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Fine-scale Geographical Structure, Intra-individual Polymorphism and Recombination in Nuclear Ribosomal Internal Transcribed Spacers in *Armeria* (Plumbaginaceae)

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• *Background and Aims* Isolation and drift are the main causes for geographic structure of molecular variation. In contrast, the one found in a previous survey in *Armeria* (Plumbaginaceae) for nuclear ribosomal ITS multicopy regions was species-independent and has been hypothesized to be due to extensive gene-flow and biased concerted evolution. Since this was inferred from a genus-level phylogenetic analysis, the aim of this study was to check for the occurrence of such structure and the validity of the proposed model at a local scale, in a southern Spanish massif (Sierra Nevada), as well as to examine the evolutionary implications at the organism level.
• *Methods* In addition to 117 sequences of direct PCR products from genomic DNA, 50 sequences of PCR products from cloned DNA were obtained to analyse cases of intragenomic polymorphisms for the ITS regions.
• *Key Results* Sequence data confirm the occurrence of a species-independent structure at a local scale and reveal insights through the analysis of contact areas between different ITS copies (ribotypes). A comparison between cloned and direct sequences (*a*) confirms that, within these contact areas, ITS copies co-occur both in different individuals and within single genomes; and (*b*) reveals recombination between different copies.
• *Conclusions* This study supports the utility of direct sequences for detecting intra-individual polymorphism and for partially inferring the ITS copies involved, given previous knowledge of the variability. The main evolutionary implication at the organism level is that gene-flow and concerted evolution shape the geographic structure of ITS variation. © 2004 Annals of Botany Company

Key words: *Armeria*, nuclear ribosomal ITS, intragenomic polymorphism, recombination, geographical structure, gene-flow, biased concerted evolution.

INTRODUCTION

Evidence is increasingly accumulating that shows that intra-individual variation for nuclear ribosomal internal transcribed spacer (ITS) regions should not be considered as an exceptional occurrence, in spite of the homogenizing mechanisms known as concerted evolution (Roelofs *et al*., 1997; Brasier *et al*., 1999; Harris and Crandall, 2000; Chiang *et al*., 2001; Gandolfi *et al*., 2001). Because of the influence of concerted evolution, the occurrence of ancestral polymorphisms is not the most likely ultimate cause for intragenomic variability in this marker. Instead, a more frequent origin is the merging of different ITS copies within the same genome as a consequence of gene-flow. Once the two copies meet, the fate of the polymorphism depends on genetic, reproductive and population-level factors: specifically, the number and location of ribosomal loci (in the same or different chromosomes), the occurrence of polyploidy and/or apomixis (Buckler *et al*., 1997; Campbell *et al*., 1997; Hershkovitz *et al*., 1999; Zhang and Sang, 1999) and the relative abundance of different ITS copies in the breeding populations.

ITS regions are the most widely used molecular markers for reconstructing plant phylogenies at the intrafamily level (Baldwin *et al*., 1995). Because of this, most reports discussing intragenomic variability for these regions stress the negative implications when estimating phylogenies (for a recent review, see Álvarez and Wendel, 2003). In contrast, the work described here focuses on the insights that studying intragenomic variability at a fine level might produce when addressing questions that affect the organism, specifically hybridization.

Hybridization is a major force in plant evolution, as evidenced by the proportion of living angiosperms that show a basic chromosome number of polyploid origin (Masterson, 1994; Arnold, 1997). Hybridization at the homoploid level lacks the evolutionary advantages of poliploidy for readjusting the genetic architectures of the two genomes involved in a hybridization event into a single one (Wendel, 2000). Consequently, the examples at this level are much less scarce (Rieseberg, 1997). Still, in the cases that have been thoroughly studied there is evidence of the selective advantage of introgressed genomes (Rieseberg *et al*., 1996; Lexer *et al*., 2003), thus revitalizing the old ideas of Anderson (1949) about the adaptive role of introgression. ITS markers have been used to address questions of hybridization mostly through detection of additive patterns (Sang *et al*., 1995; Quijada *et al*., 1997; Vargas *et al*., 1999; Rauscher *et al*., 2002; Wichman *et al*., 2002), but also by combining evidence from other differently inherited markers (Hughes *et al*., 2002).

The investigations described here have combined different markers to try to understand how representatives of a

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genus of diploid, self-incompatible plants with weak internal reproductive barriers (*Armeria*, Plumbaginaceae) can cope with frequent hybridization and introgression without a resulting loss of diversity at the species level. The most convincing evidence for hybridization and introgression in *Armeria* has come from the discovery of a geographical structure of ITS variation, which is partly independent of, and conflicts with, taxonomy (Fuertes Aguilar *et al*., 1999*b*). To explain this pattern, a model has been proposed that takes into account the molecular evolution of this marker (Nieto Feliner *et al*., 2001) and the reproductive features of *Armeria* (Nieto Feliner *et al*., 1996). Such a model has to encompass an important amount of gene-flow between congeners and biased concerted evolution of multicopy regions within areas (Fuertes Aguilar *et al*., 1999*a*). Although the best explanation for the available data, the model does not provide a full understanding of the situation because the sampling was focused at the genus level. The present paper adopts a finer scale approach to check for the occurrence of such geographic structure within a southern Spanish massif (Sierra Nevada), as well as to address the validity of the model and to try to throw light on its genetic background.

Elsewhere the scarce but meaningful occurrence of intragenomic additive polymorphisms in estimating the ITS phylogeny of the whole genus is addressed (Fuertes Aguilar and Nieto Feliner, 2003). Here, the fine-scale geographical distribution of this kind of polymorphisms is explored to see if they are consistent with the geographic structure. Further, sequences from clones were examined and compared with those of direct PCR products from genomic DNA to test for the occurrence of intragenomic recombination. This also serves to address the extent to which co-occurrence of two ribotypes within the same genome can be deduced from direct sequences.

