**Dissimilar molecular and morphological patterns in an introgressed peripheral population of
a sand-dune species (*Armeria pungens*, Plumbaginaceae)**

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Running title: Molecular and morphological patterns in introgressed *Armeria pungens*

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key message of the paper: Morphometric and molecular evidence is consistent with the
occurrence of nuclear and plastid introgression in a coastal peripheral population, but as in
most hybridization scenarios, new data is required for a thorough understanding of specific
features such as degree and direction of introgression, and underlying ecological factors

ABSTRACT

- Introgression is a poorly understood evolutionary outcome of hybridization because it may remain largely undetected whenever it involves the transfer of small parts of the genome from one species to another. A putative case of introgression from the southernmost border of *Armeria pungens* range from its congener *A. macrophylla* is revisited following the discovery of a subpopulation that does not show phenotypic signs of introgression since it resembles typical *A. pungens*.
- Aiming to understand the origin and characteristics of this subpopulation, we analyze morphometrics, nuclear ribosomal DNA ITS and plastid DNA (*trnL-trnF*) sequences, genome size, 45S and 5S rDNA loci-FISH data and nrDNA IGS sequences.
- Within the study site, most individuals match morphologies of either of the two hybridizing species, particularly the new subpopulation, with intermediate phenotypes being scarce. This pattern does not fully fit molecular evidence revealing two ITS ribotypes co-occurring intragenomically in most plants from the study site and one single plastid haplotype. Genome size and structural features of the IGS sequences both indicate that *A. pungens* from the study site is genetically more similar to its sympatric congener than to the remainder of its conspecifics.
- Introgression of *A. macrophylla* into *A. pungens* and plastid capture explain all the evidence analyzed. However, important features to understand the origin and fate of introgressed population, such as the degree and direction of introgression, which are important for understanding early stages of hybridization in plants with low reproductive barriers, should be addressed with new data.

INTRODUCTION

Evidence for the wide occurrence of natural hybridization is nowadays overwhelming not only in plants (Soltis & Soltis, 2009; Folk *et al.* 2018) but also in other organisms (Green *et al.* 2010; Leducq *et al.* 2016; Elgvin *et al.* 2017; Lamichhaney *et al.* 2018). Over the last years it has also become evident that the outcomes of hybridization are more varied and complex than previously realized (Baack & Rieseberg 2007; Abbott *et al.* 2013; Saetre 2013) and some contribute to generate new organismal diversity (Heliconius Genome Consortium 2012; Meier 2017; Wagner 2018). One of those outcomes is introgression, the transfer of alleles or traits through repeated backcrossing following hybridization. There is current debate (Butlin & Ritchie 2013) because some authors argue that such a transfer does not provide enough new genetic variation for natural selection and eventual hybrid speciation (Barton 2013; Servedio *et al.* 2013). Understanding the diversity of scenarios and outcomes where introgression is involved remains elusive and documenting the adaptive nature of this mechanism is even more puzzling, with very few studies addressing all the critical components of adaptive introgression (Suarez-Gonzalez *et al.* 2018). However, a number of available studies report differential introgression across genomes that results in mosaicism. Although this pattern seems to be due to both deterministic and stochastic factors, there is evidence that positive selection can drive the spread of alleles from one species on to the genomic background of the other species (Arnold & Martin 2009; vonHoldt *et al.* 2016; Suarez-Gonzalez *et al.* 2016). Because of our poor understanding of the evolution of hybridized genomes (excluding allopolyploids), focusing on seemingly recently introgressed populations to address their origin and possible evolutionary outcomes is a suitable approach to contribute to this burgeoning field.

Armeria pungens (Link) Hoffmanns. & Link (Plumbaginaceae) is a coastal sand-dune species restricted to the southwestern quadrant of the Iberian Peninsula (from the Setubal Peninsula in Portugal to near the strait of Gibraltar) and to two disjunct areas: an offshore

island in NW Spain, and Corsica - Sardinia. In a previous work, Piñeiro *et al.* (2011) unveiled introgression in the southernmost populations of *A. pungens*, located in a coastal promontory in the Gibraltar Strait area near Tarifa (Punta Camarinal, Spain) and suggested to be associated to its peripheral geographic location. In this study *A. pungens* was reported to be introgressed by a closely related species (*A. macrophylla* Boiss. & Reut.) on molecular and morphological grounds. Specifically, haplotypes defined from sequences of three plastid DNA regions in *A. pungens* plants from this site were found to be markedly different (19 mutational steps away) from the geographically closest ones —some 24 km west of Camarinal in Cape Trafalgar— and equal to those present in sympatric *A. macrophylla* (Piñeiro *et al.* 2011). Since no other haplotypes were found within the few samples sequenced from Camarinal, it was also hypothesized that this could represent a plastid capture (Rieseberg *et al.* 1995; Tsitrone *et al.* 2003). In addition, the introgressed *A. pungens* population from Camarinal showed double peaks in the electropherograms for the nuclear ribosomal DNA ITS nucleotides differing between *A. macrophylla* and the remaining populations of *A. pungens* (Piñeiro *et al.* 2011). This pattern is indicative of intragenomic coexistence of more than one ITS sequence (ribotype) (Nieto Feliner *et al.* 2004). Inference of introgression in *A. pungens* from Camarinal was also consistent with previous AFLP data that distinguished this population from the remainder of the species (Piñeiro *et al.* 2007). Finally, the introgressed population fits the overall morphology of *A. pungens*, but exhibited deviant characters such as a more compact life-form (less shrubby) with shorter rootstock branches, and finely pubescent organs (leaves, scapes, involucral bracts) resembling *A. macrophylla* phenotype. Piñeiro *et al.* (2011) hypothesized that such hybridization scenario could facilitate extending the range and ecological niche of *A. pungens*.

However, a recent discovery in the Camarinal site has posed new questions and increased the interest of the study case: a subpopulation that resembles *A. pungens* phenotypically was discovered in this site growing in its classic environment (open sandy

places, including dunes, by the shoreline). This contrasts with the previously known introgressed subpopulation, which is morphologically deviant and occurs c. 1 km east on consolidated terrain, which however is also sandy and lacks shrub or tree vegetation cover. The new subpopulation was not known previously since the study site is located within a military area with restricted access. The facts that the newly discovered subpopulation occurs on the classic habitat for *A. pungens* and is indistinguishable from it in its overall morphology raise the question whether it could represent genetically pure *A. pungens* compared to the introgressed one that was reported in Piñeiro *et al.* (2011). If this were the case, we could be facing an ongoing process with one subpopulation introgressed while the other —the newly discovered— is not. In addition to the interest of the new subpopulation, the fact that the previous report of introgression was based on a limited number of samples from Camarinal has motivated the present study, which revisits the introgressed subpopulation together with the newly discovered *A. pungens* subpopulation aiming to understand its origin and characteristics using a broader sampling for nuclear ribosomal DNA internal transcribed sequences (ITS) and plastid sequences, together with morphometrics, genome size, 45S and 5S ribosomal DNA loci-FISH data and nrDNA intergenic spacer (IGS) sequences.

Morphometrics, allowing phenotype analysis —particularly of characters under polygenic control and therefore able to provide intermediate phenotypes— have proved useful for exploring hybrid zones in *Armeria* (Tauleigne-Gomes & Lefèbvre 2005; 2008) and in other groups (Hardig *et al.* 2000; Runyeon-Lager & Prentice 2000). Within *Armeria*, morphometric evidence was also informative in distinguishing among synthesized early hybrid generations such as F_1 , F_2 , Bc_1 , Bc_2 (Nieto Feliner *et al.* 1996; Nieto Feliner 1997).

Nuclear ribosomal DNA ITS sequences have been widely used to inform about species delimitation and relationships (Kress *et al.* 2005; Nieto Feliner & Rosselló 2007; Yao *et al.* 2010). Also, correlations have been found between environmental factors and nrDNA variation (Sharma *et al.* 2004). Due to the multicopy nature of ribosomal genes, the quality of sequence

information benefits from knowing structural features such as the number and location of ribosomal loci (5S and 45S, the latter also known as 35S cf. Hemleben & Zentgraf 1994). This is because homogenization of different ribotypes merged within a single genome is less effective and can be slowed down when occurring on different ribosomal loci on different chromosomes (Schlötterer & Tautz 1994). The number of loci has been found to vary inter- and intraspecifically in many plant groups; a fact that requires exploring possible within-species variation for this feature (Olanj *et al.* 2015; Rosato *et al.* 2017). In addition, although the most popular regions of the ribosomal loci are the ITS, other components such as the intergenic spacers (IGS), of the same 45S ribosomal family, can host higher levels of variation and provide information on recent evolutionary events.

