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Development and Characterization of SSR Markers in Proso Millet Based on Switchgrass Genomics

Santosh G. Rajput, Tammy Plyler-Harveson, Dipak K. Santra*

Panhandle Research and Extension Center, University of Nebraska-Lincoln, Scottsbluff, USA.
Email: *dsantra2@unl.edu

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ABSTRACT

Proso millet (*Panicum miliaceum*) has high water use efficiency (WUE), a short growing-season, and is highly adapted to a semi-arid climate. Genomic resources for proso millet are very limited. Large numbers of DNA markers and other genomic tools in proso millet can readily be developed by using genomic resources in related grasses. The objectives of the present report were to 1) test and characterize switchgrass SSR markers for use in proso millet, and 2) elucidate repeat-motifs in proso millet based on new SSR marker analysis. A total of 548 SSR markers were tested on 8 proso millet genotypes. Out of these, 339 amplified SSR markers in proso millet. This showed that 62% of the switchgrass SSR markers were transferable to proso millet. Of these 339 markers, 254 were highly polymorphic among the 8 proso genotypes. The resolving power of these 254 polymorphic SSR markers ranged from 0.25 - 14.75 with an average of 2.71. The 254 polymorphic SSR markers amplified 984 alleles in the ranges of 50 bp to 1300 bp. The majority of the SSR markers (221 of 254) amplified dinucleotide repeats. Based on SSR marker analysis, AG/GA was the most abundant repeat-motifs in proso millet. Switchgrass genomic information seems to be the most useful for developing DNA markers in proso millet. Markers developed in this study will be helpful for linkage map construction, mapping agronomic traits and future molecular breeding efforts in proso millet.

KEYWORDS

Millet; Minor Crops; Comparative Genomics; Molecular Breeding

1. Introduction

Proso millet (*Panicum miliaceum* L.) is reported to have been domesticated about 10,000 years ago in central and Eastern Asia and made its way from China to the Black Sea region of Europe by 5000 BC [1]. German-Russian immigrants introduced proso millet to North America in 1875 [2]. Proso millet is known as common millet, millet, and hog millet in the United States of America (USA); broomcorn millet in China; common millet in Japan, Korea, and other Pacific Asian countries; "hersey" millet in Germany; and French white in France [3]. It is currently grown in Asia, Australia, North America, Europe, and Africa [3-5].

Proso millet is the most suitable rotational crop in majority of dryland wheat producing areas in semi-arid High Plains of the USA [6]. It is grown mostly in Colorado, Nebraska, and South Dakota and to a lesser extent in North Dakota, Montana, Wyoming, Kansas, Oklahoma, Texas, Minnesota, Wisconsin, Iowa, Michigan, North Carolina, Georgia, and Florida [7]. Proso millet is highly diversified, has excellent nutritional properties and could become an important crop for food diversification [8]. It is used primarily for human consumption in Asia. In the USA, it is grown primarily for birdseed and livestock [8,9].

A very limited number of DNA markers are reported in proso millet. A few RAPD and AFLP markers were reported in proso millet for genetic diversity analysis [10-12]. Among all PCR-based markers, microsatellites or simple sequence repeat (SSR) markers are the most

*Corresponding author.

informative and useful because they are co-dominant, multi-allelic, highly reproducible, abundant and are evenly distributed in the plant genome [13,14]. Hu *et al*. (2009) [15] identified 46 SSRs markers from rice, wheat, oat and barley for genetic diversity analysis in proso millet. Cho *et al*. (2010) [16] developed 25 SSR markers from proso millet BAC library. To date only about 100 SSR markers are available, which is inadequate for proso millet genetic and breeding research. But unfortunately, developing SSR markers is expensive and a time-consuming task for any crop [17,18]. Comparative genomics has great potential to speed up development of genomic tools in minor crops such as proso millet using genome resources of other major crops such as rice, maize, sorghum and foxtail millet [19]. SSR markers developed in related species were successfully used in sorghum, and bermudagrass [20,21].

Switchgrass, where enormous genomic resource is available, is taxonomically the closest species to proso millet [22]. A detailed genome map consisting of more than 2000 SSR markers is available in switchgrass [23-25]. Therefore, switchgrass genomic data are the most suitable for developing DNA markers for proso millet in a rapid and inexpensive way. The objectives of this report were to 1) test and characterize switchgrass SSR markers for using in proso millet, and 2) elucidate repeat-motifs in proso millet based on the new SSR marker analysis.

