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Morphological and molecular characters: Congruence or conflict in the phylogeny of *Sulla* species?

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Abstract

Sulla genus (Hedysarea) includes six Mediterranean species constituting an important phytogenetic patrimony primarily used to promote feed production chiefly in arid and semi arid regions. In order to perform a phylogenetic analysis of *Sulla* species**,** both morphological and molecular data were used. The morphological data was comprised of 10 characters, while the molecular characters were comprised of 52 phylogenetically informative sites from ITS ribosomal DNA and 85 informative ISSR markers and 295 AFLP markers. Phylogenetic reconstruction was performed by an analysis of separate and combined molecular and morphological data. Three separate topologies' baselines were evaluated. Three species, from a *Hedysarum* genus, were used as outgroup to validate the findings and the monophyly of *Sulla* species. The combined analysis showed a genetic convergence between the southern *S. spinosissima* and the northern *S. coronaria*. Moreover, the ability to differentiate species by these various techniques was confirmed by Pearson correlation coefficients. This test established the incongruence between morphological and molecular markers revealed in *Sulla* genus. In summary, the findings show a recent evolution of the Mediterranean *Sulla* species and confirm that the analysed species are descended from a common ancestor.
The main goal of this study is to provide a robust tool for showing the phylogeny *of Sulla* species utilizing distinct characteristics.

Key words: conflict; congruence; molecular markers; morphological parameters; relationships; *Sulla*
Abbreviations: AFLP_Amplified fragment length polymorphism; ISSR_Inter Simple Sequence Repeats; ITS_Internal Transcribed Spacer; PCA_Principal component analysis; PCR_Polymerase Chain Reaction; RFLP_Restriction fragment length polymorphism; UPGMA_Unweighted pair-group method of averages.

Introduction

The *Sulla* genus (# *Hedysarum*, section Spinosissima B. Fedtsch.), belonging to Leguminous family, consists of six distinct species widely distributed throughout the Mediterranean basin. These species include: *S. capitata* (Desf.) B.H. Choi & H. Ohashi, comb. nov., *S. carnosa* (Desf.) B.H. Choi & H. Ohashi, comb. nov., *S. flexuosa* (L.) Medik., *S. pallida* (Desf.) B.H. Choi & H. Ohashi, comb. nov., *S. spinosissima* (L.) B.H. Choi & H. Ohashi, comb. nov. and the only domesticated species *S. coronaria* (L.) Medik. (Choi and Ohashi, 2003). They represent vast potential fodder and feed resources because of their high protein rate and palatibility (Boussaïd et al., 1995; Trifi-Farah et al., 2002). In addition, these species support a significant number of rhizobium that promotes a high level of soil enrichment, potentially playing a crucial role in the improvement of damaged grasslands in the area (Baatout et al., 1985). Some *Sulla* species are also used as ornamental plants in various Mediterranean countries (Boussaïd et al., 1995; Trifi-Farah et al., 2002).

The *Sulla* species may be diploid or tetraploid, with a basic chromosome number of n = 8 (Baatout et al., 1985). The mating system is predominantly or strictly allogamous except for *S. spinosissima* (which is for the most part autogamous). They are well adapted to the various environmental conditions and soil types of the Mediterranean climate in which they are found. However, diverse abiotic and biotic stressors are currently threatening *Sulla* phytogenetic resources. Consequently, a progressive lack of some taxa has occurred in the region. An example of this is phenomenon is the scarcity of *S. flexuosa* in Tunisia (Trifi-Farah et al., 2002). Therefore, it is imperative to immediately develop strategic processes aimed at the preservation and the amelioration of these genetic resources. For this purpose, many research and cataloging teams have been established and permitted to create an exsitu collection currently maintained in the "Laboratory of Molecular Genetics, Immunology and Biotechnology of Tunis University El Manar II, 2092". In addition, several studies focused on the estimation of the genetic diversity of this collection have been performed using morphological, isoenzymatic and molecular markers. It was demonstrated that *Sulla* species (which include both natural and domesticated varieties), are characterized by morphological traits and isoenzymatic markers depending on the mating system and geographical origin of the plant (Trifi-Farah et al., 1989; Baatout et al., 1990; Boussaid et al., 1995). Moreover, RFLP diversity of some species showed high genetic variations (Trifi-Farah and Marrakchi, 2001). It is important to note that previous reports elucidated the genetic diversity and evolutionary relationships among some *Sulla* species. Recently, though, other studies tried to show phylogenetic relationships among all six species of the *Sulla* genus, in order to have a deeper insight into the complete genetic diversity of these plants. Chennaoui et al. (2007) and Chennaoui-Kourda et al. (2007; 2012) performed similar molecular phylogenetic reconstructions among these species based on ITS, ISSR and AFLP markers. ITS sequences have

Table 1. Definition and percentage of inertia absorbed associated with the three principal components of the PCA based on morphological traits

Principal component	Axis 1	Axis 2	Axis 3
Absorbed inertia (%)	38.011	31.707	20.376
Cumulated inertia (%)	38.011	69.718	90.094
Contribution of morphological parameters	LT (+0.975) LA (+0.939) LG (+0.903) VP (-0.887)	NB (+0.771) WF (+0.861) NF (+0.896) LS (+0.848)	LO (+0.840) NI (+0.972)

