GENETIC VARIABILITY BETWEEN SOME ALBANIAN NATURAL POPULATIONS AND COMMERCIAL CULTIVARS OF *SALVIA OFFICINALIS* L. BASED ON RAPDs

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ABSTRACT

Dalmatian sage (Salvia officinalis L.) represents one of the most significant aromatic plants that naturally grow in Albania and most of Mediterranean region. Nowadays, due to the essential oils and because of economical, medicinal, aromatic and culinary importance, it is cultivated in some areas of the country. Different molecular markers have been successfully used for years to evaluate genetic variability of common sage. The present study evaluates the genetic relationships between six natural populations and six commercial cultivars of Salvia officinalis in Albania. Genomic DNA was isolated based on a modified CTAB protocol and PCR amplification was completed, using ten decameric random primers from Operon Technologies. The PcoA software was used to perform cluster analysis and determine genetic distances among the populations. Results showed a clear separation between the indigenous and cultivated groups, and higher genetic diversity is found within natural populations. These data show that native common sage plants represent a genetic pool different from that of commercial ones sharing a similarity of only 25%.

Keywords: *sage, molecular markers, genetic variability, RAPD markers*

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1. INTRODUCTION

Salvia officinalis L. is an outcrossing, insect-pollinated, long-lived sub-shrubby plant of the family *Lamiaceae*, and it has been known for its medicinal and culinary uses since ancient times. Common sageis native to the east side of Adriatic (Ristić *et. al*., 1999) and Ionian seas with a habitat reaching south into northwest Greece (Karousou *et. al*., 2000).

These species have been investigated mainly for the content and composition of the essential oil, since this has proved to be responsible for most of the pharmacological effects attributed to the plant (Jug-Dujaković *et. al*., 2012; Liber *et al*., 2014). Sage is valued because of its antiseptic, anti-inflammatory, antioxidant, carminative, cholagogue and diaphoretic properties (Pluhár *et. al*., 2012).

Its autochthonous region is the Mediterranean basin, along with a large number of endemic species that make it one of the world plant biodiversity centers (Medail and Quezel, 1999; Sales *et al*., 2001). It has been harvested mostly from wild populations in Croatia, Bosnia and Herzegovina, Montenegro and Albania.

Meanwhile, Sage (*Salvia officinalis*) naturally grown in Albania, is studied and used extensively for commercial purposes. It is also cultivated in a number of private companies for the high quality of the essential oils. Considering the appropriate conditions, the market demand and the economic interest of cultivation, sage is successfully cultivated in a considerable area of around 1500 ha, giving these areas a considerable opportunity for economic and social development.

Molecular markers provide a powerful tool for proper characterization of plant germplasm and their management. Among developed genetic markers, RAPDs, AFLPs and SSR, RFLP, have been widely used for genetic diversity analyses (Bacu *et. al*., 2005; Khalil 2005; Adhikari 2013; Papa *et. al*., 2016) among sage populations of Albania.

Previous studies of molecular characterization with RAPD markers, AFLP and SSR markers for 80 genotypes of Albanian sage populations (Bacu *et al*., 2005; Bacu *et al*., 2011) proved that genotypes of the same populations shared from 30% to 60% and to 80% similarity; that the populations of the near geographical locations grouped together giving this way a strong indication on the important role of the environmental conditions into the genome of this species.

In other studies (Papa *et al*., 2016; Papa *et al*., 2017), 43 genotypes for 13 natural populations of northern Albania were evaluated for the diversity of monoterpene synthases coding genes and PCR-RFLP of cp-

DNA. Results from both methods showed that genotypes of the same populations shared 65% to 80% similarity, leaving aside as the most distinctive one the populations of Kruja, Torovica and Prostriba (Papa *et al*., 2016; Papa *et al*., 2017).

However, sage has been cultivated intensively in North and South of the country for export purposes dedicated to their essential oils. This commercialization has reduced the appraisal of vegetation only in the GC definition of the essential oils content, which accompanies this product as a companion certificate to foreign markets (Kathe *et al*., 2003; Kongjika *et al*., 2005). Thus, the genetic variation between natural populations and cultivated ones with molecular markers are less exploited.

