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Designing ex-situ conservation strategies through the assessment of neutral genetic markers: Application to the endangered *Androcymbium gramineum*

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Abstract

Recent anthropogenic habitat perturbations related to the unsustainable development of the two areas of distribution of *Androcymbium gramineum* in Almería (South of Spain) and the Atlantic coast of Morocco are outweighing the intrinsic biological survival assets of this narrow Ibero-Moroccan endemic. We used population genetic data on 18 isozyme loci for 13 populations to design a comprehensive sampling strategy for ex-situ conservation that straddles the results of independent theoretical developments and the indications of several genetic polymorphism parameters. Regressions based on the probabilities of loss estimate that sampling one population would be insufficient to represent the variation attributable to the rare alleles found in the populations surveyed from Almería or Morocco and suggest that the relationship between the number of populations sampled in either area should conform to the proportion 0.6:0.4, respectively. The estimate of the number of populations that need be targeted to represent 99% of the genetic variation detected indicates that it would be necessary to sample eight populations intensively. Five populations from Almería and three from Morocco were selected through the ranked values of the average number of alleles per locus and the expected heterozygosity on the grounds that these parameters provide unambiguous indications of polymorphism that, in the case of heterozygosity, are less likely to be influenced by sampling error. Spatial autocorrelation surveys in the largest population known indicate that seeds should be collected at a minimum separation of 15 m to avoid the sampling of close relatives.

Introduction

Androcymbium gramineum (Cav.) McBride (Colchicaceae) is a xerophytic, perennial, hermaphroditic, cormose monocot strictly endemic to Almería (southern Spain) and to the Atlantic coast and the western interior of Morocco (Figure 1). Based on recent phylogeographical and chronological estimates from cpDNA RFLP data, Caujapé-Castells et al. (2001) estimated the probable origin of this species in the Mid-Miocene, some 11.7 ± 2.1 million years ago (mya). Its present distribution range, with two allopatric population sets separated by the Straits of Gibraltar, was construed by Caujapé-Castells and Jansen (2003) as the likely consequence of a dispersal event from Southern Spain to Northern Africa during the Mediterranean dessication period in the Messinian (late Miocene). Although the recurrent glacial cycles that followed upon each other all through the Pliocene and Pleistocene must have affected dramatically the distribution ranges and variation levels of *A. gramineum* and other endemic organisms distributing in the Ibero-Moroccan biodiversity hot spot (Caujapé-Castells and Jansen 2003), the present degree of

habitat degradation in this area seems to be faster and more efficient at extirpating many floristic elements of the area.

It seems unlikely that the spread of agriculture and human population growth are the only factors that account for the extinction processes that are operating in Almería at present. Yet, the substantial impact of these recent perturbations on the landscape has certainly aggravated the situation for many endemics. In particular, the rapid upward surge of intensive crops and the construction of infrastructures associated with tourism are leading swiftly to an impoverishment of the plant biodiversity of the region. Furthermore, lack of planning and of strict legislation has facilitated the recent establishment of economically successful industries, which have made the situation more dramatic by radically transforming the physical landscape and contaminating the aquifers.

Many Almerian populations of *A. gramineum* have already disappeared, and the one at the 'Cortijar de Charco del Lobo', which holds the larger number of individuals and is a crucial reference for understanding the population dynamics of the species (Caujapé-Castells and Pedrola-Monfort 1997), is seriously threatened by the advance of intensive cultivars and soil erosion, that accentuate its fragmentation and demographic impoverishment. Although this species is only considered vulnerable by the UICN directory of Spanish plants (V.V.A.A. 2000), it was decreed to be endangered with extinction by the Junta of Andalucía – the autonomous government of the Spanish region where Almería belongs – (Boletín Oficial de la Junta de Andalucía 1994). Similarly, the Moroccan area where *A. gramineum* occurs is subjected to the major impact of the increase in human population and livestock, which destroys the soils, prevents regeneration, and poses a major threat to its Flora (Médail and Quézel 1997).

Protection and restoration of natural habitats (i.e. in situ conservation) is agreed to be the best method of preserving biological diversity (Lande 1988; Francisco-Ortega et al. 2000). However, this objective is very often not possible to undertake for various reasons, and less ideal management strategies have to be foreseen. In Almería, the research of the scientific staffs at the Parque Natural de Cabo de Gata and at the Estación Experimental de Zonas Áridas is focused on protecting the endangered Flora since the late 1980s. In addition, an integrated conservation plan was launched by the Junta of Andalucía in 1993 to limit human activities in the mountain and coastal areas (Hernández-Bermejo and Clemente-Muñoz 1993). Although these important efforts hint at an optimistic future for the biodiversity of the area, only the population of *A. gramineum* at the Playa de Mónsul (within the Nature Preserve of Cabo de Gata) is receiving habitat protection. Furthermore, even in the case that habitat protection was decreed for the whole range of *A. gramineum*, this would not guarantee the maintenance of all biotic and genetic diversity of this endangered Ibero-Moroccan endemic. The intense and continued habitat degradation of *A. gramineum*'s distribution area poses a serious short-term threat to its survival and is one outstanding reason to implement ex-situ conservation strategies.

Ex-situ conservation in germplasm banks is one viable alternative to prevent the immediate extinction of a species through affording a representation of its genetic makeup. When necessary, seeds conserved in germplasm banks may be instrumental to restore destructed habitats, reinforce declining populations or establish microreserves for adequate monitoring.

The knowledge of the amounts and distribution of genetic variation in natural populations of target species is one important requisite to design a sampling strategy to efficiently carry out ex-situ conservation in seed banks (Hamrick et al. 1991; Frankel et al. 1995; Chamberlain 1998; Francisco-Ortega et al. 2000; Batista et al. 2001). In this paper, we use population genetic data to offer guidelines for the genetic sampling of the endangered genetic variation of the Ibero-Moroccan endemic *A. gramineum*. On the basis of this concrete case, we raise the issue of how to assess a conservation strategy from a survey of genetic diversity for neutral markers. The general methodology underlying this assessment will be based on an approach developed by the senior author through his collaboration with the seed bank of the Jardín Botánico Canario "Viera y Clavijo" (Caujapé-Castells 2002; Vilches et al. 2002) that straddles the results of independent theoretical developments (Bengtsson et al. 1995; Hamrick et al. 1991; Sokal 1979) and the indications of several basic estimates of population genetic polymorphism to answer quantitatively three basic questions. First, how many populations should be sampled intensively to capture a substantial percentage of the genetic variation of a species?. Second, what populations should be targeted to fulfill this objective with a minimum effort?. And third, how should the intra-populational sampling at the target populations be carried out to minimize the probability of sampling related individuals?

