PHYlogenetic Evaluation Of Some *Pinus Species* From Different Genetic Resources Using Protein, Isozymes, Rapd And Issr Analyses

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Abstract: In the present study, we attempted to characterize eight different genotypes of three species of *Pinus*. *Pinus* were collected from different sources (Turkey, Tunisia and Syria) which were discriminated by using protein, isozymes (Peroxidase and Alkohol Dehydrogenase), RAPD and ISSR analyses. The generated profiles of protein and isozymes revealed high levels of polymorphism among the eight studied species genotypes represented as present ; absent fragments and differences in banding patterns density. The generated profiles of eight RAPD primers and five ISSR primers successfully generated reproducible polymorphic products. Results of these primers recorded a sum of 170 fragments, which were identified as 86 polymorphic fragments and 84 monomorphic ones in all genotypes under study. The polymorphic fragments were scored 19 unique fragments. These unique fragments were used to discriminate among the three *Pinus* species and their genotypes. The constructed dendrogram based on a combined data of protein, isozymes, RAPD and ISSR-PCR markers separated the three *Pinus* species and their genotypes into two major groups. The first group included *Pinus brutia* (from Syria) and *Pinus brutia* (from Tunisia) genotypes, while the second group is divided into two subgroups. The first subgroup included only *Pinus halepensis (*From Syria*)* genotypes, while the other subgroup included *Pinus brutia (*From Turkey*), Pinus halepensis (*From Tunisia*), Pinus pinea (*From Turkey*), Pinus pinea (*From Syria*)* and *Pinus halepensis (*From Turkey*)* genotypes In general the overall results indicated to the possible use of protein, isozymes, RAPD and ISSR analyses to detect some species-specific markers for the three *Pinus* species and their genotypes that can be used to discriminate among them and also, to detect genetic relationships among these three species and their genotypes which can be used in breeding programs.

[Gad M.A. and Mohamed S.Y. **PHYlogenetic Evaluation Of Some *Pinus Species* From Different Genetic Resources Using Protein, Isozymes, Rapd And Issr Analyses.** Journal of American Science 2012;8(3):311-321]. (ISSN: 1545-1003). http://www.americanscien. 42

Keywords: *Pinus sp.*, plant genetic resources, K (Turkey), S (Syria), T (Tunisia), protein, peroxidase, Alcohol Dehydrogenase, RAPD-PCR, ISSR-PCR.

1. Introduction

Trees have long been transferred from one country to another for economic, aesthetic, scientific and other reasons. The maintenance of biodiversity and wood production should be considered as equally valuable objectives. It is of primary importance to consolidate the existing knowledge and promotion of new research on the effects of tree species introduction and their influence on ecosystem sustainability and biodiversity **(**Engelmark *et al*., 2001).

Pine forests constitute some of the most important renewable resources supplying timber, paper and chemical industries, among other functions (Santos *et al*., 2006).

Surprisingly, *Pinus brutia* subspecies *brutia* was found to be more distant from *P. brutia* subspecies *eldarica* than from *P. halepensis* (Kaundum *et al*., 1997). It was concluded that the Tunisian and French *Pinus pinea* provenances seemed to be the most suitable for afforestration in crystalline provenance in France compared to those from other provenances such as Turkey, Spain, Greece, Lebanon, Morocco, and Italy (Court-Picon *et al*., 2004). Moreover, Weinstein (1989) found that phenological characters of *P. halepensis* and *P. brutia* behaved in variable patterns according to their original races, while those came from the same race origin characterized similarly. Besides, provenance of *P. halepensis*, found within the East European race, were influenced by introgression. The relationship between seed origin of *P. halepensis* and susceptibility to the Israeli pine bast scale, and the infestation of *P. brutia* and *P. eldarica* by this pest, were studied by Mendel (1984). He noticed that the Greek and Israeli provenances displayed a lower susceptibility to attacks by the pest than did the North African and Spanish provenances. It is possible that the resistance was transferred from *P.brutia* to *P. halepences* in Greece, where the natural distribution of these pine species overlapped. On the other hand, the relative resistance of the Israeli provenances may be due to a long coexistence in Israel of injuring pest and *P. halepensis*.

The response to climate change will include changes in species composition, but adaptation through genetic change may also be possible (Savolainen *et al*., 2004).

The past limitation associated with pedigree data ; morphological, physiological and cytological markers for assessing genetic diversity in cultivated and wild plant species have largely been circumvented by the

development of DNA markers such as randomly amplified polymorphic DNAs (RAPD) (Williams *et al*., 1990). This method has proven to be useful for germplasm identification and elucidation for genetic relationships of numerous plant cultivars and species (Halward *et al*., 1992 ;Levi and Rowland, 1997). Also, such technique is simple to use and does not require the use of radioactive materials, as well as, it enables to detect a significant degree of polymorphism. However, the generated DNA polymorphism reflects both the distance between two annealing sites and the pattern of their distribution throughout the genome of a particular cultivar or species (Williams *et al*., 1990).

Khadari *et al*. (2003) used molecular markers to characterize 100 accessions of olive and to study genetic relationships among them. A total of 497 olive trees were genotyped using 32 RAPD markers. They identified 114 RAPD fragments and detected several cases of mislabeling, synonymy and homonymy. This study allowed to construct a molecular database for the reference collection and to analyze genetic diversity for further prospecting, and for introducing new olive accessions. Kim and Ko-Kwang (2004) used RAPD technique to identify 33 Asian pears (*Pyrus* spp.). Nine primers out of 18 used primers were produced distinct and reproducible fragments. Most of these Asian pears could be identified. Neis genetic distance was used to construct the dendrogram, which differentiated the Asian pears to four clusters.

