2017

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Genet Resour Crop Evol
https://doi.org/10.1007/s10722-017-0596-z

RESEARCH ARTICLE

Genetic diversity of *Danthonia spicata* (L.) Beauv. based on genomic simple sequence repeat markers

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Received: 2 August 2017 / Accepted: 29 November 2017

Abstract *Danthonia spicata* (L.) Beauv., commonly known as poverty oatgrass, is a perennial bunch-type grass native to North America. *D. spicata* is often found in low input turfgrass areas on the East Coast of the United States and has potential for development as a new native low input turfgrass species. Roche 454 sequenced randomly sheared genomic DNA reads of *D. spicata* were mined for SSR markers using the MIcroSAtellite identification tool. A total of 66,553 singlet sequences (approximately 37.5 Mbp) were examined, and 3454 SSR markers were identified. Trinucleotide motifs with greater than six repeats and possessing unique PCR priming sites within the genome, as determined by Primer-BLAST, were evaluated visually for heterozygosity and mutation consistent with stepwise evolution using CLC Genomics software. Sixty-three candidate markers were selected for testing from the trinucleotide SSR marker sites meeting these in silico criteria. Ten primer pairs that amplified polymorphic loci in preliminary experiments were used to screen 91 individual plants composed of at least 3–5 plants from each of 23 different locations. The primer pairs amplified 54 alleles ranging in size from 71 to 246 bp. Minimum and maximum numbers of alleles per locus were two and 12, respectively, with an average of 5.4. A dendrogram generated by unweighted pair group method with arithmetic mean cluster analysis using the Jaccard's similarity coefficient was in agreement with the grouping obtained by Structure v2.3. The analyses were dominated by clonal groupings and lack evidence for gene flow with some alleles present in a single plant from a single location. Fourteen multilocus genotype groups were observed providing strong evidence for asexual reproduction in the studied *D. spicata* populations.

Keywords *Danthonia* · Germplasm · Genetic diversity · Microsatellites · Poaceace · Polymorphism · Turfgrass

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Published online: 16 December 2017

Abbreviations

AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
MISA	MIcroSAtellite identification tool
MLGs	Multilocus genotypes
NCBI	National center for biotechnology information
RAPD	Random amplified polymorphic DNA
SSR	Simple sequence repeat
UPGMA	Unweighted pair group method with arithmetic averaging

Introduction

The genus *Danthonia* (Poaceae) grows in a diversity of tropical, semi-tropical and temperate habitats. At present, there are nine species in South America, eight in North America, three in Europe, two in Africa, and two in Asia (Linder and Verboom 1996). It is a cool-season genus in the PACCAD clade more closely related to warm-season turf species (Grass Physiology Working Group 2001). The genus *Danthonia* has a base chromosome number of $x = 6$ and in North America, seven of the eight species (*D. californica* Bol., *D. compressa* Austin, *D. intermedia* Vasey, *D. parryi* Scribn., *D. sericea* Nutt., *D. spicata* (L.) Beauv. and *D. unispicata* (Thurb.) Munro ex Macoun) are native while *D. decumbens* (L.) DC., is introduced from Europe (Darbyshire 2003). *D. spicata* L., (poverty oatgrass) is a native perennial, polyploid, C_3 bunch grass that is widespread throughout the eastern and northern United States and southern Canada (Clay 1982). It is mainly present in impoverished or low-fertility soils and hence the name, "poverty oatgrass". It is a very shade tolerant, drought tolerant, low maintenance grass, frequently found on unimproved turf sites (Warnke 2010). However, it has some disadvantages with respect to cultivation such as poor seed yield, shattering of seed heads prior to harvest, and seed dormancy (Warnke 2010). *D. spicata* has potential as a low input turf species, however, a more complete understanding of its reproductive biology and genetic diversity is needed.

Danthonia spicata produces two types of seed heads terminal "chasmogamous" seed heads that have the potential for outcrossing yet often remain closed exhibiting no signs of allogamy and axial "cleistogamous" seed heads at the nodes of the flowering stem that never open and therefore have no potential for outcrossing. Weatherwax (1928) reported that both seed heads exhibit rudimentary lodicules, short styles with few stigma hairs and small-undeveloped anthers. Several papers have suggested that the dimorphic seed heads evolved due to a symbiotic relationship with the epiphytic fungus *Atkinsonella hypoxylon* (Clay 1984), that can produce a mass of fungal hyphe at the node of the flowering stem, refered to as "choke", and abort seed head production. Apomixis has also been suggested as a reproductive method in *D. spicata* (Philipson 1986) however no experimental evidence is available.

