

DISPERSAL ACROSS SOUTHERN IBERIAN REFUGIA? INTEGRATING RAPDs, SEQUENCE DATA AND MORPHOMETRICS IN *ARMERIA* (PLUMBAGINACEAE)

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Abstract: A southern Spanish massif (Tejeda/Almijara range, Málaga province, SE Spain) has been previously identified as a contact zone for genotypes of a rare taxon, *Armeria villosa* subsp. *bernisii*, and a frequent one, *A. filicaulis*, based on (1) the discovery of a species-independent geographically structured pattern of variation for nuclear ribosomal ITS sequence data, and (2) the sharing of chloroplast haplotypes, which reveal horizontal transfer between the species. This study uses RAPD data, as a total DNA marker, and morphometrics, as potentially revealing hybridization and introgression, to throw further light on the origin of the above mentioned contact zone. Individuals of the two taxa sampled from the range do not show a F1 hybrid profile for RAPD or for morphometrics. To integrate these results with the previously published sequence data (ITS and chloroplast spacer *trnL-F*) it is proposed that introgressive hybridization has occurred in *A. villosa* subsp. *bernisii*, whereas for *A. filicaulis* the contact zone occurs at the intraspecific level. With the available data, the contact between individuals of *Armeria* with different genotypes in the two taxa may have implied westward migration from a biodiverse massif like Sierra Nevada, and this may apply to other organisms although further data are needed to confirm it.

Keywords: Contact zone, Glaciations, ITS, Migrations, Morphometrics, Phylogeography, RAPD, Refugia, *trnL-F*

INTRODUCTION

The last years have witnessed an important research effort to infer changes produced by severe Quaternary climatic oscillations on species distributions (HEWITT 2000). Most studies have aimed at identifying refugia and pathways by which the northern territories were recolonized after the glacial ages and during interglacial periods following the leading edge model (PETIT et al. 1997, 2002, TABERLET et al. 1998, COMES & KADEREIT 1998, ABBOTT et al. 2000, STEHLIK et al. 2002).

In contrast, a clear gap has become apparent concerning similarly oriented studies aiming to trace spatial changes in species ranges within southern European refugia. The main reason for a scarcity of studies in southern regions is the difficulty of “unraveling the spatial genetic history of species in these refugia” as compared to northern regions (HEWITT 2004). A number of causes are ultimately responsible for such difficulty. Contraction-expansion cycles took place with limited geographical displacement as compared to northern territories (HEWITT 2001). The occurrence of a varied topography in most southern European areas facilitated, or even forced, altitudinal shifts in plant species ranges (HEWITT 1996, FERRIS et al. 1999, CARRIÓN et al. 2001). Such altitudinal migrations might have resulted in population

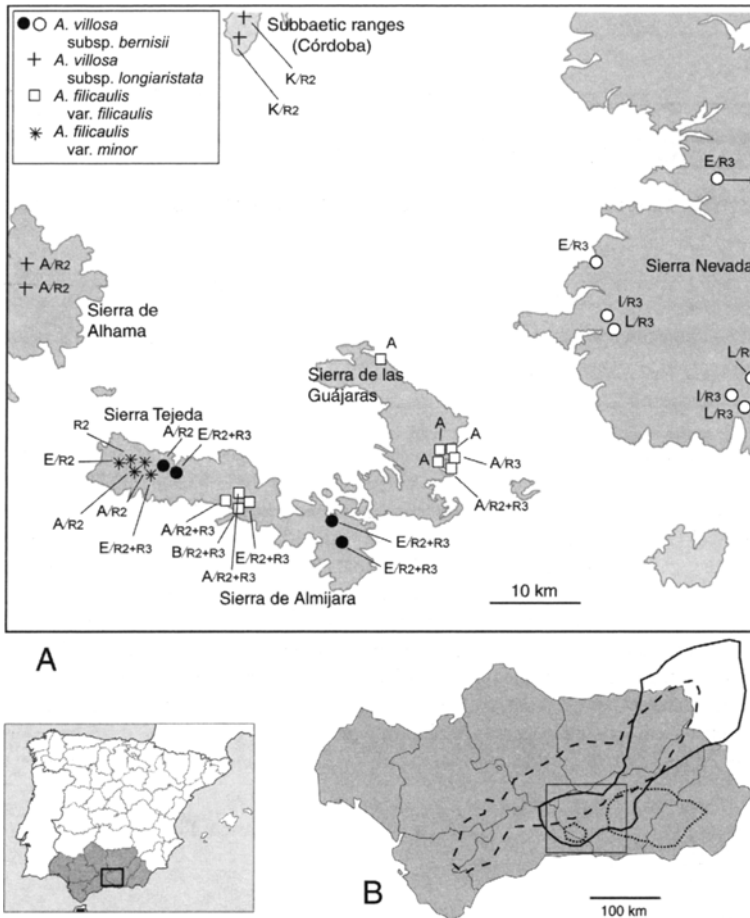


Fig. 1. A – Distribution of *trnL-F* chlorotypes (A, B, E, I, K, L) and ITS ribotypes (R2, R3) in the sampled area (SE Spain) in *Armeria*. Samples of *A. villosa* subsp. *bernisii* from the Tejada/Almirajara range are marked as solid circles. B – Distribution of the taxa sampled in Andalusia (*A. villosa* subsp. *bernisii* – dotted line; *A. villosa* subsp. *longiaristata* – dashed line; *A. filicaulis* s.l. – solid line).

subdivision and eventually differentiation. However, due to the recurrence of the climatically driven spatial shifts during the Quaternary, gene flow between previously isolated populations must have also been frequent (GUTIÉRREZ LARENA et al. 2002). As compared to northern regions, where glaciations provoked severe genetic bottlenecks, preservation of allelic richness is another feature of southern regions (WIDMER & LEXER 2001). The preservation of such diversity within a limited space combined with the dynamic nature of plant distributions caused by Quaternary climatic oscillations must have fostered interactions between genomes and resulted in complex scenarios. This prediction seems to be concordant with available data (HEWITT 2000) and thus justifies the need for a fine-scale geographic approach to try to uncover changes in the distributions in these southern areas.

