Dispersal across southern Iberian refugia? Integrating RAPDs, sequence data and morphometrics in *Armeria* (Plumbaginaceae)

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Running title: Dispersal across southern Iberian refugia

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 hybridisation 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 this results with the previously published sequence data (ITS and chloroplast spacer *trn*L-F) it is proposed that introgressive hybridisation has occurred in *A. villosa* subsp. *bernisii,* while 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 involved westward migration from a biodiversity-rich massif like Sierra Nevada, and this may apply to other organisms although further data are needed to confirm it.

Keywords *Armeria*, Plumbaginaceae, glaciations, phylogeography, ITS, *trn*L-F, RAPD, morphometrics, migrations, contact zone, refugia

INTRODUCTION

The last years have witnessed an important research effort on inferring changes produced by severe Quaternary climatic oscillations on species distributions (Hewitt 2000). Most of the studies have aimed at identifying refuges and pathways from which the northern territories were recolonised 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 refuges. The main reason for a scarcity of studies in southern regions is the difficulty of "unraveling the spatial genetic history of species in these refuges" 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 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 been also 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 the fact that several lines of evidence support the occurrence of 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 transfer between species of *Armeria* that were 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 homogenisation of ITS copies within regions (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). Since evidence of rapid homogenisation of ITS copies was found in artificial hybrids (Fuertes Aguilar et al. 1999a), the occurrence of two ribotypes both within populations and intraindividually is an indication of recent or persistent contacts between individuals from adjacent areas that bear different ribotype. Therefore, as long as a full homogenisation of these multicopy regions is not achieved, the ITS conveys information on the geographic origin of plants in *Armeria* and thus potentially trace migrations.

In this paper, we focus on the southern Spanish Tejeda/Almijara range as a contact zone inferred from (1) the co-occurrence of ITS ribotypes (at the intraindividual and intrapopulational 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 hybridisation 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 Tejeda/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 Tejeda/Almijara range, whose NW portion is called Sierra Tejeda 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 Tejeda/Almijara range (Laza 1946, Nieto Caldera & Cabezudo 1988), as 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 varieties in the massif: a small pink-flowered plant with few scapes (var. *minor* Boiss.) in Sierra Tejeda and a white-flowered with multiple scapes (var. *filicaulis)* in Sierra de Almijara. There are three additional subspecies within *A. filicaulis* (Gutiérrez Larena et al. 2005). The other species in the

Tejeda/Almijara 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 Tejeda/Almijara, 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 subsp. *longiaristata* (Boiss. et Reut.) Nieto Fel. (Fig. 1B; Nieto Feliner 1990).

Populations from neighboring mountain ranges have been considered and sampled as well, in order to minimize confounding molecular uniqueness with hybridisation between related species (Comes & Kadereit 1998). This is particularly needed in a genus where hybridisation 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 specially 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: *trn*L-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 Tejeda/Almijara range (three of *A. filicaulis* var. *minor* Boiss. from Sierra Tejeda, one of *A. filicaulis* var. *filicaulis* from Sierra de Almijara, one of *A. villosa subsp. bernisii* also from Sierra de Almijara); one population to Sierra de las Guájaras (*A. filicaulis* var. *filicaulis*) located in between the Tejeda/Almijara range and Sierra Nevada; two to Sierra Nevada (both *A. villosa* subsp. *bernisii);* and one to Sierra de Alhama (*A. villosa* subsp. *longiaristata* (Boiss. et Reut.) Nieto Fel.) north of the Tejeda/Almijara range (Table 1).

Fifty-nine primers of the Roth Random Primer Kits C, D, M, and 180 (Carl Roth Gmbh) were assayed. To select for 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 a variable and well-defined banding pattern. These selected primers were then used to amplify all the samples. A second set of primers were discarded at this point on the basis of poor definition of the bands, and/or evident problems of homology assessment. Those primers that resulted in lack of reproducibility 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 µl volumes containing 2.5 ng/µl genomic DNA, 2.5 mM $MgCl_2$, 10 mM primer, 0.2 mM dNTPs and 0.5 U Taq DNA polymerase (Genecraft, Münster, Germany). PCR were performed in a PTC-100^{TM} Peltier-Effect Cycling (MJ Research, Inc.), under the following conditions: incubation at 94º C for 3 min, followed by 40 cycles at 94º C for 20 sec, 40º C for 30 sec and 72º C for 1 min, followed by a final elongation phase at 72º 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 visualised under UV light and photographed. For band scoring, those bands appearing in both amplifications 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 x 41 presence/absence matrix was constructed by hand for further analysis. Phenetic similarity was analysed 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 which 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 (Pavlicek 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 *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

involve 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).

