RESEARCH ARTICLE (Open Access)

Genetic diversity in cultivated *Salvia officinalis* L. using molecular markers

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Abstract:

Albania continues to be a significant supplier of wild Medicinal and Aromatic Plants to the world markets of which Sage remains the major export item accounting for about 70% of the total sage imports to the US in 2013. Sage plants were randomly picked from different cultivation sites in Albania (North/Koplik; Southeast/Skrapar and South/Libohove) in order to screen genetic diversity amongst them employing Randomly Amplified Polymorphic DNA markers using twenty decameric oligonucleotide primers. A total of 2132 DNA bands were generated of which notably clear and scorable were 1555 (from 150 to 1999bp). Primers produced between 63 and 156 bands per Sage plant with an average of 107 bands per primer. Cultivated Sage plant generated between 112 to166 DNA bands with an average of 143 bands per plant. DNA banding patterns, obtained from the Shimadzu Multina PCR-RAPD analysis, were quite polymorphic and were used to carry out hierarchical cluster analysis using the average linkage between groups method of SPSS version 22. The dendrogram showed splitting of the North cultivated Sage from the Southern (southeast and south) group due to (dis)similarity in climate and soil structure/texture. Southeast cultivated Sage plants exhibited some genetic diversity within the group (intrinsic factors driven). This study indicates that RAPDs were fast and easy to use and proved to be efficient discriminatory tools detecting a high level of polymorphism within the same species (intraspecific level) which is explained with ecological variation and the genetic make-up of each individual.

Keywords: Sage; cultivation; Albania; PCR-RAPDs; electropherogram, dendrogram.

1. Introduction

Albania, situated in the southeast region of Europe, on the Balkan Peninsula, along the Adriatic and the Ionian Seas, is endowed by nature with a vast and unique diversity of medicinal and aromatic plants (MAPs) 30% of which are known European plant species.British traveler, Edith Durham, was astonished by the knowledge of northern Albanians on the use of MAPs [5] which have long been used as remedies for various diseases [1]; as peoples' names/surnames and residential locations, and in Albanian traditional folk costumes [4]; they also comprise a significant income source to rural families. Sage is Albania's most strategic export item [1]. The great demand worldwide is due to its medicinal, culinary and decorative uses. Purpose of this paper is to shed light on the genetic relationships of Sage cultivated in different regions of Albania using RAPD markers. Objectives are to (1) screen and analyze genetic diversity among cultivated Sage plants under study, and (2) add to current pool of research work on *Salvia officinalis* L.

2. Material and Methods

Plant material was obtained from seedlings (produced from imported *Salvia officinalis* L. seeds) cultivated in different geographic locations being North/Koplik-Koplik Eperm (annual min.temp. 10.3^{0}C, ann.max.19.7^{0}C; rain 1761mm; Ca^{2+} rich; dry thermic soils), South/Gjirokaster-Libohove (annual min.temp 9.2^{0}C, ann.max.19.9^{0}C; rain 1491mm; Ca^{2+} rich; wet mesic soils), Southeast/Skrapar-Strenec (annual min. temp.5.6^{0}C, ann.max.15.2^{0}C; rain 1079 mm; Ca^{2+} rich; wet thermic soils). Sage plants were randomly picked in each cultivation site in June prior to the flowering stage. Fresh leaves were fully dried prior to genomic DNA extraction.

2.1. Isolation of genomic DNA

Total genomic DNA was extracted from 25mg of dried Sage leaves of each plant following the Qiagen DNeasy® Plant Mini Kit [7]. Initial experiments with once purified DNA did not yield satisfactory results. A second clean-up of all DNA samples, using a second DNA spin column from the above kit, aided in the amplification of the desired PCR products. Amplification reactions following the second clean-up were successful (high quality of extracted DNA).

2.2. PCR Amplification

Polymerase chain reactions were carried employing the Qiagen HotStarTaq® PCR Handbook [8]. The reaction mixture was 25µl containing 2.5µl of PCR Buffer 10x [Tris-Cl, KCl, $(NH4)_2SO_4$, 15mM $MgCl_2$, pH 8.7 (200C)]; 2.5µl of template DNA; 5µl of Q solution 5x (part of Qiagen kit); 1µl of dNTPs mix (5mM each; AbgeneAB-0196/Thermo Scientific); 1µl of Primer (100µM; Sigma Aldrich); 0.125µl of HotStarTaq Polymerase (5 units/µl; Qiagen) and 12.875 1µl of Water (Abgene). Twenty random decameric oligonucleotide primers were initially desalted and HPLC purified for higher quality amplification products. Samples were then placed on a PCR machine for about 5 hours and a half. Thermal cycle used was 96^0C for 15min, 96^0C for 1min, 40^0C for 1min, 72^0C for 3min; return to step2 for an additional of 46 cycles, 72^0C for 10min and hold at 4^0C. Amplified products were run onto a Shimadzu Multina machine using a DNA-1000kit. The ladder, composed of PhiXDNA (supplied by Promega), has been treated with the restriction enzyme HindIII. Size of each amplified product was determined by comparison with the DNA laser run on each chip.

