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Andrew L. Hipp
University of Wisconsin, Madison

Paul E. Rothrock
Taylor University, Upland, Indiana

Anton A. Reznicek
University of Michigan, Ann Arbor

Paul E. Berry
University of Wisconsin, Madison

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CHROMOSOME NUMBER CHANGES ASSOCIATED WITH SPECIATION IN SEDGES: A PHYLOGENETIC STUDY IN *CAREX* SECTION *OVALES* (CYPERACEAE) USING AFLP DATA

ANDREW L. HIPP,[1,4,5] PAUL E. ROTHROCK,[2] ANTON A. REZNICEK,[3] AND PAUL E. BERRY[1,6]

[1]*University of Wisconsin, Madison, Department of Botany, 430 Lincoln Drive, Madison, Wisconsin 53706, USA;* [2]*Taylor University, Randall Environmental Studies Center, Upland, Indiana 46989-1001, USA;* [3]*University of Michigan Herbarium, Department of Ecology and Evolutionary Biology, 3600 Varsity Drive, Ann Arbor, Michigan 48108-2287, USA*

[4]*Corresponding author (ahipp@mortonarb.org)*

ABSTRACT

Phylogenetic analysis of amplified fragment length polymorphisms (AFLP) was used to infer patterns of morphologic and chromosomal evolution in an eastern North American group of sedges (ENA clade I of *Carex* sect. *Ovales*). Distance analyses of AFLP data recover a tree that is topologically congruent with previous phylogenetic estimates based on nuclear ribosomal DNA (nrDNA) sequences and provide support for four species groups within ENA clade I. A maximum likelihood method designed for analysis of restriction site data is used to evaluate the strength of support for alternative topologies. While there is little support for the precise placement of the root, the likelihood of topologies in which any of the four clades identified within the ENA clade I is forced to be paraphyletic is much lower than the likelihood of the optimal tree. Chromosome counts for a sampling of species from throughout sect. *Ovales* are mapped onto the tree, as well as counts for all species in ENA clade I. Parsimony reconstruction of ancestral character states suggest that: (1) Heilborn's hypothesis that more highly derived species in *Carex* have higher chromosome counts does not apply within sect. *Ovales,* (2) the migration to eastern North America involved a decrease in average chromosome count within sect. *Ovales,* and (3) intermediate chromosome counts are ancestral within ENA clade I. A more precise understanding of chromosomal evolution in *Carex* should be possible using likelihood analyses that take into account the intraspecific polymorphism and wide range of chromosome counts that characterize the genus.

Key words: agmatoploidy, amplified fragment length polymorphisms (AFLP), *Carex,* chromosomal evolution, maximum likelihood, minimum evolution, speciation.

INTRODUCTION

Chromosomal Evolution and Sedge Speciation

The sedge genus *Carex* L. (Cyperaceae) is one of the most species rich of angiosperm genera, with 2000 species recognized worldwide (Reznicek 1990). The genus makes up nearly half of Cyperaceae and is eight times as species-rich as its putative sister group *Scirpus* L., including the segregate *Amphiscirpus* Oteng-Yeb., *Eriophorum* L., and *Dulichium* Pers. combined (Simpson 1995; Muasya et al. 2000; Roalson et al. 2001). The diversity of the genus has long excited interest and speculation as to evolutionary relationships (Kükenthal 1909; Heilborn 1924; Mackenzie 1935; Savile and Calder 1953; Egorova 1999; Starr et al. 1999; Yen and Olmstead 2000; Roalson et al. 2001). At the same time, the subtle differences within species groups provide taxonomic riddles (Reznicek 1989) and frustrate attempts to reconstruct phylogeny from morphological data alone (Crins 1990).

Sedges have holocentric chromosomes, which are generally presumed to evolve rapidly by fission and fusion rather than by reciprocal translocation or duplication. This form of chromosomal evolution is referred to as agmatoploidy or quantitative aneuploidy (Greilhuber 1995). The potential for rapid chromosomal evolution has been hypothesized to play an important role in the diversification of sedges, either through reduced hybrid fitness (Whitkus 1988*b*) or the rearrangement of ecologically significant linkage groups (Whitkus 1988*a*). Addressing this hypothesis for increased sedge speciation using cytogenetic, ecological, and biosystematic methods is certain to be one of the central goals of sedge systematics in the coming decades.

Before the role of chromosomal evolution in sedge speciation can be addressed directly, a better understanding of the mechanism of chromosomal evolution in the family is needed. Recent work has demonstrated that chromosomal evolution may proceed by both polyploidy and agmatoploidy in at least the genus *Rhynchospora* Vahl (Luceño et al. 1998; Vanzela et al. 2000), but examples of simple polyploids are uncommon in *Carex. Carex siderosticta* Hance is the best-documented case of intraspecific polyploidy within the genus, but polyploidy appears not to be associated with speciation (Tang and Xiang 1989). The only seemingly clear-cut case of polyploid speciation with invariant chromosome numbers in *Carex* is in sect. *Chlorostachyae* Tuck. ex Meinsh. (*Capillares* (Asch. & Graebn.) Rouy) (Löve et al. 1957), but this study is based on mitotic counts, which are difficult to interpret given the small chromosomes found in most *Carex* species, and it is not clear that the sampling in

Present addresses: [5] The Morton Arboretum, 41 Illinois Route 53, Lisle, Illinois 60532-1293, USA. [6] University of Michigan Herbarium, Department of Ecology and Evolutionary Biology, 3600 Varsity Drive, Ann Arbor, Michigan 48108-2287, USA.

the study is adequate to justify the claim of invariant chromosome numbers within the species studied. In general, the confusion of haploid chromosome numbers in *Carex* and long series of consecutive counts found in many species suggests that agmatoploidy dominates chromosomal evolution in the genus.

This paper reconstructs the phylogeny of a single clade within *Carex* sect. *Ovales* Kunth to address both the evolution of morphological diversity in the section and the pattern of changes in chromosome number associated with speciation. Two hypotheses are tested from Otto Heilborn's classic (1924) paper on chromosomal evolution in the genus: (1) that chromosome number should be more similar among closely related species than among distantly related species, such that ''the chromosome numbers of a group of related species may be estimated approximately, when the number of one species of the group is known,'' and (2) that chromosomal evolution in the genus has proceeded from lower to higher numbers, such that lower chromosome counts should characterize the more ''primitive'' species.

