Fourteen Polymorphic Microsatellite Markers for a Widespread Limestone Endemic, Carex eburnea (Cyperaceae: Carex sect. Albae)

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Primer Note

Fourteen polymorphic microsatellite markers for a widespread limestone endemic, *Carex eburnea* (Cyperaceae: *Carex* sect. *Albae*)[1]

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- *Premise of the study:* Microsatellite primers were developed for a widespread limestone endemic sedge, *Carex eburnea*, to facilitate investigation of the genetic diversity and phylogeography of this taxon and its closest relative, *C. mckittrickensis*.
- *Methods and Results:* Forty-eight primer pairs were designed from Illumina sequence data and screened for suitability. Fourteen of these primer pairs were polymorphic and generated one to seven alleles per locus. Cross-species amplifications were conducted for all four members of *Carex* sect. *Albae*.
- *Conclusions:* These primer pairs can be used to assess the genetic diversity and population structure in future studies of *C. eburnea* and *C. mckittrickensis*, and likely in other members of *Carex* sect. *Albae*.

Key words: *Carex eburnea*; *Carex mckittrickensis*; *Carex* sect. *Albae*; Cyperaceae; genetic diversity; limestone endemic.

Carex L. is a taxonomically challenging, cosmopolitan genus comprising approximately 2000 species (Reznicek, 1990), many of which possess unusually small (Nishikawa et al., 1984) but labile genomes (Lipnerová et al., 2013). This complexity presents challenges at all taxonomic levels. *Carex* sect. *Albae* (Asch. & Graebn.) Kük., like most *Carex* sections, has no microsatellite markers developed to address evolutionary dynamics among recently diverged species, where many taxonomic issues occur. One small but challenging group is the *C. eburnea–C. mckittrickensis* complex. Species boundaries between *C. eburnea* Boott and *C. mckittrickensis* P. W. Ball are unclear based on randomly amplified inter-simple sequence repeat (ISSR) markers (Gillespie, 2005) and on *trnS*$^{(GCU)}$-*trnG*$^{(UUC)}$ and 3′*trnV*$^{(UAC)}$-*ndhC* chloroplast intergenic spacer data (E. Gillespie, Marshall University, unpublished data). Additionally, morphological characters vary continuously (Ball, 1998) across the two species, making this taxon an excellent target for microsatellite marker development.

Carex eburnea is a diploid species (Löve, 1981) that occurs across North America, from Alaska to Newfoundland and southward into the Ozark Mountains, the Cumberland Plateau, and the southern Appalachian Mountains. Disjunct populations occur in the southern Appalachian Mountains and in the Sierra Madre Mountains in Mexico. Based on herbarium specimens and fieldwork (by E.L.G.), *C. eburnea* occurs nearly exclusively on limestone and exists on rock outcrops, in cedar glades and bogs, and in treeless habitats such as alvar and tundra. Co-occurring dominant tree species include spruce (*Picea* A. Dietr. spp.) in the American Northwest and northern white cedar (*Thuja occidentalis* L.) in the upper Midwest and eastern North America. In the southwestern United States and in Mexico, *C. eburnea* co-occurs with junipers (*Juniperus* L. spp.) and oaks (*Quercus* L. spp.). The closest relative of *C. eburnea* is *C. mckittrickensis*, which occurs at a single locality in the Guadalupe Mountains National Park (Culberson County, Texas, USA). Two Eurasian species (*C. alba* Scop. and *C. ussuriensis* Kom.) are the only other members of *Carex* sect. *Albae*. Development of microsatellite markers will be helpful in clarifying the species boundaries and evolutionary history of this recently diverged, widespread, limestone-limited lineage and could be useful within the two Eurasian members of *Carex* sect. *Albae*.

