Biochemical and Molecular genetic Evaluation of some conifers genetic resources

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Abstract: Genetic polymorphism was investigated in six conifers representing four Pinus species, i.e (*P.halepensis, P.canariensis, P.pinea, and P.roxburghii)* which belong to family *Pinaceae* and two members of family *Taxodiaceae, i.e. (Sequoia sempervirens and Taxodium distichum).* In this respect, genetic biochemical (proteins and isozymes), as well as molecular (RAPDs and ISSRs) analysis were investigated. Proteins and peroxidase banding patterns resulted in extensive polymorphism among conifers under investigation, however, Adh isozyme banding patterns were not satisfactory in this concern. RAPD analysis exhibited a total of 66 bands, out of them 25 bands were polymorphic (37.88%). Five ISSR primers generated reproducible and informative amplified products. those were used to distinguish between the six conifers, since 38 bands were polymorphic out of total 81 bands with 47.95% of polymorphism which can be considered as useful markers for identifying conifers. Based on combined data obtained by proteins, peroxidase, RAPD and ISSR analysis, it was possible to discriminate between the six conifer trees under investigation. The present study indicates that the application of biochemical and molecular fingerprinting of the six conifers provided a solid ground that will allow an easier and faster genetic identification of other woody trees species.

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1. Introduction:

In forestry, genomic discovery will support genetic improvement of tree varieties for solid wood, pulp and paper, biofuels, and biomaterials through integration into traditional breeding approaches in domesticated tree population (Neale, 2007).

Characterization of the genetic diversity and examination of the genetic relationship among conifers are important for the sustainable conservation and increase use of plant genetic resources. Traditionally, comparative vegetative anatomy and plant systematic were two common strategies to assess the relationships among conifers (Wang, *et al.*, 2009).

Tree breeding generally involves recurrent selection and population improvement in each cycle of breeding and each cycle can take many years (White, *et al.*, 2007). In trees, breeding populations are often large and genetically diverse in their composition, as opposed to line breeding in many agricultural crops where the number of genotype is often limited. Tree breeders could then use genomic selection directly for population improvement or, more likely, combine genomic selection approach to maximize genetic gain per unit time (Neale, *et al.*, 2007).

The genus *Pinus* is among the most widely distributed and prominent genera of trees in the world, including many of the most economically valuable species of forest trees. The taxonomy of *Pinus* is based mainly on morphological and partially on molecular data that are incomplete for many taxa (Strauss and Doerksen, 1990).

Taxodium disticum is a deciduous conifer in the family *Taxodiaceae*, which has numerous attributes that qualify it as a supreme urban landscape tree and as a species to mediate harsh coastal wetland and flood plains of major rivers in the south (Zhou, 2007). *Sequoia sempervirens* has a very decay and fire resistant wood, as well, resistant to weather, insects and fungus attacks. The bark is used as hog fuel, insulation, or garden mulch. This species lives up to 1500 years, and reaches 116 m height (as the tallest stands record). The economic value of the tree is so high, however, little attention has yet been paid to it (Clark and Scheffer, 1983). There is only one *Sequoia sempervirens* tree in Egypt which had been threatened, never produces seeds, neither vegetatively propagated, however, it was possible to propagate through tissue culture techniques (Gad, *et al.*, 2006).In Egypt, no attention has been paid to evaluate the genetic relationships between such conifers which were introduced and grew well under the local environmental conditions. This study was designed to assess the pattern of genetic variation between different genetic resources of conifers genera and species. This was achieved through the use of electrophoretic (protein and isozymes) and molecular (RAPD and ISSR) techniques which have been increasingly applied to the study of tree species in

recent years, to provide information in support of conservation planning and management. Therefore, the objective of this study is to identify and characterize species-specific biochemical and molecular markers among the conifers of the two families (*Pinaceae* and *Taxodiacea*e) in order to establish genetic relationships among them. Moreover, the resulted genetic relationships can be directed towards tree genetic resources sustainable conservation as well as to exploit these investigations in breeding programs to maximize genetic gain per unit time in order to support genetic improvement of tees under investigation.

2.Materials and Methods:

2.1.Materials:

2.1.1.Plant materials

This investigation was achieved during the period from 2006 to 2010 at Biotechnology Research Laboratory, Horticulture Research Institute (HRI), Agricultural Research Center (ARC), Ministry of Agriculture, Egypt.

Genetic resources of six mother trees, morphologically identified conifers located at Orman Botanic Garden, Giza, were used as germplasm sources for this research work, namely *Pinus halepensis*, *Pinus canariensis*, *Pinus pinea*, *Pinus roxburghii*,(belong to family *Pinaceae*), *Sequoia sempervirens* and *Taxodium distichum*,(belong to family *Taxodiacea*)

2..2.2.Primers:

Table (1): List of the RAPDs primers codes and their nucleotide sequences

No	Code	Sequence	No	Code	Sequence
1	OP - F01	5` ACGGATCCTG 3`	4	OP-C11	5` AAAGCTGCGG 3`
2	OP-F05	5` CCGAATTCCC 3`	5	OP-Z01	5` TCTGTGCCAC 3`
3	OP-F08	5` GGGATATCGG - 3`			`

Table (2): List of used ISSR primer codes and their nucleotide sequences.

No.	Code	Sequences	No.	Code	Sequences
1	A98	5` ACACACACACACA 3	4	HB 12	5`CACCAC CAC GC 3`
2	HB 8	5` GTGTGTGTG TGTGG 3`	5	HB 13	5`GAGGAGGAGGC 3`
3	HB 10	5`GAGAGAGAGAGACC 3`			

2.2.Methods:

2.2.1.Biochemical genetic identification.

