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SHORT COMMUNICATION

Lack of genetic variability in the rare and endangered *Limonium cavanillesii* (Plumbaginaceae) using RAPD markers

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Abstract

***Limonium cavanillesii* is an extremely endangered plant species endemic to the east Mediterranean region of Spain. Regarded as extinct for several years, the recent discovery of a small population (only 29 individuals) has prompted the adoption of measures for its conservation by official agencies. As part of this effort, we have analysed genetic variation in this population by means of random amplified polymorphic DNA (RAPDs). The analysis of 29 individuals with 11 different primers produced 131 monomorphic bands. To our knowledge, this is the lowest level of genetic variation detected in plants using RAPD markers. This result could be explained both by the apomictic reproductive system of this species and by the passage through a severe bottleneck in recent times, after which there has been no chance for mutation to restore detectable genetic variation.**

Keywords: Limonium, RAPDs, genetic variation, endangered species, apomixis

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Introduction

The Iberian peninsula is very rich in plant biodiversity, mainly due to its geographical, orographical and geological status. There are many endemic taxa, or taxa with a restricted range of distribution, and some of them are under conservation measures of protection.

The genus Limonium (*nomen ambiguum Statice)* of the family Plumbaginaceae is one of the plant genera with the most endemic, rare or endangered species in Spain. The genus comprises ≈ 400 species, distributed world-wide, with the highest diversity in the Old World. The largest number of species is found in the western Mediterranean area (Erben 1979). In Spain there are 120 recognized species, ≈ 75% of which are endemic to that area.

Extensive human impact on the Mediterranean coast of the Iberian peninsula has resulted in a high incidence of rarity throughout the highly endemic flora of the region, and extinction events are frequent (Gómez-Campo 1987; Laguna *et al.* 1994). Conservation policies must be based on the understanding of the biology of the particular species and of the factors acting against its survival in the wild (Rossetto *et al.* 1995). The preservation of biodiversity and genetic diversity is a fundamental aim of conservation, as maintenance of genetic variability is essential for populations to respond to present and future environmental changes (Frankel & Soulé 1981). Knowledge of the amount and distribution of genetic variability can help in the design of conservation strategies and in the control of their effectiveness. Consequently, the study of population genetics has been identified as one of the main priorities for conservation (Holsinger & Gottlieb 1991).

In recent years, detection of genetic variability has been improved with the use of new molecular techniques. Random amplified polymorphic DNA (RAPD) analysis, despite its drawbacks, has provided a powerful tool for investigation of genetic variation within species (Hadrys *et al.* 1992; Huff *et al.* 1993; Williams *et al.* 1993; Yu & Pauls 1993; Gibbs *et al.* 1994), especially in a conservation context (Rossetto *et al.* 1995). Although Williams *et al.* advise the use of RFLP probes for studies of genetically diverse outbreeding populations, they affirm that RAPD markers would be suitable for analyses of more uniform inbred species, such as *L. cavanillesii.*

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Limonium cavanillesii

Limonium cavanillesii Erben is a perennial species with leaves arranged in a basal rosette and racemose inflorescences, some of them sterile and others with pink flowers. The triploid chromosome number (2n = 27) in all the individuals, the very high presence (> 95%) of sterile and malformed pollen grains, and the presence in all individuals of a self-incompatible pollen-stigmate combination (Erben 1979), have led to the classification of *L. cavanillesii* as an apomictic species (Rosselló *et al.* unpubl. data). This reproductive system is very common in other species of the genus which share with *L. cavanillesii* the features mentioned above (Baker 1966).

The species was first described by M. Erben through herbarium material of Sennen (1913). It is endemic to a restricted part of the Iberian Mediterranean coast, where it occurs on salty soils. It was declared under protection by the Valencia regional government in 1986 (DOGV 336, Generalitat Valenciana).

L. cavanillesii was thought to be extinct in the natural environment because it had not been detected during two decades and the old populations, situated between Peñíscola and Benicarló (Castellón), were destroyed by human activities or sea erosion (Aguilella 1994). In 1994 the species was rediscovered in Serra d'Irta, near Peñíscola (200 km north of Valencia, on the Spanish Mediterranean coast) as a population of 29 individuals distributed in a small area around Torreón de Badún.