Accumulated molecular data, particularly in southeastern Spain, shows that *Armeria* WILLD. (*Plumbaginaceae*) may be a suitable system for tracing migrations within glacial refugia despite complex patterns derived from extensive reticulate evolution within the genus. This suitability is based on two sources of data. First, patterns of chloroplast DNA haplotype sharing have provided support for horizontal gene transfer between species of *Armeria* that was possible thanks to altitudinal migrations driven by Quaternary climatic oscillations in several southern Spanish ranges (GUTIÉRREZ LARENA et al. 2002). One of the ranges where these sharing patterns were detected is the southern Spanish massif, on which the present paper is focused. Second, a striking taxonomic-independent geographical structure detected in the variability of the nuclear ribosomal ITS was attributed to extensive gene flow and biased homogenization of ITS copies (FUERTES AGUILAR et al. 1999b, NIETO FELINER et al. 2001). Such structure was documented both at a continental level (FUERTES AGUILAR & NIETO FELINER 2003), and at a fine scale level in southern Spain (NIETO FELINER et al. 2004). The massif studied in the present paper represents a clear contact zone for two of the ITS copies (ribotypes) found in *Armeria*: one is almost exclusive to Sierra Nevada (SE Andalusia) while the other spans along a 100 km strip westwards in central Andalusia (Fig. 1). Because evidence of rapid homogenization of ITS copies was found in artificial hybrids (FUERTES AGUILAR et al. 1999a), the occurrence of two ribotypes both within populations and between individuals is an indication of recent or persistent contacts between individuals from adjacent areas that bear a different ribotype. Therefore, as long as full homogenization of these multicopy regions is not achieved, the ITS conveys information on the geographic origin of plants in *Armeria* and thus potentially traces migrations.

In this paper, we focus on the southern Spanish Tejada/Almijara range as a contact zone inferred from (1) the co-occurrence of ITS ribotypes (at the intraindividual and intrapopulation level) and (2) the patterns of sharing of chloroplast haplotypes detected in two species of *Armeria* occurring there, *A. villosa* subsp. *bernisii* NIETO FEL. and *A. filicaulis* (BOISS.) BOISS. Supporting sequence data have been published in the papers by GUTIÉRREZ LARENA et al. (2002) and NIETO FELINER et al. (2004). Here we use RAPD data, as a total DNA marker, and morphometrics, as potentially revealing hybridization and introgression (NIETO FELINER et al. 1996), to see if further light is thrown on the origin of the above mentioned contact zone in the Tejada/Almijara range. Specifically, we aimed to explore whether RAPDs can support a hybrid or introgressed nature of populations from this range, and if, together with the rest of the available evidence (morphometrics, sequence data), convey information on migrations within *Armeria*.

MATERIAL AND METHODS

Study site and plants

The study is focused on the Tejada/Almijara range, whose NW portion is called Sierra Tejada and the SE part is known as Sierra de Almijara. It is a predominantly xeric range 40 km west of Sierra Nevada. Two species of *Armeria* occur there. Well adapted to dry habitats and sandy soils, *A. filicaulis* is frequent in the Tejada/Almijara range (LAZA 1946, NIETO CALDERA & CABEZUDO 1988), as it is in most of the Andalusian region, also reaching the Rif mountains in Morocco (Fig. 1, NIETO FELINER 1990). This species is represented by two

Table 1. Origin of the samples of *Armeria* from southern Spain used for sequence data (mtDNA ITS, chloroplast *trnL-F*), RAPDs and morphometrics. Ribotypes (ITS) and chlorotype (*trnL-F*) ascription of the sequences is according to NIETO FELINER et al. (2004) and GUTIÉRREZ LARENA et al. (2002), respectively. Accessions of *A. villosa* subsp. *bernisii* from the Tejeda/Almijara range in boldface. ¹⁾ – in NIETO FELINER et al. (2004), ²⁾ – in GUTIÉRREZ LARENA et al. (2002).

Population/Taxon Locality	Voucher specimen	Specimen code	Ribotypes ¹⁾	ITS (GenBank Acc. no.)	Chlorotypes ²⁾	<i>trnL-F</i> (GenBank Acc. no.)	RAPD	Morphometrics
1. <i>A. filicaulis</i> (BOISS.) BOISS. var. <i>filicaulis</i> Málaga: Camillas de Albaída, Sierra Tejada, road to puerto Blanco, 30SVF1479, 900 m, dolomitic sands, <i>Pinus</i> forest understorey	GN 4026	1F	R2 + R3	AY444065	ChA	AJ417266	+	+
	GN 4027	2F	R2 + R3	AY444066	ChB	AJ417267	+	+
	GN 4028	3F						
	GN 4029	4F					+	+
	GN 4030	5F					+	+
	GN 4031	6F					+	+
2. <i>A. filicaulis</i> var. <i>filicaulis</i> Granada: Camillas de Albaída, Sierra Tejada, road to puerto Blanco, 30SVF1680, 1200 m, open dolomitic sands	GN 4032	7F	R2 + R3	AY444067	ChE	AJ417268		
	GN 4035	8F	R2 + R3	AY444068	ChA	AJ417269		
3. <i>A. filicaulis</i> var. <i>filicaulis</i> Granada: Albuñuelas, Sierra de las Guájaras, 30SVF3884, 1180 m, open dolomitic sands	GN 4041	9F	R2 + R3	AY444069	ChA	AJ417270		+
	GN 4042	10F	R3	AY444070	ChA	AJ417271	+	+
	GN 4043	11F			ChA	AJ417272	+	+
	GN 4044	12F			ChA	AJ417273	+	+
	GN 4045	13F			ChA	AJ417274	+	+
4. <i>A. filicaulis</i> var. <i>minor</i> BOISS. Málaga: Alcaucín, Sierra Tejada, path to pico Maroma, 30SVF08344, 1550 m, dolomitic crevices	GN 4006	14M	R2	AY444084				+
	GN 4007	15M						+
	GN 4008	16M						+
	GN 4009	17M						+
	GN 4010	18M						+
	GN 4015	19M						+
5. <i>A. filicaulis</i> var. <i>minor</i> Málaga: Alcaucín, Sierra Tejada, path to pico Maroma, barranco de los Polvilleros, 30SVF0584, 1700 m, limestone rock crevices	GN 4016	20M					+	
	GN 4017	21M	R2	AY179780	ChA	AJ417263	+	+
	GN 4018	22M	R2	AY444085	ChA	AJ417264	+	+
	GN 4019	23M					+	+
	GN 4020	24M						+
	GN 4021	25M						+
6. <i>A. filicaulis</i> var. <i>minor</i> Málaga: Alcaucín, Sierra Tejada, path to pico Maroma, 30SVF0285, 1200 m, limestone rock crevices	GN 4022	26M					+	+
	GN 4023	27M					+	+
	GN 4024	28M					+	+
	GN 4025	29M					+	+
7. <i>A. filicaulis</i> var. <i>minor</i> Málaga: Alcaucín, Sierra Tejada, pico Maroma, 30SVF0684, 1800 m, limestone rock crevices	AP-1	29M	R2 + R3	AY444087	ChE	AJ417277	+	+
	Apfil 1	30M					+	+
	Apfil 2	31M					+	+
	Apfil 4	32M					+	+
	Apfil 5	33M					+	+

