Genetic and morphological diversity in *Armeria* (Plumbaginaceae) is shaped by glacial cycles in Mediterranean refugia

by

Javier Fuertes Aguilar[1], Belén Gutiérrez Larena & Gonzalo Nieto Feliner

Real Jardín Botánico, CSIC, Plaza de Murillo 2, E-28014 Madrid, Spain
[1] jfuertes@rjb.csic.es

Abstract

Fuertes Aguilar, J., Gutiérrez Larena, B. & Nieto Feliner, G. 2011. Genetic and morphological diversity in Armeria (Plumbaginaceae) is shaped by glacial cycles in Mediterranean refugia. *Anales Jard. Bot. Madrid* 68(2): 175-197.

Little is known of the direct effects of Quaternary glaciation-deglaciation cycles in plants within southern European refugia. This study, centered in the Sierra Nevada (S Spain), used RAPD and morphometric data from 36 populations of *Armeria* (Plumbaginaceae) from five taxa belonging to three species that are endemic to that region: *A. filicaulis* subsp. *nevadensis*, *A. filicaulis* subsp. *trevenqueana*, *A. filicaulis* subsp. *alfacarensis*, *A. splendens*, and *A. villosa* subsp. *bernisii*. The results based on genetic analyses at the population level (AMOVA, genetic diversity, genetic distance) and genetic and morphological analyses at individual level (haplotype phenetic distance, PCO, morphometrics) indicate that: (1) genetic diversity decreases with altitude, probably as a result of the postglacial recolonization processes, except in some secondary contact zones between taxa; (2) gene flow among interspecific populations, most likely facilitated by contraction of vegetation belts, led to the formation of hybrid taxa; (3) genetic distances among populations provide a useful basis for studying scenarios with frequent interspecific gene-flow since it allows distinguishing eventual cases of introgression from hybridogenous taxa.

Keywords: altitudinal gradient, glacial refugia, hybridization, Iberian Peninsula, phylogeography, reticulate evolution, Sierra Nevada.

Resumen

Fuertes Aguilar, J., Gutiérrez Larena, B. & Nieto Feliner, G. 2011. La diversidad genética y morfológica en Armeria (Plumbaginaceae) se debe a los ciclos glaciales en los refugios mediterráneos. *Anales Jard. Bot. Madrid* 68(2): 175-197 (en inglés).

Poco se sabe de los efectos directos de los ciclos de glaciación-deglaciación del Cuaternario sobre las plantas de los refugios glaciales del S de Europa. En el presente estudio, centrado en Sierra Nevada (S de España), hemos empleado RAPD y datos morfométricos de 36 poblaciones de *Armeria* (Plumbaginaceae) de cinco táxones pertenecientes a tres especies endémicas de esa región: *A. filicaulis* subsp. *nevadensis*, *A. filicaulis* subsp. *trevenqueana*, *A. filicaulis* subsp. *alfacarensis*, *A. splendens* y *A. villosa* subsp. *bernisii*. Los resultados basados en el análisis genético a nivel poblacional (AMOVA, diversidad genética, distancia genética) y los análisis genéticos y morfológicos a nivel individual (distancia fenética genotipo haploide, PCO, morfometría) indican que: (1) la diversidad genética se reduce con la altitud, probablemente como consecuencia de los procesos de colonización posglaciales, salvo en zonas de contacto entre táxones; (2) el flujo génico entre poblaciones de distinta especie, probablemente facilitado por la contracción de los cinturones de vegetación, resultó en la formación de táxones híbridos; (3) la distancia genética entre poblaciones nos proporciona una base útil para el estudio de escenarios con frecuente flujo interespecífico de genes, ya que permite distinguir casos eventuales de introgresión de táxones hibridógenos.

Palabras clave: gradiente altitudinal, refugios glaciales, hibridización, Península ibérica, filogeografía, evolución reticulada, Sierra Nevada.

Introduction

Glaciations have shaped present-day plant species distributions in the Northern Hemisphere (Hewitt, 1996, 2000) through four main direct effects on populations: isolation, expansion, migration and extinction (Comes & Kadereit, 1998). During glacial periods, species that were adapted to arctic-alpine conditions colonized large extensions of tundra and steppe south of the ice sheets and alpine enclaves, while those adapted to temperate conditions were confined to southern refugia (Ferris & al., 1999). In contrast, interglacial periods favoured the recolonization of newly open habitats in deglaciated areas, thereby inducing migratory movements towards northern areas that allowed the admixture of previously fragmented populations (Gabrielsen & al., 1997). In some of the southern European areas, including high-altitude massifs, a more complex pattern is added to these latitudinal refugia dynamics. Due to the great diversity of habitats along high-elevation mountains, altitudinal shifts in plants induced by climatic changes took place at a much more reduced scale than latitudinal changes (Hewitt, 1999). As a consequence of overlapping latitudinal and altitudinal shifts associated with contraction-expansion cycles, southern refugia became extraordinary arenas for plant evolution that fostered a variety of outcomes including fragmentation, competition, hybridization and speciation (Brochmann & al., 1998; Steen & al., 2000). Most of the phylogeographic and genetic research on European refugia has focused on extinction and recolonization processes in the arctic-alpine flora of northern and central glaciated areas (Abbott & Comes, 2004; Ronikier & al., 2008), the identification of central and southern European refugia (Tribsch & Schönswetter, 2003; Schönswetter & al., 2005), and the pathways of recolonization from such refugia into deglaciated areas (Petit & al., 2003). However, little work has been done on how glaciation-deglaciation cycles affected the plants within the refugia of southern Europe (Kropf & al., 2006, 2008; Petit & al., 2002; Nieto Feliner, 2011).

The Sierra Nevada, the highest massif in the Betic system (SE Iberian Peninsula), emerged during the Langhian (earliest middle Miocene) and estimations from sedimentary basins indicate that as early as in the Tortonian-Messinian transition (7.1 Mya), the massif had already reached 2000 m above sea-level (Braga & al., 2003). Since then, despite erosion forces, it has suffered a progressive uplift until its present-day highest elevation at the Mulhacén peak (3480 m). Climatic oscillations in the Sierra Nevada during the last 5 My (Tertiary-Quaternary) are well documented from paleosol evidence within adjacent basins (Günster

& Skowronek, 2001) as well as from palynological records in caves and lacustrine sediments (Pons & Reille, 1988; Carrión & al., 2001a). Paleosol studies in periglacial areas reveal that at least three cold Quaternary episodes took place in this massif, identified as Riss, Würm, and Late Glacial, each one with a decreasing glacial activity (Gómez Ortiz & Salvador i Franch, 1996; Simón & al., 2000; Schulte, 2002). As a consequence of its orography, during Quaternary ice-ages the Sierra Nevada paradoxically contained the southernmost glaciers in Europe and at the same time the surrounding area was a refugial area for plants (Carrión, 2002; Carrión & al., 2003).

Currently, the Betic and Sub-betic regions in Southern Spain form one of the richest centres for plant endemism in Europe and the western Mediterranean basin (Blanca & al., 2002; Lobo & al., 2001; Médail & Quézel, 1997; Molero Mesa, 1994). Plant genera for which the Sierra Nevada region is a diversity "hot-spot" include *Erodium* (6 endemic taxa) (Salinas, 2009), *Arenaria* (8) (López González, 1990), *Centaurea* (13) (Blanca & al., 2002), and *Erysimum* (7) (Nieto Feliner, 1993). The subject of our study, the Sierra Nevada thrifts *(Armeria)*, is another good example of such a diversity pattern (Salazar, 2009). The massif harbours three species of *Armeria*: *A. splendens* (Lag. & Rodr.) Webb, *A. filicaulis* (Boiss.) Boiss. and *A. villosa* Girard (Table 1). *Armeria splendens* is a high-altitude (above 2880 m) endemic occurring in alpine meadows (locally known as "*borreguiles*"). *A. filicaulis* is represented by three endemic subspecies: *A. filicaulis* subsp. *trevenqueana* Nieto Feliner is endemic to dolomites from Cerro Trevenque and Alayos del Dílar, in the western part of the Sierra Nevada (1700 m), *A. filicaulis* subsp. *alfacarensis* Nieto Fel., Gut. Larena & Fuertes, also occurs on white dolomitic substrates but in the north-east of the Sierra Nevada, on the Sierra de Alfacar and its surroundings, and *A. filicaulis* subsp. *nevadensis* Nieto Fel., Rosselló & Fuertes, is restricted to a narrow area on schist between 2100-2550 m. *A. villosa* is represented by *A. villosa* subsp. *bernisii* Nieto Fel., which is endemic to the eastern Betic mountains, including the Sierra Nevada, and is the most common taxon of *Armeria* inhabiting *Pinus* and *Quercus* forests between 1250 and 2550 m (Blanca & al., 2002; Fuertes Aguilar & al., pers. observations).

Previous studies based on *trnL-trnF* sequences (Gutiérrez Larena & al., 2002) revealed a shared pattern of plastid haplotypes among species suggesting an interspecific horizontal transfer in Sierra Nevada, with an initial colonization process being the establishment of *Armeria splendens*. The study of ITS se-

Table1. Taxonomic synopsis of *Armeria* taxa involved in this study.

Species	Subspecies	Distribution
A. villosa Girard	*longiaristata* (Boiss. & Reut.) Nieto Fel.	Central, Eastern Andalusia
	bernisii Nieto Fel.	S. Tejeda-Almijara, S. Nevada, S. Filabres, S. Gádor
A . filicaulis (Boiss.) Boiss.	*filicaulis*	Eastern Andalusia, S.Tejeda-Almijara, Rif
	trevenqueana Nieto Fel.	Western S. Nevada (Cerro Trevenque, Agujas del Dílar)
	nevadensis Nieto Fel., Roselló & Fuertes	S. Nevada
	alfacarensis Nieto Fel., Gut. Larena & Fuertes	North S. Nevada (up to S. de Alfacar)
A. splendens (Lag. & Rodr.) Webb		S. Nevada

quences (Fuertes Aguilar & Nieto Feliner, 2003; Nieto Feliner & al., 2004), revealed the prevalence of a ribotype (R3) that was almost restricted to the Sierra Nevada and which was present in all samples of the five taxa. However, in a few individuals from populations of *A. filicaulis* and *A. villosa* R3 co-occurred intra-genomically with other ribotypes, either R1 or R2, which were also detected outside the Sierra Nevada. Both studies discovered a species-independent geographically structured pattern of variation of sequence data that was also partially independent from morphological characters. Moreover, the geographical patterns of the nuclear and plastid sequences were also independent from each other. This finding, together with the emerging evidence of genetic exchange at a local scale between the different species (Nieto Feliner & al., 1998), underlined the necessity of a total genome marker study to detect local introgression and putative late generation hybrids. Due to the independence of morphological variation from DNA sequence variation (Fuertes Aguilar & al., 1999) in southern Iberian *Armeria* spp., a complementary morphometric analysis of the involved taxa was also considered relevant to help interpret the molecular data.