Rolim *et al*., (2011) said that random Amplified Polymorphic DNA (RAPD) technology is a fast procedure for studying genetic diversity using polymerase chain reaction. The poor reproducibility in early RAPD analysis has been largely overcome through improved laboratory techniques and band scoring procedures (Khalil *et al*., 2012). Since RAPD markers are dominant, they can be used to identify genetic variation (Solyman *et. al*., 2014), genetic diversity analysis (Maric *et al*., 2004; Papa *et al*., 2016) and phylogenetic relationships (Goriunova *et al*., 2004; Papa *et al*., 2016) by taking into account the fact that profiles are scored for the presence or absence of a single allele.

This study considers six natural and six cultivated populations of northern and southern Albania, which generally exhibit a low morphological diversity, aiming to exploit their genetic diversity.

2. MATERIALS AND METHODS

Plant material: Fresh young leaves from 12 populations of *Salvia officinalis* of northern and southern Albania (six natural and six cultivated) were used to extract total genomic DNA (Table 1). The cultivated plant material was taken from the 'ATC Natural' company, which deals with the cultivation of medicinal plants in different cities of Albania and their export to Europe.

Table 1. Sage germplasm used in this study, their source and origin

No.	Populations	Latitude	Longitude	GPS coordinates
1	Shkodër	42.066447	19.428860	42° 3' N, 19° 25' E
2	Skrapar	40.5	20.216667	40° 30′ 0″ N, 20° 13′ 0″ E
3	Leskovik	40.15	20.6	40° 9′ 0″ N, 20° 36′ 0″ E
4	Lezhë	41.777724	19.658028	41° 46' N, 19° 39'E
5	Himarë	40.101667	19.744722	40° 6′ 6″ N, 19° 44′ 41″ E
6	Koplik	42.213611	19.436389	2° 12′ 49″ N, 19° 26′ 11″ E

Isolation of genomic DNA: Equal amounts (0.1 g) of leaf tissue were placed in a mortar chilled with liquid nitrogen and were ground to fine powder. Total genomic DNA was extracted as described in (Doyle and Doyle 1987). Quality and quantity of DNA was measured based on (Sambrook *et al*., 2000).

PCR amplification: Ten arbitrary RAPD primers chosen from different references as described in Table 2 were used: OPA20, OPA09, OPA11, OPA08, OPA19, OPB8, OPB12, OPC15, OPB17 and OPB13.
The PCR amplifications were carried out in Veriti 96-Well Thermal Cycles (Applied Biosystem) in a total volume of 25 µl containing Master mix (Cinnagen, Tehran, Iran), genomic DNA (30 ng), 50 pmoles of decameric primer and Taq DNA polymerase. The PCR program started with an initial phase of 1 minute at 95^{o}C, followed by 45 cycles of 30 s at 95^{o}C, 30 s at 39^{o}C, 2 min at 72^{o}C and 10 min final elongation at 72^{o}C (Williams *et al*., 1990).

Table 2. RAPD primers sequences based on (Scoula *et al*., 1999)

Primer	Primer Sequence
OPA20	5’ -GTTGCGATCC- 3’
OPA09	5’ -GGGTAACGCC- 3’
OPA11	5’ -CAATCGCCGT- 3’
OPA8	5’ -GTGACGTAGG- 3’
OPA19	5’ -CAAACGTCGG- 3’
OPB8	5’ -GTCCACACGG- 3’
OPB12	5’ -CCTTGACGCA- 3’
OPC15	5’ -GACGGATCAG- 3’
OPB17	5’ -AGGGAACGAG- 3’
OPB13	5 ’-TTCCCCCGCT- 3’

The amplified products were separated by electrophoresis in horizontal 1.5% agarose gels in 10XTBE. Fragments were scored as a binary variable, (1) for the presence, and (0) for the absence of each band. The most distinct well-resolved stable bands were considered for statistical analysis.The variables were used to build genetic dendrograms with UPGMA method of PcoA software.

