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Polyphyly of *Limoniastrum* (Plumbaginaceae): evidence from DNA sequences of plastid *rbc*L, *trn*L intron and *trn*L-F intergene spacer

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Phylogenetic relationships of *Limoniastrum* and other genera of subfamily Staticoideae (Plumbaginaceae) were studied using parsimony analysis of the plastid gene *rbc*L, the intron of *trn*L and the intergene spacer of *trn*L-*trn*F. Our analysis showed that *Limoniastrum* was polyphyletic. *Limoniastrum ifniense*, in both *rbc*L and combined data analyses, is sister to *Armeria* and *Psylliostachys*, whereas in the *trn*L-F (intron and spacer combined) analysis it is sister to a clade composed of *Acantholimon*, *Dictyolimon* and the remaining species of *Limoniastrum*. In all analyses, the five remaining species of *Limoniastrum* (excluding *Limoniastrum ifniense*) formed a clade with two groups of species: *L. monopetalum* + *L. guyonianum* and those sometimes considered as the segregate genus *Bubania* (*L. feei*, *L. weygandiorum* and *L. rechingeri*). Levels of sequence divergence among these three groups of *Limoniastrum* were greater than for other well supported genera in the family and, in combination with morphological differences and paucity of synapomorphies, led us to conclude that separate generic status for each of the three clades is warranted.

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CONTENTS

Introduction 176
Material and methods 177
Total DNA extraction 177
PCR amplification and sequencing 177
Cladistic analyses 179
Molecular evolution 179
Results 180
Relationships according to *rbc*L 180
Relationships according to *trn*L-F 181
Relationships indicated by combined data 183
Limoniastrum clade 184
Data set comparisons 184
Discussion 187
Molecular evolution 187
The polyphyly of *Limoniastrum* 188
Taxonomic implications 189
Acknowledgements 190
References 190

INTRODUCTION

The genus *Limoniastrum* Heist. ex Fabr. is often considered to comprise seven species of shrubs, found mostly in coastal and saline dry areas of the Mediterranean and sub-Saharan Africa (Ozenda, 1983). Morphologically they are distinct from other genera of Plumbaginaceae Juss. in having petals that form a long tube, convolute inner bracts and styles that are connate for half their length (Boissier, 1848).

Limoniastrum has been divided into two sections (Battandier, 1890; Maire & Wilczek, 1935) or subgenera (Maire, 1936): (i) sect. *Limoniastrum* ('*Eulimoniastrum*') for tall shrubs with alternate leaves and 3-bracteate spikelets with a hornless inner bract, and (ii) sect. *Bubania* Batt. for dwarf shrubs with rosulate leaves and 2-bracteate spikelets with a horned inner bract (Girard, 1848). Another genus, *Lerrouxia* Caball. (Caballero, 1935), was described from the western Sahara and included a new species, *Lerrouxia ifniensis* Caball. related to *Limoniastrum*. This name later proved to be illegitimate, and the genus was renamed *Caballeroa* Font Quer (Font Quer, 1935), although this taxon has been accepted by many authors as a species of *Limoniastrum*. Some authors recognized all three groups as genera (e.g. Linczevski, 1968), whereas others have either united them under *Limoniastrum* (Kubitzki, 1993) or segregated only *Bubania* (Brummitt, 1992).

In a molecular study of Plumbaginaceae and related families, *Limoniastrum monopetalum* (L.) Boiss. was sister to *Limonium* Mill. and the clade formed by *Acantholimon* Boiss. and *Dictyolimon* Rech.f., although the relationships of these four genera were not well supported (Lledó *et al.*, 1998). The clade formed by all these taxa was sister to *Armeria* Willd. and *Psylliostachys* (Job. & Spach) Nevski. The subfamily Staticoideae Kostel. was a strongly supported group sister to Plumbaginoideae Burnett, and together these two clades were sister to Polygonaceae Juss. (Lledó *et al.*, 1998).

This paper examines molecular support and morphological characters of the genera among taxa formerly included in *Limoniastrum* and their relationships to other genera of Plumbaginaceae. We sampled three plastid DNA regions; *rbc*L, which has

generally been used at the interfamilial level in angiosperms but is evolving significantly faster in the Caryophyllales *s.l.*, to which Plumbaginaceae belong (Fay *et al.*, 1997; Lledó *et al.*, 1998) and the region consisting of the *trn*L intron, the 3′ *trn*L exon (only 51 bp) and the intergene spacer of *trn*L-*trn*F (hereafter *trn*L-F) which have been suggested to exhibit more variation than conserved coding regions such as *rbc*L (Taberlet *et al.*, 1991).

MATERIAL AND METHODS

Total DNA extraction

Twenty species, representative of the range of morphological diversity of Staticoideae, including six species of *Limoniastrum* (*s.l.*) and two species of Plumbaginoideae (as the outgroup) were chosen for this study (Table 1). Fresh tissue, silica gel-dried material (Chase & Hills, 1991), seeds or leaf fragments obtained from herbarium specimens (Savolainen *et al.*, 1995) were used for DNA extraction. Total DNA was isolated using a modified CTAB method (Doyle & Doyle, 1987) and purified by caesium chloride-ethidium bromide density-gradient centrifugation (1.55 g/ml density).

PCR amplification and sequencing

The *rbc*L exon was amplified using a set of primers described in Lledó *et al.* (1998). The *trn*L-F region was amplified using the universal primers c and f described in Taberlet *et al.* (1991). For most intact DNAs, both regions were amplified as a single fragment. For degraded DNA from herbarium specimens, we used internal primers to amplify each region in two fragments. For *rbc*L we used the combination of primers 1F-724R and 636F-1367R described in Lledó *et al.* (1998); for *trn*L-F we used the combination of primers c-d and e-f described in Taberlet *et al.* (1991).

Bovine serum albumin (BSA, 0.004–0.016% w/v) was added to the PCR reactions for all taxa to bind inhibitory compounds (Savolainen *et al.*, 1995). Amplified DNA products were purified using Wizard mini columns (Promega, Madison, Wisconsin, U.S.A.) following manufacturer's protocols.

