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Evaluation of biodiversity and conservation strategies in *Pancratium maritimum* L. for the Northern Tyrrhenian Sea

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Abstract. *Pancratium maritimum* L. is an Amaryllidaceous species whose presence is severely endangered in its original range, the sandy coasts of the Mediterranean sea. A molecular analysis has been performed to evaluate the genetic distance among populations coming from different locations, in order to define the best repopulating strategy. The plant genome, analysed by AFLP markers, was found to be extremely homogeneous and conserved, evoking vegetative or autogamous reproductive habits. Seeds from two different locations showed a good germination capability in greenhouse tests, indicating the potential presence of an efficient sexual reproduction. The combination of molecular data and germination tests would support the hypothesis of an autogamous reproduction for this species.

Abbreviations: AFLP – Amplified Fragment Length Polymorphism

Introduction

Pancratium maritimum L. is an Amaryllidaceous typical species of the sandy coasts of the Mediterranean Sea. Its range extends out of the Strait of Gibraltar along the Portuguese, Spanish and French coasts up to 46°56′ latitude North.

The adult plant is 30–60 cm high on sand surface, and produces in Summer white scented flowers; the stem forms big bulbs that sink down to 80 cm in the sand. The mean number of ovules per flower is 54, but generally the seeds (10×5 mm) are produced in number of 15–20 by each flower. They are black and extremely light ($\sim$50 mg each) because of a large presence of aeriferous parenchyma; dissemination is thus favoured by bird nutrition, wind and sea streams as the seeds can easily float over the water (Arcangeli 1896).

Pollination is reported to be due to different organisms depending on the region, i.e. the Lepidopteron *Sphinx convolvuli* L., in Southern France (Thompson 1914; Leraut 1997), sphingid moths in Israel, or the lizard *Podarcis lilfordi*, on Balearic Islands (Perez-Mellado et al. 2000). The pollination spectrum for the species is variable: it is relatively broad in Eastern Mediterranean while, in geographically peripheral populations, pollinators very rarely are reported to visit flowers (Eisikowitch and Galil 1971; Medrano et al. 1999).

In natural conditions, seeds and fruits are reported to be the principal nourishment for the larvae of another Lepidopteron, the sphinx *Britys crini* subsp. *pancratii*. Natural populations from marginal areas can have a long reproductive period indicating a 'satiation strategy' for protection against insect attack (Medrano et al. 1999).

From a vegetational point of view, *P. maritimum* is very common in the perennial vegetation of coastal dunes and back-dunes but sometimes can reach the seashore, in embryonic dunes or among the shrubby vegetation of consolidated dunes. In all cases, young plantlets surround old established ones.

The diffusion of this species, once conspicuous, is now sensibly reduced in environments subjected to high anthropic pressure. Its disappearance from extended regions is a conceivable risk.

In some cases, the plant presents phenotypical differentiation, expressing differences in the wideness and length of the leaf blade, in the dimension of bulbs or in the extension and diameter of roots. Such differences seem to be stable, as they are expressed also when seeds are sown in greenhouse and plants are grown under identical conditions (Minuto et al. unpublished data). However, it is known that epigenetic variations can occur, due to environmental factors, and influence only phenotypical expression, such as via methilation of DNA sequences. It is debated if the progeny inherits such variations.

Genetic data may play a significant role in the formulation of appropriate management strategies for the conservation of the species (Cardoso et al. 1998). Anyway only a molecular analysis of individuals collected from different populations can definitely highlight polymorphisms at the genome level.

Molecular analysis are based on the use of molecular markers. Among these, AFLP-derived markers are recognised as the most efficient in revealing genetic diversity within a single species (Karp et al. 1997). In fact, AFLP analysis has the potential to screen a large number of genetic loci per experiment and does not require particular knowledge about the genome under investigation. Thus, it is a very useful technique for conservation studies concerning endangered species. Molecular marker analysis is also an important tool to identify particular populations that merit separate management and high priority for conservation.