2.2.1.1. Protein electrophoresis:

Young fresh leaves were collected from conifers under investigation and were ground into a fine powder by using liquid nitrogen (-196 °C) and a mortar and pestle. A sample of 0.5 g was homogenized with 0.9 ml extraction buffer (10 ml 0.5 M Tris pH 6.8, 16 ml 10% SDS, 30 ml D.W.). The extracts were transferred into Eppendorf tubes and centrifuged for 10 min. at 1000 rpm under cooling (4 ºC). Supernatants (containing protein extract) were transferred into clean tubes and used for SDS-PAGE analysis. Isozymes were extracted, as described by Jonathan and Weeden (1990). A volume of 120 µl of protein extract was loaded into sodium dodecyle sulphate polyacrylamide gel (SDS-PAGE), 12% W/V vertical slab using BIORAD Techware 1.5 mm according to the method of Laemmli (1970) and modified by Studier (1973). The molecular weights of proteins were estimated relative to a standard protein marker with a wide range of molecular weight (Fermentas .com).

2.2.1.2. Isozymes electrophoresis

Native – polyacrylamide gel electrophoresis (Native - PAGE) was conducted to identify isozyme variations among the six studied conifers using two isozyme systems using 12% (W/V) slab gel according to Stegmann *et al.* (1985). Isozymes were extracted from 0.2 g of fresh and young leaves samples in a 1 ml of 0.125 M Tris – borate buffer, pH 8.9. A volume of 50 µl extract of each sample was mixed with 12.5 µl of glycerol, and 60 µl from this mixture was applied to each gel well.

2.2.1.2.1. Peroxidase Detection:

Peroxidase was detected by incubating the gel in a darkness for one hour at 37°C in a mixture of 15 ml of 10% benzidine (in 95% ethanol); 50ml of 1mM potassium acetate and 1 ml of 1% H_2O_2 (pH 4.7). After the incubation period the gel was rinsed in a distilled water and fixed in a 50% glycerol for one hour. The gel was placed into this solution and 5 drops of hydrogen peroxide solution were added. The gel was incubated at room temperature until bands appeared, (Brown, 1978).

2.2.1.2.2 Alcohol dehydrogenase (*Adh*):

Gel was placed in a solution composed of 100 ml of 0.1M Tris-pH (7.5), NAD 30 mg, MTT 20 mg, phenazine methosulfate (PMS) 5 mg and ethanol 6 ml, and incubated at 30 ºC for 30 min. until bands appeared.

2.2.1.2.3 Gel documentation

Gels were digitally photographed and analyzed using Gel Doc Viller Lourmat system to capture the image and to calculate band intensities.

2.2.2.Molecular genetic identification

DNA extraction:

Total genomic DNA was extracted and purified from 0.1 g of freeze – dried powered samples as described by Dellaporta *et al.* (1983). DNA present in the supernatant was precipitated according to the

described protocol, re-dissolved in a sterile, distilled water (D.W.) and quantified.

2.2.2.1. Randomly amplified polymorphic DNA (RAPD).

Amplification of genomic DNA using polymerase Chain Reaction (PCR)

Different preliminary experiments were carried out in order to optimize the factors leading to clear reproducible amplification products. A total of ten random DNA oligonucleotide primers were independently used according to Williams *et al.* (1990) in each PCR reaction.

Only five primers (Operon biotechnologies, Inc. Germany) were succeeded to generate reproducible polymorphic DNA products. Table (1) displays the base sequence of these DNA primers those produced informative polymorphic bands.

PCR was performed in a 30µl reaction volume containing the following : 3.0µl of dNTPs (2.5 mM), 3.0µl Mg-Cl_2 (25mM), 3.0 µl of 10 x buffer, 2.0 µl of primer (10 ρmol), 0.20 µl of Taq DNA polymerase (5u/ µl), 2.0 µl of template DNA (50.0 ng/ µl), 16.80 µl H_2o (sterile D.W.). The DNA amplifications were performed in an automated thermal cycle (Techn.TC-512 PCR system). The reaction was subjected to one cycle at 94ºC for 4 min. followed by 45 cycles of 1 min. at 94 ºC, 1 min. at 36 ºC, and 2 min. at 72 ºC. The reaction was finally stored at 72 ºC for 10 min.

The amplification products were separated in 1% (w/v) agarose gel in 1 x TBE buffer and visualized by staining with ethidum bromide. Reproducibility of DNA profiles was determined by replicating all RAPD reactions at least three times.

After electrophoresis, the RAPD patterns were visualized with UV transilluminator and photographed by gel documentation system (Gel Doc Bio Rad 2000). RAPD markers were scored from the gel as DNA fragments present or absent in all lanes. Each experiment was repeated twice and only stable products were scored.

2.2.2.2. Intersimple sequence repeats (ISSR)

ISSR- PCR reactions were conducted using five primers. PCR was performed according to Wang, *et al.* (2002) in 30 µl reaction volume containing 2.5 µl dNTPs (2.5 mM), 2.5 µl Mg Cl_2 (25 mM), 2.5 µl buffer (10 X), 3.0 µl primer, 2.5 µl Taq DNA polymerase (1 U/1 µl), 2.0 µl Template DNA (25 ng) and 11 µl H_2O (sterile D.W.).

The PCRs were programmed for one cycle at 94 ºC, for 4 min. followed by 45 cycles for 1 min. at 94 ºC, 1 min. at 57 ºC and 2 min. at 72 ºC then 12 min. at 72 ºC for one cycle and the reaction was finally hold at 4 ºC. The PCR products were separated on 1.5 % agarose gels and fragments sizes were estimated with the 100 bp ladder marker. Table (2) illustrates the base sequence of the DNA primers, those produced informative polymorphic bands.

2.2.3.Data analysis:

The similarity matrices were done using Gel works ID advanced software UVP- England Program.

