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Phylogenetic relationships and biogeographic patterns in North American members of *Carex* section *Acrocystis* (Cyperaceae) using nrDNA ITS and ETS sequence data

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Abstract. *Carex* section *Acrocystis* currently includes 27 taxa in North America. Recent phylogenetic studies have suggested that the North American and some but not all of the Eurasian species form a clade. Relationships and biogeographic patterns among species in this core-*Acrocystis* group are explored here using nuclear ribosomal (nrDNA) internal transcribed spacer region (ITS) and nrDNA external transcribed spacer region (ETS) sequence data. While maximum parsimony analysis of the ITS and ETS data provides only a moderately resolved branching structure for species relationships within the core-*Acrocystis* clade, maximum likelihood analysis provides a more resolved hypothesis of relationships in the section. The core-*Acrocystis* clade consists of a grade of Eurasian and primarily western North American species, with a well-supported clade of only eastern North American species nested within this grade. ITS and ETS types do not coalesce within many species or species complexes. Possible explanations for the non-coalescent nature of ITS and ETS copies in *Acrocystis* are explored, including lineage sorting, hybridization, and cryptic species.

Key words: *Acrocystis*, *Carex*, Cyperaceae, ETS, ITS, molecular phylogenetics, nuclear ribosomal DNA.

Section *Acrocystis* Dumort. (= *Montanae* (Fries) Christ) traditionally has included species of *Carex* L. that can be characterized by: (1) pubescent perigynia, (2) three stigmas (rarely two), (3) triangular fruits (rarely lenticular), (4) bracts subtending spikes sheathless or subsheathing, (5) strongly 2-keeled perigynia, (6) absence of a ring-shaped ridge at the apex of the fruit, and (7) low chromosome numbers relative to the rest of the genus (2n = 16–48). Additionally, these species are relatively diminutive (< 0.5 m tall) with short pistillate spikes (< 2 cm long). This section ranges across temperate North America and Eurasia, with one species in the Andes Mountains of South America, and predominantly inhabits dry forest understory.

Twenty-seven taxa currently are recognized as members of *Acrocystis* in North America (Crins and Rettig 2003). Mackenzie revised this group (1913a, b, 1935) and described eight new taxa. North American *Acrocystis* has been divided into several species complexes, with several orphan species not included in any of these complexes. The complexes that have been defined explicitly include the *C. pensyl-*

vanica (Mackenzie 1913a, Crins and Ball 1983), the *C. nigromarginata* (Rettig 1990, Rettig and Giannasi 1990), the *C. deflexa* (B. Ford, University of Manitoba, pers. comm.), and the *C. umbellata* complexes (Mackenzie 1913b, Table 1). Several North American species have not been included in these complexes (Table 1). The relationship among these complexes and the orphan species has not been addressed explicitly, and the North American species have not been examined in reference to the Eurasian or South American members of *Acrocystis*, with few exceptions other than the recent *Carex*-wide phylogenetic studies (Roalson et al. 2001). For example, Fernald (1902) suggested that the Eurasian *C. pilulifera* was difficult to distinguish from the North American *C. communis*, and Egorova (1999) suggested a close phylogenetic relationship between the Asian *C. vanheurckii* Müller and North American *C. pensylvanica* Lam. Since Mackenzie's revision, revisions of several species complexes have been completed without an overall revision. These studies have focused on multivariate numerical analysis of morphological characteristics (Crins and Ball 1983), foliar flavonoid chemistry (Rettig and Giannasi 1990), and achene micromorphology (Rettig 1990).

Circumscription of species and species complexes in North American *Acrocystis* is based upon few characters that are generally continuous in variation (e.g. size, shape, and nervation of the perigynia and perigynia beak length). Some characters are sometimes considered discrete, but are better described as continuous characters with somewhat distinct states (e.g. elongate rhizomes and development of basal pistillate spikes). A detailed morphometric or morphological cladistic analysis is beyond the scope of this paper, so morphological variation will be discussed but not coded.

Studies of relationships in the Cariceae (Roalson et al. 2001) support a core-*Acrocystis* clade that is nested in a large grade within *Carex* subg. *Carex* (Roalson et al. 2001). Potential sister groups of the core-*Acrocystis* clade include members of sections *Glaucae* (Ascherson) Christ. (*C. flacca* subsp. *serrulata*), *Hispidae* Mack. (*C. spissa*), *Acrocystis* s.l. (*C. grioletii* Roem., *C. mandshurica*, and *C. tomentosa* L.), *Aulocystis* Dumort. (*C. firma*

Table 1. Composition of species complexes in North American *Carex* section *Acrocystis*. Asterices denote those taxa sampled in this study

