IDENTIFICATION OF SALICORNIA POPULATION: ANATOMICAL CHARACTERIZATION AND RAPD FINGERPRINTING

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Abstract - Anatomical and Random Amplified Polymorphic DNA (RAPD) analysis of two typical populations of Salicornia europaea from Montenegro and Greece (Lesvos), one typical population of S. ramosissima from Spain and one population that belongs to the Salicornia genus from Serbia, was undertaken to develop a new strategy for identifying Salicornia plants. Anatomical variability and differentiation were examined using Principal Component Analysis (PCA) and Multivariate Discriminant Function Analysis (MDA). On the basis of the anatomical measurements, the four populations were classified into three groups: one joining the plants from Serbia and Spain, one comprising the Montenegrin group and one comprising the Lesvos group. RAPD analysis indicated that populations from Spain and Serbia were closely related to each other and the Lesvos group was quite different from all the other investigated populations. These results opened up the possibility that the specimens from Serbia belonged to S. ramosissima and not to S. europaea, as reported previously.

Key words: DNA fingerprinting, glasswort, halophyte, shoot anatomy

INTRODUCTION

The genus Salicornia is among the most diverse genera of the Salicornieae tribe. Salicornia grows on periodically wet saline coastal and inland habitats such as: salt marshes, salt lake shores, mud flats and salt pans. The genus currently comprises 25 to 30 species (Kadereit et al., 2007).

The taxonomy of the genus Salicornia is still far from satisfactory, although numerous species aggregates, species and microspecies have been described over the last 250 years (Davy et al., 2001). Frequently the name Salicornia europaea is used in a very broad sense to include most of the species of the genus. Additionally, the plants show a high level of phenotypic plasticity (Ingrouille and Pearson, 1987; Sagane et al., 2003). The salinity of their habitats fluctuates greatly due to different factors - tidal cycles, evapotranspiration, precipitation and availability of fresh groundwater. This is the reason why Salicornia develops high physiological plasticity which causes phenotypic variation (Kadereit et al., 2007). Morphological distinction between the taxa is only possible when the plants are fresh, between flowering and fruiting (Gehu et al., 1979). Morphometric studies using all phenotypic differences available, irrespective of whether they have a genetic basis or not, could not reveal distinct taxa even on a small regional scale (Ingrouille and Pearson, 1987; Ingrouille et al., 1990).
The Flora of the British Isles recognizes the species: *S. pusilla* J. Woods, *S. europaea* L. agg. (*S. ramosissima* J. Woods, *S. europaea* L. and *S. obscura* P. W. Ball and Tutin) and *S. procumbens* Smith. agg. (*S. nitens* P. W. Ball and Tutin, *S. fragilis* P. W. Ball and Tutin and *S. dolichostachya* Moss) (Stace, 1997). However, on the Atlantic coast of France Lahondère (2004) recognized eight *Salicornia* species. The diploid species are named: *S. disarticulata* Moss (or *S. pusilla* J. Woods), *S. obscura* P. W. Ball and Tutin, *S. ramosissima* J. Woods, *S. brachystachya* D. König and *S. x marshallii* D. H. Dalby (later suggested to be a hybrid of *S. disarticulata* and *S. ramosissima*). The tetraploid species are named: *S. dolichostachya* Moss, *S. fragilis* P. W. Ball and Tutin and *S. emerici* Duval-Jouve. Murakéözy et al. (2007) cited that Géhu and Géhu-Franck suggested that the taxon name *S. brachystachya* D. König is equivalent of *S. europaea* L. whose name should be replaced because it is ambiguous. According to König (1960) *S. ramosissima* from western and northern salt-marches and salt-contaminated inland sites is closely related to the exclusively coastal *S. stricta* Dumort. However, Stace (1997) considered these two taxa conspecific with *S. europaea* L. and treated them as synonyms. On the other hand, Davy et al. (2001) considered that *S. ramosissima* and *S. obscura* are perhaps variants of *S. europaea*. Numerical analysis of morphological variation in the field failed to support a distinction between the species *S. ramosissima* and *S. europaea* (Ingrouille and Pearson, 1987), although Jefferies and Gottlieb (1982) had found consistent differences at the loci coding for six enzymes. Previous results from molecular studies imply near 100% inbreeding in *Salicornia*, which certainly contributes greatly to the taxonomic difficulties in the group because of inbreeding lines with minute but fixed phenotypic differences (Noble et al., 1992). DNA polymorphism was detected among the three Spanish populations of *Salicornia* using Random Amplification of Polymorphic DNA (RAPD) approach (Luque et al., 1995). The other study using RAPD technique showed correlations between DNA polymorphism and geographical distribution in *S. ramosissima* (Krüger et al., 2002).

