Genetic Diversity of *Pinus halepensis* and *Juniperus phoenicea* Trees Grown at Mediterranean Forests in East of Libya

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ABSTRACT

The relationship between genetic diversity and eco-geographical parameters in wild trees, suggests that it may be a source of useful genes related to adaptation and stress responses. The present work was designed to survey three locations of different altitude in El-Jabel El-Akhdar (Green Mountain) area, Libya, with the aim at acquiring information on the genetic diversity of *Pinus halepensis* Mill and *Juniperus Phoenicea* L. in that region. Random Amplified Polymorphic DNA (RAPD) was used to compare genetic diversity between trees under this study. Results of *P.halepensis* gave 24 polymorphic bands from 95 amplified bands varied in molecular weight and intensity. On the other hand, results of *J. phoenicea* showed that among the four primers tested, 19 polymorphic bands from 84 bands were useful to characterize the samples, ranging from 200 to 1500 bp. To study the relationship between genetic diversity and wood formation under the influence of environmental differences, tracheid diameters analysis was conducted on both earlywood and latewood for the tested wood species at different altitudes. The results of *P. halepensis* indicated that there are significant differences among the three tested altitudes. For *J. phonicea*, the results revealed a significant difference among the recorded tracheid diameters in the earlywood area at different altitudes. Dendrograms were constructed using UPGMA algorithm based on the similarity index values for each species. They indicated that some trees from different altitude were nearly identical which confirmed by the results of tracheid diameters analysis.

Key words: ***Juniperus phoenicea*; *Pinus halepensis*; RAPD; genetic diversity; tracheid diameters.**

INTRODUCTION

The natural vegetation of El-Jabel El-Akhdar (Green Mountain) area comprises most of plant forms that exist in the Mediterranean area. The topography of this area includes three classes of different levels of altitude. There is a significant variation in the topography of the El-Jabel El-Akhdar area which includes valleys, hills and plains. These levels differ from each other in their climate. The first level close to sea shore represents plain lands and Mediterranean climate. The mean of its height above sea level does not exceed 200 m. The second level, with its maximum height of about 460 m above sea level, represents an intermediate case between the first and the third levels. The maximum height of the third level of the mountain is about 880 m above the sea level. This level is characterized by cold winter climate, but it is hot in the most of its parts during summer (El-Zwaam, 1995).

The genus *Juniperus* L. (Cupressaceae), an aromatic evergreen plant, consists of up to 68 species around the world was evaluated by Kasaian *et al*. (2011). *J. phoenicea* is a small tree that is native to the northern lands bordering the Mediterranean Sea from Portugal to Palestine. It is also native to North Africa found in Libya, Algeria, Morocco and Canary Islands (Gaussen, 1968). This plant species is a conspicuous constituent of the vegetation of the Mediterranean basin, particularly in Al-Jabel Al-Akhdar region. It constitutes about 80% of the total number of the trees and evergreen shrubs that exist in Al-Jabel Al-Akhdar area (Anonymous, 2005).

Pinus halepensis is evergreen tree widespread basin Mediterranean Sea from

Spain and Morocco to the eastern Mediterranean in Greece, Jordan, Libya (Green Mountain area), it has been growing in pure natural forests, the most widespread species in the Green Mountain area and grow at different heights from sea level. (Zunni, 1977).

The subsequent effect of domestication on the composition of genetic diversity of economically important tree species has received little attention. Genetic diversity is crucial because it allows species to adapt to local conditions and evolve in new environments (Koskela *et al*., 2007). Genetic diversity is being impacted by natural and human factors. With changing climate, genetic depletion through plantations and changes in populations' genetic structure can have detrimental consequences for the adaptive potential of tree populations to novel environments (Pandey *et al*., 2004).

Random Amplified Polymorphic DNAs (RAPDs) have been used in several studies and have proved a validity in systematics (Adams, 1999, 2000a-b, 2001). Besides its technical simplicity, the most important advantage is that no previous knowledge of the genome is required for its application, which makes this method suitable for the analysis of not well-studied species.

