Plant Ecology and Evolution 150 (3): 293–303, 2017
https://doi.org/10.5091/plecevo.2017.1309

PLANT ECOLOGY EVOLUTION

REGULAR PAPER

Phenological and genetic characterization of *Sedum hispanicum* (Crassulaceae) in the Italian peninsula at the western margin of its distribution

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Background and aims – *Sedum hispanicum* acts as a pioneer species on the gypsum outcrops of Emilia Romagna region (Northern Italy). The species was studied in two Sites of Community Importance through a comparative phenological and genetic diversity analysis to provide insights into strategies adopted by the species for successful reproduction in the harsh conditions of these rocky outcrops.
Methods – Phenology was examined in individuals from four sampling sites chosen in one study area (Gessi Bolognesi e Calanchi dell'Abbadessa, GB) from March to June in two years (2013 and 2015) with different spring temperatures. Reproductive and vegetative phenophases were compared among sites and over years. ISSR-based molecular analysis was performed to obtain genetic diversity measures on individuals collected at the same four GB sites and, for comparison, in a second area (Gessi di Monte Rocca, Monte Capra e Tizzano).
Key results – Individuals at the four GB sites showed inter-annual differences in full flowering and leaf senescence, but intra-annual synchrony of all monitored phenophases. Moderate amounts of genetic diversity were observed in the two areas (*P%* = 79.67, 67.48; *I* = 0.209, 0.205; He = 0.118, 0.122). Low genetic structure (Φst = 0.05; *Gst* = 0.06) and high gene flow (*Nm* = 3.92) resulted within the GB area. Genetic differentiation was higher between the two areas (Φst = 0.37, *Gst* = 0.22). Evidence of inbreeding (*s* = 0.61) was found in the GB area.
Conclusions – Inter-annual differences in timing of full flowering phenophase seem to be related chiefly to spring temperatures. The estimates of genetic diversity in *S. hispanicum* are comparable to those of autogamous and clonal species, while the partitioning of genetic diversity reflects the low structure typical of outcrossing species. Intra-annual flowering synchrony is likely to promote sexual reproduction. *Sedum hispanicum* seems to adopt multiple reproductive modes to overcome environmental extremes of gypsum outcrops.

Key words – Stonecrop, Natura 2000, Gypsum Vein, Northern Apennine, flowering phenology, synchrony, ISSR, genetic variation.

INTRODUCTION

Sedum hispanicum L. (Spanish stonecrop) is a tiny succulent plant, up to 15 cm tall, morphologically characterized by ascending, branched and densely foliated stems, alternate cylindrical leaves (1.5 × 8 mm), (5–)6(–9)-merous subsessile obdiplostemonous flowers grouped in unilateral cymes, with white pink-veined petals and stellate patent follicles developing small seeds dispersed by gravity and wind (Stephenson 1994, 't Hart 2003, Thiede & Eggli 2007, Gallo 2017). Nevertheless, *S. hispanicum* is a very polymorphic species (Niculae & Bârcă 2005), displaying a wide variation in many characters such as leaf hairiness, inflorescence and fruit or leaf colour/shape. The range of variation makes it a taxonomically complex species. Italian accessions were segregated into *S. hispanicum* L. and *S. pseudohispanicum* Strobl. (Pignatti 1982), but the latter is at present synonymized with the valid species *S. hispanicum* L. (The Plant List 2013).

Native to the eastern Mediterranean region, *S. hispanicum* occurs over a large geographic area throughout central and southeastern Europe. It is common on the Balkans, extends

ISSN: 2032-3913 (print) – 2032-3921 (online)

to Asia Minor and the Near East, and is naturalized in northern regions of Europe. Its altitudinal range extends from 0 to over 2 000 m a.s.l. From an ecological standpoint, the species is a typical thermophilous saxicolous xerophyte, which usually prefers calcareous substrates, although it is able to grow in a variety of habitats and soils (Niculae & Bârcă 2005). *Sedum hispanicum* has features such as extended cover, rapid and consistent growth even on thin substrates, abundant dissemination at the end of each seasonal cycle, high levels of water retention, CAM physiology, fleshy leaves and succulent habitus, which make it a drought resistant plant and hence a good colonizer of environments characterized by water deficiency, thin soil, high temperatures and intense solar radiation (Getter & Rowe 2009).

Life cycle of *S. hispanicum* is described as usually annual, sometimes biennial or perennial (Webb et al. 1993, Pyšek & Prach 2003, Niculae & Bârcă 2005). The arrangement of the obdiplostemonous androecium, where stamens of the outer whorl bend over petals and stamens of the inner whorl remain erect or bend over carpels, is related to the possibility to promote allogamy or autogamy respectively. Melittophily is assumed for *Sedum* species in general (Thiede & Eggli 2007) and has been observed in *S. hispanicum* (Grozeva 2011). Diptera belonging to Syrphidae and Bombylidae (genera *Episyrphus*, *Scaeva*, *Sphaerophoria*, *Bombylius*) and Lepidoptera belonging to Pieridae (*Pieris* spp.) were also seen on plants growing in the study areas (A. Velli, personal observation). As most of the congeneric species, *S. hispanicum* can reproduce sexually or asexually. According to Gudžinskas (2000) the species reproduces mainly by seed, but also by shoots detached from dying fertile stems. Although more energy seems to be allocated to sexual than asexual reproduction (Getter & Rowe 2009), the relative importance of each reproductive method in natural populations has never been analysed.

The western geographic extreme of its natural range falls in Switzerland and Italy. In the Italian peninsula, the species is common in the central-southern regions, where floristic affinities with the Balkan and Aegean territories are frequent; its range becomes fragmented and interrupted by wide gaps in the rest of the peninsula, where the species is considered infrequent (Viciani et al. 2013) or rare (Pignatti 1982, Gallo in press).

