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Comparative analysis of different molecular markers used for evaluation of Salix sp. variability

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Abstract The aim of this work was to analyze different types of molecular markers usually used for evaluation of plant variability. Three types of markers were considered, each of them being distinctive for a specific part of the genome. Therefore the RAPD (Random Amplified Polymorphic DNA) recognized random sequences and amplified that DNA regions, ISSR (Inter Simple Sequence Repeats) are specific for the fragments located between two neighboring microsatellites and DAMD (Directly Amplified Minisatellite DNA) are specific for minisatellites regions. As biological materials five different Salix species that are morphologically different were analyzed. Five markers from each category were used for PCR amplification, the obtained data being statistically evaluated. Therefore, the similarity coefficients and dendrograms were determined for each type of markers and also for all three together. It was pointed out that combining multiple categories of markers leading to obtain more accurate results when genetically variability is studied.

Key words

molecular markers, DAMD, ISSR, RAPD, dendrogram

The study of plants variability based on molecular markers which generate complex fingerprints it is of great interest all over the world, because they generate a large number of data which can be statistically evaluated. Therefore it is possible to determine the degree of relatedness between different genotypes, to establish the genetic fingerprint or even to establish the lineage.

In our work three types of molecular markers were used, namely RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeats) and DAMD (Directly Amplified Minisatellite DNA).

RAPD markers (Random Amplification Polymorphic DNA) are based on one short primer (8-12 nucleotides) with random sequence, which recognize different complementary sequences from genome and amplify the fragments between them, if the distance is no longer than 3kb [3, 4, 15].

Another type of markers is DAMD - direct amplification of minisatellite DNA. It is known that minisatelittes are repeated sequences, with lengths from 10 to 100bp which are repeated in tandem 20-50 times, dispersed in all chromosomes, occupying precise regions, relatively short (1-5 kb) [10, 14]. The developed markers are based on the inversions which can appear among them; therefore a single primer can recognize complementary sequences with opposite orientation, making possible the PCR amplification [7, 9, 12, 13].

The ISSR markers - Inter Simple Sequence Repeats are based on microsatellites sequences. These are 1-13bp length (usually 2, 3 or 4), repeated 10-100 times. Frequently they contain repetitions of dinucleotides, but can be three or tetra-nucleotides sequences with a variable structure, with a uniform distribution in the genome. CA sequence is very common in the human genome and can be found in every thousand base pairs. The developed markers consist of single primers, which found complementary sequences in two neighboring microsatellites on opposite chains, amplifying that region [1, 6].

All three types of molecular markers are highly polymorphic due to their small sequence, the number of generated bands is usually high, taking in consideration the propriety of randomly binding, From the genetic point of view they are dominants, because they don't allow to differentiate the homo and heterozygote genotypes [2, 8].

Our interested was focused to evaluate Salix sp. variability due to its high economically importance. Willow is a tree that belongs to Salicaceae family genus Salix found primarily on moist soils in cold and temperate regions. It has very flexible branches, leaves usually alongate also oval and round arranged alternately, with roots that can occur even from aerial parts. It has many uses both physical like weaving strings to get baskets or other tools, chemical, due to its contain of substances like salicylic acid (salicin is the precursor to aspirin and has been used in many medical cases). It also can produce BioDizel due its high energy

ration; the strong roots determine its using in earth stabilization and even heavy metal soil biofiltration. In March- May, when it bloom the pollen is the main source of food that bees have during the flowering period.

The aim of this work was to comparative analyze the three types of molecular markers for evaluation of Salix sp. variability.

Materials and Method

Biological materials

Five different Salix species, from our experimental field were analyzed: 1- Salix fragilis, 2- Salix purpurea, 3- Salix pentandra, 4- Salix alba, 5-Salix daphnoides. From each genotype leaf tissue was collected and samples of 50mg were processed.