The Study Group: Carex *sect.* Ovales

Section *Ovales* Kunth is the largest section in *Carex* subgen. *Vignea* (P. Beauv. ex T. Lestib.) Peterm., comprising approximately 20% of species in the subgenus (Song-Yun and Yan-Cheng 1990; Egorova 1999). Section *Ovales* contains 72 North American species (Mastrogiuseppe et al. 2002), ten additional species from South and Central America, and three species that are confined to the Old World (Reznicek 1993), for a total of about 85 species worldwide (Mastrogiuseppe et al. 2002). The section is ecologically widespread, ranging from floodplain forests to alpine tundra, and contains many regionally endemic, morphologically homogeneous species complexes (Hermann 1970; Whitkus and Packer 1984; Whitkus 1988*a*, *b*; Reznicek and Rothrock 1997; Rothrock et al. 1997; Rothrock and Reznicek 2001).

Analysis of nrDNA sequences from the internal transcribed spacer regions (ITS1 and ITS2), 5.8S, and the 3′-end of the external transcribed spacer region (ETS) show strong support for an eastern North American clade that comprises portions of Mackenzie's subsects. ''Festucaceae'' and ''Alatae'' and all of his subsect. ''Tribuloideae'' (ENA clade I: Fig. 1, adapted from Hipp and Rothrock 2002; Hipp in press). However, nrDNA sequences have not been sufficient to resolve relationships within this clade. Extensive taxonomic work in the eastern North American members of sect. *Ovales* over the past decade (Rothrock 1991; Reznicek 1993; Reznicek and Rothrock 1997; Rothrock et al. 1997; Rothrock and Reznicek 2001) has made it possible to sample what is believed to be every species within ENA clade I, and extensive chromosome counting across a wide phylogenetic range of the section (Whitkus 1991; Rothrock and Reznicek 1996, 1998) allows us to address Heilborn's hypotheses at several phylogenetic scales. Future work using the data presented in this paper will address analytical issues related to phylogenetic reconstruction using AFLP data and reconstruction of character evolution that can only be touched upon in this paper, as well as patterns of ecological, biogeographic, and morphological evolution.

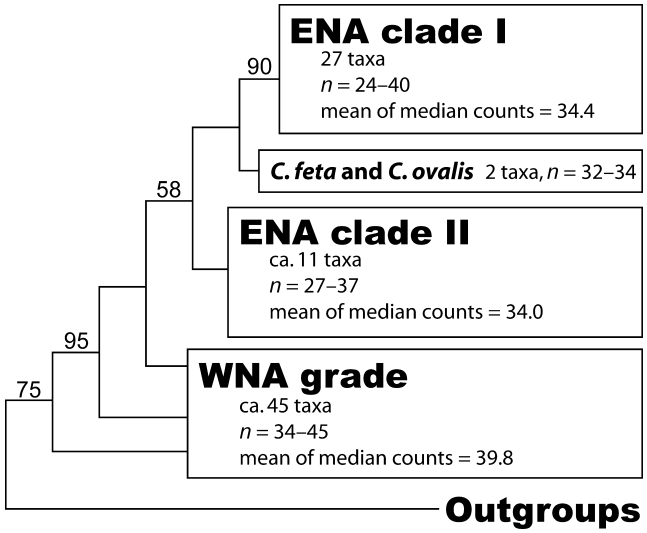

Fig. 1.—Summary of the phylogeny inferred using nuclear ribosomal DNA (ITS, 5.8S, ETS) (Hipp et al. 2002; Hipp in press). The topology shown is based on Bayesian analysis of 101 taxa, including 17 outgroups, 81 named taxa of *Carex* sect. *Ovales,* and three unnamed taxa within sect. *Ovales.* Chromosome counts are from sources cited in the text.

MATERIALS AND METHODS

Sampling

Previous work on the section has identified 27 members of sect. *Ovales,* which occur within ENA clade I (Fig. 1, 2) (Hipp in press). Because of the nearly exhaustive sampling of eastern North American taxa and the strong geographic signal found in the data, it is believed that these 27 taxa represent virtually all taxa within ENA clade I. All 27 taxa within the clade have been sampled for this study (Table 1). At least two individuals from two separate populations per taxon were sampled for 19 of the ingroup taxa; preliminary analyses indicate that the topology is robust to sampling in all but the *C. tenera* clade, in which slight differences in topology result from removal of duplicate individuals. Because of the potential for increased density of taxon sampling to reduce phylogenetic error, even using distance methods (Poe 2003), we present the topology recovered with all populations sampled. Sixteen additional members of sect. *Ovales* were sampled from outside of ENA clade I, representing all of the Mackenzian subsections and a broad morphological and geographical range of the section. A single outgroup species was included from sect. *Stellulatae* Kunth, as the limits of the section have already been addressed using a wider range of outgroups (Hipp in press).

DNA Extraction

DNA was extracted from live, silica-dried, and frozen tissue of single individuals. Initial trials indicated that the type of tissue preservation did not significantly affect results of AFLP analysis, and final results (Fig. 2) show samples from herbarium material consistently falling next to conspecific samples from fresh, frozen, or silica-dried material. Extractions were performed using DNeasy (QIA-

