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Unraveling the taxonomic complexity of *Eryngium* L. (Apiaceae, Saniculoideae): Phylogenetic analysis of 11 non-coding cpDNA loci corroborates rapid radiations

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With 3 figures and 3 tables

Abstract

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The evolution of the genus *Eryngium* L. combines a history of rapid radiations, long distance dispersals, and hybridizations. To corroborate whether the polytomies estimated in the phylogeny of *Eryngium* based on previous analyses of cpDNA *trnQ-trnK* and nrDNA ITS sequence data are due to rapid radiations, phylogenetic relationships of a subset of *Eryngium* species representing all major clades identified in our previous study were inferred using sequence data from 11 non-coding cpDNA regions (*trnQ-rps16*, *rps16* intron, *rps16-trnK, rpl32-trnL, ndhF-rpl32, psbJ-petA, 3'trnV-ndhC, trnfM-trnS, trnT-trnD, trnC-rpoB,* and *trnG-trnS)*. In total, 20 accessions representing seven informal and unranked groups of *Eryngium* subgenus *Monocotyloidea* and *E. maritimum* (*E.* subgenus *Eryngium*) were analyzed using maximum parsimony. Analysis of these 11 loci permitted an assessment of the relative utility of these non-coding regions in providing a more resolved and better supported phylogeny of the genus. The combined analysis of all cpDNA regions recovered the same informal groups previously recognized based on *trnQ-trnK* data alone: "New World s.str.", "North American monocotyledonous", "South American monocotyledonous", "Pacific", "Mexican", and "Eastern USA". The relationships among these groups, however, remained unresolved. Resolution in other portions of the tree and most bootstrap support values increased as a result of simultaneous analysis of all data. A cost/benefit examination indicated that maximum parsimony analysis of *trnQ-trnK* plus 3 regions (*trnG-trnS, rpl32-trnL*, and *3'trnV-ndhC)* results in the same number of clades and similar bootstrap support values than in the combined analysis of all cpDNA regions. The present study continues to support that the major polytomies of *Eryngium* are due to rapid radiations, and the screening of 11 non-coding cpDNA regions allowed an efficient selection of the most informative loci and the minimum amount of regions necessary for increasing resolution and support within other portions of the phylogeny.

Keywords: Apiaceae, *Eryngium*, cpDNA, phylogeny, rapid radiation.

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Introduction

Eryngium L. is the largest and probably the most taxonomically complex genus in the family Apiaceae. Many researchers, such as J. Decaisne, H. Wolff, J. M. Turmel, M. T. Cerceau-Larrival, and L. Constance, have devoted much of their professional lives contributing to the present day knowledge of the genus. Because of their hard work, we now have considerable information on the morphological diversity, distribution, karyology, and ecological preferences of *Eryngium* species. Moreover, the expertise of these authors culminated in several hypotheses of phylogenetic relationships and historical biogeography (Decaisne 1873, Wolff 1913, Turmel 1948, 1949, Cerceau-Larrival 1971, Constance 1977). However, despite the competency of these researchers, many expressed their frustrations to fully understand the evolutionary relationships or species delimitations within this species-rich group. The words of Constance in a letter to a colleague in reference to *Eryngium* probably exemplify this feeling best: "It is hard to believe that I've spent as much of my time on this ungrateful genus as I have had, and still have such a weak grasp of it …".

As new methods of phylogenetic reconstruction and related technology are developed, one supposes that the more difficult problems surrounding *Eryngium* can be unraveled. It was only recently that the first explicit phylogenetic hypothesis of *Eryngium* was estimated (Calviño et al. 2008). This study, based on phylogenetic analyses of DNA sequences from three non-coding chloroplast DNA (cpDNA) loci and the nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) region, corroborated some of the hypotheses of relationships and biogeography previously formulated but also rejected many others. Many questions, however, remained unanswered. These molecular characters have been useful to corroborate the monophyly of *Eryngium*, divide it into two redefined and monophyletic subgenera (*E.* subgenus *Eryngium* and *E.* subgenus *Monocotyloidea*), identify clades (treated as informal taxonomic groups) that share several morphological, biogeographical and/or ecological traits, estimate morphological synapomorphies, and infer a new hypothesis about the biogeographical history of the genus (Fig. 1). Moreover, the results of our earlier phylogenetic investigations enabled a postulation on the main biological processes involved in the evolution and diversification of *Eryngium*: rapid radiations, long distance dispersals, and hybridizations. The complexity and array of data sources and analytical techniques required to decipher these biological processes explain why it has been so difficult, and continues to be difficult, to understand the evolutionary history of *Eryngium* and to produce a natural classification that reflects this evolutionary history.