Because of the limited number of individuals and continuous reduction of its habitat, *L. cavanillesii* is one of the most endangered species of this genus (Laguna *et al.* 1994). Long-term *in situ* survival of the species depends upon the development of a recovery plan, which should include the quantification of its genetic variability.

The wide use of RAPD markers for population analyses of plant species (Russell *et al.* 1993) and the biological traits and the small quantity of tissue available for analysis of *L. cavanillesii* (see below), led us to apply this technique for a preliminary study of the level of genetic variation in this species as a part of its conservation programme.

Materials and methods

Plant sampling and template DNA isolation

A total of 29 wild plants were used in this study. These comprise all the known individuals of *L. cavanillesii* and thus represent the species as a whole.

DNA was extracted from leaves (from 26 to 594 mg of tissue, depending on the availability of material) using a CTAB protocol (Doyle & Doyle 1991), with the only modification being that one more chloroform–isoamylalcohol (24 : 1) extraction step was carried out when samples were still turbid after the first extraction step.

The DNA content of each sample was assessed by direct comparison with known standards of three different quantities of another species of *Limonium*, run in 0.8% agarose gels stained with ethidium bromide (final concentration 0.5 μg/mL). After this quantification, DNA was diluted in accordance with the dilution of the standard DNA samples, which had been previously tested in another RAPD study of *Limonium*. The final approximate DNA concentration in the dilution was 80 pg/μl.

DNA amplification and fragment visualization

To generate RAPD profiles we used 20 10-bp primers (OPA-1–OPA-20) from the Operon Technologies Primer Kit A in PCR amplifications. Amplification reactions were carried out in 20 μL total volume containing 1X *Taq* buffer (Pharmacia), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 15 ng of primer (Operon), 1.0 U of *Taq* DNA polymerase (Pharmacia), 5 μL of DNA previously diluted, and deionized and distilled water. Each reaction mix was overlaid with mineral oil (Sigma) when using Robocycler (Stratagene) (see below). Negative controls in which water was added instead of DNA were included in each run in order to verify the absence of contamination.

DNA amplification was initially performed for each primer in a Stratagene Robocycler Gradient 96. When the pattern obtained with this thermocycler was not reproducible, a PE-2400 thermocycler was used instead, and only if the results were reproducible in this machine the RAPD markers obtained with this particular primer were considered. The programme used was the same for both thermocyclers; an initial melting step at 94 °C (5 min), followed by 45 cycles each at 94 °C (1 min), 39 °C (2 min) and 72 °C (2 min). A final extension step at 72 °C (7 min) was performed after the 45 cycles.

Amplification products were separated on 1.4% agarose gels stained with ethidium bromide to a final concentration of 0.5 μg/mL. Gels were run in 0.5 × TBE buffer for approximately 4 h at 7.5 V/cm and then visualized under ultraviolet light. Monochrome photographic negatives (Agfapan, APX100) were taken of the gels using a Polaroid camera.

Initially we surveyed all primers for a sample of three randomly chosen individuals, in order to evaluate their suitability with the Stratagene Robocycler. Thirteen primers gave a clear profile in this pilot study, nine of which gave reproducible marker patterns (Table 1). The other four were tested for reproducibility in the PE 2400 and only two of them gave reproducible banding patterns (Table 1). Hence, a total of 11 primers were used in the final study.

Fragments included in the final analysis were tested for reproducibility. Whenever a new RAPD band appeared in the final study and it was not present in the pilot study,

Table 1 Summary of data obtained in RAPD analysis with 11 primers for *Limonium cavanillesii*. The number of fragments for each primer represents the number of reproducible, scored bands in at least two independent assays. All analyses were made using Robocyler as the thermocycler, except for primers OPA-08 and OPA-11 for which PE-2400 was used (see text for more details).