2. Materials and Methods

2.1. Plant Materials and SSR Markers

A set of 8 proso millet genotypes were used for testing efficacy of the switchgrass SSR markers. Plants were grown in 4-inch pots filled with Sunshine Mix 2 Basic Professional Growing Mix (Westco, Morill, NE, USA) in a greenhouse at the University of Nebraska, Panhandle Research and Extension Center, Scottsbluff. Leaf tissues of 21-day-old plants were used for DNA extraction.

A total of 548 SSR markers (540 genomic & 8 EST) of switchgrass were used [23,25] These SSR markers are evenly distributed throughout the switchgrass genome. Primers of these SSR markers were synthesized from MWG Biotech (Huntsville, AL) based on sequence reported by Wang *et al*. 2011 [25]. These 548 SSR markers were tested on 8 proso millet genotypes (Earlybird, Huntsman, Panhandle, Horizon, Plateau, PI436625, PI436626 and PI 463473) to determine potential polymorphic markers. Polymorphic SSR motif repeats were analyzed based on the data published by Wang *et al*. 2011 [25] and Okada *et al*. 2010 [24].

2.2. DNA Extraction and SSR Marker Analysis

Genomic DNA was isolated by micro prep CTAB DNA isolation method using about 100 mg leaf tissues [26]. DNA quality and quantity were checked on a 0.8% agarose gel using 0.5XTBE buffer and visualized under UV transillumination. Concentration of DNA in each sample was determined comparing intensity of bands of Lambda/HindIII DNA markers. PCR consisted of 10 ulreaction volume containing 1× reaction buffer, 1 U Taq polymerase, 200 uMdNTPs, 2 mM $MgCl_2$, 5 pmoles primers, 20 ng DNA. PCR cycle was: initial denaturation of 5 min at 94˚C; followed by 39 cycles of 45 s at 94˚C, 50 s at 55˚C, and 45 s at 72˚C and a final 10 min extension at 72˚C. PCR was done using BioRad S1000 Thermal cycler. PCR amplified products were separated in a 2% agarose gel using 0.5XTBE buffer and visualized under UV transillumination after staining with ethidium bromide @0.5 ug/ml. Markers (>50 bp) were scored manually using gel image and band size was determined considering 25 bp size difference between the bands. A SSR marker was considered polymorphic if two DNA bands of different sizes were observed at least in two genotypes. DNA bands of different sizes were considered different alleles and DNA bands of same size were considered as the same allele. Resolving power (Rp) of each SSR marker was calculated by following formula $Rp = \sum I_b$ and $I_b = 1 - [2 \times (0.5 - p)]$ where I_b is the allele informativeness and p is the proportion of accessions sharing the I allele [27]. The NCBI nucleotide database was searched for homology analysis with nucleotide sequences in other crops using primers sequences of the polymorphic SSR markers.

3. Results and Discussion

3.1. Switchgrass SSRs in Proso Millet

Of the 548 switchgrass SSR markers screened, 339 (62%) amplified and 209 markers (38%) did not amplify in proso millet. Of the 339 amplified markers, 254 (46%) were polymorphic. Many of these 254 markers were found to be highly polymorphic among the set of 8 proso millet genotypes as represented by one such SSR marker PVCA 21-22 (**Figure 1**). The high polymorphic nature of these markers was observed also from their high resolving power of each SSR marker, which ranged from 0.25 - 14.75 with an average 2.71 (**Figure 2**). A smallest resolving power of 0.25 was shown by six markers (PVCA 1431-32, PVCA 299-300, PVCA 985-86, PVCA 151-52, PVCA 415-16 and NFSG-246) while the highest resolving power of 14.75 was observed for PVCA 1387-88. As reported by Prevost and Wilkinson (1999) [27], higher resolving power is directly linked to the informativeness of the marker. Based on our data it seems that our SSR markers have moderate resolving power which can be useful to select the efficient marker set for future studies. The names of the 254 polymorphic SSR markers and their characteristics (allele sizes and Resolving pow-

er (Rp) values) are given in **Table 1**. We consider that the 339 SSR markers covered most of the proso millet genome since these markers were selected from most of the chromosomal regions of switchgrass. Lack of amplification of the proso millet genome by 38% of 548 switchgrass SSR markers could be due to insertion and/or deletion present in SSR motifs, or point mutations and/or deletions in flanking repeats [28].