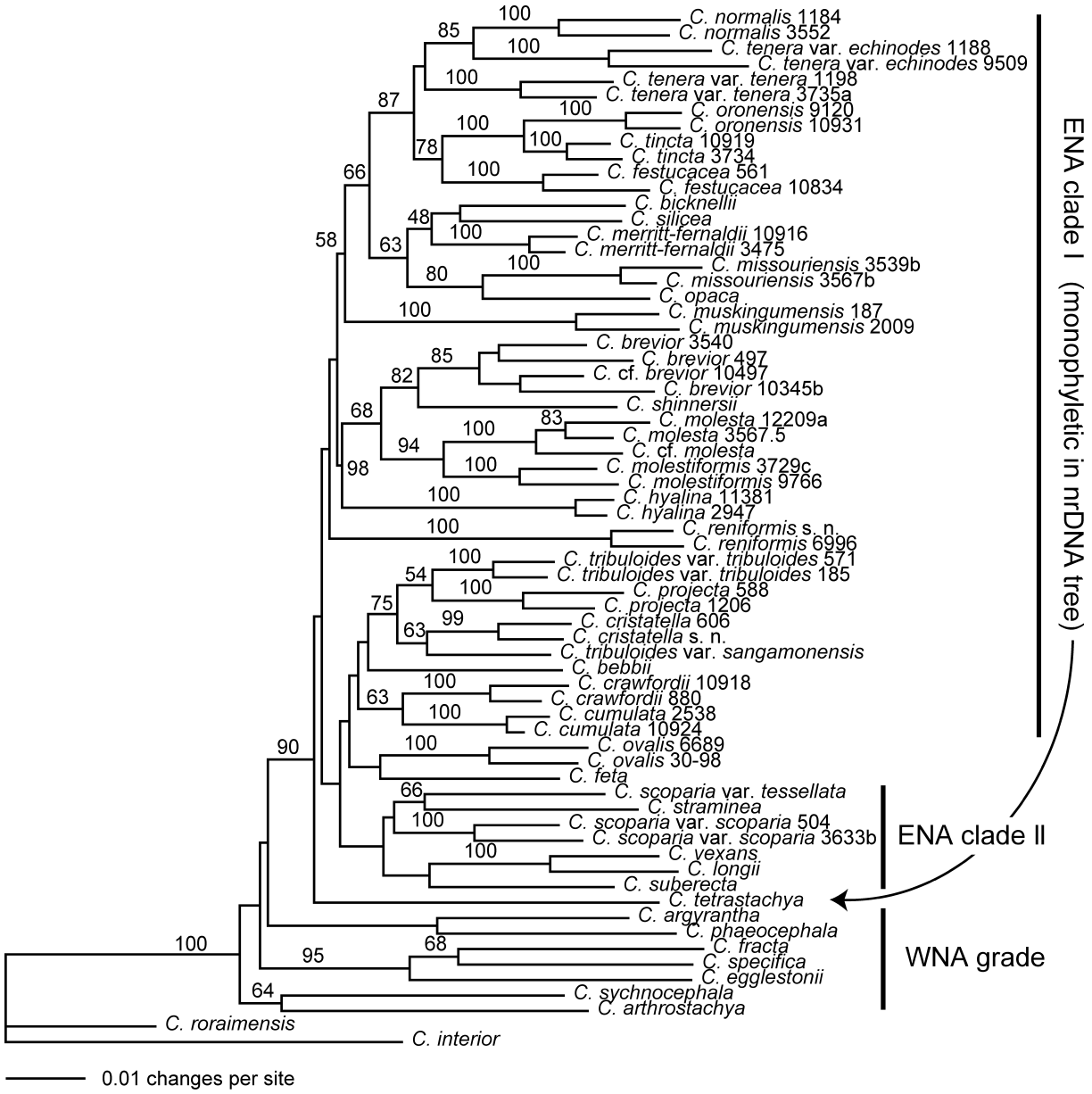

Fig. 2.—Minimum-evolution tree based on all AFLP data, with branch lengths optimized using maximum likelihood. The failure to recover a monophyletic ENA clade I in this analysis is discussed in the text. Branch lengths optimized in RESTML show less obvious bias than those optimized using least-squares (the method of branch length optimization used in the minimum evolution method) on a Nei-Li pairwise distance matrix.

GEN, Inc., Valencia, California, USA), a filter-based method that includes a 1% RNase treatment during cell lysis. Initial trials demonstrated no difference between AFLP banding patterns from DNeasy extractions vs. 6% CTAB extractions (Doyle and Doyle 1987), suggesting that the AFLP method may be robust to extraction methods, at least in *Carex*. Recommendations that only filter-based methods be used in plants (M. Berres pers. comm.) due to fluorescence of secondary compounds do not seem to be an issue in sedges, which possess minimal secondary compounds. Extracted DNA was resuspended in QIAGEN elution buffer and stored at −4°C.

AFLP Reactions

AFLP protocols are modified from the original method (Vos et al. 1995) following M. Berres (pers. comm. and Berres 2001), with details as follows:

Restriction digestion.—Samples were digested in 20 μL reactions containing 20 units of MseI; 20 units of EcoRI; 20 ng BSA; EcoRI buffer; and 5–100 ng of DNA. Initial trials indicated that the AFLP protocol gives consistent results across this range of DNA concentrations. Digestions were performed at 37°C for 3 hr.

Table 1. Collection data for individuals included in study.