Genome size (GS) is known to vary not only between species (Bennett & Leitch 2005) but to some extent also intraspecifically (Murray 2005; Inceer *et al.* 2018) in plants, despite the early notion that C-value was constant within a species (Swift 1950). Expectedly, hybridization and introgression between species differing in GS is one underlying source of GS variation but others include the proliferation of transposable elements (Ungerer *et al.* 2006). In early generations of synthetic hybrids, genome size variation does not usually follow a fully consistent pattern. The most predictable one is intermediate values between the progenitors but this comes with ample ranges of variation (Garnatje *et al.* 2006; Garcia *et al.* 2008; Marques *et al.* 2011). Also when comparing wild hybrid species with their corresponding synthetic hybrids at least during the time frames explored, i.e., a finite number of early synthetic hybrid generations, patterns are by no means coincident (Baack *et al.* 2005). In stabilized or older hybrids ranges of variation are narrower (Bureš *et al.* 2004; Leitch *et al.* 2008). At any rate, GS variation is a useful parameter to explore in recent and evolutionary dynamic scenarios such as hybridization.

With this background, the present study addresses the following questions to analyze a likely ongoing introgression process. (1) Is the newly discovered subpopulation that

resembles *A. pungens* phenotypically also genotypically *pungens*, i.e., genetically closer to populations outside the Camarinal site? (2) Can the phenotypes be used to predict the genetic composition of all *Armeria* individuals from Camarinal? (3) Can we confirm the hypothesis of a plastid capture in the *A. pungens* population originating from *A. macrophylla* as suggested by the previous finding of a single plastid haplotype in Camarinal? (4) Does genome size differ between populations of *A. pungens* and *A. macrophylla* and are size differences consistent with introgression in Camarinal? (5) What is the most likely scenario for the history of the introgression of *A. pungens* in Camarinal?

MATERIAL AND METHODS

Morphometrics. To assess phenotypic variation across the study site, we performed a morphometric analysis of two datasets using sixteen quantitative characters that had been useful in exploring morphological variation in *A. pungens* previously (Table S1; Piñeiro *et al.* 2011). The first dataset was used to check if phenotypes are distinguishable independently of genotypes within the study site. Therefore, specimens were selected to sample variation across the habitats of Camarinal, i.e. on the shoreline and elsewhere in open parts as well as on the more densely vegetated zones, under pine woods or machia shrubland (Fig. 1). To accomplish this, roughly two transects were followed: one parallel to the coast on the west and the other along a north-south direction on the central part of the Camarinal promontory. Twelve specimens from the closest *A. pungens* population (Cape Trafalgar) were also included in this dataset, which amounted to 87 specimens. To maximize independence of characters, one of the two original variables of each of the ratios used was removed. A principal components analysis (PCA) was performed to reduce the dimensionality of the data and facilitate comparison with molecular evidence (ITS, IGS, cpDNA *trnL-trnF*, GS). Accordingly, we saved the scores of the 87 samples on the first PC axis and took them as a rough summary of the morphological phenotype. The second dataset was constructed adding 211 specimens

from Piñeiro *et al.* (2011), which represent the whole range of *A. pungens*, totaling 298 specimens. Analyses were performed with IBM SPSS Statistics for Windows v. 23.0 (IBM Corp., Armonk, N.Y., USA).

nrDNA ITS and trnL-trnF sequences. Sampling for newly generated sequences —26 from the nuclear ribosomal ITS regions (ITS1, ITS2 and the 5.8S gene) and 20 from the plastid *trnL-trnF* intergenic spacer— was focused on Camarinal and geographically closer populations (Fig. 2, Table 1). Both species occur along a coastal fringe westwards into the Gulf of Cádiz in which *A. pungens* occupies the open seashore habitats whereas *A. macrophylla* grows primarily in subcoastal pinewoods. In contrast, eastwards from Camarinal the whole genus *Armeria* is absent throughout the Spanish Mediterranean coastal fringe up to nearly the French border (Nieto Feliner 1990). DNA isolation was performed from dried or fresh leaves using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The ITS region was amplified with universal primers P1A and P4 (Fuertes Aguilar *et al.* 1999a) and the plastid *trnL-trnF* intergenic spacer with primers e and f (Taberlet *et al.* 1991). Sequencing reactions were performed at Macrogen Inc. (Seoul, Korea) using the same PCR primers. In addition, 43 sequences obtained in former studies (Fuertes Aguilar & Nieto Feliner 2003; Piñeiro *et al.* 2011) —26 of these from other geographic origins— were also analyzed. In total, 89 sequences were analyzed, 46 from the ITS region and 43 from the *trnL-trnF* (Table 1). Sequences were edited using BioEdit version 7.0.9.0 (Hall 1999) and aligned by hand.

Due to the multicopy nature of ITS regions, polymorphic sites, detectable as double peaks in electropherograms, reveal the occurrence of more than one ITS different ribotype in a single genome. In fact, double peaks are usually considered as an indication of hybridization (e.g., Rauscher *et al.* 2002; Garcia *et al.* 2011) and this can be safely inferred in *Armeria* based on comprehensive knowledge of ITS variation (Fuertes Aguilar *et al.* 1999a; Fuertes Aguilar & Nieto Feliner 2003; Piñeiro *et al.* 2011) including cloned sequences (Nieto Feliner *et al.* 2004). Different ITS sequences present in an individual following hybridization tend to be

homogenized along a process that is not instantaneous (e.g., Xu *et al.* 2017) and thus in recently introgressed populations we would expect to find different stages of homogenization represented by different amounts of copies of the progenitors' ITS repeats. Because of the likely complex structure derived from introgression, in this work we have not quantified the relative amounts of each of the ITS repeat sequences co-existing intragenomically based on the relative heights of the peaks in the electropherograms as we did in Fuertes Aguilar *et al.* (1999b). Instead, we have only recorded if there was polymorphism or a fixed nucleotide for each of the two informative ITS sites (Piñeiro 2011) that differ consistently between Camarinal and the closest *A. pungens* population from Cape Trafalgar. Accordingly, double peaks in the variable nucleotide sites are represented here with IUPAC symbols and may correspond to a range of relative amounts of each of the underlying ITS repeats. For the plastid sequences, due to the non-recombinant nature and maternal inheritance, we have recorded variation as haplotypes.

To explore if intragenomic polymorphisms for the ribosomal sequences (ITS and IGS) could be slowed down by the number and different genomic location of ribosomal loci, we determined the number of ribosomal loci, both 45S and 5S rDNA, through FISH analysis following the procedures described in Rosato *et al.* (2016). We analyzed seven samples of *A. macrophylla* from Camarinal, seven samples of *A. pungens* from Cape Trafalgar, as representative of the non-introgressed populations, and one from distantly related and widespread *A. maritima* Willd. (Table S2).

IGS amplification, cloning and sequencing. We amplified the IGS regions with the universal primers 25SF and 18SR following Galián *et al.* (2014). PCR was performed using the TaKaRa LA Taq polymerase (Takara Biotechnology Inc.), specially designed to amplify long DNA fragments. PCR procedures are also described in Galián *et al.* (2014). We typically obtained two PCR products of about 4.7 and 6.0 kb. Products were excised from gel and purified using the UV-Free gel purification kit (Invitrogen, Paisley, UK) and subsequently cloned using the PCR XL-

TOPO (TA Cloning kit, Invitrogen). After cloning, plasmids were extracted and purified using the REAL Miniprep Turbo Kit (REAL Durviz, SL, Valencia Spain), and sequenced by an ABI 3100 Genetic Analyser at the Centres Científics i Tecnològics (Universitat de Barcelona); the first sequencing was initially done with M13F and M13R vector-specific primers, and we later proceeded to a primer-walking strategy with internal primers. We analyzed sequences using BioEdit (to organize and align the sequences), standard nucleotide BLAST (GenBank) to identify and locate the 26S and 18S rRNA gene termini in our sequences, and MAFFT to improve the alignments. The YASS tool was utilized to produce self-comparison of DNA sequences and detect repetitive regions visually. The Primer3Plus online program was used to design convenient primers for primer walking. In order to identify regulatory sequences, identify repetitive regions, CpG islands and GC% content, we used several tools from EMBOSS (EMBL-EBI site) as well as the program Geneious (Invitrogen), which was also used to depict the IGS structures. The CENSOR software (GIRI repbase) was used to mask sequence portions homologous to transposable elements.