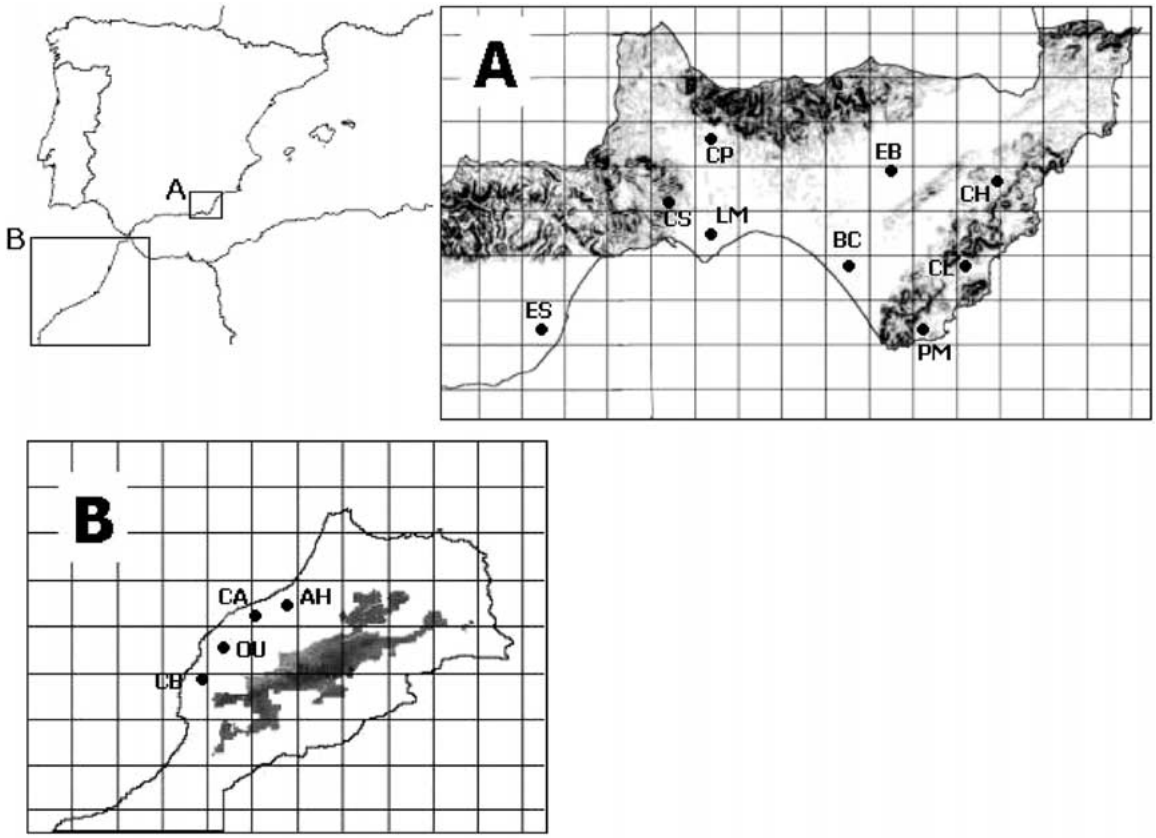

Figure 1. Physical-geographical site map of the populations of *A. gramineum* sampled in its only areas of occurrence in the Spanish province of Almería (A) and along the Atlantic coast of Morocco (B). Population codes correspond to Table 2.

Because of the existence of two allopatric units in the South of the Iberian Peninsula and the Morocan Rif and the fast population impoverishment linked to the increasing habitat degradation and fragmentation in these areas, *A. gramineum* offers a good biological model to represent the situation of many taxa of this region. Therefore, this paper may provide insight for the ex-situ conservation of other endangered floristic elements in the area.

Material and methods

Sampling. Thirteen populations of *A. gramineum* representing its distribution areas (Figure 1) in the southern Spanish province of Almería (9 populations) and in Morocco (4 populations) were collected as explained in Caujapé-Castells and Jansen (2003). Corms were carefully unearthed, put into paper bags and transported to the Estació Internacional de Biología Mediterránia-Jardí Botánic Marimurtra (EIBM), where they were planted in a research greenhouse. Three reasons compelled us to collect live corms instead of leaves or seeds. First, the time elapsed from the collection of samples in Almería to their arrival in Blanes would have been too long for the leaves to keep their enzymatic activity unaltered. Second, more than three years are necessary for seeds to develop seedlings with enough leaf material to carry out electrophoretic assays. And third, the environmental degradation of many of these populations (especially in Almería) made it advisable to collect live individuals lest the populations underwent a sudden irreversible decay. The collected corms are still kept alive in a research greenhouse at the EIBM and could thus serve to undertake eventual population reinforcements or reintroductions. Protein extracts, electrophoretic conditions and enzyme interpretations followed Caujapé-Castells and Pedrola-Monfort (1997) and Caujapé-Castells and Jansen (2003).

Table 1. Allele frequencies at the 15 populations surveyed. Population codes are those of Table 2. Dashes in some cells of locus GOT-2 mean that it was not resolved in the corresponding population. Asterisks label the 38 alleles that fulfilled the criteria for inclusion in the calculations of the probability of loss stated in the methods. Framed frequencies correspond to alleles exclusive to Almería (blank filling) and Morocco (gray filling). Bolded values are single-population exclusive alleles. Numbers in parentheses are sample sizes per locus per population