In this paper AFLP analysis has been applied to investigate gene flow, genetic structure and genetic diversity of 10 populations of *P. maritimum*, grown in different locations of the species range, in order to define conservation strategies for the species. The same technique has also been employed to

examine two groups of seedlings, grown in greenhouse, originated from two Northern Italian populations.

Materials and methods

Plant material

Leaves were collected from separate individuals in 10 different locations: Ile Hoëdic (France, Atlantic coast, 47°19′N, 02°52′W – FA), La Coruña (Spain, Atlantic coast, 43°22′N, 8°23′W – SP), Hyères (France, Tyrrhenian coast, 43°06′N, 6°10′E – FR), Varigotti (Italy, Northern Tyrrhenian coast, 44°11′N, 8°24′E – VA), Finale Ligure (Italy, Northern Tyrrhenian coast, 44°09′N, 8°20′E – FL), Punta Ala (Italy, central Tyrrhenian coast, 42°48′N, 10°43′E – TO), Marina di Sorso (Italy, Northern Sardinian coast, 40°48′N, 8°34′E – SS), Siniscola (Italy, Eastern Sardinian coast, 40°34′N, 9°41′E – NU), Cefalù (Italy, Northern Sicilian coast, 38°03′E, 14°03′ – PA) and Corfu (Greece, Western Cyclades islands, 39°36′N, 19°36′E – GR). In all localities the presence of plantlets was recorded. An exception was Varigotti with few old and large samples living in a stony soil.

Two adjunctive groups of samples were composed of plants derived from seeds, collected in Varigotti (S-V) and in Albenga (Italy, Northern Tyrrhenian coast, 44°02′N, 8°13′E – S-A). DNA was extracted from ten samples per group and analysed by the AFLP method. Seeds from Varigotti and Albenga were also tested for germination and development capability, in greenhouse, simulating natural conditions.

AFLP analysis

DNA was extracted according to Doyle and Doyle (1990). The AFLP analysis was performed as described in the European Patent 0534858 (Keygene). Genomic DNA was digested (3 h) with *Eco*RI (0.5 U) and *Mse*I (0.5 U) and ligated with *Eco*RI (5 pmol) and *Mse*I adapters (50 pmol). Primer pairs used in the preamplification reaction were M01 and E01. Amplification was carried out using six pairs of selective primers (Table 1). The *Eco*RI primers were labelled with [γ-^{33}P]-ATP (Amersham, Italy).

Genetic data analysis

AFLP markers have been considered as a unit character and scored as binary code (1/0). The binary matrixes obtained have been scored by the software Popgene version 1.31 (Yeh et al. 1997) for statistical analysis. The programme allowed to calculate a genetic distance matrix among the various populations

Table 1. Polymorphic variation among and within populations.

Primer code	Selective sequence	No. of Polymorphic loci	$H_T \pm SD$	$H_S \pm SD$	G_{ST}
E32-M33	AAC-AAG	11	0.0037 ± 0.0005	0.0007 ± 0.0000	0.8108
E35-M36	ACA-ACC	8	0.0058 ± 0.0019	0.0013 ± 0.0000	0.7758
E38-M01	ACT- A	2	0.0003 ± 0.0000	0.0002 ± 0.0000	0.3334
E41-M37	AGG-ACG	8	0.0079 ± 0.0015	0.0044 ± 0.0004	0.4430
E44-M38	ATC-ACT	2	0.0026 ± 0.0007	0.0012 ± 0.0001	0.5384
E34-M32	AAT-AAC	3	0.0005 ± 0.0000	0.0004 ± 0.0000	0.2000
G_{ST} mean value 0.5169					

H_T, total variation of polymorphic loci; H_S, within populations variation at polymorphic loci; $G_{ST} = 1 - (H_S/H_T)$.

(Nei 1978), to estimate the gene flow level and calculate the Shannon index (SH) (Shannon and Weaver 1949) and the gene diversity (GD) within the single populations and the groups of seedlings.