2.3. Hierarchical Cluster Analysis

RAPD banding patterns of cultivated Sage plants under study were used to assess genetic diversity amongst them. DNA molecule sizes were obtained reading the electropherogram (Fig.2) of each Sage plant under study. Molecular weight of amplified DNA bands (only those intense and above 150bp) and name of each Sage plant were inserted into a binary matrix; fragments were scored as (1) for presence, and (0) for absence of band. Hierarchical cluster analysis was carried out employing the Average linkage (between groups) method using the squared Euclidian distance measure (as they are binary data) of SPSS (Statistical Product and Service Solutions)-version 22.

3. Results and Discussion

Sage plants cultivated in different geographic locations being North/Koplik Eperm, South/Libohove and Southeast/Strenec were examined for the extent of the genetic variability. Plants exhibited morphological differences (leaf size and shape) between and within geographic location (Fig.1). Moreover, Sage plants of South/Libohove were the least healthy.

Figure 1. Morphological differences within location /Koplik Sage

3.1. RAPDs Analysis

Twenty decameric primers were employed for the evaluation of their genetic relationships based on the RAPD banding patterns obtained by extracting genomic DNA from each cultivated Sage plant. A total of 2132 bands was generated by the twenty primers of which 1555 (73%) were highly polymorphic; this percentage of polymorphism agrees with previously reported Lamiaceae related studies [2;6;9]. DNA bands considered for reading ranged between 150-1999bp. An average of 107 bands per primer was generated of which informative were only 78 (Fig.2. only 2 out of 6 DNA bands generated by OPB2 were considered sccorable). OPB4 yielded the highest (156) number of bands; OPB3 the lowest (63). Sage of Southeast origin (Strenec-ST3) generated the highest (166) number of bands whereas Sage of South origin (Libohove-Lb5) generated the lowest (112). An average of 143 bands per plant was obtained of which only 103 were considered scorable. Cultivated Sage plants of North (Koplik/Koplik Eperm) generated a total of 719 bands; Southeast (Strenec/Skrapar) 753; South (Libohove/Gjirokaster) 660. Some bands shared between genotypes, however primers allowed for noticable genetic diversity between sage plant groups of different cultivation areas.

SHIMADZU BIOTECH

Data File Properties

Instrument Name	: MultiNA
Date Analysis Started	: 19/12/2013 12:20:21
Project Name	: DNA-1000_Premix
Project Comment	:
Operator	: Shimadzu
Data File Name	: Sage_OPB2_OPB20.mlt
Data File Comment	:
Original Data File Name	: Sage_OPB2_OPB20
Separation Buffer	: DNA-1000
Marker Mixing Mode	: Premix
Dye	: SYBR® Gold
Ladder Type	: Standard (STD)

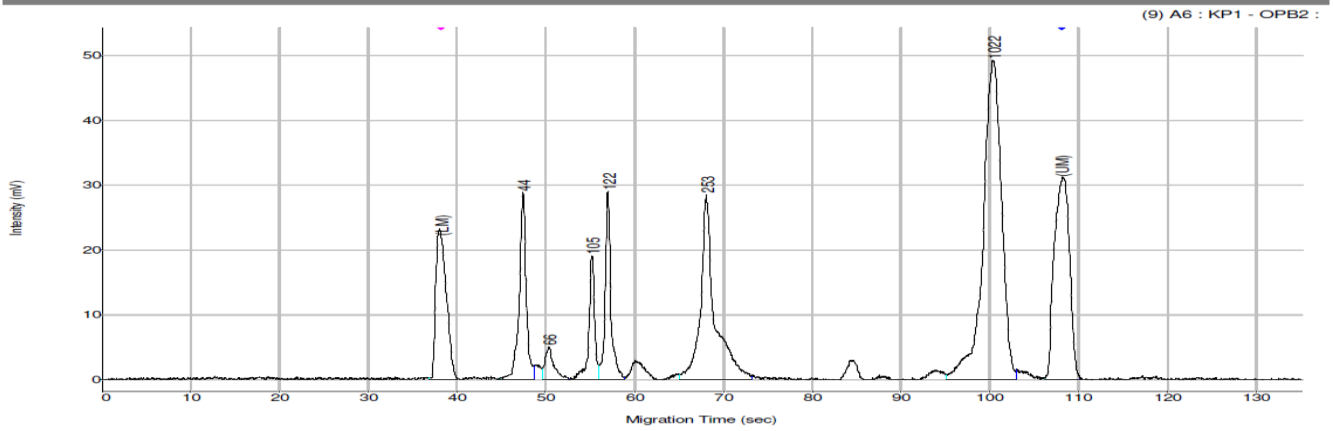
Chip and Ladder Information

Chip4: ID: 5353, Used 293 times (3) X1-3 (STD)

Sample Sheet

	Well	Sample Name	Comment	Type	Chip
(9)	A6	: KP1 - OPB2		Sample	4

Electropherogram - Analyzed Data (Migration Time)



