Phylogenetic and phylogeographic analysis of the western Mediterranean *Arenaria* section *Plinthine* (Caryophyllaceae) based on nuclear, plastid, and morphological markers

Virginia Valcárcel[1], Pablo Vargas[2] & Gonzalo Nieto Feliner[2]

[1] *Pablo de Olavide University, Ctra. Utrera km 1, 41013 Sevilla, Spain. vvalnun@upo.es (author for correspondence)*
[2] *Botanical Garden of Madrid, Pza. Murillo nº 2, 28014 Madrid, Spain.*

Arenaria sect. *Plinthine* is an easily distinguishable morphologically cohesive group encompassing 14 species and five additional subspecies. To explore phylogenetic relationships and search for phylogeographic patterns in a group that is restricted to the western Mediterranean, independent and combined analyses of four datasets (morphological characters and sequences from nuclear and plastid DNA: nrITS, *trnT-trnL*, *trnL-trnF*) were conducted using Maximum Parsimony, Neighbour-Joining, Maximum Likelihood and Bayesian Inference. Monophyly of the section is supported by the data, the ITS sequences show full agreement with taxonomy for one third of the taxa, and the high number of *trnL-F* haplotypes found in SE Spain (7 of 11) is consistent with the fact that this region is one of the hotspots of biodiversity in the Mediterranean. However, a number of less easily interpretable results have been obtained, despite considerable ITS variation (43 parsimony informative characters within the ingroup) and a reasonable intraspecific sampling for this marker. These results are: low resolution at deep nodes of the ingroup, partial discordance between taxonomy and ITS sequences particularly for widespread taxa, and lack of congruence not only between the topologies of the plastid and nuclear trees but also between the two plastid trees. It is proposed that a combination of factors (rampant aneuploidy, polyploidy, hybridization, lineage sorting, and concerted evolution of ITS sequences) are responsible for the incongruent but not totally unexpected results.

KEYWORDS: *Arenaria*, Iberian diversity, incongruence, ITS, *trnL-F*, *trnT-L*.

INTRODUCTION

The use of molecular markers, particularly sequence data, in phylogeny reconstruction of plant groups above the genus level has proven successful in the last decade (Soltis & al., 1998, 2000). Expectedly, as we move down in the systematic hierarchy towards micro-evolutionary levels, conclusive and resolved phylogenies become rare. This is due to several interconnected reasons. Scarcity of markers with an appropriate level of variability accounts for part of limited phylogenetic signal. In many instances, lack of resolution stems from the low mutation rates in plastid DNA and from the limited number of reliable nuclear markers available to date (Small & al., 1998; Doyle & Gaut, 2000; but see Cronn & al., 2002). A second crucial reason is that since hybridization among species remains common at those lower systematic levels, distribution patterns of markers in species and geographic areas become more complex. This is just a consequence of hybridization interrupting divergence (i.e., reticulation) and thus the independent accumulation of exclusive markers along lineages. The implications of a significant presence of reticulate (tokogenetic, Hennig, 1966) relationships in phylogeny reconstruction, especially when using parsimony methods and morphological characters, have been already discussed (McDade, 1997). The third fundamental cause behind the scarcity of low-level well-resolved phylogenies is the discordance between gene trees and species trees (Doyle, 1997; Maddison, 1997). Its occurrence is apparent using all kinds of molecular markers (Rieseberg & Brouillet, 1994; Rosenberg, 2003), and particularly strong when other mechanisms are involved, such as the homogenization of multicopy genes like the nuclear ribosomal DNA (Sanderson & Doyle, 1992; Buckler & al., 1997). The alternative use of fingerprinting techniques when trying to reconstruct the evolutionary history of closely-related species is no wonder frequent (Purps & Kadereit, 1998; Soltis & al., 1998) but not devoid of problems (Wolfe & Liston, 1998).

As studies get closer to closely-related species, phylogeographic approaches become more appropriate (Avise, 2000). This is related to the possibility (and relevance) of revealing population-level historical shifts in distributions inferred from gene genealogies and not just synapomorphies characterizing monophyletic groups.

Phylogeographic approaches have proven to be also useful for the above-species level (Crandall, 1994) and, if successful, have the advantage of being specifically concerned with the geographic distribution of genetic variation. However, for groups differentiated in the Mediterranean Basin, phylogeographic approaches appear to convey intrinsic difficulties due to a number of historical factors (Hewitt, 1996). Unlike northern territories, the absence of severe climatic episodes, like the *tabula rasa* hypothesis, increases the number of alternative scenarios. The consequence of recurrent events is the accumulation of various shifts in distributions of Mediterranean species and genomes instead of extinctions (Hewitt, 2001; Gutiérrez Larena & al., 2002; Vargas, 2003).

The study group, *Arenaria* section *Plinthine*, gathers a number of potential advantages to address phylogenetic and phylogeographic questions. Based on the consistency of morphological characters, it is most likely a natural group (McNeill, 1962; López González, 1990). At least four synapomorphies are shared by the representatives of the section (see below). On taxonomical grounds, the group has been quite thoroughly studied (Font Quer, 1948; López González & Nieto Feliner, 1986; Goyder, 1987, 1988; López González, 1990; Chater & Halliday, 1993; Nieto Feliner, 1994) and the most recent taxonomic treatments recognise between six and 14 species. The group is geographically restricted to the western Mediterranean, where it occurs from the Maritime Alps (SE France, NW Italy) to N Africa (N Morocco, NE Algeria). The highest number of species is found in the Iberian Peninsula. Therefore, neither the systematic nor the geographic limits of section *Plinthine* are sources of uncertainty. The group has also received attention from a cytotaxonomic point of view because of chromosome-number variation and cytological mechanisms presumably involved (Nieto Feliner, 1985, 2000; Favarger & Nieto Feliner, 1988). Specifically, a polyploid series ($2x$, $3x$, $4x$, $5x$, $6x$, $7x$) based on $x = 20$ is found in the different subspecies of *A. tetraquetra* and in *A. alfacarensis*, while aneuploidy has been very active in *A. erinacea* and not so much in other species of section *Plinthine*.

However, the existence of disparate taxonomic treatments cast some doubt on the level of genetic divergence that we could expect between some species and consequently on the possibilities of obtaining a resolved phylogeny. At any rate, such taxonomic discrepancies, coupled with a significant cytotaxonomic complexity, anticipate richness in evolutionary processes. In fact, Font Quer (1948) early realized the interest of the group and put forward a number of hypotheses on the evolution of section *Plinthine*. Therefore, *Arenaria* sect. *Plinthine* offers stimulating ingredients for unravelling the evolutionary history of a primarily Mediterranean group using molecular markers. The main objective is to estimate phylogenetic relationships within section *Plinthine* using molecular markers (organellar and nuclear) and morphology as well as to search for phylogeographic patterns within the group in a biodiversity rich area like the western Mediterranean. Secondarily we aimed to assess the impact of aneuploidy and allopolyploidy in the evolution of this section.

MATERIALS AND METHODS

Plant materials and sampling strategy. — Sixty-nine samples, representing all (19) taxa of *Arenaria* sect. *Plinthine*, were sampled as the ingroup for phylogenetic reconstructions (Appendix 1, Fig. 1A). Two species from section *Grandiflorae* (*A. grandiflora*, *A. valentina*) were selected as the outgroup since it was proposed to be the closest relative to section *Plinthine* (McNeill, 1962; López González & Nieto Feliner, 1986). Additionally, one ITS sequence from sect. *Arenaria* (*A. serpyllifolia* AY438320) and one ITS-2 partial sequence from sect. *Leiospermae* (*A. lycopodioides*, AY857978) were taken from the GenBank and included in some analyses to increase the outgroup sample. Within the ingroup, sampling strategy was based on morphological and cytogenetic studies (Nieto Feliner, 1985, 2000; Favarger & Nieto Feliner, 1988). The 72 samples were chosen in order to cover the whole distribution range of each taxon as well as their morphological and chromosomal variability. Nine specimens of *A. erinacea* were included, as this is the most widely distributed species of section *Plinthine* and displays the greatest chromosome number variability (Nieto Feliner, 2000).

Based on the results of the ITS phylogeny and the caryological data, a selection of some of the samples was made for the phylogenetic analyses of plastid sequences. To choose the most variable markers, four different plastid spacers were tested in a pilot study (*trnT-trnL*, *trnL-trnF*, *trnS-trnG*, *trnH-psbA*). Two of these spacers (*trnT-L*, *trnL-F*) provided the greatest number of variable sites. Accordingly, sequencing of both spacers was extended up to 18 and 35 specimens, respectively (see results).

Molecular study. — DNA extractions were performed using both herbarium specimens and fresh material collected in the field and dried with silica-gel. DNA was extracted using the CTAB method (Doyle & Doyle, 1987) as modified by Loockerman & Jansen (1996), for fresh material and recently-collected herbarium material. For older herbarium specimens (including cytotological vouchers), DNA was extracted using the DNeasy Plant Mini Kit, specially designed for plant DNA (QIAGEN, Germany Laboratories). Samples of 20–25 mg of dried leaves from a single individual each were used.

