In Silico and Fluorescence In Situ Hybridization Mapping Reveals Collinearity between the Pennisetum squamulatum Apomixis Carrier-Chromosome and Chromosome 2 of Sorghum and Foxtail Millet

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Abstract

Apomixis, or clonal propagation through seed, is a trait identified within multiple species of the grass family (Poaceae). The genetic locus controlling apomixis in Pennisetum squamulatum (syn Cenchrus squamulatus) and Cenchrus ciliaris (syn Pennisetum ciliare, buffelgrass) is the apospory-specific genomic region (ASGR). Previously, the ASGR was shown to be highly conserved but inverted in marker order between P. squamulatum and C. ciliaris based on fluorescence in situ hybridization (FISH) and varied in both karyotype and position of the ASGR on the ASGR-carrier chromosome among other apomictic Cenchrus/Pennisetum species. Using in silico transcript mapping and verification of physical positions of some of the transcripts via FISH, we discovered that the ASGR-carrier chromosome from P. squamulatum is collinear with chromosome 2 of foxtail millet and sorghum outside of the ASGR. The in silico ordering of the ASGR-carrier chromosome markers, previously unmapped in P. squamulatum, allowed for the identification of a backcross line with structural changes to the P. squamulatum ASGR-carrier chromosome derived from gamma irradiated pollen.

Introduction

Most current grass species are found to be derived from a common ancestor that lived about 50–80 million years ago. Despite the relatively recent and monophyletic origin of the grass genomes, there is considerable divergence in genome size and chromosome number [1, 2]. Yet sequenced members of the Poaceae clade have shown conservation of gene order (collinearity) among species such as rice, sorghum, maize, and foxtail millet [3]. Pearl millet (Pennisetum
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Pluto (P. glaucum) shares a common ancestor with foxtail millet (Setaria italica) ~8.3 million years ago and with maize and sorghum ~26 million years ago [4].

There are several apomictic species in the genus Cenchrus/Pennisetum. Apomixis has been defined as asexual reproduction through seed [5]. Apomixis in *P. squamulatum* (syn Cenchrus squamulatus) and the closely related species *C. ciliaris* (syn Pennisetum ciliare) was found to be controlled by a dominant and hemizygous genetic locus named the apospory-specific genomic region (ASGR) [6, 7]. The ASGR in *P. squamulatum* is identified as a large (>50 Mb in size), heterochromatic chromosomal block localized near the telomere on the short arm of the ASGR-carrier chromosome by fluorescence in situ hybridization (FISH). The ASGR contains a region of low copy DNA flanked by regions of high copy DNA [8]. Physical mapping of the ASGR between *P. squamulatum* and *C. ciliaris* identified an inversion but conservation of ASGR-BAC order between the two species [9]. Within the ASGR, partial sequencing of ASGR-linked BAC clones showed the presence of multiple regions of small-scale, but not large-scale, collinearity with the rice and sorghum genomes [11].

An apomictic backcross (06-A-58) of *P. glaucum*, originating from a cross between *P. squamulatum* and tetraploid *P. glaucum*, was identified by FISH to carry one alien chromosome, the ASGR-carrier chromosome from *P. squamulatum* [12]. Forty-nine contigs, generated from the assembly of 454 sequences derived from dissected apomictic ovules, were mapped to the ASGR-carrier chromosome via SCAR, CAPS or SSCP markers [13]. Contig PS26_c9369 demonstrated tight linkage to the ASGR. Three other contigs (PS26_c5080, PS26_c33813, and PS26_c2552) mapped as unlinked to the ASGR using CAPS markers. The remaining contig SCAR markers failed to identify polymorphisms for mapping within a segregating F1 population. However, in silico mapping of the 49 ASGR-carrier chromosome contigs to the sorghum reference genome identified 21 sequences (BlastN, e value ≤ e^{-20}) with similarity to the sorghum genome of which 17 had a unique or highest similarity to sorghum chromosome 2.

