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Original Citation:

New insights in Salicornia L. and allied genera (Chenopodiaceae) inferred from nrDNA / A. Papini; G. Trippanera; F. Maggini; R. Filigheddu; E. Biondi. - In: PLANT BIOSYSTEMS. - ISSN 1126-3504. - STAMPA. - 138(3):(2004), pp. 215-223.

Availability:

This version is available at: 2158/341835 since:

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23 September 2024

Plant Biosystems, Vol. 138, No. 3, November 2004, pp. 215 – 223

Taylor & Francis
healthsciences

ORIGINAL ARTICLE

New insights in *Salicornia* L. and allied genera (*Chenopodiaceae*) inferred from nrDNA sequence data

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Abstract

A phylogenetic analysis was performed based on ITS DNA sequences of fourteen samples from different sources of six species of *Salicornia*, the three allied genera *Arthrocnemum*, *Sarcocornia* and *Halocnemum* of the same tribe *Salicornieae*, and other genera of the subfamily *Salicornioideae* used in previous studies. *Bassia hirsuta*, *Camphorosma monspeliaca* (subfamily *Chenopodioideae*) and four species of *Suaeda* (subf. *Suaedoideae*) were chosen as outgroups. Results show that the annual genus *Salicornia* is a sister group to the perennial genera *Sarcocornia*, *Arthrocnemum* and *Halocnemum*. Moreover, the phylogenetic analysis based on ITS results distinguished two groups of *Salicornia* species which fitted with ploidy level: one group consisted of diploid species, and the second of tetraploid ones. *Sarcocornia* and *Arthrocnemum* are shown to be closely related, even though the species investigated here exhibited an evident distance between their ITS sequences. On the basis of our results, these two genera should be united. *Bienertia* (already separated as *Bienertieae*) was confirmed as probable outgroup to the subf. *Salicornioideae*, while *Kalidium* (subf. *Salicornioideae*, tribe *Halopeplideae*) was an outgroup to the rest of the *Salicornioideae* (tribe *Salicornieae*). The group *Allenrolfea* plus *Halocnemum* was the most basal of the tribe *Salicornieae* amongst those investigated in this study. The two samples of *Halocnemum strobilaceum* used in this work displayed numerous changes (transitions and transversions) in their respective sequences, probably related to their morphological and chorological differentiation. On the basis of our analysis, the most probable basal chromosome number for *Salicornieae* appears to be 2n = 18. The same number would also be the base number for the annual genus *Salicornia* and the perennial *Arthrocnemum* (+ *Sarcocornia*), with polyploidy arising independently in the two groups.

Key words: Arthrocnemum, Chenopodiaceae, *ITS, phylogeny, Salicornia, Sarcocornia.*

Abbreviations: ITS = Internal Transcribed Spacers; PCR = Polymerase Chain Reaction; BS = Bootstrap Support; nrDNA = nuclear ribosomal DNA

Introduction

Recent studies on the order *Caryophyllales* have raised doubts as to the autonomy of the family *Chenopodiaceae* from the *Amaranthaceae* (APG II, 2003; Cuénoud et al., 2002). For the purposes of our study, we preferred to maintain the name *Chenopodiaceae* as in Kühn, (1993) and Edmonson (1993). This recent classification of the *Chenopodiaceae* has divided the family into four subfamilies: *Chenopodioideae*, *Salicornioideae* (with the tribes *Salicornieae* and *Halopeplideae*), *Salsoloideae* and *Polycnemoideae*, while Schütze et al. (2003) considered the *Suaedoideae* as separate from the *Salsoloideae*. Despite a clear delimitation of the tribe *Salicornieae* [family *Salicorniaceae* according to some authors, e. g., Agardh (1858) and Scott (1977)], this tribe displays complex patterns of variation among different genera, and a controversial taxonomical classification in the genus *Salicornia* (glassworts). Morphological aspects of *Salicornia* were recently investigated in Europe by Géhu (1992), Iberite (1996) and Lahondere *et al.* (1992). The most evident characters of the *Salicornieae* are their succulent, articulated and apparently leafless stems, and the spike-like inflorescence of sessile, 3-flowered cymes, reduced flowers, usually consisting of a 2 – 4 lobed

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ISSN 1126-3504 print/ISSN 1724-5575 online © 2004 Società Botanica Italiana
DOI: 10.1080/11263500400006977

calyx tube with 1 – 2 stamens, and the subannular or curved embryo (Scott, 1977).

Present day classifications circumscribe *Salicornia* to annual species, while the perennial species are separated in other genera. Ball (1964) recognised two distinct sections of the genus in Europe, *Salicornia* and *Dolichostachyae*, the former diploid and the latter tetraploid. Several morphological characters were associated to this separation. The diploid series contains extremely different forms, and this variability is considered to be caused by the frequent autogamy of the species belonging to it (Ball, 1964; Iberite, 1996; Cristofolini and Chiapella, 1970).