Genome size assessments. To explore patterns of GS variation and assess how they have been affected by introgression, we sampled 118 individuals: 47 of *A. macrophylla* from Camarinal (hereafter *A. macrophylla* CAM), 44 of *A. pungens* from Camarinal (*A. pungens* CAM), and 27 of *A. pungens* from Cape Trafalgar (*A. pungens* TRA). The subpopulation from the shoreline was pooled with that of the consolidated terrain once it was found that there are no significant differences in GS between the two (results not shown) and both exhibit a *pungens* phenotype; we thus consider a single group including all *A. pungens* plants from Camarinal. Fresh leaf samples were collected from 98 individuals grown in the greenhouse from seeds collected in the field in 2014 and also from 20 individuals directly in the field (2016). To explore the inheritance of GS and facilitate the interpretation of GS variation detected in wild plants, 36 synthetic hybrids were obtained in the greenhouse by crossing plants from the three above groups. Specifically, synthetic crosses were obtained from crosses between *A. macrophylla*

CAM and *A. pungens* CAM (14), *A. pungens* CAM and *A. pungens* TRA (9), *A. macrophylla* CAM and *A. pungens* TRA (13), using both progenitors as pollen and ovule donors.

Genome size was assessed by flow cytometry. *Petunia hybrida* Vilm. 'PxPc6' (2C = 2.85 pg, Marie & Brown, 1993) was used as internal standard. The leaf tissue of three samples per individual was chopped up using a razor blade with the leaf tissue of the internal standard in 1000 µl of LB01 isolation buffer supplemented with 8% Triton (Doležel *et al.* 1989) and 5 µl of 100 mg/ml of ribonuclease A (RNase A, Boehringer). Each sample was measured twice. The nuclei suspension was stained with 40 µl of propidium iodide (1 mg/ml) (Sigma-Aldrich Química, SA, Madrid, Spain), kept on ice for 5 min and measured in a CyAn ADP cytometer (BeckMan-Coulter Life Sciences). Measurements were made at the Centres Científics i Tecnològics (Universitat de Barcelona). The instrument was set up with the standard configuration: excitation of the sample was carried out using a 488-nm laser. Forward scatter, side scatter, and red (613/20 nm) fluorescence for propidium iodide were acquired. The total nuclear DNA content (2C) was calculated by multiplying the known DNA content of the standard by the quotient between the peak positions (mode) of the target species and the standard in the histogram of fluorescence intensities. This is performed by assuming that there is a linear correlation between the fluorescent signals from the stained nuclei of the unknown specimen, the known internal standard, and the DNA amount (Doležel 1991). A minimum of 8000 particles were measured in each run.

RESULTS

Morphometrics. The first three principal components extracted in the PCA of the first dataset explained 56.83% of the overall variance whereas the first two PCs account for 33.42% and 13.54%, respectively (Table S1). Variables that contributed most to the first PC were the lengths of scape and calyx limb as well as the scape diameter and the width of the bracts

scarious margin whereas calyx tube length contributed most to the second PC. The scatterplot of the 87 samples against the first two PC axes (Fig. 1A) produced a quite consistent separation with respect to PC1, with *A. pungens* phenotypes occupying the space on the negative values and those of *A. macrophylla* on the positive values for this axis. To further summarize morphometric information we separated specimens by their scores for the PC1: values < 0 depicting an overall *A. pungens* morphology (black circles in Fig. 1) and values > 0 depicting overall *A. macrophylla* morphology (white circles in Fig. 1). Exceptions involve four samples, three of which are from Punta Camarinal and one from Cape Trafalgar. The samples from the newly discovered *A. pungens* coastline subpopulation are intermingled with those of the previously known subpopulation from the open habitat. Mapping the samples on the habitats of the study site shows that individuals exhibiting *A. pungens* phenotype occur in open vegetation —either on the sands by the shoreline or on the sandy consolidated terrain— whereas those with *A. macrophylla* phenotype occur in the understory either of the machia vegetation or the pinewoods (Fig. 1B). In the PCA of the dataset including a representation of all *A. pungens* throughout its range, the first three PCs account for 52.16% of the overall variance. The first two PCs account for 22.24% and 19.21% of the variance, respectively. The higher contributions on the first PC are by lengths of scape and calyx limb, as in the other analysis, but also by lengths of external involucre bracts and calyx lobe. For the second PC, involucre diameter provides the higher loading. The scatter diagram against the first two PCs shows that the overall separation between the two species found in the first analysis holds, but the closest specimens between them are the *A. pungens* specimens from Camarinal (Fig. S1).

ITS and trnL-trnF sequences. In accordance with our previous study, for the ITS sequences, two positions were found to be polymorphic in Camarinal with respect to other populations of *A. pungens*. Three state combinations were found based on the two polymorphic positions. The C,T combination was only detected in six individuals all from

Camarinal, five showing *A. pungens* morphology and habitat, and one showing *A. macrophylla* morphology (Table 1; blue rectangles in Fig. 2). This combination was not found amongst sequences from almost 300 samples from the whole genus. The T,C combination was detected in 13 individuals, all out of Camarinal, four of them in *A. macrophylla* and the rest in *A. pungens* (Table 1; yellow rectangles in Fig. 2). The Y,Y combination, revealing intragenomic co-occurrence of the two above ITS repeats, was confined to Camarinal, where it was detected in 27 individuals of the 33 sampled from there, 14 showing *A. pungens* phenotype and 13 *A. macrophylla* (Table 1; magenta rectangles in Fig. 2). Among the available ITS sequences from throughout the genus, except for Camarinal we have only found this combination in a sample of *A. hirta* Willd. from Cádiz (AJ225572).

In the *trnL-trnF* spacer, three polymorphic positions were identified that varied concertedly so that only two haplotypes could be recognized (Piñeiro *et al.* 2011). One ('Camarinal haplotype'; G, G, T) was found in 30 samples, 29 of them in Camarinal (17 in *A. pungens* phenotypes and 12 in *A. macrophylla* phenotypes) and one in a *A. macrophylla* sample from the closest sampled population of this species, 25 km west, at Caños de Meca (Table 1; green rectangles in Fig. 2). The other haplotype ('Gulf of Cádiz haplotype'; T, C, G) was found in 13 samples, all outside Punta Camarinal (Table 1; brown rectangles in Fig. 2).

The FISH study revealed a consistent number and position of ribosomal loci in *A. macrophylla* from Camarinal and *A. pungens* from Cape Trafalgar: two subterminal 45S loci and three 5S rDNA loci, two in interstitial and one in subterminal positions (Fig. S2). The two types of ribosomal loci were located in different chromosome pairs. The sample of *A. maritima* showed the same pattern although lacking the subterminal 5S locus (not shown).

