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Glacial-induced altitudinal migrations in *Armeria* (Plumbaginaceae) inferred from patterns of chloroplast DNA haplotype sharing

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Abstract

In contrast to northern European areas where large-scale migrations occurred to recolonize territories after glacial periods, species in southern regions survived and diverged without large geographical displacements. As a result of the importance of orography in much of the southern areas, such displacements must have involved populations ascending or descending mountains. The present study provides support for glacial-induced altitudinal migrations from chloroplast phylogeographic patterns in *Armeria* (Plumbaginaceae) in southeast Spain. One hundred and five sequences of the *trnL-F* spacer were obtained from seven species. Fifteen different haplotypes were recognized, their genealogy was inferred, and associations with geography were explored using nested clade analysis. Seven instances were detected in which the same haplotype is shared by two or three species within a particular massif. In all the cases, at least one of the species involved displayed different haplotypes in other areas; in most, the haplotype shared is predominant either in one of the species involved or in the massif. These patterns of haplotype sharing strongly suggest horizontal transfer between species. In one of the massifs (Sierra Nevada) the three species involved in haplotype sharing (*A. splendens*, *A. filicaulis* ssp. *nevadensis*, *A. villosa* ssp. *bernisii*) occur at markedly different altitudinal belts. It is argued that altitudinal migrations within the contraction–expansion model provide the best explanation for the current pattern, and that at least in one case it resulted in the formation of a new hybrid taxon, *A. filicaulis* ssp. *nevadensis*.

Keywords: *Armeria*, chloroplast DNA, glaciations, hybridization, phylogeography, *trnL-F*

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Introduction

A number of phylogeographic studies are now available on glacial refuges for plant species in Europe (Petit *et al*. 1997; Comes & Kadereit 1998; Taberlet *et al*. 1998; Abbott *et al*. 2000). These have mainly focused both on identifying the refugia and on tracing the pathways by which the central and northern European glaciated territories were recolonized. However, much less is known about how plants and genomes survived and moved during Pleistocene glaciations within southern territories. The main reason for this is probably the difficulty in unravelling the spatial genetic history of species in these refuges as compared to the northern regions (Hewitt 2001). The complexity of patterns expected in southern territories is significantly contributed by the fact that the contraction–expansion cycles took place with limited geographical displacement as compared to northern territories (Hewitt 2001). Although in these southern regions severe effects were limited to high elevation in the mountains, climatic changes must have provoked shifts in the suitable altitudinal range for each species along each massif. As a consequence, in mountainous regions like southern Spain, contraction–expansion most likely provoked the species to ascend or descend mountains.

Phylogeography, dealing with the analysis of spatial distributions of gene genealogies provides an appropriate frame to explore this kind of scenario (Schaal *et al*. 1998). Phylogeographic studies have mostly concentrated on

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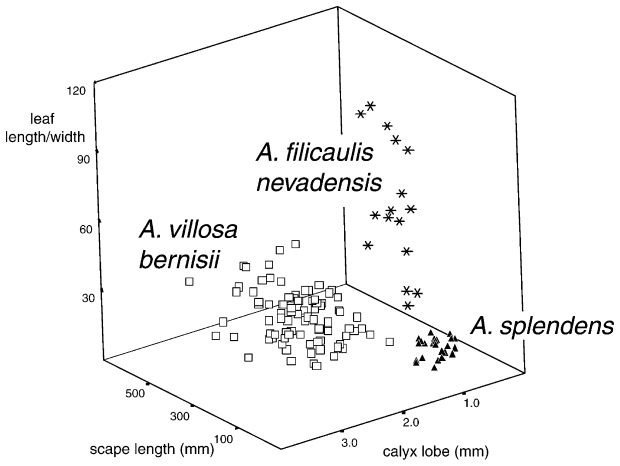

Fig. 1 Scatter diagram for three morphological features in 129 specimens of *Armeria* inhabiting Sierra Nevada (Gutiérrez Larena *et al*. unpublished data).

intraspecific problems partly because it was at the population level where a strong demand existed to incorporate a historical framework (Avise 2000; Posada & Crandall 2001). However, when methods of this discipline are applied above the species boundary, they provide a source of analysis of evolutionary patterns that is not prejudiced by taxonomic criteria (Schaal *et al*. 1998). In plants, where the assumption of a gene flow–drift equilibrium seems to be generally unrealistic (Schaal & Olsen 2000), analysing together several sympatric or parapatric species seems a reasonable approach.

Phylogeographic approaches have been successfully applied in case studies that involve interspecific gene flow or to address putative hybridization specifically (Whittemore & Schaal 1991; Jackson *et al*. 1999; Matos & Schaal 2000; Belahbib *et al*. 2001). Since internal reproductive barriers in *Armeria* are weak (Nieto Feliner *et al*. 1996), to infer the dynamics that might have brought species into contact, we need to consider all the species potentially involved or contributing to the scenario.

