Study genetic diversity between *trichophyton rubrum* isolates using ISSR and RAPD markers

دراسة التغايرات الوراثيه بين عزلات الفطر *Trichophyton rubrum* بواسطه مؤشرات الـ ISSR وRAPD

Milad A. Mezher Sanaa Hussein Mohammad* Khalil Muoload Wahab**

ZainabAnas Salman* * * Suzan Adil Rashid** Bilal Abdulrahman Tuama*****

College of Education for Pure Science/ Tikrit University,

* College of Science/ Kirkuk University

**Insititute of Medical Technology/ Al Mansour,

* * * College of Science/ University of Baghdad

****Kirkuk Technical Insititute / Northern Technical University

*****Collage of Applied Sciences/ University of Samarra

ميلادعدنان مزهر سناء حسين محمد* خليل مولود وهاب **

زينب أنس سلمان* * * سوزان عادل رشيد ** بلال عبدالرحمن طعمه *****

كلية التربيه للعلوم الصرفه/ جامعة تكريت

* كلية العلوم/ جامعة كركوك

**المعهد التقني الطبي/ المنصور

***قسم التقنيات الاحيائيه كلية العلوم /جامعة بغداد

****الكلية التقنية الشماليه/ المعهد التقني الطبي

***** كلية العلوم التطبيقيه/ جامعة سامراء

E-mail: murugasanr@annauniv.edu

Abstract

The present study included detection of genetic variability, and identification of genetic relationship and finding a fingerprint of the ten clinical isolates related to *Trichophyton rubrum* using RAPD and ISSR markers. The experiment was carried out and the results performed using six primer of the RAPD markers these primers showed 239 amplified band , out of these band 90 of them was considered as a main band, and 149 was Polymorphic band , the largest number of bands was 30 in the isolate TR6 and less number of bands in the isolate TR7. The results clear the value of genetic diversity based on RAPD analysis the lowest value of genetic diversity (0.13005) between the isolate TR3 and TR9while the highest genetic diversity (0.55941) was between the isolates TR5 and TR8. The analysis of the relationship shows that there are three main groups: the first group include isolate TR8, while the second group included three isolates are isolate TR2, isolate TR10 and isolates TR3, The third group included three subgroups included the first isolate TR1,isolates TR4 and second isolate TR3 and isolate TR9 and the third included the isolate TR5 and isolates TR6.The results of ISSR experiments: the use six a primers in the of the ISSR showed 192 bands, in the isolate of *Trichophyton rubrum*, two of these primers showed monomorphic bands (in number and location) and six primers showed monomorphic and polymorphic bands, while one showed only polymorphic bands among *Trichophyton rubrum* isolates. And the largest number of bands was 24 in the *TR5* and less number of bands 16 in isolate TR3 and isolate TR8 were finding DNA fingerprint to isolate TR1, isolate TR5, and isolate TR3. The ISSR markers showed lowest genetic polymorphism was (0.05561) between the isolates TR2 and isolate TR7 and the largest genetic distance was (0.40501) between the isolate TR4 and isolate TR8. The analysis of the relationship of genetic showed that there are three groups key first included isolates and only one isolate TR8 and the second involving isolates TR2 and isolate TR7, isolate TR4 and isolate TR3, while the group included three sub-groups, the first included isolate TR1 and isolate TR6, and the second involving isolate TR5, isolate TR10 and isolate TR9. The results confirmed the efficiency markers of the RAPD and ISSR in contrast, find a genetic fingerprint to the isolates of *Trichophyton rubrum*, and these markers differ in mechanical detection contrast, and coverage is Genome. Therefore Complementary to each other, although the ISSR markers were more efficient in terms of the number of binding sites in each type of the isolates of *Trichophyton rubrum* and so can the initiator of the discovery of a much larger area from Genome.

