

ORIGINAL ARTICLE

Introgression in peripheral populations and colonization shape the genetic structure of the coastal shrub *Armeria pungens*R Piñeiro¹, A Widmer², J Fuertes Aguilar¹ and G Nieto Feliner¹¹Real Jardín Botánico, CSIC, Plaza de Murillo 2, Madrid, Spain and ²ETH Zurich, Institute of Integrative Biology (IBZ), Universitätstrasse 16, Zurich, Switzerland

The coastal shrub *Armeria pungens* has a disjunct Atlantic-Mediterranean distribution. The historic range expansion underlying this distribution was investigated using the nuclear internal transcribed spacer region, three plastid regions (namely *trnL-F*, *trnS-FM* and *matK*) and morphometric data. A highly diverse ancestral lineage was identified in southwest Portugal. More recently, two areas have been colonized: (1) Corsica and Sardinia, where disjunct Mediterranean populations have been established as a result of the long-distance dispersal of Portuguese genotypes, and (2) the southern part of the Atlantic range, Gulf of Cadiz, where a distinct lineage showing no genetic differentiation among populations occurs. Genetic consequences of colonization seem to have been more severe in the Gulf of Cadiz than in Corsica-Sardinia. Although significant genetic divergence is

associated with low plastid diversity in the Gulf of Cadiz, in Corsica-Sardinia, the loss of plastid haplotypes was not accompanied by divergence from disjunct Portuguese source populations. In addition, in its northernmost and southernmost populations, *A. pungens* exhibited evidence for ancient or ongoing introgression from sympatric congeners. Introgression might have created novel genotypes able to expand beyond the latitudinal margins of the species or, alternatively, these genotypes may be the result of surfing of alleles from other species in demographic equilibrium into peripheral populations of *A. pungens*. Our results highlight the evolutionary significance of genetic drift following the colonization of new areas and the key role of introgression in range expansion.

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Introduction

Genetic divergence of populations frequently occurs as a consequence of long periods of allopatry. However, founder effects during the colonization of new areas might also lead to rapid divergence, in addition to diversity loss, associated with random genetic drift in small populations.

The idea that founder effects may promote evolutionary divergence was first proposed by Mayr (1942) in his ‘genetic revolution’ model of speciation. However, empirical data from a number of documented natural colonization events assessed with molecular markers, such as postglacial range expansions (Tregenza, 2002; Pruett and Winker, 2005; Alsos *et al.*, 2007) and colonizations of islands (Grant *et al.*, 2001; Clegg *et al.*, 2002; Abbott and Double, 2003; Barker *et al.*, 2009) suggest that divergence occurs only after severe founder effects. The severity of a founder effect seems to depend on the dispersal and establishment characteristics that modulate the importance of the genetic drift, in particular (1) the mode of dispersal determines different

patterns of gene flow according to the number of propagules, probability of dispersal based on the availability of vectors and geographical distance; and (2) the success of establishment is directly linked to limiting ecological factors and is driven by natural selection (Baker and Moeed, 1987; Pruett and Winker, 2005).

The role of hybridization in colonization has also been made evident in current and classic evolutionary research by the recurring observation of introgressed genotypes in marginal and/or recently colonized areas in plant and animal studies, including meta-population studies and hybrid zones (Abbott, 1992; Fuertes Aguilar *et al.*, 1999b; Ellstrand and Schierenbeck, 2000; Moody and Les, 2002; Choler *et al.*, 2004; Petit *et al.*, 2004; Albert *et al.*, 2006; Lavergne and Molofsky, 2007; TiéCré *et al.*, 2007).

In this study, we investigate the colonization history of the Atlantic-Mediterranean coastal shrub *Armeria pungens* (Plumbaginaceae). This species is distributed along a 500 km coastal belt in southern Atlantic Iberia, from the mouth of the Tagus River to the Gibraltar Strait. It also occurs on two disjunct archipelagos: in the Atlantic, in the Cíes islands (off the Galician coast, Northern Spain), and in the Mediterranean, in Southern Corsica and Northern Sardinia (Figure 1a). The Atlantic-Mediterranean disjunction was previously assessed with amplified fragment length polymorphisms (AFLPs) and bioclimatic envelope modeling. It was shown to result from long-distance dispersal from the Portuguese Atlantic coast into the Mediterranean islands of Corsica

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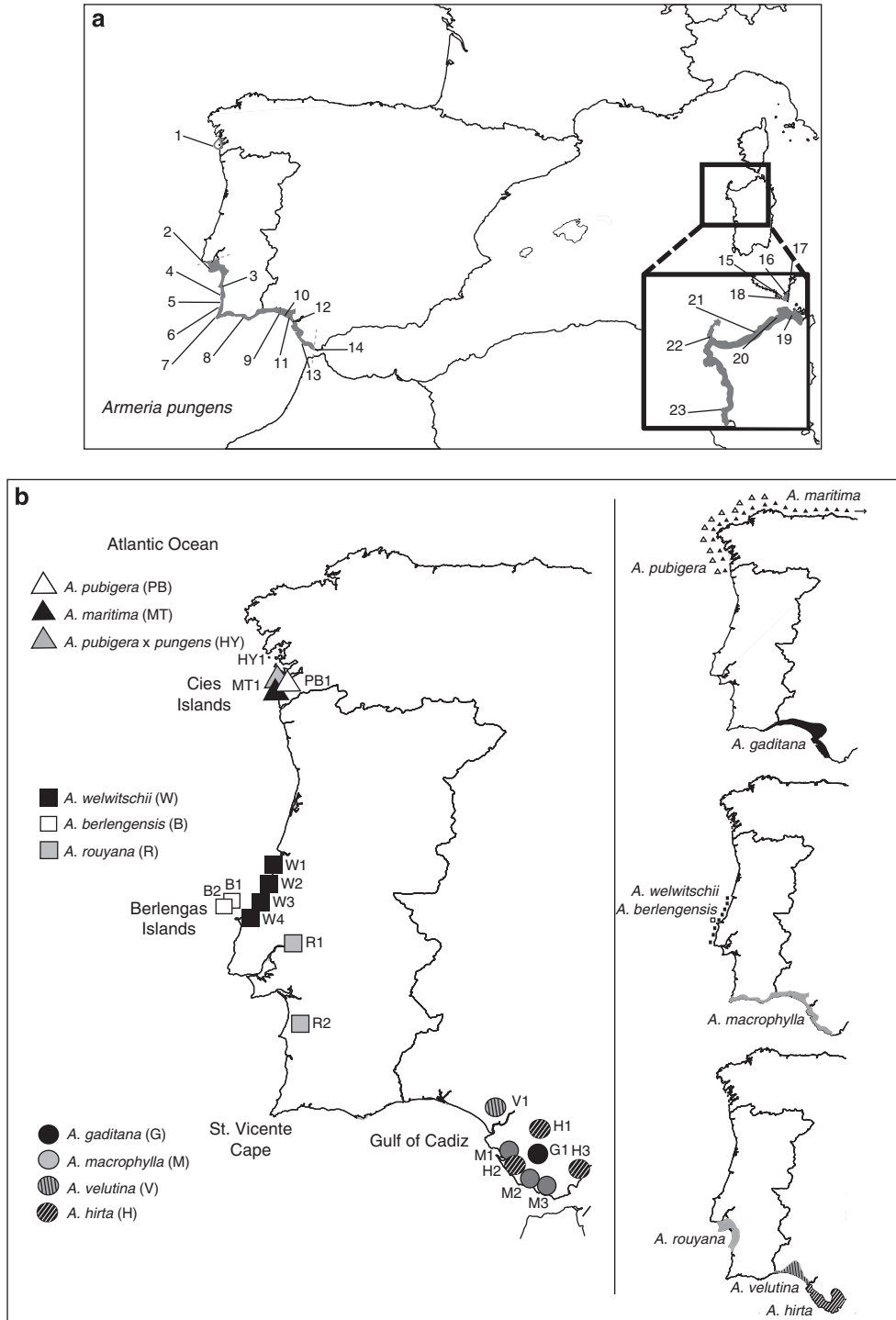


