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

Authors: Gillespie, Emily L., Pauley, Annabella G., Haffner, Megan L., Hay, Nikolai M., Estep, Matt C., et al.

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FOURTEEN POLYMORPHIC MICROSATELLITE MARKERS FOR A WIDESPREAD LIMESTONE ENDENN, CAREX EBURNEA (CYPERACEAE: CAREX SECT. ALBAE)\(^1\)

EMILY L. GILLESPIE\(^2,4\), ANNABELLA G. PAULEY\(^2\), MEGAN L. HAFFNER\(^2\), NIKOLAI M. HAY\(^3\), MATT C. ESTEP\(^3\), AND ZACK E. MURRELL\(^3\)

\(^2\)Department of Biological Sciences, Marshall University, One John Marshall Drive, Huntington, West Virginia 25755 USA; and
\(^3\)Department of Biology, Appalachian State University, 572 Rivers Street, Boone, North Carolina 28607 USA

- **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 sec. 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 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 ceder 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) was limited to 50%, and a PIG-tail sequence (GTTT) (Brownstein et al., 1996)
Forty-eight primer pairs were selected and screened in seven *C. eburnea* individuals (Appendix 1), prioritizing motif diversity and melting temperature difference $\leq 1^\circ$C. PCRs were prepared in a 10-$\mu$L reaction consisting of 1× GoTaq Flexi Buffer, 2.5 mM MgCl$_2$, 800 $\mu$M dNTPs, 0.5 $\mu$M 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 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) | $T_a$ ($^\circ$C) | GenBank accession no. |
|---------|-----------------------------|-----------------|--------------|------------------------|------------------|-----------------------|
| CEB005  | F: TAACCCGATCCTGAAATGGCG    | VIC             | (AG)$_{16}$  | 236–242                | 59.5             | KX760143              |
|         | R: GGTTGCGATCACTCCGACC      |                 |              |                        |                  |                       |
| CEB006  | F: TAATCAACCTTGGCGGAGCAGC   | 6-FAM           | (AG)$_{11}$  | 120–132                | 59.0             | KX760144              |
|         | R: GGTTGACATTTCTGCGATTTG    |                 | (AT)$_{10}$  | 202–222                | 59.2             |                       |
| CEB009  | F: TGTTGGAAATGTAAGGCTATC    | VIC             | (AT)$_{10}$  | 154–176                | 59.3             |                       |
|         | R: GGTTTGAACATGCGAGACAC     |                 | (AT)$_{10}$  | 162–166                | 58.4             |                       |
| CEB010  | F: GTTCTCTCGTCATGCCTCTC     | NED             | (AT)$_{10}$  | 151–168                | 58.8             |                       |
|         | R: GTTAAAACATGACCAAGGCTAG   |                 |              |                        |                  |                       |
| CEB015  | F: CAAAGGCTTTGTTGTTGTTG     | 6-FAM           | (AAC)$_{10}$ | 151–168                | 58.8             |                       |
|         | R: GTTTCAAGGCTGATGTTCAATG   |                 | (AAC)$_{10}$ | 151–163                | 59.2             | MF001352              |
| CEB016  | F: TCTCATGATGGCCATAAAGAGG   | NED             | (AAG)$_{10}$ | 147–165                | 59.7             |                       |
|         | R: GTTTAATGATGCGAGACAC      |                 |              |                        |                  |                       |
| CEB021  | F: GTTACGAGATGACCTTTG       | PET             | (ACT)$_{10}$ | 204–210                | 59.9             |                       |
|         | R: GTTTGTTGAGCTGATAGAAGCCC  |                 | (ACT)$_{10}$ | 215–230                | 58.8             |                       |
| CEB024  | F: TTTTAGGGTGGTTTTATCCGCG   | PET             | (AATC)$_{10}$ | 204–210                | 59.9             |                       |
|         | R: GTTTTACGTTGAGATGACATGC   |                 | (AATC)$_{10}$ | 215–230                | 58.8             |                       |

Note: $T_a =$ annealing temperature.

$^a$PIG-tail sequence is underlined on the reverse primer sequences.