Results Table

No.	Size (bp)	Conc. (ng/µL)	Molar. (nmol/L)	FWHM (sec.)	Resolution	No. of TP	Start (%)	End (%)	Start H.(mV)	End H.(mV)	History
1	(LM)	-	-	1.36	5.206	4334	-1.89	2.63	0.00	0.00	
2	44	3.38	115.45	0.72	2.110	24031	9.53	14.88	0.00	0.00	
3	66	0.68	15.41	0.92	2.110	16600	16.23	20.44	0.00	0.00	
4	105	1.35	19.33	0.58	1.913	50180	21.35	25.50	0.00	0.00	
5	122	1.86	22.89	0.54	1.913	61770	25.50	29.59	0.00	0.00	
6	253	3.18	18.89	1.00	8.453	25677	38.47	50.11	0.00	0.00	
7	1022	3.39	4.98	2.12	2.190	12415	81.45	92.67	0.00	0.00	
8	(UM)	-	-	2.04	2.190	15585	97.14	102.95	0.00	0.00	

Figure 2. DNA banding pattern of Sage/North/Koplik **(**KP1) using primer OPB2

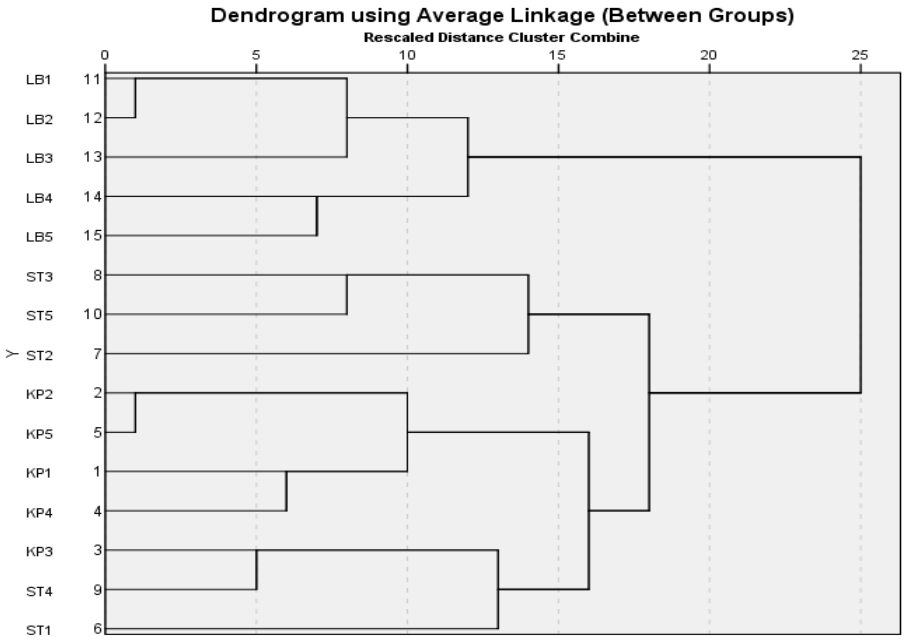


Figure 3. Genetic relationships of cultivated Sage plants based on respective RAPD-PCR banding patterns

3.2. Hierarchical Cluster Analysis

Cluster analysis (Fig.3), produced based on the RAPD banding patterns of cultivated Sage plants, allowed for the separation of the North from the Southeast and South Sage (ecological variation). South Sage plants clustered together (due to soil pattern and improper cultivation practices excercised). Southeast Sage plants linked to some Koplik Sage ones which, in agreement with [3], may be explained with terpenoid synthesis within Salvia.

4. Conclusions

Sage plants, cultivated in different geographic locations (North, South, Southeast) of Albania, exhibited remarkable range of morphological differences (leaf sizes and shapes) between and within cultivation site. South Sage plants (Libohove) were the least healthy (due to inappropriate drainage; hence cultivation techniques must be revised for this location for succesfull Sage plants growth and development and commercial cultivation viability).

RAPD patterns produced by twenty different decameric oligonucleotide primers were easily scored, clearly distinct and highly informative; they allowed characterizing many Sage genotypes and determining genetic relationships among them. The dendrogram, produced based on the RAPD banding pattern of each Sage plant, showed splitting of the North cultivated Sage from the Southeast and South Sage; the latter outgrouped notably together. This ecological variation may be due to (dis)similarity in weather patterns and soil structure/texture. Southeast cultivated Sage plants exhibited genetic diversity within the group (intrinsic factors driven).

Results of this investigation indicate that RAPDs were fast and easy to use and proved to be reliable discriminatory tools detecting a high level of polymorphism within the same species (intraspecific level) which is explained with (a) ecological variation and (b) genetic make-up of each individual plant.

Further research work is presently being carried out to assess and compare chemical profiles of the cultivated Sage plants under study in order to identify whether differences in cultivated Sage chemotypes reflect the genetic differences of these individuals; as well as how do the cultivation techniques affect chemical profiling and genetic variability amongst cultivated plants.

5. Acknowledgements

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6. References

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