Recently, inter-simple sequence repeats (ISSR) markers have been emerged as an alternative system with reliability and advantages of microsatellites (SSRs) (Cregan, 1992). Comparison of ISSR markers with other PCR-based makers have shown their efficiency in plant breeding (Adams *et al*., 2003; Archak *et al*., 2003; Galvan *et al*., 2003; Mogg and Bond, 2003; Javidfar *et al*., 2006). As a result of these advantages, their universality and easiness of development, ISSRs markers are more and more utilized. Wolf (2005) reported that inter-simple sequence repeat (ISSRs) markers were originally devised for differentiating among closely related plant cultivars but have become extremely useful for studies of natural populations of plant.

In this study, three pine species (viz., *P.brutia, P. halepensis* and *P.pinea*) from three different geographical origins (Syria, Tunis and Turkey) have been investigated by using some biochemical and molecular markers concerning their genetic and phytogeographical origin relationships.

2.Material and Methods

2.1. Plant material

This investigation was carried out during the period of 2007-2009 at Biotechnology Research Laboratory, Horticulture Research Institute (HRI), Agricultural Research Center (ARC), Giza.

Eight plants represented three *Pinus* species (*Pinus brutia, P. halepensis*, and *P. pinea)* which were used and imported from Turkey, Syria and Tunisia, as seeds, and raised till the age of 2 years in the nursery of Timber Trees Dept., (HRI) as listed in Table (1).

2.2. Methods

2.2. 1. Biochemical Analyses

1-Protein electrophoresis

a. Protein extraction:

Total soluble protein was extracted by grinding a 0.25g of each fresh needle leave sample in a 0.9 ml of extraction buffer (10 ml 0.5 M Tris pH 6.8, 16 ml 10% SDS, 30 ml D.W.) with shaking thoroughly. The extracts were transferred to eppendorf tubes and centrifuged for 10 min. at 10000 rpm under cooling. Supernatants were transferred to new tubes to be used for SDS-PAGE analysis.

b. Protein related index:

Fractionation electrophoresis was performed under identical conditions on sodium dedocylsulphate polyacrylamide gel (SDS-PAGE) (12%W/V) in vertical slabs using BIORAD Techware 1.5 mm according to the method of Laemmli (1970) as modified by Studier (1973). The molecular weights of proteins were relatively estimated to a wide range molecular weight protein marker (Fermentas comp.).

2- Isozymes electrophoresis:

Extraction of isozymes was adopted as described by **Jonathan *et al*. (1990).** Native–polacrylamide gel electrophoresis (Native-PAGE) was performed in 12% (W/V) slab gels (Davis 1964). Then, gels were stained according to Tanksely and Rick (1980) for Alcohol dehydrogenase (Adh) isozyme and Grahan *et al*. (1964) for peroxidase isozyme. The stained gels were incubated at 37 °C in dark conditions for complete staining after adding the appropriate substrates and staining solutions.

3- Gel documentation:

Gels were photographed, scanned, and analyzed by using Gel Doc Vilber Lourmat system to capture the image and to calculate band intensities.

2.2. 2. Molecular analyses

a. DNA Extraction

Young and freshly excised leaves were separately collected from the eight *Pinus* species plants. DNA extraction was performed as described by Dellaporta *et al*. (1983). About 0.1 gm (fresh weight) of plant

tissues was ground to a fine powder in liquid nitrogen in a mortar. Before the tissue thawed, a 1 ml of extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA and 0.5 M NaCl) and a 0.2 ml of 20% SDS were added. The mixture was incubated at 65°C in a water bath for 20 minutes. Then, a 1 ml of phenol, chloroform and isoamyl alcohol (25: 24: 1) was added. Centrifugation was performed at 10.000 rpm for 10 minutes. The supernatants of each sample were separately transferred to new tubes, and then a 1 ml of chloroform and isoamyl (24: 1) was added. Centrifugation was performed at 10.000 rpm for 10 minutes. The supernatants of each sample were separately transferred to new tubes, then a 1 ml of isopropanol was added, and then kept overnight in a freezer. Centrifugation was performed at 10.000 rpm for 10 minutes. The resulted pellets containing DNA were re-suspended in a 1 ml of ethanol. Centrifugation was performed at 10.000 rpm for 2 minutes. The DNA pellets were re-suspended in a 200µl **(**TE (10 mM Tris-HCl pH 8.0 and 1 mM EDTA) of buffer. DNA was quantitatively determined and gel electrophoresis was adopted.

b. Randomly amplified polymorphic DNA-Polymerase chain reaction (RAPD-PCR) analysis:

In order to obtain clear reproducible amplification products, different preliminary experiments were carried out in which a number of factors were optimized. These factors included PCR temperature cycle profile and concentration of each of the template DNA, primer, $MgCl_2$ and Taq polymerase. A total of twenty-one random DNA oligonucleotide primers were independently used according to Williams *et al*. (1990) in the PCR reaction. Only eight primers succeeded to generate reproducible polymorphic DNA fragments as shown in Table (2).

