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Genetic diversity in *Silene sennenii* Pau (Caryophyllaceae) assayed through DNA-based techniques

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Abstract
GENETIC DIVERSITY IN *SILENE SENNENII* PAU (CARYOPHYLLACEAE) ASSAYED THROUGH DNA-BASED TECHNIQUES.— *Silene sennenii* is a narrow endemic species from the NE of the Iberian Peninsula. It is considered as EN ("Endangered") according to the IUCN criteria and is under legal protection in Catalonia. In the present work we report an assay using three different approaches for surveying the genetic diversity in this rare, endangered campion: analysis of chloroplast haplotypes, AFLPs and transferability of microsatellites previously developed for *Silene latifolia*. None of the nine chloroplast regions sequenced showed any variability. Five out of the 21 microsatellite loci tested were polymorphic although more loci are required in order to perform a robust population genetics study. Regarding the AFLP analysis, five out of the 26 pairs of primers tested exhibited moderate levels of variability and therefore they could be useful for further investigating the genetic structure of *S. sennenii*. Although preliminary, our results based on three different genetic markers are in agreement with the low values of genetic variation already reported for this species employing allozymes.

Key words: AFLPs; chloroplast haplotype; cross amplification; endemism; microsatellites; SSRs.

Resumen
DIVERSIDAD GENÉTICA DE *SILENE SENNENII* PAU (*CARYOPHYLLACEAE*) A PARTIR DE TÉCNICAS BASADAS EN ADN.— *Silene sennenii* es una especie endémica, circunscrita a un área extremadamente reducida al NE de la Península Ibérica. Está catalogada como EN («En Peligro») según criterios UICN y se encuentra legalmente protegida en Cataluña. En el presente trabajo se expone el ensayo de tres aproximaciones diferentes al estudio de su diversidad genética: análisis de haplotipos cloroplásticos, AFLPs y transferibilidad de microsatélites diseñados previamente para *Silene latifolia*. Ninguna de las nueve regiones cloroplásticas secuenciadas ha presentado variabilidad. Se han obtenido cinco *loci* microsatélites polimórficos de los 21 ensayados, cantidad insuficiente para llevar a cabo un estudio robusto sobre genética poblacional. En cuanto a AFLPs, cinco combinaciones de cebadores de las 26 probadas han mostrado niveles moderados de variabilidad siendo así útiles para posteriores trabajos sobre la estructura genética de *S. sennenii*. Aun siendo preliminares, los resultados aquí expuestos, fruto del uso de tres tipos de marcadores basados en ADN, confirman la escasa diversidad genética anteriormente obtenida para esta especie mediante la técnica de electroforesis de aloenzimas.

Palabras clave: AFLPs; amplificación cruzada; endemismo; haplotipo cloroplástico; microsatélites; SSRs.

INTRODUCTION

Silene sennenii Pau (Caryophyllaceae) is a narrow endemic species restricted to the Empordà Plain (Catalonia, northeastern Iberian Peninsula; Fig. 1). According to the last census conducted in 2008 (Martinell *et al.*, 2010) it has only five small, scattered and highly fragmented populations containing fewer than 5000 individuals; its area of occupancy is 26.2 ha (see Fig. 1 and Table 1). The species grows in dry perennial grasslands (Phoenician torgrass swards) installed on deep soils, at an elevation below 100 m. This habitat, currently associated with margins of fields, slopes and pathways, has been severely affected by urban, industrial, and agricultural activities during recent decades (López-Pujol *et al.*, 2007; Martinell *et al.*, 2011). Hence, taking into account this situation, *S. sennenii* has been classified as EN ("Endangered") according to the IUCN (2001) criteria in both regional and international red lists (Font *et al.*, 2004; Moreno, 2008; Sáez *et al.*, 2010; IUCN, 2011). In addition, it is included in the Catalogue of Threatened Flora of Catalonia (DOGC, 2008), which ensures legal protection. Several actions regarding in situ and ex situ conservation are now in progress (Massó, 2011).

Given the extreme vulnerability of *S. sennenii* previous research has focused on its genetic diversity (López-Pujol *et al.*, 2007), pollination ecology (Martinell *et al.*, 2010) and reproductive biology (Massó, 2011). Using 21 allozyme loci, López-Pujol *et al.* (2007) detected low levels of genetic diversity (P = 20.9%, A = 1.31, and H_e = 0.071) which were attributed to its small population size, isolation, and fragmentation of extant populations. The main objective of the present work is to provide additional data using DNA-based techniques to supplement the information currently available on the genetic variability of *S. sennenii*. For this purpose, three different methodologies were chosen.