Standard dideoxy methods or modified dideoxy cycle sequencing with dye terminators run on an ABI 373A or ABI 377 automated sequencer (according to the manufacturer's protocols; Applied Biosystems, Inc., Warrington, U.K.) were used to sequence the amplification products directly. For *rbc*L, sequencing was carried out in four separate reactions using primers 1F, 724R, 636F and 1367R, or primers binding in similar positions described in Lledó *et al.* (1998). When the *trn*L-F region was amplified in one fragment, sequencing was carried out using two reactions with primers c and f. When *trn*L-F was amplified in two fragments, sequencing was performed in four reactions, using the same primers as for amplification (c, d, e and f).

TABLE 1. Sources of plant material. The *trn*L-F sequences presented are cited for the first time in this paper. Some *rbc*L sequences were cited previously and literature references are given

	Voucher	*rbc*L sequence accession number	Literature reference (for *rbc*L)	*trn*L-F sequence accession number
Acantholimon acerosum Boiss.	Chase 709, K	Z79639	Fay *et al.*, 1997	AJ391314
Armeria bottendorfensis A. Schulz.	Chase 708, K	Z79640	Fay *et al.*, 1997	AJ391315
Armeria splendens (Lag. & Rod.) Rech.f.	Chase 1895, K	Y16908	Lledó *et al.*, 1998	AJ391316
Dictyolimon macrorrhabdos (Boiss.) Batt.	Chase 710, K	Y16909	Lledó *et al.*, 1998	AJ391317
Limoniastrum feei (Girard) Batt.	Chase 1630, K	AJ286357	This paper	AJ391318
Limoniastrum guyonianum Durieu ex Boiss.	ABH14933	AJ286358	This paper	AJ391319
Limoniastrum ifniense (Caball.) Font Quer	K. Gnichard, BM	AJ286359	This paper	AJ391320
Limoniastrum monopetalum (L.) Boiss.	ABH 10964	Z97642	Fay *et al.*, 1997	AJ391321
Limoniastrum rechingeri J.R.Edm.	Chase 1632, K	AJ286360	This paper	AJ391322
Limoniastrum weygandiorum Maire	Schouten 192, LIV	AJ286361	This paper	AJ391325
Limonium axillare (Forssk.) Kuntze	Chase 1637, K	AJ286362	This paper	AJ391323
Limonium delicatulum (Girard) Kuntze	ABH 10730	Y16903	Lledó *et al.*, 1998	AJ391324
Limonium dufourii (Girard) Kuntze	ABH 670	AJ286363	This paper	AJ391326
Limonium narbonense Mill.	ABH10713	AJ286364	This paper	AJ391327
Limonium rigualii M.B.Crespo & Erben	ABH 303	Z97645	Fay *et al.*, 1997	AJ391328
Limonium sinuatum (L.) Mill.	ABH 9232	Y16900	Lledó *et al.*, 1998	AJ391329
Limonium spectabile (Svent.) Kunkel & Sunding	Chase 667, K	Z97646	Fay *et al.*, 1997	AJ391330
Limonium tenellum (Turz.) Kuntze	Unknown	AJ286365	This paper	AJ391331
Limonium vulgare Mill.	ABH 15440	Y16904	Lledó *et al.*, 1998	AJ391332
Psylliostachys suworowii (Regel) Roslk.	Chase 711, K	Y16907	Lledó *et al.*, 1998	AJ391335
Outgroups				
Ceratostigma minus Stapf. ex Prain	Chase 707, K	Z97641	Fay *et al.*, 1997	AJ391333
Plumbago europaea L.	ABH 16134	Y16906	Lledó *et al.*, 1998	AJ391334

Cladistic analyses

Phylogenies were inferred for *rbc*L, *trn*L-F and both data sets combined. For the *trn*L-F region, sequences were aligned using the Clustal option of Sequence Navigator (Applied Biosystems Inc.) and this first alignment was then optimized by eye. Gaps appearing in more than one taxon which had identical length and position were coded and analysed as binary (presence/absence) characters. These characters were added to both *trn*L-F and combined data matrices.

Heuristic analyses were performed with PAUP 3.1.1 (Swofford, 1993) under the Fitch criterion (equal weights; Fitch, 1971), using 1000 random taxon additions and tree bisection–reconnection branch swapping (TBR), MULPARS, but holding only five trees per replicate to minimize the time spent in swapping on suboptimal trees. The shortest trees from all replicates were then used as starting trees for a final round of heuristic search, in which all trees were swapped to completion. Characters were then successively weighted (successive approximations weighting, SW; Farris, 1969) based on the rescaled consistency index (RC), a base weight of 1000, and their maximum value if more than one tree was found. After that, a heuristic search was performed with MULPARS and TBR in effect and simple stepwise addition. Successive rounds of weighting/searching were performed until in two successive rounds the same tree length was obtained. Internal support was examined by the bootstrap (Felsentein, 1985) with SW weights applied, 5000 replicates and subtree pruning regrafting (SPR) swapping, which is faster than TBR but in our experience just as successful in obtaining optimal trees.

The three data sets (*rbc*L, *trn*L-F and combined) were also analysed using heuristic searches with 10 random taxon-additions, but enforcing a topological constraint in which the genus *Limoniastrum* was monophyletic.

Molecular evolution

Three data sets, *rbc*L, *trn*L intron and *trn*L-F intergene spacer, were used to study the molecular evolution of the plastid DNA regions sequenced. The *trn*L-F region is in fact composed of an intron, exon and intergene spacer. For this reason, the matrix was divided into those three parts, and then the *trn*L intron and *trn*L-F intergene spacer were analysed. The *trn*L exon was excluded from these analyses due to its short length and near absence of changes. For these three regions (*rbc*L, *trn*L intron and *trn*L-F intergene spacer), heuristic analyses were performed with PAUP 3.1.1. under the Fitch criterion, 1000 random taxon additions, TBR swapping and MULPARS, but holding only five trees per replicate. Trees from all replicates were then used as starting trees for a final round of heuristic search, with all trees swapped to completion. Using these trees, the number of variable and informative characters were calculated using PAUP* 4.0d64 written by David L. Swofford, and MacClade version 3.01 (Maddison & Maddison, 1992). To calculate the number of transitions and transversions observed on one of the shortest trees (as well as their CIs and RIs), we used the following step matrix to calculate the number of transversions at each base position:

	A	C	G	T
A	–	1	0	1
C	1	–	1	0
G	0	1	–	1
T	1	0	1	–

From this number of transversions and their collective CI and RI, we could calculate the number of transitions.