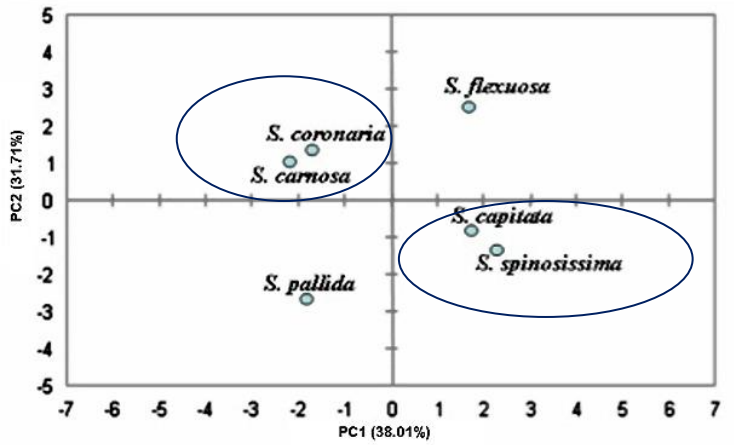

Fig 1. Principal co-ordinate analysis of 6 *Sulla* accessions using data for ten morphological traits. PC1 and PC2 are the first and second principal components explaining 38.01 and 31.71% of the total variation respectively. Details regarding groups are discussed in the text.

proven to be a useful source of information for the resolution of phylogenetic relationships at the species level (Baldwin, 1992; Baldwin et al., 1995). This region is flanked by conservative sequences based on which universal primers have been developed (White et al., 1990). The high congruence of ITS sequences obtained between *Sulla* species is indicative of a close relationship among these taxa (Chennaoui et al., 2007). ISSR markers have been generated in *Sulla* genus to detect genetic variation (Chennaoui-Kourda et al., 2007). In fact, ISSRs are another source of genetic markers that are abundant throughout the eukaryotic genome (Tautz and Renz, 1984; Kijas et al., 1995). They evolve rapidly (Levinson and Gutman, 1987) and have high reproducibility (Meyer et al., 1993; Fang and Roose, 1997). As an additional source of genetic information, amplified fragment length polymorphism (AFLP) approach (Vos et al., 1995) was used in *Sulla* genus to furnish a high number of molecular markers. It essentially consists of a combination of restriction fragment length polymorphism and PCR amplification and offers a high level of polymorphism detection with high reproducibility (Lu et al., 1996; Powell et al., 1996; Lanteri et al., 2003). AFLP is successfully applied in many plant species and it is considered a powerful tool used to detect diversity between (and within) species levels and to resolve phylogeny relationships, particularly among closely related species (Ford et al., 2006; Grati-Kamoun et al., 2006; Wang et al., 2007; Zuriaga et al., 2009; Baraket et al., 2011). Each approach produces a different result and phylogenetic topology among *Sulla* species because they target different regions of the genome. The use of more than one data set at the same time is required for reconstructing and solidifying a phylogeny as demonstrated in other species (Ferguson et al., 2004; Ferriol et al., 2004; Duran et al., 2005; Cortese et al., 2010). In general, the use of both molecular and morphological markers is recommended because each data set provides complementary data with a greater power of resolution in genetic diversity analyses (Marvaldi et al., 2002; Gomez et al., 2004; Cortese et al., 2010). Few peer-reviewed works combining and comparing the results obtained by different morphological and molecular genetics methodology have been published relative to the *Sulla* genus. The main objective of this work is to investigate the relationships between species and to show the genetic connections between various *Sulla* species utilizing morphological quantitative traits and molecular markers as ITS sequences, AFLPs and ISSRs.

Results

Morphological trait diversity and species relationships

Quantitative morphological characteristics based on vegetative and reproductive development of the six *Sulla* species were measured in order to describe their genetic diversity. For this purpose, a PCA was performed showing that the three first axes accounted for 90.094% of the global variability (Table 1). Thus, the studied parameters contributed to the majority of the diversity of these species. The first principal component (PC1) absorbed 38.011% of the total inertia and is positively correlated with Length of total axis at flowering (LT; +0.975), Length of the most developed axis at flowering (LA; +0.939), Length of great axis of pollen (LG; +0.903) and negatively with VP (Viability of pollen; -0.887). PC2 that absorbs 31.707% of the total inertia corresponded to NB (Total branches number; +0.771), WF (Waist of open flowers; +0.861), NF (Maximal number of flowers per inflorescence; +0.896) and LS (Length of small axis of pollen; +0.848). PC3 absorbed 20.376% and is mainly defined with LO (Length of orthotropic axis; +0.840) and NI (Number of items per pod (at the rate of 10 pods per plant); +0.972) (Table 1). These observations showed that the vegetative and reproductive development traits equally contributed in the variability discrimination

Table 2. Matrix of Euclidean distances between the six *Sulla* species based on morphological analysis. The bold values indicate the smallest and the largest genetic distances.