MATERIALS AND METHODS

Sampling

The sampling focused on the Sierra Nevada massif, where three different species were found to share an exclusive ITS copy in a general survey of the genus. The three species can be easily distinguished on morphological grounds (Gutiérrez Larena *et al*., 2002) and occur at different elevations: *Armeria splendens* above 2880 m; *A. filicaulis* ssp. *nevadensis* between 2100 and 2550 m, on the north-west 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. Individuals of these three species were sampled from the massif and from neighbouring areas, together with plants from seven other species: *A. bourgaei*, *A. colorata*, *A. filicaulis* (including four subspecies), *A. malacitana*, *A. splendens*, *A. trianoi* and *A. villosa* (including four subspecies) (Table 1). This sampling strategy, employed in previous studies, was justified by the frequent occurrence of hybridization. The total number of individuals sampled was 117, of which 50 were from Sierra Nevada.

DNA Isolation, PCR and sequencing

DNA isolation from fresh or silica-gel preserved material was performed using a CTAB protocol (Doyle and Doyle, 1987), with slight modifications in incubation conditions (overnight at 60 °C) (Fuertes Aguilar *et al*., 1999*b*). Double-strand amplification of the ITS region was performed on a Gene Amp PCR System 9700 (PE Biosystems, Foster City, CA, USA) with 20 µl reactions. Primers are described in Fuertes Aguilar *et al*. (1999*b*). Each reaction consisted of 10 µl of DNA plus 10 µl of cocktail. The cocktail was composed of 0·3 µl of dNTP (2·5 mM each), 2 µl of 10× buffer, 1·2 µl of 25 mM $MgCl_2$, 1 µl of each primer (10 µM), and 0·1 µl (1 U μl^{-1}) of AmpliTaq Gold (PE Biosystems). The cycle profile was: an initial cycle of 94 °C (12 min); five cycles of 94 °C (30 s), 54 °C (30 s), 72 °C (1 min); then 33 cycles with an annealing temperature of 48 °C, and a final extension step of 72 °C (10 min). PCR product was purified using MoBio 101 silica-matrix columns, checked in an agarose 1·5 % 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 (Applied Biosystems Inc., Foster City, CA, USA). The product was then separated and analysed on an ABI 377 automated DNA sequencer (Perkin-Elmer, Shelton, CT, USA/Applied Biosystems) at the Centro de Investigaciones Biológicas, CSIC. Resulting electropherograms were examined for ambiguities and overlapped 100 % to obtain a consensus sequence. Sequence alignment was performed manually using SeqApp. Of the 117 direct sequences analysed, 79 are new and have been submitted to the GenBank database (see Table 1).

Cloning

To check for the occurrence of recombination, PCR products from six individuals (see Table 1) were agarose gel purified and ligated into the vector provided with the p-GEM-T (Promega) cloning kit. Plasmid DNA from individual recombinant colonies was isolated according to a miniprep protocol (High Pure Plasmid Isolation kit; Roche Diagnostics, S.L., San Cugat del Vallés, Spain). For sequencing, cloned ITS products were cycle-sequenced with BigDye Terminator Cycle Sequencing Ready Reaction (Perkin-Elmer, Applied Biosystems) using the amplification primers. Five to fourteen clones were sequenced from each of the six isolates, resulting in 50 cloned sequences. All these have been submitted to the GenBank data base (accessions AY438482–AY438531).

Sequence analysis

IUPAC ambiguity codes were used for coding polymorphic positions. Additive polymorphic sites (APS), i.e. double peaks in the electropherograms in which both bases were also found in other accessions, were scored following the criteria in Fuertes Aguilar and Nieto Feliner (2003). Co-occurrence of more than one ribotype was deduced from APS in cases where two ribotypes only differed by

substitutions. For those cases in which an indel was involved and thus direct readings showed double peaks beyond a particular point, it was assumed that one of the ribotypes was the one exclusive to Sierra Nevada. This was justified by our previous knowledge of ITS variation in *Armeria.* Ribotype R3 is the only one showing indels (1 bp indel in site 93) and it has three additional synapomorphic substitutions (sites 129, 471 and 499, Table 1; Fuertes Aguilar *et al.*, 1999*b*; Fuertes Aguilar and Nieto Feliner, 2003). The second copy was deduced by 'subtraction' (Wichman *et al.*, 2002).

For cloned sequences, variable positions do not coincide totally with those in direct sequences. This is due to the restricted geographical sample of the clones (Fig. 1). Point mutations appearing in a single clone were not considered in the analysis since they are likely to be the result of mistakes introduced by the Taq polymerase (Smith *et al.*, 1997; Eyre-Walker *et al.*, 1998). Following the same rationale, nucleotides for which there was no known variation in our previous samplings of the genus (covering approx. 250 sequences) and do not occur in more than two clones were not scored. As a result, ten nucleotides were scored for cloned sequences characterization: 92, 128, 387, 388, 467, 495, 506, 513, 556 and 595.

RESULTS

Sequences of direct PCR products from genomic DNA

The ITS1 + 5.8S + ITS2 region is 620 or 621 bp long in the 117 genomic sequences obtained and contains 44 variable sites. However, in the context of the present sampling, the concentration has been on ten sites (Table 1). Eighty-three of the 117 sequences matched the previously defined ribotypes (Table 1 and Fig. 1; Fuertes Aguilar *et al.*, 1999*b*; Fuertes Aguilar and Nieto Feliner, 2003). R1 is the most widespread geographically of the four ribotypes represented in the current sampling. It occurs over most of the Iberian Peninsula, where it is rare only in south-eastern ranges and northern coastal areas. R2 is restricted to central Andalusia. R3 is exclusive to the Sierra Nevada massif. R4 occurs in the eastern Andalusian ranges (Cazorla-Segura). Most of the direct sequences not matching any of these ribotypes are those in which APS have been detected. Sequences containing APS have been identified as reflecting intragenomic polymorphism, i.e. the co-occurrence of more than one ribotype within the same individual. Most of these involved a mixture of two sequences that differ by 1 bp indel, i.e. including double peaks in the electropherograms beyond site 93.