We chose the genetic fingerprint marker RAPD because it represents a random sample of the entire genome (Williams & al., 1990; Lorenz & al., 1994) and because it had provided sufficient variation in studies of related taxa of *Armeria* from other areas (Nieto Feliner & al., 2002). Most RAPD is nuclear and thus biparentally inherited in angiosperms (Weising & al., 1995; Rieseberg, 1996). This implies that ad-

ditive patterns of such markers are expected in hybrids at the population level, and consequently they can be used to examine hybridization and introgression (e.g. Sale & al., 1996; Martin & Cruzan, 1999). RAPD phenotypes have been widely used to estimate the genetic structure of populations (Steward & Excoffier, 1996; Palacios & González Candelas, 1997; Jordano & Godoy, 2000; Segarra-Moragues & Catalán, 2003). Despite criticisms raised on the basis of reproducibility (Skroch & Nienhuis, 1995; Jones & al., 1997), a certain re-evaluation for this technique came from a review by Nybom (2004), who indicated that estimates of population genetic structure derived from markers such as RAPD, AFLP and ISSR produce very similar values and may be directly comparable. This view has been confirmed by several studies on plants occurring in the Mediterranean region (Morgan-Richards & Wolff, 1999; Golan-Goldhirsh & al., 2004).

Determining how morphological and genetic diversity patterns may have interacted within putative glacial refugia requires a study encompassing several techniques. Using such an approach, the aims of our study were to: (1) explore the genetic boundaries of the *Armeria* taxa inhabiting the Sierra Nevada and the possible occurrence of gene exchange among them, particularly in the hybridogenous *A. filicaulis* subsp. *nevadensis*; (2) assess the genetic structure and patterns of gene flow among populations from the different taxa; (3) compare morphometric and genetic variation across the taxa inhabiting the Sierra Nevada by verifying congruence with that previously obtained from sequence data; (4) interpret the data in the light

of the glacial history of the massif to help clarify the biogeographical role of southern European massifs as Quaternary refugia.

Material and methods

RAPD sampling

The sampling for the molecular study included a total of 197 individuals from 36 populations across the whole altitudinal and latitudinal geographical distribution of the studied species (Fig. 1, Table 2). From the Sierra Nevada, we sampled populations of *Armeria filicaulis* subsp. *nevadensis* (4 pops.), *A. filicaulis* subsp. *trevenqueana* (1 pop.), *A. splendens* (3 pops.), and *A. villosa* subsp. *bernisii* (17 pops.). In addition, three populations of *A. villosa* subsp. *bernisii* from the surrounding massifs of Sierra de Gádor, Sierra de los Filabres and Sierra de Tejeda-Almijara were included. Five populations of *A. filicaulis* from the nearby Sierra de Tejeda-Almijara (subsp. *filicaulis*) and Sierra de Alfacar (subsp. *alfacarensis*), and three populations of *A. villosa* subsp. *longiaristata* from Sub-betic localities at Cazorla and Alfacar completed the sampling, For each population, samples of leaves from at least 5 individuals were collected and preserved in silica-gel until DNA isolation. One to five individuals per population were collected for the morphometric study and as a voucher for identification. Additionally, 29 individuals from surrounding populations not included in RAPD analysis were included to complete the morphometric study (Table 2). Vouchers were deposited in the MA herbarium.

Primer selection and pilot study

In order to select those primers yielding an informative and reproducible banding pattern, a primer selection test was conducted from a set of 49 oligonucleotides used in previous studies in which within-species variability was observed (Swensen & al., 1995; Stewart & Excoffier, 1996; Jordano & Godoy, 2000; Nieto Feliner & al., 2002). Each primer was tested with nine samples obtained from three individuals belonging to three different taxa. Nine of the tested primers were selected based on number (30>n>5), resolution and intensity of bands. To assess the reproducibility of the PCR products, a survey was performed testing each of the nine primers with one individual per population. Two PCRs separated by at least 24 hours were performed and their respective banding pattern compared. As a result of this test of reproducibility, one primer was discarded and eight were chosen for the final study (OPC-06, OPC-15, OPC-16, OPD-12, OPJ-05, OPJ-09 from Operon Technologies, and UBC-111, UBC-489 from University of British Columbia catalogue). For further reliability, bands considered reproducible were restricted to a range between 100 and 1200 bp.

DNA isolation, PCR and RAPD analysis

DNA was isolated from leaf tissue using a modified method of Doyle & Doyle (1987) within 30 days after collection. 0.2 g of silica-dried leaf tissue samples were homogenized and added to 0.20 ml of CTAB buffer (1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 2% CTAB, 1% PVPP, 0.25% ß-mercaptoethanol). Samples were incubated at 65 °C for 60 min, and 0.5 ml of chloroform-isoamyl alcohol (24:1) was then added and the mixture was gently mixed for 10 min. After centrifugation at 15,000 rpm for 10 min, the aqueous layer was removed and the process repeated. The aqueous layer was subsequently precipitated with 2.5 volumes of −20 °C ethanol. The DNA was spooled, air-dried, and re-suspended in 50 µl TE. DNA was quantified using a Gene Quant II UV spectrophotometer (Amersham Biosciences Europe GmbH, Cerdanyola, Barcelona, Spain). DNA samples were adjusted to a concentration of 35 ng/µl. RAPD reactions were performed in 50-µl aliquots containing 25 ng of template DNA, PCR buffer (50
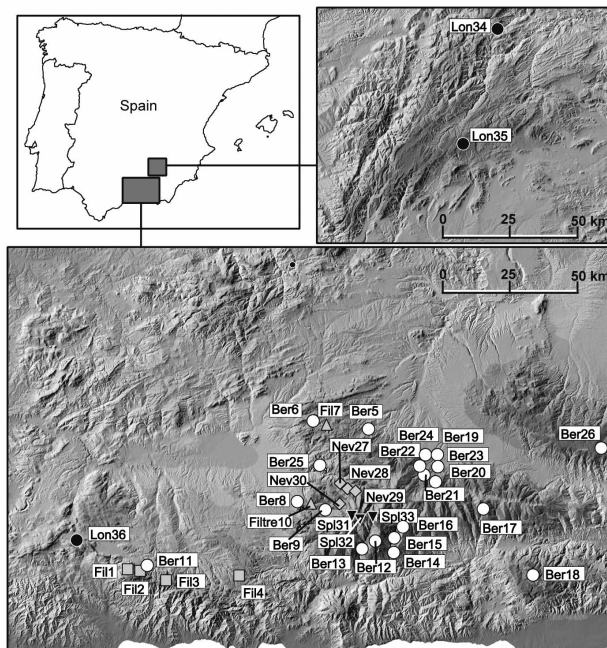

Fig. 1. Geographic location of the *Armeria* populations included in the study. *A. splendens*: black inverted triangles; *A. filicaulis* subsp. *filicaulis*: grey squares; *A. filicaulis* subsp. *nevadensis* grey diamonds; *A. filicaulis* subsp. *trevenqueana* and *A. filicaulis* subsp. *alfacarensis*: grey triangles; *A. villlosa* subsp. *longiaristata*: black circles; *A. villosa* subsp. *bernisii*: white circles.

Table 2. List of populations and individuals sampled for RAPD and morphometric studies of *Armeria* spp.

Taxon	Population	Locality	Indiv. RAPD	Indiv. morphology
A. filicaulis subsp. alfacarensis	Fil7	Granada: Beas de Granada, Puerto de la Mora, road to Pozuelo, 3 km before the pass, 30SWG6124, 1400 m, rocky slopes with crystalline dolomites.	36A-40A	297A
A. filicaulis subsp. alfacarensis		Granada: La Peza, Cerro del Salto del Caballo, 30SVG62, 1300 m, dolomitic sands.		295A
A. filicaulis subsp. alfacarensis		Granada: Sierra de Alfacar, El Puerto, 30SVF52.		296A
A. filicaulis subsp. filicaulis		Málaga: Canillas de Albaida, Sierra Tejeda, road to puerto Blanquillo, 30SVF1479, 900 m, dolomitic sands, *Pinus* forest understory.	11F-15F	11F-15F, 257F
A. filicaulis subsp. filicaulis	Fil3	Málaga: Canillas de Albaida, Sierra Tejeda, road to puerto Blanquillo, 30SVF1680, 1200 m, open dolomitic sands.	16F-19F	
A. filicaulis subsp. filicaulis	Fil4	Granada: Albuñuelas, Sierra de las Guájaras, 30SVF3884, 1180 m, open dolomitic sands.	21F-25F	21F-25F
A. filicaulis subsp. nevadensis	Nev27	Granada: Güéjar-Sierra, Sierra Nevada, Barranco de las Ánimas, 30SVG6407, 2280 m, low-scrub on schist.	138N-142N	139N-142N
A. filicaulis subsp. nevadensis	Nev28	Granada: Güéjar-Sierra, Sierra Nevada, cerca del Albergue de San Francisco, 30SVG6506, 2200 m, low-scrub on schist.	143N-147N	147N, 277N- 278N
A. filicaulis subsp. nevadensis	Nev29	Granada: Monachil, Sierra Nevada, Peñones de San Francisco, 30SVG6407, 2340 m, low-scrub on schist.	148N-151N	149N-152N
A. filicaulis subsp. nevadensis		Granada: Dílar, Sierra Nevada, Hoya de la Mora, 30SVG650, 2550 m, xeric pastures.		279N
A. filicaulis subsp. nevadensis		Granada: Güéjar-Sierra, Sierra Nevada, dirt road to Loma de los Cuartos, Barranco de las Castillas, 30SVG7010, 2200 m.		276N
A. filicaulis subsp. nevadensis	Nev30	Granada: Dílar, Sierra Nevada, Peñón del Dílar, 30SVG6103, 2460 m, xeric pastures, on schist.	153N-157N	154N-157N
A. filicaulis subsp. nevadensis		Granada: Güéjar-Sierra, Sierra Nevada, dirt road to Loma de los Cuartos, right after the control barrier, Loma de las Cunas de los Cuartos, 30SVG6910, 2100 m.		158N
A. filicaulis subsp. trevenqueana		Granada: Monachil, Cerro Trevenque base, 30SVG5803, 1850 m, sandy NE slopes of crystalline dolomites.	51T-55T	51T
A. filicaulis subsp. trevenqueana	Filtre10	Granada: Monachil, Cerro Trevenque, 30SVG5703, 1900 m, sandy NE slopes of crystalline dolomites.	56T-58T	

Table 2. List of populations and individuals sampled for RAPD and morphometric studies of *Armeria* spp. (Continuation).