Accordingly, the present study examines chloroplast haplotype variation in Southern Spanish massifs in conjunction with geographical data and taxonomic boundaries. It is our aim to analyse the relationships between those three dimensions of the data (haplotypes, geography, taxa) in inferring the effects of contraction–expansion Pleistocene cycles in the evolutionary history of congeneric species from southern European massifs.

Although the sampling covers a large portion of southeast Spain to assess appropriately chloroplast diversity, the study is focused in Sierra Nevada. This is because three species of our study group, *Armeria*, inhabit that massif at different elevations: *Armeria splendens* above 2880 m; *A. filicaulis* ssp. *nevadensis* between 2100 and 2550 m, in the northwest part of the range; and *A. villosa* ssp. *bernisii* between 1250 and 2300 m (rarely up to 2600 m on a southern slope), throughout the massif. The three species can be easily distinguished on morphological grounds. Qualitative characters that separate them include petal colour, shape, consistency and colour of involucral bracts, and leaf shape. Continuous characters are also useful to discriminate among the three species occurring in Sierra Nevada (see Fig. 1 for a scatter plot of three continuous characters). Yet, the three species share the same nuclear ribosomal DNA internal transcribed spacer sequence within the massif but display other sequences elsewhere (Fuertes Aguilar *et al*. 1999b; Fuertes Aguilar & Nieto Feliner 2002). Such a pattern of three altitudinally structured and morphologically distinct taxa that share a ribosomal marker demands an evolutionary explanation, which was probably influenced by the history of Pleistocene climatic changes in southern Europe.

Materials and methods

Sampling

To obtain a reliable estimation of haplotype distribution (both geographical and taxonomic) and to explore patterns of haplotype sharing, the sampling covered seven species and a large portion of southeast Spain: *Armeria bourgaei*, *A. colorata*, *A. filicaulis* (including three subspecies), *A. malacitana*, *A. splendens*, *A. trianoi* and *A. villosa* (including four subspecies) (Table 1). Such a sampling strategy helped to minimize confounding molecular uniqueness with hybridization between related species (Comes & Kadereit 1998). The total number of individuals sampled was 105. Because of our focus on altitudinal displacements in Sierra Nevada, 46 of the 105 sequences were from that massif.

Geographic areas

To facilitate the analysis of the geographical component, 12 areas were defined that cover the whole sampled region, except for a single individual from the Moroccan Rif mountains (Table 1). These areas represent biogeographically defined units and match, to some extent, those proposed by Peinado Lorca & Rivas-Martínez (1987) mainly on the basis of floristic and vegetation affinities. As a result of the orography of the whole region sampled, most of those areas correspond to massifs. Each of the 104 southern Spanish sequences was assigned to one of the 12 geographical units.

DNA isolation, polymerase chain reaction and sequencing

DNA isolation from fresh or silica-gel preserved material was performed using a hexadecyltrimethylammonium bromide

Table 1 Distribution of *trnL-F* haplotypes from *Armeria* in taxa and geographical areas in southeast Spain (Sierra Nevada and neighbouring territories)