		Populations												
		Almería									Morocco			
Locus/allele		LM	CH	CL	PM	BC	EB	ES	CP	SC	AH	CA	OU	CB
ACO-2	a*	0.00	**0.01**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	b	0.72	0.06	0.34	0.28	0.00	0.09	0.18	0.02	0.50	0.17	0.42	0.26	0.23
	c	0.28	0.87	0.66	0.72	1.00	0.91	0.82	0.98	0.50	0.83	0.58	0.74	0.73
	d*	0.00	**0.06**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		(18)	(282)	(22)	(29)	(49)	(32)	(13)	(31)	(21)	(41)	(25)	(21)	(28)
ACO-3	a*	0.00	0.01	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	b	0.77	0.94	1.00	0.94	0.83	0.09	0.96	0.30	0.81	0.99	0.98	0.98	0.98
	c*	0.00	0.01	0.00	0.00	0.00	0.87	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	d*	0.23	0.04	0.00	0.06	0.17	0.00	0.04	0.40	0.00	0.00	0.00	0.00	0.00
	e*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.02	0.02	0.02
	f*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.30**	0.00	0.00	0.00	0.00	0.00
	g*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.19**	0.00	0.00	0.00	0.00
		(18)	(297)	(22)	(51)	(49)	(35)	(13)	(20)	(21)	(42)	(26)	(21)	(32)
ADH	a*	0.09	0.00	0.00	0.00	0.00	0.00	0.27	0.00	0.00	0.05	0.10	0.07	0.08
	b	0.91	1.00	1.00	1.00	1.00	1.00	0.73	1.00	1.00	0.95	0.90	0.93	0.92
		(23)	(298)	(25)	(51)	(47)	(36)	(13)	(36)	(23)	(41)	(21)	(36)	(19)
FDH	a	0.95	0.69	1.00	0.97	0.91	1.00	1.00	0.95	1.00	1.00	1.00	1.00	1.00
	b*	0.05	0.31	0.00	0.03	0.09	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00
		(21)	(277)	(16)	(48)	(38)	(15)	(6)	(42)	(12)	(18)	(20)	(17)	(6)
GDH-1	a	1.00	0.92	1.00	1.00	0.95	0.78	1.00	0.88	1.00	1.00	1.00	1.00	1.00
	b*	0.00	0.00	0.00	0.00	0.00	**0.07**	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	c*	0.00	0.08	0.00	0.00	0.05	0.15	0.00	0.12	0.00	0.00	0.00	0.00	0.00
		(21)	(290)	(16)	(55)	(38)	(30)	(6)	(42)	(12)	(18)	(20)	(17)	(6)
GOT-1	a*	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.06	0.04	0.00
	b	0.83	1.00	1.00	0.88	0.99	1.00	0.50	0.45	0.79	0.37	0.56	0.84	0.88
	c	0.17	0.00	0.00	0.12	0.00	0.00	0.50	0.55	0.21	0.52	0.38	0.12	0.12
		(23)	(294)	(25)	(33)	(47)	(36)	(9)	(29)	(10)	(38)	(16)	(25)	(14)
GOT-2	a	0.50	0.95	–	0.99	1.00	1.00	0.67	1.00	–	–	–	–	–
	b	0.50	0.05	–	0.01	0.00	0.00	0.33	0.00	–	–	–	–	–
		(23)	(294)	(25)	(33)	(47)	(36)	(9)	(29)	(–)	(–)	(–)	(-)	(–)
MDH-1	a	0.00	0.46	0.00	0.04	0.57	0.40	0.00	0.27	0.00	0.31	0.11	0.12	0.00
	b	1.00	0.64	1.00	0.96	0.43	0.60	1.00	0.73	1.00	0.69	0.89	0.88	1.00
		(17)	(298)	(19)	(57)	(49)	(35)	(13)	(47)	(23)	(29)	(27)	(33)	(31)
MDH-3	a*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.03**	0.00
	b	0.88	1.00	1.00	0.99	1.00	1.00	0.77	0.94	0.87	1.00	0.98	0.97	1.00
	c*	0.12	0.00	0.00	0.01	0.00	0.00	0.23	0.06	0.13	0.00	0.02	0.00	0.00
		(17)	(298)	(19)	(56)	(49)	(35)	(13)	(47)	(23)	(29)	(27)	(34)	(31)
ME	a*	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.05	0.04	0.00
	b	1.00	1.00	1.00	1.00	0.98	1.00	1.00	1.00	1.00	1.00	0.95	0.96	1.00
		(17)	(298)	(19)	(57)	(49)	(35)	(13)	(47)	(23)	(41)	(21)	(23)	(31)
PGI	a*	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00
	b	0.97	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	0.98	0.89	1.00

