Molecular Characterization and Genetic Variability of *Trichoderma Harzianum* Isolates by Using PCR-RAPD Markers

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Abstract

The PCR-based technique of randomly amplified polymorphic DNA (RAPD) was used to characterize and assess the genetic relatedness of eight *Trichoderma harzianum* isolates. Genomic DNA of each species was extracted at a final concentration of 400 - 600 µg / 2-3 g of wet mycelium and at a purity of 1.6-1.8. Each DNA sample was amplified with each of 15 primers and the products were resolved electrophoretic ally on 1.2% agarose gel, stained with ethidium bromide and photographed under UV. Six primers failed to support amplification while the remaining nine primers produced a total of 128 main bands (11-20 per primer) across the eight isolates. Of these bands, 120 (9-19 per primer) were polymorphic. The least efficient primer was OPY-19 (8.59), while the most efficient one was OP-B14 (15.63%). Primer OP-Y19 had the lowest (7.5%) discriminatory power while primer OP-B14 had the highest (15.8%). RAPD analysis fingerprinted six of the eight isolates through unique bands with one or more of the 9 primers. Cluster analysis based on the genetic distances split the eight isolates into two major group genotypes.

Keywords: *Trichoderma harzianum*, Genetic variability, RAPD, PCR.

Introduction

Trichoderma, commonly available in soil and root ecosystems has gained immense importance since last few decades due to its biological control ability against several plant pathogens [1]. Antagonistic microorganisms, such as *Trichoderma,* reduce growth, survival or infections caused by pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions and enzyme secretion. In addition, the release of biocontrol agents into the environment has created a demand for the development of methods to monitor their presence or absence in soil. Therefore, monitoring population dynamics in soil is of much importance. Previous methods employed to identify strains of *Trichoderma* spp. in soil samples have included the use of dilution plates on selective media. However, this method does not distinguish between indigenous strains and artificially introduced ones [2,3].

The *Trichoderma* isolates were differentiated by mycelial growth rate and colony appearance, as well as microscopic morphological features, including phialides and phialospores [4]. These can also be distinguished by randomly amplified polymorphic DNA (RAPD)-PCR, restriction fragment length polymorphisms in mitochondrial DNA and ribosomal DNA and sequence analysis of ribosomal DNA [5-7]. Molecular characterization of the potential bio-control agents using RAPD-PCR, helps to determine the diversity and characterization.

This study aimed to determine DNA fingerprinting and genetic variability of *Trichoderma harzianum* mutants and their parents by different RAPD markers.

Materials and Methods

Trichodrma harzianum isolates

Two *T.harzianum* isolates and their six mutants tolerant to fungicides were used in this study. The first isolate named T1, was isolated from Iraqi commercial product called Al-Tahady ; the second isolate named T2, was kindly obtained from Dr. Hadi Mahdi Aboud (Biotechnology Center, Directorate of Agriculture Research, Ministry of Science and Technology) isolated from agriculture soil. The six *T.harzianum* mutants tolerant to fungicides named Th1, Th2 (tolerant to Beltanol fungicide), Th3, Th4, Th5 and Th6 (tolerant to Bavistin fungicide). By irradiated isolate T1 and T2 with UV light, the mutants Th1, Th3 and Th5 were obtained from T1 while Th2, Th4 and Th6 were obtained from

T2 (obtained by a previous study carried out by Alaa M. Al-araji) [8, 9].

Extraction and purification of DNA

The applied eight isolates of *T.harzianum* under study were grown for 10 days at 28+2˚C in 250 ml flasks containing 100 ml Potato dextrose broth medium. Each mycelium was harvested by filtration, washed several times with sterile distilled water and blotted dry. The genomic DNA was isolated from eight *T.harzianum* according to Cetyl-trimethyl amonium Bromide (CTAB) protocol by (10) as described below. Up to 3g of dried mycelium was (grinded by liquid nitrogen), crushed with 10ml of CTAB extraction buffer {for 250ml CTAB extraction buffer; 70ml of CTAB, 25ml of Tris-HCl (PH 8.0), 20ml of EDTA and complete the volume to 250ml with Sterile distilled water} and incubated at 65ºC water bath shaker from 60-90 minutes with shaking. The flasks were removed from the water bath and allowed to cool at room temperature and chloroform isoamly alcohol (6.5ml) was added with shaking for 15 min. The mixture was centrifuged at 4000 rpm for 15 minutes at 4ºC and upper supernatant phase was collected in new sterile tubes and chloroform isoamly alcohol (6.5ml) was added, also the mixture was centrifuged at 4000 rpm for 15 min at 4ºC and upper supernatant phase was collected in new sterile tubes. Then DNA was precipitated by adding 6ml of ice cold isopropanol. DNA was pelleted by centrifuging at 4000 rpm for 15 min and upper supernatant phase was removed. Then washing buffer was added to pellets. The pellets were air dried and suspended in TE buffer (pH 8.0). The quality and quantity of DNA was analyzed both spectrophotometrically and in 1% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA. Then part of DNA was diluted at 20ng / µl for PCR reaction.