Area	Location	Taxa	Haplotype	EMBL acession
1	Sierra Bermeja, Palmitera, Alpujata, Mijas, and Ronda (Málaga)	*A. colorata*	**M**(3)	AF281332, AF281333, AF281334
		A. malacitana	**M**(1)	AF281353
		A. villosa carratracensis	**M**(2)	AF281336, AF292076
		A. villosa longiaristata	**M**(1), N(2)	AF281339, AF281341, AF281344
		A. villosa villosa	L(1), **M**(1)	AF281338, AF281337
2	Sierra de Huma, Alhama, Gorda (Málaga)	*A. villosa longiaristata*	A(2), I(1), K(1)	AJ417327, AF281345, F281342, AF281343
3	Subbaetic mountains in Córdoba (Córdoba)	*A. trianoi*	**K**(2)	AJ417253, AJ417286
		A. villosa longiaristata	**K**(3)	AJ417324, AF281340, AF281346
4	Sierra Tejeda, Almijara (Málaga)	*A. filicaulis filicaulis*	**A**(4), **E**(3), B(1)	AJ417263, AJ417264, AJ417266, AJ417269, AJ417265, AJ417268, AJ417277, AJ417267
		A. villosa bernisii	**A**(1), **E**(1)	AJ417320, AJ417319
5	Sierra Cázulas, Guájaras (Granada)	*A. filicaulis filicaulis*	A(6)	AJ417270-AJ417274, AJ417279
6	Sierra Nevada (Granada)	*A. filicaulis nevadensis*	A(1), **I**(3), **L**(2)	AJ417258, AJ417254, AJ417257, AJ417259, AJ417255, AJ417256
		A. filicaulis trevenqueana	A(1), **E**(1), F(1)	AJ417262, AJ417260, AJ417261
		A. splendens	**I**(6)	AJ417280-AJ417285
		A. villosa bernisii	**E**(3), **I**(14), K(1), **L**(12), O(1)	AJ417326, AJ417311, AJ417321, AJ417287, AJ417288, AJ417292, AJ417296, AJ417299-AJ417301, AJ417303, AJ417304, AJ417325, AJ417313-AJ417315, AJ417328, AJ417307, AJ417293-AJ417295, AJ417297, AJ417298, AJ417302, AJ417308, AJ417309, AJ417312, AJ417316-AJ417318, AJ417310
7	Sierra de Gádor (Almería)	*A. villosa bernisii*	I(2)	AJ417305, AJ417306
8	Sierra Harana (Granada)	*A. filicaulis filicaulis*	A(2)	AJ417275, AJ417276
		A. villosa bernisii	L(2)	AJ417290, AJ417291
9	Sierra de Filabres, Baza (Granada)	*A. villosa bernisii*	A(2)	AJ417322, AJ417323
		A. villosa longiaristata	G(1)	AJ417334
10	Mountains S of Jaen (Jaén)	*A. bourgaei*	G(1)	AJ417333
		A. villosa longiaristata	L(2)	AJ417329, AF281348
11	Cazorla-Segura range, Javalcón (Jaén, Albacete, Granada)	*A. filicaulis filicaulis*	C(1), G(1)	AF292078, AJ417332
		A. filicaulis filicaulis	J(1)	AJ417336
		A. villosa longiaristata	L(1), H(3), E(1)	AJ265612, AJ417335, AJ417337, AF292077, AJ281347
12	Moratalla, Jumilla (Murcia)	*A. filicaulis filicaulis*	D(1), A(1)	AJ417330, AJ417331
—	Rif (Morocco)	*A. filicaulis filicaulis*	A(1)	AJ417278

Area number refer to those in Fig. 2.
Number of individuals per haplotype, area and taxon are in parentheses.
Haplotypes shared by more than one species within the same area are in bold.

(CTAB) protocol (Doyle & Doyle 1987), with slight modifications in incubation conditions (overnight at 60 °C) (Fuertes Aguilar *et al*. 1999b). *TrnL-trnF* chloroplast intergenic spacer was amplified using primers described in Taberlet *et al*. (1991). Polymerase chain reaction (PCR) was performed in a Gene Amp PCR system 9700 (AB Biosystems) using 20-μL reactions. Ten microlitres containing 10–20 ng of total DNA was mixed with a cocktail containing 2 μL of 10× Gold Buffer, 0.5 U of AmpliTaq Gold, 1.2 μL of 25 mm $MgCl_2$, 0.3 μL of a 2.5-mm each dNTP mix and 1 μL of each primer at 10 μm. The amplification profile began with an initial cycle of 10 min at 94 °C, plus 35 cycles each one with 1 min at 94 °C, 1 min at 48 °C, and 45 s (increasing 1 s in each cycle) at 72 °C, and a final step of 10 min at 72 °C. The PCR product was purified using MoBio 101 silica-matrix columns, checked in an agarose 1.5% tris-acetate-EDTA (TAE) minigel

and sequenced. Sequencing was performed on both strands, with the same primers used in amplification through cycle-sequencing reactions with fluorescently labelled dideoxynucleotide terminators (AB Inc.). The product was then separated and analysed on an ABI 377 automated DNA sequencer at the Centro de Investigaciones Biológicas, CSIC. The resulting electropherograms were examined for ambiguities and overlapped 100% to obtain a consensus sequence using SEQUENCE NAVIGATOR. Sequence alignment was performed manually because of the absence of indels, homology determined directly in SEQAPP, and then converted to NEXUS format for further analyses. All the new sequences were submitted to the EMBL database (see Table 1).

Analysis

The *trnL-F* region as defined by Taberlet *et al*. (1991) includes the *trnL* 5′exon, the *trnL* intron, a class I intron, the *trnL* 3′exon, and the intergenic spacer. We have focused on the intergenic spacer sequence between *trnL*3′ and *trnF* exons.

Before examining patterns of haplotype sharing, we assessed association between haplotypes and geography following the nested clade analysis approach, i.e. testing the null hypothesis of no association between geography and the haplotype network (Templeton *et al*. 1995). First, a haplotype unrooted cladogram was constructed based on the *trnL-F* using the program TCS 1.13 (Clement *et al*. 2000). This program applies statistical parsimony by implementing the algorithm described in Templeton *et al*. (1992). Under this method, unrooted cladograms that have a high probability (> 0.95%) of being true based on a finite-site model of DNA evolution are identified. The program was run both with gaps coded as missing and as a fifth state. However, to allow recognition of one haplotype (D) that differs from A only by a 1-bp deletion, the second option was chosen. To try to resolve loops obtained in the haplotype network, we applied hypotheses from coalescent theory that have received some support from empirical data (Crandall & Templeton 1993). These hypotheses result in two criteria.

