Research Article

Advances towards a Marker-Assisted Selection Breeding Program in Prairie Cordgrass, a Biomass Crop

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1. Introduction

Recent world issues associated with fuel consumption and supply have turned attention towards biofuel production, especially cellulosic biofuel. Perennial grasses provide an optimal source of cellulosic biomass due to their high yield potential. Prairie cordgrass (Spartina pectinata Bosc ex Link) is an indigenous, perennial grass of North America and can be found as a native from Texas to near the Arctic Circle [1]. Ongoing studies on prairie cordgrass in comparison with switchgrass (Panicum virgatum L.) indicate that prairie cordgrass could produce more biomass than switchgrass [2]. Furthermore, results from the comparison of prairie cordgrass and switchgrass performed by Boe and Lee in 2007 [2] indicated that prairie cordgrass has a wider environmental amplitude and is adapted to poorly drained wet areas which can have high salinity and be poorly aerated, regions not suitable for the production of conventional crops such as maize (Zea mays) [2, 3]. These results are indicative of the potential of prairie cordgrass as a source of biomass for cellulosic biofuel production. A research program at South Dakota State University (SDSU) is underway to develop native prairie cordgrass into a viable cellulosic biomass crop. The development of a new crop species requires a multidisciplinary approach; examining and validating each step before commercialization can occur. These steps include, but are not limited to, the assembling of a germplasm collection, the development of an accelerated breeding program, and the optimization of cultivation practices. A fundamental step to accelerate the breeding of prairie cordgrass is to determine and optimize a crossing protocol. Prairie cordgrass has been intrinsically believed to be a protogynous outcrossing species, based on the mode of reproduction of its maritime relative smooth cordgrass (Spartina alterniflora) [4, 5]. From work performed upon other members of the Spartina genus, it has been conjectured that prairie cordgrass will have a similar method of reproduction. In the majority of other Graminaceae species, breeding is performed via the initial emasculation of the floret to ensure that only cross
pollination can occur. Prairie cordgrass has an inflorescence composed of between 0 and 31 short paracladia and 11–13 long paracladia [6], bearing a total of 10–80 fertile spikelets, of single florets [7]. Physical emasculation of prairie cordgrass is essentially impractical, but this technique may not be necessary as with protogyny and ascertaining the appropriate timing, directed cross pollination is feasible. Self pollination has not been identified previously in prairie cordgrass and assumptions have been made that prairie cordgrass may be self incompatible, if this is the case then the classic mapping technique of producing recombinant inbred lines will be fundamentally impossible and alternate strategies will need to be utilized.

An initial stage of the prairie cordgrass project is to develop a molecular map of the species. Only limited molecular analyses have been performed upon prairie cordgrass, notably in contrast to its maritime relative S. alterniflora. In the National Centre for Biotechnology Information (NCBI), only 57 prairie cordgrass sequences have been deposited (predominantly regions of the nuclear and organelle genomes utilized for diversity analysis, that is, Waxy (AY508655 and AF372461), ITS (AF019843, AJ489796, and EF153082) and the chloroplast trnL-trnF intergenic spacer region (EF137568, EU056305, and AF372625)). A recent publication on the analysis of the transcriptome of prairie cordgrass has increased the available knowledge on the genome of the species [8]. Constructed from the analysis of the expression sequence tags (ESTs) and identified from the transcriptome of prairie cordgrass, a total of 26,302 contigs and 71,103 singletons were assembled with all sequence information available as supplemental data [8]. Additional molecular analysis of genetic diversity of prairie cordgrass in natural populations in Minnesota with amplified fragment length polymorphism (AFLP) has been performed [9].