In Emilia-Romagna region, *S. hispanicum* occurs mostly on the outcrops of the "Gypsum-Sulphurous Bank" (Ferrari 1974, Bassi & Montanari 2015), a gypsum-sulphur formation of Messinian origin that crosses the entire Apennine chain in the Italian peninsula and is called Gypsum Vein in this region, where it emerges more clearly than elsewhere on the hills along the outer edge of the Apennines. The species has been sporadically encountered in other gypsum rocky surfaces of Triassic origin (Alessandrini & Branchetti 1997) or in environments different from the gypseous ones (Romani & Alessandrini 2001, Bracchi & Romani 2010).

It is known that peripheral populations of plant species, by virtue of their small dimensions, and/or scarcity of suitable environments, often show an altered sexual/asexual reproduction ratio, with an increase in vegetative propagation, autogamy and even the evolution of self-compatibility (Beatty & Provan 2011).

Reproductive phenology (i.e. flowering and fruiting events) has an important influence on reproductive success, as it determines reproductive synchrony among potential mates and impacts the opportunity for gene flow and sexual reproduction by influencing the attraction of pollinators and seed dispersers (Goulart et al. 2005). Additionally, vegetative phenology is important as timings of vegetative growth may be intimately related to clonal activity experienced by plants (Sampaio & Scariot 2008).

Neutral ISSR molecular markers, already used to analyse Crassulaceae species (Kozyrenko et al. 2011, György et al. 2013), are especially useful in detecting the level and distribution of genetic variation among related individuals. Indirectly, they also provide insight into reproductive modes and gene flow shaping the partitioning of genetic diversity in plant populations.

Consequently, to better understand the reproductive strategies of edge populations, it is advisable to integrate several sources of data (e.g. phenology and genetic structure).

Sedum hispanicum represents an interesting model to study the implications of distributional ranges in a peripheral region characterized by extreme environmental conditions. In the present study, the species was examined in two areas of the Gypsum Vein belonging to Sites of Community Importance (SCIs, Council Directive 92/43/EEC). To date, only few studies on the native taxa of these sites have been conducted (Aleffi et al. 2014), although their flora is of great conservation interest.

Specifically, phenological patterns and genetic composition of *S. hispanicum* were analysed with the following aims: (1) to describe for the first time the phenology of the species; (2) to assess intra- and inter-annual differences regarding the main growth phases; (3) to detect the amount of genetic diversity harboured by *S. hispanicum*; (4) to analyse the distribution pattern of genetic variation in the same and in different gypsum outcrops; and (5) to infer information about gene flow and reproductive strategies, taking into account the different methodological approaches, and search for correspondences between them.

MATERIAL AND METHODS

Study areas

The two areas of the Gypsum Vein selected for the present investigations are respectively included in two Sites of Community Importance extending over the first hillsides southeast and southwest of Bologna, about 14 km apart (fig. 1). The Sites are characterized by the presence of gypsum outcrops with particular habitats of conservation interest and identified as SCI IT4050001 - Gessi Bolognesi e Calanchi dell'Abbadessa (hereafter GB), and SCI IT4050027 - Gessi di Monte Rocca, Monte Capra e Tizzano (hereafter GMR).

Sedum hispanicum occurs in scattered and discontinuous patches, from 190 to 220 m a.s.l., in both Sites, and characterizes the priority habitat 6110* "Rupicolous calcareous or basophilic grasslands of the *Alysso-Sedion albi*" together

Table 1 – **Characteristics of *Sedum hispanicum* sites sampled in the Gypsum Vein south of Bologna.**
Slope, exposure and rockiness are averaged over plots for each site. *: sites used in the phenological analysis; N: sample size for the ISSR analysis.

Study area	Site code	Latitude	Longitude	Slope (°)	Exposure (°)	Rockiness (%)	N
GB (SCI IT4050001 Gessi Bolognesi e Calanchi dell'Abbadessa)	GB1*	44°26′49″N	11°22′45″E	21 ± 6.8	193 ± 18.7	79 ± 7.5	10
	GB2*	44°26′42″N	11°22′26″E	16 ± 8.2	153 ± 30.3	83 ± 8.9	10
	GB3*	44°26′45″N	11°22′28″E	18 ± 7.2	196 ± 24.0	82 ± 7.6	10
	GB4*	44°26′45″N	11°22′29″E	25 ± 5.8	219 ± 18.6	76 ± 5.6	10
GMR (SCI IT4050027 Gessi di Monte Rocca, Monte Capra e Tizzano)	GMR	44°27′52″N	11°13′05″E	37 ± 19.9	205 ± 55.2	64 ± 27.6	20

with *Sedum album* L., *S. sexangulare* L., *S. acre* L., *S. rupestre* L., *Saxifraga tridactylites* L., *Triticum ovatum* (L.) Raspail, *Petrorhagia saxifraga* (L.) Link, *Geranium molle* L., *Catapodium rigidum* (L.) C.E.Hubb., *Trifolium scabrum* L. and *Poa bulbosa* L. As a whole, plant cover in the patches ranges from 20 % to 50 % due to the presence of bare rocks and habitats different from the above mentioned 6110* (*Quercus pubescens* Willd. woodlands, shrublands dominated by *Spartium junceum* L. or *Prunus spinosa* L., meso-xerophilous grasslands). The distribution of *S. hispanicum* can therefore be considered sparse and globally scarce.

The study areas were chosen within the Sites where *S. hispanicum* forms the most abundant and stable populations at regional level, its presence having been documented since the nineteenth century (Bertoloni 1839, Cocconi 1883). Phenological surveys were carried out in the GB study area in the years 2013 and 2015, whereas for the genetic investigations *S. hispanicum* was sampled both in this area and in GMR.