As the 454 transcriptome data was 3’ biased due to T7 amplification of the ovule RNA, we generated additional transcriptome assemblies using RNA-seq data from an apomictic backcross and screened these to extend the length of the 454 ASGR-carrier chromosome contigs for additional comparison to the sorghum and foxtail millet genomes. The predicted in silico positions of 7 contigs from the *P. squamulatum* ASGR-carrier chromosome, based on hits to the sorghum and foxtail millet genome, were verified by cytogenetic mapping of BAC clones containing the SCAR marker for the ASGR-carrier chromosome contigs. Our analysis demonstrates that the ASGR-carrier chromosome from *P. squamulatum*, outside the ASGR boundary, is collinear with chromosome 2 of sorghum and foxtail millet. Using the established ASGR-carrier chromosome SCAR markers and physical mapping results, a screen of gamma irradiated offspring was tested to identify lines with structural changes to the ASGR-carrier chromosome. This screen identified a sexual line which has lost the ASGR, but retained most of the long arm of the ASGR-carrier chromosome.

**Materials and Methods**

**Plant material**

Apomictic *P. glaucum* backcross 8 (BC_8) (06-A-58) derived seedlings carrying the ASGR-carrier chromosome [12] were used for FISH and tissue collection for RNA extraction. Backcross 06-A-58 is a facultative apomict and therefore produces progeny derived through both modes of reproduction, apomictic and sexual. DNA from seedlings was extracted using a modified
CTAB method [14] and screened for the ASGR using the ASGR-linked SCAR marker p787/788 [12].

Extension of ASGR-carrier chromosome transcript information

RNA was extracted from unfertilized ovaries of apomictic and sexual BC$_8$ (06-A-58) derived plants collected on the day of anthesis (anther exsertion). Approximately 50 ovaries were collected from individual plants based on their mode of reproduction. RNA was isolated from 12 apomictic and 12 sexual plants using Qiagen Plant RNAeasy kit. RNA from individuals was pooled based on mode of reproduction to make an apomictic and sexual RNA sample for sequencing. Sequencing libraries were constructed according to manufacturer’s instructions and Illumina sequenced to yield 2 x 76 paired-end reads. Sequences from both libraries were quality trimmed, separated into paired-end and single-end reads (~100 million reads for each library), and assembled together with the Velvet de novo [15] assembly algorithm. Two different assemblies were used (varying in K-mer value) to identify the longest ASGR-carrier chromosome transcript available. PS contig sequences used for in silico analysis are located in S1 File.

Identification of ASGR-carrier chromosome BACs

BACs linked to the ASGR-carrier chromosome were identified by screening the polyhaploid BAC library [16] with probes derived from the ASGR-carrier chromosome transcripts [13]. Hybridizing BAC clones were confirmed as linked to the ASGR-carrier chromosome via PCR amplification with the respective ASGR-carrier chromosome SCAR marker. Markers with isolated BACs are indicated in Table 1.

Southern blot hybridization of HindIII digested fragments of BAC clones with $^{32}$P label genomic DNA from apomictic BC$_8$ (06-A-58) was used to assess the level of DNA repetitiveness within the BAC clones based on signal intensity. A centromeric BAC clone was identified from the polyhaploid BAC library using a 160 bp KpnI repeat probe [17].

FISH

**FISH probes.** BAC DNA for nick translation was extracted using an alkaline lysis method http://www.protocolpedia.com/component/sobipro/?pid=69&sid=2209:BAC-DNA-Isolation-from-200-ml-Cultures-by-a-Cleared-Lysate-Method-Followed-by-Double-Acetate-Precipitation&Itemid=0 with the following modifications. The RNase treatment was done with 10 μl Ambion® RNase cocktail (Life Technologies, Grand Island, NY) consisting of 5 U of RNase A and 200 U of RNase T1. The BAC DNA was suspended in a final volume of 50 μl Buffer EB (QIAGEN Inc., Valencia, CA, USA). 1–2 μg of BAC DNA was labeled with biotin (bio)-11-dUTP (Roche, Indianapolis, IN) or digoxigenin (dig)-11-dUTP (Roche), using the nick translation kit (Roche) according to manufacturer’s instructions.

PCR centromere probes were prepared by labeling with biotin-11-dUTP using primers 5’-GGTACCCCGTAATAGTGCATTC-3’ and 5’-GAAAATGGTTTCGCAACAAAAG-3’ designed from the 160 bp KpnI repeat family sequence [17].

