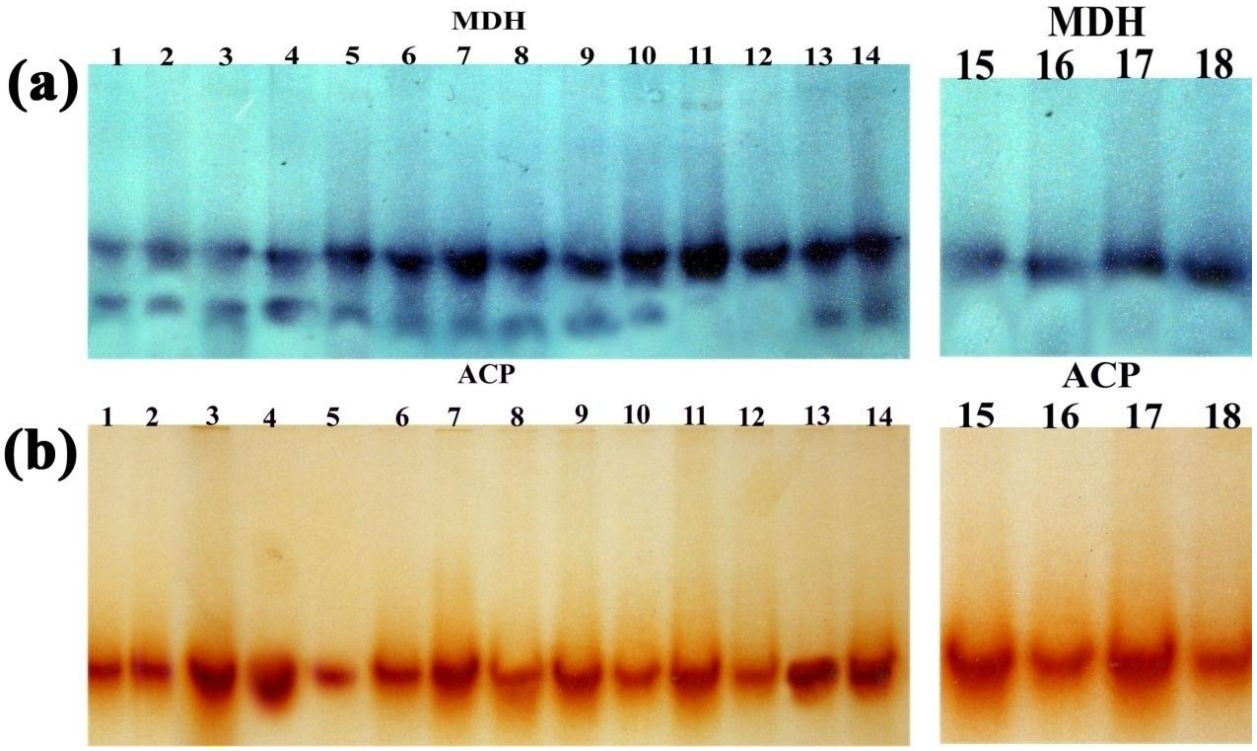

Figure 5. Electrophoretic isozyme patterns detected in all tested fungal isolates. a, Malate dehydrogenase (MDH); b, Acid phosphatase (ACP).

on the basis of similarity coefficient matrix among all different isolates involved in the cluster analysis. The most noticeable results are that the dendrogram separated the tested fungi according to their taxonomic order rather than geographical origin, that is, all species of the same genera were clustered together. All isolates fell into two major clusters, labeled as A and B (Figure 6). Cluster-A was further subdivided into four sub-clusters comprising 12 isolates of dermatophytes and Cluster-B contained the six isolates of *Candida*. Cluster-B was distinctly away from the Cluster-A with a similarity of GS = 0.791 (Figure 6). These results suggest that the genetic differences between the genera and species of dermatophytes and *Candida* were higher than that between the isolates of the same species. Close relationship between the isolates of the same specie supports the theory of a common lineage. Also RAPD approach showed considerable potential for identifying and discriminating dermatophytes and *Candida* spp.

