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The influence of the Miocene Mediterranean desiccation on the geographical expansion and genetic variation of *Androcymbium gramineum* (Cav.) McBride (Colchicaceae)

JULI CAUJAPÉ-CASTELLS* and ROBERT K. JANSEN†
Laboratorio de Biodiversidad Molecular, Jardín Botánico Canario 'Viera y Clavijo', Ap. de correos 14 de Tafira Alta, 35017 Las Palmas de Gran Canaria, Spain, †Section of Integrative Biology and Institute of Cellular and Molecular Biology, University of Texas, Austin, TX 78713–7640, USA

Abstract

Chloroplast DNA (cpDNA) restriction site and isozyme data were combined to explore the spatial–temporal influence of the Messinian desiccation in the Mediterranean on the disjunct distribution of *Androcymbium gramineum* in Almería and Morocco (north and south of the straits of Gibraltar, respectively). Lack of evidence for different selective pressures, divergence time estimates based on the calibration of the isozyme molecular clock with the cpDNA data, the basal position of Almerian populations in the *A. gramineum* clade, and the much higher isozyme polymorphism in Almería suggest that (i) only a southern European range of *A. gramineum* existed before the Messinian [≈ 11.2 million years ago (Ma), in the middle Miocene] and (ii) the desiccation of the Mediterranean basin about 5.5–4.5 Ma induced the migration of *A. gramineum* from Almería to Morocco (between 4.9 and 4.6 Ma, according to our time estimates). After the split into two allopatric units following the refilling of the Mediterranean, the major influence of drift associated with Plio-Pleistocene recurrent glaciation cycles and range expansions/contractions probably fostered the substantial interpopulation genetic differentiation observed within Almería (CG_{ST} = 0.41, average D_{Nei} = 0.185) and, to a lesser extent, within Morocco (CG_{ST} = 0.24, average D_{Nei} = 0.089), but did not hinder the maintenance of considerable levels of genetic variation in either geographical area (A = 2.14, H_E = 0.230 and A = 1.90, H_E = 0.213, respectively).

Keywords: *Androcymbium gramineum*, biogeography, cpDNA phylogeny, isozyme diversity, Messinian desiccation, molecular clock

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Introduction

Sudden environmental changes that we designate as 'natural catastrophes' may cause the extinction of groups of organisms; however, natural catastrophes often trigger the appearance of novel genetic adaptations or foster the enhancement of geographical distribution ranges. The desiccation of the Mediterranean sea as a result of the closing of the straits of Gibraltar (Hsü *et al*. 1977) in the Messinian (late Miocene) about 4.5–5.5 million years ago (Ma) was one recent catastrophic event the impact of which on the Mediterranean landscape and on the distribution of plant groups in this area is only beginning to be explored using molecular markers. The Messinian desiccation induced a continued change towards a cooler and more arid climate in the circum-Mediterranean area (Hsü *et al*. 1973) that probably determined the evolutionary success of many xeric plant groups related to the Flora of southern Africa (Caujapé-Castells *et al*. 2001) and north-eastern Africa (Bramwell 1972). Furthermore, the conversion of an interior sea into a desert territory established a land bridge between southern Europe and northern Africa (Hsü 1974) and among the islands of the Mediterranean (Wijmstra 1969; Beerli *et al*. 1996) that promoted the migration and colonization of new territories by organisms that, otherwise, would have remained within narrower geographical areas because of intrinsic biological

Correspondence: Juli Caujapé-Castells. Fax: 34 928219581; E-mail: julicaujape@cabgc.org

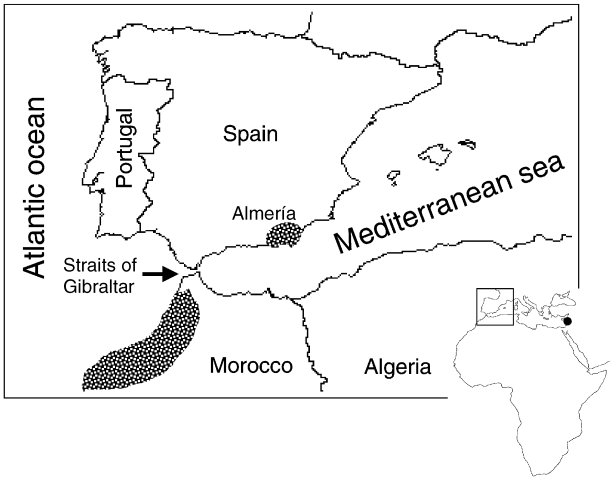

Fig. 1 Geographic distribution of *Androcymbium gramineum* (dotted areas in Almería and Morocco). The black dot in the Middle East on the map of Africa pinpoints the distribution of *A. palaestinum*.

limitations (e.g. excessive geographical separation to allow dispersal or an inability to cross salt-water barriers). The subsequent refilling of the Mediterranean basin following the reopening of the Gibraltar Straits (some 4.5 Ma) brought about the irreversible geographical fragmentation of those organisms that used the Mediterranean basin to expand their range during the period of dryness (Beerli *et al.* 1996).

The distribution of *Androcymbium gramineum* (Cav.) McBride (Colchicaceae) exhibits a geographical discontinuity between the province of Almería (southern Spain) and the area in the Atlantic fringe of Morocco from Casablanca to Tan-Tan (Fig. 1), thus embracing the westernmost region affected by the Messinian event. Greuter (1967) assigned different taxonomic categories to the populations distributed in either geographical area: *A. gramineum* (Cav.) McBride for Morocco and *A. europaeum* (Lange) K. Richter for Almería. However, a survey of morphological traits and isozyme variation for a thorough representation of these taxa (Pedrola-Monfort & Caujapé-Castells 1994) indicated that their taxonomic separation might be more related to a biased sampling of their high intrapopulation variation than to their morphological distinctiveness. A chloroplast (cpDNA) restriction fragment length polymorphism (RFLP) survey of *Androcymbium* (Caujapé-Castells *et al.* 1999, 2001) provided a phylogenetic basis to support that conclusion by placing the Moroccan and Spanish populations in a strongly supported monophyletic group that does not suggest a 'Straits of Gibraltar effect' because some Moroccan populations are much closer to their Spanish congeners than to other Moroccan ones. Hence, morphological, isozyme and cpDNA data strongly indicate that the 'taxonomic disjunction' between these two sets of populations is biologically unjustified, regardless of the distributional hiatus represented by the Mediterranean.