Genetic diversity of *Pinus halepensis* Mill. was analysed by Gómez *et al.*,(2001) in nine populations (six Spanish populations and one each from Tunisia, France and Greece). Twenty four RAPD loci were amplified with 60 mega-gametophyte DNA samples from each population. Their results showed genetic variation which suggested that RAPD markers are valuable for the estimation of genetic diversity in *P. halepensis* and for the study of the divergence among population, allowing thinking that eastern Mediterranean populations of *P. halepensis* have undergone a different history from those of the western Mediterranean area.

Genetic structure and diversity of ten natural populations of *Juniperus phoenicea* L. from the western part of the species was studied using RAPD markers by Dzialuk *et al*. (2011). Their results and discussion revealed that among 10 analyzed primers only 3 reproduced consistently across successful PCR reactions and gave 45 loci. The significant level of differences between European and African populations can result the earlier divergence and considerably low level of gene flow between them, or a different mutation rate within population of different continent.

Determining the genetic differences shows the extent of environmental impact and geographical diversity on the genetic composition of the type one as well as linking resulting from DNA, and this helps in identifying models distinctive qualities undesirable such as drought tolerance. Meanwhile, it can be used for genetic improvement and the propagation processes. The aim of this research was to study the genetic diversity of *Pinus halepensis* and *Juniperus phoenicea* trees using RAPD technique and determine the degree of genetic kinship within the two types in order to rapid propagation of genetic models distinct from them. The relationship between genetic diversity and Tracheid diameters under the influence of environmental differences was investigated.

MATERIALS AND METHODS

1- Plant material and study locations:

Plants under this study are from the two species of *Pinus halepensis* Mill and *Juniperus Phoenicea* L. Samples were collected from three locations of different altitude in El-Jabel El-Akhdar (Green Mountain) area, Libya, i.e. 284 m, 413 m and 830m for *P.halepensis* and 284 m, 413 m and 780 for *J. Phoenicea* (Table, 1).

2- Samples preparation

One tree from each location (i.e. six trees), from *Juniperus phoenicea* and *Pinus halepensis* was selected from AL-Jabel AL-Akhder area, Libya, Tabel (1). The leaves were collected randomly from each tree, well-isolated parts of their crowns at 1 m ground level.

Scion's standard DNA extraction procedure was don adapted to Cato and Richardson (1996). Chopped needle tissue (300mg) was homogenized with a mortar and pestle under liquid nitrogen, placed into a 2mL tube and 1 mL of pre-warmed (65 ºC) CTAB buffer added. After one hour incubation at 65 ºC, cellular debris was pelleted by centrifugation at 18000x g, and 700 µl of supernatant was transferred to a fresh tube containing RNase A to a final concentration of 100mg/mL. After a 30 min incubation at 37 ºC, 1/5 volume 5 M NaCl and 1x volume chloroform: isoamyl alcohol (24:1) were added, the tube was mixed by gentle inversion and centrifuged at 18000x g for 20 min. The aqueous phase was removed and re-extracted with another 1x volume chloroform: isoamyl alcohol. After centrifugation at 18000x g for 20 min, the aqueous phase was transferred to a fresh tube, an additional ethanol/sodium acetate precipitation to the re-suspended DNA. 1/10x volume 3M sodium acetate and 2.5x volumes 96% ethanol added, mixed by gentle inversion and incubated at -5 ºC for 2 hours. DNA was pelleted at 18000x g for 10min, washed with 1x volume 96% ethanol, and air-dried before resuspension in 50µL sterile water. (Telfer *et al*., 2013).

4. RAPD Profiling

Four primers (Metabion, Germany) were used in this study (Table 2). Polymerase chain reaction (PCR) amplifications were carried out in a 25 µl volume (Table 3). Amplification was performed in BiometraT Gradient Thermocycler. PCR steps are shown in Table (4).

Table 1: Description of the Location study.

Level	Location	Longitude	Latitude	Altitude
First	Alwsita	32° 510 ' 9.4"	21° 39 ' 60.5"	248 m
Second	Wadi Alkuf	32° 40 ' 23.9"	21° 33 ' 00.1"	413 m
Third	Sidi Alhomre *P. halepensis*	32° 32 ' 165"	21° 47 ' 466"	830m
	Ashnishen *J. phooenices*	32° 36 ' 435"	21° 56 ' 000"	780m

Table 2: Code and sequence of four different RAPD primers used in the present study.