Taxon	Population	Locality	Indiv. RAPD	Indiv. morphology
A. filicaulis subsp. *filicaulis*		Málaga: Alcaucín, Sierra Tejeda, trail to Pico Maroma, 30SVF08344, 1550 m, dolomitic crevices.		252M-256M
A. filicaulis subsp. *filicaulis*	Fil1	Málaga: Alcaucín, Sierra Tejeda, trail to Pico Maroma, Barranco de los Polvilleros, 30SVF0584, 1700 m, limestone rock crevices.	1M-4M	1M, 5M
A. filicaulis subsp. *filicaulis*	Fil2	Málaga: Alcaucín, Sierra Tejeda, trail to Pico Maroma, 30SVF0285, 1200 m, limestone rock crevices.	6M-10M	6M-9M
A. splendens		Granada: Dílar, Sierra Nevada, dirt road to Veleta and Laguna de las Yeguas, km 42, 30SVG6602, 2280 m, near alpine meadows, on schist.		280S-284S
A. splendens	Spl31	Granada: Dílar, Sierra Nevada, near Laguna de las Yeguas, 30SVG6601, 2870 m, on schist.	163S-165S, 167S	163S-167S
A. splendens		Granada: Capileira, Sierra Nevada, Laguna de Aguas Verdes, 30SVG6800, 2990 m, alpine meadows, near lagoon.		285S
A. splendens	Spl32	Granada: Capileira, Sierra Nevada, Laguna de Río Seco, 30SVG6900, 3100 m, alpine meadows between the road and the lagoon.	168S-172S	169S-172S, 289S
A. splendens		Granada: Capileira, Sierra Nevada, Laguna de la Caldereta, 30SVG7001, 3040 m, on schist.		287S-288S
A. splendens	Spl33	Granada: Capileira, Sierra Nevada, Laguna de la Caldera, 30SVG7001, 3040 m, on schist.	173S-177S	173S-177S, 286S
A. splendens		Granada: Güéjar-Sierra, Sierra Nevada, near Laguna Juntillas, from Los Cervatillos to Lagunilla de la Mojonera, 30SVG7607, 3000 m, alpine meadows apart from the creek.		178S-179S
A. villosa subsp. *bernisii*	Ber5	Granada: La Peza, road to Quéntar, 1 km before Puerto Blancares, 30SWG6723, 1250 m, developed soils on dolomites, in *Quercus* forest understory.	26B-30B	212B-215B, 26B-30B
A. villosa subsp. *bernisii*	Ber6	Granada: Beas de Granada, Puerto de la Mora, road to Pozuelo, 1 km before the pass, 30SWG5925, 1300 m, pastures, rocky soils on limestone.	31B, 33B-35B	31B, 33B-35B
A. villosa subsp. *bernisii*		Granada: Beas de Granada, road to Pozuelo, 1 km before the pass, 30SWG6025, 1350 m, pine forest understory, rocky soils on limestone.		216B-218B
A. villosa subsp. *bernisii*	Ber8	Granada: La Zubia, dirt road to Cerro Trevenque, 30SVG5404, 1350 m, broom heath on limestones, near the creek.	41B-45B	41B-45B
A. villosa subsp. *bernisii*	Ber9	Granada: Monachil, E of Cerro Trevenque, near Collado Ruquino, 30SVG5803, 1800 m, pineforest understory, on dolomites.	46B-50B	46B-50B

Table 2. List of populations and individuals sampled for RAPD and morphometric studies of *Armeria* spp. (Continuation).

Taxon	Population	Locality	Indiv. RAPD	Indiv. morphology
A. villosa subsp. bernisii		Granada: Cáñar, Sierra Nevada, Barranco del río Chico, near camping area Fuente Palo, 30SVF6391, 1800 m, schistose slope near the creek.		219B–222B
A. villosa subsp. bernisii		Granada: Soportújar, Sierra Nevada, Barranco Frío, 30SVF6489, 1590 m, on schistose slope, near rush marsh and a creek.		223B
A. villosa subsp. bernisii	Ber12	Granada: Bubión, Sierra Nevada, dirt road from Capileira to Veleta, 30SVF6991, 1950 m, low shrub on schist.	64B–68B	64B–65B, 67B
A. villosa subsp. bernisii		Granada: Pórtugos, Sierra Nevada, Loma de Piedra Blanca, 30SVF7192, 2230 m, rocky xeric pastures, on schist.		224B
A. villosa subsp. bernisii		Granada: Pórtugos, Sierra Nevada, dirt road from Capileira to Veleta, Cascajar Negro, 30SVF7295, 2630 m, thorn cushion shrubs, on schist.		225B
A. villosa subsp. bernisii	Ber13	Granada: Pórtugos, Sierra Nevada, dirt road from Capileira to Veleta, Loma de Piedra Blanca, 30SVF7192, 2230 m, rocky xeric pastures, on schist.	69B–73B	69B–72B
A. villosa subsp. bernisii	Ber14	Granada: Bérchules, Sierra Nevada, dirt road above the village, 30SVF8092, 1830 m, broom heath, on schist near a depression.	74B–78B	74B–78B
A. villosa subsp. bernisii	Ber15	Granada: Bérchules, Sierra Nevada, dirt road above the village, 30SVF8194, 1920 m, broom heath, on schist.	79B–83B	80B–83B
A. villosa subsp. bernisii	Ber16	Granada: Bérchules, Sierra Nevada, dirt road, barranco de las Angosturas above the village, 30SVF8199, 2060 m, nitrified meadows, on schist.	84B–88B	84B–88B
A. villosa subsp. bernisii		Granada: Bérchules, Sierra Nevada, dirt road, near La Cruz de los Caminos, 30SVF8496, 1950 m, on schist.		226B–227B
A. villosa subsp. bernisii		Granada: Sierra Nevada, Puerto de la Ragua, Barranco del Hornillo, 30SVG9705, 1880 m, broom heath, on schist.		228B
A. villosa subsp. bernisii	Ber17	Granada: Bayárcal, Sierra Nevada, near Las Minillas, 30SWG0101, 1920 m, clearings in broom heath, on schist.	89B–93B	89B–93B, 229B
A. villosa subsp. bernisii	Ber19	Granada: Ferreira, Sierra Nevada, road to Puerto de la Ragua, 30SVG9513, 1600 m, dry pastures on schist.	99B–103B	99B–102B
A. villosa subsp. bernisii	Ber18	Almería: Berja, Sierra de Gádor, Balsa Bermeja, 30SWF1587, 1800 m, rocky slopes, on limestones.	94B–95B, 97B–98B	230B, 94B–98B
A. villosa subsp. bernisii		Granada: Aldeire, Sierra Nevada, dirt road, 30SVG9510, 1790 m, roadside with dry pastures, under pineforest.		231B–235B

Table 2. List of populations and individuals sampled for RAPD and morphometric studies of *Armeria* spp. (Continuation).

Taxon	Population	Locality	Indiv. RAPD	Indiv. morphology
A. villosa subsp. bernisii		Granada: Aldeire, Sierra Nevada, dirt road, 30SVG9307, 1920 m, pineforest clearings, on schist, in firewall.		236B-240B
A. villosa subsp. bernisii		Granada: Aldeire, Sierra Nevada, dirt road, near Barranco de los Tejos, 30SVG9007, 2060 m, clearings, on schist, near the creek.		241B
A. villosa subsp. bernisii	Ber20	Granada: Aldeire, Sierra Nevada, dirt road, near Barranco de los Tejos, 30SVG9007, 2100 m, disturbed zones and roadsides, on schist.	104B-108B	104B-108B, 242B
A. villosa subsp. bernisii	Ber21	Granada: Aldeire, Sierra Nevada, dirt road, Loma del Collado del Pino, 30SVG8909, 1990 m, clearings among thorn cushion shrubs, on schist.	109B-113B	109B-113B
A. villosa subsp. bernisii	Ber22	Granada: Lanteira, Sierra Nevada, dirt road, Loma del Collado del Pino, 30SVG8910, 1900 m, rocky slopes, on schist.	114B-117B	114B-117B
A. villosa subsp. bernisii	Ber23	Granada: Aldeire, Sierra Nevada, dirt road, 30SVG9010, 1820 m, rocky slopes, on schist.	118B-122B	118B-122B
A. villosa subsp. bernisii	Ber24	Granada: Lanteira, Sierra Nevada, dirt road, Hoya de las Monjas, 30SVG9011, 1720 m, rocky slopes, on schist.	123B-127B	123B-126B, 243B
A. villosa subsp. bernisii ,	Ber25	Granada: Monachil, Sierra Nevada, El Dornajo, 30SVG6008, 2020 m, on limestones NW slope.	128B-132B	128B, 130B-131B
A. villosa subsp. bernisii	Ber26	Almería: Bacares, Sierra de los Filabres, Fuente de la Venta Luisa, 30SWG4320, 1950 m, roadside, dry pastures in afforested pineforest on shales.	133B-137B	134B, 136B
A. villosa subsp. bernisii	Ber11	Granada: Sedella-Alhama de Granada, "Las Llanadas", 1600 m, understory of *Quercus pyrenaica* and *Sorbus aria* forest.	59B-63B	211B
A. villosa subsp. longiaristata		Albacete: Villaverde de Guadalimar, dirt road from creek to La Cañada, 30SWH4654, 1200 m, pineforest clearings.		265L
A. villosa subsp. longiaristata		Albacete: Villaverde de Guadalimar, Fuente de las Raigadas, 30SWH4653, 1240 m, disturbed zone close to dirt road.		266L
A. villosa subsp. longiaristata	Lon34	Jaén: Siles, road from Segura de la Sierra, crossroad with JF-7012, 7 km from Camp Río Madera, 30SWH3540, 1300 m, pineforest clearings.	183L-187L	183L, 187L
A. villosa subsp. longiaristata		Jaén: Segura de la Sierra, secondary road JF-7012, near El Yelmo, 30SWH3134, 1300 m.		261L

Table 2. List of populations and individuals sampled for RAPD and morphometric studies of *Armeria* spp. (Continuation).