The origin of the current distribution of *A. gramineum* north and south of the straits of Gibraltar is still unresolved. Thus far, molecular population genetic evidence for this species is restricted to a study with 10 isozyme loci for only its Almerian range that led Pedrola-Monfort & Caujapé-Castells (1995) to suggest that interpopulation diversification in this region probably proceeded during the Quaternary. However, one important criticism to this hypothesis is that it was based solely on the inference of divergence times using the values of Nei's distance (D_{Nei}, Nei 1972) and an assumed mutation rate. In general, this procedure results in time estimates that may be 'compatible' with almost any possible fossil or geological data, even if overly incorrect (Avise & Aquadro 1982), because the mutation rates for isozymes may span huge ranges (in most cases between 10^{-5} and 10^{-7}). Empirical studies indicate that chronological interpretations of isozymes are best resolved using calibrations based on independent data, either molecular or geological (Britten 1986; Beerli *et al.* 1996; Membrives *et al.* 2001).

The large number of cpDNA restriction site changes detected at the interspecific level in *Androcymbium* (Caujapé-Castells *et al.* 1999) and the adequacy of these molecular markers to estimate divergence times (Parks & Wendel 1990; Wendel & Albert 1992; Wen & Jansen 1995) allowed us to date some of the main events in the evolutionary history of the genus (Caujapé-Castells *et al.* 2001). These calculations showed that the more recent common ancestor of *A. gramineum* was related to the Middle Eastern species *A. palaestinum* Baker and originated some 11.7 ± 2.1 Ma. Although cpDNA variation was not very informative at the intraspecific level in *Androcymbium*, these data provided crucial reference points for calibrating the isozyme molecular clock for the southern African representatives of this genus (Membrives *et al.* 2001). Given the availability of divergence time estimates based on a thorough cpDNA RFLP survey for *Androcymbium* (Caujapé-Castells *et al.* 1999, 2001) and the existence of a lower limit to the time of isolation between the populations north and south of the straits of Gibraltar, *A. gramineum* is an excellent model system for assessing the possible influence of known historical events on interpopulation diversification using a proper calibration of the isozyme molecular clock. In this study, we combine cpDNA RFLP data with evidence on 18 isozyme loci to discuss the spatial–temporal interpopulation diversification of *A. gramineum*.

Materials and methods

Sampling

We sampled 13 populations of *Androcymbium gramineum* representing the distribution areas of this species (Fig. 1) in

the southern Spanish province of Almería (nine populations) and in Morocco (four populations). Given the continuous distribution of plants in all stands of *A. gramineum*, population sampling for isozyme electrophoresis was carried out at regular intervals along transects, although when physical clumps of individuals were detected we sampled the corms of several (or all) of them. This sampling design is likely to be more informative either if stochastic forces account for population variability or if the detected variation is attributable largely to environmental heterogeneity (Heywood 1991). A much more exhaustive sampling (286 individuals) was carried out in the stand of Charco del Lobo in Almería because of its very large size (see Caujapé-Castells & Pedrola-Monfort 1997 for details). A total of 613 corms (525 corresponding to the nine populations from Almería and 88 to the four populations from Morocco) were carefully unearthed, put into paper bags and transported to the Estació Internacional de Biología Mediterránia-Jardí Botánic Marimurtra, where they were planted in a research greenhouse. Phylogenetic analyses with cpDNA RFLP data used a single individual of each of these 13 populations. We also included two populations of *A. palaestinum*, a species from the Middle East that was shown to be the closest relative of *A. gramineum* based on a previous cpDNA RFLP phylogeny (Caujapé-Castells *et al*. 1999). Due to the large amount of leaf tissue needed for the RFLP analyses, we could not check whether there was intrapopulation cpDNA variation.

Electrophoretic analyses. Protein extracts were made from the tips of young leaves that were collected in the greenhouse on the same day as the electrophoresis. The composition of the extraction buffer and the electrophoretic conditions followed those described by Caujapé-Castells (1995). The resulting enzyme patterns were analysed on an allelic basis. For each enzyme, gene loci and isozymes were labelled 1, 2, 3 … and, for each locus, alleles were labelled a, b, c … , beginning with the fastest anodally migrating bands. We did not carry out a programme of crosses to ascertain the genetic basis of the banding patterns because of the time required (3–4 years) for plants grown from seed to develop leaves suitable for electrophoresis. However, the number and intensities of all interpreted bands were coincident with what is expected under the hypothesis of Mendelian co-dominance in all loci. Intrapopulation, interpopulation and interspecific verification of enzyme mobilities was determined by side-by-side comparisons of allelic variants on the same gel. Interpretations followed Caujapé-Castells & Pedrola-Monfort (1997).

RFLP analyses. DNA isolation, digestion with 21 restriction endonucleases and filter hybridizations were carried out as described in Jansen & Palmer (1987) and Caujapé-Castells *et al*. (1999). Three outgroups from the Colchicaceae were included (two populations of *Colchicum lusitanum* Brot. and a population of *Merendera montana* (Pourret) P. Fourn. based on their close phylogenetic relationship to *Androcymbium* (Buxbaum 1936; Nordenstam 1982; Persson 1993). The dataset for this work is available at http://www.biosci.utexas.edu/IB/faculty/jansen.htm.