Species Complex	Included Species
C. pensylvanica complex	**C. inops* L.H.Bailey subsp. *heliophila* (Mackenzie) Crins, *C. inops* subsp. *inops*, *C. lucorum* Willd. ex Link var. *austrolucorum* Rettig, **C. lucorum* var. *lucorum*, *C. pensylvanica*.
C. nigromarginata complex	**C. albicans* Willd. ex Spreng. var. *albicans*, *C. albicans* var. *australis* (L.H.Bailey) Rettig, *C. albicans* var. *emmonsii* Dewey ex Torr.) Rettig, **C. floridana* Schw., **C. nigromarginata* Schw., **C. peckii* Howe.
C. deflexa complex	**C. brevicaulis* Mackenzie, **C. deflexa* Hornem. var. *bootii* L.H.Bailey, *C. deflexa* var. *deflexa*, **C. geophila* Mackenzie, *C. pityophila* Mackenzie, **C. rossii* Boott.
C. umbellata complex	**C. tonsa* (Fern.) E.P.Bicknell var. *rugosperma* (Mackenzie) Crins, *C. tonsa* var. *tonsa*, **C. umbellata* Schkuhr ex Willd.
Orphan species	**C. brainerdii* Mackenzie, *C. communis* L.H.Bailey var. *amplisquamata* (F.J.Hermann) Rettig, **C. communis* var. *communis*, **C. globosa* Boott, **C. novae-angliae* Schw., **C. turbinata* Liebm., **C. serpenticola* Zika.

Host), *Rhomboidales* Kük. (*C. wahuensis* subsp. *robusta*), and *Mitratae* Kük. (*C. umbrosa* subsp. *sabynensis*). This core group of *Acrocystis* is derived within *Carex* subgenus *Carex* and is composed of all North American *Acrocystis* species, the Eurasian *C. ericetorum* and *C. pilulifera*, and the east Asian *C. oxyandra*. Several species of *Acrocystis* have yet to be sampled, so their position cannot be inferred.

Nuclear ribosomal DNA (nrDNA) has been used widely as a phylogenetic tool in plants (Baldwin et al. 1995, Soltis and Soltis 1998, Hershkovitz et al. 1999, Hershkovitz and Lewis 1996, Mayer and Soltis 1999, Roalson et al. 2001, Starr et al. 2003). This region of DNA is generally considered to be evolving by concerted evolution (unequal crossing over of the tandem DNA repeat array leading to gene conversion and homogenization, Arnheim et al. 1980, Dover 1982). It has been found that concerted evolution does not always act over a short enough period of time to remove infraspecific polymorphisms and can occur in multiple tandem repeat loci or have pseudogene copies present in the genome (Mayol and Rosselló 2001 and references therein). While these factors can have a negative influence on the usefulness of nrDNA for phylogenetic inference, nrDNA spacer sequences are among the few sequence markers of easy use in exploring phylogenetic relationships among closely related taxa, and therefore are explored for phylogenetic utility here. Specifically, this study explores sequence variation in the nrDNA internal transcribed spacer region (ITS) and the 5′ external transcribed spacer region (ETS) within the core-*Acrocystis* clade and the patterns of diversification implied by this genetic variation.

Materials and methods

Plant samples. Samples were selected from live material grown at Rancho Santa Ana Botanic Garden (RSABG), live material provided by Barb Wilson (OSC) and Takuji Hoshino (OKAY), and herbarium material at ARIZ, BRCH, OSC, NMCR, and RSA. Outgroup sampling included five species that are members of the potential sister groups to the core-*Acrocystis* clade as inferred in Roalson et al. (2001). Twenty-nine individuals representing 22 *Acrocystis* taxa were included. Voucher information is provided in Table 2. Sequences have been deposited in GenBank (new accessions AY325429 to AY325487).

DNA sequencing. DNA was isolated using a modified 2X CTAB buffer method (Doyle and Doyle 1987, Porter 1997). Templates of the nrITS region were prepared using a 1:1 ratio of primers ITS5i (5′-AGG TGA CCT GCG GAA GGA TCA TT-3′) and ITS4i (5′-GGT AGT CCC GCC TGA CCT GG-3′). Templates of the nrETS region were prepared using a 1:1 ratio of primers ETSf (5′-CTG TGG CGT CGC ATG AGT TG-3′) and 18Sr (5′-AGA CAA GCA TAT GAC TAC TGG CAG G-3′, Starr et al. 2003). Polymerase chain-reaction (PCR) amplifications follow the procedures described by Roalson et al. (2001) and Starr et al. (2003). In order to reduce the likelihood of selective amplification of psuedogenes/paralogous ITS/ETS copies, 10% DMSO was included in all amplifications (Buckler and Holtsford 1996, Buckler et al. 1997).

The PCR products were electrophoresed using a 0.8% agarose gel in a 0.5X TBE (pH 8.3) buffer, and subsequently stained with ethidium bromide to confirm a single product and purified using the PEG precipitation procedure (Johnson and Soltis 1995).

Sequencing was performed using an Applied Biosystems Model 373A Automated DNA Sequencing System or an Applied Biosystems Model 377 Automated DNA Sequencing System. Direct cycle-sequencing of purified template DNAs followed manufacturer's specifications, using the PRISM® DyeDeoxy™ Terminator Kit (PE Biosystems) or the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems).