Figure 1 (a) Distribution and sampling of *A. pungens*. Locations sampled for the morphological and molecular studies (three cpDNA regions *trnL-F*, *trnS-fM* *matK* and nrDNA ITS) are numbered as populations from 1 to 23, and the total distribution of the species is represented in the background (in gray). (b) Distribution and sampling of the congeners sympatric to *A. pungens*. A total of 19 populations of coastal and subcoastal *Armeria* species were sampled for sequencing of three cpDNA regions (*trnL-F*, *trnS-fM* and *matK*) and partly for the nrDNA ITS. The approximate distribution of the nine species studied is represented according to Bernis (1955); Arrigoni (1970) and Nieto Feliner (1990). Population names and sample information are provided in Supplementary information S1 and S2B.

and Sardinia, probably favored by similar climatic conditions in both areas (Piñeiro et al., 2007).

The populations in the southern part of the Atlantic range (Gulf of Cadiz), which has drier summers than the remaining localities of the species range, were shown to form a distinct and less diverse genetic group, based on

AFLP, despite the absence of physical barriers with Portugal. However, the unordered AFLP data did not allow us to assess whether this distinct group is the result of long-term isolation or recent colonization (Piñeiro et al., 2007). In this study, we address this question using genealogical data, that is, DNA sequences

that allow identification of the closest relative to the distinct populations in the Gulf of Cadiz. For this purpose, we sequenced three plastid regions—*trnL-trnF*, *matK* and *trnS-trnfM*—and the nuclear ribosomal internal transcribed spacers (ITSs) across the whole range of *A. pungens*. We also performed a detailed morphological study. The use of plastid markers is highly suitable for assessing the genetic effects of colonization due to maternal inheritance and low effective population size (Clegg *et al.*, 1994) of the haploid chloroplast genome, which make it more sensitive to genetic drift than the nuclear genome. In agreement with this prediction, patterns of variation of the low-copy nuclear gene *GapC* in *A. pungens* were shown to be partially blurred by incomplete lineage sorting (Piñeiro *et al.*, 2009). The use of ITS sequences in phylogeography is less straightforward, given that the concerted evolution of different ITS copies usually results in low levels of variation within reproductive groups (Baldwin *et al.*, 1995). In addition, the multicopy nature of these markers and their specific mode of evolution frequently results in a mixture of orthologous and paralogous rDNA copies within a genome, which complicates phylogenetic inference (Álvarez and Wendel, 2003). Nevertheless, we also surveyed this marker, because accumulated data in *Armeria* (Fuertes Aguilar *et al.*, 1999b; Fuertes Aguilar and Nieto Feliner, 2003; Nieto Feliner *et al.*, 2004) provide a useful background against which new data can be interpreted. Furthermore, following Nieto Feliner and Rosselló (2007), careful use of these markers that includes representative sampling, careful lab protocols and thoughtful analyses can provide useful and even insightful information into species-level evolutionary studies.

Hybridization is very common in natural populations of *Armeria* owing to weak interspecific reproductive barriers, even between distantly related species, as shown in artificial crossing experiments (Baker, 1966; Lefèbvre, 1974; Nieto Feliner *et al.*, 2001). This process is well documented to affect the structure of populations long after the divergence of species has started (Gutiérrez Larena *et al.*, 2002; Tauleigne-Gomes and Lefèbvre, 2005), and thus needs to be taken into account in any evolutionary study within the genus. In particular, biparentally inherited ITS markers have been useful for documenting reticulate evolution in *Armeria*, based on the identification of individual additive polymorphisms and a pattern of variation consistent with geography but not with taxonomy (Fuertes Aguilar *et al.*, 1999b). In this study, we addressed the putative incidence of hybridization in the evolution of *A. pungens* through a comparison of ribosomal DNA markers with maternally inherited plastid sequences. The identification of putative maternal donors of introgression events was also attempted through sequencing of plastid markers from congeners sympatric or parapatric to *A. pungens* (Tauleigne-Gomes and Lefèbvre, 2005).

The specific objectives of this study were as follows:

- To assess whether the proposed long-distance dispersal into Corsica and Sardinia is confirmed with morphological data, plastid DNA and ITS sequences;
- to test for colonization versus isolation in the Gulf of Cadiz;
- to assess the genetic consequences of colonization; and

- to test for introgression from other species of *Armeria* into *A. pungens*.

Materials and methods

Study system

A detailed description of *A. pungens* and its suitability for phylogeographic studies is provided in the study by Piñeiro *et al.* (2007). The remaining coastal and subcoastal species of *Armeria* in the Iberian Peninsula are restricted to the western and northern Atlantic coasts (Bernis, 1955; Nieto Feliner, 1990). The western Atlantic coast is the center of diversity for sect. *Macrocentron*, including *Armeria welwitschii*, *Armeria berlengensis*, *Armeria pinifolia*, *Armeria rouyana*, *Armeria hirta*, *Armeria gaditana*, *Armeria macrophylla* and *Armeria velutina* (Figure 1b). In addition, the non-*Macrocentron* species *Armeria pseudoarmeria* occurs locally in Cabo da Roca and the surrounding areas. In contrast, the northern Atlantic coast is dominated by the *maritima* aggr., represented by the widely distributed *Armeria maritima* and the closely related Iberian endemic *Armeria pubigera*. The Cíes islands are the only location where sect. *Macrocentron* and *maritima* aggr. meet, as *A. maritima*, *A. pubigera* and *A. pungens* coexist in the archipelago. The genus *Armeria* is largely absent from the coasts of Eastern Spain, Southern France and Italy, with the notable exception of *Armeria soleirolii* in Northern Corsica, as well as *A. pungens* in Southern Corsica and Northern Sardinia.

Sampling strategy

For the molecular study, dried leaves and seeds were collected from 23 populations spanning the entire range of the species (Figure 1a; Supplementary information S1). A distance of 10 m between plants was maintained. Seeds were germinated after a cold treatment of 1 month, and seedlings were cultivated under greenhouse conditions. The average sampling size was two individuals per population for the ITS region ($N=59$) and five individuals per population for the three plastid regions ($N=112$). For the morphological study, voucher specimens were collected for 21 of the populations and deposited in the herbarium of the Real Jardín Botánico (MA). The average sampling size was 13 individuals per population ($N=241$).