Defining *Salicornia* taxa by conspicuous morphological characters could also be misleading even when they are genetically fixed. Other morphological parallelisms are certainly less obvious and even more difficult to detect, especially when they appear in characters related to growth form, branching angle, segment and flower shape. It is therefore very difficult to realize in the field that plants occurring in the same region and sharing a similar morphology possess different genotypes, such as for instance *S. ramosissima* and *S. europaea*. Anatomical parameters proved to be taxonomically useful in many taxa, especially when morphological differentiation was difficult (Metcalfe and Chalk, 1957; Morris et al., 1996; Klopper and Wyk, 2001; Polić et al., 2009; Zorić et al., 2009). Therefore, anatomical characters may provide additional evidence to assess with the delimitation of different taxa (Klopper and Wyk, 2001). As such, an analysis of the internal structure of plants may contribute much to our understanding of their adaptive strategies. However, little anatomical investigation of *Salicornia* species shoot has been performed (Metcalfe and Chalk, 1957; Fahn, 1972, Fahn and Cutler, 1992; Redondo-Gómez et al., 2005).

According to Kadereit et al. (2007), the main reason for the taxonomic confusion are the young age of the extant lineages, the rampant dispersal of *Salicornia* which has led to widespread genotypes with high phenotypic plasticity. This is the reason why *Salicornia* plants have different names in different regions, and morphological parallelism resulted in the fact that different genotypes have the same name in one region.

The first finding of *Salicornia* plants in Serbia was around Bečej in northern Serbia in 1929 by Kovács (1929). These plants were identified as *Salicornia europaea*. Later on it was found at several localities: Senta (Slavnić, 1939, 1943; Atanacković, 1958), Senta, Martonoš (Slavnić, 1948), Novi Bečej (Slavnić, 1948; Adrejević, 1976), Novo Miloševo (Slavnić, 1948, 1952), Dragutinovo, Melenci (Slavnić, 1972), however it was dominant at the locality where it was first found - Slano Kopovo (Janjatović, 1980; Janjatović and Andelić, 1979; Janjatović and Kastori,
Nevertheless, these authors did not carry out detailed taxonomic study, but they marked the plants as *Salicornia europaea*. Slavnić (1972) pointed out that in Serbia *S. ramosissima* also grows, but he did not specify the localities. Due to reclamation of saline and habitat loss, this species was found only at one locality – Slano Kopovo (Vučković, 1999).

The random amplified polymorphic DNA (RAPD) method (Williams et al., 1990) has been successfully used for the differentiation of bacteria species (Campbell et al., 2000). Sagane et al. (2003) used this method for the identification of *Salicornia* populations from Japan. It also facilitates the characterization of DNA polymorphisms in plants (Crockett et al., 2000, Xi et al., 2001) and animals (Beeman and Brown, 1999; Oliver et al., 1999).

Because of the difficulties in the morphological determination of *Salicornia* species, we performed genetic and anatomical analyses as possible alternative methods for identification of species from this group. In order to develop a strategy for identifying *Salicornia* plants using RAPD techniques, we analyzed two typical populations of *Salicornia europaea* from Montenegro and Greece (Lesvos), one typical population of *S. ramosissima* from Spain and one population that belongs to the *Salicornia* genus from Serbia. To assess the specific limit of RAPD, we compared the genotypic classification based on the RAPD patterns with anatomical features. Also, the aim of our research was to analyze the anatomical variability of *Salicornia* plants in their natural habitats which differed by their origin and to establish the level of anatomical differentiation between the populations.