Thirty-four accessions with APS are interpreted as involving mixtures of both R2 and R3 (19 cases), R3 and R1 (seven cases), R2 and R4 (one case) or one identified ribotype and an unidentified one (six cases, commented on below). Ribotypes R2 and R3 differ in five positions (Table 1), R3 and R1 differ in six positions, R2 and R4 differ in four or five positions depending on whether it is a sub-ribotype of R4 (distinguished by position 471) or not. Direct sequences resulting from these samples containing more than one ribotype do not always display APS for all the differing positions. This pattern is probably due to incomplete homogenization and results in the following features: eight of the 19 accessions that have co-occurring R3 and R2 show APS for all the five differing positions, eight show four APS, and three show three APS. Of the seven accessions with co-occurring R3 and R1, two display APS for all the six differing positions, four show five APS, and one shows four APS. The single accession with co-occurring R2 and R4 has two APS. The six remaining accessions showing APS need additional explanation (marked with a dagger in Table 1 and a pink dot in Fig. 1). They show one single APS, otherwise matching either ribotype R1 (one), R2 (one), R3 (two) or R4 (two). They may be the result of a more advanced homogenization stage following the merging of two ribotypes. But in all the six accessions the single APS does not allow the identification of the second ribotype involved. Alternatively, these single APS might be due to point mutations, although the fact that they appear in informative positions (Table 1) makes such a possibility less likely. The only accession not fully fitting any of the previous ribotypes, while lacking APS, is a sample of *A. villosa* ssp. *longiaristata* from Córdoba (accession no. 112, Table 1). It is interpreted as a mixture of ribotypes R2 and R4 in which two of the five differing positions (471 and 599) have been homogenized towards R4, while three (169, 367 and 517) have been homogenized towards R2.

Bias in homogenization of the variable nucleotide positions has been explored by examining sequences containing at least two APS, but results are not conclusive. This may be partly due to the difficulty of properly accounting for the effect of crossing towards the predominant R3. Nevertheless, there was some indication for bias favouring an 'A' in position 471, representing a synapomorphic character for ribotype 3 exclusive to Sierra Nevada. Ten of the 11 accessions with two or more APS in which that position was homogenized displayed an 'A'. Other cases were less conclusive, e.g. position 499. Five of the six accessions with two or more APS in which the position was homogenized displayed a 'G'.

Sequences of PCR products from cloned DNA

Sixteen different sequences were obtained from the 50 clones sampled. After discarding autapomorphies and probable artefactual substitutions (see above), the sequence in 28 of the clones matched ribotype R3 of Sierra Nevada (Fig. 2). Other replicated sequences were c2 (three times), c7 (twice), c8 (three times) and c10 (three times). The remaining 11 sequences were detected each in a single clone.

Once the sequences matching previously defined ribotypes were located (R3, 28 samples; R1, one sample; R2, one sample), each of the remaining 20 sequences was examined by comparison with all known ITS sequences in *Armeria* (Fuertes Aguilar and Nieto Feliner, 2003). Of the 16 different cloned sequences, two (c7 and c9) differed by one base from R3 and one (c12) did from R2. These sequences were interpreted as arising from point mutations

TABLE 1. *Informative positions for sequences of direct PCR products from genomic DNA of the nuclear ribosomal ITS1 + 5.8S + ITS2 regions in 117 samples of* Armeria *from southern Spain. To facilitate ascription to previously defined ribotypes in the genus, the diagnostic nucleotides based on Fuertes Aguilar and Nieto Feliner (2003) are given at the end of the table*

Accession no.	Taxon	Voucher no.	93	129	169	367	388	389	471	499	517	599	Ribotype	Observations	GenBank accession no.
1	*A. bourgaei*	Vogt3328	–	C	T	T	C	C	G	G	G	A	R4		AY179766
2–4	*A. colorata*	GN3683, MGC44192, MGC44195	–	C	T	A	T	T	G	G	A	G	R1*		AJ225592, AF233316, AF233317
5	*A. filicaulis* ssp. *alfacarensis*	GN2722	–	C	A	A	C	C	G	G	A	G	R2		AY179779
6		GN4070	A/–	T/C	T/A	A	C	C	A	T/G	A	G	R3/R2	1 site homogenized towards R3 (471)	AY444063
7		GN4074	A/–	T/C	T/A	A	C	C	A/G	G	A	G	R3/R2	1 site homogenized towards R2 (499)	AY444064
8	*A. filicaulis* ssp. *filicaulis*	157PV00	–	C	T	A	T	T	G	G	A	G	R1		AY444075
9		GN4042	A	T	T	A	C	C	A	T	A	G	R3		AY444070
10–14		105PV99, Castrov.14566, MA490911, MA319954, MA208105	–	C	T	T	C	C	G	G	G	A	R4		AY444074, AJ225579, AY444073, AY444072, AY444071
15–17		GN4026, GN4032, GN4041	A/–	T/C	T/A	A	C	C	A/G	T/G	A	G	R3/R2		AY444065, AY444067, AY444069
18		GN4027	A/–	T/C	T/A	A	C	C	A	T/G	A	G	R3/R2	1 site homogenized towards R3 (471)	AY444066
19		GN4035	A/–	T/C	T/A	A	C	C	A/G	G	A	G	R3/R2	1 site homogenized towards R2 (499)	AY444068
20–24	*A. filicaulis* ssp. *nevadensis*	BG6, BG14, **BG15,** BG28, BG33	A	T	T	A	C	C	A	T	A	G	R3		AY444076, AY444078, **AY444079,** AY444080, AY444081
25		**BG10**	A/–	T/C	T	A	T/C	T/C	A	T/G	A	G	R3/R1	1 site homogenized towards R3 (471)	**AY444077**
26		BG121	A/–	T/C	T	A	T/C	T/C	A/G	T/G	A	G	R3/R1		AY444082
27–29	*A. filicaulis* ssp. *trevenqueana*	GN4084, GN4090, CN2248	A	T	T	A	C	C	A	T	A	G	R3		AY444083, AY179781, AY179782
30–32	*A. filicaulis* var. *minor*	GN4006, GN4017, GN4020	–	C	A	A	C	C	G	G	A	G	R2		AY444084, AY179780, AY444086
33		GN4018	–	C	T/A	A	C	C	G	G	A	G	R2[†]	1 polymorphic site (169)	AY444085
34		AP1	A/–	T/C	T/A	A	C	C	A/G	T/G	A	G	R3/R2		AY444087
35	*A. malacitana*	GN1733	–	C	T	A	T	T	G	G	A	G	R1*		AJ225595
36–42	*A. splendens*	**BG17,** BG23, BG43, BG44, BG52, BG57, IA1388	A	T	T	A	C	C	A	T	A	G	R3		**AY179817,** AY444088, AY444089, AY444090, AY444091, AY444092, AJ225591
43		BG135	A	T/C	T	A	T/C	T/C	A	T/G	A	G	R3/R1	2 sites homogenized towards R3 (93, 471)	AY444093