Sequence data

Protocols for isolation, amplification, alignment, sequencing and data analysis of the ITS and *trn*L-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), twenty seven morphological characters were measured (including four qualitative ones) in 3 to 6 specimens per population (43 individuals in total) and subjected to ordination 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 spanning tree based on the Euclidean distances was superimposed on the scatter plot of the samples against the first three PCA axes. The PCA analysis was 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* s.l. although not present in all samples, five (6.5 %) to *A. villosa* s.l., 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. filiacaulis and var. minor) were on the positive values. Along the minimum 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 *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 (24.47%) when groups are considered as taxa than when groups are considered as regions (19.36%). Conversely, the among-populations variance component was larger (38.38% vs. 33.51%) when

groups are areas. This result indicates that the Tejeda/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 70.19 % of the total variance (47.90%, 14.22% and 8.07%, 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 internal involucral bracts, ratio of involucral bract length to spike bract length and petal color. 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 Tejeda, were separated from the rest with respect the second axis, a pattern that is agreement with previous studies (Nieto Feliner et al. 2001). On the basis of the minimum spanning tree, the Tejeda/Almijara samples of *A. villosa* subsp. *bernisii* were connected to other samples of the same subspecies but one of them was relatively apart from the other two with respect to the third axis.

DISCUSSION

RAPD matches morphology and taxonomy

Analysis of intragenomic additive polymorphisms in ITS has proven useful in studying contact zones (Marshall and Sites 2001). Together with the geographic structure of variation detected in this marker, they 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 at throwing additional light at the complex reticulate scenario envisaged through sequence data by 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 F_1 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 hybridisation of a F_1 profile (i.e., clear additivity of bands) between *A. villosa* subsp. *bernisii* and *A. filicaulis* in the Tejeda/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 Tejeda/Almijara range (Table 3).

These RAPD results could be questioned based on criticisms of poor reproducibility against 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 has performed a thorough test of reproducibility even at the risk of loosing true signal. An indication for relying on RAPDs in *Armeria* is that an unpublished study (Fuertes Aguilar et al. unpubl.) based on a different sampling and primers allowed distinguishing taxa of hybrid origin from more localized cases of introgression in *Armeria***.** It is noteworthy the high number of bands and high intraspecific variability but this has been also 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 pattern (Hamrick & Godt 1996, Nybom & Bartish 2000), together with the occurrence of interspecific hybridisation, 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, it does not allow an individual-by-individual comparison with sequence data.

Variation in RAPDs is consistent with taxonomy, since 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

Even if representing genetic variation from a limited portion of the genome, as compared to a total DNA marker like RAPD, ITS sequence data 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 for a contact zone including those in the Tejeda/Almijara range are strong. In fact, an alternative scenario where incomplete lineage sorting could have generated the ITS patterns was conclusively discarded as unrealistic since it would involve multiple selective loses of the same ITS copy in different species within the same territory (Fuertes Aguilar et al. 1999b). Therefore, the question is how do we harmonise the RAPD and morphometric data presented here with the previous ITS sequence data and patterns of chloroplast haplotype sharing (summarised in Figure 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 hybridisation between *A. villosa* subsp. *bernisii* and *A. filicaulis*; (2) gene flow between individuals from the same species bearing different genotypes.

This 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 Tejeda/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 Tejeda/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 Tejeda, hybridisation 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 Tejeda (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*. Homogenisation 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 Tejeda/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 hybridisation 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 Tejeda/Almijara range are caused by contacts between populations from the same species not with *A. villosa* subsp. *bernisii*.

The two proposed hypotheses involve contacts between *Armerias* with different genotypes. With the available data, these might have implied westwards migration from Sierra Nevada into Tejeda/Almijara both of *A. villosa* subsp. *bernisii* and *A. filicaulis.* The latter species is represented in Sierra Nevada by two subspecies, 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 morphological traces of hybridisation 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 *(trn*L-F, ITS) has to be done with caution since 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 *trn*L-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) and the occurrence of some sort of boundary and exchange zone for Sierra Nevadan 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 for other organisms. A high percentage of common plant species between Eastern 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), theTejeda/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 been also 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 boundary.

