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Genomic Linkage Analysis of Iranian Clinical Isolates of Dermatophytes Fungi Using the RAPD-PCR

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ABSTRACT

Dermatophytes are a group of keratinophilic fungi capable of invading keratinized tissues (skin, hair and nails). They cause dermatophytosis (commonly known as tinea or Ring worm) in human and animals. In this report, DNA similarities and genomic linkage of 40 dermatophytes strains was obtained from different universities, were studied by random amplified polymorphic DNA (RAPD–PCR) using 11 random primers. The similarity of *Microsporum* genus with two other genera was 13%, and the similarity of *Trichophyton* with *Epidermophyton* was 20.8%. These results provide the basis for the rapid identification of dermatophytes at the genetic level, in additions to the existing laboratory methods. *Iran. Biomed. J. 4 (4): 123-128, 2000*

Keywords: Genomic linkage, Dermatophytes, RAPD-PCR

INTRODUCTION

Tinea is a common disease in human and animals, invading skin, hair and nails and caused by keratinophilic fungi (dermatophytes). Dermatophytes consist of *Epidermophyton*, *Microsporum* and *Trichophyton*. Both *Microsporum* and *Trichophyton* have multiple species/subspecies, and many of them are pathogenic for human. [1]

However Epidermophyton, has only one recognized pathogenic species (*E. floccosum*). Clinically, it is difficult to differentiate dermatophytosis caused by various dermatophyte species. The availability of rapid and accurate laboratory techniques for identifying the dermatophytes is important for early treatment and control strategies. The current laboratory diagnosis of dermatophytosis is based on microscopic examination and *in vitro* culture. The microscopic identification of hyphae directly from the lesion samples is rapid, but non-specific and is relatively insensitive. Up to 15% of false-negative results can be attributed to this technique [2]. *In vitro* culture is capable of giving a specific diagnosis on the basis of morphological and biochemical criteria in 10-15 days in over 95% of cases.

However, for some unusual and atypical isolates, identification can be very slow and may take weeks to produce a definite result [3]. Recent development of polymerase chain reaction (PCR) technology, in particular random amplified polymorphic DNA (RAPD), has greatly enhanced the molecular detection and identification of various organisms, including fungi [4-7]. Through random amplification and identification of distinct DNA band patterns in the RAPD-PCR, common dermatophyte such as *T. rubrum* and *T. mentagrophytes* isolates can be readily distinguished [8].

In the present study, we show the use of a random decamer UBC 400 (5'-GCC CTGATAT-3') in RAPD-PCR, for rapid determination of *T. rubrum* isolates on the basis of characteristic band patterns in gel electrophoresis.

MATERIALS AND METHODS

We have used 8 strains of dermatophytes supplied from Tehran University of Medical Sciences, 7 species from Isfahan University of Medical Sciences and 25 species routinely isolated from patients in our mycology laboratory in Shiraz University of Medical Sciences. The isolated

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samples were examined in a drop of KOH 10% and then cultured in Mycobiotic agar slopes and incubated at 26°C for 3 weeks. The dermatophytes were identified using the microscopic examination and culture techniques on the basis of morphological and biochemical criteria [4, 8, 9].

Totally 40 dermatophyte species including 13 *Microsporum* species (4 *M. canis*, 8 *M. gypseum* and 1 *M. cookei*), 24 *Trichophyton* species (11 *T. mentagrophytes of that* 2 *Var. interdigitale and* 9 *Var. mentagrophytes*, 4 *T. rubrum*, 3 *T. violaceum*, 3 *T. tonsurans*, 2 *T. verrucosum and* 1 *T. schoenlinii*) and 3 *Epidermophyton floccosum* were examined.

The methods for calculating similarity of a single primer was as follow:

$$S_1 = a/(a + b + c) \qquad (I)$$

Where a, number of similar bands to both patterns; b, number of bonds present in one and absent in the other; c, vice versa.

The average similarity between two species was then given by:

$$S = (S_1 + S_2 + ... + S_n)/n \qquad (II)$$

Where n, number of primers used for comparing the samples [10].