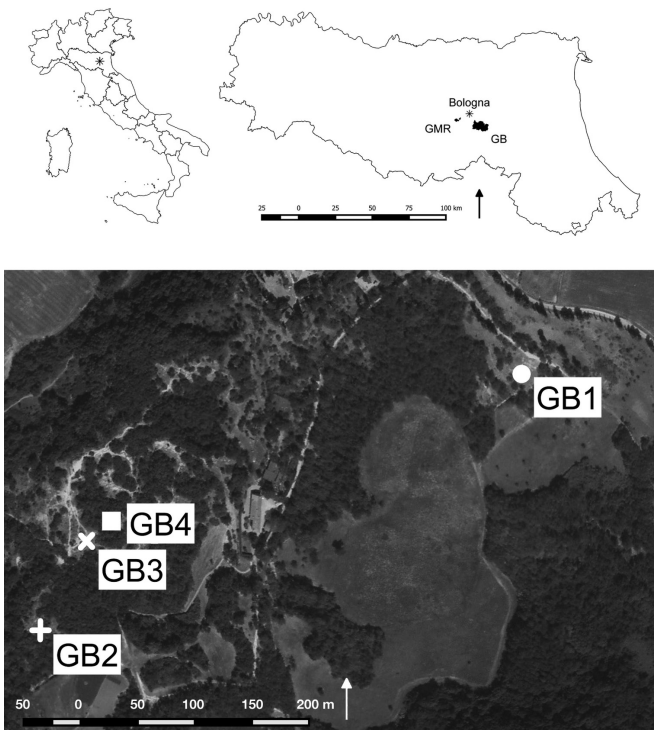

Figure 1 – Locations of the SCI Sites (SCI IT4050027 including GMR area, SCI IT4050001 including GB area) and sampling sites (GB1 to GB4) in the Gypsum Vein south of Bologna (Northern Italy, Emilia Romagna Region). For further details see also table 1.

In the GB area the climate is characterized by cold winters, with one or more periods of frost, and warm and arid summers. Available climatic data from the weather stations of Settefonti (Ozzano dell'Emilia, Bologna; c. 8 km far from the area, 336 m a.s.l.) and Borgo Panigale (Bologna, c. 7 km from the area, 42 m a.s.l.) indicate a sub-Mediterranean annual pattern of temperatures: mean annual = 13.6 °C; T mean min = 4.1 °C in the coldest month (January); T mean max = 24.2 °C in the warmest one (August). The rainfall (mean annual: 805 mm) has two seasonal peaks, the main one in November and the other one in April, and an absolute low in July, during the xerothermic period.

As regards the sampling periods, the spring of 2013 was particularly cold and rainy, with average maximum temperatures of 17.3 °C and rainfall of 328 mm; instead in 2015 it was exceptionally warm and dry, with average maximum temperatures of 19.3 °C and rainfall of 228 mm. Concerning the monthly spring temperature, the difference between 2015 and 2013 was stronger in March (+2.2 °C), nearly absent in April (+0.1 °C), then became notable in May (+1.7 °C) and decreased in June (+1.1 °C).

Phenological sampling and analysis

Within the GB area, phenological analysis was carried out in four sampling sites selected for similar topographic features: moderate slope, southwards exposure and pretty high rockiness (fig. 1, table 1). They were 0.03–0.48 km apart, and fell into the potential distance for pollen dispersal by insects, estimated to be within 1 or 2 km (Chifflet et al. 2011, Couvillon et al. 2014). The four sites were on average 200 m^2 wide and counted about one thousand individuals fragmented in small patches some meters apart from each other.

Five 50 cm × 50 cm plots containing *S. hispanicum* were randomly located within each sampling site and up to twenty randomly selected individuals were observed for each plot. Attention focused on flowering and fruiting stages, recorded from late March until the first half of June. Vegetative

Table 2 – Summary of phenological events recorded for *Sedum hispanicum* over the periods March–June 2013 and 2015 in the sampling sites from GB area.
Phenophases are indicated and descripted according to BBCH code.

BBCH code	phase	description
20	main shoot	leaves developed with no side shoot
21	tillering	first side shoots detectable
55	pre-flowering	flowers visible and still closed on compressed inflorescence
57	advanced pre-flowering	flowers visible and still closed on extended inflorescence
61	beginning of flowering	first flowers open
65	full flowering	at least 50 % of flowers open
67	flowers withering	majority of petals fallen or dry
69	end of flowering	no petals, fruit visible
89	full ripening	follicles ripe, seeds dark and hard
95	leaf senescence	about 50 % of leaves discoloured or fallen

phases (defined as absence of pre-flowering and anthesis on developed plants) detectable in the same period were also recorded. The monitoring was made on the epigeous portions of plants, the status of the belowground part being undetermined; therefore, what we refer to henceforth as "individuals" are the emergent stems.

Data were collected for two years (2013 and 2015) at 7(–10) day intervals and ten phenological stages were observed on each individual. The BBCH (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie; Meier 2001) scale was used. This is a widely adopted system for describing growth stages in plant species. A two-digit code identifies the different stages: the first digit (from 0 to 9) defines the principal stages, while the second one (also between 0 and 9) refers to the partitions of the previous ones in secondary stages. The ten stages recorded in this study are listed in table 2.

Six phenophases were chosen as significant for depicting the growth cycle of *S. hispanicum* since they represent the beginning and the end of the vegetative stage (20, 21, 95), of the reproductive stage (55, 89), and the full flowering (65). The dates of occurrence of the selected growth phases were expressed in days of the year (DOY = number of days counted from January 1st) and averaged by individuals to a site-level. A two-way ANOVA was used to test for intra-annual differences as well as inter-annual differences among the four sampling sites for the occurrence of the six selected phases. DOY values were log transformed before comparisons to achieve normality; the analysis was performed using PAST 3.14 software (Hammer et al. 2001).

The phenophases 55, 57 (flower buds development) and 61, 65, 67, 69 (flowering) were used to produce the phenograms of the mean date of flowering in the two years. Data were calculated on the whole data set of GB area as percentage of individuals in each phenophase. The resulting diagrams showed inter-annual differences in the time of occurrence of pre-anthesis and anthesis stages.