Taxon	Population[a]	Locality	Indiv. RAPD	Indiv. morphology
A. villosa subsp. *longiaristata*		Jaén: La Matea, road to El Cerezo, 30SWH3515, 1280 m, roadside.		262L
A. villosa subsp. *longiaristata*		Jaén: Cazorla, Sierra de Cazorla, road from la Nava de San Pedro to El Cerezo, km 27-28, 30SWG1193, 1380 m, pine forest clearings.		263L–264L
A. villosa subsp. *longiaristata*	Lon35	Jaén: Cazorla, Sierra de Cazorla, 200 m from la Nava de San Pedro, 30SWG1093, 1320 m, pine forest clearings.	188L–192L	192L
A. villosa subsp. *longiaristata*		Córdoba: Cabra, La Nava, "El Registro", 30SUG7851, 1000 m, meadows.		267L–269L
A. villosa subsp. *longiaristata*		Córdoba: Rute, Sierra de Rute, 30SUG8133, 1200 m, rocky pastures on limestones, northern slope.	198L–199L	
A. villosa subsp. *longiaristata*	Lon36	Málaga: Alfarnate, Sierra de Alhama, Puerto del Sol, western slopes above the pass, 30SUF9292, 1250 m, crevices in limestone rocks.	193L–197L	

[a] Missing codes under Population column correspond to individuals exclusively used in the morphometric study.

mM KCl; 10 mM Tris-HCl pH 8.8; 0.1% Triton X-100), 3.0 mM $MgCl_2$, 0.25 mM each dNTP, 0.2 mM primer, and 1.25 U Taq DNA polymerase (Biotools, B&M Labs, Madrid, Spain). Amplification reactions were performed in a Perkin-Elmer Gene Amp 9700 Thermal Cycler (PE Biosystems, Foster City, California, USA) programmed for 45 cycles of 94 ℃ for 30 s, 45 ℃ for 60 s, and 72 ℃ for 60 s, with a final 72 ℃ hold for 7 min after the completion of cycles. A negative control with DNA substituted by ddH_2O was included in each PCR. PCR products were separated by electrophoresis on 2.5% high resolution agarose (MS-8, Pronadisa, Madrid, Spain) 1× TAE gels. Aliquots of 15 µl of PCR were loaded in each well and run for 5 hrs at 90 v in a refrigerator at 4 ℃. A well was loaded every 7 lanes with a 100 bp standard (100-3000 bp, 30 bands, BioRad Laboratories SA, Madrid, Spain) for band length calibration. Gels were stained in a 3 µg/ml ethidium bromide solution for 10 min, destained in distilled water for 35 min, visualized on a UV transilluminator and digitized using a Gel documentation system Gel Doc 2000 (BioRad Laboratories SA, Madrid, Spain) equipped with a Kodak DC-290 digital camera. Digitized gels were exported for band analysis using Quantity One 4.2.1 software package (BioRad Laboratories SA, Madrid, Spain). Homology was assigned between bands from different lanes based on size and performed automatically, allowing a 1.5-2 % tolerance depending on primers. This procedure made feasible the comparison among different gels and the use of a uniform criterion for the large amount of processed samples. Variations in intensity between bands of equal molecular weight across samples were not considered. Those bands that could not be reproduced twice in the same individual during the pilot study were discarded. A final 1/0-matrix representing band presence/absence was then generated and exported to .xls format for further analysis.

Statistical and genetic analysis of RAPD

A multivariate analysis of the binary RAPD matrix by Principal Coordinates Analysis (PCO) was performed using NTSYS-pc 2.11a (Rohlf, 2002). To avoid bias produced by false negatives the Dice coefficient (S) (Dice, 1945), also known as the Sørensen coefficient, was used to construct the individual pairwise similarity matrix, as suggested by Wolfe & Liston (1998). The Jaccard coefficient was also calculated but results did not differ significantly (data not shown). A genetic distance version of the Dice coefficient was generated transforming the similarity matrix in NTSYS-pc using the formula $D = (1-S)^{1/2}$, as pro-

posed by Legendre & Legendre (1998). This distance matrix was then imported into PAUP*4.0b10 (Swofford, 2002) to produce a Neighbour-Joining tree (Saitou & Nei, 1987) with support values of branches estimated through 1000 bootstrap analyses. The partitioning of the genetic variance within and among populations was evaluated through the analysis of molecular variance (AMOVA), as presented in the package ARLEQUIN 2.0 (Schneider & al., 2000). The analysis was performed at three hierarchical levels using two hypotheses: (1) considering the three species as separate groups (*Armeria filicaulis*, *A. villosa*, and *A. splendens*); and (2) considering the seven identified taxa as separate groups (*A. filicaulis* subsp. *filicaulis*, *A. filicaulis* subsp. *trevenqueana*, *A. filicaulis* subsp. *nevadensis*, *A. filicaulis* subsp. *alfacarensis*, *A. villosa* subsp. *bernisii*, *A. villosa* subsp. *longiaristata*, and *A. splendens*). The significance values for each variance component were obtained through non-parametric permutations using 1000 replicates. Pairwise genetic distances between populations were calculated based on the Φ_{ST} statistics values generated by AMOVA among RAPD phenotypes. The distance matrix was then used to produce a tree using the Neighbour-Joining algorithm. Branch support was also assessed through 1000 bootstrap replicates in PAUP* 4.0b10. Gene diversity (H_{pop}) was estimated for each population from the frequency of RAPD bands using Nei's (1973) algorithm adopted for RAPD by Peever & Milgroom (1994). Genetic distances between populations using Nei's genetic distance (1972) with an estimation of support for each cluster, were also inferred using TFPGA (Miller, 1997).

To further identify the genetic structure underlying our dataset, a Bayesian non-hierarchical clustering method was applied using STRUCTURE version 2.3 (Hubisz & al., 2009), which calculates the likelihood for the individuals being assigned to a given number (K) of groups based on minimizing linkage between groups, and maximizing linkage within them. Ten replicates of each value of K (1 to 15) were run under the correlated allele model (Falush & al., 2007) with a MCMC and burn-in settings of 10^6 and 10^5 iterations, respectively. We followed two criteria to detect the optimal number of K groups: First, the method suggested in the original STRUCTURE paper, which consists of comparing mean log likelihoods from the different cluster assignments penalized by one-half of their variance (Pritchard & al., 2000); Second, the highest increment in DK values (Evanno & al., 2005) from the different cluster assignments.

The correlation analysis between genetic diversity and altitude was performed using a Pearson product-moment correlation with SPSS 12.0.1 statistical software package (SPSS, Chicago, IL)

Morphometric analysis

A total of 30 characters were measured (Table 3) for 212 individuals, including at least one individual from each population surveyed in the RAPD study. Characters were selected based on previous studies (Nieto Feliner & al., 1996, 2001), and included sixteen quantitative continuous, one quantitative discrete, seven qualitative discrete (2 or 3-state), and five ratios of quantitative continuous characters (Table 3). For quantitative continuous characters three measurements were taken in each individual and the mean value was calculated. Standardization of variables was performed before the analyses. To display relative positions of individual phenotypes in the morpho-space, a Principal Component Analysis (PCA) was performed as an ordination method. A minimum spanning tree (MST) based on Euclidean distance was superimposed on the scatter-plot to help identify the closest relatives among different swarms. All these analyses were carried out with NTSYS-pc 2.11a.

Results

RAPD variation and genetic relationships among taxa

A total of 62 RAPD bands, ranging between 200 and 1200 bp were scored, with an average of 7.7 bands per primer. After processing, no replicated RAPD profiles were found, so the number of RAPD multiband phenotypes was equal to that of individuals (N = 197). Only two primers generated products that were useful to distinguish taxa. Primer OPC-06 produced a 950 bp band that discriminated *Armeria villosa* subsp. *bernisii* from the remaining taxa. Two more bands (760 and 360 bp) generated by OPC-15 grouped, exclusively, individuals of *A. villosa* together with *A. filicaulis* subsp. *trevenqueana*. No diagnostic markers suitable for the discrimination of any of the three species were identified. A marker was considered diagnostic if it was present at a frequency equal to or greater than 90% in all individuals of a given taxon and present at a frequency lower than or equal to 10% in all individuals of other taxa. We considered this relaxed criterion to be justified due to the frequent occurrence of hybridization within this genus (Fuertes Aguilar & Nieto Feliner, 2003). In the PCO analysis, the first three coordinates explained 20.8% of the variance (8.6%, 7.0% and 5.2% respectively). Phenotypes spread across the space defined by the first two axes, which together accounted for 15.6% of the total

Table 3. Morphometric characters used in the multivariate analysis of *Armeria* spp. individuals.

Number	Character description
1	Leaf length
2	Leaf width
3	Ratio of leaf length to leaf width
4	Scape length
5	Diameter of the scape at base
6	Diameter of the involucre
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 outer to the longest inner involucral bracts
12	Width of inner involucral bracts
13	Length of mucro of inner involucral bracts
14	Length of mucro of intermediate involucral bracts
15	Length of spikelet bracts

Number	Character description
16	Ratio of the length of spikelet bracts to the length of inner involucral bracts
17	Calyx length
18	Calyx lobe length (including awn)
19	Ratio of calyx lobe length (including awn) 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	White salt crystals (absence, 0; presence, 1)
25	Cilia on leaf margins (absence, 0; presence, 1)
26	Glabrous (0) vs. pubescent (1) leaves
27	Slender (0) vs. firm (1) involucral bracts
28	Calyx with 10 (0) vs. 5 (1) rows of longitudinal hairs
29	Involucral bracts colour: yellowish-cream (0), tawny or copper (1), brown (2)
30	Petal colour: white (0), pink (1), purplish (2)
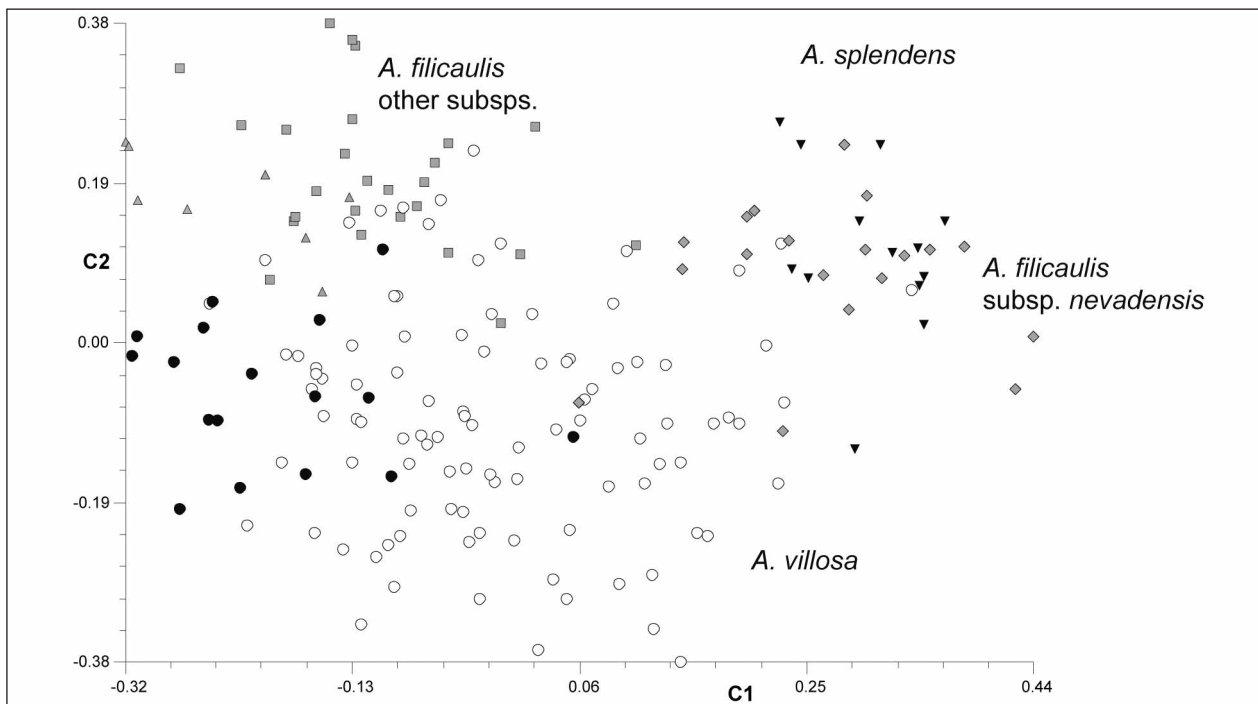
Fig. 2. Genetic variation among 197 studied individuals of *Armeria* spp. as displayed by a bi-dimensional scatter-plot using the two first coordinates of a principal coordinates analysis based on a Dice similarity matrix of RAPD's phenotypes. Symbols as in Fig. 1.

