RESEARCH

Big Bluestem Gene Pools in the Central and Northeastern United States

David L. Price,* Paul R. Salon, and Michael D. Casler

ABSTRACT

Big bluestem (*Andropogon gerardii* Vitman) is a native grass of the North American tallgrass prairie, used as a forage crop and as a possible biofuel feedstock. Understanding the genetic diversity of big bluestem can enable better classification of accessions and increase the ability to recognize unique genetic variation for use within breeding programs. Previous studies of the genetic diversity of big bluestem have generated mixed conclusions; further study is warranted to provide clarification of the genetic structure. Genetic diversity was measured both among and within three groups of big bluestem populations: natural populations collected in Wisconsin, natural populations from the northeastern United States, and cultivars or accessions available through various gene banks (mostly originating in the Great Plains). A total of 417 amplified fragment length polymorphism loci were used for principal coordinate analysis, analysis of molecular variance, and Mantel tests. Results indicate that germplasm groups represent three distinct genetic pools with overlapping regions. Partitioning of genetic variance for each of the three groups revealed significant variance among ecoregions and hardiness zones and among populations within ecoregions and hardiness zones. Unique big bluestem germplasm is present outside of the Great Plains, providing opportunities to increase genetic variation within breeding germplasm. Efforts should be made to preserve these genetic resources.

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Abbreviations: AFLP, amplified fragment length polymorphism; AMOVA, analysis of molecular variance; GRIN, Germplasm Resources Information Network; HZ, hardiness zone; NRCS, Natural Resources Conservation Service; PCoA, principal coordinate analysis; WDNR, Wisconsin State Department of Natural Resources.

The prairies of North America and their flora have historically provided abundant resources to many peoples. Uses of prairie land have been as varied as wildlife habitat, fertile farmland, and recreation areas (Curtis, 1959). These lands are also a valuable resource for native plant germplasm, acting as in situ gene banks (Boller and Veteläinen, 2010). All released cultivars of prairie species originate directly or indirectly from collections made at remnant prairie sites. The importance of these lands as a source of genetic variation warrants further study of the germplasm that exists within their boundaries; increased knowledge of the underlying genetics will facilitate improved preservation and utilization.

Big bluestem (*Andropogon gerardii* Vitman) is a warm season (C4), perennial grass; it is largely self-incompatible, accounting for a high degree of outcrossing within the species (Norrmann et al., 1997). The species has a base chromosome number of $n = 10$, and is typically found as a hexaploid ($2n = 6x = 60$) (Gould, 1967; Norrmann et al., 1997). As a high-quality forage species, big bluestem can fill the forage availability gap between peak spring

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and autumn growth of cool season grass species (Moser and Vogel, 1995). More recently it has been suggested that big bluestem could be a valuable as a biofuel feedstock (Weimer and Springer, 2007; Anderson et al., 2008).

Big bluestem is found growing natively in areas throughout the United States and was once the dominant species of the tallgrass prairie, making up greater than 80% of the vegetation at some prairie sites (Weaver, 1954). The native range extends from east of the Rocky Mountains from northern Mexico to southern Canada and to the eastern seaboard (Gould and Shaw, 1983). Within the state of Wisconsin, big bluestem is credited with contributing much of the character to the tallgrass prairie of the state and is still found in many undisturbed prairies (Fassett, 1951), mostly in the southern and western parts of the state, with some growing on sandy soils in the north.

Historical accounts by the first European explorers provide descriptions of large grasslands within the northeastern United States before colonial times; big bluestem was specifically observed in accounts of the Hempstead Plains, in Long Island, New York (Denton, 1670; Allen, 1847; Vickery and Dunwiddie, 1997). In addition to these accounts, archeological evidence dating to the 14th century suggests that the Native Americans of the northeastern United States lined food storage pits with big bluestem, similar to practices of the Great Plains inhabitants, suggesting a long-term presence before European settlement (Bendremer et al., 1991).

Currently there are a limited number of improved cultivars of big bluestem available. Many of these cultivars consist of composites of a limited collection of plants, usually from a source-identified location (Anderson et al., 2008). Most of these collections were made by the Natural Resources Conservation Service (NRCS) and have been focused in the Great Plains region and areas west of the Mississippi River; exceptions to this include released accessions from Indiana, Michigan, and New York. Previous work has demonstrated significant variation in big bluestem that can be used for the improvement of both yield and quality traits (Mitchell et al., 2005).

Understanding the genetic diversity within a crop species can enable better classification of accessions and increase the ability to recognize unique genetic variation for use within breeding programs (Mohammadi and Prasanna, 2003). Amplified fragment length polymorphism (AFLP) markers (Vos et al., 1995) have been an invaluable tool for assessing genetic diversity in both wild and cultivated plant species, particularly in species with limited previous DNA marker information. Previous studies have demonstrated the utility of the AFLP procedure for measuring genetic diversity in natural populations of grasses (Fu et al., 2004; Moncada et al., 2007; Wang et al., 2010).

Previous studies of the genetic diversity of big bluestem have generated mixed conclusions. Analysis of plants from northern Arkansas, using random amplified polymorphic DNA markers, revealed few genetic differences among natural populations, but significant differences between plants from Arkansas and those from distant areas (Gustafson et al., 1999). In contrast, measurements of genetic diversity, using random amplified polymorphic DNA markers, between natural populations and Conservation Reserve Program plantings (seed source Missouri and Texas) in Ohio demonstrated no significant differences between the two groups (Selbo and Snow, 2005). Fine-scale genotyping using AFLP markers within a single natural population revealed most plants within the population to be of a common genotype, with some intermediate frequency genotypes and a small number of rare genotypes (Avolio et al., 2011). Phenotypic studies of morphology and physiology have suggested that released cultivars of big bluestem are more fit in some environments than collections made from local populations (Gustafson et al., 2001; Lambert et al., 2011). A major limitation of the current studies of big bluestem is that only populations from small geographic areas were surveyed and comparisons to released cultivars were conducted with a limited number of accessions. On account of these limitations, we suggest that further study of the genetic variation in big bluestem on regional scale is warranted to provide clarification of the genetic structure of big bluestem in the United States.