Furthermore, in 2015, vegetative shoots in an early stage of development falling into BBCH 20, but shorter than 2 cm, and easily distinguished from flowering shoots, were also monitored. They are henceforth defined as plantlets, and their proportions were calculated with respect to both total number of recorded individuals and total number of individuals falling in the same phenophase (BBCH 20), and graphically represented.

Molecular sampling and analysis

The evaluation of DNA polymorphism by means of the ISSR-PCR technique was carried out on ten individuals randomly collected in each sampling site in the GB area (for a total amount of forty sampled individuals). For comparison, twenty individuals collected in the GMR area in a single site were included in the survey (fig. 1, table 1); therefore, for the purpose of this analysis, the terms "site" and "area" are interchangeable for the GMR sampling location. Leaves were collected from plants located at a distance of at least 1 m, to reduce the likelihood of sampling clonal individuals, and placed in silica gel. After lyophilisation, leaf tissues were stored at -20 °C until DNA extraction.

Genomic DNA was extracted using the Invisorb Spin Plant Mini Kit (Invitek) according to the manufacturer's protocol. DNA quality and quantity were assessed by spectrophotometry (absorbance at 260 nm; BioPhotometer, Eppendorf). Three ISSR primers from the UBC set no. 9 (Biotechnology Laboratory, University of British Columbia) were used: 868 [$(GAA)_6$], 888 [$BDB(CA)_7$] and 890 [$VHV(GT)_7$]. 30 ng of template DNA and 40 pM of the required primer were added to each dried reaction mixture purchased by "PuRe Taq Ready-To-Go PCR Beads" kit (GE Healthcare Life Sciences) along with distilled water, up to a final volume of 25 ml.

PCR reactions were performed in an MJ Mini thermal cycler (Bio-Rad) programmed as follows: 94 °C for 7 min, 45 cycles of 1 min at 94 °C, 2 min at 52 °C (UBC 868) or 58 °C

Table 3 – **Dates of occurrence of six phenophases representative of the growth cycle of *Sedum hispanicum* over the periods March–June 2013 and 2015 in the sampling sites from GB area.**
Site and phase codes follow table 1 and table 2 respectively. x: average DOY (day of year from January 1st); s: standard deviation; different letters in the same column indicate a significant ($P < 0.01$) F value for the year factor in a two-way (site × year) ANOVA.

Year	Site	Main shoot (BBCH 20)		Tillering (BBCH 21)		Pre-flowering (BBCH 55)		Full flowering (BBCH 65)		Full ripening (BBCH 89)		Senescence (BBCH 95)	
		x	s	x	s	x	s	x	s	x	s	x	s
2013	GB1	110.5	23.3	127.2	17.2	137.1	8.1	147.7 [a]	1.8	161.0	0.0	157.6 [a]	5.8
	GB2	110.1	25.0	129.5	20.1	136.3	8.3	148.4 [a]	2.4	161.0	0.0	158.9 [a]	4.8
	GB3	114.2	22.3	126.0	18.6	132.9	7.5	150.2 [a]	5.3	161.0	0.0	159.5 [a]	4.2
	GB4	108.0	23.8	127.4	19.6	130.3	4.4	149.4 [a]	4.3	161.0	0.0	155.8 [a]	6.4
	average	110.5	23.8	127.6	18.8	134.4	7.8	148.4	2.9	161.0	0.0	158.0	5.5
2015	GB1	108.9	22.6	119.3	23.2	136.1	23.2	141.4 [b]	4.8	157.2	6.0	151.9 [b]	6.1
	GB2	109.4	23.6	118.9	21.9	134.3	7.1	139.8 [b]	3.8	156.3	6.3	154.6 [b]	6.6
	GB3	111.1	23.2	109.6	21.8	132.8	6.8	139.3 [b]	4.5	160.1	3.3	151.8 [b]	9.4
	GB4	113.1	24.1	116.4	20.1	136.2	9.2	141.8 [b]	4.9	160.1	0.0	156.9 [b]	6.2
	average	110.5	23.4	116.1	21.8	134.9	9.3	140.4	4.6	158.5	5.1	153.6	7.5

(UBC 888 and 890), 2 min at 72 °C, followed by a final extension of 7 min at 72 °C. PCR amplified products were size-separated by standard horizontal gel electrophoresis, using 1 % agarose gel in 1x TAE buffer at 90 V for 2 h 45 min, and visualized by SYBR Safe staining (Invitrogen). The molecular weight standard pBR322 DNA - BstN I Digest (New England Biolabs) was run in each gel as a size reference. For each gel, a permanent scanned record was obtained and used for subsequent automated scoring using the image analysis software GelAnalyzer 2010a (http://www.gelanalyzer.com).

ISSR data are dominant and, therefore, each band represents the phenotype at a single bi-allelic locus. Due to their dominant behaviour, these markers provide less direct information per locus than co-dominant markers. This drawback was overcome by using software packages able to handle both co-dominant and dominant markers or specifically developed for dominant markers.

Reproducible bands of similar molecular weight and migration distance across individuals were assumed to be homologous (Adams & Rieseberg 1998), and scored as either present (1) or absent (0) for all sampled individuals; then the binary matrix of ISSR data was processed in GenAlEx 6.5 software (Peakall & Smouse 2006) to evaluate genetic diversity as percentages of private bands (*pb%*) and polymorphic loci (*P%*), Shannon diversity index (*I*) and Nei's gene diversity (*He*). These parameters were measured both for each GB sampling site and on the whole data sets from the two areas. With the same software, the hierarchical ISSR frequency distribution was described through an analysis of molecular variance (AMOVA) and by computing the coefficient of genetic differentiation (*Gst*) both among GB sites and between GB and GMR areas. The level of gene flow was estimated as $Nm = (1 - Gst)/4Gst$.