1 Frequencies of haplotypes contain information about their position in the network. Specifically, rare haplotypes (including singletons) occur preferentially at the tips of the cladograms and more frequent haplotypes in the interior. Therefore, more frequent haplotypes have a greater number of mutational connections in the network.
2 Geographical location can be used to resolve ambiguities because singletons are more likely to be connected to haplotypes from the same population than to haplotypes from different populations.

Once loops were resolved, a nested design was superimposed to the haplotype unrooted cladogram following the rules in Templeton *et al*. (1987) and Templeton & Sing (1993). Finally, an exact permutational contingency test (1000 permutations) was conducted for any resulting clade at each nesting level using the program GEODIS (Posada *et al*. 2000).

Results

Haplotypes

The *trnL-F* spacer is 399 or 400 bp long in the 105 sequences obtained and contains 10 variable sites. Fifteen haplotypes are distinguished. Eight haplotypes are exclusive to a single species (the singletons B, C, D, F, J, O, plus N and H), and the rest are found in either two or three species (Fig. 2). Haplotype K occurs in *Armeria trianoi* and *A. villosa*, haplotypes A, L, E occur in *A. filicaulis* and *A. villosa,* haplotype I occurs in *A. filicaulis*, *A. villosa* and *A. splendens,* haplotype G occurs in *A. filicaulis*, *A. villosa* and *A. bourgaei,* and haplotype M occurs in *A. colorata*, *A. malacitana* and *A. villosa.*

Nested clade analysis

The statistical parsimony analysis of the *trnL-F* data yielded a single network including all of the 15 haplotypes (Fig. 3A). However, the network contains several loops, which represent ambiguities. The two criteria from the coalescence (tip-interior rule and geographical location) can be invoked to break the loops formed by haplotypes I, L, M and N as well as that by haplotypes I, L, O and K as proposed in Fig. 3(B). Breaking the loop that affects haplotypes A, H, G and E cannot be solved unambiguously but some support for the proposed breakage comes from the fact that haplotype G spans a larger area some 150 km in diameter involving several massifs (Mágina, Cazorla–Segura, Baza) as compared to haplotype H. The nested design incorporated to the haplotype network contains only up to two-steps clades because haplotype variation is low (Fig. 3B). The results of the nested contingency test using GEODIS show that only one of the one-step clades reveals significant geographical structure (1–1), while both two-step clades as well as the total network reveal significant association with geography (Table 2).

Haplotype sharing

Seven cases of haplotype sharing between two (or even three) species within the same area are revealed by this study (Fig. 2, Table 1). From west to east, these are the following. Haplotype M is shared by *A. colorata*, *A. malacitana* and three subspecies of *A. villosa* within area 1. Haplotype K is shared by *A. trianoi* and *A. villosa* in area 3.