Extraction and purification of fungal DNA. Chitin is one of the most abundant compounds in the cell wall of the most fungi. Because chitinase was not available for us, DNA extraction was used. For this purpose, we used two simple methods together. TELT lysing buffer (Tris, EDTA, Licl, Triton X_{100}) and freeze-thaw under liquid nitrogen. [8, 11].

Fungal isolates grown on Mycobiotic agar slopes in primary cultures were collected using a sterile loop into a 1.5 ml tube with 500 µl of TELT and 500 µl sterile distilled water. The mycelia were disrupted using mechanical shearing by repeated passage through needle (No. 19). Then the passage is repeated three times, freeze-thawed under liquid nitrogen and incubated at 75°C. Equal volume of phenol/chloroform (25:25) was added and mixed well by inversion and then centrifuged at 4,000 rpm for 10 min. The concentration of DNA was determined by measuring the absorbance of the samples at 260 nm.

primers. Eleven random decamer primers had been provided from University of British Colombia (UBC) in Canada and Advanced Biotechnology company of England (AB-1). Sequences of the applied primers are as follows:

UBC 310	(5'-GAG CCA GAA G-3')
UBC 313	(5'-ACG GCA GTG G-3')
UBC 337	(5'-TCC CGA ACC G -3')
UBC 340	(5'-GAG AGG CAC C-3')
UBC 359	(5'-AGG CAG ACC T-3')
UBC 361	(5'-GCG AGG TGC T -3')
UBC 389	(5'-CGC CCG CAG T-3')
UBC 400	(5'-GCC CTG ATA T-3')
AB1-04	(5'-GGA GTG TAG T-3')
AB1-11	(5'-GTA GAC CCG T-3')
AB1-20	(5'-GGA CCC TTA C-3')

RAPD–PCR. The RAPD-PCR was performed in a total reaction volume of 25 µl. The reaction mixture comprised about 50 ng of genomic DNA. PCR buffer containing (50 mmol/L KCl, 10 mmol/L NaCl, 10 mmol/L Tris-HCl pH 9, DTT 0.5 mmol/L, Triton X_{100} 0.1%), $MgCl_2$ 2.5 mmol/L and 200 µmol/L of each dNTPs (dATP, dCTP, dGTP and dTTP), 1 u/100µl of taq DNA polymerase and 15 pmol of one of the random primers [11]. The reaction mixture was incubated in a progene PCR system (Techne, model, FPROG05d) using the following program:

Three cycles of 94°C for 60 s, 36°C for 45 s, 72°C for 90 s, and 32 cycles of 94°C for 30 s, 36°C for 45 s, and 72°C for 90 s [11].

A tube without template DNA was included as a negative control. Upon completion of PCR, 3 µl of loading buffer containing (bromophenol and glycerol) was added to each tube, and about 10 µl of the PCR products was electrophoresed (at 5 V/cm) in a 1.5% agarose gel. In the presence of ethidium bromide, and subsequently detected under UV light [3].

Phylogenic analysis. The banding patterns were compared to determine the genetic relatedness of species. Separate data matrix was constructed for each primer by scoring each isolate for the presence or absence of each band. The results obtained for 11 primers were pooled and these data were clustered using the unweighted pair-group method arithmetic average (UPGMA) [12].

RESULTS AND DISCUSSION

Figure 1 shows RAPD-PCR using some random primer that amplified characteristic band patterns in DNA from different dermatophyte species from three distinctive areas. The UBC-361 primer amplified polymorphic bands of 0.84 kb, 0.81 kb and 2.0 kb for recognition *T. mentagrophytes*, *T. rubrum* and *T. violaceum* respectively (Fig.1a). The UBC-389 primer is the best primer for the identification of three genera of dermatophytes making quite distinct band patterns for *Trichophyton*, *Microsporum* and *Epidermophyton* (Fig.1b). This primer is useful in distinction of different species. The comparison of different *Microsporum* and *Epidermophyton* species with this primer showed the similarity in genomic sequence species isolated from the patients in three different areas. The UBC-313 primer identified *T. schoenlinii* from other dermatophytes by making a single band 0.315 kb (Fig. 1c). The UBC-359 primer differentiated *T. violaceum* from other species by making a unisharp band 0.79 kb. This primer is suitable for calculation and genomic relationship between different dermatophytes (Fig.1d).

