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Original Research Article

Characterization of *Sulla* species via SRAP markers: polymorphism and systematic analyses

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Abstract

The use of molecular markers is effective to evaluate the genetic variation. The chosen biological material in this study is the Legume genus *Sulla*, which is threaten by an ecologic dequilibrium, the genetic erosion. *Sequence-related amplified polymorphism* (SRAP) markers were used to assess the genetic diversity and to schematize the relationships among and between the species of *Sulla*, endemic to mediterranean provinces. Twelve SRAP primer combinations generated 94 bands and showed a high inter-specific variability. Actually, (Φpt) value is relatively high 0.477 and the AMOVA analysis revealed that 62% of the diversity is inter-specific. These results were confirmed by the topology of the (NJ) cladogramm. The systematic examination of the genus of *Sulla* revealed the relatedness of the north african species, suggesting that the Maghreb is a hotspot of diversity. The use of these co-dominant markers allowed us to estimate the genetic diversity and then to improve the adaptability of the crops in the Mediterranean soils. SRAP markers could also be anchored to virtual Linkage Groups and then used for gene mapping.

Keywords: Sulla, Genetic diversity, SRAP, Phylogeny

Abbreviation

AFLP: Amplified Fragment of Length Polymorphism
AMOVA: Analysis of Molecular Variance
ISSR: Inter Sequence Simple Repeats
MCMC: Markov Chain Monte Carlo
ORFs: Open Reading Frames
PCoA: Principal Coordinate Analysis
QTL: Quantitative Trait Loci
RFLP: Restriction Fragment of Length Polymorphism
SRAP: Sequence Related Amplified Polymorphism
SSR: Simple Sequence Repeats

INTRODUCTION

In 2003, Choi and Ohashi reviewed the systematic of the genus *Hedysarum* L. and according to morphological characetrization, regrouped six mediterranean species into a seperategenus*Sulla* :*S. capitata* (Desf.) B.H. Choi and H. Ohashi. comb. nov., *S. carnosa* (Desf.) B.H. Choi and H. Ohashi. comb. nov., *S. flexuosa* (L.) Medik., *S. pallida* (Desf.) B.H. Choi and H. Ohashi. comb. nov., *S. spinosissima* (L.) B.H. Choi and H. Ohashi. comb. nov.

and *S. coronaria* (L.) Medik. *Sulla* is exploited as fodder, hay for animals and pastoral production since they are highly nutritious and highly palatable to sheep. The species are all annuals and diploids, with a basic chromosomic composition of n = 8. However, in extreme conditions of freeze and high altitude, *S. pallida* is prestented as a tetraploid species, growing by vegetative propagation (suckering). The habit of the species of *Sulla* is predominantly or strictly allogamous except for *S. spinosissima,* which is strictly autogamous (Zitouna et al. 2013).

A better knowlege of the amount and the distribution of the genetic history could be an effcient way to predict the reactions and then to face-up all ecological threats and to ensure the long-term success of *Sulla* in breeding programs. In this optic, many cataloging teams were established and allowed to create *ex-situ* collections currently maintained in a 8°C room in the “Laboratory of Molecular Genetics, Immunology and Biotechnology - Faculty of Sciences of Tunis-Tunisia”. Severalmolecular studies were undertaken to investigate the genetic diversity and the phylogenyof these North African species viaAmplified Fragment of Length Polymorphism AFLP (Chennaoui-Kourda et al. 2012), Inter Sequence Simple Repeats ISSR (Chennaoui-Kourda et al. 2007), Simple Sequence Repeats SSR (Zitouna et al. 2013) andRestriction Fragment of Length Polymorphism (Trifi-Farah and Marrakchi 2002). The variability among species was previously studied with morphological characteristics and isozymes analyses (Trifi-Farah and Marrakchi 2002). The aim of these studies is to conserve the germplasm threatened by a loss of biodiversity caused by climatic fluctuations in the Mediterranean region and by human extensionsrecently (*S. flexuosa* and *S. carnosa* are nowadays completly and partially exctincted from Tunisian areas, respectively). Nevertheless, information about relationships and the characterization of the genus are still unsufficient and additional markers are needed for gene discovery and diversity analyses.