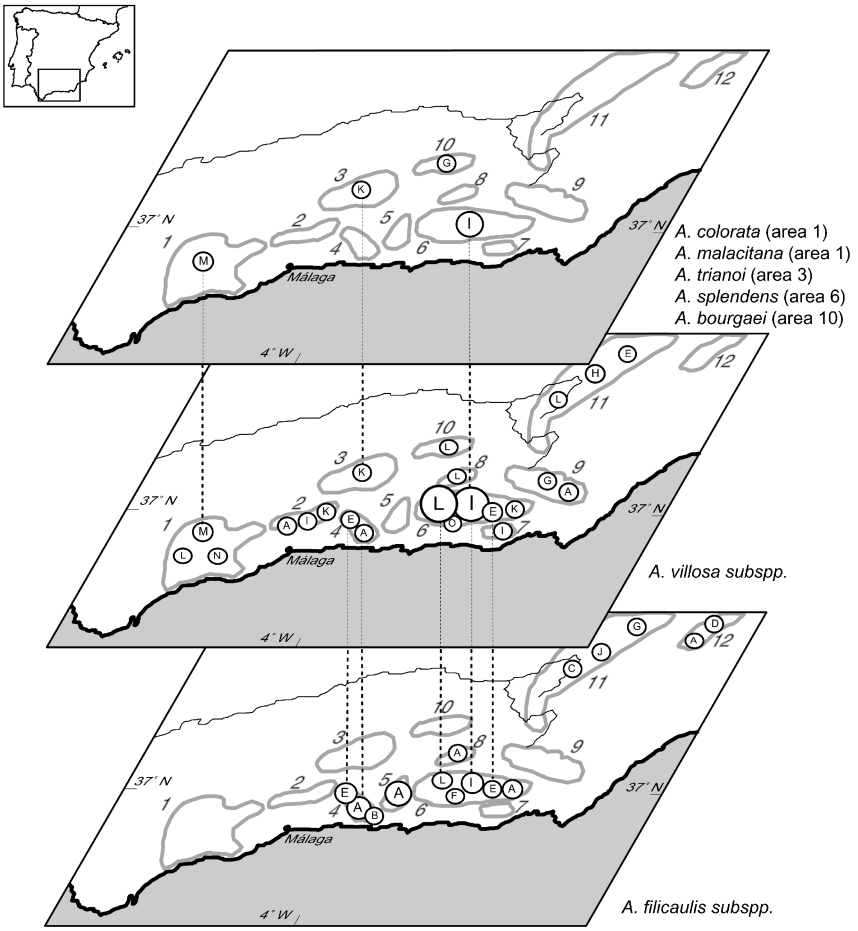

Fig. 2 Distribution of *trnL-F* haplotypes in *Armeria* in southeastern Spain. For convenience, the seven species sampled are separated into three different layers. Dashed lines indicate the occurrence of a given haplotype in the same area in two or three species. Numbered areas (in grey) correspond to the following biogeographically homogeneous units: (1) west of the Málaga province (Sierra Bermeja, Ronda, Mijas, etc.); (2) north of the Málaga province (Sierra de Alhama, Gorda, etc.); (3) subbaetic massifs in the Córdoba province; (4) Sierra Tejeda/Almijara; (5) Sierra de Cázulas/Guájaras; (6) Sierra Nevada; (7) Sierra de Gádor; (8) Sierra Harana; (9) Sierra de Baza/Filabres; (10) Sierra de Mágina and mountains S of Jaén; (11) Cazorla-Segura massif, Calar de Mundo, Cerro Javalcón; (12) mountains west of the Murcia province. Size of the haplotype symbol roughly reflects its frequency within each area.

Area 4 (Sierra Tejeda, Sierra Almijara) provides two more cases. Haplotypes E and A are shared between *A. villosa* and *A. filicaulis* (Fig. 2). Of the eight individuals sequenced from that chain, four have haplotype A, three have haplotype E, and one has the singleton B, which is likely to be derived from A (Fig. 3). Unlike *A. filicaulis*, *A. villosa* is very rare in the Tejeda/Almijara chain and occurs on *Quercus pyrenaica* understorey, a habitat that differs from the prevailing drier ecological conditions. The two individuals of *A. villosa* sequenced have haplotypes A and E, respectively. These two haplotypes are rare within *A. villosa* (8.2% each of the 61 sequences) and common or not so rare in *A. filicaulis* (51.6% and 12.9%, respectively, of the 31 sequences).

The remaining cases of haplotype sharing between species within the same area all refer to Sierra Nevada (area 6). Three haplotypes (I, L, E) are shared within that massif by *A. villosa* and *A. filicaulis* (Fig. 2). One of them (I) is also shared by all the six sampled individuals of the local high-altitude endemic *A. splendens*. Of the 20 occurrences of haplotype L, 18 correspond to *A. villosa*, 12 of them within Sierra Nevada (area 6, Table 1). The remaining two individuals belong to the endemic *A. filicaulis* ssp. *nevadensis*. Haplotype E is also interior but, unlike haplotype L, it is not predominant in one of the two species (four individuals of *A. filicaulis*, five individuals of *A. villosa*). Haplotype I is frequent in *A. villosa* (27.9% of the

Table 2 Permutational χ^2 probabilities for geographical structure of the clades shown in Fig. 3(B) from 1000 resamples; probabilities below 0.05 suggest significant geographical structure

Clade	Permutational χ^2 statistic	Probability
1–1	67.000	0.008*
1–2	5.833	0.868
1–3	21.000	0.200
1–4	55.238	0.136
1–5	6.000	1.000
1–6	4.444	1.000
2–1	103.44	0.002*
2–2	60.916	0.013*
Total cladogram	95.812	0.000*

*Significant at the 0.05 level.