DISCUSSION

Identification of dermatophytes at the species level is essential because of the therapeutic and epidemiological importance. Conventional methods on the basis of phenotype variations and molecular methods on the basis of molecular differences were used for identification of dermatophytes species. Due to some limitations in traditional methods such as the high degree of phenoltypic similarity between these relative species identification and time-consumption, many isolates reveal unusual characteristics (Weiyzman et al., 1995; Graser et al., 2008; Shehata et al., 2008; De Baere et al., 2010). To overcome these problems, in recent years, molecular marker approaches relying on genetic makeup are regarded as useful in the exact and rapid recognition of dermatophytes (Mochizuki et al., 2003; Kanbe et al., 2003; Liu et al., 2000; Graser et al., 1998).

During the present work, three molecular marker systems based on PCR were applied to study the genetic relationships among 18 selected isolates of dermatophytes (12 isolates) and yeasts (6 isolates). Four PCR-RAPD primers and two PCR-ISSR primers were used to amplify randomly the DNA fragments of target fungi. After gel electrophoresis, the total number of bands per fungal isolate ranged from 35 to 43 bands depending on the primer structure and number of annealing sites in the fungal genome. Polymorphic bands were detected with the different primers used to amplify the DNA of the fungal isolates.

These bands accounted for 55.2% of total bands and were analyzed to establish a relationship between the genotypes of fungal isolates. Establishment of a dendrogram revealed that *M. canis* was separated in a single branch (0.787 GS) reflecting a relatively longer genetic distance from other isolates of dermatophytes and yeasts. It was also observed that both *C. krusei* and *C. tropicalis* are closely related showing 0.933 genetic simila-

Table 5. The electrophoretic banding patterns of the EST, PER, URA, PROT, MDH, GOT and ACP isozymes in fungal isolates.

		Esterase banding patterns																	
Number	**R.F**	***T. mentagrophytes* (L)**	***T. mentagrophytes* (E)**	***T. verrucosum* (L)**	***T. verrucosum* (E)**	***E. floccosum* (L)**	***E. floccosum* (E)**	***M. canis* (L)**	***M. canis* (E)**	***T. rubrum* (L)**	***T. rubrum* (E)**	***C. albicans* (L)**	***C. albicans* (E)**	***T. violaceum* (L)**	***T. violaceum* (E)**	***C. krusei* (L)**	***C. krusei* (E)**	***C. tropicalis* (L)**	***C. tropicalis* (E)**
EST1	0.21	+	+	+	+	--	--	--	--	+	+	--	--	+	+	--	--	--	--
EST2a	0.38	+	+	+	+	--	--	+	+	+	+	+	+	+	+	+	+	+	+
EST2b	0.43	+	+	+	+	+	+	+	+	+	+	--	--	+	+	--	--	--	--
EST2c	0.48	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
EST3a	0.69	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
EST3b	0.73	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
EST3c	0.76	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		Peroxidase banding patterns																	
PRX1	0.47	+	+	+	+	+	+	+	+	+	+	--	--	+	+	--	--	--	--
PRX2	0.57	+	+	+	+	--	--	+	+	+	+	+	+	+	+	+	+	+	+
PRX3	0.71	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PRX4	0.78	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PRX5	0.89	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PRX6	0.94	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		Protease banding patterns																	
PROT1	0.62	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PROT2a	0.79	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PROT2b	0.82	+	+	+	+	--	--	+	+	+	+	--	--	+	+	--	--	--	--
PROT2c	0.84	+	+	+	+	+	+	--	--	+	+	--	--	+	+	--	--	--	--
		Urease banding patterns																	
URA1	0.27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
URA2	0.34	+	+	+	+	+	+	+	+	--	--	--	--	+	+	--	--	--	--
URA3	0.57	+	+	+	+	+	+	+	+	--	--	--	--	+	+	--	--	--	--
URA4	0.70	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
URA5	0.76	+	--	+	--	--	--	--	--	--	--	--	--	+	--	--	--	--	--
		Glutamate oxaloacetate transaminase banding patterns																	
GOT1	0.44	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GOT2	0.57	+	+	+	+	+	+	+	+	+	+	--	--	+	+	--	--	--	--
		Malate dehydrogenase banding patterns																	
MDH1	0.48	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MDH2	0.54	+	+	+	+	+	+	+	+	+	+	--	--	+	+	--	--	--	--
		Acid phosphatase- banding patterns																	
ACP1	0.58	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

