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Amplified fragment length polymorphism analysis of genetic relationships among the serpentine barrens endemic *Cerastium velutinum* Rafinesque var. *villosissimum* Pennell (Caryophyllaceae) and closely related *Cerastium* species[1]

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GUSTAFSON, D. J. (Department of Biology, University of Pennsylvania, Philadelphia, PA 19104), G. ROMANO (Morphotek, Inc., 210 Welsh Pool Rd., Exton, PA 19341), R. E. LATHAM (Continental Conservation, P.O. Box 57, Rose Valley, PA 19086), AND J. K. MORTON (Department of Biology, University of Waterloo, Waterloo, ON, N2L 3G1 Canada). Amplified fragment length polymorphism analysis of genetic relationships among the serpentine barrens endemic *Cerastium velutinum* Rafinesque var. *villosissimum* Pennell (Caryophyllaceae) and closely related *Cerastium* species. J. Torrey Bot. Soc 130:218–223. 2003.—Amplified fragment length polymorphism (AFLP) banding patterns were used to examine the genetic relationships among the long-haired barrens chickweed (*Cerastium velutinum* var. *villosissimum*), members of the *Cerastium arvense* complex, and *Cerastium* species native to Europe. *Cerastium velutinum* var. *villosissimum* is genetically similar to the widespread *C. velutinum* and this association clustered with *C. arvense* spp. *strictum* and *C. viride*. *Cerastium fontanum*, *C. glomeratum*, and *C. tomentosum*, species native to Europe, were distinct from the North American group. *Cerastium velutinum* var. *villosissimum* is globally endangered, is genetically more similar to *C. velutinum* than other members of the North American *C. arvense* complex and is deserving of conservation efforts.

Key words: AFLP, serpentine barrens, *Cerastium* spp., phylogenetic analysis.

The genus *Cerastium* L. (Caryophyllaceae) includes more than 100 species worldwide, with *C. arvense*, *C. viride*, and *C. velutinum* comprising the native North American *C. arvense* complex (Gleason and Cronquist 1991; Morton, unpublished data). The long-haired barrens chickweed (*Cerastium velutinum* Raf. var. *villosissimum* [Pennell]) is a perennial member of the *C. arvense* complex in North America, separated from the typical *C. velutinum* by its densely white-villous, rather than green and merely pubescent, leaves (Pennell 1930; Latham 1993; Tyndall and Hull 1999; Morton, unpublished data). The villous leaf character appears to be under genetic control and segregation has been observed in plants grown from seed in ordinary soil (Morton, unpublished data). The habitat in which *C. velutinum* var. *villosissimum* occurs, eastern North American serpentine grasslands, is considered a globally rare and endangered community due to urban sprawl, fire suppression, and encroachment by greenbriers (*Smilax rotundifolia*, *S. glauca*), eastern redcedar (*Juniperus virginiana*), Virginia pine (*Pinus virginiana*), and black locust (*Robinia pseudoacacia*) (Tyndall 1992; Latham 1993; Barton and Wallenstein 1997; Tyndall and Hull 1999). Herbarium records indicate that *C. velutinum* var. *villosissimum* has historically occurred at three sites in Pennsylvania and there is a more recent unvouchered report of a population in Maryland (Latham 1993). Determining genetic relationships among *C. velutinum* var. *villosissimum* and other members of the North American *C. ar-*

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vense complex is an essential first step in establishing whether *C. velutinum* var. *villosissimum* should be a conservation priority. Three non-native *Cerastium* species, which occur in the mid-Atlantic region, were included for comparison with the North American species.

In this study, genetic relationships among *C. velutinum* var. *villosissimum*, closely related *Cerastium* spp., and three European species (*C. glomeratum*, *C. tomentosum*, and *C. fontanum*) were assessed using amplified fragment length polymorphism (AFLP) analysis. AFLP analysis is a DNA fingerprinting technique first introduced in 1995 (Vos et al. 1995); it has already been applied widely to a range of plant species. Recent studies show that AFLP is a reliable method for generating many highly polymorphic DNA markers and inferring phylogenetic relationships among closely related taxa (Aggarwal et al.1999; Anamthawat-Jonsson et al. 1999; Angiolillo et al. 1999; Loh et al. 1999; Mace et al. 1999; Mueller and Wolfenbarger 1999; Zhang et al. 1999; Gaudeul et al. 2000; Le Thierry d' Ennequin et al. 2000; Shim and Jorgensen 2000; Tosto and Hopp 2000; Zawko et al. 2001).