In this study, we take an exploratory approach to test, with additional data, whether the polytomies estimated in the phylogeny of *Eryngium* based on cpDNA *trnQ-trnK* and nrDNA ITS sequence data are due to rapid radiations. Polytomies may reflect artifacts of the methods or data used, or evolutionary processes that are not congruent with a bifurcating pattern of species diversification. Calviño et al. (2008) reported that the three major polytomies in the phylogeny of *Eryngium* (indicated by grey lines in Fig. 1) are the result of lack of accumulated molecular changes on those portions of the tree and concluded that these polytomies are evidence of rapid radiations and not of insufficient or inadequate data. However, these scenarios are difficult to distinguish and

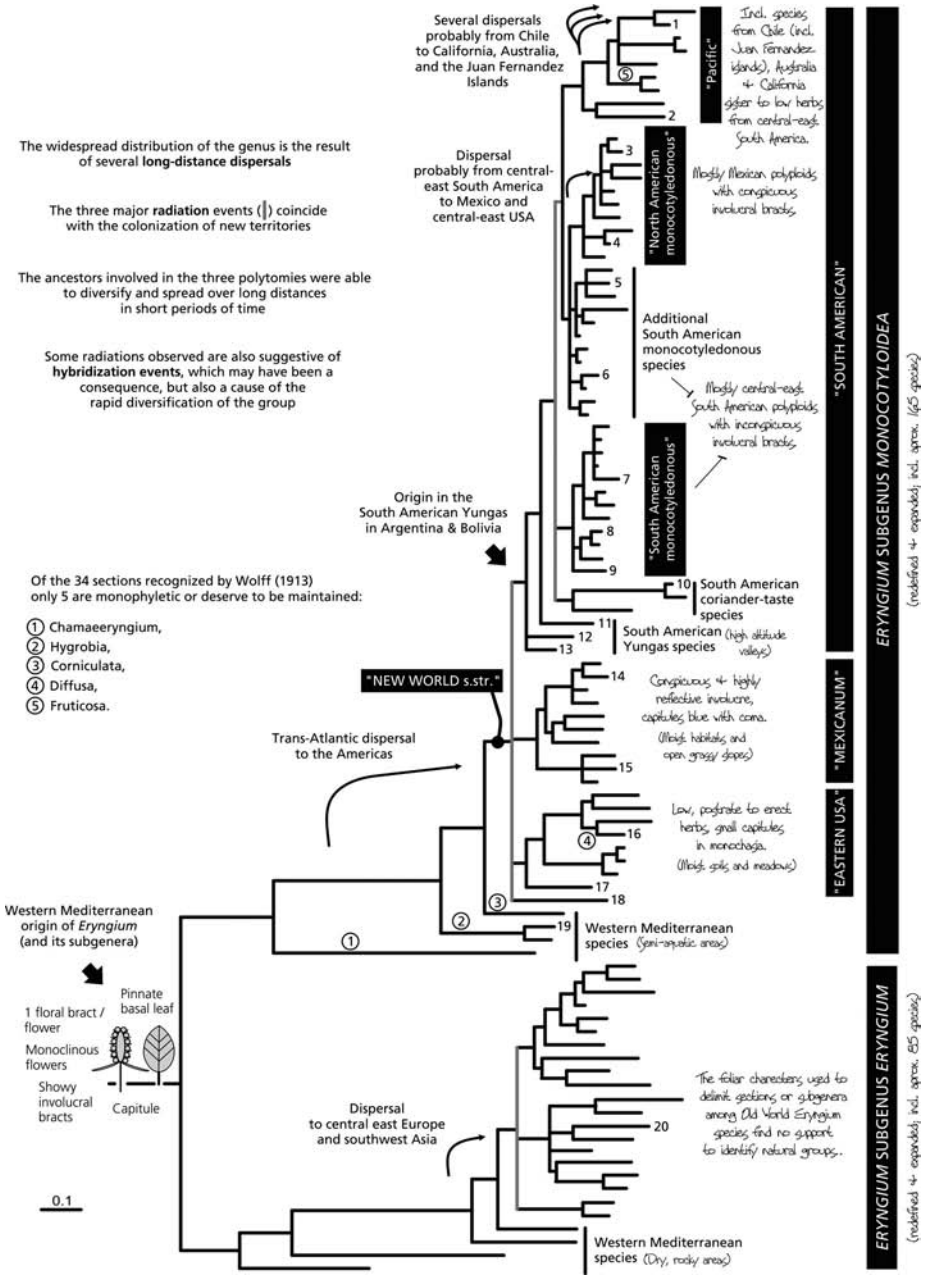

Fig. 1. Summary of the main results inferred from previous molecular phylogenetic analyses of *Eryngium* (Calviño et al. 2008). The tree backbone corresponds to a majority-rule consensus of 200,000 trees derived from Bayesian analysis of 112 *trnQ-trnK* and ITS sequences of *Eryngium* and outgroups (the latter not shown). Black boxes show the two monophyletic subgenera and the seven subclades within *Eryngium* subgenus *Monocotyloidea* that are treated as informal and unranked groups. Comments about shared ecological, biogeographical and/or morphological traits are provided in italics. Evolutionary processes that explain the taxonomic complexity of *Eryngium* are highlighted in bold. Monophyletic or monotypic sections that deserve to be maintained are identified with circled numbers above branches: (1) Chamaeeryngium; (2) Hygrobia; (3) Corniculata; (4) Diffusa; and (5) Fruticosa. Morphological synapomorphies for *Eryngium* are drawn and indicated on the root of the tree. Dispersal events are represented with arrows. Gray lines show three polytomies that are interpreted as major radiation events. Numbers from 1–20 at the end of branches indicate the placement on this tree of the 20 taxa examined in this study (Table 1; Fig. 3).

with DNA sequencing becoming easier and less expensive, we asked if these polytomies are resolvable by the addition of extra molecular characters.