To test for the origin of the divergent plastid haplotypes in populations from the margins of the distribution of *A. pungens*, an extended interspecific sampling of coastal and subcoastal species of *Armeria* was undertaken. To examine the northernmost disjunct population, we sampled all congeners from the Cíes islands (*A. maritima*, *A. pubigera* and putative hybrids between *A. pungens* and *A. pubigera*), and three of four species from the central portion of the Iberian Atlantic coast (*A. welwitschii*, *A. berlengensis* and *A. rouyana*). In the vicinity of the southernmost population, all extant subcoastal species (*A. hirta*, *A. velutina*, *A. macrophylla* and *A. gaditana*) were studied. The locally distributed species *A. pseudoarmeria* and the sublittoral species *A. pinifolia* were the only coastal species from Atlantic Iberia not included in the study. Therefore, in addition to the samples of *A. pungens*, a total of 37 individuals from 19 populations belonging to 10 taxa were included (Figure 1b; Supplementary information S2).

DNA isolation, PCR amplification and sequencing

DNA isolation from dried or fresh leaves was performed using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The ITS region (ITS1, ITS2 and the 5.8S gene) was amplified and sequenced with universal primers P1A and P4 (Fuertes Aguilar *et al.*, 1999b). The *trnL-trnF* intergenic spacer was amplified with primers e and f (Taberlet *et al.*, 1991) and sequenced with primer e. The *trnS-fM* intergenic spacer was amplified with universal primers *trnS* and *trnFm* (Demesure *et al.*, 1995) or, in two fragments, using primer combinations *trnS-intM* and *intS-trnFm* for amplification and *trnS* and *intS* for sequencing. The *matK*-encoding gene was amplified and sequenced with specific primers intK2 and intK3. For recalcitrant samples, PCR products obtained with primers extK2 and extK3 were used for nested amplification with intK2 and intK3. Specific primers intM, intS, extK2, extK3, int2 and intK3 (see Supplementary information S3) were designed using the Primer3 software (Rozen and Skaletsky, 2000). PCR reactions contained 20–200 ng of template DNA, $10 \times$ or $5 \times$ PCR buffer, 1.5 mM MgCl₂, 1 mM dNTPs, 0.4 μM of each primer and 0.4 μl Taq (Promega, Madison, WI, USA), or were performed with Ready-To-Go beads (Amersham Biosciences Europe GmbH, Cerdanyola, Barcelona, Spain) with 10–500 ng template DNA, 0.2–0.8 mM of each primer and 4% dimethyl sulfoxide or bovine serum albumin. The PCR conditions for ITS followed those described in the study by Lihová *et al.* (2004), with a 52–54 °C annealing temperature. The *trnL-F* profile followed the procedure of Gutiérrez Larena *et al.* (2002), with a 50 °C annealing temperature. The *trnS-fM* and *trnK-K* profiles are reported in Supplementary information S4. Sequencing reactions were run on an ABI Prism sequencer 377 or 3730 (Applied Biosystems, Foster City, CA, USA).

Armeria pungens

cpDNA: Three plastid regions—*trnL-F*, *trnS-fM* and *matK*—were chosen. A total of 112 concatenated sequences of *A. pungens* were obtained and manually aligned with Bioedit 5.0.9 (Hall, 1999) following the guidelines proposed by Kelchner (2000). The same individuals were sampled for *matK* and *trnS-fM*, whereas different individuals from the same populations were included for *trnL-F* in 69 cases. The three plastid regions were analyzed together in the same network after the finding that 19 of 23 populations were monomorphic for *trnL-F*. To control for putative artificial consensus sequences in the four populations found to be polymorphic for *trnL-F*, we checked two cases (populations 3 and 23), in which concatenations of different individuals produced singletons (haplotypes L and D). These two haplotypes were removed from an alternative analysis without producing any significant difference. Genealogical relationships among haplotypes were estimated in a statistical parsimony network using TCS 1.21 (Clement *et al.*, 2000). For mononucleotide repeats of different lengths, each contiguous gap position was coded as a single substitution. Indels were coded as a single mutation.

Population genetic parameters were calculated using dnaSP 4.10.7 (Rozas *et al.*, 2003) and Haplonst (Pons and Petit, 1996). The within-population diversity was estimated first in terms of allele frequencies with Nei's

haplotypic diversity, Hd, and second in terms of the weighted sequence divergence with the nucleotide diversity, π , the average number of nucleotide differences per site between two sequences (Rozas *et al.*, 2003). Population differentiation was estimated for different geographical regions based on haplotype frequencies only, G_{st} , and both on haplotype frequencies and genetic distance between haplotypes, N_{st} (Pons and Petit, 1996). The difference between the two estimates was assessed with a haplotype identity permutation test (10 000 permutations) implemented in PERMUT 1.0 (<http://www.pierroton.inra.fr>). The regional allelic richness, A , corrected for sample size ($N=28$) using the rarefaction method of El Mousadik and Petit (1996), was calculated using FSTAT 2.9.3.2 (Goudet, 2001).

Internal transcribed spacers: A total of 59 ITS consensus sequences of *A. pungens* were generated from forward and reverse sequences and aligned with Bioedit 5.0.9. Intraindividual polymorphisms, detected as double peaks in both the forward and reverse electropherograms, were carefully checked and coded using ambiguity codes of the IUPAC (The International Union of Pure and Applied Chemistry) according to Fuertes Aguilar *et al.* (1999a).

Morphometrics: In all, 19 morphological variables (15 quantitative continuous and 4 qualitative, Supplementary information S5A and B) were recorded. We performed a principal components analysis based on a correlation matrix of the quantitative characters and four derived ratios (Supplementary information S5A) with SPSS 14 for Windows (SPSS Inc.). To maximize independence of characters, one original measure of each ratio was removed. Qualitative characters (Supplementary information S5B) were examined by calculating the percentage of individuals exhibiting each character state per population.

Sympatric congeners

cpDNA: A chloroplast DNA (cpDNA) matrix containing 71 sequences was constructed by adding 36 *Armeria* sp. sequences to a reduced matrix of 34 sequences of *A. pungens* (selected out of 112 by deleting identical sequences of each population). One specimen of *Psylliostachys suworowi* was used as the outgroup following the study by Lledó *et al.* (1998). Gaps resulting from alignment were treated as missing values.

Phylogenetic analyses were performed using Maximum Parsimony and Bayesian approaches. Maximum Parsimony was carried out using PAUP v. 4.0b10 (Swofford, 2002) under Fitch parsimony through heuristic searches with 100 random taxon addition replicates, holding 100 trees at each step and TBR branch swapping on all trees (options steepest descent off and multrees off). Branch support was calculated using the fast bootstrap option with 10 000 replicates under two different treatments of indels: by simply treating gaps resulting from alignment as missing values and by adding an additional binary character representing the presence/absence of the indel. Before the Bayesian analyses, the cpDNA matrix for each plastid region was imported into Modeltest 1.1b (Nylander, 2003) to select the model of evolution under the Akaike information criterion. Subsequently, a Bayesian analysis was run

with Mr Bayes 3.1 (Ronquist and Huelsenbeck, 2003), setting three parallel runs for five million generations. Convergence was diagnosed using the s.d. of split frequencies. A total of 1250 000 trees (25%) were discarded and the remaining trees were compiled into a majority rule consensus tree, using the posterior probability as a measure of clade support.