Random Amplified Polymorphic DNA (RAPD) analysis

RAPD analysis was done using fifteen different random primers (Operon Technologies Inc., Almenda, CA, USA). The different random primers used for amplifications were (OPA-01, -03,-10, -12; OPB-14; OPC-05; OPD-06, -07; OPE-20, OPF-16, OPG-8, OPN-16, OPW-14 and OPY-02, -19) Table (1). PCR was performed in 25µl reaction mixture, containing 10X PCR buffer (2.5µl), 200mM dNTPs mix (2.5µl), 1U/µl Taq DNA polymerase (0.15µl), 20ng/µl template DNA (2µl), 1.6mM $MgCl_2$ (0.8µl), 10 pM Primer (2µl) and PCR water (15µl). The PCR was done with an initial heat denaturation of DNA at 94℃ for 5 min was followed by 35 cycles consisting of 1min at 94℃, 1 min at 37℃ and 1 min at 72℃ and the final extension for 5 min at 72℃. PCR product (25µl) was mixed with loading buffer (3µ1) and then loaded in 1.2% Agarose gel. The Agarose gel was stained in ethidium bromide, visualized under UV and photographed using digital camera standard molecular weight markers also used in each electrophoresis run (11).

Data Analysis

RAPD results were analyzed depending on the presence or absence of amplified DNA bands, total number of amplified bands across all isolates of *T. harzianum*, the number of polymorphic bands, which can be detected horizontally. The Numerical Taxonomy System (NTSYS) 1.8 software, using the Jaccard coefficient of similarity (12), and Unweight pair group method arithmetic (UPGMA) averages cluster analysis were used to calculate genetic distance and obtaining phylogenetic tree (13,14). Primer efficiency and discriminatory power were calculated for each primer using two equations as described by (15, 16).

Results and Discussion

DNA isolation

The DNA was extracted efficiently by using CTAB method depending upon extraction protocols (10). Purity and concentration of DNA were measured using the standard method (17). The yield of the DNA extracted from the *T. harzianum* isolates was in range of (500-750) µg per 3g with purity of 1.8-2.

RAPD-PCR analysis

This study may be a pioneer one accomplished in Iraq that used Random Amplified Polymorphic DNA (RAPD) markers in the characterization of

T. harzianum isolates. RAPD-PCR technique used in this study as a PCR-based molecular technique in an attempt to determine DNA fingerprinting and detect genetic variation of eight *T. harzianum* isolates.

Although there are several ways for the genetic characterization of *T. harzianum* isolates, RAPD technique was used successfully for *T. harzianum* and is less costly and time consuming than other techniques. The RAPD technique is straightforward and does not require previous knowledge of nucleotide sequence of target organism. Furthermore, it is very quick and convenient to perform, since a strain can be typed within 48hrs of harvesting the cells from the medium. It is highly sensitive, requires minimum amount of template DNA. Furthermore, RAPD-PCR is discriminatory because it analyzes the whole genome. However due to its sensitivity and reproducibility, can be affected by small variations in reaction mixture and temperature cycles. Thus, care is needed to standardize the procedure if it is to be used for routine analysis. Positive (type strain template DNA) and negative (no template DNA) should always be included (18,19).

Table (1)
Random primers used in the current study and their sequences.