The internal transcribed spacer (ITS) region is part of the transcriptional unit of the nrDNA cistrons and is constituted by the two spacers ITS1 and ITS2 which separate the 5.8S subunit from the 18S and 26S regions. The nrDNA genes are examples of a multigene family: they are tandemly repeated in thousands of copies at a chromosomal locus or at multiple loci (Rogers & Bendich 1987; Hamby & Zimmer, 1992), and are subjected to concerted evolution so that they do not evolve independently but in a concerted manner (Arnheim, 1983).

The utility of ITS sequences in plants for evaluating systematic relationships, even at the species level, is now well established (Baldwin et al., 1995; Hershkovitz & Lewis, 1996) also for the *Chenopodiaceae* (Pyankov et al., 2001; Schütze et al., 2003; Shepherd et al., 2004), even though Alvarez & Wendel (2003) proposed a broader investigation of the rDNA evolutionary process to evaluate the possibility of misleading results due to paralogy, compensatory base exchanges, and alignment problems due to indel accumulation.

The aim of our work was to ascertain the systematic relationships between the annual genus *Salicornia* and the allied perennial genera of the *Salicornieae*: *Sarcocornia, Arthrocnemum, Halocnemum* and other genera of the subfamily Salicornioideae. Moreover, we tested the phylogenetic relationships based on ITS sequence variations within the genus *Salicornia* by sampling taxa from the Mediterranean and Atlantic European coasts.

Materials and methods

Collection of samples

Fresh material was collected from 1999 to 2002, and identified by E. Biondi and R. Filigheddu of the Universities of Ancona and Sassari for the Italian samples, and by J. Izco and M. Herrera of the Universities of Santiago de Compostela and Bilbao for the Atlantic samples. A herbarium sample for each DNA sequence entry is deposited at the Universities of Ancona and Sassari and available from the authors. Species and samples investigated are listed, with their collection sites, in Table I. Five species and 14 samples (from different sites) of *Salicornia*, 1 species of *Arthrocnemum*, 2 species of *Sarcocornia*, 1 species and 2 samples (from different sites) of *Halocnemum* from the *Salicornioideae*, and 2 species of *Chenopodioideae* (*Bassia hirsuta* and *Camphorosma monspeliaca*) were sequenced. The other species included in the analysis were 4 species of *Suaeda* (*Suaedoideae*), the problematic *Bienertia cycloptera*, and other 6 species from the subf. *Salicorniodeae*: *Kalidium foliatum, Allenrolfea occidentalis, Microcnemum coralloides, Sclerostegia moniliformis and Tecticornia australasica*. These sequences were available in Genbank, and already used in an earlier study focused on the *Suaedoideae* (Schütze et al., 2003).

The nomenclature for Salicornieae followed Castroviejo et al. (1990) and Edmonson (1993).

DNA Extraction

DNA was extracted from vegetative branch segments following the extraction procedure described by D'Ovidio (1992).

PCR conditions

PCR reactions were carried out with 10 ng of genomic DNA in a total volume of 50 μl with 1.25 U of Taq polymerase (Perkin Elmer) for each reaction. The primers on the 18S sequence were 5′-CGTAACAAGGTTTCCGTAG, and on the 25S 5′-AGTCCGCCCTGATGGGCGA. The adopted thermal cycling profile consisted of 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C and a final extension step of 7 min at 72°C. Single-banded fragments were visualised on 1% agarose gels. The resulting single-banded amplification products were purified and directly sequenced in both directions by using the above described primers with an automated sequencer Perkin Elmer 310. Cycle Sequencing and the BigDye Terminator Ready reaction kit (Applied Biosystems) were used.

Sequence and phylogenetic analysis

The resulting ITS sequences were checked by eye with the software CHROMAS 1.43 (C. McCarthy, School of Biomolecular and Biomedical Sciences, Brisbane, Australia), assembled and aligned for several standard descriptive parameters (including size, percentage of G + C content, base substitution at conserved sites, percentage of pairwise divergence) with the Sequence Analysis Software DNAMAN-1999 (Lynnon Biosoft). A BLAST (Altschul et al., 1997) search was performed to exclude the sequencing of any contaminant organism.

Table I. Species, population (with geographical locality) and origin of the sample, with Genbank accession number of each sequence deposited by the authors (other authors are indicated) and available chromosome number with literature reference (not referred to the single population indicated).