variance (Fig. 2). No clear-cut groups matched any of the species. In the scatter-plot, three clusters could be distinguished on the basis of taxonomic ascription. One large cluster encompassed *A. villosa* samples (subsp. *longiaristata* and subsp. *bernisii*), a second included all *A. filicaulis* representatives except those of *A. filicaulis* subsp. *nevadensis,* and a third grouped representatives of *A. splendens* and *A. filicaulis* subsp. *nevadensis.* Individuals of *A. villosa* subsp. *bernisii* and

A. filicaulis subsp. *alfacarensis* connected the first and second groups.

The neighbour-joining tree based on the Dice coefficient better visualized the genetic relationships among RAPD phenotypes (Fig. 3). Despite low bootstrap support, the unrooted NJ-tree showed 6 clusters. Clusters 1 (Boostrap Support [BS] 52%), 2 (BS < 50 %), and 3 (BS < 50 %) were formed by geographically close populations of *Armeria villosa* subsp.
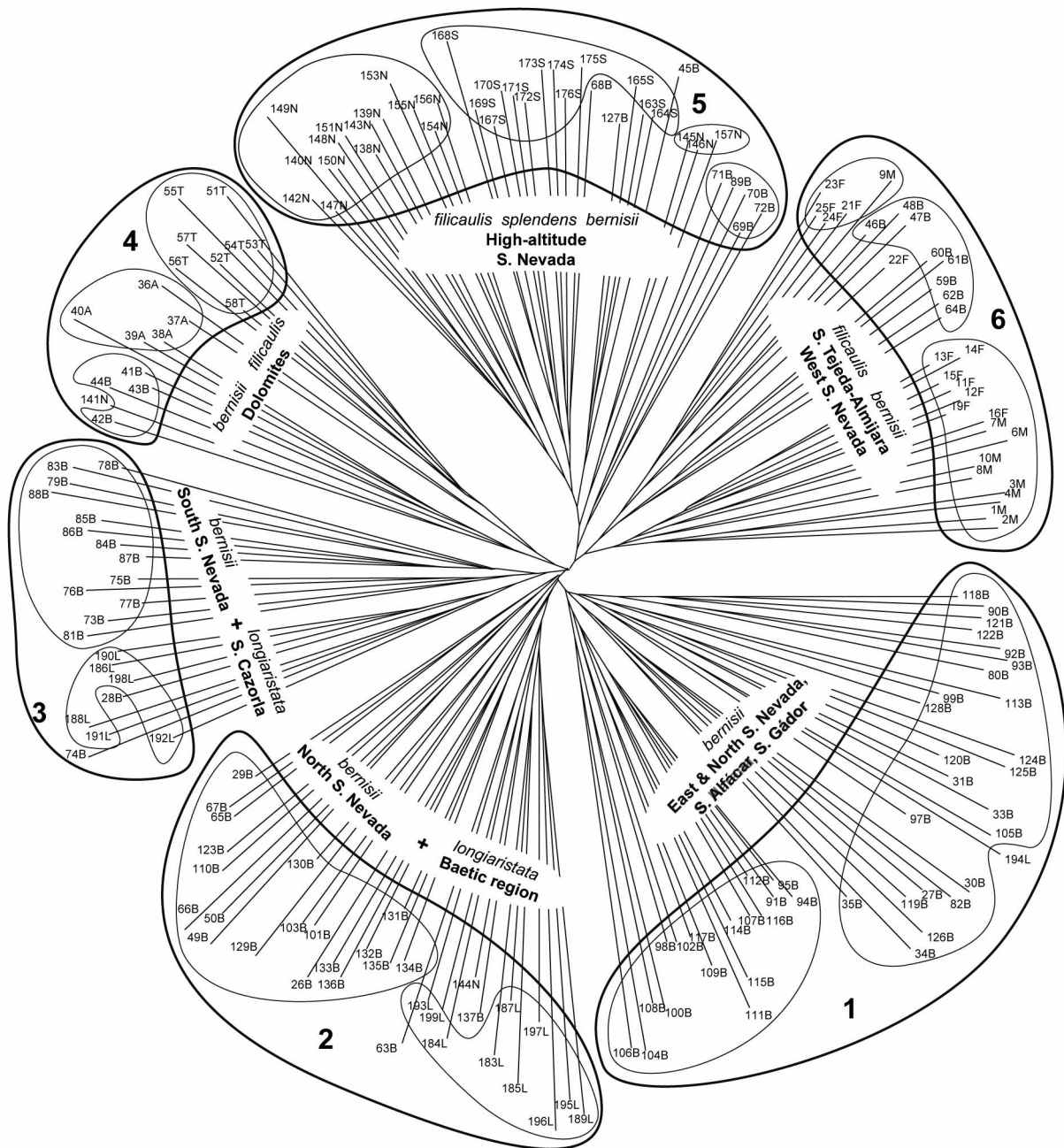

Fig. 3. Neighbour-joining tree based on distance transformed from Dice similarity matrix from all analysed *Armeria* spp. individuals. Taxonomic ascription and individuals identified as in Table 2.

bernisii, joined by Betic (Lon36) *A. villosa* subsp. *longiaristata* samples in cluster 2, and by Sierra de Cazorla (Lon36) *A. villosa.* subsp. *longiaristata* populations in cluster 3. Cluster 4 was almost exclusively formed by *A. filicaulis* populations that thrive on crystalline dolomites (Fil7, Filtre10) in northern (Sierra de Alfacar) and western (Cerro Trevenque, Alayos del Dílar) Sierra Nevada. Interestingly, individuals of *A. villosa* subsp. *bernisii* from dolomites (Ber8) also joined this cluster. Cluster 5 was the most heterogeneous taxonomically; it included all *A. splendens* populations, all *A. filicaulis* subsp. *nevadensis;* a few high-altitude individuals of *A. villosa* subsp. *bernisii* (mostly from Ber13, 2230 m) also fell within it. Cluster 6 was exclusively formed by *A. filicaulis* and *A. villosa* subsp. *bernisii* from the Tejeda-Almijara mountain range.

Morphological variation

Since morphological characters are the basis of the present taxonomy of the studied taxa, it is not surprising that morphology was in accordance with the taxonomic ascription. In the PCA analysis, the first three components explained 62.4% of total variance (43.3%, 11.6, and 7.6%, respectively). The projections of individuals onto the morpho-space created by the first three axes (Fig. 4) displayed four main groups. The first three (A, B, C) each corresponded to one of the species occurring in the Sierra Nevada: *Armeria villosa*, *A. filicaulis* and *A. splendens*, while the fourth (D) was composed of *A. filicaulis* subsp. *nevadensis* individuals, which were connected to the *A. splendens* and *A. filicaulis* groups in the MST. Individuals of *A. villosa* subsp. *bernisii* clustered with those of *A. villosa* subsp. *longiaristata*, thus supporting the coherence of the species. The position of *A. villosa* subsp. *bernisii* was intermediate between *A. villosa* subsp. *longiaristata* and *A. filicaulis* subsp. *filicaulis*. Three outlier individuals of *A. filicaulis* (295A, 296A, 297A) from the Alfacar population (Fil7) were placed closer to *A. villosa* subsp. *longiaristata* than to their remaining conspecific individuals, especially when PC1 and PC2 were considered. The plants from this locality and its surroundings have recently received taxonomic recognition as *A. filicaulis* subsp. *alfacarensis*
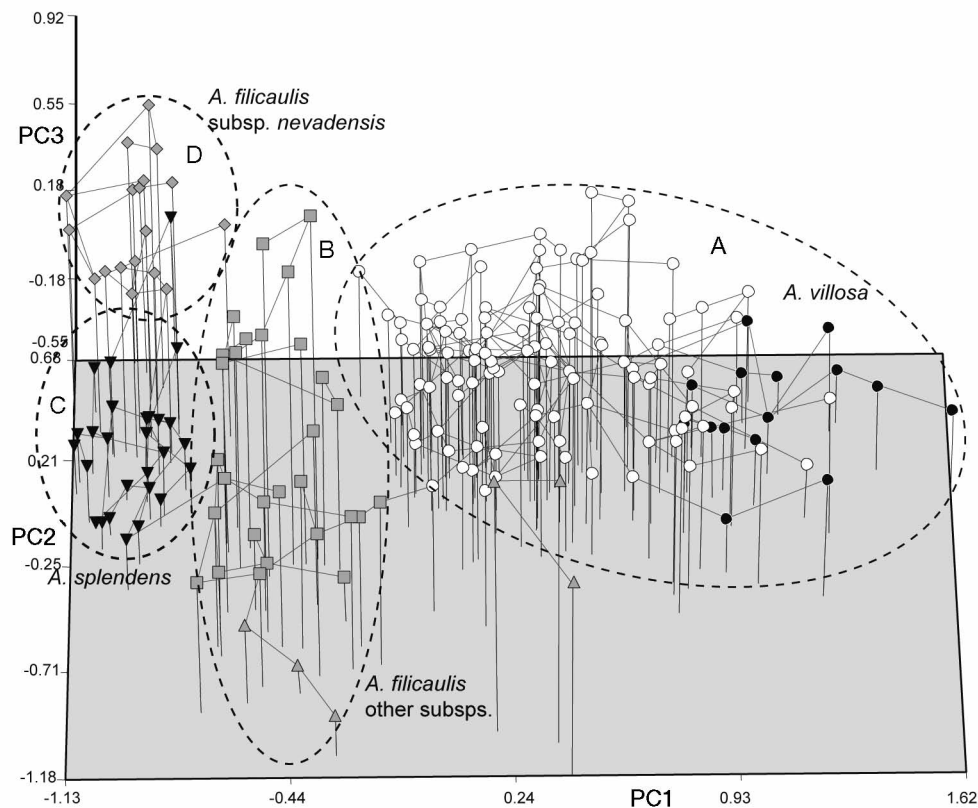

Fig. 4. Morphological variation among 212 studied *Armeria* spp. individuals, as displayed by tri-dimensional scatter-plot using, the three first components of a principal components analysis based on 30 morphological characters (Table 3). A minimum-spanning tree based on the Euclidean distance helps to identify connection between groups. Centroids identify clusters for major taxa in the morphospace. Symbols as in Fig. 1.