An optimal marker system for the development of a molecular map of prairie cordgrass are SSRs or microsatellites. Microsatellites are highly utilized molecular markers and have been developed for the majority of agriculturally and economically important crop species [10]; their efficacy arises predominantly due to their reproductivity, codominant inheritance, and abundance [11]. Modern techniques allow the rapid identification of microsatellite regions in species which have limited sequence information, and by using proprietary genomic library screening techniques, microsatellites have been developed for numerous non-model organisms, encompassing insects [12], birds [13], fish [14], mammals [15], and plants, including other species in the Spartina genera [16]. Previous studies on other members of the Spartina genera have developed 35 markers for microsatellite loci in S. alterniflora [16, 17]. Of these S. alterniflora markers, three have been found to amplify in prairie cordgrass [16]. SSRs have been used extensively in numerous crop species, in wheat (Triticum aestivum L.), and another Poaceae species; SSRs have been used to enhance molecular maps begun with restriction fragment length polymorphism (RFLP) and to identify genes of interest [18]. Furthermore, Wilde et al. in 2007 [19] found that the incorporation of MAS with SSR markers into a traditional breeding program can result in a substantial increase in the incorporation of Fusarium head blight resistance, a quantitative trait, in wheat with the shortest possible time. The results of Wilde et al. [19] are indicative of the potential of MAS for the breeding of prairie cordgrass.

This paper details the first investigation into initial breeding and crossing of prairie cordgrass, specifically the production of F1 individuals and the validation of a crossing protocol. Furthermore, the characterization of 35 microsatellite loci in prairie cordgrass from genomic DNA is discussed. The validation of the microsatellite loci occurred in a prairie cordgrass germplasm collection and a reciprocal cross as an initial step in the development of a molecular map, which can be further utilized during marker-assisted selection of lines with traits desirable for the improvement of prairie cordgrass as a biofuel crop.

2. Materials and Methods

2.1. Genomic Library Construction. Genomic DNA was extracted from four lines of prairie cordgrass using the method as described by Karakousis and Langridge in 2003 [20], with minor modifications. A mixture of the four random prairie cordgrass clones DNA, totaling >100 µg, was sent to Genetic Identification Services (GIS) (http://www.genetic-id-services.com/) for the development of genomic libraries. The genomic libraries were enriched for the four simple sequence repeat (SSR) motifs (CA)n, (GA)n, (AAG)n, and (CAG)n. Subsamples of 100 clones were sequenced and primers were designed to flank the SSR regions using DesignerPCR, version 1.03 (Research Genetics, Inc.) and PRIMER 3 [21]. The SSR regions were classified according to Jones et al. [22] as being pure repeats (i.e., [N1N2]n), compound repeats (i.e., [N1N2]n[N3N4]n), and interrupted repeats (i.e., [N1N2]nX[N3N5]n).

2.2. Plant Material. Wild germplasm has been collected from throughout the mid-western states of the United States, creating a core germplasm collection to be utilized in prairie cordgrass breeding (unpublished data). A geographically diverse sample of sixteen plants, collected from South Dakota, North Dakota, Minnesota, and Iowa, was characterized using the identified SSR loci. A sample from the closely related species S. spartinae was also included to examine cross species amplification. The germplasm collection was grown in standard greenhouse conditions.

Crosses between and amongst these genotypes were performed. Crossing between two plants of prairie cordgrass was performed in the following manner; two inflorescence at the appropriate stage of development were placed inside a crossing bag for seven days, producing F1 plants (Figure 1). A reciprocal cross between two genotypes (designated RR2 and RR21) from the Red River morphotype was produced. The reciprocal cross produced 45 F1 plants from the RR21 × RR2 directed cross and 49 F1 plants from the RR2 × RR21 directed cross, a total of 94 plants. Two genotypes identified as clones (designated SP1.1B and SP1.2A) from the SSR evaluation were crossed via the same technique, producing a total of
2.3. DNA Extraction and Evaluation of SSR Primers. DNA was extracted from all examined plants using the method as described by Karakousis and Langridge in 2003 [20], with minor modifications. Evaluation of the primers was performed in a PCR reaction consisting of 2 U Taq DNA polymerase (Promega GoTaq), 1 × PCR Buffer (Promega GoTaq), 2 mM MgCl₂, 0.6 mM dNTPs, and 0.6 mM of each primer, with a final volume of 20 µL [23]. PCR reactions were performed in a BioRad MyCycler (BioRad, Hercules, CA). Initially a gradient of annealing temperatures was used to determine optimal $T_a$, as these primers were designed to be utilized in a prairie cordgrass breeding program, a robust and high-throughput protocol was developed. Specific thermocycling conditions for the primers used in this study were as follows: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 53 or 55°C for 1 minute, 72°C for 1 minute, followed by an extension step of 72°C for 10 minutes, and a 10°C hold. PCR product was visualized on 8% nondenaturing PAGE; bands were scored and sized by comparison to a 100 bp ladder using AlphaEaseFC Software (Alpha Innotech, San Leandro, CA).