Data analysis

Isozymes. The number of alleles per locus (A), percentage of polymorphic loci (P), observed and expected heterozygosity (H_O and H_E) and genetic distances (Nei 1978) were calculated using BIOSYS-1 Version 1.7 (Swofford & Selander 1989). For *A. gramineum*, all calculations were made at the population level from genotype data corresponding to each locus. An UPGMA tree was constructed with Nei's (1978) genetic distance using NTSYS-pc version 1.80 (Rohlf 1993). We used Nei's (1978) distance because simulations in Nei *et al.* (1983) showed this estimator to outperform other genetic distance calculation methods.

We applied a sign test for heterozygosity excess (Cornuet & Luikart 1996) to detect whether the populations have experienced recent historical bottlenecks. This test compares expected heterozygosity (H_E) under Hardy–Weinberg expectations to the heterozygosity expected at mutation-drift equilibrium (H_{EQ}) in a sample that has the same size and the same number of alleles as the sample used to measure H_E (Luikart & Cornuet 1998). The rationale of the test is that because low-frequency alleles are lost at a much faster rate than heterozygosity in a bottleneck situation, bottlenecked populations are expected to have a heterozygote excess. Calculations were made based on allele frequency data under the Stepwise Mutation Model and the Independent Allele Model using the program BOTTLENECK-PC (Piry *et al.* 1998).

Nei's (1973) population structure statistics (both unmodified and unbiased for sample size) were calculated for *A. gramineum* as a whole and for its Almerian and Moroccan distribution areas using the computer program GENESTAT-PC version 3.31 (Lewis 1993). Alternative estimates of D_{ST} and G_{ST} to be used in cases of large amounts of divergence among the considered populations (CD_{ST} and CG_{ST}) were also calculated with this program.

cpDNA RFLPs. The *g1* statistic (Hillis & Huelsenbeck 1992) was calculated for 100 000 random trees to evaluate the amount of phylogenetic signal in the data. Parsimony analyses were performed using branch-and-bound searches with MULPARS and furthest addition sequence using Wagner (Farris 1970) parsimony in PAUP* version 3.1.1 (Swofford 1991). Bootstrap values (Felsenstein 1985) were obtained from 100 branch-and-bound replicates. Differences in rates of cpDNA evolution were assessed by pairwise

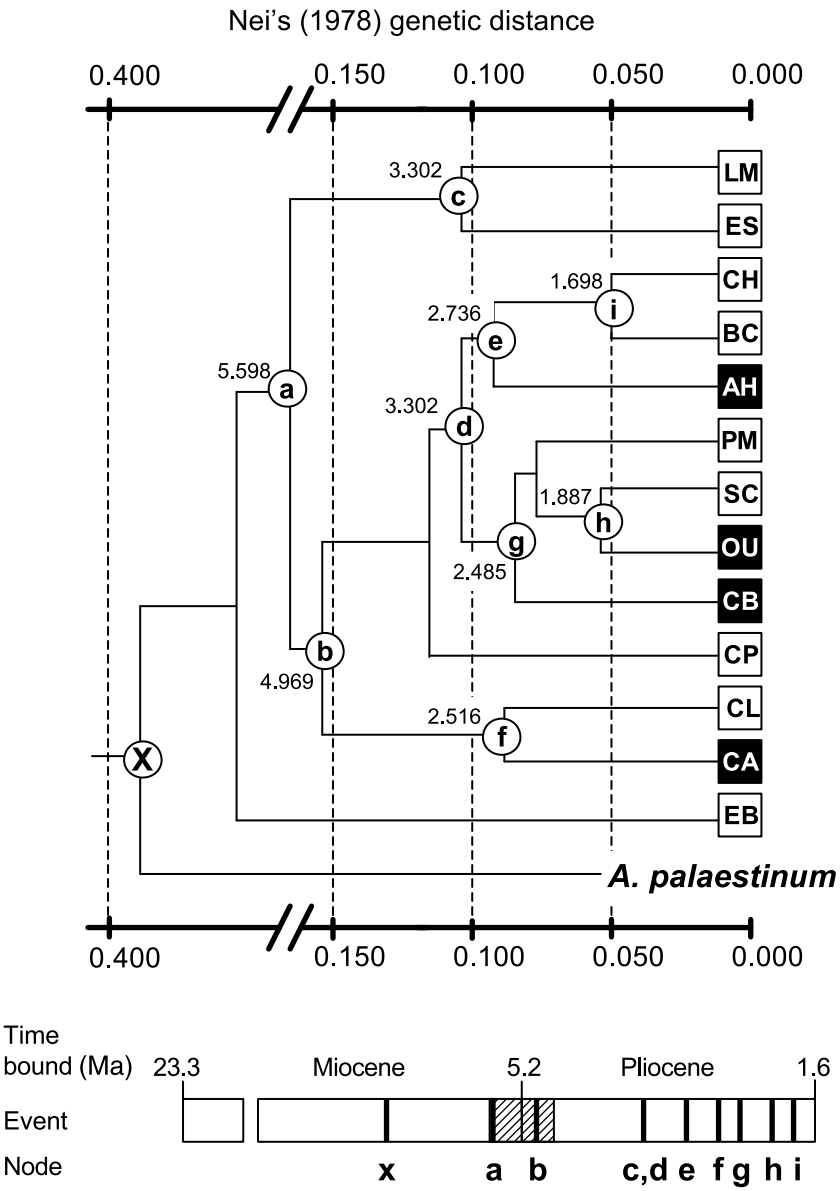

Fig. 2 Chronological relationships among the populations of *Androcymbium gramineum* mapped onto the UPGMA tree with Nei's 1978 distance. The nodes labelled with letters 'a' to 'i' represent the diversification events mapped onto the chronology at the bottom. The node labelled 'X' is the one used to calibrate Nei's (1978) distance with the time estimates from the cpDNA RFLPs. Numbers to the left of the nodes are the average divergence time estimates (in Ma). The notched area in the chronology corresponds to the period of Mediterranean dryness during the Messinian. ■, Moroccan populations; □, Almerian populations.

comparisons involving three representative populations from Almería, Morocco and the Middle Eastern *A. palaestinum* using the two-tailed Wilcoxon matched-pair signed rank test (Templeton 1983).