In the only study to evaluate *D. spicata* genetic diversity Clay and Antonovics (1985b) evaluated the variation of quantitative characters in chasmogamous (CH) and cleistogamous (CL) progeny raised in their native habitat and in the greenhouse. Significant genetic variation existed for twelve quantitative characters measured in greenhouse-grown progeny. Two morphologically similar groups of families were identified and most of the genetic variation was between families with only 5% of the variation within families. In field-grown plants, variance component analysis suggested that CL progenies were genetically more similar to each other than were CH progenies from the same plant and that selective differentials would be larger among families than within families. The use of marker loci as a method of estimating outcrossing rates was suggested however no polymorphic marker loci could be identified.

DNA-based molecular markers like random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), and amplified fragment length polymorphism (AFLP) have been used in fields such as taxonomy, plant breeding, genetic engineering etc. (Chai and Sticklen 1998; Joshi et al. 2011). Among these markers; SSR markers are widely distributed across genomes, highly polymorphic, codominant, and can be used to reproducibly fingerprint organisms in different laboratories (Kalia et al. 2011; Powell et al. 1996; Thammina et al. 2014). SSR markers have been used to examine genetic diversity in bahiagrass, *Paspalum notatum* Flüggé (Cidade et al. 2008), bermudagrass, *Cynodon dactylon* (L.) Pers. (Wu et al. 2006), creeping bentgrass, *Agrostis stolonifera* L. (Kubik et al. 2009), Kentucky bluegrass, *Poa pratensis* L. (Honig et al. 2010), *Paspalum* L. (Cidade

Table 1 List of *D. spicata* (L.) Beauv. germplasm, used for assessing molecular diversity with genomic simple sequence repeat (SSR) markers

Plant ID	Abbreviated plant ID[a]	Collection site
B05-16-001, B05-16-002, B05-16-004, B05-16-005	B051, B052, B054, B055	Beltsville Agricultural Research Center, Beltsville, MD
BRP1-2, BRP1-3, BRP1-4, BRP1-5	BRP12, BRP13, BRP14, BRP15	Blue Ridge Parkway, location 1
BRP2-1, BRP2-3, BRP2-4, BRP2-5	BRP21, BRP23, BRP24, BRP25	Blue Ridge Parkway, location 2
BRP3-2, BRP3-3, BRP3-4, BRP3-5	BRP32, BRP33, BRP34, BRP35	Blue Ridge Parkway, location 3
BRP4-1, BRP4-3, BRP4-4, BRP4-5	BRP41, BRP43, BRP44, BRP45	Blue Ridge Parkway, location 4
BRP5-1, BRP5-2, BRP5-3, BRP5-4, BRP5-5	BRP51, BRP52, BRP53, BRP54, BRP55	Blue Ridge Parkway, location 5
BRP6-1, BRP6-2, BRP6-3, BRP6-4	BRP61, BRP62, BRP63, BRP64	Blue Ridge Parkway, location 6
CR-16-002, CR-16-003, CR-16-004, CR-16-005	CR162, CR163, CR164, CR165	Crossroads Cemetery, Carroll County, MD
EGCAP10-001, EGCAP10-002, EGCAP10-003, EGCAP10-004	CAP1, CAP2, CAP3, CAP4	United States National Arboretum, Washington D.C.
EGFES10-001, EGFES10-002, EGFES10-003, EGFES10-004	FES1, FES2, FES3, FES4	United States National Arboretum, Washington D.C.
EGMDT10-002, EGMDT10-003, EGMDT10-004, EGMDT10-004 c1	MDT2, MDT3, MDT4, MDT4 c1	United States National Arboretum, Washington D.C.
EGMTH10-001, EGMTH10-002, EGMTH10-003, EGMTH10-004	MTH1, MTH2, MTH3, MTH4	United States National Arboretum, Washington D.C.
EGMTO10-001, EGMTO10-002, EGMTO10-003, EGMTO10-004	MTO1, MTO2, MTO3, MTO4	United States National Arboretum, Washington D.C.
EGSTR10-001, EGSTR10-003, EGSTR10-005, EGSTR10-008	STR1, STR3, STR5, STR8	United States National Arboretum, Washington D.C.
GW1-1, GW1-2, GW1-4	GW11, GW12, GW14	George Washington and Jefferson National Forest, Location 1
KM-16-002, KM-16-003, KM-16-004, KM-16-005	KM162, KM163, KM164, KM165	Klees Mill Road, Carroll County, MD
SK1-2, SK1-3, SK1-4, SK1-5	SK12, SK13, SK14, SK15	Skyline Drive, location 1
SK2-1, SK2-2, SK2-4, SK2-5	SK21, SK22, SK24, SK25	Skyline Drive, location 2
SK3-1, SK3-2, SK3-3, SK3-5	SK31, SK32, SK33, SK35	Skyline Drive, location 3
SK4-1, SK4-2, SK4-4, SK4-5	SK41, SK42, SK44, SK45	Skyline Drive, location 4
SK5-1, SK5-2, SK5-3, SK5-5	SK51, SK52, SK53, SK55	Skyline Drive, location 5
SK6-1, SK6-2, SK6-3, SK6-4	SK61, SK62, SK63, SK64	Skyline Drive, location 6
WH-16-002, WH-16-003, WH-16-005	WH162, WH163, WH165	White House, Beltsville, MD