2.4. SSR Markers Analysis. Polymorphic bands in the two examined populations described above were scored on a presence (1) or absence (0) basis. An estimation of genetic distance was calculated with Nei and Li’s algorithm [24] and the resulting matrix was clustered with unweighted pair group method with arithmetic mean (UPGMA) [25]. Analysis was performed using multivariate statistics package (MVSP) [26].

3. Results

3.1. Germplasm and Crossing. The crossing of the germplasm collection was successful with the production of a total of 14,813 putatively viable seeds from 110 crosses, performed in lines and collected from 48 distinct collection locations (Table 1). Numerous nonviable seeds were produced from each cross. A seed was determined to be viable if the actual seed (endosperm and embryo) was visible within the glumes (Figure 2). The number of viable seeds produced per head varied from 6 to 642, with an average of 134 seeds per cross. The crosses can be grouped into two, dependent upon the parents, those that were outcrossed and those that were selfed. Crosses that were designated “out” were between genotypes that were geographically diverse, while crosses designated “self” were produced from members of the population collected at the same location. The average number of seeds produced from outcrossed individuals was 143, with a range of 12 to 250, while selfed individuals produced an average of 112 seeds per cross, with a range of 6 to 642 (Figure 3).

3.2. Microsatellite Characterization. Of the total 100 genomic clones sequenced, 70 contained microsatellite motifs, 26 from the (CA)$_n$ enriched library, 25 from the (GA)$_n$ enriched library, 3 from the (AAG)$_n$ enriched library, and 16 from the (CAG)$_n$ enriched library. All 70 loci were examined and 35 were amplified with the standardized conditions (Table 2). The repeat SSR structure that occurred most frequently was the pure repeat (27), followed by the compound repeat (7) and only one interrupted repeat sequence was found (Table 2).

Only two primers produced monomorphic profiles from the analysis of the sixteen lines, SP5D004 from the (GA)$_n$ library and SP5D048 from the (AAG)$_n$ library. The remaining primers produced between 1 and 12 scorable bands. Of the examined primers, 11 were amplified between 1 and 7 bands in $S$. $s$artinae (Table 2).

All sequences were examined using BLASTn [27] on the National Center for Biotechnology Information (NCBI) server for similarities to other recorded sequences. Only two of the prairie cordgrass sequences displayed homology ($e < e^{-20}$) to recorded sequences, SP5D003 to six sequences from rice, wheat, and field mustard ($B$. $r$ap$\alpha$) (accession numbers: AP008214.1, AP005495.3, NM_001067901.1, AK111788.1, EU660901.1, and AC189183.2) and SP5D050 to five sequences all from rice (accession numbers: CR855236.1, AP008210.1, AL606607.3, NM_001059819.1, and AK108706.1) [25].

3.3. Crossing Population. A total of 94 individuals from the RR2 × RR21 and the RR21 × RR2 crosses were examined with 35 SSR primers. The amplified bands were scored based on presence or absence, the resulting data was analyzed with Nei and Li’s coefficient producing a similarity matrix (data not shown), and the data was clustered with UPGMA producing a dendogram (Figure 4). The results of the dendogram indicate that each individual was a cross

**Figure 1:** Two prairie cordgrass heads undergoing crossing, contained within a single bag.
Table 1: Summary of crosses performed amongst the core prairie cordgrass germplasm collection. The type of cross is designated “Out” for crosses between geographically diverse members and “Self” for members of the population collected at the same location.