The main objectives of this study are to test whether the major polytomies in the phylogeny of *Eryngium* are resolvable by increasing the amount of cpDNA molecular data and whether these additional data result in greater resolution and/or branch support in other unresolved portions of the phylogeny. Because it is important to obtain robust phylogenies from independent data sources for *Eryngium* (e.g., cpDNA and nuclear DNA), this study analyzes only cpDNA evidence to eventually compare it with a similarly robust nuclear data set. Ancillary objectives include a corroboration of the recovery of the informal groups of *Eryngium* subgenus *Monocotyloidea* identified by Calviño et al. (2008) and an evaluation of the cost/benefit (in terms of effort vs. expected results) to produce a more resolved and robust phylogeny of *Eryngium*. To resolve these questions, we examine 11 non-coding cpDNA regions (for a total of 13,412 aligned nucleotide positions) for a subset of 20 species that represents the seven informal and unranked groups of *Eryngium* and their allies identified previously by phylogenetic analyses of combined cpDNA *trnQ-trnK* and nrDNA ITS sequence data (Calviño et al. 2008; Fig. 1). The fulfillment of these objectives will elucidate further studies on unraveling the complex evolutionary history and taxonomy of *Eryngium*.

Materials and methods

Accessions and cpDNA regions examined

Twenty accessions representing the seven informal groups of *Eryngium* and their allies identified in previous phylogenetic analyses of combined cpDNA *trnQ-trnK* and nrDNA ITS sequence data (Calviño et al. 2008; Fig. 1) were examined for sequence variation in 11 non-coding cpDNA regions. The plastid genome of *Eryngium* has the same consensus structure and gene order as found in other Apiaceae and the vast majority of flowering plants (Plunkett & Downie 1999, 2000, Ruhlman et al. 2006), and the locations of these 11 loci are mapped on this circular genome (Fig. 2). These regions include the *trnQ-rps16* intergenic spacer, *rps16* intron, and *rps16-trnK* intergenic spacer that constitute the *trnQ-trnK* data partition used previously for *Eryngium* (Calviño & Downie 2007, Calviño et al. 2008), and the *rpl32-trnL*$^{(UAG)}$, *ndhF-rpl32, psbJ-petA, 3'trnV*$^{(UAC)}$*-ndhC, trnfM*$^{(CAU)}$*-trnS*$^{(UGA)}$, *trnT*$^{(GGU)}$*-trnD*$^{(GUC)}$, *trnC*$^{(GCA)}$*-rpoB,* and *trnG*$^{(UUC)}$*-trnS*$^{(GCU)}$ intergenic spacers that were selected because they provided more parsimony informative characters than any other of the 34 non-coding cpDNA regions evaluated for phylogenetic utility in angiosperms by Shaw et al. (2005, 2007). DNA sequences for the *trnQ-trnK* data partition were obtained from our previous studies; data for the remaining eight regions were specifically obtained for this study.

Experimental strategy

Total genomic DNAs for the 20 accessions examined herein were the same as used in our earlier study (Calviño et al. 2008). The strategies used to obtain these sequence data are presented elsewhere (see Shaw et al. 2007, for PCR amplifications, and Calviño et al. 2006, and Calviño & Downie 2007, for DNA purification and sequencing). Simultaneous consideration of both DNA strands across all cpDNA regions permitted unambiguous base determination in all taxa. All newly obtained sequences have been submitted to GenBank (Table 1).

