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# Additive polymorphisms and reticulation in an ITS phylogeny of thrifts (*Armeria*, Plumbaginaceae)

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**Abstract**

A parsimony analysis of 133 sequences of the nuclear ribosomal DNA ITS1 + 5.8S + ITS2 region from 71 taxa in *Armeria* was carried out. The presence of additive polymorphic sites (APS; occurring in 14 accessions) fits the reticulate scenario proposed in previous work for explaining the ITS pattern of variation on a much smaller scale and is based mainly on the geographical structure of the data, irrespective of taxonomic boundaries. Despite the relatively low bootstrap values and large polytomies, part of which are likely due to disruptive effects of reticulation and concerted evolution in these multicopy sequences, the ITS analysis has phylogenetic and biogeographic implications. APS detected in this study are consistent with hypothesized hybridization events, although biased concerted evolution, previously documented in the genus, needs to be invoked for specific cases and may be responsible for a possible ''sink'' effect in terminals from a large clade. The causes for sequences of the same species appearing in different clades (here termed transclade) are discussed.


## 1. Introduction

Divergence and reticulation have opposite effects in shaping the topology of phylogenetic trees. Divergence originates hierarchical patterns of taxa and characters that are appropriately represented in a tree-like form. A predominance of reticulate (tokogenetic) relationships among organisms renders most methods of phylogeny reconstruction and their tree-like representations unjustifiable (Humphries and Funk, 1984). However, a moderate degree of reticulation may only partially obscure hierarchical patterns resulting from a predominantly divergent scenario and allow the tracing of reticulate events (McDade, 1992, 1995), particularly if different sources of evidence are available (Arnold, 1997).

Unfortunately, the development of methods for phylogenetic reconstruction from sequence data that take reticulation into account is still limited. Either these methods are able to detect only hybrids between terminal branches (Rieseberg and Morefield, 1995) or they require genetic frequency data (Xu, 2000). Reticulation is, thus, difficult to demonstrate conclusively, and most molecular studies of groups with putative hybrid species rely on one or more of the following types of evidence: (1) incongruence between phylogenies based on maternally (e.g., cpDNA restriction fragment length polymorphism, chloroplast spacers) and biparentally (e.g., nrDNA) inherited genes (Wendel et al., 1995b), (2) additivity patterns from dominant (RAPD, ISSR; Brochmann et al., 1996; Wolfe et al., 1998) or codominant (e.g., nrDNA; Soltis and Soltis, 1991; Kim and Jansen, 1994; Sang et al., 1995; Whittall et al., 2000) markers, and (3) significant conflict among morphological, biogeographical, and molecular evidence (Sytsma, 1990; Fuertes Aguilar et al., 1999b).

The ITS region from nuclear ribosomal DNA is the most widely used genetic marker for phylogenetic reconstruction below the family level in angiosperms (Baldwin et al., 1995). The way that reticulation affects the sequences from this particular region has been discussed in the recent literature. The two main outcomes that reticulation produces on ITS sequences are additive patterns (both parental ribotypes present) and biased homogenization toward one of the parental lineages. The predominance of either one depends on the intensity and direction of concerted evolution, which is proposed to act through the recombinational mechanisms occurring

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during meiosis (Li, 1997). This is consistent with its retardation or absence in cases of apomixis (Campbell et al., 1997) or recent hybridization (Fuertes Aguilar et al., 1999a). On the other hand, an active biased concerted evolution results in the hybrid lineage presenting a ribotype with the same sequence as that of one of its parents (Wendel et al., 1995a; Fuertes Aguilar et al., 1999a; Marshall and Sites, 2001; Schilthuizen et al., 2001). Intermediate situations between complete additivity and total homogenization are found (Sang et al., 1995; Whittall et al., 2000). Further complications to an additive vs a homogenized pattern can be found when molecular mechanisms of correction, deletion and/or compensatory mutation balancing the secondary structure of RNA occur (Whittall et al., 2000).

The genus *Armeria* L. (''thrifts;'' Plumbaginaceae) with ca. 120 described species is, after *Limonium* and *Acantholimon*, the most species rich within the family. *Armeria* is primarily Mediterranean, occurring from Turkey to Portugal and Madeira, with a major center of diversity in the Iberian peninsula (Greuter et al., 1989). One species or species complex, *A. maritima*, has a predominantly holarctic distribution with a disjunct area located in the Chile–Patagonian region (Moore and Yates, 1974). Like most genera of Plumbaginaceae, thrifts are stress-tolerant plants due to the possession of osmoprotective compounds (Hanson et al., 1994) and thus can grow under extreme ecological conditions. Coastal dunes and cliffs, salt marshes, heavy-metal-rich soils, and alpine rock fissures are some of the habitats in which species occur (Arrigoni, 1970; Izco et al., 1989; Nieto Feliner, 1990). Geographical ranges of the species are usually restricted, sometimes even to mountains or single islands (*A. splendens*, *A. colorata*, *A. berlengensis*, and *A. maderensis*). However, exceptions, such as *A. maritima*, occur in both the Northern and the Southern Hemispheres. The number of individuals per population varies from several hundred, as in *A. maritima*, to less than 20 in some of the most restricted endangered species (Woodell and Dale, 1993; Philipp et al., 1999; pers. observ.).

*Armeria* has a basic chromosome number of $n = 9$, and virtually all the chromosome counts are diploid (Moore, 1982; Castroviejo and Valdés-Bermejo, 1991). All the species of *Armeria*, except some subspecies of *A. maritima*, have a well-studied self-incompatibility system (Baker, 1966). Internal reproductive barriers among species are weak (Nieto Feliner et al., 1996). The pollination system is based on nonspecialized insects (Herrera, 1988; Woodell and Dale, 1993). Dispersal by wind is suggested by a parachute-like or dart-shaped fruit (Philipp et al., 1999), but ornithochory may also occur (see below). All these facts facilitate gene flow among species via pollen or seeds. The actual occurrence of gene exchange and hybridization has been supported on morphological, biogeographical, population genetic, and molecular evidence in addition to experimental hybridization (Bernis, 1954; Lefèbvre, 1969; Philipp, 1974; Nieto Feliner et al., 1996; Nieto Feliner, 1997; Fuertes Aguilar et al., 1999a,b).

Probably because of the pervasiveness of gene flow among taxa, the systematics of *Armeria* has been studied by several authors, with dramatic differences in their findings. An extreme view is represented by Bernis (1950) in his first work, which accommodated the whole genus into 1 species. Lawrence (1940) recognized 35 species, 12 of which form section *Macrocentron*. In the most comprehensive revision of the genus, achieved by Bernis (1954, 1955, 1957), the number of species is restricted to 7, 6 of which are the members of section *Macrocentron*. The 7th species, *A. maritima*, encompasses the remaining variability of the genus. In this work, a liberal criterion for the infraspecific taxonomic categories (subspecies, varieties, subvarieties) is used to accommodate a large part of the morphological variability of the genus within *A. maritima*. No additional treatment is available except for the accounts in ''Flora Europea'' (Pinto da Silva, 1972b) and ''Med-Checklist'' (Greuter et al., 1989), in which more analytical approaches record 34 species for the European territory and 92 species for the Mediterranean basin, respectively. On a more restricted geographical scale, but covering the most diverse area, Nieto Feliner (1990) recognized 54 species for the Iberian peninsula. Western Hemisphere taxa, traditionally considered in *A. maritima*, have also been the object of recent revisionary work (Lefèbvre and Vekemans, 1995).

From a molecular perspective, previous work has shown that the ITS region has a rather low substitution rate within *Armeria*, suggesting that it is a recently diversified group. However, this marker has been particularly fruitful in the study of reticulation. Incongruence with morphology, presence of individual additive polymorphisms, and a pattern of variation consistent with geography but not with taxonomic arrangement support the occurrence of reticulate evolution (Fuertes Aguilar et al., 1999b). The scarcity of additive polymorphic sites (APS) detected was explained by the finding that concerted evolution is very active and biased in ITS sequences of experimental $F_2$ hybrids of *Armeria* (Fuertes Aguilar et al., 1999a).

In this paper we undertake a phylogenetic analysis of the ITS region covering the whole geographical range of the genus and ca. 70% of the species. The main objectives of our study are to examine how the composition of the additive polymorphic sites obtained fit the reticulate scenario previously documented, to identify which of those polymorphic sites might be attributed to hybridization and thus could allow tracing of the origin of specific reticulation events, to explore the phylogenetic conclusions at the species level based on the ITS data despite disruptive effects of reticulation and concerted

evolution in these multicopy sequences, and to discuss the biogeographic implications of the data.

## 2. Material and methods

### 2.1. Sampling

The sampling strategy was designed to cover as many described taxa as possible. A total of 133 individuals from 71 taxa of *Armeria*, covering the whole range of morphological variation, geographic distribution, and subgeneric classification, were included (Table 1, Fig. 1). *Psylliostachys suworowii* was used as the outgroup, given its sister position to *Armeria*, as inferred from a generic phylogeny of the Plumbaginaceae based on *rbcL* and *trnL-F* (Lledó et al., 1998, 2000). Attempts to add ITS sequences from species of *Limonium* and *Limoniastrum* to the outgroup failed because it was impossible to align the sequences with those of *Armeria*. Intraspecific sampling focused on those taxa with large and/or disjunct distributions (e.g., *A. maritima*) and those taxa showing taxonomically recognized intraspecific diversity (i.e., *A. villosa*, *A. filicaulis*, *A. arenaria*).