METHODS AND RESULTS

DNA was extracted from one individual of *C. eburnea* using a QIAGEN Plant Mini Kit (QIAGEN, Valencia, California, USA) (Appendix 1). A microsatellite sequencing library (MiSeq v2 protocol) was constructed and 2 × 250 paired-end sequencing was performed on an Illumina MiSeq at the Cornell Life Sciences Sequencing and Genotyping Facility (Ithaca, New York, USA). A total of 2,093,696 raw sequence reads (GenBank Short Read Archive accession SRA557216) were trimmed to remove vectors and low-quality sequence. The resulting reads were queried by MSATCOMMANDER version 1.0.8 (Faircloth, 2008) with default settings, except that mononucleotide repeats were not included in the search, minimum primer size was set at 20 bp, maximum primer GC content was limited to 50%, and a PIG-tail sequence (GTTT) (Brownstein et al., 1996)

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TABLE 1. Characteristics of 16 microsatellite primer pairs developed for *Carex eburnea*.

Locus	Primer sequences (5′–3′)[a]	Fluorescent dye	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
CEB005	F: TAACCCGAATCTGAAATGGCG R: GTTTCGTCTCACACACCCTTTG	VIC	$(AG)_{16}$	236–242	59.5	KX760143
CEB006	F: TATATCAACTTCGTCGGCAGC R: GTTTGACATTTCCTGCGCTTTG	6-FAM	$(AG)_{11}$	120–132	59.0	KX760144
CEB009	F: TGTGGGAAATGTAAGGCGTATC R: GTTTGTGAACCATGCAGAGACG	VIC	$(AT)_{10}$	202–222	59.2	KX760145
CEB010	F: ACTTCATGAAACACGGTAGCC R: GTTTCTTCCCATTGCAGCTCTC	NED	$(AT)_{11}$	154–176	59.3	KX760146
CEB012	F: AATTGGATGGAAAGCAACAGC R: GTTTAACACATGACCAAGGAGCTAG	6-FAM	$(AT)_8$	162–166	58.4	KX760147
CEB015	F: CAAAGGCTTGGTTGTGGTTG R: GTTTCACAGGCTGTAGTCCAATG	6-FAM	$(AAC)_{10}$	151–166	58.8	KX760148
CEB016	F: TCATGCATGGCCATAAAGAGG R: GTTTAATGATACGGCGACCACC	NED	$(AAG)_3$	151–163	59.2	MF001352
CEB021	F: CGTAGAGATTAGGACTTTGGCC R: GTTTGGTAGGCTAGAAATGAAACCC	PET	$(ACT)_{10}$	215–230	58.8	KX760149
CEB024	F: TTGAAGGTTGTTATTCGGCGG R: GTTTACCGTAACCAACTCCGAG	PET	$(ACT)_3$	204–210	59.9	MF001353
CEB025	F: TTTCCAACTCATCAAGCACGG R: GTTTCCAAGCCTCAAGAAGCTG	VIC	$(ATC)_{11}$	245–266	59.7	KX760150
CEB032	F: ACAATCCAACCATCATCACCTG R: GTTTCACTTACAGGAGTTGCGTAC	VIC	$(AATC)_6$	216–226	58.9	KX760151
CEB033	F: TGTGAACTGCTCTGAATGATGC R: GTTTAGCGGTGACAGATATTGACTAC	PET	$(AATG)_7$	204–220	59.2	KX760152
CEB037	F: TCCGCCGTGAAATTTACCTTG R: GTTTGGAAATCGATGCCATCCC	NED	$(ATCC)_8$	147–165	59.8	KX760153
CEB039	F: CGAGCTTCTTGTTGTCTTTACC R: GTTTACCATCTCTCCACAGAACAAG	6-FAM	$(AAAAAC)_{10}$	127–169	58.2	KX760154
CEB043	F: AATTGGCAATTGGGAAACGG R: GTTTCCAAGTTGACGGTTTGAGAC	NED	$(AATAGG)_6$	148–172	58.7	KX760155
CEB048	F: GCGTGGCACTTGAATTTAGG R: GTTTCCGATCTCACAAACTTAAGCC	PET	$(AAAG)_6$	202–214	58.8	KX760156

Note: T_a = annealing temperature.
[a]PIG-tail sequence is underlined on the reverse primer sequences.

was added to one primer. Out of 312,744 identified microsatellites, unique DNA suitable for primer design flanked 89,413.