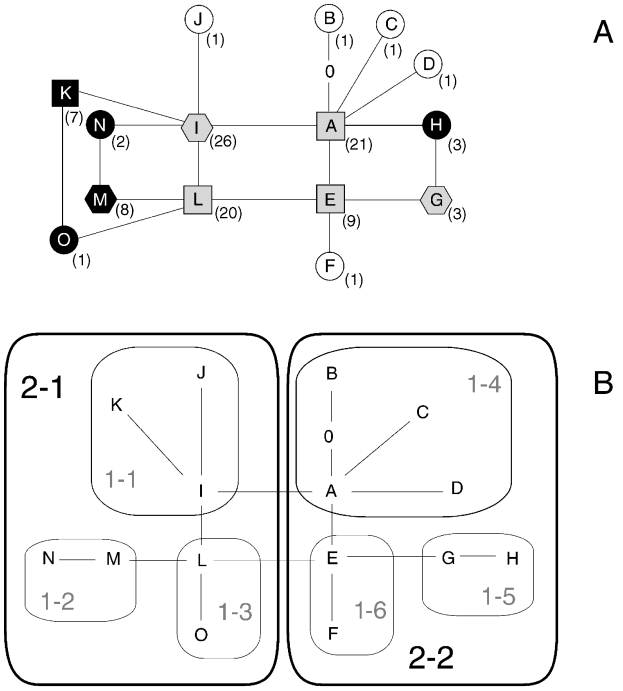

Fig. 3 (A) Haplotype parsimony network for 15 haplotypes defined on the basis of chloroplast *trnL-F* sequences in southern Spanish *Armeria*. Bars represent single mutational steps. Haplotypes in circles were found in a single species while squares indicate occurrence in two species and hexagon in three species. Black background indicates that the haplotype is present in *A. villosa* but not in *A. filicaulis*, white background indicates the opposite (present in *A. filicaulis* but not in *A. villosa*), grey background corresponds to haplotypes found both in *A. filicaulis* and *A. villosa*. Frequencies for each haplotype in parenthesis. (B) Nested design applied to the haplotype *trnL-F* network following the rules of the nested clade analysis. Narrow-lined boxes enclose one-step clades. Thick-lined boxes enclose two-step clades. Three loops in (A) have been broken using ancillary criteria from coalescent theory.

61 individuals), but moreover it is frequent in Sierra Nevada. Of the 26 individuals with this haplotype, only 3 were found elsewhere and two of them were in a close massif (Sierra de Gádor). Like haplotypes L and E, I is interior.

Discussion

Species-independent haplotype distribution

Because we are using organellar DNA, we may ask what conclusions can be drawn from our data independently of the taxonomic identification of each sample. This approach has been followed in addressing different questions (Wolf *et al*. 1997; Dumolin-Lapègue *et al*. 1999). With this restriction, we report low diversity for the *trnL-F* spacer in our sampling (15 haplotypes), which contributes to the occurrence of uncertainties (loops) in the statistical parsimony network. Three of those haplotypes (A, I, L) are much more frequent than the rest and all three occur in the interior of the network. This is in accordance with coalescent theory, which also predicts that these interior frequent haplotypes should be older (Hudson 1990; Castelloe & Templeton 1994; Fu & Li 1999). When the geographical origins of the samples are added to the results, we conclude that two of the 12 areas are rich in haplotypes (Fig. 2). These correspond to two massifs (Sierra Nevada with seven haplotypes, Cazorla-Segura with six haplotypes) holding rich biotas probably resulting from complex biogeographic histories.

At this taxonomy-free level of analysis, we also report from the nested clade analysis significant associations between haplotypes and geography when two-step clades (and the whole network) are considered (Table 2). Thus the geographical distribution of haplotypes does not seem to be merely the result of random sorting of ancestral polymorphisms.

Patterns of haplotype sharing across species in different areas

The fact that haplotypes are shared between more than one species suggests hybridization or introgression and/or shared ancestral polymorphisms (Schaal & Olsen 2000). The possibility of shared ancestral polymorphisms cannot be excluded in a genus with low molecular variation and a likely recent origin (Neigel & Avise 1986; Fuertes Aguilar & Nieto Feliner 2002; this work). However, when the occurrence of the same haplotype in two (or three) species coincides in the same area, as in the seven cases described in this study, horizontal transfer is much more likely. Schaal *et al*. (1998; see also Matos & Schaal 2000) have suggested introgression based on chloroplast haplotype sharing but focused on tip haplotypes. They argued that because tip haplotypes are usually younger than interior, it is less likely that they represent ancestral variation. However, we think that putative instances of horizontal transfer should not be restricted to shared tip haplotypes in the present study. Two facts are relevant in this respect. First, in all the seven cases, at least one of the species displays different haplotypes in other geographical areas (Table 1, Fig. 2). Second, when the results are analysed in detail it turns out that in most of these cases, the haplotype shared is predominant either in one of the species involved or in the area (see Results and Table 1). For instance, haplotypes A and L are predominant in species (*Armeria filicaulis* and *A. villosa*, respectively) while haplotypes M, K and I predominate in areas (1, 2–3, and 6, respectively). Support for the horizontal transfer interpretation provided by a taxon-predominant haplotype appearing marginally in another taxon is clear (e.g. haplotype L predominant in