### 2.2. DNA isolation, PCR amplification, and sequencing

Total DNA was isolated from fresh, silica gel, and herbarium specimens following a modified CTAB method (Doyle and Doyle, 1987). Double-strand amplification of the ITS region was performed on a Gene Amp PCR System 9700 (PE Biosystems) with 20-μl reactions. Primers are described in Fuertes Aguilar et al. (1999b). 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) of Ampli*Taq* Gold (PE Biosystems). The cycle profile was an initial cycle of 94 °C (12 min), 5 cycles with 94 °C (30 s), 54 °C (30 s), and 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 products were purified through silica matrix columns following the supplier protocol (PCR Clean-Up Kit; MoBio Laboratories) and checked for concentration on a 1.5% TAE agarose gel. From 20 to 50 ng of purified product was used for sequencing reactions. Automatic sequencing with fluorescently labeled dyes was carried out following BigDye Terminator (PE Applied Biosystems) recommendations. Sequencing reaction products were run on a 5% polyacrylamide denaturing gel in an ABI Prism 377 (PE Applied Biosystems) system. Each amplified product was sequenced in both forward and reverse directions with the same primers used for amplification, achieving a 100% overlap of both strands. Sequences were analyzed and processed with Sequence Navigator (PE Applied Biosystems) and aligned manually in SeqApp.

IUPAC ambiguity codes were used for coding polymorphic positions. A site is designated polymorphic (PS) when more than one peak was present in the electropherogram and the weakest signal reached at least 25% of the strength of the strongest signal (Fuertes Aguilar et al., 1999a). To minimize the inclusion of bad reads as polymorphisms, we added the restriction that double peaks had to occur on the same position on both direct and reverse strands. Additionally, 2 non-Watson–Crick bp occurring on the same position in forward and reverse strands are also here considered polymorphic sites. Because we aimed to identify individuals containing different ITS repeats in the same genome that might result from hybridization, we focused on additive polymorphic sites. APS were recorded in those instances when the two bases involved in a polymorphic site were also found separately in other accessions of the data set. For example, those sites with a Y(C/T) were scored as additive when C and T were independently detected in the same position in any other accession of the data matrix. Whittall et al. (2000) proposed the term superimposed nucleotide additive pattern (SNAP) for what we here call parsimony-informative APS. However, we need a slightly broader concept for our study. The use of SNAP is restricted to those present in parsimony-informative sites. This criterion excludes situations in which one of the bases is present in a single accession in addition to the polymorphic site. Also, it excludes the occurrence of an indel in some of the ITS repeats within the same individual (see Section 3). We think that, in working with reticulate scenarios, it is worth tracing APS whether or not they are strictly parsimony informative. Additionally, SNAP can be confused with the widespread molecular biology term SNP (i.e., single nucleotide polymorphism), which, although related, denotes a different concept (Jordan and Humphries, 1994). The data set for the phylogenetic analysis included the complete sequences of ITS1, 5.8S, and ITS2. Nucleotide statistics were scored with MEGA (Kumar et al., 1993), MacClade 3.05 (Maddison and Maddison, 1992), and PAUP* 4.1b3 (Swofford, 1998). New sequences are available at the EMBL database (Table 1).

### 2.3. Phylogenetic analysis

Removal of hybrids from a phylogenetic analysis has been advocated on the basis of their negative effects on the signal and their origin, which violates assumptions of cladistic analysis (Hennig, 1966; Funk, 1985). An increase in levels of homoplasy and number of trees and a decrease in resolution of the resulting tree topology are the most common outcomes of inclusion of hybrids in an analysis (McDade, 1995; Campbell et al., 1997; Whittall et al., 2000). However, analysis of putative

Table 1
Sampled *Armeria* taxa and outgroup

| *Armeria* taxa | Locality and voucher |
|---|---|
| *A. alliacea* (Cav.) Hoffmanns. & Link | [1] Alicante (SP) Vogt s/n. |
| *A. alpina* Willd. | [2] Huesca (SP) 38Vargas97; |
| | [3] Trento (IT) Alvarez 1348; |
| | [4] Salzburg (AU) USBG 1055. |
| *A. arenaria* (Pers.) Schultes | |
| subsp. *arenaria* | [5] Bouches-du-Rhône (FR) Martin 10498. |
| subsp. *bilbilitana* (Bernis) Nieto Feliner | [6] Guadalajara (SP) Nieto s/n. |
| subsp. *confusa* (Bernis) Nieto Feliner | [7] Lleida (SP) Aedo 2304. |
| subsp. *segoviensis* (Bernis) Nieto Feliner | [8] Valladolid (SP) Rico 6767; |
| | [9] Madrid (SP) Nieto 3925. |
| *A. beirana* Franco | [10] Minho (PO) Alvarez 1329; |
| | [11] Algarve (PO) Nieto 3933. |
| *A. berlengensis* Daveau | [12] Estremadura (PO) Nieto 3969; |
| | [13] Estremadura (PO) Nieto 3971. |
| *A. bigerrensis* (C. Vicioso & Beltrán) Rivas Martínez | |
| subsp. *bigerrensis* | [14] Avila (SP) Nieto 3790; |
| | [15] Avila (SP) Nieto 3791; |
| | [16] Avila (SP) Nieto 3846. |
| subsp. *losae* (Bernis) Rivas Martínez et al. | [17] Soria (SP) Nieto 3843. |
| *A. bourgaei* Boiss. ex Merino | |
| subsp. *bourgaei* | [18] Almería (SP) MA306105; |
| | [19] Jaén (SP) Vogt 3328. |
| subsp. *lanceobracteata* (Lawrence) Nieto Feliner | [20] Granada (SP) Nieto 1365-1; |
| | [21] íd. Nieto 1365-2. |
| subsp. *willkommiana* (Bernis) Nieto Feliner | [22] Murcia (SP) Alvarez 1138. |
| *A. bubanii* Lawrence | [23] Huesca (SP) Castroviejo 13256; |
| | [24] Huesca (SP) Alejandre 1097/86. |
| *A. caballeroi* (Bernis) Donadill | [25] Zamora (SP) Aldasoro s/n. |
| *A. caespitosa* (Gómez Ortega) Boiss. | [26] Segovia (SP) Martinez s/n. |
| *A. canescens* (Host) Boiss. | [27] Basilicata (IT) Jury 17379; |
| | [28] Arkadia (GR) García 951; |
| | [29] Thessalia–Macedonia (GR) Nieto 2798. |
| *A. cantabrica* Willk. | [30] Cantabria (SP) Nieto 3807. |
| *A. cariensis* Boiss. | [31] Irmiz (TU) Nydegger 47530. |
| *A. choulettiana* Pomel | [32] High Atlas (MO) Güemes 1595; |
| | [33] Mid Atlas (MO) Rico 6048. |
| *A. colorata* Pau | [34] Málaga (SP) Nieto 3683-2; |
| | [35] Málaga (SP) Nieto 3683-9. |
| *A. ebracteata* Pomel | [36] High Atlas (MO) Jury 11881. |
| *A. euscadiensis* Donadille & Vivant | [37] Vizcaya (SP) MA 477925; |
| | [38] Guipúzcoa (SP) MA 532232. |
| *A. filicaulis* (Boiss.) Boiss. | |
| subsp. *filicaulis* | [39] Murcia (SP) Castroviejo 14566; |
| | [40] Granada (SP) Nieto 2722; |
| | [41] Málaga (SP) Nieto 4017. |
| subsp. *nevadensis* Nieto Feliner, Rosselló, & Fuertes | [42] Granada (SP) Alvarez 1365. |
| subsp. *trevenqueana* Nieto Feliner | [43] Granada (SP) Nieto 4090; |
| | [44] Granada (SP) Navarro 2248. |
| *A. fontqueri* Pau | [45] Tarragona (SP) Nieto 3927. |
| *A. gaditana* Boiss. | [46] Cádiz (SP) Nieto 3879. |
| *A. genesiana* Nieto Feliner | [47] Toledo (SP) MA 509191; |
| | [48] Toledo (SP) MA 311234. |
| *A. hirta* Willd. | [49] Málaga (SP) Nieto 3856; |
| | [50] Cádiz (SP) Nieto 3865; |
| | [51] Cádiz (SP) Nieto 3874. |
| *A. hispalensis* Pau | [52] Cádiz (SP) Nieto 3788. |
| *A. humilis* (Link) Schultes | [53] Minho (PO) Alvarez 1328. |
| *A. langei* Boiss. | |
| subsp. *daveaui* (Coutinho) Pinto da Silva | [54] Tras-Os-Montes (PO) Castroviejo 14633/1. |
| *A. leucocephala* Koch | [55] Corse (FR) Lambinon 91/Co/336. |
| *A. linkiana* Nieto Feliner | [56] Alto Alentejo (PO) MA 506643. |