The objective of this study was to measure among- and within-population genetic variation in three groups of big bluestem populations: natural populations collected in Wisconsin, natural populations from the northeastern United States (Maryland, Maine, New York, Pennsylvania, Vermont, and West Virginia), and a diverse collection of cultivars or accessions available through various gene banks (NRCS, Germplasm Resources Information Network [GRIN], and commercial seed producers). Due to the limited collection area of current released accessions, we hypothesize that a significant amount of regional adaptation and unique genetic variation exists within natural populations outside of the region from which most current released accessions originate. The identification of this new genetic diversity and its subsequent use in germplasm development will introduce new variation, forming a basis for potentially improved performance and adaption. The continued improvement of big bluestem will be necessary to facilitate increased use both as forage and as a biofuel feedstock. Increased understanding of the genetic diversity of wild populations will be a valuable resource for organizations interested in maintenance and restoration of local prairie sites.

MATERIALS AND METHODS

Plant Materials

Plant material for Wisconsin seed accessions in this study was obtained by seed collections from natural populations throughout Wisconsin during September and October 2008 (Fig. 1). Seed collection sites mainly consisted of State Natural Areas identified by the Wisconsin State Department of Natural Resources (WDNR)

Figure 1. Central and eastern United States showing 34 sites in the state of Wisconsin and 24 sites in the northeastern United States that were the source of natural populations of big bluestem used for DNA analysis. 28 sites are shown as the place of origin for released accessions or cultivars of big bluestem, based on release information and collectors' notes.

as containing native prairie grasses (Table 1). Additional seed was collected from a small number of wildlife areas maintained by the WDNR. Seed was collected from numerous plants at each site, with a minimum of 20 m between plants (seed from each maternal plant was stored individually). Efforts were made to collect from the maximum amount of both phenotypic and landscape diversity observed at each site. Seed from seven different maternal plants from each site was germinated in a greenhouse and a single leaf was collected from one plant from each maternal line.

The plant materials used in this study from the northeastern United States were originally collected for use by the Big Flats Plant Material Center in Corning, NY, between 1998 and 2007 (Fig. 1, Table 2). Seed from these collections was bulked by collection site and then subsequently planted in a common nursery at Corning, NY. Tissue was collected from these plants in September 2009 by removing a single leaf from approximately seven distinct plants from each population.

Seed from cultivars and released accessions of big bluestem was obtained by donation from NRCS, GRIN, and commercial seed producers (Table 3, Fig. 1). Cultivars and released accessions are defined as seed accessions that are readily available from commercial sources or plant materials centers (many of these accessions are broad-based germplasm with limited improvement). Seed was obtained from as diverse geographic origins as possible to best assess geographic effects on genetic diversity (Fig. 1). Seed was germinated within a greenhouse and a single leaf was collected from about four plants of each cultivar or accession.

Collected leaf tissue was lyophilized for approximately 3 d and thereafter ground to a fine powder. Total DNA extraction was done following a modified sorbitol extraction protocol

Table 1. Seed source for 34 natural populations of big bluestem from Wisconsin analyzed for amplified fragment length polymorphism (AFLP) diversity.

Site	n†	Lat. °N	Long. °W	County	HZ‡	Ecoregion§
Arena Pines	7	43.18	89.93	Iowa	4	Driftless area
Avoca Prairie/Savanna	7	43.20	90.29	Iowa	4	Driftless area
Bald Bluff	7	42.84	88.59	Jefferson	5	Southeastern Wisconsin till plains
Battle Bluff Prairie	6	43.46	91.21	Vernon	4	Driftless area
Blue River Sand Barrens	7	43.19	90.55	Grant	4	Driftless area
Brady's Bluff	5	44.02	91.48	Trempealeau	4	Driftless area
Buena Vista Quarry	6	44.32	89.63	Portage	4	North central hardwood forests
Chiwaukee Prairie	4	42.50	87.81	Kenosha	5	Central Corn Belt plains
Colburn State Wildlife Area	5	44.11	89.68	Adams	4	North central hardwood forests
Crex Sand Prairie	7	45.87	92.66	Burnett	3	Northern lakes and forests
Dewey Heights Prairie	7	42.73	91.02	Grant	4	Driftless area
Fish Lake Wildlife area	7	45.70	92.70	Burnett	3	Northern Lakes and forests
Five Mile Bluff	6	44.47	92.07	Pepin	4	Driftless area
Gasner Hollow	7	42.92	91.12	Grant	4	Driftless area
Grand River Wildlife Area	7	43.70	89.22	Green Lake	4	Southeastern Wisconsin till plains
Kessler Railroad Prairie	6	42.63	89.13	Rock	5	Southeastern Wisconsin till plains
Kettle Moraine Low Prairie	7	42.91	88.48	Waukesha	5	Southeastern Wisconsin till plains
LaCrosse River Trail	5	43.89	90.96	La Crosse	4	Driftless area
Mazomanie Oak Barrens	7	43.24	89.73	Dane	4	Driftless area
Morgan Coulee	6	44.61	92.31	Pierce	4	Driftless area
Muir Park	7	43.69	89.39	Marquette	4	North central hardwood forests
Muralt Bluff Prairie	7	42.70	89.49	Green	4	Southeastern Wisconsin till plains
Puchyan Prairie	7	43.89	89.02	Green Lake	4	Southeastern Wisconsin till plains
Rocky Run Oak Savanna	7	43.46	89.33	Columbia	4	Southeastern Wisconsin till plains
Scuppernong Prairie	7	42.90	88.50	Waukesha	5	Southeastern Wisconsin till plains
Sehered Slough	7	43.10	90.77	Grant	4	Driftless area
Smith Slough and Sand Prairie	5	43.19	90.22	Richland	4	Driftless area
Solon Springs	5	46.30	91.83	Douglas	3	Northern Lakes and forests
Sterling Barrens	6	45.56	92.83	Polk	3	Northern Lakes and forests
Swenson Wet Prairie	7	42.56	89.36	Rock	4	Southeastern Wisconsin till plains
Waterloo Prairie	4	43.17	88.94	Jefferson	5	Southeastern Wisconsin till plains
Westport Drumlin	5	43.18	89.39	Dane	5	Southeastern Wisconsin till plains
Woodman Lake Sand Prairie	6	43.10	90.79	Grant	4	Driftless area
Young Prairie	7	42.84	88.62	Walworth	5	Southeastern Wisconsin till plains