DNA extraction

For DNA extraction the modified CTAB method was applied [5]. The leaf tissue was grinded, than 600 µl extraction buffer (3% CTAB (w/v), 100 mM Tris-HCl (pH 8), 20 mM EDTA (pH 7.5), 1.4M NaCl, 3% PVP (3% mercaptoethanol) was added. The mixture was incubated for 1h at 65^0C, stirring from time to time. 600µl of chloroform: isoamylalcohol (24:1) mixture was added and stirred for 5 min until an emulsion was obtained. The mixture was centrifuged for 10 min, 8000rot/min. The supernatant was transferred in clean tubes and the chloroform purification was repeated. The supernatant was transferred in clean tubes; a volume of 200µl NaCl (5M) was added, followed by vortexing. Than 800 µl etanol 95% was added, the mixture was vortexed and centrifuged 10 min at 14000 rot/min. The supernatant was removed and the pellet was washed with 70% ethanol. The DNA samples were dried and re-suspended in 50µl TE solution (10 mM Tris- HCl pH 8) and 1mM EDTA (pH 8).

The DNA concentration and quality was determined by spectrophotometric method with NanoDrop 8000, Thermo Scientific. The DNA concentrations were different, therefore all of them were diluted to 100 ng/µl to make the amplification results comparable.

PCR analysis

For PCR analysis the following primers were used (Table 1).

Table 1

The sequences of the three types of markers used in the experiments

Primer	Primer sequence 5'-3'	Primer	Primer sequence 5'-3'	Primer	Primer sequence 5'-3'
ISSR		**DAMD**		**RAPD**	
A2	(ACTG)5	URF 2F	GTGTGCGATCAGTTGCTGGG	OPW13	CACAGCGACA
A13	(GT)6CC	URF 2R	CCCAGCAACTGATCGCACAC	OPW 19	CAAAGCGCTC
A17	(GTG)3GC	URF 9F	ATGTGTGCGATCAGTTGCTG	OPX 01	CTGGGCACGA
UBC818	(CA)7G	URF 6R	GGCAAGCTGGTGGGAGGTAC	P 7	CTGCATCGTG
UBC811	(GA)8C	14 C2	GGCAGGATTGAAGC	P 11	CCATTCCCCA

The amplification mix had the following composition: 12.5 µl GoTaq Green Master Mix Fermentas 5x, 1 µl $MgCl_2$ 10µM, 5 µl, PVP, 1,5 µl primer 20µM for RAPD and ISSR and 2.5 µl for DAMD, 1 µl DNA and ultrapure water till 25µl.

All the amplifications were performed in Eppendorf Master Cycler ProS, with the following programs, specific for each type of marker.

DAMD markers: denaturation 94°C-5 min, 45 cycles: denaturation 94°- 1 min, primer annealing 53-56°C 1 min (according to the primer sequence), synthesis 72°C-2 min and final extension 72°C-5 min.

ISSR markers: denaturation 94°C-3 min, 45 cycles: denaturation 94°- 1 min, primer annealing 54°C-1,5 min, synthesis 72 °C-2 min and final extension 72 °C-7 min

RAPD markers: denaturation 94°C-3 min, 35 cycles: denaturation 94°- 1 min, primer annealing 36 °C-1 min, synthesis 72 °C-2 min and final extension 72 °C-7 min.

The amplification products were analyzed in 1,8% agarose gel electrophoresis (1.8 g agarose in 100ml TAE solution) in ethidium bromide presence.

The obtained images were interpreted with the VisionWorks®LS, (UVP, Anglia) software to determinate the length and the polymorphism for each band. The obtained data were evaluated statistically **http://genomes.urv.cat/UPGMA/index.php [19].**

Results and Discussions

In our experiments five Salix species with different morphological traits were selected, therefore the expected genetic variability among them was high.

First, the five ISSR primers were used to amplify the DNA extracted from the five different Salix species (Fig. 1). Only the results obtained for the first two primers are shown for exemplifying.