SRAP (Sequence-related amplified polymorphism) markers are PCR-based co-dominant markers that may identify unique genotypes as potentially important new sources of alleles for enhancing the characterization of *Sulla* and analyzing the evolutionary and historical development of genotypes at the genomic level. SRAP markers were developed by Li and Quiros (2001) and target Open Reading Frames (ORFs). This technique was used for genetic diversity and phylogenetic studies in different legume crops: lentil, alfalfa and pea. The primers are designed according to the genomic characteristic that exons are GC-rich (the forward primer), while the promoters and introns (reverse primer) are AT-rich (Liu et al. 2013). Polymorphism is generated because of the length differences of introns, promoters and intervals of the analyzed specimens. Having the advantages of simplicity, high polymorphism and co-dominance ; SRAP markers have been used to study genetic diversity (Guo et al. 2012; Abedian et al. 2012). Their use has been extended to genetic mapping (Xie et al. 2011) and QTL detection (Zhang et al. 2011).

In this report, we used SRAP markers to evaluate genetic diversity, bayesian structure and and systematic of the *Sulla* genus and species, which originated from North Africa.

MATERIAL AND METHODS

Plant Materials and DNA Extraction

DNA of Ten-days seedlings from sixty *Sulla* genotypes was isolated using the protocol described by the commercial kit (PureLink Plant, Total DNA Purification Kit). Each species is represented by ten genotypes. Agronomic and eco-edaphic characteristics of the species are represented in the supplementary data- Table 1.

The DNA samples were dissolved in 100 µL of TE and stored at -20°C. The quality and concentration of the isolated DNA were checked using a spectrophotometer before utilisiation.

SRAP-PCR assay

A total of twenty different SRAP primer pairs were used to amplify polymorphisms, following the nucleotide sequences of primer sets from Li and Quiros (2001). The tested primers are listed in the supplementary data- Table 2. The PCR reaction mixtures (total volume of 25 µL) consisted of 10 mM *taq* Buffer, 50 mM $MgCl_2$, nucleotides dATP, dTTP, dCTP, and dGTP (200 µM each), 0.2 µM of each primer, 30 ng of template DNA and 2 units of *Taq* DNA polymerase (Biomatik).

The thermal cycler profile for PCR amplification was set on a TPROFESSIONAL TRIO thermocycler (Biometra, An Analytic Jena Company) and programmed as follow: 94 °C for 5 min, followed by five cycles of 94 °C for 1 min, 35 °C for 1 min, and 72 °C for 1 min. In the remaining 30 cycles, the annealing temperature was increased to 50 °C for 1 min followed by a final elongation step at 72 °C for 7 min. The touch-down PCR allows us to detect multiple loci with a single pair of SRAP primers. For the electrophoresis, the 25 µL of PCR products were fractionated on a 3% agarose gel stained by Ethidium Bromide and running in 0.5x TBE buffer (Tris Boric acid EDTA, pH = 8) at a constant voltage of 90 V for 3 h. The concentrated agarose gel aimed to identify homozygous and heterozygozous geneotypes. The electrophoretic patterns of the PCR products were photographed digitally using a Gel-Doc 2000 image analysis system (Bio-Rad. USA).

Data scoring and statistical analysis for phylogenetic analyses

The clear and polymorphic DNA bands sized between 100 bp and 3000 bp were scored as1 for presence or 0 for absence and compiledin a binary qualitative data matrix for further analysis. Twelve SRAP primer combinations exhibited high polymorphism estimated by various indices of diversity calculated by both POPGENE version 3.2 (Yeh et al. 1999) and Genalex software ver. 6.5 (Peakall and Smouse 2012). The following parameters were calculated assuming the Hardy-Weinberg Equilibrium: (1) the observed number of alleles per locus (Ao)and the effective number of alleles per locus (Ae); (2) Nei's gene diversity or the expected heterozygosity (He) and Shannon's information index (I); (3) the estimation of population genetic differentiation (Φpt) and (4) the Nei's (1972) genetic distances among the six species (GD). Polymorphism information content (PIC) was calculated using the formula : $PIC = 1-\sum Pi^2_{(i=1)}$; where Pi is the frequency of the i^{th} allele in the set of genotypes investigated. Effective Multiplex Ratio (EMR) was estimated as follow EMR = np. β, where np is the total number of polymorphic bands and β is the ratio of polymorphic bands to total bands. Marker Index (MI) was calculated using the formula MI = PIC. EMR.