RESULTS

Relationships according to rbc*L*

The matrix analysed had 1310 characters, of which 265 characters were variable and 122 potentially phylogenetically informative (Table 4). Three most parsimonious trees were found of 438 steps, consistency index (CI) of 0.69 and retention index (RI) of 0.62. After SW, one of these most parsimonious trees was retained (weighted length = 235136, CI = 0.92 and RI = 0.85; Table 2). This tree is shown in Figure 1 with its Fitch lengths shown above the branches (ACCTRAN optimization) and bootstrap percentages below.

TABLE 2. Lengths and fit measures of unit-weighted and SW trees obtained for *rbc*L, *trn*L-F and combined analyses

		*rbc*L	*trn*L-F	Combined
Unit-weighted	Number of trees	3	15	1
	Length	438	434	874
	CI	0.69	0.83	0.76
	RI	0.62	0.82	0.72
SW	Number of trees	1	3	1
	Length (Fitch)	235 136 (438)	316 562 (434)	550 611 (874)
	CI (Fitch)	0.92 (0.69)	0.95 (0.83)	0.94 (0.76)
	RI (Fitch)	0.85 (0.62)	0.95 (0.82)	0.91 (0.72)

The following three groups were found: (i) a clade formed by *Psylliostachys* and *Armeria* (99% bootstrap) that is sister to *Limoniastrum ifniense*; (ii) a clade with *Acantholimon* and *Dictyolimon* (82% bootstrap) that is sister to the rest of the species of *Limoniastrum*, although this branch is not present in the strict consensus of the equally weighted trees; (iii) the genus *Limonium* with two well defined subclades, respectively corresponding to subgenus *Pteroclados* (Boiss.) Pignatti (100% bootstrap) and the remaining species (94% bootstrap). Relationships among these main three clades were unresolved before SW (see open-headed arrows in Fig. 1). The *Limoniastrum* clade (excluding *L. ifniense*) has moderate bootstrap support (89%) and is divided in two subclades, sect. *Limoniastrum* and sect. *Bubania* (Fig. 1) which are strongly supported by the bootstrap (100% and 97%, respectively).

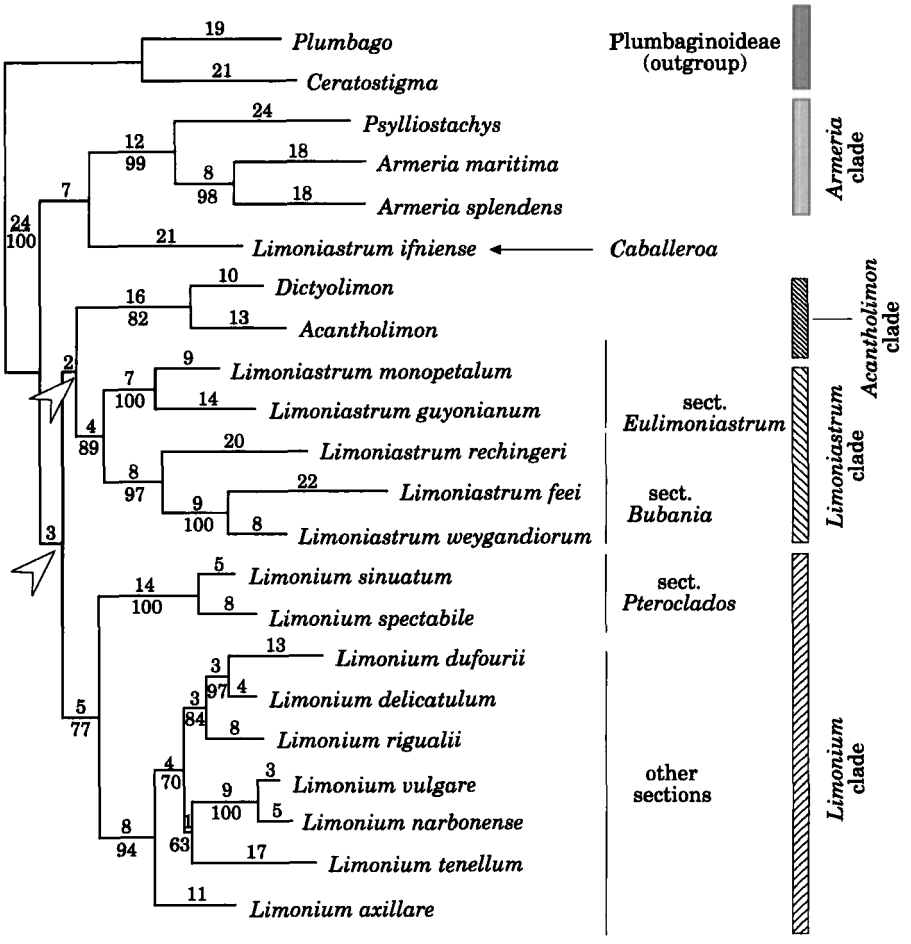


Figure 1. The single most parsimonious SW tree found with *rbc*L. Fitch lengths (ACCTRAN optimization) are shown above the branches, and bootstrap values are shown below. Branches not found in the Fitch strict consensus are marked with an open-headed arrow. This tree is one of those found with Fitch (unit-weighted) parsimony (tree length = 438 steps; CI = 0.69; RI = 0.62).