Isozyme molecular clock calibration. To estimate the time of the divergence between Spanish and Moroccan populations of *A. gramineum* and assess the temporal sequence of diversification within Almería and Morocco, we calibrated the isozyme molecular clock using the average divergence time between *A. gramineum* and *A. palaestinum* as estimated from the RFLP data (11.7 ± 2.1 Ma, Caujapé-Castells *et al.* 2001). We obtained a conversion factor of Ma per Nei's (1978) distance (D_{Nei}) unit dividing the value of the cpDNA RFLP time estimate of the split between *A. gramineum* and *A. palaestinum* by D_{Nei} at the corresponding node in the UPGMA cluster (labelled 'X' in Fig. 2, D_{Nei} = 0.370). This calibration was used to estimate the times of divergence at nine nodes in the UPGMA tree (labelled 'a' to 'i' in Fig. 2) and between all pairwise combinations of populations from the values of D_{Nei}. Unfortunately, the absence of a fossil record for *Androcymbium* and the lack of geological age data for the areas of these populations' distribution hindered other calibrations.

Results

Isozyme analyses

Out of the 74 isozyme alleles scored, 15 were exclusive to *Androcymbium gramineum* from Almería, four to *A.*

Table 1 Basic descriptors of isozyme variation in the Almerian and Moroccan populations of *Androcymbium gramineum*

								Bottleneck tests				
								IAM		SMM		
Area/population	Code	*N*	*P*	A	*F*	H_E	H_O	*PL*	H_d/H_e	*S*	H_d/H_e	*S*
Almería												
Los Molinos	LM	25	76.5	2.18	0.185	0.224 (0.050)	0.187	13	8/5	0.312	10/3	0.026
Cortijar de Charco del lobo	CH	286	70.6	3.12	0.249	0.349 (0.075)	0.231	12	3/9	0.059	3/9	0.112
Cerro de los lobos	CL	18	11.8	1.12	−0.269	0.029 (0.027)	0.042	2	1/1	0.726	1/1	0.715
Playa de Mónsul	PM	49	76.5	2.29	0.432	0.196 (0.053)	0.106	13	10/3	0.072	10/3	0.008
Barranco de Curriá	BC	43	76.5	2.35	0.284	0.266 (0.061)	0.162	13	4/9	0.099	9/4	0.096
El Barranquete	EB	34	64.7	2.29	0.549	0.268 (0.058)	0.132	11	5/6	0.565	6/5	0.306
El Solanillo	ES	11	53.0	1.65	0.437	0.184 (0.055)	0.071	9	5/4	0.559	5/4	0.308
Cerro de los peligros	CP	42	76.5	2.35	0.235	0.304 (0.065)	0.219	13	5/8	0.270	6/7	0.567
Cerro de San Cristobal	CS	17	58.8	1.88	0.296	0.247 (0.062)	0.182	10	3/7	0.188	5/5	0.499
Average Almería		58.3	62.8	2.14	0.262	0.230 (0.056)	0.148					
Morocco												
Aïn Harrouda	AH	28	64.7	2.12	0.145	0.276 (0.068)	0.229	11	2/9	0.038	6/5	0.371
Casablanca	CA	21	76.5	1.82	0.300	0.192 (0.046)	0.127	13	6/7	0.446	10/3	0.037
Oualidia	OU	22	76.5	2.12	0.441	0.229 (0.055)	0.090	13	9/4	0.138	9/4	0.071
Cap Beddouza	CB	17	47.1	1.53	0.391	0.156 (0.050)	0.071	8	5/3	0.405	5/3	0.268
Average Morocco		22	66.2	1.90	0.319	0.213 (0.055)	0.129					

N, total sample size; *P*, per cent of polymorphic loci; *A*, mean number of alleles per locus; *F*, inbreeding coefficient; H_E, expected heterozygosity; H_O, observed heterozygosity; *PL*, number of polymorphic loci on which bottleneck tests are based; H_d, H_e, number of loci with heterozygosity deficiency and excess, respectively; *S*, significance of the H_d/H_e ratio under the independent allele model (IAM) and the stepwise mutation model (SMM). Numbers between brackets are standard deviations.

gramineum from Morocco, and four to the Middle Eastern *A. palaestinum* (the table of allele frequencies is available upon request to J.C.C.). There were 31 alleles shared by the three geographical areas. We detected five exclusive shared alleles between Almería and the Middle East, 15 between Almería and Morocco, and one between Morocco and the Middle East. In several cases, not all the populations of *A. gramineum* possessed the alleles shared between Morocco and Almería exclusively. Locus *Got-2* was not resolved in any of the Moroccan populations and therefore was not considered either in these comparisons or in the calculations of variation levels.

The basic estimators of isozyme variation (Table 1) showed that the populations from Almería had higher expected heterozygosity (average $H_E = 0.230$, between $H_E = 0.029$ in CL to $H_E = 0.349$ in CH) and mean number of alleles per locus (average $A = 2.14$) than their Moroccan relatives (average $H_E = 0.213$, ranging from $H_E = 0.156$ in CB to $H_E = 0.276$ in AH, and average $A = 1.90$), although these differences were not statistically significant. In contrast, Moroccan populations had a higher (although nonsignificant) proportion of polymorphic loci ($P = 66.2$) than Almería ($P = 62.8$). Only one significant deviation of the expected heterozygosity (corresponding to the Moroccan AH) was detected through the bottleneck test under the independent allele model, and three deviations (two in Almería and one in Morocco) under the stepwise mutation model (Table 1). The values of D_{Nei} between all pairwise combinations of populations (Table 2) spanned from 0.048 between SA and AH (Morocco) to 0.339 between CA (Morocco) and ES (Almería). The average D_{Nei} within Almería, Morocco and the Middle Eastern *A. palaestinum* were 0.185, 0.089 and 0.149, respectively. The population structure statistics (Table 3) show that the interpopulation component explains a large part of the genetic variation detected in *A. gramineum* ($G_{ST} = 0.32$, $CG_{ST} = 0.37$). The value of G_{ST} unbiased for sample size and population number in Almería ($G_{ST} = 0.35$, $CG_{ST} = 0.41$) is similar to that of *A. gramineum* as a whole, but much larger than that for the Moroccan populations ($G_{ST} = 0.21$, $CG_{ST} = 0.24$).