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FIGURE LEGENDS:

Fig. 1.—A) Distribution of *trn*L-F chlorotypes (A, B, E, I, K, L) and ITS ribotypes (R1, R2) in the sampled area (SE Spain) in *Armeria* Willd. Samples of *A. villosa* subsp. *bernisii* from the Tejeda/Almijara range are marked as solid circles. B) Distribution of the taxa sampled in Andalusia (*A. villosa* subsp. *bernisii* Nieto Fel, dotted line; *A. villosa* subsp. *longiaristata* (Boiss. et Reut.) Nieto Fel., dashed line; *A. filicaulis* (Boiss.) Boiss. s.l., solid line).

Fig. 2..--Principal coordinate analysis of RAPD bands of *Armeria* Willd. 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* (Boiss.) Boiss. var*. filicaulis* (squares), *A. filicaulis* var. *minor* Boiss. (asterisks), *A. villosa* subsp. *bernisii* Nieto Fel. (circles, those from the Tejeda/Almijara range are solid), *A. villosa* subsp. *longiaristata* (Boiss. et Reut.) Nieto Fel. (crosses).

Fig. 3--UPGMA phenogram of 41 samples of *Armeria* Willd. 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* (Boiss.) Boiss. var*. filicaulis*), M (*A. filicaulis* var. *minor* Boiss.), B (*A. villosa* subsp. *bernisii* Nieto Fel*.,* those from the Tejeda/Almijara marked with an asterisk), L (*A. villosa* subsp. *longiaristata* (Boiss. et Reut.) Nieto Fel.).

Fig. 4.—Principal component analysis of 30 morphometric characters in *Armeria* Willd. 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* (Boiss.) Boiss. var*. filicaulis* (squares), *A. filicaulis* var. *minor* Boiss. (asterisks), *A. villosa* subsp.

bernisii Nieto Fel. (circles, those from the Tejeda/Almijara range are solid), *A. villosa* subsp. *longiaristata* (Boiss. et Reut.) Nieto Fel. (crosses).

Table 1. Origin of the samples of *Armeria* Willd. from southern Spain used for sequence data (nrDNA ITS, chloroplast *trn*L-F), RAPDs and morphometrics. Ribotypes (ITS) and chlorotype (*trn*L-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.

Population	**Taxon**	**Locality**	**Voucher specimen**	**Specimen code**	Ribotypes in Nieto Feliner et al. 2004.	ITS (GenBank Acc. no.)	Chlorotypes in Gutiérrez Larena et al. 2002	*trn*L-F (GenBank Acc. no.)	**RAPD**	**morphometrics**
1	*A. filicaulis* (Boiss.) Boiss. var. *filicaulis*	Málaga: Canillas de Albaida, Sierra Tejeda, road to puerto Blanquillo, 30SVF1479, 900 m, dolomitic sands, Pinus forest understory	*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	*A. filicaulis* var. *filicaulis*	Granada: Canillas de Albaida, Sierra Tejeda, road to puerto puerto Blanquillo, 30SVF1680, 1200 m, open dolomitic sands	*GN 4032*	7F	R2 + R3	AY444067	ChE	AJ417268		
			GN 4035	8F	R2 + R3	AY444068	ChA	AJ417269		
3	*A. filicaulis* var. *filicaulis*	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	*A. filicaulis* var. *minor* Boiss.	Málaga: Alcaucín, Sierra Tejeda, 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						+
5	*A. filicaulis* var. *minor*	Málaga: Alcaucín, Sierra Tejeda, path to pico Maroma, barranco de los Polvilleros, 30SVF0584, 1700 m, limestone rock crevices	*GN 4015*	19M					+	+
			GN 4016	20M					+	
			GN 4017	21M	R2	AY179780	ChA	AJ417263	+	
			GN 4018	22M	R2	AY444085	ChA	AJ417264	+	
			GN 4019	23M					+	+
6	*A. filicaulis* var. *minor*	Málaga: Alcaucín, Sierra Tejeda, path to pico Maroma, 30SVF0285, 1200 m, limestone rock crevices	*GN 4020*	24M	R2	AY444086	ChE	AJ417265	+	+
			GN 4021	25M					+	+
			GN 4022	26M					+	+
			GN 4023	27M						+
			GN 4024	28M					+	
7	*A. filicaulis* var. *minor*	Málaga: Alcaucín, Sierra Tejeda, pico Maroma, 30SVF0684, 1800 m, limestone rock crevices	*AP- 1*	29M	R2 + R3	AY444087	ChE	AJ417277	+	
			Apfili 1	30M					+	
			Apfili 2	31M					+	
			Apfili 4	32M					+	
			Apfili 5	33M					+	
8	*A. villosa* subsp. *bernisii* Nieto Fel.	Granada: Otívar, Sierra de Almijara, pico Navachica W slope, 30SVF2976, *Quercus pyrenaica* and *Sorbus aria* forest	*MGC 44193*	34B	R2 + R3	AY444133	ChE	AY444147		+
9	*A. villosa* subsp. *bernisii*	Granada: Alhama de Granada, Sierra de Almijara, road to Navachica, 30SVF2781, *Quercus pyrenaica* and *Sorbus aria* forest	*MGC 44202*	35B	R2 + R3	AY444132	ChE	AY444148		+

Table 1. cont.