IGS sequences. After an initial screening of the two IGS variants (present in all populations), only the longer ones were subsequently cloned and sequenced, since a preliminary sequencing of some of the shorter products showed that they lacked important regulatory regions and likely represented pseudogenized versions of the functional IGSs. Of

each of the groups, four, three and two clones were partially to fully sequenced corresponding to *A. macrophylla* CAM, *A. pungens* CAM and *A. pungens* TRA, respectively. Because some of the clones were only partially sequenced due to the difficulty of direct sequencing repetitive regions, consensus sequences for each group were constructed for subsequent analyses (GenBank numbers MK560848, MK560849, MK560850). At the 5' and 3' ends, the selected clones were very similar to the 26S and 18S coding regions, according to searches by BLAST (GenBank). After removing the coding regions, the remaining consensus sequences were 4947 (*A. macrophylla* CAM), 4890 (*A. pungens* CAM) and 4952 (*A. pungens* TRA) bp long. The G+C content was 51.3% (*A. macrophylla* CAM), 51% (*A. pungens* CAM) and 50.7% (*A. pungens* TRA). The analysis of sequences found the highest percent of similarities between *A. macrophylla* CAM and *A. pungens* CAM (90.05% and 90.64%, respectively) whereas the other two comparisons gave similar results: *A. pungens* CAM vs. *A. pungens* TRA (71.30% and 72.38% respectively) and *A. macrophylla* CAM vs. *A. pungens* TRA (72% and 72.87%, respectively). Complete results on the analysis of similarity are shown in Supplementary Appendix 1. The dotplots generated by the genomic similarity search tool for each IGS sequence revealed that most of the sequence is composed by repetitive regions in the three groups, and that the IGS patterns of *A. macrophylla* CAM and *A. pungens* CAM are more similar between them than any of the two with respect to *A. pungens* TRA (Fig. S3). The repetitive part accounts for 33.37% in *A. macrophylla* CAM, 42.37% in *A. pungens* CAM and 34.28% in *A. pungens* TRA. A display of the IGS structure of the three taxa is shown in Figure S4, and a table listing the most abundant repeats for each taxon is presented in Supplementary Appendix 1. Conserved transcription termination sites, transcription initiation sites and other regulatory motives were found in all three consensus sequences. In addition, several regions between 41-349 bp with 68.7-83.7% similarity with transposable elements were found in the three groups (Supplementary Appendix 1). Overall, the differences between the IGS of the three taxa were mainly structural, due to the presence, absence or variation of certain repeats.

Genome size. Genome size estimations (Supplementary Appendix 2) differed significantly among the three groups sampled (Kruskal-Wallis chi-squared= 56.207, df = 5, p <0.0001). *Armeria macrophylla* CAM showed the highest average (8.87 ± 0.5 pg) followed by *A. pungens* CAM (8.63 ± 0.39 pg) and *A. pungens* TRA (7.99 ± 0.47 pg) (Fig. 3). For synthetic hybrids, the GS averages were between those of their respective progenitors: *A. macrophylla* CAM x *A. pungens* CAM (8.69 ± 0.30 pg), *A. pungens* CAM x *A. pungens* TRA (8.28 ± 0.29 pg) and *A. macrophylla* CAM x *A. pungens* TRA (8.61 ± 0.33 pg). With regards to the ranges of variation, with two exceptions the absolute ranges of variation of GS in each of the three synthetic hybrid classes were within the values defined by the 50% quartiles of their progenitors (Fig. 3). No significant differences were found in the GS of synthetic hybrids from reciprocal crosses between the same progenitors.

DISCUSSION

Evidence for introgression

Our study is consistent with previous data (Piñeiro *et al.* 2011) in concluding that molecular and morphological peculiarities of the southernmost population of *Armeria pungens* with respect to the remainder of the species are due to introgressive hybridization with *A. macrophylla*. The newly discovered subpopulation in the same site, Camarinal, also fits the introgression scenario since despite exhibiting a morphological phenotype and habitat of *A. pungens*, it shows similar molecular signatures of introgression to the remainder *A. pungens* individuals from Camarinal.

Morphometric results depict an overall separation of *A. pungens* and *A. macrophylla* in the study site and a similar behavior of the two *A. pungens* subpopulations (Fig. 1). However, in the morphometric analysis of the first dataset the samples from each species do not form compact clusters and the picture is compatible with different degrees and/or direction of

introgression affecting morphometric characters; four samples even showing scores that we consider characteristic of the alternative species in the PC1 (Fig. 1). Interestingly, in the PCA that includes a representation of all *A. pungens* populations from throughout its range, the Camarinal samples of this species bridge the point clouds from *A. pungens* and *A. macrophylla* (Fig. S1). Since selected morphometric characters have proved to distinguish among different synthetic early hybrid generations, this phenotypic pattern supports introgression into *A. pungens* (Nieto Feliner et al. 1996).

Although ITS sequences have not been cloned, based on the knowledge of ITS variation in the genus *Armeria* throughout its range, we can assume that the ribotype from Camarinal (showing C,T in the variable nucleotide positions, Fig. 2) is exclusive to this site. The other ribotype (T,C), causing the polymorphic pattern (Y,Y) when coexisting intragenomically with the Camarinal ribotype, is prevailing in *A. pungens* populations throughout the southwestern Atlantic coasts of Iberia up to Cape S. Vicente (Fig. 2; Piñeiro et al. 2011). The finding of just six samples showing the Camarinal ribotype compared to 27 containing the two ribotypes (Y,Y) out of 33 (81.8 % of the samples of this site) is also consistent with the scenario of introgression. The finding that 13 of those samples are from *A. macrophylla* suggests that introgression is not unidirectional and it has occurred into this species too. But since morphometric analysis does not include samples of *A. macrophylla* from other populations we lack morphometric support for the reciprocal direction of introgression.

The fact that all samples sequenced from Camarinal from both species exhibit the same *trnL-trnF* haplotype (Fig. 2) not only suggests introgression but the occurrence of plastid capture (Rieseberg et al. 1995; Tsitrone et al. 2003). The combined findings of two very divergent haplotypes, one occurring in a close location and the other in a single site in two species (Camarinal) makes introgression and capture of *A. macrophylla* plastid genome by *A. pungens* much more likely than incomplete lineage sorting.

Our genome size estimates are also consistent with an introgression scenario. The average GS in *A. pungens* CAM (8.63 pg), intermediate between that in *A. macrophylla* CAM (8.88 pg) and *A. pungens* TRA (7.99 pg) but closer to the former, is likely the result of introgression of *A. macrophylla* CAM into *A. pungens* CAM. Immediately after hybridization, GS is usually intermediate between the parental species (in homoploid hybrids). Over time GS tends to undergo changes that range from downsizing in polyploid hybrids to even upsizing in the longer term, both in polyploid and homoploid hybrids (Baack *et al.* 2005; Leitch *et al.* 2008). The intermediate values obtained when estimating GS in synthetic hybrids, compared to their progenitors, also lend support to this interpretation by showing that this parameter is heritable in early hybrid generations. Given the minor differences between GS of natural and equivalent artificial hybrids, we hypothesize that the introgression process is recent. Homoploid hybrid species in *Helianthus* have 50% more nuclear DNA than parental species but first to sixth-generation synthetic hybrids did not show such increase (Baack *et al.* 2005) and synthetic hybrids in *Artemisia* show values close to the expected averages of GS from parentals (Garnatje *et al.* 2006).

Based on the functional elements found in the three IGS consensus sequences, including transcription initiation and termination sites, regulatory and repetitive regions, the IGS structures here reported are comparable with the IGS patterns found in other angiosperms such as *Solanum* (Komarova *et al.* 2008, genbank accession number: AY366528), *Vicia sativa* (Macas *et al.* 2003, AY234366) and *Artemisia absinthium* (Garcia *et al.* 2009, EU649668), among others, and therefore likely functional. Because IGS sequences were initially intended to explore transposable elements activity, the results from IGS sequencing require some caveats when used as evidence for the main focus of this paper. In multicopy regions orthology is a complex issue (Doyle & Davis 1998) and thus the degree to which those IGS sequences may reflect individual genome differences associated to introgression is uncertain. What we can report is that the relationships among IGS sequences from *A. macrophylla* CAM, *A. pungens*

CAM and *A. pungens* TRA is largely consistent with introgression (Fig. S3, S4, Suppl. Appendix 1). The sequence similarity values, as well as the internal repeat structure of the IGS point to a much closer relationship between *A. macrophylla* CAM and *A. pungens* CAM than with *A. pungens* TRA. Among the traces of transposable elements found in the three sequences, one fragment similar to the LTR transposon Sabrina is also shared between the former two, while the latter contains a relatively large fragment (349 bp) similar to the DNA transposon EnSpm (CACTA), absent in the other two. It is well known that the IGS region is prone to insertion of transposable elements and length variation of this sequence can be caused by activity of these repetitive elements (Chester *et al.* 2010). The comparison of the IGS features in the three taxa point to a likely gene conversion process to the rDNA type of *A. macrophylla* CAM in *A. pungens* CAM, although data from more clones would be needed to confirm that point.