Table 1 (*continued*)

| *Armeria* taxa | Locality and voucher |
|---|---|
| *A. macrophylla* Boiss. & Reuter | [57] Cádiz, (SP) Nieto 3877; |
| | [58] Cádiz (SP) Nieto 3870; |
| | [59] Cádiz (SP) Nieto 387; [60] Cádiz, (SP) Nieto 3872. |
| *A. macropoda* Boiss. | [61] Calabria (IT) Jury 17383. |
| *A. maderensis* Lowe | [62] Madeira (PO) Santos s/n; |
| | [63] Madeira (PO) Güemes s/n. |
| *A. majellensis* Boiss. | [64] Basilicata (IT) Jury OI-8 1816. |
| *A. malacitana* Nieto Feliner | [65] Málaga (SP) Nieto 1733. |
| *A. maritima* Willd. | |
| subsp. *andina* (Boiss.) David M. Moore & B. Yates | [66] Cerro Torre (AR), Galán. |
| subsp. *californica* | [67] California (US) Nieto 2122. |
| subsp. *elongata* (Hoffman) Bonnier | [68] Saxen-Anhalt (GE) Podlech 52001. |
| subsp. *maritima* | [69] Santander (SP) Nieto 2866; |
| | [70] Pontevedra (SP) García-Martínez 6915; |
| | [71] Normandie (FR) IS Nantes; |
| | [72] Brighton (UK) 30PV97. |
| *A. merinoi* (Bernis) Nieto Feliner & Silva Pando | [73] La Coruña (SP) Nieto 2934. |
| *A. multiceps* Wallr. | [74] Corse (FR) Martínez 434. |
| *A. nebrodensis* Guss. | [75] Sicily (IT) IS Palermo. |
| *A. pauana* (Bernis) Nieto Feliner | [76] Jaén (SP) Nieto 3885. |
| *A. pinifolia* (Brot.) Hoffmanns & Link | [77] Estremadura, (PO) IS Lisbon 552. |
| *A. pseudarmeria* (Murray) Mansfeld | [78] Estremadura, (PO) IS Lisbon 511; |
| | [79] Estremadura (PO), Jury s/n. |
| *A. pubigera* (Desf.) Boiss. | [80] Lugo (SP) Aedo 3410; |
| | [81] Pontevedra (SP) García-Martínez 6932; |
| | [82] Pontevedra (SP) Carcía-Martínez 6941; |
| | [83] Pontevedra (SP) García-Martínez 6942. |
| *A. pungens* (Link) Hoffmann | [84] Cádiz (SP) Nieto 3867; |
| | [85] Algarve (PO) Nieto 3936. |
| *A. rouyana* Daveau | [86] Baixo Alentejo (PO) IS Lisbon 555. |
| *A. rumelica* Boiss. | [87] Perister Planina (MA) Frost-Olsen 2554. |
| *A. ruscinonensis* Girard | |
| subsp. *ruscinonensis* | [88] Pyrenées Orientales (FR) Sáez s/n. |
| *A. salmantica* (Bernis) Nieto Feliner | [89] Salamanca (SP) Nieto 3804; |
| | [90] Salamanca (SP) Nieto 3805. |
| *A. sardoa* Spreng. | |
| subsp. *sardoa* | [91] Sardinia (IT) Lambinon 81/176. |
| *A. simplex* Pomel | [92] Kenitra (MO) Vogt 6085. |
| *A. soleirolii* Duby | [93] Corse (FR) Martinez 518. |
| *A. splendens* (Lag. & Rodr.) Webb | [94] Granada (SP) Alvarez 1388; |
| | [95] Granada (SP) Gutiérrez 17. |
| *A. trachyphylla* Lange | [96] Cuenca (SP) MA 616230. |
| *A. transmontana* (Samp.) Lawrence | [97] Tras-Os-Montes (PO) Sequeira 2805. |
| *A. trianoi* Nieto Feliner | [98] Córdoba (SP) Nieto 3992; |
| | [99] íd. Nieto 3993; |
| | [100] íd. Nieto 3995; |
| | [101] Córdoba (SP) SB 17550. |
| *A. velutina* Welw. ex Boiss. | [102] Huelva (SP) Nieto 3883. |
| *A. villosa* Girard | |
| subsp. *alcaracensis* Nieto Feliner | [103] Albacete (SP) MA 586743. |
| subsp. *bernisii* Nieto Feliner | [104] Granada (SP) Nieto 1370; |
| | [105] Almería (SP) MA 503922; |
| | [106] Granada (SP) Nieto 3975; |
| | [107] Granada (SP) Nieto 3976; |
| | [108] Granada (SP) Nieto 3981; |
| | [109] Granada (SP) Nieto 3982; |
| | [110] Granada (SP) Nieto 3984; |
| | [111] Granada (SP) Nieto 3985. |
| subsp. *carratracensis* (Bernis) Nieto Feliner | [112] Málaga (SP) Nieto 3803-1; |
| | [113] íd. Nieto 3803-2; |
| | [114] íd. Nieto 3803-3/1; |
| | [115] id. Nieto 3803-3/2. |

Table 1 (*continued*)

| *Armeria* taxa | Locality and voucher |
|---|---|
| subsp. *longiaristata* (Boiss. & Reuter) Nieto Feliner | [116] Albacete (SP) 3346-2;<br>[117] íd. 3346-13;<br>[118] Jaén (SP) 3675-5;<br>[119] íd. 3675-7;<br>[120] Córdoba (SP) Nieto 3850;<br>[121] Granada (SP) Nieto 3678-2;<br>[122] íd. Nieto 3678-3;<br>[123] Cádiz, 3862;<br>[124] Jaén (SP) Nieto 3676-2;<br>[125] íd. Nieto 3676-8;<br>[126] Sevilla (SP) Nieto 3853;<br>[127] Jaén (SP) Nieto 3679-5;<br>[128] íd. Nieto 3679-7;<br>[129] Córdoba. (SP) Nieto 4002. |
| subsp. *provillosa* (Bernis) Nieto Feliner | [130] Jaén (SP) MA 403427. |
| subsp. *villosa* | [131] Málaga (SP) Nieto 1423. |
| *A. welwitschii* Boiss. | [132] Estremadura, (PO) IS Lisbon 515;<br>[133] Estremadura (PO) Nieto 3964. |
| *Psylliostachys suworowii* (Regel) Roshk. | [134] Insbrück Bot. Gart. IS 623. |

*Note*. All the vouchers, seeds, and living specimens are deposited at herbarium (MA), seedbank, and research greenhouse from the Royal Botanical Garden in Madrid. AR, Argentina; AU, Austria; FR, France; GE, Germany; GR, Greece; IT, Italy; MA, Makedonija; MO, Morocco; PO, Portugal; SP, Spain; TU, Turkey; UK, Great Britain; US, United States.

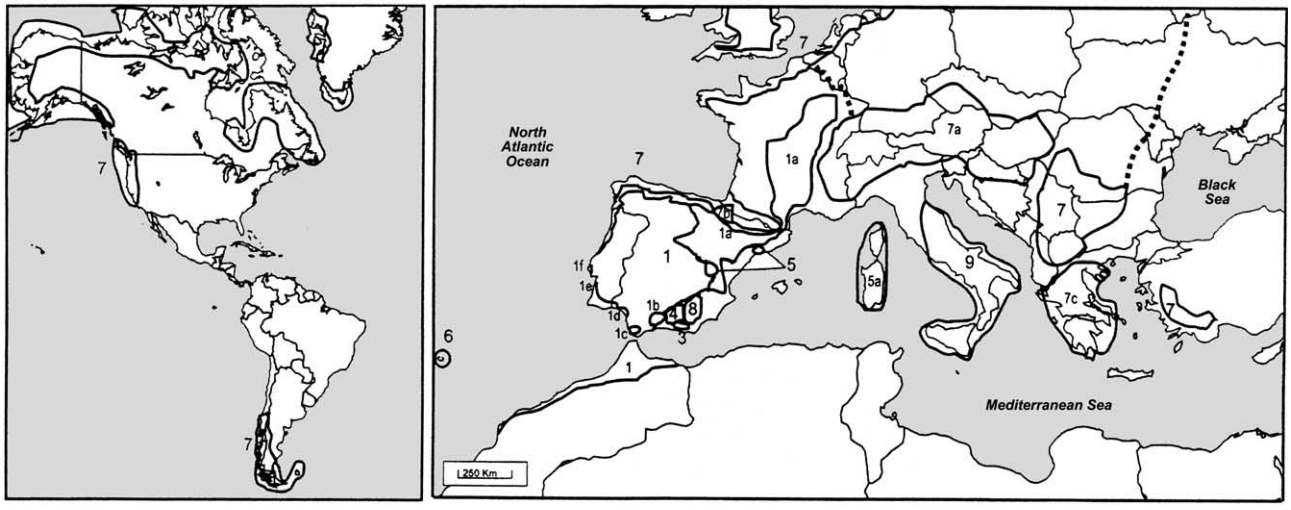


Fig. 1. Main distribution areas covered by the sampling of the genus *Armeria*. The numbers refer to major clades in Fig. 2.

hybrids with no distinction from the rest of the terminals has been defended also (Skála and Zrzavý, 1994). We followed the latter approach in previous analyses (Fuertes Aguilar et al., 1999b). Additionally, we have found it useful to examine the relative positions of terminals of putative hybrid origin either in all the fundamental trees or in a majority of them. In addition to the combination of character states, hybrids may be detected by the substantial changes in their relative positions among fundamental trees (McDade, 1992). We have used two data sets in the phylogenetic analysis to allow an examination of the behavior and possible origin of putative hybrids and to assess their effects on the topology of the most parsimonious trees, while minimizing disruptive effects of reticulation. The first excludes those taxa with APS (data set A) and the second includes all the accessions sampled (data set B).