**Chromosome preparation.** Root tips from apomictic BC$_8$ (06-A-58) derived seedlings were collected, washed, placed in a 0.5ml Eppendorf tube with a hole in the lid in 300 μl distilled water and treated with nitrous oxide at 1 to 1.5 Mpa for 3 to 4 hours at room temperature in a Nitrous Oxide gas chamber [18] prior to fixing in 3:1 (V:V) ethanol to acetic acid solution. Root caps were removed and 2–3 mm of the meristematic region was incubated in an enzyme mix containing 2% (w/v) cellulose RS (Karlan Research, Santa Rosa, CA), 1% (w/v), pectolyase Y23 (Karlan Research, Santa Rosa, CA), 1% (w/v) macerozyme R 10 (Desert Biologicals,
Table 1. Information for in silico and deletion mapping line.

| SCAR Primers | PS26 contig | 454 contig length (bp) | 454 contig hit to sorghum genome | SCAR hit | Velvet contig length (bp) | Sorghum hit | e-value | FT Millet Hits | e-value | FT Millet start | Presence of SCAR marker in the 312 line |
|--------------|-------------|------------------------|----------------------------------|---------|--------------------------|-------------|--------|---------------|--------|----------------|----------------------------------------|
| 1538/1539    | c17388      | 209                    | n                                | 527     | No hit                   | No hit      |        |               |        |                | yes                                     |
| 1498/1499    | c30691      | 219                    | n                                | 615     | No hit                   | No hit      |        |               |        |                | N/A                                     |
| 1514/1515*   | c9369       | 330                    | y                                | 515     | chr-3, 4, 6, 8, 10      | –6.83E-35   | 9.88E-45|               |        |                |                                         |
|              |             |                        |                                  |         | chr-1                   | 3.00E-46    |        | 23,795,001   | no     |                |                                         |
| 1476/1477    | c10331      | 301                    | n                                | 1635    | chr-9 chr-2             | 4E-38 6E-35 | 59,306,986| 71,178,293 | chr-6  | 4.00E-113     | 5,498,118                             |
| 1478/1479    | c11544      | 237                    | n                                | 544     | chr-1                   | 8.00E-75    |        | 30,659,990   | chr-9  | 7.00E-92     | 20,435,700                             |
| 1604/1605*   | c194        | 478                    | n                                | 1425    | No hit                  | 0           |        | 5,554,462    | no     |                |                                         |
| 1567/1568    | c1422       | 397                    | y                                | 512     | chr-2                   | 4.00E-114   | 8,168,376| chr-2     | 0      | 7,323,838    |                                         |
| 1658/1659    | c6744       | 321                    | y                                | 638     | chr-2                   | 1.00E-90    |        | 8,178,536   | chr-2  | 2.00E-118    |                                         |
| 1704/1705    | c28392      | 230                    | n                                | not found| No hit                  | chr-2       | 9.00E-30 | 8,412,330   | no     |                |                                         |
| 1573/1574    | c1472       | 456                    | y                                | 613     | chr-2                   | 6.00E-41    | 9,308,984| chr-2     | 4.00E-139 | 8,573,318    | N/A                                     |
| 1642/1643*   | c2838       | 199                    | n                                | 1116    | chr-2                   | 2.00E-77    | 11,446,435| chr-9**    | 2.00E-121 | 44,511,972   | –10,400,000                           |
| 1692/1693*   | c19109      | 235                    | n                                | 657     | chr-2                   | 3.00E-78    | 20,718,640| chr-2     | 2.00E-88  | 15,545,094   | no                                      |
| 1510/1511**  | c583        | 408                    | y                                | 967     | chr-2                   | 7.00E-114   | 21,622,996| chr-2     | 0       | 15,729,820   | no                                      |
|               |             |                        |                                  |         |                         |             |        | Centromere region* | –30,000,000–35,000,000| chr-2 | Centromere region* | –17,000,000–20,000,000 |
| 1542/1543*   | c1312       | 332                    | y                                | 2430    | chr-10                  | 5.00E-171   | 1,611,963| chr-2     | 0       | 21,705,415   | no                                      |
| 1664/1665*   | c9776       | 333                    | n                                | 6066    | chr-7                   | 0.00E+00    | 18,750,193| chr-2     | 0       | 22,231,687   | no                                      |
| 1530/1531*   | c1279p      | 535                    | y                                | 1669    | chr-2                   | 1.00E-85    | 45,159,033| chr-2     | 3.00E-140 | 24,209,378   | no                                      |
| 1666/1667    | c14318      | 366                    | y                                | 1799    | chr-2                   | 1.00E-83    | 49,436,750| chr-2     | 0       | 24,923,646   | N/A                                     |
| 1534/1535*   | c2785       | 313                    | n                                | 887     | chr-2                   | 5.00E-33    | 52,122,450| chr-2     | 4.00E-104 | 26,193,530   | N/A                                     |
| 1492/1493*   | c2448       | 367                    | n                                | 595     | chr-2                   | 4.00E-102   | 59,163,077| chr-2     | 0       | 30,619,621   | no                                      |
| 1571/1572*   | c6131       | 377                    | n                                | 1406    | chr-2                   | 0.00E+00    | 62,313,486| chr-2     | 0       | 34,471,990   | yes                                     |
| 1480/1481*   | c13157      | 249                    | n                                | 1392    | chr-2                   | 4.00E-30    | 62,939,050| chr-2     | 6.00E-111 | 34,862,608   | yes                                     |
| 1512/1513*   | c8165       | 200                    | n                                | 2167    | chr-2                   | 4.00E-128   | 63,654,052| chr-2     | 0       | 35,734,036   | yes                                     |