**MATERIALS AND METHODS**

**Plant material**

Plant analyses were done on samples from four localities: *S. ramosissima* from Saladar de Aguamarga (Spain), *S. europaea* from the Ulcinj salt pans (Montenegro) and Kalloni salt pans (Lesvos) and *Salicornia* sp. from Slano Kopovo (Serbia) (Fig. 1). In Serbia, Montenegro and Lesvos *Salicornia* plants were found at only one locality. The Ulcinj and Kalloni salt pans are considered to be the largest salt pans in Montenegro and Lesvos, respectively.

The plants were determined at the Department of Biology and Ecology, University of Novi Sad. Ten plants of each population were used for anatomical investigations. In the RAPD study, 20 plants from Serbia, Montenegro and Lesvos populations and 18 plants from Spain population were used.

**Anatomical comparison among populations**

Shoot segments from the middle part of the plants were separated and fixed in 50% ethanol. Cross sections were made using a Leica CM 1850 cryostat, at temperature from 18°C to 20°C, and at cutting intervals of 25 μm. The structures of the shoots were observed and measurements made using Image Analyzing System Motic 2000. Relative proportions were calculated for the shoot tissues, and expressed as a ratio to the whole shoot cross-section area.

Data were statistically processed by analysis of variance and means, standard errors, and coefficients of variation were calculated using STATISTICA for Windows version 10.0 (StatSoft, 2011). The significance of differences in the measured parameters between the populations was determined using Duncan’s test (p≤0.05). Multivariate Discriminant Function Analysis (MDA) was done in order to check the hypothesis that the analyzed sample was composed of groups which differentiated from each other. The general structure of sample variability was established by Principal Component Analysis (PCA), based on a correlation matrix.

**DNA extraction and RAPD fingerprinting**

The total DNA content of 50 mg silica-gel-dried and crushed plant material of individual plants was extracted using a Genomic DNA Purification Kit (Fermentas) according to the manufacturer’s instruction.
Random amplified polymorphic DNA (RAPD) assays were performed in 25µl reaction mixture containing 100 ng of template DNA, 2.5µl 10 x Buffer (with 15 mM Mg), 2.5µl dNTP Mixture (2mM), 2.5 µl of each primer (10pM), 1.5 µl MgCl₂ (25mM) and 1 unit of REDTaq Genomic DNA Polymerase. Primers selected for the analysis were K01 (5'-CATTC- GAGCC-3’), K15 (5'-CTCCTGCACAA-3’) and M02 (5'-ACAACGCCTC-3’) used by Krüger et al. (2002) for S. ramosissima, while primers OPA01 (5'-CAG- GCCCTTC-3’), OPB06 (5'-TGCTCTGCC-3’), OPB11 (5'-GTAGACCCGT-3’) and OPB12 (5'- CCTTGACGCA-3’) (Operon Technologies Inc.) were used for the first time in this study of *Salicornia* species. The thermal cycler was programmed as follows: 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 36°C, 90 s at 72°C, and finally by one cycle of 10 min at 72°C. Amplification products were separated by electrophoresis on 3% agarose gels. Gels were stained with ethidium bromide while DNA bands were visualized under UV light and documented using a Polaroid gel camera. The bands that commonly appeared in each population were defined as monomorphic band. On the other hand, the bands whose presence or absence varied among the plant individuals were consider as polymorphic bands. The RAPD markers were scored as 1 for a positive marker and as 0 for a negative marker. To assess the genetic distance among each population, we compared the similarity matrix with the Nei and Li method (Nei and Li, 1979) using the scores, and then created a neighbor-joining (NJ) dendrogram. The calculation of the matrix and construction of the tree were performed using Freetree software (Hampl et al., 2001).