Species	Collector	Collection locality	Herbarium
C. argyrantha Tuck. ex Dewey	*Reznicek 10921*	Maine (Washington)	WIS
C. arthrostachya Olney	*Hipp et al. 794*	California (Nevada)	WIS
C. bebbii (L. H. Bailey) Fernald	*Hipp 516*	Wisconsin (Dane)	WIS
C. bicknellii Britton var. *bicknellii*	*Hipp 549*	Wisconsin (Dane)	WIS
C. brevior (Dewey) Mack. ex Lunell	*Reznicek 10345b*	Texas (Kaufmann)	MICH
C. brevior (Dewey) Mack. ex Lunell	*Hipp 497*	Wisconsin (Dane)	WIS
C. brevior (Dewey) Mack. ex Lunell	*Rothrock 3540*	Illinois (Fayette)	MICH
C. cf. *brevior* (Dewey) Mack. ex Lunell	*Reznicek 10497*	Mexico: Chiapas	MICH
C. crawfordii Fernald	*Reznicek & Reznicek 10918*	Maine (Hancock)	WIS
C. crawfordii Fernald	*Zimmerman 880*	Wisconsin	WIS
C. cristatella Britton	*Hipp & Zimmerman 606*	Wisconsin (Rock)	WIS
C. cristatella Britton	*McCullougy & Morehouse s. n. 8/15/2001*	Wisconsin (Monroe)	WIS
C. cumulata (L. H. Bailey) Mack.	*Rothrock 2538*	Indiana (Newton)	MICH
C. cumulata (L. H. Bailey) Mack.	*Reznicek 10924*	Maine (Washington)	WIS
C. egglestonii Mack.	*Hipp 1594*	Colorado (Grand Co.)	WIS
C. festucacea Schkuhr ex Willd.	*Hipp et al. 561*	Wisconsin (Juneau)	WIS
C. festucacea Schkuhr ex Willd.	*Reznicek & Reznicek 10834*	Illinois (Iroquois)	WIS
C. feta L. H. Bailey	*Hipp 457*	California (Humboldt)	WIS
C. fracta Mack.	*Hipp 635*	California (Mariposa)	WIS
C. hyalina Boott	*Bryson 11381*	Mississippi (Coahoma)	WIS
C. hyalina Boott	*Rothrock 2947*	Mississippi (Tunica)	MICH
C. interior L. H. Bailey	*Thompson 399*	Wisconsin (Trempealeau)	WIS
C. longii Mack.	*Zamudio et al. 11237*	Mexico: Michoacan	MICH
C. merritt-fernaldii Mack.	*Rothrock 3475*	New Hampshire (Strafford)	MICH
C. merritt-fernaldii Mack.	*Reznicek & Reznicek 10916*	Maine (Cumberland)	WIS
C. missouriensis P. E. Rothrock & Reznicek	*Rothrock 3539b*	Illinois (Fayette)	MICH
C. missouriensis P. E. Rothrock & Reznicek	*Rothrock 3567b*	Missouri (Macon)	MICH
C. molesta Mack. ex Bright	*Bryson 12209a*	Mississippi (Bolivar)	MICH
C. molesta Mack. ex Bright	*Rothrock 3567.5*	Missouri (Macon)	MICH
C. cf. *molesta* Mack. ex Bright	*Reznicek 10461*	Arkansas (Marion)	MICH
C. molestiformis Reznicek & P. E. Rothrock	*Reznicek 9766*	Oklahoma (Mays)	MICH
C. molestiformis Reznicek & P. E. Rothrock	*Rothrock 3729c*	Tennessee (Jackson)	MICH
C. muskingumensis Schwein.	*Hipp 187*	Wisconsin (Iowa)	WIS
C. muskingumensis Schwein.	*Hipp & Biggs 2009*	Wisconsin (Iowa)	WIS
C. normalis Mack.	*Rothrock 3552*	Missouri (Boone)	MICH
C. normalis Mack.	*Hipp & Rothrock 1184*	Wisconsin (Ozaukee)	WIS
C. opaca (F. J. Herm.) P. E. Rothrock & Reznicek	*Reznicek 10856*	Illinois (Washington)	MICH
C. oronensis Fernald	*Reznicek et al. 10931*	Maine (Penobscot)	WIS
C. oronensis Fernald	*Reznicek & Reznicek 9120*	Maine (Penobscot)	WIS
C. ovalis Gooden.	*Judziewicz 6689*	Wisconsin (Ashland)	WIS
C. ovalis Gooden.	*Ford 30-98*	New Zealand: Westland Land District	MICH
C. phaeocephala Piper	*Hipp 1642*	Colorado (Gunnison)	WIS
C. projecta Mack.	*Hipp et al. 588*	Wisconsin (Jackson)	WIS
C. projecta Mack.	*Hipp et al. 1206*	Wisconsin (Adams)	WIS
C. reniformis (L. H. Bailey) Small	*Bryson & Goodlett s. n. 5/6/2002*	Mississippi (Holmes)	MICH
C. reniformis (L. H. Bailey) Small	*Hyatt 6996*	Arkansas (Dallas)	MICH
C. roraimensis Steyerm.	*Reznicek 11054*	Venezuela: Roraima	MICH
C. scoparia Schkuhr ex Willd.	*Rothrock 3633b*	Indiana (Newton)	MICH
C. scoparia Schkuhr ex Willd.	*Hipp 504*	Wisconsin (Dane)	WIS
C. scoparia Schkuhr ex Willd. var. *tessellata* Fernald & Wiegand	*Reznicek 10923*	Maine (Washington)	WIS
C. shinnersii P. E. Rothrock & Reznicek	*Reznicek 10367*	Texas (Delta)	MICH
C. silicea Olney	*McNeilus 88-472*	Canada: Newfoundland	WIS
C. silicea Olney	*Reznicek & Reznicek 10915*	Maine (Cumberland)	WIS
C. specifica L. H. Bailey	*Hipp 861*	California (Eldorado)	WIS
C. straminea Willd. ex Schkuhr	*Hipp et al. 561*	Wisconsin (Juneau)	WIS
C. suberecta (Olney) Britton	*Hipp & Zimmerman 598*	Wisconsin (Rock)	WIS
C. sychnocephala J. Carey	*Hipp s. n. 2578*	Wisconsin (Waushara)	WIS
C. tenera Dewey var. *echinodes* (Fernald) Wiegand	*Reznicek 9509*	Canada: Ontario	MICH
C. tenera Dewey var. *echinodes* (Fernald) Wiegand	*Hipp & Rothrock 1188*	Wisconsin (Ozaukee)	WIS

Table 1. Continued.

Species	Collector	Collection locality	Herbarium
C. tenera Dewey var. *tenera*	*Rothrock 3735a*	Maine (Washington)	MICH
C. tenera Dewey var. *tenera*	*Hipp et al. 1198*	Wisconsin (Sauk)	WIS
C. tetrastachya Scheele	*Reznicek 10411*	Texas (Jefferson)	MICH
C. tincta (Fernald) Fernald	*Reznicek & Reznicek 10919*	Maine (Hancock)	WIS
C. tincta (Fernald) Fernald	*Rothrock 3734*	Maine (Hancock)	MICH
C. tribuloides Wahlenb. var. *sangamonensis* Clokey	*Rothrock 2941*	Mississippi (Tunica)	MICH
C. tribuloides Wahlenb. var. *tribuloides*	*Hipp et al. 571*	Wisconsin (Juneau)	WIS
C. tribuloides Wahlenb. var. *tribuloides*	*Hipp 185*	Wisconsin (Iowa)	WIS
C. vexans F. J. Herm.	*Rothrock 2379*	Florida (Paseo)	MICH

Adapter ligation.—Double-stranded adapters were ligated to digestion product in 40 μL reactions using 160 units T4 DNA ligase, 0.75 picomoles of the MseI adapter, 0.75 picomoles of the EcoRI adapter, 1× ligase buffer, and 20 μL of digestion product. The reaction was held at 16°C for 12–16 hr, after which the ligation reaction product was diluted 5-fold.

Preamplification.—10 μL of diluted ligation reaction product was amplified in 50 μL reactions using 0.30 μM MseI + C primer, 0.30 μM EcoRI + A primer, 0.20 μM dNTP, 1.25 units Taq DNA polymerase, 1× $MgCl_2$-free buffer, 1.5 μM $MgCl_2$. Cycling regimen was: initial extension of 94°C for 60 s; 20 cycles of 94°C for 50 s, 56°C for 60 s, 72°C for 80 s; final extension of 72°C was held for 2 min, followed by an indefinite hold at 4°C.

Selective amplification..—Final amplification was conducted in 25 μL reactions with 1.0 μM of selective primer MseI + Cxx, 0.20 μM of fluorescently labeled primer EcoRI + Axx (where each ''x'' denotes a nucleotide), 0.30 mM dNTP, 1.25 units Taq DNA polymerase, 1× $MgCl_2$-free buffer, and 1.5 mM $MgCl_2$. Nine initial touch-down cycles of 94°C for 50 s, annealing temperature for 60 s, and 72°C for 120 s were conducted to bring the annealing temperature from 65°C to 56°C, followed by 20 additional cycles at 56°C. Final extension of 72°C was held for 10 min, followed by an indefinite hold at 4°C. In selective amplifications, the EcoRI + Axx primer was labeled with the fluorescent dye 6-FAM.