For the GB area, the Bayesian inbreeding index *f* (analogous to Wright's F_{IS} for dominant markers) was estimated including the forty individuals sampled in the four sites using HICKORY 1.1 (Holsinger & Lewis 2007). The ISSR data were fitted to four models: 'full model', which allows for inbreeding; '$f = 0$' model, which implies lack of inbreeding; '*theta* = 0' model, which implies a zero-valued F_{ST} analogous; '*f* free' model, which decouples the estimates of *f* and *theta*. Computations were carried out using the default values: burn–in = 5 000, number of samples = 25 000 and thinning factor = 5. To estimate the best fit of the four models, the parameter *DIC* (deviance information criterion) was used and the model with the smallest *DIC* was preferred. The selfing rate for the GB area was estimated as $s = 2f / (1 + f)$ (Hartl & Clark 1989).

A Mantel test was performed in the software GenAlEx to verify the correlation between genetic and geographical distances for the four GB sampling sites, while a principal component analysis (PCA) tested with 999 permutations was produced from a variance-covariance matrix with PAST 3.14 (Hammer et al. 2001) to represent the genetic relationships among all sampled individuals from both the areas.

RESULTS

Phenological pattern

The occurrence of six phenophases chosen as significant to represent the growth cycle of *S. hispanicum*, and recorded in 2013 and 2015 in the four sampling sites from GB area, is presented in table 3.

Based on average values across plots, it results that the vegetative growth had already begun in the second half of March, with leafy plants without side shoots; then, in the fol-

Table 4 – **Genetic diversity statistics for *Sedum hispanicum* from GB sites. Estimates for GB and GMR areas are also reported.** Site and area codes follow table 1. *pb%*: percentage of private bands; *P%*: percentage of polymorphic loci; *I*: Shannon diversity index; *He*: Nei's gene diversity; *Φsc*: diversity within sites/areas and *Φst*: diversity among sites/areas from AMOVA; *Gst*: coefficient of genetic differentiation; *Nm*: gene flow; *f*: Bayesian inbreeding index.

Site / Area	*pb%*	*P%*	*I*	*He*	*Φsc*	*Φst*	*Gst*	*Nm*	*f*
GB1	9.86	60.58	0.232	0.141					
GB2	9.72	62.50	0.216	0.129	0.95	0.05	0.06	3.92	0.44
GB3	6.35	52.88	0.192	0.115					
GB4	9.46	64.42	0.234	0.140					
average	8.85	60.10	0.219	0.131					
GB	37.5	79.67	0.209	0.118	0.63	0.37	0.22	0.89	
GMR	22.6	67.48	0.205	0.122					

lowing weeks, side shoots progressively developed. The reproductive cycle started with flowers visible but still closed between the end of April and the beginning of May and ended within mid June with fully ripe fruits and seed dispersal. Leaf senescence began between late May and early June, ahead of the full ripening.

In particular, plants without side shoots (BBCH 20) were found on DOY 110 (i.e. 20 April) both in 2013 and 2015, tillering (BBCH 21) was detectable on DOY 127 (i.e. 7 May) in 2013, but occurred earlier, at DOY 115 (25 April), in 2015. First flower buds (BBCH 55) were observed on DOY 134 (14 May) in both years, while the mean date of full flowering (BBCH 65) differed in 2013 (DOY 148, i.e. 29 May) and 2015 (DOY 140, i.e. 21 May). Fruit ripening (BBCH 89) was at DOY 161 (11 June) in 2013 and three days earlier in 2015 (DOY 158, 8 June). Senescence (BBCH 95), detected on DOY 158 (8 June) in 2013 and on DOY 153 (3 June) in 2015, occurred before the stage of full ripening.

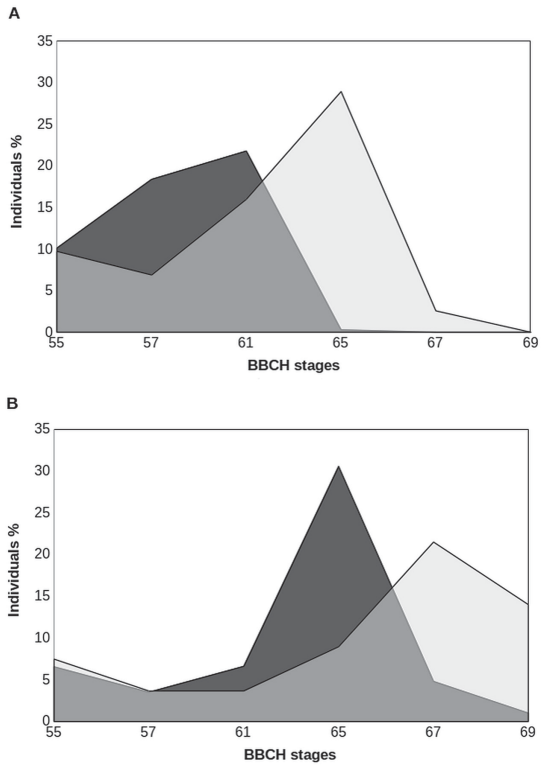

Figure 2 – Phenograms of *Sedum hispanicum* at GB area on 18 May (A) and 28 May (B) in 2013 (black areas) and 2015 (grey areas). Phase codes follow table 2.

Plants from the different sampling sites exhibited the same intra-annual trend: no significant differences occurred in the mean date of the six considered phenophases among the four sites of GB area, both in 2013 and in 2015. By contrast, dates of full flowering (ANOVA; $F_{1, 23} = 82$, $P < 0.01$) and leaf senescence ($F_{1, 75} = 13$, $P < 0.01$) differed significantly in the two years.