Approximately 98% of the polymorphic markers were genomic SSR markers and only 2% were EST SSR markers. This is similar to other reports in proso millet and in switchgrass [16,25]. High percentages of transferability of switchgrass markers into proso millet supports the utilization of cross species SSR markers as a useful resource for developing markers in minor crops where very limited or no work has been done at the molecular level. Similar results were obtained in other crops such as sorghum, bermudagrass, napiergrass, finger millet and other grasses [20,21,29,30]. Such high level of transferability is probably because many genomic regions are

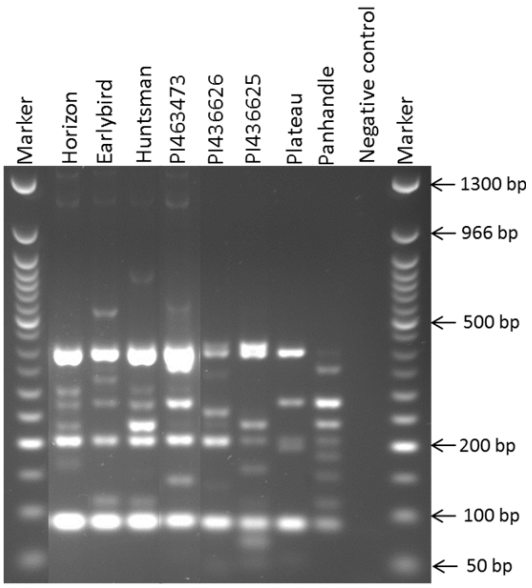

Figure 1. Polymorphic profile of switch grass SSR marker PVCA 21-22 in 8 proso genotypes (M = 50 bp NEB DNA Ladder, NC = Negative control).

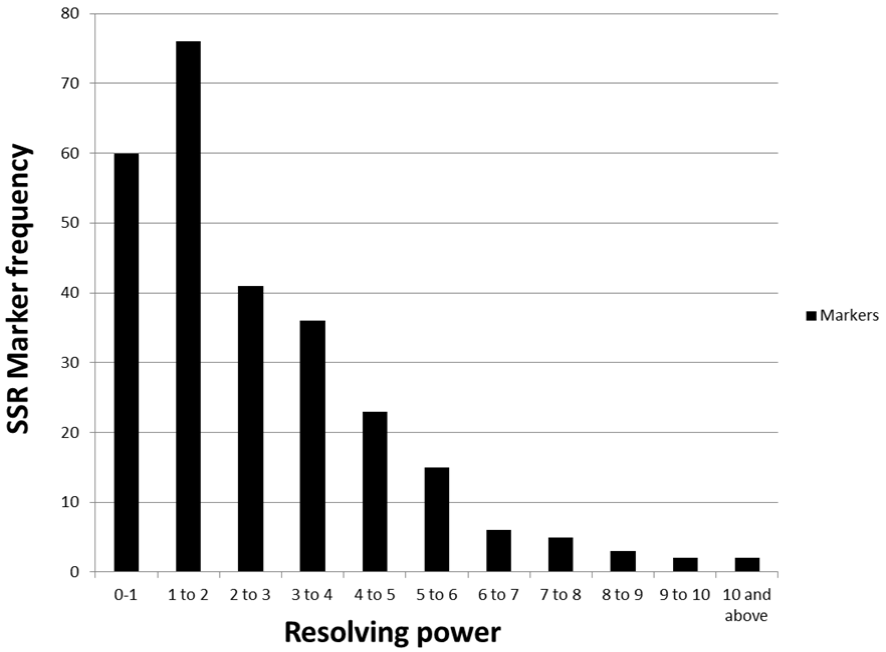

Figure 2. Resolving power values of 254 polymorphic SSR markers in proso millet.

Table 1. Characteristics of switchgrass SSR markers, which were polymorphic in proso millet.