Twenty-four primer pairs were screened to identify primers that would provide a range of information both across sect. *Ovales* and within the *C. tenera* group. From this screening, nine primer pairs were selected for amplification on all taxa (Table 2). PCR product was purified using the CleanSEQ dye-terminator removal system (Agencourt Bioscience Corporation, Beverly, Massachusetts, USA).

AFLP Fragment Analysis

Purified PCR product was electrophoresed in an ABI 3700 automated sequencer (Applied Biosystems, Foster City, California, USA) with a ROX-labeled internal lane standard, fragments of known size ranging from 50–625 base pairs (bp) in 25-bp intervals. The resulting gel image was analyzed in GeneScan vers. 3.7 for Windows, eliminating bands below 50–85 bps in length, according to what the smallest unambiguous bands were for each primer. Tabular data from GeneScan were exported to Microsoft Excel and manually edited with reference to the ABI chromatographs to correct for discrepancies in fragment size-calling and inconsistencies in the intensities of bands between different lanes. Markers that could not be unambiguously scored (i.e., could not be positively determined to be present or absent for every individual represented) were excluded from the data set. Edited markers were scored as binary data matrices. The edited data matrix and trees reported in this study are deposited in TreeBASE (accession number SN1908-6169).

Phylogenetic Analyses

Topologies were recovered from the data using the minimum evolution (ME) criterion (Rzhetsky and Nei 1992) on

Table 2. Selective nucleotides of the nine primer pairs employed in this study. Sequences of the primers used for PCR were complementary to the adapters ligated to cut restriction sites, with three added selective nucleotides. Markers were determined to be variable and/or informative by removing character sets in PAUP*.

EcoRI	MseI	Total markers	Variable markers	Potentially informative markers	Potentially informative within ENA Clade
AGC	CAG	191	177 (92.7%)	103 (53.9%)	75 (39.3%)
ATG	CAG	186	177 (95.2%)	126 (67.7%)	85 (45.7%)
ACT	CTT	160	152 (95.0%)	97 (60.6%)	79 (49.4%)
ACT	CCG	157	150 (95.5%)	87 (55.4%)	66 (42.0%)
ATG	CTC	148	143 (96.6%)	86 (58.1%)	55 (37.2%)
ATG	CGA	144	138 (95.8%)	92 (63.9%)	70 (48.6%)
ATT	CGT	141	135 (95.7%)	77 (54.6%)	56 (39.7%)
AGA	CGG	136	132 (97.1%)	65 (47.8%)	43 (31.6%)
ATT	CCG	131	124 (94.7%)	66 (50.4%)	46 (35.1%)
SUM TOTALS		1394	1328 (95.3%)	799 (57.3%)	575 (41.2%)

a pairwise distance matrix calculated using the restriction site distance of Nei and Li (Nei and Li 1979). Although Nei and Li's distance is based on the relatively simple Jukes-Cantor model of nucleotide substitution (Jukes and Cantor 1969) and is typically not tailored to the large number of nucleotides that make up the recognition site of a typical AFLP marker (16 base pairs in this study and most other published AFLP studies), it nonetheless recovered topologies more congruent with nrDNA trees and with consistently higher likelihood scores than distance analyses conducted using the more complex distance metric implemented in the RESTDIST program of PHYLIP vers. 3.6 (Felsenstein 1989). Heuristic searches were conducted in PAUP* vers. 4.0b10 (Swofford 2002) with a neighbor-joining start tree, tree-bisection-reconnection (TBR) branch swapping, and steepest descent activated. Branch support was estimated using nonparametric bootstrapping, 200 ME heuristic search pseudoreplicates. Bootstrap support within ENA clade I was estimated without outgroups. This method is occasionally used in likelihood or parsimony analysis to reduce the effects of long-branch attraction between ingroup and outgroup taxa (Hendy and Penny 1989; Jordan et al. 2003), but the method also is well suited to distance analyses, in which topology is estimated by optimizing the fit of all pairwise distances to a single tree. Inclusion of highly divergent sequences in this case may strongly affect estimates of clade support. Bootstraps along the three branches involving outgroups were estimated in a second set of analyses including outgroups.

A maximum likelihood method developed for analysis of restriction site data (Felsenstein 1992) was used to optimize branch lengths and evaluate the likelihood of trees recovered in the distance analyses, and the Shimodaira-Hasegawa (SH) test was used to test the support for optimal trees relative to alternative hypotheses (Shimodaira and Hasegawa 1999). These analyses were performed in RESTML (PHYLIP vers. 3.6), treating each AFLP band as a 16 base-pair restriction site (Felsenstein 2004). Performing these analyses required changing MAXCUTTER in the PHYLIP header files PHYLIP.H and SEQ.H from 8 to 16 (base pairs); this constant is used only in dimensioning arrays and therefore has no effect on likelihood calculations. The program was recompiled using the Microsoft Visual C++ compiler and results double-checked to verify that likelihood calculations were not affected.

Analysis of Chromosomal Data

Heilborn's hypothesis that ''chromosome numbers of a group of related species may be estimated, approximately, when the number of one species of the group is known'' can be interpreted more formally as the hypothesis that there will be less variation in chromosome number within a group of closely related species than among several less closely related groups. We evaluated this hypothesis at the subsectional and sectional levels by using single-factor ANOVA and two-sample *t*-tests to ascertain chromosome number means within and between WNA and ENA *Ovales* lineages and among the three strongly supported clades in ENA clade I. Means for each lineage were calculated as the average of the median chromosome count found within each taxon sampled. Chromosome numbers were taken from the three most recent and thorough reviews of chromosome numbers in the section (Whitkus 1991; Rothrock and Reznicek 1996, 1998) and unpublished counts by P. E. Rothrock. Species were assigned to each clade based on nrDNA data (Hipp and Rothrock 2002; Hipp in press). All analyses were conducted in Microsoft Excel. Tabulated chromosome data are available from the first author on request.

Heilborn's second hypothesis is that higher chromosome numbers are the derived condition in *Carex*. If this is true, we expect the chromosome number inferred for the most recent common ancestor of any clade within *Carex* to be lower than the average for that group, provided that the ancestral chromosome number is inferred correctly. To evaluate Heilborn's hypothesis at the sectional level, we tested whether the mean chromosome counts among species of the WNA grade differed significantly from those of the ENA clades using a two-sample *t*-test. If Heilborn's hypothesis is true, we would expect mean chromosome numbers within the western grade to be significantly lower than the more highly derived ENA clades, implying that the chromosome count for the most recent ancestor common to the species of sect. *Ovales* is lower than the average for the section. Within ENA clade I, we mapped all chromosome counts onto the tree and marked taxa in which populations were known with the four highest (n = 37–40, HI) and the four lowest (n = 24–27, LO) chromosome counts; the remaining taxa contain only populations with a medium number of chromosomes (n = 28–36, MED). The ancestral chromosome count for the clade was inferred using maximum parsimony in MacClade (Maddison and Maddison 1992).