The four ITS sequencing primers provide sequences for overlapping fragments that collectively cover the entire spacer and 5.8S rDNA regions along both strands. Sequencing of the ITS region made use of primers ITS5i, ITS4i, ITS2 (5′-GCT GCG TTC TTC ATC GAT GC-3′), and ITS3 (5′-GCA TCG ATG AAG AAC GCA GC-3′). Sequencing of the ETS region employed the primers ETSf and 18Sr, providing overlapping fragments that covered the 3′ end of the 5′ ETS region (Starr et al. 2003) and a portion of the 5′ end of the nrDNA 18S gene.

Table 2. Taxa of *Carex* section *Acrocystis* and outgroups sampled and voucher information. Collections from USA unless otherwise noted. Specimens are deposited in RSA unless otherwise noted. Designations in parentheses after taxon names refer to designations used to identify samples in the figures when there is more than one sample per taxon

Species	Geographic location	Voucher	Genbank accessions (ITS; ETS)
albicans Willd. ex Spreng. var. *albicans*	West Virginia, Grant Co.	*Reznicek 8150* (BRCH)	AY325479; AY325454
brainerdii Mackenzie	California, Shasta Co.	*Vincent 8588*	AY325485; AY325460
brevicaulis Mackenzie	Oregon, Lincoln Co.	*Wilson s.n.* (OSC)	AY325471; AY325446
communis L.H.Bailey var. *communis*	Canada, Quebec	*Roalson 1334*	AY325469; AY325444
deflexa Hornem. var. *boottii* L.H.Bailey	Wyoming, Teton Co.	*Morse 1795*	AY325480; AY325455
ericetorum Pollich	Sweden, Småland	*Snogerup & Snogerup 3214*	AF284974; AY325437
floridana Schw.	Texas, Jasper Co.	*Jones 6261* (BRCH)	AY325482; AY325457
geophila Mackenzie (r1409)	Arizona, Cochise Co.	*Roalson 1409*	AY325474; AY325449
(m1296)	Colorado, Boulder Co.	*Morse 1296*	AY325481; AY325456
globosa Boott	California, Marin Co.	*Zika 12316* (OSC)	AY325487; AY325462
inops L.H.Bailey subsp. *heliophila* (Mackenzie) Crins	Wyoming, Crook Co.	*Marriott 6575*	AY325484; AY325459
lucorum Willd. ex Link var. *lucorum*	Ohio, Lucas Co.	*Roalson 1336*	AY325464; AY325436
nigromarginata Schw.	Georgia, Rabun Co.	*Roalson 1310*	AY325478; AY325453
novae-angliae Schw.	Canada, Quebec	*Roalson 1333*	AY325475; AY325450
oxyandra Kudo	Japan	*Ikeda 15896* (OKAY)	AF285061; AY325443
peckii Howe	Minnesota, Clearwater Co.	*McNeilus 89-178* (BRCH)	AY325483; AY325458
pilulifera L.	Sweden, Uppland	*Alm 805*	AF284975; AY325438
rossii Boott (r975)	New Mexico, Sierra Co.	*Roalson 975* (NMCR)	AY325470; AY325445
(m1525)	Idaho, Custer Co.	*Morse 1525*	AY325466; AY325440
(w8101b)	California, Siskiyou Co.	*Wilson 8101b* (OSC)	AY325473; AY325448
(cw)	Oregon, Benton Co.	*Wilson s.n.* (OSC)	AY325463; AY325435
serpenticola P. Zika	California, Marin Co.	*Zika 12319* (OSC)	AY325476; AY325451
turbinata Liebm. (r1224)	Arizona, Santa Cruz Co.	*Roalson 1224*	AF284973; AY325434
(l1398)	Mexico, Chihuahua	*Laferrière 1398* (ARIZ)	AY325465; AY325439
umbellata Schkuhr ex Willd. (r1320)	North Carolina, Montgomery Co.	*Roalson 1320*	AY325477; AY325452
(j8963)	Michigan, Washtenaw Co.	*Jones 8963* (BRCH)	AY325486; AY325461
(r1307)	Texas, Burleson Co.	*Roalson 1307*	AY325472; AY325447
sp. nov. (sc)	South Carolina, Barnwell Co.	*Hyatt 5823*	AY325468; AY325442
sp. nov. (nc)	North Carolina, Buncombe Co.	*Rothrock 3011*	AY325467; AY325441
flacca Schreb. subsp. *serrulata* (Biv.) Greuter	Greece	*Hartvig & Franzén 8709*	AF284982; AY325429
mandshurica Meinsh.	Korea, Kangwon Province	*Tyson 5044* (POM)	AF285045; AY325432

Table 2 (continued)

Species	Geographic location	Voucher	Genbank accessions (ITS; ETS)
spissa L.H.Bailey	California, San Diego Co.	*Tilforth & Wisura 2140*	AF285040; AY325431
umbrosa Host subsp. *sabynensis* Less. ex Kunth	U.S.S.R., Siberia	*Murray et al. 344*	AF285042; AY325430
wahuensis C.A.Mey. subsp. *robusta* (Fr. & Sav.) T. Koyama	Japan, Shizuoka Pref.	*Amano s.n.*	AF285023; AY325433

Automated DNA sequencing chromatograms were proofed, edited, and contigs were assembled using Sequencher 3.0 and 4.0 (Gene Codes Corporation, Inc.). The sequences were truncated to include only ITS1, 5.8S, ITS2, the 5′ ETS, and a portion of the 5′ end of the nrDNA 18S gene. Identification of the terminal ends of ITS1, ITS2, and ETS were based on comparisons with other species of Cyperaceae (Roalson and Friar 2000, Roalson et al. 2001, Starr et al. 2003). The sequences were aligned manually.