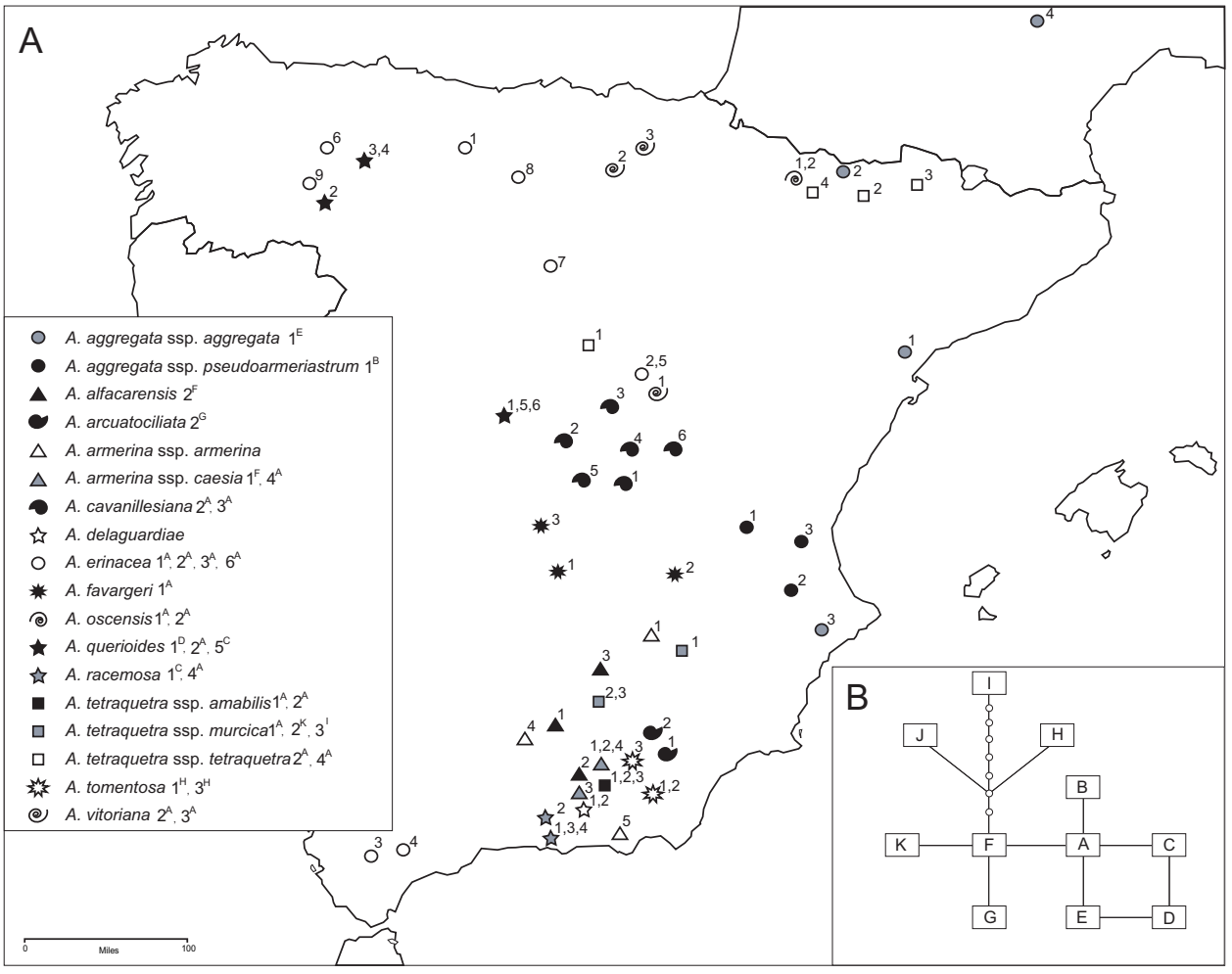

Fig. 1. A, Distributional map of the 66 European populations belonging to 18 taxa (represented by different symbols) in *Arenaria* sect. *Plinthine* sampled in this study for the molecular data. Samples numbered as in Appendix 1. Haplotypes in each population indicated (as superindices) after each taxon name; B, Statistical parsimony network of 11 plastid haplotypes found in *Arenaria* section *Plinthine*, defined on the basis of plastid *trnL-trnF* sequences. Lines represent single mutational steps, small circles are haplotypes not found in any sample. Two haplotypes detected in section *Grandiflorae* (*A. grandiflora* and *A. valentina*, respectively) were unconnected to this network and are not represented.

Basic PCR conditions and primers used to amplify the Internal Transcribed Spacer (ITS) nuclear region as well as the four plastid spacers are shown in Table 1. PCR amplicons were purified using spin filter columns (PCR Clean-up kit, MoBio Laboratories, California). Cleaned products were sequenced using dye terminators (Big Dye Terminator v 2.0, Applied Biosystems, California). For cycle sequencing of forward and reverse strands the following primers were used: ITS-4 and ITS-5 (White & al., 1990) for the ITS spacer; trna and trnb for the *trnT-L* spacer; and trne and trnf for the *trnL-F* spacer (Taberlet & al., 1991). Sequencing reactions were carried out under the following conditions: 95°C for 2 min followed by 25 cycles of 95°C for 10 s, 50°C for 5 s, and

Table 1. General PCR conditions for the amplification of the molecular markers sequenced: forward and reverse primers, annealing times and temperatures, and total number of cycles.

Molecular marker	Forward primer	Reverse primer	Annealing temperature	Annealing time	No. cycles
ITS[a]	17SE	26SE	50º C	30 s	24
trnT(UGU)-*trnL*(UAA)[b]	trna	trnb	52º C	120 s	35
trnL(UAA)-*trnF*(GAA)[b]	trne	trnf	51º C	90 s	35
trnH(UGU)-*psbA*[c]	trnh	psbA	53º C	60 s	35
trnS(GCU)-*trnG*(UCC)[c]	trns	trng	52º C	60 s	40

[a] Sun & al., 1994; [b] Taberlet & al., 1991; [c] Hamilton, 1999

60°C for 4 min. Polyacrylimide gel (at a concentration of 7%) electrophoresis of sequencing products was conducted by using a Perkin-Elmer/Applied Biosystems model 377 automated sequencer.

Three different matrices were constructed to perform the phylogenetic analyses. One of them included the 72 ITS sequences (69 of the ingroup plus three of the outgroup), the second one contained 18 *trnT-L* sequences (16 of the ingroup, plus two from the outgroup), the third one included 35 *trnL-F* sequences (33 from the ingroup plus two from the outgroup). Alignments were accomplished using the ClustalW multiple sequence alignment program (Thompson & al., 1994) as implemented in ClustalX 1.62b (Thompson & al., 1997) followed by manual revision.

Phylogenetic analyses were conducted using Maximum Parsimony (MP) under no constraints (Fitch parsimony), Neighbor-Joining (NJ), and Maximum Likelihood (ML) as implemented in PAUP* (Swofford, 1999). Bayesian Inference (BI) was also implemented to infer phylogenetic relationships using the program MrBayes v.3.0 (Huelsenbeck & Ronquist, 2001). Heuristic searches (100 replicates) for MP analyses were performed using the Stepwise Addition algorithm using the "random starting trees" option. Branch swapping was performed by Tree Bisection-Reconnection (TBR), with MULPARS, and STEEPEST DESCENT options. The heuristic search conducted with 100 replicates and retaining all best trees was not completed because a memory fault interrupted the analysis at first replicate of the ITS and *trnT-L* analyses. The final heuristic search for the MP analyses of both regions was eventually carried out retaining no more than 500 trees per replicate with a total score less or equal to the one detected in the previously interrupted heuristic search (Schultheis, 2001). Branch supports were obtained both by fast (10,000 replicates) and full (100 replicates) bootstrapping, and Decay index (Bremer, 1994) using the heuristic strategy detailed above.

Models of DNA evolution were estimated for each molecular marker via bottom-up strategy of hierarchical likelihood ratio test and Akaike Information Criterion (AIC; Akaike, 1979), as implemented in Modeltest v.3.06 (Posada & Crandall, 1998). The best fit models predicted by Modeltest (of those available in PAUP and MrBayes) were used for conducting the ML and BI. When different evolutionary models were obtained by different criteria, each dataset was analysed under both models. For each dataset, analyses based on different models displayed the same topologies only differing in support values. Therefore, pairwise differences, tree topologies, and clade supports presented herein were those obtained from applying the evolutionary model selected by AIC.

Heuristic searches for the ML analyses were conducted with 100 Stepwise Addition replicates and TBR branch swapping. Bootstrap supports for ML analyses were obtained performing 20 replicates. Bayesian Inference was run for 5,000,000 generations. Four Markov Chain Monte Carlo (MCMC) were run simultaneously at intervals of 100 generations. Burn-in was evaluated by examination of likelihood scores over generations. The stationary level was reached at 30,000 generations for the ITS dataset, 5,000 for *trnT-L*, and 15,000 for *trnL-F*. Thus, the first 300, 50, and 150 trees were discarded respectively, to compute consensus trees. Posterior probabilities (pp) for clades support were estimated by a majority rule consensus.

Taking advantage of its potential uniparental inheritance and lack of recombination, the relationships among plastid haplotypes detected in section *Plinthine* were also estimated applying statistical parsimony. A haplotype unrooted cladogram was constructed using the program TCS 1.13 (Clement & al., 2000), which implements the algorithm described in Templeton & al. (1992). Under this method, unrooted cladograms that have a high probability (>0.95%) of being true based on a finite-site model of DNA evolution are identified. The program was run with gaps coded as missing. Only the 35 *trnL-F* sequences were used to construct the parsimony haplotype network because the *trnT-L* sequences required a high number of gaps to align the sequences, which generated an excess of uncertainties in haplotype definition when using the TCS software. In addition, technical limitations precluded a representative sampling of the *trnT-L* spacer. Particularly, a poly-A between sites 266–277 in the aligned matrix hindered obtaining quality sequences in many cases.

Morphological study. — A morphological matrix, with 16 characters coded as unordered, was compiled for the phylogenetic analysis (Appendices 2, 3). This matrix included 20 taxa, representing the outgroup and all taxa of section *Plinthine*, except for *A. aggregata* ssp. *mauritanica*, a poorly known taxon of which we lack information of three of the 16 characters (chromosome number, capsule relative size, seed ornamentation). Two of the 16 characters were coded as multi-state, whereas the remaining 14 were coded as binary. Fourteen of the 16 morphological characters were qualitative and two quantitative (pedicel length, chromosome number). The morphological matrix of 20 taxa × 16 characters was analyzed applying MP, using the same heuristic search strategy detailed above for molecular data, as implemented in PAUP.