The PCR amplification was performed in a 25 µl of reaction volume containing the following: a 2.5 µl of dNTPs (2.5 mM), a 1.5µl of $MgCl_2$ (25 mM), a 2.5 µl of 10x buffer, a 2.0 µl of DNA primer (2.5 µM), a 2.0 µl of DNA template (50ng/µl), a 0.3 µl of Taq polymerase (5U/µl) and a 14.7 µl of sterile ddwH_2O. Amplification was carried out in Techni TC-512 PCR thermocycler. The reaction was subjected to one cycle at 95 ºC for 5 minutes, followed by 35 cycles at 96 ºC for 30 seconds, 37 ºC for 30 seconds, and 72 ºC for 30 seconds. Then, a final cycle at 72 ºC for 5 minutes was performed. PCR products were run at 100 V for one hour on 1.4 % agarose gels. After electrophoresis, the RAPD fragments were visualized with an UV transilluminator. RAPD fragments were scored from the gels as present or absent in all lanes.

c. Inter Simple Sequence Repeats- polymerase chain reaction (ISSR-PCR) analyses

ISSR-PCR reactions were conducted using five primers. Amplification was conducted in a 25 µl of reaction volume containing the following reagents: 2.5 µl of dNTPs (2.5 mM), a 2.5 µl of $MgCl_2$ (2.5 mM), and a 2.5 µl of 10 x buffer, a 3.0 µl of primer (10 pmol), a 3.0 µl of DNA template (25 ng/ µl), a 1 µl of Taq polymerase (1U/ µl) and a 12.5 µl of sterile ddw H_2O. The reaction was programmed to one cycle at 94º C for 4 min. followed by 45 cycles for 1 min. at 94 ºC, 1 min. at 57 ºC, and 2 min, at 72 ºC. The reaction was finally stored at 72 ºC for 10 min., then the PCR products were separated on a 1.5 % agarose gels and fragments sizes were estimated with the 100bp ladder marker. Table (3)showed codes and sequences of these ISSR primers that produced informative polymorphic bands.

Table (1): List of Pinus species, their code number in gel lanes, and their seeds sources

Code Number	*Pinus* species	Source
1	*Pinus brutia*	K
2	*Pinus brutia*	S
3	*Pinus brutia*	T
4	*Pinus halepensis*	K
5	*Pinus halepensis*	S
6	*Pinus halepensis*	T
7	*Pinus pinea*	K
8	*Pinus pinea*	S

K =Turkey, S =Syria, T =Tunisia

Table (2): List of RAPD primers used and their nucleotide sequences.

No.	Primer code	Sequence
1	OP-A10	5´ TCGGCCATAG 3`
2	OP-A19	5´ CCTTGACGCA 3`
3	OP-B17	5´ AGGGAACGAG 3 `
4	OP-F04	5´GGTGATCAGG 3`
5	OP-L12	5` GGG CGG TAC T 3`
6	OP-L16	5` GGACCCAACC 3`
7	OP-M17	5` AAGCCTCGTC 3`
8	OP-Q15	5` GGACCCAACC 3`

Table (3): List of ISSR primers used and their nucleotide sequences.

No.	Primer code	sequence
1	HB09	5` GTGTGTGTGTGTGG 3`
2	HB10	5` GAGAGAGAGAGACC 3`
3	HB12	5` CACCACCACGC 3`
4	HB13	5` GAGGAGGAGGC 3`
5	HB14	5` CTCCTCCTCGC 3`

2.2.3. Statistical analysis:

RAPD and ISSR gels were processed using Quantity One software (Bio-Rad) which identifies DNA fragments using an optimized set of parameters (as reported in Quantity One user guide for version 4.2 Windows Bio-Rad Laboratories) which was manually adjusted by visual inspection.

Fragments identification was then used to create a qualitative data matrix of presence (1) or absence (0) that was processed using SPSS software program (version 10). Pairwise similarities among the eight genotypes were calculated using Jaccard's coefficient for qualitative data (Jaccard, 1908) according to the formula: Jaccard's coefficient =a (n-d), where n: is the total number of polymorphic bands, a: the present bands in the studied genotypes and d: the absent bands in the studied genotypes. The resulting similarity matrix (calculated with the Jaccard coefficient) was used to construct a dendrogram by means of the UPGMA (unweighted pair-group method with arithmetical averages) algorithm (Sensi *et al*., 2003).

3. Results and Discussion:

3.1.SDS-Protein banding patterns:

The electrophoretic banding pattern of proteins extracted from leaves of the eight *Pinus* species genotyprs are shown in Figure (1) and their denstrometric analysis is illustrated in Table (4). The presence and absence of these bands were assessed with (1) and (0), respectively.

SDS-PAGE analysis revealed a total number of 15 bands with molecular weights (MW) ranging from about 13.0 to 78.0 KDa. Analysis of data showed nine common bands while, the remaining six bands were polymorphic with a 40 % of polymorphism.

Leaf protein electrophoresis provides valuable evidence for taxonomic and evolutionary relationships of plant species**.** It is worthy to note that leaf protein profiles are often species – specific, highly stable and unlikely to be not influenced by environmental conditions and seasonal fluctuations (Yates *et al*., 1990**)**.

3.2.Peroxidase banding patterns:

Figures (2 and 4) represent leaf peroxidase electrophoresis banding patterns of the eight *Pinus* species genotypes. A total of five bands were characterized for the eight studied genotypes, which four of them (monomorphic) were represented in all *Pinus* species genotypes with differences in their banding patterns densities and they could be considered as common bands. The remaining band (polymorphic) was absent in only one genotype (*P. brutia* (T)) and present in the other genotypes.

3.3. Alkohol Dehydrogenae banding patterns:

Figures (3 and 5) demonstrated Alkohol dehydrogenas (Adh) banding patterns of the eight *Pinus* species genotypes. The banding patterns exhibited four bands, which two of them were present in some genotypes and absent in the others (polymorphic), while the other two bands were present in all genotypes (monomorphic) with differences in their banding patterns densities which could be considered as common bands for all *Pinus* genotypes.

