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BRIEF COMMUNICATION

Genetic variability in the endemic *Leucojum valentinum*

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Abstract

The genetic variability of *Leucojum valentinum* Pau (*Amaryllidaceae*), a vulnerable endemic species restricted to a small area in the region of Valencia (Eastern Spain), has been studied using random amplified polymorphic DNA (RAPD) markers. A total of 197 individuals from eleven populations were studied using 13 RAPD primers. Our results show high variability for the species, low differentiation among populations and uncorrelated levels of genetic variability and population size. Four groups in which three populations (SAG, PUG and COL) are separated from all the others were found, but without connection to geographical location.

Additional key words: conservation, fragmented populations, Mediterranean region, molecular markers, populations' management, RAPD, threatened plants.

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Leucojum valentinum Pau is an endemic species restricted to 11 populations located in an area of 600 km^2 in the region of Valencia (Eastern Spain). Plants grow on limestone fissures and populations size range from nine to almost a thousand individuals. The species is included in the Spanish Red List. The petaloid perianth and the bulbous biotype suggest that the plant shares allogamous sexual reproduction with vegetative multiplication but no data are available neither on the species' reproductive biology nor on other species of the genus. The empirical evaluation of genetic variability is now considered a fundamental step in the conservation programs of endangered species (Fenster and Dudash 1994, Knapp and Rice 1996). Knowledge on the genetic variability may guide seed banks in their sampling strategies, studies on the effect of environmental impact on the fragmentation of habitats and help institutions responsible for making decisions on the populations to be conserved.

RAPD analysis has been widely used in population genetics studies to assess levels and structure of variability (Refoufi and Esnault 2008), mainly in rare or threatened plant species (Xena de Enrech 2000, Wroblewska *et al.* 2003, Zeng *et al.* 2003, Mohapatra and Rout 2006, Narasimhan *et al.* 2006, Padmesh *et al.* 2006, Rout 2006). The present project aims to estimate the levels of genetic diversity of the species, as well as its partition, through the study of the DNA markers generated with 13 RAPD primers in eleven populations of *L. valentinum*. Our data will contribute to the efforts being made to the conservation of this endemic species, giving scientific support to the design of recovery plans and to the selection of the most suitable strategies for maintaining populations and developing reintroduction programs.

Material was sampled in the eleven known populations of the species: PUG - El Puig, MAG -

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Abbreviations: RAPD - random amplified polymorphic DNA, UPGMA - un-weighted pair-group method arithmetic average.
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Magdalena, RCA - Rambla Carmadai, ALM - Almenara, ART - Artana, VUX - Vall d'Uxó, PE1 - Pedriza 1, PE2 - Pedriza 2, TOC - Torre Cordá, SAG - Sagunto and TCO - Torre Colomera. A total of 197 individuals were studied. Total genomic DNA was prepared by a modification of Cenis (1992) protocol from 0.5 g of dried leaves per individual.

PCR reactions were carried out in a final volume of 0.025 cm^3 in a *PTC 100 Peltier Thermal Cycler* (*MJ Research*, Waltham, MA, USA). The PCR program was as follows: an initial denaturalization step of 5 min at 94 ºC, followed by 45 cycles of 1 min at 94 ºC, 1 min at 36 ºC and 2 min at 72 ºC.

A set of 40 random decamer oligonucleotides (series A, B, C and D) purchased from *Operon Technologies* (Alameda, USA) was tested. Amplification products were separated on 1.8 % agarose gels in TAE buffer, and stained with ethidium bromide (0.5 mg cm^{-3}). DNA bands were visualized under UV radiation and compared against 100 bp DNA ladder (*MBI Fermentas*, Amherst, USA*)* to determine fragment size. Only 13 primers yielding reliable and reproducible bands in four independent DNA samples were selected; their sequences are detailed in Table 1. A total of 127 bands ranging between 300 - 2 000 bp were generated. The number of scored bands obtained across the total of individuals ranged from 4 for primer OPA5 to 16 for OPC13 (Table 2). OPB10 (0.515) and OPA19 (0.799) were the primers which generated the set of markers with the lowest and highest average levels of genetic variability, respectively. The percentage of polymorphic bands within populations ranged from 29 % in PUG to 73.2 % in ALM. Bands of identical size, amplified with the same primer, were considered to be homologous. The RAPD bands were scored as 1 (present) or 0 (absent), and a binary matrix was generated with 127 polymorphic RAPD bands and 197 individuals. The pairwise difference distance among populations (Excoffier *et al.* 1992) was computed with *Arlequin vs. 2.00* (Schneider *et al.* 2000) and a cluster analysis was carried out on pairwise distances using the un-weighted pair-group method arithmetic average (UPGMA) using *SPSS 10.0* (Norusis 2002). Bivariate correlation of Pearson between genetic variation and population size was analyzed using *SPSS 10.0* (Norusis 2002). A Mantel test was performed to check for correlation between genetic and geographical distances divided by 100, with *NTSYSpc vs. 2.22* (Rohlf 2004).