Species	Locality	Genbank accession number	Chromosome number	Voucher (Herbarium)
Camphorosma monspeliaca L.	Porto Ferro (Sassari)	AJ577270	2n = 12 (1)	Available from the authors
Bassia hirsuta (L.) Ascherson	Foggia (FG)	AY641531		Available from the authors
Suaeda splendens (Pourr.) Gren. & Godr.	(16)	AY181844 (15)		(16)
Suaeda pannonica (Beck) Graebn.	(16)	AY181839 (15)		(16)
Suaeda monoica Forssk.	(16)	AY181859 (15)	2n = 18 (15)	(16)
Suaeda linifolia Pall.	(16)	AY181870 (15)	2n = 18 (2)	(16)
Bienertia cycloptera Bunge	(16)	AY181873 (15)		(16)
Microcnemum coralloides (Loscos & Pardo) Buen	(16)	AY181876 (15)		(16)
Sclerostegia moniliformis Wilson	(16)	AY181878 (15)		(16)
Tecticornia australasica (Moq.) Wilson	(16)	AY181877 (15)		(16)
Kalidium foliatum (Pall.) Moq.	(16)	AY181874 (15)	2n = 18 (2)	(16)
Allenrolfea occidentalis (S. Watson) Kuntze	(16)	AY181875 (15)	2n = 18 (3)	(16)
Halocnemum strobilaceum (Pall.) M. Bieb.	Stagno Santa Gilla (CA)	AJ578061	2n = 18 (4)	Available from the authors
Halocnemum strobilaceum (Pall.) M. Bieb.	Sacca di Bellocchio (RA)	AJ577568		Available from the authors
Salicornia veneta Pign. et Lausi	Palude la vela (TA)	AJ578064	2n = 36 (5)	Available from the authors
Salicornia veneta Pign. et Lausi	S'Ena Arrubia (OR)	AJ578065		Available from the authors
Salicornia veneta Pign. et Lausi	Sacca di Bellocchio (RA)	AJ578062		Available from the authors
Salicornia veneta Pign. et Lausi	Marina di Romea (RA)	AJ578063		Available from the authors
Salicornia emerici Duval-Jouve	S'Ena Arrubia (OR)	AJ578055	2n = 36 (6)	Available from the authors
Salicornia dolichostachya Moss	Stagno Santa Gilla (CA)	AJ578056	2n = 36 (7)	Available from the authors
Salicornia dolichostachya Moss	Bilbao (SP)	AJ578057		Available from the authors
Salicornia dolichostachya Moss	Foggia (FG)	AY641533		Available from the authors
Salicornia patula Duval-Jouve	Ancona (AN)	AJ578058	2n = 18 (9)	Available from the authors
Salicornia patula Duval-Jouve	Palude del Capitano (LE)	AJ578060		Available from the authors
Salicornia patula Duval-Jouve	S'Ena Arrubia (OR)	AJ578059		Available from the authors
Salicornia patula Duval-Jouve	Foggia (FG)	AY641532		Available from the authors
Arthrocnemum macrostachyum (Moric..) Moris.	S'Ena Arrubia (OR)	AJ577565	2n = 36 (10)	Available from the authors
Sarcocornia fruticosa (L.) A.J. Scott	S'Ena Arrubia (OR)	AJ577566	2n = 36, 54, 72 (11; 8)	Available from the authors
Sarcocornia fruticosa (L.) A. J. Scott var. *deflexa* (Rouy) C.H. Lahondère & J. Gamisans	Tenuta di Tombolo (Tuscany)	AJ577567		Available from the authors
Sarcocornia perennis Miller (= *Arthrocnemum perenne* (Miller) Moss = *Salicornia radicans* Sm.)	Galicia (SP)	AY641534	2n = 18 (11*; 8*; 12**; 13**; 14**)	Available from the authors

* As *Sarcocornia perennis* Miller. ** As *Arthrocnemum perenne* (Miller) Moss. (1) Ball & Akeroyd, 1993; (2) Lomonosova & Krasnikov, 1993; (3) Ward & Spellenberg, 1988; (4) Al-Turki et al., 2000; (5) Lausi, 1969; (6) Castroviejo & Valdés, 1990; (7) Huiskes et al., 1985; (8) Castroviejo & Lago, 1992; (9) Iberite, 1996; (10) Runemark, 1996; (11) Pastor & Valdés, 1986; (12) Queirós, 1985; (13) Luque, 1985; (14) D'Amato & Pavesi, 1990; (15) Subramanian, 1988; (16) Schütze et al., 2003. (AN) = Ancona; (CA) = Cagliari; (FG) = Foggia; (LE) = Lecce; (OR) = Oristano; (RA) = Ravenna; (SP) = Spain; (TA) = Taranto.