Sr. no.	Marker Name[#]	Observed allele size in proso millet (bp)	Resolving power (Rp)
1	PVCA1763-64	400, 350	0.75
2	PVCA1761-62	600, 500, 350	0.75
3	PVCA1751-52	400, 350, 300	1
4	PVCA1737-38	600, 200	0.5
5	PVCA1717-18	1000, 500, 250	0.75
6	PVCA1525-26	325, 250, 175	4.5
7	PVCA1513-14	650, 250, 200	2.5
8	PVCA1431-32	650	0.25
9	PVCA1429-30	75, 50	1
10	PVCA1263-64	500, 400, 250, 75	1.75
11	PVCA1217-18	150	1
12	PVCA1181-82	600, 150	1.25
13	PVCA993-94	1300, 250	1
14	PVCA989-90	1000, 800, 550, 525, 325, 225, 150, 75	3.5
15	PVCA751-52	900, 750, 200, 100	4
16	PVCA733-34	750, 275	2
17	PVCA445-46	600, 300	1.25
18	PVCA407-08	450, 250, 200	2.5
19	PVCA401-402	1300, 400, 350, 250, 200	6
20	PVCA1647-48	1200, 400, 325, 250, 125	3.75
21	PVCA215-16	525, 500, 75, 50	3.5
22	PVCA201-02	750, 700, 350, 225, 200, 175, 100	7.75
23	PVCA155-56	175, 150, 100	4.5
24	PVCA137-38	450, 300, 335, 175, 150	3
25	PVCA255-56	1000, 700, 600, 350, 200, 100	4
26	PVCA343-44	1300, 500, 250, 200	4.25
27	PVCA1535-36[*]	375, **350**, 300, 150, 75, 50	6.25
28	PVCA1119-20	300, 275, 225, 150	4.5
29	PVCA1107-08	1000, 150	0.5
30	PVCA1091-92	300, 75	2
31	PVCA47-48	1300, 450, 400	1
32	PVCA1799-1800	700, 600, 425, 400, 325, 253, 175, 125, 75	6.5
33	PVCA1659-60	250	1
34	PVCA1701-02	900, 400, 300, 250, 225, 200, 175, 75	3.75
35	PVCA1687-88	1000, 800, 650, 425, 200, 100, 75, 50	6
36	PVCA1257-58	1300, 1200, 650, 450, 400, 325, 225	7.25
37	PVCA1679-80	1000, 750, 600, 400, 250, 200	4.75
38	PVCA21-22	700, 600, 500, 400, 350, 150	4.25
39	PVCA57-58	475, 300, 175, 150, 100	4.5
40	PVCA167-68	1300, 1200, 350, 250, 200, 150, 75	7.5
41	PVCA285-86	750, 550, 450, 400, 300, 275, 200, 175, 150, 100	7.5
42	PVCA299-300	125	0.25
43	PVCA429-30	700, 500, 150, 125, 100, 75, 50	4
44	PVCA1259-60	800, 750, 700, 600, 550, 350, 150, 100, 75	4.5
45	PVCA639-40	900, 700, 450, 425, 325, 300, 250, 175, 150, 100	6.5
46	PVCA687-88	800, 750, 700, 650, 475, 450, 400, 300, 175, 125	5.25
47	PVCA183-84	1200, 1000, 750, 650, 375, 350, 200, 150, 100, 50	4.25
48	PVCA191-92	1300, 1200, 1000, 700, 400, 300, 250, 225, 175, 100	9