The genetical distance matrix has been used to design an UPGMA dendrogram showing genetic relationships among populations.

Gene diversity ($GD = (n/(n-1))(1 - \sum P_i^2)$ where P_i is the frequency of the ith allele at each AFLP locus in each population and n is the number of individuals for populations) was determined using the method of Nei (1987). Gene diversity within each population was calculated as the mean genetic diversity over all loci from all populations.

Gene flow analysis

Gene flow is a term that includes all mechanisms resulting in the movement of genes from one population to another. There are various ways to estimate the level of gene flow among populations. Slatkin (1985) called such methods 'indirect methods' and contrasted them with 'direct methods' that depend on observations of dispersed individuals or gametes. The starting point for these indirect methods is a list of gene frequencies of different loci, determined by electrophoretic surveys. In this study genetic frequencies have been calculated on the basis of the data obtained from the dominant AFLP markers, considering each band as a gene. The gene flow among the analysed populations have been approximated as Nm, where N indicates the actual population dimension, and m, calculated with the expression $G_{ST} = 1/(1 + 2Nm)$, represents the migration rate (Slatkin 1987). The degree of AFLP variation among populations (G_{ST}) was calculated using Wright's F-statistics (Wright 1978) where H_t and H_S represent the total and partial heterozygosity.

Values of $Nm < 1$ have been indicated as a 'reduced gene flow', denoting a gene isolation among the considered populations. Our gene flow estimation thus represents an average level of isolation among different populations.

Germination tests and ex situ cultivation

Seeds were sown in the Botanical Garden (Genova University) in large pots with sandy soil. The soil was smoothed and irrigated (only at dry conditions) under greenhouse conditions (15–22° C temperature, exposed to sunlight). The seeds were equally spaced in each pot and the emergence number was measured periodically.

Three months after first germination, all seedlings were transplanted in pots, each consisted of 4 bottom perforated plastic (15 cm height × 10 cm diameter), filled with the same sandy soil used before. Seedlings were irrigated only on rainy days. Seven months later young plants were transplanted in a sandy flowerbed (40 cm depth) in the open air. Plant dimension was measured 3, 9 and 15 months after transplantation in flowerbed.

Results

Genetic analysis

AFLP analysis of the 10 *P. maritimum* populations generated 956 bands on polyacrilamide gels. Of these, 34 (3.56%) turned out to be polymorfic. Table 1 reports the number of polymorphic loci and the variability of populations related to the single couples of primers. The size of AFLP fragments was determined by comparing a standard marker to AFLP patterns.

The values of Shannon index (SH) and the gene diversity (GD) for the 10 populations and for the groups of seedlings are reported in Table 2. Seedlings from Varigotti (S-V) show both of the values, SH (0.029) and GD (0.020) relatively high with respect to all the natural populations and to seedlings from Albenga (S-A). The reduced genetic variability, with loss of allelic diversity in the natural populations, will significantly increase the inbreeding depression and the size of populations in future generations.

The G_{ST} value (Table 1) for the whole population was 0.5169, indicating a substantial variation in the entire population. Such a high value for G_{ST} indicates a reduced gene flow among the different populations. A number of 0.4673 migrants (*Nm*) per generation has been estimated. Therefore, our results suggest that the gene flow among the *P. maritimum* populations considered in this study is low.

The genetic distance matrix (Table 3) has been calculated on the basis of gene frequency values. The UPGMA dendrogram (Figure 1) shows the higher genetic distance for all the populations collected in the central Mediterranean area. On the contrary, westward populations show high genetic affinity. The only exception results to be the population from Varigotti. Moreover, the genetic distance among populations revealed that individuals collected at Siniscola (NU) are more closely related to those collected at Marina di Sorso (SS), due to the geographical position on the Sardinian island.

Table 2. Gene diversity (GD) and Shannon (SH) index for populations.