RESULTS

Molecular Markers

A total of 1394 unambiguous loci were scored across all taxa (Table 2). Of these, 18 markers appeared sufficiently differentiated from two adjacent markers that they were assigned noninteger fragment lengths. While real DNA fragments cannot be composed of a noninteger number of base pairs, sparing use of this method of scoring permitted the recognition of markers that may be shifted on the chromatograph due to differing base-pair composition. Such markers would not then be homologous with either of the adjacent markers, though their recognition sites might or might not be homologous. Markers ranged from 50–616 bp in length, with a mean length of 220 bp, and a strong bias toward smaller fragments (note that these are PCR fragment lengths, which include the PCR primers complementary to the adapters; the original restriction fragments are 24 bp shorter than the fragment that shows up on the chromatographs.) This is consistent with previous findings that demonstrate a bias toward smaller fragments in simulated AFLP data (Vekemans et al. 2002). Across the entire data set, 799 markers are potentially informative, 575 of which are potentially informative within the ENA clade alone (Table 2). Seven individuals sampled from ENA clade I plus one of the *C. ovalis* individuals failed to amplify successfully for one of the nine primer pairs utilized in the study. Data sets corresponding to the failed primer pairs were scored as ambiguities (''?'') in the data matrix. Analyses performed with these individuals

deleted recovered trees in which *C. ovalis* and *C. feta* were not monophyletic, which stands at odds with the nrDNA results (data not shown). Consequently, these individuals were included despite the failed primer pairs.

Sectional Phylogeny

The AFLP topology of sect. *Ovales* (Fig. 2) supports the nrDNA analysis (Fig. 1) in three regards. First, *C. roraimensis,* a species endemic to tepuis of the Guayana Shield in Venezuela, groups more closely with *C. interior* (sect. *Stellulatae*) than with sect. *Ovales.* This relationship is one of the major points of incongruence between ITS and ETS data for sect. *Ovales* and is currently under investigation. The position of *C. roraimensis* is particularly interesting in light of chromosomal evolution, because it has the highest count known in *Carex* (n = 62: Rothrock unpubl. data). Second, the western North American taxa form a grade paraphyletic to the ENA taxa. Third, a monophyletic ENA clade II, composed of members of Mackenzie's subsects. ''Alatae,'' ''Festucaceae,'' and ''Fetae,'' is recovered with moderate bootstrap support.

AFLP data strongly support the monophyly of eastern species as a clade (90%), but they do not support the monophyly of ENA clade I, the focus of this study. However, the bootstrap support along the spine of the tree is for the most part very weak, suggesting that AFLP data do not provide strong evidence regarding the relationship among clades within the larger eastern clade. Given the strong support for ENA clade I in the nrDNA analysis (90% bootstrap: Fig. 1) and weak support for most of the deeper nodes in this study, including the nodes required to evaluate the monophyly of ENA clade I (Fig. 2, 3), ENA clade I was analyzed as a clade for the remainder of the study.

Relationships within ENA Clade I

Rooting ENA clade I with ENA clade II as the outgroup recovers a topology that is identical to the topology recovered when *C. feta* and *C. ovalis* are the designated outgroup. Based on the nrDNA topology (Hipp in press), *C. feta* and *C. ovalis* were designated as outgroups for purposes of this study. To evaluate the strength of support for the position of this root, we shifted the root found in the Nei-Li tree to eleven alternative positions (marked in Fig. 3) and evaluated the likelihood of these trees. By the SH test, none are significantly worse than the optimal tree, which turns out in this case to be not the minimum evolution tree but a tree in which *C. muskingumensis* is sister to the remainder of ENA clade I (Table 3). Importantly, the three trees with the lowest likelihood are the only trees evaluated that fail to support the clades discussed below. In a series of random-addition heuristic searches conducted in RESTML, one tree was recovered with a higher likelihood (logL = −29,667.4) than any reported in this study. This tree (not shown) recovers the four major clades discussed below and most of the same relationships within them, but places the root along a branch not recovered in the ME searches. Because of the computational time required for each search, it is not clear that this tree represents the ML topology for ENA clade I and is consequently not discussed further in this paper.

Three major clades are recovered with over 70% bootstrap support:

1. *The* Carex tribuloides *group.*—A morphologically homogeneous group of three species that have elongated vegetative culms capable of rooting at the nodes; loose leaf sheaths with the ventral hyaline band poorly defined or lacking; narrow, scale-like perigynia; and narrow achenes. The group comprises most of Mackenzie's subsection ''Tribuloideae,'' excluding *C. muskingumensis.*
2. *The* Carex brevior *group.*—A group of six species with broad perigynia that tend to be widest near the middle of the body and achenes that are broadly elliptic to round. *Carex molesta, C. molestiformis,* and *C. brevior* share a close resemblance and are frequently misidentified. *Carex* cf. *molesta* represents a taxonomically ambiguous collection from a valley in Marion Co., Arkansas, and *C.* cf. *brevior* is a population of this group from Chiapas, Mexico, whose taxonomic status has not been determined. *Carex hyalina* is a morphologically highly distinctive species with creeping rhizomes, found in bottomland forests of the south central USA. *Carex shinnersii* is a recently described species from the Great Plains that had previously been confused with *C. bicknellii.*
3. *The* Carex tenera *group.*—A group of six species with lanceolate to ovate perigynia mostly less than 2 mm wide and elliptical achenes. *Carex tenera* var. *tenera, C. tenera* var. *echinodes,* and *C. normalis* form a very coherent clade morphologically. These three can be difficult to distinguish from one another and are the subject of another paper currently in preparation. The other three species are morphologically more diverse and their alliances had previously not been clear.

In addition to these well-supported clades, a fourth clade is supported with 63% bootstrap support in the large-scale analysis (Fig. 2) and 51% support in the ENA clade I analysis (Fig. 3):

4. *The* Carex bicknellii *group.*—Includes four species that are morphologically rather similar, two of which (*C. missouriensis* and *C. opaca*) have only recently been teased apart from *C. bicknellii* (Rothrock and Reznicek 2001; reflected in Mastrogiuseppe et al. 2002). The fifth species, *C. silicea,* is a species of the north Atlantic Coast that was placed in subsect. ''Alatae'' by Mackenzie but is morphologically peculiar enough that its placement was uncertain. Its position in this study could not be verified with a second specimen. Because this position is so far at odds with our expectation based on morphology, *C. silicea* is not included in chromosomal analyses presented below.