Maximum parsimony analysis. Maximum parsimony (MP) analysis was performed using PAUP* 4.0b10 (Swofford 2001). Congruence of the ITS and ETS datasets was assessed using the partition homogeneity test (PHT, Farris et al. 1995) as implemented by PAUP*4.0b10 (Swofford 2001). As the PHT found a non-significant result (see Results), the ITS and ETS datasets were combined in all subsequent analyses. Heuristic searches were employed (ACCTRAN, 1000 random addition cycles, TBR branch swapping, STEEPEST DESCENT, MULTREES in effect). Clade support was estimated using 1000 heuristic bootstrap replicates (10 random addition cycles per replicate, 10 trees saved from each addition cycle, TBR branch swapping, STEEPEST DESCENT, Felsenstein 1985, Hillis and Bull 1993).

Maximum likelihood analysis. Maximum likelihood (ML) analysis was performed using PAUP* 4.0b10 (Swofford 2001) on the combined ITS/ETS data set. Heuristic searches were employed (ACCTRAN, starting tree based on neighbor-joining reconstruction, TBR branch swapping, STEEPEST DESCENT, MULTREES in effect). The Tamura and Nei (1993, TrN) model of evolution with rate heterogeneity and among-site rate variation was used in the ML analysis based on the results of analysis using Modeltest 3.0 (Posada and Crandall 1998). The Modeltest analysis tests the fit of various ML models to the data set and estimates base change frequencies, proportion of variable characters, and shape of the gamma distribution, and chooses the model that best fits the data using the Hierarchical Likelihood Ratio Tests (Posada and Crandall 1998). The parameters assigned to the data set for this analysis were as follows: empirical base frequencies ($A = 0.1767$, $C = 0.2800$, $G = 0.2966$, $T = 0.2468$), three substitution types, proportion of sites assumed to be invariable $= 0.5198$, rates for variable sites assumed to follow a gamma distribution with shape parameter $= 1.0100$, and a substitution rate matrix of A/C: 1.0000, A/G: 2.9230, A/T: 1.0000, C/G: 1.0000, C/T: 6.6853, and G/T: 1.0000.

Results

The four ITS sequencing primers produced overlapping fragments that collectively covered the entire spacer and 5.8S rDNA regions along both strands. The aligned ITS data matrix was 615 bp long and the aligned ETS data matrix was 645 bp long. The combined ITS/ETS dataset included 242 variable sites, of which 118 potentially were parsimony-informative. The length of the unaligned ITS sequences varied from 581 to 613 bp and the length of the unaligned ETS sequences varied from 549 to 642 bp. Two ITS sequences are missing a portion (16 and 25 base pairs) of the 5′ end of the ITS1 spacer due to poor sequencing of that region. Eight ETS sequences are missing a portion (1 to 28 base

pairs) of the 5′ end of the ETS region due to poor sequencing of that region. Twenty-three ETS sequences are missing a portion (2 to 62 base pairs) of the 3′ end of the ETS region/5′ end of the 18S gene due to poor sequencing of that region. These regions of missing sequence total 1.12% of the total dataset. The alignment was unambiguous and there were eight gaps ranging from 1 to 4 bp in length in the ITS region and six gaps ranging from 1 to 2 bp in length in the ETS region.

Maximum parisimony analysis. Maximum parsimony analysis of the ITS/ETS *Acrocystis* data set resulted in 480 most-parsimonious trees (length = 380 steps, CI = 0.708, RI = 0.784, RC = 0.555). Figure 1 is the strict consensus of the most-parsimonious trees.

The strict consensus tree has moderate resolution and supports the grouping of several of the core-*Acrocystis* species (Fig. 1). The consensus topology suggests that the Eurasian species (*C. ericetorum*, *C. oxyandra*, and *C. pilulifera*) form a grade (Eurasian Grade [EG]) and diverged from the rest of *Acrocystis* prior to the radiation in North America (Fig. 1). Within the North American lineages, there is a grade and polytomy of primarily western North American species/clades (Western North American Clades [WNAC]) with a large and well-supported clade composed of only eastern North American species (Eastern North American Clade 1 [ENAC1], Fig. 1) and a second clade of two predominately eastern North American species (ENAC2) nested within the WNAC. The WNAC can be split into three sections (WNAC1, 2, and 3, Fig. 1). *Carex turbinata* (r1224) appears to be sister to the rest of the North American *Acrocystis* species, followed by an unresolved polytomy of the rest of the North American collections. Several other nodes are well supported for groups of two or three taxa. Species represented by multiple samples often do not group together (e.g. *C. rossii* and *C. turbinata*) and taxa considered to form a species complex often do not group together either (e.g. the *Carex nigromarginata* species complex). One species complex (*C. umbellata* species complex) is suggested to be monophyletic given current sampling (Fig. 1).