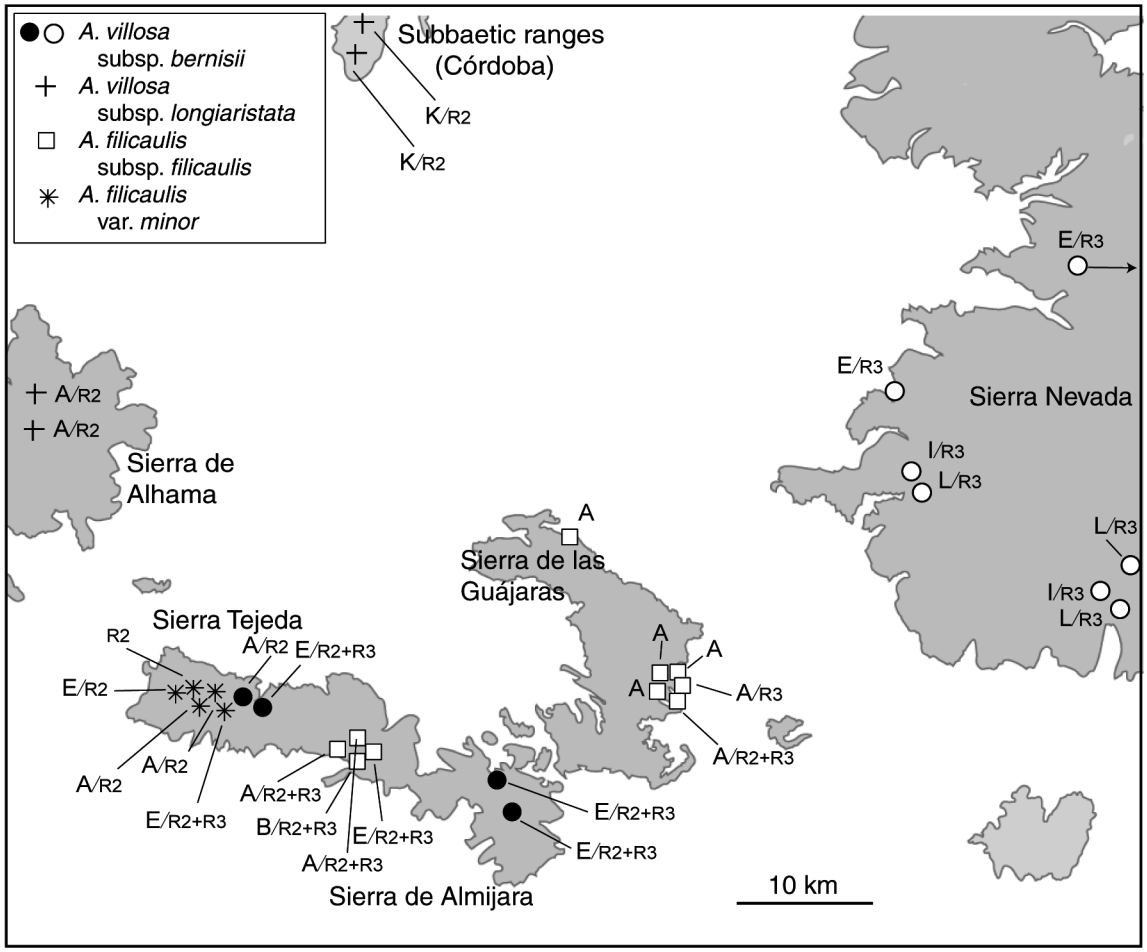
	Taxon	**Locality**	**Voucher specimen**	**Specimen code**	Ribotypes in Nieto Feliner et al. 2004.	ITS (GenBank Acc. no.)	Chlorotypes in Gutiérrez Larena et al. 2002	*trn*L-F (GenBank Acc. no.)	**RAPD**	**morphometrics**
10	*A. villosa* subsp. *bernisii*	Granada: Sedella- Alhama de Granada, Sierra de Almijara, "Las Llanadas", 1600 m, *Quercus pyrenaica* and *Sorbus aria* forest	*AP- 2*	36B	R2 + R3	AY444130	ChE	AJ417319		+
			Apbern 1	37B	R2	AY444131	ChA	AJ417320	+	
			Apbern 2	38B					+	
			Apbern 3	39B					+	
			Apbern 5	40B					+	
11	*A. villosa* subsp. *bernisii*	Granada: Monachil, E slope of Cerro Trevenque, near collado Ruquino, 30SVG5803, 1800 m, *Pinus* forest	*GN 4094*	41B					+	+
			GN 4095	42B	R3	AF270510	ChI	AJ417292	+	+
			GN 4096	43B	R3	AY444102	ChL	AJ417293	+	+
			GN 4097	44B					+	+
			GN 4098	45B					+	+
12	*A. villosa* subsp. *bernisii*	Granada: Bubión, Sierra Nevada, road Capileira-Veleta, 30SVF6991, 1950 m, scrub on schist	*GN 4112*	46B	R3	AY444104	ChI	AJ417296	+	+
			GN 4113	47B	R3	AY444105	ChL	AJ417297	+	+
			GN 4114	48B					+	
			GN 4115	49B					+	+
			GN 4116	50B					+	
13	*A. villosa* subsp. *bernisii*	Granada: Pórtugos, Sierra Nevada, road Capileira-Veleta, Loma de Piedra Blanca, 30SVF7192, 2230 m, open pasture and scrub on schist	*GN 4121*	51B						+
			GN 4123	52B	R3	AY444106	ChL	AJ417298		+
			GN 4124	53B						+
14	*A. villosa* subsp. *bernisii*	Granada: Bérchules, Sierra Nevada, 30SVF8194, 1920 m, scrub on schist	*GN 4134*	54B						+
			GN 4135	55B						+
			GN 4136	56B						+
			GN 4137	57B						+
15	*A. villosa* subsp. *bernisii*	Granada: Dílar, Sierra Nevada, La Zubia, 30SVG5404, 1350 m, scrub on limestone	*GN 4078*	58B	R3	AY444101	ChE	AJ417326		
16	*A. villosa* subsp. *bernisii*	Granda: Aldeire, Sierra Nevada, 30SVG9007, 2060 m, clearings on schist	*GN 4186*	59B	R3	AY444119	ChE	AJ417311		
17	*A. villosa* subsp. *longiaristata* (Boiss. et Reut.) Nieto Fel.	Málaga: Alfarnate, Sierra de Alhama, puerto del Sol, 30SUF9292, 1250 m, limestone rock crevices, W slope	*GN 4232*	60L					+	
			GN 4235	61L					+	
			GN 4237	62L	R2	AF270505	ChA	AF281345	+	
			GN 4239,1	63L	R2	AY444136	ChA	AJ417327	+	
			GN 4239,2	64L					+	
18	*A. villosa* subsp. *longiaristata*	Córdoba: Cabra, La Nava, "el Registro", 30SUG7851, 1000 m, wet pastures, clay	*GN 4002*	65L	R2	AY179831	ChK	AF281336		+
			GN 4003	66L						+
			GN 4004	67L	R2	AY444135	ChK	AF292076		+