First, chloroplast DNA (cpDNA) haplotypes are a valuable tool to investigate genetic variability at species level and to infer directions of gene dispersal through seeds (McCauley, 1997; Irwin, 2002). The latter is extremely valuable since, as stated by Prentice *et al.* (2008), broad-leaved herbs are poorly represented in the pollen record and their migrational history can usually only be inferred with the help of genetic markers. Using allozyme markers, López-Pujol *et al.* (2007) offered two alternative hypotheses about the origin of the largest population of *S. sennenii* (CSF): (1) a founder effect from a few propagules coming from the second largest population (BAS), or (2) from an already extinct nearby locality with a similar allelic composition. Based on the present occurrence of some very small patches with a few individuals in some field margins very close to CSF population, these authors favoured the second hypothesis and cpDNA haplotypes may provide additional insight to this and other questions regarding the phylogeographic history of *S. sennenii*.

Second, microsatellites (SSRs) are codominant and hypervariable markers widely used to investigate genetic variability and structure both at the population and species levels. They provide data similar to that obtained with allozymes but SSRs are more variable and thus provide more resolution and are more informative (Sunnucks, 2000). However, their main weakness is that the process of SSR isolation is expensive and time-consuming because they must be specifically designed for each species (Zane *et al.*, 2002; Squirrell *et al.*, 2003). To avoid this drawback, the use of primers developed for one species (source species) into others (target species), known as transferability or cross-amplification, is desirable in terms of cost-efficiency (Peakall *et al.*, 1998). Therefore, as a starting point for a population genetic study in a given species using SSRs, it is worth testing markers previously developed for congeneric plants—if available—in few individuals. If they work, then there is no need to look for new loci; however, when amplification fails or these loci are monomorphic, development of specific microsatellites will be necessary.

Third, amplified fragment length polymorphisms (AFLPs) are dominant markers also used in phylogeographic and population genetics studies (Meudt & Clarke, 2007). Their main advantage compared to microsatellites is that they are not species-specific and therefore they do not need to be designed *ex novo* for each species.

MATERIAL AND METHODS

Plant material

Silene sennenii is a hemicryptophyte which produces several rosettes and flowering stems with numerous flowers every year (Fig. 2A–D). It is a diploid