All the amplified fragments were analyzed, scoring with 1 the presence and with 0 the absence of the bands. Based on ISSR markers it was pointed out that they generated a number of 128 fragments, of what 114 were polymorphic (89.1%).

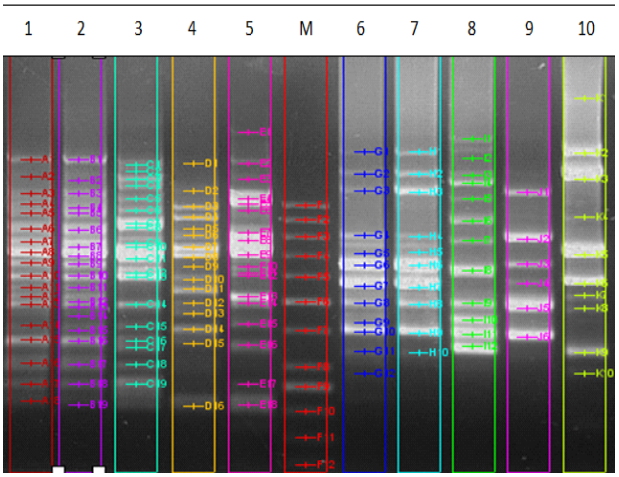

Fig. 1 The agarose gel electrophoresis for the amplification with the ISSR primers A 17 (1-5) and UBC 18 (6-10)
1,6 -S. fragilis; 2,7 -S. purpurea; 3,8 -S. pentandra; 4,9 -S. alba; 5,10 -S. daphnoides; M- GeneRuler 50 bp DNA Ladder.

Next, the five DAMD primers were used to amplify the DNA extracted from the five different Salix species (Fig. 2). Only the results obtained for the first three primers are shown for exemplifying.

All the amplified fragments were analyzed, scoring with 1 the presence and with 0 the absence of the bands. Based on DAMD markers it was pointed out that they generated a number of 94 fragments, of what 87 were polymorphic (92.6%).

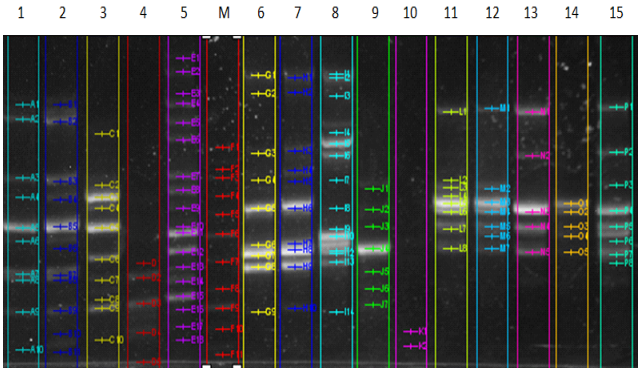

Fig. 2 The agarose gel electrophoresis for the amplification with the DAMD primers URF 9F (1-5), URF 6R (6-10) and 14 C2 (11-15)
1, 6, 11- S. fragilis; 2,7,12 - S. purpurea; 3, 8, 13 - S. pentandra; 4, 9, 14 - S. alba; 5, 10, 15-S. daphnoides; M- GeneRuler 50 bp DNA Ladder .

Next, the five RAPD primers were used to amplify the DNA extracted from the five different Salix species (Fig. 3). Only the results obtained for the first three primers are shown for exemplifying.

All the amplified fragments were analyzed, scoring with 1 the presence and with 0 the absence of the bands. Based on RAPD markers it was pointed out that they generated a number of 103 fragments, of what 97 were polymorphic (94.2%).