The analysis of molecular variance (AMOVA) was used to calculate the total SRAP variation into within-population and between populations (Excoffier et al. 1992). Variance components and the sum of all the squared differences were calculated.

The systematics of the *Sulla* genus was elucidated using a Neighbor Joining cladogram shematized using standardized Jaccard's Distance Index (JDI). The matrix was subjected DARWIN Software to perform a phylogenetic dendrogram (Perrier and Jacquemoud-Collet 2006). Additionally, we performed a Principal Coordinate Analysis (PCoA) to plot all analyzed accessions of *Sulla*, on the basis of the dissimalirity index.

The bayesian barplots was realized using STRUCTURE program ver.2.2 (Pritchard et al. 2000). The number of clusters (K) is estimated after ten independent runs for each K varying from 2 to 9. The admixture model with 10.000 Markov Chain Monte Carlo (MCMC) repetitions and 100.000 burn-in periods is applied. The convenient K is determined using the *ad-hoc* parameter (ΔK) of Evanno et al. (2005) implemented in STRUCTURE HARVESTER webserver.

RESULTS AND DISCUSSION

Investigating the genetic variation among *Sulla* species is crucial for the effective conservation and utilization of these resources in breeding programs. These species are threatened by an ecological desequilibrium caused by a genetic erosion. Genotyping them using performant molecular markers could be a reliable tool to detect interresting QTLs, later.

PCR-SRAP efficiency

Over thirty twenty combinations of forward and reverse SRAP primers, seven were negative, one monomorphic and twelve positively amplified. The combinaison F13-R7 produced a monomorphic pattern represented by two bands (600 bp and 850 bp). Using the twelve primers, 94 polymorphic bands were recorded in a binary matrix for statistical analysis. The number of amplified fragments varied from 5 (F8-R8) to 12 (F8-R7) among the *Sulla* species. Despite the relatively low number of detected alleles, Alghamdi et al. (2012) confirmed that 50 to 100 markers are sufficient for genealogic and phylogenic studies. The number of polymorphic bands is important but lower than those obtained in other genetic diversity' studies of *Sulla* using binary matrices (dominant markers) such as ISSR and AFLP approaches which detected respectively 85 and 295 total polymorphic bands (Marghali et al. 2014). Using eight ISSR primers, Chennaoui-Kourda et al. (2007) got between 6 and 16 polymorphic bands. However, 24 to 51 bands were scored using the AFLP technique, with an average of 36.8 bands per combination (Chennaoui-Kourda et al. 2007).

The number of polymorphisms amplified in this study is also lower than SRAPs of Faba Bean (Alghamdi et al. 2012), which reported a total of 1036 bands using fourteen primers. However, many SRAP studies congurate with the low number of alleles (Jing et al. 2013). In fact, some labeled SRAP oligonucleotides such as FC1, BG23 and SA7 amplify more than ten polymorphic loci while Em2 and DC1 produc less than six (Li et al. 2013). Thus, the potency of this technique is correlated with the set of used primers.

Primers utility was estimated by the indices of PIC, EMR and MI. The polymorphism information content (PIC) values for primers ranged from 76.03% to 87.67% (Table 1). The average values of Effective Multiplex Ratio (EMR) in this study correspond to the number of generated alleles, which is between 5 and 12. The yielded value of Marker Index (MI) ranged between 3.8 and 10.51. High, medium or low polymorphism is in accordance with PIC > 0.5, 0.5 > PIC > 0.25 and PIC < 0.25, respectively (Xie et al. 2011). All the PIC values in this analysis are higher than 0.5, the observed polymorphism is supposed to be high. The average values of PIC, EMR and MI highlighted a considerable genetic variation of *Sulla* germplasm, which should be considered as a background for future breeding programs.

Herein, the observed number of allele (Ao) is equal to 1.244 and is larger than (Ae) the effective number (1.158). This result confirms the polymorphism of these molecular markers and at the same time the specificity of the used

Table 1. Polymorphism Information Content (PIC) values, Effective Multiplex Ratio (EMR), Marker Index (MI) of used primers in present study.