Phylogenetic analyses were performed with unweighted parsimony with PAUP* (Swofford, 1998). Heuristic searches for most parsimonious reconstructions included 100 replicates, each with random addition of sequences, TBR and ACCTRAN options in branch swapping and character optimization, respectively, and no maxtrees limit. Support for clades was evaluated with heuristic bootstrap analyses with 100 replicates, each with no maxtrees limit and with random sequence addition with TBR and MULPARS in effect. Divergence was calculated under the Kimura two-parameter model

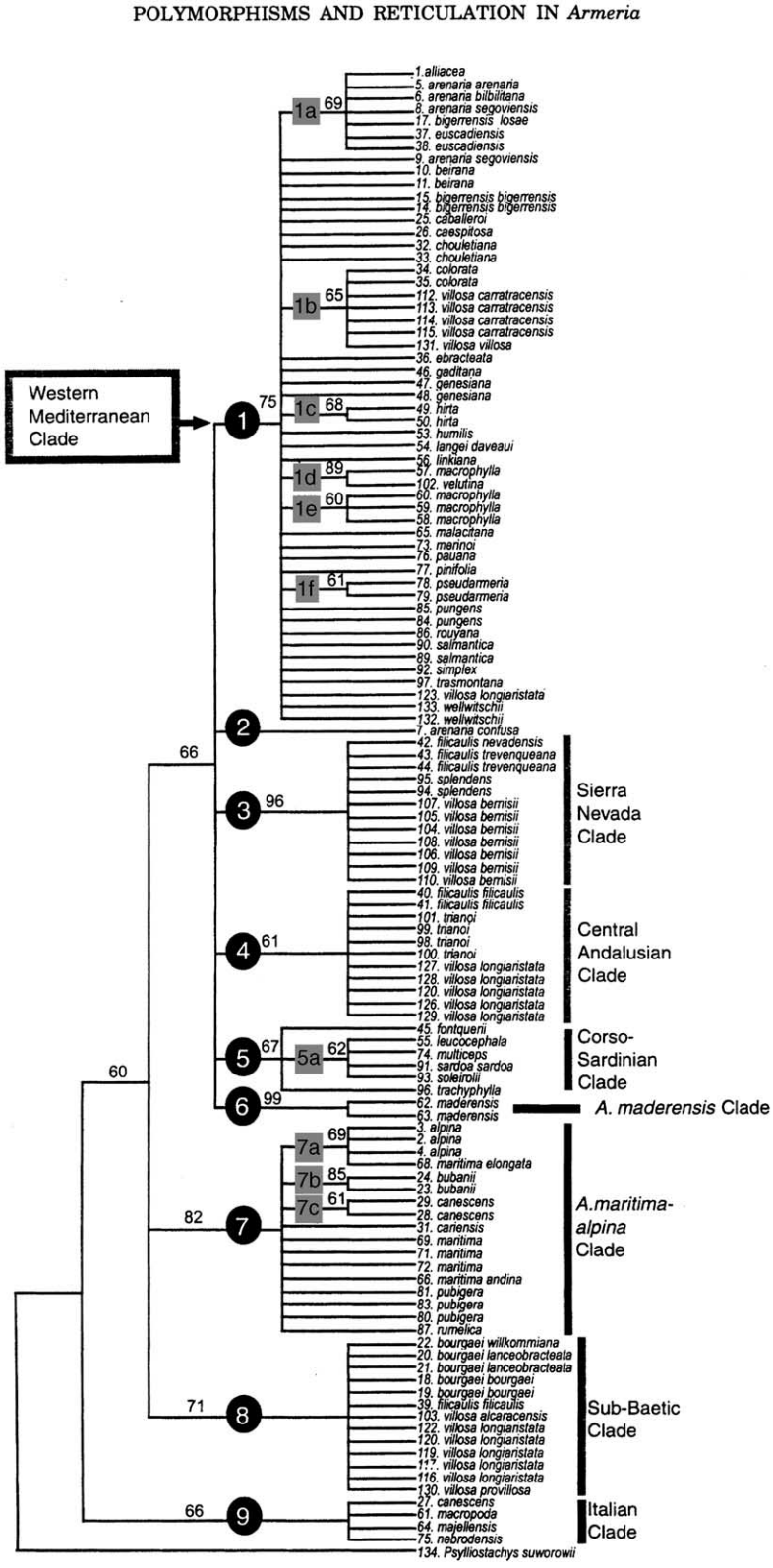


Fig. 2. Single most parsimonious tree from a parsimony analysis of 119 nrDNA internal transcribed spacer (ITS1 + 5.8S + ITS2) sequences in *Armeria* (data set A). Numbers above branches indicate bootstrap values. Terminals containing additive polymorphic sites are excluded. Major clades are numbered as explained in the text.

(Kimura, 1980) in PAUP. Mapping of nucleotide substitutions and insertion/deletions (indels) and calculation of average transition/transversion (ti/tv) ratios from the most parsimonious trees were conducted with MacClade version 3.05 (Maddison and Maddison, 1992).

## 3. Results

### 3.1. ITS sequence analysis

The total length of the ITS1 + 5.8S + ITS2 region varies between 609 and 611, with the length variation produced by the presence of two 1-bp indels in ITS1, which ranges from 207 to 209 bp. One of these indels is synapomorphic for the terminals belonging to clade 3 (Fig. 2), whereas the other is exclusive to one population of *A. bigerrensis*. The G + C content was significantly higher in the 5.8S region than in both internal spacers. As observed in other angiosperms, the G + C value for ITS2 is higher than that for ITS1 (Hershkovitz and Zimmer, 1996; Hershkovitz and Lewis, 1996). Intrageneric variability of ITS is rather low compared with the average values in other families (Baldwin et al., 1995). Thus, sequence divergence is also low, the highest values of pairwise divergence being those estimated between *A. maderensis* and *A. cariensis* (1.4%). Distribution of the variability along the region follows a pattern similar to that of other families of angiosperms, i.e., ITS2 > ITS1 > 5.8S (Table 2).

The number of parsimony-informative sites (35) is considerable, despite the relatively low sequence variability, and the ratio of informative to variable sites is comparable to that in other groups of angiosperms. In total, 65% of the variable sites are parsimony informative. Ten of the informative sites are located in ITS1, 22 are in ITS2, and 3 are in the 5.8S region. Forty sites (6.5% of the total sites and 83% of the variable sites) contain polymorphisms in at least one sequence (Table 2). Fifty-two individuals (38.8% of data set B) display at least one polymorphism in their ITS sequences.

Three types of APS are relevant for hybrid detection. First, those sites containing parsimony-informative characters suggest either reticulation between taxa from different clades or preservation of ancestral polymorphisms. Second, different sequences of the same individual differing with respect to the occurrence of an indel also can be considered an APS, as long as sequences both with and without the insertion are found elsewhere in the data set. This type may be located at a parsimony-informative site and, thus, have the same implications as those of the first type. Third, those sites where the APS involves an autapomorphy, i.e., one of the bases involved occurs elsewhere in a single sample, may be particularly relevant for hybrid detection because they may pinpoint one of the possible parents.

In our data set there are 15 APS (i.e., nucleotide sites for which an additive polymorphism was recorded in at least one sequence), 3 in ITS1, and the remainder in ITS2; 11 are in parsimony-informative sites, 3 involve an autapomorphy, and 1 is registered as the occurrence of repeats differing in an insertion within a single individual. Fourteen accessions (H1 to H14, Table 3) possess APS. They belong to *A. berlengensis* (2 accessions), *A. bigerrensis*, *A. cantabrica*, *A. maritima* (2 accessions), *A. pubigera*, *A. hirta* (2 accessions), *A. ruscinonensis*, *A. villosa* subsp. *bernisii*, and *A. villosa* subsp. *longiaristata* (3 accessions). The number of informative APS per individual varies from one to four (Table 3). Among the APS, a majority (19 cases, summed across sites) involves a purine and a pyrimidine. Therefore, if the observed

Table 2
Main ITS sequence characteristics in *Armeria* spp.[a]

| Parameter | ITS-1 | 5.8S | ITS-2 | Complete sequence |
|---|---|---|---|---|
| Length range (bp) | 207–209 | 157 | 245 | 609–611 |
| Aligned length (bp) | 209 | 157 | 245 | 610 |
| G + C content range (%) | 47.1–49.0 | 53.5–54.2 | 47.7–50.0 | 50.0–51.3 |
| G + C content mean (%) | 48.0 | 54.1 | 48.8 | 50.5 |
| Sequence divergence (%)[b] with complete deletion of gaps and missing data | 0.0–2.6 | 0.0–2.0 | 0.0–2.9 | 0.0–1.4 |
| No. of variable sites | 16 | 4 | 34 | 54 |
| No. of parsimony-informative changes | 10 | 3 | 22 | 35 |
| No. of polymorphic sites[c] | 6 | 4 | 30 | 40 |
| No. of additive polymorphic sites (APS)[d] (with indel) | 2 (3) | 0 | 12 | 14 (15) |
| No. of APS at parsimony-informative sites[e] | 1 | 0 | 10 | 11 |

[a] Data set B, excluding the outgroup.
[b] Based on Kimura two-parameter substitution model.
[c] Sites with more than one nucleotide in the same individual.
[d] Sites with two nucleotides in the same individual, both also occurring independently in another individual.
[e] A shared 1-bp indel has been coded as missing and therefore is here considered noninformative.