Relationships according to trn*L-F*

The length of the *trn*L-F region varied from 850 bp in *Armeria bottendorfensis* A.Schulz to 953 bp in *Limoniastrum rechingeri* J.R.Edm. The final length of the matrix after alignment was 1080 bp. The number of variable characters was 157 for the *trn*L intron and 138 for the *trn*L-F spacer, of which 83 and 94 characters, respectively, were potentially informative (Table 4). Fifteen most-parsimonious trees were found with 434 steps, CI = 0.83, and RI = 0.82. Successive weighting identified three of these 15 as optimal (weighted length = 316562, CI = 0.95 and RI = 0.95). One of these trees is shown in Figure 2 with its Fitch lengths shown above the branches (ACCTRAN optimization) and bootstrap percentages below.

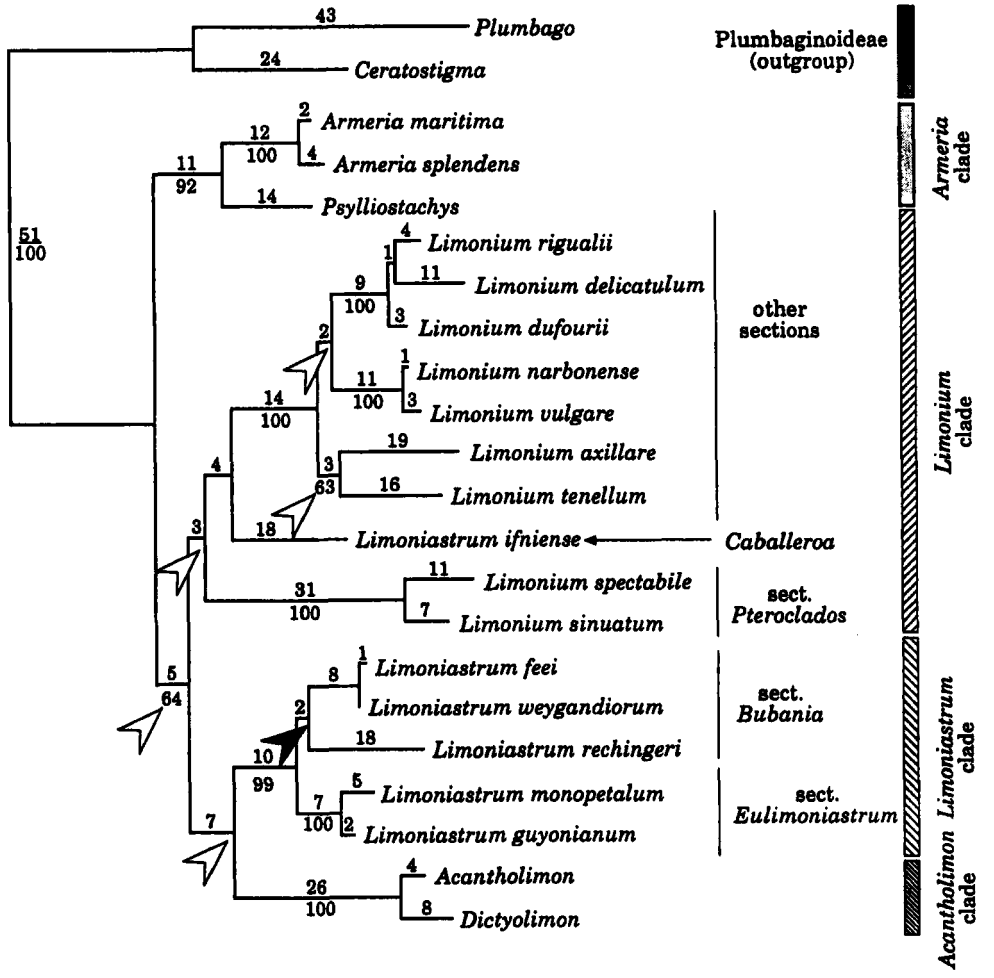


Figure 2. One of the three most parsimonious SW trees found with *trn*L-F. Fitch lengths (ACCTRAN optimization) are shown above the branches, and bootstrap values are shown below. Branches not found in the weighted strict consensus are marked with a solid arrow and those not found in the Fitch strict consensus are marked with a open-headed arrow. This tree is one of those found with Fitch (unit-weighted) parsimony (tree length = 434 steps; CI = 0.83; RI = 0.82).

The main clades present in the *rbc*L trees are also present in these trees, although relationships among them differ. *Limoniastrum ifniense* is sister to the clade formed by all *Limonium* species excluding subgenus *Pteroclados* (represented by *L. sinuatum* (L.) Mill. and *L. spectabile* (Svent.) Kunkel & Sunding). The position of *Limoniastrum rechingeri* is not resolved within the *Limoniastrum* clade. Bootstrap values are high in each clade (*Limoniastrum* clade 99%; *Acantholimon* clade 100%, *Limonium* subgenus *Pteroclados* 100%, other sections of *Limonium* 100%, *Armeria* clade 92%). Relationships among these clades are not supported, and these branches are unresolved in the strict consensus of the Fitch trees (see open-headed arrow in Fig. 2).