Combined analyses. — In order to check for the adequacy of a combined analysis, a Templeton Less Parsimonious test (based on Wilcoxon Signed-ranks test; Templeton, 1983) was performed in PAUP to test for

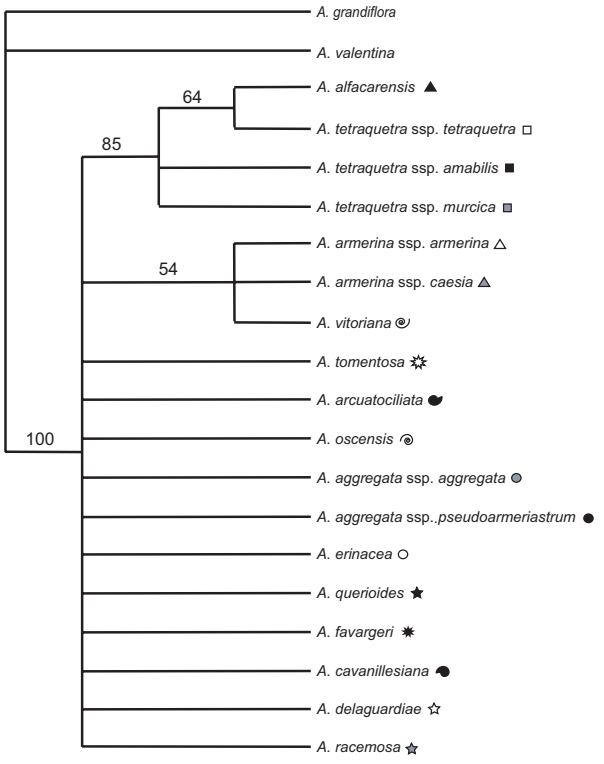

Fig. 2. Strict consensus of the 4,623 most-parsimonious trees (length = 32; CI excluding uninformative characters = 0.71; RI = 0.86) of *Arenaria* sect. *Plinthine* based on 16 morphological characters coded as unordered, using *A. grandiflora* and *A. valentina* as outgroup. Full heuristic bootstrap values over 50% are indicated above branches. Taxon symbol follows each sample.

congruence between datasets (morphological, nuclear, plastid). Sequential Bonferroni correction (Rice, 1989) was performed on the significance levels obtained. Maximum parsimony analyses were conducted in PAUP for reduced, compatible matrices of the ITS, *trnT-L*, *trnL-F*, and the morphological datasets. To facilitate comparison with morphology, only one sequence per species for each of the molecular matrices was used, giving a combined matrix of 16 taxa. Selection was made by choosing sequences from the same sample. Since the number of most parsimonious trees obtained from the independent analyses of the four matrices was high, datasets were optimized on the strict consensus topology from the alternative dataset instead of on fundamental trees.

RESULTS

Morphological analyses. — Cladistic analyses of morphological data revealed 4,623 most parsimonious trees of 32 steps with a consistency index excluding uninformative characters (CI) of 0.71 and a retention index (RI) of 0.86. Morphological data supports taxonomical circumscription of section *Plinthine* with 100% bootstrap (Fig. 2). The strict consensus tree (Fig. 2) recovered only two clades, one with *A. alfacarensis* and the three subspecies of *A. tetraquetra* with a bootstrap support (bs) of 85% and a second with *A. vitoriana* and the two subspecies of *A. armerina* (54% bs).

ITS sequence analyses. — The 72 ITS sequence length ranges from 619 bp in *A. grandiflora*2 (622 bp in section *Plinthine*, found in *A. favargeri*1, 2, 3) to 624 bp (*A. erinacea*8; *A. oscensis*2; *A. racemosa*1, *A. vitoriana*3; *A. querioides*1, 2, 3, 4; *A. tomentosa1*, 2, 3): 243–247 bp for ITS-1; 144–146 bp for 5.8S; and 230–232 bp for ITS-2. The number of the variable/potentially-informative characters (140/102) in *Arenaria* was distributed as follows: 87/62 bp for ITS-1; 7/6 bp for 5.8S; and 46/34 bp for ITS-2. The number of the variable/potentially-informative characters (65/43) in section *Plinthine* was distributed as follows: 34/23 bp for ITS-1; 5/4 bp for 5.8S; and 26/16 bp for ITS-2. A+T content was 0.43. Corrected pairwise GTR+I+G divergences of the Internal Transcribed Spacer (see below) within sect. *Plinthine* vary between 0 (62 pairwise comparisons) and 3.7% (*A. erinacea*1 and *A. racemosa*4). Double nucleotide peaks in the chromatograms, as a result of direct ITS sequencing were observed at 32 of the 65 variable sites.

Differences from the MP, NJ, ML (GRT+I+G model), and BI (GTR+I+G model) were obtained in terms of phylogenetic topologies and support. The MP analysis yielded the least resolved phylogeny and was chosen to discuss fundamental incongruencies. The semistrict consensus of 50,000 optimal trees of 202 steps (CI = 0.77; RI = 0.90) is shown in Figure 3. *Arenaria* sect. *Plinthine* is a monophyletic group (100% bs, 100% pp) when using its morphologically closest relatives (*A. grandiflora* and *A. valentina*) as outgroups, as hypothesized in previous taxonomic studies. Monophyly of sect. *Plinthine* is also highly supported (>94% bs, >98% pp) when including two more species (*A. serpyllifolia* and *A. lycopodioides*) (results not shown). A large basal-most polytomy is retrieved, which prevents from describing major clades. Distribution of sequences from the same taxon across the ITS phylogeny varied depending on the taxon (Fig. 3). Relative to well-supported clades joined all accessions of the same taxon in *A. alfacarensis* (76% bs), *A. favargeri* (91% bs), *A. tetraquetra* ssp. *amabilis* (< 50% bs), *A. oscensis* (92% bs), *A. vitoriana* (64% bs), and *A. tomentosa* (97% bs). Accessions from nine species did not form monophyletic groups: *A. aggregata*, *A. cavanillesiana*, *A. erinacea*, *A. tetraquetra*, *A. arcuatociliata*, *A. armerina*, *A. delaguardiae*, *A. querioides*, *A. racemosa*, although part of the conspecific sequences grouped together (see discussion). To explore the impact

Table 2. Results of the Templeton (1983) Less Parsimonious test for topological congruence of the phylogenetic hypotheses inferred from each dataset. Significant *P*-values given are those obtained after sequential Bonferroni correction. An asterisk indicates statistical significance and β indicates non-significant results that could not be explicitly tested because fewer than six characters vary.

Dataset	Alternative topologies	Number of steps: Increase	Decrease	N	*P*
ITS	*trnL-trnF*	9	0	9	0.0027*
	trnT-trnL	9	1	8	0.0033*
	Morphology	9	0	9	0.0027*
Morphology	ITS	7	0	7	0.0158*
	trnL-trnF	8	0	8	0.0103*
	trnT-trnL	8	0	8	0.0103*
trnL-trnF	ITS	1	2	-1	β
	trnT-trnL	1	0	1	β
	Morphology	1	0	1	β
trnT-trnL	ITS	5	0	5	β
	trnL-trnF	5	0	5	β
	Morphology	5	0	5	β

of sample error and number of accessions, a limited matrix of sequences (marked as number 1 of each taxa) was analysed retrieving again large polytomies (results not shown).

Nucleotide additivity (double peaks) from direct ITS sequencing was observed in 28 accessions. Most cases occurred within 24 of the 43 parsimony-informative positions. Although it could not be determined whether in some cases double-peak patterns were the result of sequencing artifacts, equimolar proportions of alternative nucleotide peaks suggested the presence of different ITS copies in other cases. This view is supported by the fact that 21 of the 43 parsimony-informative sites presented additivities in which the two nucleotides involved were present elsewhere in the dataset (additive polymorphic sites, APS). Only three additivities located at parsimony-informative positions involved one nucleotide not found in any other sequence. Because multiple, alternative inheritance pathways of nucleotides are equally possible, no clear phylogenetic signal can be extracted. Inferring the origin of the additivities from hybridization events was precluded since, for most sequences, not all the APS from the same sequence suggested the same putative progenitor sequences.

Plastid sequence analyses. — The pilot study of four *Arenaria* species rendered a low number of nucleotide substitutions: 10 in the *trnT-L* intergenic spacer; 5 in the *trnL-F* spacer; 3 in the *trnS-G* intergenic spacer; 0 in the *trnH-psbA* intergenic spacer. Sampling was extended for *trnT-L* (18 sequences) and *trnL-F* (35 sequences) in 14 species of section *Plinthine* and two accessions of *A. grandiflora* and *A. valentina*. The lower sampling for *trnT-L*, despite a higher number of substitutions detected in the pilot study as compared to *trnL-F*, is due to serious doubts on the phylogenetic signal within this region. Limiting the sampling has been ultimately due to large partially overlapping indels of different length, a strong contrast between the number of variable vs. informative characters, as well as technical problems in the amplification and sequencing of *trnT-L* spacer caused by long poly-A or poly-T stretches.