(Continued)
### Table 1. (Continued)

| SCAR Primers<sup>a</sup> | PS26 contig | 454 contig length (bp) | 454 contig hit to sorghum genome | Velvet contig | Sorghum hit length (bp) | Sorghum hit e-value | FT Millet Hits start | FT Millet Hits e-value | FT Millet start | Presence of SCAR marker in the 312 line |
|-------------------------|-------------|------------------------|---------------------------------|---------------|-------------------------|---------------------|---------------------|---------------------|----------------|--------------------------------------|
| 1502/1713<sup>*</sup>  | c3993       | 723                    | y                               | smaller       | chr-6                   | 1.00E-91            | 55,228,864          | chr-2               | 3.00E-102       | 36,457,704                          | yes                        |
| 1724/1725              | c33813      | 229                    | n                               | 692           | chr-2                   | 1.00E-81            | 64,442,929          | chr-2               | 5.00E-70        | 36,736,857                          | N/S                        |
| 1640/1641              | c2807       | 331                    | n                               | smaller       | No hit                  | 1.00E-63            | 65,545,284          | chr-2               | 3.00E-128       | 37,436,112                          | yes                        |
| 1486/1487              | c13922      | 360                    | n                               | 571           | chr-2                   | 1.00E-63            | 65,545,284          | chr-2               | 3.00E-128       | 37,436,112                          | yes                        |
| 1630/1631<sup>*</sup>  | c10535      | 243                    | n                               | 3144          | chr-2                   | 6.00E-115           | 65,959,535          | chr-2               | 6.00E-172       | 40,711,360                          | yes                        |
| 1482/1483<sup>*</sup>  | c13655      | 242                    | n                               | 1592          | chr-7                   | 5.00E-156           | 59,786,300          | chr-2               | 0              | 38,785,829                          | yes                        |
| 1656/1657              | c6373       | 257                    | y                               | not found     | chr-2                   | 1.00E-28            | 67,249,943          | chr-2               | 4.00E-100       | 39,733,092                          | yes                        |
| 1650/1651              | c4150       | 497                    | y                               | 1245          | chr-2                   | 0.00E+00            | 67734730            | chr-2               | 0.00E+00        | 40,261,848                          | yes                        |
| 1532/1533<sup>*</sup>  | c7587       | 460                    | y                               | 2203          | chr-2                   | 0.00E+00            | 68,388,845          | chr-2               | 0.00E+00        | 40,983,241                          | yes                        |
| 1581/1582              | c32589      | 240                    | n                               | 921           | chr-2                   | 4.00E-41            | 69,077,003          | chr-2               | 4.00E-136       | 41,654,883                          | yes                        |
| 1505/1516              | c4364       | 223                    | n                               | 569           | chr-2                   | 3.00E-122           | 69,975,526          | chr-2               | 0.00E+00        | 42,480,974                          | N/S                        |
| 1548/1549<sup>*</sup>  | c338        | 518                    | y                               | 754           | chr-2                   | 0                  | 70,344,223          | chr-2               | 0              | 42,859,260                          | N/S                        |
| 1690/1691              | c1878       | 287                    | n                               | 1583          | chr-2                   | 4.00E-160           | 70,622,801          | chr-2               | 0              | 43,138,453                          | yes                        |
| 1575/1576<sup>*</sup>  | c2388       | 207                    | n                               | 1118          | chr-2                   | 0.00E+00            | 71,136,317          | chr-2               | 0              | 43,636,319                          | yes                        |
| 1506/1507<sup>*</sup>  | c5080       | 383                    | y                               | 1136          | chr-2                   | 3.00E-144           | 72,066,759          | chr-2               | 4.00E-169       | 44,450,743                          | yes                        |
| 1500/1501<sup>*</sup>  | c3546       | 309                    | n                               | 1538          | chr-2                   | 1.00E-125           | 72,255,129          | chr-2               | 3.00E-140       | 44,616,393                          | yes                        |
| 1646/1647              | c3609       | 406                    | y                               | 465           | chr-2                   | 7.00E-26            | 72,610,707          | chr-2               | 3.00E-103       | 44,941,727                          | yes                        |
| 1652/1653              | c5210       | 398                    | y                               | 940           | chr-2                   | 0.00E+00            | 73,140,227          | chr-2               | 0              | 45,462,728                          | yes                        |
| 1670/1671              | c2552       | 614                    | y                               | not found     | chr-2                   | 7.00E-95            | 73,707,837          | chr-2               | 0              | 45,964,151                          | yes                        |
| 1583/1681<sup>*</sup>  | c1406       | 505                    | y                               | 1405          | chr-2                   | 0.00E+00            | 73,764,842          | chr-2               | 0              | 46,006,364                          | yes                        |
| 1496/1497              | c30198      | 225                    | y                               | 2061          | chr-2                   | 0.00E+00            | 74,461,686          | chr-2               | 0.00E+00        | 46,598,403                          | N/A                        |
| 1484/1485              | c1372       | 441                    | y                               | 1843          | chr-2                   | 0.00E+00            | 75,033,568          | chr-2               | 0              | 47,026,836                          | yes                        |
| 1540/1541<sup>*</sup>  | c3455       | 327                    | n                               | 4130          | chr-2                   | 0                  | 75,576,016          | chr-2               | 0              | 47,430,665                          | yes                        |