The phenological structure of the GB area in the second half of May (fig. 2) shows inter-annual differences in the time of occurrence of pre-anthesis and anthesis stages (BBCH 55 to 69). Only one flowering peak was observed in both years: on 18 May (DOY 138), pre-anthesis was the most represented phenophase in 2013, with 41 % of the individuals in stages BBCH 57–61. Two years later, at the same date, the relatively prevailing phenophase was full flowering (BBCH 65, 29 % of the individuals). On 28 May (DOY 148), full anthesis (BBCH 65, 31 % of the individuals), and flowers withering (BBCH 67, 22 % of the individuals) were the most represented stages respectively in 2013 and 2015.

In 2015 plantlets (vegetative shoots distinguishable from flowering shoots and shorter than 2 cm) were present throughout the entire survey period (fig. 3). Their proportion with respect to the total number of individuals diminished from 94 % (DOY 79) to less than 6 % (DOY 163) with a rapid decrease after DOY 112. When compared to the total number of individuals in BBCH 20, their proportion decreased gradually from 98 % (DOY 79, March 20) until less than 50 % on DOY 138 (May 18), but, from this date until mid-June, the trend became positive again and, on DOY 163 (June 13), plantlets represented the majority (80 %) of the vegetative phase BBCH 20.

Genetic pattern

For the sixty *S. hispanicum* samples taken in the two areas GB and GMR, the three selected primers generated a total of 123 bands (see electronic appendix 1), all of which were polymorphic. A subtotal of 104 bands could be referred to

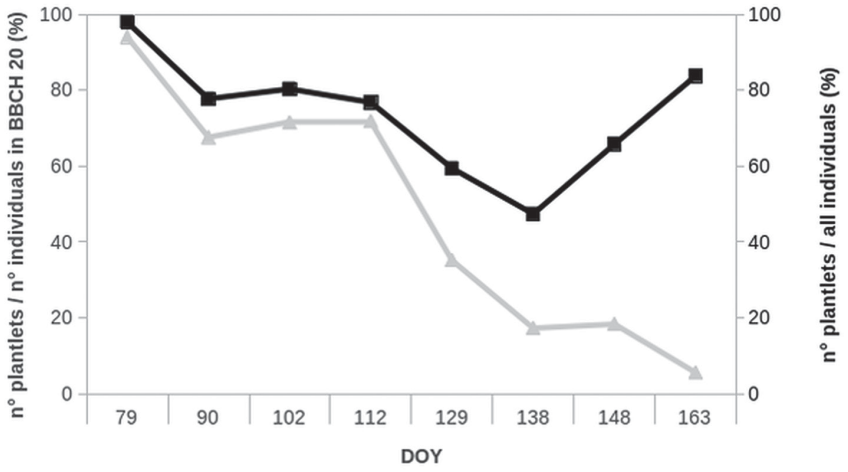

Figure 3 – Variation in the proportion of plantlets with respect to total number of individuals monitored (grey line), and total number of individuals falling in BBCH 20 (black line) in the period March–June 2015 at GB area (DOY: day of year from January 1st).

the forty samples taken in the GB area (see electronic appendix 2).

Genetic diversity measures for the four GB sampling sites based on 104 loci are given in table 4, together with their averaged estimates. Measures for the GB area as a whole and for the single site from GMR area based on 123 loci are also reported. Individuals at the four GB sampling sites exhibited the same pattern of genetic diversity. The mean frequency of private bands (*pb%*) unique to a single group was 8.85 and the mean percentage of polymorphic loci (*P%*) was 60.10 %. Values of the Shannon index and Nei's gene diversity (or expected heterozygosity) were respectively $I = 0.219$ and $He = 0.131$, averaged over the four sites. All individuals sampled from GB area had higher percentages of private bands (*pb%* = 37.5) and polymorphic loci (*P%* = 79.67), compared to those from GMR area (*pb%* = 22.6 and *P%* = 67.48 respectively) while estimates of Shannon index ($I = 0.209$, 0.205) and Nei's gene diversity ($He = 0.118$, 0.122) were comparable in the two areas.

Measures of genetic differentiation are also given in table 4 and referred both to the four GB sampling sites and to the two areas. Based on AMOVA analysis, a high proportion of the total diversity ($\Phi sc = 95$ %) was attributable to variation among individuals of the four GB sampling sites, while moderate genetic differences among them were detected according both to AMOVA ($\Phi st = 5$ %) and Nei's gene diversity ($Gst = 6$ %). In agreement with these results, the estimate of gene flow ($Nm = 3.92$) exceeded the critical value of 1, above which weak structure and high variability are expected among groups of conspecific individuals (Khan et al. 2010). When considering samples from the two areas, a lower proportion of the total diversity ($\Phi sc = 63$ %) depended on variation among individuals and the two areas appeared more differentiated ($\Phi st = 37$ %, $Gst = 22$ %), with higher isolation ($Nm = 0.89$).

In the Bayesian approach, the smallest deviance information criterion ($DIC = 1327.08$) was obtained from the 'full model' and the resulting index ($f = 0.44$) indicated pronounced inbreeding within individuals in the GB area. The estimated selfing rate was $s = 0.61$. When clonality was tested, as percentage of distinguishable genotypes, no identical genotypes were detected, pointing at a complete absence of clonal propagation within the GB study area (data not shown).

According to the Mantel test result, a low but significant correlation between matrices of genetic and geographical distances was observed in the four GB sampling sites ($r = 0.17$; $P = 0.020$). In the PCA analysis (fig. 4), the first two components accounted for 23.13 % and 5.65 % of total variation. Along the first axis, individuals from the four GB sampling sites grouped into overlapping clusters that were separated from individuals sampled at GMR site.