Table 1. Continued

Locus/allele		Populations: Almería LM	CH	CL	PM	BC	EB	ES	CP	SC	Morocco AH	CA	OU	CB
	c*	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.00
	d*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.04**	0.00
		(37)	(298)	(19)	(59)	(49)	(36)	(30)	(47)	(23)	(40)	(27)	(33)	(19)
PGM-1	a*	0.00	0.10	0.00	0.00	0.06	0.60	0.00	0.21	0.00	0.01	0.00	0.00	0.00
	b	0.18	0.53	0.98	0.25	0.74	0.37	0.23	0.42	0.34	0.50	0.19	0.13	0.12
	c	0.80	0.33	0.02	0.27	0.20	0.03	0.73	0.30	0.59	0.40	0.81	0.87	0.79
	d	0.02	0.04	0.00	0.48	0.00	0.00	0.04	0.07	0.07	0.09	0.00	0.00	0.09
		(22)	(301)	(26)	(52)	(48)	(35)	(13)	(43)	(16)	(35)	(26)	(16)	(25)
PGM-2	a	0.00	0.40	0.00	0.26	0.16	0.04	0.04	0.25	0.19	0.00	0.00	0.00	0.00
	b	1.00	0.60	1.00	0.74	0.84	0.96	0.96	0.61	0.81	0.85	0.71	1.00	1.00
	c*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.15	0.29	0.00	0.00
	d*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.07**	0.00	0.00	0.00	0.00	0.00
		(23)	(296)	(25)	(49)	(47)	(35)	(13)	(50)	(16)	(39)	(26)	(21)	(21)
6PGD-1	a	0.05	0.23	0.00	0.16	0.53	0.53	0.00	0.06	0.06	0.40	0.18	0.47	0.33
	b	0.95	0.21	1.00	0.81	0.06	0.05	1.00	0.50	0.36	0.24	0.82	0.53	0.67
	c*	0.00	0.34	0.00	0.00	0.36	0.42	0.00	0.44	0.09	0.24	0.00	0.00	0.00
	d*	0.00	0.17	0.00	0.03	0.05	0.00	0.00	0.00	0.27	0.00	0.00	0.00	0.00
	e*	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00
		(41)	(256)	(3)	(48)	(33)	(31)	(6)	(42)	(11)	(17)	(17)	(17)	(28)
6PGD-2	a*	0.00	**0.21**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	b*	0.00	0.05	0.00	0.04	0.49	0.00	0.00	0.00	0.00	0.31	0.00	0.00	0.17
	c	0.05	0.32	1.00	0.96	0.42	0.50	0.00	0.42	0.42	0.69	1.00	0.47	0.83
	d*	0.00	0.01	0.00	0.00	0.08	0.02	0.00	0.08	0.13	0.00	0.00	0.24	0.00
	e	0.95	0.25	0.00	0.00	0.00	0.48	1.00	0.50	0.46	0.00	0.00	0.29	0.00
	f*	0.00	**0.16**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		(41)	(266)	(12)	(45)	(38)	(29)	(6)	(42)	(12)	(8)	(17)	(17)	(6)
6PGD-3	a	0.03	0.05	1.00	0.02	0.05	0.00	0.00	0.00	0.00	0.20	0.50	0.15	0.00
	b	0.83	0.21	0.00	0.03	0.03	0.03	0.00	0.02	0.00	0.20	0.00	0.50	0.00
	c	0.03	0.26	0.00	0.56	0.27	0.08	0.17	0.88	0.33	0.60	0.36	0.35	0.25
	d	0.11	0.24	0.00	0.22	0.53	0.48	0.67	0.00	0.67	0.00	0.00	0.00	0.75
	e*	0.00	0.01	0.00	0.01	0.12	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.00
	f*	0.00	0.17	0.00	0.16	0.00	0.40	0.16	0.10	0.00	0.00	0.00	0.00	0.00
	g*	0.00	**0.06**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		(30)	(249)	(12)	(45)	(37)	(31)	(6)	(42)	(12)	(10)	(9)	(13)	(6)
6PGD-4	a	0.07	0.10	1.00	0.02	0.00	0.00	0.00	0.02	0.00	0.17	0.78	0.13	0.00
	b*	0.00	0.02	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50
	c*	0.00	0.12	0.00	0.00	0.10	0.10	0.00	0.12	0.00	0.00	0.00	0.00	0.00
	d	0.42	0.36	0.00	0.07	0.45	0.69	0.00	0.80	0.00	0.17	0.22	0.16	0.50
	e	0.17	0.21	0.00	0.09	0.08	0.02	0.00	0.06	0.25	0.41	0.00	0.00	0.00
	f	0.25	0.04	0.00	0.64	0.35	0.15	0.50	0.00	0.75	0.25	0.00	0.71	0.00
	g*	0.09	0.15	0.00	0.18	0.00	0.04	0.50	0.00	0.00	0.00	0.00	0.00	0.00
		(30)	(250)	(12)	(45)	(30)	(26)	(6)	(42)	(9)	(12)	(18)	(16)	(6)
SDH-1	a*	0.00	0.05	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	b*	0.33	0.35	0.00	0.00	0.15	0.80	0.00	0.00	0.06	0.00	0.18	0.00	0.00
	c	0.63	0.60	1.00	1.00	0.86	0.10	1.00	1.00	0.92	1.00	0.82	1.00	1.00
	d*	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00
		(27)	(250)	(16)	(48)	(38)	(30)	(6)	(24)	(24)	(18)	(20)	(17)	(6)

Data analysis. Basic estimators of genetic variation (number of alleles per locus, percentage of polymorphic loci (99% criterion), and observed and expected heterozygosities) were calculated using BIOSYS-1 Version 1.7 (Swofford and Selander 1989) from genotype data corresponding to each locus and each population.

The probability of loss L is defined as the probability that a sample of size N fails to include an allele with population frequency p. This parameter was estimated using the expression $L = (1 - p)^{2N}$ (Bengtsson et al. 1995) for the alleles that (1) had an overall frequency lower than 0.5, and (2) were present in 50% or less of the populations of *A. gramineum* considered. Both the expected probabilities of loss, L_e (i.e., taking for p the mean allele frequency over all the populations considered), and the observed probability of loss, L_o, were calculated and plotted. These values were used for two linear regression analyses where the frequency of each allele was the x-axis and $-\log L_o$ and $-\log L_e$ were the respective y-axes. Following Bengtsson et al. (1995), we obtained a value for the representativity (R) of sampling only one population of *A. gramineum* relative to the total sample of rare alleles dividing the slope of the observed regression line (based on the values of L_o) by the slope of the expected regression line (based on the values of L_e). These calculations were carried out with *A. gramineum* on the whole and with its Moroccan and Almerian contingents to assess the representativity of sampling a single population in each of these geographical subdivisions, to estimate the proportion of populations that should be sampled from either area and to compare the values of the probability of loss of rare alleles with those of the degree of inter-population subdivision as measured by G_{ST}.

The mean values of H_T and H_S over all loci were used to calculate G_{ST} (that quantifies the amount of isozyme variation attributable to the inter-population component) according to Nei (1973) using the computer program Genestat-PC version 3.31 (Lewis 1993). Although there are other methods to estimate the among-population apportionment of genetic variation (i.e., Hamrick and Godt 1989), Nei's procedure seems more meaningful biologically, since it is more sensitive to population differentiation as estimated from the variation in allele frequencies across populations (Culley et al., 2002). The value of G_{ST} unbiased for population size and number was used in the relationship $P = (1 - G_{ST})^n$ (Hamrick et al. 1991) to estimate the number of populations (n) necessary to represent a given proportion (P) of the among-population isozyme variation detected (represented by G_{ST}). Calculations entailing the value of G_{ST} were carried out considering the same geographical subdivisions of *A. gramineum* as for the probability of loss. The probabilities of loss and the different file formats needed to run the population genetic programs used to obtain the values of intra- and inter-population genetic variation parameters were generated by the computer programs Transformer-1 (Caujapé-Castells 2001) and Transformer-2 (Caujapé-Castells and División de Sistemas de Información ITC 2003) after the matrix of individual genotypes per locus per population.

Results

Out of the 69 isozyme alleles scored, 20 were exclusive to *A. gramineum* from Almería, and 4 to *A. gramineum* from Morocco (Table 1). The remaining 45 alleles were shared by the two geographical areas, although only 15 of these were detected in all the populations surveyed (Table 1). Locus *Got-2* was not resolved in any of the Moroccan populations and therefore was not considered either in these comparisons or in the quantitative genetic analyses.

The basic estimators of isozyme variation (Table 2) showed that the populations from Almería had higher average expected heterozygosity (H_e = 0.230) and number of alleles per locus (A = 2.14) than their Moroccan relatives (H_e = 0.213 and A = 1.90). In contrast, Moroccan populations displayed a slightly higher proportion of polymorphic loci (P = 66.2) than the Almerian ones (P = 62.8).