†Number of plants analyzed from a collection site, each plant represents seed from a unique maternal line.

‡HZ (hardiness zone) as defined by USDA plant hardiness zone map (Cathey, 1990).

§Level III ecoregions (Omernik, 1987; U.S. Environmental Protection Agency, 2010).

(Storchova et al., 2000). Extracted DNA was normalized to 100 ng μL^{-1} for AFLP reactions.

AFLP Analysis

Individual DNA samples were randomly distributed in 96-well plates (Meudt and Clarke, 2006) to minimize plate-to-plate variation. Negative controls (samples with only water) were randomly distributed within the plates to control for failed amplifications (Bonin et al., 2004). A total of 81 individual samples were randomly selected, and replicate DNA samples were independently extracted and randomly distributed throughout the plates. Replicate samples represented 17.6% of the total individuals tested.

Fragments of DNA were amplified using the AFLP method (Vos et al., 1995), following the protocol by Clarke and Meudt (2005). *Mse*I selective primers used were *Mse*I+*CGA*, *Mse*I+*CTG*, and *Mse*I+*CTT*. *Eco*RI selective primers used were *Eco*RI+*AAG*, *Eco*RI+*AGC*, and *Eco*RI+*AGG*, fluorescently labeled with FAM, TAMARA, and HEX, respectively. Nine selective *Eco*RI–*Mse*I primer combinations were used for amplification. After selective amplification, products were combined by *Mse*I primer, to multiplex analysis runs. Amplified fragments were separated using ABI 3730 automatic capillary DNA sequencer (Applied Biosystems, Foster City, CA) and electropherograms were processed using Peak Scanner v1.0 (Applied Biosystems) using default parameters. Fragment sizes were measured relative to a GeneScan 500 ROX Size Standard (Applied Biosystems). Fragment data was scored for presence (1) or absence (0) using the RawGeno package v1.1-2 (Arrigo et al., 2009) in the freely available statistical package R (http://www.r-project.org; Ihaka and Gentleman, 1996). Scoring parameters used were as follows: scoring range 100–400 bp; minimum intensity, 100 relative fluorescence units; minimum bin width, 0 bp; maximum bin width, 2 bp. Closely sized bins were removed. Bins with rare alleles (less than three present alleles) were removed from further analysis. All monomorphic bands were removed from subsequent analyses. Samples having similar banding patterns to the negative controls were removed from the analysis.

Table 2. Seed source for 24 natural populations of big bluestem from the northeastern United States analyzed for amplified fragment length polymorphism (AFLP) diversity.

NRCS accession no. †	*n*‡	Lat. °N	Long. °W	State	County	HZ§	Ecoregion¶
9085193	5	39.68	77.24	MD	Carrol	6	Northern piedmont
51990	6	44.64	70.09	ME	Franklin	4	Laurentian plains and hills
51992	5	45.00	70.00	ME	Somerset	4	Northeastern highlands
9086108	2	41.90	75.26	NY	Delaware	5	Northern Appalachian plateau
9106115	6	41.00	72.04	NY	Suffolk	7	Atlantic coastal pine barrens
9106124	5	43.14	75.28	NY	Oneida	4	Eastern Great Lakes lowlands
9106126	4	44.59	75.32	NY	St. Lawrence	4	Eastern Great Lakes lowlands
9106201	7	42.97	78.66	NY	Erie	6	Eastern Great Lakes lowlands
9106207	7	42.50	77.71	NY	Steuben	6	Northern Appalachian plateau
9106212	6	42.76	73.67	NY	Rensselaer	5	Northeastern coastal zone
9106213	6	42.80	73.84	NY	Saratoga	5	Northeastern coastal zone
9106215	7	43.52	73.82	NY	Warren	6	Northeastern highlands
9106220	7	41.49	75.89	PA	Wyoming	5	Northern Appalachian plateau
9106243	7	41.32	78.14	PA	Cameron	5	North central Appalachians
9106245	6	39.72	79.87	PA	Fayette	6	Western Allegheny plateau
9106247	7	40.99	80.28	PA	Lawrence	5	Erie drift plain
9106248	6	41.26	77.33	PA	Lycoming	5	Ridge and valley
9106250	7	40.66	76.93	PA	Snyder	6	Ridge and valley
9106251	7	41.62	79.12	PA	Forest	5	North central Appalachians
9106252	7	39.74	76.05	PA	Chester	6	Northern piedmont
9106253	7	39.81	76.32	PA	York	6	Northern piedmont
9106260	5	41.01	80.00	PA	Butler	5	Western Allegheny plateau
51993	2	43.44	72.62	VT	Windsor	4	Northeastern highlands
9078781	6	39.28	77.89	WV	Jefferson	6	Ridge and valley

†Natural Resources Conservation Service, Big Flats Plant Materials Center, Corning, NY.