The UBC-310 primer can clearly differentiate common *T. ment* varieties (*Var. interdigital* and *Var. mentagrophytes*), but is not a suitable primer for the separation of different *Trichophyton* species (Fig.1e). The UBC-400 primer is a valuable primer for recognition of *T. rubrum* with distinctive band pattern (Fig.1f). According to the obtained results, it seemed that RAPD-PCR using aforementioned primers is a sensitive method for rapid differentiation of dermatophytes. Table 1 shows genomic analysis of dermatophytes using the 10 decamer, except UBC-400 primer that used in *T. rubrum* only. Table 1 indicates results of genomic similarity for different dermatophytes by using UPGMA method. Table 2 is calculated from genomic similarity group average of each genus. In these cases, the similarity of *Microsporum* genus with two other genera was nearly 13% and the similarity of *Trichophyton* with *Epidermophyton* was 20.8% (Table 2).

Finally, the phylogenetic relationships among different dermatophyte species are estimated by determining the degrees of similarity between their DNA sequences (Fig. 2).

Dermatophytes are a common and important cause of morbidity in humans. In cases of scalp Ringworm or *tinea corpris*, it is particularly important to determine whether the infection is anthropophilic or zoophilic as soon as possible, for treatment and prevention [8]. In fact, many dermatophytes share common genetic structures and show similar cultural characteristics [11, 13]. Despite their close genetic relationship, various dermatophyte fungi have enough differences at the molecular level to be exploited for the rapid identification of several common dermatophyte species [8]. The findings in the present study provide further evidence that most dermatophyte species are distinguishable by their formation of characteristic electrophoretic band patterns in the RAPD-PCR [14, 15]. The molecular determination of various dermatophyte species through the RAPD-PCR has advantages over conventional laboratory techniques such as microscopic examination and *in vitro* culture. The RAPD-PCR is not only rapid but also precise, as it is based on the measurement of genotypic rather than phenotypic differences. It is well known that various dermatophyte isolates show considerable phenotypic variations in terms of culture characteristics and colony morphology. Furthermore, phenotypic characteristics are often subject to outside influences (including chemotherapy) that affect the metabolism of the fungus. Therefore, the RAPD-PCR provides a rapid and precise means for the improved determination of dermatophyte fungi, and represents a useful addition to the current laboratory diagnostic techniques for human dermatophytosis. Currently, the RAPD-PCR is carried out with cultured organisms. The eventual refinement of the test for the detection from clinical specimens particularly nails where culture often fails, would make it a valuable tool in the diagnosis and epidemiological investigation, as well as control, of dermatophytosis. In conclusion, it is possible to assign special primers for making probe for improved diagnosis of particular species of dermatophytes. [16]