Maximum likelihood analysis and comparison of MP and ML analyses. The ML analysis examined 71,388 rearrangements with a single (−ln = 4099.32698) most-likely topology (Fig. 2).

While the degree of resolution of relationships within North American *Acrocystis* and its allies based on the MP analysis is only moderate, the ML tree corresponds well with some of the MP trees. Both analyses include the same major clades (Figs. 1–2). While not as well-resolved as the ML topology, all branches present in the MP strict consensus are present in the ML topology (Figs. 1–2). The ML analysis provides more resolution for the grouping of species into the WNAC1, 2, and 3 than the MP analysis (Figs. 1–2), and was used for the creation of these groupings where branches were unresolved in the MP analysis.

Discussion

Traditional circumscription of species groups. The *Carex pensylvanica* complex includes three species, two with two subspecies each. Two of these species were sampled in this analysis (Table 1, Crins and Ball 1983). Collections representing this complex do not form a monophyletic group (Figs. 1–2), although both *C. inops* subsp. *heliophila* and *C. lucorum* var. *lucorum* are in the ENAC1. The primary character supporting the grouping of the *C. pensylvanica* complex is the presence of long rhizomes, however, it appears this character might be homoplastic.

The *Carex nigromarginata* complex includes six taxa, four of which were sampled here (Table 1, Rettig 1990, Rettig and Giannasi 1990). The species of this complex are scattered across the entire core-*Acrocystis* clade (Figs. 1–2). *Carex peckii* occurs in the ENAC2, while *C. albicans* var. *albicans*, *C. nigromarginata*, and *C. floridana* occur in the ENAC1. While the scattering of this species complex across the tree is surprising, the only species sampled that occurs west of