The number of variable/parsimony-informative characters was of 128/42 for *trnT-L* and 91/54 for *trnL-F* across *Arenaria*; 51/7 for *trnT-L* and 16/6 for *trnL-F* across section *Plinthine*. The longest *trnT-L* sequence within *Arenaria* was found in *A. grandiflora*1 (662 bp), and in *A. oscensis*2 (622 bp) within section *Plinthine*; the shortest in *A. tetraquetra* ssp. *murcica*3 (543 bp). The highest pairwise difference of the corrected *trnT-L* sequence divergence using the TrN +I model selected by Modeltest, was between *A. tomentosa*1 and *A. armerina* ssp. *caesia*1 (3.74%), and the lowest one (0%) between the following accessions: *A. racemosa*4-*A. vitoriana*2, *A. racemosa*4-*A. arcuatociliata*2, *A. vitoriana*2-*A. arcuatociliata*2, and *A. cavanillesiana*2-*A. armerina* ssp. *caesia*4. The longest *trnL-F* sequence within *Arenaria* was found in *A. valentina* (395 bp), and within section *Plinthine* in *A. armerina* ssp. *armerina*2 (335 bp); the shortest in *A. tetraquetra* ssp. *tetraquetra*4 (331 bp). The highest pairwise TrN +I corrected *trnL-F* sequence divergence (TIM+I was selected by Modeltest, but could not be implemented in PAUP) was between *A. querioides*1 and *A. tetraquetra* ssp. *murcica*3 (3.21%) and the lowest one (0%) between 214 pairwise comparisons. A+T content was 0.79 for the *trnT-L* and 0.63 for the *trnL-F*, the latter of which is in accordance with the range (0.63–0.69) reported in Bakker & al. (2000).

The four phylogenetic analyses (MP, NJ, ML, BI) performed on the matrix of 18 *trnT-L* sequences depicted different trees, being MP tree resolution more limited than that provided by NJ, followed by ML and BI (results not shown). The same is true for the analyses of the *trnL-F* matrix of 35 sequences. When analysing two submatrices of *trnT-L* and *trnL-F* sequences from the same 18 DNAs, different phylogenetic relationships were retrieved irrespective of the approach used (MP, NJ, ML, BI). For instance, in the MP analysis some support was found for a single *trnL-F* clade (70% bs), which includes *A. tetraquetra* ssp. *murcica*3 and *A. tomentosa*1 (Fig. 4). In contrast, the only *trnT-L* clades with support over 50% consisted of the following accession pairs: *A. alfacarensis*2-*A. oscensis*2 (55% bs), *A. racemosa*1-*A. tetraquetra* ssp. *tetraquetra*2 (64% bs); and *A. tomentosa*1-*A. favargeri*1 (90% bs) (Fig. 4).

The statistical parsimony analysis of the *trnL-F* data yielded a single network including the 11 plastid haplotypes from section *Plinthine* and containing one loop (Fig. 1B). The two haplotypes detected in section *Grandiflorae* were both unconnected to the *Plinthine*

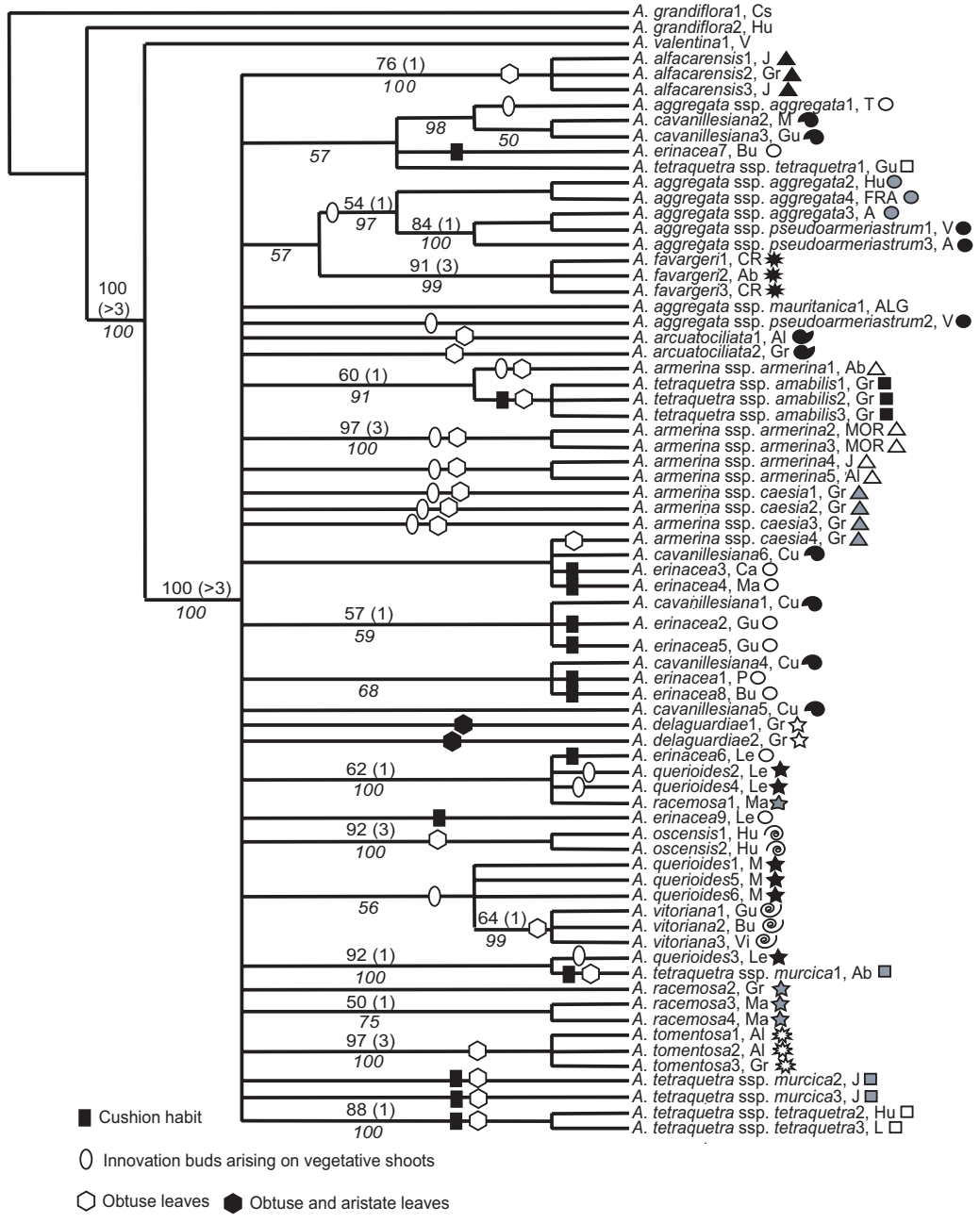

Fig. 3. Semistrict consensus of the 50,000 optimal trees (length = 202; CI excluding uninformative characters = 0.77; RI = 0.90) based on 69 ITS sequences of *Arenaria* sect. *Plinthine* plus two sequences of *A. grandiflora* and one of *A. valentina* as outgroup. Bootstrap values over 50% are shown above branches, decay indices are specified in parenthesis and posterior probabilities from Bayesian Inference are supplied in italics below branches. Cushion habit, innovation buds arising on vegetative shoots and characters of leaves are mapped on clades. Abbreviations for Spanish provinces and taxon symbol follow each sample.

network. Geographical and network distribution of plastid haplotypes is shown in Fig. 1. Two major results are inferred from the plastid haplotype analysis: (1) the highest haplotype variation (7) is found in SE Iberia; (2) the interior (ancient) haplotype A is distributed throughout the range of the section, including North Africa, and is shared by 10 species of section *Plinthine*.

Congruence among datasets. — The nonparametric Templeton parsimony test conducted for assessing topological congruence among nuclear, plastid and morphological datasets, revealed statistically significant incongruences. The *trnT-L*, *trnL-F*, and morphological topologies tested were rejected by the ITS dataset (Templeton's tests, $P < 0.05$; see Table 2). The same

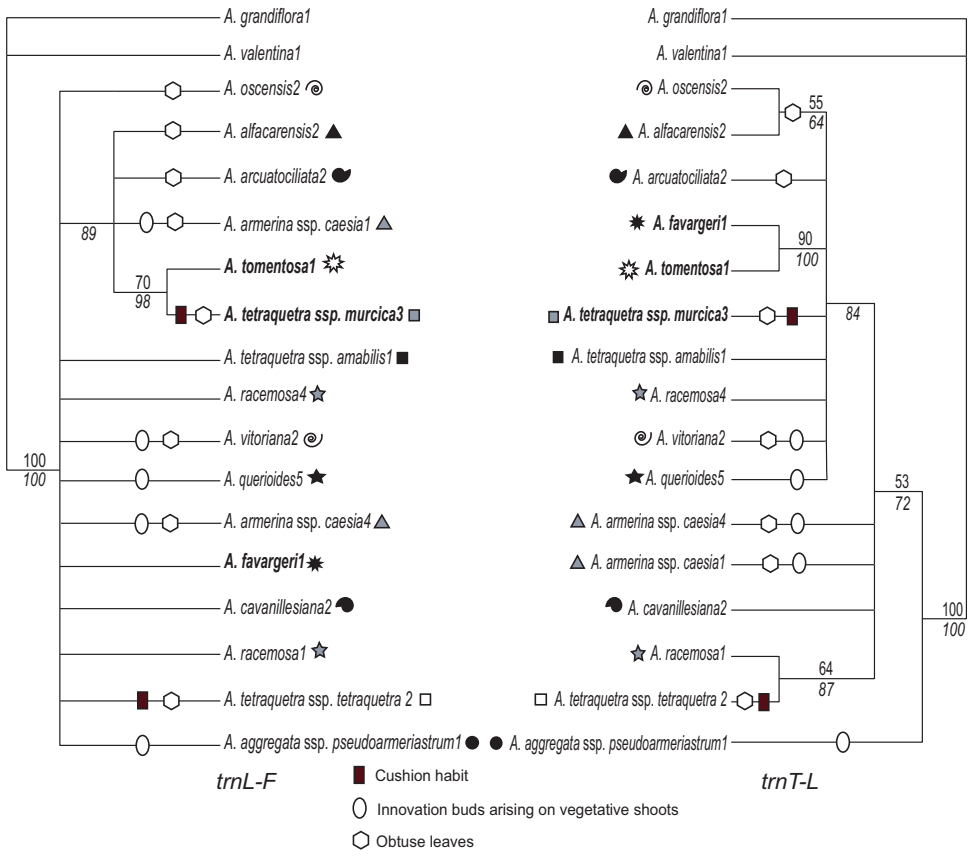

Fig. 4. Comparison of the two majority rule consensus trees obtained from the Bayesian analyses of *trnT-trnL* and *trnL-trnF* plastid sequences using the TrN+I and TIM+I evolutionary models, respectively. Posterior probabilities >50% are indicated in italics below branches for each particular node. Bootstrap values >50% from the Parsimony analysis are specified above branches. Taxa in bold highlight strong discrepancies between the two topologies. Cushion habit, innovation buds arising on vegetative shoots and characters of leaves are mapped on clades. Taxon symbol follow each sample.