Primer	Nucleotide sequence	Thermocycler used	No. of fragments	Band size range (bp)
OPA-04	AAT CGG GCT G	Robocycler	21	550–1800
OPA-07	GAA ACG GGT G	Robocycler	18	300–2500
OPA-09	GGG TAA CGC C	Robocycler	15	700–3500
OPA-10	GTG ATC GCAG	Robocycler	13	550–2400
OPA-15	TTC CGA ACC C	Robocycler	14	600–2400
OPA-16	AGC CAG CGA A	Robocycler	7	900–2900
OPA-18	AGG TGA CCG T	Robocycler	13	300–2500
OPA-19	CAA ACG TCG G	Robocycler	10	700–2700
OPA-20	GTT GCT ATC C	Robocycler	5	1700–1300
OPA-08	GTG ACG TAG G	PE 2400	9	600–1700
OPA-11	CAA TCG CCG T	PE 2400	6	800–1300
Total			131	300–3500

two replicate PCR reactions, one with the same DNA dilution and one with a new DNA dilution, were performed.

Results and Discussion

Figure 1 shows examples of RAPD profiles obtained with some primers. The negative controls, in which DNA was omitted, were always free of DNA fragments. Table 1 summarizes the data on the number of fragments detected per primer. The total number of fragments scored was 131, ranging in size from 300 bp (OPA-07 and OPA-18) to 3500 bp (OPA-09), with an average of 11.9 fragments per primer.

Primers OPA-04, OPA-08, OPA-09, OPA-10, OPA-15 and OPA-19 showed the same banding pattern for all the individuals of *L. cavanillesii* (Fig. 1). However, primers OPA-07, OPA-11, OPA-16, OPA-18 and OPA-20 gave some bands that were different between individuals but that were not reproducible in a second test. Problems with band reproducibility, even using standardized conditions and reagents, seem to be the norm rather than the exception in RAPDs studies. They may be due merely to a random difference among PCR reactions or to the sensitivity of the technique to small differences in template DNA concentration between samples, which is a quite common drawback in DNA fingerprinting methods (Vos *et al.* 1995). Once all the possible differences were tested and nonreproducible bands were discarded from the results, we concluded that no RAPD variation was found in *L. cavanillesii*. To our knowledge, this is the most extreme case of low genetic variability detected in a natural plant population by means of the RAPD technique.

The ability of RAPD technique to identify variation within populations when it exists has been well demonstrated (Huff *et al.* 1993; Gibbs *et al.* 1994; M'Ribu & Hilu 1994; Rossetto *et al.* 1995). We also have evidence for the ability of RAPD analysis to detect within species variability in the genus *Limonium*. A preliminary RAPD survey on three populations of the apomictic species *L. dufourii* showed an average band diversity of 0.444 and a highly structured polymorphism, where 83% of the variation was due to among populations variation, and 17% to within populations variation (C. Palacios *et al.* unpubl. data).

Therefore, the absence of RAPD variation in *L. cavanillesii* is a reflection of its isogenic state and it is in agreement with the reproductive mechanism of this species, apomictic and without possibility of sexual reproduction due to its uneven number of chromosomes. In this situation, mutation is the only force that can cause genetic differentiation between parents and offspring. This indicates that the recently rediscovered, unique population of the species must have gone through a recent bottleneck, and there has been no time for mutation to restore detectable genetic variation.

Prospects for the future

Now that the biology of *Limonium cavanillesii*, and the causes leading to its decline are better understood, and the genetic variability of the only wild population of the species has been studied, decisions for future *in-situ* and *ex-situ* conservation of the species can be made.

The adoption of conservation measures for apomictic species can be controversial. Questions about their status as proper species arise very often, especially when they have a hybrid origin as it is the case in the genus *Limonium*. Besides, the taxonomy of endemic apomictics can be quite confounded, as clearly distinct ecotypes may have been classified as different species on the basis of morphological characters, but the use of different markers, such as DNA fingerprinting, can lead to very different conclusions (van Heusden *et al.* 1991; Kraft & Nybom 1995; Kraft *et al.* 1996). This situation is frequent in the genus *Limonium*, for which a revised systematics using molecular markers is under way. Until this revision is completed, the