B05 Beltsville agricultural research center; *BRP* blue ridge parkway; *CAP* capitol columns; *CR* Crossroads Cemetary; *FES* Fescue Varietal Trial; *GW* George Washington and Jefferson National Forest; *MDT* Maryland Turf Center; *MTH* Mount Hamilton; *MTO* Mount Olivet; *STR* Grove of State Trees; *KM* Klees Mill Road; *SK* Skyline Drive; *WH* White House

[a]Abbreviated plant ID's were used as plant labels in Figs. 1, 2A–C

et al. 2013), zoysia grass, *Zoysia* spp. (Guo et al. 2012). The objectives of this study were to (a) utilize Roche 454 sequencing data from genomic DNA of *D. spicata* to develop SSR primer pairs and (b) utilize the SSR primer pairs to assess the levels of variability within and among native *Danthonia* populations.

Materials and methods

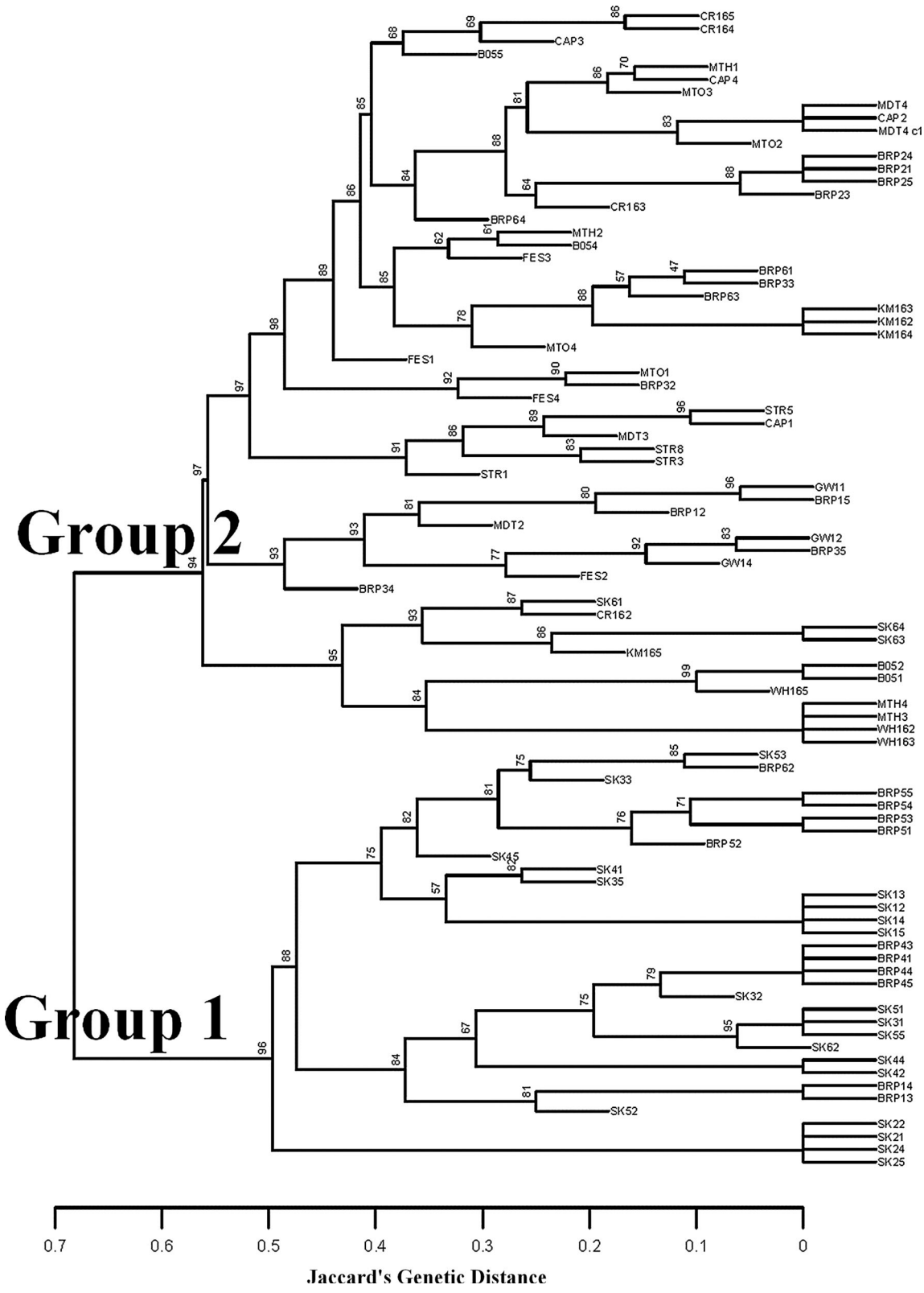
Plant materials

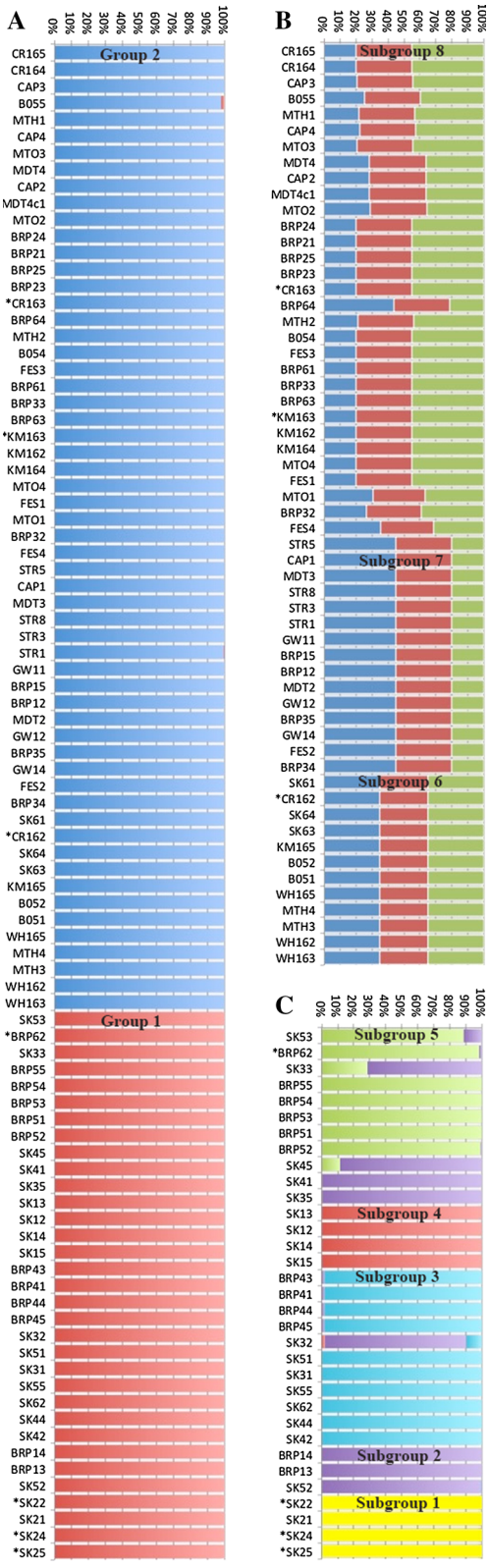
Ninety-one individual *D. spicata* genotypes composed of 3–5 plants from each of 23 different locations in Maryland, North Carolina, Virginia, and Washington, D.C. were collected between spring 2010 and spring 2016 (Table 1). These collection sites were chosen randomly from the natural populations of *D. spicata.* The United States National Arboretum collection sites were all distinct populations located within the 182.1 ha that make up the facility. The Beltsville, MD sites are approximately a half-mile from each other and 24.1 km from the Arboretum. The Carroll County, MD locations are approximately 8.04 km apart and 80.4 km from the Arboretum and Beltsville. The Skyline drive collections were made by starting at the northern entrance to Shenandoah National Park in Front Royal, VA and collecting at picnic sites approximately 32.2 km apart throughout the Park. Blue Ridge Parkway collections were also collected at picnic sites from the northern end of the Parkway through approximately 64.4 km into North Carolina. Plants were maintained in a greenhouse in 4-inch clay pots containing PRO-MIX® HP MYCORRHIZAE™ (Premier Horticulture Inc; Quakertown, PA, USA) at the Beltsville Agricultural Research Center, Beltsville, MD. Plants were maintained at a day temperature of 13–19 °C and night temperature of 17–23 °C under natural light. Peters Professional® Acid Special 21-7-7 fertilizer (Scotts Company LLC; Oxford, PA, USA) was applied every 30 days and irrigated as required. Genomic DNA was extracted from fresh young leaf tissue of *D. spicata* genotype using a PowerPlant® Pro DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's recommendations, and quantified with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Genomic sequencing and primer design