result was obtained when ITS, *trnT-L*, and *trnL-F* trees were tested against the morphological dataset (Templeton's tests, $P < 0.05$; Table 2). Congruence of *trnT-L* and *trnL-F* matrices with the alternative topologies from morphology and ITS could not be tested, as the number of varying characters in each comparison were fewer than six (Sokal & Rohlf, 2003). After correcting for multiple test using the sequential Bonferroni corrections (Rice, 1989), the same comparisons remained significant. In addition, as visual inspection of *trnT-L* and *trnL-F* topologies reveals that they are significantly discordant not only with those of morphology and ITS but also between each other, no combined analysis was finally conducted.

DISCUSSION

Discordance between datasets. — ITS data provide poor phylogenetic resolution as judged from both the BI and the MP trees (Fig. 3). As in many phylogenetic analyses based on ITS sequences, this is particularly true for deep nodes of the tree and not so much for shallow clades, particularly when coupled with high CIs (Fuertes Aguilar & al., 1999a; Torrell & al., 1999). The same pattern of irresolution at deep nodes is found when analysing morphological (Fig. 2) and plastid matrices (Fig. 4). This result has been related to ancient reticulation or radiation processes in other plant groups (Soltis & al., 1998).

The fact that the Templeton Less Parsimonious test reveals significant incongruence between ITS nrDNA and morphology suggests that they recover different phylogenetic histories. Although incongruence of the *trnT-L* and *trnL-F* matrices against the other datasets (Table 2) could not be checked with Templeton test, evident incongruences can be detected from direct observation of topologies. This includes discordant placement of three taxa, when comparing *trnT-L* and *trnL-F* tree topologies. *Arenaria favargeri*1, *A. tetraquetra* ssp. *murcica*3, and *A. tomentosa*1 are clustered in different clades and supported by bootstrap and posterior probability values greater

than 50% (Fig. 4). Fundamental incongruence between species trees and gene trees has been traditionally interpreted as the result of biological phenomena, such as reticulation, lineage sorting, and mistaken orthology (Doyle, 1992). In addition to such phenomena, particular characteristics in section *Plinthine*, such as polyploidy and aneuploidy may have contributed to major phylogenetic conflicts, that prevent us from recovering a fully resolved phylogenetic hypothesis.

It is intriguing to observe that the two spacers located in nearby loci in the plastid suggest different evolutionary courses, in spite of plastid DNA being a non-recombinant uniparentally inherited molecule. Alternatively, the common assumptions that underlie the use of different plastid DNA sequences in phylogenetic analyses may not hold for section *Plinthine* as it is starting to be realized for other groups (Fitter & al., 1996; Wolfe & Randle, 2004). Disproportionate number of variable/informative characters across accessions of the *trnT-L* sequences (51/7) and large gaps of different length in the alignment (positions 136–165 and 629–685) indicate that this plastid region may not be adequate for retrieving a reliable phylogeny in *Arenaria* sect. *Plinthine*, as compared to other plant groups where the ratio of variable/informative characters is much lower (14/6 in *Hedera*, Valcárcel & al., 2003; 61/20 in *Solanum*, Bohs, 2004; 19/11 in *Narcissus*, Pérez & al., 2004). In contrast, lower ratio of variable/informative characters in the *trnL-F* sequences (16/6) and a low number of gaps (3 indels of 1–6 bp) suggest that this region contains a more reliable albeit limited phylogenetic signal (Bakker & al., 2000).

Morphology and ITS divergence. — Section *Plinthine* is an easily identifiable morphologically cohesive group restricted to the western Mediterranean. At least, four morphological synapomorphies (Appendices 2 and 3) support the taxonomical identity of this section (Fig. 2), when compared to sect. *Grandiflorae*: presence of imbricate bracts surrounding calyx (character 5), thickened sepals (character 11), hygrochastic capsules dehiscence (character 13), and the existence of abcission joints on stems (character 16). A significant number of additional characters allow recognizing over 14 species and 19 taxa (López González, 1990). However, patterns of variation are not fully understood within some of species and 12 cladistically informative characters do not provide a resolved phylogeny, although seemingly natural groups are recovered: *A. tetraquetra* + *A. alfacarensis* and *A. armerina* + *A. vitoriana* (Fig. 2).

The ITS phylogeny resolved a well-defined clade (100% bs and pp) containing the 69 accessions from section *Plinthine*, and thus strongly supported the monophyly of this section. Low resolution within the section was retrieved despite a high number of ITS variable/potentially-informative characters within section *Plinthine* (65/43). The measures of fit are rather high (CI, e.u.c. = 0.77; RI = 0.90). However, deeper resolution in the tree is precluded by the distribution of informative characters, which are shared primarily, at most, by 16 terminals (within the section) and in general by much less than that. Therefore, the ITS dataset lacks informative characters to cluster together large subsets of the 69 terminals in section *Plinthine*.

Not only morphological and nuclear ITS datasets provided low internal phylogenetic resolution, but also the few natural groups reported by morphological data have no molecular support. For instance, the strong morphological affinity between *A. tetraquetra* spp. and *A. alfacarensis*, is not supported by the ITS phylogeny.

The level of ITS sequence divergence found within section *Plinthine* (a maximum K-2p distance of 4.1%) falls within the range of typical Mediterranean plants with similar number of species: in *Saxifraga* sect. *Saxifraga* (0–9.4%, Vargas & al., 1999), in *Teucrium* sect. *Polium* (1–12.5%, Oualidi & al., 1999), in *Antirrhinum* (0–4.37%, Vargas & al., 2004). Interestingly, another Alsinoideae genus in the Caryophyllaceae with a Euro-Australasian disjunct distribution (*Scleranthus*) displays somewhat higher divergence (0–7.7%, Smissen & al., 2003). The fact that most substitutions are not shared by a significant number of sequences suggests that the variation found may be the result of a recent diversification. Chromosome number variation across the section may also reflect active speciation processes. As most of the representatives of section *Plinthine* occur on Mediterranean habitats, association between diversity and Quaternary climatic influences in the Iberian Peninsula has been stated (Font Quer, 1948). ITS sequence divergence (below 4.1% K-2p) between most species comparisons also supports the hypothesis of a recent divergence and lead us to argue that most diversification in section *Plinthine* may have not predated the onset of summer drought in the Mediterranean region (3.2 Myr; Suc, 1984), as already tested in another plant groups (Vargas, 2003).

ITS phylogeny and taxonomy. — In this study, particular attention has been paid to cover a representative sample of cytotaxonomical diversity and distribution of the taxa (see Material and Methods, Appendix 1). However, agreement between the ITS phylogeny (Fig. 3) and taxonomic origin of the sequences is limited. Taxa whose ITS sequences are fully monophyletic are *A. alfacarensis*, *A. tetraquetra* ssp. *amabilis*, *A. favargeri*, *A. oscensis*, *A. tomentosa*, and *A. vitoriana* (i.e., six out of 18 taxa with more than one accession). In five other cases, a majority of the conspecific sequences fall within the same clade in the semistrict consensus tree (Fig. 3). For instance, there is a clade containing three of the four sequences of *A. aggregata* ssp. *aggregata*, plus two of

the three of *A. aggregata* ssp. *pseudarmeriastrum*. There are also other results that do not show strong discrepancies with the taxonomy although not involving fully monophyletic conspecific sequences. They include the terminal clade comprising the two Pyrenean samples of *A. tetraquetra* ssp. *tetraquetra*, but not the disjunct population from central Spain, which differs in ploidy level and, to a lesser extent, in morphological features (Favarger & Nieto Feliner, 1988). Also, the sequences from two pairs of morphologically related taxa (*A. vitoriana* - *A. querioides*; *A. erinacea* - *A. cavanillesiana*) are grouped in more than one clade (Fig. 3).

In contrast, there are several other conflicts between taxonomy and ITS. Species with large distribution areas, such as *A. erinacea*, *A. tetraquetra* and *A. querioides*, are the ones whose sequences are more scattered throughout the ITS tree. This pattern is compatible with more than one explanation. The main causes for disagreement between gene phylogeny and taxonomy at this level appear to be horizontal transfer and lineage sorting (deep coalescence; Doyle, 1992). Since comparison with plastid DNA trees is relatively unsuccessful in our study, possible cases of horizontal transfer might be suggested based on the detection of additive polymorphic sites (APS; Rauscher & al., 2002; Wichman & al., 2002) and the concurrence of ITS similarity and geographic sympatry (Fuertes Aguilar & al., 1999b).