The relationships among genotypes and species as revealed by dendrograms were done by using SPSS windows (Version 10) program. Dice computer package was used to calculate the pairwise difference matrix and plot the phenogram among conifers genotype under investigation. The resultant similarity matrix was employed to construct a dendrogram using Sequential Agglomerative Hierarchical Nesting (SAHN) based Unweighted Pair-Group Method with Arithmetic Means (UPGMA) to infer genetic relationships and phylogeny(Sensi, et al., 2003)

3.Results:

3.1.Biochemical genetic identifications

3.1.1. SDS- PAGE protein banding patterns of conifers leaves.

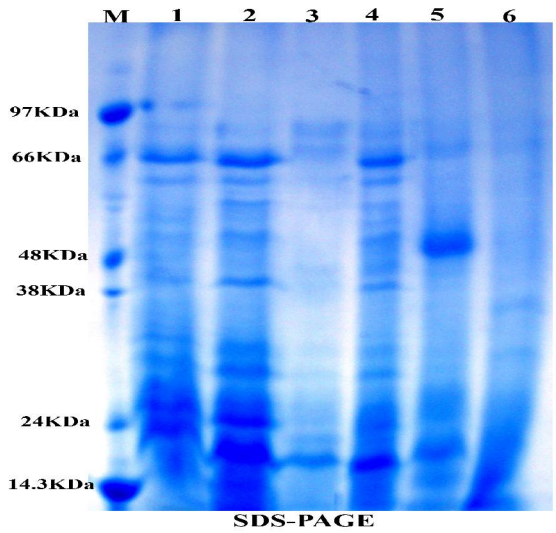
The needle leaves protein banding profile which was separated using SDS-PAGE of the six genera and species of conifers are illustrated in Fig. (1). The total number of bands was 23 with molecular weights ranged from 15.798 KDa to 100.751 KDa (Table 3). The highest number of bands was 16, detected in *Pinus roxburghii* while the lowest number of bands was 8, identified in *Taxodium distichum.*

Demonstrative analysis of the presence and absence of bands were assessed with (1) and (0), respectively, are illustrated in Table (3). It is observed that two bands were monomorphic (29.677 and 21.287 KDa), while 18 bands were polymorphic, giving 91.304% polymorphism and 3 unique bands (100.751, 92.825 and 24.963 KDa) among the examined conifers.

The matrix of similarity index for the six conifers germplasm is presented in Table (4). The highest coefficient was 86.7 recorded between *Pinus canariens*is and *P.roxburghii*, followed by 80.0 recognized between *Sequoia sempervirens* and *Taxodium distichum*, followed by 75.9 between *P.roxburghii* and *P.halepensis* and finally 74.1 between each of (*P.halepensis* and *P.canariensis*) and (*P.roxburghii* and *P.pinea*). On the other hand, the lowest coefficient value was 28.6 observed between *P.halepensis* and *Taxodium distichum.*

3.1.2. Isozymes banding patterns.

The isozymes banding pattern of peroxidase and alcoholdehydrogenase (*Adh*) isozymes of the six conifers genotypes is presented in Fig. 3 (a and b), while data are scored in Table (5).



Fig(1): SDS-PAGE protein banding patterns of leaves of the six conifers

[M :standard protein (KDa) ,
1-Pinus halepensis,
2- P.canariensis,
3- P.pinea,
4-P.roxburghii,
5-Sequoia sempervirens,
6-Taxodium distichum].

The peroxidase patterns exhibited a total number of 20 bands, 13 of them are polymorphic, with (100%) polymorphism while 7 bands are unique, though, no monomorphic bands were scored. Polymorphism exhibited by this pattern completely discriminated between the studied conifers.

Table (4) A Proximity matrix of protein banding patterns

Case	Matrix file input					
	1	2	3	4	5	6
1		0.741	0.583	0.759	0.400	0.286
2	0.741		0.720	0.867	0.538	0.455
3	0.583	0.720		0.741	0.522	0.316
4	0.759	0.867	0.471		0.500	0.333
5	0.400	0.538	0.522	0.500		0.800
6	0.286	0.455	0.316	0.333	0.800	

Pinus roxburghii was discriminated by the presence of the unique bands with Rf values (0.105 and 0.361). Besides, *Sequoia sempervirens* was distinguished by the absence of the unique negative bands with Rf values (0.279, 0.336 and 0.387) and the presence of unique bands with Rf values (0.731, 0.790, 0.836 and 0.900). As well, *Taxodium distichum* was identified by the presence of (0.055) and the absence of (0.597) unique bands, in addition, to other polymorphic bands recognized in each conifer.

Table (3): Data matrix illustrating the presence or absence of bands in the leaves protein electrophoresis banding patterns for the six conifers.

Band number	MW (KDa)	P.halepensis	P canariensis	P.pinea	P.roxburghii	S.sempervirens	T.distichum
1	100.751	0	0	0	1	0	0
2	92.825	1	0	0	0	0	0
3	84.94	1	1	1	1	0	0
4	77.725	0	0	0	0	1	1
5	77.196	0	0	1	0	1	0
6	73.593	1	1	0	1	0	0
7	65.826	1	1	1	1	0	0
8	59.283	0	1	0	1	0	0
9	54.993	1	1	1	1	0	0
10	49.639	1	1	0	1	1	0
11	49.414	0	0	0	0	1	1
12	43.600	0	0	1	1	1	0
13	40.445	1	1	1	1	1	0
14	36.341	0	0	0	0	1	1
15	31.847	1	0	0	1	0	0
16	29.677	1	1	1	1	1	1
17	26.727	0	1	1	1	0	0
18	24.963	1	0	0	0	0	0
19	23.690	1	1	0	1	1	1
20	21.287	1	1	1	1	1	1
21	19.171	0	1	1	1	1	1
22	17.662	1	1	1	1	0	0
23	15.798	0	1	0	0	1	1
Total		13	14	10	16	12	8
Polymorphism %	91.304						

In Fig (3-b) and Table (5) there is a quite evidentence that only four polymorphic bands were identified out of five total scorable bands with 80% polymorphism, while one monomorphic band (Rf 0.863) was recognized as well as 1 unique band was scored in the illustrated *Adh* profile. Only *Taxodium distichum* was discriminated with the presence of the unique band with (0.205) and the absence of (0.268) Rf values, while the other taxa shared the presence and absence of several bands. Each of *P. halepensis* and *P. canariensis* shared the presence of the bands with RF values (0.268, 0.363 and 0.863) and the absence of (0.205 and 0.739) bands. Besides, each of *P. pinea*, *P.roxburghii* and *S. sempervirens* shared the presence of bands with Rf values (0.268, 0.739 and 0.863) and the absence of the bands (0.205 and 0.363) Rf values. Therefore, *Adh* isozyme patterns were not satisfactory to detect

phylogenetic relationships among conifers genotypes used in this study.