Forty-eight primer pairs were selected and screened in seven *C. eburnea* individuals (Appendix 1), prioritizing motif diversity and melting temperature difference ≤1°C. PCRs were prepared in a 10-µL reaction consisting of 1× GoTaq Flexi Buffer, 2.5 mM $MgCl_2$, 800 µM dNTPs, 0.5 µM each primer, 0.5 units GoTaq Flexi DNA Polymerase (Promega Corporation, Madison, Wisconsin, USA), and ~20 ng DNA. PCR was completed using a touchdown thermal cycling program on an Eppendorf Mastercycler (Eppendorf, Hauppauge, New York, USA) or an MJ Mini Thermal Cycler (Bio-Rad, Hercules, California, USA) with annealing

TABLE 2. Descriptive statistics for 14 polymorphic microsatellite loci in *Carex eburnea*.[a]

	Johnson Co., TN (N = 24)			SE Fairbanks Co., AK (N = 22)			Rockbridge Co., VA (N = 22)		
Locus	A	H_o	H_e[b]	A	H_o	H_e[b]	A	H_o	H_e[b]
CEB005	4	0.400	0.368***	3	0.059	0.258***	4	0.450	0.581^{NS}
CEB006	2	0.043	0.043^{NS}	4	0.313	0.434^{NS}	2	0.045	0.044^{NS}
CEB009	7	0.211	0.722***	3	0.000	0.623***	3	0.143	0.643***
CEB010	5	0.278	0.600***	3	0.211	0.421***	3	0.091	0.334***
CEB012	1	0.000	0.000^{M}	3	0.364	0.549^{NS}	1	0.000	0.000^{M}
CEB015	2	0.000	0.083***	1	0.000	0.000^{M}	3	0.286	0.516^{NS}
CEB021	4	0.571	0.649***	2	0.133	0.444**	4	0.100	0.615***
CEB025	5	0.238	0.638***	3	0.095	0.503***	6	0.412	0.730^{NS}
CEB032	2	0.000	0.091***	2	0.000	0.455***	2	0.000	0.408***
CEB033	3	0.048	0.217***	2	0.000	0.484***	2	0.091	0.087^{NS}
CEB037	5	0.435	0.692*	3	0.455	0.368^{NS}	6	0.952	0.761***
CEB039	2	0.348	0.340^{NS}	4	0.318	0.412***	3	0.227	0.599***
CEB043	3	0.273	0.376***	4	0.591	0.526^{NS}	3	0.273	0.577**
CEB048	3	0.043	0.124***	1	0.000	0.000^{M}	1	0.000	0.000^{M}
Mean	3.43	0.206	0.353	2.71	0.181	0.391	3.07	0.219	0.421

Note: A = number of alleles detected across all individuals; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals.
[a]Voucher and locality information are provided in Appendix 1.
[b]Statistically significant deviation from Hardy–Weinberg equilibrium is indicated as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; NS = not statistically significant; M = monomorphic marker.