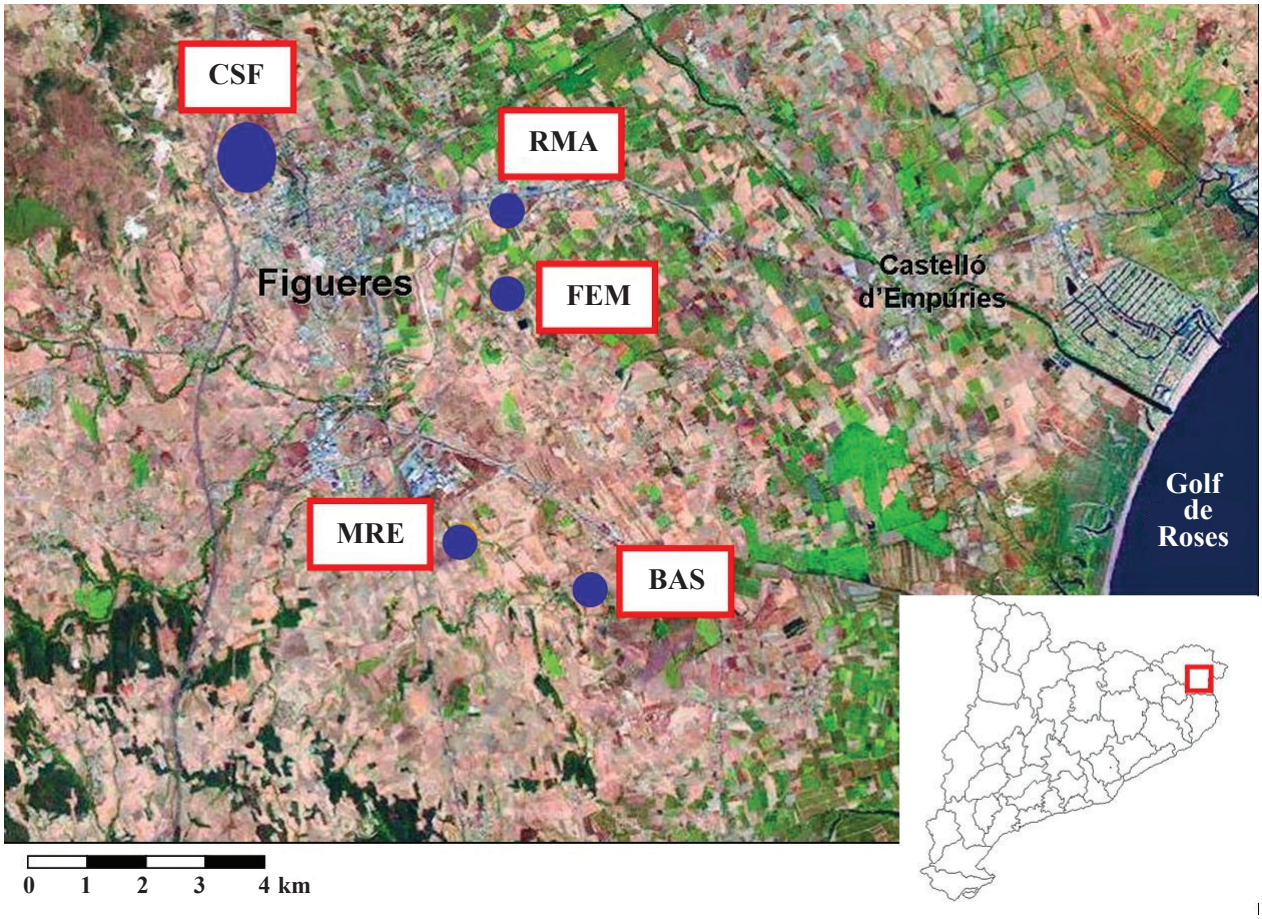

Figure 1. Location of the five extant populations of *Silene sennenii*.

species with $2n = 24$ chromosomes (López-Pujol *et al.*, 2007). It is a hermaphrodite entomophilous plant with strongly protandrous flowers mainly pollinated by nocturnal or crepuscular moths, although a limited capacity for self-fertilization has also been reported (Martinell *et al.*, 2010; Massó, 2011). According to Martinell *et al.* (2010) the system composed by the plant and its pollinators show evidences of disruption in some populations.

The five extant populations of *S. sennenii* are highly irregular in terms of size. According to the last census performed in 2008 (Martinell *et al.*, 2010), the largest populations, CSF (Castell de Sant Ferran; Fig. 2E) and BAS (Baseia), comprise 72% and 24% of the total number of individuals of this species, respectively. Thus, the remaining three populations are extremely small; RMA (Riu Manol), FEM (Far d'Empordà; Fig. 2F) and MRE (Mas Renart), contain 3, 2 and less than 1% of the individuals, respectively.

Leaf material was collected by non-destructive methods in November 2009 from four of the five known wild populations of *S. sennenii*. No samples were taken from the smallest population (MRE) as it contained a single individual. Depending on the size of each population, between 28 and 46 individuals were sampled (Table 1) in order to minimize the potential damage to populations. Sampled localities and population information are provided in Table 1. Genomic DNA was extracted from silica dried leaf tissue using the CTAB method of Doyle & Doyle (1987) as modified by Cullings (1992) and Tel-Zur *et al.* (1999). The extracted DNA was checked on 1.2% agarose gels stained with SYBR.

Chloroplast DNA (cpDNA) haplotypes

Nine chloroplast regions were selected according to previous works reporting polymorphism for different species of *Silene* (Ingvarsson & Taylor,

Table 1. Studied populations of *Silene sennenii.* The location of sampled populations, their area of occupancy and size (2008 census; Martinell, 2010), and the number of individuals sampled (*N*) for each population are provided.

Population code	Population location	Area (m^2)	Population size	*N*
BAS	Baseia, Siurana	17,522	1011	35
CSF	Castell de Sant Ferran, Figueres	233,890	3097	46
FAR	El Far d'Empordà	7260	69	28
RMA	Left bank of Manol River, Vilatenim	3340	130	33

2002; Prentice *et al.*, 2003; Štorchová & Olson, 2004; Malm & Prentice, 2005; Prentice *et al.*, 2008; Hathaway *et al.*, 2009; Naciri *et al.*, 2010). These markers were *trnL*^{UAG}*-rpl32*, *trnT*^{UGU}*-trnL*^{UAA}, *trnL*^{UAA}*-trnF*^{GAA}, *trnH-trnK*, *trnS-trnT*, *trnH-psbA* and *trnS-trnG* intergenic spacers, *trnL*^{UAA} intron, and *trnK* exon. See more detailed information regarding references and primers used for polymerase chain reaction (PCR) and sequencing in Table 2. For this assay, 32 specimens collected from the four studied populations were processed.