Most APS present in the data might be interpreted as evidence of hybridization, although available data does not allow us to propose it as the major mechanism involved in the evolution of the section. The 11 APS displayed by *A. racemosa*2 most likely indicate hybridization. But identifying the progenitors is not straightforward because alternative nucleotides of different APS do not suggest the same putative progenitor sequences. For instance, the G/T polymorphism in *A. racemosa*2 at site 458, where T was only found in a sequence of *A. delaguardiae*2 from the same massif, would suggest that the latter species is one of the progenitors, and the other is a standard *A. racemosa*. However, when other parsimony informative positions are considered, based on our dataset, the progenitor could be *A. cavanillesiana* (c. 400 km north). Although we considered ITS sampling appropriate for reconstructing lineages at the species level (see above), it may not be detailed enough in section *Plinthine*, and additional data sources are needed to understand hybridization events revealed by APS.

Clades including different taxa from the same or close localities is a pattern that may also suggest hybridization. However, this pattern is not consistently found in our data. In particular, clades including more than one taxon unexpected from a morphological point of view, displayed no clear sympatric situations. For instance, *A. querioides*3 and *A. tetraquetra* ssp. *murcica*1 are from the distant provinces of León (NW Spain) and Albacete (SE Spain), respectively (Figs. 1A, 3). Although not attributable to hybridization either, the clade formed by three samples from León (*A. erinacea*6, *A. querioides*2, 4) and Málaga (*A. racemosa*1) deserves comment. The tetraploid individuals of *A. querioides* from NW Spain are disjunct to the core of the species located in central Spain, which contains only diploid populations (Nieto Feliner, 1985). The former individuals lack the morphologically distinct features of the diploid plants and were postulated to be tetraploid deviant forms (Nieto Feliner, 1985). These tetraploid forms tend to present a more cespitose habit, with less pronounced calyx median nerves, and less dimorphism between leaves from flowering stems and innovation shoots. The placement of two tetraploid samples of *A. querioides* together with one accession of *A. erinacea* from the same area, in the ITS tree, might suggest an allopolyploid origin for these northwestern populations of *A. querioides*, a possibility that is consistent with the above mentioned morphological features.

An alternative explanation for discrepancies between taxonomy and topology of gene trees is lineage sorting (deep coalescence). Telling apart horizontal transfer from lineage sorting is difficult in general (Wendel & Doyle, 1998). When using ribosomal data a further complication is provided by homogenization of ITS repeat copies (concerted evolution) within individuals and reproductive groups thereby gradually erasing possible traces of hybridization (Alvarez & Wendel, 2003). The scarcity of cases that, based on our data, can unequivocally be attributed to recent (or traceable) hybridization, suggests that lineage sorting may have also contributed to the discrepancies between taxonomy and phylogenetic placement of the sequences in this study.

Another possible source of discrepancies between ITS phylogeny and taxonomy may be the high karyological variation between species and also within certain species. Despite a particular effort has been made in this work to sequence 18 cytological vouchers plus 11 individuals from populations with previously-reported chromosome numbers (Appendix 1; Nieto Feliner, 2000), the results are not good enough to describe clear patterns of chromosome evolution. *Arenaria* section *Plinthine* exhibits a wide range of chromosome numbers that encompass several basic numbers (Nieto Feliner, 1985, 2000). Such variability is extreme in *A. erinacea* ($2n$ = 20 to $2n$ = 68) where evident aneuploid shifts have taken place but also allopolyploidy is suspected. It has been postulated that such variability may have been due to periods of karyotype instability as those inferred at the genus level in *Arenaria* and *Minuartia* (Favarger, 1962). If this is true and has involved allopolyploidy, it is con-

ceivable that different ITS copies from different chromosome complements have merged within the same genome. Ultimately, the spread of different samples of *A. erinacea* in the ITS tree may be caused by general mechanisms like those affecting the fate of duplicated genes in allopolyploids (Wendel, 2000; Liu & Wendel, 2003). In addition, specific mechanisms affecting multicopy genes like ITS, such as in biased homogenization of different repeat types (Hillis & al., 1991; Fuertes Aguilar & al., 1999a) or failure to detect all copies by direct sequencing (Rauscher & al., 2002) may have also contributed. It is here proposed that aneuploidy may have also added to the dispersion of *A. erinacea* accessions across the topology of the ITS tree. In particular, aneuploid changes are so remarkable in *A. erinacea*, that there is the possibility of losses affecting the NOR regions thereby eliminating ITS copies. Since the chromosome number has been found to vary even within populations of *A. erinacea* (Nieto Feliner, 2000), such possibility is compatible with the observation of conspecific accessions from the same locality exhibiting different ITS sequences (e.g., samples 1 and 4 of *A. racemosa*, 3 and 4 of *A. querioides*).

Phylogeography and previous evolutionary hypotheses. — The amount of data acquired by sequencing (three markers from two genomes) is not conclusive to confirm or rule out all previous evolutionary hypotheses. An increase in ploidy level through hybridization in *A. tetraquetra* was previously postulated based on cytotaxonomical data (Favarger & Nieto Feliner, 1988). Although our sample is not comprehensive enough, relative position of accessions from the three subspecies (*tetraquetra*, *amabilis*, *murcica*) in all phylogenetic trees agrees with a non-monophyletic origin. Unrelated haplotypes in *A. tetraquetra* ssp. *murcica* (A, I, K) as inferred in the plastid haplotype network (Fig. 1B) further support the postulated multiple production of polyploids by hybridization (Favarger & Nieto Feliner, 1988). Notice in Figure 1B that seven plastid haplotypes have not been found in any accession, although this may be due to insufficient sampling or extinction. An ISSR study with an extended number of samples (already in progress) may shed further light on the evolutionary mechanisms responsible for a ploidy series of $2n = 40$, 60, 80, 100, 140 in *A. tetraquetra*.

Font Quer (1948) put forward a number of hypotheses on the evolution within section *Plinthine*. Some of them cannot be tested with our data, e.g., primitive vs. derived nature of obtuse leaves (Figs. 3 and 4). Among the others, he proposed that cushion-shaped plants mainly occurring on alpine habitats were derived from lax forms from lower-elevation montane plants. Since cushion-shaped habit occurs within several species (Appendices 2 and 3, Figs. 3 and 4), Font Quer hypothesized the independent origin of such habit in different lineages. Although our data are insufficient to test all the cases, they do support such hypothesis in one case. *Arenaria aggregata* shows a lax habit throughout its range with a single exception: the population 4 of subspecies *aggregata* from Mt. Ventoux (SE France). The placement of this sample within the core of an *A. aggregata* clade in the ITS tree (Fig. 3), suggests the derived nature of cushion habit and also solves a previous controversy on the identification of such French population as *A. erinacea* (Nieto Feliner, 1994). This result agrees with the evolutionary pattern of differentiation of alpine forms from lowland congeners documented in several plant groups from Europe (Comes & Kadereit, 2003).

The analysis of plastid sequences across the range of sect. *Plinthine* reveals a pattern (highest haplotype variation in SE Iberia) that is fully congruent with other data from the section (comparatively high diversity of taxa, morphological characters, and ITS lineages) as well as from other groups since the Baetic mountains are well-known as a Mediterranean hotspot for diversity (Medail & Quezel, 1997; Myers & al., 2000). This point was already stressed by Font Quer (1948) based exclusively on the richness of morphological characters in this area. Seven of 11 *trnL-F* haplotypes were found in this area of Andalusia, including some interior plastid haplotypes (A, C, F) and singletons (G, H, I, K) in the network reconstruction (Fig. 1, Appendix 1). The occurrence of several interior plastid haplotypes in SE Iberia is related with a refugium of ancient genetic variation while the singletons may be indicative of an active area for recent diversification (Hewitt, 2000). The haplotype parsimony network indicates that haplotype A is an old one predating the diversification within section *Plinthine*. Besides the interior position in the network (suggestive of primitiveness in coalescence theory; Hudson, 1990), its occurrence across 12 of 17 taxa from Iberia and N Africa supports an ancient status, although a partial spread of this haplotype through horizontal transfer after differentiation of lineages within the section cannot be excluded. Specifically, the detection of a single haplotype A across five different taxa, along the Pyrenean–Cantabrian ranges spanning c. 700 km, is noteworthy.

Concluding remarks. — General evolutionary forces as well as particular characteristics of section *Plinthine* may have contributed to lack of phylogenetic resolution and to discrepancies found between taxonomy and nuclear and plastid trees. Such discrepancies, specifically the placement of both ITS and *trnL-F* sequences from the same taxon in different clades, affect primarily the widespread taxa. A considerable mutation rate in ITS sequences, combined with organism-level processes (lineage sorting) and gene or genome-level processes (biased concerted evolution, loss or silencing of genes duplicated by polyploidy, karyotype rearrangements

affecting ribosomal loci) may have significantly contributed to such discrepancies. After reading recently published critical reviews on the use of ribosomal DNA for phylogenetic reconstruction of plant taxa, one draws the conclusion that a count-down for the use of ITS may have started (Alvarez & Wendel, 2003; Bailey & al., 2003). The unsatisfactory resolution obtained within section *Plinthine* seems to agree with that conclusion. However, we argue that fully resolved gene phylogenies (based on ITS or any other regions) congruent among themselves and with species phylogenies are not a realistic expectation, not only in our study group but also in most plant groups. Several causes at the molecular and organismic level contribute to it, primarily the pervasiveness of hybridization and its consequences on the evolution and inheritance of molecular markers. In any case, a significant degree of discord between different sources of evidence (morphological characters, cytological diversity, three molecular markers from two genomes) lead us to conclude that unparallel molecular and organismal evolution may have been operating in *Arenaria* sect. *Plinthine*.

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Appendix 1. List of studied material including natural distribution of the taxon (in parenthesis); sample number (in parenthesis); locality; voucher reference; chromosome numbers (between semicolons) are given in bold for individuals also used in sequencing, in italics for counts obtained from the same population but different individual as that sequenced, and in roman for counts inferred from a different population; *trnL-trnF* plastid haplotype (in capitals); and GenBank accession numbers followed by corresponding molecular marker (in parenthesis).