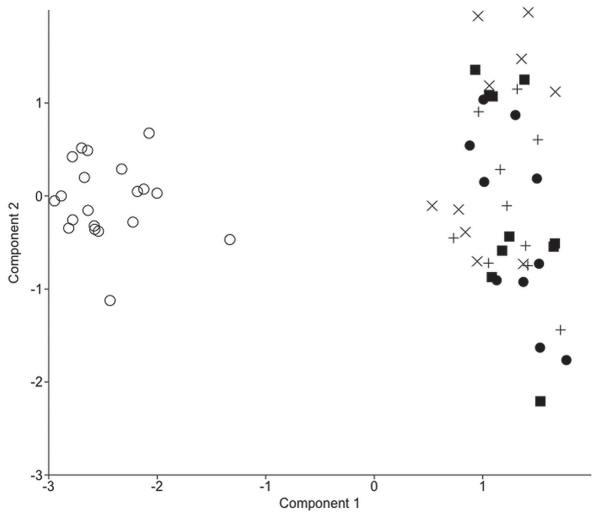

Figure 4 – Plot of the first two axes of the PCA from the ISSR data representing relationships among sixty individuals of *Sedum hispanicum* sampled at GB and GMR sites. Axis 1 accounts for approximately 23 % of the total variation and axis 2 for 6 %. Site symbols: GB1 ●, GB2 +, GB3 ×, GB4 ■, GMR ○.

DISCUSSION

Reproductive and vegetative phenology

This study provides for the first time data on the phenological behaviour of *Sedum hispanicum*. The growing season is brief and quite precocious: it begins by March and ends within mid-June, with similar observations in both years.

Both in 2013 and 2015, plants at the four sites from GB overlapped extensively in their growth and phenological traits, with synchronous full flowering phase spanning few days. It is worth noting that, although the succession of phenological stages is quite regular, leaf senescence (BBCH 95) begins before complete fruit ripening (BBCH 89), implying that when plants begin to die, seeds are protected intact inside the follicles, where they remain during the harsh conditions of the summer months. Seed retention, reported in annual desert plants (Hegazy & Kabiel 2010), as well as in understory geophytes (La Rocca et al. 2014), is known to occur also in other *Sedum* species as a specific adaptation for survival throughout the summer conditions of abiotic stress (Sharitz & McCormick 1973).

Phenological shifts were observed between the two years, highly likely related to the different temperatures registered in springs of 2013 and 2015 (17.3 °C and 19.3 °C being the respective average maximum temperature) and, possibly, even if to a lesser extent, to the different rainfall amounts observed in the same periods (328 mm and 228 mm respectively).

In temperate regions plants deal with highly seasonal climate and tune their growing phases according to particular conditions of each year. In Mediterranean ecosystems, temperature and water availability are primary factors influencing phenological stages. Gordo & Sanz (2010), on the basis of 200 000 records for six phenological events of 29 perennial plant species (period: 1943–2003), demonstrated that maximum/mean values of temperature have the most remarkable effects on plant phenology and overpass the role of precipitations.

Certainly, additional data would be needed to fully understand how different aspects of the climate affect phenology of *S. hispanicum*. Nevertheless, for localities and years considered in this study, the species seems to follow the reported pattern, providing flexible responses to inter-annual climate change.

Inter-annual differences in timing of phenophases regarded in particular the reproductive phase of full anthesis (BBCH 65) and leaf senescence (BBCH 95); no significant differences between the two sample years were observed for the other phases, both vegetative and reproductive. Phenophases appear often to be differently influenced by warmer and drier conditions, in fact they react in different ways to climate change because they cope with environmental cues in different times of the season (Davies et al. 2013).

Prostrate stems of *S. hispanicum* may lose leaves, root and break up into segments, thus acting as shallow rhizomes (A.L. Mandolfo, personal observation). Besides, in the sample sites from the GB area, plantlets, that is short vegetative shoots distinguishable from flowering shoots, were present throughout the whole survey period and, when monitored (2015), their proportion constantly decreased in comparison to the total number of individuals, as expected; however they showed only a slight decrease in mid-May followed by an increase when compared to the total number of individuals in the vegetative stage BBCH 20. It is possible that at least part of the plantlets detected from March to June developed by some means of vegetative propagation.

The ability of *Sedum* to spread vegetatively is well known: simple plant fragments can easily give rise to new individuals and, in perennial species, propagation occurs through prostrate multi-branched rhizomes, that die after the production of new shoots (Stephenson 1994). Vegetative growth through shoots detached from dying fertile stems has been already reported for *S. hispanicum* (Gudžinskas 2000).

Ecological data support the abundance of annuals rather than that of perennials in extreme habitats since the former rapidly convert resources obtained during the vegetative phase into seeds, yet the capacity to adapt life history traits according to the environmental conditions exists among annual species (Hegazy & Kabiel 2010). It is known that *S. hispanicum* exhibits annual, biennial or perennial life cycles (Webb et al. 1993, Pyšek & Prach 2003, Niculae & Bârcă 2005). The observations here referred show that the species may shift from annual to a different habit type on the Gypsum Vein. As the observed rhizomes are superficial and thin, the species may be said to adopt a sub–perennial life form in this difficult environment.

Amount and distribution of genetic diversity

The mean level of genetic diversity encountered in *S. hispanicum* from the GB area (*P%* = 60.10, *I* = 0.219, *He* = 0.131) was low compared to other Crassulaceae analysed with similar markers: *P%* = 87, *I* = 4.80, *He* = 0.182 in *Sedum ussuriense* Komarov (Ku et al. 2011); *P%* = 96.4 or 92.45, *I* = 0.291 or 0.412, *He* = 0.176 or 0.267 in *Rhodiola rosea* L. (Kozyrenko et al. 2011, György et al. 2013) and, more broadly, compared to outcrossing (*He* = 0.260) species, while resulted closer to values reported for autogamous species (mean *He* = 0.091, Csergö et al. 2009). Likewise, genetic variation was quite low when considering the two areas (*He* = 0.118 for GB and *He* = 0.122 for GMR), with estimates of Nei's gene diversity similar to the mean value reported for plant taxa with both sexual and asexual modes of reproduction (*He* = 0.123, Dev et al. 2010).