3.4. Genetic similarity and cluster analysis based on protein and isozymes markers

The protein and isozymes data were used to estimate the genetic similarity among the eight genotypes of *Pinus* species by using UPGMA computer analysis (Table 5 and Fig.8). The highest similarity value (1.0) was recorded between *Pinus brutia* (T) and *Pinus halepensis* (K), while the lowest similarity value (0.1) was detected between *Pinus brutia* (K) and *Pinus halepensis* (T) genotypes and also between *Pinus halepensis* (K) and *Pinus halepensis* (T) genotypes. A dendrogram for the genetic relationship among the eight *Pinus* species genotypes is illustrated in Fig. (8) which separated them into two major groups. The first group included each of *Pinus brutia* (K), *Pinus brutia* (T) and *Pinus brutia (S)* genotypes, while the second group included the other samples (*Pinus halepensis* (T), *Pinus pinea* (K), *Pinus pinea (S), Pinus halepensis (K) and Pinus halepensis* (S)) genotypes.

3.5.Molecular genetic identification

3.5.1. Randomly amplified polymorphic DNA (RAPD) markers

The eight 10-*mer* arbitrary primers succeeded in amplifying DNA fragments for the eight genotypes of *Pinus* species as illustrated in Table (9) and Fig (9). Polymorphism levels differed from one primer to another. Only OP-L16 primer did not show any polymorphism among samples, while OP-A19 primer exhibited low level of polymorphism (25%). On the other hand, OP-A10 (38.4 %), OP-Q15 (41.7%) and OP-M17 (30%) primers exhibited moderate levels of polymorphism. However, OP-L12 (64.3%), OP-B17 (77.7%) and OP-F04 (81.8%) primers exhibited high levels of polymorphism.

The number of total amplified fragments (TAF), polymorphic fragments (PF), mononmorphic fragments (MF) and specific markers (SM) for each sample using the eight primers are shown in Table (9).

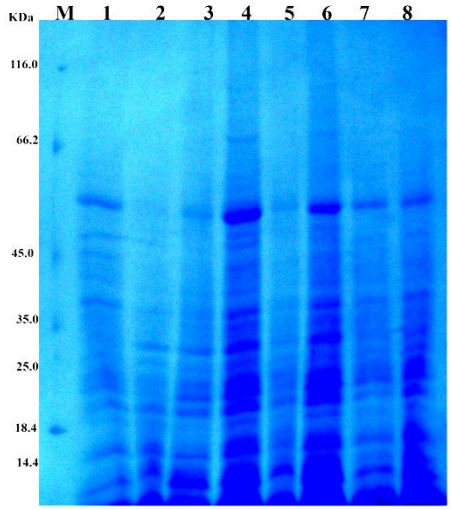

Fig. (1): SDS- leaf protein banding patterns of the eight *Pinus species* genotypes.

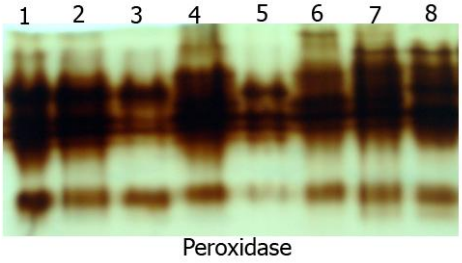

Fig. (2): Leaf Peroxidase isozyme banding patterns of eight *Pinus species genotypes*.

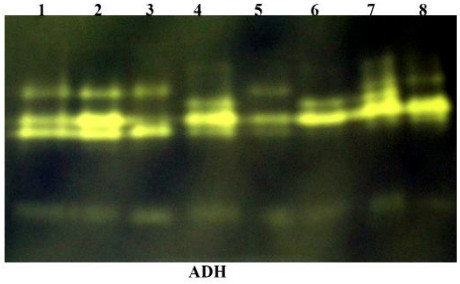

Fig. (3):Leaf Alkohol Dehydrogenase (Adh) isozyme banding patterns of eight *Pinus species* genotypes.

Table (4) : Densitometric analysis for SDS leaf proteins of the eight *Pinus species* genotypes.

Band No.	MW KDa	*Pinus brutia(k)*	*Pinus brutia(S)*	*Pinus brutia(T)*	*Pinus halepensis(K)*	*Pinus halepensis(S)*	*Pinus halepensis(T)*	*Pinus pinea(K)*	*Pinus pinea(S)*
		genotypes							
1	78.0	0	0	0	1	0	1	0	0
2	65.0	1	0	0	1	1	1	1	1
3	57.0	1	1	1	1	1	1	1	1
4	48.0	1	1	1	1	1	1	1	1
5	45.0	1	1	1	1	1	1	1	1
6	41.0	1	1	1	1	1	1	1	1
7	39.0	1	0	1	1	0	1	0	1
8	33.0	1	0	0	1	1	1	1	1
9	28.0	1	1	1	1	1	1	1	1
10	25.0	1	0	0	1	1	1	1	1
11	23.0	1	1	1	1	1	1	1	1
12	18.0	1	1	1	1	1	1	1	1
13	16.0	1	1	0	1	1	1	0	1
14	14.0	1	1	1	1	1	1	1	1
15	13.0	1	1	1	1	1	1	1	1
	Total	14	10	10	15	13	15	12	14

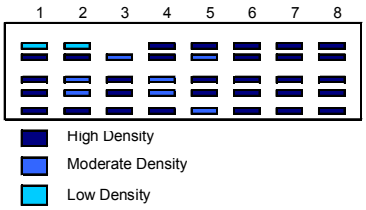

Fig. (4): Edeogram analysis for leaf Peroxidase isozyme banding patterns of eight *Pinus species* genotypes.