	E32-M33		E35-M36		E38-M01		E41-M37		E44-M38		E34-M32		Mean	
	GD	SH	GD	SH	GD	SH	GD	SH	GD	SH	GD	SH	GD	SH
NU	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SS	0.002	0.004	0.004	0.007	–	–	0.007	0.011	0.003	0.004	0.001	0.001	0.003	0.005
GR	0.005	0.010	–	–	–	–	0.007	0.011	0.002	0.003	–	–	0.005	0.005
VA	–	–	–	–	0.001	0.001	–	–	–	–	0.001	0.002	0.001	0.002
PA	–	–	0.009	0.013	0.001	0.002	0.019	0.027	0.001	0.002	–	–	0.008	0.011
FR	–	–	–	–	0.001	0.001	0.009	0.013	–	–	0.003	0.004	0.004	0.006
FL	–	–	–	–	–	–	0.003	0.005	0.001	0.002	–	–	0.002	0.004
TO	–	–	–	–	–	–	–	–	0.002	0.004	–	–	0.002	0.004
FA	–	–	–	–	–	–	–	–	0.004	0.005	–	–	0.004	0.005
SP	–	–	–	–	–	–	–	–	–	–	–	–	–	–
S-A	0.002	0.005	0.006	0.010	–	–	–	–	0.002	0.004	–	–	0.003	0.005
S-V	0.007	0.010	0.036	0.053	0.013	0.010	0.021	0.033	0.024	0.039	–	–	0.020	0.029

FA, Ile Hoëdic (France); FL, Finale Ligure (Liguria, Italy); FR, Hyères (France); GR, Corfu (Greece), NU, Siniscola (Sardinia, Italy); PA, Cefalù (Sicily, Italy); SP, La Coruña (Spain); SS, Marina di Sorso (Sardinia, Italy); TO, Punta Ala (Tuscany, Italy); VA, Varigotti (Liguria, Italy); S-A, seedlings from Albenga; S-V, seedlings from Varigotti.

Table 3. Genetic distance among populations was calculated by Nei (1978) index.

	NU	SS	GR	VA	PA	FR	FL	TO	FA	SP
NU	–									
SS	0.0051	–								
GR	0.0095	0.0065	–							
VA	0.0131	0.0105	0.0134	–						
PA	0.0099	0.0046	0.0108	0.0143	–					
FR	0.0103	0.0073	0.0095	0.0141	0.0045	–				
FL	0.0113	0.0091	0.0121	0.0156	0.0044	0.0015	–			
TO	0.0075	0.0061	0.0092	0.0127	0.0045	0.0015	0.0030	–		
FA	0.0067	0.0062	0.0095	0.0130	0.0047	0.0018	0.0032	0.0001	–	
SP	0.0088	0.0063	0.0091	0.0126	0.0045	0.0014	0.0029	0.0001	0.0004	–

FA, Ile Hoëdic (France); FL, Finale Ligure (Liguria, Italy); FR, Hyères (France); GR, Corfu (Greece); NU, Siniscola (Sardinia, Italy); PA, Cefalù (Sicily, Italy); SP, La Coruña (Spain); SS, Marina di Sorso (Sardinia, Italy); TO, Punta Ala (Tuscany, Italy); VA, Varigotti (Liguria, Italy).

Germination tests

Both *P. maritimum* seed sets revealed first emergencies 20–30 days after sowing. The medium germination value reached 95%. Emergencies show two demographic maxima: the first after 10–15 days, the second after 17–20 days. This phenomenon seems to be a constant of the species. Plants from Varigotti show a different phenotype if compared with plants from Albenga (Table 4) and from other locations (Minuto et al. unpublished data). Their mean dimensions almost double Albenga group dimensions, at any development time considered.

Seedling growth in pots showed 79 and 96% survival in two successive transplantations, repsectively. After 10–12 days from the first transplantation, all seedlings presented at least 4–5 secondary roots, showing a good adaptation to the new pot condition. The overcoming of this critical stage allowed the second transplantation in flowerbed to be successful.