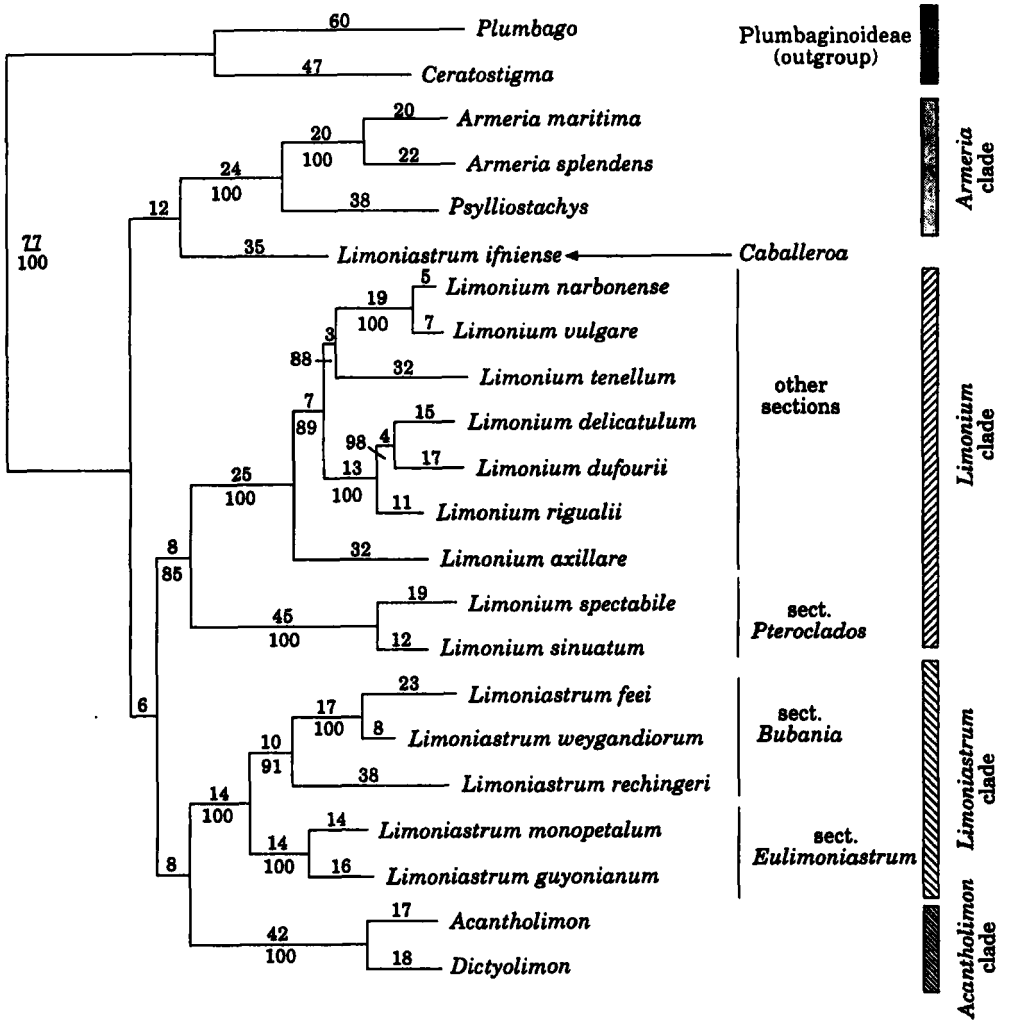


Figure 3. The single most parsimonious found in the combined analysis (SW and Fitch). Fitch lengths (ACCTRAN optimization) are shown above the branches, and bootstrap values are shown below (tree length = 874 steps; CI = 0.76; RI = 0.72).

Relationships indicated by combined data

When both data sets were combined, a single tree of 874 steps was obtained (CI = 0.76, RI = 0.72). Successive weighting produced the same tree (550611 steps, CI = 0.94 and RI = 0.91; see Table 2). This tree (Fig. 3) is similar to the trees obtained for the *rbc*L data, and it confirms the diphyly of *Limoniastrum* found in the previous analyses. There are four main clades within Staticoideae. The (i) *Limonium* clade (85% bootstrap) with the same subclades described for *rbc*L and *trn*L-F is sister to the (ii) *Limoniastrum* clade (excluding *Limoniastrum ifniense*) which has a bootstrap value of 100% plus (iii) *Acantholimon* and *Dictyolimon* (bootstrap 100%). These three clades form a major clade that is sister to the (iv) *Armeria* clade, and *Limoniastrum ifniense* is sister to the last (Fig. 3). Bootstrap values are higher for each clade than

TABLE 3. Lengths and fit measures comparisons for *rbc*L, *trn*L-F and combined trees, as well as the trees obtained from enforcing a topological constraint in which the genus *Limoniastrum* was monophyletic

	*rbc*L	*rbc*L constraint	*trn*L-F	*trn*L-F constraint	Combined	Combined constraint
Number of trees	3	1	15	54	1	5
Length	438	441	434	435	874	878
CI	0.69	0.69	0.83	0.83	0.76	0.76
RI	0.62	0.61	0.82	0.82	0.72	0.72

in the previous analyses and also for relationships among the main clades, which were not supported in previous analyses or had weak or moderate bootstrap values.

Limoniastrum *clade*

In all analyses, the genus *Limoniastrum* as currently recognized is diphyletic. If *L. ifniense* is excluded, the remaining species of *Limoniastrum* form a monophyletic group, in which two subclades are well supported by bootstrap: (i) sect. *Limoniastrum* with *L. monopetalum* and *L. guyonianum*; and (ii) sect. *Bubania* that includes *L. rechingeri*, *L. weygandiorum* Maire & Wilczek, and *L. feei* (Girard) Hook.f. ex Pax. Although in the *trn*L-F tree the position of *L. rechingeri* is not resolved, both *rbc*L and combined data trees place it in the *Bubania* clade with strong bootstrap support.

When the monophyly of *Limoniastrum* was enforced, tree lengths increased by one step for *trn*L-F, three steps for *rbc*L and four steps for the combined analysis (Table 3). The constrained *Limoniastrum* clade was defined by one molecular synapomorphy in the *rbc*L matrix, five in the *trn*L-F matrix and eight in the combined matrix.

Data set comparisons

In the *rbc*L analysis, the bootstrap does not strongly support the relationships among the main four clades (*Limonium*, *Limoniastrum*, *Acantholimon* and *Armeria* clades; see Fig. 1), although the internal branches within these clades are well supported. The *trn*L-F data do not give strong support for relationships among the main clades and lack resolution within *Limoniastrum*, producing a trichotomy in the strict consensus tree (see solid arrow in Fig. 2). Some taxa show different positions in each analysis. *Limoniastrum ifniense* is related to the *Armeria* clade in *rbc*L, whereas in the *trn*L-F tree it is related to *Limonium* clade. Moreover, *Limonium* is monophyletic for *rbc*L but is polyphyletic in the *trn*L-F tree. The major clades resulting from analyses of both data sets separately are strongly supported by the bootstrap, but relationships among these clades still have no bootstrap support.