(Continued)
Phoenix, AZ) in citrate buffer (10 mM sodium citrate, 10 mM sodium EDTA, pH 5.5) [19] for 90 minutes at 37°C. Slide preparation for chromosome spreads after digestion was done either through air-drying [20] or a "SteamDrop" method [21].

**Fluorescence in situ hybridization and detection.** FISH was performed according to Zhong [22] with modifications. Slides with chromosomal spreads were treated with 5 μg/ml pepsin in 0.01M HCl for 5 to 10 minutes, fixed in 1% formaldehyde with 50 mM MgCl₂ in 2× SSC and dehydrated in a series of 70, 90 and 100% (v/v) ethanol. The hybridization mix consisted of 1–5 ng/μl of each probe, 50% formamide, 10% dextran sulfate, 10–50 ng/μl salmon sperm DNA and 2× SSC in a final volume of 18–20 μl. If necessary, *P. squamulatum* blocking DNA (10–50 ng/μl) was added to the hybridization mix to block signal from minor repetitive sequence within BAC clones. Hybridization mixtures were denatured at 80°C for 5 minutes, snap chilled on ice, applied to the chromosome spread, and covered with a 22 × 30 mm coverslip. Slides were placed on an 80°C heat block for 3 minutes then incubated in a moist chamber at 60°C for 90 minutes followed by a 37°C incubation for 64–67 hours. Two post-hybridization washes were done in 50% formamide in 2× SSC at 37°C for 10 minutes each. Slides were blocked in TNB (100 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1× DIG blocking solution, Roche) for 30 minutes at 37°C, and blocked again in 5% (w/v) IgG-free bovine serum albumin (Sigma) in TN (100 mM Tris-HCl, pH 7.5; 150 mM NaCl) for 30 minutes at 37°C. Two-color detection was carried out according to Zhong et al. (1996) with modifications. The biotin-labeled probes were detected with Texas red using a three step amplification and DIG-labeled probes were detected with FITC with a two-step amplification. Antibodies were diluted in TNB. Preparations were counterstained by mounting in Vectashield (Vector Laboratories) containing 1.5 μg/ml DAPI. Slides were examined with a Zeiss Axioskop 2 plus fluorescence microscope. Fluorescent signals were detected for DAPI (λ<sub>ex</sub> = 360 nm, λ<sub>em</sub> = 420 nm), FITC (λ<sub>ex</sub> = 480 nm, λ<sub>em</sub> = 515 nm), and Texas red (λ<sub>ex</sub> = 560 nm, λ<sub>em</sub> = 645 nm). Monochrome digital images were captured using a charge-coupled device AxioCam camera and stored using AxioVision, version 4.8 for Windows. Composite images were constructed using Adobe® Photoshop CS2 version 9.0.