An alternative to introgression would be convergence or incomplete lineage sorting. However, considering all the evidence, these possibilities are not the most parsimonious explanation. Specifically, convergence of morphological characters that would result in *A. macrophylla* population in Camarinal being phenotypically close to *A. pungens* is unlikely because more than half of the characters contributing to PC1 —and thus distinguishing the two species— involve reproductive organs (Table S1). It would be unlikely that quantitative characters such as proportions of different parts of the calyx as well as size and arrangement of involucral bracts would suffer environmental pressures as habit and leaf size probably do and/or vary concertedly with them as a result of convergence. For the same reason phenotypic plasticity is improbable. Incomplete lineage sorting (ILS) affecting molecular markers is likewise unlikely compared to the introgressive hybridization scenario. Plastid haplotypes were as much as 19 mutational steps away when three regions were considered (Piñeiro *et al.* 2011). As to the ribosomal regions (ITS, IGS), the sharing of the same ribotype by *A. macrophylla* and *A. pungens* from Camarinal could in principle be due to ILS. However, the confluence of facts such as that *A. pungens* is sympatric with *A. macrophylla* in Camarinal and that they also share the

plastid haplotype in the same site make the possibility of ILS almost negligible. A stochastic phenomenon should not concomitantly affect markers with different inheritance. In addition, the GS pattern, being a reflection of the whole genome, cannot be explained either by a process like ILS that should affect loci randomly and not have whole genome effects.

Considering all the evidence gathered here, one of the most interesting patterns observed is the association between morphology, and thus species adscription, and vegetation cover. This contrasts with the fact that molecular patterns of variation do not seem to be tightly associated to morphology, particularly in the newly discovered subpopulation. A non-homogenous introgression across the whole genome may explain this pattern. There is ample evidence for the porosity of the genomes (e.g., Baack & Rieseberg 2007; Minder & Widmer 2008) where some genomic regions are more permeable than others to introgression and this is compatible with maintaining species integrity (Gailing and Curtu, 2014). However, selection for backcrossed phenotypes and against intermediate phenotypes and hybrid generations with no introgression (F_1 , F_2 , F_3) could also play a role. For our case study, the possibility of underlying adaptive factors needs to be addressed in future studies.

Historical scenario

Although the conclusion that introgression is behind the patterns observed in Camarinal is straightforward, inferring a detailed historical scenario is more complicated. Yet, reconstructing the origin of a recently introgressed population could help understand how groups with low reproductive barriers, e.g. syngameons, face early stages of population contact. A first hypothesis is that a strictly shoreline population of *A. pungens* pre-existing in the site came in contact with *A. macrophylla* from the pine woods and machia shrubland existing landwards. Human activities may have fostered or at least intensified such a contact since, as can be observed in available aerial orthophotos (Instituto Geográfico Nacional;

<https://fototeca.cnig.es/>), the *Pinus pinea* woods currently existing in the area was planted in the 1960s on terrains that previously consisted of open vegetation. Currently, the pinewoods extend from the inner part of the Camarinal promontory almost to the shoreline (Fig. 1) and thus the possibilities for hybridization between the two species —pollinated by generalist pollinators like other *Armeria* species (Herrera, 1988; García-Camacho et al., 2009)— were greatly facilitated for the last three to four decades. Hybridization and subsequent introgression could have led to plastid capture extirpating the original plastid DNA of the coastal *A. pungens* population. Plastid capture requires repeated backcrossing towards hybrids bearing the same plastid genome, i.e., *A. macrophylla* plastid genome (Rieseberg et al. 1995; Tsitrone et al., 2003).

In line with this hypothesis, Piñeiro *et al.* (2011) suggested that introgression in this peripheral population could have facilitated extending its niche (Abbott *et al.* 2003; Ellstrand 2003), colonizing consolidated terrain although still in open vegetation. This would imply that introgression was adaptive. The newly discovered shoreline subpopulation of *A. pungens*, with molecular signatures of introgression but occurring on typical sand dune and beach environments apparently did not involve a new niche and the adaptive nature of introgression, if any, should be based on other aspects that we have not detected in this study and thus require further research.

An alternative hypothesis, also pointed out in Piñeiro *et al.* (2011), is that 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 & Excoffier, 2009). This hypothesis would fit well with the arrival of a single or a few *A. pungens* individuals to the site. As most species in this genus (Baker 1966; Nieto Feliner *et al.* 1996), *A. pungens* is self-incompatible, so it would tend to produce offspring through crossings with congeners, in this case *A. macrophylla*. This would be consistent with simulation studies by Currat *et al.* (2008) predicting that introgression of neutral genes during an invasion

occurs from the local to the invading species. This alternative hypothesis involves long-distance dispersal for which there is evidence based on two disjunct occurrences of *A. pungens* in islands. There is also circumstantial evidence of the establishment limitations of one *A. pungens* individual following long-distance into Eastern Spain, which resulted in its ephemeral existence due to lack of congeners (López Espinosa *et al.* 2009). That six *A. pungens* samples from Camarinal show the fixed *A. macrophylla* pattern (C,T) is consistent with introgression into *A. pungens* (Table 1, Fig. 2). However, not all the ITS patterns are fully understood. Despite suggesting that *A. macrophylla* has also been introgressed, the occurrence of the polymorphic condition (Y,Y) in 13 out of 14 samples from Camarinal might have been influenced also by other factors such as the differential tempos for nuclear genome and molecular forces driving ITS sequence homogenization.

Concluding remarks

We report evidence from various sources that introgression has taken place at Punta Camarinal, particularly into *A. pungens*, a finding that is consistent with preliminary unpublished genotyping-by-sequencing (GBS) data. However, in accordance with the diversity of outcomes and complexity of patterns that may result from hybridization and introgression (Abbott *et al.* 2013), details of the origin, determinant factors and fate of introgressed populations are not fully understood.

Features that will be addressed in the future include the direction and degree of introgression. Although asymmetrical introgression is frequent in other plant groups due to cyto-nuclear incompatibilities, habitat selection and differential abundance of the two progenitors (Burgess *et al.* 2005; Arnold *et al.* 2010; Scascitelli *et al.* 2010), asymmetry is usually referred more to the differential amount of genes transferred from the hybrids to one of the parents than to the

nonexistence of backcrossing towards one of them (Brubaker *et al.* 1993; Tagane *et al.* 2008; Arnold *et al.* 2010). In Camarinal, introgression into *A. macrophylla* is yet poorly understood. Interactions with the habitat are another relevant feature for maintenance of introgressed scenarios (Arnold 1997). The association between phenotype and habitat rather than with molecular data (Fig. 1) is not rare. In Corsica, *Cyclamen balearicum* Willk. phenotypes introgressed by *C. repandum* Sm. show differences in substrate and tree canopy although sharing climatic conditions with *C. repandum* (Debussche & Thompson 2003). In Camarinal, selection for backcrossed phenotypes that are close to *A. pungens* in open habitats is one possible explanation, which is compatible with demographic factors if a few immigrants of *A. pungens* were the origin of the contact (Currat *et al.* 2008). But other crucial features for understanding the introgressed population include more intrinsic genetic factors such as genetic incompatibilities and porosity of genomes.

To better understand this system we have started a project based on genomic (GBS) data aiming to determine the degree and direction of introgression, confirm plastid capture, explore the association between genotypes and environment searching for specific selective pressures, and reconstructing demographic historical scenarios.

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792

Tables:

Table 1.- Sequence evidence used in analyzing introgression in *Armeria pungens* at Punta Camarinal (Cádiz, Southern Spain)

Figure legends:

Fig. 1.-Morphometric analysis of *Armeria* in Punta Camarinal, Cádiz, Spain. A) Principal component analysis of 87 samples: 75 samples from four habitats of the Camarinal and 12 from the closest population of *A. pungens* (Cape Trafalgar, TRA). B) Distribution across habitats of the overall *A. pungens* and *A. macrophylla* phenotypes represented by negative and positive scores, respectively, for the first PCA axis in A. The dashed line marks a newly discovered subpopulation of *A. pungens*.

Fig. 2.-Nuclear ribosomal DNA (ITS) and plastid DNA (*trnL-trnF*) sequence variation in *Armeria* in Punta Camarinal, Cádiz, Spain (below) and other populations in the region. Two variable nucleotide sites in the ITS region occur either as fixed bases (C, T— T, C) or as polymorphisms (Y, Y), which reveal the existence of more than one ITS copy. Two plastid haplotypes are recognized. Phenotypes of the samples from Camarinal are labeled according to the scores for the PC1 in Fig. 1. Identification of the samples from other populations is uncontroversial. The samples on the western part of the site along the sea shore have been discovered recently.