Discussion

The presence of *P. maritimum* on Tyrrhenian Sea coasts has been strongly reduced during the last century, particularly in the Northern regions constituting the margin of the species range in the Mediterranean Sea. The reduction is mainly due to human pressure where tourist resorts have occupied the sea littorals.

Considering the importance of the plant as one of the main component of an endangered habitat, the necessity arises to establish a suitable repopulating strategy, in order to limit the loss of biodiversity. To this purpose, two are the main topics to be considered: (1) a population study, including reproductive and genetic variability level considerations, in order to evaluate the degree of

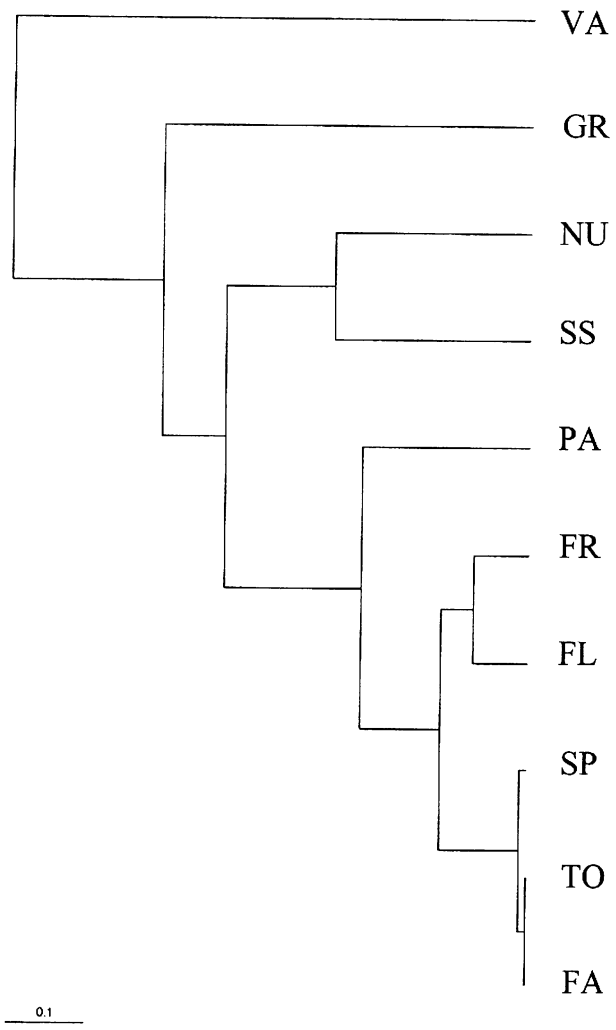

Figure 1. Dendrogram generated by UPGMA analysis based on genetic distance Nei (1978) with the Popgene software. FA, Ile Hoëdic (France); FL, Finale Ligure (Liguria, Italy); FR, Hyères (France); GR, Corfu (Greece); NU, Siniscola (Sardinia, Italy); PA, Cefalù (Sicily, Italy); SP, La Coruña (Spain); SS, Marina di Sorso (Sardinia, Italy); TO, Punta Ala (Tuscany, Italy); VA, Varigotti (Liguria, Italy).

differentiation among populations; (2) the germination capability of seeds and the percentage of seedling survival after transplantation. The results of such analyses will enable the repopulation of degraded areas with the best fitting samples.

Seed germination tests in greenhouse, simulating natural conditions, gave good results, indicating the potential presence of an efficient sexual reproduction.

Table 4. Dimensions (in cm) of seedlings from Varigotti and Albenga in flowerbed cultivation, compared with plantlets from other populations.

Time after transplantation (months)	Varigotti			Albenga			Others		
	Lvs	Rts	Blb	Lvs	Rts	Blb	Lvs	Rts	Blb
3	20	30	1.5	10	15	0.5	15	20	1
9	35	35	2	20	15	1	25	20	1.5
15	40	40	3	20	20	2	30	30	2.5

Lvs, leaves; Rts, roots; Blb, bulbs.