Total genomic DNA was extracted from fresh leaf tissue of a *D. spicata* genotype at the United States National Arboretum (USNA, Washington, D.C., USA) and quantified using the aforementioned procedure. Genomic library construction and sequencing were performed at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign on a Roche 454 sequencer (Roche Diagnostics, Branford, CT, USA); (Tamaki et al. 2011). Later, singlet sequences were mined for SSR markers using the MIcroSAtellite identification tool (MISA) (Coello Coello and Cortés 2005) to detect tandem repeats of two to six nucleotides for at least five perfect repeat core motifs. Trinucleotide motifs with greater than six repeats and possessing unique polymerase chain reaction (PCR) priming sites within the genome, as determined by Primer-BLAST (Ye et al. 2012), were evaluated visually for heterozygosity and mutation consistent with stepwise evolution using CLC Genomics software. From the trinucleotide SSR sites meeting this in silico criteria, 63 candidate markers were selected for gradient-PCR tests. PCR primer pairs were designed with the following settings: primer length of 20 ± 2 nucleotides, GC content of 40–60%, and a PCR product size ranging from 70 to 300 bp. PCR primers were manufactured by Integrated DNA Technologies (Coralville, Iowa, USA). The forward primers had an additional M13 (–21) universal sequence (TGTAAAACGACGGCCAGT) attached to the 5′ end to allow indirect fluorescent labeling of PCR products using just one universal FAM (6-carboxy-fluorescine)–labeled M13 primer (Schuelke 2000). These tests were conducted on EGFES10-002 and BRP2-3 clones, which were chosen randomly from the collected genotypes. PCR was carried out in a Bio-Rad T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The 20-μL PCR reaction mixture contained 20 ng of template genomic DNA, 0.25 μM of each reverse and universal FAM-labeled M13 (–21) primer, and 0.0625 μM of the forward primer with 1 × Bioline MangoMix and 2.5 mM Bioline MgCl2 (Bioline, Taunton, MA, USA). PCR profiles consisted of initial denaturation at 94 °C for 5 min; followed by 35 cycles of 94 °C for 30 s, 58–63 °C annealing temperature range for each primer pair for 45 s, and 72 °C for 45 s; followed by 8 cycles of 94 °C for 30 s, 53 °C for 45 s,

Fig. 1 Dendrogram of 91 *D. spicata* (L.) Beauv. genotypes ▶ based on 10 simple sequence repeat (SSR) markers generated by unweighted pair group method with arithmetic averaging cluster analysis. Confidence levels greater or equal to 95% from bootstrap analysis (20,000 replicates) are indicated at the nodes


Group 2
Group 1
CR165
CR164
CAP3
B055
MTH1
CAP4
MTO3
MDT4
CAP2
MDT4 c1
MTO2
BRP24
BRP21
BRP25
BRP23
CR163
BRP64
MTH2
B054
FES3
BRP61
BRP33
BRP63
KM163
KM162
KM164
MTO4
FES1
MTO1
BRP32
FES4
STR5
CAP1
MDT3
STR8
STR3
STR1
GW11
BRP15
BRP12
MDT2
GW12
BRP35
GW14
FES2
BRP34
SK61
CR162
SK64
SK63
KM165
B052
B051
WH165
MTH4
MTH3
WH162
WH163
SK53
BRP62
SK33
BRP55
BRP54
BRP53
BRP51
BRP52
SK45
SK41
SK35
SK13
SK12
SK14
SK15
BRP43
BRP41
BRP44
BRP45
SK32
SK51
SK31
SK55
SK62
SK44
SK42
BRP14
BRP13
SK52
SK22
SK21
SK24
SK25
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Jaccard's Genetic Distance



◀**Fig. 2** Structure analysis of 91 *D. spicata* (L.) Beauv. genotypes based on 10 simple sequence repeat (SSR) markers. **A** Separation of *D. spicata* genotypes into two groups by Structure v2.3. **B**, **C** Further analysis of the two main groups yielded eight sub-groups represented by different colors. Posterior probability is represented in percentages. Genotypes marked with (*) exhibited choke from *Atkinsonella hypoxylon* upon flowering

and 72 °C for 45 s; and a final extension at 72 °C for 10 min. Products were analyzed on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using 1.0 µL of PCR product, 10 µL of formamide (Applied Biosystems), and 0.2 µL of GeneScan 500 LIZ Size Standard (Applied Biosystems). Amplified products were scored using GeneMarker version 2.6.3 (SoftGenetics, State College, PA, USA) based on the analysis parameters published by Riley et al. (2014).

Germplasm characterization

A set of 10 SSR primer pairs that amplified clear, easily distinguishable polymorphic peaks were selected for genetic diversity analysis among the 91 individual *D. spicata* genotypes. PCR amplification and ABI capillary electrophoresis conditions were the same as described for primer prescreening with the exception that the annealing temperature was based on clean amplification profiles (Table 3). Allele sizes and number of alleles per locus were determined with GeneMarker version 2.6.3. To avoid false negatives, primers that resulted in null alleles in some of the samples were tested at least twice. The polymorphism information content (PIC) for each SSR marker was calculated based on the formula published by Botstein et al. (1980). We used this parameter instead of the measure of heterozygosity (P_{het}), as P_{het} is utilized only for co-dominant markers (Cordeiro et al. 2003).