**RESULTS**

**Anatomical characterization of *Salicornia* plants**

The shoot cross sections were rounded in shape, which was a common feature of the plants from all...
four populations (Fig. 2). The epidermal cells were fairly large, formed a single layer and had a cuticle with visible cuticular folds (Fig. 3A). The outer part of the shoot cortex was differentiated into palisade tissue. The palisade tissue consisted of 2-3 rows of elongated and densely arranged cells. The peripheral palisade tissue cells were smaller, with a higher chloroplast density compared to inner cells (Fig. 3B). Beneath the palisade tissue several layers of thin-walled parenchymatous cells (water-storage tissue) occurred. Branches of leaf vascular bundles were arranged in a circle between the palisade and water-storage tissue (Fig. 4A). In the palisade tissue, there were elongated elements similar to tracheids - trachoid idioblasts, with characteristic spiral thickenings (Figs. 4A, B). The functions of these cells are the transport of water to peripheral shoot layers or the accumulation of air or water (Fahn, 1972). The last layer of cortex was a uniseriate endodermis. The endodermal cells were thin-walled, elongated along the tangential axis, oval or elliptical in shape and occasionally contained starch grains (Fig. 5A). A single layer of pericycle, whose cells were thin-walled and smaller compared to the endodermal cells, was present beneath the endodermis (Fig. 5B). In the central cylinder, collateral vascular bundles were arranged in a circle, with well-developed sclerenchyma tissue above them (Fig. 6A). Above the vascular bundles, in the zone of the pericycle, there were individual fibers (Fig. 6B). The pith parenchyma was compact, composed of relatively large parenchyma cells. In the centre of the shoot cross-section there was a small central cavity (Fig. 2).

Duncan’s test showed that the plants of the Lesvos population had a significantly higher shoot cross-
section area and percentage of pith parenchyma, and a significantly lower proportion of epidermis than the plants of the other three populations (Table 1). The Serbian and Spanish populations had a significantly higher proportion of palisade tissue and the highest value of cortex/cylinder ratio (18.7 in both populations). The Montenegrin plants had the highest proportions of vascular bundles with sclerenchyma and the smallest proportion of cortex parenchyma as well as the lowest cortex/cylinder ratio.

The variation of the anatomical parameters of the *Salicornia* plants was examined by Principal Components Analysis (PCA). According to anatomical characters the first principal component accounted for 46.11% of total variation and the second component represented 41.35% (Fig. 7). The cumulative contribution percentage of the first two PCs was 87.46%. The projection of the cases of the first two components showed that the investigated populations could be clearly separated into three groups according to the variability of anatomical characters. Specimens from Serbia and Spain formed a single and homogeneous group, distinctive from the specimens from Lesvos and Montenegro.
The results of the Multivariate Discriminant Analysis (MDA) also pointed to a very high similarity in shoot anatomical parameters between the populations from Serbia and Spain (Fig. 8). These two populations clearly separated along the first discriminant axis from the other two populations. The second discriminant axis further separated the population from Lesvos.

**RAPD fingerprinting of Salicornia plants**

The RAPD analysis of 78 Salicornia plants from four populations with seven different primers yielded 58 polymorphic bands: five for OPB11, seven for OPB06 and OPB12, eight for K01 and K15, 10 for OPA01 and 15 for M02 (Table 2).

The analysis performed indicated that primer M02 has the highest number of polymorphic bands (15), while primer OPB11 has the lowest number of polymorphic bands (five). The population from Spain has the highest number of polymorphic bands for primers K01, K15, M02, OPB11 (as observed in the population from Serbia) and OPB12. The popu-
Table 1. Anatomical characteristics (mean value ± standard error and coefficient of variation %).