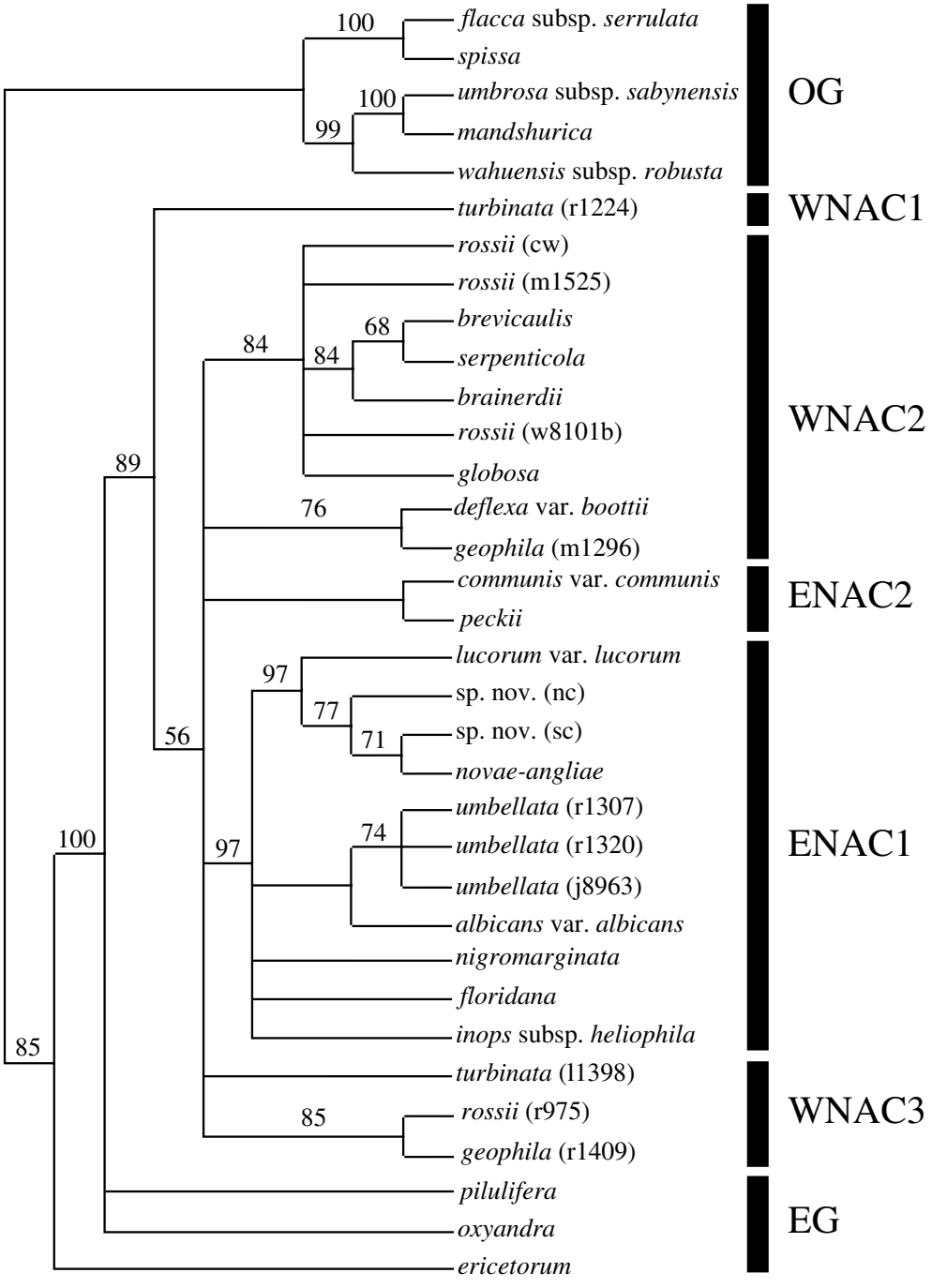

Fig. 1. Core-*Acrocystis* strict consensus tree of 480 most parsimonious trees of 380 steps from the combined data MP analysis (CI = 0.708, RI = 0.784, RC = 0.555). Numbers above the branches are bootstrap percentages. All species in the tree are members of the genus *Carex*. The "OG," "ENAC1," "ENAC2," "WNAC1," "WNAC2," "WNAC3," and "EG" to the right of species names refers to the outgroups, eastern North American clades 1 and 2, the western North American clades 1, 2, and 3, and the Eurasian grade, respectively, as discussed in the text

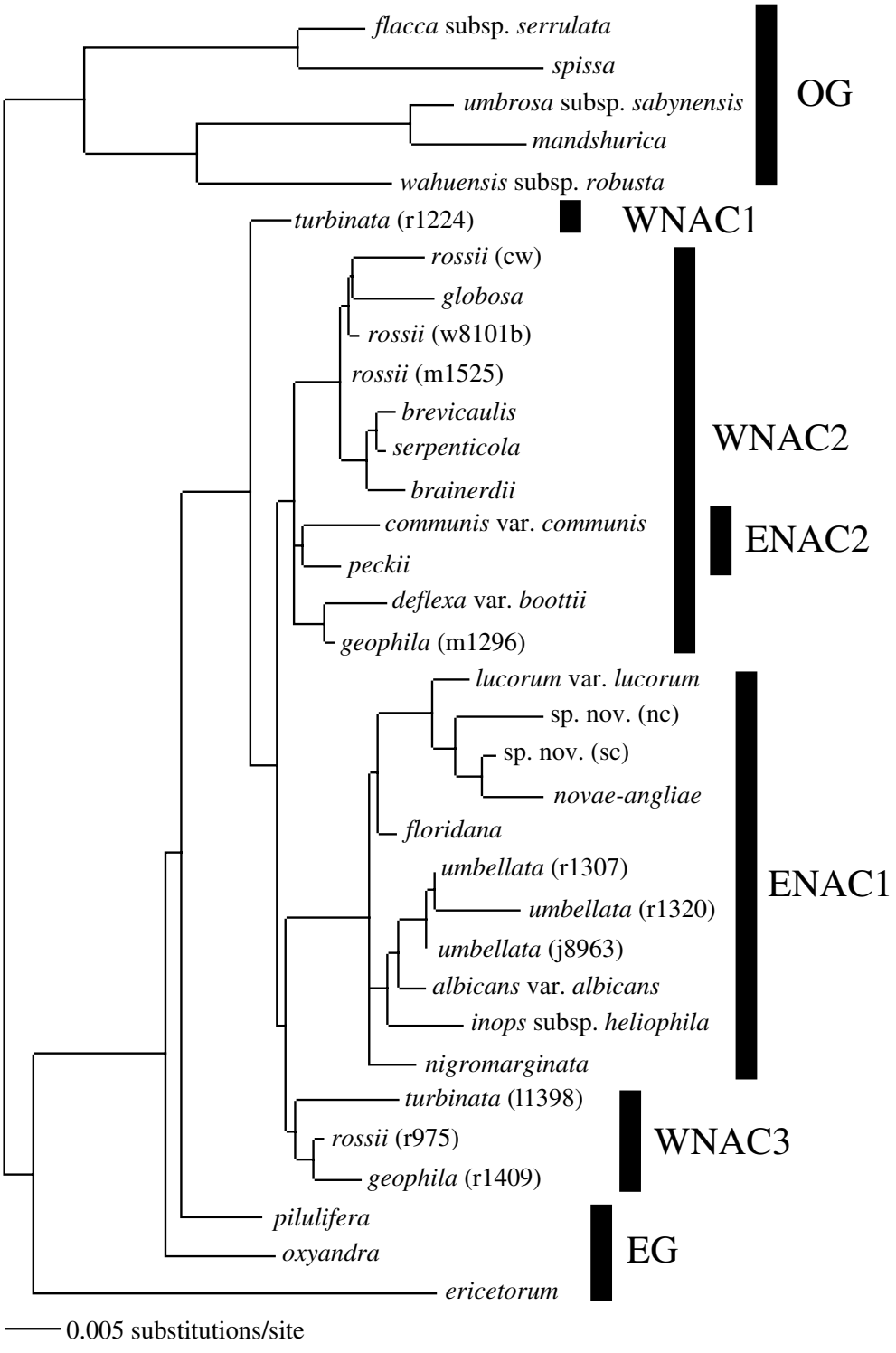

Fig. 2. Core-*Acrocystis* maximum likelihood tree (−ln = 4099.32698). All species in the tree are members of the genus *Carex*. The "OG," "ENAC1," "ENAC2," "WNAC1," "WNAC2," "WNAC3," and "EG" to the right of species names refers to the outgroups, eastern North American clades 1 and 2, the western North American clades 1, 2, and 3, and the Eurasian grade, respectively, as discussed in the text

the Rocky Mountains is *C. peckii* which is found as far west as the Yukon Territory of Canada.