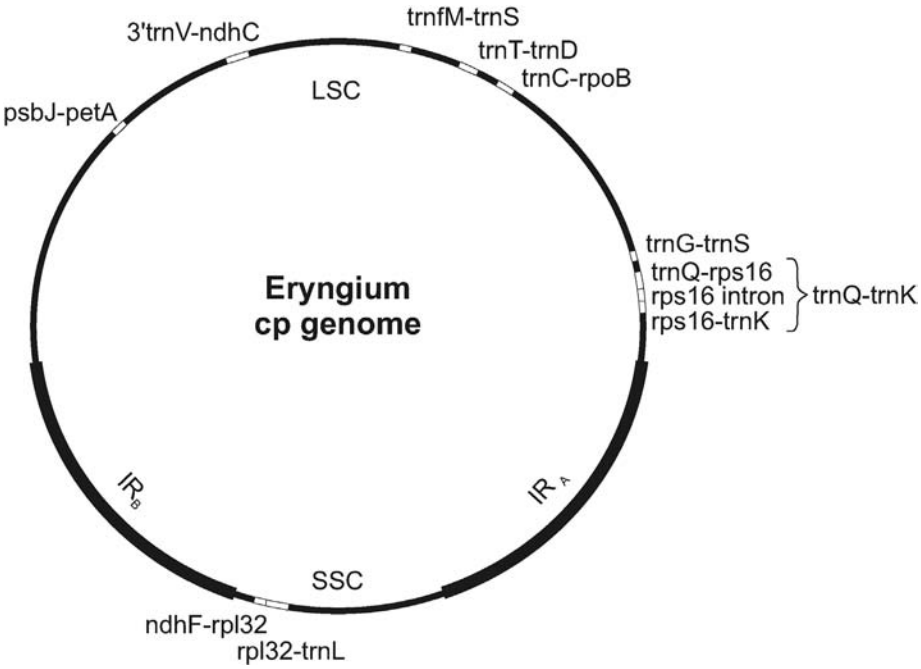

Fig. 2. Generalized map of an *Eryngium* chloroplast genome showing the relative position of the 11 non-coding regions (in nine data partitions) explored for *Eryngium* and characterized in Table 2. The thick lines indicate the extent of the inverted repeats (IR_A and IR_B), which separate the genome into small (SSC) and large (LSC) single copy regions.

Sequence comparisons and phylogenetic analyses

Editing and alignment of DNA sequences for the 11 non-coding cpDNA regions were carried out following the same strategies as described in Calviño et al. (2008). Likewise, a matrix of binary-coded indels was constructed for each of the nine data partitions (i.e., *trnQ-trnK* and the eight newly obtained intergenic spacers) to incorporate length mutational information into the phylogenetic analysis.

Characterization of each cpDNA data partition was facilitated using BioEdit version 6.0.7 (Hall 1999) and PAUP version 4.0b10 (Swofford 2002). Uncorrected pairwise nucleotide distances of unambiguously aligned positions were determined using the distance matrix option of PAUP*.

All nine cpDNA data partitions (with and without their corresponding scored indels) were analyzed simultaneously using maximum parsimony (MP), as implemented by PAUP*. The results of these total evidence analyses were compared to equivalent analyses based only on the *trnQ-trnK* data partition to investigate whether more resolution and higher support values than those found in Calviño et al. (2008) are possible by increasing the number of cpDNA regions examined. Heuristic searches were performed for 100,000 replicates with random addition of taxa and tree-bisection-reconnection (TBR) branch swapping. Bootstrap values (Felsenstein 1985) were calculated from 10,000 replicate analyses using "fast" stepwise-addition of taxa and only those values compatible with a 50% majority-rule consensus tree were recorded. The relative utility of adding extra cpDNA regions to the *trnQ-trnK* data matrix in resolving phylogenetic relationships was assessed by comparing the results of MP analyses of the *trnQ-trnK* region plus one to three extra regions against those clades with bootstrap values >50% inferred from MP analysis of all cpDNA regions (i.e., the nine cpDNA data partitions).

Table 1. Accessions of *Eryngium* from which 11 non-coding cpDNA regions were obtained, with corresponding DNA accession numbers. GenBank reference numbers are provided for each of the eight data partitions newly sequenced. Voucher information, and GenBank reference numbers for the *trnQ-rps16, rps16* intron, and *rps16-trnK* regions are provided in Calviño et al. 2008.