‡Number of plants analyzed from an accession.

§Hardiness zone (HZ) as defined by USDA plant hardiness zone map (Cathey, 1990).

¶Level III ecoregions (Omernik, 1987; U.S. Environmental Protection Agency, 2010).

Results of the scoring were used to create a binary matrix table indicating the presence or absence of a fragment at each locus (within this paper a locus is defined as a single bin). Error rates were calculated using 81 replicated samples. For each AFLP locus a locus-specific error rate was calculated by dividing the number of mismatches by the total number of samples (Bonin et al., 2004). Using the locus-specific error rate, associations between error rate and the number of present alleles were examined and a threshold was set for locus-specific error optimizing for the number of present alleles. Loci with an error rate greater than the threshold were removed from subsequent analyses. Overall error rates were calculated using the mean error rate for all loci used in our analyses.

Data Analysis

Analyses of AFLP binary marker data were completed using GENALEX6 (Peakall and Smouse, 2006). Pairwise genetic distances for binary data were calculated and used to create Euclidean genetic distance matrices used in subsequent analyses. Differences between samples were visualized using principal coordinate analysis (PCoA); this procedure transformed the binary variables into new sets of uncorrelated variables (principal coordinates). Those principal coordinates explaining the most variation were subsequently used to create scatterplots representing the relationship between the respective coordinates (Mohammadi and Prasanna, 2003).

The analysis of molecular variance test (AMOVA; Excoffier et al., 1992) was performed on all samples in a hierarchal manner to partition variance among and within the three groups of samples, collections sites or accession, and individual samples. Significance of the AMOVA was tested using 999 permutations. Differences between samples from varying ecoregions and plant hardiness zones were tested using AMOVA. Ecoregions were defined as level III ecoregions, which emphasizes features that make an area unique from the surrounding areas (Omernik, 1987; U.S. Environmental Protection Agency, 2010). Variance was similarly partitioned using USDA hardiness zone (Cathey, 1990) for the samples from all groups.

To measure the relationship between genetic distance and geographic distance, a geographic distance matrix was computed based on GPS data from individual collection sites. Correlations between the geographic distance matrix and the genetic distance matrix were tested using a Mantel test with 999 permutations (Mantel, 1967; Sokal and Rohlf, 1995; Escudero et al., 2003). Separate tests were conducted for the collections from Wisconsin and the northeastern United States. Further associations between weather and genetic distance were also measured using the Mantel test. Annual average (1971–2000) weather data were obtained for each collection site from the National Climatic Data Center using the Climatography of the United States No. 20, Monthly Station Climate Summaries data (National Climatic Data Center, 2010). The weather data were then used to create a Euclidean distance matrix for each climatic variable using the Proc Distance function in SAS (SAS Institute, Cary, NC). The climatic distance matrix was then tested for correlations with the genetic distance

Table 3. Seed source for 30 released accessions of big bluestem analyzed for amplified fragment length polymorphism (AFLP) diversity.

Accession name†	Seed source	n‡	State of Origin	Lat.°N§	Long. °W§	HZ¶	Ecoregion#
9063121	GRIN- PI 635103	4	MN, SD	44.38	96.45	4	Northern glaciated plains
9063122	GRIN- PI 635104	4	MN, SD	44.38	96.45	4	Northern glaciated plains
Bison-N	NRCS Bismarck, ND	4	MN, ND	47.08	100.94	4	Northwestern Great Plains
Bison-G	GRIN- PI 537589	3	MN, ND	47.08	100.94	4	Northwestern Great Plains
Bonanza	University of Nebraska	3	NE	40.11	96.15	5	Western Corn Belt plains
Bonilla	NRCS Bismarck, ND	4	SD	44.58	98.50	4	Northern glaciated plains
Central Iowa	NRCS Elsberry, MO	3	IA	42.08	93.59	5	Western Corn Belt plains
Champ-U	University of Nebraska	3	NE, IA	42.13	96.35	4	Western Corn Belt plains
Champ-A	Arrow Seed, Broken Bow, NE	3	NE, IA	42.13	96.35	4	Western Corn Belt plains
Earl	NRCS Knox City, TX	3	TX	32.82	97.80	7	Cross timbers
Goldmine	University of Nebraska	2	KS	39.09	96.58	5	Flint hills
Hampton	NRCS Booneville, AR	3	MO	37.04	90.53	6	Ozark highlands
HZ4††	University of Nebraska	4	Great Plains	–	–	4	–
HZ5††	University of Nebraska	3	Great Plans	–	–	5	–
Kaw-N	NRCS Manhattan, KS	4	KS	39.09	96.58	5	Flint hills
Kaw-G	GRIN- PI 421276	4	KS	39.09	96.58	5	Flint hills
Niagara	GRIN- PI 601478	4	NY	42.85	78.64	5	Eastern Great Lakes lowlands
Northern Iowa	NRCS Elsberry, MO	4	IA	43.15	93.93	4	Western Corn Belt plains
Northern Missouri	NRCS Elsberry, MO	4	MO	39.89	93.26	5	Central irregular plains
OH 370	NRCS Elsberry, MO	4	MO, AR, OK, IL	36.49	94.62	6	Ozark highlands
OZ-70	NRCS Elsberry, MO	3	MO, AR, OK, IL	36.49	94.62	6	Ozark highlands
Pawnee-U	University of Nebraska	2	NE	40.11	96.15	5	Western Corn Belt plains
Pawnee-S	Stock Seed, Murdock, NE	4	NE	40.11	96.15	5	Western Corn Belt plains
Prairie View Indiana-N	NRCS East Lancing, MI	4	IN	39.73	86.14	5	Eastern Corn Belt plains
Prairie View Indiana-G	GRIN- PI 642389	4	IN	39.73	86.14	5	Eastern Corn Belt plains
Refuge	NRCS Elsberry, MO	3	AR	36.17	93.87	6	Ozark highlands
Rountree	NRCS Elsberry, MO	4	IA	41.92	95.85	5	Western Corn Belt plains
Southern Iowa	NRCS Elsberry, MO	3	IA	40.71	93.61	5	Central irregular plains
Southlow Michigan	NRCS East Lancing, MI	4	MI	42.29	84.91	5	Southern Michigan and Northern Indiana drift plains
Sunnyview	GRIN- PI 603289	4	SD	42.72	96.72	4	Western Corn Belt plains