Table 1. Characteristics of the polymorphic RAPD markers generated using 13 primers across 197 individuals of *L. valentinum.*

Primer	Sequence of nucleotides 5'- 3'	Number of markers	Size range [bp]
OPA-5	AGGGGTCTTG	4	600-1000
OPA-7	GAAACGGGTG	11	450-2000
OPA-10	GTGATCGCAG	9	350- 590
OPA-11	CAATCGCCGT	12	490-1500
OPA-16	AGCCAGCGAA	5	450-1250
OPA-17	GACCGCTTGT	10	350-1700
OPA-19	CAAACGTCGG	9	410-1400
OPB-7	GGTGACGCAG	10	400-1500
OPB-10	CTGCTGGGAC	10	300-1300
OPB-13	TTCCCCCGCT	7	500-1500
OPC-5	GATGACCGCC	12	460-2000
OPC-11	AAAGCTGCGG	12	390-1900
OPC-13	AAGCCTCGTC	16	300-1700
Total		127	300-2000

Genetic diversity was estimated using Nei's algorithm (Nei 1978, Nybom and Bartish 2000). The observed diversity was calculated for two levels: the average diversity within populations (H_{pop}) and the diversity within species (H_{sp}). The proportion of diversity within populations was then estimated as H_{pop}/H_{sp}, and the proportion of diversity among populations as $(H_{sp}-H_{pop})/H_{sp}$. Partitioning of molecular variance (*AMOVA*; Excoffier *et al.* 1992) was performed at three levels, among groups, among populations within groups, and within populations, assigning populations to groups according to UPGMA cluster analysis. *AMOVA* analysis was performed using *Arlequin version 2.0* (Schneider *et al.* 2000).

Two population-specific bands were present in all the studied individuals of SAG and another two bands were specific to some individuals of COL. Individuals of SAG shared one band with one individual of ALM. VUX shared one band with SAG and PE2, another band with RCA and ALM and a third with MAG and ART. MAG, RCA and ART shared one band. The genetic variability of *L. valentinum* was high in all the studied populations (Table 2); ART (0.756) and SAG (0.490) were the populations with the highest and lowest levels of genetic variability across loci. A low and non-significant correlation (r = 0.157, S = 0.644) existed between population size and within population level of variation.

The UPGMA cluster of the populations (Fig. 1) showed that SAG is separated from the other populations, as are PUG and COL. Two subgroups can be observed in the main group of populations, with MAG, RCA, ALM and ART included in one of them, and VUX, PE1, PE2 and COR in the other. The Mantel test showed low and non-significant correlation among genetic and geographic distances (r = 0.41, P = 0.99). *AMOVA* analysis resulted in a 38.32 % of variability among groups being of 21.34 % the proportion of variability among populations within groups.

Genetic variability was closely related to both population size and the reproductive system. Allogamous species are expected to show higher variability, as levels of heterozygosity are maintained through out-breeding, whereas selfing leads to homozygosity thus reducing levels of variation (Ellstrand and Elam 1993). Small population size increased the level of inbreeding and genetic drift, thus reducing genetic variability; therefore, in species with a small range and a reduced number of individuals, low levels of variability are expected (Barrett and Kohn 1991). Despite the theoretical predictions, high levels of diversity, assessed through RAPDs, have been frequently reported in narrow range threatened species

Table 2. Estimation of genetic diversity (Ho) in the 11 studied populations of *L. valentinum*.