Reactions were performed in 25.0 µl volumes with 10% 10x AmpliTaq buffer, 10% 50 mM $MgCl_2$, 10% of 2 mM dNTP mix, 4% of each primer at a concentration of 5 pmol/µl, 1.0 unit (0.2 µl) AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA), and 5.0 µl of template DNA (30–60 ng/µl). The volume was brought to 25.0 µl with distilled sterilized water. Double-stranded DNA was amplified by PCR following four different profiles depending on the marker. The intergenic spacer *trnL*^{UAG}*-rpl32* was amplified as explained in López-Vinyallonga *et al.* (2011). The profile used for the amplification of *trnT*^{UGU}*-trnL*^{UAA}, *trnL*^{UAA}*-trnF*^{GAA} and *trnL*^{UAA} consisted of a previous denaturation step of 1 min 35 s at 95°C, followed by 35 cycles of 93°C for 1 min, 58°C for 1 min and 72°C for 2 min. The amplification of *trnH-trnK*, *trnS-trnT* and *trnK* consisted of a previous denaturation step of 4 min at 94°C, followed by 30 cycles of 92°C for 45 s, 61°C to 62°C for 45 s and 72°C for 4 min. Finally, the amplification of *trnH-psbA* and *trnS-trnG* consisted of a previous denaturation step of 5 min at 96°C, followed by 35–40 cycles of 96°C for 45 s, 53°C and 52°C respectively for 1 min and 72°C for 1 min. All reactions were given a final 10 min extension time at 72ºC.

Sequencing of the amplified DNA segments used the same primers as PCR and was performed on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the Interdisciplinary Center for Biotechnology Research (ICBR) facility at the University of Florida. Nucleotide sequences were edited with BioEdit v7.0.1 (Hall, 1999) and aligned manually by sequential pairwise comparison (Swofford & Olsen, 1990).

Microsatellites (SSRs)

For this interspecific cross-amplification assay, SSR loci were selected when they met the maximum number of the following features: showing high variability (in terms of allele number, observed heterozygosity, and polymorphic information content), belonging to independent linkage groups, and having a successful transferability into other species of *Silene*. According to these criteria, 21 microsatellites previously developed for *S. latifolia* Poir. were chosen (Teixeira & Bernasconi, 2007; Moccia *et al.*, 2009; see Table 3 for details). For this assay, 48 specimens collected from the four *S. sennenii* populations were processed.

All SSR loci were amplified using FAM, NED, PET and VIC fluorescently labeled forward primers as explained in López-Vinyallonga *et al*. (2010). Two different profiles, based on the conditions established for each locus in the original publications with slight modifications, were used for PCR reactions. Amplification of the six markers of Teixeira & Bernasconi (2007) used PCR cycling conditions with a preheating step at 95°C for 15 min, followed by 30 cycles composed of 30 s denaturation at 94°C, 90 s annealing at 48ºC–62ºC depending on the loci and 60 s elongation at 72°C, followed by a final elongation step of 30 min at 60°C. Amplification of

Figure 2. *Silene sennenii* and its habitat: (A), anthesis in the nightfall; (B), flowers in different phenologic status; (C), individuals in Manol River locality; (D), open flower during its second male night; (E), Castell de Sant Ferran locality in summer 2009; (F), el Far d'Empordà locality in summer 2012.

Table 2. Details of primers examined in *Silene sennenii*. F: forward primer; R: reverse primer.