Internal transcribed spacers: ITS sequences of *A. pungens* were reanalyzed within the framework of a previous ITS phylogenetic study of the genus *Armeria* representing 70% of the species (Fuertes Aguilar and Nieto Feliner, 2003). In that study, most coastal Iberian species of sect. *Macrocentron* were shown to harbor a set of closely related ITS ribotypes falling within the same clade. For simplicity, the whole clade will be referred to here as ribotype R1. This ribotype was also detected in many inland western Iberian species. In contrast, coastal species from the *maritima* aggr. formed an independent clade that will be referred to as ribotype R7. Finally, the Corso-Sardinian species, *Armeria leucocephala*, *Armeria multiceps*, *A. soleirolii* and *Armeria sardoa*, shared a single ITS ribotype (ribotype R5) remarkably distant from ribotype R1.

In populations in which *A. pungens* is known to be sympatric with congeners from other ITS clades, individuals were checked for putative polymorphic sites revealing hybridization (Fuertes Aguilar and Nieto Feliner, 2003; Keller *et al.*, 2008). In particular, additive polymorphic sites revealing the coexistence of ribotype R1 (characteristic of *A. pungens*) and ribotype R7 (characteristic of the *A. maritima* clade) were checked in the Cíes Islands, whereas in Corsica and Sardinia, additive polymorphic sites between ribotypes R1 and R5 were sought. For this purpose, 10 additional sequences from congeners *A. pubigera*, *A. maritima*, *A. pungens* × *A. pubigera*, and *A. macrophylla* were obtained.

Results

Molecular and morphological variation within *A. pungens*

The sequencing of *trnL-F*, *trnS-fM* and *matK* in *A. pungens* resulted in a final matrix of 112 individuals and 3037 bp. In all, 20 nucleotide substitutions, 3 mononucleotide repeats and one 6-bp indel defined 16 haplotypes (Supplementary information S6). Identical networks, only showing differences in branch lengths, were produced by adding or excluding the presence/absence characters. The geographic distribution of haplotypes and relationships among them are represented in Figures 2a and b.

The ITS matrix for *A. pungens* encompassed 59 individuals and 629 bp. *A. pungens* presented only ITS sequences corresponding to ribotype R1, also present in many western Iberian species (Fuertes Aguilar and Nieto Feliner, 2003), across its whole range, even in Corsica and Sardinia (Table 1). However, five within-individual polymorphic sites were detected. On the basis of our previous knowledge of the variation of this marker in *Armeria*, these polymorphic sites are interpreted as the intragenomic occurrence of more than one ITS sequence (Fuertes Aguilar *et al.*, 1999a; Nieto Feliner and Rosselló, 2007). Therefore, populations that share the same polymorphic sites are interpreted as having the same

additional ITS sequence besides R1. Accordingly, the five ITS profiles recorded through direct sequencing are named R1a, R1b, R1c, R1d and R1e (Table 1; Figure 2c), meaning that the R1a profile contains only R1, whereas the other four contain different additional ITS sequences. Some of the least frequent ITS copies may not have been sequenced, given that the amplified products were not cloned. Nonetheless, the low levels of ITS variation observed through direct sequencing discouraged us from a more thorough sampling of the ITS variation.

The first three principal components extracted in the principal components analysis of quantitative morphological variables explained 68.89% of the overall variance (Supplementary information S5A). The scatterplot of 241 individuals against PC1 and PC2 (Supplementary information S7) showed no morphometric discontinuities, revealing the absence of morphological differentiation of *A. pungens* across geographical areas.

The main genetic and morphological groups within

A. pungens

cpDNA and ITS markers both revealed two main genetic groups comprising most of the populations within *A. pungens*: the 'Portuguese-Corso-Sardinian group' (populations 2–7 and 15–23) and the 'Gulf of Cadiz lineage' (populations 8–13). The two highly divergent haplotypes corresponding to populations on the margins of the range of *A. pungens* (populations 1 and 14) are treated below.

The Portuguese and Corso-Sardinian areas shared three plastid haplotypes (namely A, C and G; Figures 2a and b) and two ITS profiles (the standard one, R1a, and R1b) (Figure 2c; Table 1). Portuguese populations (populations 2–7) harbored the highest number of plastid DNA variants within the species, 10 of 16 (haplotypes A, B, C, F, G, H, I, J, K and L; Figures 2a and b). In contrast, Corso-Sardinian populations (populations 15–23) had five plastid haplotypes: a subset of haplotypes from Portugal (the dominant haplotype A and the local haplotypes C and G) and two exclusive local haplotypes (haplotype M in population 15 and haplotype D in population 23; Figures 2a and b). The Gulf of Cadiz lineage harbored an inferred ancestral haplotype in one individual (population 8, haplotype E), closely related to Southern Portugal haplotypes (B, G and H) and the exclusive haplotype N, present in all six populations, derived from the same lineage but separated by several mutational steps (Figures 2a and b). Two ITS profiles (R1c and R1d; Figure 2c; Table 1) were also exclusive to this lineage. Nonetheless, the standard ITS profile appeared in a single population and was shared with the Corso-Sardinian and southern Portuguese populations (R1a; Figure 2c; Table 1).

The nature of the ITS variation detected in *A. pungens*, involving the coexistence of more than one ITS repeat within individuals, did not allow for estimations of population diversity parameters. Genetic diversity and differentiation were thus calculated based only on plastid markers. The overall level of plastid differentiation in *A. pungens* was high with $G_{st} = 0.73$ (average for angiosperms: $G_{st} = 0.63$; Petit *et al.*, 2005). Portuguese populations exhibited high intrapopulation (average $h_s = 0.48$; Table 2) and regional ($A = 10$; $h_t = 0.90$; Table 2)