TABLE 1. *Continued*

Accession no.	Taxon	Voucher no.	93	129	169	367	388	389	471	499	517	599	Ribotype	Observations	GenBank accession no.
44	*A. trianoi*	GN3992	–	C	A	A	C	C	G	G	A	G	R2		AY179821
45	*A. villosa* ssp. *bernisii*	AP bern 1	–	C	A	A	C	C	G	G	A	G	R2		AY444131
46–71		GN4048, GN4054, GN4078, GN4095, **GN4096,** GN4104, GN4109, GN4112, GN4113, GN4123, GN4126, GN4151, GN4152, GN4155, GN4172, GN4175, GN4181, GN4186, GN4190, GN4193, GN4198, GN4203, GN4208, GN4213, GN1370, MA503922	A	T	T	A	C	C	A	T	A	G	R3		AY444096, AY444097, AY444101, AF270510, **AY444102**, AF270507, AY444103, AY444104, AY444105, AY444106, AY444107, AY444111, AY444112, AY444113, AY444116, AY444117, AF270508, AY444119, AY444121, AY444122, AY444123, AY444124, AY444125, Y444126, AY444095, AY444134
72, 73		GN4144, GN4176	A	T	T	A	C	C	A	T/G	A	G	R3[†]	1 polymorphic site (499)	AY444110, AY444118
74, 75		BG37, BG40	A/–	T/C	T	A	T/C	T/C	A	T/G	A	G	R3/R1	1 site homogenized towards R3 (471)	AY444128, AY444129
76		**GN4139**	A/–	T/C	T	A	T/C	T/C	A/G	T/G	A	G	R3/R1		**AY444109**
77, 78		GN4068, MGC44202	A/–	T/C	T/A	A	C	C	G	G	A	G	R3/R2	2 sites homogenized towards R2 (471and499)	AY444100, AY444132
79		GN4066	A/–	T/C	T/A	A	C	C	A	G	A	G	R3/R2	1 site homogenized towards R3 (471) and 1 towards R2 (499)	AY444099
80		BG1	A	T/C	T/A	A	C	C	A/G	T/G	A	G	R3/R2	1 site homogenized towards R3 (93)	AY444127
81, 82		GN4133, **GN4187**	A/–	T/C	T	A	C	C	A/G	T/G	A	G	R3/R2	1 site homogenized towards R3 (169)	AY444108, **AY444120**
83		GN4061	A/–	T/C	T/A	A	C	C	A	T/G	A	G	R3/R2	1 site homogenized towards R3 (471)	AY444097
84–87		GN4160, GN4164, AP-2, MGC44193	A/–	T/C	T/A	A	C	C	A/G	T/G	A	G	R3/R2		AY444114, AY444115, AY444130, AY444133
88	*A. villosa* ssp. *longiaristata*	GN3862	–	C	T	A	T	T	G	G	A	G	R1		AJ225601

TABLE 1. *Continued*

Accession no.	Taxon	Voucher no.	93	129	169	367	388	389	471	499	517	599	Ribotype	Observations	GenBank accession no.
89, 90		MGC44200, MGC46065	–	C	T	A	C	C	A	G	A	G	R1*		AF270504, AF270502
91		GN3676-2,	–	C	T	A	T	T/C	G	G	A	G	R1[†]	1 polymorphic site (389)	AJ225605,
92-101		GN4002, GN4004, GN4239, GN4237, GN4249-1, GN3679-5, GN3679-7, GN3850, GN3853, MGC44196,	–	C	A	A	C	C	G	G	A	G	R2		AY179831, AY444135, AY444136, AF270505, AY444138, AJ225617, AJ225616, AJ225603, AJ225602, AF270503
102–107		GN3346-2, GN3346-13, BG108, GN3675-5, GN3675-7, GN3678-3,	–	C	T	T	C	C	G	G	G	A	R4		AJ225586, AJ225611, AY444141, AJ225607, AJ225609, AJ225606,
108		BG87	–	C	T	T	C	C	A/G	G	G	A	R4	The two nucleotides detected in position 471 occur within R4 (Fuertes Aguilar *et al*., 1999*b*)	AY444140
109 110		GN3676-8, GN3678-2	–	C	T	T	C	C	G	G	A/G	A	R4[†]	1 polymorphic site (517)	AJ225612, AJ225608
111		MA508698	A/–	T/C	T	A	T/C	T/C	A	T/G	A	G	R3/R1	1 site homogenized towards R3 (471)	AY444142
112		GN4257-3	–	C	A	A	C	C	A	G	A	A	R2/R4	3 sites homogenized towards R2 (169, 367, 517) and 2 towards R4 (471, 599).	AY444139
113		GN4243	–	C	T/A	A	C	C	A/G	G	A	G	R2/R4	3 sites homogenized towards R2 (367, 517 and 599)	AY444137
114,11	*A. villosa* ssp. *villosa*	MGC46063, MGC46064	–	C	T	A	T	T	G	G	A	G	R1*		AF233318, AF233319
116 117	*A. villosa* ssp. *carratracensis*	GN3803-1, GN3803-2	–	C	T	A	T	T	G	G	A	G	R1*		AY179828, AY179830

Accessions in bold are those from which cloned sequences have been obtained.

* A subtype of ribotype 1 (i.e. falling within a subclade of the large one encompassing ribotype1 in Fuertes Aguilar and Nieto Feliner, 2003).

[†] Sequences with only one polymorphic informative site, which probably reflect an advanced homogenization stage after the merging of two ribotypes, and where the second ribotype is unclear.