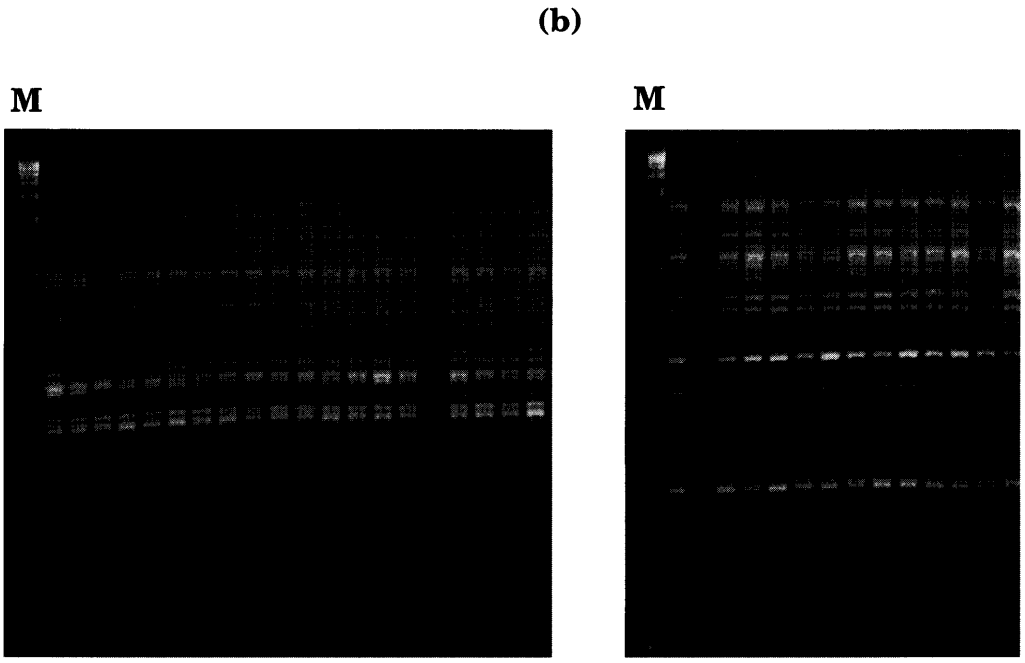

Fig. 1 Representative RAPD results with primers (a) OPA-04 and (b) OPA-09 showing lack of genetic variation in *Limonium cavanillesii* individuals. Lanes labelled M represent different size markers.

conservative approach for conservation would be to preserve as much as it is possible, because this policy can always be revised, whereas the opposite one cannot.

A separate issue for obligate apomictic species is how to decide what to preserve: populations, clones, or individuals. Given the genetic homogeneity of the only extant population of *L. cavanillesii*, it seems obvious that the whole population should be preserved. However, the problem is likely to arise in cruder terms in other species of this genus (e.g. *L. dufourii*). Is every single genotype worth of preservation under economic shortage? Probably not, but it is not clear how the evolutionary potential of these species can be preserved, as each genotype represents a separate alternative. In these cases, the identification of those populations that hold the most genetic variability and their subsequent preservation becomes a priority.

Due to its isogenic state, *L. cavanillesii* is likely to be very sensitive to environmental changes. Conservation of the unique wild population of the species must be an urgent measure, via preservation and protection of the area where it is currently established. Some authors have stressed the potential value of small reserves to provide a wider choice of sites for protection and emphasize that they can play an important role in plant conservation (Lesica & Allendorf 1991; Reznicek 1987).

Re-establishment of the species in suitable and ecologically secure areas, creating new self-sustaining populations, would also be desirable. It could be carried out through random selection of seeds from wild plants, as the current homogeneity of the species makes a priori selection of individuals unnecessary.

Ex-situ conservation is another measure for preservation of the species in the future that should be considered. Conservation of seeds in germplasm banks has not been possible in some species of the genus (M. D. Lledó, personal communication), but good seed germination results have been obtained with material from *L. dufourii*, after one year of being dormant (J. A. Rosselló, personal communication). Although costly and time consuming, conservation by micropropagation of some individuals might be desirable. At the same time, a study of the germination capacity of the seeds, after being maintained in germplasm banks for 1 or more years, may be of importance when considering the possibility of replacing micropropagation by this cheaper technique in the future.

We believe that the critical situation of *L. cavanillesii* deserves attention and that it should be included in the IUCN Red book, where it is not currently present, perhaps because it was considered to be extinct.

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