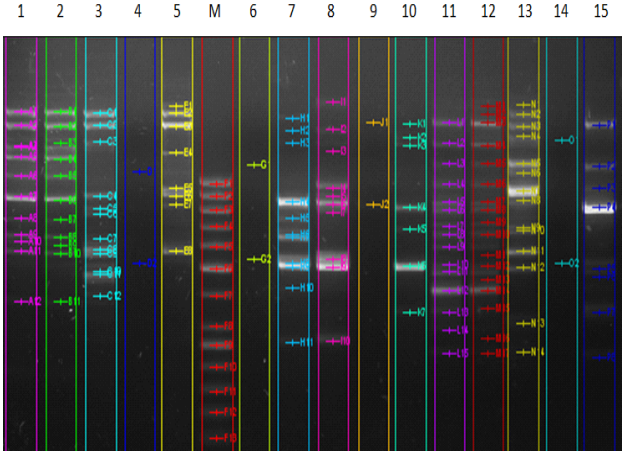

Fig. 3 The agarose gel electrophoresis for the amplification with the RAPD primers OPW13 (1-5), OPW 19 (6-10) and OPX01 (11-15)
1, 6, 11- S. fragilis; 2,7,12 - S. purpurea; 3, 8, 13 - S. pentandra; 4, 9, 14 - S. alba; 5, 10, 15-S. daphnoides; M- GeneRuler 50 bp DNA Ladder .

The number of bands for each primer was counted and the monomorphic/polymorphic condition was evaluated in accordance with their presence on the same length locus (Table 2).

Table 2

The number of bands / polymorphic bands for each primer

ISSR			**DAMD**			**RAPD**		
Primer	Total bands	Poly bands	Primer	Total bands	Poly bands	Primer	Total bands	Poly bands
A2	25	24	URF 2F	23	20	OPW 13	30	29
A13	30	25	URF 2R	20	19	OPW 19	19	18
A17	29	23	URF 9F	24	23	OPX 01	13	11
UBC 818	21	20	URF 6R	17	17	P7	25	24
UBC 811	23	22	14C2	10	8	P11	16	15
Total	**128**	**114**		**94**	**87**		**103**	**97**
Polymprphism		**89.1%**	**Polymprphism**		**92.6%**	**Polymprphism**		**94.2%**

It was emphasized that ISSR markers generated the highest number of fragments (128), followed by RAPD (103) and DAMD (94). The polymorphism was higher for RAPD (94.2%) and lowest for ISSR (89.1%), but it was around 90% for each type of markers.

Further on, the data were introduced in a binary matrix and statistically analyzed to design the dendrograms. First, the dendrograms were done separately, with each type of markers (Fig. 4). Some differences were observed between the different markers dendrograms. But in all three, the samples B (S. purpurea) and A (S. fragilis) were in the same subcluster, with the highest degree of similarity.