Primer	PUG	MAG	RCA	ALM	ART	VUX	PE1	PE2	COR	SAG	COL	Average
OPA5	0.150	0.667	0.667	0.465	0.794	0.666	0.503	0.503	0.667	0	0.803	0.535
OPA7	0.872	0.852	0.716	0.779	0.730	0.425	0.350	0.194	0.586	0.448	0.767	0.611
OPA10	0.556	0.637	0.599	0.699	0.775	0.642	0.704	0.763	0.682	0.761	0.780	0.691
OPA11	0.805	0.637	0.649	0.816	0.853	0.751	0.682	0.687	0.720	0.728	0.571	0.718
OPA16	0.800	0.750	0.792	0.642	0.679	0.550	0.582	0.550	0.753	0.114	0.400	0.601
OPA17	0.445	0.683	0.661	0.906	0.839	0.656	0.667	0.657	0.517	0.375	0.530	0.630
OPA19	0.885	0.772	0.752	0.839	0.865	0.907	0.886	0.883	0.830	0.464	0.710	0.799
OPB7	0.802	0.570	0.673	0.725	0.839	0.789	0.776	0.649	0.781	0.792	0.593	0.726
OPB10	0.515	0.631	0.261	0.570	0.648	0.556	0.334	0.275	0.442	0.804	0.627	0.515
OPB13	0.580	0.643	0.666	0.552	0.611	0.625	0.416	0.444	0.515	0.157	0.466	0.516
OPC5	0.491	0.772	0.851	0.752	0.844	0.924	0.797	0.821	0.756	0.530	0.568	0.757
OPC11	0.497	0.426	0.494	0.650	0.519	0.678	0.602	0.635	0.494	0.471	0.437	0.537
OPC13	0.858	0.805	0.610	0.659	0.837	0.787	0.852	0.804	0.769	0.772	0.687	0.767
Average	0.635	0.680	0.645	0.696	0.756	0.689	0.627	0.605	0.655	0.490	0.611	

(Cotrim *et al.* 2003, Segarra-Moragues and Catalán 2003, Wroblewska *et al.* 2003). Accordingly, analysis of RAPD markers revealed high levels of genetic diversity in all the studied populations of *L. valentinum* but there was no correlation between population size and genetic variability.

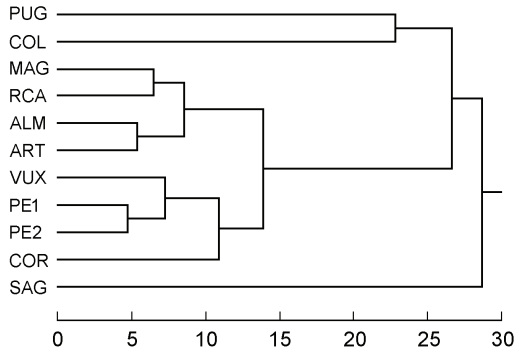

Fig. 1. UPGMA cluster of 11 populations of *L. valentinum*. using 127 RAPD markers based on pairwise distance. Cophenetic correlation is 0.95.

As reported by Hamrick *et al.* (1979), out-crossing and perennial characters reduce the effect of genetic drift, increase the migration rate, and thus increase the specific levels of variation with low differentiation among populations. On the other hand, selfing and annual characters lead to lower levels of variation and higher differentiation among populations. Habitat fragmentation has several genetic consequences, such as erosion of genetic variability and increased inter-population divergence (Young *et al.* 1996), depending on several factors: such as size of the remnant subpopulations, distance and connectivity (Saunders *et al.* 1991).

The lack of correspondence between genetic and geographic distance suggests low levels of gene flow among populations and the existence of a recent fragmentation process affecting their recent fragmentation, so that high levels of variability are remnants of a more continuous and larger population. Actually, most of the populations are close to villages, castles, churches and the seashore, all affected by human activity, which is one of the most important threats to Spanish plant species (Domínguez *et al.* 1996). Following Allphin *et al.* (1998), the absence of correlation between geographic and genetic distances may be influenced by habitat fragmentation due to human activity, which is the case for *L. valentinum*.

Our results have practical consequences for recovery plans, management of populations, and population reinforcement or reintroduction programs for this threatened species. The high level of genetic variability should be preserved through *ex situ* conservation of bulbs and seeds. These materials should be suitably labelled to preserve original population data, especially those originating from SAG, PUG and COL. As the small size of populations can produce a rapid reduction of genetic variability through inbreeding and genetic drift, the development of reinforcement programs in the smaller populations would be convenient. Reinforcements should be addressed to keep population sizes able to maintain the present levels of genetic variability, and should be performed by using material (bulbs, seeds or plants) from the same population.

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