Fig. 3.-Genome size variation in *Armeria* at Punta Camarinal (Cádiz, Spain). Boxplots for six categories: *A. macrophylla* and *A. pungens* from Camarinal, *A. pungens* from nearest populations (Cape Trafalgar) and synthetic hybrids obtained by crossing plants from the first three categories.

Supplementary material

Table S1.- Principal Components Analysis of 87 samples of *Armeria* from southern Spain based on 16 morphometric variables. Loadings of these variables of the first three PCs and % of variance explained.

Table S2.- Number of ribosomal 45S and 5S loci assessed by FISH. Pattern of chromosomes bearing 5S rDNA sites according to the chromosome position. I: Interstitial. St: subterminal. n: sample size. n.a: not available.

Fig. S1.- Morphometric analysis of *Armeria* from Punta Camarinal, Cádiz, Spain (75 samples) and a representation of *A. pungens* from throughout its range (211).

Fig. S2.- Karyological 45S and 5S rDNA site phenotypes in *Armeria* assessed by FISH analysis. 45S rDNA sites are shown as green fluorescent signals and 5S rDNA are shown as red fluorescence sites: A) Types of chromosomes landmarks revealed by the presence of ribosomal loci, B) *A. macrophylla*, C) *A. pungens*. One chromosome bearing the 45S rDNA site was missed. Scale bar: 10 μ m.

Fig. S3.- Internal structure of the IGSs. Green lines represent repeats in the forward direction and red lines represent repeats in the reverse direction. Dotplots obtained by the YASS genomic similarity search tool (<http://bioinfo.lifl.fr/yass/yass.php>)

Fig. S4.- Structure of the IGS of the three taxa. Orange icons represent repeat and subrepeat internal structure. Transcription Initiation Sites (TIS) and some pyrimidine rich regions indicated in green, and Transcription Termination Sites (TTS) indicated in fuchsia.

Supplementary Appendix 1.- Analysis of identity and similarity of nr DNA IGS sequences.

Supplementary Appendix 2.- Genome size estimates of 154 samples of *Armeria*

TABLE. 1

Sequence evidence used in analyzing introgression in *Armeria pungens* at Punta Camarinal (Cádiz, Southern Spain)

	Collection code	ITS (GenBank Acc. no.)	ITS pattern	<i>trn</i> L- <i>trn</i> F (GenBank Acc. no.)	<i>trn</i> L- <i>trn</i> F pattern
<i>A. macrophylla</i> (Camarinal site, pinewoods)	4669GN	GQ901336.1	Y, Y	GQ901336.1	G, G, T(Cam)
	4670GN	GQ901337.1	Y, Y	GQ901191.1	G, G, T(Cam)
	4671GN	GQ901338.1	Y, Y	GQ901192.1	G, G, T(Cam)
	4701GN	MK547326	C, T		
	4725GN #1	MK547333	Y, Y	MK559072	G, G, T(Cam)
	4725GN #2 (s/n)	MK547334	Y, Y	MK559073	G, G, T(Cam)
	4725GN #3	MK547335	Y, Y	MK559074	G, G, T(Cam)
	4725GN #4	MK547336	Y, Y	MK559075	G, G, T(Cam)
<i>A. macrophylla</i> (Camarinal site, macchia shrubland)	4724GN s/n	MK547327	Y, Y		
	4724GN #1	MK547328	Y, Y	MK559067	G, G, T(Cam)
	4724GN #2	MK547329	Y, Y	MK559068	G, G, T(Cam)
	4724GN #3	MK547330	Y, Y	MK559069	G, G, T(Cam)
	4724GN #4	MK547331	Y, Y	MK559070	G, G, T(Cam)
	4724GN #5	MK547332	Y, Y	MK559071	G, G, T(Cam)
<i>A. macrophylla</i> (Caños de Meca, Cadiz)	3870GN	AY179793.1	T, C*	GQ901189.1	G, G, T(Cam)
	3871GN	AY179792.1	T, C*		
	3872GN	AY179791.1	T, C*		
<i>A. macrophylla</i> (Chiclana, Cadiz)	3876GN			GQ901187.1	T - C - G (Port)
	3877GN	AY179790.1	T, C*	GQ901188.1	T - C - G (Port)
	3878GN			GQ901186.1	T - C - G (Port)
<i>A. pungens</i> (Camarinal site: open terrain in the southern part)	4362GN	GQ901376.1	Y, Y	GQ901376.1	G, G, T(Cam)
	4363GN	GQ901378.1	Y, Y	GQ901278.1	G, G, T(Cam)
	4364GN			GQ901279.1	G, G, T(Cam)
	4365GN	GQ901380.1	C, T	GQ901280.1	G, G, T(Cam)
	4373GN	GQ901379.1	C, T	GQ901281.1	G, G, T(Cam)

	4374GN	GQ901377.1	Y, Y	GQ901377.1	G, G, T(Cam)
	4702GN	MK547337	Y, Y		
<i>A. pungens</i> (Camarinal site: sand beach and open terrain in the western part)	4723GN s/n	MK547339	Y, Y		
	4723GN#1	MK547340	Y, Y		
	4723GN#2	MK547341	Y, Y	MK559076	G, G, T(Cam)
	4723GN#3	MK547342	C, T	MK559077	G, G, T(Cam)
	4723GN#4	MK547343	Y, Y	MK559078	G, G, T(Cam)
	4723GN#5	MK547344	Y, Y	MK559079	G, G, T(Cam)
	4723GN#6	MK547345	C, T	MK559080	G, G, T(Cam)
	4723GN#7	MK547346	Y, Y	MK559081	G, G, T(Cam)
	4723GN#8	MK547347	Y, Y	MK559082	G, G, T(Cam)
	4723GN#9	MK547348	Y, Y	MK559083	G, G, T(Cam)
	4723GN#10	MK547349	Y, Y	MK559084	G, G, T(Cam)
	4723GN#11	MK547350	C, T	MK559085	G, G, T(Cam)
	4723GN#12	MK547351	Y, Y	MK559086	G, G, T(Cam)
<i>A. pungens</i> (cape Trafalgar)	4379GN	GQ901374.1	T, C*	GQ901272.1	T, C, G (Cadiz Gulf)
	4380GN	GQ901373.1	T, C*	GQ901273.1	T, C, G (Cadiz Gulf)
	4381GN	GQ901375.1	T, C*	GQ901274.1	T, C, G (Cadiz Gulf)
	4382GN			GQ901275.1	T, C, G (Cadiz Gulf)
	4384GN			GQ901276.1	T, C, G (Cadiz Gulf)
	4703GN #2	MK547338	T, C		
<i>A. pungens</i> (Gulf of Cadiz)	4394GN	GQ901367.1	T, C	GQ901263.1	T, C, G (Cadiz Gulf)
	4406GN	GQ901361.1	T, C	GQ901257.1	T, C, G (Cadiz Gulf)
	4421GN	GQ901359.1	T, C	GQ901252.1	T, C, G (Cadiz Gulf)
	4590GN	GQ901371.1	T, C	GQ901267.1	T, C, G (Cadiz Gulf)
<i>A. pungens</i> (Algarve)	4433GN	GQ901355.1	T, C	GQ901246.1	T, C, G (Cadiz Gulf)
No. of sequences		46 (26)		43 (20)	

New sequences in bold

* additional change in other nucleotides

Table S1

Principal Components Analysis of 87 samples of *Armeria* from southern Spain based on 16 morphometric variables. Loadings of these variables of the first three PCs and % of variance explained.

Morphometric variables	PC1	PC2	PC3
Leaf width (mm)	.714	.194	-.322
Ratio leaf length to leaf width	.478	-.431	.364
Scape length (cm)	.892	.068	-.214
Diameter of scape base (mm)	.746	.375	-.252
Diameter of the involucre (mm)	.431	.407	.097
Ratio involucre diameter to length internal involucre bract	-.378	-.227	.447
Number of involucre bracts	-.728	.079	-.058
Width of internal involucre bract	.293	.513	-.044
Length of external involucre bract	.647	-.253	-.286
Ratio length external involucre bract to internal involucre bract	.051	-.639	-.282
Internal involucre bracts: ratio of scarious margin (x2) to whole width	.737	-.301	-.106
Length of calyx spur	.014	.244	.138
Length of calyx tube	-.159	.761	.000
Length of calyx limb	.749	-.254	.005
Length of calyx lobule including awn	.660	.118	.639
Ratio calyx lobule + awn to calyx length	.571	.037	.670
% of variance	33.42	13.54	10.05
% of cumulative variance	33.42	46.77	56.83

Table S2

Number of ribosomal 45S and 5S loci assessed by FISH in *Armeria*. Pattern of chromosomes bearing 5S rDNA sites according to the chromosome position. I: Interstitial. St: Subterminal. n: sample size.