Table 1. (Continued)

| SCAR Primers<sup>a</sup> | PS26 contig length (bp) | 454 contig hit to sorghum genome | Velvet contig length (bp) | Sorghum hit | e-value | Sorghum start | FT Millet Hits | e-value | FT Millet start | Presence of SCAR marker in the 312 line |
|------------------------|------------------------|---------------------------------|--------------------------|-------------|--------|---------------|---------------|--------|----------------|-------------------------------------|
| 1654/1655* (m)         | c5851                  | 228                             | n                        | chr-2 chr-1 | 2E-36 2E-29 | 75871067 58867780 | chr-2 | 2.00E-55 | 47,643,065 | yes                                    |
| 1708/1709              | c704                   | 675                             | y                        | 1830 chr-2  | 0.00E+00 | 77,649,034 | chr-2 | 0         | 49,009,115 | N/S                                    |
| 1528/1529              | c2339                  | 383                             | n                        | 3652 chr-2  | 0.00E+00 | 77,783,329 | chr-2 | 0         | 49,087,867 | No                                     |
| 1696/1697              | c22381                 | 185                             | n                        | 1265 chr-2  | 1.00E-63 | 77,862,634 | chr-2 | 3.00E-53 | 49,107,338 | N/S                                    |

<sup>a</sup>Primer information from [13].

<sup>*</sup>BAC clones were isolated for these markers. (m) and (h) denote BAC clones with medium and high repetitive DNA. Underlined PS26 contig BAC clones were used for physical mapping.

<sup>b</sup>BAC clone did not give a single FISH signal. N/A–marker not scored. N/S–marker not specific in 312 segregating line.

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Deletion Study

**Pollen irradiation.** Pollen was collected in glassine bags from individual heads each morning between 10:30 and 11:00 am from greenhouse-grown plants. Individual plants were derived from six backcross 8 lines and one backcross 7 line, genotyped as ASGR positive. Pollen was irradiated within 30 minutes of collection in the glassine bags using a J.L. Shepherd Model 109-GR-12 self-contained Cobalt-60 irradiator set for either 2 or 3 Kr of Cobalt-60 gamma radiation. After irradiation, the pollen was immediately used to pollinate inflorescences of sexual tetraploid pearl millet which had stigmas exerted, but at least a day before pollen shed.

**Molecular screen of plant lines derived from irradiated pollen crosses.** DNA was extracted from equal amounts of tissue from 4 green plants within a line using a modified CTAB method [14]. DNA was then amplified with ASGR specific primers 787/788 and ASGR-carrier chromosome CAPS marker p1670/71 and SCAR marker 1656/1657 [13].

Results and Discussion

Longer sequences were identified for 44 of the 49 ASGR-carrier chromosome 454 contigs within the Velvet assemblies (Table 1, S1 File). The Velvet-assembled contigs had to share at least 90% or greater sequence identity to the ASGR-carrier chromosome 454 contigs [13]. The longest available ASGR-carrier chromosome contig was used for BlastN (cutoff of $e^{-20}$) in silico analysis against the foxtail millet (NW_004675962.1) and sorghum (NC_012877.1) genomes at the National Center for Biotechnology Information (NCBI). With the additional contig lengths, 47 and 44 of the ASGR-carrier chromosome contigs showed similarity to the foxtail millet and sorghum genomes, respectively. Forty-three (88%) and 37 (82%) of the ASGR-carrier chromosome contigs showed a unique or highest similarity to foxtail millet and sorghum chromosome 2 with individual contig hits distributed along the length of chromosome 2 of both species (Table 1). Contig PS26_c9369, tightly linked to the ASGR [13], had similarity to chromosome 1 in foxtail millet and chromosomes 3, 4, 6, and 10 in sorghum. PS_c194, PS_c28392 and PS_c2807 had similarity to foxtail millet chromosome 2 but did not have corresponding BlastN hits to the sorghum genome. PS_c9776, PS_c9993 and PS_c13655 had hits to foxtail millet chromosome 2 but identified more significant similarity to genes on sorghum chromosomes 6 or 7. These noted PS contigs did not tightly cluster in a particular area of the foxtail millet chromosome. PS26_c283, aligned on sorghum chromosome 2 but to foxtail millet chromosome 9, although it was identified on a scaffold mapped to chromosome 2 in the Beijing Genomics Institute (BGI) foxtail millet genome assembly (http://foxtailmillet.genomics.org.cn). The identification of large-scale collinearity between sorghum and foxtail millet for chromosome 2 was expected based on whole-genome dot plot comparisons which show that chromosome 2 in sorghum and foxtail millet share large degrees of similarity except at the centromeric region [4]. While the pearl millet genome is not yet available, comparative mapping revealed that pearl millet linkage group 7 is homoeologous to foxtail millet 2 [23] and is likely the homoeologous chromosome for the *P. squamulatum* ASGR-carrier chromosome.