Data analysis

Due to polyploidy we did not make any assumptions about the genetic nature of examined alleles so each SSR allele was treated as dominant (Cordeiro et al. 2003). We have found 1–3 alleles amplified per plant for each SSR marker. The amplified allele data was converted to a binary matrix (presence/absence) for each allele and a genetic distance matrix was

Table 2 Summary statistics for genomic simple sequence repeat (SSR) mining of *D. spicata* (L.) Beauv. singlet sequences

Genomic-SSR mining	No.
Total number of sequences examined	66,553
Total size of examined sequences (bp)	37,563,475
Total number of identified SSR markers	3454
Mono-nucleotide SSRs	1584
Di-nucleotide SSRs	997
Tri-nucleotide SSRs	791
Tetra-nucleotide SSRs	57
Penta-nucleotide SSRs	18
Hexa-nucleotide SSRs	7
Number of SSR containing sequences[a]	3219
Number of sequences containing more than 1 SSR	215
Number of SSR markers present in compound formation	138

[a]Number of SSR containing sequences: these are the singlet sequences containing various types of SSRs

generated based on Jaccard's similarity index implemented in the 'pvclust' package version 1.3-2 of R software (Suzuki and Shimodaira 2015). The Jaccard's distance was chosen since it does not use the absence of an allele as a shared characteristic (Legendre and Legendre 1998). Accessions were then clustered by using the unweighted pair group method with arithmetic averaging (UPGMA) algorithm implemented in 'pvclust.' The UPGMA algorithm was chosen because in a phenetic analysis, it makes no assumptions about evolutionary rate or phylogenetic relationships. Finally, the confidence levels for branches of the dendrogram were determined by calculating "approximately unbiased" (AU) *p* values using multiscale bootstrap resampling based on 20,000 replications implemented in the 'pvclust' package version 1.3-2 of R software. All bootstrap values are shown in the Fig. 1. A hierarchical Bayesian method (structure, Pritchard et al. 2000) was also tested to determine higher-level groupings. Following Evanno et al. (2005), Structure v2.3 was run with a range of groups (one to twelve) and results for the most parsimonious number of groups were examined in detail. Following the first split, this methodology was repeated on each subset, and the probabilities of individuals belonging to particular groups were examined. Analysis of molecular variance (AMOVA) was performed in GenAlEx software (version 6.502; Peakall and Smouse 2012) to perform population pairwise comparisons and calculate the significance of variation among and within populations. We considered *D. spicata* genotypes collected at each of the 23 locations as a population. Finally, multilocus genotype analysis was also performed in GenAlex to identify unique combination of alleles across two or more loci.

Results

Sequencing results and microsatellite discovery

Roche-454 sequencing produced 66,553 singlet sequences (approximately 37.5 Mbp). These sequences were examined with the MISA algorithm, and 3454 SSR markers were identified, including 1584 mono-, 997 di-, 791 tri-, 57 tetra-, 18 penta-, and seven hexa-nucleotide repeats (Table 2). Number of SSR containing sequences were 3219; while 138 SSR markers were present in compound formation, indicating multiple SSR markers were found in certain sequences (Table 2). Out of the 63 SSR primer pairs designed with Primer-BLAST, 10 proved to be polymorphic and resulted in expected amplification profiles. From the remaining 53, 21 amplified multiple regions or an unexpected size product, 12 failed to amplify, 11 primer pairs amplified monomorphic loci, and 9 amplified stutter peaks. We spot sequenced some of the amplified products and they aligned with the original Roche 454 sequence reads (data not shown).

Germplasm characterization

The 10 SSR markers showing clear amplification products and a high degree of polymorphism were

Table 3 Characteristics of the 10 genomic-SSR loci used in the analysis of 91 *D. spicata* (L.) Beauv. genotypes

Locus	Forward primer[a]	Reverse primer	Repeat motif	Ta (°C)	Allele size range (bp)[b]	TA	PIC
GRYJBMV02JWIUR	5′M13-TCTCGATGTAATGCGCTGGA	AATCTTCAGCCTCCCAAGCT	GTG	62	159–196	7	0.70
GRYJBMV02HM1E6	5′M13-ACGTACCAGGAAATTCACTGC	TTTCATGGCGGCAGATCAAC	TAG	62	185–197	5	0.61
GRYJBMV02GSBOQ	5′M13-ATCACCTCCGTCATCACTGG	CTATGGCTGCTTGGTATGCG	CAT	62	219–246	5	0.64
GRYJBMV02JD637	5′M13-ACCGTTCATCAGTCCAGAGC	CTGGACCTCAGATACGGCCT	GCC	62	118–121	2	0.37
GRYJBMV02IEVI0	5′M13-GTCGGGTGGGAAGAAACGAA	GGCATGCATGTTGCAGATGT	GCC	62	204–217	4	0.60
GRYJBMV01B481U	5′M13-GCTCTTGTCCTTTGGGGGAG	CGAGTACGCAACAGCACAAA	AAG	62	82–126	12	0.81
GRYJBMV02IEHKN	5′M13-GGGAATATGACCATTGTGGCA	AGCCTACCCCAACTTGCTTG	AAC	62	71–92	7	0.72
GRYJBMV02I1FTJ	5′M13-TTGCTCAGTAGCAGCCCTTT	ACTTGCTTGGGACAAAACGC	AAC	62	94–112	7	0.77
GRYJBMV02GSDBB	5′M13-ACCAGACCAACCGAAACCAG	GACGGTCACGCACTTGATGA	AGC	62	116–128	3	0.41
GRYJBMV01CLVIA	5′M13-TTGCTTTCTGCACTTTACCAGG	CCATTGGTTCTTCGTCCACAT	GGA	62	113–116	2	0.22