Materials and Methods. PLANT SPECIES. Four members of the North American *Cerastium* arvense complex were included in this study: *C. arvense* ssp. *strictum*, *C. velutinum*, *C. velutinum* var. *villosissimum*, and *C. viride* (Table 1). *Cerastium arvense* L. ssp. *strictum* Gaudin is a perennial diploid (2n = 36) taxon that occurs in a variety of habitats (dunes, sea cliffs, prairies, arctic and alpine tundra) and has a cosmopolitan distribution. Differences in chromosome number represent an effective reproductive barrier with other *C. arvense* subspecies and several closely related species such as *C. velutinum* Rafinesque and *C. viride* (A. A. Heller) Muhl. (Morton, unpublished data; Rhoads and Block 2000). *Cerastium velutinum* Rafinesque is a perennial tetraploid (2n = 72) native of North America, occurs on serpentine barrens, limestone outcrops and woodlands (Morton, unpublished data; Argus et al. 1987). *Cerastium velutinum* var. *villosissimum* Pennell is a densely villous variety with broad leaves, currently confirmed from two serpentine barrens, in Chester County, Pennsylvania, U.S.A. and in Cecil County, Maryland, U.S.A. (Latham, unpublished data). *Cerastium viride* (A. A. Heller) Muhl. is a native perennial tetraploid (2n = 72) species that inhabits grassy slopes along the California and Oregon coast of North America (Morton, unpublished data).

Three non-native *Cerastium* species that occur in the mid-Atlantic vascular flora were included in this analysis (Table 1). *Cerastium tomentosum* L. is a perennial mat-forming tetraploid (2n = 72) herb with densely white tomentose stems and leaves, which is native to southeastern Europe (Morton 1973; Morton, unpublished data; Rhoads and Block 2000). *Cerastium glomeratum* Thuill. is an annual tetraploid (2n = 72) weed of roadsides and waste places, native to Europe (Morton, unpublished data; Rhoads and Block 2000). *Cerastium fontanum* Baumg. ssp. *vulgare* (Hartman) Greuter & Burdet is a short-lived perennial (2n = 144) weed of cultivated ground, native to Europe (Boscaiu et al. 1999; Morton, unpublished data; Rhoads and Block 2000).

Genetic Analysis. Twenty individuals representing five species, one subspecies, and one variety of *Cerastium* were analyzed using three primer pair combinations. Total genomic DNA was extracted from approximately 0.1 g of silica-dried leaves from field-collected and herbarium specimens (provided by Morton) using E.Z.N.A. plant DNA miniprep kit (Omega Biotek, Doraville, GA). AFLP fingerprints were generated following the AFLP System II (Cat. Nos. 10717–015, 10719–011, Gibco BRL/Life Technologies) protocol and using a fluorescently-labeled forward primer (5′NED-GACTGCGTACCAATTCAA-3′) and three reverse primers (5′GATGAGTCCTGAGTAA(M)-CAT, M-CTA, M-CTC) during the selective PCR amplification. Approximately 120 ng of total genomic DNA was digested with *EcoRI/MseI* (2U) in a 20 μl reaction vessel and incubated at 22 °C overnight. Restriction enzyme activity was halted by incubating at 70 °C for 20 min and then placing the tube on ice. Adapter ligation solution (19.2 μl) and DNA ligase (0.8 μl) were added, incubated at 22 °C for two hours, and the product diluted 1:50 with TE buffer for preselective amplification. Preselective and selective amplifications followed AFLP System II protocol. Electrophoresis was performed on final PCR products in 5% denaturing acrylamide on an ABI Prism 377 sequencer where profiles were recorded digitally.

Relative mobility of fragments was calculated by the inclusion of an internal size standard within each sample. ABI Genescan software was used to visualize and score profiles. Binary profiles from the absence or presence of fragments were constructed for each taxa. Relationships

Table 1. Collection information on four *Cerastium* taxa native to North America (N.A.) and three non-native *Cerastium* species.