8. <i>A. villosa</i> subsp. <i>bernisii</i> NIETO FEL. Granada: Otívar, Sierra de Almijara, pico Navachica W slope, 30SVF2976, <i>Quercus pyrenaica</i> and <i>Sorbus aria</i> forest	MGC 44193	34B	R2 + R3	AY444133	ChE	AY444147	+
9. <i>A. villosa</i> subsp. <i>bernisii</i> Granada: Alhama de Granada, Sierra de Almijara, road to Navachica, 30SVF2781, <i>Quercus pyrenaica</i> and <i>Sorbus aria</i> forest	MGC 44202	35B	R2 + R3	AY444132	ChE	AY444148	+
10. <i>A. villosa</i> subsp. <i>bernisii</i> Granada: Sedella-Alhama de Granada, Sierra de Almijara, "Las Llanadas", 1600 m, <i>Quercus pyrenaica</i> and <i>Sorbus aria</i> forest	AP- 2 Aphern 1 Aphern 2 Aphern 3 Aphern 5	36B 37B 38B 39B 40B	R2 + R3 R2	AY444130 AY444131	ChE ChA	AJ417319 AJ417320	+ + + +
11. <i>A. villosa</i> subsp. <i>bernisii</i> Granada: Monachil, E slope of Cerro Trevenque, near collado Ruquino, 30SVG5803, 1800 m, <i>Pinus</i> forest	GN 4094 GN 4095 GN 4096 GN 4097 GN 4098	41B 42B 43B 44B 45B	R3 R3	AF270510 AY444102	ChI ChL	AJ417292 AJ417293	+ + + + +
12. <i>A. villosa</i> subsp. <i>bernisii</i> Granada: Bubión, Sierra Nevada, road Capileira-Veleta, 30SVF6991, 1950 m, scrub on schist	GN 4112 GN 4113 GN 4114 GN 4115 GN 4116	46B 47B 48B 49B 50B	R3 R3	AY444104 AY444105	ChI ChL	AJ417296 AJ417297	+ + + + +
13. <i>A. villosa</i> subsp. <i>bernisii</i> Granada: Portugos, Sierra Nevada, road Capileira-Veleta, Loma de Piedra Blanca, 30SVF7192, 2230 m, open pasture and scrub on schist	GN 4121 GN 4123 GN 4124	51B 52B 53B	R3	AY444106	ChL	AJ417298	+ + +
14. <i>A. villosa</i> subsp. <i>bernisii</i> Granada: Bérchules, Sierra Nevada, 30SVF8194, 1920 m, scrub on schist	GN 4134 GN 4135 GN 4136 GN 4137	54B 55B 56B 57B					+ + + +
15. <i>A. villosa</i> subsp. <i>bernisii</i> Granada: Dílar, Sierra Nevada, La Zubia, 30SVG5404, 1350 m, scrub on limestone	GN 4078	58B	R3	AY444101	ChE	AJ417326	
16. <i>A. villosa</i> subsp. <i>bernisii</i> Granada: Aldeire, Sierra Nevada, 30SVG9007, 2060 m, clearings on schist	GN 4186	59B	R3	AY444119	ChE	AJ417311	
17. <i>A. villosa</i> subsp. <i>longiaristata</i> (BOISS. et REUT.) NIETO FEL. Málaga: Alfarnate, Sierra de Alhama, puerto del Sol, 30SUF9292, 1250 m, limestone rock crevices, W slope	GN 4232 GN 4235 GN 4237 GN 4239.1 GN 4239.2	60L 61L 62L 63L 64L	R2 R2	AF270505 AY444136	ChA ChA	AF281345 AJ417327	+ + + + +
18. <i>A. villosa</i> subsp. <i>longiaristata</i> Córdoba: Cabra, La Nava, "el Registro", 30SUG7851, 1000 m, wet pastures, clay	GN 4002 GN 4003 GN 4004	65L 66L 67L	R2 R2	AY179831 AY444135	ChK ChK	AF281336 AF292076	

varieties in the massif: a small pink-flowered plant with few scapes (*A. filicaulis* subsp. *filicaulis* var. *minor* BOISS.) in Sierra Tejada and a white-flowered one with multiple scapes (subsp. *filicaulis* var. *filicaulis*) in Sierra de Almirajara. There are three additional subspecies within *A. filicaulis* (GUTIÉRREZ LARENA et al. 2004). The other species in the Tejada/Almirajara range is *A. villosa* GIRARD here represented by subsp. *bernisii*, which is frequent on the schistose substrates of Sierra Nevada, usually under pine forests (MOLERO MESA & PÉREZ RAYA 1987), but is rare in Tejada/Almirajara, where it occurs in relatively fresh forest patches under *Quercus pyrenaica* and *Sorbus aria* (Fig. 1). In other parts of its area, mostly Andalusia, this species is represented by five additional subspecies. The largest range is that of *A. villosa* subsp. *longiaristata* (BOISS. et REUT.) NIETO FEL. (Fig. 1B; NIETO FELINER 1990).