Diagnostic nucleotides based on Fuertes Aguilar and Nieto Feliner (2003) are given below:

93	129	169	367	388	389	471	499	517	599	Ribotype
–	C	T	A	T	T	G	G	A	G	R1
–	C	A	A	C	C	G	G	A	G	R2
A	T	T	A	C	C	A	T	A	G	R3
–	C	T	T	C	C	G (A)	G	G	A	R4

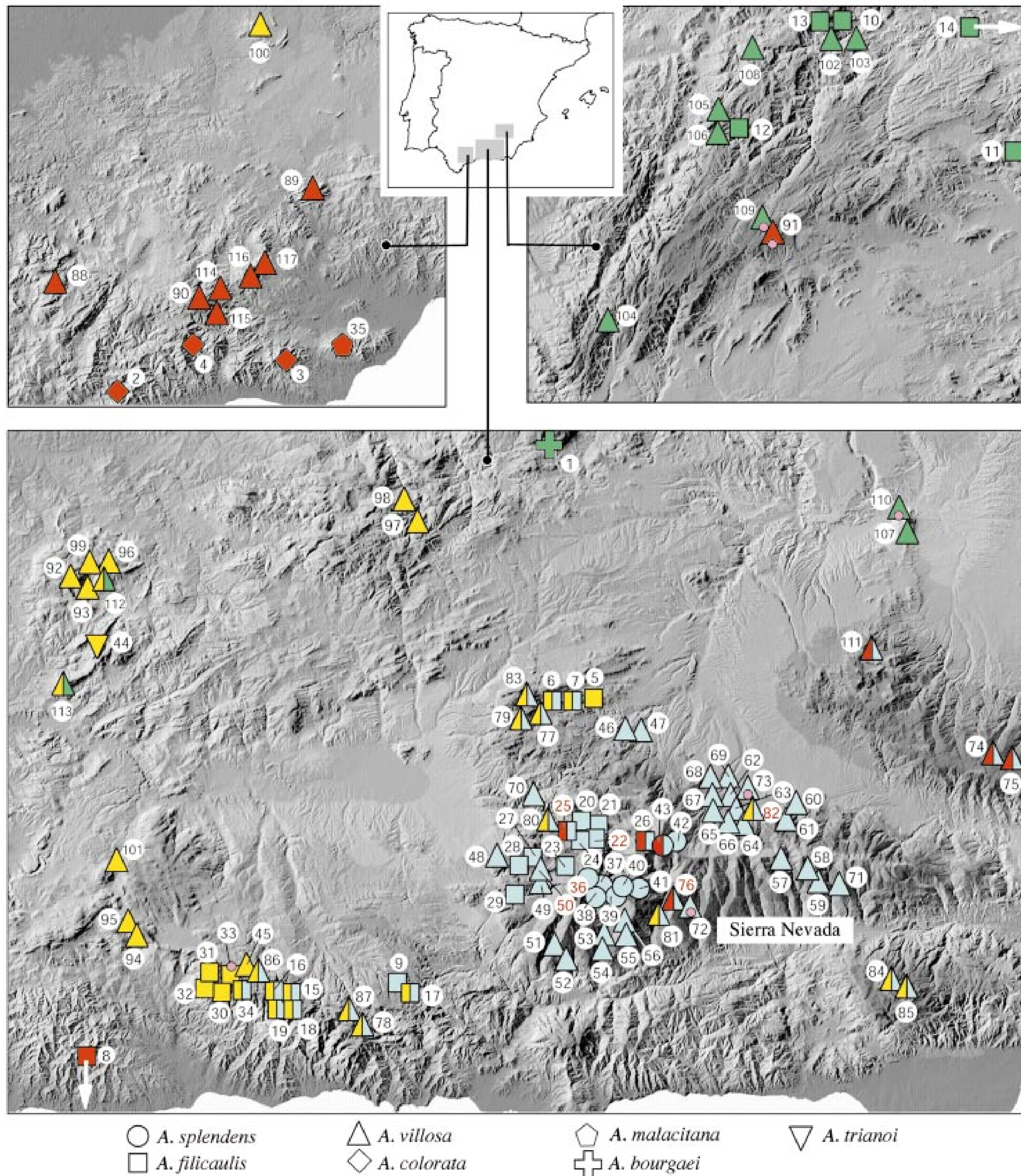

FIG. 1. Distribution of ITS copies (ribotypes) in seven species of *Armeria* in south-eastern Spain: R1 (red); R2 (yellow); R3 (blue); R4 (green). Double colours indicate intragenomic mixtures of two ribotypes; all those in which R3 is involved differ by 1 bp indel. Those bearing a small dot denote partially homogenized ribotypes via concerted evolution, in which only one site shows additive polymorphisms. Samples from which cloned sequences have been obtained have their accession numbers in red. R1 corresponds to clade 1, R2 to clade 4, R3 to clade 3, and R4 to clade 8 in Fuertes Aguilar and Nieto Feliner (2003).

(Fig. 3). Ten of the sequences are likely to be recombinants, and four of them may result from double recombination.

Comparing direct with cloned sequences, the three individuals with co-occurring ribotypes (as identified by direct sequencing) produced five, seven and ten different clones, respectively, whereas the three individuals without APS produced a single cloned sequence: R3 (Fig. 2). The exception to this consistency was the recombinant sequence c2, appearing in two individuals (accessions nos 25 and 76) with co-occurring ribotypes (as identified by direct