No.	Operon code	Nucleotide sequence	No.	Operon code	Nucleotide sequence
1	OPA-01	$^{5'}$CAGGCCCTTC$^{3'}$	9	OPE-20	$^{5'}$AACGGTGACC$^{3'}$
2	OPA-03	$^{5'}$AGTCAGCCAC$^{3'}$	10	OPF-16	$^{5'}$ GGAGTACTGG $^{3'}$
3	OPA-10	$^{5'}$GTGATCGCAG$^{3'}$	11	OPG-08	$^{5'}$ TCACGTCCAC $^{3'}$
4	OPA-12	$^{5'}$TCGGCGATAG$^{3'}$	12	OPN-16	$^{5'}$ CCAAGCTGCC $^{3'}$
5	OPB-14	$^{5'}$TCCGCTCTGG3$^{3'}$	13	OPW-14	$^{5'}$ CTGCTGAGCA$^{3'}$
6	OPC-05	$^{5'}$GATGACCGCC$^{3'}$	14	OPY-02	$^{5'}$ CATCGCCGCA$^{3'}$
7	OPD-06	$^{5'}$GGGGTCTTGA$^{3'}$	15	OPY-19	$^{5'}$ TGAGGGTCCC$^{3'}$
8	OPD-07	$^{5'}$TTGGCACGGG $^{3'}$			

Table (2)
Total bands, polymorphic bands, efficiency and discriminatory power of each RAPD primers that observed in eight isolates.

No.	Primer name	No. of bands amplified in all 8 isolates of *Trichoderma harzianum*			Primer efficiency	discriminatory power %	Isolates name	Unique bands at molecular weight (base pair)
		Total bands	polymorphic bands	polymorphic loci (%)				
1	OPA-01	12	12	100%	9.38	10.0	T1; T2	3700; 2600
2	OPA-03	15	15	100%	11.72	12.5	T2; Th2; Th1	4300, 2800, 2500, 1100; 2300; 700
3	OPA-10	16	15	94%	12.50	12.5	T1; T2; Th2; Th5	750, 200; 2600,400; 4100; 1800, 1100
4	OPB-14	20	19	95%	15.63	15.8	T1; T2; Th2	1650; 2500, 1200; 2750
5	OPD-06	12	12	100%	9.63	10.0	T2; Th1; Th5	3200, 1350; 1550; 3000, 500
6	OPD-07	15	14	93%	11.72	11.7	T2; Th2; Th5	4200, 300; 3800; 3400, 2400
7	OPW-14	14	12	86%	10.94	10.0	T2; Th5; Th6	600; 3900, 900; 2400
8	OPY-02	13	12	92%	10.16	10.0	Th6	3600,50
9	OPY-19	11	9	82%	8.59	7.5	T1;T2; Th5	2100; 1150; 2550
Total		128	120					

The RAPD technique was found to be advantageous over other molecular techniques for the genetic characterization of *Trichoderma* spp. due to the possibility of detecting DNA polymorphisms for very closely related strains (20, 21). RAPD-PCR detects differences along the entire fungus genome, not only in particular sequences. Thus, this system is helpful in characterizing fungus isolates over long periods (22).

The experiments of RAPD-PCR for genomic DNA of *T. harzianum* isolates were started after obtaining the optimum condition and the following results have been obtained. In this study, 15 primers were used; nine primers of 15 random primers Table (2) amplified their targets across all the isolates. The products of RAPD were analyzed as shown in Table(2) and six primers did not give result (OPA-12, OPC-05, OPE-20, OPF-16, OPG-08 and OPN-16) because there primers did not find any complementary region on the template DNA of *T. harzianum* isolates (20).

The nine RAPD primers generated a total of 128 amplified main bands and 120 polymorphic bands, this indicate that there is a significant genetic diversity among the isolates of *T. harzianum*. Based on the number of revealed bands per primer, they varied between 11 and 20 main bands Table (2). The high number of RAPD patterns was shown in primer OPB-14 (20 main bands), while the lowest number was shown with primer OPY-19 (only 11 main bands). The high number of bands may be attributed to the presence of high number of primer annealing sites on the template DNA of the tested isolates. The highest number of bands usually gives a better chance for detecting polymorphisms among individuals (23).