The observed and expected probabilities of loss (L_o and L_e) for the 38 alleles of *A. gramineum* (sensu lato) that fulfilled the two conditions stated in the Methods are shown in Table 3. The quotient between the slope of the regression lines (Figure 2) corresponding to $-log(L_o)$ and $-log(L_e)$ gave representativity values of R = 0.461 (*A. gramineum*), R = 0.503 (Moroccan populations), and R = 0.619 (Almerian populations), indicating that sampling one population from these geographical subdivisions would represent roughly 46%, 50% and 62% of the alleles that fulfilled the two criteria of rarity stated in the methods. Considering *A. gramineum* sensu lato, the preferred sampling area (i.e., the area where the allele holds a lower probability of loss) was Almería in 27 cases (that included the 20 alleles exclusive to this Spanish region) and

Table 2. Basic descriptors of isozyme variation in the Almerian and Moroccan populations of *A. gramineum* (Caujapé-Castells and Jansen 2003). *P*: percent of polymorphic loci; *A*: mean number of alleles per locus; H_e: expected heterozygosity; H_o: observed heterozygosity; Numbers between brackets are standard deviations

Area/population	Code	*P*	*A*	H_e	H_o
Almería					
Los Molinos	LM	76.5	2.18	0.224 (0.050)	0.187
Cortijar de Charco del lobo	CH	70.6	3.12	0.349 (0.075)	0.231
Cerro de los lobos	CL	11.8	1.12	0.029 (0.027)	0.042
Playa de Mónsul	PM	76.5	2.29	0.196 (0.053)	0.106
Barranco de Curriá	BC	76.5	2.35	0.266 (0.061)	0.162
El Barranquete	EB	64.7	2.29	0.268 (0.058)	0.132
El Solanillo	ES	53.0	1.65	0.184 (0.055)	0.071
Cerro de los peligros	CP	76.5	2.35	0.304 (0.065)	0.219
Cerro de San Cristobal	CS	58.8	1.88	0.247 (0.062)	0.182
Average Almería		62.8	2.14	0.230 (0.056)	0.148
Morocco					
Aïn Harrouda	AH	64.7	2.12	0.276 (0.068)	0.229
Casablanca	CA	76.5	1.82	0.192 (0.046)	0.127
Oualidia	OU	76.5	2.12	0.229 (0.055)	0.090
Cap Beddouza	CB	47.1	1.53	0.156 (0.050)	0.071
Average Morocco		66.2	1.90	0.213 (0.055)	0.129

Morocco in the remaining 11 cases. If we use these figures to estimate the proportion of populations to be sampled at Almería and Morocco, the result is 0.6:0.4, respectively.

The inter-population component explains a substantial part of the genetic variation detected in *A. gramineum* sensu lato (G_{ST} = 0.371). Following Culley et al. (2002), values of G_{ST} for individual loci are reported in Table 4, along with the values of H_T, H_S and D_{ST}, to allow a survey of the patterns of variation in these statistics and to facilitate eventual recalculations using other methods. The value of this parameter within Almería was much larger than that within Morocco (G_{ST} = 0.413 vs G_{ST} = 0.240, data not shown). Using the average unbiased G_{ST} value for *A. gramineum* sensu lato in the relationship $P = 1 - {G_{ST}}^n$ (Hamrick et al. 1991) to estimate how many populations should be sampled to represent 99% of the isozyme variation detected resulted in n = 5 populations (4.63 in exact figures). According to the proportions 0.6:0.4, this would entail sampling three and two populations in Almería and Morocco, respectively. Applying Hamrick et al's (1991) equation in Almería and Morocco separately gives estimates of n = 5 and n = 3, respectively.

Discussion

Despite the considerable debate regarding the kind of biological information that is most important for conservation purposes (Hamrick et al. 1991; Brussard 1991; Schemske et al. 1994), there seems to be a widespread consensus in that habitat preservation should be a top priority (Francisco-Ortega et al. 2000; Batista et al. 2001). Because the present degree of habitat degradation is not likely to lessen and outweighs the intrinsic survival attributes of *A. gramineum*, the implementation of ex-situ conservation strategies seems fully justified to warrant a representation of the (still quite considerable) genetic variation of this Ibero-Moroccan endemic. The significance of these populations as relict reservoirs of genetic variation is stressed by a recent chronology based on cpDNA data (Caujapé-Castells and Jansen 2003) that showed that *A. gramineum* is one survivor of the Pliocene-Pleistocene glacial cycles that affected the northern quarter of Africa.

Ecological and historical aspects notwithstanding, another paramount reason to suggest *ex-situ* managing strategies for *A. gramineum* is that a large proportion of genetic variation would be missed if all our conservation efforts were devoted to the only population that

Table 3. Probabilities of loss for the 38 alleles that fulfilled the conditions of rarity stated in the methods when *A. gramineum* is considered as a single management unit. *N*: number of populations where each allele was detected; L_o: observed probability of loss; L_e: expected probability of loss under the assumption of equal frequency in all populations (see text for explanation). 'Preferred sampling area' refers to the geographical area where it would be more probable to capture the corresponding allele