The combined-data tree resembles the *rbc*L trees, in which *Limoniastrum ifniense* is sister to the *Armeria* clade and *Limonium* is monophyletic. Consistency and retention indices are intermediate compared to those obtained in individual analyses (Table

TABLE 4. Lengths, fit measures, and statistics of the three cpDNA regions analysed

	*rbc*L	*trn*L intron	*trn*L-F spacer
Sequence length	1310	580	449
Number of trees	3	8	1166
Tree length	438	222	198
CI	0.69	0.81	0.86
RI	0.62	0.77	0.87
Number of variable characters (%)	265 (22.2%)	157 (27.1%)	138 (30.7%)
Number of informative characters (%)	122 (9.3%)	83 (14.3%)	94 (20.9%)
Transitions (ts)	211	92	72
CI (ts)	0.75	0.90	0.95
RI (ts)	0.67	0.80	0.92
Transversions (tv)	227	130	126
CI (tv)	0.63	0.72	0.77
RI (tv)	0.57	0.74	0.82
ts/tv	0.93	0.71	0.57
A+T/G+C ratio	1.29	2.04	1.97

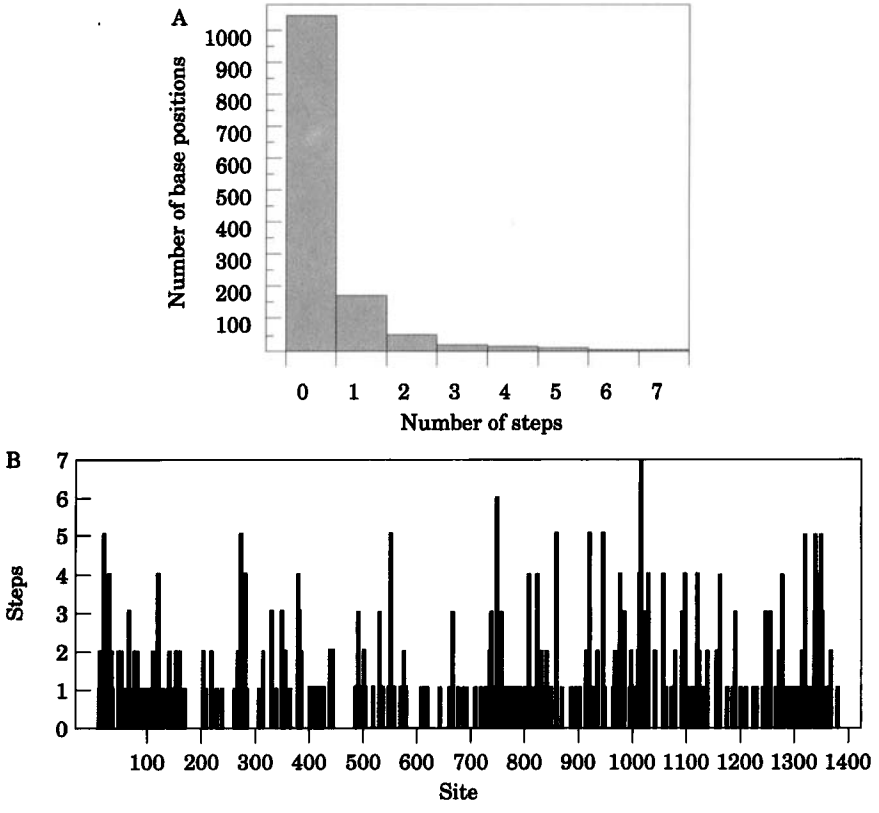


Figure 4. A, number of base positions plotted against the number of times these change on the *rbc*L tree. B, Nucleotide substitutions along the length of the *rbc*L exon used in this analysis.

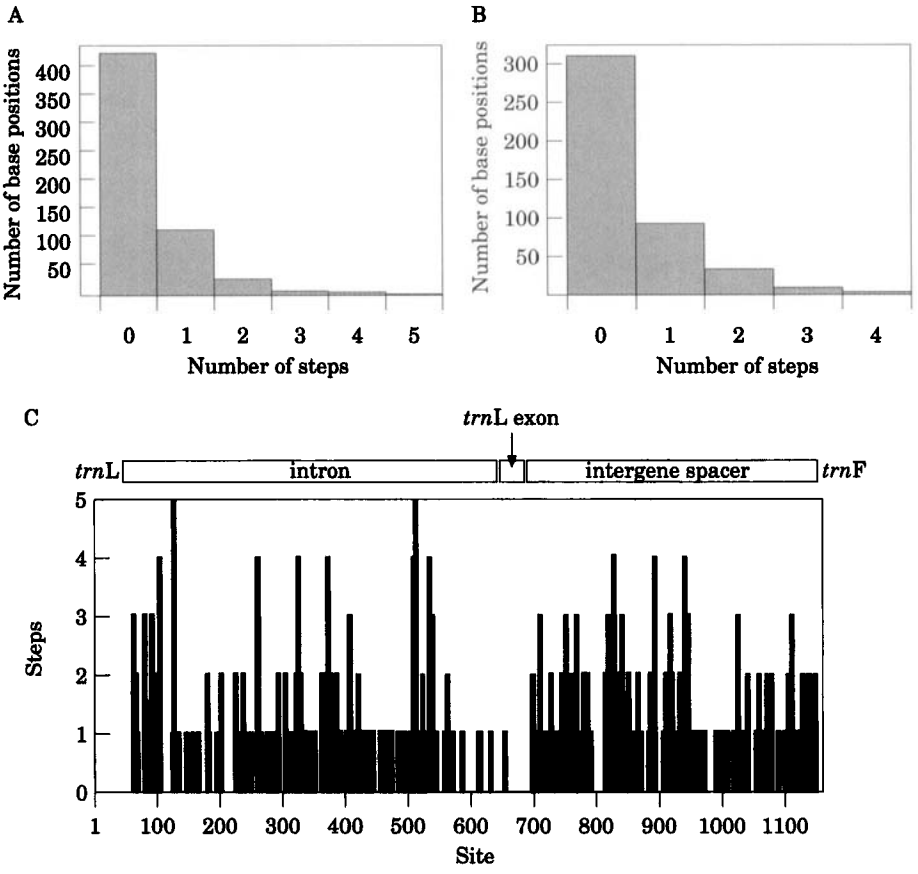


Figure 5. The number of base positions plotted against the number of times these change on the (A) *trn*L intron tree and (B) *trn*L-F spacer tree. (C) Nucleotide substitutions along the length of the *trn*L-F region used in this analysis.