Region	Primer	Sequence (5'–3')	Reference
trnL^{UAG}*-rpl32*	rpl32 (F)	CAGTTCCAAAAAAACGTACTTC	Shaw *et al.* (2007)
	trnL$_{UAG}$ (R)	CTGCTTCCTAAGAGCAGCGT	
trnL^{UAA} intron	trnLa (F)	CATTACAAATGCGATGCTCT	Taberlet *et al.* (1991)
	trnLb (R)	TCTACCGATTTCGCCATATC	
trnT^{UGU}*-trnL*^{UAA}	trnLc (F)	CGAAATCGGTAGACGCTACG	
	trnLd (R)	GGGGATAGAGGGACTTGAAC	
trnL^{UAA}*-trnF*^{GAA}	trnLe (F)	GGTTCAAGTCCCTCTATCCC	
	trnLf (R)	ATTTGAACTGGTGACACGAG	
trnK exon	trnK1D$_{UUU}$ (F)	GGGTTGCCCGGGACTCGAAC	Demesure *et al.* (1995)
	trnK2D$_{UUU}$ (R)	CAACGGTAGAGTACTCGGCTTTTA	
trnH-trnK	trnH-Dem (F)	ACGGGAATTGAACCCGCGCA	
	trnK-Dem (R)	CCGACTAGTTCCGGGTTCGA	
trnS-trnT	trnS-Dem (F)	CGAGGGTTCGAATCCCTCTC	
	trnT-Dem (R)	AGAGCATCGCATTTGTAATG	
trnH-psbA	trnH (F)	ACTGCCTTGATCCACTTGGC	Hamilton (1999)
	psbA (R)	CGAAGCTCCATCTACAAATGG	
trnS-trnG	trnS$_{GCU}$ (F)	GCCGCTTTAGTCCACTCAGC	
	trnG$_{UUC}$ (R)	GAACGAATCACACTTTTACCAC	

the 15 markers of Moccia *et al.* (2009) used PCR cycling conditions with a preheating step at 94°C for 5 min, followed by 30 cycles composed of 30 s denaturation at 94°C, 45 s annealing at 51ºC–62ºC and 45 s elongation at 72°C, followed by eight additional cycles consisting of 30 s denaturation at 94°C, 45 s annealing at 52°C and 45 s elongation at 72°C. Annealing temperature was dependent upon primers used, as shown in Table 3. A final extension step at 72°C for 10 min was added.

Genotyping was performed on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the Interdisciplinary Center for Biotechnology Research (ICBR) facility at the University of Florida. Fragment analysis was performed with GENEMARKER v1.5 (SoftGenetics, LLC, State College, PA, USA) software using LIZ600 size standard, and data was scored manually.

Amplified fragment length polymorphisms (AFLPs)

Digestion ligation, pre-amplification and selective amplification reactions were carried out as explained in Vilatersana *et al.* (2007). Table 4 shows the 26 selective primer combinations used. Fragments were separated using 1.2% agarose gel electrophoresis and were visualized with SYBR staining. Genotyping was performed as explained in the SSR analysis. For this assay, 48 specimens collected from the four studied populations of *S. sennenii* were processed.

RESULTS AND DISCUSSION

Chloroplast DNA (cpDNA) haplotypes

All amplifications were of high quality and the total aligned chloroplast data set was 4958 bp, ranging from 265 bp for *trnH-psbA* to 1006 bp for *trnH-trnK*. Nevertheless, none of the nine cpDNA regions sequenced showed any variability. On the contrary, previous works on other *Silene* species based on the same cpDNA markers employed in the present study report high haplotype variability. As an example, Ingvarsson & Taylor (2002) amplified three chlo-

Table 3. Marker information: locus names, repeat motifs, references and number of alleles (*NA*) reported in the original publications for *Silene latifolia* and observed in the present work for *S. sennenii*, and optimal annealing temperature (Tm) for *S. sennenii*. –: failed reaction.

Loci	Repeat motif	Reference	*NA* *S. latifolia*	*NA* *S. sennenii*	Tm (ºC) *S. sennenii*
SL1	$(CA)_{31}$	Teixeira & Bernasconi (2007)	43	–	–
SL4	$(CT)_{19}CC(CT)_3T(CT)_2$		33	–	–
SL6	$(GT)_{40}$		29	–	–
SL8	$(GA)_{37}$		25	–	–
SL14	$(GT)_{41}$		41	–	–
SL15	$(GT)_{46}$		40	–	–
SL_eSSR01	$(AACCCA)_{4.7}$	Moccia *et al.* (2009)	4	1	60
SL_eSSR06	$(AAT)_9$		9	5	60
SL_eSSR07	$(TCAAT)_9$		3	3	60
SL_eSSR08	$(TAA)_{21}$		–	3	51
SL_eSSR10	$(GT)_9$		9	–	–
SL_eSSR11	$(TTA)_{8.7}$		12	–	–
SL_eSSR12	$(TC)_8$		8	3	60
SL_eSSR14	$(AGC)_{9.7}$		7	–	–
SL_eSSR17	$(ATT)_{13.3}$		10	–	–
SL_eSSR20	$(TGA)_5$		4	1	60
SL_eSSR22	$(ACA)_{8.3}$		6	1	60
SL_eSSR24	$(CT)_{16.5}$		7	–	–
SL_eSSR25	$(TCA)_{12.3}$		7	–	–
SL_eSSR29	$(AAG)_8$		12	–	–
SL_eSSR30	$(CTT)_5$		10	2	59.6