Code	Species	Accession	Collected	County	State	Native
A1	*C. velutinum* var. *villosissimum*	Gustafson & Romano 2201	25 April 2001	Cecil	Maryland	N.A.
A2	*C. velutinum* var. *villosissimum*	Gustafson & Romano 2202	25 April 2001	Cecil	Maryland	N.A.
A3	*C. velutinum* var. *villosissimum*	Gustafson & Romano 2502	25 April 2001	Cecil	Maryland	N.A.
A4	*C. velutinum* var. *villosissimum*	Gustafson & Romano 401	4 April 2001	Chester	Pennsylvania	N.A.
A5	*C. velutinum* var. *villosissimum*	Gustafson & Romano 402	4 April 2001	Chester	Pennsylvania	N.A.
B1	*C. velutinum*	Gustafson 4601	14 May 2001	Delaware	Pennsylvania	N.A.
B2	*C. velutinum*	Morton NA3500/Gustafson 4801[b]	20 June 1972	Richmond	New York	N.A.
B3	*C. velutinum*	Gustafson & Romano 601	4 April 2001	Chester	Pennsylvania	N.A.
B4	*C. velutinum*	Gustafson & Romano 901	4 April 2001	Chester	Pennsylvania	N.A.
B5	*C. velutinum*	Gustafson & Romano 903	4 April 2001	Chester	Pennsylvania	N.A.
C	*C. viride*	Morton s.n./Gustafson 4901[b]	20 June 1973	Clatsop	Oregon	N.A.
D1	*C. arvense* spp. *strictum*	Morton & Venn NA18577/ Gustafson 5101[b]	2 May 1995	Idaho	Idaho	N.A.
D2	*C. arvense* spp. *strictum*	Morton NA3313/Gustafson 5201[b]	31 May 1975	Sheridan	Wyoming	N.A.
D3	*C. arvense* spp. *strictum*	Morton/Gustafson 5301[b]	2 June 1971	Lincoln	Oregon	N.A.
E	*C. fontanum*	Gustafson 1201	19 April 2001	Delaware	Pennsylvania	Europe
F1	*C. tomentosum*	Gustafson & Romano 3601	3 May 2001	Bucks	Pennsylvania[a]	Europe
F2	*C. tomentosum*	Gustafson & Romano 3602	3 May 2001	Bucks	Pennsylvania[a]	Europe
F3	*C. tomentosum*	Gustafson & Romano 4401	5 May 2001	Delaware	Pennsylvania[a]	Europe
F4	*C. tomentosum*	Gustafson & Romano 4402	5 May 2001	Delaware	Pennsylvania[a]	Europe
G	*C. glomeratum*	Gustafson 3901	3 May 2001	Bucks	Pennsylvania	Europe

[a] Purchased at local plant vendor.

[b] Corresponding Gustafson code.

among taxa were investigated using principal components analysis (PCA) and parallel analysis (PA) to establish which PCA axes were appropriate for interpretation (SAS Institute 1989). Parallel analysis was used to derive the 95th percentile eigenvalues for each successive PCS axis, based on Monte Carlo analysis of Longman et al. (1989) regression equations. Only axes with eigenvalues greater than the PA eigenvalues were retained for interpretation (Franklin et al. 1995).

Results. The analysis yielded 178 bands, of which approximately 26% were unique to North American taxa. Three (*C. viride*), four (*C. arvense* ssp. *strictum*), five (*C. velutinum* var. *villosissimum*), and ten (*C. velutinum*) bands were unique to only one North American taxon. The three European species had 32.6% unique bands, with four (*C. fontanum*), 13 (*C. glomeratum*) and 30 (*C. tomentosum*) species-specific bands.

The first three axes of the AFLP—PCA accounted for 16.6% (axis 1, eigenvalue = 76.18), 11.4% (axis 2, eigenvalue = 52.46), and 9.9% (axis 3, eigenvalue = 45.36) of the variance (Fig. 1). Parallel analysis indicated that the first three axes were statistically significant and appropriate for interpretation. PCA analysis revealed predictable associations, with the North American and European taxa forming roughly two groups. Our AFLP analysis supports the North American *C. arvense* complex consists of *C. arvense*, *C. velutinum*, and *C. viride* based on morphological, cytological and breeding experiment (Morton, unpublished data). *C. velutinum* and *C. velutinum* var. *villosissimum* were genetically more similar to one another than they were to *C. viride* and *C. arvense* spp. *strictum*, which may reflect *C. velutinum* var. *villosissimum*'s origin from *C. velutinum*, long isolation of eastern and western North American taxa, or some combination of phylogenetic and biogeographical factors (Fig. 1). Ploidy levels do not correspond with genetic relationships: *C. tomentosum* (2n = 72) associated more closely with *C. fontanum* (2n = 144) than with *C. glomeratum* (2n = 72).