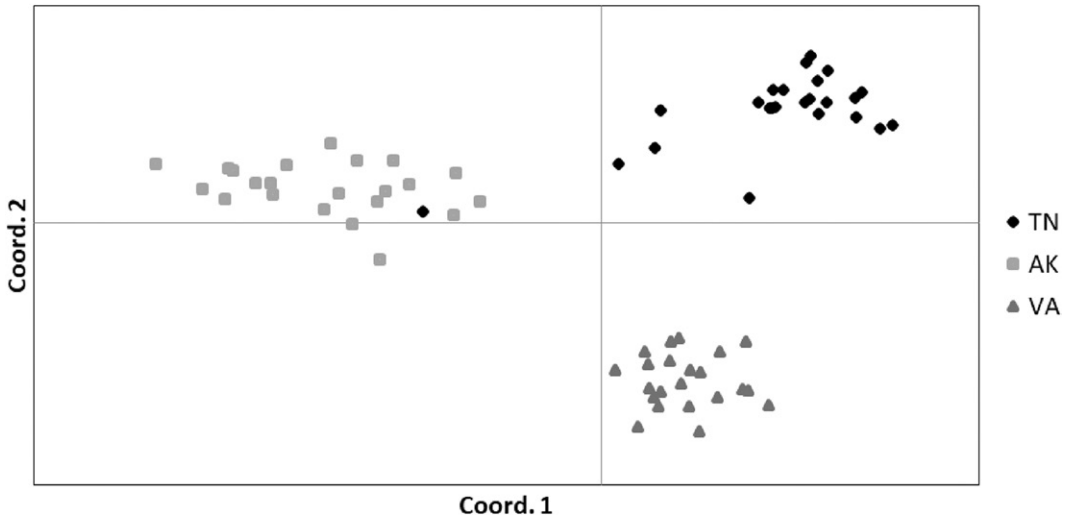

Fig. 1. Principal coordinates analysis (PCoA) of 68 *Carex eburnea* individuals and 14 microsatellite loci. Axis loadings for coordinates 1, 2, and 3 were 16.6%, 15.5%, and 7.3%, respectively. ♦ = Johnson Co., Tennessee, USA; ■ = southeast Fairbanks Co., Alaska, USA; ▲ = Rockbridge Co., Virginia, USA.

temperatures ranging from 68°C to 55°C. Initial denaturation was 94°C for 5 min, followed by 13 cycles (45 s at 94°C, 2 min at touchdown temperature, and 1 min at 72°C), followed by 24 cycles (45 s at 94°C, 1 min at 55°C, and 1 min at 72°C), followed by 5 min at 72°C. PCR products were examined on a 1% agarose gel in 1× TBE and scored for the presence or absence of an appropriately sized PCR product and uniform amplification. Sixteen primer pairs produced repeatable amplicons across all seven individuals. These 16 pairs were screened for polymorphisms in 68 individuals from three populations (Appendix 1).

PCR reaction conditions for screening polymorphisms were the same as above, except that the forward primer concentration was reduced to 0.25 µM and replaced with 0.25 µM M13 primer (5′-CACGACGTTGTAAAACGAC-3′), labeled with 6-FAM, VIC, NED, or PET (Life Technologies, Grand Island, New York, USA). PCR products labeled with different fluorescent dyes were pooled in equal amounts, and 2 µL of the pooled reactions were submitted along with a GeneScan 500 LIZ Size Standard (Life Technologies) for genotyping on an ABI 3730xl DNA Analyzer at the Georgia Genomics Facility (Athens, Georgia, USA). Resulting chromatograms were scored using Geneious 9.1.5 (Kearse et al., 2012; Biomatters Ltd., Auckland, New Zealand). Genotypic data were analyzed using GenAlEx version 6.503 (Peakall and Smouse, 2006, 2012) to obtain standard descriptive statistics, to test for deviations from Hardy–Weinberg equilibrium (HWE) assumptions, to examine the utility of the markers to distinguish among populations, and to evaluate the level of clonality within each population.

Of the 16 primer pairs, 14 loci revealed chromatograms that were consistent with a diploid taxon (Table 1), and two markers (CEB016 and CEB024) did not amplify consistently across all populations. The number of alleles per locus ranged from one to seven with an average of 3.071 across all three populations (Table 2). Observed heterozygosity ranged from 0.0 to 0.952 (mean 0.202). Twelve (86%) loci failed to meet the expectations of HWE in at least one population. Of these, four (29%) loci failed to meet HWE assumptions in all three populations. In almost all cases, excess homozygosity is evident, which may indicate inbreeding or genetic drift. Genetic distance followed by principal coordinates analysis (Orloci, 1978) (Fig. 1) demonstrated that the 14 loci distinguish among the populations, with the first three axes explaining 39.4% of the variation. A multilocus match analysis (Peakall and Smouse, 2006, 2012) revealed no identical individuals across all 14 loci within or among populations.

Cross-amplification of 14 primer pairs was conducted on three additional *C. eburnea* population representatives from across the range (Arkansas, USA; Ontario, Canada; and Querétaro, Mexico), five *C. mckittrickensis* individuals (all from the only known locality in Texas), and single representatives of *C. alba* and *C. ussuriensis* (Table 3). Twelve primer pairs amplified well in all three additional *C. eburnea* representatives (the remaining two pairs failed in two different *C. eburnea* individuals). All but two individual reactions were successful in the *C. mckittrickensis* individuals. Eight and 10 primer pairs cross-amplified successfully in the more distantly related *C. alba* and *C. ussuriensis*, respectively.