| Female parent | Male parent | Number of crosses | Type | Total no. of seeds |
|---------------|-------------|------------------|------|-------------------|
| SP1 SP21      | 2           | Out              | 126  |
| SP1 SP71      | 1           | Out              | 71   |
| SP21 SP30     | 1           | Out              | 51   |
| SP21 SP31     | 6           | Out              | 484  |
| SP21 SP4      | 2           | Out              | 248  |
| SP21 SP42     | 1           | Out              | 78   |
| SP21 SP66     | 2           | Out              | 233  |
| SP21 SP7      | 1           | Out              | 82   |
| SP24 SP30     | 1           | Out              | 180  |
| SP29 SP22     | 1           | Out              | 146  |
| SP29 SP30     | 1           | Out              | 42   |
| SP3 SP77      | 1           | Out              | 282  |
| SP30 SP31     | 1           | Out              | 164  |
| SP30 SP52     | 1           | Out              | 246  |
| SP31 SP21     | 4           | Out              | 408  |
| SP31 SP30     | 1           | Out              | 28   |
| SP31 SP32     | 1           | Out              | 38   |
| SP31 SP46     | 3           | Out              | 211  |
| SP31 SP5      | 1           | Out              | 81   |
| SP31 SP66     | 1           | Out              | 46   |
| SP31 SP79     | 1           | Out              | 198  |
| SP32 SP21     | 1           | Out              | 173  |
| SP32 SP31     | 2           | Out              | 310  |
| SP32 SP45     | 1           | Out              | 296  |
| SP32 SP66     | 1           | Out              | 240  |
| SP32 SP67     | 1           | Out              | 193  |
| SP32 SP78     | 1           | Out              | 94   |
| SP35 SP78     | 1           | Out              | 279  |
| SP41 SP42     | 1           | Out              | 6    |
| SP41 SP45     | 1           | Out              | 86   |
| SP44 SP54     | 1           | Out              | 312  |
| SP44 SP59     | 1           | Out              | 274  |
| SP45 SP31     | 1           | Out              | 282  |
| SP45 SP49     | 1           | Out              | 225  |
| SP45 SP67     | 1           | Out              | 138  |
| SP49 SP40     | 1           | Out              | 203  |
| SP49 SP45     | 1           | Out              | 176  |
| SP49 SP66     | 2           | Out              | 237  |
| SP5 SP31      | 1           | Out              | 189  |
| SP5 SP78      | 1           | Out              | 407  |
| SP53 SP67     | 2           | Out              | 43   |
| SP54 SP44     | 1           | Out              | 145  |
| SP54 SP59     | 2           | Out              | 81   |
| SP56 SP58     | 1           | Out              | 235  |
| SP65 SP57     | 1           | Out              | 70   |
| SP66 SP31     | 1           | Out              | 93   |
| SP67 SP32     | 1           | Out              | 99   |

between the two parents. When the population derived from the cross between the two plants (SP1.1B and SP1.2A) and determined to be clones was examined, bands present in both parents were found to segregate in the progeny (data not shown), indicative of sexual recombination; this result provides validation that successful crossing occurred.

4. Discussion

The primary requirement of any breeding program is to ensure that accurate crosses are made; in many other members of the Poaceae this is achieved by physical or chemical emasculation. The prolific numbers of flowers per head in addition to the small size of the flowers make physical emasculation unfeasible. Furthermore, due to limited knowledge about the nature of the fertility of this species, chemical emasculation has not been developed for prairie cordgrass. The results from the SSR analysis indicate that utilizing the inherent protogyny of prairie cordgrass allows successful crossing between two individuals without the need for emasculation, confirming the validity of the breeding methodology used. The presence of individuals in the F1 mapping populations which show limited genetic dissimilarity from the parents could be evidence of selfing; further investigations are required. The presence of these potential selfed individuals indicates that future breeding and/or mapping populations should be examined with the molecular markers devised in this research to remove suspect individuals. Subsequently, the successful crossing between two clones is indicative that prairie cordgrass may not be self-incompatible and that it may be possible to develop in this species conventional mapping populations, such as recombinant inbred lines. Further studies into self-compatibility
with investigations into potential apomictic prairie cordgrass plants are underway. The number of seeds observed in this research appears to be larger than what was previously described by Clayton et al. [7]. The variation between the two studies can be attributed to both environmental and genetic variations. Genetic variation in seed set in prairie cordgrass, although at this stage not quantified, is demonstrated by the range in viable seed set observed in this research.