	Species	1	2	3	4	5	6
1	*S. capitata*	0.000					
2	*S. carnosa*	0.225	0.000				
3	*S. flexuosa*	0.227	0.257	0.000			
4	*S. pallida*	0.217	0.180	**0.370**	0.000		
5	*S. coronaria*	0.266	0.162	0.248	0.267	0.000	
6	*S. spinosissima*	**0.048**	0.257	0.234	0.255	0.292	0.000

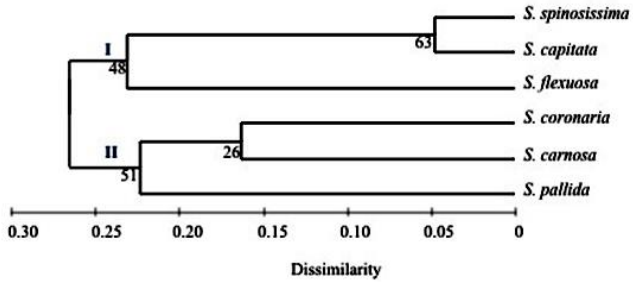

Fig 2. Euclidean distances among *Sulla* species revealed by UPGMA, cluster analysis based on morphological parameters data provided in the computer program NTSYS-pc software version 2.02i. The values between branches are the bootstrap values generated by 2000 resamplings in the program WinBoot. Details regarding groups I and II are discussed in the text.

between the *Sulla* species. The projection of species in (1-2) plot exhibited a continued genetic diversity (Fig. 1). It is worth to note, the convergence of *S. spinosissima* and *S. capitata*, which were classified in the former nomenclature as two subspecies as well as the convergence of *S. carnosa* and *S. coronaria*. The opposition of these two groups revealed their extreme divergence on the morphological vegetative level. On the whole, the obtained PCA highlighted a species clustering independently from their bioclimatic origin and mating system.

In order to estimate the phenetic relationships of the *Sulla* species, Euclidean distances based on these morphological traits were calculated. The resultant matrix revealed values ranged from 0.048 to 0.370 with an average of 0.233 (Table 2). Thus, the analyzed species are characterized by a high degree of genetic diversity at the morphological level. The smallest value was observed between the two species *S. spinosissima* and *S. capitata*, suggesting great similarities in their phenotypic traits (narrow leaves, rigid cloves, violet corolla, 6-12 flowers per inflorescence). In fact, these species have long been considered as two sub-species, and were a subject of debate for taxonomists until 2003, when Choi and Ohashi reconstructed the taxonomy of the Hedysarea tribe. The maximum distance value suggested a high divergence between *S. pallida* and *S. flexuosa*. The ensuing UPGMA phylogram revealed two major divergent clusters (Fig. 2). The first one included *S. spinosissima S. capitata* and *S. flexuosa*. The only cultivated species, *S. coronaria,* was included with *S. carnosa* and *S. pallida;* and constituted the second cluster which was morphologically different from the other species.

Phylogenetic analysis of the combined ITS, ISSR and AFLP datasets

The search for genomic variability and phylogenetic relationships among *Sulla* species was carried out by different molecular markers such as ITS, ISSR and AFLP (Chennaoui et al., 2007; Chennaoui-Kourda et al., 2007; 2012).

The ITS region (ITS1, 5.8S and ITS2) has been amplified for these species, with the use of universal primers specific to extremities of respectively both ribosomal genes 18S and 26S. These sequences have been referenced in GenBank as AY772223-AY772226, AY772229 and AY775312. The alignment of theses sequences revealed little variations relative to the sequences' length (637-643 bp) and the GC percent (52-55%) with 52 informative sites. Their characteristics were similar to those reported for others angiosperms (Baldwin et al., 1995). The estimation of the genetic distances showed divergent values between the ITS sequences which ranged from 0.000 to 0.061 (Table 3). *S. coronaria* and *S. flexuosa* had identical sequences in both ITS1 and ITS2 regions. These minor scored variations indicated that the ITS regions are quite conserved over time in this genus.

Concerning the ISSR approach, eight primers were used to study the *Sulla* species. A total of 85 ISSR fragments were scored with a percentage of polymorphic bands of 98.8% (Table 4). Depending on the primer, 6 to 16 polymorphic bands were generated with the average of 10.5 bands per primer. The analysis of these data revealed that the Rp values ranged from 4.18 to 11.88 with a collective value of 60.06 (Table 4). The primer $(AG)_{10}G$ revealed the highest value of Rp (11.88). A high level of polymorphism was also detected among species, ranging from 0.317 to 0.742 respectively, with an average of 0.550 (Table 3).

The analysis of these *Sulla* species was also investigated using eight AFLP primer combinations. A total of 295 AFLP fragments were generated with a percent of polymorphic bands of 99.6% (Table 4). Depending on the primer combination used, 24 to 51 bands were scored, with an average of 36.8 bands per combination (Table 4). The resolving power (Rp) values ranged from 13.80 to 32.60 with a total collective value of 175.12 (Table 4). E_{AAC}/M_{CAT} showed the highest Rp value and constituted the more powerful AFLP primer combination for the assessment of genetic diversity among the analyzed species. The resultant AFLP genetic distance matrix revealed values ranged from 0.525 to 0.839 with an average of 0.743 (Table 3).

Table 3. Average and range of pairwise genetic distances among *Sulla* species obtained with different data sets. The bold values indicate the smallest and the largest genetic distances.