Populations from neighboring mountain ranges have been considered and sampled as well to minimize confounding molecular uniqueness with hybridization between related species (COMES & KADEREIT 1998). This is particularly needed in a genus where hybridization is frequent and thus molecular rarity may not be only the result of isolation but also of horizontal transfer from a differentiated species. Origin of the samples is shown in Table 1. Populations sampled for RAPDs and morphometrics were the same as those for previously published sequence data although use of the same individuals was not possible in many cases due to exhaustion of some samples and to rarity of individuals in their natural sites. This is especially so in *A. villosa* subsp. *bernisii* from the studied massif. The number of individuals in natural populations of *Armeria* vary from several hundred, e.g., in coastal species as *A. maritima* WILLD., to less than twenty in some of the most restricted endangered species (WOODELL & DALE 1993, PHILIPP et al. 1999, NIETO FELINER et al., pers. observ.), a circumstance that limits the availability and precludes heavy intrapopulation sampling. Sequence data have been published in GUTIÉRREZ LARENA et al. (2002) and NIETO FELINER et al. (2004) with two single exceptions: *trnL-F* sequences of individual no. 34B and 35B (for GenBank accession numbers see Table 1). Voucher specimens are kept in herbarium MA except for those corresponding to populations 8 and 9 in Table 1, which are in MGC.

RAPD molecular study

Five individuals per population were sampled in nine populations, resulting in a total of 45 individuals. Five populations belonged to the Tejada/Almirajara range (three of *A. filicaulis* var. *minor* from Sierra Tejada, one of *A. filicaulis* var. *filicaulis* from Sierra de Almirajara, one of *A. villosa* subsp. *bernisii* also from Sierra de Almirajara); one population to Sierra de las Guájaras (*A. filicaulis* var. *filicaulis*) located in between the Tejada/Almirajara range and Sierra Nevada; two to Sierra Nevada (both *A. villosa* subsp. *bernisii*); and one to Sierra de Alhama (*A. villosa* subsp. *longiaristata* north of the Tejada/Almirajara range (Table 1)).

Fifty-nine primers of the Roth Random Primer Kits C, D, M, and 180 (Carl Roth GmbH) were assayed. To select the most useful ones, a series of amplifications of four samples were made using the 59 primers. A first selection of primers was achieved with those producing variable and well-defined banding patterns. These selected primers were then used to amplify all the samples. A second set of primers were discarded at this point because of poor band definition, and/or evident problems of homology assessment. Those primers that could not be

reproduced when comparing banding patterns with those of the first four samples were also discarded. The third phase consisted of a full test of reproducibility by amplifying every single sample with the selected primers. Five primers were finally used: 180-05 (ACCCCAGCCG), C-01 (TTCGAGCCAG), C-06 (GAACGGACTC), C-09 (CTCACCGTCC), and D-03 (GTCGCCGTAA).

PCR reactions were carried out in 20 μ l volumes containing 2.5 ng/ μ l genomic DNA, 2.5 mM MgCl₂, 10 mM primer, 0.2 mM dNTPs and 0.5 U Taq DNA polymerase (Genecraft, Münster, Germany). PCR were performed in a PTC-100TM Peltier-Effect Cycling (MJ Research, Inc.), under the following conditions: incubation at 94 °C for 3 min, followed by 40 cycles at 94 °C for 20 sec, 40 °C for 30 sec and 72 °C for 1 min, followed by a final elongation phase at 72 °C for 8 min. Negative controls were included in every amplification. RAPD products were run in 1.4% agarose (NuSieve, LEEO) gels in 1X TBE accompanied by Amersham Pharmacia 100 bp ruler and then stained with ethidium bromide. The banding pattern was visualized under UV light and photographed. For band scoring, those bands appearing in both amplifications (in the reproducibility protocol each sample was amplified twice; see above) were recorded as present regardless of the intensity of the band as recommended by GROSBERG et al. (1996). Four samples were discarded during this process, thus 41 individuals were finally scored for RAPDs.

Based on the scoring, a 77 \times 41 presence/absence matrix was constructed by hand for further analysis. Phenetic similarity was analyzed by ordination (principal coordinates analysis, PCO) and classification techniques (cluster analysis) applied to a similarity matrix constructed using the Dice coefficient (DICE 1945), thus scoring only the shared presence of RAPD bands (WOLFE & LISTON 1998). A minimum spanning tree (MST) based on the Euclidean distances was superimposed on the scatter plot of the samples against the first three PCO axes to help detect local distortions (pairs of points that look close together but actually are far apart if other dimensions are taken into account). The unweighted pair group method algorithm (UPGMA) was used to construct the phenograms. Both the PCO and the UPGMA analyses were made with the NTSYS-PC vers. 2.1 computer package (ROHLF 2000). To estimate the level of confidence of each cluster in the UPGMA phenogram, 1000 bootstrap pseudoreplicates were generated using the FREETREE program (PAVLÍČEK et al. 1999).

An analysis of molecular variance (AMOVA) was performed to explore how the genetic variation was partitioned among areas and taxa, and if these two aspects matched. Two different designs were tested. The first considered three groups coinciding with three mountain ranges: Sierra Nevada (including two populations of *A. villosa* subsp. *bernisii*), Tejeda/Almijara range (five populations of *A. filicaulis* and one of *A. villosa* subsp. *bernisii*) and Sierra de Alhama (one population of *A. villosa* subsp. *longiaristata*). The second design considered three groups corresponding to taxa, and involved a single change as compared to the first design: the population of *A. villosa* subsp. *bernisii* from Tejeda/Almijara changed to the group containing the other two populations of this taxon from Sierra Nevada. AMOVA analyses were carried out using ARLEQUIN 2.0 (SCHNEIDER et al. 2000).

Table 2. Characters used in the morphometric analysis of *Armeria*.

1	Leaf length
2	Leaf width
3	Leaf length to leaf width ratio
4	Scape length
5	Scape diameter at base
6	Involucral diameter
7	Ratio of the involucral diameter to the length of the involucral sheath
8	Number of involucral bracts
9	Length of outer involucral bracts
10	Length of longest inner involucral bracts
11	Ratio of the shortest to the longest involucral bracts
12	Width of inner involucral bracts
13	Mucro length of inner involucral bracts
14	Mucro length of intermediate involucral bracts
15	Length of spikelet bracts
16	Ratio of the spikelet bract length to the inner involucral bract length
17	Calyx length
18	Calyx lobe length (including awn)
19	Ratio of calyx lobe length to total calyx length
20	Calyx tube length
21	Calyx limb length
22	Ratio of calyx tube length to calyx limb length
23	Length of calyx pedicel scar
24	Presence (vs. absence) of white salt crystals on leaf epidermis
25	Presence (vs. absence) of cilia in leaf margin
26	Pubescent (vs. glabrous) leaves
27	Petal colour (white vs. pink)

Sequence data

Protocols for isolation, amplification, alignment, sequencing and data analysis of the ITS and *trnL-F* regions are described in GUTIÉRREZ LARENA et al. (2002) and FUERTES AGUILAR et al. (1999b). Terminology follows NIETO FELINER et al. (2004) for ITS copies (ribotypes) and GUTIÉRREZ LARENA et al. (2002) for cpDNA haplotypes.