No	Oligo Name	Sequence
1	01	5'-CCG ACA AAC C 3'
2	02	5'-TCA ACG GGA C 3'
3	03	5'-TCC CCA TCA C 3'
4	04	5'- ACG CTG TGC T 3'

Table 3: Components of RAPD reaction.

Component	Quantity/Reaction (µL)
Master mix (Thermo)	12
Primer	2
DNA	2
H_2O	9
Total	25

Table 4: The amplification of used protocol.

Step	Temperature ºC	Time	Number of cycles
Initial denaturation	95	4min	1
Denaturation	95	30s	40
Annealing	35	30s	
Extension	72	1 min	
Final Extension	72	5min	

5- Electrophoresis

Reaction products were resolved by electrophoresis in 1% agarose gel with 0.5 X TBE buffer (Brody *et al*., 2004). Run was started at 80 volts for about 30 min. Fractionated bands were visualized by staining with ethidium bromide, and tramsillumination of the gel under short-wave UV light. A Gene Ruler™ 1000bp ladder (Promega®) was used as a molecular weight standard.

6- Data analysis

RAPD data were scored for presence (1) or absence (0), and used to create a data matrix. The Phoretix ID image analysis system (Phoretix International, London) was used in order to integrate the data of RAPD bands. A similarity dendrogram among the species was produced using UPGMA cluster analysis program.

7-Tracheid diameters determination

The wood samples were taken from the same tree species under study (*J. phoenicea* and *P. halepensis*). Discs (5 cm thickness) from each tree were sawn at breast height (1.3 m) of the stem to represent all sample trees (nine tree) at the three tested altitudes. The samples to be measured were prepared by sawing a bar from the pith to the bark of each disk, and the bars were then air dried. Each bar was divided to three equal blocks. The wood blocks were saturated with distilled water prior to sectioning. Thin cross sections (20-30 µm) were obtained using a laboratory digital microtome. Sections were stained with Safranin and images were captured with digital camera attached to a light microscope. Cross-sectional dimensions of individual tracheid for earlywood and latewood were measured by calibrated slide by a length of 2 mm (Irbe *et al.*, 2013).

RESULTS AND DISCUSSION

The DNA fingerprints as revealed by RAPD have been found to be useful in a number of approaches in botanical research. RAPD markers are DNA fragments generated by the amplification of genomic DNA through PCR reaction using a single primer of arbitrary nucleotide sequences in each reaction.

1- Pinus halepensis

In the present study, agarose gel electrophoresis for the RAPD-PCR amplified DNA products of *P. halepensis* had shown in Figure (1) and Table (5).

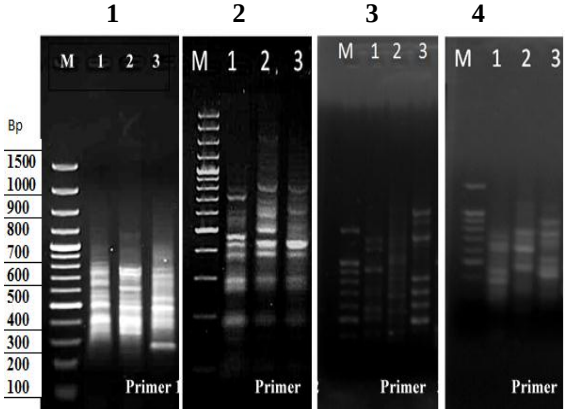

Figure 1: RAPD fingerprints profiles of the accessions of *P. halepensis*, generated by PCR amplification using primers No. 1, 2, 3 and 4.

The data obtained were constructed as a matrix of the binary character states. The presence or absence of amplified fragments of a certain size in RAPD patterns was considered as state 1 or 0, respectively. The results revealed that, the four useful primers gave 24 bands from 95 amplified bands detected ranging from 100 to 900 bp.

Table 5: Total No. of bands for *P. halepensis*, using 4 RAPD primers and the total No. of polymorphic bands.

No. of primer	Total no. of bands			Total bands	polymorphic bands	% of polymorphic
	1	2	3			
1	8	9	7	24	5	20.8
2	12	11	8	31	6	25
3	8	5	9	22	10	41.7
4	5	6	7	18	3	12.5
Total	33	31	31	95	24	100

Primer No. 2 had the highest total number of total bands (31 bands), while Primer No. 4 gave the lowest number of total bands (18 bands). Primer No. 3 had the highest total polymorphic number of different PCR bands (10 bands), while primer No.4 showed the lowest total polymorphic number of bands (3 bands). However, Primer No. 3 showed high percent of polymorphic bands 41.7%.