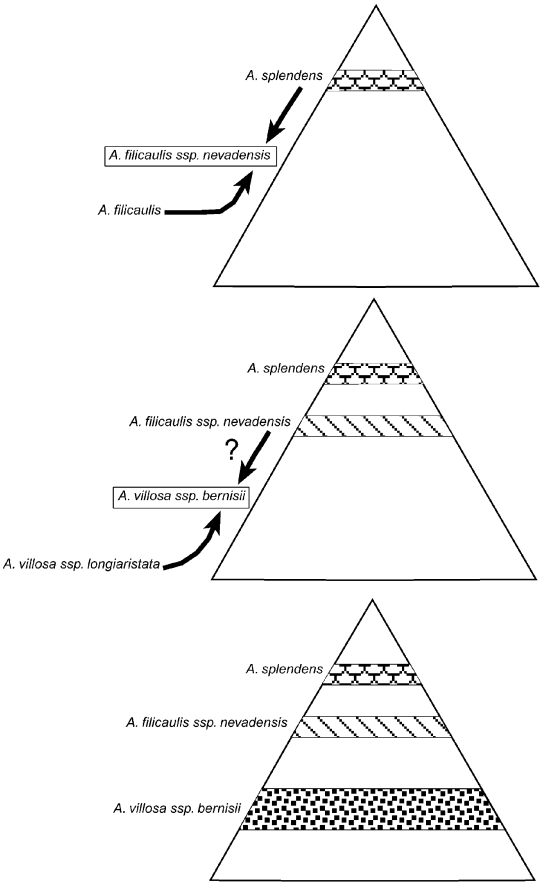

Fig. 4 Scheme representing a likely scenario for altitudinal migrations of *Armeria* in the Sierra Nevada massif, during glacial and interglacial Pleistocene periods, that resulted in horizontal transfer and possibly in the origin of hybrid taxa (see text).

A. villosa transferred horizontally to *A. filicaulis* in area 6; Table 1, Fig. 2). However, the support provided by the other pattern (haplotypes predominant or exclusive to an area) is less straightforward. The occurrence of a single haplotype in a single massif on two or more nonsister species suggests a single origin within the massif. The alternative explanation, persistence of ancestral polymorphisms, is unlikely because it requires that the same haplotype be sorted out independently in the same place in two different species. However, because it is found in several species, some kind of horizontal transfer must be involved. Similar cases of chloroplast haplotype sharing in certain areas, attributable to horizontal transfer, have been reported in Mexican *Pinus* (Matos & Schaal 2000), Australian *Eucalyptus* (McKinnon *et al*. 2001), and *Quercus* (Belahbib *et al*. 2001), among other studies.

In summary, the first conclusion from the patterns of haplotype sharing presented, i.e. co-occurrence of different species with the same haplotype in the same massif, is that they are probably a result of horizontal transfer all over southern Andalusia. This conclusion is reinforced when coincident patterns from several haplotypes are taken into account (e.g. *A. villosa* and *A. filicaulis* share two and three haplotypes in areas 4 and 6, respectively). This concordance diminishes the likeliness of lineage sorting in explaining haplotype distributions.

Glacial-induced altitudinal migrations

There is a second implication from the patterns of haplotype sharing in southern Spain, which relates to phylogeography in southern European territories. This is the main aim of this paper, and for this we need to focus on the Sierra Nevada massif.

The three species, on which we have documented horizontal transfer in Sierra Nevada (*A. splendens*, *A. filicaulis* ssp. *nevadensis*, *A. villosa* ssp. *bernisii*), currently occur in three different altitudinal layers. Therefore, contacts between them must have occurred in the past. The best explanation that we can provide for such a pattern is that contacts took place by glacial-induced migrations along altitudinal belts. It is known that one of the effects of climatic changes during Quaternary glacial periods was alteration of the current altitudinal belts of vegetation and concomitant contacts between previously isolated populations (Ferris *et al*. 1999). At the molecular level, repeated contraction and expansion would accumulate different genotypes in southern European refugia (Hewitt 1996; Taberlet *et al*. 1998), and this is also consistent with our chloroplast data showing that two of the three species presumably involved in such altitudinal migrations display a variety of haplotypes.

There is fossil pollen evidence to support the occurrence of migrations along glacial and interglacial periods in Southern Europe in concordance with the contraction–expansion model (Hewitt 1996). Based on molecular markers, there is also ample evidence of migrations provoked by climatic changes during the Pleistocene both in Europe and North America (Soltis *et al*. 1997; Taberlet *et al*. 1998). The altitudinal gradient is also the basis of well-studied cases of gene flow and hybrid zones in plants (Arnold 1997; Brochmann *et al*. 2000). However, to our knowledge there is no explicit report documented on molecular grounds of glacial-induced migrations across altitudinal belts in a given massif.