3. RESULTS AND DISCUSSIONS

The RAPDs based analysis of six natural populations and six cultivated populations of sage of Albania displayed a variable level of similarity for different primers used to amplify arbitrary primed regions. Six decameric primers of Operon technologies out of ten gave best stable polymorphic profiles, respectively OPA11, OPA08, OPA09, OPB12, OPC15 and OPB08, which were further processed (Fig 1-4). The other 4 primers, respectively OPA20, OPA19, OPB17, OPB13, did not amplify or present good amplification pattern. Table 3 shows the sequences of RAPD primers with the best polymorphic profiles, their sequence, total number of bands and polymorphic bands as well as the range of the size of fragments.
The number of amplification products per primer varied from 10 (OPB-08 and OPC15) to 12 (OPA-11). Six out of ten primers used were able to distinguish all the populations, and produced a result according to which 88% of the bands were polymorphic. This percentage of polymorphism was within the range previously reported for *Salvia fruticosa* (Skoula *et al.*, 1999), and other medicinal and aromatic species of the *Lamiaceae* family such as *Ocimum gratissimum* (Vieira *et al*., 2001), *Lavandula angustifolia* (Echeverrigaray and Agostini 2000).

Table 3. List of decamer oligonucleotide primers with best polymorphic profiles

Primer	Primer sequence 5’ - 3’	Total number of bands	Nr of polymorphic bands	Size of fragments (bp)
OPA11	5’ -CAATCGCCGT- 3’	12	11	250-1200
OPA08	5’ -GTGACGTAGG- 3’	11	10	300-1500
OPA09	5’ -GGGTAACGCC- 3’	9	9	200-1200
OPB12	5’ -CCTTGACGCA- 3’	11	9	250-1400
OPC15	5’ -GACGGATCAG- 3’	10	8	150-850
OPB08	5’ -GTCCACACGG- 3’	10	9	150-1200
Total	-	63	56	-

Figure 1-4 show the best amplified bands from six RAPD primers involved in the present investigation (Table 3).

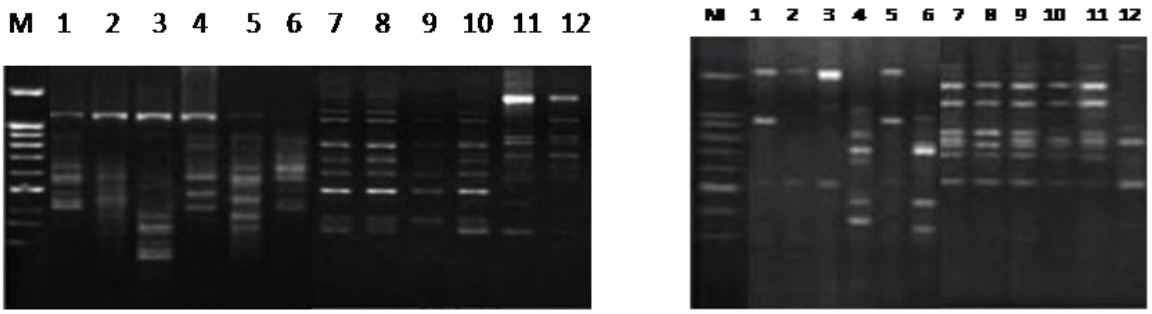

Fig. 1: RAPD profiles from 12 populations of sage from amplification with primer OPA11 (left) and

OPA08 (right). From left to right populations: (M) marker 100bp, **1-** Himarë (natural population), **2-** Koplik (natural population) **3-** Leskovik (natural population), **4-** Lezhë (natural population), **5-** Shkodër (natural population), **6-** Skrapar (natural population), **7-** Himarë. (commercial cultivar), **8-** Koplik (commercial cultivar), **9-** Leskovik (commercial cultivar), **10-** Lezhë (commercial cultivar), **11-** Shkodër (commercial cultivar), **12-** Skrapar (commercial cultivar).

OPA11 generated 12 fragments in total, 11 of them as polymorphic. Most of the cultivated samples, based on the bands of gel electrophoresis, have similarities between them, which is expressed by the presence of bands at the same size, approximately 500-100 bp. Cultivated populations of Leskovik and Lezha (respectively 9 and 11) appear as more polymorphic among others. The size of bands generated by OPA 11 ranged from 250bp to 1200 bp. The percentage of polymorphism generated for all primer was 91%.