Table 3
Summary of nucleotide site variation in *Armeria* for the ITS region

| | 31 | 71 | 74 | 94 | 99 | 125 | 130 | 163 | 168 | 170 | 197 | 198 | 214 | 236 | 370 | 379 | 381 | 390 | 391 | 392 | 398 | 408 | 415 | 416 | 417 | 445 | 461 | 474 | 502 | 520 | 539 | 554 | 564 | 567 | 595 | 603 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Clade 1 | T | C | G | — | G | A | C | T | A | T | A | A | T | C | A | G | C | C | T | T | T | A | C | G | G | G | C | G | G | A | G | G | C | G | T | G |
| subcl. 1a | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | T | . | . |
| subcl. 1b | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | T | . | . | . |
| 38. *euscadiensis*[a] | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | G | A | . | T | . | A | . |
| subcl. 1c | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | G | . | T | . | . | . | . |
| subcl. 1d | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . | C | . | . | . | G | . | . | . | . | . | . |
| subcl. 1e | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | A | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . |
| subcl. 1f | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . |
| 97. *transmontana*[a] | . | . | . | — | T | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . |
| 133. *welwitschii*[a] | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | G | . | . | . | . | . | . |
| Clade 2 | . | . | . | — | . | T | . | . | . | . | G | . | . | . | . | . | . | . | C | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Clade 3 | . | . | . | A | . | . | T | . | . | . | . | . | . | . | . | . | . | . | C | C | . | . | . | . | . | . | . | A | T | . | . | . | . | . | . | . |
| Clade 4 | . | . | . | — | . | . | . | . | . | A | . | . | . | . | . | . | . | . | C | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Clade 5 | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | C | . | . | . | . | T | . | . | . | . | . | . | . | . | . | . | . |
| subcl. 5a | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | C | . | . | . | T | T | . | . | . | . | . | . | . | . | . | . | . |
| Clade 6 | . | T | . | — | . | . | . | . | . | . | . | T | A | T | . | . | . | . | C | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T |
| Clade 7 | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | T | T | C | C | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . |
| subcl. 7a | . | . | . | — | . | . | . | G | . | . | . | . | . | . | . | . | T | T | C | C | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . |
| subcl. 7b | . | . | C | — | . | . | . | . | . | . | . | . | . | . | . | . | T | T | C | C | . | . | . | . | . | . | T | . | . | G | . | . | . | . | . | . |
| subcl. 7c | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | T | T | C | C | . | . | . | . | . | . | . | . | . | G | . | . | . | C | . | . |
| Clade 8 | . | . | . | — | . | . | . | . | . | . | . | . | . | . | T | . | . | . | C | C | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | A |
| Clade 9 | C | . | . | — | . | . | . | . | G | . | . | . | . | . | . | . | . | . | C | C | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . |
| H1 *berlengensis* | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | Y | . | . | . | . | . | . | . | . | . | . | . | . | . |
| H2 *berlengensis* | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | Y | . | . | . | . | . | . | . | . | . | . | . | . | . |
| H3 *bigerrensis* | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | R | . | . | . | . | . |
| H4 *cantabrica* | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | R | . | . | . | . | . |
| H5 *maritima* | . | . | . | — | Y | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| H6 *maritima* | . | . | A | — | Y | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| H7 *pubigera* | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | Y | Y | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| H8 *hirta* | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | Y | . | . | . | . | . | . | . | . | S | . | . | . | . | . | . | . | . | . | . |
| H9 *hirta* | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | Y | . | Y | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| H10 *ruscinonensis* | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | . | Y | Y | . | . | . | . | . | . | . | . | . | . | . | . | Y | . | . | . |
| H11 *v. bernissi* | . | . | . | —/A | . | . | Y | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | R | K | A | . | . | . | . | . | . |
| H12 *v. longiaristata* | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | Y | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| H13 *v. longiaristata* | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | R | . | . | . | . | . | . |
| H14 *v. longiaristata* | . | . | . | — | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | R | . | . | . | . | . | . |

*Note*. Only sites containing either synapomorphies that support clades in Fig. 2 or APS are shown. Terminals are represented by clades except for those including APS (H1–H14).
[a] Terminals presenting autapomorphies involved in an APS.

Table 4
A comparison of nucleotide substitutions in the phylogeny of *Armeria* and the hypothetical substitutions needed to produce the observed additive polymorphisms (APS)

| Nucleotide substitutions and corresponding polymorphism | C ↔ T Y | A ↔ G R | Total ti | A ↔ C M | A ↔ T W | G ↔ C S | G ↔ T K | Total tv |
|---|---|---|---|---|---|---|---|---|
| No. of substitutions inferred from phylogeny | 12 | 7 | 19 | 1 | 7 | 3 | 6 | 17 |
| No. of substitutions inferred to explain the observed APS | 14 | 5 | 19 | 0 | 0 | 1 | 1 | 2 |

APS were all the result of nonhomogenized substitutions, a majority of them would have originated from transversions (Table 4).

### 3.2. Phylogenetic analysis of data set A

The unweighted parsimony analysis of data set A yielded one most parsimonious tree with a CI of 0.936, excluding uninformative characters, and a RI of 0.83. Eight major clades (labeled 1 and 3 to 9) are represented in the tree. Additionally, one single terminal has been labeled as branch 2 only for descriptive purposes (Fig. 2).

In general, species are grouped in clades consistent with their geographic provenance (Fig. 1). Branch 1 (75% bootstrap) includes species occurring almost exclusively in the Iberian peninsula and North Africa. The resolution of the clade is low, consisting of a large polytomy in which six subclades, 1a to 1f (Fig. 2) nest. Subclade 1a includes populations from different species, distributed from the Pyrenees to the Iberian System. Subclade 1b contains species from a small area close to Sierra de Grazalema located in the southern tip of the Iberian peninsula. Each subclade from 1c to 1f includes 1 or 2 species from the southwestern coast of the Iberian peninsula. The remaining species of clade 1 form a polytomy with all the above subclades. In total, clade 1 encompasses 30 species, of which 28 (all except *A. arenaria* and *A. villosa*) are exclusive to this clade. Clade 3 (96% bootstrap) includes populations from all 3 species occurring in the Sierra Nevada massif. Clade 4 (61% bootstrap) is restricted to a series of small ranges in central Andalusia (southern Spain), whereas clade 5 (67% bootstrap) includes species from Corsica, Sardinia, and eastern Spain. Clade 6 (99% bootstrap) contains only the accessions from the Madeiran endemic *A. maderensis*. Branches 1 to 6 are grouped, forming a polytomy in a clade with bootstrap support of 66%.

Clades 7 and 8 (82 and 71% bootstrap, respectively) lie in a trichotomy with the large clade that includes branches 1 to 6. Clade 7 encompasses species and subspecies related to *A. maritima* and *A. alpina*. Subclade 7a groups all the *A. alpina* terminals plus an inland subspecies of *A. maritima* (subsp. *elongata*), subclade 7b includes both accessions of *A. bubanii*, and 7c groups the Greek accessions of *A. canescens*. The distribution of clade 7 is holarctic, with a disjunct area in the southern tip of South America (Fig. 1). Clade 8 includes populations from different species occurring in the Baetic ranges of Segura, Alcaraz, and Cazorla in eastern Andalusia (Spain). Clade 9 (66% bootstrap) is sister to the remaining terminals of *Armeria* and contains species from southern and central Italy. Most of the clades described above, apart from sharing synapomorphies in ITS sequences, span a distinct geographic area in which some of the included terminals are sympatric.

A common concern in phylogenetic studies based on molecular markers that include intraspecific sampling is the nonmonophyletic nature of the conspecific terminals. However, in a scenario in which interspecific gene flow is frequent and ITS homogenization after hybridization has been documented (Nieto Feliner, 1997; Nieto Feliner et al., 1996; Fuertes Aguilar et al., 1999a), the defining as poly- or paraphyletic of those species whose terminals appear in different clades of the ITS gene tree can be misleading. This is due not only to the fact that different populations of the same species may be paraphyletic for a gene tree (Neigel and Avise, 1986) but also because inclusion in the same clade does not necessarily mean common ancestry at the organismic level, because of hybridization and gene exchange. Therefore, we prefer to apply the term ''transclade'' species (from Latin for beyond) to those species possessing ITS sequences unconstrained to a single clade, i.e., *A. villosa*, *A. filicaulis*, and *A. arenaria* (Fig. 2). In transclade species, samples appearing in different clades have different geographic origins. Sequences from *A. villosa* occur in four clades (1, 3, 4, and 8). Those in clade 1 belong to *A. villosa* subsp. *villosa*, *A. villosa* subsp. *longiaristata*, and *A. villosa* subsp. *carratracensis*. Different sequences from *A. villosa* subsp. *longiaristata* fall in clades 1, 4, and 8, whereas those of *A. villosa* subsp. *bernisii* are placed in clade 3. Likewise, sequences of *A. filicaulis* from different geographic areas appear in different clades: *A. filicaulis* subsp. *filicaulis* falls in clade 8 except for populations from Sierra Tejeda, which fall in clade 4. *A. filicaulis* subsp. *nevadensis* and subsp. *trevenqueana* appear in clade 3. *A. arenaria* is another transclade species. Some of the populations are in the basal

polytomy within clade 1, such as one of two samples of *A. arenaria* subsp. *segoviensis*, whereas three other samples from different subspecies are placed in subclade 1a. *A. arenaria* subsp. *confusa* is placed on its own independent branch (branch 2, Fig. 2), as part of the polytomy gathering branches 1 to 6.