	PIC	EMR	MI
F8-R7	87.67%	12	10.51
F8-R8	76.03%	5	3.8
Me4-R8	80.31%	7	5.62
F8-R9	81.46%	6	4.88
F9-R15	80.57%	7	5.63
F13-R14	83.51%	8	6.68
F8-R15	83.22%	8	6.65
F8- Em2	86.68%	9	7.79
F10-R9	86.10%	10	8.61
F10- Em2	84.13%	8	6.72
F9-R9	83.83%	7	5.86
Me4-R14	76.47%	7	5.34

PIC: polymorphic Information Content; EMR: Effective Multplex Ratio; MI: Marker Index

Table 2. The genetic diversity observed across the six species of *Sulla*

Species		%P	I	He
Sulla capitata	Mean	27.66%	0.162	0.111
	SE		0.028	0.019
Sulla carnosa	Mean	43.62%	0.234	0.158
	SE		0.030	0.021
Sulla coronaria	Mean	42.55%	0.222	0.147
	SE		0.028	0.019
Sulla flexuosa	Mean	30.85%	0.173	0.116
	SE		0.027	0.019
Sulla pallida	Mean	26.60%	0.155	0.106
	SE		0.027	0.019
Sulla spinosissima	Mean	35.11%	0.206	0.142
	SE		0.030	0.021
Total	Mean	34.4%	0.192	0.130
	SE	3%	0.012	0.008

%P: Polymorphism Percent; I: Shannon's index; He: Gene Diversity

primers. Therefore, a higher level of genetic diversity is expected to be observed because of the mating system predominantly allogam for five species (except *S. spinosissima*).

In the current study, despite the non-exploitation of the revealed genotypes, SRAP markers were abled to characterize the analyzed species as well ass the co-dominant SSR markers (Zitouna et al. 2013). The low number of revealed alleles was enough informative to retrace the relationships of the six North African species of *Sulla*. Nevertheless, SRAP markers would be more performant if they are deeped with other molecular markers as founded in other crops taxa (Guadagnuolo et al. 2001).

Polymorphism levels and molecular variance

The precision and accuracy of SRAP markers in detecting genetic diversity at a molecular level is important to discriminate between closely related species. The species segregating for the highest number of alleles (60) are *S. carnosa* and *S. coronaria* compared to 51 alleles for *S. flexuosa*. All these alleles occure at a minimum frequency of 5%. However, the species amplifying the most specific alleles was *S. pallida* (4 alleles), while *S. spinosissima* did not amplify any specific band. *S. spinosissima* is the only autogamous species, which could explain its low rate of specificity.

In this study, the six analyzed species are native to different eco-geographic areas and have a wide geographical distribution around the Maghreb region. Therefore, a higher level of genetic diversity is expected to be observed at the species level. This result could be explained by the mating system which is predominantly allogam for five (from the six) species. Table 2 presents the polymorphic percent (%P), (He) and (I) indices for the six species composing the genus *Sulla*. The mean values of %P, I and He are 34.4%, 0.192 and 0.130, respectively. The minimum was observed within *S. pallida* (26.6%,

Table 3. The molecular covariance indices calculated

Source of variation	df	SSD	MSD	Est. Var.	% *
Among *Sulla* species	5	629.700	125.940	11.869	62%
Within *Sulla* species	54	391.600	7.252	7.252	38%
Total	59	1021.300		19.121	100%

d.f. = degrees of freedom; SSD = sum of squared deviations; MSD = mean squared deviation * Significant at alpha 0.001 (after 999 random permutations)

0.155 and 0.106) and the maximum within *S. carnosa* (43.62%, 0.234 and 0.158). Usually, species presenting a high genetic variability will have a stronger environmental adaptability and wider natural distribution (Liu et al. 2013). In this case, *Sulla carnosa* is the most polymorphic species, and is also the only species represented by an Algerian population. Algeria is the only country including all the Mediterranean *Hedysarea* species and seems to be unique as a diversity center (Trifi-Farah and Marrakchi 2002). In the other side, *S. pallida* is the only species which could reproduce vegetativly in extreme conditions of freeze and altitude (Marghali et al. 2014).