2). Combining data sets improves support for the topology; more clades are strongly supported by bootstrap. The tree from the combined analysis is two steps longer than the sum of steps for trees from the individual data sets. Calculating the length of the individual trees based on the combined tree, but alternately excluding each region, these two extra steps were found to belong to the *trn*L-F data set.

The number of phylogenetically informative characters is higher in *trn*L-F (177 characters) than in *rbc*L (122), although the former tree is shorter than the latter (Table 4). The number of character changes per nucleotide site are shown in Figures 4B and 5C. In *rbc*L, the changes are distributed evenly along the exon (Fig. 4B) whereas in the *trn*L-F region, the exon (51 bp long) and slightly upstream region show fewer changes compared with the intron in general and intergene spacer (Fig. 5C).

The *trn*L-F matrix was broken into two parts (*trn*L intron and *trn*L-F spacer). Comparisons of the statistics of these two regions and *rbc*L are shown in Table 4. The percentage of phylogenetically informative characters is highest in the spacer region (30.7%) followed by intron (27.1%) and *rbc*L (22.2%), but although *rbc*L has few variable sites, each changes more often on average (see Figs 4A, 5A,B).

For all three regions the number of transversions was higher than of transitions. Transition/transversion ratios (ts/tv, Table 4) in all cases were less than one. The lowest ratio was found in the spacer region, which shows double the number of transversions. For *rbc*L the ts/ts ratio was close to one. Consistency and retention indices (CI and RI) were calculated separately for transition and transversion changes, and both were higher for transitions. The three regions analysed are AT rich, with A+T/C+G percentages of 2.04 for the intron, 1.97 for the spacer and 1.29 for *rbc*L (Table 4).

DISCUSSION

Molecular evolution

Phylogenetic analyses using sequence data have typically been based on a single DNA region. In this study, three data sets are used to resolve taxonomic problems, as well as to compare the phylogenetic signal and molecular evolution between coding and non-coding regions. Contrary to published data (Morton, 1997), ts/tv ratios in Plumbaginaceae do not correspond to those expected for plastid coding and non-coding regions. In general, transitions should occur more frequently than transversions, and the ratio expected for coding regions should be much higher than one. For non-coding regions the expected ts/tv ratios are close to one. In our data, *rbc*L has a ts/tv ratio of 0.93, the *trn*L intron of 0.71 and the *trn*L-F intergene spacer of 0.57 (Table 4).

The unexpectedly high proportion of transversions in our data sets could be related to the high content of A+T in these sequences. G+C rich sequences favour a bias towards transitions, whilst A+T rich environments favour transversions (Morton, 1997). The regions analysed in this paper are A+T rich, especially *trn*L intron (Table 3). Nevertheless, *rbc*L in Plumbaginaceae is not specially A+T rich. The A+T/G+C ratio for the 499 taxa *rbc*L matrix published by Chase *et al.* (1993) is 1.27 (M.W. Chase, unpublished), very close to the 1.29 found in our data set, but the ts/tv ratio is close to 1.6 (M.W. Chase, unpublished). The explanation for this higher proportion of transversions in our data seems not to be related with its AT richness.

Each data set (*rbc*L and *trn*L-F) has a slightly different phylogenetic signal. Consistency and retention indices are higher in *trn*L-F (Table 2), as well as the number of variable and potentially informative characters (Table 4). Nevertheless, when *rbc*L and *trn*L-F were analysed together, the single most-parsimonious tree obtained resembled more the *rbc*L topology. Assuming that the combined data matrix is providing the more accurate phylogenetic pattern because of its generally higher bootstrap percentages, the *rbc*L data set, which produced a topology similar to the combined data matrix, is the better one. Although the *trn*L-F matrix has more variable sites than *rbc*L, the length of the most-parsimonious trees found in each analysis is similar (438 and 434 steps respectively). Fewer *rbc*L characters change, but they change more often. Moreover, *trn*L-F is missing evidence for some substitutions; this data set required two additional steps to produce the combined tree. In other words, although the statistics for *trn*L-F are better than for *rbc*L, the combined results paradoxically show that the information of *rbc*L is more accurate.

Simulations (Hills, 1998; Yang, 1998) have shown that faster evolving regions are more accurate, and here the more rapidly evolving *rbc*L sites provide better evidence than those of *trn*L-F.

The polyphyly of Limoniastrum

In all analyses of combined and separate data sets, *Limoniastrum* is diphyletic. In both *rbc*L and combined data trees, *L. ifniense* is sister to the *Armeria* clade, and with *trn*L-F it is placed within *Limonium*. However when *L. ifniense* was forced to form a monophyletic group with the rest of *Limoniastrum*, the loss of parsimony was low. Additionally, the number of molecular synapomorphies defining the larger *Limoniastrum* clade (with all the species) was only one.

Three principal morphological synapomorphies have been used to delimit *Limoniastrum s.l.* The convolute inner bract is present in all species, but it is not an exclusive character: taxa such as *Muellerolimon* (F.Muell.) Lincz. or *Limonium* sect. *Myriolepis* Boiss. also exhibit this condition. Petals connate for half of their length are characteristic of *Limoniastrum* and also of *Limonium* sect. *Myriolepis*, *Psylliostachys* and genera of subfamily Plumbaginoideae (*Plumbago* L., *Ceratostigma* Bunge among others). Finally, only the possession of styles fused to half of their length is an exclusive character of *Limoniastrum s.l.*

Several autapomorphies differentiate *L. ifniense*, a species from western Sahara, from the rest of *Limoniastrum*, among others the presence of an outer bract longer than the inner, the anther forms a 90° angle with the filament (180° in the rest of *Limoniastrum*) and staminal filaments adnate only to the petal base (not to the apex). Relationships of *L. ifniense* with other genera of Plumbaginaceae are not clear. The bootstrap does not support the inclusion of *L. ifniense* in any clade. Its position outside the *Limoniastrum* clade is confirmed not only by the results obtained in each search, but also by the paucity of substitutions defining the *Limoniastrum* clade if monophyly is enforced. Analysis including more genera will probably help to clarify relationships with other members of the family.