roplast intergenic spacers (*trnL-trnF*, *trnH-psbA*, and *trnG-trnS*) and one intron (*trnL*) for *S. vulgaris* (Moench) Garcke and *S. latifolia* from Europe. Ignoring variation caused by insertions/deletions, they found 14 and 28 exclusive polymorphisms, respectively. Further, Štorchová & Olson (2004) reported 24 haplotypes for *S. vulgaris* from central Europe amplifying one single region (*trnH-psbA* intergenic spacer). Although we expected much lower values for *S. sennenii* considering its narrow distribution area and the low number of populations and individuals, the absolute lack of variation using nine markers was unexpected.

Microsatellites (SSRs)

Fifteen of the 21 studied loci could not be amplified in *S. sennenii* at the annealing temperatures advised in the source publications (Teixeira & Bernasconi, 2007; Moccia *et al.*, 2009). After assaying at many different temperatures and testing different DNA concentrations and proportions of reagents, only two of these 15 regions were successfully amplified. Therefore, 13 of the 21 markers had to be discarded and the remaining eight were used for genotyping. Three of the eight correctly amplified loci were monomorphic across individuals and populations and, thus, they would be of limited use for further works. The remaining five loci were polymorphic for *S. sennenii* with 3.2 alleles on average, ranging from two (SL_eSSR30) to five (SL_eSSR06). The global number of alleles obtained for *S. sennenii*, using 48 individuals from four different populations, was 19 considering the eight regions amplified (both monomorphic and polymorphic) and 16 considering only the five polymorphic loci (Table 3). These values of allelic richness are much lower than those reported for *S. latifolia* in the source publications for these loci. Teixeira & Bernasconi (2007) found up to 211 alleles in 60 individuals belonging to four different populations using six loci (that is, a mean of 35.2 alleles per locus at the species level),

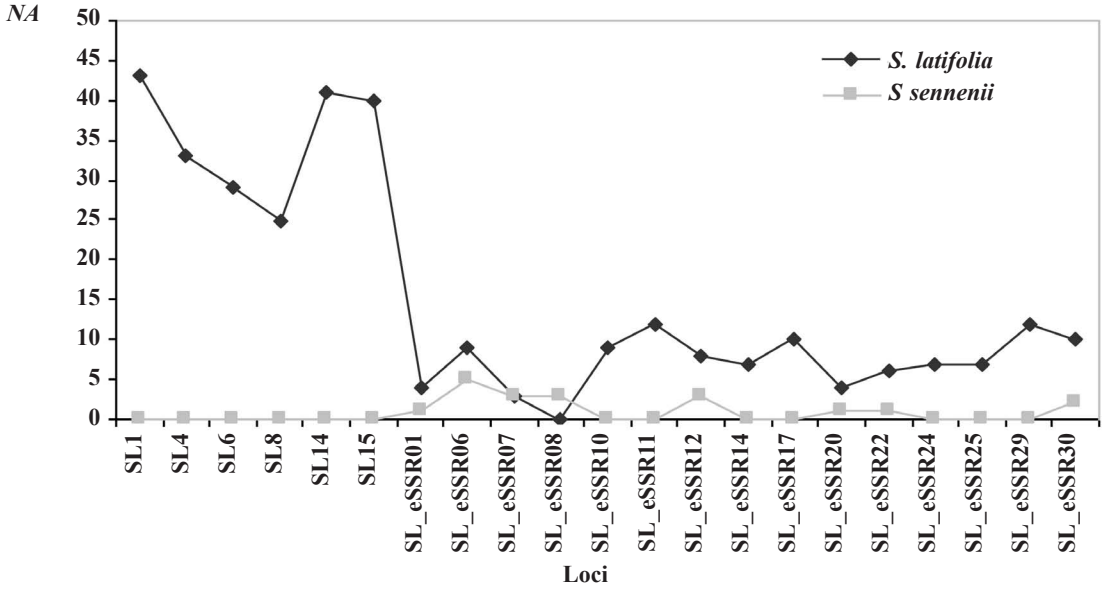

Figure 3. Number of alleles (*NA*) recovered for *Silene latifolia* (black dots) in Teixeira & Bernasconi (2007) using loci SL1 to SL15 and in Moccia *et al.* (2009) using loci SL-eSSR01 to SL-eSSR30, as well as for *S. sennenii* (grey squares) for the 21 loci tested in the present cross-amplification assay.

whereas we were unable to amplify any of their six loci. Moccia *et al.* (2009) recovered 108 alleles in 30 individuals from a single population employing 14 loci (mean of alleles/locus = 7.7), whilst we obtained only 19 alleles using more individuals and populations although we used only eight loci (i.e. a mean of 2.4 alleles per locus).