The genetic differentiation among *Sulla* genus using AMOVA analysis was highly significant ($P < 0.001$). The results showed that 62% of the total genetic variation existed among the populations and 38% within the species (Table 3). A similar level of genetic differentiation among the populations was obtained using the (Φpt) which was approximately 0.447. The high inter-specific divergence has also been shown in *Sulla* using SSR, AFLP, iso-enzymes (Trifi-Farah and Marrakchi 2002 ; Chennaoui-Kourda et al. 2012; Zitouna et al. 2013). This is explained by the high level of conservation of the genomes of these species, and the recent and slow evolution of this genus (Marghali et al. 2014). On the other hand, the low rate of intraspecific variation could be due to the specificity of the used primers, and the low mutations rates of the amplified ORF.

Genetic diversity is essential for the long-term survival of a species and its adaptability to the environment. Thus, it is important to be aware of a species' inter- and intraspecific diversities before developing any protection and management strategies for an endangered species (Hamrick and Godt, 1996). In the case of *Sulla* genus, the species requiring the major interest are *Sullacarnosa* and *S. flexuosa*, both threatned by extinction in the Maghreb areas.

Intra and inter-specific systematic

The genetic distance (GD) among the six species composing the genus *Sulla*, obtained from SRAP data ranged from 0.164 to 0.463. The lowest values were observed between *S. capitata* and *S. spinosissima* while the highest GD values was observed between *S. capitata* and *S. pallida*. Different results (ie., ISSR, AFLP) provide a strong support for the closeness of *S. coronaria* and *S. flexuosa* (Marghali et al. 2014), which suggest their inclusion in breeding programs. In front of this, the analysis using SRAP markers revealed a GD equal to 0.390 and confirms then the analysis via SSR markers (Zitouna et al. 2013). SRAP and SSR are both co-dominant, and these markers are reminded for their efficiency and dependability. The pairwised GD highlight a recent evolution of the *Sulla* species and confirm that the analysed species are issued from a common ancestor. Actually, the relatively low values of genetic distances obtained by SRAP anlysis evidenced the relatedness of the six species. The same result was traduced by the recent evolution of these species (Chennaoui-Kourdaet al. 2007, 2012). It's worth to remember that *Sulla* was recently (2003) derived from *Hedysarum* genus, which is considered as the ancestal taxa.

The other relevant result is the proximity of all maghrebian species and the inclusion of *S. carnosa* (Algeria) and *S. flexuosa* (Morocco) with the 4 Tunisian species. The north of Africa is a center of diversity for crop species. Reports have also suggest the spread of the alleles through egological barriers such the straits and moutains (Magri et al. 2006)

The evolutionary potential of a species and its ability to withstand adversity depends on the degree of intra-specific genetic variation (Liu et al. 2013). In this study, the investigated diversity was not high, which suggests that the level of genetic structure will be not very high. Intraspecific relationships were clarified through the Neighbor-Joining cladogrambased on the Jaccard Index (Figure 1).

The clusters revealed a moderate bootstrap support values ranging between 30 and 100. This could be explained by the relatively small number of loci sampled. The topology of the obtained tree, reveals the similarity and the grouping of each 10 analyzed genotypes per species. The cladogram suggests the relatedness of *S. capitata* and *S. spinosissima,* with a bootstrap support equal to 98. The combining work of Marghali et al. (2014) using several molecular markers, also report the similarity of these two species. The two species were considered