Taxon	**DNA accession no.**	**GenBank reference no.**							
		trnG-trnS	***rpl32-trnL***	***3'trnV-ndhC***	***trnC-rpoB***	***trnT-trnD***	***psbJ - petA***	***trnfM-trnS***	***ndhF-rpl32***
Eryngium buchtienii H. Wolff	2818	FJ686651	FJ686591	FJ686531	FJ686611	FJ686671	FJ686571	FJ686631	FJ686551
Eryngium coquimbanum Phil. ex Urb.	2820	FJ686652	FJ686592	FJ686532	FJ686612	FJ686672	FJ686572	FJ686632	FJ686552
Eryngium coronatum Hook. & Arn.	508	FJ686653	FJ686593	FJ686533	FJ686613	FJ686673	FJ686573	FJ686633	FJ686553
Eryngium eburneum Decne.	2323	FJ686654	FJ686594	FJ686534	FJ686614	FJ686674	FJ686574	FJ686634	FJ686554
Eryngium elegans Cham. & Schltdl.	786	FJ686655	FJ686595	FJ686535	FJ686615	FJ686675	FJ686575	FJ686635	FJ686555
Eryngium galioides Lam.	2954	FJ686656	FJ686596	FJ686536	FJ686616	FJ686676	FJ686576	FJ686636	FJ686556
Eryngium glossophyllum H. Wolff	2965	FJ686657	FJ686597	FJ686537	FJ686617	FJ686677	FJ686577	FJ686637	FJ686557
Eryngium incantatum Lucena, Novara & Cuezzo	2363	FJ686658	FJ686598	FJ686538	FJ686618	FJ686678	FJ686578	FJ686638	FJ686558
Eryngium juncifolium (Urb.) Mathias & Constance	2364	FJ686659	FJ686599	FJ686539	FJ686619	FJ686679	FJ686579	FJ686639	FJ686559
Eryngium leavenworthii Torr. & Gray	2832	FJ686660	FJ686600	FJ686540	FJ686620	FJ686680	FJ686580	FJ686640	FJ686560
Eryngium madrense S. Watson	2955	FJ686661	FJ686601	FJ686541	FJ686621	FJ686681	FJ686581	FJ686641	FJ686561
Eryngium maritimum L.	2957	FJ686662	FJ686602	FJ686542	FJ686622	FJ686682	FJ686582	FJ686642	FJ686562
Eryngium mesopotamicum Pedersen	2485	FJ686663	FJ686603	FJ686543	FJ686623	FJ686683	FJ686583	FJ686643	FJ686563
Eryngium nudicaule Lam.	2486	FJ686664	FJ686604	FJ686544	FJ686624	FJ686684	FJ686584	FJ686644	FJ686564
Eryngium pristis Cham. & Schltdl.	2367	FJ686665	FJ686605	FJ686545	FJ686625	FJ686685	FJ686585	FJ686645	FJ686565
Eryngium prostratum Nutt. ex DC.	2329	FJ686666	FJ686606	FJ686546	FJ686626	FJ686686	FJ686586	FJ686646	FJ686566
Eryngium sanguisorba Cham. & Schltdl.	790	FJ686667	FJ686607	FJ686547	FJ686627	FJ686687	FJ686587	FJ686647	FJ686567
Eryngium spiculosum Hemsl.	559	FJ686668	FJ686608	FJ686548	FJ686628	FJ686688	FJ686588	FJ686648	FJ686568
Eryngium vaseyi J.M. Coult. & Rose	562	FJ686669	FJ686609	FJ686549	FJ686629	FJ686689	FJ686589	FJ686649	FJ686569
Eryngium yuccifolium Michx.	807	FJ686670	FJ686610	FJ686550	FJ686630	FJ686690	FJ686590	FJ686650	FJ686570

The additional regions added to the *trnQ-trnK* data partition were selected to maximize the total number of parsimony informative nucleotide substitutions in each data set. Comparisons were made of the number of major clades recovered in each of these analyses and their corresponding bootstrap support values.

Results

Sequence comparisons and phylogenetic analyses

Sequence characteristics of the nine cpDNA data partitions, separately and combined in a total evidence analysis, are presented in Table 2. Of the eight newly obtained cpDNA loci examined, the *trnG-trnS* intergenic spacer is the largest region, whereas the *ndhF-rpl32* intergenic spacer is the smallest. Alignment of all partitioned regions for 20 accessions of *Eryngium* resulted in a matrix of 13,412 positions. Of these, 418 were excluded from the analysis because of alignment ambiguities (see Table 2 for number of positions eliminated from each data partition). The remaining 12,994 aligned positions yielded 158 parsimony informative nucleotide substitutions. In addition, 148 unambiguous alignment gaps were inferred, of which 15 were parsimony informative. The latter ranged in size from 1 to 40 base pairs (bp). Besides *trnQ-trnK*, the next two regions with the highest number of parsimony informative characters are *trnG-trnS,* and *rpl32-trnL*; a ranking of all data partitions, ordered from most to least total number of parsimony informative characters (substitutions plus gaps), is presented in Table 2. Regions *3'trnV-ndhC* and *trnC-rpoB* have the same total number of parsimony informative characters; however, the latter region displayed many alignment ambiguities (232 nucleotide positions, or approx. 15% of aligned positions, were eliminated). Maximum pairwise sequence divergence estimates within *Eryngium* subgenus *Monocotyloidea* are much lower than between the two subgenera. The *3'trnV-ndhC* intergenic spacer had the highest levels of sequence divergence among all accessions examined, with a maximum divergence value of 7.2%, whereas the *rpl32-trnL* spacer displayed the highest levels of sequence divergence within *Eryngium* subgenus *Monocotyloidea*, with a maximum divergence value of 2.6%.

MP analysis of the 3465 unambiguously aligned *trnQ-trnK* nucleotide positions resulted in 144 trees, each of 308 steps (consistency index, CI = 0.6714 without uninformative characters; retention index, RI = 0.7013). The strict consensus of these trees is presented in Fig. 3A. This tree is congruent with the relationships inferred previously for 117 accessions using the same cpDNA region (Fig. 2 in Calviño et al. 2008). The same topology was recovered when scored indels were included in the analysis as additional characters (strict consensus tree not shown); bootstrap values were similar, except for an increased support from 69% to 80% for the "Mexican" clade when indels were considered. In all *trnQ-trnK* derived trees, *Eryngium galioides* is sister group to the "New World s.str." clade with high bootstrap support (97%). The "New World s str." clade includes five subclades previously designated as "North American monocotyledonous", "South American monocotyledonous", "Pacific", "Mexican", and "Eastern USA". These five subclades show mostly poor to moderate bootstrap support

Table 2. Sequence characteristics of the nine cpDNA data partitions, separately and combined, examined for 20 accessions of *Eryngium* that represent the seven informal groups of *Eryngium* subgenus *Monocotyloidea* identified in previous study (Calviño et al. 2008; Fig. 1), with *Eryngium maritimum* (*Eryngium* subgenus *Eryngium*) used to root the trees. Data partitions ordered from most to least total number of parsimony informative characters (left to right).