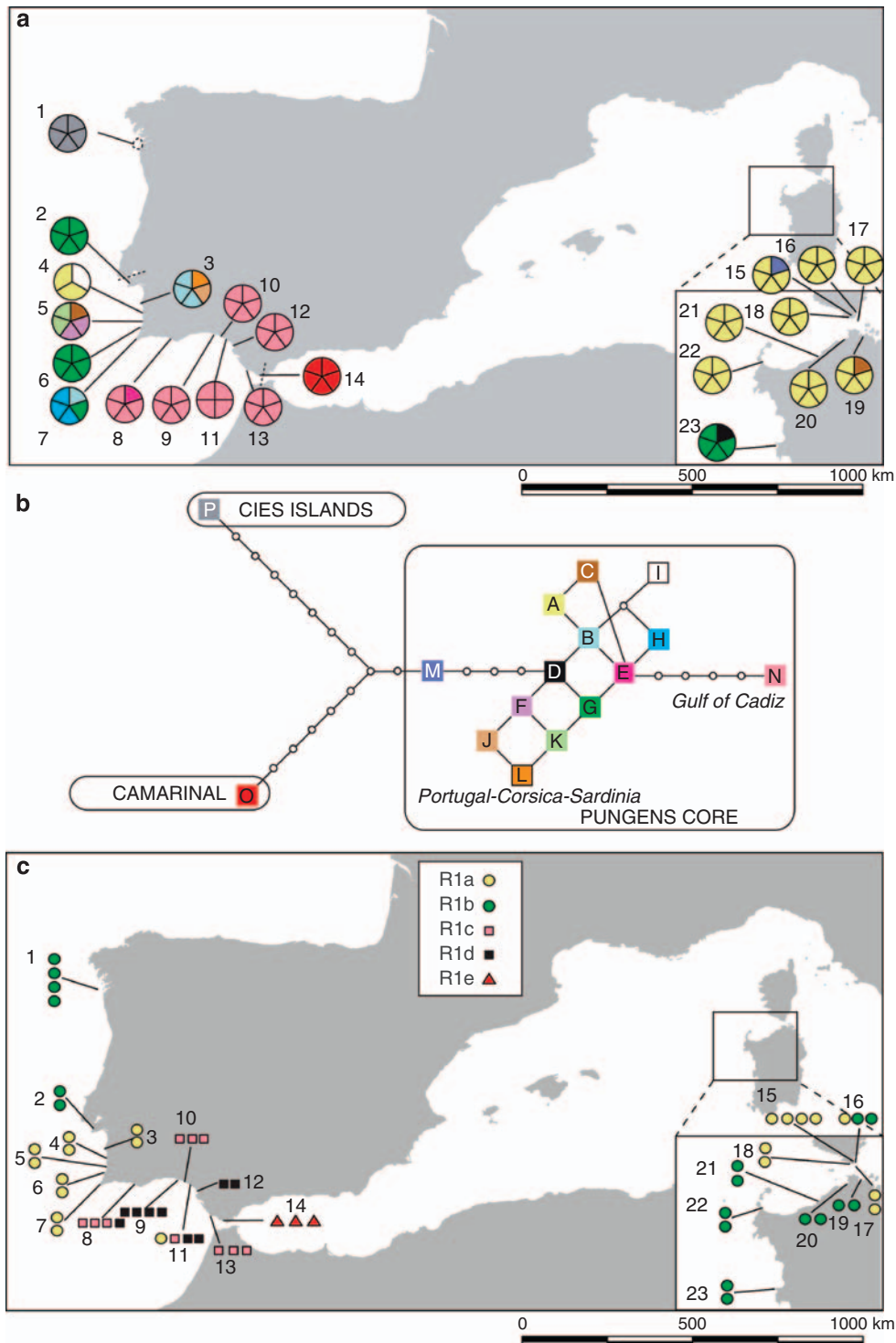


Figure 2 Main genetic groups in *A. pungens*. (a) Geographic distribution of the 16 plastid haplotypes (*trnL-F*, *trnS-FM*, *matK*) found in 112 individuals from 23 populations of *A. pungens* (numbered as in Supplementary information S1). (b) Statistical parsimony network showing the genealogical relationships among the 16 plastid haplotypes. (c) Geographic distribution of ITS profiles across 59 individuals from 23 populations of *A. pungens*: R1a denotes typical Western Mediterranean ribotype (Fuertes Aguilar and Nieto Feliner, 2003), without polymorphic sites in direct sequencing; R1b, R1c, R1d, R1e indicate polymorphic sites in different positions that are interpreted as intragenomic cooccurrence of more than one ITS sequence (Table 1).

diversities, resulting in a moderate level of regional differentiation ($G_{st}=0.47$; Table 2). The haplotype diversity within populations was high in populations 3, 4, 5 and 7 ($H_d=0.67-0.80$; Table 3), whereas populations 2 and 6 were fixed for a single haplotype ($H_d=0$;

Table 3). The diversity in Corsica-Sardinia was moderate ($h_s=0.09$; $H_d=0$; $h_t=0.26$; $A=4$), also resulting in moderate regional differentiation ($G_{st}=0.66$; Table 2). Finally, the genetic variation was almost zero along the Gulf of Cadiz ($H_d=0.07$; $A=2$; $h_t=0$; $G_{st}=0$; Tables 2

Table 1 Nucleotide variation across the aligned ITS1-5.8S-ITS2 sequences found in *Armeria pungens* populations

	62	99	380	381	390	391	392	416	417	448	520
<i>Armeria</i> spp. (Fuertes Aguilar and Nieto Feliner, 2003)											
Western Iberian clade R1	T	C	T	C	C	T	T	G	G	C	A
Corsica-Sardinia clade R5a	C	C	T	T	.	.
<i>A. maritima-alpina</i> clade R7	.	.	.	T	T	C	C	.	.	.	G
<i>Armeria pungens</i> (N = 59)											
R1a	T	C	T	C	C	T	T	G	G	C	A
R1b	.	Y
R1c	Y	Y
R1d	Y
R1e	.	.	Y	Y	.
Putative hybrids or introgressed individuals from sympatric populations (N = 10)											
<i>A. pungens</i> × <i>A. pubigera</i> (Cíes)	.	Y	.	Y	Y	Y	Y	.	.	.	R
<i>A. maritima</i> , <i>A. pubigera</i> (Cíes)	.	.	.	Y	Y	Y	Y	.	.	.	R
<i>A. macrophylla</i> (Camarinal)	.	.	Y	Y	.

Abbreviations: IUPAC, The International Union of Pure and Applied Chemistry; pop., population; ITS, internal transcribed spacer. Clade nomenclature for reference purposes follow Fuertes Aguilar and Nieto Feliner, 2003. The five ITS profiles recorded through direct sequencing in *A. pungens* are named R1a, R1b, R1c, R1d and R1e, meaning that the R1a profile only contains R1, whereas the other four contain different additional ITS sequences. Within-individual additive sites are coded according to the IUPAC code. Nucleotides identical to the top sequences are indicated by dots.

Table 2 Regional cpDNA diversity and differentiation (*trnL-F*, *trnS-fM*, *matK*) in *Armeria pungens*

Region	N individuals (N pops.)	A (N = 28)	h_s	h_t	G_{st}	v_s	v_t	N_{st}	$N_{st perm}$
Portugal	28 (6)	10	0.48 (0.1522)	0.90 (0.1131)	0.47 (0.1066)	0.47 (0.1561)	0.90 (0.1668)	0.48 (0.0701)	0.45
Gulf of Cadiz	29 (6)	2	0	0	0	0	0	0	—
Corsica-Sardinia	45 (9)	4	0.09 (0.0588)	0.26 (0.1751)	0.66 (0.0648)	0.04 (0.0238)	0.27 (0.1947)	0.86 (0.0452)	0.65 ^a

Abbreviations: A, allelic richness corrected for sample size (N = 28); cpDNA, chloroplast DNA; G_{st} , genetic differentiation based on haplotype frequencies only; h_s , average within-population diversity based on haplotype frequencies only; h_t , total genetic diversity based on haplotype frequencies only; N, sampling size; N_{st} , genetic differentiation based on haplotype frequencies and genetic distance between haplotypes; $N_{st perm}$, permuted N_{st} based on permuted distances between pairs of haplotypes; pop., population; v_s , average within-population diversity based on haplotype frequencies and genetic distance between haplotypes; v_t , total genetic diversity based on haplotype frequencies and genetic distance between haplotypes.

s.d. is indicated in parentheses.