TA total number of alleles, *PIC* polymorphism information content

[a]The forward primers had an additional M13 (–21) universal sequence (TGTAAAACGACGGCCAGT) attached to the 5′ end to allow indirect fluorescent labeling of PCR products using just one universal FAM (6-carboxy-fluorescine)–labeled M13 primer

[b]Allele size ranges for each locus were obtained by deleting 18-bp M13 (–21) universal sequence from the size observed on the samples

Table 4 Analysis of molecular variance (AMOVA) showing the partitioning of genetic variation within and between the populations of *D. spicata* (L.) Beauv. genotypes

Source of variation	Df	SS	MS	Est. var.	%	PhiPT	p^a
Among populations	22	371.175	16.872	3.304	46	0.465*	> 0.001
Within populations	68	258.583	3.803	3.803	54	0.541*	> 0.001
Total	90	629.758		7.107	100		

df degree of freedom; *SS* sum of squares; *MS* mean squares; *Est. var.* estimate of variance; *%* percentage of total variation

*Not significant at the 0.001 probability level

[a]*p* value is based on 999 permutations

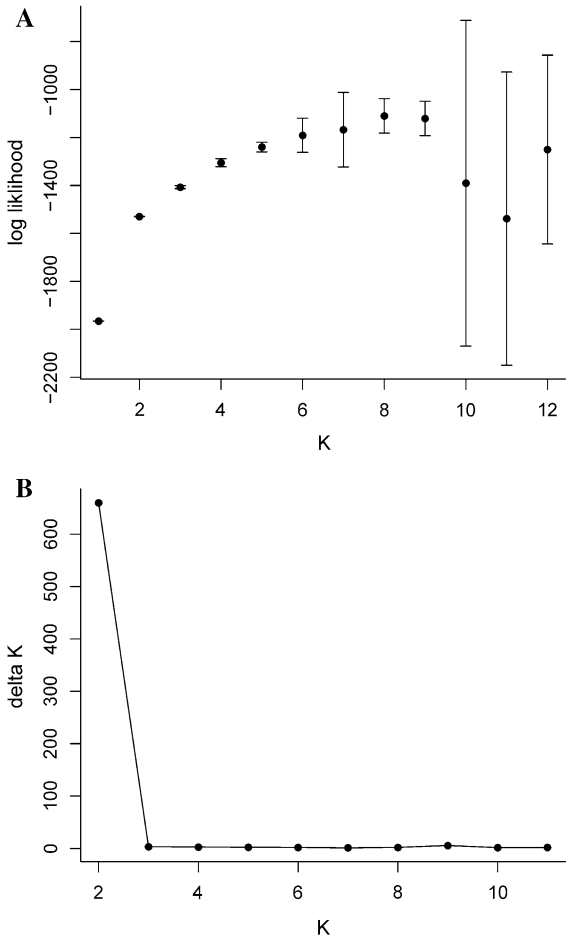


Fig. 3 Description of the two steps for the graphical method allowing detection of the true number of groups K^*. **A** Absolute values of the second order rate of change of the likelihood distribution (mean ± SD) calculated according to the formula: $|L''(K)| = |L'(K+1) - L'(K)|$. **B** ΔK calculated as $\Delta K = m|L''(K)|/s[L(K)]$. The modal value of this distribution is the true $K(*)$ or the uppermost level of structure, here two clusters

selected for further analysis on the germplasm collection. They amplified 54 alleles ranging in size from 71 to 246 bp (Table 3). Minimum and maximum number of alleles per locus were two and 12, respectively, with an average of 5.4 (Table 3). PIC values (Table 3), ranged between 0.22 and 0.81 indicating the usefulness of these markers in determining polymorphisms among the *D. spicata* genotypes.