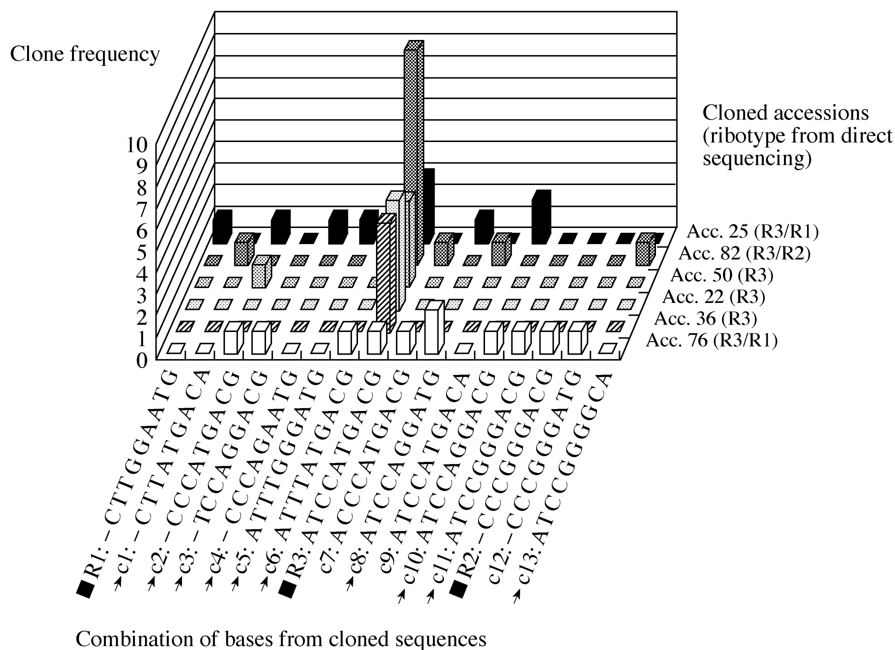

FIG. 2. Frequency of cloned ITS sequences across six individuals. The *x*-axis shows the 16 different sequences obtained from 50 clones, represented by positions used to define ribotypes (see text and Fig. 3). The *y*-axis displays the ribotype profile inferred from direct sequencing from the six accessions sampled for cloning. Black boxes indicate sequences that match previously defined ITS copies (R1, R2 and R3). Arrows indicate possible recombinants as interpreted in Fig. 3.

sequencing) but also in a clone from one individual (accession no. 50) with no signs of intragenomic polymorphisms in its genomic sequences. Other inconsistencies include cases in which a position that appeared invariant by direct sequencing was shown to be polymorphic when the cloned sequences were obtained. For example, a G in position 467 was only detected against the A (characteristic of R3) in clones (R1 and c5) from an individual (*A. filicaulis nevadensis*, accession no. 25) but not when PCR products were sequenced directly from genomic DNA amplifications.

DISCUSSION

Geographical structure and intragenomic polymorphisms for ITS

The study described here confirms the species-independent geographic structure of ITS variation in *Armeria* when analysed at a local scale. There is a ribotype almost exclusive to the Sierra Nevada massif (R3), which has been found co-occurring with other ribotypes in neighbouring massifs (Fig. 1). It has been detected as the only ITS copy in 43 of the 50 direct sequences from Sierra Nevada and in one sample from a nearby range (accession 9, Table 1). But when cases of intragenomic polymorphisms are also considered, R3 is present in the remaining seven accessions from the massif as well as in 19 from neighbouring massifs. Furthermore, R3 is the ribotype found in 28 of 50 cloned sequences. Areas other than Sierra Nevada examined in this study mostly show predominance or even exclusivity of one ITS repeat, and the occurrence of two ITS copies within a single individual in those areas where individuals with different ribotypes are found (Fig. 1).

By direct sequencing, co-occurrence within the same genome of ITS copies differing exclusively in substitutions can only be inferred by APS (Whittall *et al*., 2000; Fuertes Aguilar and Nieto Feliner, 2003). Inferring intragenomic variability from direct sequences displaying double peaks beyond a site is quite straightforward, but deducing which sequences are involved is not. The assumption that one of them is the ribotype R3 has been supported by the cloned sequences obtained here (see below). Twenty-six individuals bearing a second copy besides the one from Sierra Nevada have been detected (Table 1 and Fig. 1). Seven of these individuals are from the massif itself while the remaining 19 are from neighbouring massifs.

Gene-flow has been demonstrated to be frequent in the genus (Larena *et al*., 2002), and thus can cause different ITS copies to meet within the same genome. However, the relatively high number of cases involving intragenomic polymorphism detected in the Sierra Nevada and adjacent massifs as compared with previous studies (Fuertes Aguilar and Nieto Feliner, 2003) calls for an explanation. There is a possible artefactual cause, since copies differing by indels are more easily detected by direct sequencing than those copies differing only by substitutions and, as stated above, R3 is the only ribotype known in *Armeria* to differ by indels. The alternative possibility, that gene-flow and concomitant contacts between different ribotypes have been particularly active in the Sierra Nevada massif and surroundings, has some support (Gutiérrez Larena *et al*., 2002). But, since concerted evolution of ITS sequences can proceed in a fast fashion (Fuertes Aguilar *et al*., 1999*a*), such a possibility