Marker system	Number of polymorphic markers	Euclidean distances*	
		average	Range
Morphological	-	0.233	0.048-0.370
ITS	52	0.033	0.000-0.061
ISSR	85	0.550	0.317-0.742
AFLP	295	0.743	0.525-0.839
Combined molecular	432	0.686	0.452-0.807
Molecular vs Morphological	-	0.243	0.190-0.316

*: For all markers, The genetic dissimilarity matrices were calculated using Euclidean distances (Goodman, 1972).

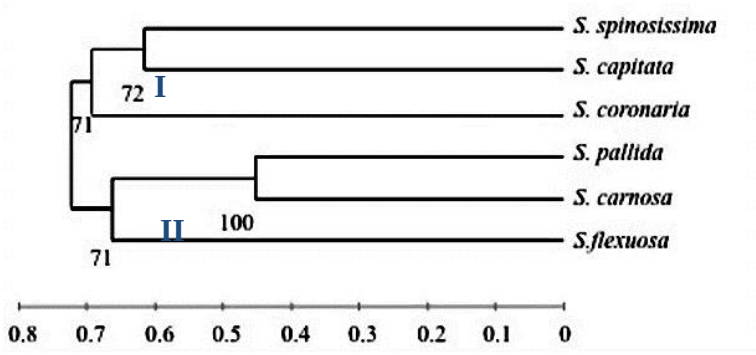

Fig 3. Tree of 6 Sulla species based on molecular combined UPGMA analysis. The values between branches correspond to the bootstrap values generated by 2000 resamplings in the program WinBoot. Details regarding groups I and II are discussed in the text.

These results showed the fluctuation either of the primers' efficiency or of the levels of polymorphism detected by different marker approaches. Overall, the rate of polymorphism detected by individual marker system and the resolving power of primers used displayed their efficiency.

The different molecular data sets (ITS, ISSR and AFLP) were combined into a single analysis for a total data matrix of 432 polymorphic molecular markers. These combined data produced genetic distance values ranging from 0.452 to 0.807 (Table 5) with an average of 0.686 (Table 3). The smallest distance value was observed between *S. pallida* and *S. carnosa* which appeared to be the most similar species (Table 5). The maximum distance value was obtained between the most divergent species *S. pallida* and *S. spinosissima* (Table 5). The dendrogram constructed from combined molecular data produced two main clusters with a strong bootstrap value (average of 78.5% (Fig. 3). The first cluster was represented by *S. spinosissima, S. capitata* (bootstrap support of 72%) and *S. coronaria*. Finally, *S. carnosa, S. flexuosa* were pooled with *S. flexuosa* into the second cluster. Therefore, this topology showed no correlation with the climatic stages origin of the *Sulla* species.

Molecular markers versus morphological traits

Different polymorphism levels and dendrogram topologies have been shown with the various techniques used. Such divergence is often observed between molecular and morphological analyses in different Leguminosae species (Bremer and Struwe, 1992; Degtjareva et al., 2006; Arraouadi et al., 2009). The combination of all data sets is shown in this work for the six *Sulla* species considered. The analysis of the resulting data revealed a genetic distances matrix with values varying from 0.190 to 0.316 and an average of 0.243 (Table 6). Considering both molecular and morphological data, the species *S. spinosissima* and *S. carnosa* were the most genetically divergent. *S. spinosissima* and *S. capitata* were found to be the most similar species. The derived UPGMA dendrogram, illustrated in Fig. 4, showed two main clusters. The first one is represented by *S. spinosissima* and *S. capitata* with a highest bootstrap value of 93%. These two species are pooled to the *S. pallida*. *S. coronaria*, *S. carnosa* and *S. flexuosa* came of the other species and constructed the second cluster with a bootstrap value of 67%. It is worth to note that this topology was also independent of climatic factors. In order to test the monophyletic of *Sulla* genus, which was recently differentiated from the *Hedysarum* genus, the three Mediterranean hedysarea species were included in this combined analysis as: *Hedysarum aculeolatum* Munby, *H. humile* L. and *H. membranaceum* Coss. & Bal. The obtained dendrogram represented in Figure 5 highlights three mains clusters with some divergence compared to previous results. The first one includes *S. spinosissima*, *S. capitata* and *S. pallida* with a bootstrap value of 71%. This clustering is supported again. The second cluster is subdivided in two sub-clusters: the first regrouping *S. coronaria* and *S. flexuosa* (Bootstrap equal to 100%) while the second is constituted by the meridional *S. carnosa* and the two *Hedysarum humile* and *H. aculeatum*. The third cluster was composed by the monophyletic *H. membranaceum*.

Correlation coefficients

The ability to differentiate species by these various techniques was confirmed by Pearson coefficients correlation. The lowest correlation values of the test were observed between the Euclidean distance matrix (based on the 10 morphological traits) and all the other matrices based either on molecular tools or on combined analysis (Table 7). This result confirmed the incongruence between morphological

Table 4. Used dominant markers' primers and obtained banding patterns among *Sulla* species (Adopted Chennaoui-Kourda et al., 2007; 2012)