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49	PVCA199-200	750, 700, 450, 400, 250, 225, 175, 125, 100, 75	8.25
50	PVCA951-52	1000, 650, 600, 550, 375, 350, 300, 275, 250, 100, 75, 50	4.75
51	PVCA1055-56	900, 400, 350, 300, 250, 200, 150	5.25
52	PVCA1105-06	650, 600, 500, 400, 275, 250, 175, 150	3
53	PVCA1221-22	1000, 750, 700, 400, 350, 300, 275, 250, 225, 200, 175, 150, 125,100,75, 50	8.75
54	PVCA1267-68	950, 600, 125, 100	1.75
55	PVCA1279-80	900, 550, 500, 350, 325, 250, 150	7
56	PVCA1387-88	1300, 650, 500, 375, 350, 300, 225, 175, 150, 125, 100, 75, 50	14.75
57	PVCA1421-22	500, 250, 125, 100, 75	1.5
58	PVCA1449-50	275, 250, 225, 200, 50	2.5
59	PVCA1479-80	1300, 900, 550, 300, 200, 175, 75, 50	3
60	PVCA1555-56	650, 600, 400, 375, 225, 200, 150, 100	9.25
61	PVCA1565-66*	650, 600, **350**, 325, 300, 275, 250, 225, 150, 100	9.25
62	PVCA1571-72	1100, 900, 850, 800, 750, 650, 625, 550, 400, 350, 325, 175, 150, 125, 75	7.25
63	PVCA1573-74	500, 400, 325, 300, 250, 200, 175, 150, 100	6
64	PVCA1651-52	900, 700, 525, 450, 400, 325, 300, 275, 250, 100, 75, 50	5.5
65	PVCA1731-32*	750, 600, 500, 425, 400, 350, 300, 325, 300, **225**, 175, 150, 125, 100, 75, 50	10.5
66	PVCA1-2	700, 250	0.75
67	PVCA109-10	700, 325, 300, 200	1.5
68	PVCA161-62	500, 400, 150	1.75
69	PVCA231-32	700, 500, 300, 150	2.75
70	PVCA259-60	600, 500, 300, 150	3.25
71	PVCA271-72	500, 325, 250, 100	1.25
72	PVCA275-76	1300, 750, 300, 200, 150, 100	4.25
73	PVCA297-98	500, 350, 300, 250	1.25
74	PVCA301-02	750, 250, 200	3
75	PVCA345-46	700, 350	0.5
76	PVCA347-48	325, 200, 150	1
77	PVCA445-46	175, 125, 100	1.5
78	PVCA495-96	800, 300, 150	1
79	PVCA533-34	700, 500, 400	0.75
80	PVCA541-42	750, 700, 350, 200, 150	2.75
81	PVCA557-58	550, 500, 300	1.25
82	PVCA571-72	400, 300	0.75
83	PVCA579-80	700, 650, 600, 500, 200	2.75
84	PVCA609-10	750, 650, 450, 350, 150	4.75
85	PVCA615-16	500, 300, 150, 100	2.25
86	PVCA669-70	500, 200, 150, 75	3.25
87	PVCA683-84	750, 650	1
88	PVCA705-06	1300, 750, 700, 350, 150	2.25
89	PVCA723-24	425, 300, 250, 150	1.25
90	PVCA985-86	200, 75	0.25
91	PVCA1065-66	300, 100	1.25
92	PVCA1077-78	700, 350, 300	2.5
93	PVCA1083-84	500, 200, 100, 75	3.75
94	PVCA1149-50	1300, 75	1
95	PVCA1191-92	1300, 500, 250	2
96	PVCA1209-10*	600, 500, 400, 350, 300, **250**	3.5
97	PVCA1251-52	500, 450, 400, 300	1.5
98	PVCA1291-92	700, 200	0.5

Continued

99	PVCA1333-34	400, 225	2.5
100	PVCA1347-48	400, 225	4.25
101	PVCA1353-54	350, 300, 150	2.5
102	PVCA1355-56	500, 300, 250, 125, 75	6.25
103	PVCA1383-84	650, 300, 200	1
104	PVCA1385-86	700, 500, 450	1
105	PVCA1403-04	800, 750	0.75
106	PVCA1409-10	600, 350, 200, 175, 100	6
107	PVCA1413-14	300, 150	1.25
108	PVCA1455-56	700, 125	2.75
109	PVCA1477-78	400, 250, 200, 150	2.75
110	PVCA1511-12	750, 500, 400, 200, 100	4.5
111	PVCA1531-32	600	0.5
112	PVCA1533-34	350, 150	0.75
113	PVCA1541-42	600, 500, 400, 200	1
114	PVCA1557-58	600, 500, 400, 200	3.25
115	PVCA1591-92	600, 400, 300, 200	1.25
116	PVCA1593-94	500, 300, 150, 100	3.25
117	PVCA1631-32	400, 350, 100	1.25
118	PVCA1667-68	400, 250, 200, 50	1.75
119	PVCA1685-86	700, 100	1
120	PVCA1691-92	500, 300	0.5
121	PVCA1705-06*	700, **350**, 150, 100	2.75
122	PVCA1753-54	700, 500, 150	1
123	PVCA1759-60	200, 75	0.75
124	PVCA1769-70	600, 250, 150, 100	1
125	PVCA1781-82	250, 200	1.25
126	PVCA1783-84	600, 500, 250, 150	1.5
127	PVCA1811-12	300, 250, 125, 75	5.5
128	PVCA1813-14	400, 150	1.5
129	PVCA1819-20	700, 600, 550, 450, 400, 300, 150	5.5
130	PVCA1827-28	1300, 500, 300, 150	5.75
131	PVCA1835-36	1300, 550, 150, 100	1.75
132	PVCA1851-52	200, 175, 125	2.5
133	PVCA1853-54	300, 250, 200, 150, 100	6
134	PVCA1867-68	400, 300, 150	2.75
135	PVCA1869-70*	**325**, 250	2.25
136	PVCA1881-82	325, 250, 150	1.75
137	PVCA1883-84	350, 100	0.5
138	PVCA1915-16	300, 250, 150	2.25
139	PVCA1925-26	700, 400, 300, 150, 100,	5.5
140	PVCA1937-38	400, 350, 250, 100	3.5
141	PVCA1939-40	700, 500, 450, 250,	3.25
142	PVCA1941-42	400, 300, 200, 150,	4.75
143	PVCA1947-48	400, 300	1.75
144	PVCA1955-56	600, 500, 400, 350	1.5
145	PVCA1957-58	325, 300	1.75
146	PVCA1963-64	200, 175	2
147	PVCA1997-98	600, 400, 300	1
148	PVCA2003-04	500, 450, 300, 250, 100	2.25