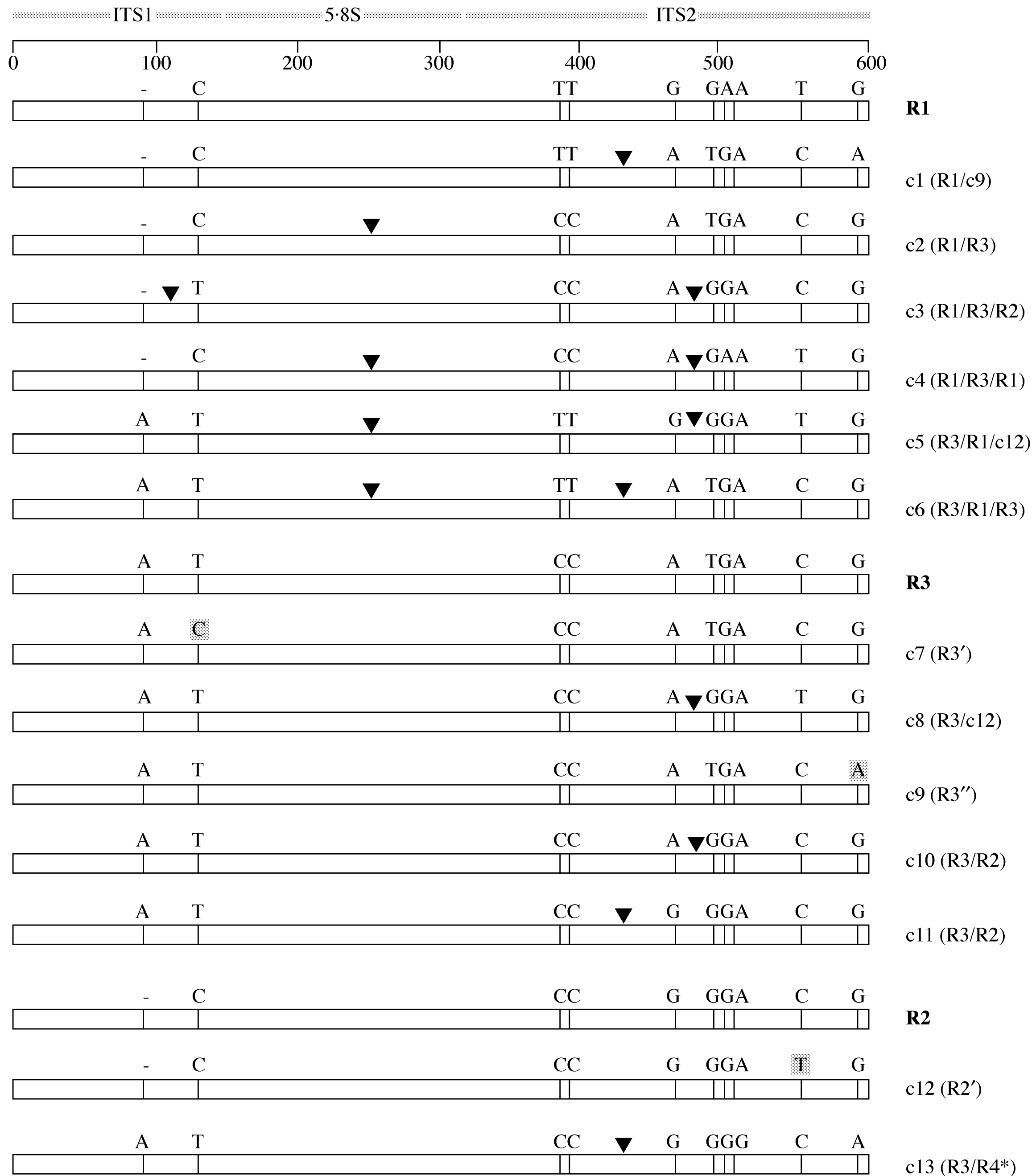

FIG. 3. Sequences schematized by ten variable positions (92, 128, 387, 388, 467, 495, 506, 513, 556 and 595) obtained from 50 clones produced from six samples of *Armeria* (accession nos 22, 25, 36, 50, 76 and 82). Autapomorphies have been disregarded, as explained in the text. Arrows mark proposed recombination events, but recombination points are arbitrarily placed mid-way between flanking diagnostic nucleotide sites. Shaded bases indicate substitutions compared with one of the defined haplotypes. Possible interpretations for each sequence are given in parenthesis with slashes indicating recombinants. Ribotype 4 (R4) is not shown because it has not been obtained as previously defined from any of the clones. See Fig. 2 for the number of clones matching each sequence.

would require that the origin of these cases of intragenomic polymorphism are recent, or have occurred recurrently.

When searching for their origin, not all the co-occurring ribotypes detected can be interpreted in the same way. A large proportion (14 out of 18) of them fit the geographic structure since they occur in areas where two different ribotypes are supposed to meet, and involve the two contacting ribotypes. For instance, in Sierra de Almijara and las Guájaras where ribotype R2 and R3 apparently meet, individuals with either ribotype, R2 or R3, are found as are individuals with both copies (Fig. 1, lower left). As commented above, these are likely to be the result of recent (and maybe also recurrent) contacts between congeners bearing different ribotypes.

In contrast, some of the individuals showing intragenomic polymorphism do not readily fit the geographical

structure of ITS variation, since they occur in areas that are not marginal, specifically in the core of the Sierra Nevada massif (accessions 25, 26, 43, 76 and 80, Fig. 1). Therefore, a recent event of gene-flow from sympatric congeners bearing other ribotypes is an unsatisfactory explanation for this pattern. Furthermore, in these same cases, but also in others (accessions 74, 75 and 111 from Sierra de Baza and Filabres), the second ribotype is not the one that would be expected on the basis of the geography. Four possible hypotheses for these cases, that are not fully concordant with the geographical structure, can be envisaged.

First, it is possible that long-distance dispersal brought about the contact of individuals with non-adjacent ribotypes (e.g. R1 with individuals bearing the Sierra Nevada ribotype R3). This possibility cannot be rejected, but strong independent evidence for such a large dispersal capacity is not available (Woodell and Dale, 1993). Secondly, insufficient sampling can never be excluded as a contributing factor. In particular, examination of areas south of Sierra Nevada with relatively low elevations, where *A. villosa* is much rarer, might help to interpret the occurrence of intragenomic polymorphisms involving R2/R3 in Sierra de Gádor (Fig. 1, lower right, accession nos 84 and 85). However, our sampling was concentrated in Sierra Nevada and the nearest massifs to the west and north of the chain.

In contrast to these two hypotheses, the remaining possible causes are related to molecular mechanisms. Retardation in concerted evolution might, in principle, cause old gene-flow events to remain tractable. However, factors that are known to be associated with such retardation, such as polyploidy or apomixis (O'Kane *et al*., 1996; Campbell *et al*., 1997), do not occur in *Armeria*. In addition, experimental evidence is available for substantial homogenization occurring as rapidly as within two generations in this genus (Fuertes Aguilar *et al*., 1999*a*). Location of ribosomal loci on different chromosomes that would also contribute to retarding homogenization (but see Wendel *et al*. 1995) has not been checked in *Armeria*. The fourth possibility is bias in homogenization after the merging of two ITS copies in the same genome. This phenomenon could result in mixed ITS copies that cannot be properly identified with direct sequencing, but also in preserving copies that are rare within an area if favoured by bias. Bias in homogenization of ITS sequences from artificial hybrids have been reported, depending on the nucleotide sites (Fuertes Aguilar *et al*., 1999*a*). Further, it has been suggested that such bias might ultimately result in favouring one specific ribotype (R1), a hypothesis that explains why this is the most frequent and geographically extended in the main centre of diversity for the genus, i.e. the Iberian Peninsula (Fuertes Aguilar and Nieto Feliner, 2003). A previous study concluded that there was a clear bias in artificial hybrids for five of the six positions in which the progenitors differed (Fuertes Aguilar *et al*., 1999*a*). However, in that experiment, progenitors contributed R1 and R4. Since data on experimental hybrids from progenitors with R3 and R1 are lacking, the outcome of merging the same ribotypes in the wild and in artificial hybrids cannot be compared. For the same reason, the strength of R1 against other ribotypes cannot be conclusively addressed. At first sight, the data given here do not seem to support a bias favouring R1, but rather a bias towards R3 because of its predominance in the area (Fig. 1). However, the occurrence of small pockets of R1 (as a rare repeat copy co-occurring intra-individually) within an area of striking predominance of R3 would be better explained if there is a bias towards R1 that counteracts to some extent the 'dosage' effect of R3. A question this raises is why there are no traces of R1 in Sierra Tejeda/Almijara (lower left in Fig. 1), despite it being close to areas where R1 predominates.