A range of one to five BAC clones were isolated from the polyhaploid BAC library [15] for 25 of the 49 ASGR-carrier chromosome transcripts (Table 1). The relative amount of repetitive DNA within each BAC clone was assayed by the signal strength and number of restriction fragments of the BAC DNA hybridizing to labeled 06-A-58 total DNA when compared to ASGR BAC clones p109 and p800 which were used as low and high copy controls, respectively [8]. BAC clones with moderately (m) or highly (h) repetitive DNA are noted in Table 1. The ASGR-carrier chromosome BAC clones were selected for physical mapping based on the in silico mapping of the contig to the foxtail millet and sorghum genomes and their level of repetitive DNA. Seven BAC clones were physically mapped to the ASGR-carrier chromosome in
The ASGR on the ASGR-carrier chromosome was detected using either a high copy ASGR-BAC clone (red pseudo-color) or a combination of a high (red pseudo-color) and low (green pseudo-color) ASGR-BAC clone. The red arrow denotes the centromere signal and the green arrow denotes the mapped ASGR-carrier chromosome BAC signal from the following: a) p285J18/PS_c2838, b) p220A02/PS_c583, c) p236E19/PS_c10535 (*), d) p036L06/PS_c2448, e) p258L05/PS_c3993, f) p236E19/PS_c10535, g) p057M05/PS_c5080, and h) p142D19/PS_c5851.

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Collinearity of a Pennisetum apomixis Carrier-Chromosome to Sorghum and Foxtail Millet Chromosome 2
of the chromosome, was unsuccessful, even with blocking, due to repetitive DNA, which hybridized as a large signal on both sides of the centromere on the ASGR carrier chromosome. Positions determined with cytogenetic mapping along the ASGR-carrier chromosome corresponded to the predicted *in silico* locations based on the sorghum/foxtail millet genomes as shown in Figs 1 and 2. Based on the *in silico* and cytogenetic mapping data, the collinearity of the *P. squamulatum* ASGR-carrier chromosome outside the ASGR and foxtail millet starts from ~5.5 Mb of foxtail millet and continues across the entire chromosome. Whether the synteny extends to the very beginning of chromosome 2 to right outside the ASGR remains unknown until additional BACs can be identified. Of the seven *P. squamulatum* ASGR-carrier chromosome BACs used for physical mapping, none showed additional FISH signals on the
pearl millet chromosomes. The lack of signal on the pearl millet genome could be due to FISH hybridization conditions or to the divergence of the non-genic regions between the *P. glaucum* and *P. squamulatum* chromosomes. Additional research will be required to determine if the ASGR-carrier chromosome markers will be useful to identify similar regions in other apomictic *Pennisetum* species. If the ASGR-carrier chromosome BACs show signal on other *Pennisetum* species, this may allow us to more fully understand the evolution of the apomixis locus within the *Pennisetum* species.

A published RFLP mapping study placed the apospory locus on linkage group 7b in buffelgrass (*C. ciliaris*) [24]. Sequence information could be found for 3 RFLP markers within the 6 marker linkage group covering 78.8 cM. Two markers, HHU27 (gb|H54993.1) and pPAP3A07 (gb|BM048123.1) flanked the aposporous locus at 10.7 cM and 1.4 cM, respectively. The third marker, pPAP08H05 (gb|BM048577.1) resided 43.2 cM from HHU27. All 3 markers showed highest similarity in a linear order to chromosome 7 in foxtail millet and chromosome 6 in sorghum. In foxtail millet, pPAP3A07 was located at ~14 Mb, HHU27 at ~24 Mb and pPAP08H05 at ~31 Mb. In sorghum, pPAP3A07 was located at ~19.1 Mb, HHU27 at ~50.8 Mb and pPAP08H05 at ~58.4 Mb. While limited in data points, the marker sequence comparison and karyotype differences identified between the ASGR-carrier chromosomes in *C. ciliaris* and *P. squamulatum* [9, 10] further supports the idea of translocation of the ASGR to different chromosomes between apomictic *Pennisetum/Cenchrus* species. Changes at the apomixis-controlling-locus (ACL) in the *Paspalum* genus have also been identified. Markers from the telomeric portion of the long arm of rice chromosome 12 flank the ACL in mapping studies with *P. simplex* [25] and *P. malacophyllum* [26]. However, *P. notatum* showed both rice chromosome 2 and chromosome 12 markers flanking the ACL [26, 27]. Markers identified as ACL linked in *P. simplex* were not linked to the ACL in *P. procurrens* [28]. Sorghum chromosome 2 shows synteny blocks with rice chromosome 3, 7, 8 and 9, but not with rice chromosome 2 or 12 [29].