Morphometric study

Based on our previous results showing that morphometric characters are reliable indicators of different levels of introgression in other taxa of *Armeria* (NIETO FELINER 1997, NIETO FELINER et al. 1996), we decided to measure twenty-seven morphological characters (including four binary ones) in three to six specimens per population (43 individuals in total) and to subject them to both ordination and clustering analyses (Table 2). Metric characters below 10 cm were measured with the aid of a Brown & Sharpe Plus digital calliper (model 599-571-3).

A principal components analysis (PCA), based on the correlation matrix, was used to examine structural relationships among characters and among species and to reduce the number of original variables to a few representative uncorrelated ones (the PCs). A minimum

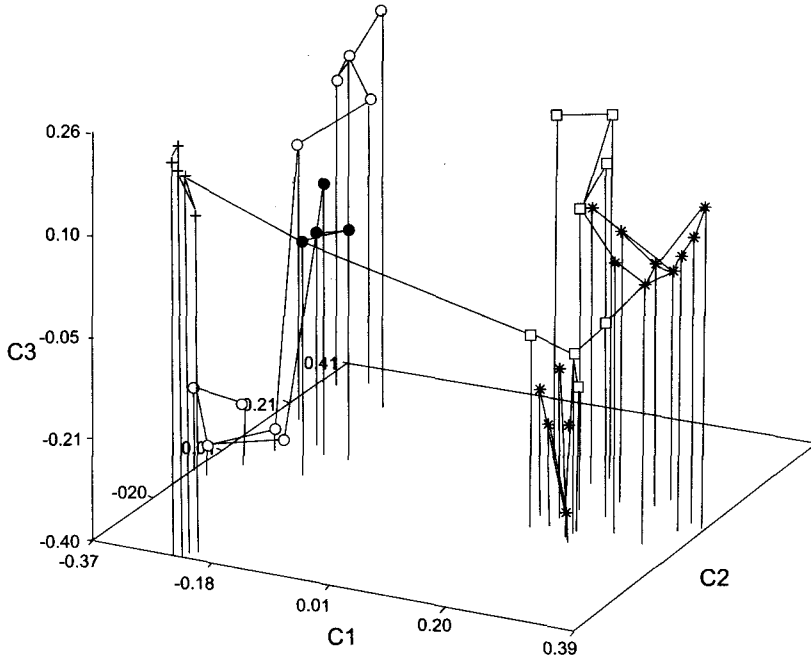


Fig. 2. Principal coordinate analysis of RAPD bands of *Armeria* from SE Spain based on a Dice similarity matrix. Plot of 41 samples in the space defined by the first three principal coordinate axes with a superimposed minimum spanning tree (based on Euclidean distance): *A. filicaulis* var. *filicaulis* (squares), *A. filicaulis* var. *minor* (asterisks), *A. villosa* subsp. *bernisii* (circles, those from the Tejada/Almijara range are solid), *A. villosa* subsp. *longiaristata* (crosses).

spanning tree based on the Euclidean distances was superimposed on the scatter plot of the samples against the first three PCA axes. A UPGMA cluster analysis was conducted based on a similarity matrix constructed using Euclidean distance. Both the PCA and the UPGMA analyses were performed with the NTSYS-PC vers. 2.1 computer package (ROHLF 2000).

RESULTS

RAPD

Only five of the 59 primers assayed in the pilot study produced reproducible banding patterns (180-05, C-01, C-06, C-09, D-03). A total of 77 bands were scored ranging from 300 to 1350 bp. Banding patterns were very variable so that no shared phenotypes were found. Of the total number of bands, eight (10.3%) were exclusive to *A. filicaulis* (incl. both varieties) although not present in all samples, five (6.5%) to *A. villosa* (incl. both subspecies), and eight (10.3%) to *A. villosa* subsp. *bernisii*.

In the PCO, the first three axes accounted for 39.12% of the variance (20.67%, 9.90% and 8.55%, respectively). The scatter diagram of the samples against the first three axes revealed two clusters that match the taxonomic arrangement (Fig. 2). Samples of *A. villosa* (subsp. *bernisii* and *longiaristata*) were clustered on the negative values for the first axis, while those of *A. filicaulis* (var. *filicaulis* and var. *minor*) were on the positive values. Along the minimum