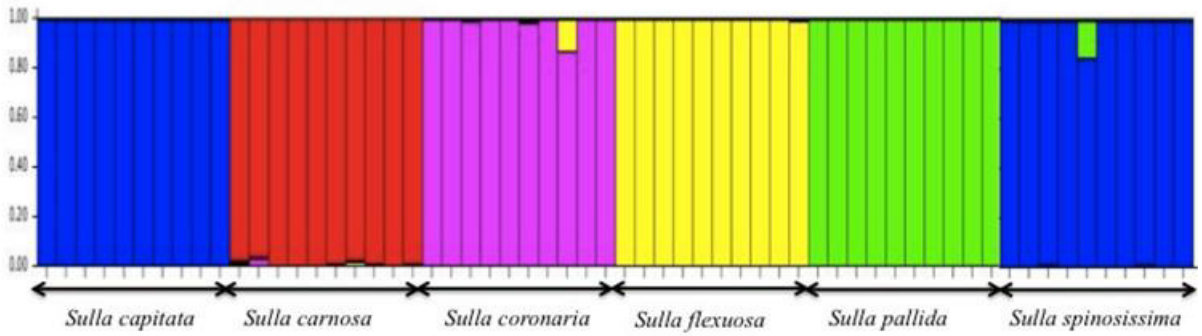

Figure 1. Dendrogram obtained by NJ cluster analysis constructed according to the Jaccard Index algorithm. Numbers next to nodes indicate boots trap support percentages in 1000 pseudo-replicates.

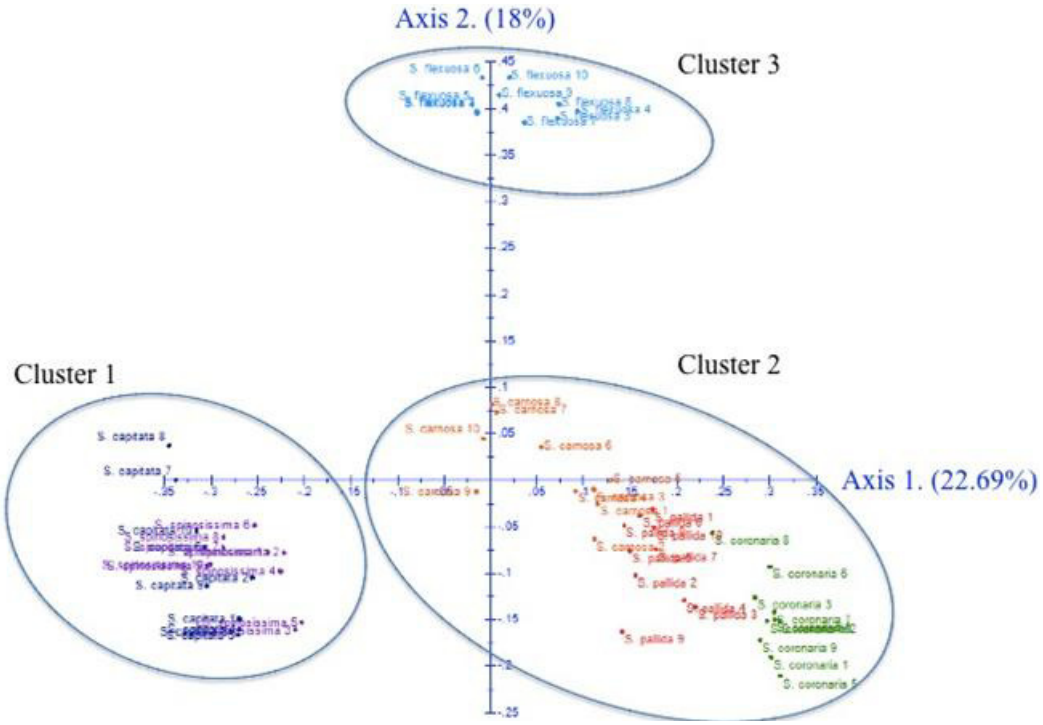

Figure 2. PCoA Plot showing the genetic differentiation among the six species of *Sulla*.
Principal co-ordinate analysis of 6 *Sulla* accessions using data from 12 SRAP primers-combinations. PC1 and PC2 are the first and second principal components explaining 22.69 and 18% of the total variation respectively. Details regarding groups are discussed in the text.

before the new taxonomy of Choi and Ohashi (2003) as two sub-species of *Hedysarum euspinosissimum*. Furthermore, *S. carnosa* and *S. flexuosa* were pooled with together with the other species. These two species, originated from Algeria and Morocco. Therefore, the topology of the Nighbor Joining cladogram showed no correlation with the climatic stages of the six species. Such finding, is confirmed by the topology of the dendrogram revealed by the co-dominant SSR markers (Zitouna et al. 2013).