Key words: *Trichophyton rubrum*, genetic diversity, ISSR, RAPD

الملخص

تم في هذه الدراسة تحديد العلاقة الوراثية ، تحديد البعد الوراثي، وإيجاد البصمة الوراثية لعشرة عزلات من الفطر *Trichophyton rubrum* باستخدام مؤشرات RAPD وISSR. حيث درست تفاعلات الـ RAPD باستخدام 6 بادئات واظهرت البادئات 239 حزمة، منها 90 حزمة رئيسيهmain band و 149 حزمة متباينة Polymorphic band, وكان اكبر عدد من مواقع الارتباط 30 موقع في العزله (6)، وأقل عدد من كان 20 موقع في العزله (7) . واستثمرت تلك النتائج لدراسة التباين الوراثي بين العزلات الداخلة في الدراسة.ظهر من خلال نتائج البعد الوراثي لتفاعلات الـ RAPD ان اقل قيمة كانت (0.13005) بين العزله (3) و (9) ،وأعلى قيمة كانت (0.55941) بين العزلات (5) و (8) .بينما اظهر تحليل العلاقة الوراثية ان هناك ثلاث مجاميع رئيسة ضمت الاولى العزله رقم (8) ،اما المجموعة الثانية فقد ضمت ثلاثة عزلات من الفطر هي (2) و(10) و(7)، وشملت المجموعة الثالثة ثلاث مجاميع فرعية ضمت الاولى العزله (1) و(4)، والثانية العزلات (3) و(9)،والثالثة العزلات (5) و (6) . اما نتائج تفاعلات الـ ISSR والتي استخدام فيها 6 بادئات أظهرت 192 حزمة، حزماً متماثلة في البادئين ISSR2وISSR9، واربع أظهرت حزم متباينة ومتماثلة، بينما اظهر البادئ ISSR10 حزما متباينة فقط بين انواع عزلات الفطر*Trichophyton rubrum*. واكبر عدد من الحزم ظهر كان 24 في العزله (5) واقل عدد من الحزم 16 في العزلتين (3) و (8) ، وتم ايجاد البصمة الوراثية للعزله (1) (5) و (8). وأظهرت مؤشرات الـ ISSR ان اقل بعد وراثي كان (0.05561) بين العزلتين (2) و (7)، واكبر بعد وراثي كان (0.40501) بين عزلتي الفطر*Trichophyton rubrum*(4) و (8) . اما تحليل العلاقة الوراثية فأظهر ان هناك ثلاث مجاميع رئيسة ، الاولى ضمت عزله واحده فقط هي (8) ، والثانية ضمت (2) و(7) و (4) و (3) بينما ضمت المجموعة الثالثة مجموعتين فرعيتين ، الأولى ضمت العزلتين (1) و(6)، والثانية ضمت (5) و(10) و(9). وقد اكدت النتائج كفاءة مؤشرات الـ RAPD و ISSR في ايجاد التباين والبصمة الوراثية لعزلات الفطر*Trichophyton rubrum*، وان هذه المؤشرات تختلف في ميكانيكية اكتشاف التباين، وتغطية المجين ، لذلك تعتبر مكملة لبعضها، بالرغم من ان بعض بادئات مؤشر الـ ISSR كانت اكثر كفاءة من حيث عدد مواقع الارتباط المتباينة بين عزلات الفطر *Trichophyton rubrum*.

الكلمات الدالة: الفطريات الجلديه ، التغايرات الوراثيه ، تفاعل البلمرة المتسلسل العشوائي.

Introduction

Trichophyton rubrum is one of the most commonly encountered dermatophytes that infect human keratinized tissue such as skin, nail, and possibly hair. This pathogen causes well characterized superficial infections, and also produced skin infections in unusual parts of the body in immuno depressed patients [1].

One of the diagnostic methods for *T. rubrum*is Polymerase Chain Reaction (PCR), which can amplify some specific fragment of DNA into millions of copies (McPherson and Møller, 2001; Abdulbaqi and DheebBI *et al*., 2018). Different molecular typing techniques have been applied to study the genetic diversity of *Trichophyton spp.* and the possible occurrence of similarity and difference between them, Random Amplified Polymorphic DNA (RAPD) analysis can be performed as a method for study genetic diversity with large number of different strains of microorganisms. It is inexpensive and requires less amount of DNA [2,3]. Moreover, RAPD analysis is technically being commonly used as an indicator for determination the genetic diversity, while Inter-spread sequence repeat technique ISSR analysis based on variation found in the regions between microsatellites it has been used in genetic fingerprinting gene tagging and detection of clonal variation [4,5]. This technique which involved amplification of DNA segment present in between two identical microsatellite repeat regions by addition the oriental in opposite direction with suitable distances ISSR method has been reported produce more complex markers patterns than the RAPD markers In addition, ISSR method are more reproducible than RAPD method Because ISSR primers designed to anneal temperature to a microsatellite sequences are long than RAPD primers, allowing higher annealing temperature to be used. It also because of multilocus fingerprinting profile obtained ISSR has been found to be an efficient, low cost, simple operation, high stability and abundance of [6,7].The aim of the studyDetection of the Genetic Diversity between isolates of *Trichophyton rubrum*and Comparative study between RAPD and ISSR markers.