RFLP analyses

We detected a total of 66 apomorphic restriction site changes in *A. gramineum* and *A. palaestinum*. Of these, 11 were shared by Almería, Morocco and the Middle East, 18 by Almería and Morocco, eight by *A. palaestinum* and *A. gramineum* from Almería and only one by *A. palaestinum* and *A. gramineum* from Morocco. *Androcymbium palaestinum*, *A. gramineum* from Morocco and *A. gramineum* from Almería exhibited 21, three and two unique restriction site changes, respectively. Four of the changes were length

Table 2 Inter-population Nei's (1978) genetic distance values among the populations *Androcymbium gramineum* and *Androcymbium palaestinum* (below diagonal) and divergence times (in Ma) estimated after applying the calibration discussed in the text (above diagonal)

	Androcymbium gramineum														
	Almería									Morocco				*A. palaestinum*	
	LM	CH	CL	PM	BC	EB	ES	CP	SC	AH	CA	OU	CB	BS	DI
LM	—	5.46	7.56	5.52	8.12	9.33	3.26	6.76	3.69	6.42	5.36	2.98	4.90	10.94	13.89
CH	0.176	—	5.77	3.63	1.67	4.81	5.92	3.99	3.63	3.13	5.05	3.69	3.81	6.85	8.49
CL	0.244	0.186	—	4.09	6.39	11.22	7.84	8.09	6.05	4.71	2.48	5.27	5.18	13.08	16.40
PM	0.178	0.117	0.132	—	3.97	8.80	4.28	4.53	2.02	2.45	2.95	2.14	2.26	8.06	11.32
BC	0.262	0.054	0.206	0.128	—	5.02	7.07	4.46	3.75	2.60	6.29	3.94	3.60	9.36	9.30
EB	0.301	0.155	0.362	0.284	0.162	—	10.76	7.04	7.87	8.53	10.51	8.68	7.87	13.49	12.46
ES	0.105	0.191	0.253	0.138	0.228	0.347	—	5.86	2.60	5.49	5.67	3.47	4.09	10.57	12.52
CP	0.218	0.129	0.261	0.146	0.144	0.227	0.189	—	5.36	3.32	5.58	5.15	5.08	12.34	14.04
SC	0.119	0.117	0.195	0.065	0.121	0.254	0.084	0.173	—	3.13	4.31	1.86	2.57	6.73	8.74
AH	0.207	0.101	0.152	0.079	0.084	0.275	0.177	0.107	0.101	—	2.67	2.36	3.19	10.51	13.52
CA	0.173	0.163	0.080	0.095	0.203	0.339	0.183	0.180	0.139	0.086	—	2.91	2.82	10.70	16.80
OU	0.096	0.119	0.170	0.069	0.127	0.280	0.112	0.166	0.060	0.076	0.094	—	2.51	8.68	12.03
CB	0.158	0.123	0.167	0.073	0.116	0.254	0.132	0.164	0.083	0.103	0.091	0.081	—	9.86	14.63
BS	0.353	0.221	0.422	0.260	0.302	0.435	0.341	0.398	0.217	0.339	0.345	0.280	0.318	—	4.62
DI	0.448	0.274	0.529	0.365	0.300	0.402	0.404	0.453	0.282	0.436	0.542	0.388	0.472	0.149	—

Population codes correspond to Table 1.

Table 3 Nei's (1973) unmodified and unbiased population structure statistics for *Androcymbium gramineum* as a whole and for its two allopatric units in Almería and Morocco

	Unmodified					Unbiased for sample size and population number				
	H_T	D_{ST}	CD_{ST}	G_{ST}	CG_{ST}	H_T	D_{ST}	CD_{ST}	G_{ST}	CG_{ST}
A. gramineum	0.33	0.11	0.15	0.32	0.37	0.34	0.11	0.15	0.32	0.37
Almería	0.34	0.12	0.16	0.34	0.39	0.35	0.13	0.18	0.35	0.41
Morocco	0.27	0.05	0.07	0.18	0.21	0.29	0.06	0.08	0.21	0.24

variations (one exclusive to the Middle East, two shared by Almería and Morocco and the other one shared by all three geographical regions). Except for two haplotypes shared between Barranco de Curriá and San Cristóbal (*A. gramineum* from Almería) and between Cap Beddouza and Oualidia (Moroccan *A. gramineum*), all populations were characterized by a unique array of cpDNA restriction site changes.

The *g1* statistic for 100 000 randomly generated trees was –2.370, indicating that the data are skewed significantly from random and therefore they contain an informative phylogenetic signal (Hillis & Huelsenbeck 1992). Branch-and-bound searches on the RFLP data generated 10 equally parsimonious trees of 271 steps with a CI = 0.854, HI = 0.146, and RI = 0.923, excluding auto-apomorphies. The cpDNA phylogeny (Fig. 3) strongly supported the monophyly of *A. gramineum* (100% bootstrap value) and showed considerable intermixing between Almerian and Moroccan populations. Assuming complete lineage sorting at speciation, if these two sets of populations were different taxonomic entities we would expect them to appear in separate clades in the phylogeny. Constraining the Moroccan and the Spanish populations to appear in separate clades required 13 additional steps on the most parsimonious cpDNA tree, thus arguing for their conspecific status. Constraining the Moroccan populations to form a monophyletic group within the general *A. gramineum* clade required no extra steps on the most parsimonious tree. The average patristic distances (i.e. distances between taxa as computed by the number of steps separating them in the most parsimonious tree) within Almería, Morocco and the Middle Eastern *A. palaestinum* were 10.8, 2.5 and 20, respectively. According to the results of Templeton's (1983) tests, there were no significant deviations from the assumption of a molecular clock.