Primers	ISSR-PCR bands			
	Total	Polymorphic		Resolving power (Rp)
		Number	PPB (%)	
$(AG)_{10}T$	16	16	100	8.360
$(AG)_{10}G$	12	12	100	11.880
$(AG)_{10}C$	8	8	100	5.720
$(TC)_{10}C$	15	15	100	4.400
$(AGG)_6$	8	7	87.5	9.020
$(ACTG)_4$	7	7	100	4.180
$(GACA)_4$	14	14	100	6.820
$(GACAC)_4$	6	6	100	9.680
Total	85	84	98.8	60.06
	AFLP-PCR bands			
E_{AGC}/M_{CAA}	30	29	96.6	17.56
E_{AAC}/M_{CAA}	45	45	100	25.30
E_{AGG}/M_{CAA}	24	24	100	13.80
E_{ACG}/M_{CAG}	29	29	100	16.21
E_{AGC}/M_{CAG}	30	30	100	18.60
E_{AAC}/M_{CAG}	44	44	100	25.30
E_{AAC}/M_{CAC}	43	43	100	25.75
E_{AAC}/M_{CAT}	51	51	100	32.60
Total	295	294	99.6	175.12

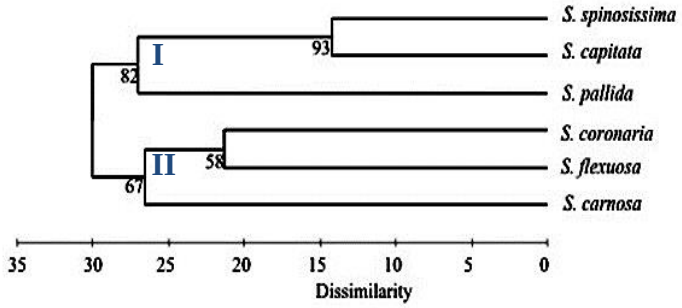

Fig 4. UPGMA phenogram of the genetic relationships among 6 Mediterranean *Sulla* species constructed from estimated Euclidean distances based on combined molecular and morphological markers. The values below branches correspond to the bootstrap values generated by 2000 resamplings in the program WinBoot. Details regarding groups I and II are discussed in the text.

and molecular markers revealed in *Sulla* genus. Moreover, Pearson's correlation coefficient was calculated (0.097; p=0.302) between environnemental factors and combinated molecular markers. The obtained results shown that no correlation exists between these two traits (Chennaoui-Kourda et al., 2012).

Considering the three molecular dissimilarity matrices (ITS, ISSR and AFLP), the obtained Pearson's correlation coefficient (0.315, p=0.065) supported the absence of correlation between the three different types of markers. In addition, the obtained values between dissimilarity matrices were consistently lower than those between each different type of markers and the combined dissimilarity matrix (ITS + ISSR + AFLP) (Table 7). The lower positive and significant correlation coefficients calculated between ITS, ISSR and AFLP distances' matrices led to the incongruence of the clustering results obtained with these three type of markers when considered separately. In fact, the obtained values confirmed the absence of correlation between the different targets regions of genome. The results obtained with ISSR data differed considerably from the others.

Discussion

The aim of the present study was to use both morphological traits and molecular markers to examine the phylogenetic relationships of *Sulla* species based on combined all the obtained data sets. The use of several marker systems with different degrees of polymorphism can resolve genetic relationships between species and give important information for efficient utilization of plant genetic resources. From always, the morphological traits have been served for the phylogenetic relationships analysis among different species (Lesins and Lesins, 1963; Taylor and Gillett, 1988; Marquez-Ortiz et al., 1996; Bulinska-Radomska 2000). Researchers became aware of the influence of the environmental factors on the morphological variations. Hence, the searches turn over the molecular markers known among others for their neutralities. A large panel of molecular taxonomy studies based on variation analyses in rDNA's internal transcribed spacers (ITS) has proved their efficiency to examine phylogenic relationships between different species (Arnheim, 1983, Baldwin et al., 1995, Katz-Downie et al., 1999). Similarly, dominant molecular markers such as ISSR and AFLP have been successfully employed for assessing genetic diversity in many plant species and drawing their genetic relationships (Huang and Sun, 2000; Pasakinskiene et al., 2000; Ghariani et al., 2003; Lanteri et al., 2004; Portis et al., 2005; Belaïd et al., 2006; Ford et al., 2006; Grati-Kamoun et al., 2006; Jaimes et al., 2006; Wang et al., 2007).

Table 5. Matrix of genetic distances between the six *Sulla* species based on combined molecular markers (ITS, ISSR and AFLP). The bold values indicate the smallest and the largest genetic distances.

	Species	1	2	3	4	5	6
1	*S. capitata*	0.000					
2	*S. carnosa*	0.691	0.000				
3	*S. flexuosa*	0.629	0.679	0.000			
4	*S. pallida*	0.690	0.646	**0.452**	0.000		
5	*S. coronaria*	0.672	0.745	0.700	0.741	0.000	
6	*S. spinosissima*	0.615	0.737	0.771	**0.807**	0.712	0.000