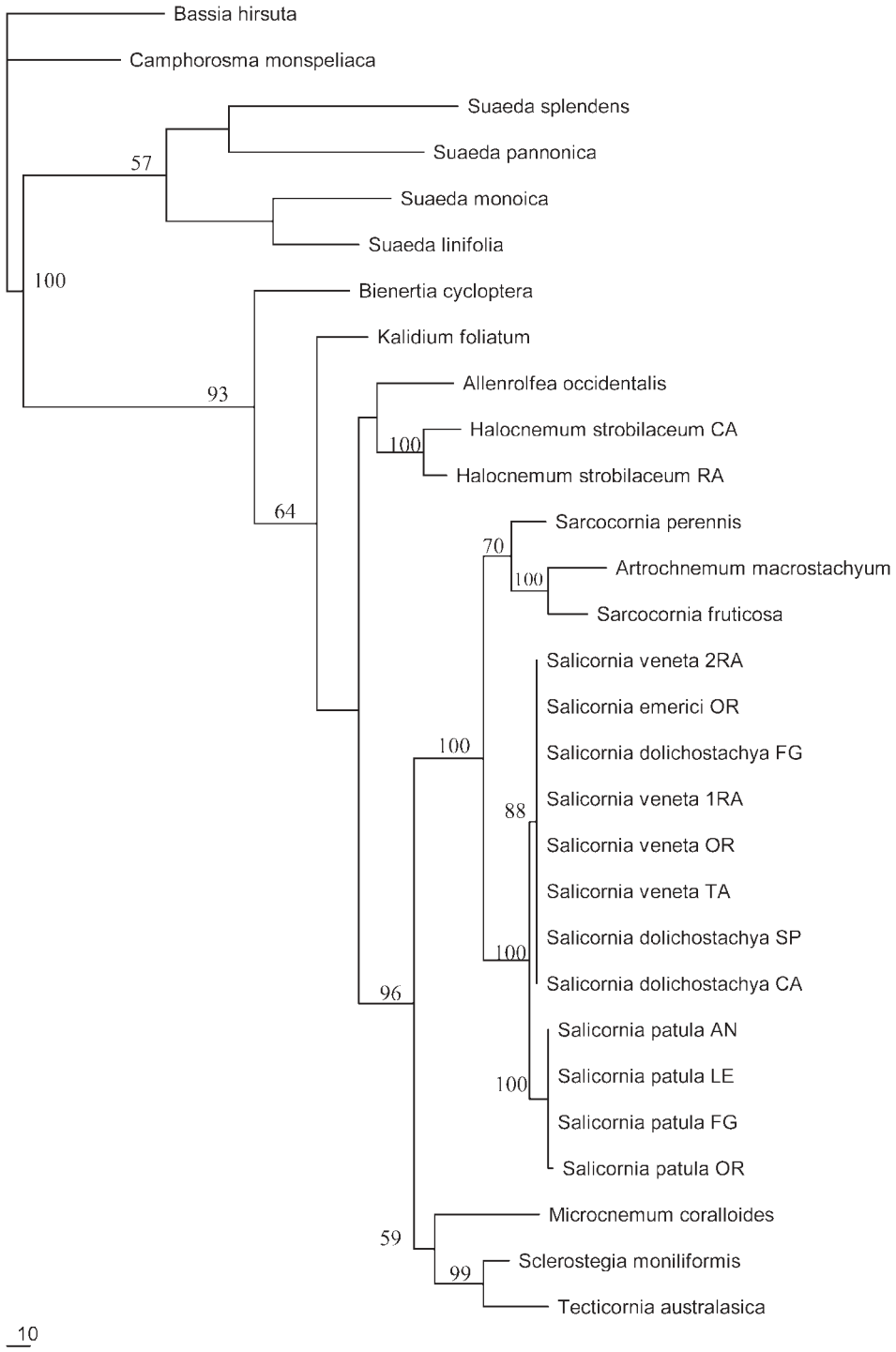

Figure 1. General alignment of *Salicorniodeae*. Numbers above the alignment indicate indel positions. Population locations of *Salicornieae*: (AN) = Ancona; (CA) = Cagliari; (FG) = Foggia; (LE) = Lecce; (OR) = Oristano; (RA) = Ravenna; (SP) = Spain; (TA) = Taranto.

The new ITS sequences produced during our investigation were deposited in Genbank (Table I). Optimal multiple alignment was obtained with CLUSTALW 1.81 (Thompson et al., 1994) and checked by eye. Parsimony analysis was performed with the PAUP 4.0b1 (Swofford, 1998) software for PC. All characters were weighted equally, and character state transitions were treated as unordered. Gaps were treated after Simmons & Ochoterena (2000), and coded with simple gap coding using the software Gapcoder (Young & Healy, 2003). This process codes indels as separate characters in a data matrix, which is then considered, along with the DNA base characters, in the phylogenetic analysis.

The maximum parsimony analysis was performed with 100 replicated heuristic searches, using random

stepwise addition of taxa, tree bisection reconnection (TBR) branch swapping, and MULPARS in effect.

A maximum likelihood (Felsenstein, 1981) search approach was carried out using Modeltest 3.06 (Posada & Crandall, 1998) to evaluate the likelihood of 56 different models of sequence evolution on the basis of our data. The likelihood ratio test option in Modeltest 3.06 was used to compare likelihood scores in a nested design. We used the most likely model of evolution from Modeltest 3.06 as settings in a maximum likelihood (ML) phylogenetic analysis in PAUP. The maximum likelihood heuristic search was done with 10 random additions and TBR branch swapping. The likelihood value of each of the previously obtained parsimony trees was calculated. Because of computational time limitations for the maximum likelihood analysis we used a data matrix containing only the data for the subf. *Salicornioideae*.