On the other hand, molecular data, obtained from plants and seeds collected in the different considered locations, would indicate a vegetative or authogamous reproduction, confirming data from the literature (Medrano et al. 1999).

The first target of genetic conservation studies is to estimate the level of genetic variability in a considered species. Currently genetic information is considered fundamental for the preparation of conservation and safeguard plans for endangered species.

AFLP analysis was chosen because at the moment is considered as the most efficient technique for DNA screening in the case of species whose genome is still rather unknown. The analysed loci showed a low polymorphism level, both within the single populations and among them. The only noticeable differences are among populations, nevertheless the analysis of data gives low values of gene diversity and of Shannon index for all the populations considered. This is an indication of low genetic variability among the different *P. maritimum* populations. Knowledge of levels of genetic variation within populations is very important for the surviving species. Reduced levels of genetic variation within populations may decrease the potential for the species persistence in front of long-term biotic or abiotic environmental changes (Soule 1980). However, the higher variability found within the groups of seedlings and the low values of *Nm* indicate that the populations are subjected to a heavy environmental pressure selecting specific genotypes. The success of outbreeding in *P. maritimum* can thus be modified and levelled by environmental factors. As cross-pollinating breeding system is the best mechanism for the maintenance of genetic variability, this species risks genetic erosion and extinction.

Currently, another important topic in conservation studies is the definition of the level of isolation of populations (Ellstrand 1992). Gene flow values calculated among the examined *P. maritimum* populations denote a low gene exchange. One migrant every about two generations has been calculated to pass among *P. maritimum* populations; thus this event appears to be extremely rare (Ellstrand and Elam 1993). Inter-population gene flow is considered beneficial in conservation (Huenneke 1991) because it can increase the effective size of populations. We can postulate at least two causes able to explain gene isolation in *P. maritimum*: (a) anthropic pressure, leading to a reduction in the number of individuals and to fragmentation of the natural habitat; (b) predominance of authogamy as the main breeding system; usually in fact, plants

with efficient pollen and seed diffusion systems have gene flow values particularly high (Govindaraju 1988).

The variability values present in *P. maritimum*'s populations geographically located near the centre of the species range (NU, SS, GR, PA), indicate that cross-pollination could be a decisive factor in increasing gene differentiation. Accordingly, populations belonging to peripheral areas (FR, FL, SP, TO, FA) present a very similar genome, suggesting an increasing presence of authogamy because of the long distance occurring among populations. Isolation can certainly be increased by an inefficient gene flow. As an hypothesis, *P. maritimum* populations located in marginal areas of the range present a more frequent authogamous reproduction as already indicated by Medrano et al. (1999).

The Varigotti population represents the only exception and it is to be considered a 'relict population' (Minuto et al., unpublished data). The relative genetic diversification could be explained by two factors: (1) this population can be considered particularly isolated because it has been composed for a long time by a few specimens under physical, geographical and ecological segregation. Plants live in a stony ground where viable seeds cannot germinate and plantlets cannot have any development; (2) the enduring segregation conditions can have caused an absence of outbreeding activity and vegetative reproduction has maintained the same samples without any seed renovation of the population.

The present genetic analysis highlights low genetic variability within the single populations and genetic isolation. Nursery activities show the presence of different phenotypes among the seedlings. Plant dimension can sensibly vary, as shown by the 'giant' specimens from Varigotti's population. Allogamy seems to be in contrast with molecular observations, while authogamy could fit with the analysis of data.

The results of this study could be useful in the set up of appropriate management strategies for *in situ* and *ex situ* conservation. They also could represent the bases for the reconstitution of protected areas, in which human influence could be minimal. Moreover, the *ex situ* propagation would be beneficial for conservation programs of selected individuals. The results also suggest the uselessness of collecting a wide number of seeds in different geographical areas, because of the great genetic homogeneity of the species.

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