Species	Origin	Code	n	45S rDNA loci	5S rDNA loci	
					I	St
<i>A. macrophylla</i>	Cádiz, Camarinal site	4701GN	7	2	2	1
<i>A. pungens</i>	Cádiz, Cape Trafalgar	4703GN	7	2	2	1
<i>A. maritima</i>	unknown	-	1	2	2	-

A **B** **C**

A. macrophylla CAM

A. pungens CAM

A. pungens TRA

Supplementary Appendix 1

1. Data on sequence identity and similarity (tool Ident and Sim from the Sequence Manipulation Suit http://www.bioinformatics.org/sms2/ident_sim.html). Regions from 26S and 18S ribosomal RNA genes have been trimmed for the analysis)

Results for *A. macrophylla* CAM vs *A. pungens* CAM:

- Alignment length: 4966
- Identical residues: 4472
- Similar residues: 29
- Percent identity: 90.05
- Percent similarity: 90.64

Results for *A. macrophylla* CAM vs *A. pungens* TRA:

- Alignment length: 5410
- Identical residues: 3895
- Similar residues: 47
- Percent identity: 72.00
- Percent similarity: 72.87

Results for *A. pungens* CAM vs *A. pungens* TRA:

- Alignment length: 5366
- Identical residues: 3826
- Similar residues: 58
- Percent identity: 71.30
- Percent similarity: 72.38

2. Results of the Repbase/CENSOR tool regarding the search of homologous portions to transposable element fragments, in a given sequence (<https://www.girinst.org/censor/>). Regions from 26S and 18S ribosomal RNA genes have been trimmed for the analysis)

Dir: values in column Dir indicate orientation (D for direct and C for complementary

Sim: Column Sim contains value of similarity between two aligned fragment.

Name	From	To	Name	Class	Dir	Sim
<i>A. macrophylla</i> CAM	86	127	Sabrina3 TM LTR	LTR	C	0.8372
<i>A. macrophylla</i> CAM	693	786	LTR-2 Mad-1	LTR	D	0.7312
<i>A. macrophylla</i> CAM	1348	1434	Copia 10 ALY I	LTR/Copia	C	0.7558
<i>A. macrophylla</i> CAM	4658	4718	Caulimovirus 9 ATr	Integrated virus/Caulimovirus	C	0.7931
<i>A. pungens</i> CAM	86	127	Sabrina 3 TM LTR	LTR	C	0.8372
<i>A. pungens</i> TRA	2176	2525	EnSpm 14 CCri	DNA/EnSpm/CACTA	D	0.6869

3. Results of the Etandem (EMBOSS) tool to search for tandem repeats in a sequence (parameters: minimum repeat size: 2; threshold: 20)

***Armeria macrophylla* CAM**

Start	End	Strand	Score	Size	Count	Identity	Consensus
1258	1545	+	122	32	9	76.7	A
3693	4144	+	92	226	2	85.2	B
2692	2871	+	82	90	2	97.8	C
379	609	+	42	33	7	66.2	D
722	849	+	40	32	4	78.1	E
2571	2634	+	24	32	2	93.8	F
2168	2295	+	24	32	4	71.9	G
2387	2566	+	20	60	3	72.2	H

Consensus repeats:

- A. TGGCTTGTCTTGGGGTGGCATGGGCATTGGCA
- B. CTTTCGAAGTCTGTACGGATTGGCTTNTCTTTTGTGATCGTCGGCCCTCTTGGATTCCGCTGTAGCACAAATG
GTGTGGCTTGGTACTGGTATTTGGTGTGACTNCGCCATGATTTTGTGACCAAGGTATTTGCTCCTTGGCCA
TGCTCNTGGGTTGGCACGTCAATCTCCNCGCTNTGTTACGCCATGTTTTATGTTGTGGTCTTGCGGGGCTGT
NGTTCACGC
- C. CCAAGAGGTGGCATGGGCATTGGCATGCGTTGCCTTGCCTTGGTGTGGGCGTTGGCATGCCTTGCCTTGTG
TTGGCATGAGCTTGTCTTG
- D. GCGTGGGCATTGGCATGTCTTGCCTTGCTTTG
- E. TGCCTTGCCGTGTGTTGGCATGGGCGTTTGCA
- F. TTGGCATGTCTTGCCGGGTGTTGGTGTGGGCG
- G. ATGGCATGTCTTGCCTTGCTATGGGCATGGCC
- H. GGNCACTGTCTGNCTGCCCTTGACTTGGTATAGGCATGGCTAGTCATGAGGTGGCTTG

***Armeria pungens* CAM**

Start	End	Strand	Score	Size	Count	Identity	Consensus
3517	4191	+	88	225	3	73.2	A
2671	2850	+	82	90	2	97.8	B
1458	1697	+	80	120	2	91.7	C
310	702	+	60	131	3	74.3	D
2865	3096	+	52	116	2	86.2	E
715	842	+	42	32	4	78.9	F
2458	2617	+	32	32	5	70.0	G
205	268	+	24	32	2	93.8	H

- A. GTGTCTCTATAATTATTTGATGAAGATGGTTTGCTGCTCCTTGCCATGCTCTTGGGTGGCACGTCATTCTCC
GCGCTTTGTTACGCCATGTCTTTGTTGGTCTTGGGGGGCTGTTGTTACCCCTTTCGAAGTCTGTACGGAATTG
GCTTCTCTTGTGTGGATGTTGGCCCTCTGGATCTACCGTGAAACAACGGTGTGGCTTGGTTTGGGTTTTGGG
- B. CAAGAGGTGGCATGGGCATTGGCATGCGTTGCCTTGC GTTGGTGTGGGCGTTGGCATGCCTTGCCTTGTGTT
GGCATGAGCTTGTCTTG
- C. GTGTTACATGGCATGGACATGGCTAGTCTTGAGGTGGCATGGCAATTGGCATGGCTTGTCTAGGTATGGC
ATGGGCATTGTCACCTCTTGGCTTGTCTTGACATGGCTAAATTATGGG
- D. CATGGGCATTGACACCTCNTGGCTTGTCTTGACATGNCGTGGGCATGACTAGTCTTGAGGTGGCGTGGGC
ATNGGCATGTCTTTCCTTGTGTTGGNGTGGGNATTGAGACATGGCGGGCCTAGCTTNGGG
- E. TAGTAGTTGGGGTCCGATTTTGAAAATTCGAAATTTATGATTGTTTCATCCGTGTGAAGGGCCAGTGTTCACTA
CGAGAGTTCCTAAAAAGTTACATAGACTACTATAGGGGGGTAG
- F. TGCCTTGCCGTGTGTTGGTATGGGCGTTTGCA
- G. ATGTCTTGCCTTGAGTTGGTTTGGGCGTTGGC
- H. TGTGGGCGTTTCCATGACTCGCCTTGTGTTGG

***Armeria pungens* TRA**

Start	End	Strand	Score	Size	Count	Identity	Consensus
842	1285	+	82	148	3	75.9	A
2238	2519	+	81	141	2	89.4	B
2552	2785	+	49	117	2	85.5	C
563	786	+	46	32	7	67.4	D
3857	4306	+	33	225	2	78.7	E
1305	1368	+	24	32	2	93.8	F