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

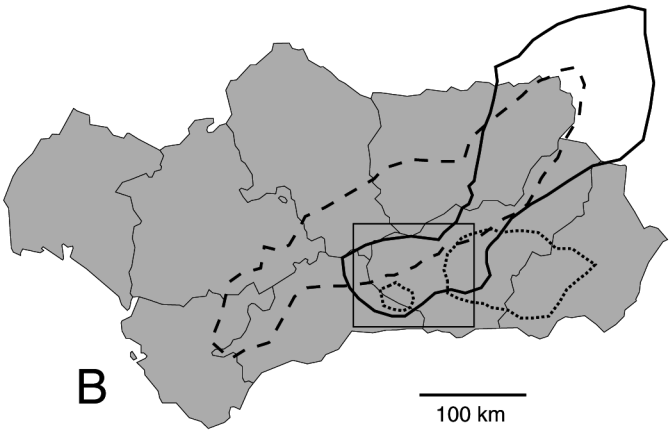
1	Leaf length
2	Leaf width
3	Leaf length to leaf width ratio
4	Scape length
5	Scape diameter at base
6	Involucre diameter
7	Ratio of the involucral diameter to the length of the involucral sheath
8	Number of involucral bracts
9	Length of outer involucral bracts
10	Length of longest inner involucral bracts
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 bracts length to the inner involucral bracts 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)