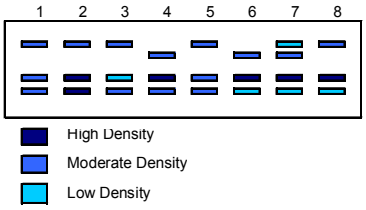

Fig. (5): Edeogram analysis for leaf Alkohol Dehydrogenase (Adh) isozyme banding patterns from different *Pinus species* and genetic resources.

M= Protein Marker (KDa), 1= Pinus brutia(k), 2= Pinus brutia(S) , 3= Pinus brutia (T), 4= Pinus halepensis(K), 5= Pinus halepensis(S), 6= Pinus halepensis(T), 7= Pinus pinea(K), 8= Pinus pinea(S).
K =Turkish source, S =Syrian source, T =Tunisian source.

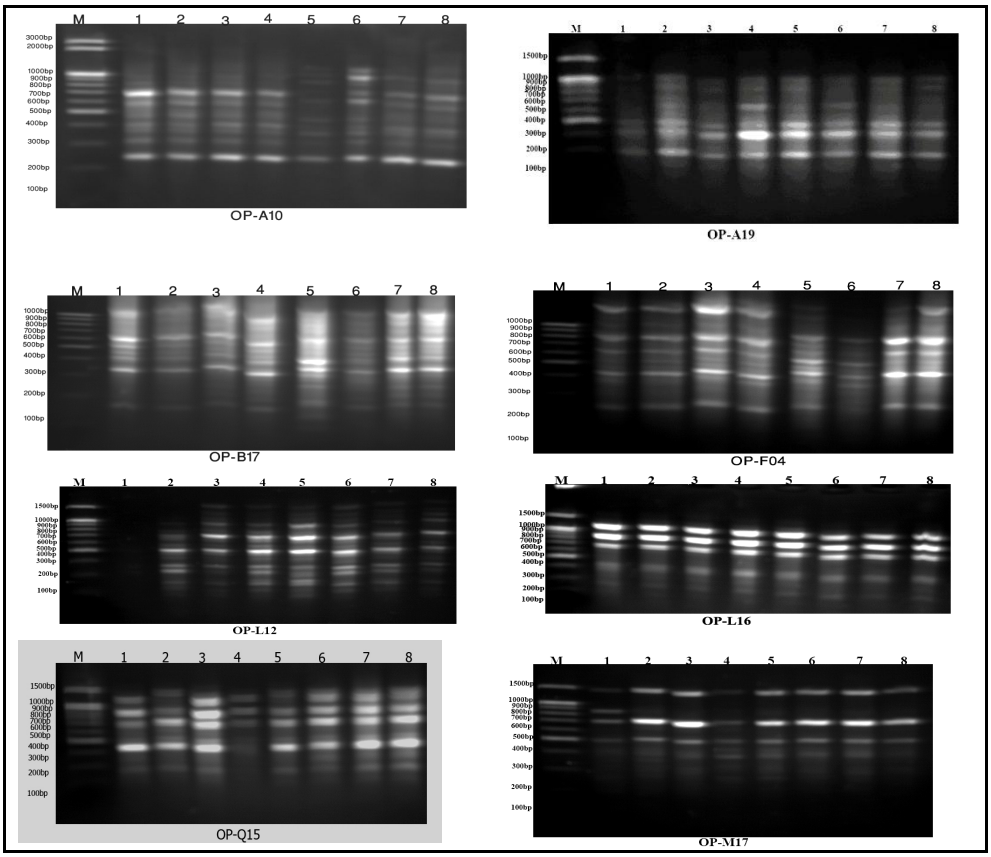

Fig (6): RAPD fragments of eight *Pinus* species genotypes amplified using eight primers.
{1= *Pinus brutia(k), 2= Pinus brutia(S) , 3= Pinus brutia (T), 4= Pinus halepensis(K), 5= Pinus halepensis(S), 6= Pinus halepensis(T), 7= Pinus pinea(K), 8= Pinus pinea(S).*
K =Turkish source, S =Syrian source, T =Tunisian source}.

OP-A10 primer produced thirteen fragments with molecular size ranging from 260 to 1130bp (Fig.6). Five fragments were polymorphic (38.4%) and the other eight bands were present in all genotypes which is considered as common fragments, OP-A19 primer indicated to the amplification of twelve fragments with molecular size ranging from 186 to 418bp, which three fragments of them were polymorphic (25.0%), while, the other nine fragments were represented in all genotypes which are considered as common fragments. OP-B17 primer resulted in the amplification of nine fragments with molecular size ranging from 220 to 945bp, in which seven fragments were polymorphic (77.7 %) and the other two fragments were presented in all genotypes which are considered as common fragments. OP-F04 primer resulted in eleven DNA fragments with molecular size ranging from 120 to 1140bp, in which nine fragments were polymorphic (81.8 %) and three of them were species - specific markers at 160bp for *Pinus brutia(K)*, 790bp for *Pinus halepensis (S)* and 800bp *Pinus pinea* (S), while the other two fragments were present in all genotypes which are considered as common fragments. OP-L12 primer resulted in fourteen DNA fragments with molecular size ranging from 112 to 1620bp, nine fragments were polymorphic (64.3%) in which one of them was species- specific marker at 980bp for *P. halepensis* (T) and the other five fragments were present in all genotypes which are considered as common fragments. Primer OP-L16 resulted in nine fragments with molecular size ranging from 140-1000bp, all fragments were present in all genotypes which are considered as common fragments. OP-M17 primer resulted in ten DNA fragments with molecular size ranging from 185 to 1290bp, three fragments were polymorphic (30 %), and one of them was species-specific marker at 830bp for *Pinus brutia (K)* and the other seven fragments were presented in all genotypes which are considered as common fragments. OP-Q15 primer resulted in twelve DNA fragments with molecular size ranging from 200 to 500bp, five fragments were polymorphic (41.6 %), and the other seven fragments were presented in all genotypes which are considered as common fragments.