- A. ATGGCATGGGCATTGGCATGGCTTGTCTTGC GTTGGCATGGGCGTTGGCATGCCTTGCCTTGTGTTGGCATG
GGCTGGCCTTTGTATGGCATGGTCCTTGGCACCTCTTGGCTTGCCCTTGACATGGCATGGACAGGGTAATCA
TGAG
- B. TGGCATGGGCGTTGGAAGCNCTTGGCTTGTCTTGACATGGCTATGGACATGGCTAGTCAAGAGGTGGCAT
GGGCATTGGCATGCGTTGCCTTGC GTTGGTGTGGGCGTTGGCATGCCTTGCCTTGTGTTGGCATGAGCTT
- C. GTCCGATTTTGAAAATTCGAAATTTATGATTGTTTCATCCGTGTGAAGGGCCAGTGTTCACTACGAGAGTTCCT
AAAAAGTTACATAGACTACTATAGGGGGGTAGGTATGTTGAAA
- D. CTTGCTCTGGCCTGGGCATTGGCATGTCTTGT
- E. TTACACCATGNNTTATGTTGNGGNCTTGGNGGGCTGTTGTTTCANACCTTTTGAAGTCTGNACCGGATTGGC
TTCTCTTNTGTGAATGTTAGACCTCTTAGATCTACCGTGAAACTACGGTGTGGCTTGGTTTGGATTGGCG
TGGCTTTCATGATTTTGTGACCAAGGTGTTTTGCTCCTTGACCATACTTTTTGAGATAGCAGGCGTTGCTCTT
GGTCTT

F. TGTCTTGCCGGGNGTTGGTGTGGGCGTTGGCA

	Average 2C	SD	HCPV standard	HCPV sample
<i>Armeria macrophylla</i> CAM (A)				
1	7.73	0.01	6.73	4.98
2	7.75	0.05	5.88	2.97
3	8.01	0.16	5.81	3.85
4	8.12	0.64	3.79	1.92
5	8.22	0.06	5.90	2.13
6	8.45	0.06	5.51	0.64
7	8.45	0.03	5.55	2.66
8	8.53	0.21	5.65	1.51
9	8.57	0.35	6.77	1.92
10	8.6	0.04	4.45	2.15
11	8.63	0.05	3.48	4.09
12	8.69	0.19	5.66	2.12
13	8.71	0.01	2.57	1.38
14	8.72	0.24	4.40	1.27
15	8.77	0.2	5.13	0.21
16	8.78	0.02	3.45	2.81
17	8.79	0.2	2.89	1.57
18	8.8	0.33	5.74	3.02
19	8.81	0.02	6.29	4.96
20	8.81	0.02	1.90	3.21
21	8.84	0	5.91	5.54
22	8.84	0.06	1.66	0.12
23	8.84	0.17	2.86	3.16
24	8.84	0.04	5.79	1.41
25	8.86	0.24	7.21	3.05
26	8.86	0.04	0.46	0.17
27	8.87	0.09	1.55	0.23
28	8.89	1.05	3.10	3.33
29	8.89	1.05	2.42	0.49
30	8.91	0.21	4.07	2.92
31	8.93	0.22	3.47	4.04
32	8.94	0.22	4.80	3.33
33	8.94	0.24	2.69	2.41
34	9	0.22	4.84	4.11
35	9.02	0.08	3.88	1.43
36	9.05	0.16	3.38	1.96
37	9.11	0.18	5.85	0.60
38	9.12	0.36	1.12	0.10
39	9.23	0	4.95	0.51
40	9.23	0.28	0.48	0.12
41	9.25	0	2.75	3.70
42	9.38	0.18	1.28	0.26
43	9.51	0.53	3.65	2.72
44	9.6	0.41	0.48	0.20
45	9.61	0.27	2.72	0.16
46	10.3	0.97	1.38	2.62
47	10.39	0.85	2.38	1.25

Armeria pungens CAM (B)				
1	7.96	0.25	1.40	0.21
2	8.15	0.08	6.59	2.40
3	8.18	0.14	5.61	4.13
4	8.22	0.61	2.88	0.59
5	8.23	0.41	7.40	1.39
6	8.23	0.01	4.05	3.63
7	8.23	0.01	7.17	1.16
8	8.30	0.19	4.17	3.34
9	8.33	0.08	4.18	4.11
10	8.35	0.03	3.41	0.17
11	8.35	0.06	7.68	1.94
12	8.37	0.09	6.07	2.66
13	8.40	0.10	6.06	3.14
14	8.40	0.78	4.53	1.24
15	8.42	0.01	3.69	3.11
16	8.44	0.25	7.44	0.51
17	8.45	0.31	1.59	1.94
18	8.46	0.13	6.36	0.82
19	8.52	0.18	6.75	1.02
20	8.53	0.42	2.13	1.21
21	8.54	0.15	3.89	1.85
22	8.54	0.09	0.50	0.13
23	8.56	0.07	3.70	4.42
24	8.61	0.52	6.03	3.06
25	8.62	0.38	2.58	0.67
26	8.62	0.42	4.11	3.46
27	8.64	0.03	7.11	2.22
28	8.66	0.04	3.73	3.83
29	8.67	0.49	4.05	2.90
30	8.70	0.23	3.23	0.73
31	8.78	0.02	4.61	3.28
32	8.78	0.07	2.72	0.74
33	8.79	0.48	7.33	2.39
34	8.79	0.15	4.14	1.61
35	8.80	0.10	6.92	0.98
36	8.82	0.33	4.44	0.46
37	8.94	0.09	5.12	0.68
38	9.00	0.86	3.22	0.52
39	9.18	0.05	5.83	1.87
40	9.23	0.15	1.12	0.20
41	9.31	0.09	6.45	0.39
42	9.34	0.24	5.78	1.45
43	9.49	0.46	5.28	0.24
44	9.86	0.18	0.63	3.12
Armeria pungens TRA (C)				
1	7.08	0.35	4.31	2.12
2	7.09	0.60	4.83	0.29
3	7.48	0.04	3.99	0.62
4	7.54	0.02	1.97	0.58

5	7.54	0.16	2.39	2.12
6	7.54	0.00	4.68	0.69
7	7.56	0.09	4.38	0.46
8	7.59	0.18	4.74	1.41
9	7.63	0.15	6.84	1.59
10	7.64	0.20	3.37	0.57
11	7.91	0.39	5.74	1.64
12	8.03	0.29	3.80	0.81
13	8.04	0.27	4.83	0.35
14	8.04	0.69	7.43	2.94
15	8.06	0.32	4.59	1.57
16	8.13	0.00	4.69	0.17
17	8.18	0.00	3.80	0.41
18	8.20	0.20	3.88	3.33
19	8.25	0.11	6.35	2.04
20	8.35	0.06	4.53	3.44
21	8.37	0.22	5.07	0.68
22	8.40	0.15	6.72	0.57
23	8.44	0.21	3.33	2.34
24	8.52	0.13	4.18	0.24
25	8.53	0.34	6.33	1.11
26	8.72	0.00	0.52	0.18
27	8.81	0.00	0.78	0.28
Synthetic hybrid A x B (F)				
1	7.88	0.04	6.05	0.36
2	8.28	0.12	4.86	0.27
3	8.54	0.22	4.61	1.04
4	8.55	0.23	2.43	0.42
5	8.69	0.10	4.62	0.23
6	8.71	0.00	4.50	0.41
7	8.79	0.02	6.12	0.33
8	8.81	0.00	4.69	0.11
9	8.81	0.23	5.78	0.15
10	8.85	0.58	1.95	0.30
11	8.86	0.03	2.92	0.26
12	8.94	0.01	4.02	0.22
13	8.94	0.31	2.99	0.19
14	8.99	0.24	6.49	0.40
Synthetic hybrid C X B (G)				
1	7.82	0.01	3.09	0.35
2	8.07	0.08	3.31	2.35
3	8.09	0.13	0.76	1.04
4	8.10	0.14	3.65	2.53
5	8.26	0.02	3.82	0.64
6	8.42	0.06	4.20	0.49
7	8.49	0.16	4.69	2.05
8	8.61	0.37	7.24	2.24
9	8.69	0.05	2.11	2.86
Synthetic hybrid A x C (H)				
1	7.96	0.25	3.02	0.72

2	8.13	0.20	0.56	1.96
3	8.20	0.40	3.31	0.55
4	8.47	0.24	4.07	1.60
5	8.55	0.25	1.30	0.22
6	8.66	0.30	1.82	0.22
7	8.72	0.45	6.85	0.36
8	8.73	0.15	1.48	0.51
9	8.79	0.38	3.66	0.55
10	8.81	0.07	4.26	1.54
11	8.89	0.31	0.83	0.39
12	8.90	0.27	1.28	0.67
13	9.09	0.28	5.86	0.68