Isozyme molecular clock calibration

Taking 11.7 ± 2.1 Ma as a reasonably accurate divergence time reference for the split between *A. gramineum* and *A.*

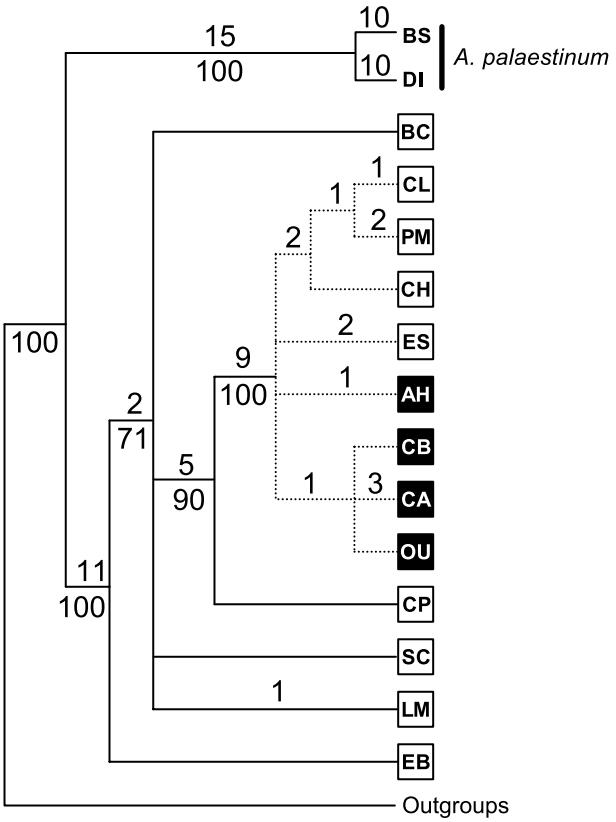

Fig. 3 Cladogram showing the phylogenetic relationships in *Androcymbium gramineum* based on cpDNA RFLPs. Dashed lines indicate nodes that collapse in the strict consensus tree. Numbers above the branches are restriction site changes. Numbers below the branches indicate bootstrap support of the corresponding clades (bootstrap values below 70% are not shown). ■, Moroccan populations; □, Almerian populations.

palaestinum (Caujapé-Castells *et al*. 2001), 0.1 D_{Nei} units represent 3.1 ± 0.6 Ma (see node 'X' in Fig. 2, corresponding to a D_{Nei} = 0.370). Although there is no fossil record for *Androcymbium*, the time calibration reference is consistent with the palaeobotanical evidence discussed in Caujapé-Castells *et al*. (2001), which shows that the northern quarter of Africa was occupied by a subtropical woodland savanna that must have prevented colonization by xerophytic taxa like *Androcymbium* until the middle-late Miocene (Berggren & van Couvering 1974), when part of that area began acquiring typically arid conditions. This calibration of the isozyme molecular clock dated the split between the Spanish and the Moroccan populations (node 'b' in Fig. 2, D_{Nei} = 0.158) at about 4.9 ± 0.9 Ma. Applying the calibration to the average D_{Nei} between Morocco and Almería (D_{Nei} = 0.147) resulted in an average divergence time of 4.6 ± 0.8 Ma. Applying the calibration to D_{Nei} within Almería and within Morocco (Table 2), the differentiation of the genus in the former area spanned from 11.2 ± 2.2 Ma (middle Miocene) to 1.7 ± 0.3 Ma (late Pliocene), whereas the diversification of the Moroccan populations was restricted to the Pliocene, between 3.2 ± 0.6 Ma and 2.4 ± 0.4 Ma (Fig. 2). The resulting calibration was roughly one unit larger than that for a comprehensive representation of southwestern African populations of *Androcymbium* where the same loci and enzyme systems were used (Membrives *et al*. 2001). Thus, the diversification of the genus in North Africa proceeded at a slower rate than in southern Africa.

Discussion

The cpDNA RFLP and isozyme data are important for inferring the probable sequence of events that explains the present distribution of *Androcymbium gramineum* in northern Africa. A previous cpDNA phylogeny based on extensive geographical sampling of the entire genus (Caujapé-Castells *et al*. 2001) showed that the Middle Eastern *A. palaestinum* is basal to the *A. gramineum* clade, and estimated the split of both taxa to have occurred during the middle-Miocene, ≈ 11.7 ± 2.1 Ma. By that time, the mountain chains that had formed during arid phases of the Oligo-Miocene in the present Moroccan Maghreb were already settled (Quézel 1978) and constituted a milieu especially favourable to the speciation and migration of the flora, which proceeded in an east to west direction (Stebbins 1974). The basal position of *A. palaestinum* in the cpDNA RFLP phylogeny (Fig. 3) suggests that *Androcymbium* also colonized the Mediterranean area in an east–west sense.