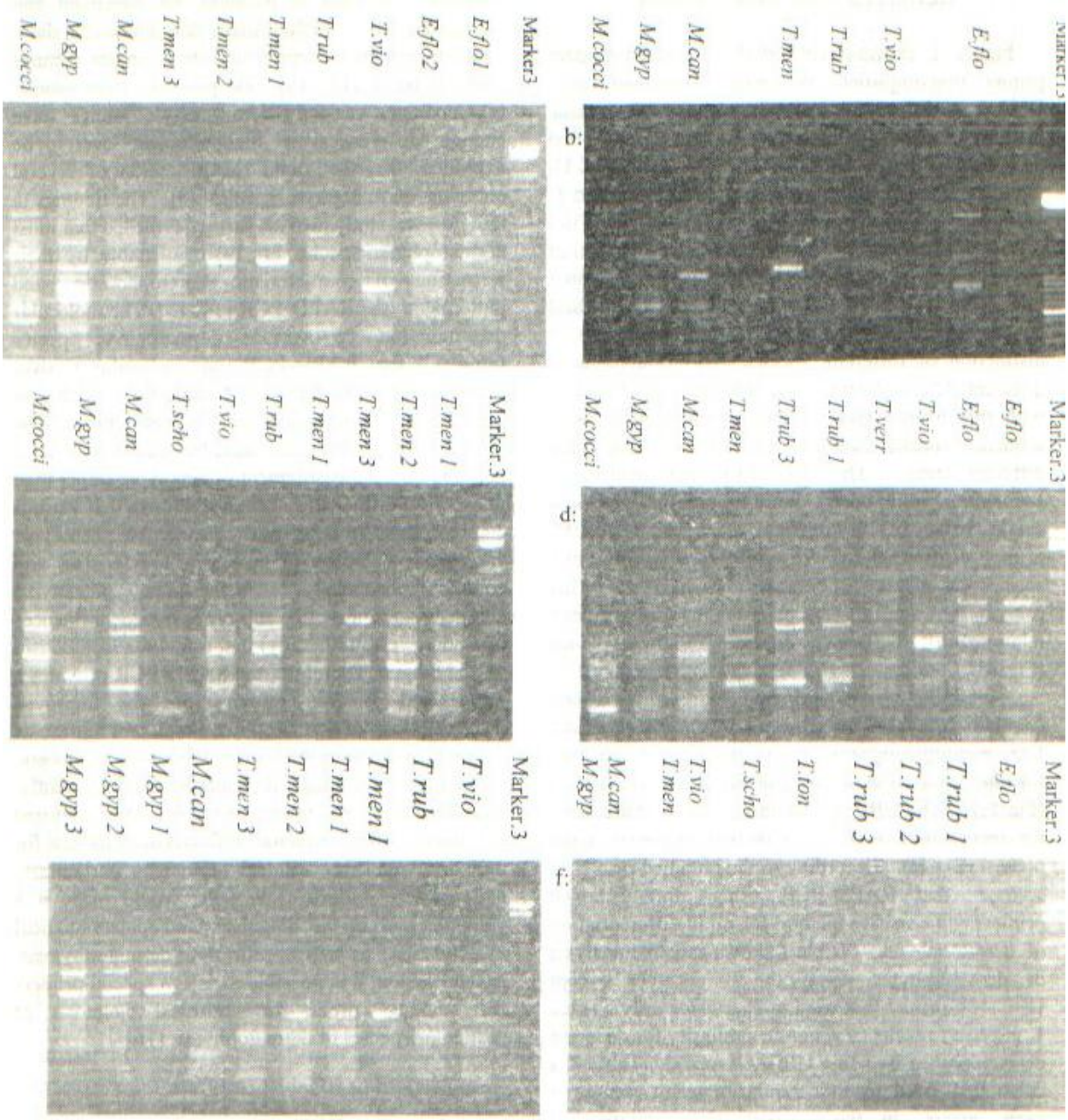

Fig. 1. (a), primer UBC 361 lane 4,5,6, band of 0.84 kb for recognition of *T.men*, lane 7 band of 0.81kb for *T.rub;* lane 8, band of 0.2 kb for *T.vio*. (**b),** primer UBC 389 lane1,2,3, band patterns for *Microsporium*; lane5,6,7 band patterns for *Trichophyton;* lane9, band patterns for *Epidermophyton*. (**c),** primer UBC 313 lane 4, identified *T.schoenlinii* from other dermatophytes by a single band 0.315 kb. (**d),** primer UBC 359 lane 8, differentiated *T.violaceum* from other species by making a unisharp band 0.79 kb. (**e),** primer UBC 310. This primer can clearly differentiate *T.men.varieties*(lane 8, *var.Interdigital* & lanes 5,6,7, *var.mentagrophytes* from three different areas). (**f),** primer UBC 400 lanes 7,8,9, *T.rubrum* isolated from three different areas. This primer does not created any visible bands with other dermatophytes.x

Table 1. Results of genomic similarity for different dermatophyte species.