Sequence Characteristic	*trnQ-trnK*	*trnG-trnS*	*rpl32-trnL*	*3'trnV-ndhC*	*trnC-rpoB*	*trnT-trnD*	*psbJ-petA*	*trnfM-trnS*	*ndhF-rpl32*	**all cpDNA**
Length variation (range in bp)	3180-3330	1513-1534	810-1116	1097-1124	1291-1416	964-1025	813-1143	1135-1178	660-731	11969-12289
No. aligned positions	3479	1604	1270	1209	1572	1084	1179	1263	752	13412
No. positions eliminated	14	8	91	0	232	62	2	9	0	418
No. positions not variable	3199	1469	1066	1086	1249	936	1092	1195	698	11990
No. positions autapomorphic	226	101	90	108	76	74	73	52	46	846
No. positions parsimony informative	40	26	23	15	15	12	12	7	8	158
No. unambiguous alignment gaps	38	16	19	11	18	17	9	13	7	148
No. unambiguous alignment gaps parsimony informative	5	0	0	1	1	3	1	4	0	15
Sequence divergence (range in %)										
All taxa included	0.1-4.7	0.1-4.5	0.2-5.4	0-7.2	0-3.5	0-4.9	0-4.9	0.1-2.7	0-3.9	0.1-4.3
Within *Eryngium* subgenus *Monocotyloidea*	0.1-1.5	0.1-1.6	0.2-2.6	0-2.5	0-1.4	0-2.5	0-2.4	0.1-1.4	0-2.3	0.1-1.6
Total no. parsimony informative characters[a]	45	26	23	16	16	15	13	11	8	173

[a] Number of parsimony informative nucleotide substitutions plus number of parsimony informative gaps