†For five cultivars (Bison, Champ, Kaw, Pawnee, and Prairie View Indiana), two seed sources were used and analyzed separately. Multiple seed sources are identified by a single letter attached to the accession name: "-A" = Arrow Seed, "-G" = Germplasm Resources Information Network, "-N" = Natural Resources Conservation Service, "-S" = Stock seed, and "-U" = University of Nebraska.

‡Number of plants analyzed from an accession.

§Geographic coordinates for cultivars and accessions comprised of composite seed from multiple sites are general to the region where seed originates and not specific to a collection site.

¶Hardiness zone (HZ) as defined by USDA plant hardiness zone map (Cathey, 1990).

#Level III ecoregions (Omernik, 1987; U.S. Environmental Protection Agency, 2010).

††Accessions HZ4 and HZ5 were developed from collection from broad regions of hardiness zones 4 and 5 respectively, as such specific geographic coordinates are not given, these accessions are not included in ecoregion or hardiness zone analyses.

matrix using a Mantel test (Mantel, 1967); significance was tested using 999 permutations.

For the samples collected in Wisconsin, additional soil data were also collected from each site. Seven soil cores (~25-cm depth) were collected and bulked from each site. The University of Wisconsin Soil and Plant Analysis Lab conducted all soil analyses (pH, organic matter, total phosphorus, total potassium, total nitrogen, and soil texture). A Euclidean distance matrix was calculated for each soil variable and compared to the genetic distance matrix for those samples from Wisconsin using the Mantel test. Significance was tested using 999 permutations.

RESULTS

Using nine primer combinations, 2711 AFLP polymorphic loci were identified and scored for each sample as having a present or absent allele. Calculations of error rate using replicate samples resulted in an estimated initial mean error rate of 12% error per locus for all loci. Of the 2711 identified loci, 2294 loci had greater-than-threshold error rates (based on the expected error rate from the number of present alleles) and were removed from the data set. For the remaining 417 loci, the error rate was estimated to be 6%. This error rate is within the range of error rate per locus previously documented for AFLP experiments (Arrigo et al., 2009; Avolio et al., 2011; Holland et al., 2008). Due to the information redundancy obtained from a large number of AFLP markers, the data are expected to be robust (Pompanon et al., 2005).

The genetic relationship between the three groups of accessions analyzed in these experiments was visualized using PCoA plots. This analysis demonstrated the three groups could be separated by visualization of the first two dimensions representing 33 and 18% of the variation, respectively (Fig. 2). While differentiation of the groups

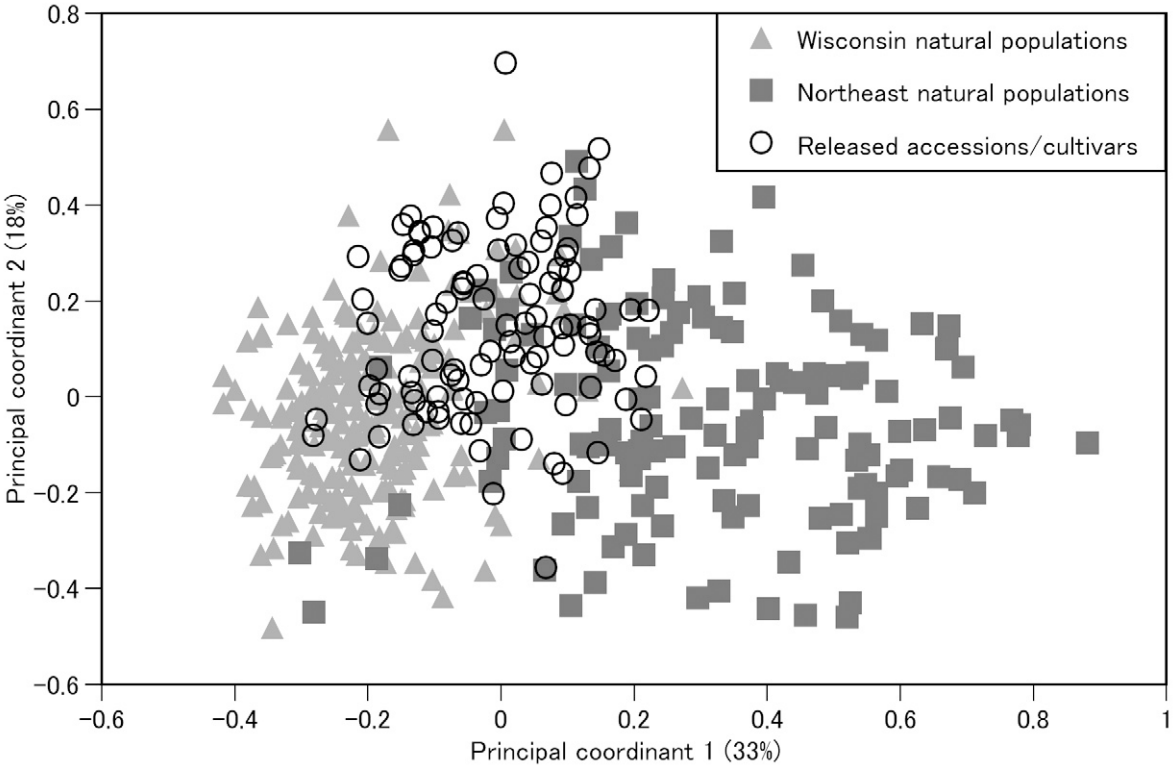

Figure 2. Principal coordinates analysis of 458 big bluestem plants based on 417 amplified fragment length polymorphism (AFLP) fragments, labeled by collection group