Allele	*N*			Average frequency	Probabilities of loss		Preferred sampling area
	A. gramineum	Almería	Morocco		L_o	L_e	
Aco-2a	1	1	0	0.010	0.980	0.770	Almería
Aco-2d	1	1	0	0.060	0.884	0.200	Almería
Aco-3a	2	2	0	0.025	0.904	0.518	Almería
Aco-3c	2	2	0	0.440	0.098	0.000	Almería
Aco-3d	6	6	0	0.157	0.129	0.012	Almería
Aco-3e	4	0	4	0.018	0.868	0.632	Morocco
Aco-3f	1	1	0	0.300	0.490	0.000	Almería
Aco-3g	1	1	0	0.190	0.656	0.004	Almería
Adh-a	6	2	4	0.110	0.247	0.048	Morocco
Fdh-b	5	5	0	0.106	0.326	0.054	Almería
Gdh-1b	1	1	0	0.070	0.865	0.152	Almería
Gdh-1c	4	4	0	0.100	0.430	0.065	Almería
Got-1a	4	1	3	0.030	0.784	0.453	Morocco
Mdh-3a	1	0	1	0.030	0.941	0.453	Morocco
Mdh-3c	6	5	1	0.095	0.302	0.075	Almería
Me-a	3	1	2	0.037	0.799	0.379	Morocco
Pgi-a	2	1	1	0.030	0.885	0.453	Morocco
Pgi-c	3	1	2	0.023	0.868	0.541	Morocco
Pgi-d	1	0	1	0.040	0.922	0.346	Morocco
Pgm-1a	5	4	1	0.196	0.113	0.003	Almería
Pgm-2c	2	0	2	0.220	0.370	0.002	Morocco
Pgm-2d	1	1	0	0.070	0.865	0.152	Almería
6Pgd-1c	6	5	1	0.315	0.011	0.000	Almería
6Pgd-1d	4	4	0	0.130	0.328	0.027	Almería
6Pgd-1e	2	1	1	0.080	0.716	0.114	Morocco
6Pgd-2a	1	1	0	0.210	0.624	0.002	Almería
6Pgd-2b	5	3	2	0.212	0.092	0.002	Almería
6Pgd-2c	6	5	1	0.093	0.309	0.078	Almería
6Pgd-2f	1	1	0	0.160	0.706	0.011	Almería
6Pgd-3e	4	3	1	0.070	0.560	0.152	Almería
6Pgd-3f	5	5	0	0.198	0.110	0.003	Almería
6Pgd-3g	1	1	0	0.060	0.884	0.200	Almería
6Pgd-4b	2	2	1	0.180	0.452	0.006	Morocco
6Pgd-4c	4	4	0	0.110	0.394	0.048	Almería
6Pgd-4g	5	5	0	0.192	0.119	0.004	Almería
Sdh-1a	2	2	0	0.075	0.732	0.132	Almería
Sdh-1b	6	5	1	0.312	0.011	0.000	Almería
Sdh-1d	2	2	0	0.030	0.885	0.453	Almería

lives in a protected habitat (PM, within the Nature Preserve of Cabo de Gata). This population is not among the more genetically variable ones (see Table 2) and, according to Ecologistas en Acción, even its protected enclave is threatened in the short term by a projected urbanization in the area of the Playa de Mónsul (http://www.indalia.es/actualidad/medio-ambiente/hemeroteca/costa/16100/).

The importance of rare alleles. A variety of reproductive, genetic and demographic factors influence the degree of population spatial clustering (Schemske et al. 1994; Chung et al. 2003). Therefore, interpreting biological attributes of endangered species in the light of spatial considerations associated with their genetic variation seems one consistent way to suggest collection designs for *ex-situ* preservation in seed banks. Based on this premise, a most crucial commitment is to adjust the cost of collection with the chance of failing to include rare alleles in the sample. Rare alleles are important in conservation biology because they represent unique evolutionary byproducts that may endow the species with advantageous properties to cope with eventual environmental shifts (Schonewald-Cox et al. 1983; Richter et al. 1994; Bengtsson et al. 1995). Thus, collection designs oriented to sampling rare alleles provide the manager of genetic diversity with adequate tools with which to reinforce declining populations or aid the survival of reintroduced plants.

In this context, a first important sampling issue is to know whether one single population would be sufficient to represent the rare alleles detected. The graph in Figure 2 shows the observed and expected relationship between average allele frequency (*x*-axes) and the negative logarithms of the observed and expected probabilities of loss for the rare alleles detected in *A. gramineum* (*y*-axes). According to the representativity value obtained for the Morocan and Almerian populations indistinctly (R = 0.461), sampling intensively within a single population would account for only about 46% of these alleles. Probably, Almería (R = 0.427) is slightly less representative than Morocco (R = 0.503) on account that most rare alleles included in the calculations for the Moroccan *A. gramineum* are present in 50% of its four populations, whereas rare alleles in Almería are less widespread (many of them are found in less than four populations out of nine) and hold lower average frequencies.

At the level of the total alleles detected, the average G_{ST} for *A. gramineum* sensu lato (G_{ST} = 0.371)

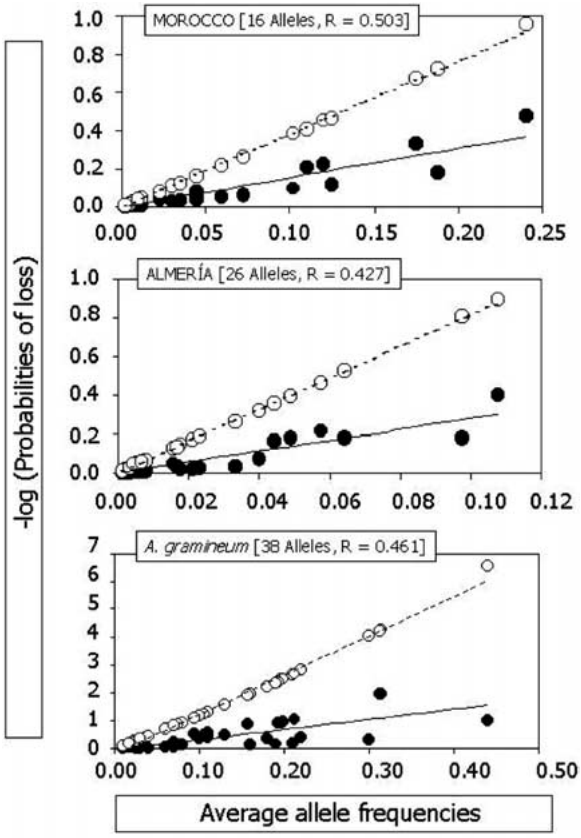

Figure 2. Regression lines of the average allele frequency (x-axis) with $-\log(L_o)$ (solid line, black circles) and $-\log(L_e)$ (dotted line, empty circles) for the alleles that fulfilled the criteria stated in the Methods in the three geographical subdivisions of *A. gramineum* considered. Boxes above each representation indicate the corresponding geographical subdivision, the number of alleles used in the representations and the representativity value (R).

(Table 4) converges with the probabilities of loss to indicate that sampling a single population of *A. gramineum* would be insufficient to account for the rare alleles detected in this Ibero-Moroccan endemic. This result is of especial application in the Almerian populations: consistent with their estimated superior antiquity (Caujapé-Castells and Jansen 2003), they hold the larger number of rare alleles and have a higher degree of inter-population differentiation as quantified by the unbiased value of G_{ST} (G_{ST} = 0.413 in Almería vs. G_{ST} = 0.241 in Morocco [data not shown]).