The relationships within and between groups were estimated by a UPGMA cluster analysis of GS matrices (Figure 2). Samples No. 2 and 3 were more closely related with each other more than the first sample. Sample No. 1 growing at (280m altitude) near of the sea level, followed by a sample No. 2 growing at (413 m altitude) then the sample No. 3 (830 m altitude), i.e., the highest altitude above sea level .These results and the genetic distance allow us to think that populations of *P. halepensis* have undergone different history at the three altitudes. These obtained data were consistent with similar studies carried out by (Gómez *et al.*, 2001).

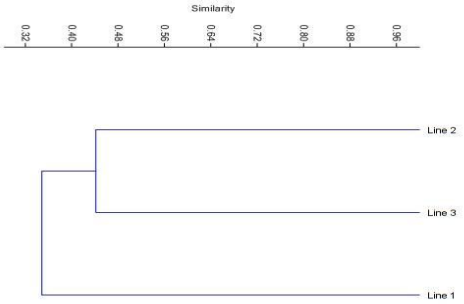

Figure 2: Dendrogram for the phylogenetics relationships among the three samples of *P. halepensis* based on RAPD data.

Where:

Line 1=248 m, Line 2 = 413 m and line3 = 830 m

In this study we have shown that RAPD markers are useful for generating candidate-specific markers of *P. halepensis*. The detection of higher level of genetic diversity and the identification of specific population alleles would provide good information about the genetic structure and could help in selecting the most variable genotypes to conserve and use them as a source for the national forestation. These obtained data were consistent with similar studies carried out by (Choumane *et al*., 2004 and Lara *et al*., 2010).

2- Juniperus phonicea

The RAPD-PCR amplified DNA products of *J. Phonicea* shown in Figure (3) and Table (6).

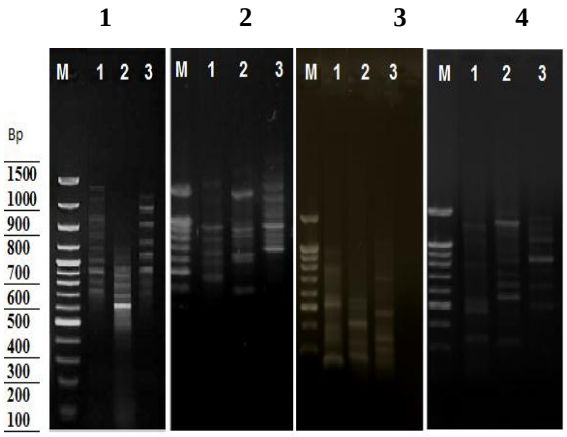

Figure 3: RAPD fingerprints profiles of the accessions of *J. Phonicea*, generated by PCR amplification using primers No. 1, 2, 3 and 4.

Among the four primers tested, 19 polymorphic bands from 84 bands proved useful to characterize the samples, ranging from 200 to 1500 bp. The highest number of polymorphic loci (7 polymorphic bands) was exhibited with primer No. 1 and the lowest number (3 polymorphic bands) was with primers No. 3 and 4. Primer No. 1 had the highest total number of total bands (32 bands), while Primer No. 4 gave the lowest number of total bands (16 bands). Moreover, Primer No. 1 showed high percent of polymorphic bands 36.8%.

The relationships within and between groups were estimated by a UPGMA cluster analysis of GS matrices (Figure 4). Samples No. 1 and 2 were more closely related with each other more than the third sample. Sample No. 1 growing at (280 m altitude) above sea level, while, sample No.2 growing at (413 m altitude). On the other hand, it is different from the sample No.3 which growing at (780 m altitude). These findings are similar to those obtained by Adams *et al*. (2006).

Table 6: Total No. of bands for *J. Phonicea* using 4 RAPD primers and the total No. of polymorphic bands.