In this cross-amplification assay there was no correlation between the number of alleles obtained for each locus for the target (*S. sennenii*) and the source species (*S. latifolia*). As an example, the maximum number of alleles in *S. sennenii* (five) is found in SL_eSSR06, a locus with a relatively low number of alleles (nine) in *S. latifolia*. On the other hand, the maximum number of alleles in *S. latifolia* (43) is found in SL1, a locus that could not been amplified in *S. sennenii* (see Fig. 3 and Table 3 for details).

As stated by many authors (e.g. Primmer *et al.*, 1996; Eujayl *et al.*, 2004; Saha *et al.*, 2004; Guo *et*

Table 4. Selective PCR primer combinations screened for AFLP analysis in *Silene sennenii* (fluorescent dye within parentheses). –: failed reaction; +: unambiguous and variable primer combinations selected.

	MseI-CAA	MseI-CAT	MseI-CTC	MseI-CTG	MseI-CAC	MseI-CAG	MseI-CTT
EcoRI-ACT(6Fam)	–		+				
EcoRI-AAG(6Fam)	–	–	–	–	+		
EcoRI-ATC(6Fam)	–	–	–	+			
EcoRI-AGC(6Fam)	–	–	–	–			
EcoRI-AGG(Ned)				–			
EcoRI-ACG(Ned)	–						
EcoRI-ACA(Ned)					–		
EcoRI-ACA(Pet)		–					
EcoRI-ACC(6Fam)	–	–	–		+	+	
EcoRI-AAC(6Fam)		–					
EcoRI-ATG(Pet)							–

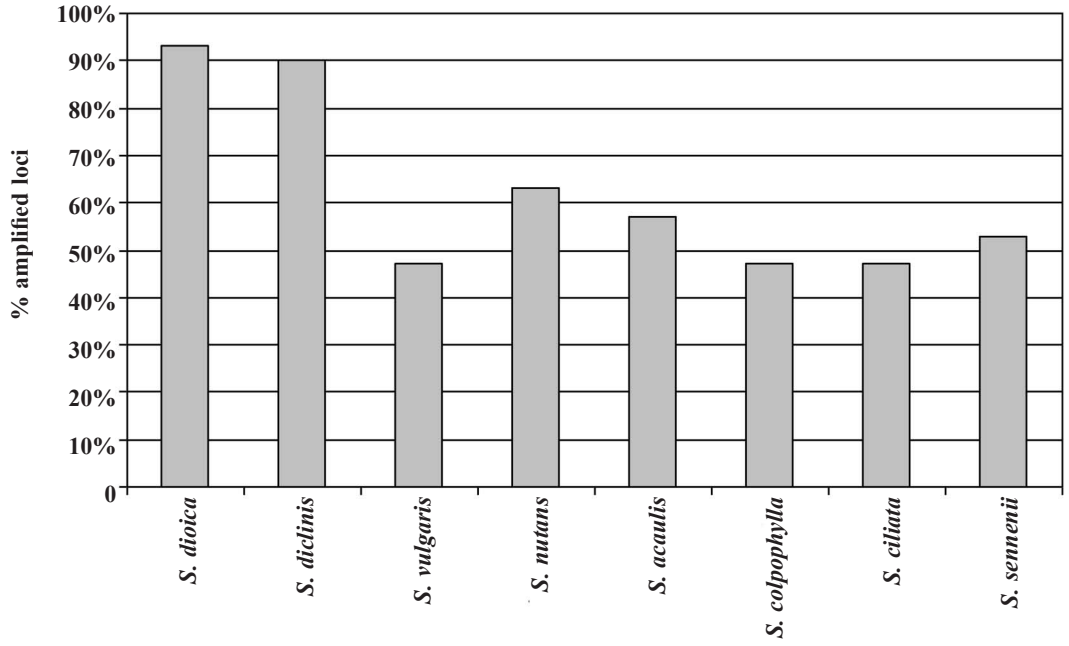

Figure 4. Percentages of transferability success for the loci by Moccia *et al.* (2009) from *Silene latifolia* into eight congeneric species.