Continued

149	PVCA2005-06	300, 200, 100	1
150	PVCA2009-10	300, 200	1
151	PVCA2013-14	200, 150, 100	1
152	PVCA7-8	350, 325	2
153	PVCA17-18	300, 150, 100, 50	2.5
154	PVCA19-20	100, 50	3
155	PVCA37-38	300, 200, 100, 50	3
156	PVCA145-46	700, 500, 300, 250, 100, 50	5
157	PVCA151-52	250, 150	0.25
158	PVCA225-26	75, 50	0.75
159	PVCA243-44	600, 500, 400, 300, 200	3
160	PVCA285-86	600, 500, 150, 100, 50	2
161	PVCA309-10	750, 700, 550, 500, 200, 100	2.75
162	PVCA341-42	250, 200, 100,	3.25
163	PVCA347-48	350, 300	1
164	PVCA349-50	700, 500, 150, 100	3.25
165	PVCA405-06	700, 650, 600, 500, 450, 300, 125	6.5
166	PVCA411-12	650, 600, 300, 100, 50	5.5
167	PVCA415-16	200, 150	0.25
168	PVCA425-26	150, 100, 75	2.25
169	PVCA765-66	550, 450, 400, 100	2
170	PVCA797-98	150, 100	2
171	PVCA863-64	350, 300, 200, 150, 100	5.25
172	PVCA931-32	150, 75	2
173	PVCA1037-38	250, 225	2
174	PVCA1045-46	175, 150, 50	2.5
175	PVCA1187-88	650, 300, 200, 150, 100	2.5
176	PVCA1197-98	450, 350	1.7
177	PVCA1271-72	250, 200	2
178	PVCA1337-38	350, 250	2.25
179	PVCA1387-88	300, 225, 75	3.5
180	PVCA1401-02	275, 225	2
181	PVCA1405-06	275, 250, 200	0.5
182	PVCA1485-86	200	1.25
183	PVCA1549-50	325, 300	2
184	PVCA1589-90	500, 225, 150, 75	2.25
185	PVCA1605-06	225, 200	1.5
186	PVCA1627-28	225, 200	2
187	PVCA1669-70	350, 300	1.5
188	PVCA677-78	325, 300	2
189	PVCA1741-42	200, 175	2
190	PVCA1883-84	250, 175	2.25
191	PVCA2059-60	300, 225, 100	1.5
192	PVCA2123-24	200, 175, 150	3.75
193	PVCA2143-44	325, 300	1.5
194	PVCA2197-98	225, 200, 175, 150	4.5
195	PVCA2199-2200	300, 275	2
196	PVCA2207-08	550, 500, 325, 200	3.75
197	PVCA2239-40	200, 175	2
198	PVCA2269-070	200, 225	2

Continued

199	PVCA2279-80	200, 175	2
200	PVCA2361-62	250	1
201	PVCA2393-94	200, 175	2
202	PVCA2437-38	150, 125	2
203	PVCA2461-62	250, 200	2
204	PVCA2471-72	200, 175	2
205	PVCA2473-74	150, 100	1.75
206	PVCA2503-04	250, 200	2
207	PVCA2527-28	200, 175	2
208	PVCA2615-16	350, 325	2
209	PVCA2623-24*	700, **150**	1.75
210	PVCA2647-48	400, 250, 200	3.25
211	PVCA2721-22	250, 225	2
212	PVCA2847-48	600, 500, 300, 200	1.25
213	PVCA2979-80	200, 150, 100	1.25
214	PVCA3119-20	500, 400, 200	3.25
215	PVCA3205-06	250, 225	2
216	PVCA3327-28	300, 250	2.5
217	PVCA3053-54	200	0.5
218	PVCA23-24	225, 200	0.75
219	PVCA247-48	150	1.75
220	PVCA249-50	300, 250, 200, 150	1
221	PVCA251-52	200	0.75
222	PVCA253-54	550, 500, 450, 150	3.25
223	PVCA259-60	750, 700, 600, 400, 250, 150, 100	4
224	PVCA261-62	300, 200	2.25
225	PVCA271-72	175, 100	0.5
226	PVCA275-76	650, 550, 500, 450, 400, 350, 200	3.5
227	PVCA281-82*	700, 350, 300, **150**	1
228	PVCA283-84	1300, 600, 400, 350, 250, 200, 100	6
229	PVCA287-88	900, 800, 650, 400, 350, 150	4.75
230	PVCA297-98	600, 400, 300, 200	3
231	PVCA299-300	750, 700, 500, 450, 400, 150	4.25
232	PVCA301-302*	800, 650, **250**	1.25
233	PVCA307-08	900, 800, 650, 450, 400, 350, 300, 150	3.5
234	PVCA337-38	150	0.75
235	PVCA343-44	600, 300, 200	2
236	PVCA345-46	450, 200	0.5
237	PVCA351-52	900, 650, 150	0.75
238	PVCA357-58	700, 550, 150	1
239	NFSG-105	200, 175	3
240	NFSG-26	150, 125	3
241	SWW-177	200, 175	2
242	SWW-108	175, 150	0.5
243	NFSG-50	175, 150	0.5
244	NFSG-125	175, 150	2
245	SWW-2945	600, 350, 300, 250	1.5
246	SWW-2906	275, 50	1
247	NFSG-36	500, 225, 175	1.5
248	NFSG-54	600, 150	2