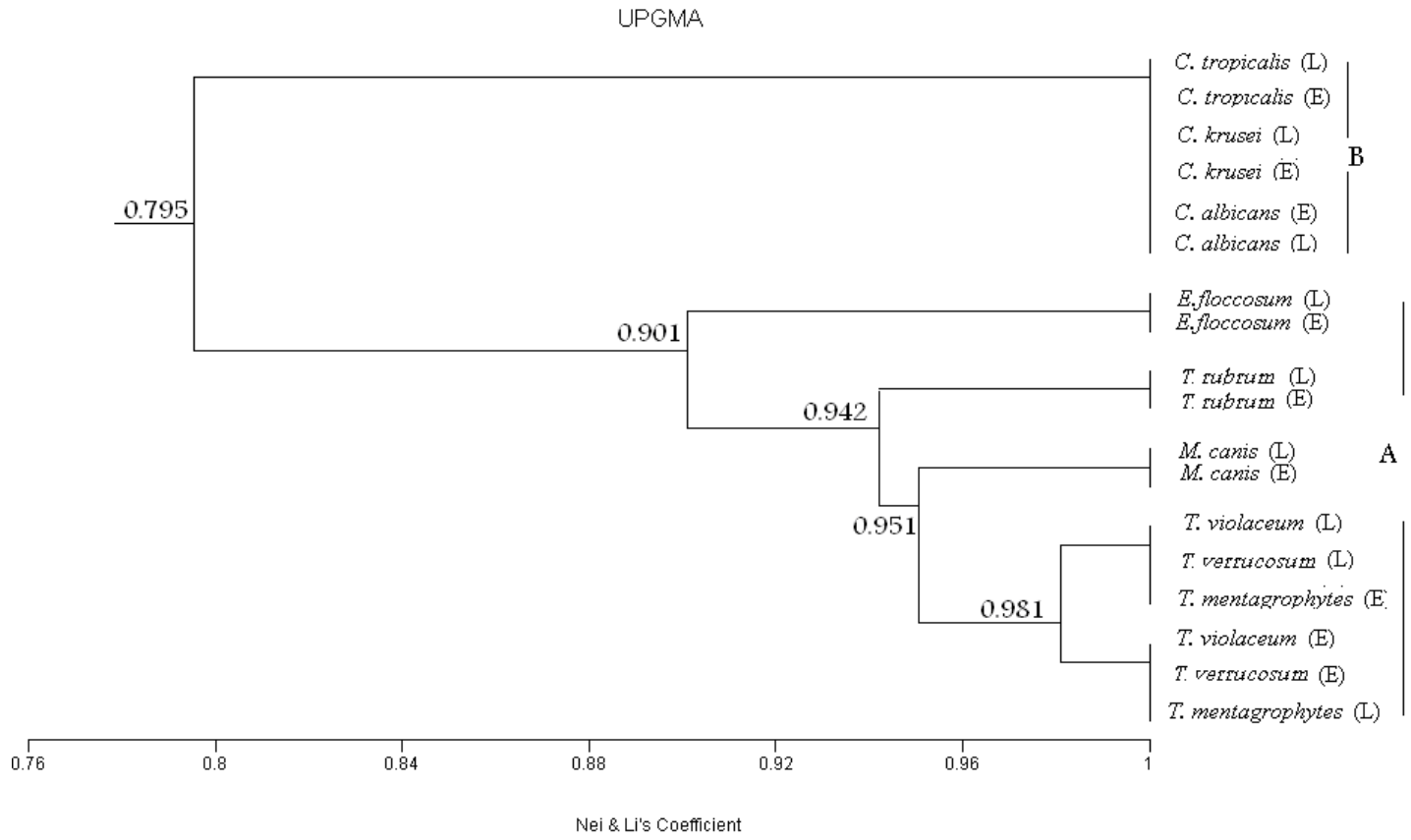

Figure 6. Dendrogram based on the UPGMA analysis of genetic similarity derived from the simple matching coefficient of Nei and Li, based on isozyme data, showing the relationships among 12 isolates of dermatophytes and 6 *Candida*.

rities *C. albicans* showed 0.909 similarities with other species of *Candida. E. floccosum* was easily separated from all *Trichophyton* species showing 0.873 similarities. It is to be noted that the monomorphic bands which accounted for 44.8% of total bands are constant bands and cannot be used to study the diversity while, polymorphic bands revealed differences and could be used to examine and establish systematic relationships among the genotypes (Hadrys et al., 1992). The presence of a unique band for a given genotype is taken as a positive marker while, the absence of a unique band is referred to as negative marker. Such bands could be used as DNA markers for isolate identification and discrimination. It is also important to observe that the results of RAPD-PCR came in complete harmony with the conventional methods employed for identification and characterization of these fungal species.