Materials and Methods

Trichophyton rubrum isolates.

A total of 10 *Trichophyton rubrum* dermatophyte were, isolated from skin, and hair infectious.as shown in Table (1) and examined according to their microscopic features, and were sub cultured on sabouraud Dextrose Agar, for using in DNA extraction.

Table (1): *Trichophyton rubrum*der matophyte isolates examined during this study

T. rubrum isolates	Samples type	Sources
TR1	Dermal scrapes	skin
TR2	Dermal scrapes	skin
TR3	Dermal scrapes	skin
TR4	Hair	hair
TR5	Hair	hair
TR6	Dermal scrapes	skin
TR7	Dermal scrapes	skin
TR8	Dermal scrapes	skin
TR9	Hair	hair
TR10	Hair	hair

Genomic DNA extraction

The DNA was extracted by small-scale method commercial kit (Bionner-Korea) DNA Purity was measured depending on optical density using Nano-drop. DNA quality was visualized by agars gel electrophoresis with ethidium bromide and visualized under UV light [8,9].

Molecular Analysis

RAPD assay

Six of RAPD primers were used in this study, the primers were synthase by (Bioneer-Korea) in lyophilized form and dissolved in sterile distilled water to get final conc. of (10pmol/ml [10,11]. The primers and their sequences are listed in Table (2).

Table (2): The names and sequences of the primers used in this study

N0.	Primer name	Sequences(´5 -´3)
1.	OPI – 06	AAGGCGGCAG
2.	OPE-16	GGTGACTGTT
3.	OPN-07	GAGCCCGAG
4.	OPQ-17	GAAGCCCTTG
5.	OPD-20	ACCCGGTCAC
6.	OPL-05	ACGCAGGCAC

Amplification of genomic DNA was performed with the following master amplification reaction:
RAPD – PCR premix (final reaction volume = 20 µI).No. of cycles = 40 cycles between initial denaturation and final extension, following Table (3) which showed the RAPD program

Table (3): RAPD program used in the study

Steps	Temperature (°C)	Time (min.)	cycle
Initial denaturation	94	5	40 cycle
Denaturation	94	1	
Annealing	36	1	
Extension	72	2	
Final extension	72	10	

Followed by a hold at 4°C [12,13]. Each PCR amplification reaction was repeated twice separately to ensure reproducibility the products analyzed by electrophoresis in 1.5% agars gels with 0.5µl stained ethidium bromide at 5v/cm for 2hours.

ISSR assay

Six of ISSR primers were used they were provided by (Bioneer – Korea) in lyophilized form and dissolved in sterile distilled water to get final conc. of (10pmol/ml) [14,15]. Recommended by provider the primers which tested in this study listed in Table (4) and master amplification as show in Table (5)

Table (4): The names and sequences of the primers used in this study

No.	Primers Name	Sequence(´5-´3)
1	$ISSR_2$	GACAGACAGACAGACA
2	$ISSR_6$	AGAGAGAGAGAGAGAGAGAGC
3	$ISSR_7$	AGAGAGAGAGAGAGAGAGAGT
4	$ISSR_8$	CTCTCTCTCTCTCTCTCTCTA
5	$ISSR_9$	CTCTCTCTCTCTCTCTCTCTG
6	$ISSR_{10}$	CTCTCTCTCTCTCTCTCTCTT

Table (5): Master amplification reaction

Materials	Final concentration	Volume for 1tube
PCR Premix	1x	5µI
Deionised D.W.	—	17 µI
Primer(10pmol/ml)	10pmol/ml	2µI
DNA template	100ng	1µI

Final concentration was performed in a volume of 25µl. PCR program for ISSR assay using the following program: No. of cycles= 40 cycles between initial denaturation and final extension, Table (6) showed the ISSR program:

Table (6): ISSR program used in the study.