3.5.2.Genetic similarity and cluster analysis based on RAPD markers

The RAPD data were used to estimate the genetic similarity values among the eight genotypes of *Pinus* species by using UPGMA computer analysis (Table 6 and Fig. 9). The highest similarity value (1.0) was recorded between *Pinus brutia* (K) and *Pinus halepensis* (K) genotypes, while the lowest similarity value (0.1) was detected between *Pinus halepensis* (S) and *Pinus halepensis* (T) genotypes and also between *Pinus pinea* (K) and *Pinus halepensis (T)* genotypes. A dendrogram for the genetic relationship among the eight *Pinus* species genotypes is exhibited in Fig. (9) which separated them into two major groups. The first group included only *Pinus brutia* (K) genotype, while the second group included two subgroups, the first subgroup involved *Pinus brutia* (S) only and the other subgroup included the other six genotypes. The results of RAPD analysis are in harmony with those obtained by Hassan *et al*., (2002).

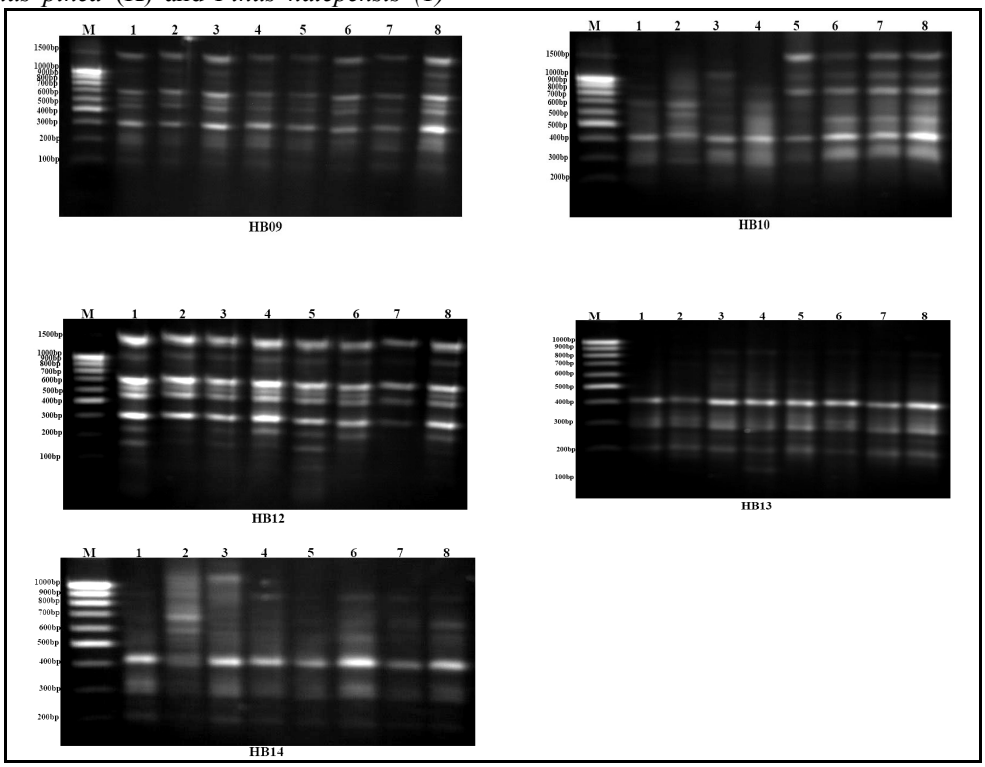

Fig (7): ISSR fragments of the eight *Pinus* species genotypes amplified by using five primers.
{1= *Pinus brutia(k), 2= Pinus brutia(S) , 3= Pinus brutia (T), 4= Pinus halepensis(K), 5= Pinus halepensis(S), 6= Pinus halepensis(T), 7= Pinus pinea(K), 8= Pinus pinea(S).*
K =Turkish source, S =Syrian source, T =Tunisian source}.

3.5.3. Inter Simple Sequence Repeats (ISSRs) markers

The five ISSR primers succeeded in amplifying DNA fragments for the eight *Pinus* species genotypes (Fig.7)**.** Polymorphism levels differed from one primer to another, *i.e.* HB-09, HB-10, HB-13 and HB-14 primers exhibited moderate levels of polymorphism (44.4%, 56.2%, 57% and 53.3%), respectively, while, HB-12 primer exhibited high level of polymorphism (70%) as exhibited in Table (9). The number of total amplified fragments (TAF), polymorphic fragments (PF), monomorphic fragments (MF) and specific markers (SM) for each primer of the five primers are shown in Table (9).

HB-09 Primer showed 18 DNA fragments with molecular size ranging from 170 to 1460bp (Fig.7 and Table 9), eight fragments were polymorphic (44.4 %), and four of them were positive species- specific markers at 170, 735, 785 and 1460bp for *Pinus halepensis (S), Pinus pinea* (S) genotypes and the last two of them for *Pinus halepensis (S)* genotype, respectively. HB-10 primer showed sixteen DNA fragments with molecular sizes ranging from 95 to 1360bp, nine fragments were polymorphic (56.2 %), and three of them were positive species- specific markers at 760, 775 and 1225bp for *Pinus brutia (K), Pinus halepensis* (S) and *Pinus pinea (S)* genotypes, respectively. However, there was a negative specific marker at 1340bp for *Pinus halepensis* (S) genotype.