(Gutiérrez Larena & al., 2004). The UPGMA-tree based on Euclidean distance (not shown) distinguished the same groups outlined in PCA.

Population structure and genetic variation

Under both AMOVAs, groups by species (3) and groups by taxa (7), variation within populations was exceedingly higher than variation among populations and among groups. The percentage of total variance explained by among-populations differences decreased (from 25.1% to 17.9%) and the among-group differences increased (from 8.75% to 16.77%) when groups were considered as taxa (Table 4). The values of genetic diversity are presented in Table 5. The highest values in genetic diversity in the Sierra Nevada were found in *Armeria villosa* subsp. *bernisii* from mid to low-elevation populations (Ber5 and Ber15 with 0.30). In contrast, the lowest values were found at the extremes of altitudinal range: at very low elevation in *A. filicaulis* (Fil3) from Puerto Blanquillo (900 m) and at high-elevation populations (2870 m) of *A. splendens* (Spl31) (0.16 and 0.15, respectively). Values of H_{pop} ranged from 0.17 to 0.21 in *A. filicaulis* subsp. *nevadensis* to 0.16-0.25 in the remaining *A. filicaulis* populations. In *A. villosa*, the values for subsp *longiaristata* (0.21-0.25) fell within the range detected for subsp. *bernisii* (0.17-0.29). The Bayesian analysis contributed to detect a genetic structure with clusters independent of their taxonomic ascription (Fig. 5). The similarity coefficients calculated from the STRUCTURE results including all individuals showed that 1 and 2 are the only values of K that gave an unambiguous distribution of the individuals. In addition, the highest likelihood was obtained for $K = 2$ and $K = 6$, and the DK values also

supported $K = 6$. The six identified groups matched the population clusters and subclusters identified by the Φ_{ST} – based NJ–tree of populations. Actually, the same geographic pattern and a moderate congruence with taxonomy could be observed in the neighbour-joining tree based on Φ_{ST} pairwise values among populations (Fig. 5). The first cluster (C1, Fig.5) included populations of *A. filicaulis* (Fil1, Fil2, Fil3, Fil4, Filtre10) and *A. villosa* subsp. *bernisii* (Ber11, Ber9) from the Tejeda-Almijara range and Western Sierra Nevada, plus Fil7, a population from the dolomites of Alfacar that was genetically linked to another dolomiticolous population (Ber8) from the Cerro Trevenque. The second cluster was divided into two subclusters, the first one (C2, Fig. 5) exclusively composed of *A. villosa*, and the second one (C3, Fig.5) encompassing all the populations of *A. splendens* and *A. filicaulis* subsp. *nevadensis* plus two of *A. villosa* subsp. *bernisii* (Ber12 and Ber26). The remaining group of *A. villosa* subsp. *bernisii* (populations Ber14, Ber 15, and Ber16), all from the southern slopes of the Sierra Nevada, fell apart from the others, being closer to the lowland *A. villosa* subsp. *longiaristata* (C4, Fig. 5).

Discussion

Genetic variation and population structure

It is generally accepted that the parameters describing the genetic variation, its partition among and within populations and the genetic divergence among populations, result from the combination of two types of factors: life history traits (e.g. breeding system, seed dispersal, life form, geographic range) of the taxon and the evolutionary history of its studied populations. Based on data in various studies using

Table 4. Analysis of molecular variance (AMOVA) based on 62 RAPD bands of *Armeria* spp. populations. Significance level after 1023 iterations : *p < 0.05; **p < 0.01; ***p < 0.001.

Source of variation	d.f.	Sum of squared deviations	Variance components	% of total variance
Among species (3 groups)[a]	2	120.19	0.90***	8.75
Among populations within species	33	643.29	2.57***	25.07
Within populations	142	963.50	6.79***	66.18
Among taxa (7 groups)[b]	6	306.18	1.74***	16.77
Among populations within taxa	29	457.30	1.85***	17.87
Within populations	142	963.50	6.78***	65.37

[a] *A. splendens*, *A. villosa*, and *A. filicaulis*; [b] *A. splendens*, *A. villosa* subsp. *longiaristata*, *A. villosa* subsp *bernisii*, *A. filicaulis* subsp. *nevadensis*, *A. filicaulis* subsp. *trevenqueana*, *A. filicaulis* subsp. *alfacarensis* and *A. filicaulis* subsp. *filicaulis*.

Table 5. Synthesis of geographical, genetic and morphological information of studied populations of *Armeria* spp. in southern Spain. Populations codes correspond to those indicated in Table 1.

Population	Population size[a]	Altitude m asl	Morphological group [b]	RAPD indiv.[c]	H_{pop}	RAPD pop.[d]	ITS ribotype[e]	trnL-F[f] haplotype
Fil1	II	1700	B	6	0.20	C1	R2	A
Fil2	II	1200	B	6	0.25	C1	R2	E
Fil3	I	900	B	6	0.16	C1	R2/R3	A, B
Fil4	II	1180	B	6	0.20	C1	R3, R2/R3	A
Filtre10	I	1875	B	4	0.21	C1	R3	E, F
Ber8	II	1350	A	4 (5)	0.24	C1	R3	E
Ber25	I	2020	A	2 (1)	0.20	C2	R2/R3	E
Fil7		1400	B	4	0.24	C1	R2/R3	A
Ber26		1950	A	2	0.20	C2	R1/R3	A
Nev30	III	2460	D	5	0.20	C3	R3	A
Nev27	II	2280	D	5 (4)	0.17	C3	R3	I
Nev28	II	2200	D	5	0.21	C3	R3, R1/R3	L
Nev29	II	2340	D	5	0.18	C3	R3	I
Spl31	II	2870	C	5	0.15	C3	R3	I
Spl32	II	3100	C	5	0.17	C3	R3	I
Spl33	II	3040	C	5	0.17	C3	R3	I
Ber5	IV	1250	A	1, 2 (3)	0.29	C2	R3	I
Ber6	II	1300	A	1	0.21	C2	R2/R3	L
Ber9	II	1800	A	6 (2)	0.23	C1	R3	I, L
Ber11	I	1600	A	6 (2)	0.25	C1	R2, R2/R3	A, E
Ber12	II	1950	A	2 (5, 6)	0.26	C3	R3	I, L
Ber13	III	2230	A	5 (3)	0.22	C3	R3	L
Ber14	III	1830	A	3	0.25	C4	R3	I
Ber15	II	1920	A	3 (1)	0.30	C4	R2/R3	I
Ber16	II	2060	A	3	0.17	C4	R3, R1/R3	I, L
Ber17	II	1920	A	1	0.22	C2	R3	I
Ber18	III	1800	A	1	0.18	C2	R2/R3	I
Ber19	I	1600	A	1(2)	0.27	C2	R3	K, L
Ber20	I	2100	A	1	0.28	C2	R3, R2/R3	I, L
Ber21	II	1990	A	1(2)	0.22	C2	R3	I
Ber22	II	1900	A	1	0.18	C2	R3	I
Ber23	II	1820	A	1	0.19	C2	R3	L
Ber24	III	1720	A	1 (2, 5)	0.26	C2	R3	L
Lon34		1300	A	2 (3)	0.21	C4	R4	I
Lon35		1320	A	3 (2)	0.27	C4	R4	G
Lon36	I	1250	A	2 (1)	0.26	C4	R2	A

[a] I: N < 50; II: 50 < N < 100 individuals; III: 100 < N < 500; IV: N > 500; [b] Groups represented in Fig. 4; [c] Groups represented in Fig. 3; [d] Clusters represented in Fig. 5; [e] Ribotype groups after Nieto Feliner & al., 2004; [f] Haplotype groups after Gutiérrez Larena & al., 2002.

molecular markers, Nybom & Bartish (2000) and Nybom (2004) estimated the average values of within-population diversity and among-population diversity for several life history traits, thus: $H_{pop} = 0.25$ and $\Phi_{ST} = 0.25$ for long-lived perennials; $H_{pop} = 0.20$ and $\Phi_{ST} = 0.26$ for endemics; $H_{pop} = 0.28$ and $\Phi_{ST} = 0.34$ for narrowly distributed plants; and $H_{pop} = 0.27$ and $\Phi_{ST} = 0.27$ for obligate outcrossers. In contrast to a study on continental populations of another species of *Armeria*, *A. maritima,* in which Baumbach & Hellwig (2003) reported low values of genetic variability per population (0.122-0.193) and high levels of differentiation ($\Phi_{ST} = 0.46$) for this perennial out-

crosser, the values reported here for part of our studied *Armeria* species are in accordance with the parameters indicated by Nybom & Bartish (2000) and Nybom (2004). The average values of genetic diversity observed in *A. filicaulis* ($H_{pop} = 0.20$, $\Phi_{ST} = 0.34$) and *A. villosa* ($H_{pop} = 0.24$, $\Phi_{ST} = 0.37$) match those of obligate outcrossers with comparable distribution areas. Indeed, the partitioning of the RAPD variation revealed the highest proportion of within-population variation (66.2 % and 65.4%), which is in accordance with the pattern in obligate outcrossers. An important implication from hierarchical analysis of variance is that differences among groups (either species or

subspecies) accounted for a small percentage of the total variance (Table 4), suggesting the importance of interspecific gene exchange. The lack of diagnostic markers represented by fixed private bands in total-genome markers for the species and subspecies is good evidence that gene flow occurs across species barriers, as detected in *Pistacia* (Yüzbasioglu & al., 2008).