^a $P < 0.01$.

and 3). The estimate of the overall genetic differentiation among *A. pungens* populations that takes into account allele similarity was significantly higher ($N_{st} = 0.85$) than the estimate based on allele frequencies (confirmed by the permutation of haplotype identities test, $U = G_{st}/N_{st} = 2.15$; $N_{st perm} = 0.70$ with $P < 0.01$; Pons and Petit, 1996). In Portugal, there was almost no difference between G_{st} (0.47) and N_{st} (0.48; Table 2), indicating mutation-drift equilibrium. In Corsica-Sardinia, N_{st} (0.86) was also significantly higher than G_{st} (0.66; Table 2).

In contrast to the principal components analysis based on quantitative characters, the leaf pubescence showed a clear geographical trend that is consistent with the main genetic groups (Figure 3). Most plants in populations along the Gulf of Cadiz displayed glabrous to subglabrous leaves, whereas those in Portugal-Corsica-Sardinia exhibited mostly ciliate leaves on middle veins. An exception is Bordeira (population 6), with dense pubescence on leaves and scapes.

Molecular variation in sympatric congeners

The cpDNA matrix including the sympatric species had 3057 bp and 94 variable sites, 23 of them parsimony

informative. The majority rule consensus of the 87 trees retained in the parsimony analysis was constructed. For the Bayesian analyses, the data were partitioned according to the model of evolution of each plastid marker revealed by Modeltest 1.1b: F81 + I for *trnL-F*, HKY for SfM and GTR + I for *matK*. The topology of the majority rule consensus tree was almost identical to that of the parsimony tree. Two weakly supported main clades, A and B, were present (Figure 4). Clade A included two subclades (labeled 1 and 2) and eight single terminals. The relationships between clades 1 and 2 were not resolved. Clade 1 (60% bootstrap; 99 posterior probability) contained the majority of *A. pungens* sequences, thus representing the *A. pungens* core lineage. Within this core, the Gulf of Cadiz lineage, comprising populations 8–13 (subclade 1a; 85% bootstrap; 100 posterior probability), was clearly differentiated from the populations in Portugal-Corsica-Sardinia, comprising populations 2–7 and 15–23 and one individual from population 8 (haplotype E). Populations RO1 and RO3 (*A. rouyana*), V1 (*A. velutina*) and M1 (*A. macrophylla*), as well as individuals from G1 (*A. gaditana*) and W1 (*A. welwitschii*), also fell close to the Portuguese-Corso-Sardinian samples. Clade 2 (70% bootstrap; 99 posterior probability) was restricted to the southern *Armeria* species,

including *A. pungens* from Camarinal (population 14), *A. macrophylla*, *A. hirta* and *A. gaditana*. The close relationship between the Camarinal haplotype and its sympatric population of *A. macrophylla* had some support

Table 3 Within-population cpDNA diversity (*trnL-F*, *trnS-fM*, *matK*) in *Armeria pungens*

Population	N	Hd	$\pi \times 10^{-3}$
Cies Islands			
Pop. 1	5	0.00	0.00
Portugal			
Pop. 2	5	0.00	0.00
Pop. 3	5	0.70	1.01
Pop. 4	3	0.67	0.48
Pop. 5	5	0.80	0.51
Pop. 6	5	0.00	0.00
Pop. 7	5	0.70	0.29
Gulf of Cadiz			
Pop. 8	5	0.40	0.29
Pop. 9	5	0.00	0.00
Pop. 10	5	0.00	0.00
Pop. 11	4	0.00	0.00
Pop. 12	5	0.00	0.00
Pop. 13	5	0.00	0.00
Camarinal			
Pop. 14	5	0.00	0.00
Corsica-Sardinia			
Pop. 15	5	0.40	
Pop. 16	5	0.00	0.58
Pop. 17	5	0.00	0.00
Pop. 18	5	0.00	0.00
Pop. 19	5	0.40	0.00
Pop. 20	5	0.00	0.14
Pop. 21	5	0.00	0.00
Pop. 22	5	0.00	0.00
Pop. 23	5	0.40	0.00
Total range	112	0.19	0.14

Abbreviations: cpDNA, chloroplast DNA; Hd, haplotype diversity; N, sample size; π , nucleotide diversity; pop., population.

(63% bootstrap; 100 posterior probability). Clade B (50% bootstrap; 70 posterior probability) encompassed *A. pungens* from the Cies islands, two putative hybrids *A. pungens* \times *A. pubigera*, *A. berlengensis* (populations B1, B2) and *A. welwitschii* (populations W1, W2, W3, W4). The congeners from Cies (*A. pubigera* and *A. maritima*) fell apart from the Cies samples of *A. pungens* as single terminals in a polytomy with the two main clades together with the remaining three putative hybrids, *A. pungens* \times *A. pubigera*. Bootstrap supports and posterior probabilities of the major clades decreased when additional binary characters were added to represent the presence/absence of indels, which indicates that indels are homoplastic above the species level.

In the Cies islands, additive patterns were found in ITSs between the R1b profile of *A. pungens* and the R7 in the putative hybrids, *A. pungens* \times *A. pubigera*. *A. maritima* and *A. pubigera* also presented the same pattern (Table 1). In Camarinal, *A. pungens* exhibited the R1e profile shared with the sympatric species *A. macrophylla*. Finally, in Corsica and Sardinia, *A. pungens* showed the western Mediterranean ribotype R1 profile (that is, the standard R1a profile) without additions with respect to the ribotype of the other Corso-Sardinian species (R5).

The genetic divergence of peripheral populations of *A. pungens*

The southernmost population and the disjunct northernmost population of *A. pungens* were found to be significantly different from the two main genetic groups. The southernmost population (population 14—Camarinal) displayed two ITS polymorphic sites at positions 380 and 448 (profile R1e: Table 1; Figure 2c), which were lacking in the remaining populations, together with an exclusive divergent plastid haplotype (O; Figures 2a and b) and puberulous leaves (Figure 3) that were also lacking elsewhere within *A. pungens*. In the northernmost island population (population 1—Cies), conflict between the maternally inherited plastid sequences and



Figure 3 Geographic distribution of the leaf pubescence in *A. pungens*. The percentages of ciliate (black), glabrous (white), dense pubescent (gray) and puberulous (patterned) leaves are indicated for each population. *A. pungens* individuals are labeled with population numbers as described in Supplementary information S1 and Figure 2a.