Table 3. Cross-amplification of 14 primer pairs in additional representatives from *Carex* section *Albae*.[a]

Locus	C. ebur (AR)	C. ebur (Mexico)	C. ebur (Ontario)	C. mck 1	C. mck 2	C. mck 3	C. mck 4	C. mck 5	C. alba	C. uss
CEB005	+	+	+	+	+	+	+	+	+	—
CEB006	+	+	+	+	+	+	+	+	+	+
CEB009	+	+	+	+	+	+	+	+	+	+
CEB010	+	+	+	+	+	+	+	+	—	+
CEB012	+	+	—	+	+	+	+	+	—	—
CEB015	+	+	+	—	+	+	+	+	+	+
CEB021	+	+	+	+	+	+	+	+	—	—
CEB025	+	+	+	+	+	+	+	+	+	+
CEB032	+	+	+	+	+	+	+	+	+	+
CEB033	+	+	+	+	+	+	—	+	+	+
CEB037	—	+	+	+	+	+	+	+	+	+
CEB039	+	+	+	+	+	+	+	+	—	—
CEB043	+	+	+	+	+	+	+	+	—	+
CEB048	+	+	+	+	+	+	+	+	—	+

Note: + = positive amplification; — = no observable amplification; C. ebur = *Carex eburnea*; C. mck = *Carex mckittrickensis*; C. uss = *Carex ussuriensis*.
[a]Voucher and locality information are provided in Appendix 1.

CONCLUSIONS

The markers reported here will likely be useful in population studies within *C. eburnea*; despite elevated levels of homozygosity generally, these markers discriminated among three populations (including two from the same physiographic region). Cross-amplification experiments confirmed that these markers should be applicable in the *C. eburnea–C. mckittrickensis* species complex and potentially in additional members of *Carex* sect. *Albae*, providing a novel population genetic tool in *Carex*.

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Appendix 1. Voucher information for *Carex* individuals included in this study.

Species	Voucher (Herbarium)[a]	Geographic coordinates		Elevation (m)	State (Country)	County	*N*
		Latitude	Longitude				
Carex eburnea Boott	Gillespie s.n. (BOON)[b]	36.30	−81.93	598	Tennessee (USA)	Johnson	1
Carex eburnea	Gillespie 16-156 (MUHW)[c]	36.30	−81.93	598	Tennessee (USA)	Johnson	24
Carex eburnea	Mason 16-001 (MUHW)[c]	64.02	−145.72	362	Alaska (USA)	SE Fairbanks	22
Carex eburnea	Gillespie 16-157 (MUHW)[c]	37.63	−79.54	343	Virginia (USA)	Rockbridge	22
Carex eburnea	Gillespie 03-230 (BOON)[d]	35.96	−92.18	250	Arkansas (USA)	Stone	1
Carex eburnea	Reznicek s.n. (MICH)[d]	21.28	−99.18	1110	Querétaro (Mexico)	NA	1
Carex eburnea	Richardson s.n. (OAC)[d]	45.18	−81.61	180	Ontario (Canada)	NA	1
Carex mckittrickensis P. W. Ball	Gillespie 04-001 (BOON)[d]	31.98	−104.79	1900	Texas (USA)	Culberson	1
Carex alba Scop.	Hendrichs 3705 (TUB)[d]	49.07	10.01	600	Bayern (Germany)	NA	1
Carex ussuriensis Kom.	Elias 10982 (ALA)[d]	48.31	135.09	153	Khaborovsk (Russia)	NA	1

Note: *N* = number of individuals; NA = not applicable.

[a]Vouchers are deposited at the following herbaria: I. W. Carpenter Jr. Herbarium, Appalachian State University (BOON), Boone, North Carolina, USA; Marshall University Herbarium (MUHW), Huntington, West Virginia, USA; University of Michigan Herbarium (MICH), Ann Arbor, Michigan, USA; Guelph University Herbarium (OAC), Guelph, Ontario, Canada; Universität Tübingen (TUB), Tübingen, Germany; and University of Alaska Museum of the North (ALA), Fairbanks, Alaska, USA.

[b]Voucher for Illumina sequencing.

[c]Voucher for marker development (separate collection effort).

[d]Voucher for cross-amplification (five individuals from Culberson County, Texas, USA).