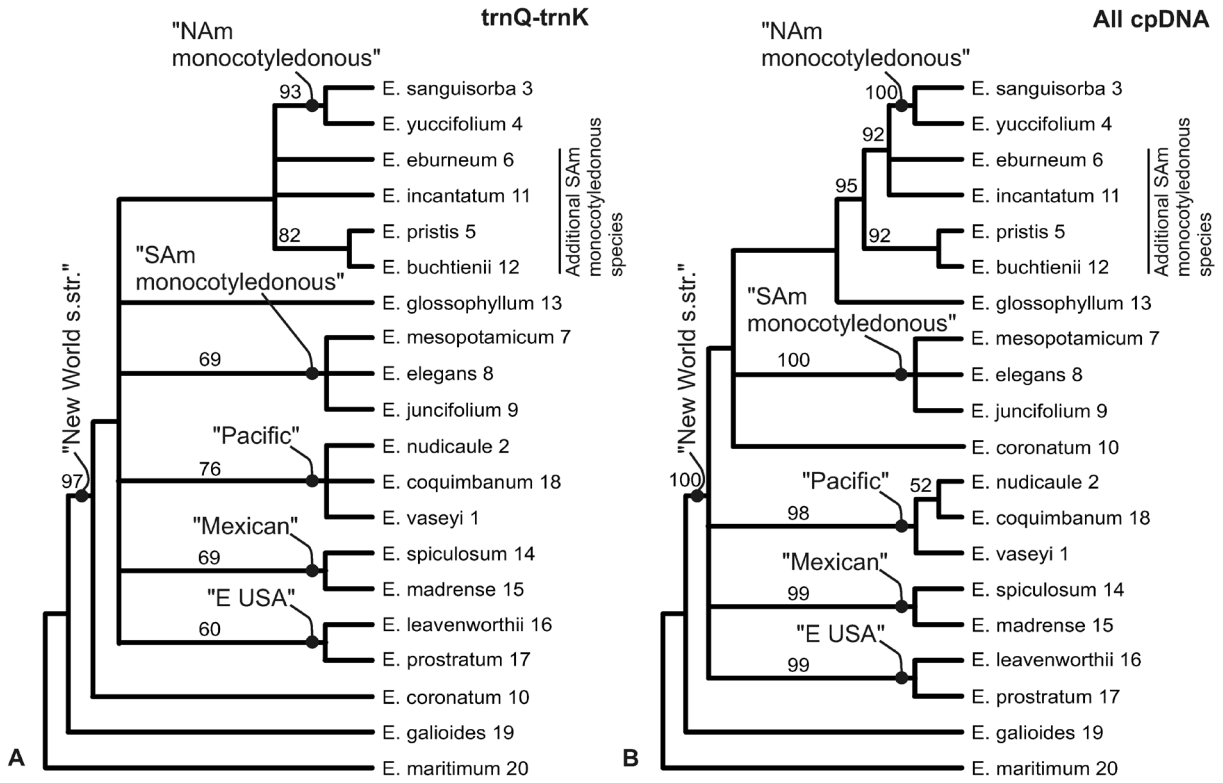

Fig. 3. Comparison of the strict consensus trees derived from maximum parsimony analysis of 20 accessions of *Eryngium* that represent the seven informal groups of *Eryngium* subgenus *Monocotyloidea* identified in previous study (Calviño et al. 2008; Fig. 1), with *Eryngium maritimum* (*Eryngium* subgenus *Eryngium*) used to root the trees. (A) CpDNA *trnQ-trnK* data partition only (tree length=308; CI=0.6714, without uninformative characters; RI=0.7013); (B) All 11 non-coding loci, representing nine cpDNA data partitions (tree length=1135; CI=0.6883, without uninformative characters; RI=0.7148). Numbers above branches are bootstrap estimates for 10,000 replicate analyses. The numbers at the terminals indicate the placement of these 20 accessions in Fig. 1.

(60–76%), with only one subclade with high support (93%). The "North American monocotyledonous" subclade comprises one branch of a polytomy that is made up of additional South American monocotyledonous species (with *E. pristis* and *E. buchtienii* being sister species). This assemblage along with *Eryngium glossophyllum* and the "South American monocotyledonous", "Pacific", "Mexican", and "Eastern USA" subclades form a large polytomy that is sister to *Eryngium coronatum*.

MP analysis of 12,994 unambiguously aligned nucleotide positions from all nine cpDNA data partitions resulted in 65 trees, each of 1135 steps (CI = 0.6883 without uninformative characters; RI = 0.7148). The strict consensus of these trees is presented in Fig. 3B. The same topology and similar bootstrap values were recovered when indels were included in the analysis as separate characters (strict consensus tree not shown). Once more, *Eryngium galioides* is sister group to the "New World s.str." clade with high bootstrap support (100%). Within the "New World s.str." clade the same five subclades identified in the *trnQ-trnK* trees are evident: "North American monocotyledonous", "South American monocotyledonous", "Pacific", "Mexican", and "Eastern USA". In contrast with the *trnQ-trnK* results, these five subclades show higher bootstrap support values (98–100%) when all nine cpDNA data partitions are analyzed simultaneously. The assemblage formed by the "North American monocotyledonous" subclade plus additional South American monocotyledonous species is slightly more resolved than in the *trnQ-trnK* strict consensus tree and finds higher bootstrap support (<50% *trnQ-trnK*, 95% all cpDNA). This assemblage is sister to *E. glossophyllum* and, along with the "South American monocotyledonous" subclade and *Eryngium coronatum,* comprises a weakly supported monophyletic group. This new clade, together with the "Pacific", "Mexican", and "Eastern USA" subclades, comprises a polytomy at the base of the "New World s.str." clade. In total, the following ten clades show bootstrap values >50% when all cpDNA data partitions are considered: "New World s.str.", *E. sanguisorba* to *E. buchtienii* (comprising *E. sanguisorba, E. yuccifolium, E. eburneum, E. incantatum, E. pristis,* and *E. buchtienii*), *E. sanguisorba* to *E. incantatum* (comprising *E. sanguisorba, E. yuccifolium, E. eburneum,* and *E. incantatum*), "North American monocotyledonous", *E. pristis* plus *E. buchtienii,* "South American monocotyledonous", "Pacific", *E. nudicaule* plus *E. coquimbanum,* "Mexican", and "Eastern USA" (Fig. 3B).

The results of MP analyses of the *trnQ-trnK* region plus one to three extra regions, and their comparisons to the results of the aforementioned analyses are presented in Table 3. The *trnQ-trnK* plus two (*trnG-trnS* + *rpl32-trnL*) or three (*trnG-trnS* + *rpl32-trnL* + *3'trnV-ndhC*) regions recovered the 10 clades inferred by analysis of all nine cpDNA regions, whereas the *trnQ-trnK* alone or *trnQ-trnK* plus one region (*trnG-trnS*), did not recover two of these clades (i.e., clade *E. nudicaule* plus *E. coquimbanum,* and clade *E. sanguisorba* to *E. incantatum*). Considering all analyses, bootstrap support values are higher as more regions are considered; the only exception is *E. nudicaule* plus *E. coquimbanum* with a lower bootstrap value when compared to the results of *trnQ-trnK* plus two or three regions. Average bootstrap values for the *trnQ-trnK* plus three regions and all cpDNA regions are high (90% and 93%, respectively).

Table 3. A comparison of bootstrap support values for the 10 clades of *Eryngium* subgenus *Monocotyloidea* with bootstrap values >50 % shown in Fig. 3B, resulting from MP analysis of data matrices constructed by combining additional cpDNA regions to the *trnQ-trnK* data partition: + 1 region = *trnQ-trnK* + *trnG-trnS*; + 2 regions = *trnQ-trnK* + *trnG-trnS* + *rpl32-trnL*; + 3 regions = *trnQ-trnK* + *trnG-trnS* + *rpl32-trnL* + *3'trnV-ndhC*; all cpDNA regions = the nine cpDNA data partitions examined in Table 2. The additional regions chosen for analyses were selected to maximize the total number of parsimony informative nucleotide substitutions in each data set.