Chromosomal Patterns

Tests of differences in mean chromosome number between the WNA grade (mean n = 39.8), ENA clade I (mean n = 34.4), and ENA clade II (mean n = 34.0) show highly significant differences between the WNA grade and each of the ENA clades ($P < 0.0001$ for ANOVA and two-sample t-tests), but no difference between the ENA clades (P = 0.758 in a two-tailed t-test assuming equal variance). Overall differences among the three clades identified within ENA

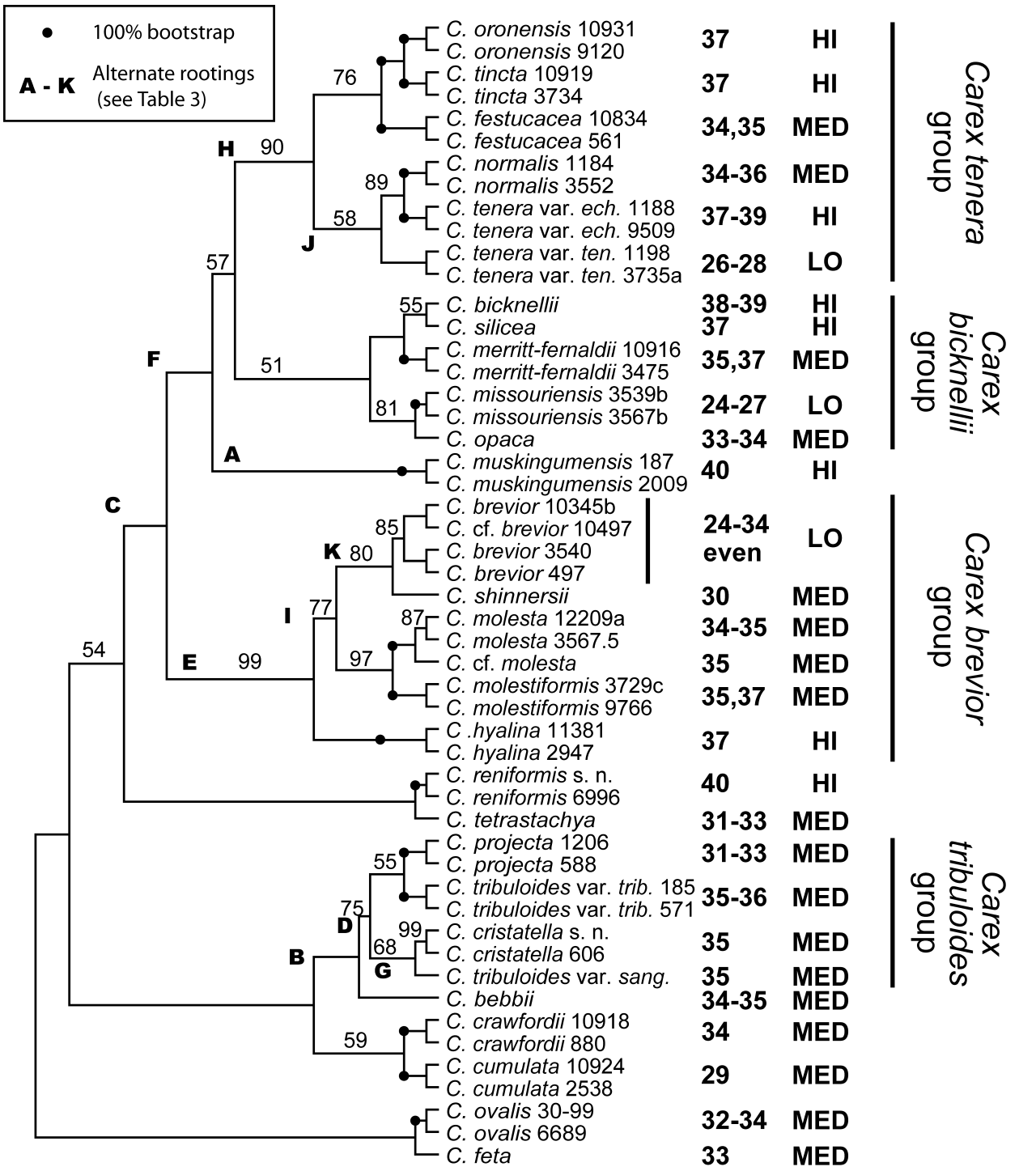

Fig. 3.—Minimum-evolution topology for ENA clade I, with *C. feta* and *C. ovalis* as outgroups. The same tree topology is found when ENA clade II is designated as the outgroup. Haploid chromosome counts are categorized as high (n = 37–40, HI), medium (n = 28–36, MED), and low (n = 24–27, LO) as discussed in the text.

clade I are insignificant (P = 0.946 in a single-factor ANOVA). Parsimony mapping of the three-state chromosome character (HI–LO–MED) onto the AFLP phylogeny inferred for ENA clade I (Fig. 3) requires nine steps, with six independent gains of the HI state and three of the LO state. The MED state is recovered as the most-parsimonious ancestral character state for this tree.

DISCUSSION

Utility of AFLP Data in Inferring Phylogenies

AFLPs are generally not expected to be as reliable across large phylogenetic distances as they will be across smaller phylogenetic distances. Even data analyzed with a correction for nucleotide saturation using the Nei and Li (1979) restric-

Table 3. Likelihood of ME tree recovered in PAUP* relative to 11 alternate rootings, with significance of likelihood difference evaluated using the Shimodaira-Hasegawa (SH) test. Likelihoods and SH *p*-values were calculated in RESTML.

Root position	Log likelihood	Difference	SH *p*-value
A	−29833.0	BEST	n/a
ME–Fig. 3	−29836.9	−3.9	0.905
B	−29845.0	−12.0	0.891
C	−29846.6	−13.6	0.890
D	−29847.4	−14.4	0.919
E	−29866.2	−33.2	0.905
F	−29875.8	−42.8	0.896
G	−29893.7	−60.7	0.896
H	−29957.4	−124.4	0.898
I	−29981.8	−148.8	0.872
J	−29988.8	−155.8	0.874
K	−30025.0	−192.0	0.876

tion site distance frequently recover phylogenies with long terminal branches and extremely short internal branches (Albertson et al. 1999; Richardson et al. 2003), suggesting that either comigrating, nonhomologous bands, or inadequate correction for base pair substitution are biasing phylogenetic reconstruction.