A neighbour-joining analysis (Saitou & Nei, 1987) was also performed on the complete data set. Gaps were excluded in the sequence divergence calculation. The neighbour-joining tree was produced using Kimura's two-parameter method (Kimura, 1980). This method assumes that all sites in a sequence evolve at the same rate and follow the same substitution scheme, and assumes a different frequency rate for transitions with respect to transversions. For sequences shorter than 1000 bp and which are not too

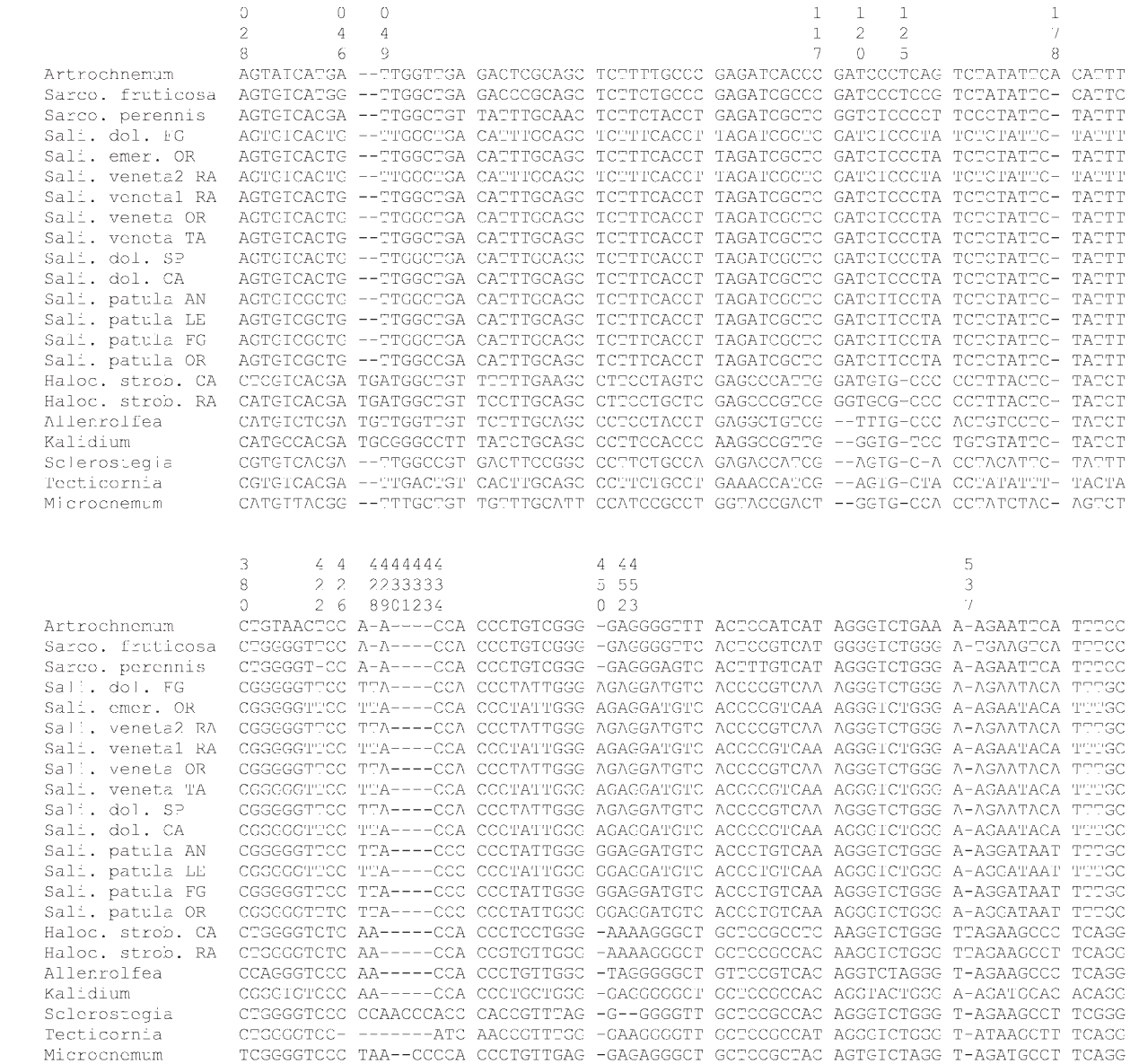

Figure 2. One of the most parsimonious trees is described (962 steps long, CI = 0.639, RI = 0.739). Bootstrap support is indicated on branch if higher than 50%. ITS tree of *Salicornioideae* and *Suaedoideae* (plus *Bienertia*) with *Bassia* and *Camphorosma* (*Chenopodioideae*) as outgroups, based on parsimony criterion with bootstrap support on branches. Gaps treated as separate characters (simple gap coding). Population locations of Salicornieae: (AN) = Ancona; (CA) = Cagliari; (FG) = Foggia; (LE) = Lecce; (OR) = Oristano; (RA) = Ravenna; (SP) = Spain; (TA) = Taranto.

divergent, this method gives acceptable results compared with models with more parameters and more time-consuming calculations also (Li, 1997).