### 3.3. Phylogenetic analysis of data set B

The inclusion of sequences from those individuals bearing APS (H1 to H14, Table 3) in the data set affects substantially the topology of the obtained trees (Fig. 3). The resulting analysis raises the number of trees to 2436,

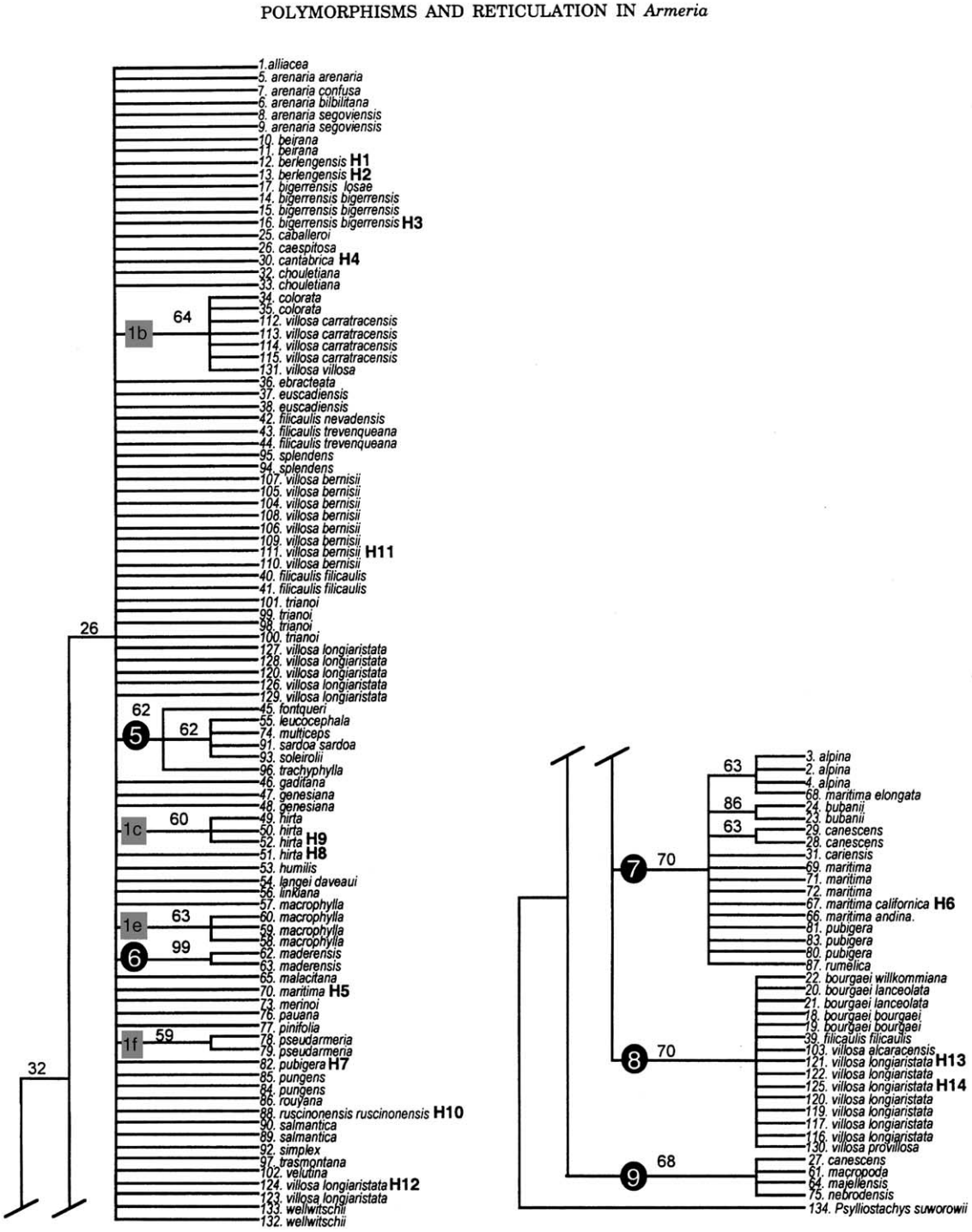


Fig. 3. Strict consensus tree from a parsimony analysis of 133 nrDNA internal transcribed spacer (ITS1 + 5.8S + ITS2) sequences in *Armeria* (data set B). Numbers above branches indicate bootstrap values. Terminals (H1 to H14) containing additive polymorphic sites (APS) are included. Clade numbers refer to those in Fig. 2, regardless of the inclusion of one or more terminals with APS.

and the CI falls to 0.757. The topology of the strict consensus tree differs from that obtained from data set A in the collapse of clades 1, 3, and 4, which results in a large polytomy that encompasses the terminals included in clades 1 to 6 (Figs. 2 and 3). The changes in topology also affect subclades 1a and 1d, which collapse into the large polytomy. Most of the terminals with APS are lumped into the large polytomy containing terminals included in clades 1 to 6, i.e., *A. berlengensis* (H1, H2), *A. bigerrensis* (H3), *A. cantabrica* (H4), *A. ruscinonensis* (H10), *A. villosa* subsp. *bernisii* (H11), and *A. villosa* subsp. *longiaristata* (H12). Whereas accession H6 of *A. maritima* subsp. *californica* appears with the remaining conspecific terminals in clade 7, the other polymorphic accession of *A. maritima* (H5) and that of *A. pubigera* (H7) fall in the largest polytomy. Both H5 and H7 are placed within clade 1 in 86% of the most parsimonious trees recovered. Accession H8 of *A. hirta*, which in the strict consensus tree falls in the large polytomy, is the sister to subclade 1d in 52% of the most parsimonious trees. Accessions H13 and H14 are placed in the polytomy of clade 8 together with other samples of *A. villosa* subsp. *longiaristata* from the Subbaetic ranges.

## 4. Discussion

### 4.1. ITS sequences with additive polymorphic sites

Additive polymorphic sites in nuclear ribosomal genes have been used as evidence of reticulation events (Sang et al., 1995; Campbell et al., 1997; Whittall et al., 2000). However, our conclusion of a reticulate scenario in *Armeria* was not based on the occurrence of such polymorphisms. Instead, the scenario was inferred from the geographical structure of the ITS data, largely independent of taxonomic boundaries, which was very unlikely to be explained by radiation and lineage sorting alone (Fuertes Aguilar et al., 1999b). In fact, the interpretation given to explain the ITS data was congruent with the scarcity of APS if a number of the polymorphisms produced after hybridization events had been homogenized by concerted evolution. Because APS that have not yet been homogenized within genomes can be the result of reticulation and thus may represent a different way to look at the same marker, we focus here on these APS. We discuss below how the composition of APS detected in this extended sampling fits the scenario documented before and the insights that these polymorphism may provide.

APS may constitute evidence for reticulation under the assumption that polymorphism has resulted from the merging of divergent ITS repeats in a single genome. However, we considered two additional aspects before hypothesizing reticulation on the basis of an APS. First, we looked for current biogeographical congruence (sympatry or parapatry) between the two putative parental taxa, such as an intermediate position between the two areas of distribution. Second, different sites in a sequence from a hybrid lineage need not follow the same pattern with regard to polymorphisms because homogenization after hybridization can act in a biased direction for only some nucleotides (Fuertes Aguilar et al., 1999a). Therefore, polymorphisms need not be currently present in every site in which the two hybridizing parents differ. This is important when we try to track down the putative parents.

Previous studies in *Armeria* failed to detect a large percentage of individuals bearing APS in the ITS region in taxa such as A *villosa*, for which interspecific gene flow has been documented (Fuertes Aguilar et al., 1999b). In that study only 2 accessions, here also recorded under *A. villosa*, of 54 examined displayed such a pattern. In our present study there are 14 accessions with APS of a total sample of 133 accessions. Although the proportion is slightly higher than that in the first study, the number still seems rather low if we consider the evidence for reticulation in different species represented in our sampling. Scarcity of APS can be due either to rapid concerted evolution acting on ITS sequences in homoploid hybrids or to introgression toward one of the parents. Both forms of homogenization have been demonstrated in *Armeria* (Fuertes Aguilar et al., 1999a). Additional evidence supporting the origin of those polymorphisms from a reticulation event is that in at least 5 (H6 to H11) of the 14 sequences there is more than one additive polymorphism per sequence, all of them consistent with the same parentage (Table 3). It seems unrealistic to hypothesize that those polymorphisms originated independently in the same sequence.

Based on the occurrence of APS, all the major clades contain taxa that may have been involved in interclade reticulation, except 6 and 9, the latter of which may have been insufficiently sampled (Fig. 4). This suggests that hybridization between species from different clades is a rather common event in *Armeria* and thus conforms to the previously proposed reticulate scenario. For example, the two studied accessions (H1 and H2, Table 3, Fig. 4) of *A. berlengensis* show a polymorphic site (C/T) in position 415. Except for this site, the general ribotype for *A. berlengensis* is that of Clade 1. *A. berlengensis* is endemic to the Berlengas Islands, a small archipelago 10 km offshore central Portugal. The only sample with an autapomorphic T in that position belongs to *A. welwitschii*, a morphologically well-differentiated taxon endemic to the Portuguese coast from Cape Mondego to Cascais, including the Peniche peninsula near the Berlengas (Pinto da Silva, 1972a). Bernis (1957) and Nieto Feliner (1987) pointed out the existence of forms intermediate between those of these two species. Movement of seeds between these two ranges seems possible via ornith-

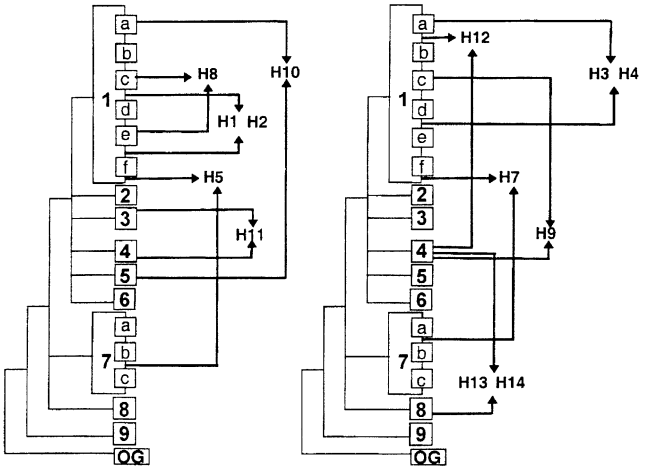


Fig. 4. Putative parental relationships of polymorphic terminals (H1 to H14) as inferred from APS. Topology and branch numbers are those of Fig. 2. Polymorphic terminals are placed on two different trees for clarity purposes.

ochory. Each day seagulls (*Larus argentatus argentatus*) from nesting populations within the *A. berlengensis* patches visit the continent, where *A. welwitschii* grows, for feeding on fisheries by-products (Tauleigne-Gomes, pers. comm.; J. Fuertes Aguilar and G. Nieto Feliner, pers. obs.). Another example is represented by *A. ruscinonensis*. The only sequenced accession (H10, Table 3, Fig. 4) of this taxon endemic to northeast Spain and the adjacent French coast bears three APS in sites 391 (C/T), 392 (C/T), and 564 (C/T). These polymorphisms are consistent with the possibility of introgression from *A. arenaria* from subclade 5.