The lower genetic distance between Almería and the Middle East (D_{Nei} = 0.341) than between Morocco and the Middle East (D_{Nei} = 0.390) and the shared alleles between these geographical regions (six between Almería and the Middle East vs. one between the Middle East and Morocco) hint that the extant Spanish range of *A. gramineum* may be more ancient than the extant Moroccan populations. Both patristic distances and D_{Nei} are also much lower within Morocco than within Almería (Fig. 4), supporting the hypothesis that the Almerian populations have had much more time to diverge. Thus, according to the topology of the cpDNA RFLP phylogeny (Fig. 3) and to the isozyme data, the extant Almerian populations of *A. gramineum* must be much closer to the common ancestor shared by this taxon and *A. palaestinum* than their Moroccan congeners. If the Moroccan populations had been closer to the Middle Eastern *A. palaestinum* than to the Almerian *A. gramineum*, we would have predicted a closer phylogenetic relationship between these two areas, a lower isozyme genetic distance between Morocco and the Middle East than between Morocco and Almería, and a higher number of shared alleles and RFLP changes between Morocco and the Middle East.

According to our time estimates, the split between the Almerian and Moroccan populations of *A. gramineum*

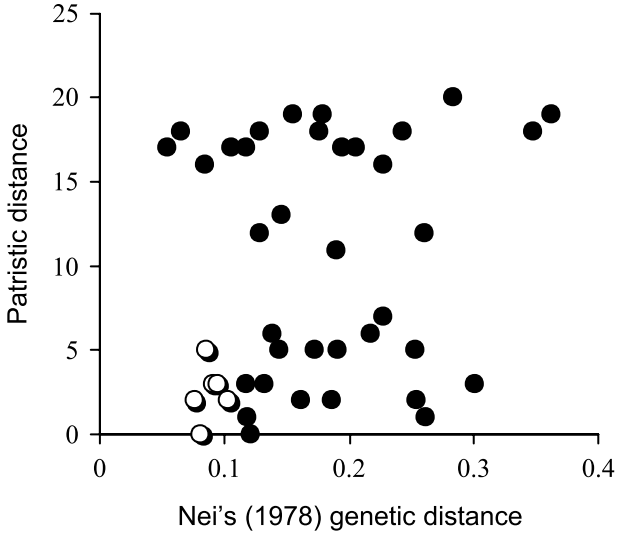

Fig. 4 Relationship between Nei's (1978) genetic distances and patristic distances (based on RFLPs) for the geographical areas surveyed in the paper. ●, Almerian populations; ○, Moroccan populations.

occurred approximately 4.6–5 Ma (based on the average D_{Nei} between both areas and on D_{Nei} at node 'b' in Fig. 2, respectively), well within the period of Mediterranean dryness. Our time estimates also indicate that interpopulation diversification of *A. gramineum* in Almería spans a long time period (Table 2) starting much earlier than the Messinian (at about 11.2 ± 2.2 Ma) and proceeding until 1.7 ± 0.3 Ma. In contrast, the Moroccan *A. gramineum* diversified within a relatively narrow time period in the Pliocene, between 3.2 ± 0.6 and 2.4 ± 0.4 Ma (Table 2), considerably later than the refilling of the Mediterranean basin.

There is undoubtedly an error attached to divergence time estimates, but these data can provide useful information to confirm or refute the more unlikely historical scenarios, and sometimes (as in *Androcymbium*) it is all that is available. Although extreme caution should be used when inferring past historical events from extant taxa, two possible historical scenarios can be proposed to explain our results. First, *A. gramineum* may have existed only in areas of Mediterranean southern Europe earlier than the Messinian. Second, it is feasible that Moroccan populations gave rise to the Spanish range of *A. gramineum* during the Messinian, then died off, and finally a back-colonization from southern Spain gave rise to the present Moroccan range of the species before the Mediterranean refilling. In both cases, a progressive range expansion along the land bridge represented by the dry Mediterranean seems a more feasible alternative than assuming long-distance dispersal across the Gibraltar straits. Although *Androcymbium*'s capsules do not have adaptations for long-range dispersal (Pedrola-Monfort 1993) and its seeds are not eaten by birds because of their high colchicine content, this does not rule out long-distance dispersal as a possible explanation for this Ibero-Moroccan relationship, because this mechanism was probably responsible for the origin of the Canary Island species.

Because we can only sample in geographical space and not through geological time, our data do not allow us to discriminate between these two alternatives; phylogenetic resolution is always constrained by historical ignorance and by biased sampling in the dimensions of time, space and data sources. However, lack of evidence for different selective pressures in either geographical area, the much higher isozyme polymorphism detected in Almería, and the fact that all the enzyme variation (except for alleles *Mdh2-a*, *Pgi-a*, *Pgi-e* and *Pgm2-c*) in Morocco is a subset of that in Almería favour the hypothesis that the extant Moroccan populations of *A. gramineum* originated from their Spanish relatives. This hypothesis is also more consistent with the basal position of only Spanish populations in the clade of *A. gramineum* (Fig. 3) and with our divergence time estimates. Significantly, these time estimates strongly conflict with the scenario of the founding of the Almerian range from Morocco during the Messinian, because they show that population divergence in Almería has proceeded since 11.2 ± 2.2 Ma vs. only 3.4 ± 0.6 Ma in Morocco.

Our RFLP and allozyme data firmly suggest that only a southern European range of *A. gramineum* existed before the Messinian and that the desiccation of the Mediterranean basin about 4.5–5.5 Ma (Hsü *et al*. 1977) was important in the range expansion of this species from the south of Spain to Morocco. This result is counterintuitive, as the other northern hemisphere species of the genus are strictly African, with extant ranges in the Middle East (*A. palaestinum*), on the coast of Libya and the Greek islet of Elafonisos (*A. rechingerii*), in arid areas from Tunisia to Mauritania (*A. wyssianum*), and in the Canary Islands (*A. hierrense* and *A. psammophilum*). Furthermore, although the south of Spain is regarded as the geographical origin of a series of postglacial colonization events involving different species of plants and animals presently distributed in Europe (see Hewitt 1999, 2001 for comprehensive reviews), in none of these cases did the proposed routes entail dispersal towards Morocco, as found in *A. gramineum*. Because of the possible bias associated with all divergence time estimates, our interpretation needs be tested using molecular evidence from other DNA markers. *Androcymbium*'s internal transcribed spacer (ITS) showed only very few changes between species that were separated clearly by the RFLP analysis (Caujapé-Castells and Jansen, unpublished results), and that is the reason why an ITS phylogeny has not been attempted with the genus. Sequence analysis under way for other phylogenetically informative nuclear

and chloroplast DNA regions (Del Hoyo *et al*. in preparation) is expected to provide additional data to support or refute the hypotheses proposed here.