Bootstrap (Felsenstein, 1985) resampling was performed using TBR branch-swapping with ten random taxon entries per replicate, and multrees option in effect both for parsimony (with 100 replicates) and neighbour joining (10000 replicates). The MacClade version 3.1 (Maddison & Maddison, 1992) was finally used to trace and map character states onto the consensus tree, and to evaluate less parsimonious positions of some taxa.

To test the significance of the difference of less parsimonious trees relative to the most parsimonious solution, the Templeton test (Templeton, 1983) was used as implemented in PAUP. The congruence of ITS1 and ITS2 data sets was evaluated using the incongruence-length difference (ILD) test of Farris et al. (1995) using PAUP.

Bassia hirsuta and *Camphorosma monspeliaca* (subfamily *Chenopodioideae*) were chosen as outgroups in the phylogenetic analyses. Some representatives of *Suaeda* were also included since the close relationship between *Suaedoideae* and *Salicornioideae* has been proposed in previous studies (Schütze et al., 2003)

Results

Analysis of ITS sequences

Nucleotide sequences of the internal transcribed spacers (ITS1 and ITS2) and the 5.8S coding region of nuclear ribosomal DNA repeats were visually inspected after the alignment. A sequence of *Sarcocornia fruticosa* var. *deflexa* corresponded completely to that of *Sarcocornia fruticosa* var. *fruticosa*, and hence was excluded from the subsequent phylogenetic analysis.

The ITS1 sequences vary from 234 bp (*Camphorosma monspeliaca*) to 241 bp (*Arthrocnemum macrostachyum*), the 5.8S is 165-bp long, and ITS2 varies from 219 bp (*Arthrocnemum* and *Sarcocornia*) to 229 bp (*Camphorosma*). A relevant difference between the two groups of *Salicornia* is the length of the ITS1 sequence: 239 bp for the diploid entities and 240 bp for the tetraploid ones. The two transversions in positions 542 and 543 in ITS2 are an important marker separating the two groups of *Salicornia*.

The two populations of *Halocnemum strobilaceum* exhibited 3 transversions and 11 transitions in ITS1, one transition in the 5.8S sequence, and 3 transversions and 7 transitions in ITS2. For each sequence, the G + C content of ITS1 was less than that of ITS2. The alignment of the ITS sequences of the species belonging to the subf. *Salicornioideae* is shown in Figure 1. Indels can be easily observed, and their position is indicated.

Phylogenetic analysis

For the purpose of our maximum parsimony phylogenetic analysis of the *Salicornioideae*, the ITS1 and ITS2 data sets (excluding indels) were found to be combinable according to the ILD test ($P = 0.109$).

The simplest maximum likelihood model identified for our data with Modeltest 3.06 assumed equal base frequencies, six substitution categories, and gamma distributed rate heterogeneity partitioned into four rate categories. These settings correspond to the General Time Reversible Model (GTR + G, Huelsenbeck & Crandall, 1997; Posada & Crandall, 1998). The maximum likelihood analysis produced a tree with a topology compatible with that obtained by parsimony. Maximum likelihood differed from maximum parsimony in that it was unable to separate the tetraploid clade of *Salicornia* s. s. (data not shown).

The maximum parsimony analysis of *Salicornioideae* was done with a heuristic search. Out of 670 characters (excluding the 74 gap-derived ones) 319 were constant, 109 parsimony-uninformative, and 243 parsimony-informative (319, 144 and 283 including gap characters, respectively). The maximum parsimony search produced four trees, 962 steps long, $CI = 0.639$, $RI = 0.739$ (simple gap coding was applied, and gaps treated as separate characters). One of the most parsimonious trees is described in Figure 2. Bootstrap support is indicated on the branch if higher than 50%. Omitting gaps from the analysis produced similar phylogenetic results, but with lower bootstrap support (data not shown).

Alternative phylogenetic hypotheses were tested with MacClade, starting from the maximum parsimony tree. We tested older or more recent taxonomic treatments not completely corresponding to our phylogenetic reconstruction. Putting *Sarcocornia fruticosa* together with *Salicornia* s. s. produced an 18-step longer tree, and a statistically significant difference using the Templeton test. Constraining *Arthrocnemum macrostachium* with *Salicornia* s. s. produced a 19-step longer tree, and a statistically significant difference (Templeton test).