Steps	Temperature(°C)	Time (min.)	cycle
Initial denaturation	94	5	40 cycle
Denaturation	94	1	
Annealing	50	1	
Extension	72	1	
Final extension	72	10	

Each PCR amplification reaction was repeated twice to ensure reproducibility the products analyzed by electrophoresis in 1.5% agars gels with stained ethidium bromide 0.5µl at 5v/cm for 1.5 hour.

Data analysis

Estimation of molecular weight

Computer software Photo-Capture M.W. program was used to determine molecular size based on comparing the RAPD-PCR and ISSR-PCR products depending on molecular size of bands and number bands of a 2000bp DNA ladder Bioneer (which consist of 13 bands from 100 to 2000 bp.)

Estimation of polymorphism, efficiency and discriminatory power

Data generated for molecular weight RAPD and ISSR markers result bands were a score for each bands on the molecular size (1 for present, 0 for absence) the commercial soft word[16,17]. Only major bands consistently amplified were scored. Polymorphism of each primer was calculated based on the following formula:

$$\textbf{Polymorphism \%} = (Np / Nt) \times 100$$

Where Np = the number of polymorphic bands of random primer, Nt = the total number of bands of the same primer. Efficiency and discriminatory power of each primer calculated according to the formula below:

• Efficiency =number of polymorphic bands to each primer / total number of bands to the same primer.

• Discriminating power= number of polymorphic band to each primer / total number of polymorphic band to all primer X100 %.

Primer efficiency ranged between (0-1). Discrimination power of each primer.

Results and discussion

RAPD-PCR analysis

Based on RAPD assay the data developed from the PCR analysis demonstrated that some primers generate several bands, while other generates only a few bands. A total of six RAPD primers were used for study the genetic differences between seven *Trichophyton rubrum* isolates, amplified 341 bands,126 bands were polymorphic, with average of (3-43) polymorphic bands, that OPD-20 produce 3 polymorphic bands only ,were OPN-07 can be produce 43 polymorphic bands, Figure (1). Some isolates could be distinguished from all other isolates with selection of these primers, for instance OPN-07 primers can be produce higher discrimination power 19.1 bands only, while OPL-05 gave 2 unique bands patterns Table (7).

Table (7): Values of genetic distance among *Trichophyton rubrum* Isolates calculated according to Nei and Lei, 1979).

	1	2	3	4	5	6	7	8	9	10
1	0.00000									
2	0.20273	0.00000						,		
3	0.21052	0.30133	0.00000							
4	0.15394	0.28768	0.19011	0.00000						
5	0.30809	0.34484	0.30133	0.28768	0.00000					
6	0.21899	0.19858	0.25039	0.15413	0.15413	0.00000				
7	0.39925	0.31430	0.27080	0.37884	0.31430	0.20273	0.00000			
8	0.38566	0.42588	0.38237	0.42588	***0.55941***	0.31430	0.33472	0.00000		
9	0.24275	0.32770	***0.13005***	0.17355	0.22234	0.19415	0.29370	0.34811	0.00000	
10	0.32175	0.24418	0.31845	0.30133	0.42650	0.30168	0.27080	0.32175	0.28420	0.00000

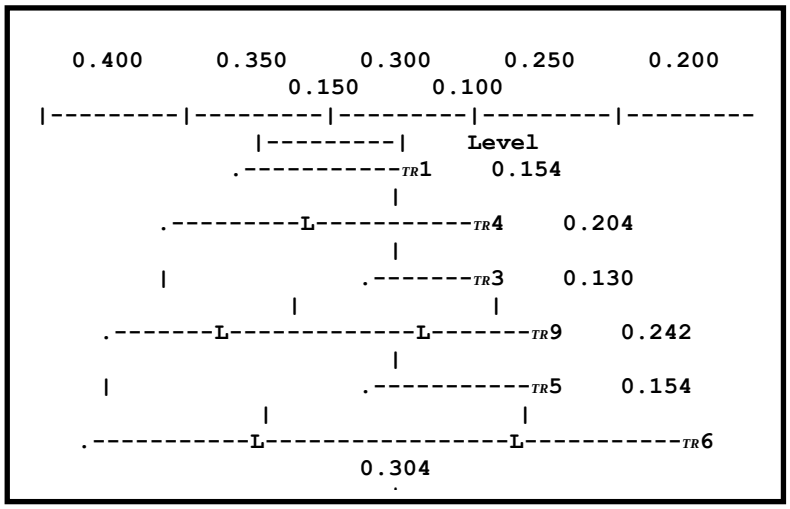

Fig. (1): Dendrogram illustrated genetic fingerprint and relationship between *Trichophyton rubrum* isolates developed from RAPD data