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<th>Characters</th>
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<tr>
<td>Shoot cross section area (mm²)</td>
<td>4.9±0.3ᵇ</td>
<td>8.5±0.4ᵃ</td>
<td>2.7±0.3ᶜ</td>
<td>4.8±0.3ᵇ</td>
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<tr>
<td>Cortex</td>
<td>(19.3)</td>
<td>(16.0)</td>
<td>(33.3)</td>
<td>(16.9)</td>
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<td>% Epidermis</td>
<td>4.9±0.2ᵃ</td>
<td>2.9±0.1ᵇ</td>
<td>4.9±0.2ᵃ</td>
<td>4.7±0.2ᵇ</td>
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<tr>
<td>(12.2)</td>
<td>(14.4)</td>
<td>(11.0)</td>
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<td>% Pallisade tissue</td>
<td>29.8±1.7ᵃ</td>
<td>20.1±1.1ᵇ</td>
<td>23.8±1.4ᵇ</td>
<td>29.8±1.7ᵃ</td>
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<td>(17.6)</td>
<td>(15.8)</td>
<td>(18.9)</td>
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<td>% Parenchyma</td>
<td>59.8±1.6ᵃ</td>
<td>58.3±1.4ᵇ</td>
<td>42.0±1.7ᵇ</td>
<td>59.8±1.6ᵃ</td>
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<td>(8.3)</td>
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<td>(13.1)</td>
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<td>Cylinder</td>
<td>3.9±0.2ᶜ</td>
<td>10.4±0.5ᵇ</td>
<td>19.5±1.5ᵃ</td>
<td>3.9±0.2ᶜ</td>
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<tr>
<td>(20.4)</td>
<td>(15.2)</td>
<td>(24.4)</td>
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<td>% Pith parenchyma</td>
<td>0.9±0.1ᵇ</td>
<td>7.2±0.3ᵃ</td>
<td>5.8±0.7ᵇ</td>
<td>0.9±0.1ᵇ</td>
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<tr>
<td>(3.4)</td>
<td>(1.4)</td>
<td>(3.1)</td>
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<tr>
<td>Cortex/cylinder ratio</td>
<td>18.7ᵃ</td>
<td>4.4ᵇ</td>
<td>2.6ᵇ</td>
<td>18.7ᵃ</td>
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I – Serbia (Slano Kopovo), II - Lesvos (Kalloni), III – Montenegro (Ulcinj salt pans), IV – Spain (Saladar de Aguamarga).
Different superscripts indicate that differences between localities are significant according to Duncan’s test (p≤0.05).

Table 2. Number of RAPD profiles and polymorphic bands.

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I – Serbia (Slano Kopovo), II – Lesvos (Kalloni), III – Montenegro (Ulcinj salt pans), IV – Spain (Saladar de Aguamarga).
N – number of specimens, n – number of RAPD profiles analyzed per populations and per primer, P – number of polymorphic bands found per primer in each populations.

Table 3. Genetic Distance between investigated populations based on RAPD analysis (Nei and Li, 1979).

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<tr>
<td>1</td>
<td>Serbia</td>
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<td>2</td>
<td>Lesvos</td>
<td>0.57692</td>
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<td>-</td>
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<td>3</td>
<td>Montenegro</td>
<td>0.75000</td>
<td>0.54545</td>
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<tr>
<td>4</td>
<td>Spain</td>
<td>0.46875</td>
<td>0.60000</td>
<td>0.67857</td>
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I – Serbia (Slano Kopovo), II – Lesvos (Kalloni), III – Montenegro (Ulcinj salt pans), IV – Spain (Saladar de Aguamarga).

Table from Montenegro has the highest number of polymorphic bands for the primer OPA01, while the group from Lesvos as well as the group from Serbia has the highest number for primer OPB06. The lowest numbers of RAPD bands analyzed per population and per primer were detected in the following groups: Serbian group for primer OPA01 and Montenegrin group for primer K01, M02, OPB12, as well as K15 and OPB11, like in the population from Lesvos.

Values of the pairwise comparisons of Nei and Li (1979) genetic distance (D) between the populations computed from combined data for the seven primers, ranged from D=0.469 (Serbia-Spain) to D=0.750 (Serbia-Montenegro) (Table 3).
The relationships among the studied populations are represented by a neighbor-joining dendrogram using the Freetree software (Fig. 9). The robustness of the tree topology was assessed by bootstrap analysis with a repetition value of 5000. The population from Lesvos was separated as a distinct group from the other investigated populations with a bootstrap value of 100%. Furthermore, the Montenegrin population was separated from the Lesvos, Serbia and Spanish populations with a bootstrap value of only 23%. The last phenogram division included the Serbian population and the population from Spain with a bootstrap value of 47%.