Putting *Sarcocornia perennis* (= *Arthrocnemum perenne* = *Salicornia radicans*) as outgroup to *Salicornia* s. s. costed 3 steps more, and yielded a statistically not significant difference (Templeton test), whereas combining the two representatives of *Sarcocornia* produced a 14-step longer tree, which was statistically different from the most parsimonious one. Grouping together the four samples of *Salicornia dolichostachya* produced a maximum parsimony tree, and no significant difference according to the Templeton test.

In 50% of the maximum parsimony trees *Allenrolfea* clustered together with *Halocnemum*; in all trees these two species resulted as outgroup to the rest of

the tribe *Salicornieae*, and shared common insertions in 47–48 and 214 with *Kalidium* (*Halopeplideae*). *Microcnemum*, *Sclerostegia* and *Tecticornia* clustered together with 59% bootstrap support and an insertion in 568. This group was the outgroup to *Salicornia* s. l. (*Salicornia* + *Arthrocnemum* + *Sarcocornia*) with 96% bootstrap support. *Arthrocnemum* and *Sarcocornia* are related (70% bootstrap and a common deletion in 428), and occupy a basal position relative to *Salicornia*. *Sarcocornia perennis* was basal with respect to *Arthrocnemum macrostachyum* and *Sarcocornia fruticosa* (grouping with 100% bootstrap).

Salicornia turned out to be monophyletic, with a bootstrap index of 100%. The sequences obtained from 14 samples of *Salicornia* belonging to 5 different recognised species clustered in two groups in each of which further phylogenetic relationships were not clear on the basis of the ITS sequences. These two groups, obtained with the ITS sequence analysis, fitted with the caryological data: one group comprised the four populations of the diploid *Salicornia patula* (100% bootstrap), while the second (88% bootstrap and an insertion in 242) consisted of the tetraploid entities (*Salicornia veneta, S. dolichostachya* and *S. emerici*). With the neighbour-joining analysis (not shown), the two populations of *Salicornia patula* from Ancona and the one from Lecce clustered together with a bootstrap support of 82%.

Using the same reduced data set for the *Salicornioideae*, under the parsimony criterion (data not shown) the maximum likelihood tree was only one step longer than the maximum parsimony one, with the same CI and RI values, and the two were not statistically different according to the Templeton test.

Discussion

The identity of the sequence of *Sarcocornia fruticosa* var. *deflexa* with the sequence of *Sarcocornia fruticosa* confirmed that the former is only a rooting ecotype of the latter growing mainly in areas more frequently subjected to salt-water level variations during the year. Hence, the value given to the rooting system in the taxonomy of perennial *Salicornia* s.l. has probably been overemphasised (Géhu & Biondi, 1992).

The phylogenetic analysis produced quite robust results, confirmed both by high bootstrap values and by checking alternative phylogenetic hypotheses with MacClade.

The monophyly of the tribe *Salicornieae* was supported by a 64% bootstrap index, but by no indel, while the monophyly of the group *Salicornia* + *Arthrocnemum* + *Sarcocornia* was supported by 100% bootstrap and two deletions in 430–433 and 566–568. In this study, *Halocnemum* and *Allenrolfea* turned out to be the most basal representatives of the *Salicornieae*; this position was reinforced by two plesiomorphic insertions common to *Kalidium* (*Halopeplideae*).

Results also indicate that the annual genus *Salicornia* is derived from the perennial taxa. The closest perennial ancestors of *Salicornia* amongst those investigated in this study are *Sarcocornia* and *Arthrocnemum*, while *Halocnemum* appears to be sister to the rest of the *Salicornieae*. This result can be related to the ecological analyses on salinity gradients (Andreucci *et al.*, 2000), indicating that the annual species of *Salicornia* s.s. occupy the soils with the highest concentration of salt (which were previously empty ecological niches). The perennial species also followed the salinity gradient, which represents the fundamental ecological factor influencing the phylogenetic radiation of the *Salicornieae*.

The monophyly of the annual genus *Salicornia* has 100% bootstrap support. Despite the high morphological heterogeneity of the genus, *Salicornia* showed only one evident separation on the basis of the ITS sequences, i.e., between diploid (2n = 18) and tetraploid (2n = 36) species.

The four populations of the diploid *Salicornia patula* clustered together with 100% bootstrap, while the monophyly of the tetraploid species had 88% bootstrap support. The fact that the two Italian samples of *Salicornia patula* clustered together with 82% bootstrap, as revealed by neighbour-joining analysis, can be easily explained on phytogeographical grounds.

The maximum likelihood tree differed from the maximum parsimony one in not being able to cluster together the tetraploid clade. Since this phylogenetic hypothesis cost, under the parsimony criterion, only one step more, with no significant difference according to the Templeton test, the relationships between the two groups of *Salicornia* might require further investigation. The fact that the maximum likelihood analysis produced a very similar tree to the maximum parsimony ones lends support to our results.