al., 2006; Heesacker *et al.*, 2008), cross-amplification is expected to work better for phylogenetically close species. In Moccia *et al.* (2009) the transferability of the markers was higher for *S. dioica* (L.) Clairv. (28 loci; 93% of amplification success) and *S. diclinis* (Lag.) M. Laínz (27 loci; 90% of amplification success) than for the other five species of *Silene* assayed (Fig. 4), because of their shorter evolutionary distance respect to the source species, *S. latifolia*. The exact phylogenetic relationship between our target and source species (*S. sennenii* and *S. latifolia*, respectively) is unknown, but the low success achieved in the present cross-amplification assay may indicate that *S. latifolia* is phylogenetically more closely related to the pair *S. dioica*-*S. diclinis* than to *S. sennenii*. Nevertheless, since they are conspecific we expected a much higher transferability success for the rare Iberian campion (only eight out of 21 loci were successfully amplified). Besides, the markers by Moccia *et al.* (2009) are EST-SSRs, that means they are located in transcribed regions of the genome; these regions are widely conserved in plants and therefore are expected to function in distantly related species, even over different taxonomic levels (e.g. Scott *et al.*, 2000; Cordeiro *et al.*, 2001). In addition, given the high amplification success obtained with their markers, Moccia *et al.* (2009) suggested that the flanking regions of these loci were sufficiently conserved, and that these markers could be used for comparative analyses of genetic diversity in the genus *Silene*. Related to this, Pashley *et al.* (2006) found that EST-derived microsatellites were more than three times more transferable across species than the anonymous SSRs derived from genomic DNA (73% vs. 21%, respectively). This trend is verified in our cross-amplification assay (Fig. 3) since the transferability success was higher for EST-SSR loci of Moccia *et al.* (2009) than for the markers developed by Teixeira & Bernasconi (2007).

To sum up, the amplification optimization carried out for *S. sennenii* was not successful and to continue working on it seems not cost-effective. At this point, more loci could be tested, although development of specific microsatellites is likely the best option to perform a population genetic study.

Amplified fragment length polymorphisms (AFLPs)

After testing 26 primer pairs, seven of them were chosen because they were easy to amplify (Table 4). Two of these primer pairs were discarded because one of them was difficult to score (EcoRI-AGC-

6Fam/MseI-CTG) and the other showed low variability (EcoRI-ACT-6Fam/MseI-CAA). Therefore, the five final primer combinations for the selective PCR (all EcoRI oligos fluorescently labeled with 6-Fam dye) were EcoRI-ACT/MseI-CTC, EcoRI-ATC/MseI-CTG, EcoRI-ACC/MseI-CAG, EcoRI-AAG/MseI-CAC, and EcoRI-ACC/MseI-CAC (Table 4). Thus, the present work provides these five primer pairs, which could be used for genotyping 10 to 15 individuals from the four sizable populations of *S. sennenii* in order to carry out a future population genetic study.

CONCLUDING REMARKS

Although more accurate studies are required, the absolute uniformity of the cpDNA regions amplified and the low polymorphism detected with SSRs suggest that *S. sennenii* fits the pattern of low genetic variability reported for this species in a previous work based on allozymes (López-Pujol *et al.*, 2007) and which is characteristic of many narrow endemic species (e.g. Hamrick & Godt, 1990; Gitzendanner & Soltis, 2000; López-Pujol *et al.*, 2009). This low diversity is probably the consequence of multiple factors such as demographic constraints (scarce number of individuals and populations), small range (extremely narrow area of distribution), habitat fragmentation and evolutionary history (e.g. putative bottlenecks).

According to the results obtained in the present work, the most appropriate approximations for understanding the genetic structure of *S. sennenii* would be SSR and AFLP analyses. However, and given the low efficiency obtained in the transferability assay, we were only able to select five SSR markers which would provide insufficient information to conduct a statistically robust study. The low success obtained with the cross-amplification of microsatellites also prevented us from performing additional assays with more markers published for other congeneric species. This suggests that the design of specific SSR primers *ex novo* for *S. sennenii* could be a better strategy. The AFLP technique has provided more encouraging results and therefore this approximation seems the best suited for a further study of the genetic structure of *S. sennenii*. Hence, the five primer pairs selected here could be a good starting point for further works.

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