### Table 2. Descriptive statistics for 14 polymorphic microsatellite loci in *Carex eburnea*.$^a$

| Locus   | Johnson Co., TN (N = 24) | SE Fairbanks Co., AK (N = 22) | Rockbridge Co., VA (N = 22) |
|---------|--------------------------|-------------------------------|-------------------------------|
|         | $A$ $H_e$ $H_o$ $H_e$ | $A$ $H_e$ $H_o$ $H_e$ | $A$ $H_e$ $H_o$ $H_e$ |
| CEB005  | 4 0.400 0.368*** | 3 0.059 0.258*** | 4 0.450 0.581NS |
| CEB006  | 2 0.043 0.043NS | 4 0.313 0.434NS | 2 0.045 0.044NS |
| CEB009  | 7 0.211 0.722*** | 3 0.090 0.623*** | 3 0.143 0.643*** |
| CEB010  | 5 0.278 0.664*** | 3 0.211 0.421*** | 3 0.091 0.334*** |
| CEB012  | 1 0.000 0.000M | 3 0.364 0.549NS | 1 0.000 0.000M |
| CEB015  | 2 0.000 0.083*** | 1 0.000 0.000M | 3 0.286 0.516NS |
| 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 0.730NS |
| CEB032  | 2 0.000 0.091*** | 2 0.090 0.455*** | 2 0.090 0.408*** |
| CEB033  | 3 0.038 0.217*** | 3 0.000 0.484*** | 3 0.091 0.087NS |
| CEB037  | 5 0.435 0.692*  | 3 0.455 0.368NS  | 6 0.952 0.761*** |
| CEB039  | 2 0.348 0.340NS  | 4 0.318 0.412*** | 3 0.227 0.599*** |
| CEB043  | 3 0.273 0.376*** | 4 0.591 0.526NS  | 3 0.273 0.577** |
| CEB048  | 3 0.043 0.124*** | 1 0.000 0.000M  | 1 0.000 0.000M |
| 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.
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 GenAIEx 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.

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

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

| 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 mckittrckensis; C. alba = Carex alba; C. uss = Carex ussuriensis.

*Voucher and locality information are provided in Appendix 1.
CONCLUSIONS

The markers reported here will likely be useful in population studies within *Carex 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) | Geographic coordinates | Elevation (m) | State (Country) | County | N |
|--------------------------|---------------------|------------------------|---------------|-----------------|--------|---|
| *Carex eburnea* Boott    | Gillespie s.n. (BOON) | 36.30 | −81.93 | 598 | Tennessee (USA) | Johnson | 1 |
| *Carex eburnea*          | Gillespie 16-156 (MUHW) | 36.30 | −81.93 | 598 | Tennessee (USA) | Johnson | 24 |
| *Carex eburnea*          | Mason 16-001 (MUHW) | 64.02 | −145.72 | 362 | Alaska (USA) | SE Fairbanks | 22 |
| *Carex eburnea*          | Gillespie 16-157 (MUHW) | 37.63 | −79.54 | 343 | Virginia (USA) | Rockbridge | 22 |
| *Carex eburnea*          | Gillespie 03-230 (BOON) | 35.96 | −92.18 | 250 | Arkansas (USA) | Stone | 1 |
| *Carex eburnea*          | Reznicek s.n. (MICH) | 21.28 | −99.18 | 1110 | Querétaro (Mexico) | NA | 1 |
| *Carex eburnea*          | Richardson s.n. (OAC) | 45.18 | −81.61 | 180 | Ontario (Canada) | NA | 1 |
| *Carex mckittrickensis* P. W. Ball | Gillespie 04-001 (BOON) | 31.98 | −104.79 | 1900 | Texas (USA) | Culberson | 1 |
| *Carex alba* Scop.       | Hendrichs 3705 (TUB) | 49.07 | 10.01 | 600 | Bayern (Germany) | NA | 1 |
| *Carex assuvieriensis* Kom. | Elias 10982 (ALA) | 48.31 | 135.09 | 153 | Khabarovsk (Russia) | NA | 1 |

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

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

2. Voucher for Illumina sequencing.

3. Voucher for marker development (separate collection effort).

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