The distribution of genetic variation, however, does not show the marked genetic structure often displayed by autogamous and/or clonal species: differentiation between groups was moderate. In fact, the extent of differentiation among GB sites, based on AMOVA and Nei's gene diversity was quite low, ranging from 5 % to 6 %. Low genetic isolation by distance was recorded also by the Mantel test, showing a weak though significant correlation between genetic and geographical distances (r = 0.17; P = 0.020). Consistent with this, the value of gene flow found in the GB area is high enough (*Nm* = 3.92) to support the hypothesis that the four sites should be within the homogenizing capability of local gene flow (Khan et al. 2010). As expected, individuals from GB and GMR areas, separated by a distance of 14 km, including natural and anthropogenic barriers (the valleys of

the rivers Reno and Savena, peripheral districts and villages around Bologna), are more differentiated and less involved in recurrent gene flow (Nm = 0.89), as assessed by differentiation indices (Φst = 37 %, Gst = 22 %) and by their clustering according to geographic location in the PCA plot.

The close relationship between plant reproductive traits and levels of genetic variation and genetic structure has been documented in many studies using different methods. The following outcomes can be drawn from those surveys: within-population diversity is, in general, negatively correlated with the level of population differentiation; long-lived, outcrossing taxa retain most of their variation within populations, whereas selfing and clonal taxa allocate more variation among populations; isolation by distance occurs in many different kinds of outcrossing plant species, in which gene flow among populations decreases inbreeding (Nybom et al. 2014, Wu et al. 2015).

Contrary to the general expectations, both low levels of genetic variation and low structure among sampling sites were indicated by ISSR fingerprinting in *S. hispanicum* analysed in the GB area. This pattern may be explained by different and not mutually exclusive reasons.

The estimated level of inbreeding was quite high (f = 0.44, s = 0.61), and included in the range 0.2–0.8 referred to intermediate selfing populations (McClure & Whitlock 2012), suggesting that uni- or biparental inbreeding seems to take an important role in the reproductive mode adopted by *S. hispanicum* on the Gypsum Vein, and that it may be implicated in decreasing the level of genetic variation of *S. hispanicum*. By contrast, its known effect of structuring the distribution of genetic variation among groups (Beatty & Provan 2011) is quite negligible in the GB area, where the four study sites lie at short distances (0.027–0.476 km) from each other and are subjected to the merging action of a short–distance gene flow (Nm = 3.92).

It is known that inbreeding, though reducing genetic variation, can be adopted by peripheral populations of plant species for occupying open areas by one or a few individuals (Michalski & Durka 2007). The private bands detected in four sampled groups (from 6.35 % to 9.86 %) may be attributable to fixation of specific alleles through modes of reproduction different from outbreeding; also, they may represent unique genotypes harbouring some degree of adaptive potential necessary to survive changing climatic conditions (Beatty et al. 2008).

Increased levels of clonal propagation at the expense of the benefits of sexual reproduction, such as the generation of new genotypes via recombination, have been also observed in range-edge populations of different species (Beatty et al. 2008).

Clonal recruitment could allow individuals of *S. hispanicum* to persist in the adverse conditions encountered at the margins of the species' range on the Gypsum Vein. Unfortunately, the estimate of clonal reproduction, based on the ISSR markers here used, did not agree with our morphological and phenological observations in the GB area. It must be taken into account, however, that the genotypic diversity (measured as percentage of distinguishable genotypes) of clonal species may vary within wide limits (0.02–1.0, Reisch & Kellermeier 2007), depending on both theoretical and experimental factors.

The "Genotypic Identity", for instance, is one of the mechanisms proposed to explain the genotypic variation of clonal species; it states that "a population which was started by a number of sexually derived propagules may thus retain its initial genotypic variation for a very long period of time, even if it later reproduces almost exclusively asexually" (Bengtsson 2003). Another explanation is related to the analysed spatial level: clonal propagation could be detected only at smaller distances than those adopted in the sampling procedure.

CONCLUSIONS

At the periphery of the distribution range and in the extreme environmental conditions of the Gypsum Vein, *Sedum hispanicum* shows high flexibility to adjust the timing of phenological events in response to variations of environmental conditions: full anthesis and senescence in particular appear related to local year-to-year changes in temperature, whereas the possible effect of the precipitations on these phenophases could be assessed only with further years of study.

The observed intra-annual flowering phenology and synchrony among individuals produces a large floral display, essential in promoting sexual reproduction. *Sedum hispanicum* appears to have a mixed-mating system in which selfing contributes to loss of genetic variation, and outcrossing, implemented by regular gene flow, weakens the spatial genetic structuring. The capacity of clonal growth stands for a subsidiary role of vegetative propagation coexisting with sexual reproduction.

Because of the limited study areas and sample sizes, we are far from determining the general properties of the species; nonetheless, field and experimental data here reported put forward the ability of *S. hispanicum* to act as a pioneer species on the Gypsum Vein through the modulation of its phenological traits and reproductive strategies.

SUPPLEMENTARY DATA

Supplementary data related to this article are available at *Plant Ecology and Evolution*, Supplementary Data site (http://www.ingentaconnect.com/content/botbel/plecevo/supp-data) and consist of the following Excel spreadsheets: (1) binary matrix generated by ISSR markers in sixty individuals of *Sedum hispanicum* from GB plus GMR areas; and (2) binary matrix generated by ISSR markers in forty individuals of *Sedum hispanicum* from GB area.

ACKNOWLEDGMENTS

This research was supported by the Project Life Natura Gypsum (LIFE +08NAT/IT/00039), which promotes suitable conservation measures and management techniques.

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Manuscript received 10 Nov. 2016; accepted in revised version 3 Jul. 2017.

Communicating Editor: Myriam Heuertz.