is obvious, there was considerable overlap of the released accessions and the samples from both Wisconsin and the northeastern United States. Of particular note the cultivar Niagara, which originates in eastern New York, clearly overlapped with other samples originating from the New York region (data not shown). AMOVA partitioned 4% of the variance among groups and 10% of the variance among populations within groups; the remaining 86% of the variance was attributed to variation within groups (Table 4).

Independent PCoA of the Wisconsin populations resulted in slight discrimination among accessions (data not shown). The AMOVA of the Wisconsin accessions resulted in significant ($P < 0.001$) variance among ecoregion and hardiness zone, although only small amounts of the variance were explained: 1%, respectively (Table 5). Due to the small amounts of variance explained, labeling samples by ecoregion and hardiness zone similarly did not reveal observable visual patterns in the PCoA (data not shown). Only 3% of the variance could be attributed to populations within ecoregions and hardiness zones, while the remaining 96% of variance was partitioned within populations. The Mantel correlation between genetic distance and geographic distance for individuals within Wisconsin was $r = 0.036$ ($P = 0.156$). Correlations between genetic distance and environmental variables were not significant (Table 6). Degree days cooling, mean annual precipitation, soil pH, organic matter, and total soil nitrogen each had Mantel correlation coefficients with $P < 0.20$.

For the samples collected in the northeastern United States, PCoA of samples by population demonstrated distinct clustering of populations, but labeling by ecoregion and hardiness zone did not reveal distinct patterns (data not shown). The AMOVA of the northeast group confirmed the results observed in the PCoA (Table 5). Using a hierarchal analysis, ecoregion and populations within ecoregions accounted for 3 and 20% of the variance ($P < 0.01$). Hardiness zone did not account for a significant portion of the variance. In contrast to the samples from Wisconsin, a Mantel test indicated a highly significant correlation between genetic distance and geographic distance ($r = 0.205$, $P < 0.001$) for the samples collected in the northeastern United States. Correlations for all climatic variables were also significant ($P < 0.001$; Table 6).

An independent analysis of the released accessions of big bluestem was also conducted. Principal coordinate analysis of 30 released accessions did not reveal visually distinct patterns (data not shown). The AMOVA partitioned 2% of the variance

Table 4. Analysis of molecular variance (AMOVA) for 34 Wisconsin collections, 24 northeastern U.S. collections, and 30 released accessions of big bluestem (458 individuals), based on 417 AFLP fragments.

Source of variation	df	MS	Variance component	% of variance	*P* value
Among groups	2	330.76	1.85	4	<0.001
Populations within groups	85	59.87	4.22	10	<0.001
Plants within populations	370	37.96	37.96	86	<0.001
Total	457		44.03	100	

Table 5. Analysis of molecular variance (AMOVA) for 34 Wisconsin collections, 24 northeastern U.S. collections and 28 released cultivars and accessions, based on 417 amplified fragment length polymorphism fragments. Two hierarchal AMOVA tests were conducted for each group; the first partitions the variance among and within ecoregions and populations, the second partitions variance between hardiness zone and populations.

Source of variation	df	MS	Variance component	% of variance	*P* value
Wisconsin natural populations					
Among ecoregions	4	62.21	0.45	1	<0.001
Populations within ecoregions	29	46.77	1.08	3	<0.001
Plants within populations	179	39.92	39.92	96	
Total	212		41.45	100	
Among hardiness zone	2	77.26	0.57	1	<0.001
Populations within hardiness zone	31	46.79	1.10	3	<0.001
Plants within populations	179	39.92	39.92	96	
Total	212		41.59	100	
Northeastern U.S. natural populations					
Among ecoregions	10	103.3	1.13	3	<0.001
Populations within ecoregions	13	83.55	8.90	20	<0.001
Plants within populations	116	34.21	34.21	77	<0.001
Total	139		44.24	100	
Among hardiness zone	3	88.23	0.00	0	0.104
Populations within hardiness zone	20	92.76	10.11	23	<0.001
Plants within populations	116	34.21	34.21	77	<0.001
Total	139		44.32	100	
Released cultivars and accessions					
Among ecoregions	9	53.60	0.92	2	<0.001
Populations within ecoregions	18	44.77	1.69	4	<0.001
Plants within populations	70	38.98	38.98	94	<0.001
Total	97		41.59	100	
Among hardiness zone	3	59.63	0.68	2	<0.001
Populations within hardiness zone	24	46.22	2.06	5	<0.001
Plants within populations	70	38.98	38.98	93	<0.001
Total	97		41.72	100	

among ecoregions and 4% among populations within ecoregions (Table 5). In AMOVA with hardiness zones, 2% of the variance was partitioned to among hardiness zones and 5% among populations within hardiness zone (Table 5).