Taxon (natural distribution): Sample number; Locality, followed in some cases by UTM coordinates; Voucher; Chromosome number (2*n*); *trnL-F* plastid haplotype (capital letters); GenBank accession (molecular marker).

***Arenaria* sect. *Plinthine* (Reichenb.) Pau:** *Arenaria aggregata* (L.) Loisel. ssp. *aggregata* (NE Spain, SE France): (1) Tarragona, track from Fredos to Monteclaro; *C. Navarro & al. 2409CN* (626802MA); 30; E; AY691562 (ITS), AY692377 (*trnL-F*). (2) Huesca, Aínsa, 30SYM4590; *D. Gómez & C. Aseginolaza s.n.* (616295MA); 30; AY691563 (ITS); (3) Alicante, Sierra Serella 30SYH38; *G. Mateo & R. Figueroa s.n.* (296966MA); 30; AY691564 (ITS). (4) France, Bedoin up Mt. Ventoux; *B. Girerd s.n.* (497397MA); 30; AY691565 (ITS). *A. aggregata* ssp. *pseudoarmeriastrum* (Rouy) G. López & Nieto Feliner [E Spain (Valencia)]: (1) Valencia, Sierra de Enguera 30SXJ8314; *G. López 9500GL* (502906MA); **28**; B; AY691567 (ITS), AY692413 (*trnT-L*), AY692379 (*trnL-F*); (2) Valencia, Ayora; *J. Riera & al. s.n.* (589121MA); 26, 28; AY691568 (ITS). (3) Valencia, Valldigna, 30SVJ32; *R. Figuerola s.n.* (430119MA); 26, 28; AY691569 (ITS). *A. aggregata* ssp. *mauritanica* Batt. (Algeria): (1) Algeria; *V. Valcárcel & al. 118VV00* (MA); ?; A; AY691566 (ITS), AY692380 (*trnL-F*). *A. alfacarensis* Pamp. (SE Spain): (1) Jaén, Sierra de Almadén; *A. Izuzquiza & al. 876AI* (426912MA); 40; AY691559 (ITS). (2) Granada, Sierra de Alfacar, Cueva del Agua; P. Vargas 106PV00 (MA); *40*; F; AY691560 (ITS), AY692409 (*trnT-L*), AY692370 (*trnL-F*). (3) Jaén, Sierra de Cazorla; *F. Valle & G. Blanca s.n.* (220209MA); 40; AY691561 (ITS). *A. arcuatociliata* G. López & Nieto Feliner (SE Spain). (1) Almería, road Chirivel, Vertientes; *A. Pallarés s.n.* (488126MA); 30; AY691570 (ITS). (2) Granada, Baza 30SWG2048; *P. Vargas 114PV00* (MA); 30; G; AY691571 (ITS), AY692407 (*trnT-L*). *A. armerina* Bory ssp. *armerina* (N Morocco and SE Spain): (1) Albacete, Riopar, Chorros del río Mundo; *V. J. Arán* (545610MA); 28, 30; AY691572 (ITS). (2) Morocco, Chefchaouen, Bab l'ars; *P. Vargas 158PV00* (MA); 28, 30; J; AY691573 (ITS), AY692387 (*trnL-F*). (3) Morocco, Great Atlas 32º31'54"N 6º00'56"W; *Cirujano & al. s.n.* (624763MA); **18**; AY691574 (ITS). (4) Jaén, Valdepeñas; *C. Fernández s.n.* (312778MA); 28, 30; AY691575 (ITS). (5) Almería, Sierra de Gádor; *A. Pallarés s.n.* (542102MA); 28, 30; AY691576 (ITS). *A. armerina* ssp. *caesia* (Boiss.) C. Díaz, C. Morales & F. Valle (SE Spain): (1) Granada, La Peza; *G. Nieto Feliner 4057GN* (MA); 36; F; AY691577 (ITS), AY692406 (*trnT-L*), AY692388 (*trnL-F*). (2) Granada, Víznar, Lobo pass; *V. Valcárcel & al. 15VV00* (MA); 36; AY691578 (ITS). (3) Granada, Sierra Nevada, Capileira; *G. López & R. Morales 2470* (296504MA); 36; AY691579 (ITS). (4) Granada, road from Cuéntar to La Peza; *G. Nieto Feliner 4047GN* (MA); 36; A; AY691580 (ITS), AY692419 (*trnT-L*), AY692400 (*trnL-F*), AY692265 (*trnS-G*). *A. cavanillesiana* (Font Quer & Rivas Goday) Nieto Feliner (CE Spain): (1) Cuenca, Uclés, Bedija river; *V. J. Arán & M. J. Tohá 14-06-1997* (593613MA); 20, 23; AY691581 (ITS). (2) Madrid, Villalbilla 30TVK7477; *Lansac & Nieto Feliner 1106GN* (346790MA); **23**; A; AY691582 (ITS), AY692416 (*trnT-L*), AY692394 (*trnL-F*). (3) Guadalajara, Yebra 30TWK0466; *Galán & Nieto Feliner 1093GN* (296508MA); **20**; A; AY691583 (ITS), AY692375 (*trnL-F*). (4) Cuenca, Huete, Saceda del Río 30TWK2949; *P. Galán & G. Nieto Feliner 1092GN* (346798MA); **20**; AY691584 (ITS). (5) Cuenca, Zarza de Tajo; *V. J. Arán & M. J. Tohá s.n.* (593615MA); 20, 23; AY691585 (ITS). (6) Cuenca, Carrascosa del Campo, Peña de la Saceda; *V.J. Arán & M. J. Tohá s.n.* (593617MA); 20, 23; AY691586 (ITS). *A. delaguardiae* G. López & Nieto Feliner (SE Spain): (1) Granada, Sierra de Cázulas, Cerro del Tranco, 30SVF3393; *G. López & G. Nieto Feliner 9494GL* (294673MA); 30; AY691587 (ITS). (2) Granada, Sierra de Cázulas, road from Granada to Almuñécar 30SVF3593; *G. López & G. Nieto Feliner 9496GL* (294672MA); 30; AY691588 (ITS). *A. erinacea* Boiss. [Spain (N, E and S)]: (1) Palencia, Santibáñez de Resoba 30TUN6652; *G. Nieto Feliner & Lansac 1152GN* (296932MA); **44**; A ; AY691589 (ITS), AY692403 (*trnL-F*). (2) Guadalajara, Sacecorbo - Ocentejo 30TWL5115; *G. Nieto Feliner & Lansac 1098GN* (296945MA); **52**; A, AY691590 (ITS), AY692373 (*trnL-F*). (3) Cádiz, Grazalema, Las Palomas pass 30YTF8874, *G. Nieto Feliner & Lansac 1088GN* (296919MA); **60**; A; AY691591 (ITS), AY69237889 (*trnL-F*). (4) Málaga, Sierra de la Nieves, Los Pilones pass; *C. Aedo & al. 1096CN* (526199MA); ?; AY691592 (ITS). (5) Guadalajara, Cifuentes - Canredondo 30TWL4016; *G. Nieto Feliner & Lansac 1094GN* (296944MA); **c. 52**; AY691593 (ITS). (6) León, Luna Reservoir; *E. Bayón & al. 8652SC* (296936MA); *c. 28*; A; AY691594 (ITS), AY692374 (*trnL-F*). (7) Burgos, Ciruelos de Cervera, 30TVM5743; *J.A. Alejandre 892* (485550MA); **40**; AY691595 (ITS). (8) Burgos, Humada, San Martín de Humada, Monte Portillo, 30TVN1521; *G. Nieto Feliner & Lansac 1147GN* (423124MA); **20**; AY691596 (ITS). (9) León, Ponferrada, Montes Aquilianos, Peñalba de Santiago, 29TQG0299; *Lansac & Nieto 302* (296933MA); **28**; AY691597 (ITS). *A. favargeri* (Nieto Feliner) G. López & Nieto Feliner (C Spain): (1) Ciudad Real, Herencia; *G. Nieto Feliner 4334GN* (MA); 34; A; AY691599 (ITS), AY692420 (*trnT-L*), AY692391 (*trnL-F*). (2) Albacete, La Pulgosa; *J.M. Herranz s.n.* (488094MA); 34; AY691600 (ITS). (3) Ciudad Real, La Guardia; *G. Nieto Feliner 4335GN* (MA); 34; AY691601 (ITS). *A. oscensis* (Pau) P. Monts. (C Pyrenees): (1) Huesca, San Juan de la Peña; *V. Valcárcel 115VV00* (MA); *28*; A; AY691602 (ITS), AY692390 (*trnL-F*). (2) Huesca, Sierra Javier, Mont Repós; *Aseginolaza & al. s.n.* (454389MA); 28; A; AY691603 (ITS), AY692410 (*trnT-L*), AY692389 (*trnL-F*). *A. querioides* Pourret ex Willk. (C and NW Spain): (1) Madrid, Zarzalejo, 30TVK9990; *G. Nieto Feliner 4263.1GN* (MA); *26*; D; AY691604 (ITS), AY692399 (*trnL-F*). (2) León, Sierra Cabrera 29TQG1879; *A. Barrá, R. Morales & Nieto Feliner 854GN* (296738MA); *52*; A; AY691605 (ITS), AY692395 (*trnL-F*). (3) León, Sierra Carbajal, 29TQG0282; Alamillo, Castroviejo 2501SC, *Fernández-Queirós & Nieto Feliner s.n.* (296748MA); **52**; AY691606 (ITS). (4) León, Carbajal pass; *E. Monasterio & J. J. Aldasoro 744MH* (531022MA); 52; AY691607 (ITS). (5) Madrid, Zarzalejo, 30TVK9905; *Nieto Feliner 838GN* (296743MA); **26**; C; AY691608 (ITS), AY692412 (*trnT-L*), AY692372 (*trnL-F*). (6) Ávila, Puerto del Pico; *P. Vargas 166PV01bis* (MA); 26; AY691609 (ITS). *A. racemosa* Willk. (SE Spain): (1) Málaga, Sierra Tejeda, Alcaucín; *G. Nieto Feliner 4011GN* (MA); 30; C; AY691610 (ITS), AY692417 (*trnT-L*), AY692396 (*trnL-F*). (2) Granada, Suspiro del Moro; *V. Valcárcel & al. 07VV00* (MA); 30; AY691611 (ITS). (3) Málaga, Competa, Sierra de la Almijara, Collado pass, 30SVF19; *P. Cubas & G. López 1980GL* (312788MA); 30; AY691612 (ITS). (4) Málaga, Sierra de Tejeda, Alcaucín; *G. Nieto Feliner 4013GN* (MA); 30; A; AY691613 (ITS), AY692411 (*trnT-L*), AY692401 (*trnL-F*), AY692266 (*trnS-G*), AY829339 (*trnH-psbA*). *A. tetraquetra* ssp. *amabilis* (Bory) H. Lindbl. fil. [Spain (Sierra Nevada)]: (1) Granada, Sierra Nevada, Mulhacén SVG7299; *Pizarro & al. s.n.* (577188MA); 40; A; AY691617 (ITS), AY692418 (*trnT-L*), AY692376 (*trnL-F*). (2) Granada, Sierra Nevada, Aguas verdes 30SVG60; *Molero Mesa & Pérez Raya s.n.* (298275MA); 40; A; AY691618 (ITS), AY692384 (*trnL-F*). (3) Granada, Sierra Nevada, Capileira, VG7001; *Betoño & al. 2281* (351192MA); 40; AY691619 (ITS). *A. tetraquetra* ssp. *murcica* (Font Quer) Favager & Nieto Feliner (SE Spain): (1) Albacete, Calar del río Mundo 30SWH45; *G. López & G. Nieto Feliner 1206* (298281MA); 60, 80, 100; A; AY691620 (ITS), AY692383 (*trnL-F*). (2) Jaén,