Limoniastrum ifniense is in none of the trees sister to the rest of *Limoniastrum*, and with them it uniquely shares only one quantitative character: the degree of stylar fusion. Other characters of *Limoniastrum s.l.* are present in other genera of the family (e.g. the presence of a rolled inner bract also occurs in *Muellerolimon* and in *Limonium* section *Myriolepis*). There is no reason to suspect that the DNA trees are wrong because the rest of the patterns make sense in terms of morphology, so we accept that this species must be recognized as a distinct genus apart from *Limoniastrum*.

The transfer of *L. ifniense* to a separate genus is indicated by its peculiar autapomorphies, such as the angle of insertion of the anthers in the filament and the size of the outer bract, as stated before, as well as some synapomorphies (e.g. the absence of calcium carbonate deposits on the leaves) shared with other genera in the family (e.g. *Armeria, Acantholimon*) but not with *Limoniastrum*.

If *L. ifniense* is regarded as a distinct genus, then the remaining five taxa of *Limoniastrum* form a well supported monophyletic group (89%). Of the few synapomorphic characters defining this clade (see above) the most important is stamens being adnate for half of their length (only at the petal base in *L. ifniense*). All other morphological features are also present either *Limoniastrum* or in other genera of Staticoideae.

However, the *Limoniastrum* clade is composed of two groups well defined by morphological characters; these correspond to sect. *Limoniastrum* and sect. *Bubania*, which also are supported by these molecular analyses (100% and 97%). Species of *Limoniastrum* sect. *Limoniastrum* are tall branching shrubs of the Mediterranean coastal zone with alternate leaves and 3-bracteate spikelets with the smooth inner bract much longer than the outer one. Within the genus, *L. monopetalum* is the only species widely distributed in the Mediterranean region and has also been recently reported from Fuerteventura, Canary Islands (Barone, Scholz & Mesa, 1995). *Limoniastrum guyonianum* is similar morphologically to *L. monopetalum* but differs in having narrower, subcylindrical leaves (not wide and flattened) and a filiform middle bract (not three-angulate). The latter is endemic to the subdesert areas of northern Tunisia and Algeria.

The four species of *Limoniastrum* sect. *Bubania* are dwarf shrubs with rosulate leaves, spikelets without the middle bract, and with peculiar long curved horns on the rolled inner bract, a character not found in the rest of the family. They are narrow endemics growing in subdesert saline regions of northern Africa and rarely occur with plants of sect. *Limoniastrum*. Moreover, they show an unusual disjunct distribution in the Sahara Desert: *L. feei* and *L. weygandiorum* are found in Morocco and Algeria, *L. migiurtinum* (Choiv.) Maire (the only species of *Limoniastrum* not included in this analysis) in Somalia and *L. rechingeri* in Yemen and northern Somalia. The former two species are similar in morphology (with widely branched, silky-haired panicles) and are together sister to *L. rechingeri* which is morphologically similar to *L. migiurtinum* (long, shortly branched panicles with almost glabrous spikelets).

Once *L. ifniense* has been excluded, the great majority of morphological characters defining *Limoniastrum* clade are not exclusive features. There is no combination of characters that can easily define this clade. Such characters are also present either in *L. ifniense* or in other genera of the family. The only synapomorphy is the adnation of staminal filaments up to half of the petal length (they are fused to the petal base in *L. ifniense* and free in the rest of the genera of the family). However, the six species of *Limoniastrum* clade can be easily divided into two groups, corresponding to sect. *Bubania* and sect. *Limoniastrum*, each exhibiting an exclusive syndrome of several characters explained above, some of them unique in the family. Both sections are monophyletic, and although they are sister to each other they can be regarded as distinct genera, as recognized by some authors (e.g. Linczevski, 1968).

The two subclades of *Limoniastrum* differ by many more characters than they share, so on the basis of maximizing phylogenetic information, stability and ease of identification (Backlund & Bremer, 1998) we would argue that taxonomic recognition of both clades and distinct genera is warranted.

Taxonomic implications

Our molecular results, together with morphological data, indicate that *Limoniastrum s.l.* should be classified as follows:

(i) *Limoniastrum*, consisting only of *L. monopetalum* and *L. guyonianum*. These taxa form a consistent and well supported group in all analyses and significant synapomorphies coincide with the molecular trees.

(ii) *Bubania*, consisting of *L. rechingeri*, *L. weygandiorum*, *L. feei* and *L. migiurtinum* (the last not included in this analysis). This group is sister to the *Limoniastrum* clade.

The characteristics shared by all taxa in this group (e.g. the horned inner bract and the absence of a middle bract), as well as the level of molecular divergence lead us to conclude that they are best treated as a distinct genus from *Limoniastrum*, as originally described.

(iii) *Caballeroa. Limoniastrum ifniense*, although commonly included in *Limoniastrum*, does not form a monophyletic group with the other species of *Limoniastrum*. In all trees it is sister to other groups within Staticoideae; either the *Armeria* clade (consisting of *Armeria* and *Psylliostachys*) in the *rbc*L and combined trees or sister to the *Acantholimon–Limoniastrum* clade in the *trn*L-F trees. We recommend that *L. ifniense* should be regarded as a separate genus.

These three genera, *Limoniastrum*, *Bubania* and *Caballeroa*, have been previously accepted by several authors (Linczesvki, 1968). They are supported here by molecular data and have the greatest number of easily identified morphological characters whereas *Limoniastrum s.l.* is either not monophyletic or defined by trivial and quantitative characters. This is neither desirable nor practical. The appropriate names that should apply to each group require revision. Both *Caballeroa* and *Bubania* were originally described contradicting several rules of the International Code of Botanical Nomenclature. The nomenclatural discussion which derives from these analyses is presented in the preceding paper (see pp. 165–274).

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