No. of primer	Total no. of bands			Total bands	polymorphic bands	% of polymorphic
	1	2	3			
1	13	10	9	32	7	36.8
2	5	6	8	19	6	31.6
3	7	5	5	17	3	15.8
4	5	6	5	16	3	15.8
Total	30	27	27	84	19	100

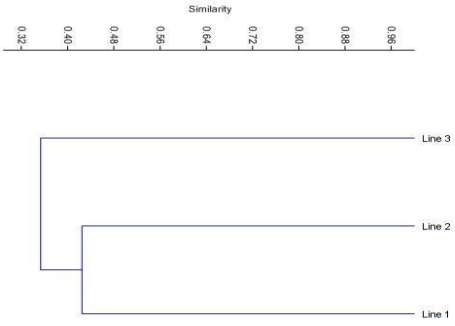

Figure 4: Dendrogram for the phylogenetics relationships among the three samples of *J. Phonicea* based on RAPD data.

Where:

Line 1=248 m, Line 2= 413 m and line3= 780 m

Furthermore, our study showed that RAPD markers are useful for generating candidate-specific markers of *J. Phonicea*. RAPD markers can be used to distinguish among *J. phonicea* at different altitudes. Where the origin of a specific juniper cultivar is uncertain, analysis of genetic distance can pinpoint close relatives. These obtained data were consistent with similar studies carried out by (Adams *et al*., 2006 and Dzialuk *et al*., 2011).

3- J. Phonicea* and *P. halepensis

We have investigated the possible relationship between the two tree species that both relate to the same place in figure (5). Each species represents a total independent group and there is no relationship between them, while the relations are between the highs only which confirms previous findings.

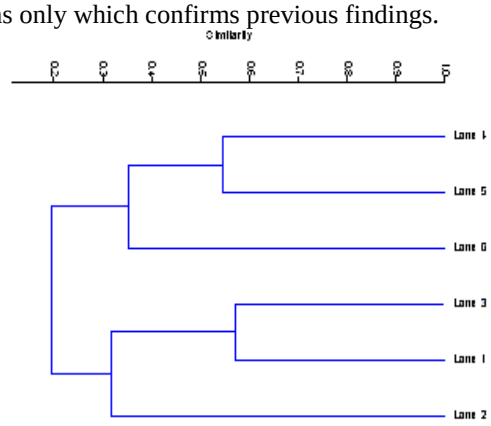

Figure 5: Dendrogram for the phylogenetics relationships among the samples of *P. halepensis* and *J. phonicea* based on RAPD data.

Where:

P. halepensis Line 1 = 248 m, Line 2 = 413 m and Line 3 = 830 m

J. phonicea Line4 = 284 m, Line 5 = 413 m and Line 6 = 780 m

***4-* Tracheid diameters determination**

The average of tracheid diameters (µm) in both earlywood (EW) and latewood (LW) of *P. halepensis* and *J. phonicea* growing at different altitudes were reported in Table 7. For *P. halepensis*, the results indicated that there are significant differences among the three tested altitudes. However, it is clear that the tracheid diameters are associated with climatic changes with the highest value at the altitude of 830 m, which were 27.26 and 10.00 µm of early wood and latewood, respectively. In the meantime, the lowest value 13.60 and 3.6 µm for the tracheid diameters were observed at the altitude of 280 m in early wood and late wood, respectively. Based on the obtained data, it can be noted a good correlation the tracheid diameters and the climatic factors, which certainly varied up rise on the surface of the sea. These obtained data were consistent with similar studies carried out by Xu *et al*. (2013), Xu *et al*. (2014), and Irbe *et al*. (2013). Interestingly, it is found that the tracheid diameter at the altitudes of 830 m and 413 m were too convergent despite the differences in the environmental factors in terms of temperature, precipitation and soil characteristics. This was interpreted genetically by testing the relationships within and between groups estimation using UPGMA cluster analysis of GS matrices. It was found that the samples obtained from the second altitude (413 m) and the third one (830 m) were approximately similar and more closely with each other when compared with the samples obtained from the first altitude (280m) (Fig. 2). On the other hand, for *J. phonicea* the results revealed significant differences among the recorded tracheid diameters in the early wood area at different altitudes which showing again the effect of the climatic factors. On the contrary, no significant differences were observed among tracheid diameters of late wood area at the three tested altitudes where the recorded value was 10.00 µm. This probably could be attributed to the genotype of tested species, which emphasized using UPGMA cluster analysis of GS matrices (Fig.4).