Consequently, isozyme profile permitted the identification of three conifers under investigation, *i.e. Pinus roxburghii*, *Sequoia sempervirens* and *Taxodium distichum* by the presence of two unique markers found in peroxidase isozyme with *P. roxburghii*, four with *Sequoia sempervirens* and absence of three bands and the presence of one with *Taxodium distichum* with absence of one unique band.

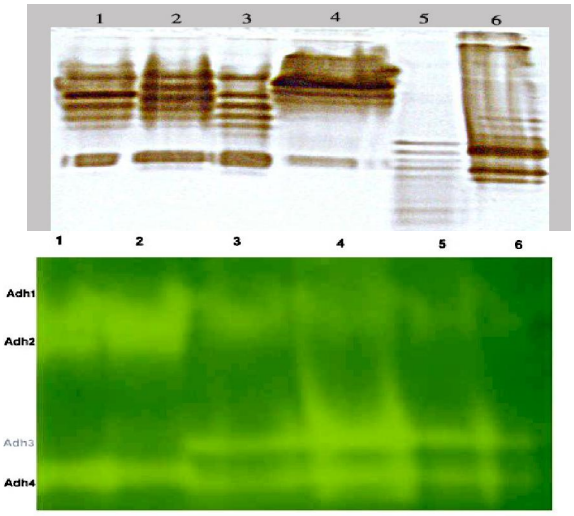


Fig. (3): Zymogram of: (a) peroxidase and (b) Adh isozymes of the six conifers :
1-*Pinus halepensis*
2-*P.canariensis*
3-*P.pinea*
4-P.roxburghii
5-*Sequoia sempervirens*
6-*Taxodium distichum*

3.2.Molecular markers:

3.2.1. Randomly Amplified Polymorphic DNA (RAPDs) analysis

Out of ten decamer (RAPD) primers tested, five revealed distinct polymorphism among the six conifers under investigation. A total of 66 DNA bands were detected; 25 of them showed polymorphism. Out of these polymorphic bands, 10 unique bands were scored (Table 6). However, polymorphism ranged between 18.182% (primers OP-F01, OP-F05 and OP-C11) and 58.824% (primer OP-Z01). The range of DNA bands size was between 97.071 and 882.55 bp (Fig 4).

From Table (6) and Fig (4) it could be noticed that a maximum of two polymorphic (18.182% polymorphism) and nine monomorphic DNA bands were recorded in the RAPD profiles generated by the primer OP-C11, while no polymorphic-unique bands were scored. The first polymorphic band (about 350 bp) was observed in all conifers genera and species, except in *P.halepensis*, while the second polymorphic band (313.4 bp) was identified in all conifers, except in *S.sempervirens*. The absence of the forementioned polymorphic bands could be considered as negative unique bands for both genera.

It could be observed that the primer OP-F01 generated a total of two polymorphic bands, inducing 18.182% polymorphism and nine monomorphic DNA bands in the studied species and genotypes, while one unique band (positive) was identified out of the total polymorphic bands. This unique band (molecular size 587.065 bp) was detected in *Sequoia sempervirens*, while the absence of the (negative) band (molecular size 473.151 bp) was observed in *Pinus canariensis*. These unique bands (positive and negative) clearly discriminate both of *Sequoia sempervirens* and *P.canariensis* from the pool of conifers investigated.

Table (6) and Fig (4) illustrate the RAPD profile generated by the primer OP-F05, which produced nine monomorphic and two polymorphic DNA bands with 18.182% polymorphism. One polymorphic band was identified as a unique band (with fragment size 337.427 bp) in *Taxodium distichum*. The other polymorphic, non-unique band, was detected at about 592.501 bp distinguishing the four *Pinus* species by its presence, while it was absent in each of *S.sempervirens* and *T.distichum*. This primer could discriminate each of *S.sempervirens* and *T.distichum* from the group of *Pinus* species, since *S.sempervirens* exhibited no polymorphic bands while *T.distichum* was distinguished by the presence of 337.427 bp unique band.

A total of nine polymorphic bands out of 16 total observed bands with 56.25% polymorphism were generated by the primer OP-F08, three bands were scored as unique, out of the polymorphic bands. All the three unique bands (with 562.785, 366.313 and 341.174 bp) discriminated *S.sempervirens* from the other genera. On the other hand, seven monomorphic bands were scored at about 386.7, 328.8, 290.1, 271.8, 212.2, 180.4 and 158.8 bp. Each of the six conifers is characterized by polymorphic non unique bands, *i.e. P.halepensis* recorded 521.2, 441.9 and 97.07 bp polymorphic bands, *.canariensis* scored only 97.07 bp as polymorphic band while *P.pinea* and *P.roxburghii* involved 235.06 and 97.07 bp. On the other hand, each of 443.2, 343.1 and 235 bp bands were observed in both of *S.sempervirens* and *T.distichum*, in addition to 521, 441.9, 212.2, 180.4 and 158.8 bp bands in *S.sempervirens*.

The primer OP-Z01 generated a total of ten polymorphic (58.824% polymorphism) and seven monomorphic DNA bands in the studied conifers genotypes and species. Five unique bands were scored out of the total identified polymorphic bands in the conifers under investigation. *S.sempervirens* was characterized by the presence of the unique bands with fragment size 838.8, 483.3 and 98.9 bp while *T.distichum* was distinguished by the unique bands (235.5 and 219.5 bp). Moreover, *P.pinea* scored the polymorphic band (364.7 bp) as well as *P.roxburghii*,

S.sempervirens and *T.distichum*. In addition, the polymorphic band (453 bp) was recorded in each of *P.roxburghii*, *S.sempervirens* and *T.distichum*. Besides, *S.sempervirens* and *T.distichum* were both discriminated by the polymorphic bands (546.6 and 269.66 bp), while each of *P.roxburghii* and *T.distichum* were characterized by the presence of (270.4 bp) polymorphic band. However, non of *P.halepensis* and *P.canariensis* scored polymorphic bands. The forementioned polymorphic and unique bands generated by the primer OP-Z01 represent the most distinct ones, therefore, these bands provide additional potentiality for discrimination among the studied genotypes and conifers species.

Table (5): Isomers of peroxidase and Adh enzymes (0/1) and their Rf value.