RAPD and ISSR-PCR methods have frequently been used for phylogenetic analysis and identification of dermatophytes (Kim et al., 1999, 2001; Cano et al., 2005; Leibner-Ciszak et al., 2010). Spesso et al. (2013) demonstrated that the detection of intra-species polymorphisms in isolates of *M. canis* by RAPD-PCR may be applied in future molecular epidemiological studies in order to identify endemic strains, the route of infection in an outbreak and the coexistence of different strains in a single infection. In contrast to that reported and using the same method, Leibner-Ciszak et al. (2010) were not able to detect genetic variations in 13 clinical isolates of *M. canis*. These discrepancies could have been due to different conditions in the PCR reaction or that the strains of *M. canis* used by these authors had no clonal diversity.

RAPD-PCR as well as phenotype was also used by Resende et al. (2004) to identify 242 yeasts isolated from hospitalized patients. There was agreement and consistency between phenotypic and genotypic analysis using RAPD, demonstrating that it is possible to apply this method for the identification of *Candida* species. While, Rocha et al. (2008) concluded that the RAPD proposed might be used to confirm *Candida* species identified by microbiological methods. On the other hand, the ITS regions of the fungal ribosomal DNA (rDNA) were frequently used for species identification because it is a faster, accurate species determination, specific, and are less feasible to be affected by exterior effects such as temperature changes and chemotherapy (Girgis et al., 2006; Kong et al., 2008). Ellis et al. (2007), Aala et al. (2012) and Samuel et al. (2013) used both conventional

and molecular methods to identified dermatophytes species. They revealed that the conventional methods are generally prolonged and may be indecisive. However, molecular studies based on ITS sequencing provide a very accurate result.

In our study, PCR-RFLP two universal primers (ITS1 and ITS4) were used to amplify the ITS region of the rDNA gene in all fungal isolates studied followed by digestion of the PCR product with restriction enzymes (*Hinf*I and *Hae*III endonucleases). Digestion with *Hinf*I created 5 fragments for *T. mentagrophytes*, *T. verrucosum* and *E. floccosum*, 4 fragments for *T. rubrum, T. violaceum* and *C. tropicalis*, 3 fragments for *C. krusei* and *C. albicans*. Application of *Hae*III endonuclease resulted in the cutting of 2 to 3 fragments of varying size (90 to 450 bp) depending on the fungal isolate. The cutting pattern was similar for *C. albicans* and *C. tropicalis* (420 and 90 bp) showing differences from *C. krusei* (370, 90 and 40 bp). It is worthy to mention that the ITS region in *M. canis* was not digested by *Hinf*I but the same region produced three fragments when *Hae*III endonuclease was used.

Therefore, PCR-RFLP technique can be considered as a powerful technique for discrimination and identification of fungal species especially when the proper restriction endonucleases are selected. These results also suggest that dermatophytes and *Candida* species isolated from different geographical regions (Assiut and Tripoli) are closely related, supporting the theory of a common lineage. Also, RAPD and ISSR approaches showed considerable potential for identifying and discriminating dermatophytes and *Candida* spp.

The identification results are in agreement with established and recent taxonomical insights into the dermatophytes; for example, highly related species also had closely related and sometimes difficult-to-discriminate ITS2-RFLP patterns. Analysis of ITS region based on RFLP-PCR was used to identify and discriminate between 57 *T. rubrum* clinical isolates (Hryncewicz-Gwóźdź et al., 2011) and between different species or varieties of *Trichophyton*, *Microsporum* and *Epidermophyton* (Gräser et al., 1999, 2000a and 2000b; Mirzahoseini et al., 2009). They reported that PCR-RFLP serves as a rapid and reliable method for the identification of *T. rubrum* isolates and other species of dermatophytes, while the RAPD analysis is rather a disadvantageous tool for *T. rubrum* strain typing while, De Baere et al. (2010) reported that ITS2-RFLP analysis proved to be most useful for identification of species of the genera *Arthroderma*, *Chrysosporium* and *Epidermophyton*, but could not distinguish between several *Trichophyton* species. Recently, Rezaei-Matehkolaei et al. (2012) used PCR-RFLP assay to find the exact differentiating restriction profiles for each dermatophyte species. They reported that the ITS-PCR cut by restriction enzymes *MvaI*-RFLP is a useful and reliable schema for identification and differentiation of several pathogenic species and can be used for rapid screening of even closely related species of dermatophytes in clinical and epidemiological settings.