The amplification of S. spartinae with the SSR primers developed in this research are indicative of the potential colinearity amongst the genomes of Spartina spp. and other grass species; this colinearity will allow easier identification and characterization of genes. The colinearity between prairie cordgrass and other Poaceae is currently utilized to examine genes identified in related species in prairie cordgrass, specifically genes utilized to examine phylogeny (i.e., the waxy gene for granule bound starch synthase).

The prevalence and distribution of SSR regions across plant genomes are extremely variable. Variation in SSRs is not limited to their location, but also their motif, putative function, abundance, and repeat number [28]. The results of this analysis indicate that the two dinucleotide repeats (CA)$_n$ and (GA)$_n$ are more prevalent in prairie cordgrass (31% and 46%, resp.), than the two trinucleotide repeats (AAG)$_n$ and (CAG)$_n$ (6% and 17%, resp.). The prevalence of the dinucleotide motif in prairie cordgrass is similar to what was observed in the characterization of SSRs in other Spartina sp., where Blum et al. [16] found 82% of isolated SSRs contained dinucleotide repeats and Sloop et al. [17] found 71% containing similar motifs. In all three

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| Locus  | Library | Primer sequence (5’3’) | SSR motif | Expected size (bp) | Size range (bp) | T<sub>a</sub> (°C) | GenBank ID |
|--------|---------|------------------------|-----------|--------------------|-----------------|-----------------|------------|
| SPSD001| (CA)<sub>n</sub> | F: GCTGCTCCTCTCTCTCTGCT<br>R: ACGCCACACTTGGTTTTTGG | (CA)<sub>13</sub> | Perfect | 180 | 212–386 | 55 | GQ354531 |
| SPSD002| (CA)<sub>n</sub> | F: GCACCTTTTGGTGATGCTC<br>R: CTGACGCAAGGTGTATGAG | (CA)<sub>13</sub> | Perfect | 249 | 100–655 | 55 | GQ354532 |
| SPSD009| (CA)<sub>n</sub> | F: ATGGTTCTCAAAGTGGCAAGT<br>R: CAGGGCTGCCTACAGTATG | (GT)<sub>15</sub> | Perfect | 166 | 215–432 | 55 | GQ354533 |
| SPSD010| (CA)<sub>n</sub> | F: AACAAAAAGATAGGACCCCTA<br>R: ACGAAATATGTGACCGATAC | (CA)<sub>28</sub> | Perfect | 168 | 127–310 | 53 | GQ354534 |
| SPSD011| (CA)<sub>n</sub> | F: CTAAACGTATGCTGTTCTG<br>R: AAGGGCGTTTTAAGGGCTAAG | (GT)<sub>11</sub> | Perfect | 135 | 147–251 | 53 | GQ354535 |
| SPSD013| (CA)<sub>n</sub> | F: GGATGCTTTGTAGATAGATA<br>R: TCTTCCTCTTACTCTGTCAC | (CA)<sub>41</sub> | Perfect | 157 | 85–173 | 55 | GQ354536 |
| SPSD016| (CA)<sub>n</sub> | F: CTCGTTCTCCTTCTTCTCCGTC<br>R: ACCCTTTTTCTTTTGGTCTC | (CA)<sub>16</sub>(GA)<sub>38</sub> | Compound | 134 | 220–365 | 55 | GQ354538 |
| SPSD019| (CA)<sub>n</sub> | F: ATGAGACGATAGCAGGATGAC<br>R: ACGGAGTTAGCTTAGATGG | (CA)<sub>23</sub> | Perfect | 178 | 146–288 | 55 | GQ354539 |