However, some of our studied taxa showed deviations from the expected values for obligate outcrossers. *Armeria splendens* ($H_{pop} = 0.17$) and, to a lesser extent, *A. filicaulis* subsp. *nevadensis* ($H_{pop} = 0.19$) were closer to those of facultatively allogamous plants than to the values expected for obligate outcrossers with a self-incompatibility system, like most *Armeria* species (Baker, 1966). However, no evident signs of breakdown in the incompatibility system were observed in these species since the two morphs associated to the incompatibility system are found (Fuertes Aguilar & Nieto Feliner, unpubl. data). The possible occurrence of polyploids in *A. filicaulis* subsp. *nevadensis* and *A. splendens*, which could break the diallelic incompatibility system, was also discarded after a cytological survey of high-alti-

tude populations of the two taxa (Fuertes Aguilar & Nieto Feliner, unpubl. data). Rather, we think that the low genetic diversity values observed in populations of *A. splendens* and *A. filicaulis* subsp. *nevadensis*, and especially in the case of the former, are not related to life history traits but to the recent history of their habitats. The small-sized scattered populations of *A. splendens* strictly depend on habitats surrounding lakes created by morraines after deglaciation. Present populations of *A. splendens* were therefore in the frontline of re-colonization of deglaciated environments after the last stadial, and might have suffered a loss of genetic variation due to founder effects and bottlenecks during migration (Gabrielsen & al., 1997; Piñeiro & al., 2011). In the case of *A. filicaulis*, the populations of subsp. *nevadensis*, concentrated in N-NW slopes of the Sierra Nevada, are altitudinally and geographically isolated from the remaining populations of *A. filicaulis*, and therefore their inter-populational gene flow might be restricted and so favouring genetic drift and stronger genetic differentiation, as reported in other alpine taxa by Aægisdóttir & al. (2009), and Winkler & al. (2010).
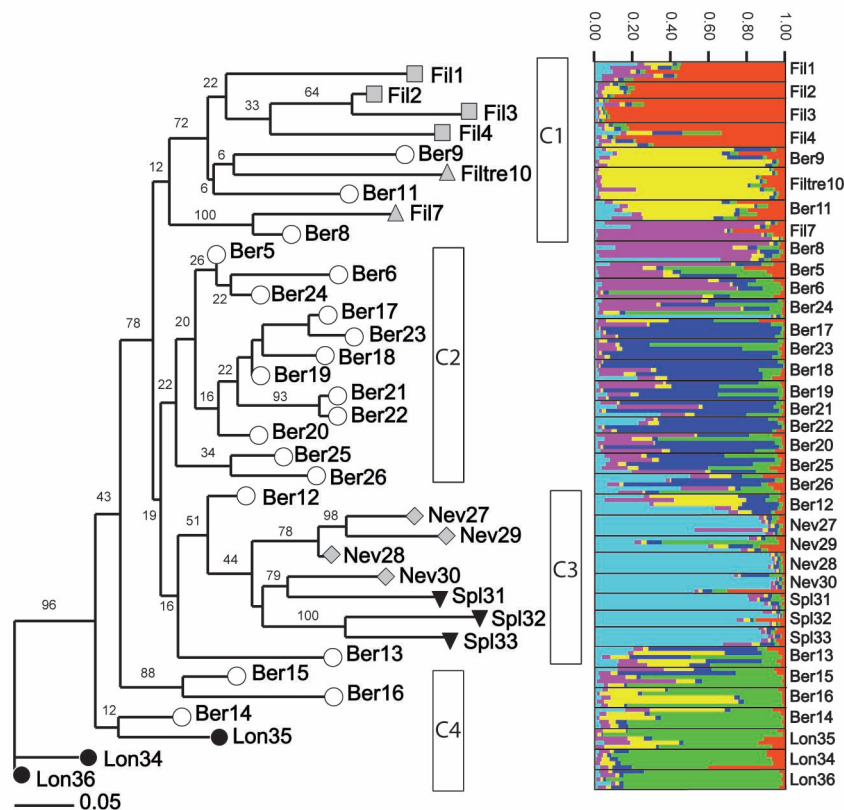

Fig. 5. Left, Neighbour-joining tree of studied *Armeria* spp. populations using Φ_{ST} as an estimation of genetic distance showing four main clusters. Symbols as in Fig. 1. Right, genetic structuring of *Armeria* spp. individuals detected by genetic admixture analysis in STRUCTURE for K = 6.

Genetic diversity along an altitudinal gradient

Correlation analyses between populational parameters and geographic distances have been used to study the history of postglacial migrations (Gabrielsen & al., 1997, Holderegger & al., 2002). In the present study, the linear distances are so small, the topography is so irregular, and the groups are so heterogeneous that any kind of corrected geographic distance would be inaccurate to describe true among-population relationships. Nevertheless, the distribution of the populations along an elevation gradient perpendicular to the direction of the glaciation/deglaciation front allows us to explore whether recolonization influenced any of the population parameters. The correlation analysis (Fig. 6) produced a significant negative correlation ($r^2 = 0.410$, d.f. = 18, p = 0.003) between elevation and population genetic diversity (H_{pop}) for *Armeria villosa* subsp. *bernisii*. The populations of *A. splendens* and *A. filicaulis* subsp. *nevadensis* analysed together (to compensate their low number) gave no significant result ($r^2 = 0.497$, d.f. = 7, p = 0.051), probably due to the low number of populations sampled, but they showed a trend towards a lower H_{pop} values as the altitude increased. To perform the analysis on *A. villosa* subsp. *bernisii*, a correction for elevation values was needed for populations on the southern slope of the Sierra Nevada. Climatic and vegetation conditions for Supra-Mediterranean and Oro-Mediterranean thermotypes on the southern slopes appear approximately 200 m higher than on the northern slopes (Lorite & Valle, 1999; Rivas-Martínez & al., 1986). Therefore, for these specific correlation analyses, we compensated the elevation values by decreasing the respective altitudes by 200 m for the nine populations of *A. villosa*. subsp. *bernisii* from the southern slope (Ber12 to Ber20, Table 5). The continuous decrease of H_{pop} for such populations as the elevation increases is clear: populations below 1850 m were in the 50% percentile, whereas the lowest values were found above that elevation. No other common factor groups these populations with low H_{pop} except for the population of *A. villosa* subsp. *bernisii* from Gador (Ber18), where the isolation from neighbouring populations (15 km to the closest one) and habitat degradation might be determinant. The fact that the lowest values for *A. villosa*. subsp. *bernisii* are similar to those exhibited by *A. splendens* and *A. filicaulis* subsp. *nevadensis* suggests that glaciation events could have influenced the genetic depauperation of those populations. However, the trend observed in *A. villosa* subsp. *bernisii* suddenly ends for the high-altitude populations (Ber13, Ber20, Ber21, Ber25), which depart from the common trend, showing values of H_{pop} 25-50% higher than those of populations immediately below in altitude. It is also noteworthy that the altitudinal belt between 1400 and 1900 m, where *A. villosa*. subsp. *bernisii* reaches its highest diversity values is the only gap in the elevational distribution of *A. filicaulis*. A possible explanation for this pattern is that *A. villosa* subsp. *bernisii* genetically assimilated previously existing populations of *A. filicaulis* subsp. *nevadensis* in this range.

We suggest that the pattern of genetic diversity along the altitudinal gradient in the Sierra Nevada is congruent with that presented in previous evidence based on plastid (Gutiérrez Larena & al., 2002) and ITS sequences (Nieto Feliner & al., 2002), and could be explained by two parallel waves of altitudinal colonization into deglaciated habitats, one by *Armeria filicaulis* subsp. *nevadensis* plus *A. splendens* and another by *A. villosa* subsp. *bernisii* and these are reflected by the two different regression slopes (Fig. 6). The higher than expected values in high-altitude populations of *A. villosa*. subsp. *bernisii* may be due either to the presence of populations of this taxon prior to glaciation (*nunatak* hypothesis) or, alternatively, to recent gene flow between *A. filicaulis*. subsp. *nevadensis* and *A. splendens* that created an additive effect in border populations produced via hybridization. Another possible indication of the genetic assimilation of other congeners' populations by *A. villosa* subsp. *bernisii* is the observed increase of genetic diversity in high-altitude populations (Ber12, Ber13, Ber26) (Fig. 6), two of which cluster with *A. filicaulis* subsp. *nevadensis* in the NJ-tree (Fig. 5). The process of assimilation of taxa by genetically "aggressive" species has been previously reported for *A. villosa* (Fuertes Aguilar & al., 1999) and it conforms to the compilospecies concept (Harlan & De Wet, 1963). A similar evolutionary scenario has been reported to occur during overlapping postglacial migration of different taxa in Arctic areas (Brochmann & al., 1996).

Genetic distance and morphological cohesion

The NJ-tree produced by analysing individual RAPD phenotypes shows a pattern analogous to that obtained with sequence data (Gutiérrez Larena & al., 2002; Nieto Feliner & al., 2004): few (6) recognizable groups that are taxonomically heterogeneous, mostly with a geographical and geological common ground, and with low BS (Fig. 3). The situation changes dramatically when the analysis is made on a populational basis, using estimates of Φ_{ST} as genetic distance (Fig. 5). The three main clusters detected are largely congruent with the morphological variation and taxonomy, except for those taxa with a putative hybrid origin and a few idiosyncratic populations, already detected through morphological and molecular incongruence
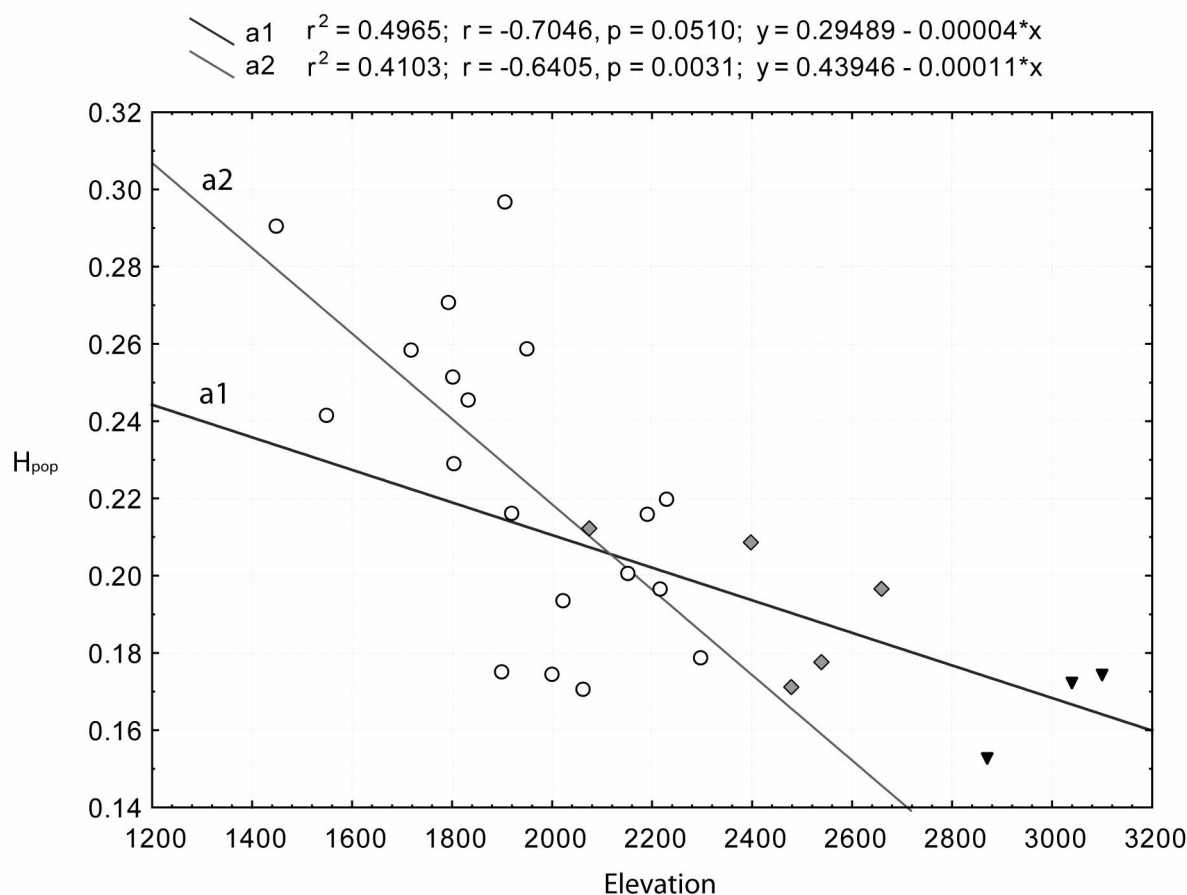
Fig. 6. Correlation analysis graphs between *Armeria* spp. populational genetic diversities and altitudes, considering only the Sierra Nevada populations. a1 = *A. filicaulis* subsp. *nevadensis* + *A. splendens*, a2 = A. *villosa* subsp. *bernisii*. Symbols as in Fig. 1.