Continued

249	NFSG-288	175, 150	2
250	NFSG-246	100, 50	0.25
251	NFSG-145	250, 225	2
252	SWW-1394	250, 200	2.25
253	NFSG-133	175, 150	0.75
254	NFSG-132	325, 300	1.75

[#]Data published by Wang *et al*. 2011 [25] and Okada *et al*.2010 [24]. [*]Amplified allele (marked bold) size in proso millet = allele size in switchgrass [25].

conserved and syntenic between switchgrass and proso millet, which suggests amplification of either orthologous or paralogus genes [31].

Most of the polymorphic markers (181 out of 254) produced 2, 3 or 4 alleles and 73 markers have produced 1, 5, 6, 7 or 8 alleles. Total numbers of alleles amplified by each SSR marker on the set of 8 lines ranged from 1 to16. Highest alleles (16) were amplified by marker PVCA 1221-22. High frequency of SSR markers with 2 alleles also indicates diploid nature of proso millet genome. These indicate that proso millet genome is not 100% tetraploid but rather partially tetraploid and partially diploid although proso millet was reported as allotetraploid [32]. Amplification of multiple loci by a few SSR markers might be due to the multiple primer binding sites throughout the genome or within a single locus, which may be due to gene duplication or duplicated genomic regions [17]. This is in agreement with previous reports on proso millet, potato, and zoyagrass species [15, 27,33].

The 254 polymorphic SSR markers amplified a total of 984 alleles (amplified DNA markers) among the set of 8 proso millet genotypes. Sizes of alleles by the switchgrass SSRs in proso millet ranged from 50 bp to 1300 bp with varied number of bands of each size. Amplified DNA bands in ranges of 50 - 200 bp, 200 - 400 bp, 400 - 600 bp, 600 - 800 bp, 800 - 1000 bp, >1000 bp were 377 (38%), 325 (33%), 138 (14%), 98(10%), 23 (2%) and 23(2%), respectively. When SSR marker sizes in proso millet were compared with expected switchgrass sizes, only 9 markers (PVCA1535-36PVCA1565-66, PVCA1731-32, PVCA1209-10, PVCA1705-06, PVCA1869-70, PVCA2623-24, PVCA281-82, PVCA301-302) showed exact matches with that of switchgrass and majority of marker sizes were different in proso millet. This high level of differences indicates significant genome reorganization in proso millet after speciation during evolution which was reported in wheat [34].

3.2. SSRs and Possible Association with Abiotic Stresses

We found that eight SSR markers are part of the mRNA, UniGenes or genomic sequences in other crops (**Table 2**). The SSR marker PVCA1017-1018 has 100% sequence similarity with the mRNA sequence involved in glutathione metabolism in sorghum. Another SSR marker PVCA723-724 is located on the rice BAC clone from where *Sub*-1 gene was cloned. Glutathione metabolism in sorghum and *Sub*-1 gene in rice were reported to be associated with drought and flood tolerance, respectively [35,36]. It is likely that, these genes are still present in the proso millet genome and are showing different mechanism for drought tolerance. This evolutionary conservation for these genes provides a valuable source for further marker development for abiotic stresses in proso millet.