Identification of *Candida* species and isolates using PCR-RFLP for amplification of the ITS region has been applied by Pento et al. (2004), Mirhendi et al. (2005 and 2006) and Saltanatpouri et al. (2010). Isozymes analysis is more reliable than traditional methods since the expression of isozymes loci are co-dominant, in addition this technique provides a fairly rapid and inexpensive alternative tool for taxonomic studies and for identifying fungi species (Ryan and Scowcroft, 1987; Bonde et al., 1993; Bragaloni et al., 1997). Klaas (1998) reported that the isozyme markers can correctly identify several levels of taxa, accessions, and individuals, since the assumption of homology can be more accurate than for some genomic DNA markers.

In this study, analysis of isozyme profiles was based on the data from all seven isozymes pattern. The combined data of the present work give more complete information than if each isozyme was to be analyzed separately. All isolates fell into two major clusters. First cluster (A) was further subdivided into four sub-clusters comprising 12 isolates of dermatophytes and second cluster (B) of 6 isolates of *Candida*. Cluster-B was distinctly away from the Cluster-A with a similarity of GS = 0.791. However, two disadvantages were observed. One is inability to differentiate the three different species of *Candida* from each other. The second is the confusion resulting from the grouping of three different *Trichophyton* species in the same sub cluster. These are *T. mentagrophytes, T. violaceum* and *T. verrucosum* which can be easily identified with the conventional culturing and microscopic examination. It can be suggested to try more different isozymes to design a set of isozymes suitable for successful clustering and identification of the different medically important yeasts and dermatophytes. It is also very important to compare the results of isozyme profiles with those of conventional and molecular based identification methods in order to achieve correct identification and characterization of the pathogenic fungi.

In *Trichoderma*, the first characterization was done by Zamir and Chet (1985), who reported that the 23 geographically diverse isolates of *T. harzianum* were grouped into 5 types according to their isozyme profiles. The results indicate that enzyme electrophoresis was useful for distinguishing *Trichoderma* at the intraspecies level. Rosa et al. (2000) used multilocus enzyme electrophoresis (MLEE) and numerical taxonomic methods to establish the degrees of relatedness among five *Candida* species commonly isolated from humans oral cavities. Of 20 enzymic systems assayed, five showed no enzymic activity (aspartate dehydrogenase, mannitol dehydrogenase, sorbitol dehydrogenase, glucosyl transferase and *a*-amylase). The obtained data revealed that some of these enzymes are capable of distinguishing strains of different species, but most of them could not organize all strains in their respective species-specific clusters. Numerical classification based on MLEE polymorphism must be regarded for surveys involving just one *Candida*

species.

Isozyme and protein electrophoresis data from mycelia extracts of 27 isolates of *T. harzianum*, 10 isolates of *T. aureoviride*, and 10 isolates of *T. longibrachiatum* from Southern Peninsular Malaysia were investigated by Siddiquee et al. (2010). The eight enzyme and a single protein pattern systems were analyzed. Three isozyme and total protein patterns were shown to be useful for the detection of three *Trichoderma* species. The isozyme and protein data were analyzed using the Nei and Li Dice similarity coefficient for pairwise comparison between individual isolates, species isolate group, and for generating a distance matrix. The UPGMA cluster analysis showed a higher degree of relationship between *T. harzianum* and *T. aureoviride* than to *T. longibrachiatum*. They suggested that the *T. harzianum* isolates had high levels of genetic variation compared with the other isolates of *Trichoderma* species.

Generally, the utilization of genetic markers such as isozymes, RAPD, ISSR and PCR-RFLP together with morphological analysis to study dermatophytes and *Candida* spp. help alleviate confusion in the identification of isolates at the species level. It is to be emphasized that data obtained from molecular analysis (RAPD ISSR and RFLP) came in harmony with identifications based on conventional morphological examination of all fungal species studied

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