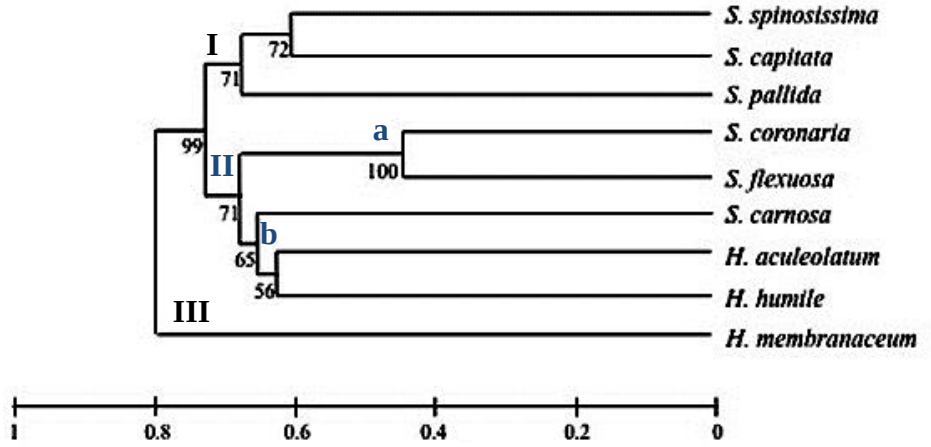

Fig 5. UPGMA phenogram of the genetic relationships among 6 Mediterranean Hedysarea species constructed from Euclidean distances matrices based on combined molecular and morphological markers. The values below branches correspond to the bootstrap values generated by 2000 resamplings in the program WinBoot. Details regarding groups I, II and III are discussed in the text.

Marker efficiency in interspecific phylogeny

The morphological traits related either to reproductive or to structural development of plants contributed equally to resolving variability within the *Sulla* genus. In fact, the length of total axis at flowering, the length of the most developed axis at flowering, the length of great axe of pollen and the viability of pollen contributed the most in the discrimination between these species. Besides their high discriminative power, the length of orthotropic axis and the number of items per pod contributed the least in the discrimination between these species.

Polymorphisms obtained with ITS, ISSR and AFLP markers have different underlying sources at the molecular level, and may differ in their effectiveness for use in the research of genetic diversity and the establishment of genetic relationships between species. A total of 295 polymorphic AFLP fragments were scored from eight primer combinations in front of 85 and 52 polymorphic markers generated respectively from eight ISSR primer pairs and ITS analysis. Hence, it is of great interest to the scientific community to determine whether these techniques contributed the most to the observed diversity. Operationally, the recorded collective AFLP resolving power of 175.12 is higher than the ISSR (60.06) and ITS (25.55) ones. In addition, it is useful to compare the absolute levels of polymorphisms shown by the three classes of markers (Moyano et al., 2003; Gaudeul et al., 2004; Powell et al., 1996). This was made possible throughout the assessment of correlation's Pearson and Spearman coefficients. These were tested by computing matrices based either on separate or combined ITS, ISSRs and AFLPs. Moreover, the correlation test exhibited consistently lower values between ITS, ISSR and AFLP dissimilarity matrices. Consequently, the clustering results obtained with these three markers, when considered independently, would not be very convincing (in spite of their suitability to establish genetic relationships in these closely related species). In fact, the distribution of each class of markers along the genome is critical to determine its effectiveness to assess the relationships among genotypes in relation to their level of linkage disequilibrium (Powell et al., 1996). As reported in other genus crops as *Triticum*, *Hordeum*, *Aegilops*, *Elmus* and *Agropyron* (Sun et al., 1999; Guadagnuolo et al., 2001), the combination of different kinds of markers would be more useful to establish genetic relationships between *Sulla* species. Normally, different marker systems should able to uniquely fingerprint the studied species; however, the three approaches detected different amounts of polymorphism. DNA divergence values between the ITS sequences of *Sulla* species ranged from 0.00 to 0.06, indicating that the ITS regions are quite conserved in these species. Low ITS sequence divergence in these species precludes its application for resolution of genetic relationships among species. In fact, the species *S. coronaria* and *S. flexuosa* have identical sequences in both ITS1 and ITS2 regions. In contrast both AFLP and ISSR approaches reveal substantial amount of variation among species averaging 74.3% and 55.0%, respectively.

Interspecific relationships

Data analyses of these different markers showed that these methods are useful to assess the genetic diversity in this plant species. The analysis of the data obtained provides evidence of a high degree of polymorphism. The considerable variability among some of these species has been also previously detected with morphological characteristics and isoenzymes analyses (Trifi-Farah et al., 1989; Trifi-Farah and Marrakchi, 2002). On the whole, the resulting UPGMA trees show minor topological differences (despite some minor inconsistencies). However, the UPGMA based on ISSR is the mostly incompatible with the other tests and have lower

Table 6. Matrix of genetic distances between the six *Sulla* species based on molecular and morphological markers.

	Species	1	2	3	4	5	6
1	*S. capitata*	0.000					
2	*S. flexuosa*	0.235	0.000				
3	*S. pallida*	0.208	0.242	0.000			
4	*S. carnosa*	0.239	0.238	0.215	0.000		
5	*S. coronaria*	0.214	0.227	0.229	0.255	0.000	
6	*S. spinosissima*	**0.190**	0.287	0.285	**0.316**	0.264	0.000

Table 7. Correlation coefficients between the different types of used molecular markers (Mantel Test).