3.3. Repetitive DNA in Proso Millet

Repeat motifs of the 254 polymorphic SSR primers in proso millet were analyzed to elucidate the type of repetitive DNA in proso millet. Of the total 254 polymorphic markers 221 (87%) were di-nucleotide repeats and the remaining 33 (13%) were tri- and other composite repeats (**Table 3**). Of all these repeat-motifs, 196 were perfect (77%) and 58 were compound (23%) repeats. Of the 221 di-nucleotide motifs, the most abundant repeat-motif was AG/GA (47%) followed by AC/CA (22%) repeat motif in proso millet. Together the AG/GA and AC/CA repeat motif contributed 69% of all the polymorphic markers in proso millet. The third most abundant di-nucleotide repeats in proso millet were GT/TG and CT/TC repeats, which together was 11%. All the remaining di-nucleotide repeats accounted for 7% of all the repeats.

A high level of di-nucleotide repeats in polymorphic SSRs in proso millet matches with other reports where $(CA/GT)_n$ and $(CT/AG)_n$ were predominant repeat motifs and were found in the euchromatin and hetrochromatin regions, respectively [37]. The AG/GA repeats were shown to be related to drought tolerance in rice [38]. Therefore, it may not be surprising that AG/GA repeats are the pre-dominant repeat motifs in proso millet since proso millet is known to have good tolerance to low soil moisture condition. This result is similar to the report presented by Cho *et al*. and 42% AC/CA repeats. However, it is opposed to AT/TA and CT/GA repeats as predominant repeats in other plants [39,40].

Table 2. Eight switchgrass SSR markers, which are polymorphic in proso millet and their possible functional association in other crops based on sequence similarities available in NCBI data base.

SSR markers	Crop	Accession No.	Sequence type (gDNA/mRNA)	Putative protein /gene/location
PVCA275-276	Sorghum	XM_002466569.1	mRNA	Hypothetical protein
PVCA275-276	Oat	JN390967.1	mRNA	Lipoxygenase
PVCA429-430	Wheat	GU985444.1	gDNA	Mitochondrion
PVCA495-496	Maize	BT065995.2	mRNA	Uncharacterized
PVCA723-724	Rice	DQ453964.1	gDNA	*Sub*1 locus
PVCA1017-1018	Sorghum	XM_002465397.1	mRNA	Hypothetical protein (predicted to be involved in glutathione metabolism)
PVGA1119-1120	Barley	AK373276.1	mRNA	Predicted protein
PVCA579-580	Arabidopsis	CP002687.1	gDNA	Peroxidase 38 Tetratricopeptide repeat domain-containing protein Myosin heavy chain-like protein

Table 3. 254 polymorphic SSR markers and their type and repeat-motifs, and its relative frequency in proso millet.

Repeat unit	Repeat type[#]	Number of markers	Frequency (%)
Di-Nucleotide (221)	AG or GA	120	47.24
	AC or CA	57	22.44
	GT or TG	18	7.09
	CT or TC	10	3.94
	AC/AG, CA/GA, CA/AG, GA/CA	7	2.76
	AC/AT	2	0.79
	AG/GT, GT/GA	2	0.79
	CT/AG	1	0.39
	CT/GT	1	0.39
	GT/AC	1	0.39
	TA/AT	1	0.39
	TA/TG	1	0.39
	CT/AC	1	0.39
Sub-total		**221**	**87%**
Tri-Nucleotide (30)	GAA/AAG	3	1.18
	AGC,CAG,GCA, ACA	5	1.97
	TGC,GCT,CTG	6	2.36
	ATC,CAT	2	0.79
	CCG,CGC	2	0.79
	TCT	4	1.57
	GGT	1	0.39
	$(TGC)_5$-$(CTG)_5$,$(TGC)_9$-$(TGC)_6$	2	0.79
	$(TCC)_5$-$(TCC)_5$,$(TCC)_7$-$(TGC)_8$	2	0.79
	$(GCT)_8$-$(CTG)_5$	1	0.39
	$(CAG)_5$-$(GCA)_6$	1	0.39
Others (tetra,penta, hexa-nucleotide) (3)		3	1.18
Sub-total		**33**	**13%**
Total		**254**	**100%**

[#]Repeat motifs from Wang *et al.* 2011 [25] and Okada *et al.* 2010 [24].

4. Conclusion

To the best of our knowledge, this is the first report of such a large set (339) of SSR markers for proso millet using cross species genomic resources. This will help to advance proso millet genomic research and will ultimately lead to genetic improvement of this crop.

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