OPA08 generated 11 fragments totally, all of them as polymorphic. Natural populations are more polymorphic that the cultivated ones. The size of bands generated in the twelve natural populations ranged from 300bp to 1500bp. A similarity between the cultivated populations (respectively 7, 8, 9, 11 and 12) was observed, which were almost identical with each other, based on the size of bands.

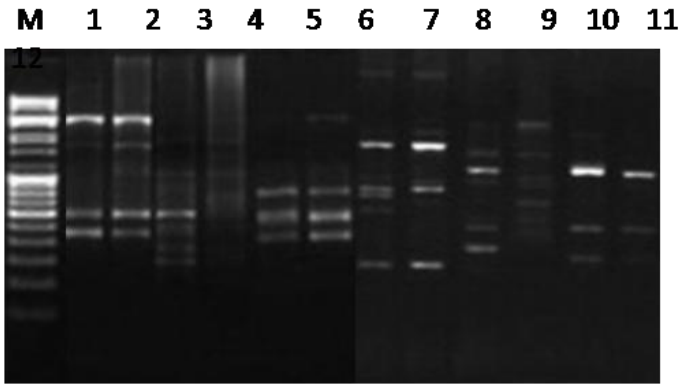

Fig. 2: RAPD profiles from 12 populations of sage from amplification with primer OPA09. From left to right populations: (M) marker 100bp, **1-** Himarë (natural population), **2-** Koplik (natural population) **3-** Leskovik (natural population), **4-** Lezhë (natural population), **5-** Shkodër (natural population), **6-** Skrapar (natural population), **7-** Himarë (commercial cultivar), **8-** Koplik (commercial cultivar), **9-** Leskovik (commercial cultivar), **10-** Lezhë (commercial cultivar), **11-** Shkodër (commercial cultivar), **12-** Skrapar (commercial cultivar).

OPA09 generated in total 9 fragments, all of them polymorphic. Natural populations showed a higher polymorphic level compared to the commercial cultivars. The size of bands generated ranged from 200bp to 1200bp.

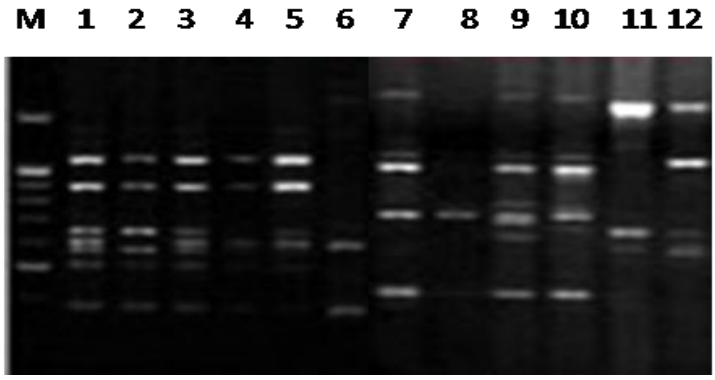

Fig.3: RAPD profiles from 12 populations of sage from amplification with primer OPB12: From left to right populations: (M) marker 100bp, **1**-Himarë (commercial cultivar), **2**-Koplik (commercial cultivar) **3**- Leskovik (commercial cultivar), **4**- Lezhë (commercial cultivar), **5**- Shkodër (commercial cultivar), **6**- Skrapar (commercial cultivar), **7**- Himarë (natural population), **8**- Koplik (natural population), **9**- Leskovik (natural population), **10**- Lezhë (natural population), **11**- Shkodër (natural population), **12**- Skrapar (natural population).

OPB12 generated in total 11 fragments, all of them polymorphic. A similarity between the commercial cultivars of Himara, Koplik, Leskovik and Shkodra (respectively 1, 2, 3, and 5) was observed, which were almost identical with each other. Likewise, the cultivated populations of Himara, Leskovik and Lezha (respectively 7, 9 and 10) showed a high similarity between each other. The size of bands generated from OPB12 ranged from 250 to 1400bp. The percentage of polymorphism generated for primers OPA08, OPA09 and OPB12 was 100%.
OPC15 generated in total 10 fragments, eight of them polymorphic. The size of bands generated in the twelve populations ranged from 150bp to 850bp. The percentage of polymorphism generated for primer OPC15 was the lowest compared to the others, 80 %.
OPB08 generated in total 10 fragments, 9 of them polymorphic. The natural population of Himara and the cultivar of Lezha showed higher polymorphic level compared to the rest of natural and cultivated populations. The size of bands generated from OPB08 ranged from 100bp to 1200bp and the percentage of polymorphism generated for primer OPC15 was approximately 90%.