The DNA content of the *P. squamulatum* chromosome was roughly estimated at ~200 Mbp [8] of which ~50 Mbp is the ASGR. Therefore the *P. squamulatum* ASGR-carrier chromosome has expanded roughly 2 to 3 times when compared to the corresponding sorghum and foxtail millet chromosome 2. It is likely that much of that expansion is caused by transposable elements as has been shown when comparing the number of predicted genes within a genome to genome size in many plant species [30].

With the location of the ASGR-carrier chromosome markers identified, a screen to identify backcross lines with potential structural changes to the ASGR-carrier chromosome was undertaken. We sought to identify a line where a functional ASGR locus had been moved from the *P. squamulatum* ASGR-carrier chromosome to a pearl millet chromosome via irradiated pollen (S1 Fig). For the study, irradiated pollen from offspring derived from six different apomictic backcross 8 and one apomictic backcross 7 tetraploid pearl millet lines was used to pollinate 71 sexual tetraploid IA4X heads. As the ASGR is a single-dose locus, approximately half of the pollen used in the crosses would not carry the ASGR. To help identify lines which reproduced sexually, heads from 1962 plants derived from the irradiated pollen by IA4X cross were pollinated with Red IA4X pollen and seed collected. Red IA4X pollen contains the dominant Rp1 allele which confers a red midrib color [31]. Plants producing only red progeny, indicating obligate sexuality, were not screened by DNA markers. Seventy-eight lines were initially tested for structural changes to the ASGR-carrier chromosome using ASGR SCAR marker p787/788 and ASGR-carrier chromosome SCAR markers from PS26_c6373 (*in silico* mapped approximately half way between the centromere and telomere on the long arm of foxtail millet and sorghum) and CAPS marker from PS26_c2552 (*in silico* mapped close to the telomere of the long arm in foxtail millet and sorghum) based on their production of green, and therefore
potentially apomictic, progeny. The markers chosen for screening are co-dominant and therefore would eliminate false negative results in the PCR-based screen. Fifty-one lines did not carry either the ASGR or ASGR-carrier chromosome markers tested. These lines producing green progeny were generated by self-pollination after unsuccessful crossing with Red IA4X pollen. Twenty-six lines carried all three markers and therefore did not contain large structural changes to the ASGR-carrier chromosome. One line, 312, contained both ASGR-carrier chromosome markers but not the ASGR marker. Line 312 was derived from 2 Kr irradiated pollen from the BC$_7$ line. Plant 312 was screened for reproductive phenotype by ovary clearing [32]. As expected, plant 312 formed mature sexual embryo sacs. Further screening using the ASGR-carrier chromosome SCAR markers showed that the 312 plant inherited most of the long arm of the ASGR-carrier chromosome from *P. squamulatum* (Table 1).

As shown in our preliminary screen of gamma irradiated apomictic pollen offspring, the mapped ASGR-carrier chromosome markers can be used to identify structural changes in the ASGR-carrier chromosome as found in plant 312. Additional screening of more plants may allow us to identify apomictic lines with large deletions in the *P. squamulatum* ASGR-carrier chromosome. If found, these plants could be subjected to both genomic and transcriptional sequencing which could help as a process of elimination to identify the genes controlling apomixis in *P. squamulatum*.

Supporting Information

**S1 Fig. Graphical Overview of Deletion Study Screen.** Graphical overview detailing the steps of the deletion study screen.

(TIF)

**S1 File. PS_Contigs.txt.** Fasta file of PS_contigs.

(TXT)

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Author Contributions

Conceived and designed the experiments: JAC PO-A WWH KF SD MC. Performed the experiments: SS JAC WWH BS KF SD. Analyzed the data: SS JAC WWH SD KF. Wrote the paper: JAC SS PO-A.

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