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

Source of variation	d.f.	Sum of squares	Variance components	% of variation	*P*
Among three regions	2	135.472	2.99654	19.36	<0.001
Among populations within regions	6	197.373	5.94092	38.38	<0.001
Among individuals within populations	32	209.350	6.54219	42.26	<0.001
Total	40	542.195	15.47965		
Among three taxa	2	153.629	3.80931	24.47	<0.002
Among populations within regions	6	179.216	5.21644	33.51	<0.001
Among individuals within populations	32	209.350	6.54219	42.02	<0.001
Total	40	542.195	15.56794		


A. villosa subsp. bernisii
A. villosa subsp. longiaristata
A. filicaulis subsp. filicaulis
A. filicaulis var. minor
Subbaetic ranges (Córdoba)
K/R2
K/R2
E/R3
E/R3
Sierra Nevada
I/R3
L/R3
L/R3
I/R3
L/R3
A/R2
A/R2
Sierra de Alhama
A
Sierra de las Guájaras
A
A
A
A/R3
A/R2+R3
Sierra Tejeda
R2
A/R2
E/R2+R3
E/R2
A/R2
A/R2
A/R2+R3
E/R2+R3
B/R2+R3
E/R2+R3
A/R2+R3
E/R2+R3
E/R2+R3
Sierra de Almijara
10 km