High number of polymorphic bands was presented with primer OPB-14 (19 bands), while the lower level of polymorphism was shown with primer OPY-19 (9 bands) Table(2). The differences in molecular weights of polymorphic bands reflect the number of targets for each primer site locus within the DNA in question (24). Polymorphisms at DNA level may occur as a result of several types of mutations such as single base change in the primer-annealing site in the genome that prevents amplification by introducing a mismatch at 3‘end of a DNA segment (25), this can be considered the main reason for polymorphisms at DNA of *T. harzianum* isolates because the isolates Th1, Th2, Th3, Th4, Th5 and Th6 are mutants obtained by UV radiation. Other sources of polymorphisms may include deletion of a priming site, insertion that render priming sites to be too distant to support amplification, or they may change the size of DNA segment without preventing its amplification (26). The failure of many primers to amplify DNA may be due to their need to special requirements for amplifications in terms of PCR-reagents or temperature profile, since all of the reaction parameters were identical for all primers. Moreover, differences in banding patterns are likely due to specific requirements of a given primer. The G+C content of the primer may further interfere with PCR yield (27).

Furthermore, the current study focused on determining the efficiency of each primer Table (2). The primer OPB-14 had the highest calculated efficiency (15.63), while primer OPY-19 demonstrated the lowest efficiency of (8.59). The discriminatory power of the various primers varied greatly. The highest discriminatory power showed with primer OPB-14 (15.8), which produced distinct fingerprints. However, primers OPY-19 revealed the minimal discriminatory power of 7.5 Table (2). This can be explained by primer capacity to reflect variations in the genomic of microorganisms to the total number of variations. The primer with high discriminatory power has a capacity to obtain high number of polymorphic bands to the total number of polymorphisms (28). Determining the efficiency and discriminatory power of any primer is important, since it can be used to reduce the extent of blind screening of the primer used to analyze the molecular bases of other individuals of the same species. This will eliminate the possibility of producing monomorphic patterns between genetically different individuals. It will also increase the possibility of obtaining proper fingerprints of individual strains (29).

On the other hand, the results in the Table (2) showed that all primers in this study gave unique bands, these unique bands may indicate as a fingerprinting or marker for these

isolates. So that all of *T. harzianum* isolates (T1, T2, Th1, Th2, Th5 and Th6) in this study were distinguished by different primers which produced unique bands except isolate Th3 and Th4 have not unique bands, and all primers in this study gave unique bands for isolate T2 except OPY-02 Fig.(1).

The origin of these unique markers maybe attributed to mutation at the priming site of the primers or to insertion/deletion mutation in the distance between the reverse and forward priming sites of the primers. The presence of unique RAPD markers among the various *T. harzianum* genotypes confirms the utility of the approach for fingerprinting purposes. RAPD fingerprinting has a number of potential applications including the determination of isolate purity, efficient use and management of genetic resources collection, particularly in identification of mislabeled accessions (30).