According to Ball (1964), two distinct series of *Salicornia* species are present in Europe, one of which is diploid and the other tetraploid. Associated with each series are several morphological character states, such as the capacity to produce red pigmentation, the number of stamens, and the position of the three flowers. Other quantitative character states which fit with this division, after Ball & Tutin (1959), are seed diameter, anther size, and pollen diameter. Our results (under maximum parsimony) confirm Ball's (1964) opinion on the autonomy of the two series. Based on our analyses, the closest relatives to the annual *Salicornia* are *Sarcocornia* and *Arthrocnemum*. The chromosome numbers of the three species included in the analysis are, respectively, 2n = 36, 54, 72 for *Sarcocornia fruticosa* (Pastor & Valdés, 1986; Castroviejo & Lago 1992), 2n = 36 for *Arthrocnemum macrostachyum* (Runemark, 1996), and 2n = 18 for

Sarcocornia perennis (Pastor & Valdés, 1986; Castroviejo & Lago, 1992; Queiròs, 1985; Luque, 1985; D'Amato & Pavesi, 1990). The most basal genera of the *Salicornieae* included in the analysis are *Halocnemum strobilaceum* with chromosome number 2n = 18 (Al-Turki et al., 2000), or 2n = 36 (Castroviejo *et al.*, 1990) and *Allenrolfea occidentalis*, with n = 9 (Ward & Spellenberg, 1988), while for the representative of the other tribe of the *Salicornioideae* (tribe *Halopeplideae*) included in the analysis, *Kalidium foliatum*, 2n = 18 was reported by Lomonosova & Krasnikov (1993). As for the two *Chenopodioideae* inserted in our preliminary analysis, 2n = 12 was reported (Ball & Akeroyd, 1993) for *Camphorosma monspeliaca*, and 2n = 18 for *Suaeda linifolia* (Lomonosova & Krasnikov, 1993) and *S. monoica* (Subramanian, 1988). On the basis of our analysis, the most probable basal number for the tribe *Salicornieae* appears to be 2n = 18; it is the number of the most basal genera of the investigated *Salicornieae*, and it is also present in the *Halopeplideae*. The difficulty in clearly segregating samples belonging to different species in the tetraploid clade, such as *S. veneta, S. emerici* and *S. dolichostachya*, might depend on a too recent species separation, on a too slowly evolving ITS sequence in these phylogenetic branches, or on an incomplete sexual separation in these species.

Lausi (1969) described *Salicornia veneta* (all samples in the tetraploid clade) in peculiar formations of the Venice Lagoon called ''barene'', and indicated 2n = 36 as the chromosome number of this species. The species was considered endemic of the North Adriatic sector (Pignatti, 1982), but it was recently found by Filigheddu et al. (2000) in the S'Ena Arrubia Lagoon in the gulf of Oristano (Sardinia). According to Iberite (1996), *S. emerici* is considered to be closely related to *S. veneta*, and also tetraploid.

The fact that, by comparing the sequences from two samples of *Halocnemum strobilaceum*, we found 6 transversions and 18 transitions as well as a transition in the 5.8S sequence could be explained by it being a more ancient species than the other investigated *Salicornieae.* Thus, further infraspecific morphological investigations for this taxon are necessary. A preliminary analysis revealed some interesting differences in habitus (probably corresponding to chorological separation) among the two populations.

The two species *Arthrocnemum macrostachyum* and *Sarcocornia fruticosa* clustered together with 99% bootstrap support, thus confirming the strict relationship between these two genera. However, these two sequences appeared to be divergent, with various transitions and transversions both in the ITS1 and in the ITS2, and also a transition in the highly conserved 5.8S region. *Sarcocornia perennis* clustered basally to these two species. The topology of this subtree is in better accord with Ball (1993) and Moss (1954), who kept these three taxa united (genus *Arthrocnemum*), than with the proposal of Scott (1977) or Castroviejo et al. (1990) of keeping *Sarcocornia* separated from *Arthrocnemum*. The position of *Arthrocnemum perenne* would indicate this species as the closest to the common ancestor of *Salicornia* + *Arthrocnemum* (+ *Sarcocornia*). Since 2n = 18 is the basal chromosome number of *Sarcocornia perennis,* it is probably the basal chromosome number of the whole group *Salicornia-Arthrocnemum* (-*Sarcocornia*), with polyploid series arising separately in the perennial and annual groups.

The identity of the ITS sequences of *Sarcocornia fruticosa* var. *deflexa* (found in Corsica by Jeanmonod & Burdet, 1988) with these of *Sarcocornia fruticosa* var. *fruticosa* indicate that the morphological differences between the two varieties are probably of poor taxonomical value.

Acknowledgements

The authors would like to thank Prof. Jesus Izco of the University of Santiago de Compostela for the collection of samples of *Sarcocornia perennis*, Prof. Mercedes Herrera of the University of Bilbao for the samples of *Salicornia dolichostachya*, and two anonymous reviewers for their useful comments.

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