Sequences from genomic vs. *cloned DNA*

In determining instances and composition of intragenomic polymorphisms, the cloned sequences confirmed, to a large extent, the results from direct sequencing. With a single exception (clone c2 appearing from accession no. 50), only those individuals in which direct sequences revealed co-occurring ribotypes rendered cloned sequences matching ribotypes other than R3. On the other hand, cases like those reported here of undetected site polymorphisms that were revealed in the cloned sequences have also been noted by Gernandt *et al*. (2001). This result highlights the limitations of direct sequencing to detect rare ITS repeats (Rauscher *et al*., 2002).

Recombination in ITS

Either resulting from hybridization (Roelofs *et al*., 1997; Brasier *et al*., 1999; Chiang *et al*., 2001) or not (Gandolfi *et al*., 2001; Gernandt *et al*., 2001), intragenomic recombination between different homologous copies within the same genome has been reported or suggested for nuclear ribosomal multicopy regions. The use of software for detecting recombination has been avoided because a minimum of 5 % sequence divergence is needed for the different programs to be effective (Posada and Crandall, 2001). However, our detailed knowledge of the ITS variation in *Armeria*, extending to more than 250 sequences, facilitates determining non-recombinant sequences (Cronn *et al*., 2002). Despite this fact, recombination has to be suggested with caution because at least three other factors may be involved. First, point mutations may be responsible for sequences differing by one nucleotide. Secondly, biased homogenization affecting individual sites also cannot be excluded as a possible explanation for some of the cloned sequences. Thirdly, some clones may be the result of PCR recombination, i.e. *in vitro* chimeras originating from non-identical templates (Bradley and Hillis, 1997; Cronn *et al*., 2002). In those individuals where more than one ITS copy has been detected by direct sequencing there are homologous ITS copies, and thus it is possible that part of the amplicons detected after cloning are PCR chimeras. However, of the ten cloned sequences interpreted as recombinants, four (c3, c4, c5 and c6) seem to involve two recombination events or, alternatively, coincident Taq errors at the few diagnostic positions. While it is very difficult to discard the possibility of *in vitro* recombination (Cronn *et al*., 2002), double *in vitro* recombination seems unlikely, as do Taq errors affecting the same positions in

independent PCR reactions from different templates (Gandolfi *et al*., 2001). Therefore, it is most likely that at least some of the cloned sequences are not PCR artefacts but true recombinants.

Evolutionary implications

Geographical structuring of genetic variation is mainly the result of isolation and drift, while gene-flow between populations counteracts differentiation (Schaal *et al*., 1998). However, gene-flow between sympatric species (i.e. within a limited geographical frame) may also allow geographical structuring. Species-independent geographical structure has been reported for chloroplast DNA (Wolf *et al*., 1997; Dumolin-Lapègue *et al*., 1999) where it is attributed to the fact that the maternally inherited chloroplast genome depends exclusively on seeds for dispersal. Also, genetic drift is expected to be higher for the chloroplast haploid genome due to its smaller effective size as compared with the nuclear genome. The geographical structure in *Armeria* is of interest not only because it is found in a nuclear marker like the ITS regions but also because its cause, as explained, does not seem to be simply isolation and drift.

When exploring the evolutionary implications of the geographical structure of ITS variation as described here, the study of the contact zones between different ribotypes is of particular interest. The fact that, in those areas (see Fig. 1) two ribotypes are found, irrespective of the species confirms that the origin of this pattern is gene-flow, either within species bearing different ribotypes or between species. Besides, evidence of past gene-flow between species is provided by cases of haplotype sharing in the same area (Gutiérrez Larena *et al*., 2002). Therefore, fine-scale analysis of the geographical pattern of ITS variation is congruent with the occurrence of gene-flow in these contact areas irrespective of species identification. From this, it is most parsimonious to assume that intragenomic polymorphism for ITS found in these contact areas has the same origin as the co-occurrence of ribotypes in different individuals. This ought to be true at least in those cases in which the involved ribotypes are the ones expected on the basis of the geographical structure. Therefore, the extensive gene-flow proposed as one of the elements of the model of evolution of ITS for *Armeria* is supported by the data presented here. A different question arising from our data is what are the causes for the unexpected cases of intragenomic polymorphism, i.e. those appearing in the core of one of the ITS areas and/or involving one ribotype that is not expected on the basis of its known geographical distribution. As mentioned above, our results are not conclusive on this point, but bias in the homogenization of ITS copies following gene-flow events is the most plausible explanation.

There is no evidence that recombination has contributed to the known ITS diversity and thus the evolutionary implications of recombinants arising from the mixture of two ribotypes are unclear. None of the three recombinants (c2, c8 and c10) obtained in more than one individual have been detected by direct sequencing in other samples of *Armeria*.

The interest of trying to link molecular mechanisms and evolution of ITS regions with organism evolution rests on the wide use of such markers for phylogeny as well as on the increasing number of cases documenting reticulate evolution in plants. Some of them involve economically important genera such as *Quercus*, *Eucalyptus* or *Pinus* and are based on extensive samplings of chloroplast and nuclear markers (Whittemore and Schaal, 1991; Dumolin-Lapègue *et al*., 1999; Jackson *et al*., 1999; Matos and Schaal, 2000; McKinnon *et al*., 2001). But some studies use also nuclear ribosomal DNA to document reticulate evolution (Fuertes Aguilar *et al*., 1999*b*; Gernandt *et al*., 2001). It is expected that as sequences from other molecular markers are added to the wealth of existing ITS phylogenies, instances of reticulation will be confirmed and new cases will be uncovered. It is thought that this work will help to reinterpret existing ITS data sets and facilitate interpreting new ones.

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