Possibly because of its latitude, east–west orientation, and the marked altitudinal gradient, Sierra Nevada represents a cul-de-sac for many species of temperate areas. Under this framework, it is possible that the current situation for *Armeria* is a result of three waves of colonization: a first one that brought *A. splendens* into the massif, a second one that involved colonization by *A. filicaulis*, and a third one, in which more temperate conditions associated with

forest expansion allowed colonization by *A. villosa* (Fig. 4). Altitudinal variation of vegetation belts associated with climatic changes in the Pleistocene may account for the introgression events and the origin of hybrid taxa. Although glaciers were restricted to higher elevations in the Sierra Nevada massif during Quaternary cold periods, the glaciated altitudinal range was 850 m downwards from the summits on the north slope and 650 m on the southern (Gómez Ortiz & Salvador Franch 1996). This suggests that direct glacial activity must have affected the suitable habitats for *A. splendens*, *A. filicaulis* and *A. villosa* and that altitudinal ranges of the three species probably moved considerably up and down during ice ages. As a result of topographic conditions (e.g. northern vs. southern orientations, valleys vs. exposed slopes) it is likely that ranges did not move evenly, thereby facilitating contact between species.

Some of these glacial-induced altitudinal migrations probably resulted not only in gene flow but in the formation of hybrid taxa. This possibility is exemplified by the case of the Sierra Nevada endemic *A. filicaulis* ssp. *nevadensis*. Since this case adds to the whole interpretation of the patterns of haplotype sharing, the relevant evidence presented here is briefly discussed. It was proposed that this subspecies is the result of hybridization between the Sierra Nevada high-altitude endemic *A. splendens* and some form of *A. filicaulis* (Nieto Feliner *et al*. 1998). The altitudinal range of ssp. *nevadensis* (2100–2550 m) as compared to the two parents (2880–3100 m in *A. splendens*, < 1900 m in most populations of *A. filicaulis* sensu stricto) is also consistent with such a hypothesis, as is morphological and nuclear ribosomal DNA internal transcribed spacer data (Fuertes Aguilar *et al*. 1999a,b). The chloroplast data presented in this study are also consistent with a hybrid origin. In contrast to other narrow endemics sampled that display a single haplotype (*A. splendens*, *A. trianoi*, *A. colourata*), *A. filicaulis* ssp. *nevadensis* displays three *trnL-F* haplotypes in an area not more than 10 km in diameter. Of the three haplotypes, two have not been found elsewhere within *A. filicaulis* (I, L) but have been found within Sierra Nevada (I in *A. splendens*, both in *A. villosa*), and the third one (A) seems to be predominant in *A. filicaulis* (Fig. 2). Chloroplast DNA is maternally inherited in *Armeria* (Nieto Feliner *et al*. 2002). It is thus likely that the *filicaulis* progenitor contributed haplotype A to ssp. *nevadensis* and the other progenitor (*A. splendens* with haplotype I) also contributed as ovule donor. This seems likely in plants with low internal reproductive barriers and contrasts with other groups where hybridizing species are highly intersterile but sporadically may originate hybrid F_1 and subsequent lineages (Arnold 1997).

This scenario would explain why *A. splendens* displays a single haplotype (I) unlike the other two species in the massif. This haplotype could be a result of common ancestry (not to horizontal transfer) and would be consistent with the finding of the same haplotype in high mountain plants like *A. alpina* and its relatives *A. bigerrensis* and *A. caespitosa* (unpublished), none of them occurring in southern Spain (Nieto Feliner 1990). The possibility that *A. splendens* was the first inhabitant of *Armeria* in Sierra Nevada is also consistent with the sharing of a single internal transcribed spacer sequence by the three species in the massif (Fuertes Aguilar *et al*. 1999b). In accordance with the latter, the wide altitudinal range displayed by haplotype I (1250–3100 m) in Sierra Nevada makes it conceivable that it was transferred downwards along the massif from *A. splendens* into *A. filicaulis* and *A. villosa*, as might have been the single internal transcribed spacer sequence from the massif.

The possibility that a hybridization event, allowed by altitudinal migrations, was in the origin of *A. villosa* ssp. *bernisii* (Fuertes Aguilar *et al*. 1999b; Fuertes Aguilar & Nieto Feliner 2002) is not contradicted by the chloroplast data presented but the pattern seems to be more complex than in *A. filicaulis* ssp. *nevadensis*. *Armeria villosa* is involved in all the seven cases of haplotype sharing, including the three in Sierra Nevada (Fig. 2). But separating instances of horizontal transfer from one that originated a new race is not easy on the basis of the *trnL-F* data. In fact, even in *A. filicaulis* ssp. *nevadensis*, there are hints that gene flow might have occurred after its hybrid origin, e.g. the occurrence of the *villosa*-predominant haplotype L in ssp. *nevadensis*.

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