Table 7: The average of tracheid diameters (µm) in both early wood (EW) and late wood (LW) of *P. halepensis* and *J. phonicea* growing at different altitudes.

Altitude (m)	*P. halepensis*		*J. phonicea*	
	EW	LW	EW	LW
280	15.60^{b}	3.6^{b}	21.33^{a}	10.00^{b}
413	25.86^{a}	10.00^{a}	20.73^{b}	10.00^{b}
780 - 830	27.26^{a}	10.00^{a}	25.60^{c}	10.00^{b}

Means with the same superscript letter are not significantly different at 0.05 level of probability.

4-Summary and Conclusion

- Random Amplified Polymorphic DNA (RAPD) was used to compare genetic diversity in *Pinus halepensis* and *Juniperus Phoenicea* grown at different altitudes in AL-Jabel Al- Akhdar region, Libya.
- For *Pinus halepensis*, the results revealed that, the four useful primers gave 24 bands from 95 amplified bands detected ranging from 100 to 900 bp. Primer No. 2 had the highest total number of total bands (31 bands), while Primer No. 4 gave the lowest number of total bands (18 bands).
- For *Juniperus Phoenicea*, among the four primers tested, 19 polymorphic bands from 84 bands proved useful to characterize the samples, ranging from 200 to 1500 bp. The highest number of polymorphic loci (7 polymorphic bands) was exhibited with primer No. 1 and the lowest number (3 polymorphic bands) was with primers No. 3 and 4.
- The averages of tracheid diameters at the altitudes of 830 m and 413 m were too convergent despite the differences in the environmental factors in terms of temperature, precipitation and soil characteristics. This was interpreted genetically by testing the relationships within and between groups estimation using UPGMA cluster analysis of GS matrices.

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الملخص العربى

التنوع الوراثي في أشجار الصنوبر الحلبي والعرعر النامية في غابات البحر الأبيض المتوسط بشرق ليبيا

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العلاقة بين التنوع الجيني والبيئة الجغرافية التي تنمو فيها الأشجار تشير إلى أنها قد تكون مصدر للجينات المتعلقة بالإستجابات للتكيف والأجهاد. تم استخدام تقنية Random Amplified Polymorphic DNA (RAPD) المعتمدة على التضخيم العشوائي لقطع DNA المتباينة شكلياً بهدف دراسة التنوع الجيني لأشجار الصنوبر الحلبي والعرعر الفينيقي النامية على ثلاثة إرتفاعات مختلفة بمنطقة الجبل الأخضر– شرق دولة ليبيا. أعطت نتائج الصنوبر الحلبي ٢٤ قطعة من الـDNA متعددة الأشكال حيث تظهر في بعض العينات ولا تظهر في عينات أخرى وذلك من مجموع ٩٥ قطعة مضاعفة من الـ DNA ظهرت مع البادئات الأربعة المستخدمة. من ناحية أخرى أظهرت نتائج العرعر الفينيقي ١٩ قطعة متعددة الأشكال من مجموع ٨٤ قطعة من الـ DNA ذات وزن جزيئي bp ٢٠٠–١٥٠٠ . ولدراسة تأثير العلاقة بين التنوع الوراثي والإختلافات البيئية على صفات وتكوين الخشب في الأشجار تحت الدراسة فقد تم قياس قطر القصيبات في كلٍ من الخشب المبكر والخشب المتأخر في كلا النوعين من الأشجار علي الإرتفاعات المختلفة حيث أظهرت النتائج وجود إختلافات معنوية في قطر القصيبات في الخشب المبكر والخشب المتأخر للنوع الواحد عند الإرتفاعات المختلفة مما يعكس تأثير التنوع الوراثي والاختلافات البيئية بفعل الإرتفاع عن سطح البحر على قطر قصيبات الخشب. وبناء على ذلك فيمكن القول أن التنوع الوراثي داخل النوع الشجري وكذلك الاختلافات البيئية لها تأثير كبير على صفات الخشب مما ينعكس على تحديد نوعية وجودة الخشب الناتج من الأشجار.

الكلمات الدليلية: العرعر الفينيقي، الصنوبر الحلبي، التنوع الوراثي، قطر القصيبة، RAPD.