Although the contrasts between the values of G_{ST} in Almería and Morocco are paralleled by the different number of rare alleles in either area (26 and 16, respectively), it is convenient to make it clear that G_{ST} does not bear a linear relationship with any of the variables related to the probabilities of loss (see

Table 4. Nei's (1973) population structure statistics unbiased for sample size and population number at the 17 loci resolved in the 13 populations of *A. gramineum* included in this paper

Locus	H_S	H_T	D_{ST}	G_{ST}
ACO-2	0.319	0.396	0.120	0.238
ACO-3	0.179	0.341	0.220	0.527
ADH	0.087	0.097	0.011	0.109
FDH	0.055	0.079	0.014	0.175
GDH-1	0.054	0.071	0.007	0.094
GOT-1	0.272	0.362	0.132	0.295
MDH-1	0.209	0.283	0.098	0.296
MDH-3	0.080	0.089	0.010	0.103
ME	0.016	0.017	0.000	0.015
PGI	0.025	0.026	0.001	0.033
PGM-1	0.440	0.535	0.428	0.425
PGM-2	0.222	0.266	0.058	0.188
6PGD-1	0.433	0.625	0.414	0.422
6PGD-2	0.356	0.624	0.538	0.550
6PGD-3	0.489	0.793	0.902	0.573
6PGD-4	0.504	0.810	0.957	0.578
SDH-1	0.159	0.282	0.158	0.476
Average	0.231	0.341	0.155	0.371

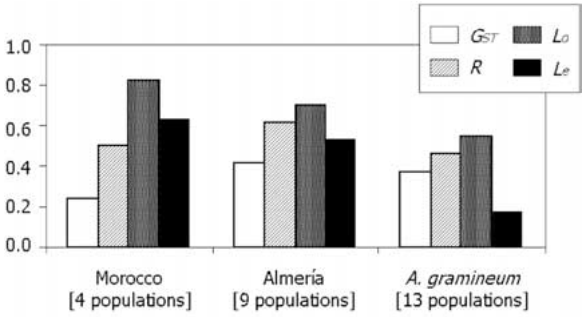

Figure 3. Relationships between the values of the interpopulation differentiation (G_{ST}) and the basic parameters related to the probability of loss (R, L_o and L_e) in the three geographical subdivisions considered in *A. gramineum*.

Figure 3 for an illustration). As defined by Nei (1973) G_{ST} can be expressed by $1 - H_s/H_t$ (Culley et al. 2002), where H_t is the total gene diversity, and H_s (diversity within populations) is the average expected heterozygosity ($H_e = 1 - \Sigma {p_i}^2$, where p_i is the frequency of allele i) across all populations. Because most rare alleles do hold low p_i values by definition, their impact on the value of G_{ST} will be minimum. Therefore, the absence of relationship between this parameter and the probabilities of loss is not unexpected; rare alleles are the only variables used for the representativity estimates in Figure 2, whereas the value of G_{ST} is mostly determined by inter-population differences in the frequencies of more common alleles.

How many populations? Given the scarce representativity of a single population of *A. gramineum* in terms of either rare or common alleles, a second general issue to address is how many populations should be sampled to have a consistent representation of the genetic variation detected. An important preliminary consideration before answering this question is whether we should merge the two geographical sets of populations of *A. gramineum* into a single conservation unit or, instead, we should consider them separately. Soltis and Gitzendanner (1999) show that biodiversity conservation is more meaningful if its biological units equate univocally with monophyletic groups. Hence, checking the assumption of monophyly is important, as molecular phylogenies have often revealed multiple lineages in population systems assumed to be monophyletic (Soltis et al. 1997).

Molecular phylogenetic studies in *A. gramineum* (Caujapé-Castells and Jansen 2003) provided compelling evidence that the Moroccan and the Spanish populations make up a single, well-supported, monophyletic assemblage regardless of the geographical hiatus represented by the Mediterranean sea. Therefore, it seems justified to consider populations from both areas indistinctly in the design of ex-situ conservation strategies. Reproductive evidence from artificial crossings (Ardanuy 1997) also results in no appreciable differences in terms of reproductive efficiency between crosses entailing one individual from either area vs. two individuals from the same area. This indirectly indicates that it would be improbable that outbreeding depression was a factor of threat in case that seeds sampled in one area of distribution were used for reintroductions or population reinforcement in the other area. These pieces of evidence bolster the joint consideration of Almerian and Moroccan populations in conservation strategies and, together with the ecological uniformity throughout *A. gramineum*'s range, justify the use of only population genetic parameters to substantiate the final sampling decisions for the ex-situ preservation of this species.

Hamrick (1983) recommends that the ideal consistency for conservation purposes is achieved when at least 99% of the isozyme variation is preserved in germplasm banks. In connection with this recommendation, the estimate of the number of populations

to be sampled (n) given by the relationship $P = 1 - {G_{ST}}^n$ (Hamrick et al. 1991) proved to be robust against different calculation methods of G_{ST} in simulations carried out by Culley et al. (2002). However, one potential drawback to the application of this equation in *A. gramineum* is that it assumes equal levels of genetic diversity across the populations considered. The degree of genetic heterogeneity in this Ibero-Moroccan endemic is highlighted by the differences between the estimate of n (setting $P = 0.99$) in *A. gramineum* sensu lato ($n = 5$) and those in Almería ($G_{ST} = 0.413$; $n = 5$) and Morocco ($G_{ST} = 0.240$; $n = 3$) considered separately. If there were no substantial differences in the levels of gene diversity, the estimated n for *A. gramineum* sensu lato should be close to the sum of the estimates for Almería and Morocco. One direct consequence of this high inter-population differentiation (especially within Almería) is that more populations will need be sampled in order to ensure that the suggested proportion of genetic diversity is retained (Hamrick et al. 1991). Therefore, it is safer to opt by heeding the estimate of n that entails a higher number of populations. Consistent with the proportions 0.6:0.4 estimated from the probabilities of loss of rare alleles (Table 3), five of these eight populations should be sampled in Almería and three in Morocco.

Which populations? The figure resulting from the application of Hamrick et al.'s equation does not convey any indication as to which populations are best suited to represent the suggested proportion of variation. Basic population genetic theory indicates that it is advisable to consider both the average number of alleles detected per locus (A) and the value of expected heterozygosity (H_e) for the selection of sampling targets. Although a direct relationship between the two parameters seems to exist in *A. gramineum* ($r = 0.896$, Figure 4), they furnish different kinds of information. While A gives an indication of the raw amount of variation per population, H_e offers an indirect measure of genetic heterogeneity that is less affected by sampling error (i.e., its value is not influenced by the frequencies of rare alleles) and whose correlation with fitness-related traits is widely accepted (Mitton and Grant 1984; Zouros and Foltz 1987) and was concluded to be statistically significant in a meta analysis (Britten 1996; but see Elliott and Pierce 1992; Savolainen and Hedrick 1995; David 1998).