Genetic similarity and population structure

Analysis of Molecular Variance (AMOVA) based on PhiPT values indicated that genetic diversity was apportioned among (46%) and within populations (54%), and the estimate of variance was 3.304 and 3.803, respectively (Table 4). In addition, multilocus genotype analysis identified 14 multilocus genotype groups (Fig. 1). A dendrogram was generated using the Jaccard's genetic distance and two major groups were identified supported by 96 and 94% bootstrap, respectively (Fig. 1). Group 1 contained a majority of BRP (Blue Ridge Parkway) and SK (Skyline Drive) plants; while Group 2 contained the plants collected from the U.S. National Arboretum, Washington D.C. (EGMDT, EGMTH, EGSTR, EGMTH etc.), Klees Mill Road, Carroll County, MD (KM), Crossroads Cemetery, Carroll County, MD (CR), White House, Beltsville, MD (WH), Beltsville Agricultural Research Center, Beltsville, MD (B05), and George Washington and Jefferson National Forest Location, Hollins, VA (GW) (Fig. 1). Structure analysis of the 91 *D. spicata* genotypes using a model-based approach (Pritchard et al. 2000) provided evidence of population structure. Utilizing the ΔK method (Evanno et al. 2005), it was determined that $K = 2$ best described the data (Figs. 2A, 3). These groups

matched well with the UPGMA dendrogram. Further analysis of the two main groups yielded eight subgroups represented by different colors (Fig. 2B, C).

Discussion

Danthonia spicata is a native grass species often found growing in low-input turf areas on the east coast of the U.S. Much of the research concerning the reproductive biology of *D. spicata* was conducted over 30 years ago. Modern molecular techniques provide an opportunity to greatly improve the current understanding of *D. spicata*'s unusual reproductive biology. *D. spicata* exhibits both axial fully cleistogamous seed heads and terminal flower heads that rarely exhibit any signs of allogamy. Both axial and terminal flower heads have very small underdeveloped anthers that produce no detectable pollen yet seed set is near 100%. The literature relative to the reproductive biology of *D. spicata* is confusing and best summarized by Darbyshire and Cayouette (1989).

The most striking finding from this diversity assessment is that there are 14 multilocus genotype groups containing 39 genotypes. This is strong evidence in support of clonal reproduction in *D. spicata*. The likelihood of this number of identical genotypes existing at 10 genomic SSR loci with 54 putative alleles, through sexual reproduction is extremely small. The UPGMA dendrogram and Structure analysis were dominated by clonal groupings that exhibit variation possibly arising through mutation or migration rather than crossing and selection. Two alleles were found in only a single plant in the study and in another case an allele was found only in all four samples from a single location that is suggestive of mutations arising and then being spread through clonal reproduction. Overall it was difficult to identify polymorphic loci in *D. spicata*; efforts to identify variation through gel based assays were ineffective and only by focusing on tri-nucleotide SSR markers with the highest numbers of repeats and using sequencer based scoring was it possible to identify reliable polymorphism. A lack of polymorphism in *D. spicata* was also reported for isozyme markers by Clay and Antonovics (1985a). These findings do not support previously reported outcrossing levels based on heritability estimates of morphological variation by Clay and Antonovics (1985b). Our data and observations of anther development also support asexual reproduction through seed. Other forms of asexual reproduction are unlikely as *D. spicata* does not form rhizomes or stolons and is well adapted to spread through seed dispersal mechanisms. These findings are in support of the suggestion by Philipson and Christey (1985) that apomixis should be considered as a reproductive mechanism in *D. spicata* and are not supportive of the suggestion by Cheplick and Clay (1989) that *D. spicata* is an example of a species with both outcrossing and obligate self-fertility operating at the same time. Weatherwax (1928) documented that *D. spicata* does not exhibit large extruded anthers or have lodicule characteristics that would be indicative of successful outcrossing. These observations were further documented by Philipson (1986) and are also supported by personal observations of the plant material collected in this study. Anther development issues support the hypothesis that *D. spicata* may be an example of an autonomous apomict (Philipson and Connor 1984). Analysis of Molecular Variance (AMOVA) of the data exhibited an approximately equal division of variation within and between populations. These results do not support a predominantly selfing or outcrossing reproductive strategy; as selfing species generally maintain diversity between populations while outcrossing species maintain variation within populations.

The fungal epiphyte *Atkinsonella hypoxylon* and its association with *D. spicata* has been reported numerous times with the implication that this association may have been an evolutionary force leading to the development of axial cleistogomous seed heads (Clay 1984). Plants infected with *A. hypoxylon* generally exhibit a fungal sclerotium, or "choke," that is generated at the initiation of host plant flowering and results in abortion of all but a few infected cleistogamous seeds at the base of much-reduced reproductive tiller (McCormick et al. 2001). However, only 7 out of the 91 samples in this study exhibited choke from *A. hypoxylon* upon flowering, and this level of association would not suggest that "choke" is a strong evolutionary force in *D. spicata*.

Conclusions

The SSR markers in this report are the first polymorphic nuclear markers available for *D. spicata* and

suggest that asexual reproduction mechanisms be further explored through progeny testing.

Acknowledgements Research presented in this article was supported in part by funds from the Floral and Nursery Research Initiative administered through the United States Department of Agriculture, Agricultural Research Service (USDA-ARS). The mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendations or endorsement by the USDA.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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