The *Carex deflexa* complex includes five species, four of which were sampled here (Table 1). This complex is difficult morphologically, and is currently under investigation utilizing morphometric analyses (B. Ford, University of Manitoba, pers. comm.). All of these species occur in the WNAC2 and 3, but are intermingled with other western species, including *C. brainerdii*, *C. globosa*, *C. serpenticola*, and *C. turbinata*, and a few eastern North American species (Figs. 1–2). *Carex rossii* is especially variable morphologically, particularly in leaf width. While the taxonomy of this variable species is uncertain, the groupings based on morphotypes and ITS/ETS-types do not correspond (E. H. Roalson, unpubl. data). This species complex is also characterized by the presence of basal pistillate spikes on an elongate peduncle (Mackenzie 1935). This character is inferred to be homoplastic based upon ITS/ETS data. This feature is also present in the ENAC1 (Figs. 1–2), primarily associated with the *Carex umbellata* complex.

While traditional species and species complex circumscription appears to be a particular problem in the *Carex deflexa* species complex, there is some biogeographic pattern to the phylogenetic topology of this complex. The samples in the WNACs appear to form clades based on geographic proximity (WNAC2 and 3). The samples *C. turbinata* (l1398), *C. rossii* (r975), and *C. geophila* (r1409) are all from southern New Mexico and northern Mexico. The samples *C. deflexa* var. *boottii* and *C. geophila* (m1296) are both from Colorado. Finally, the clade of samples including *C. brainerdii*, *C. brevicaulis*, *C. globosa*, *C. rossii* (cw, w8101b, and m1525), and *C. serpenticola* are all from the Pacific Northwest. This geographic structure could imply that there is ongoing hybridization among these species, or it could suggest that current species/species complex circumscriptions do not reflect evolutionary units. Instead, populations within geographic regions have diversified to form similar morphological forms that have been misclassified. Further study is necessary to clarify evolutionary patterns in this group.

Several recent collections from eastern and southeastern North America have been referred to as possible new species (*C.* sp. nov. [nc] and *C.* sp. nov. [sc]). They are distinct from other eastern taxa in being loosely caespitose and having basal spikes and thin, delicate leaves. These samples group in the ENAC1 with other eastern taxa and appear somewhat similar morphologically to *C. novae-angliae*, differing primarily in the presence of basal spikes in the two putative new species. In addition, both undescribed species group with *C. novae-angliae* in the ITS/ETS analysis (Figs. 1–2).

As has been discussed, the *Acrocystis* species that have not previously been considered to belong to a defined species complex are intermingled with several of the defined species complexes. Interestingly, a collection of one of these species, *C. turbinata* (r1224), is placed as sister to the rest of the North American collections, albeit weakly (bs = 56%; Fig. 1). This collection of *C. turbinata*, *Roalson 1224*, would traditionally be classified as *C. leucodonta* T.Holm, but has been recently submerged under a broader concept of *C. turbinata* (Crins and Rettig 2003). This result suggests that the circumscription of *C. turbinata* needs to be looked at carefully, as the leucodonta-type *C. turbinata* collection is the only member of WNAC1 and the turbinata-type *C. turbinata* collection falls within WNAC3.

Biogeographic patterns. The phylogenetic hypothesis presented here supports a clear biogeographic pattern in *Carex* section *Acrocystis*. The earliest branches of the core-*Acrocystis* clade include three species from Eurasia, followed by a grade of species clades restricted to western North America and two clades of species predominately in eastern North America nested within the western North American species clades (Figs. 1–2). This pattern suggests that there was one dispersal event from eastern Eurasia to western

North America followed by two separate dispersal events from western North America to eastern North America. While our preliminary hypothesis is that the Eurasian species radiated into western North America with two subsequent dispersal and radiation events into eastern North America, it may be as likely that after dispersal to North America from Eurasia, there were monophyletic radiations in each geographic region. Morphology does not clearly support either of these potential biogeographic hypotheses, so further studies will be necessary to clarify biogeographic patterns.

Possible influence of lineage sorting and gene paralogs on the ITS/ETS phylogeny. One of the most obvious results of these analyses is the non-monophyly of species and species complexes. At least three mechanisms resulting in non-monophyly could be in effect: lineage sorting/gene paralogs, hybridization, and the presence of cryptic species. Lineage sorting, the stochastic sorting of ancestral polymorphisms, is often inferred to be a cause of non-coalescence of alleles within species (Doyle 1996). If the time since divergence of the species is short and a number of alleles were present in the species before divergence occurred, lineage sorting is expected to result in non-monophyly of species. Lineage sorting of ITS/ETS types among recently diverged species is a possible explanation of non-monophyly of several *Acrocystis* species, given the short branches found in much of the tree and the morphological similarity among the species involved. It is also possible, though, that this could also be a result of homoplasy caused by recombination, although tests for recombination have found no evidence for this (maximum likelihood estimation using PLATO 2.11, data not shown).