Appendix 1 (continued.)

Taxon (natural distribution): Sample number; Locality, followed in some cases by UTM coordinates; Voucher; Chromosome number (2*n*); *trnL-F* plastid haplotype (capital letters); GenBank accession (molecular marker).

Santiago de la Espada SW2110; *Soriano & al. s.n.* (457021MA); 60; K; AY691621 (ITS), AY692382 (*trnL-F*). (3) Jaén, Sierra de la Cabrilla 30SWG1797; *F. Muñoz-Garmendia & C. Soriano 364* (457023MA); 60, 80, 100; I; AY691622 (ITS), AY692414 (*trnT-L*), AY692386 (*trnL-F*). *A. tetraquetra* L. ssp. *tetraquetra* [Spain (C Pyrenees and C Spain)]: (1) Guadalajara, Sierra de la Pela, Somolinos 30TUL9568; *Lansac & Nieto Feliner 883GN* (240369MA); 140; AY691623 (ITS). (2) Huesca, Benasque, Pirineos mountain range; *V. Valcárcel & al. 117VV00* (MA); 120; A; AY691624 (ITS), AY692408 (*trnT-L*), AY692402 (*trnL-F*), AY692268 (*trnS-G*), AY829340 (*trnH-psbA*). (3) Lérida, Pallars Sobira, 30TCH3426; *V. J. Arán & M. J. Tohá s.n.* (618566MA); 120 ; AY691625 (ITS). (4) Huesca, Tella, *V. Valcárcel & al. 119VV00* (MA); 120; A; AY692385 (*trnL-F*). *A. tomentosa* Willk. (SE Spain): (1) Almería, Almirez; *G. Nieto Feliner 4165GN* (MA); 30; H; AY691614 (ITS), AY692421 (*trnT-L*), AY692381 (*trnL-F*), AY692267 (*trnS-G*). (2) Almería, Piedra de Lúcar plateau; *Pallarés & al. s.n.* (307677MA); 30; AY691615 (ITS). (3) Granada, Baza, sur Villosa 30TWG2048; *Lansac & Nieto Feliner 1084GN* (312775MA); **30**; H; AY691616 (ITS), AY692397 (*trnL-F*). *A. vitoriana* Uribe-Echebarría & Alejandre (CN and CE Spain): (1) Guadalajara, Sacecorbo - Ocentejo 30TWL5115; *Lansac & Nieto 1101GN* (346792MA); **32**; AY691626 (ITS). (2) Burgos, Condado de Treviño; *Uribe-Echevarría s.n.* (484739MA); 30, 32; A; AY691627 (ITS), AY692415 (*trnT-L*), AY692392 (*trnL-F*). (3) Álava, Salvatierra, Sierra Entzia, Iturrieta; *Betoño & J.A. Alejandre 1067* (365100MA); 30, 32; A; AY691628 (ITS), AY692393 (*trnL-F*).
***Arenaria* sect. *Grandiflorae* McNeill:** *A. grandiflora* L. ssp. *grandiflora* (Europe and N Africa): (1) Castellón, Peñagolosa, 40º13'19"N, 0º20'9"W; *J. Jiménez & al. 42* (626633MA); 22, 44; M; AY691556 (ITS), AY692404 (*trnT-L*), AY692369 (*trnL-F*). (2) Huesca, Peñas de Riglás, 30TXM7997; *P. Monteserrat & J. L. Benito s.n.* (589118MA); 44; AY691557 (ITS). *A. valentina* Boiss. (E Spain): Valencia, Cargante; *Vila, Hernández Viadel s.n.* (599163MA); ?; L; AY691558 (ITS), AY692405 (*trnT-L*), AY692398 (*trnL-F*).

Appendix 2. Description of states of each morphological character used in the phylogenetic analysis of *Arenaria* sect. *Plinthine*. *x*' = means a secondary basic number

1) Habit: not cushion-shaped (0); cushion-shaped (1). **2)** Growth pattern: innovation buds occurring in leaf axils all along the length of the flowering shoots, well above the first nodes of the season's growth and almost up to the terminal glomerule or flower (0); flowering shoots lacking innovation buds, which arise on vegetative shoots arising below the season growth of the fertile axis (1). **3)** Leaf margins: as thick as or thinner than the median nerve (0); thicker than the median nerve (1). **4)** Leaf apex: aristate (0); not aristate (1). **5)** Bracts: calyx not surrounded by bracts (0); calyx closely surrounded by pairs of bracts (1). **6)** Inflorescence: cyme (0); few or many-flowered head, sometimes with axilary heads below (1); solitary flowers in a cushion-shaped plant (2); false raceme (3). **7)** Calyx: sepals not clearly exceeding the bracts (0); sepals clearly overtopping the bracts (1). **8)** Pedicels > 2 mm (0); < 2 mm (1). **9)** Flower whorls: pentamerous (0); tetramerous (1). **10)** Sepal apex: all sepals acute (0); outer sepals obtuse, inner acute (1); all sepals obtuse (2). **11)** Sepal margin: not thickened (0); marginal nerve thickened in the upper half (1). **12)** Capsule: exserted (0); inserted (1). **13)** Capsule dehiscence: not hygrochastic (teeth closing up very slowly when wetted); hygrochastic (teeth opening in a few seconds when wetted). **14)** Seed ornamentation: without papillae (0); with papillae (1). **15)** Chromosome number: $x = 11$ (0); $x = 10$ or 20 (1); $x = 15$ (2). **16)** Abcission joints: stems without abcission joints (0); stems with abcission joints, disarticulating after fruiting either only below the flower or glomerule or also at lower nodes (1).

Appendix 3. Character states of the morphological dataset used in the phylogenetic analysis of *Arenaria* sect. *Plinthine*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
A. aggregata ssp. *aggregata*	0	1	0	0	1	1	0	1	0	0	1	?	1	0	2	1
A. aggregata ssp. *pseudoarmeriastrum*	0	1	0	0	1	1	0	1	0	0	1	1	1	0	?	1
A. alfacarensis	1	0	1	1	1	2	1	1	1	2	1	0	1	0	1	1
A. arcuatociliata	0	0	0	1	1	1	0	1	0	0	1	1	1	0	2	1
A. armerina ssp. *armerina*	?	1	0	1	1	1	1	1	0	1	1	0	1	1	2	1
A. armerina ssp. *caesia*	0	1	0	1	1	1	1	1	0	1	1	0	1	1	?	1
A. cavanillesiana	0	0	0	0	1	1	0	1	0	0	1	1	1	0	1	1
A. delaguardiae	0	0	0	?	1	3	1	1	0	0	1	1	1	1	2	1
A. erinacea	1	0	0	0	1	?	1	1	0	0	1	?	1	0	?	1
A. favargeri	0	0	0	0	1	1	0	1	0	0	1	1	1	0	?	1
A. grandiflora	0	0	0	0	0	0	?	0	0	0	0	0	0	0	0	0
A. oscensis	0	1	0	1	1	1	0	1	0	0	1	1	1	0	?	1
A. querioides	0	1	0	0	1	1	0	1	0	0	1	1	1	0	?	1
A. racemosa	0	0	0	0	1	3	1	1	0	0	1	1	1	0	?	1
A. tetraquetra ssp. *amabilis*	1	0	1	1	1	2	1	1	0	1	1	0	1	0	1	1
A. tetraquetra ssp. *murcica*	1	0	1	1	1	2	1	1	0	2	1	0	1	0	1	1
A. tetraquetra ssp. *tetraquetra*	1	0	1	1	1	2	1	1	1	2	1	0	1	0	1	1
A. tomentosa	0	0	0	1	1	1	1	1	0	1	1	1	1	0	2	1
A. valentina	0	0	0	0	0	0	?	0	0	0	0	?	0	0	0	0
A. vitoriana	0	1	0	1	1	1	1	1	0	2	1	1	1	1	2	1