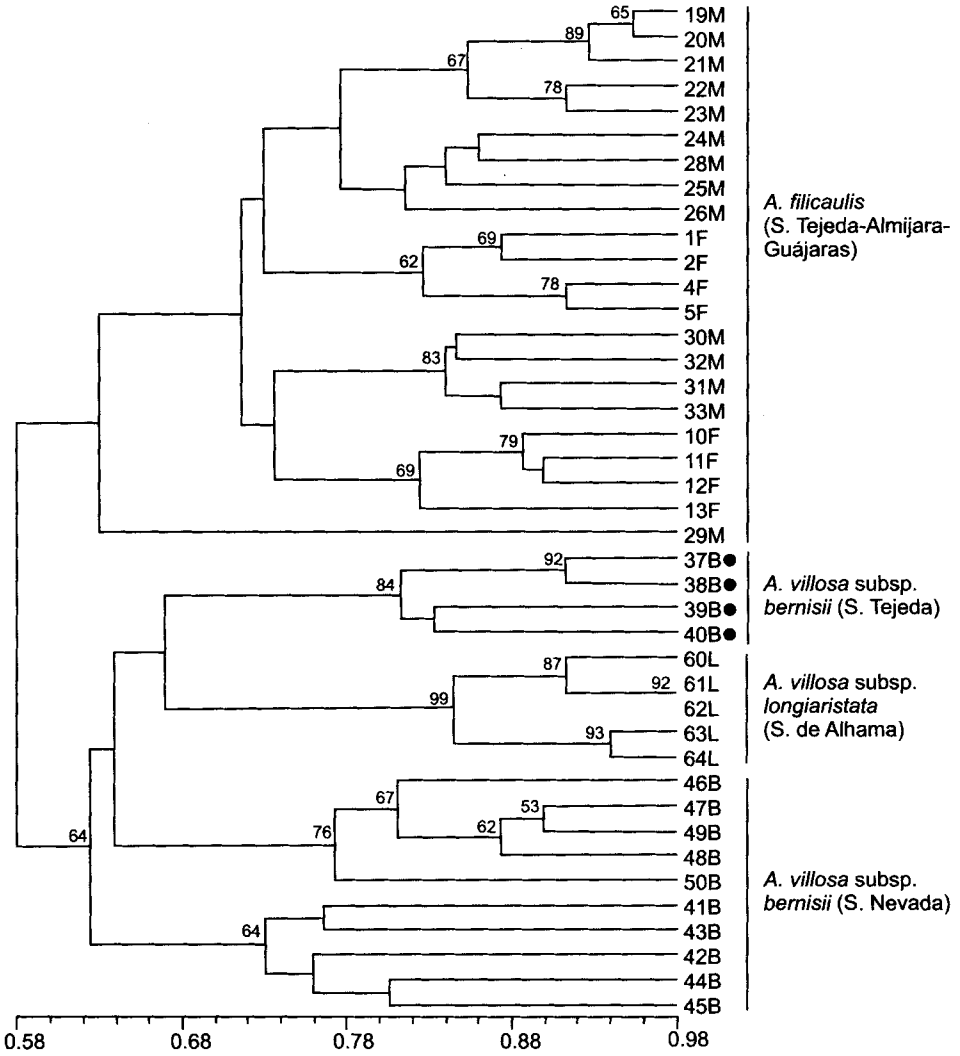


Fig. 3. UPGMA phenogram of 41 samples of *Armeria* from SE Spain based on a Dice similarity matrix from 77 RAPD bands. Numbers refer to accessions in Table 1. Bootstrap values above 50% are indicated on the branches. Acronyms: F (*A. filicaulis* var. *filicaulis*), M (*A. filicaulis* var. *minor*), B (*A. villosa* subsp. *bernisii*, those from the Tejeda/Almijara are marked with an asterisk), L (*A. villosa* subsp. *longiaristata*).

spanning tree, the Tejeda/Almijara population of *A. villosa* subsp. *bernisii* was linked to *A. villosa* subsp. *longiaristata*, to *A. filicaulis* var. *filicaulis* (through an accession from Sierra de las Guájaras), and also to western Sierra Nevada populations of *A. villosa* subsp. *bernisii*. The UPGMA cluster diagram based on the RAPD bands (Fig. 3) provided an overall similar picture with two clusters corresponding to *A. villosa* and *A. filicaulis*, respectively. There was an almost perfect agreement between cluster composition and population origin, that is, samples from the same populations clustered together. The Tejeda/Almijara population of

Table 3. Analysis of molecular variance (AMOVA) based on 76 RAPD phenotypes of *Armeria* from three regions in Eastern Andalusia (Sierra Tejada/Almijara, Sierra Nevada, Sierra de Alhama; see Fig. 1) in three taxa: *A. villosa* subsp. *bernisii*, *A. villosa* subsp. *longiaristata*, *A. filicaulis* (var. *filicaulis* and var. *minor*). Significance tests are based on 1023 permutations.

Source of variation	d.f.	Sum of squares	Variance components	% of variation	P
Among three regions	2	135.5	3.0	19.7	< 0.001
Among populations within regions	6	197.4	5.9	38.4	< 0.001
Among individuals within populations	32	209.4	6.5	42.3	< 0.001
Total	40	542.2	15.5		
Among three taxa	2	153.6	3.8	24.5	< 0.002
Among populations within taxa	6	179.2	5.2	33.5	< 0.001
Among individuals within populations	32	209.6	6.5	42.0	< 0.001
Total	40	542.2	15.6		

A. villosa subsp. *bernisii* grouped with samples of *A. villosa* subsp. *longiaristata* instead of other samples of *A. villosa* subsp. *bernisii* although with low bootstrap support (28).

The two designs tested for the AMOVA differ in the percent of variance explained by differences among groups and among populations within groups (Table 3). The among-groups variance component is higher (24.5%) when groups are considered as taxa than when groups are considered as regions (19.4%). Conversely, the among-populations variance component was larger (38.4% vs. 33.5%) when groups are areas. This result indicates that the Tejada/Almijara population of *A. villosa* subsp. *bernisii* is genetically closer to samples from its own taxon than to samples of *A. filicaulis* from the massif where it occurs.

Morphometric study

In the PCA, the first three axes accounted for 70.2% of the total variance (47.9%, 14.2% and 8.1%, respectively). Characters that contributed most significantly to the first axis were leaf width, scape diameter and calyx lobe length, while those correlated with the second axis were length of inner involucre bracts, ratio of involucre bract length to spikelet bract length and petal colour. The scatter diagram of the samples against the first three axes paralleled that of PCO based on RAPDs in depicting two clusters corresponding to *A. villosa* and *A. filicaulis*, respectively (Fig. 4). Within the latter cluster, samples of *A. filicaulis* var. *minor*, endemic to Sierra de Tejada, were separated from the rest with respect to the second axis, a pattern that is in agreement with previous studies (NIETO FELINER et al. 2001). On the basis of the minimum spanning tree, the Tejada/Almijara samples of *A. villosa* subsp. *bernisii* were connected to other samples of the same subspecies but one was relatively apart from the other two with respect to the third axis. This situation is also reflected in the UPGMA phenogram, where one of the Tejada/Almijara population samples did not cluster with the other two (results not shown). Unlike the UPGMA based on RAPD bands, a large part of the specimens from the two species did not cluster with those from the same population, so that there was not a perfect match between cluster composition and population origin.