This fact notwithstanding, the AFLP data presented here show very strong support (90% bootstrap) for a large clade of eastern North American species, comprising nearly half of the section (ca. 40 spp.). This node has only 58% bootstrap support in the nrDNA tree with complete sampling (Fig. 1), increasing to 63% bootstrap support when sequence data are pared to only taxa included in this study (tree not shown). AFLP data thus prove to be informative across a wide phylogenetic range in sedges, exceeding the utility of nrDNA at the finest levels and providing data that complement nrDNA data at deeper levels.

Support for three major clades within ENA clade I is also high, and support for most branches within those clades is over 70%. The relationships among clades, however, are not strongly supported by either nrDNA or AFLP data. A few species in this study—e.g., *C. muskingumensis, C. reniformis,* and *C. tetrastachya*—are not conclusively placed. These taxa appear to represent a gap in informative characters between nrDNA sequences (suitable at larger scales within the section) and AFLP data (suitable primarily at the species complex level and at a few deeper nodes). It may be that adding additional AFLP data will help resolve the relationships between these species and the species groups identified in this study; this prospect will be explored more thoroughly in future studies. However, the species groups identified in this study for the most part begin to resolve with only two primer pairs. Addition of another seven primer pairs has the main effect of increasing support for these clades and firming up relationships within them.

Maximum likelihood using a restriction site model had previously been suggested by Felsenstein (2004) to be applicable to AFLP data, but the method is not known to have been applied in any empirical study. While the restriction site model employed in RESTML disregards the potential for loss or gain of an AFLP band due to insertions, deletions, or changes in restriction sites within the band, the model has the potential to extract more information from the data than existing distance methods. Bayesian implementation of a more complete model of the process by which AFLPs evolve is currently under development (B. Larget and R. Luo pers. comm.). Until this model is implemented, likelihood under a restriction site model is probably the best way of evaluating the strength of support for alternative AFLP topologies.

Species Groups Identified within ENA Clade I

The four major species groups identified within ENA clade I correspond largely with expectations based on morphology and chromosome counts. Of these, the most obviously identifiable is the *C. tribuloides* group. The exclusion of *C. muskingumensis* from Mackenzie's subsect. ''Tribuloideae'' is not surprising. *Carex muskingumensis* is morphologically highly anomalous and was included in the subsection based primarily on the fact that it produces elongated vegetative culms. The species grows in forested floodplains, frequently with *C. tribuloides,* suggesting that the elongated vegetative culms may be a case of convergence in response to flooding and/or forest understory conditions that would make taller vegetative shoots adaptive. The alliance of *C. bebbii* with the *C. tribuloides* group is intriguing, if weakly supported. *Carex bebbii* can be confused with *C. cristatella* in the field and forms prominent vegetative shoots late in the season. This relationship and the apparent sister relationship between *C. tribuloides* var. *sangamonensis* and *C. cristatella* bears further study.

Within the *C. tenera* group, the placement of *C. tincta* and *C. oronensis* is surprising but very strongly supported by the data. A previous hypothesis had suggested that *C. oronensis* was related to the Old World *Carex ovalis* (Dibble and Campbell 2001). Both *C. tincta* and *C. oronensis* are confined to open, disturbed ground in the Northeast and have limited ranges that most likely underwent extreme contraction during the last glacial cycle. Resulting bottlenecks may have led to fixation of morphological anomalies in these two species. Interestingly enough, *C. tincta* was first described as a variety of *C. normalis* (under *C. mirabilis* Dewey var. *tincta* Fernald). While pains have previously been taken to distinguish *C. festucacea* from such other species as *C. brevior* and *C. albolutescens* Schwein. (Rothrock 1991), similarity between *C. festucacea* and *C. tenera* in gross inflorescence morphology suggest the placement found for these species in our study may be plausible. Moreover, *C. festucacea* often has papillose leaf sheaths, as *C. tenera* var. *tenera* always does. Low bootstrap support for the position of *C. tenera* suggests that a closer relationship with *C. festucacea* is not inconceivable.

Relationships within the *C. brevior* and *C. bicknellii* groups accord largely with expectations based on morphology with a few notable exceptions. *Carex silicea* is a maritime species of the North Atlantic with dense, obovate, relatively narrow-winged perigynia that does not have an obvious morphological relationship to *C. bicknellii.* Until additional data are available, this position seems somewhat questionable. The position of *C. hyalina* separate from the similarly broad-winged species *C. tetrastachya* and *C. reniformis* is somewhat surprising, as is the separation of mem-

bers of the *C. bicknellii* group from those of the *C. brevior* group. The low support for relationships among these clades, however, makes it plausible that these clades might cluster together into a single larger clade. This question bears closer study, perhaps using more highly resolving sequence data.

Chromosomal Patterns

Heilborn's hypothesis regarding the predictive value of chromosome numbers does seem to have some value at broad phylogenetic levels, but among the eastern species it is not clear that the trend holds. Preliminary analyses (not shown) using permutation tests suggest that there is a tendency for sister species to be more similar in chromosome number than would be expected if the chromosomal counts were phylogenetically unstructured, but these tests are inconclusive and may require larger samples or more sensitive analysis.

Analyses of chromosomal trends in sect. *Ovales* presented in this paper provide no support for Heilborn's hypothesis that chromosome number change in *Carex* trends upwards. To the contrary, the data presented in this paper suggest that there has been a decrease in average chromosome number along the branch leading to the predominantly eastern clade of species that comprises ENA clades I and II, as well as *C. feta* and *C. ovalis,* suggesting that the ancestral state for the section as a whole is a higher chromosome count. Both Reznicek (1990: 1428) and Roalson et al. (2001: 339) have argued that decreases in chromosomal number may be as common as or more common than increases within *Carex.* Our data, while crude, appear to support their argument.

Within ENA clade I, parsimony analysis of categorized chromosome counts suggests that an intermediate count is ancestral to the clade, with several independent transitions to both the highest and the lowest counts. This result also argues against Heilborn's hypothesis that chromosomes evolve in *Carex* primarily by fission; but parsimony is a biased means of reconstructing ancestral character states (Cunningham et al. 1998), making this result preliminary at best. Likelihood analysis would permit an evaluation of whether chromosome number increases are more common than chromosome number decreases without conditioning on any particular ancestral character state reconstruction. However, the wide range and intraspecific polymorphism in *Carex* chromosome counts would require optimizing the likelihood for a given set of model parameters across many combinations of ancestral character states and terminal states. Because of the large number of species that would be needed for statistical power in such a test (Pagel 1994, 1999), a well-resolved phylogeny of substantial portions of the genus will presumably be needed if we are to get a handle on the pattern of chromosomal evolution in *Carex.*

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