DISCUSSION

The analyses of *Salicornia* plants in their autochthonous habitats as well as in herbarium collections (Herbario, University of Alicante, Spain; the Herbarium Institute of Botany and Botanical Garden, Faculty of Biology, University of Belgrade, Serbia; the Herbarium Department of Biology and Ecology, University of Novi Sad, Serbia) revealed their diverse morphology in distant populations. However, all individuals were determined as a *Salicornia europaea* group on the basis of the standard determination key. This was the reason for the suspicion assumption that there could be different species or subspecies of the dominant members of the *S. europaea* group. Therefore, it became necessary to conduct further analyses, other than morphological.

The shoot of the *Salicornia* species is of a specific structure. The adaxial side of the leaves is fused with the stem, forming a single anatomic entity. These stems, without classic separate leaves, are called articulated or aphyllous stems (Fahn and Arzee, 1959; Stevanović and Janković, 2001). According to the PCA and MDA analyses based on the shoot anatomical measurements and observation, the four populations were classified into three groups: one joining the plants from Serbia and Spain, one comprising the Montenegrin group and one comprising the Lesvos group. Ingrouille and Pearson (1987) stated that *S. europaea* and *S. ramosissima* could not be clearly separated on the basis of their morphological characteristics. Our results showed that *S. europaea* (populations from Montenegro and Lesvos) differed in some shoot anatomical parameters from the *S. ramosissima* populations from Spain. These differences were more quantitative than qualitative. The shoot of this species had a well-developed central cylinder, with a significantly higher percentage of vascular tissue with sclerenchyma and percentage of pith parenchyma. The cortex of *S. europaea* had a significantly lower percentage of palisade tissue and a lower value of cortex/cylinder ratio.

*Salicornia* plants tend to have phenotypic variations depending on environmental conditions such as temperature, quality of soil, concentration of salt and population density (Ungar et al., 1979; Ellison, 1987; Boorman et al., 2001). Ball and Akeroyd (1993) suggested that the specific limits of classification of the *Salicornia* plants based on morphological fea-
tures, especially those of dried Salicornia plants, are obscure. To prove the relevance between the genotype and phenotype in Salicornia plants, genetic variability was analyzed by RAPD fingerprinting.

The use of molecular markers is considered to be the best for genetic diversity analysis since it has proved to be non-invasive in the sense that there are no negative effects on the stage of development, environment or management practices. Furthermore, these kinds of studies can be applied even on dead plants when the genomic DNA is extractable (Choudhury et al., 2001; Sagane et al., 2003). Moreover, among available molecular marker systems, the RAPD technique (Williamns et al., 1990) is the fastest and simplest one (Choudhury et al., 2001). The random amplified DNA method has been successfully used for the differentiation of bacteria species (Campbell et al., 2000) as well as the characterization of DNA polymorphisms in plants (Crockett et al., 2000; Xie et al., 2001), insects (Beeman and Brown, 1990) and animals (Oliver et al., 1999). Altogether, RAPD fingerprinting may be a reliable strategy for the identification of types of plant populations (Sagane et al., 2003).

The genotypes based on the RAPD markers indicated that the populations from Spain and Serbia are closely related to each other. The study showed that the Montenegrin group was different from both the Serbian and Spanish groups, which led to the conclusion that it might have derived from a different ancestry. On the other hand, the study pointed out that the Lesvos group was quite different from all the other investigated populations. We believe that this group was separated a long time ago, probably when islands were forming, which made it possible for the Lesvos population to develop as an individual one.

In our opinion, based on the results of this study, the high similarity in the analyzed parameters of the populations from Serbia and Spain implies that these specimens belong to the same species, S. ramosissima. These results open up suggest that S. ramosissima is the species that grows in Serbia, and not S. europaea, as was reported previously.

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