A possible alternative to reticulation as an explanation of the occurrence of APS is inheritance of two paralogues from recent ancestors, i.e, preservation of polymorphisms. This seem unlikely because of concerted evolution of rDNA loci within lineages. An APS indicates the coexistence of at least two different ITS copies (ribotypes) within the same individual. If the APS resulted from inherited polymorphisms in the absence of reticulation, we would expect that substitutions implied by the APS should conform to the same patterns inferred in the phylogenetic tree. However, this is not the case (Table 4). Whereas the substitutions inferred in the phylogenetic tree show a balance between transitions and transversions (ti/tv = 19/17), there is a strong bias toward transitions (ti/tv = 19/2) in the APS if these were to be interpreted as resulting from substitutions not yet homogenized. It is not probable also that this observation can be attributed to pseudogenes (Buckler et al., 1997), because most of the putative parental sequences have been detected elsewhere in the data set. Therefore, the occurrence of APS being due to causes other than gene flow without subsequent complete homogenization seems unlikely, although it cannot be totally ruled out.

### 4.2. Phylogenetic and biogeographic implications

Another objective of this paper was to discuss the possible phylogenetic implications of our analysis of ITS sequences, taking into account the possible disruptions caused by reticulation. Data set A has been used as the main framework for this. However, once the terminals with APS were included in the matrix (data set B), an effort was made to trace the putative parents based on the distribution of polymorphic sites. In cases in which the molecular pattern was ambiguous or there was more than one possible parental species, the morphology and the geographical proximity are also considered (Sang et al., 1995). In addition to individual scrutiny of each APS in the aligned sequences, the phylogenetic analysis of all terminals, including those with APS, has brought about the opportunity to examine changes in topology (Wiens, 1999). The topology obtained from the analysis of data set B is consistent with that obtained by the analysis of ITS sequences from artificial hybrids (Nieto Feliner et al., 2001).

The possible disruptions caused by reticulation in the ITS phylogeny make more difficult the inference of the species phylogeny from a gene tree; thus, the implications at the organismal level are tentatively discussed separately for each clade, taking into account as much evidence available. Polytomies, understood as uncertainties about relationship, restrict phylogenetic inferences. However, their predominance in our ITS tree is likely supported by active biased homogenization of ITS sequences (Fuertes Aguilar et al., 1999b). Bootstrap values for some of the clades are moderate to low but these values are combined with good fit of the characters to the tree, i.e., low homoplasy (CI = 0.936, excluding uninformative characters). This implies that few changes support each clade but congruence among characters is high, a common pattern in the analysis of multicopy genes (Sanderson and Doyle, 1992).

The position of the Italian clade (clade 9; Fig. 2) as sister to the remainder of the genus has implications for the historical biogeography of the genus. This clade, including Sicilian and Peninsular Italian species, displays a homogeneous ribotype, except for one autapomorphy in *A. majellensis*. The sampled species, *A. macropoda*, *A. nebrodensis*, *A. canescens*, and *A. majellensis*, axe rather similar morphologically, which led Bernis (1954) to consider them a group of related varieties, to which he also associated *A. rumelica* from northern Italy and the Balkans. Intermediate forms in contact areas between two of these species have been recorded (Bianchini, 1983). However, the position of the Italian clade as sister to the rest of the genus has not

been previously hypothesized, and we lack conclusive support for it.

*The A. maritima–alpina clade*. Clade 7 (Fig. 2) encompasses morphologically similar taxa, occurring mostly either on mountains or in coastal areas from temperate to subarctic regions of the world. All the taxa in this clade have been assigned either to the *A. maritima* or to the *A. alpina* species complexes. Despite their different habitats, the strong morphological similarity between *A. maritima* and *A. alpina*, which has led several authors to consider the second species a subspecies of the first (Bernis, 1954; Pinto da Silva, 1972b), is supported by the ITS data. *A. canescens* and *A. rumelica* are central Mediterranean and Balkan species occurring on mountain habitats. The two Greek collections of *A. canescens* are included in this clade, whereas the Italian accession appears in the clade with other Italian species. Clade 7 thus represents another example of the geographic structure of the ITS data, in this case extra-Iberian. The three accessions of *A. alpina*, from the Italian Alps, Austrian Alps, and Central Pyrenees, group together with *A. maritima* subsp. *elongata*, although with low bootstrap support (69%). This placement is consistent with the origin of the latter taxon from hybridization between *A. maritima* and *A. alpina*, a view defended on morphological and biogeographical grounds by several authors (Gams, 1927; Philipp, 1974). South American populations of *A. maritima* have been studied by Moore and Yates (1974), who accommodated the considerable amount of continuous morphological variability displayed in South America under subsp. *andina*. These plants are self-compatible and monomorphic for pollen and stigma. This feature points out a possible origin either from subsp. *sibirica* or from subsp. *californica*, both monomorphic and occurring in the Northern Hemisphere. Although the sampling is far from complete, no synapomorphy groups North American and South American subspecies, apart from those supporting all of clade 7.

Another Pyrenean species, *A. bubanii*, considered very close to *A. alpina* (Bernis, 1954; Nieto Feliner, 1990; Pinto da Silva, 1972b), also appears in clade 7. The two accessions of *A. bubanii* share a substitution that seems to be synapomorphic for the species. ITS variation appears to reflect the complex biogeographic nature of the Pyrenean range. In contrast to central or southern European ranges (Alps, Apenines, Sierra Nevada), from which all the species of *Armeria* sampled nest in a clade that corresponds to a particular range, the taxa found in this massif belong in three different clades: *A. euscadiensis* and *A. arenaria* subsp. *arenaria* (on northern slopes) in clade 1a, *A. alpina* and *A. bubanii* in clade 7, and *A. ruscinonensis* from the eastern coast in the basal polytomy of clade 1. A possible cause for this pattern is the accumulation of plants from different origins as a consequence of migrations that took place after the glacial periods.

*A. arenaria* subsp. *confusa*, represented by a single terminal (branch 2), also distributed in the Pyrenees, is part of the polytomy of clades 1 to 6 (Fig. 2), a position that could be due to reticulation. Based on morphological characters and biogeography, *A. arenaria* subsp. *confusa* has been hypothesized to be of hybrid origin between populations of *A. arenaria* and Pyrenean populations of *A. alpina* (Nieto Feliner, 1987). Its position in the cladogram (Fig. 2) is consistent with the ribotype being the result of concerted evolution following hybridization between taxa included in subclades 1a and 7a. However, this taxon has two autapomorphies. This finding might question the hybrid origin unless subsequent divergence after reticulation took place, a possibility that is consistent with morphological cohesiveness maintained by this taxon across its range (Nieto Feliner, 1990).

*The A. maderensis clade. A. maderensis* is the only species occurring in Macronesia and the only taxon endemic to an oceanic island. This rare endemic grows on rock fissures at the highest elevations of Madeira, between 1700 and 1800 m (Press and Short, 1994). The two individuals sampled of *A. maderensis* share three synapomorphic changes that support their own clade 6 (Fig. 2). The remaining clades forming the polytomy that includes *A. maderensis* are composed mostly of Iberian or North African representatives. Some Macaronesian endemics have been proposed to be the relicts of an ancestral Mediterranean Tertiary flora (Bramwell, 1985; Francisco-Ortega et al., 1997a). The position of clade 6 within a large polytomy does not confirm or reject such a pattern. Morphologically, *A. maderensis* possesses two distinct characters. Whereas all other species have an oblique pedicel insertion at the base of the calyx, in *A. maderensis* pedicel insertion is almost truncate. Also, involucral bracts form a single row in what appears to be a rather undifferentiated involucre. *A. maderensis*, like the vast majority of species, is heteromorphic for pollen and stigma and thus presumably self-incompatible. This would represent a violation of Baker's (1955) rule which precludes the existence of self-incompatible species on oceanic islands. The smallest distance to the continent, where several species of *Armeria* (*A. pungens*, *A. maritima*, and *A. pubigera*) occur, is more than 800 km. This paradox, which involves long-distance dispersal and self-incompatibility, is also present in other island genera such as *Argyranthemum* in the Canaries (Francisco-Ortega et al., 1997b) and the silversword alliance in Hawaii (Carr et al., 1986; Baldwin et al., 1991).

Whatever its closest relatives might be, currently *A. maderensis* is largely isolated from other species. Therefore, it provides a case with which to test our hypothesis that geographically isolated taxa should allow more substitutions in ITS to be preserved against gene flow from other congeners. This situation is dramatically different from that on the Iberian peninsula, where 54

species (and more than 20 additional subspecies) coexist within a limited territory, resulting in frequent sympatry. The fact that *A. maderensis* has three synapomorphies is consistent with our prediction that reproductive isolation from other taxa results in a higher divergence for the ITS region.