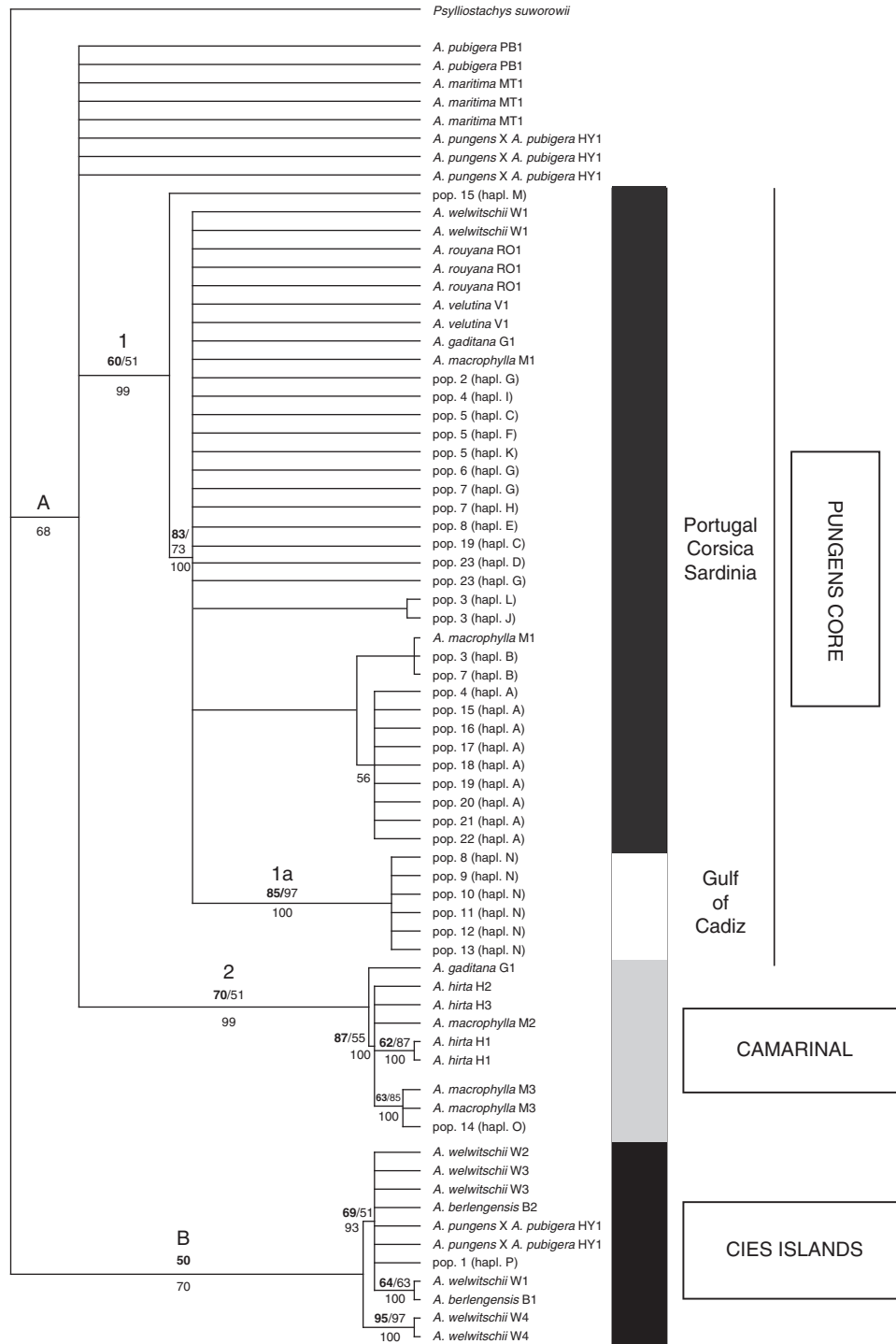


Figure 4 Chloroplast phylogenetic tree (*trnL-F*, *trnS-fM*, *matK*) of *A. pungens* and sympatric congeners. Overall, 50% majority rule consensus of 87 MP trees depicting the phylogenetic relationships between the plastid haplotypes of 23 populations of *A. pungens* and 36 individuals from 19 populations of 10 coastal and subcoastal congeners. Bootstrap supports above 50% are indicated above the branches both without (in bold) and with an additional binary character representing the presence/absence of indels. Posterior probabilities of the Bayesian analysis are indicated below branches. The names of chloroplast haplotypes and main genetic groups in *A. pungens* are indicated according to Figures 2a and b. *A. pungens* individuals are labeled with population numbers as described in Supplementary information S1 and Figure 1a. The name and population of origin of the other species follow Supplementary information S2 and Figure 1b.

biparentally inherited ITS markers was detected. Although plastid sequences revealed an exclusive haplotype (P; Figures 2a and b) that was remarkably divergent from the rest of the populations, the ITS

data indicated similarity with the Portuguese-Corso-Sardinian populations of *A. pungens* (Figure 2c). This is revealed by the sharing of the R1b profile with the closest mainland population (population 2—Albufeira),

Sardinia (populations 19–23) and two Corsican individuals (population 16). The presence of ciliate leaves related the Cies population to the Portuguese-Corso-Sardinian genetic group (Figure 3), in agreement with the ITS.

The sequencing of plastid markers in the sympatric and parapatric species provided a picture of how these congeners relate to the anomalous disjunct northernmost and southernmost populations of *A. pungens* (Figures 2a and b). In the plastid phylogeny, *A. pungens* from the Cies islands (population 1; clade B; Figure 4) appeared to be closer to *A. berlegensis* (populations B1 and B2) and *A. welwitschii* (populations W1, W2 and W3) than to mainland *A. pungens* (populations 2–14; clade A; Figure 4). Similarly, *A. pungens* from Camarinal (population 14) was placed in clade 2 together with its sympatric southern congeners *A. gaditana*, *A. hirta* and *A. macrophylla*, apart from all other populations of the species. The latter relationship was also supported by the sharing of the ITS R1e profile that involved two ITS polymorphic sites at positions 380 and 448 (Table 1) with *A. macrophylla*.

The other species from Cies, *A. pubigera* and *A. maritima*, were part of the polytomy in clade 1 in the plastid phylogeny (Figure 4). Moreover, in the Cies islands, the five putative *A. pungens* × *A. pubigera* hybrid individuals studied were shown to fall either close to the plastid haplotype P of *A. pungens* (two individuals) or close to *A. pubigera* (three individuals in a polytomy with the four major clades). Finally, these hybrids showed additive ITS polymorphic sites for all five parsimony-informative positions differing between the typical *A. pungens* ribotype R1 and the typical *A. pubigera* R7 (Table 1).

Discussion

Plastid DNA and ITS data of *A. pungens* show clear footprints of colonization and hybridization.