Ranking the populations of *A. gramineum* in terms of these two parameters indicates that, in Almería,

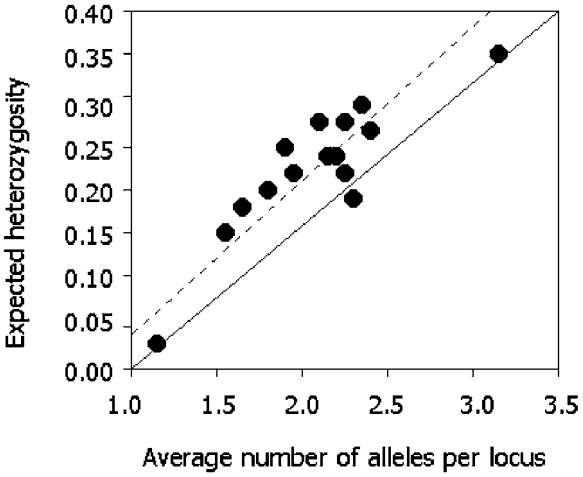

Figure 4. Relationship between the values of the average number of alleles per locus and the expected heterozygosity in *A. gramineum*. The dotted line is the linear regression trendline. The solid line is the expected trendline if the correlation between both parameters was 1.

Cortijar de Charco del Lobo ($A = 3.12$, $H_e = 0.349$), Cerro de Los Peligros ($A = 2.35$, $H_e = 0.304$), Barranco de Curriá ($A = 2.35$, $H_e = 0.266$), El Barranquete ($A = 2.29$, $H_e = 0.268$) and Playa de Mónsul ($A = 2.29$, $H_e = 0.196$) should be sampled (see Table 2). Sampling intensively within these five populations would allow us to capture 61 of the 65 alleles detected in this area (94% of the isozyme variation). In Morocco, the maximum number of alleles per locus ($A = 2.12$) occurs in both Aïn Harrouda and Oualidia, which have similar heterozygosity values ($H_e = 0.276$ and $H_e = 0.229$, respectively). Sampling in these two populations and in Casablanca ($A = 1.82$, $H_e = 0.192$) would target all alleles detected in Morocco except for *6Pgd3-d* (98% of the isozyme variation). Overall, sampling intensively in these eight populations would minimize collection effort, seed storage space and handling difficulties while representing the genetic variation of *A. gramineum* consistently. If we use the isozyme data to estimate the proportion of genetic variation that this proposed sampling design would target, the result is 97% (all the alleles detected except for *Aco3-g* and *Sdh1-d*).

Note that if we took as a reference the estimate of n corresponding to *A. gramineum* sensu lato ($G_{ST} = 0.371$; $n = 5$) and, consistent with the proportions 0.6:0.4, sampled three populations in Almería (CH, CP and BC) and 2 in Morocco (AH and OU), we would leave out six of the total 65 different alleles in the former area (thus targeting about 91% of the genetic variation detected) and five of those found in

the latter (capturing roughly 90% of the variation). On the whole, we would be able to capture 96% of the total variation (68 out of the 71 different isozyme alleles detected in *A. gramineum*). As to the rare alleles, we would leave out *Aco3-g*, *Gdh1-b* and *Sdh1-d* (Table 2). Hence, the main difference between taking $n = 8$ or $n = 5$ populations resides in that $n = 8$ allows to target a higher proportion of variation within each geographical area. However, the increase in the proportion of variation retained for *A. gramineum* sensu lato is not substantial (97% for $n = 8$ vs. 96% for $n = 5$).

Which individuals? Once the geographic aspects of sampling have been clarified, it is also important to know how it should be carried out in the target populations. Disregarding this factor could lead to a collection containing only a few genetically distinct individuals that might jeopardize the long-term success of eventual population reinforcements or reintroductions through decreasing population fitness (e.g., by the action of inbreeding depression). Thus, the avoidance to sample related individuals is of primary concern at this level. Spatial autocorrelation is a non-parametric statistical methodology (see Sokal (1979) for its applications to population biology) that is suitable for this purpose because (i) the coefficient of spatial autocorrelation (Moran's *I*, *MI*) can be related to Wright's (1922) coefficient of relationship, ρ; and (ii) when positive spatial autocorrelation (i.e. a monotonic decline from positive to negative values of *MI* as inter-individual distances increase) is detected, a direct estimate of the diameter ($\oslash$) of the genetically homogeneous area (i.e. that within which the probability of finding close relatives is maximized) can be calculated.

Spatial autocorrelation analyses carried out in the largest population of *A. gramineum* (Cortijar de Charco del Lobo, in Caujapé-Castells and Pedrola-Monfort 1997) revealed a distinct pattern of short distance spatial structuring of isozyme variation (construed as the result of capsule indehiscence, moderate vegetative reproduction and assortive mating), and estimated a homogeneous area of $\oslash = 15$ m. Although equating this estimate with the minimum separation among the seed samples to be collected in *A. gramineum* is a sensible first approach to the intra-population sampling, it would be more convenient to run similar analyses in the remaining target populations to adjust the collection distances (population CH is the largest known in *A. gramineum* and its homogeneous area might be larger than those of the other populations). Unfortunately, these analyses cannot be carried out at present, as we lack the needed spatial coordinates associated with each of the individual samples.

Final remarks. Although the general strategy of basing ex-situ conservation on the spatial analysis of genetic variability data may stall seed collections slightly, it is advantageous in that it results in a comprehensive representation of the species' genetic variation that decreases collection effort and minimizes the chance of sampling related individuals. Not infrequently, large stocks of seeds that have not been sampled according to genetic criteria may under-represent the variation of the species at issue, thereby being of scarce conservation value.

This work has shown that isozyme electrophoresis is one molecular tool that can be exploited to help represent genetic variation in germplasm banks consistently. Seed collections of *A. gramineum* undertaken by heeding the sampling design proposed would probably be useful to contribute to the success of eventual population reinforcement strategies or to the establishment of microreserves. Contacts with biodiversity managers in Almería and with the germplasm conservation facilities of Andalucía have already been initiated by the senior author in the hope that the sampling suggestions given here do result in the setup of a seed bank for *A. gramineum* that provides the basis to foster eventual reinforcements and reintroductions in its natural habitats.

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