Table (8) summarized the in formations which can be obtained from RAPD analysis, and from genetic distance the ration genetic diversity among the *Trichophyton rubrum* isolates from 0.9852 to 0.3336.The highest similarity 0.9852 (98.5%) was obtained between isolates numbers (5 and 6) while 0.48562 (48.5%) similarity between isolates numbers (2 and 6), the lowest level of similarity 0.3336 (33.3%) was obtained between isolate number (5 and 7) [18,19].

Cluster analysis illustrated genetic relationship among seven of *Trichophyton rubrum* isolates showing two major clusters Figure (1), the first cluster contained two main groups, first group, 5 and 7 isolated in one sub group cluster with low genetic distance 0.3336. These were introduced from environmental sources and isolated number 1 formed separated line due to different in isolate source, while isolate number 2 and 6 formed another sub clusters with genetic distance 0.48652 these isolates introduced from environmental and clinical source, second group contained

isolate number 3 only, during clusters analysis showing the levels of genetic relatedness also dendrogram indicates difference between isolates based on source of the isolates, present result appeared multiple difference in isolates of *Trichophyton rubrum* came from two factors including genetic factor and environment factor, also the results indicate that the clinical isolate have greater genetic variability than the environment isolates during gene distance and dendrogram, genetically different may be come from clinical ones on the other hand the clinical isolates of patients constitute one group according to genetic characteristic with the environmental isolates, genetic difference observed in this study come from adept fungi to grow and ability isolates that infected patients to reactive and general more variability in relation to the original strain [20,21]. Genetic diversity may be attributed to mutation or recombination that occurs in fungal cell into resistance to anti-mycotic treatment or under environmental stress [22,23]. Environmental and clinical isolates of *Trichophyton rubrum*may be different in genotype consisted of gene involved in transport, regulation of transcription, metabolism of molecular with 1-3 carbon and paroxysm all proteins [24,25].

ISSR-PCR analysis

In this study, ISSR-PCR technique was used to reveal the genetic diversity among different studied *A.fumigatus*isolates in order to search the genetic diversity between *A.fumigatus* isolates and study the differences that come from environment. A total of 178 use full bands were scored from the amplified products with the seven Inter Simple Sequence Repeat (ISSR), 120bands were polymorphic, with average of (6) polymorphic bands ISSR10, and ISSR6 produce 32 polymorphic bands. Figure (2). ISSR9 primers can be produce high unique bands can be produce 5 unique bands, Table (6).

Table (8): Values of genetic distance among *Trichophyton rubrum* Isolates calculated according to Nei and Lei, 1979).

	1	2	3	4	5	6	7	8	9	10
1	**0.00000**									
2	**0.40392**	**0.00000**								
3	**0.29356**	**0.09485**	**0.00000**							
4	**0.40392**	**0.12516**	**0.23795**	**0.00000**						
5	**0.22803**	**0.29758**	**0.26727**	**0.23304**	**0.00000**					
6	**0.17185**	**0.24977**	**0.37361**	**0.24977**	**0.17087**	**0.00000**				
7	**0.37949**	***0.05561***	**0.15046**	**0.11624**	**0.22803**	**0.23639**	**0.00000**			
8	**0.29356**	**0.31799**	**0.20764**	***0.40501***	**0.26727**	**0.37361**	**0.29356**	**0.00000**		
9	**0.24515**	**0.32307**	**0.36687**	**0.25408**	**0.13882**	**0.18453**	**0.24515**	**0.22377**	**0.00000**	
10	**0.26738**	**0.27630**	**0.24599**	**0.27630**	**0.11225**	**0.20675**	**0.20675**	**0.18145**	**0.11754**	**0.00000**