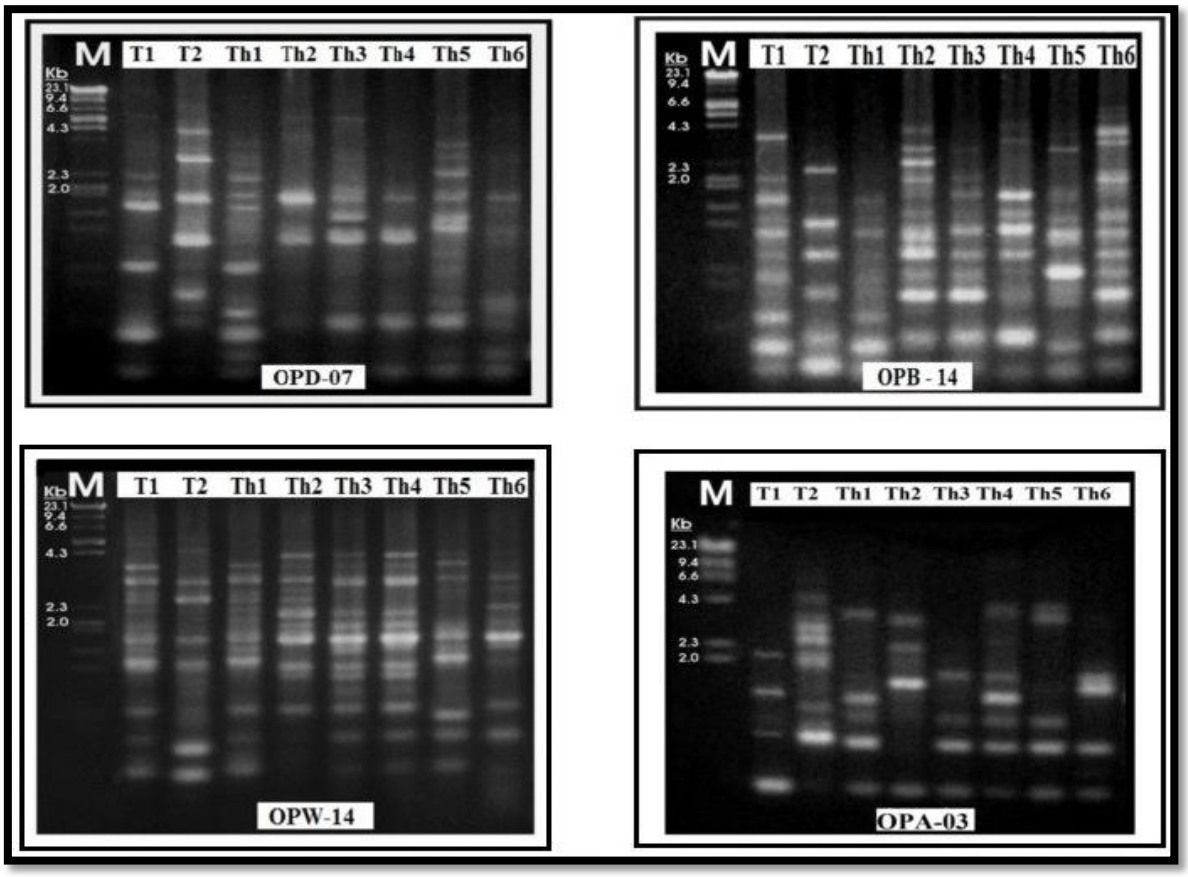

Fig.(1): Agrose gel electrophoresis of PCR-RAPD reaction for random primer OPA-03; OPB-14; OPD-07 and OPW-14 for DNA samples of Trichoderma harzianum (under optimum condition). Bands were freactionated by electrophoresis on 1.2% agrose gel (2hr, 5v/cm, 0.5x Tris-biorate buffer) and visualized by ethidium bromid staining.

Lanes of eight samples of Trichoderma harzianum.
M: λDNA

Table (3)
The genetic distance values for eight Ttichoderma harzianum isolates according to RAPD markers.

	T1	T2	Th1	Th2	Th3	Th4	Th5	Th6
T1	0.00000							
T2	0.82031	0.00000						
Th1	0.25140	0.75124	0.00000					
Th2	0.48720	0.62883	0.55533	0.00000				
Th3	0.55949	0.72286	0.57743	0.35997	0.00000			
Th4	0.54699	0.71855	0.50690	0.31930	0.11088	0.00000		
Th5	0.55949	0.96402	0.51859	0.70090	0.33024	0.42933	0.00000	
Th6	0.49272	0.76942	0.60597	0.31568	0.35445	0.38788	0.48120	0.00000

Genetic fingerprinting, phylogenetic diversity and genetic distance of *T. harzianum* isolates were evaluated using RAPD technique. Such information will be useful in its classification, epidemiological survey, ecology and diagnosis. Table (3) summarize the values of genetic distance for eight *T. harzianum* isolates. The genetic distance values ranged from 0.11088 to 0.96402. It was clear that the lowest genetic distance (0.11088) was found between isolates Th3 (Bavestin tolerant mutant obtained from T1) and Th4 (Bavestin tolerant mutant obtained from T2), whereas the highest genetic distance (0.96402) was found between isolates T2 (isolated from agricultural soil) and Th5 (Bavestin tolerant mutant obtained from T1).

Cluster analysis and phylogenetic tree depending on genetic distance in order to reveal the genetic relationship between the eight *T. harzianum* isolates were subjected RAPD-PCR analysis Fig.(2). Analysis of this dendrogram, the overall result indicated that all *T. harzianum* isolates were classified into two major group genotypes. The first major group was further separated into two subgroups, the first subgroup included isolate T1 and Th1, while the second subgroup included two divisions, the first isolate Tha5 and the second includes two divisions, the first isolate Th2 and Th6 while the second includes isolate Th3 and Th4. The second main group included only isolate T2. Maybe attributed the high level of genetic diversity among the *T. harzianum* isolates, to the fact that most of these *T. harzianum* isolates were the result of mutagenesis by UV.