Species \ Primer	UBC 310	UBC 313	UBC 337	UBC 340	UBC 359	UBC 361	UBC 389	AB1 04	AB1 11	AB1 20	S
M.cook-M.gyp	ND	18.2	20	7.14	20	23.5	16.7	11.1	0	ND	16.7
M.cook-M.can	ND	28.6	16.7	0	7.7	13.3	16.7	25	16.7	ND	15.6
M.cook-T.men	0	26.7	20	0	13.8	7.14	0	28.6	16.7	25	13.8
M.cook-T.rub	0	26.7	0	16.7	7.1	16.7	0	22.2	16.7	0	11.8
M.cook-T.vio	0	20	0	12.5	0	7.14	0	20	16.7	0	8.48
M.cook-E.flo	0	23.1	10	25	0	6.7	0	10	16.7	0	10.17
M.gyp-M.can	12.5	10	11.1	12.5	8.3	5.3	33.3	20	20	16.7	15
M.gyp-T.men	12.5	20	22.2	7.7	6.7	11.1	16.7	0	20	16.7	13.36
M.gyp-T.rub	10	0	40	9.1	7.7	13.3	40	0	0	25	14.51
M.gyp-T.vio	10	0	20	7.7	11.1	5.5	20	0	0	0	7.43
M.gyp-E.flo	0	12.5	28.6	15.4	0	11.8	33.3	0	20	0	12.16
M.can-T.men	11.1	21.4	12.5	14.3	6.3	23.1	16.7	25	33.3	16.4	18.01
M.can-T.rub	0	21.4	0	0	8.3	8.3	40	16.7	14.3	10.9	12
M.can-T.vio	0	14.3	0	0	10	15.4	20	14.3	14.3	8.8	9.7
M.can-E.flo	25	16.7	12.5	0	16.7	15.4	14.3	0	33.3	13.4	14.7
T.men-T.rub	71.4	20	25	0	28.7	9.1	50	50	33.3	28.6	31.6
T.men-T.vio	71.4	27.3	12.5	0	16.7	7.7	25	40	33.3	33.3	26.3
T.men-E.flo	33.3	20	37.5	0	18.8	27.3	0	20	33.3	16.7	20.7
T.rub-T.vio	71.4	88.9	66.7	40	10	9.1	25	28.6	60	16.7	41.6
T.rub-E.flo	12.5	7.15	60	16.7	7.7	0	16.7	14.3	14.3	25	17.4
T.vio-E.flo	12.5	8.3	40	25	0	16.7	20	12.5	33.3	25	19.3
T.men-T.men I	50	71.4	ND	ND	ND	ND	75	ND	ND	20	54.1

0, no common band was observed; ND, not determind; S, the average similarity between two species.

Table 2. Results of genomic similarity for different dermatophyte genera.

Genera \ Primer	UBC 310	UBC 313	UBC 337	UBC 340	UBC 359	UBC 361	UBC 389	AB1 04	AB1 11	AB1 20	S
Microsprom-Microsprom	12.5	18.9	15.9	6.6	12	14.1	22.3	18.7	18.3	16.7	15.7
Microsprom-Tricophyton	7.3	16.7	12.7	7.6	7.9	12	17	14.1	14.7	11.4	12.14
Microsprom-Epidermophyton	12.5	17.4	17	13.5	5.8	11.3	15.8	5.1	23.3	11.1	13.28
Tricophyton- Tricophyton	71.4	45.4	34.8	20	18.5	8.6	33.3	39.5	42.2	26.2	34

S, the average similarity between two genera.