Band number	Rf	*P.halepensis*	*P.canariensis*	*P.pinea*	*P.roxburghii*	*S.sempervirens*	*T.distichum*
Peroxidase							
1	0.055	0.00	0.00	0.00	0.00	0.00	1.00
2	0.105	0.00	0.00	0.00	1.00	0.00	0.00
3	0.138	1.00	1.00	0.00	1.00	0.00	1.00
4	0.195	1.00	1.00	1.00	0.00	0.00	1.00
5	0.235	0.00	1.00	0.00	1.00	0.00	1.00
6	0.279	1.00	1.00	1.00	1.00	0.00	1.00
7	0.336	1.00	1.00	1.00	1.00	0.00	1.00
8	0.361	0.00	0.00	0.00	1.00	0.00	0.00
9	0.387	1.00	1.00	1.00	1.00	0.00	1.00
10	0.436	1.00	1.00	1.00	0.00	0.00	1.00
11	0.477	0.00	0.00	1.00	0.00	0.00	1.00
12	0.521	0.00	0.00	0.00	0.00	1.00	1.00
13	0.559	0.00	0.00	0.00	0.00	1.00	1.00
14	0.597	1.00	1.00	1.00	1.00	1.00	0.00
15	0.654	0.00	0.00	0.00	0.00	1.00	1.00
16	0.696	0.00	0.00	0.00	0.00	1.00	1.00
17	0.731	0.00	0.00	0.00	0.00	1.00	0.00
18	0.790	0.00	0.00	0.00	0.00	1.00	0.00
19	0.836	0.00	0.00	0.00	0.00	1.00	0.00
20	0.900	0.00	0.00	0.00	0.00	1.00	0.00
Polymorphism%					100%		
Adh							
1	0.205	0.00	0.00	0.00	0.00	0.00	1.00
2	0.268	1.00	1.00	1.00	1.00	1.00	0.00
3	0.363	1.00	1.00	0.00	0.00	0.00	0.00
4	0.739	0.00	0.00	1.00	1.00	1.00	1.00
5	0.863	1.00	1.00	1.00	1.00	1.00	1.00
Polymorphism%					80%		

Table (6): Type and number of the amplified DNA bands generated by five DNA random primers (RAPD) used for the identification of the six conifers.

Primer code	Total amplicones	Monomorphic amplicones	Polymorphic amplicones	Unique amplicones	Polymorphism %
OP-C11	11	9	2	0	18.182
OP-F01	11	9	1	1	18.182
OP-F05	11	9	1	1	18.182
OP-F08	16	7	6	3	56.250
OP-Z01	16	7	5	5	58.824
Total	66	41	15	10	
Average Polymorphism %					37.88

Data illustrated in Table (7) reveal that the highest similarity coefficient was 98% between *P.roxburghii* and *P.pinea*, followed by 96.8% between *P.pinea* and *P.canariensis* then 95.7% between *P.canariensis* and *P.halepensis*. On the other hand, the lowest similarity coefficient was 80.8% between *S.sempervirens* and *P.canariensis* followed by 83% between *S.sempervirens* and *P.halepensis* then 84.1% between *S.sempervirens and P.pinea.*

Table (7): Similarity coefficient among the six conifers as estimated by RAPD analysis.

Species	*P.halepensis*	*P.canariensis*	*P.pinea*	*P.roxburghii*	*S.sempervirens*	*T.distichum*
P.halepensis	1.00					
P.canariensis	0.957	.				
P.pinea	0.947	0.968				
P.roxburghii	0.928	0.947	0.980			
S.sempervirens	0.830	0.808	0.841	0.844		
T.distichum	0.843	0.860	0.893	0.914	0.877	1.00

3.2.2. **Genotype identification by RAPD markers**

The RAPD assay permitted the identification of the six conifers under investigation by unique positive and / or negative markers, as well as the polymorphic markers, as recorded in Table (6) and Fig. (4). *Pinus halepensis* was characterized by the presence of the polymorphic markers with molecular size (521.2, 441.9 and 97.07 bp) obtained from the primer OP-F08 and the polymorphic marker (313.4 bp) obtained from the primer OP-C11. *Pinus canariensis* was distinguished by the absence of the negative unique marker (473.1 bp) revealed with primer OP-F01 and the presence of the polymorphic marker (97.07 bp) obtained with the primer OP-F08. However, *P.pinea* was poorly recognized by the presence of the polymorphic marker (364.7 bp) only as revealed by the primer OP-Z01. *Pinus roxburghii* was characterized by the presence of the polymorphic markers (453, 364.7 and 270.4 bp) all together revealed by the primer OP-Z01. On the other hand, each of *S.sempervirens* and *T.distichum* were highly discriminated from other conifers, as *S.sempervirens* was distinguished by the presence of the unique marker (587.1 bp) by the primer OP-F01, the unique markers (562.8, 366.3 and 341.2 bp) by the primer OP-F08 and the unique markers (838.8, 483.3 and 98.9 bp) out of the primer OP-Z01. Besides, eight polymorphic markers obtained by the primer OP-F08 (521.2, 443.2, 441.9, 343.1, 235, 212.2, 180.4 and 158.8 bp) provided additional potentiality for the discrimination of *S.sempervirens* from the other conifers. *Taxodium distichum*, as well, was highly differentiated by the presence of the unique marker (337.4 bp) out of the primer OP-F05, and the unique markers (235.5 and 219.5 bp) by the primer OP-C11. Moreover, the presence of the polymorphic markers (443.2, 343.1 and 235 bp) from the primer OP-F08 and the polymorphic markers (546.6, 453, 364.7, 270.4 and 269.6 bp) obtained from the primer OP-Z01 and the polymorphic markers (350 and 313.4 bp) recorded by the primer OP-C11 played a considerable role in discriminating *T.distichum*.