*The Corso–Sardinian clade*. Arrigoni (1970) recognized seven species of thrifts in Corsica and Sardinia. One of them, *A. pungens*, was originally described from disjunct populations in southwestern Portugal. The remaining species are endemic to either island. We sampled four species, *A. leucocephala*, *A. multiceps*, and *A. soleirolii*, endemic to Corsica, and *A. sardoa* subsp. *sardoa* from Sardinia, all of which form a monophyletic group (subclade 5a, Fig. 2), with one autapomorphy in *A. sardoa*. The lack of resolution within the subclade is compatible with both (1) a recent radiation resulting in morphological diversification without ITS divergence and (2) ITS homogeneity due to gene flow between species and subsequent concerted evolution. As pointed out by Bernis (1954), there are strong morphological similarities among *A. leucocephala*, *A. multiceps*, and *A. soleirolii.*

Two species from the eastern Iberian peninsula, *A. fontqueri* and *A. trachyphylla*, are grouped with the Corso–Sardinian subclade in a weakly supported branch (67% bootstrap, clade 5, Fig. 2). No link had been previously hypothesized between the Iberian species and the Corso–Sardinian endemics. Whether this link is the product of a recent migration from continental plants followed by radiation or the result of past geological connections between these areas should be investigated further. Pollen records of *Armeria* are old enough (middle Miocene; cf. Rivas Carballo et al., 1998) to suggest a vicariance relationship. Further, the Catalonian coastal ranges where *A. fontqueri* occurs were part of an arc of islands that connected the Iberian peninsula with Corsica and Sardinia by the mid-Tertiary (Jonge et al., 1993). A vicariance scenario would also be consistent with the area cladograms obtained from several groups of animals (Oesterbroek and Arntzen, 1992). The absence of the genus in the Balearic Islands and particularly in Minorca, which would be the link between the islands and the continent, is puzzling but might be explained by extinction.

Regardless of its origin, the close relationship among ITS sequences from Corsica, Sardinia, and eastern Iberia is noteworthy. Furthermore, the extended Corso–Sardinian clade 5 nests within a polytomy in which the remaining clades involve species from the Iberian peninsula, Madeira, and North Africa. Despite the closer geographical proximity to Italy than to Iberia, clade 5 is separated from clade 9 (the Italian clade) by four changes. The topology thus supports an Iberian origin for the Corso–Sardinian species. The question of whether the Corso–Sardinian populations of *A. pungens* share a ribotype with the other island species (or alternatively with the conspecific populations from Iberia) could not be resolved due to our inability to sample the Corsican populations.

*The southern Iberian clades*. Clades 3 (Sierra Nevada), 4 (central Andalusian), and 8 (Sub-baetic) group terminals from three adjacent areas in the southeastern part of the Iberian peninsula (Figs. 1 and 2). These clades have in common the presence of two transclade species, *A. villosa* and *A. filicaulis*. In each of the clades, reticulation events between species (including transclade) and colonization of intermediate habitats seem to have occurred in the origin of subspecific taxa, following a compilospecies model (Fuertes Aguilar et al., 1999b). Our extended sampling confirms the pattern described previously. Clade 3, corresponding to the Sierra Nevada massif, is the subject of a population study now in progress based on ITS, *trnL–trnF* intergene chloroplast spacer, RAPDs, and morphological and ecological variation. That study aims to elucidate the complex relationships among the taxa occurring in the Sierra Nevada, most of which share a single ITS sequence. In the present study, we added eight new sequences from four taxa that conform to the local ribotype from the massif. Also, an individual (No. 111, Table 1; H11 in Fig. 3) of *A. villosa* subsp. *bernisii* that combines ribotypes of both clades 3 and 4 and results in additive polymorphisms was detected. This individual causes the collapse of clades 3 and 4 when data set B is analyzed (Figs. 2 and 3) and supports the occurrence of hybridization between these two clades.

*The large Iberian clade*. Clade 1 encompasses 53 terminals, most of them branching from a basal polytomy. Except for two accessions from southern France and four from northern Morocco, the plants represented are restricted to the Iberian peninsula (Fig. 1). This clade is remarkable for the number of species and the morphological diversity encompassed and is divided into six subclades (named 1a to 1f in Fig. 2). Four of the subclades (1c, 1d, 1e, and 1f) mostly contain a single species from sect. *Macrocentron*, the group of plants that spans most of the diversity of the genus according to Bernis' (1954) lumping taxonomy. Despite including most accessions from section *Macrocentron* (*A. berlengensis*, *A. gaditana*, *A. macrophylla*, *A. pinifolia*, *A. pungens*, *A. rouyana*, *A. simplex*, *A. velutina*, *A. welwitschii*), all from the western Mediterranean, clade 1 leaves out two species that were included in the section by Lawrence (1940). These are *A. rumelica* and *A. cariensis*, both from the eastern Mediterranean. The main morphological character in this section is the projection of the oblique pedicel insertion of the calyx onto a long spur. The spur, with the aid of the downward-projected hairs, allows the fruit to get anchored in a sandy substrate (G. Nieto Feliner, pers. observ.). A parallel acquisition of the long spur on both ends of the Mediterranean cannot be ruled out. On the other hand, the ITS data do not argue

strongly against the monophyly of the western species of section *Macrocentron*. Clade 1 also contains the vast majority of the species occurring on noncoastal habitats of Spain and Portugal. High-altitude taxa, such as *A. bigerrensis* subsp. *bigerrensis*, with a cushion-like habit and growing on granite rocks above 2000 m, have the same ribotype as *A. gaditana*, a plant from southern Spain with scapes up to 1 m tall, typically growing on sandy soils near the sea. The combination of moderate genetic variation as estimated by ITS and significant morphological diversification associated with a number of habitats might suggest extensive radiation. However, without discarding the possibility of radiation, we think that the morphological, ecological, and, to some extent, biogeographical heterogeneity observed in clade 1 is more complex in origin. Part of it may have to do with the clade acting like a sink. Some of the samples seem to be the result of biased concerted evolution of ITS sequences after hybridization with individuals bearing any of the ribotypes included in clade 1. The bias in homogenization toward the ribotype in clade 1 thus would be consistent with the large size of this clade. Two types of independent evidence provide support for this hypothesis. First, $F_2$ artificial hybrids synthesized from individuals of *A. villosa* from clade 8 and *A. colorata* from clade 1a bear ribotypes that place them in clade 1. This is the result of an active biased homogenization process that involves the six nucleotides by which ITS clades 8 and 1a differ (Fuertes Aguilar et al., 1999a; Nieto Feliner et al., 2001). Second, four of the individuals bearing APS are suggested to be the result of hybridization between individuals from clade 1 and those from other clades: H5, H7, H10, H12 (Table 3, Fig. 4). In these four individuals, nucleotides that differ between the two involved clades present the base corresponding to clade 1, with the exception of those that maintain the APS. For instance, H7 is hypothesized to be the result of hybridization between clades 1 and 7 (Fig. 4). This individual displays APS for nucleotides 381 and 390, but for the other two nucleotides in which the two clades differ (391 and 392), it presents T, i.e., the base present in clade 1. This suggests biased homogenization toward the clade 1 pattern. Which of the species included in clade 1 are the result of true common ancestry is a question that requires additional evidence both from the organismic level and from molecular markers.

### 4.3. The significance of transclade species

In *Armeria*, two patterns of morphological variation and geographical distribution can be distinguished. Whereas most of the species are narrowly distributed, a few have wider ranges. The latter include *A. alpina*, *A. arenaria*, *A. filicaulis*, *A. maritima*, *A. transmontana*, *A. villosa*, and, to a lesser degree, *A. hirta*. All of them display intraspecific morphological variability, which is taxonomically recognized at the subspecific or varietal rank. In many cases, such morphological entities occur near the geographic boundaries of the distribution, where they are sympatric or parapatric with other species and colonize novel habitats relative to the core of the species.

In previous work (Fuertes Aguilar et al., 1999b), we suggested that *A. villosa* fitted the "compilospecies" model proposed by Harlan and de Wet (1963). This concept described situations in which a species captures portions of the genomes of other sympatric species via introgression. In our proposal of *A. villosa* as a compilospecies inferred from the ITS pattern, we underlined several facts: (1) different samples of a single subspecies, *A. villosa* subsp. *longiaristata*, appear in three of the major clades, (2) samples of at least one of the six subspecies of *A. villosa* appear in four major clades, and (3) composition of major clades shows greater congruence with the geographic origin of plants than with the traditional systematic arrangement based primarily on morphology. The relevant question is whether all the species here referred to as transclade also fit the compilospecies model. The extension of the intraspecific sampling to other species in the present study has certainly revealed a similar pattern for *A. filicaulis*, *A. arenaria*, and probably *A. maritima*, *A. alpina*, *A. canescens*, and *A. bigerrensis*, i.e., different ribotypes in different areas. Further, of all the transclade species, *A. maritima*, *A. villosa*, and *A. hirta* have accessions bearing APS in the ITS sequence (Table 3). We lack detailed information (ecological, molecular) for certain specific cases for which morphological data suggest the compilospecies model. However, with the available data it is likely that at least some of the above species also conform to the compilospecies concept. The occurrence of transclade species in ITS phylogenies is not exclusive to *Armeria*. For example, species of *Phlox* (Polemoniaceae) (Ferguson et al., 1999) and *Streptanthus* (Cruciferae) (Mayer and Soltis, 1999) exhibit the same pattern in phylogenetic analyses. In the case of *Phlox*, the role of hybridization in *P. pilosa* is explicitly discussed by the authors. In the case of *Streptanthus*, the placement of different populations of *S. glandulosus* in separate clades occurs not only in ITS but also in cpDNA trees. Further, the detection of additive polymorphic sites in ITS is consistent with reticulation.

Our work, based on natural and experimental populations, shows that standard phylogenetic analyses of ITS sequences are not enough for the understanding of mechanisms underlying the gene-based genealogies. In addition to cladistic assumptions, factors such as intraspecific sampling, concerted evolution, secondary structure constraints, recombination, and dosage effects cannot be ignored as critical elements for reconstruction of organismal relationships with ITS.

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