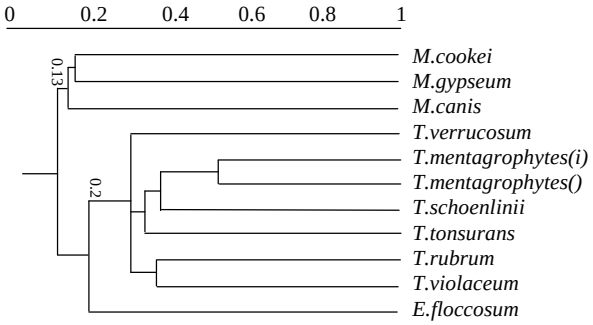

Fig. 2. Phylogram of dermatophytes fungi clustered by UPGMA method based on the major band generated using 12 primers. Scale value of 1 indicates 100% genetic similarity.

REFERENCES

1. Weitzman, I. and Summerbell, R.C. (1995) The dermatophytes. *Clin. Microbiol. Rev. 8: 240-25*.
2. Rippon, J.W. (1988) Medical mycology. The pathogenic fungi and the pathogenic actinomycetes, 3rd edition, W.B. Saunders, Philadelphia.
3. Liu, D., Coloe, S., Barid, R. and Pederson, J. (1997) PCR identification of *T. mentagrophytes Var. interdigital* and *T. mentagrophytes Var. mentagrophytes* dermatophytes with a random primer. *J. Med. Microbiol. 46: 1043-1046.*
4. Enrique, M.A. and George, A.M.C. (1933) Rapid isolation of DNA from tripanosematid protozoa, using a simple, mini-prep procedure. In: *Medical Health Care Career Prep Path*. Borrance Publishing Co. pp. 327-330.
5. Ray, W. (1995) Recombinant DNA methodology II. Copyright by Academic press, Inc.
6. Ausubel, F., Brent, R., Kingston, R.E., Moore, D., Seidman, J.G., Smith, J.A. and Struhl, K. (1995) Short protocols in molecular biology, third edition, Copyright by john Wiley & Sons, Inc.
7. Erland, S. (1995) Abundance of *Tylospora fibrillosa* ectomycorrhizas in a south Swedish spruce forest measured by RFLP analysis of the PCR-amplified rDNA its region. *Mycol. Res. 99 (12): 1425-1428*.
8. Liu, D., Coloe, S., Barid, R. and pederson, J. (1997) Molecular determination of dermatophyte fungi using the arbitrarily primed PCR. *Br. J. Dermatol. 137 (3): 351-355*.
9. Mochizuki, T., Watanabe, S. and Uehara, M. (1996) Genetic Homogeneity of *T. mentagrophytes Var. interdigitale* isolated from geographically distant regions. *J. Med. Vet. Mycol. 34: 139-143*.
10. Le Blancq, S.M., Cibulsks, R.E. and Peters, W. (1986) *Leishmania* in the old world: 5 numexical analysis of isoenzyme data. *Trans. R. Soc. Trop. Med. Hyg. 80: 517-524*.
11. Liu, D., Coloe, S. and Barid, R. (1996) Use of arbitrarily primed polymerase chain reaction to differentiate trichophyton dermatophytes. *FEMS. Microbiol. Lett. 136: 147-150.*
12. Bently, S., Pegg, K.G. and Dale, J.L. (1995) Genetic variation among a world-wide collection of isolates of *Fusarium oxysporum, F. spcubense* analysed by RAPD-PCR finger printing. *Mycol. Res. 99 (11): 1378-1384*.
13. Harmsen, D., Schwinn, A., Weig, M., Kano, R., Matshashiro, H. and Watari, T. (1995) Phylogeny and dating of some pathogenic keratinophilic fungi using small subunit ribosomal RNA. *J. Med. Vet. Mycol. 33: 299-303*.
14. Luck, J.E. and Gillings, M.R. (1995) Rapid identification of benomyl resistant strains of *Botrytis cinerea* using the PCR. *Mycol. Res. 99 (12): 1483-1488*.
15. Newton, C.R. and Graham, A. (1994) Polymerase chain reaction (PCR). BIOS scientific publishers limited, Oxford, UK.
16. Barasubiye, T. and Parenn, J.G. (1995) Discrimination between alfalfa and potato isolates of Verticillium albo-atrum using RAPD markers. *Mycol. Res. 99 (12): 1507-1512*.