PCoA was performed in a multidimensional space and revealed 3 distinct clusters (Figure 2). The first two principal components explained 22.69% and 18% of the molecular variance, respectively. *S. capitata* and *S. spinosissima* (first cluster) and *S. coronaria*, *S. carnosa* and *S. pallida* (second cluster) were plotted under the first axis, while S. flexuosa were plotted on the top of the second axis. The closeness of *S. coronaria* and *S. carnosa* are in congruence with reports of Chennaoui-Kourda et al. (2007, 2012), who suggested to incorpore these species into breeding programs aiming to enhance these crops mainly in arid climates. Aditionnaly, *S. pallida* which share the same agronomic traits and spatial repartition with *S. coronaria*, are plotted together.

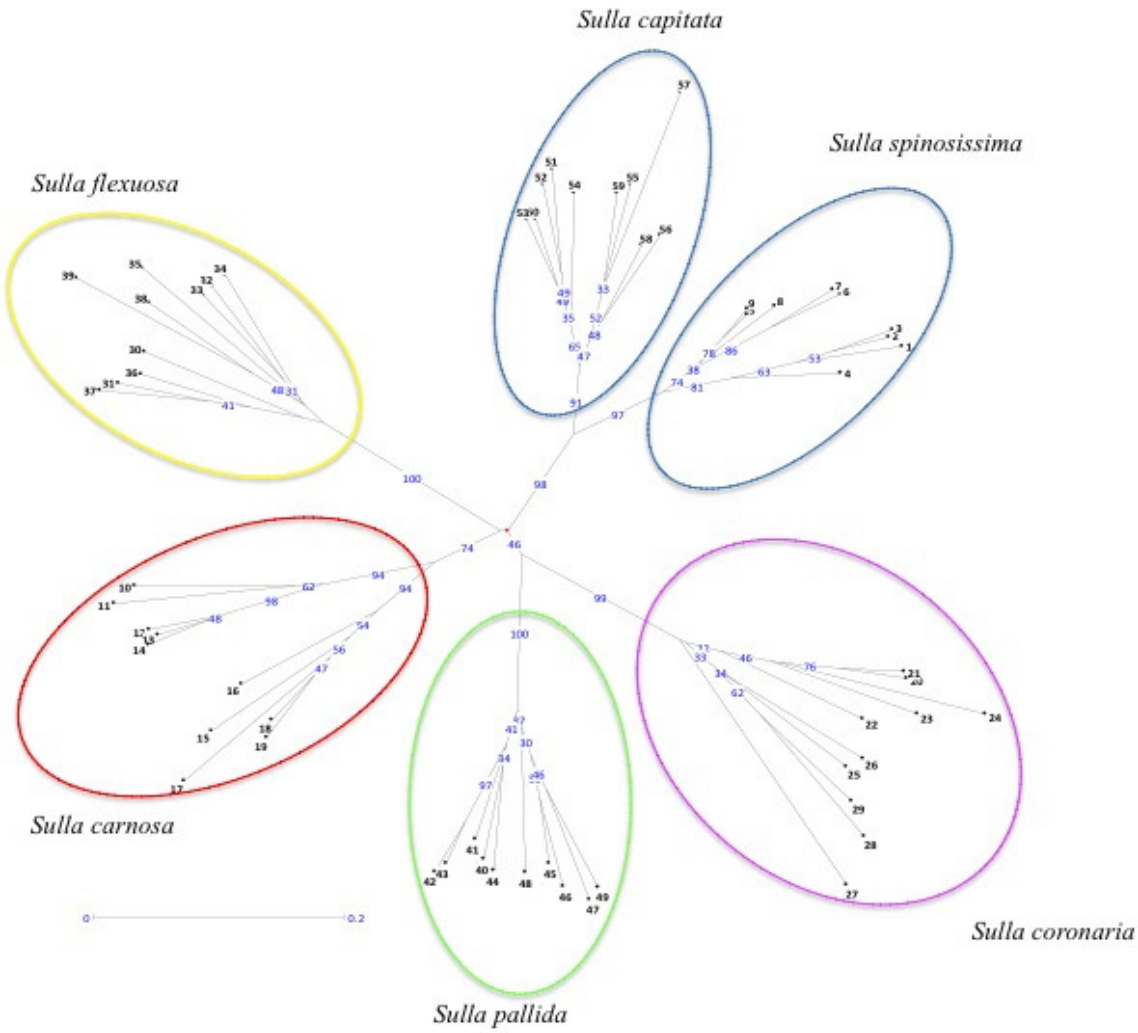

Figure 3. Estimated *Sulla*Bayesian structure. Each individual is represented by a thin vertical line, which is partitioned into K colored segments that represent the individual's estimated membership fractions in K clusters. The Figure shown indicates that the highest probability run is at K = 5, the 5 clusters are represented by 5 colors. Species are labeled below the Figure.