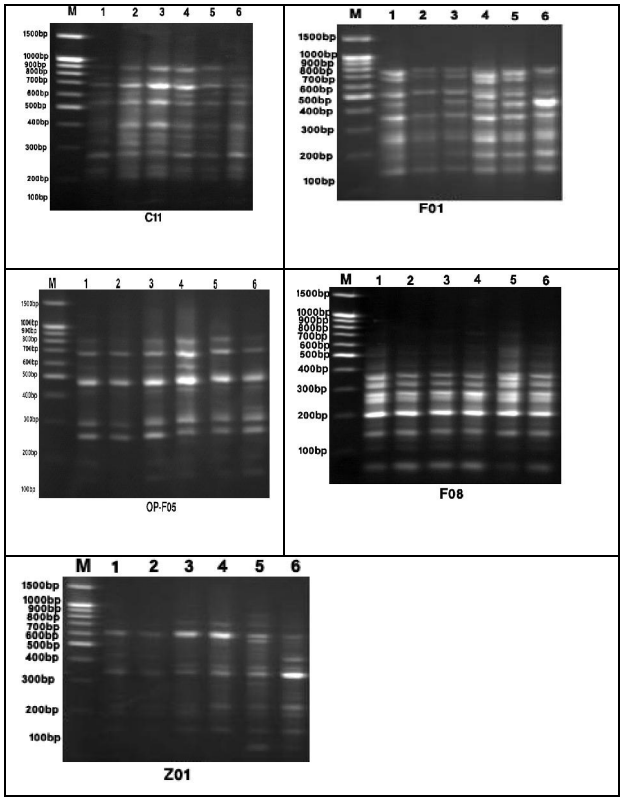


Fig. (4): RAPD profiles for the six conifers as detected with primers: OP-C11, OP-FO1, OP-F05, OP-F08 and OP-Z01 [Lanes 1to6 repr*esent: P. halepensis, P.canariensis, P.pinea, P.roxburghii, S.sempervirens* and *T.distichum.* M: bp ladder DNA marker]

3.2.3 Inter Simple Sequence Repeats (ISSRs)

Five ISSR primers successfully amplified DNA fragments of the six conifers under investigation with total number of 81 fragments producing 38 polymorphic bands (7 unique + 31 non-unique) with 47.95% of mean polymorphism, as demonstrated in Table (8). Besides polymorphism range was recorded between 42.11% (primer A98) and 55.56% (primer HB12) and the range of DNA band size was between (120 – 1460) bp.

Table (8) and Fig. (5) illustrate that primer A98 produced 8 polymorphic bands (42.11% polymorphism) and 11 monomorphic DNA bands in the studied conifers, while 1 unique band was observed out of the polymorphic bands. Four unique bands (491, 436, 305 and 276 bp) were recorded in *P.halepensis*, other four unique bands (389, 303, 278 and 215 bp) were observed in *P.canariensis*, two unique bands (214 and 135 bp) were seen in *P.pinea* and one unique band (478 bp) in *P.roxburghii*. However, *S.sempervirens* and *T.distichum* did not record any unique band concerning for the primer A98. Moreover, *P.halepensis* did not show any non-unique polymorphic band with the primer A98.

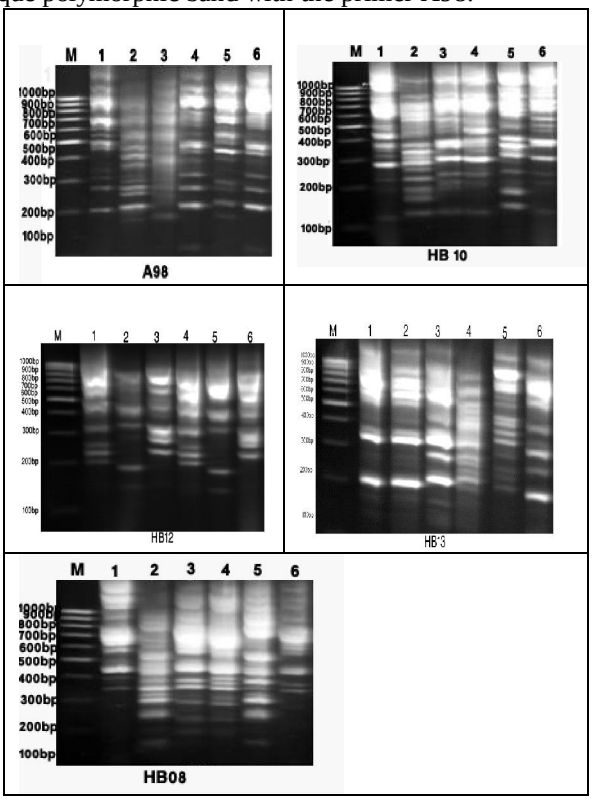


Fig. (5): ISSR profiles for the six conifers as detected with primers:OP-A98,OP-HB10,OP-HB12,OP-HB13, OP-HB08

Primer HB08 resulted in the amplification of twenty DNA fragments with molecular size range from 139 to 1460 bp, with nine polymorphic bands (45% polymorphism), two of them were unique (384 and 598 bp) in *P.roxburghii* and *T.distichum*, respectively. The primer HB 10 produced total of seventeen DNA fragments with molecular size range from 131 to 1288 bp, eight of them were polymorphic (47.06% polymorphism) with two unique DNA bands (256 and 1288 bp) considered as plant specific markers to *P.canariensis* and *T.distichum*, respectively.

Table (8): Type and number of amplified DNA bands generated by five DNA-ISSR primers used for the identification of the six conifers.

Primer code / Band type	A98	HB08	HB10	HB12	HB13	Total
Monomorphic	11	11	9	4	8	43
Unique	1	2	2	0	2	7
Polymorphic (non unique)	7	7	6	5	6	31
Total bands	19	20	17	9	16	81
Polymorphism (%)	42.11	45.00	47.06	55.56	50.00	Mean: 47.95
Fragment size range (bp)	134-1446	139-1460	131-1288	126-763	120-1018	

On the other hand, the primer HB12 resulted in nine DNA fragments with molecular size range from 126 to 763 bp, but did not produce any unique bands, only recorded five polymorphic bands (55.56% polymorphism). Besides, the ISSR profile generated by the primer HB13 (Fig. 5 and Table 8) produced eight polymorphic bands (50% polymorphism) out of them two bands were identified as unique (187 and 147 bp) both were noticed in *P.roxburghii*. The molecular size range generated by the primer HB13 was between 120 and 1018 bp