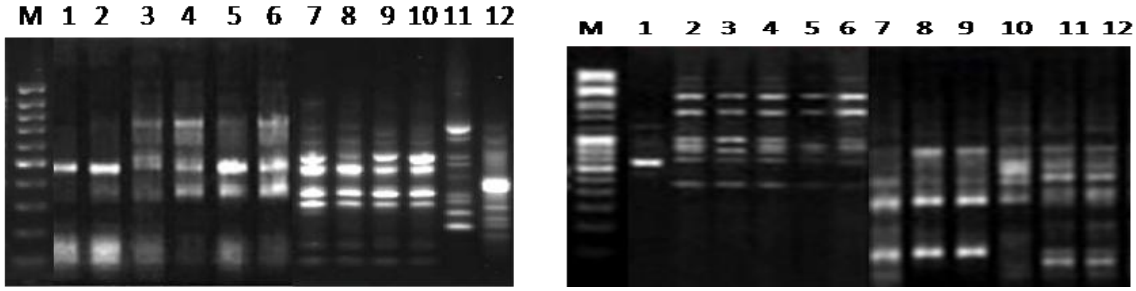

Fig.4: RAPD profiles from 12 populations of sage from amplification with primer OPC15(left) and OPB08 (right): From left to right populations: (M) marker 100bp, **1**- Himarë (natural population), **2**-Koplik (natural population) **3**- Leskovik (natural population), **4**- Lezhë (natural population), **5**- Shkodër (natural population), **6**- Skrapar (natural population), **7**- Himarë (commercial cultivar), **8**- Koplik (commercial cultivar), **9**- Leskovik (commercial cultivar), **10**- Lezhë (commercial cultivar), **11**- Shkodër (commercial cultivar), **12**- Skrapar (commercial cultivar).

The ability of the RAPD analysis to differentiate *S.officinalis* populations in the present investigation suggests that this technique can be very useful to the identification of genetic diversity among natural and cultivated populations of sage. Reproducibility of bands was good, and they were used to build clusters of similarity among the populations (Fig 5).

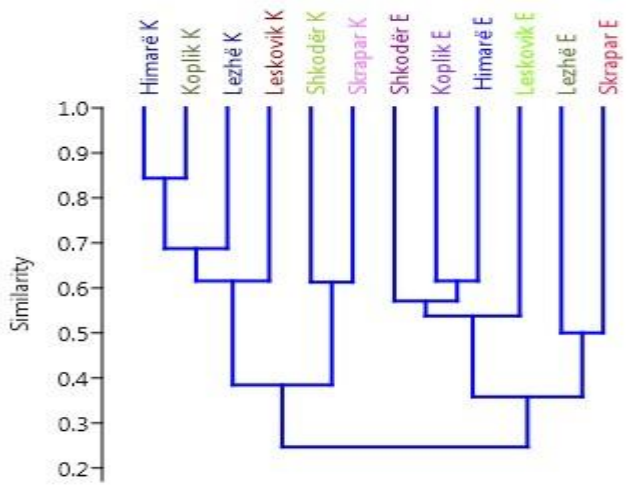

Fig.5: Dendrogram of polymorphic distances between analyzed samples, constructed by UPGMA method of PCoA software.

The dendrogram provided showed two main clusters. The first main cluster consisted of natural populations (populations to the right of the dendrogram) and the second cluster consisted of cultivated populations (populations to the left of the dendrogram). Natural populations and commercial cultivars, according to *Jaccard* coefficient, had a similarity level approximately 25% where the diversity level is more present among natural populations compared to the cultivated ones. These two groups were joined together at about 0.250 genetic distance level.