A confounding factor in the use of ITS and ETS for phylogenetic inference in several plant families is the presence of ITS/ETS intraspecific polymorphisms (Apiaceae: Vargas et al. 1998, 1999a; Asteraceae: Baldwin et al. 1995 and references therein; Gesneriaceae: Denduangboripant and Cronk 2000; Myrtaceae: Steane et al. 1999; Plumbaginaceae: Fuertes Aguilar et al. 1999; Saxifragaceae: Vargas et al. 1999b), paralogous copies of ITS in the genome (Brassicaceae: O'Kane et al. 1996, Waters and Schaal 1996, Fagaceae: Muir et al. 2001, Malvaceae: Wendel et al. 1995, Paeoniaceae: Sang et al. 1995, Rosaceae: Campbell et al. 1997), and pseudogene copies of the nrDNA repeat (Cactaceae: Hartmann et al. 2001, Fagaceae: Mayol and Rosselló 2001, Malvaceae, Poaceae, and Winteraceae: Buckler et al. 1997). In the case of paralogous loci, the number of distinct nrDNA copies is likely greatly influenced by the frequency of polyploidy and hybridization in the lineage (Mayol and Rosselló 2001).

In this study steps were taken to minimize the potential influence of these factors by using DMSO in amplifications, which has been shown to reduce the likelihood of preferential amplification of pseudogene copies of nrDNA in other organisms (Buckler et al. 1997). Additionally, when paralogs or pseudogenes are present it is often evident in the ITS/5.8S region using direct sequencing by highly polymorphic sequences (Muir et al. 2001, Denduangboripant and Cronk 2000), significant sequence variation in the 5.8S region (Buckler et al. 1997), high levels of sequence divergence among putatively closely related species (Mayol and Rosselló 2001), or conspicuous length and G+C% divergence (Buckler et al. 1997, Mayol and Rosselló 2001). Since (1) sequence polymorphisms in the *Acrocystis* ITS/ETS sequences were rare, and, when present, not at the levels expected if paralogs were involved, (2) there were not high levels of sequence variation in the 5.8S region, (3) there were not significant length differences, and (4) there was not significant G+C% divergence among the ITS/ETS copies sequenced, it is expected that paralogous and pseudogene copies of ITS/ETS are not having a major influence on the topology of the ITS/ETS phylogeny. ITS/ETS copies were not cloned as part of this study, and this and other experiments may help resolve any potential influence of paralogous copies in future studies.

The lack of coalescence of ITS/ETS-types could also be a function of an inadequate amount of sequence data. The genetic distance (uncorrected p) within the ingroup is from 0 to 6%. It is possible that sampling error is responsible for the lack of coalescence and additional data will be necessary to refute this alternative hypothesis and support actual non-coalescence of genotypes within species and species complexes.

Possible influence of hybridization and cryptic species on the ITS/ETS phylogeny. *Carex* species have been regularly inferred to hybridize (Cayouette and Catling 1992, Ford et al. 1993). Some samples were excluded from the analyses presented here due to the likelihood they had hybridized in nature (samples of *C. albicans* var. *emmonsii* and *C. pensylvanica*, data not shown). The possibility that hybridization is influencing the topologies presented here cannot be excluded and will have to be explored in more detail in future studies.

The presence of cryptic species is a possible confounding factor, as has been found in other studies in *Carex* (Naczi et al. 1998). *Carex* is also renowned for its difficulty in species determination (Webber and Ball 1984). Given the paucity of morphological features used to separate species and the presence of largely continuous variation in several characters, this could explain the non-monophyly of species, particularly the broad distribution of *C. rossii* in this study. Alternatively, there could be fewer species of *Acrocystis*, that is, there may be an over-estimation of species number based on morphology. While this concept is difficult to reconcile with the phylogenetic hypothesis presented here, it is possible that the non-monophyly of species in the ITS/ETS phylogeny is indicative of within-species population variation. This is possible, but this interpretation would require lumping of several species in *Acrocystis*.

These analyses provide a hypothesis of relationships for the North American members of *Carex* section *Acrocystis*. While resolution in this study is only moderate, the evidence suggests that there is a geographical grade from Eurasia to western North America with two subsequent dispersal events to eastern North America (Figs. 1–2). Of particular interest is the possible interactions of lineage sorting and hybridization in confounding the ITS/ETS phylogeny and species boundaries. These issues need to be addressed in greater detail with population genetic and comparative morphological studies in the future. The data presented here may support the position that non-coalescence of ITS/ETS-types within species and species complexes is more a factor of young species where sufficient time has not yet passed to achieve coalescence or the presence of cryptic species rather than the position that paralogous or pseudogene copies of ITS/ETS are influencing the phylogenies.

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