Each conifer subjected to this study could be discriminated from the others by ISSR-PCR specific markers, except *P.pinea* and *S.sempervirens*. *Pinus halepensis* was characterized by the presence of the unique band 491 bp (primer A98), *P.canariensis* was distinguished by the presence of the unique band 256 bp by the primer HB10. While *P.roxburghii* was discriminated by the unique band 384 bp (primer HB08) and (187 and 147 bp) generated by HB13 primer. Besides, *T.distichum* was distinguished by 598 bp unique band from HB08 primer and 1288 bp generated by the primer HB10

It is evident from Table (9) that the highest similarity coefficient values are (93.4%) between *P.canariensis* and *P.halepensis*, (91.2%) between *P.roxburghii* and*P.halepensis* and 91% between *P.roxburghii* and *P.pinea*. On the other hand, the lowest values are observed between *T.distichum* and *P.pinea* (78.7%) followed by *T.distichum* and *P.halepensis* (79%) and between *T.distichum* and *P.roxburghii* (79.7%).

Table (9): Similarity coefficient among the six conifers as estimated by ISSR analysis

species	*P.halepenses*	*P.canariensis*	*P.pinea*	*P.roxburghii*	*S.sempervirens*	*T.distichum*
P.halepenses	1.00					
P.canariensis	0.934	1.00				
P.pinea	.0.88	0.904	1.00			
P.roxburghii	0.912	0.885	0.910	1.0		
S.sempervirens	0.868	0.855	0.85	0.829	1.0	
T.distichum	0.790	0.840	00.878	0.797	0.865	1.0

3.3. The overall polymorphism detected by the biochemical and molecular markers:

The relationship among the six conifers based on the overall polymorphism detected by the biochemical assays (leaves protein and isozyme polymorphism) and the molecular markers (RAPDs and ISSRs) are presented in Table (10). The total produced bands were 190 out of all markers, 104 (77 non-unique polymorphic and 27 unique bands) were polymorphic bands with 54.74% polymorphism and 86 monomprphic bands. Peroxidase marker gave the highest proportion of polymorphism (100%) followed by protein marker (91.3%), while ISSR and RAPD marker resulted in lower propotion of polymorphism (46.91% and 37.9%), respectively and overall mean (54.74%) of polymorphism % out of the whole assays

Table (10): Polymorphism detected by each marker system in the six conifers

Marker number	Marker system		Monomorphic bands	Polymorphic bands	Unique bands	Total	Polymorphism %
1	Protein		2	18	3	23	91.30
2	Peroxidase isozyme		0	13	7	20	100.0
3	RAPD	OP-F01	9	1	1	11	
4		OP-F05	9	1	1	11	
5		OP-F08	7	6	3	15	
6		OP-Z01	7	5	5	17	
7		OP-C11	9	2	0	11	
	Total RAPD bands		41	15	10	66	37.9
8	ISSR	A98	11	7	1	19	
9		HB08	11	7	2	20	
10		HB10	9	6	2	17	
11		HB12	4	5	0	9	
12		HB13	8	6	2	16	
	Total ISSR bands		43	31	7	81	46.91
	Total number of bands		86	77	27	190	
	Mean polymorphism %						54.74

3.4.Genetic relationships based on protein, peroxidase, RAPD-PCR and ISSR-PCR analysis.

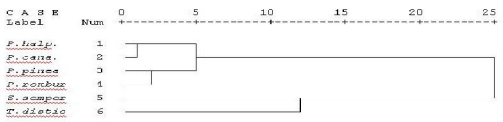
The matrix of similarity index for the six conifers under investigation is presented in Table (11). The maximum similarity coefficient value (91.8%) recorded between *P.halepensis* and *P.canariensis* followed by (91.1%) observed between *P.pinea* and *P.roxburghii*, then (90.5%) between *P. canariensis* and *P.pinea* followed by (90.0%) between *P.canariensis* and *P.roxburghii*. However, the lowest similarity coefficient value (75.3%) was noticed between *P.halepensis* and *T.distichum*. Similarity coefficient range value between *Pinus* species was observed between 91.8% (between *P.halepensis* and *P.canariensis*) and 87.7% (between *P.halepensis* and *P.pinea*), while similarity coefficient between *S.sempervirens* and *T.distichum* was 85.4%. The range of similarity coefficient between *S.sempervirens* and *Pinus* species was between 77.4% (with *P.roxburghii*) and 79.1% with *P.pinea*. On the other hand, the highest similarity coefficient value between *T.distichum* and *Pinus* species was 79.4 (with *P.canariensis*) and the lowest value (75.3%) with *P.halepensis*

Table (11): Similarity coefficient among the six conifers as estimated by protein, peroxidase isozyme, RAPD and ISSR analysis.

Species	P.halepensis	P.canariensis	P.pinea	P.roxburghii	S.sempervirens	T.distichum
P.halepensis	1.00					
P.canariensis	0.918	1.00				
P.pinea	0.877	0.905	1.00			
P.roxburghii	0.895	0.900	0.911	1.00		
S.sempervirens	0.784	0.779	0.791	0.774	1.00	
T.distichum	0.753	0.794	0.775	0.780	0.854	1.00

Data illustrated in Fig. (6) exhibit the dendrogram of the genetic distance between the six investigated conifers, they are grouped into two main clusters, at a distance of 25. The first cluster included *T.distichum* and *S.sempervirens* and the second cluster involved the four *Pinus* species which subdivided into two groups, at a distance of 0.5, the first group included *P.roxburghii* and *P.pinea* and the second group contained *P.canariensis* and *P.halepensis*.

Fig. (6): Clustering dendrogram of the genetic distance between the six conifers, based on proteins, peroxidase, RAPD and ISSR analysis data.



4. Discussion:

Characterization of the genetic diversity and examination of the genetic relationship among conifers are important for the sustainable conservation and increase use of plant genetic resources. In our study, Genetic polymorphism was investigated in six conifers representing four *Pinus*

species, i.e(P.halepensis, P.canariensis, P.pinea, and *P.roxburghii)which* belong to *family Pinaceae* and two members of family *Taxodiaceae, (Sequoia sempervirens and Taxodium distichum).* Genetic biochemical (proteins and isozymes), as well as molecular (RAPDs and ISSRs) analysis were investigated. Proteins and peroxidase banding patterns resulted in extensive polymorphism among conifers under investigation.