Discussion. We found AFLP analysis to be an effective tool for providing quantitative estimates of genetic similarity among previously recognized *Cerastium* species. Members of the North American *C. arvense* complex formed an association different from the three European species (Fig. 1). The serpentine grassland endemic, long-haired barrens chickweed (*C. velutinum* var. *villosissimum*) is closely related to the geographically more widespread *C. velutinum* and genetically different from other members of the North American *C. arvense* complex. Its narrow endemism, sharply distinct morphology from the species type variety, and retention of the distinctive traits when grown from seed in ordinary soil suggest that the long-haired barrens chickweed constitutes an entity worthy of taxonomic recognition and conservation efforts.

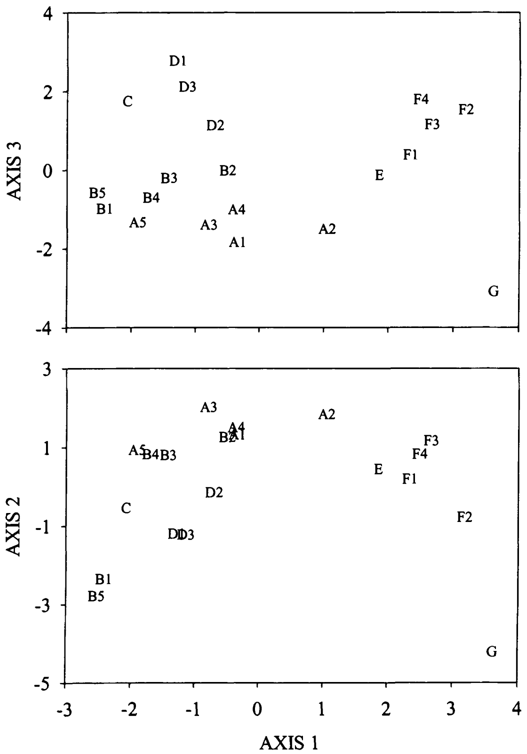

Fig. 1. Principal components analysis depicting the relationships among 20 individuals from the genus *Cerastium*, based on AFLP analysis of 178 bands generated from three primer combinations.

Conclusions. The eastern serpentine grasslands may have arisen during the warmest (and in east-central North America, the driest) interval in the Holocene so far, around 8,000 to 4,500 years ago (Deevey and Flint 1957; Wright 1976; Haas and McAndrews 2000), when grasslands expanded across eastern North America. There is evidence of these grasslands having been maintained by burning and used as hunting and foraging grounds by indigenous peoples as late as 1731 (Marye 1955; Tyndall and Hull 1999). Of the 26 serpentine barrens sites ≥2 ha in temperate eastern North America from Georgia to Vermont (Latham, unpublished data), approximately half of the total area is in the eight sites of the State Line Serpentine Barrens in Pennsylvania and Maryland, including the two known long-haired barrens chickweed locations, Goat Hill Barrens in Pennsylvania (recorded as Octoraro Creek by the botanist who originally described the variety [Pennell 1930]) and Pilot Barrens in Maryland. The State Line Serpentine Barrens harbor at least 85 plant and animal taxa classified by the two states' natural heritage programs as rare, including six that are globally rare and at risk of extinction (Pennsylvania Natural Diversity Inventory; Maryland Wildlife and Heritage Division; Latham, unpublished data).

Plants living in serpentine grasslands are limited to species and ecotypes that are tolerant of soils with near-toxic magnesium and nickel concentrations and deficient in calcium (Proctor and Woodell 1975). All such plants grow slowly. In the absence of fire, soil organic matter builds up, buffering plants from the adverse effects of serpentine mineral soil and allowing faster-growing competitors to displace the toxic-soil-tolerant plants (Latham 1993; Barton and Wallenstein 1997). Effective management to conserve these unique assemblages must include prescribed burning and selective tree species removal to reopen the forest canopy and deter the buildup of soil organic matter. These interventions are vital to the survival of this endangered plant and the unique grassland community on which it depends.

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