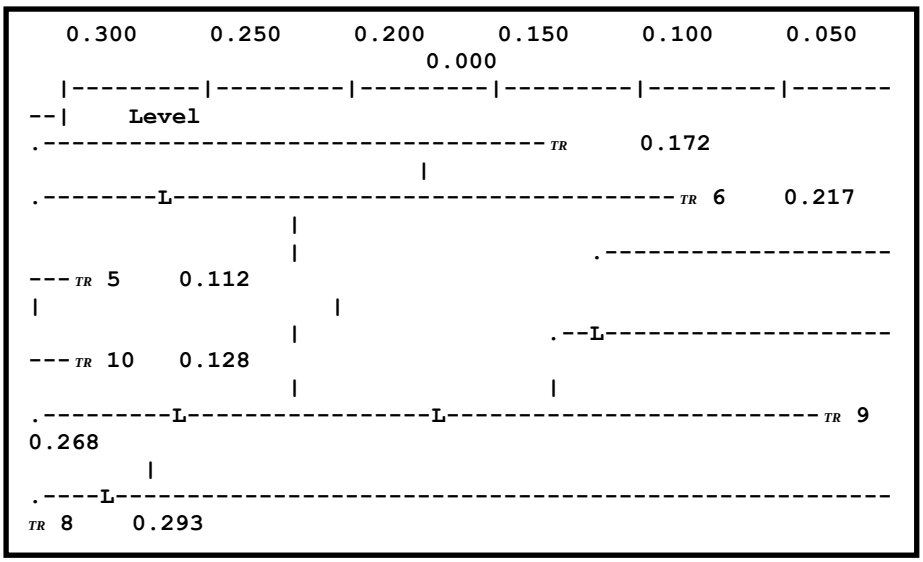

Fig. (2): Dendrogram illustrated genetic fingerprint and relationship among *Trichophyton rubrum* isolates developed from ISSR data.

From genetic distance, the ratio of genetic similarity among the ten *Trichophyton rubrum* isolates from 0.97868 to 0.1025,showing Table (6).The highest similarity 0.97868 (99.8%) was obtained between isolates number (1 and 2)while 0.21556 (21.5%) genetic similarity between isolate number 3 and 6, but lowest level of similarity 0.1025 (10.2%) was appears between isolate number (5 and 7).

During dendrogram were constructed based on [13]. Genetic distance using UPGMA cluster analysis and depicted genetic relationship among seven *Trichophyton rubrum* isolates showing two major cluster, first cluster contained two main distance, these were introduced from patients sources and isolate number 2 formed separated line that came from environmental sources, and second cluster contained isolate number (7,5) with lowest genetic similarity 0.1025.These isolates introduced from patients and environment sources, group, first group contain isolates number (1,2) with higher genetic similarity 0.9782.

In this study, each of genetic distance based on ISSR and on RAPD markers don't show geographic profiling between isolates. It has been reported that the dendrogram generated by ISSR better with genealogy and the know pedigree of the ISSR than RAPD results, on the another hand, it has been found that the data on RAPD genetic distance have more relationship with the geographic distribution in comparative with ISSR data that based on number of chromosomes, ISSR markers are highly polymorphic and are useful in studies on genetic diversity [18,20]. Numbers of analysis studies used both ISSR and RAPD technique were found that ISSR produce more information with fewer number primer than number RAPD primer, during among study found a number polymorphic bands was still higher with less number [13, 25,26]. ISSR less primers means less time, less DNA, less supplied and less samples, RAPD markers don't have the specific target comparing to ISSR markers. In fact ISSR markers are known to be more sensitive than RAPD markers, in this study it was obvious that the dendrogram based on RAPD markers was not in according with the dendrogram based on ISSR markers, thus, both dendrogram are in agreement with the groups of geographic origin, but RAPD markers greatly agree with these group than ISSR markers, the differences in clustering pattern of genotypes using RAPD and ISSR markers also may be attributed to markers sampling error and the level of polymorphic detected [16,25,26].

Conclusion

ISSR markers produced high rate from polymorphism depending on polymorphic rate, the ISSR technique can be produce high level from unique bands a comparative with another markers, ISSR less efficiency in dendrogram results.

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