Colonization of Corsica-Sardinia and the Gulf of Cadiz

Previous AFLP data suggest that the Atlantic-Mediterranean disjunction of *A. pungens* resulted from the long-distance dispersal of Portuguese plants into Corsica-Sardinia (Piñeiro et al., 2007). These data are supported by the detection in Corsica and Sardinia of the typical western Iberian *Armeria* ITS ribotype (R1a without additions with respect to R5), the presence of plastid haplotypes also found in Portugal and the morphological similarity with Portuguese populations (no differences in quantitative characters and the presence of ciliate leaves in both areas). In agreement with this scenario, the Portuguese source area exhibits a high number of plastid DNA haplotypes (10 of 16) within the same lineage, with almost identical values of G_{st} and N_{st} (Table 2), which suggests the long-term stability of these populations. No evidence for introgression from sympatric species accounting for such high plastid diversity levels in Portugal was apparent from the described pattern, even in Bordeira (population 6), which showed plastid DNA and ITS variants typical of the Portuguese lineage of *A. pungens* despite exhibiting unusually densely pubescent leaves and scapes.

A few individuals from several congeners (*A. welwitschii*, *A. rouyana*, *A. velutina*, *A. macrophylla* and

A. gaditana) showed plastid sequences closely related to those of *A. pungens* in Portugal (Figure 4; clade 1), suggesting either some incomplete sorting of ancestral polymorphisms or introgression of *A. pungens* into these populations. The fact that these closely related haplotypes occur in geographically distant populations makes the hypothesis of sorting of ancestral polymorphisms more likely. An extended population-level sample of chloroplast and nuclear markers covering the entire range of all the species may help to test this hypothesis.

Unlike previous AFLP markers, plastid sequences allowed the reconstruction of genealogical relationships between the distinct Gulf of Cadiz lineage and the remaining populations of the species. This lineage was revealed to be linked to southern Portuguese haplotypes and exhibited extremely low plastid diversity and lack of differentiation among populations. This genetic structure indicates that the Gulf of Cadiz lineage has recently colonized its current distributional area. In this context, genetic drift might have been the dominant evolutionary force, which might explain the possible extinction of the intermediate haplotypes between E and N. The attainment of such levels of differentiation through long-term isolation seems less plausible, given the lack of correlation between haplotypes and geographical locations in the Gulf of Cadiz. Still, these levels might also be attained if a regional population extinction event was followed by recolonization from, for example, remnant populations in the east near the Gibraltar strait. On the other hand, differentiation of the Gulf Cadiz lineage by means of introgression from an extinct (or not sampled) lineage of a sympatric species also seems unlikely, given the notable genetic connection to the haplotypes found in *A. pungens* from Southern Portugal. In addition to their different genetic composition, the plants of the Gulf of Cadiz tend to exhibit glabrous to subglabrous leaves. Interestingly, classic taxonomic treatments had described only the dense pubescent populations that we found in Bordeira (Bernis, 1955; Pinto da Silva, 1972) but not the glabrous populations occurring along the Gulf of Cadiz.

The comparison between the plastid genetic structure of *A. pungens* in Corsica-Sardinia and in the Gulf of Cadiz revealed dissimilar genetic consequences of colonization in the two areas. In the Gulf of Cadiz, the loss of genetic diversity was associated with a significant change in the haplotype composition and frequencies. In contrast, the loss of plastid diversity observed in Corsica-Sardinia was not accompanied by divergence from the Portuguese source populations. Therefore, the genetic founder effect was found to be more severe in the Gulf of Cadiz, where climatic conditions differ from the remaining populations, as compared with Corsica-Sardinia, where the probability of dispersal from Portugal is reduced by the much-larger physical distance (Piñeiro et al., 2007). This might indicate an important role of the establishment phase and natural selection during colonization. Nonetheless, to establish causal relationships between genotypes and ecological variables, more appropriate methods than the mere correlation between the neutral genetic variation and environmental conditions should be used. Future studies might include manipulative experiments to assess particular environmental factors as well as identification and survey of loci under selection (Karrenberg and Widmer, 2008).

Hybridization in peripheral populations

The genetic pattern of the northernmost disjunct population on the Cíes islands shows inconsistency between the typical *A. pungens* morphology (ciliate leaves) and nuclear markers (ITS and AFLP; Piñeiro *et al.*, 2007) on one side and the divergent maternally inherited plastid DNA on the other. This matches other cases of chloroplast capture following ancient hybridization (Rieseberg and Wendel, 1993; Arnold, 1997). The additional finding of plastid-haplotype sharing across species allows the rejection of the hypothesis of plastid DNA divergence through the isolation of *A. pungens* in the Cíes Islands and suggests introgression from the Portuguese coastal species *A. welwitschii* and *A. berlengensis*, or a related taxon. The habitat similarities of *A. pungens* and *A. welwitschii* (sandy coastal environments), in the context of the frequent interspecific hybridization in *Armeria* and the dynamic history of Quaternary vegetation in Europe, make it conceivable that both species showed different latitudinal boundaries in the past that favored contacts. Although the possibility that this plastid haplotype has persisted on the Cíes islands as a result of the random sorting of ancestral polymorphisms is less likely, it cannot be completely ruled out.

More straightforward is the interpretation of the origin of the plants in the Cíes islands occurring in the ecotone between sand dunes where *A. pungens* occurs and in granite cliffs where *A. pubigera* occurs. The detection of plastid haplotypes of the two species and additive ITS patterns in these individuals indicates that they may be part of a hybrid zone. Morphometric studies confirm the occurrence of such a hybrid zone containing different hybrid genotypes (Piñeiro R, Fuertes Aguilar J and Nieto Feliner G, unpublished data).

A. pungens in the disjunct population of the Cíes islands thus seems to have experienced at least two hybridization events at different times: past introgression from *A. welwitschii*, *A. berlengensis* or a closely related taxon and ongoing hybridization with *A. pubigera*. Whereas in the latter event an intermediate genetic pattern between the two parental species is still easily recognized, in the former event, the genetic traces of hybridization may have been partially erased by backcrossing toward *A. pungens* following patterns detected in the ITS and morphology. In plants, nuclear markers are frequently less introgressed and thus more congruent with morphology than plastid markers (Rieseberg and Wendel, 1993). This observation is attributed to the faster mutation rates and higher effective population size of the nuclear genome, although the effective migration of nuclear genes by pollen dispersal may be also implicated (Petit and Excoffier, 2009).

In Camarinal, recent introgression from *A. macrophylla* can also be easily inferred from the sharing of ITS polymorphisms and puberulous leaves, as well as the close relationship of plastid haplotypes. The habitat of this population (low open shrubs on fossil dunes) also supports introgression because it is intermediate between that of *A. pungens* (coastal sand dunes) and that of *A. macrophylla* (pine wood understory on sandy soils).

The observation of introgressed genotypes exclusively in the northern and southern marginal populations of *A. pungens*, despite the regular occurrence of sympatric species of *Armeria* along the Iberian coast, indicates that hybridization is favored in the margins of the distribu-

tion. This pattern may be due to selection for introgressed genotypes at the latitudinal extremes of the species' range, which may allow the colonization of novel niches (Anderson and Stebbins, 1954; Abbott *et al.*, 2003; Ellstrand, 2003). Alternatively, population dynamics in peripheral populations, characterized by reduced densities, may have favored the surfing of alleles from sympatric species in demographic equilibrium into *A. pungens* (Klopfstein *et al.*, 2006, Petit and Excoffier, 2009).

Conflict of interest

The authors declare no conflict of interest

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