The needle leaves protein banding profile was separated using SDS-PAGE of the six genera and species of conifers. The highest number of bands was 16, detected in *Pinus roxburghii* while the lowest number of bands was 8, identified in *Taxodium distichum.*

The protein assay permitted the identification of only three conifers under investigation by unique positive and negative markers. In this regard, Piovesan, *et al.* (1993) stated that the genus *Pinus* has maintained a considerable homogeneity, may be attributed to speciation processes mainly due to gene mutations in which hybridization plays a major role.

In addition, isozyme profile permitted the identification of three conifers under investigation, *i.e. Pinus roxburghii*, *Sequoia sempervirens* and *Taxodium distichum* by the presence of two unique markers found in peroxidase isozyme with *P. roxburghii*, four with *Sequoia sempervirens* and absence of three bands and the presence of one with *Taxodium distichum* with absence of one unique band.

In this respect, González-Andrés, *et al.* (1999) could distinguish between *P.canariensis, P.halepensis, P.pinaster* and *P.pinea* by using *ACP*, *GOT* and *SOD* isozymes banding patterns. They found that *P.canariensis* and *P.pinea* had similarity level of 0.6, while *P.halepensis* presented the lowest similarity level with the other species.

RAPD assay permitted the identification of the six conifers under investigation by unique positive and / or negative markers, as well as the polymorphic markers. Our results revealed that the RAPD marker produced 66 bands with mean polymorphism 37.88%, out of them 10 unique markers were recorded across the six conifers under investigation, besides of 15 polymorphic markers. Each of *S. sempervirns* and *T. distichum* produced the highest unique markers (seven positive markers with *S. sempervirens* and three positive markers with *T. distichum*) followed by *P. canariensis* (one negative marker), while the other *Pinus* species showed no unique markers. In this concern, Cuesta, *et al.* (2010) postulated that stone pine (*Pinus pinea*), the exceptionally low genetic polymorphism of the species has been confirmed in studies applying different markers, such as isozymes, chloroplast and nuclear microsatellites and RAPD. Moreover, Klaus (1989) noted that *P.pinea, P.canariensis* share many cone and vegetative characters, while Frankis (1993) combined *P.canariensis, P.halepensis* in one subsection, *Pinaster*, but he still placed *P.pinea* in a separate subsection. However, Wang, *et al.* (1999) grouped *P.pinea* together with *P.canariensis* and *P.halepensis* in the same subsection.

ISSR results confirm the role of fragments polymorphism on conifers identification. The results were in harmony with those recorded by Wang, *et al.* (2009), and Wang, *et al.* (1999), since they concluded that the ISSR markers are believed to be distributed throughout the whole genome. Most of them may have no direct effect on morphological phenotypes because these DNA sequences are not amplified from functional genes. Also, results suggested the ISSR genetic diversity did not necessarily match the morphological trait difference among the used species (*Dendrobium*). Application of multiple DNA marker systems would help reveal more accurately the phylogenetic relationships among the species based on differentiation of their whole genome organization.

In this investigation, the observed polymorphism in different biochemical (proteins and isozymes patterns) as well as in molecular patterns (RAPDs and ISSRs) recorded with the six conifers, exhibited different similarities between some *Pinus* species which does not match those of taxonomy. This could be explained on the bases of adaptations to the local environmental conditions associated with selection processes through many generations or may be attributed to the occurrence of mutational events that alter the performance of genes encoding some isozymes as well as changes in the annealing site of a random primer. This suggestion is in agreement with Rottenberg, *et al*. (2000).

Newton, *et al*. (2002) postulated that pines, genetically were among the most variable of organisms and characterized by high variation both within and between populations, as indicated by assessments of both quantitative and isozyme variation. The high diversity generally recorded within populations of pines was attributed to reproductive characteristics such as wind pollination, high reproductive capacity, effective mechanisms of seed dispersal, and flexible mating systems which permit inbreeding and selfing in isolated trees. Pines were monoecious, and in most species of the genus, mechanisms of self-incompatibility appeared to be lacking. However, rates of out crossing were generally very high, which appeared to be mentioned by partial self-sterility.

Kusumi, *et al.* (2000) concluded that *Sequoia* were formed in a clade with *Metasequoia* and *Sequoiadendron*, while *Taxodium* formed another clade together while *Glyptostrobus*, both in the family *Taxodiaceae*.

On the other hand, Gadek, *et al*. in the same year (2000) related *Taxodium* and *Seqouia* to two subfamilies (*Taxodioideae* and *Sequoioideae*), respectively under the family *Cupressaceae*.

On the other hand, Liston, *et al.* (1999) found that Himalayan *P.roxburghii* was paraphyletic to the Asian and Mediterranean hard pines, and that the strong

morphological resemblance of *P. roxburghii* to *P.canariensis* has promoted the classification of the two taxa into the same subsection, *Canarienses*. Klaus (1989) suggested that *P.roxburghii* originated from Mediterranean ancestors of P. *canariensis* which reached the Himalayan region and led to the rise of *P. roxburghii.* However, Mirove (1967) suggested an eastern Asian origin of *P.roxburghii* from where it purportedly migrated to the Himalayas and extended to the west, hence he proposed the closely related *P. canariensis* reached to the canary Islands. Moreover, Liston, *et al*. (1999) concluded that *P.roxburghii* might represent an ancestral stock to the Eurasian hard pines.

It is necessary to search extensively at genome scale for more ISSR markers or molecular markers of other types for molecular diagnosis tool specific for additional conifers genus and species.

The ISSR markers reported in the present study will facilitate the understanding of inter- species gene flow, genetic structure of species, genetic diversity and evolutionary relationships in the conifers under investigation. Remaining challenges, as incorporating additional nuclear loci into the molecular analysis, comparing and combining the molecular results with morphology-based phylogenetic analyses.

In conclusion, the present data distinguished the genetic relationship between the six conifers under investigation and established the genetic similarities. In fact, both biochemical and molecular identification were useful in the discrimination between conifers, generally characterized by a high level of polymorphism.

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