A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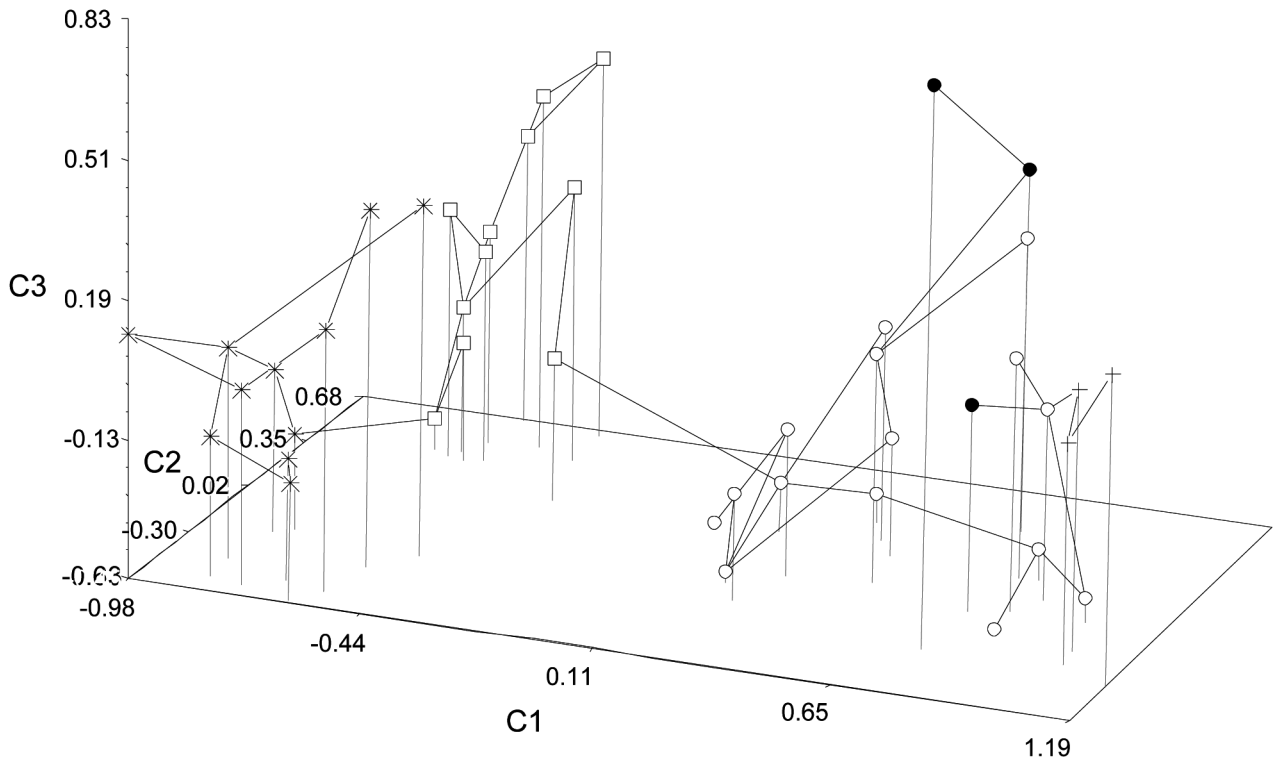
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0.19
-0.13
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C3
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-0.30
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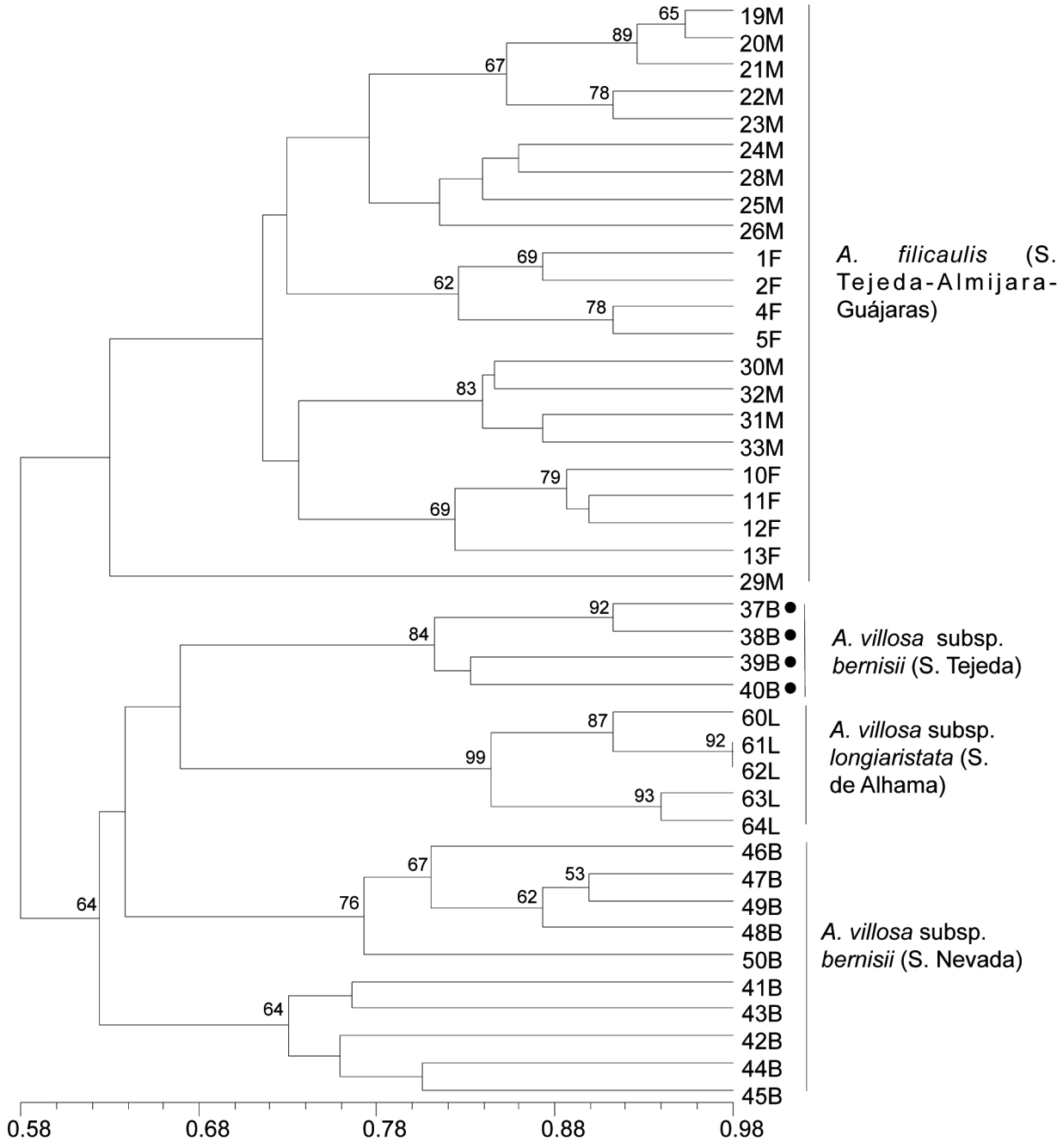
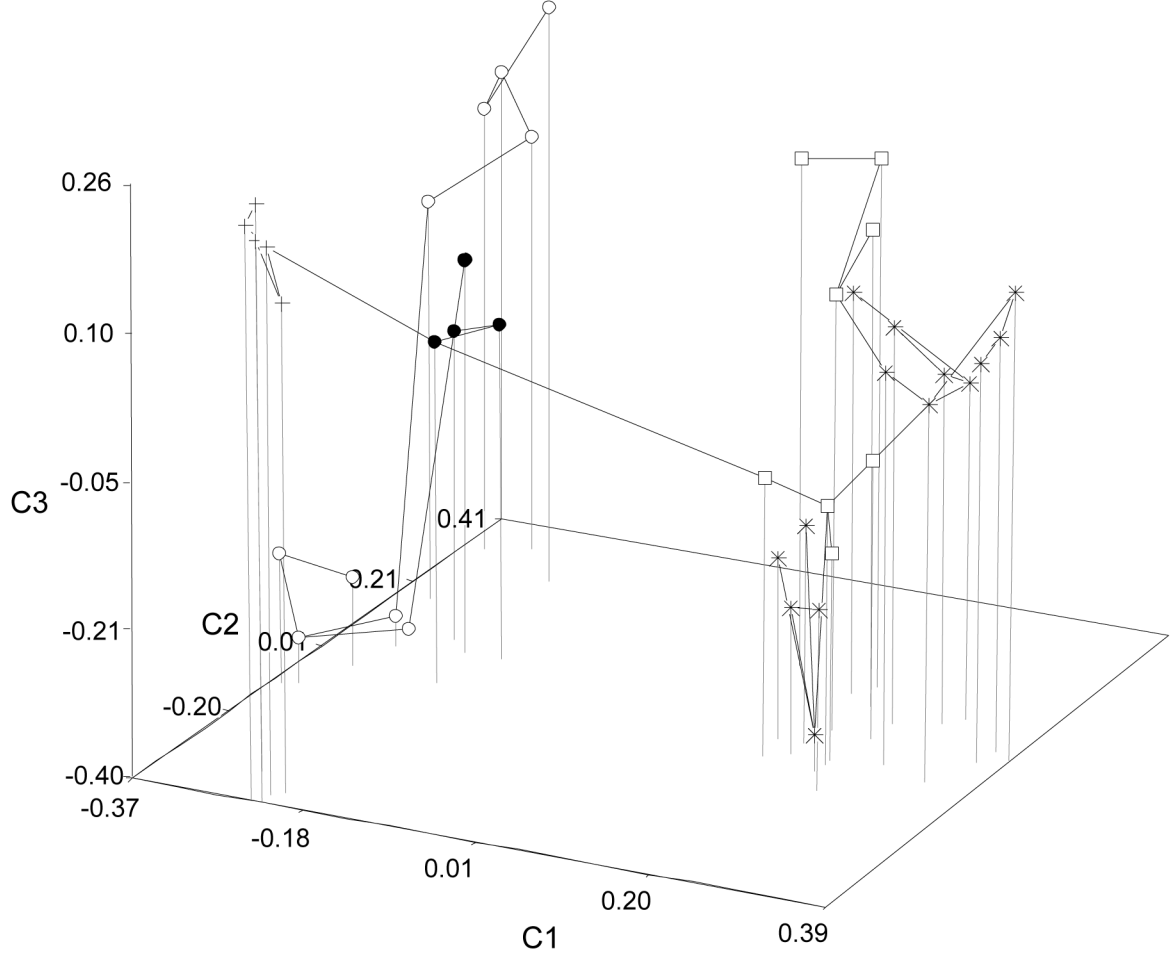
0.11
0.65
1.19
C1


19M
20M
21M
22M
23M
24M
28M
25M
26M
1F
2F
4F
5F
30M
32M
31M
33M
10F
11F
12F
13F
29M
37B
38B
39B
40B
60L
61L
62L
63L
64L
46B
47B
49B
48B
50B
41B
43B
42B
44B
45B
65
89
67
78
69
62
78
83
79
69
92
84
87
92
99
93
67
53
64
76
62
64
A. filicaulis (S. Tejeda-Almijara-Guájaras)
A. villosa subsp. bernisii (S. Tejeda)
A. villosa subsp. longiaristata (S. de Alhama)
A. villosa subsp. bernisii (S. Nevada)
0.58
0.68
0.78
0.88
0.98


0.26
0.10
-0.05
-0.21
-0.40
C3
0.41
0.21
0.01
C2
-0.20
-0.37
-0.18
0.01
0.20
0.39
C1