HB-12 primer showed seventeen DNA fragments with molecular size ranging from 60 to 1010bp, twelve fragments were polymorphic (70 %), and two of them were positive species- specific markers at 105 and 840bp for *Pinus brutia (K) and Pinus halepensis* (S) genotypes, respectively. HB-13 primer showed fourteen DNA fragments with molecular size ranging from 115 to 890bp, eight fragments of them were polymorphic (57.1 %), and one of them was positive species- specific markers at 650bp for *Pinus pinea* (S) genotype. HB-14 primer showed fifteen DNA fragments with molecular size ranging from 210 to 1020bp, eight fragments were polymorphic (53.3 %) and the other seven genotypes were present in all genotypes which are considered as common fragments.

3.5.4.Genetic similarity and cluster analysis based on ISSR markers

The ISSR data were used to estimate the genetic similarity values among the eight *Pinus* speices genotypes by using UPGMA computer analysis (Table 7 and Fig. 10). The highest similarity values were recorded (1.0) between *Pinus brutia* (K) and *Pinus pinea* (S) genotypes, as well as between *Pinus halepensis (S)* and *Pinus halepensis (T),* while the lowest similarity value (0.1) was recorded between *Pinus brutia* (T) and *Pinus halepensis* (K) genotypes and also between *Pinus pinea (K) and Pinus halepensis (T)* genotypes.

A dendrogram for the genetic relationship among the eight *Pinus* species genotypes is illustrated in Fig. (10) As they were separated into two major groups. The first group included *Pinus brutia* (K) and *Pinus brutia* (S) genotypes, while the second group was divided into two subgroups, The first subgroup included each of *Pinus brutia* (T), *Pinus halepensis* (K) and *Pinus halepensis* (S) genotypes and the other subgroup included each of *Pinus halepensis* (T), *Pinus pinea (K) and Pinus pinea* (S) genotypes.

3.5.5.Combined identification based on protein, isozymes, RAPD and ISSR analyses

Genetic similarities and phylogenetic relationships among the eight *Pinus* species genotypes based on a combined data of protein, isozymes, RAPD and ISSR-PCR markers (Table 8 and Fig. 11) were determined using UPGMA computer program.

The highest similarity value (1.0) is recorded between *Pinus brutia* (K) and *Pinus pinea* (S) genotypes, while the lowest similarity value (0.1) is recorded between *Pinus bruita* (S) and *Pinus brutia* (T) genotypes and also between *Pinus pinea(K)* and *Pinus pinea* (S) genotypes .

The dendrogram based on protein, isozymes, RAPD and ISSR-PCR markers (Fig.11) separated the eight *Pinus* species genotypes into two major groups. The first group included *Pinus brutia* (S) and *Pinus brutia* (T) genotypes, while the second group is divided into two subgroups. The first subgroup included only *Pinus halepensis (S)* genotype, while the other subgroup included *Pinus brutia (K), Pinus halepensis (T), Pinus pinea (K), Pinus pinea (S)* and *Pinus halepensis (K)* genotypes.

These results could be explained on the bases that, due to ecological variation, there is possibility that some genomic mutations could be took place. In this regard, authors have hypothesized that the absence of intraclonal RAPD polymorphism cannot guarantee genetic stability, because important variations like genomic mutations could be missed (Palombi and Damiano, 2002 and Cuesta *et al.*, 2010). Moreover, Weinstein (1989) reported that provenances of *P. halepensis*, found within the East European race, are influenced by introgression and found that phonological characters of *P. halepensis* and *P. brutia* behaved in variable patterns according to their original races, while those came from the same race origin characterized similarly. Besides, Mendel (1984) proposed that the resistance to the pest was possibly transferred from *P. brutia* to *P. halepensis* in the same location, where the natural distribution of these pine species overlapped.

On the other hand, our results indicated that protein, isozymes, RAPD and ISSR techniques are useful in the establishment of the genetic fingerprinting and estimation of genetic relationships among *Pinus* genotypes. Also, these techniques could detect enough polymorphism in the studied *Pinus* species genotypes to distinguish each species genotype from the others by at least one specific fragment. Furthermore, the use of these results in the future is important for *Pinus* germplasm management and improvement as well as for the selection strategies of parental lines that facilitate the prediction of crosses in order to produce hybrids with higher performance as was indicated by (Adawy *et al*., 2004; Ebtissam *et al*., 2005; Soliman *et al*., 2006 and Hemeid *et al.*, 2007).

In general the overall results indicated the possible use of protein, isozymes, RAPD and ISSR analyses to detect some species - specific markers for the eight *Pinus* species genotypes that can be used to discriminate among these species and their genotypes and also, to detect genetic relationships among these species and their genotypes which can be used in breeding programs. The molecular genetic results of these three *Pinus* species and their genotypes are efficient tools for the characterization of these species.

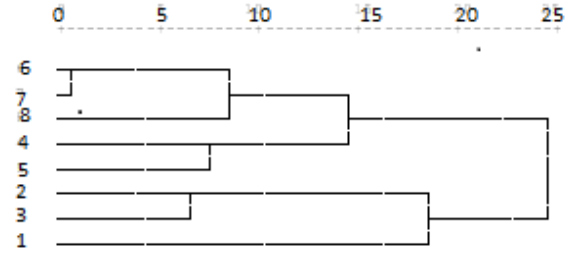

Fig. (8): A dendrogram illustrates the genetic distance among *Pinus species* genotypes based on protein and isozymes data.