DISCUSSION

This study documents genetic variation in big bluestem through analysis of DNA markers in three distinct groups of germplasm: natural populations from Wisconsin, natural populations of the northeastern United States, and a panel of currently available cultivars and prairie-remnant accessions. As hypothesized, DNA markers revealed that a large amount of genetic variation is present within both natural and cultivated populations of big bluestem. As an outcrossing species with a complex polyploid genome (Norrmann et al., 1997), it was expected that a large amount of genetic diversity would be present. It is hypothesized that the present genetic variation contributes to the success of big bluestem across its native range, containing large geographic and landscape diversity.

Comparisons of DNA markers among the three groups demonstrate that each group represents a unique genetic pool, with overlapping regions. This result is not surprising because seed from each group represents a distinct provenance, separated by considerable geographic distance. Previous studies have demonstrated significant phenotypic differences between big bluestem seed from varied provenance and seed from released cultivars (Gustafson et al., 2004). Our data suggest that regional differences exist among big bluestem on a macro level. This conclusion is in contrast to previous DNA marker work that found no significant genetic differences between local populations and Conservation Reserve Program plantings of commercial cultivars in the state of Ohio using random amplified polymorphic DNA markers (Selbo and Snow, 2005). The discrepancy between the current study and this prior work can be explained by the limited number of commercial cultivars tested and a reduced provenance in the study of Ohio populations. We propose that the current study better identifies genetic differences between regional gene pools due to the diverse panel of natural and cultivated germplasm surveyed and the large number of DNA markers screened. This finding is of considerable importance, because much of the current commercially available seed has an origin in the Great Plains region of the United States. It is expected that germplasm from outside of the Great Plains region contains unique genetic variation that can be used for future improvements of big bluestem germplasm.

Analyses of each group revealed unique characteristics that varied between groups. For the natural populations from Wisconsin, variance among populations was significant, but accounted for only a small part (3%) of the total variance. In contrast, variance among populations from the northeastern United States was highly significant and accounted for 20% of the total variance. Greater landscape diversity represented by the populations from the northeastern United States, may contribute to the observed differences. This argument is supported by the fact that a greater amount of the variance in the northeastern United States was partitioned among ecoregions (3%) compared to Wisconsin (1%). Furthermore, genetic diversity in the northeastern United States was significantly correlated with geographic distance and climatic variables.

There are other possible explanations for the differences in the genetic variance partitions within Wisconsin vs. the northeastern United States. First, fragmentation of habitat can cause genetic isolation and thus genetic divergence between populations. While the current landscape is highly fragmented in natural populations in Wisconsin, the landscape was once interconnected prairie and savanna. This prairie and savanna habitat extended throughout much of the state, with the major exception of the far north and northeastern regions of the state, and likely contributed to considerable gene flow over thousands of years (Casler et al., 2007; Zhang et al., 2011b). Frequent gene flow across a broad landscape would have negated founder effects associated with colonization of new habitats and served to homogenize the populations across a broad landscape. Because habitat fragmentation has occurred only recently in Wisconsin, since the mid-1800s, the polyploidy and outcrossing nature of big bluestem has preserved vast amounts of genetic variation within individual prairie and savanna remnants. These remnants appear to represent the relatively homogeneous population characteristics of the nonfragmented habitat.

For populations collected in the northeastern United States a different landscape history has occurred. The precolonial landscape of this area can be described as a patchwork of ecological factors resulting in a large variety of landscapes and species within small geographic areas (Cronon, 2003). During colonization many of the grasslands were converted to other uses, and since that time the overall number of grasslands has declined markedly (Whitney, 1994). The grasslands that remain today in this region are even more fragmented and isolated, while at the same time reduced in size (Vickery and Dunwiddie, 1997; Vickery et al., 1994). Based on our results, we hypothesize that increased genetic isolation in the northeastern United States has resulted in greater genetic divergence among populations. This hypothesis is supported by the difference in among population variance between samples originating in the northeastern United States and Wisconsin (20% vs. 3%).

Table 6. Mantel correlation coefficients (*r*) between genetic distance and six climatic variables tested using Mantel test for 34 natural populations in Wisconsin and 24 natural populations collected in northeastern USA. Correlation with eight soil variables also measured for Wisconsin populations. Genetic distance based on AFLP marker data and climatic variables based on annual average 1971 to 2000. Soil variables based on collected soil samples from individual collections sites.

Variable	Range (min and max)	*r*	*P* value
Wisconsin natural populations			
Climatic			
Mean annual precipitation, cm	77.7–90.4	0.032	0.157
Degree days cooling (base temp 18.3°C)	167–457	−0.049	0.124
Degree days heating (base temp 18.3°C)	3853–5227	−0.016	0.347
Mean daily max temperature (°C)	11.4–14.2	−0.013	0.361
Mean annual temperature, °C	4.4–9.0	−0.026	0.256
Soil			
Soil pH	7.8–5.0	−0.030	0.076
Organic matter, %	1.0–12.3	0.048	0.139
Phosphorus, μg g^{-1}	4.0–122.0	0.024	0.283
Potassium, μg g^{-1}	28.0–210.0	0.005	0.420
Total nitrogen, μg g^{-1}	400–6400	0.040	0.172
Soil texture			
Sand %	36–93	−0.007	0.392
Silt %	1–52	−0.018	0.287
Clay %	4–22	0.003	0.484
Northeastern U.S. natural populations			
Climatic			
Mean annual precipitation, cm	80.0–122.1	0.224	<0.001
Degree days cooling (base temp 18.3°C)	80–601	0.187	<0.001
Degree days heating (base temp 18.3°C)	2671–4892	0.159	<0.001
Mean daily max temperature, °C	11.8–18.9	0.136	<0.001