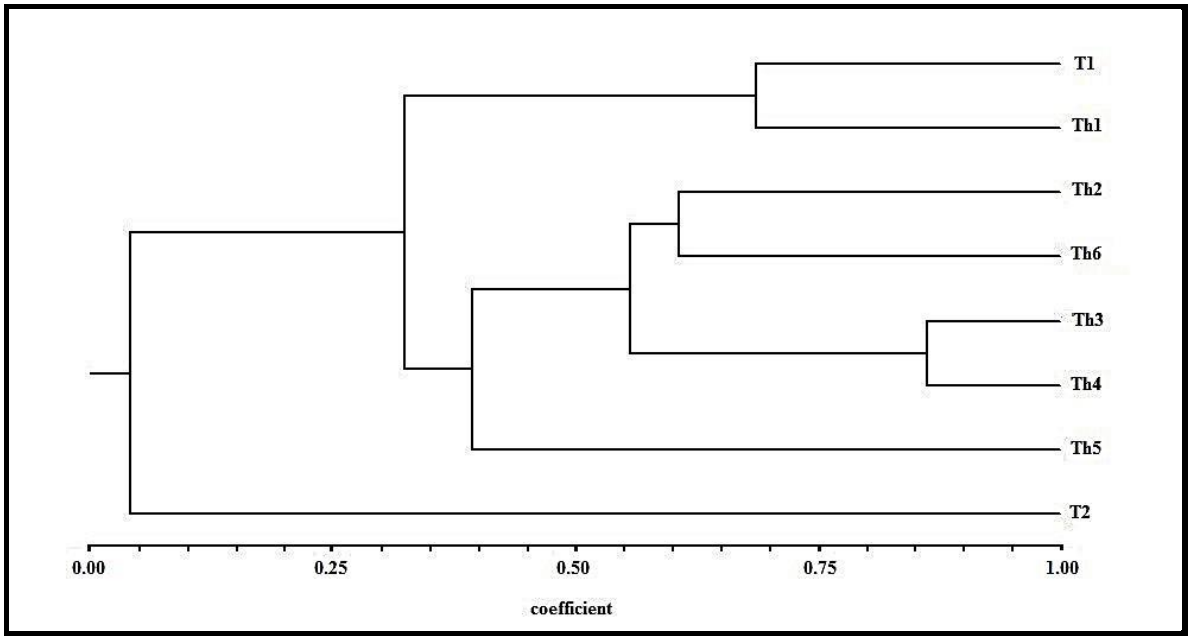

Fig.(2): Dendrogram showing Genetic diversity among eight Ttichoderma harzianum isolates according to RAPD technique.

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الخلاصة

استعملت تقنية RAPD المعتمدة على تفاعل PCR للتوصيف الجزيئي وتقييم العلاقة الوراثية لثمان عزلات من الفطر *Trichoderma harzianum*. وجرى استخلاص الدنا من كل عزلة وبتركيز نهائي قدره 400–600 مايكروغرام لكل 2–3 غم من الغزل الفطري الرطب وبنقاوة 1.6–1.8. وجرى مضاعفة كل عينة من عينات الدنا بواسطة 15 بادئا واظهرت النتائج بالترحيل كهربائيا خلال هلام الاكاروز ذي التركيز 1.2% والمصبغ بالمادة المتألقة بروميد الايثيديوم والتصوير تحت الاشعة فوق البنفسجية (U.V.). أخفقت ست بادئات في دعم عملية التضاعف لاي دنا مجيني بينما انتجت البادئات التسعة المتبقية ما مجموعه 128 حزمة دنا رئيسية (بمعدل 11–20 لكل بادئ) للعزلات الفطرية الثمانية. ومن هذه الحزم كانت 120 حزمة متباينة (بمعدل 9–19 لكل بادئ). وكان اقل البادئات كفاءة في اسناد التضاعف هو البادئ OPY–19 (بكفاءة 8.59%)، بينما الاكثر كفاءة كان OP–B14 (بكفاءة 15.63%). البادئ OP–Y19 حقق اقل قوة تمييزية (7.5%) بينما البادئ OP–B14 حقق اعلى قوة تمييزية (15.8%). ساعدت تقنية RAPD من تحديد البصمة الوراثية لستة عزلات من الثماني عزلات المدروسة من خلال ظهور حزمة مؤشرة فريدة حين مضاعفة الدنا من كل عزلة من هذه العزلات مع واحد او اكثر من البوادئ التسعة اعلاه. التحليل العنقودي للمسافات الوراثية للعزلات الثمانية قسمها الى مجموعتين رئيسيتين من الطرز العرقية.