Dendrogram cluster for the commercial cultivars showedtwo main clusters, one of them divided in 3 main sub-clusters and the other only of one sub-cluster, grouped in different genetic distances. The first sub-cluster consisted of Himara and Koplik populations, linked together at about 0.850 genetic distance. The second and the third sub-cluster consisted of Lezha and Leskovik population, respectively, which stay apart from the others in a genetic distance level approximately 0.70 and 0.63. The fourth one consisted of two populations, Shkodra and Skrapar, joined together at about 0.60 genetic distance level. Genetic distance levels of cultivated populations varied from 0.350 to 0.850. The high similarity level among cultivated populations compared to the natural populations shows that breeding programs do not explore the whole genetic variability of sage germplasms, as seen also in different studies from the Balkan and European countries (Echeverrigaray *et al*., 2006; Liber *et. al*., 2014). Reduction in the genetic variability of *Salvia officinalis* is one of the negative aspects of these kinds of breeding programs and it can affect seriously the genetic vulnerability of it or reduce sage selection profits, unless there are incorporated new sources of variation.

Dendrogram cluster for natural populations showed two main clusters, joined together at 0.350 genetic distance level. The first one was divided in four sub-clusters and the second one only in one. The first sub-cluster consisted of Shkodra population in a genetic distance level of 0.570. Likewise, the third sub-cluster consisted of Leskovik populations in a genetic distance level of 0.550. The third one consisted of two populations, Koplik and Himara, linked together at about 0.620 genetic distance level. The fourth sub-cluster consisted of two populations, Lezha and Skrapar, both of them in about 0.500 genetic distance level.

Natural populations showed significant diversity, while cultivated populations indicated a moderate diversity among one another.Our results indicate that RAPD markers are sufficiently informative to assess genetic diversity of Dalmatian sage populations. The application of molecular markers as RAPD markers, AFLP, RFLP and SSR markers have proven to be powerful enough in detecting variability even between very closely related individuals of the same aromatic plant species classified as ecotypes in previous studies (Bacu *et al*., 2005; Bacu *et al*., 2011; Papa *et al.*, 2016).

Sage has been cultivated intensively in North and South of Albania and in the Balkan countries like Croatia, Bosnia Herzegovina etc., for export purposes due to their essential oils (Kathe *et al*., 2003; Adhikari *et. al*., 2013; Liber *et al*., 2014). This commercialization has reduced the appraisal of vegetation

only in the GC definition of the essential oils content, which accompanies this product as a companion certificate to foreign markets (Kathe *et. al*., 2003; Kongjika *et. al*., 2005). Our results about the low diversity level between cultivated populations indicated that even in Albania among other Balkan and European countries, commercial gathering in the wildness has a negative impact on biodiversity conservation (Echeverrigaray *et. al*., 2006; Statovic *et. al*., 2012). Breeding programs based on detailed information on population genetic structure of aromatic and medicinal plants, such as *Salvia officinalis*, together with new accurate and appropriate *ex situ* conservation strategies would help the preservation and exploitation of these genetic resources in the country.

5. CONCLUSIONS

Genetic relationships between six natural populations and six commercial cultivars of *Salvia officinalis* L. is in the present paper evaluated. The RAPDs reactions showed that six out of ten random primers (OPA11, OPA08, OPA09, OPB08, OPB12, OPC15) were able to distinguish polymorphismsbetween *Salvia officinalis* populations.

Analysis of 12 samples in the study using 6 RAPD decameric primers generated a total of 63 fragments with an average of 60 polymorphic fragments per locus. The highest number of bands resulted in the analysis with the OPA11 primer, which generated 12 fragments, 11 of them polymorphic, while the lowest number of bands received was observed in the amplified samples with the OPA09 primer, which generated 9 fragments. The percentage of polymorphism generated for all primer varied from 80-100%.

PCoA analysis grouped populations into two major clusters, natural populations and commercial cultivars apart from each other.

Natural populations and commercial cultivars had a similarity of 25% where the level of diversity is more noticeable among the wild populations than the cultivated ones.

Among the commercial cultivars, those that showed greater similarity between them, respectively 85% according to *Jaccard* coefficient, are the cultivars of Himara and Kopliku.

All the natural populations reported significant, but moderate differentiation diversity among cultivated populations. The populations that showed greater genetic distance between them are natural populations of Leskovik and Shkodër, with about 55% according to *Jaccard* coefficient.

The high similarity level between commercial cultivars shows that commercial gathering in the wildness has a negative impact on biodiversity conservation of *Salvia officinalis* L.

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