(Fil7, Filtre10, Ber8, Ber9, Ber11). Cluster 1 gathers populations from the Tejeda-Almijara range and the nearby Western Sierra Nevada, thus suggesting connections through migration and genetic exchange between these areas (Gutiérrez Larena & al., 2006). The large cluster 2 encompasses almost exclusively populations of *Armeria villosa*, except for the recently described populations of *A. filicaulis* subsp. *alfacarensis* (Gutiérrez Larena & al., 2004). Cluster 3 groups *A. splendens* with populations of hybridogenous *A. filicaulis* subsp. *nevadensis*. This cluster also includes two populations of *A. villosa* subsp. *bernisii* (1950 m) that are sympatric to those of *A. filicaulis* subsp. *nevadensis*. This connection lends support to the hypothesis that *A. villosa.* subsp *bernisii* originated through genetic swamping of *A. filicaulis* populations with *A. villosa* invaders from outside the Sierra Nevada (Guitérrez-Larena & al., 2002).

An unexpected outcome obtained both from RAPD and morphological analyses is that populations that occur on two rather isolated crystalline dolomitic enclaves (Alfacar, Cerro Trevenque) are genetically closer than previously thought. *Armeria filicaulis* subsp. *alfacarensis* is connected in the MST to individuals of *A. villosa* subsp *bernisii* from Trevenque and all of them are placed in proximity to *A. filicaulis* subsp. *trevenqueana* in the Principal Component Analysis of morphometric data. The question arises whether there is a common origin for those dolomiticolous taxa, or whether the similarity is a result of introgression among them. A third alternative is a strong selective pressure for morphological and genetic characters adapted to such a demanding environment. Although there is a recent study supporting the relevance of substrate type as responsible for alpine plant distribution patterns the answer to these questions requires further research.

Glacial dynamics and paleoecological data

Paleoecological reconstructions for the Sierra Nevada environments during Pleistocene are still very incomplete. A significant difference in the effects of

glaciations on Mediterranean massifs such as the Sierra Nevada compared to those at higher latitudes such as the Pyrenees and the Alps is that glaciers were smaller and much more adapted to relief in the former massif. As a consequence, water availability from melting ice was more geographically limited. This feature, combined with the reduced water available from precipitation during glacial periods, promoted the predominance of arid (steppic) ecosystems in areas only moderately distant from melting-ice water. The result was an enforcement of the island effect on some alpine plants. Another effect of climatic changes during glacial maxima was the contraction of bioclimatic belts. Given that the Sierra Nevada relief has remained more or less constant since the onset of Pleistocene glaciations, we can infer that conditions for the crioromediterranean bioclimatic belt descended to c. 1800 m on the north western slope of the massif (Gómez Ortiz & Salvador i Franch, 1996). This contraction caused a more marked ecological gradient between xeric and humid conditions along the slopes (Ferris & al., 1999). The sequence of changes has been recorded at the Padul site (950 m) for the Sierra Nevada, with the appearance of steppic vegetation (*Artemisia*, *Ephedra*, Chenopodiaceae) in basal Sierra Nevada zones during Last Glacial Maximum (Pons & Reille, 1988). There is also the possibility that high-altitude plants survived during the glaciations in nunataks, i.e. areas that remained ice-free, but the existence of nunatak survival in European mountain systems and its relevance for the interglacial recolonization process is still under discussion (Stehlik, 2000, 2003; Stehlik & al., 2002). The arrival of the genus *Armeria* in the southern Iberian Peninsula is difficult to date and paleopalynological data contribute little due to the low pollen production in the genus (Barrón & al., 2010). There is no evidence of *Armeria* fossil pollen recorded in the Padul sediments (Pons & Reille, 1988) but a sample from a hyena coprolite from Late glacial (12700 yr BP) c. 20 km north of the Sierra Nevada may belong to *Armeria*, possibly *A. villosa* (Carrión & al., 2001b; Carrión, unpubl. data.). This sample was originally assigned to *Limonium*, a genus with a pollen morphology indistinguishable from that of *Armeria*. However, *Limonium* is always associated with alkaline soils and never with the *Quercus* aff. *pyrenaica* forest that the paleoecological reconstructions indicate for this deposit.

Two previous studies based on plastid DNA and ITS markers proposed a scenario of colonization of the Sierra Nevada by successive migrations (Gutiérrez Larena & al., 2002; Nieto Feliner & al., 2004). On this view, *Armeria splendens* was the first species that colo-

nized the Sierra Nevada. *A. splendens* belongs to a group of high-elevation species including *A. alpina* from the Alps and the Pyrenees, and *A. bigerrensis* and *A. caespitosa* from the Iberian Central System, with which it shares the same plastid haplotype (Gutiérrez Larena & al., 2002, Fuertes Aguilar & Nieto Feliner, unpubl. data). Of the three species present in the Sierra Nevada, *A. splendens* is also the only one that has exclusively the endemic ITS ribotype R3 (Nieto Feliner & al., 2004). In a subsequent phase, *A. splendens* would have originated *A. filicaulis* subsp. *nevadensis* by hybridization with *A. filicaulis.* This scenario is consistent with the finding that, based on RAPDs at the population level, *A. splendens* has its lowest genetic distance with *A. filicaulis* subsp. *nevadensis* populations (Nev27, Nev28, Nev29, and Nev30). The genetic diversity values of *Armeria filicaulis* subsp. *nevadensis* and *A. splendens* also decrease with increasing altitude, an effect observed in alpine plants during the colonization of glacier forelands along the successional series following ice retreat (Raffl & al., 2006). Another possible explanation for the low genetic diversity of *A. splendens* and *A. filicaulis*, as compared with *A. villosa* subsp. *bernisii*, is population size (Fig. 6), which typically is positively and significantly correlated with genetic diversity (Nybom, 2004). *Armeria splendens* and *A. filicaulis* have smaller population sizes (70-1000 individuals) than *A. villosa* subsp. *bernisii*, with populations of more than 1000 individuals (Blanca & al., 2002). Other factors responsible for genetic depauperations and local extinction, such as genetic bottlenecks caused by brief periods of glacial activity, have also been recorded in the Late Glacial and Holocene for taxa in SE Spain (Carrión & al., 2001a; Carrión, 2002).

What kind of ecological conditions could have favoured the contact between *Armeria splendens* and *A. filicaulis* to produce the hybrid taxon *A. filicaulis* subsp. *nevadensis*? Assuming that both taxa have maintained their current ecological requirements, a descent in the altitudinal range of *A. splendens* so as to converge with that of *A. filicaulis* in W and NW Sierra Nevada may have occurred during the maximum contraction of vegetation belts in the Sierra Nevada. Such conditions may have only been met during the maximum glacial stages. The same kind of predominantly periglacial xeric conditions have been recorded in the Cañada de la Cruz (Sierra de Cazorla) series by Carrión & al. (2001a). Assuming niche conservatism, at the end of that period and during the present interglacial stage, *A. splendens*, and to a lesser extent, *A. filicaulis* subsp. *nevadensis,* could have extended their range upwards following their optima,

until they reached their present distribution. In contrast to the dry and cold environmental conditions associated with the establishment of *A. splendens* and *A. filicaulis* in the Sierra Nevada, the climatic conditions for the wave of colonization in the massif by *A. villosa* are likely to have been considerably wetter and warmer. This species grows in the understory of *Pinus* and *Quercus* spp. forests, whose ranges expanded in Betic and Sub-betic areas during interglacial periods (Carrión & al., 2001a). The concomitant forest expansion across the massif probably facilitated the hybridization between *A. villosa* and *A. filicaulis*, resulting in the partial assimilation of the latter to create what is now known as *A. villosa* subsp. *bernisii,* and which is currently distributed over a large part of the forested slopes of the Sierra Nevada. This scenario is consistent with the clustering of two populations of the latter taxon (Ber12, Ber26) with those of *A. splendens* and *A. filicaulis* subsp. *nevadensis* in the NJ-tree based on genetic distances among populations (Fig. 5), as well as with the rare occurrence of the plastid haplotype L that is predominant in both *A. villosa* subsp. *bernisii* and *A. filicaulis* subsp. *nevadensis* (Gutiérrez Larena & al., 2002). A possible trace of the processes described above, which involved repeated genetic exchange between the *Armeria* congeners, is the predominance of haplotype I (from *A. splendens*) in populations of *A. villosa* subsp. *bernisii* and the occurrence of haplotype E, which is frequent in *A. filicaulis* but absent in populations of *A. villosa*. subsp. *bernisii* from the Sierra Nevada except at El Dornajo (Ber25) (Gutiérrez Larena & al., 2002). The expansion undergone by this latter taxon was probably the result of population fragmentation and long-distance dispersal, as reflected by the absence of a congruent geographic pattern among haplotype and ribotype distributions in the Sierra Nevada, and in the genetic distance tree (Fig. 5). A relevant part of this process of expansion has involved the migration of *A. villosa*. subsp. *bernisii* outside the Sierra Nevada and the ability to colonize the Tejeda-Almijara range (Gutiérrez Larena & al., 2006).

In summary, this work shows how a populational approach encompassing different species may help to unravel the evolutionary mechanisms (hybridization, genetic drift, gene flow) that were involved in the shaping of the present taxonomic and genetic diversity of the genus *Armeria* in the Sierra Nevada. In glacial refugia, such complex interactions between different taxa are often difficult to interpret using purely phylogeographic approaches. However, our present understanding of population genetics in arctic and alpine postglacial colonization, in conjunction with the infe-

rence of ecological conditions during glacial/interglacial cycles, provides an explanation to how morphological and genetic diversity was moulded in southern European refugia.

Acknowledgements

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