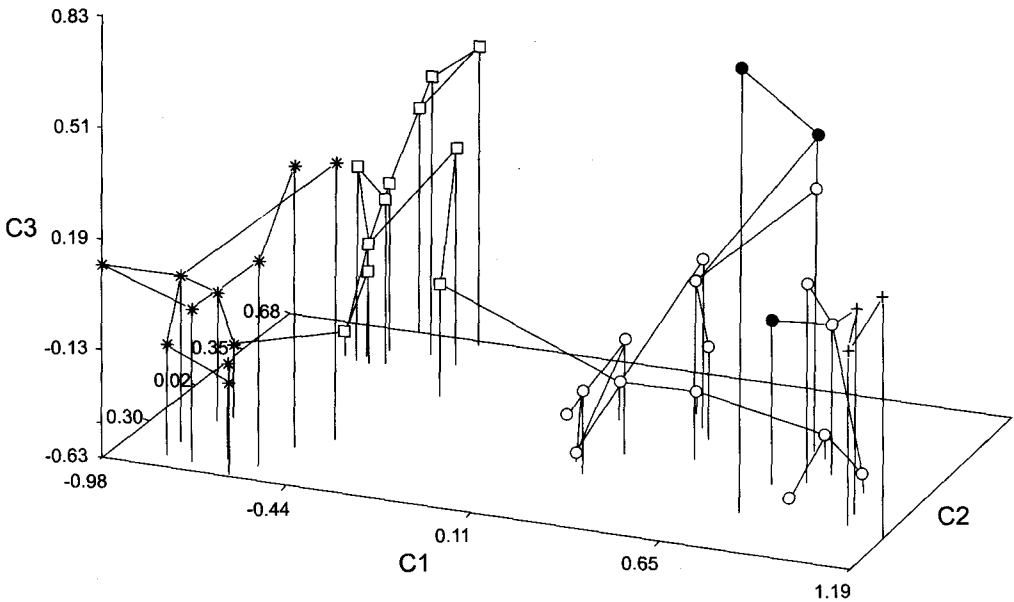


Fig. 4. Principal component analysis of 27 morphometric characters in *Armeria* from SE Spain. Plot of 43 specimens against first three principal axes, with a minimum spanning tree based on the Euclidean distance superimposed: *A. filicaulis* var. *filicaulis* – squares, *A. filicaulis* var. *minor* – asterisks, *A. villosa* subsp. *bernisii* – circles (those from the Tejada/Almijara range are solid), *A. villosa* subsp. *longiaristata* – crosses.

DISCUSSION

RAPD matches morphology and taxonomy

Analysis of intragenomic additive polymorphisms in ITS has proven useful in studying contact zones (MARSHALL & SITES 2001). ITS intragenomic polymorphisms together with the geographic structure of variation detected in this marker have indeed provided sound evidence for reticulation in *Armeria* and revealed the occurrence of contact zones of individuals bearing different ribotypes (FUERTES AGUILAR et al. 1999b, FUERTES AGUILAR & NIETO FELINER 2003, NIETO FELINER et al. 2004). This has been supported by chloroplast sequences (GUTIÉRREZ LARENA et al. 2002). However, the details of the processes occurring in those contact zones in *Armeria* are not fully understood. Therefore, this study aimed to throw additional light on the complex reticulate scenario envisaged through sequence data by completing a total genome survey of variation focused on one of those contact zones. In particular, it was expected that a fingerprinting technique like RAPDs may provide resolution to distinguish F1 or early generations and late-generation hybrids, which are frequent in a genus with low reproductive barriers like *Armeria* (NIETO FELINER et al. 1996).

In our study, RAPD data do not reveal clear signs of hybridization of a F1 profile (i.e., clear addition of bands) between *A. villosa* subsp. *bernisii* and *A. filicaulis* in the Tejada/Almijara range (Fig. 2), although phenotypes in *A. villosa* subsp. *bernisii* are compatible with introgressive or late-generation scenarios. In particular, individuals of *A. villosa* subsp.

bernisii from Tejeda/Almijara exhibit a slightly deviant behavior with respect to the remaining conspecific samples through the MST, which links one of them with *A. filicaulis* var. *filicaulis*. The AMOVA confirms that *A. villosa* subsp. *bernisii* from the Tejeda/Almijara range is genetically closer to the remaining samples of the same taxon than to populations of *A. filicaulis* from the Tejeda/Almijara range (Table 3).

These RAPD results could be questioned based on criticisms of poor reproducibility in early RAPD studies. However, this problem can be overcome by improved laboratory techniques that make the RAPD data comparable to those obtained with other fingerprinting techniques, as recently indicated (NYBOM 2004, KJØLNER et al. 2004). Our study included a thorough test of reproducibility even at the risk of losing a true signal. An indication for reliance on RAPDs in *Armeria* can be seen also in an unpublished study (FUERTES AGUILAR et al. unpubl.) based on a different sampling and primers that allowed distinguishing taxa of hybrid origin from more localized cases of introgression in *Armeria*. The high number of bands and high intraspecific variability is noteworthy, but this has also been found in three other studies that used RAPD data in *Armeria* (NIETO FELINER et al. 2002, BAUMBACH & HELLWIG 2003, FUERTES AGUILAR et al., unpubl.). The allogamous breeding system combined with the perennial life-span and dominant nature of RAPDs is likely to contribute to such a pattern (HAMRICK & GODT 1996, NYBOM & BARTISH 2000), together with the occurrence of interspecific hybridization, which is frequent in *Armeria*. Therefore, despite possible concerns about RAPD data in general, there are indications that those presented here convey information about relationships in our data even if, due to reduced sampling, they do not allow an individual-by-individual comparison with sequence data.

Variation in RAPDs is consistent with taxonomy, because samples of *A. villosa* subsp. *bernisii* from Tejeda/Almijara clustered with the remaining samples of *A. villosa* (Figs. 2, 3). Further, RAPD phenotypes are good markers for population origin and largely consistent in overall pattern with morphology (Fig. 2, 3 vs. Fig. 4). Therefore, RAPD and morphometrics both suggest that the samples of *A. villosa* subsp. *bernisii* from Tejeda/Almijara fit within the species.

RAPD vs. sequence data

ITS sequence data, even if representing genetic variation from a limited portion of the genome as compared to a total DNA marker like RAPD provide crucial information that has been confirmed by cloning (NIETO FELINER et al. 2004). This ITS evidence for extensive reticulation in Andalusian populations of *Armeria* and the implications of a contact zone including those in the Tejeda/Almijara range are strong. In fact, an alternative scenario where incomplete lineage sorting could have generated ITS patterns was conclusively discarded as unrealistic because it would involve multiple selective losses of the same ITS copy in different species within the same territory (FUERTES AGUILAR et al. 1999b). Therefore, the question is how do we harmonize the RAPD and morphometric data presented here with the previous ITS sequence data and patterns of chloroplast haplotype sharing (summarized in Fig. 1)?