| SPSD020| (CA)<sub>n</sub> | F: CAGTCCATGCAACTCAAGATA<br>R: AACCTGATAGAAGTGGTCATGC | (CT)<sub>16</sub>(CA)<sub>38</sub> | Compound | 269 | 204–748 | 55 | GQ354540 |
| SPSD025| (CA)<sub>n</sub> | F: GTGGAAATCAACAACACACAG<br>R: GTGCGTATGACCCCTAAAG | (GA)<sub>13</sub>(GT)<sub>20</sub> | Compound | 209 | 196–539 | 55 | GQ354541 |
| SPSD026| (CA)<sub>n</sub> | F: ATGGAAACTGTCTGGAACTGAC<br>R: AGCAATAACCACAGAAGACC | (CT)<sub>28</sub> | Perfect | 294 | 231–263 | 55 | GQ354542 |
| SPSD027| (GA)<sub>n</sub> | F: TCAAAACATGGCCGGAGAAG<br>R: CTGGCTCGACCCTTCTTGG | (CT)<sub>17</sub> | Perfect | 214 | 188–224 | 55 | GQ354543 |
| SPSD004| (GA)<sub>n</sub> | F: GGTGGCTGTTGCAATG<br>R: GGCACACAAAGATGAC | (GA)<sub>22</sub> | Perfect | 178 | 133 & 164 | 55 | GQ354544 |
| SPSD031| (GA)<sub>n</sub> | F: TCGCACATTTTATCTTCTTAC<br>R: TGGATGGATTAGTATTCTTGT | (CT)<sub>20</sub> | Perfect | 188 | 155–346 | 55 | GQ354545 |
| SPSD032| (GA)<sub>n</sub> | F: CTTCTGCCCCATTTCTAC<br>R: GCATTGCTGTGTGGTGGAGC | (CT)<sub>13</sub> | Perfect | 196 | 146–193 | 53 | GQ354546 |
| SPSD034| (GA)<sub>n</sub> | F: CAGGTCTAGGGAGTTCTACT<br>R: TCAAAAGAGACACATACACA | (CT)<sub>12</sub> | Perfect | 160 | 144–306 | 53 | GQ354564 |
| SPSD036| (GA)<sub>n</sub> | F: TTCAACACACACTTTATCC<br>R: GGAAGCAACAAAAACATGATG | (CT)<sub>29</sub> | Perfect | 271 | 222–260 | 53 | GQ354547 |
| SPSD039| (GA)<sub>n</sub> | F: CTTTCCAGTAGCTCCACTGATC<br>R: AGCAATAACTGTGGCATATACCTTCT | (GA)<sub>12</sub> | Perfect | 206 | 193–448 | 55 | GQ354548 |
| SPSD040| (GA)<sub>n</sub> | F: AATCGAAATGCAGACGCCAAC<br>R: CATGGCTTTTTTCTACTATGAG | (GA)<sub>11</sub> | Perfect | 214 | 190–416 | 55 | GQ354549 |
| SPSD041| (GA)<sub>n</sub> | F: CCAACAGATGTTTCTCTTCTCT<br>R: TCCAGGAACACGGATGGTCCT | (GA)<sub>26</sub> | Perfect | 135 | 104–180 | 53 | GQ354550 |
| SPSD042| (GA)<sub>n</sub> | F: ACCTCCCAGCTGTTCTG<br>R: GCATTGCTGTGTGGTGG | (CT)<sub>19</sub> | Perfect | 211 | 131–257 | 53 | GQ354551 |
| SPSD043| (GA)<sub>n</sub> | F: GTTCATTGTCAGACAAGACTCAG<br>R: ATTCGATCTCAGACTAGAAC | (GA)<sub>30</sub> | Perfect | 277 | 231–252 | 53 | GQ354552 |
studies the di- and trinucleotide repeats occurred as perfect, compound, and interrupted motifs. Based on the results found in this research, the genomic libraries enriched with the dinucleotide repeats have been extensively sequenced; the resulting sequence information will be screened to isolate additional SSR regions, primers will be designed, and the resulting markers will be used to develop a molecular map of prairie cordgrass. The molecular map will then be used to find linkage between SSR markers and traits of interest allowing future MAS to be performed.

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