	ITS	ISSR	AFLP	Morpho	ITS+ISSR+AFLP	Mol+morpho
ITS	1					
ISSR	0.462	1				
AFLP	0.487	0.283*	1			
Morpho	-0.149*	-0.451*	-0.041*	1		
ITS+ISSR+AFLP	0.891	0.673	0.827	0.170*	1	
Mol+morpho	0.579	0.574	0.386*	0.327*	0.706	1

bootstrap values. This could be caused by a relatively smaller number of ISSR fragments sampled in comparison with AFLPs and ITS sequences. The combined dataset has been proved to be the most robust to establish a relationship between species. In fact, when compared to results of separate analyses of the individual data sets, the simultaneous analysis showed greater resolution with very strong bootstrap support (>50%) in all branches. Hence, comparable arrangements were observed using data based either on AFLP, ITS or ISSR. This equivalence significantly supports many assumptions, such as the distinction between *S. spinosissima* and *S. capitata*, which their morphological characteristics have caused some taxonomists to they treat as subspecies of the same taxa (Baatout et al., 1990). Based on the whole molecular data obtained in this study, we can consider maintaining *S. spinosissima* and *S. carnosa* as separate species. Moreover, results provide very strong support for a close relationships between *S. coronaria* and *S. flexuosa* in all trees based on AFLP, ISSR, ITS and the combined dataset. This close congruence of the only cultivated *Sulla* species (i.e. *S. coronaria*) and *S. flexuosa* that is affected by a severe genetic erosion (Trifi-Farah et al., 2002) recommend these species be included in breeding programs to enhance forage production.

It's worth noting that when considering both *Hedysarum* and *Sulla* species, the obtained genetic distances values using the different approaches reflected a high level of genetic diversity among natural species and lower variability between the two genera *Sulla* and *Hedysarum* indicating inter genus similarity levels. This conclusion is supported by genus-specific bands which occurred at low frequencies. This low divergence between the genus and the large variability detected among the species could be explained by the occurrence of gene flow in the natural species or common origins of the two genera. In addition, the AFLP, ISSR, ITS and combined clustering methods showed a species clustering which is independent from the geographic origin. Such results were also previously obtained in others studies (Chennaoui et al., 2007; Chennaoui-Kourda et al., 2007; 2012). Taking into account the taxonomical classification of the *Hedysarum* and *Sulla* genus based on morphological characters (Choi and Ohashi, 2003), the indistinctiveness of the different analysis of the two analyzed genus allowed us to propose an hypothesis of a common genetic history and suggested that they may have generated from a very narrow gene pool. So far, we may theorize that the analyzed species have evolved from a common ancestor *H. membranaceum*. Moreover, our investigation suggested a nuclear lineage among the Mediterranean *Hedysarea* species, showing that their genomic DNA had likely recently evolved. In fact, the AFLP, ISSR and ITS trees clearly indicated that *H. membranaceum* is the most divergent in the *Hedysarea* species complex (Chennaoui et al., 2007; Chennaoui-Kourda et al., 2007; 2012). The combined analysis supports the distinction of this monophyletic species (99% of bootstrap support) from all the remaining species. This result is coherent taking into account that *H. membranaceum* is originating of Algeria. In fact, only this country includes all the Mediterranean *Hedysarea* species (Trifi-Farah et al., 2002) and seems to be unique as a diversity center. Although the related closeness of the arid *S. carnosa* and *H. humile* and the semi-temperate *H. aculeolatum* remained with low bootstrap support in the combined analysis (bootstrap values of 56% and 65%), it could be exploited in genetic breeding programs to improve feed crop and Tunisian grasslands especially in arid and semi arid areas.

Material and Methods

Plant material

Morphological and molecular analyses were carried out on the six *Sulla* species. Samples of seeds characterizing each species were collected from 1974 until 1986 throughout North Africa. The used accessions were obtained from an exsitu collection maintained at the Laboratory of Molecular Genetics, Immunology and Biotechnology of the University of Tunisia. The characteristics of the analysed accessions and their ecological factors were reported in Table 8.

DNA extraction

Total genomic DNA was extracted individually from fresh seedlings according to the procedure described by Dellaporta et al. (1983) with minor modifications adapted to mini-extraction. After purification, DNA samples were quantified using Gene-Quant spectrophotometer (Pharmacia) and their qualities were evaluated by electrophoresis on 0.8 % agarose gel according to Sambrook et al. (1989).

Quantitative traits analysis

The study of the morphological variability included all six *Sulla* species. Morphological measurements were taken on 25 random individuals from each of the six different *Sulla* species. Indeed, seeds of each species were pooled, and a

Table 8. Summary of the six *Sulla* species used in experiments

Sulla genus	Geographic repartition	Mating system	Climate stage	Altitude (m)	Latitude (degree)	Longitude (degree)
S. capitata (Desf.) B.H. Choi & H. Ohashi, comb. nov.	Tunisia	Predominantly allogamous	Semi-temperate	398.6	35.6	5.2
S. carnosa (Desf.) B.H. Choi & H. Ohashi, comb. nov.	Algeria	Predominantly allogamous	Arid	116.7	33.9	8.4
S. coronaria (L.) Medik.	Tunisia	Predominantly allogamous	Semi-temperate	278.6	36.9	8.4
S. flexuosa (L.) Medik.	Morocco	Strictly allogamous	Semi-temperate	360.0	36.9	4.5
S. pallida (Desf.) B.H. Choi & H. Ohashi, comb. nov.	Tunisia	Predominantly allogamous	Semi-temperate	348.6	61.0	2.8
S. spinosissima (L.) B.H. Choi & H. Ohashi, comb. nov.	Tunisia	Predominantly autogamous	Arid	370.0	35.3	4.4

random sample of fifty seeds was scarified and soaked in water for 24h before being germinated for five-seven days on moist filter paper. The experiments were conducted at the faculty of sciences of Tunis and the samples were then transferred in a greenhouse to the measurement of morphological traits. To assess quantitative genetic differentiation, ten morphological traits related to structural and reproductive development of plants were measured. Results were expressed by average for each species considering discriminating traits related to morphology such as length of the most developed axis at flowering (LA), length of total axis at flowering (LT), length of orthotropic axis (LO), total branches number (NB) and related to reproduction power such as waist of open flowers (WF), maximal number of flowers per inflorescence (NF), number of items per pod (at the rate of 10 pods per plant) (NI), length of small axis of pollen (LS), length of great axis of pollen (LG), viability of pollen (VP).