To accommodate the co-occurrence of two ribotypes even intraindividually in the Tejeda/Almijara range in both species with a taxonomic-concordant RAPD profile, two

hypothesis are proposed: (1) introgression following hybridization between *A. villosa* subsp. *bernisii* and *A. filicaulis*; (2) gene flow between individuals from the same species bearing different genotypes.

The hypothesis of introgression following hybridization is consistent with the detected combination of genotypes in individuals representing direct evidence of the contact zone (those presenting co-occurring ribotypes, R2+R3) as explained below. Three chlorotypes have been detected in those individuals: ChA in *A. filicaulis* var. *filicaulis*, hereafter FIL(R2+R3–ChA); ChB in *A. filicaulis* var. *filicaulis*, FIL(R2+R3–ChB) and ChE both in *A. filicaulis* (var. *filicaulis* and var. *minor*) FIL(R2+R3–ChE) and in *A. villosa* subsp. *bernisii*, BER(R2+R3–ChE) (Fig. 1, Table 1). When individuals with a single ribotype are considered, one combination was found in both species: FIL(R2–ChA), BER(R2–ChA). Additionally, in geographically close ranges we also found FIL(R3–ChA) (Sierra de las Guájaras), FIL(R3–ChE) and BER(R3–ChE) (Sierra Nevada; NIETO FELINER et al. 2004). Since there are no individuals including R3 exclusively in the Tejada/Almijara range and we know that R3 is predominant and mostly confined to the Sierra Nevada range (NIETO FELINER et al. 2004) it is conceivable that the contact in Tejada/Almijara range has followed after migration of *A. villosa* subsp. *bernisii* from Sierra Nevada into ecologically suitable sites. In particular, the hypothesis of a dispersal of BER(R3–ChE) from Sierra Nevada into Tejada, hybridization with *A. filicaulis* [FIL(R2–ChA)] and subsequent introgression into *A. villosa* subsp. *bernisii* fits the presence of individuals of the latter taxon with R2+R3–ChE (Fig. 1). It should be noted that the genotype R3–ChE is found in Sierra Nevada both in *A. filicaulis* and *A. villosa* subsp. *bernisii* (NIETO FELINER et al. 2004), whereas ChE does not appear westwards from Tejada (GUTIÉRREZ LARENA et al. 2002).

However, this first hypothesis is not satisfactory to explain the occurrence of intragenomic polymorphisms for ITS in the other species inhabiting the massif, *A. filicaulis*. Homogenization of different ITS sequences can be very active in *Armeria* following their merging within a single genome (FUERTES AGUILAR et al. 1999a). In the study area, six of the nine individuals of *A. filicaulis* from Tejada/Almijara (plus one of two from Sierra de las Guájaras) present co-occurring ribotypes (R2+R3). The possibility that such co-occurrence of ribotypes in *A. filicaulis* from different locations arose from a single introduction of R3 genotypes of *A. villosa* subsp. *bernisii* from Sierra Nevada is at odds with the rarity of *A. villosa* subsp. *bernisii* in the range, since backcrossing is hindered. Further, if those six individuals of *A. filicaulis* with co-occurring ribotypes were due to hybridization or introgression with *A. villosa* subsp. *bernisii*, we would probably have detected morphological traces in them, as we did in artificial hybrids from other species (NIETO FELINER et al. 1996). Therefore, it is likely that co-occurrence of ribotypes in *A. filicaulis* within the Tejada/Almijara range are caused by contact between populations from the same species not with *A. villosa* subsp. *bernisii*.

The two proposed hypotheses involve contacts between *Armeria* taxa with different genotypes. With the available data, these might have implied westward migration from Sierra Nevada into Tejada/Almijara both of *A. villosa* subsp. *bernisii* and *A. filicaulis*. The latter species is represented in Sierra Nevada by two subspecies, *A. filicaulis* subsp. *nevadensis* NIETO FEL. et al. (with chlorotypes I, L and E) and subsp. *trevenqueana* NIETO FEL.

(chlorotypes A, E and F). Both subspecies present R3 (NIETO FELINER et al. 2004). Therefore, the possibility that eastern *A. filicaulis* with R3 dispersed into the Tejeda/Almijara range would explain the occurrence of those genotypes in *A. filicaulis* with R2+R3 with no traces of hybridization with *A. villosa* subsp. *bernisii* either based on RAPDs or morphometrics.

Inferring migrations or other historical events based on sequence data from two DNA regions (*trnL-F*, ITS) has to be done with caution because sampling within populations is limited. However, sampling at the regional scale and at the genus level is accurate (more than 200 sequences of ITS, more than 100 of *trnL-F*, including a fine-scale sampling in Sierra Nevada). This background knowledge of the overall variation of the two markers allows inferences on the presence or absence of a given chlorotype or ribotype in the studied ranges, although not on the presence of a given two-marker combination.

Floristic relationships between ranges

Sierra Nevada is one the most important hot spots for biodiversity and endemism in the Mediterranean region (MÉDAIL & QUÉZEL 1997, BLANCA et al. 2002). The occurrence of some sort of boundary and exchange zone for Sierra Nevada and central Andalusian genomes of *Armeria* has been previously documented (FUERTES AGUILAR et al. 1999b, NIETO FELINER et al. 2004). Should the scenario of a westward migration by two species of *Armeria* be confirmed, this would raise the possibility that the contact zone is the result not just of stochastic dispersal of single plants but of horizontal shifts of vegetation. If this was the case, the contact zone could also apply to other organisms. A high percentage of common plant species between western Sierra Nevada and the Tejeda/Almijara range (MOTA et al. 2000, 2002) suggest that exchanges between both massifs are likely. These exchanges may have been facilitated by the occurrence of similar crystalline dolomitic substrates in western Sierra Nevada (pico del Trevenque, Agujas del Dilar), the Tejeda/Almijara range and a geographically intermediate location (Sierra de las Guájaras, Fig. 1). With such a spatially connected series of similar substrates and habitats, it is conceivable that westward (and eastward) migration has taken place following a stepping-stone mode.

The indication of a floristic boundary lying roughly along the Tejeda/Almijara range has also been suggested based on distributional data from monocots (MORENO SAIZ et al. 1998). Detailed molecular data from other organisms are needed to confirm the existence of an effective boundary for the biotas west and east of the massif, the historical processes that have caused it, as well as the direction and degree of permeability for species and genomes across such a boundary.

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