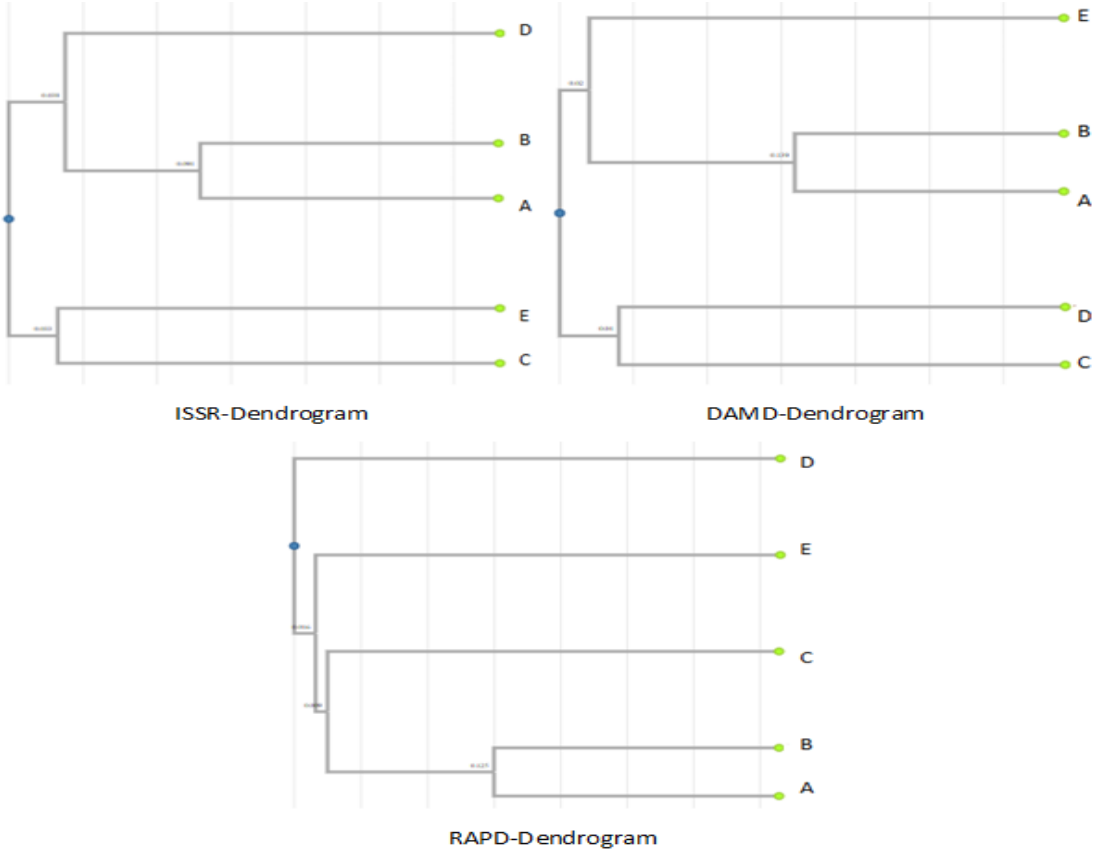

Fig. 4 Dendrogram of each type of primers.
A - S. fragilis; B - S. purpurea; C - S.pentandra; D - S. alba; E - S. daphnoides

The data obtained with all three types of markers were used to design a combined dendrogram (Fig. 5). It was pointed out that it was similar with the DAMD ones, even it also comprised data from RAPD and ISSR ones.

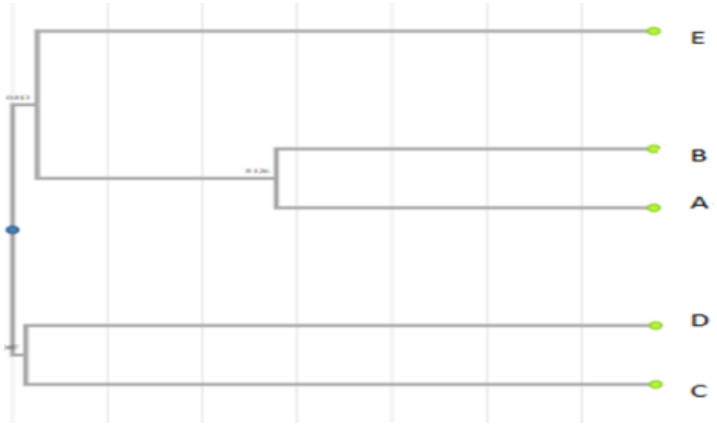

Fig. 5: Final dendrogram based on all three types of markers
A - S. fragilis; B - S. purpurea; C - S. pentandra; D - S. alba; E - S. daphnoides.

Conclusions

The three types of markers generated a high number of fragments originated from different regions of the genome. The highest number was for ISSR and the lower for DAMD (94). The polymorphism was high, being 94.2% for RAPD, 92.6 for DAMD and 89.1% for ISSR. Therefore all the markers are suitable for variability analysis.

The dendrograms obtained with each type of markers were different, pointing out the differences between the annealing sites for the specific primers. The combined dendrogram unified all the obtained data, and in this particular case was similar with the DAMD one.

To increase the accuracy and specificity of the results it is recommended to use several types of markers, generating a high amount of data which better evaluate the genome polymorphism.

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