ITS Amplification and Sequencing

The amplification and sequencing conditions were performed as described in Chennaoui et al. (2007). The PCR products were purified by spin column chromatography (QIAquick spin QIA-GENE GmbH, Hilden, Germany). Purified DNA was directly sequenced using the Ready Reaction Big Dye Terminator Cycle Sequencing Kit according the manufacturer's instructions.

ISSR analysis

The ISSR-PCR assays were described in the study of Chennaoui-Kourda et al. (2007). Eight oligonucleotides complementary to Simple Sequence Repeat (SSR) markers were used (Table 4). Amplifications were performed at least twice and only reproducible products were taken into account for further data analysis. Amplification products were separated by electrophoresis on 1.5% agarose gel and in 0.5× TBE buffer (pH 8.3), and stained with ethidium bromide as described by Sambrook et al. (1989).

AFLP analysis

Generation of AFLP fingerprints was carried out as described by Chennaoui-Kourda et al. (2012). Eight AFLP primer combinations with three selective bases were selected for the specific amplifications (Table 4). The AFLP markers were loaded on denaturing polyacrylamide gel (6%). Subsequently, the products were silver stained and were dried overnight prior to visually scoring polymorphic bands read separately by two persons and ambiguous markers were not recorded (Bassam et al., 1991; Chalhoub et al., 1997).

Statistical analysis

Morphological data analysis

Population means were used only as a descriptive tool without any statistical inferences. The 6 species means were then subjected to principal component analysis (PCA) using the Statistical Analysis System software version 6.07 (SAS 1990).

Data for morphological traits were standardized as described by Roldan-Ruiz et al. (2000) and then used to calculate Euclidean distances. Discriminant traits were used as input variables for a cluster analysis using the unweighted pair-group method of averages (UPGMA) to generate a dendrogram using the program NTSYS-pc software version 2.02i (Rohlf, 1998).

Combined data analysis

In order to derive robust and well-reconstructed relationships of species, a simultaneous analysis of the combined morphology, ITS, ISSR and AFLP data sets were considered. The polymorphic fragments for each technique (AFLP, ISSR) were scored as 0 and 1 for absence and presence of bands, respectively. The ITS sequences were treated as dominant markers and combined for the construction of a total data matrix enclosing all molecular markers. The efficiency of each kind of molecular markers in genetic diversity was estimated by calculating the percentage of polymorphic bands (PPB) and their resolving power (Rp) (Prevost and Wilkinson, 1999). Rp was calculated following the formula described by Gilbert et al. (1999). For individual and combined molecular markers and molecular versus morphological traits, genetic dissimilarity matrices were calculated using Euclidean distances (Goodman, 1972). The obtained matrices were used for cluster analysis using the Unweighted Pair-Group Mean Arithmetic method analysis (UPGMA) provided in the computer program NTSYS-pc software version 2.02i (Rohlf, 1998). The confidence limits of cluster phenograms were determined by performing bootstrap of the binary data using the program WinBoot with

2000 replicates (Yap and Nelson 1996). Correlations between individual and combined matrices were calculated using the Mantel test (Mantel, 1967) and the probability test of the correlation coefficient is estimated with 10, 000 permutations.

Conclusion

The data seem to highlight a recent evolution of Mediterranean *Hedysarea* species and confirm that the analysed species are issued from a common ancestor. The exploration of plant genetic resources and the design of plant improvement programs require a detailed knowledge of the amount and distribution of genetic diversity within species. Molecular markers can provide a relatively unbiased method of quantifying such genetic diversity. In fact, the present study provides no evidence for the hypothesis that the two genera *Hedysarum* and *Sulla* have been independently evolved from different ancestral species. Although the distinctiveness of *H. membranaceum* from the *Sulla* species is supported in the present study, there is a close genetic relationship between *H. aculeolatum* and *H. humile* with the other species. This overlapping in all AFLP, ISSR and ITS trees provide strong support for a monophyletic origin of these species. The data provided evidence by the use of either AFLP or ISSR or ITS as powerful tools to assess genetic diversity and establish genetic relationships among Mediterranean *Hedysarea* species. However, the AFLP method is the most effective generating the highest number of polymorphic fragments. Large datasets can reduce sampling errors and thus increase the accuracy and repeatability of phylogenetic estimations, as shown by the high level of congruence among the AFLP trees and the trees based on the combined data. Such information may be of great use to the scientific community and could serve as a starting point for natural feed conservation and amelioration. Moreover, species fingerprinting with proper tools is necessary for the effective management and the prioritization of these important forage species.

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