A second explanation for the observed results is the influence of land managers on the genetic landscape of an area. The idea of land restoration implies restoring land to its native state. As native prairie land within Wisconsin was converted to other uses it was recognized that portions of the prairie should be maintained and in some cases restored to their prior state. Often this maintenance or restoration involved the spreading of seed of plants native to the area, including big bluestem. When selecting seed for this work land managers are faced with two choices: seed collected at nearby locations or commercial seed, likely sourced from the Great Plains. Within the present study the addition of seed from either source to natural populations in Wisconsin would account for a dilution of among-population genetic diversity. As some sites within our study have been managed for more than 50 yr, adequate records do not exist to verify the provenance of the big bluestem plants, whether they originated from a seed bank, a nearby local collection, or from a nonlocal collection. Due to the lack of reliable information we must consider seed introduction as a possible explanation for the relatively small amount of among-population genetic variance for samples originating in Wisconsin. Finally, we

must consider that within the relatively small geographic areas considered, stochastic events can have a considerable impact on genetic diversity, and therefore may have led to observed differences in explainable variance.

Tests of genetic diversity among the released accessions and cultivars revealed a large amount of within-population variance, with limited amounts of among-population variance (Table 5), suggesting that while significant diversity exists within the currently released germplasm, it is shared between many of the populations. A small, but significant ($P < 0.001$) portion of the variance was attributed to ecoregions and hardiness zones suggesting a small geographic effect. While the amount of among-population variance explained within the released accessions is greater than that found in populations from Wisconsin, it is much less than what is found in those populations from the northeastern United States. We hypothesize that the method by which many of the accessions were created can explain the lack of divergence among populations. Several accessions were created by bulking seed from multiple natural populations within a specific geographic region; others were created by combining seed from diverse sets of plants with a variety of traits. Use of these methods to create the analyzed cultivars likely resulted in a dilution of natural genetic divergence that would have occurred over many thousand years. Additionally the coproduction of seed of varying cultivars, within neighboring areas, could contribute to cross-pollination between cultivars, resulting in additional dilution of genetic divergence. Our results of cultivar diversity are in contrast to recent studies in another tall-grass prairie species, switchgrass (*Panicum virgatum* L.). Using simple sequence repeat markers, switchgrass cultivars within in the United States can be differentiated by ploidy level, ecotype, region of origin, and cultivar with considerable separation (Zalapa et al., 2010). The source of these differences may be an increased focus on switchgrass cultivar improvement and the greater number of generations of selection that have occurred in the development of some switchgrass cultivars compared with the majority of big bluestem cultivars.

From a restoration ecology perspective, the observed genetic differences described have serious implications. Big bluestem is a commonly seeded native species in prairie restoration but little empirical evidence has been utilized to determine the source of seed used for these restoration projects (Gustafson et al., 2005). Arguments have been made for using only local seed for restoration plantings and have gone as far as requiring the use of seed collected from within a small geographic boundary. Our results confirm that regional big bluestem seed does contain unique variation, which has likely developed over thousands of years without large additions of germplasm from outside regions.

While unique local variation does exist, it should be noted that not all local sources of seed are genetically equal. In the current study we observe that local populations in both the northeastern United States and Wisconsin contain plants that are genetically similar to available cultivars and prairie-remnant accessions from other regions of the United States. Further examination of the PCoA plot representing the three groups (Fig. 2) demonstrates that both the Wisconsin and the Northeast groups overlap with the released cultivars, but overlap very little with each other. This result may be indicative of a transfer of germplasm, from the Great Plains, to natural populations in Wisconsin and the northeastern United States. Recent work on natural populations of switchgrass has hypothesized that switchgrass seed from the Great Plains was transferred to the east coast by prairie hay being transported with animals between military instillations during the early 20th century (Zhang et al., 2011a). It can be assumed that this prairie hay, in addition to switchgrass, contained big bluestem due to its abundance in the area. While this study does not provide direct evidence for the transfer of specific big bluestem germplasm from one region to another, it does demonstrate that genetic pools between the three groups analyzed overlap with each other, and therefore supports some level of gene flow between the groups.

Historically, genetic resources within natural grasslands have been a very important resource for the improvement of forage species (Boller and Veteläinen, 2010). To those seeking to improve big bluestem, both as a forage crop and a biofuel feedstock, the identification of unique germplasm represents a great opportunity to increase genetic variation within current gene pools. Currently, most germplasm originates from the Great Plains, west of the Mississippi River. Our results demonstrate that natural populations in Wisconsin and the northeastern United States are genetically unique from the currently released germplasm and, therefore, represent two new sources of germplasm for use in big bluestem improvement. These differences represent an opportunity for the development of new accessions adapted to variable environments and the introgression of new alleles into current germplasm. Increased genetic variation within big bluestem germplasm will be essential for continued efforts to increase the value of big bluestem both as a forage and biofuel crop.

From both a genetic improvement and ecological standpoint, the importance of maintaining existing genetic diversity in big bluestem is clear. This maintenance can be done in two ways: first, plant genetic resources can be maintained in situ within natural grassland populations. To maintain genetic diversity within these populations conscious efforts will need to be made by land managers to prevent the addition of outside seed sources and to continue to protect these lands as a valuable natural resource. The second method by which genetic diversity can be maintained is through continued seed collection and maintenance of diverse populations. We believe that recent suggestions of maintaining source-identified

accessions of seed for switchgrass (Zhang et al., 2011b) are equally warranted in big bluestem. Our results, along with other genetic diversity studies in forage species, clearly indicate that much of the genetic diversity of these species is within populations (Boller and Veteläinen, 2010). This result suggests that the most effective strategy for future seed collections will be sampling larger numbers from a small number of sites, rather than a few numbers from a large number of sites. By maintaining genetic diversity of local populations both in situ and by seed collections, it can be assured that local adaptations and genotypes can be maintained both as source for future plant improvement and for understanding the origins of big bluestem.

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