Andalucía (i.e. the Spanish region where Almería belongs) and the Moroccan Rif are usually grouped together in the same biodiversity hot spot because of their conspicuous floristic and bioclimatic affinities (Médail & Quézel 1997). According to Quézel (1978), more than 500 plant species are endemic to these zones, and Valdés (1991) estimates that they share about 75% of 3500 plant species. In the case of *A. gramineum*, this tight floristic connection is best explained by a considerable range expansion during the Mediterranean dryness that must have allowed this species to cover a large area that included (but may not have been restricted to) southern Spain and northwestern Africa. According to our time estimates, this continuum was irreversibly disrupted into two allopatric units (*sensu* Kornet 1993) by the subsequent refilling of the Mediterranean basin. The close phylogenetic relationship of the populations of *A. gramineum* contrasts with the case of *Saxifraga globulifera* Desf., whose areas of distribution north and south of the Gibraltar straits split into two well-supported lineages in an ITS phylogeny (Vargas *et al*. 1999).

Vegetation maps based on diverse sources of data (e.g. Frenzel 1992; Velichko & Isavea 1992) indicate that during the late Miocene–Pliocene, southern Europe was dominated by a virtually treeless semiarid steppe similar to the cool and arid montane areas found today in the pamirs of southern Russia (Tallis 1990). This scenario may have fostered expansion of xerophytic taxa like *Androcymbium* in this region in the early Pliocene. However, if the two allopatric units of *A. gramineum* attained a widespread distribution north and south of the Gibraltar straits, it is likely that most of this range was extirpated or severely fragmented by the recurrent influence of Plio-Pleistocene glaciations in the area, as described for many other elements of the North African Flora (Quèzel 1978; Maley 1980).

Most glaciations lasted for about 100 000 years and were followed by interglacial periods of favourable climatic conditions of some 10 000 years (Hewitt 1996). Several palaeobotanical studies (Quézel 1978; Grichuk 1992) show that only some mountainous areas in southern Europe and in the current Sahara may have served as refugia for plants (e.g. *Cupressus dupreziana*, *Olea laperrini* and *Myrtus nivellei*) during unfavourable climatic conditions (Bruneau de Miré & Quézel 1961). Considering the present distribution of *A. gramineum* under this 'refugium' hypothesis, survival of climatic fluctuations could have been successful in sheltered regions at the Sierra de Gata (the mountain range that surrounds Almería) and in the Atlas and anti-Atlas in Morocco. In view of the recurrence of the climate cycles during the Plio-Pleistocene glacial periods, the population history of *A. gramineum* must have been subjected to continued and considerable variation involving severe population bottlenecks and extinctions. Since the Almerian range of this species seems to have had much more time to diversify, this process possibly had a larger impact on populations from this region. It is also possible that the major action of drift associated with these complex population phenomena is diminishing the resolution of our molecular data. Most likely, the two cases where the bottleneck tests were significant (Table 1) relate to the founding of new populations in Almería and in Morocco during the more recent, favourable climatic stages. These results are unlikely to reflect the influence of the Messinian desiccation event in the range expansion of *A. gramineum* because the heterozygote excess after a bottleneck is transient, and only bottlenecks that have occurred in the recent past (i.e. 10 000 years ago at most) are detectable by the test, according to Luikart & Cornuet (1998). The high G_{ST} values (Table 3, $G_{ST} = 0.32$, $CG_{ST} = 0.37$) suggest that the two disjunct allopatric units of *A. gramineum* have maintained considerable genetic isolation. However, it is also worth noting the low degree of gene flow within Almería, where the amount of genetic variation attributable to interpopulation differentiation ($G_{ST} = 0.35$, $CG_{ST} = 0.41$) is higher than that of the species as a whole and is almost twofold higher than in Morocco ($G_{ST} = 0.21$, $CG_{ST} = 0.24$).

About 11 000–12 000 years ago, cold climate became more severe and moist in Almería. Indicators in the palynological record of the region hint at a progressive deforestation (Yll *et al*. 1994; Pantaleón-Cano 1998), which probably accelerated soil erosion and resulted in the semidesert conditions that dominate the area at the present time. The acquisition of arid conditions probably fostered the expansion of xerophytic taxa like *Androcymbium* and many other extant representatives of the flora of this region. The period about 7000 years ago is regarded as the beginning of many important plant landscape transformations in Almería as a direct consequence of climatic improvement (Pantaleón-Cano 1998). These favourable environmental conditions probably aided the final phase of establishment of *A. gramineum*, coinciding with the end of the Holocene optimum, dated some 4500 years before present (Pantaleón-Cano 1998). At present, the upward surge of intensive agriculture in Almería and the growth of human population and livestock in Morocco are threatening many constituents of the plant biodiversity of the regions, including *A. gramineum*. Hopefully, the molecular data discussed in this work, in combination with population-level biological evidence, will help propose adequate preservation strategies for the genetic variation of this endangered Ibero-Moroccan endemic (Caujapé-Castells and Pedrola-Monfort in preparation).

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The research of J. Caujapé-Castells focuses on the application of molecular population genetic and phylogenetic inference to understand floristic connections and diversification processes in the northern quarter of Africa and the Mediterranean, with a special interest in the multidisciplinary comparative study of the origin, diversification and conservation of Canarian and Mediterranean island endemics. R. K. Jansen's research focuses in several areas of plant molecular systematics and evolution, including chloroplast DNA evolution, systematics and evolution of the Asteraceae and Campanulaceae, and the origin and evolution of oceanic island floras.