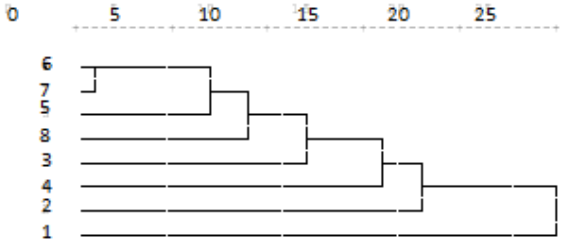

Fig. (9): A dendrogram illustrates the genetic distance among *Pinus species* genotypes based on RAPD data.

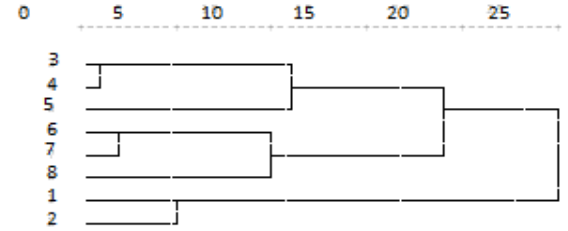

Fig. (10): A dendrogram illustrates the genetic distance among *Pinus species* genotypes based on ISSR data.

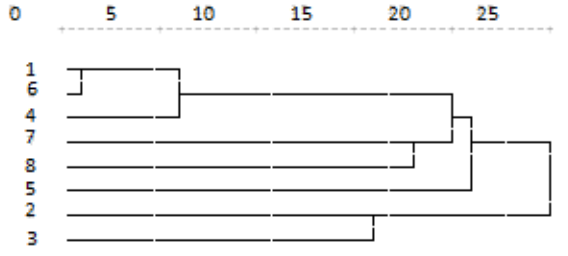

Fig. (11): A dendrogram illustrates the genetic distance among *Pinus species* genotypes based on over-combination of protein, isozymes, RAPD, and ISSR analyses.

Table (5): Similarity value (Pairwise comparison) of the eight *Pinus species* genotypes based on protein and isozymes data.

	1	2	3	4	5	6	7
1							
2	0.3						
3	0.6	0.4					
4	0.2	0.7	1.0				
5	0.5	0.5	0.8	0.6			
6	0.1	0.4	0.7	0.1	0.6		
7	0.6	0.6	0.9	0.4	0.5	0.7	
8	0.4	0.5	0.7	0.5	0.6	0.5	0.5

Table (6): Similarity value (Pairwise comparison) of the eight *Pinus species* genotypes based on RAPD data.

	1	2	3	4	5	6	7
1							
2	0.5						
3	0.5	0.2					
4	1.0	0.4	0.3				
5	0.6	0.2	0.2	0.2			
6	0.6	0.4	0.3	0.4	0.1		
7	0.5	0.5	0.2	0.4	0.2	0.1	
8	0.5	0.5	0.3	0.4	0.3	0.2	0.3

Table (7): Similarity value (Pairwise comparison) of the eight *Pinus species* genotypes based on ISSR data.

	1	2	3	4	5	6	7
1							
2	0.3						
3	0.5	0.3					
4	0.5	0.4	0.1				
5	0.4	0.7	0.4	0.2			
6	0.7	0.5	0.3	0.0	1.0		
7	0.8	0.6	0.6	0.3	0.4	0.1	
8	1.0	0.8	0.7	0.7	0.6	0.3	0.2

Table (8): Similarity value (Pairwise comparison) of *the* eight *Pinus species* genotypes based on over-combination of protein, isozymes, RAPD and ISSR analyses.

	1	2	3	4	5	6	7
1							
2	0.3						
3	0.7	0.1					
4	0.9	0.5	0.2				
5	0.7	0.6	0.4	0.2			
6	0.7	0.6	0.5	0.2	0.3		
7	0.9	0.8	0.6	0.5	0.4	0.2	
8	1.0	0.9	0.7	0.6	0.5	0.3	0.1

{1= *Pinus brutia(k), 2= Pinus brutia(S) , 3= Pinus brutia (T), 4= Pinus halepensis(K), 5= Pinus halepensis(S), 6= Pinus halepensis(T), 7= Pinus pinea(K), 8= Pinus pinea(S).*

K =Turkish source, S =Syrian source, T =Tunisian source}.

Table (9): Species-specific RAPD and ISSR markers for *Pinus* species genotypes

Primers code	Range of M.S.	TAF	MF	PF	SM	Polymorphism (%)
RAPD primers						
OP-A10	260-1130	13	8	5	-	38.4
OP-A19	186-418	12	9	3	-	25
OP-B17	220-945	9	2	7	-	77.7
OP-F04	120-1140	11	2	9	3^+ (160,790,800) bp	81.8
OP-L12	112-1620	14	5	9	1^+ (980) bp	64.3
OP-L16	140-1000	9	9	0	-	-
OP-M17	185-1290	10	7	3	1^+ (830) bp	30
OP-Q15	200-1500	12	7	5	-	41.7
Total RAPD primers		**90**	**49**	**41**	**5**	
ISSR primers						
HB09	170-1460	18	10	8	4^+ (170,735,785,1460)bp	44.4
HB10	95-1360	16	7	9	3^+ (760,775,1225)bp 1^- (1340) bp	56.2
HB12	60-1010	17	5	12	2^+ (105,840) bp	70
HB13	115-890	14	6	8	1^+ (1650) bp	57
HB14	210-1020	15	7	8	-	53.3
Total ISSR primers		**80**	**35**	**45**	**11**	
Total		**170**	**84**	**86**	**16**	

TAF = Total Amplified Fragments, MF= Monomporphic Fragments, PF= Polymorphic Fragments, SM= Specific Markers, [(+) = positive markers, (-) = negative markers].

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2/20/2012