Clade	*trnQ-trnK*	+ 1 region	+ 2 regions	+ 3 regions	All cpDNA regions
New World s.str.	97	99	99	100	100
E. sanguisorba to *E. buchtienii*	43	61	78	88	95
E. sanguisorba to *E. incantatum*	9	22	82	83	92
North American monocotyledonous	93	98	99	99	100
E. pristis plus *E. buchtienii*	82	75	81	81	92
South American monocotyledonous	69	80	87	92	100
Pacific	76	69	87	87	98
E. nudicaule plus *E. coquimbanum*	<5	22	72	72	52
Mexican	69	88	96	97	99
Eastern USA	60	75	97	99	99
Average	60	72	88	90	93

Discussion

Polytomies: is the problem solved?

Lack of resolution is a widespread problem among many published phylogenies (Hughes et al. 2006). Because molecular phylogenetic studies often serve as foundations for testing other biological hypotheses, it is crucial that the cause of these polytomies be examined thoroughly in order to distinguish artifacts of the data or method used from evolutionary processes, such as rapid radiations or hybridizations, that are not congruent with a bifurcating pattern of species diversification. Increasing the amount of cpDNA sequence data, potentially guided by selecting more variable noncoding cpDNA loci, has been successfully used to obtain greater resolution and branch support (Shaw et al. 2005, 2007), although this is not guaranteed. Calviño et al. (2008) reported three major polytomies in the evolutionary history of *Eryngium* (grey lines in Fig. 1) that were interpreted as rapid radiations that coincided with the colonization of new territories. However, because the cause of these polytomies was determined to be a lack of accumulated *trnQ-trnK* and ITS character-changes in those portions of the trees, it was desirable to test whether the polytomies are resolvable by adding a considerable amount of extra characters from new regions with different levels of variation. Therefore, in the present study, we quadrupled the amount of parsimony informative characters available for phylogenetic reconstruction. The same informal groups recognized by Calviño et al. (2008) in *Eryngium* subgenus *Monocotyloidea* were recovered, although the relationships among them remained mostly unresolved. Therefore, these results continue to support our previous hypothesis that the lack of resolution in *Eryn-*

gium is due to rapid radiations in the ancestor of the "New World s.str." clade. The polytomy within *Eryngium* subgenus *Eryngium* is equivalent (in terms of its characterization) to the "New World s.str." polytomy (Calviño et al. 2008). Consequently, we continue to support our previous hypothesis that rapid radiations within *E.* subgenus *Eryngium* are also the cause of its major polytomy. Within the "New World s.str." clade some, but not all, of the collapsed branches are resolved in the phylogenetic analysis of all cpDNA regions (Fig. 3). A new clade is uncovered that includes *Eryngium coronatum,* "South American monocotyledonous", and the subclade of "North American monocotyledonous", additional South American monocotyledonous species and *E. glossophyllum*. This clade, however, has a bootstrap value of <50%. Taking into consideration these results (i.e, that resolution for the three major polytomies is not improved), we consider that for *Eryngium* it is not worthwhile to commit to a full scale sequencing effort of these 11 cpDNA regions with the objective of resolving these polytomies. More promising results for the study of relationships among the groups that radiated rapidly in *Eryngium* will probably come from further studies of plastid and nuclear genomes using next-generation sequencing technologies.

Cost / benefit analysis: how many regions are necessary?

The major polytomies of the *Eryngium* phylogeny could not be resolved by the analysis of the 11 non-coding regions examined herein, however, the simultaneous analysis of all cpDNA data resulted in more resolution in other portions of the tree and, in general, higher bootstrap support values. These results indicate that there is still more to be done to improve our knowledge of the evolutionary history of *Eryngium* using cpDNA sequence data. The question is, is the cost (in terms of time and money) worth the benefit? In other words, is it necessary to sequence all 11 cpDNA regions to obtain a more resolved and better supported phylogeny of *Eryngium*? The comparison of number of clades recovered and bootstrap values among the 10 clades obtained by analysis of all cpDNA regions show that by adding the *trnG-trnS, rpl32-trnL*, and *3'trnV-ndhC* to the *trnQ-trnK* region (i.e., *trnQ-trnK* plus 3 regions), we obtain the same set of major clades as in the analysis of the 11 cpDNA regions and with similar bootstrap values. Therefore, our plans are to continue acquisition of these three cpDNA regions for phylogenetic analysis of all species of *Eryngium*. These characters need to be complemented with additional characters from the nucleus. Once we obtain robust phylogenies from the chloroplast and nuclear genomes we will be able to test for hybridizations (which is another important process in the evolutionary history of *Eryngium*) and, ultimately, produce a modern classification of the genus.

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