The Bayesian analysis revaled a 5 clusters-barplot, confirmed by the ad-hoc parameter and as schematized in the Figure 3. *S capitata* and *S. spinosissima* constitue the first cluster (Blue color) while the four other species are segregated into four seerate clusters. The proximity between the two first species agreed with the previous nomenclature (*Hedysarumeuspinosissimum* ssp. *capitatum* and ssp. *spinosissimum*).

Generally, the Bayesian structure, the spatial PCoA repartition and the phylgenetic analyses converge into the same results.Such findings are in congruence with the morphological studies of the genus (Marghali et al. 2014). In fact, quantitative morphological analyses based on the use of vegetative and reproductive characteristics of the six *Sulla* species exhibited a continued genetic diversity with the close spatial repartition of of *S. spinosissima* and *S. capitata,* which were classified in the former nomenclature as two subspecies. In addition, the results highlighted a clustering independently from their bioclimatic origin and mating system.

The present study highlights the usage of the co-dominant SRAP markers for studying the genetic diversity among the six species composing the genus of *Sulla*. These findings will be utilised as new informations for future breeding programs and for the gene mapping of this forage genus.

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Supplementary Data

Table 1. Characteristics of the analyzed *Sulla* species

	Geographicdistribution	Locality (Origin)	Climatic adaptation	Habit of reproduction	Agronomic traits
Sulla capitata[e]	Maghreb, Sardinia, Malta	Korbous (Tunisia)	Semi-temperate	Allogamous[a]	Forage and pasture
Sulla carnosa[e]	Maghreb	Talha (Algeria)	Arid	Allogamous[a]	Tolerance to salin soils, mowing plants, honey production
Sulla coronaria[e]	Maghreb, Sardinia, Malta	El Haouaria (Tunisia)	Semi-temperate	Allogamous[a]	Forage and pasture, cultivated in Italy
Sulla flexuosa[e]	Algeria, Morocco	Tanger (Morocco)	Semi-temperate	Allogamous[b]	Forage and pasture
Sulla pallida[e]	Maghreb	El Kef (Tunisia)	Semi-temperate	Allogamous[a]+ Vegetative[c]	Forage and pasture, growing in association with *S. coronaria* or *S. capitata*
Sulla spinosissima[e]	Maghreb, Sardinia, Malta, South of France, Sardinia	Sousse (Tunisia)	Arid/Semi-Arid	Autogamous[d]	Forage and pasture

Legend.[a] : a preferentiallyallogamous reproduction (2n = 16); [b] : a strictlyallogamous reproduction (2n = 16); [c] : The tetraploid *S.pallida*(4n=32) present a vegetative reproduction in extreme conditions of freeze and high altitudes ; [d] : a strictlyautogamous reproduction ; [e] : Tenrandomlycollectedseedswereused in thisstudy.

Table 2. Sequence of the SRAP primersused to screen *Sulla* genotypes

Forwardprimers	Reverse primers
F13: 5'-CGAATCTTAGCCGGCAC-3'	R15 : 5'-CGCACGTCCGTAATTCCA-3'
F8: 5'-GTAGCACAAGCCGGAAT-3'	R14: 5'-CGCACGTCCGTAATTAAC-3'
F9: 5'-GTA GCA CAA GCC GGA CC-3'	R9: 5'-GACACCGTACGAATTTGA-3'
F10: 5'-GTAGCACAAGCCGGAAG-3'	R8: 5'-GACACCGTACGAATTGAC-3'
Me4: 5'-TGAGTCCAAACCGGACC-3'	R7: 5'-GACACCGTACGAATTTGC-3'
	Em2: 5'-GACTGCGTACGAATTTGC-3'