Ploidy determination of buffel grass accessions in the USDA National Plant Germplasm System collection by flow cytometry

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Received 28 September 2011; received in revised form 19 December 2011; accepted 21 December 2011

Abstract

The DNA content of 568 accessions of buffel grass (Cenchrus ciliaris L. syn. Pennisetum ciliare (L.) Link) in the USDA National Plant Germplasm System was determined through flow cytometry. Based on DNA content, 308 accessions were determined as tetraploids with 36 chromosomes, 139 as pentaploids with 45 chromosomes, 20 as hexaploids with 54 chromosomes, two as septaploids with 63 chromosomes, and 99 as aneuploids.

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Keywords: Cenchrus ciliaris; Flow cytometry; Germplasm; Pennisetum ciliare; Ploidy

1. Introduction

Buffel grass (Cenchrus ciliaris L. syn. Pennisetum ciliare (L.) Link) is an economically important warm-season perennial used primarily as a forage and range grass in many of the drier regions throughout the subtropics and tropics. After conducting a buffel grass plant collection trip in the Republic of South Africa in 1976, Bashaw (1985) reported a high amount of morphological diversity and a number of different ecotypes occurred within the former Transvaal and Cape Provinces, suggesting that this geographical region was the center of origin of the species. Following the reconfiguration of the Republic of South Africa into nine smaller provinces in 1994, the area in the former Transvaal and Cape Provinces that covers maximum buffel grass diversity currently comprises roughly the southwestern third of North West, eastern quarter of Northern Cape, northwestern quarter of Eastern Cape, and northeastern quarter of Western Cape provinces. From this presumed center of origin, the species is proposed to have migrated northwards into the drier areas of tropical and subtropical Africa, and eventually spread to Madagascar, the Canary Islands, Arabia, and the drier areas of tropical and subtropical India and Pakistan (Bogdan, 1977). Its natural area of distribution is considered to be in the semi-arid areas extending from southern Africa to India (Bogdan, 1977; Hanselka, 1988). Because buffel grass has excellent drought tolerance combined with desirable forage traits, it is an important forage and range grass in parts of Africa, including South Africa. It was introduced into many of the arid subtropical regions of the world, primarily Australia, North America, and South America, and today the species is grown on more than 50 million ha worldwide (Hanselka et al., 2004).

Buffel grass reproduces primarily by apomixis through apospory followed by pseudogamy (Fisher et al., 1954; Snyder et al., 1955). For years, the species was considered an obligate apomict, but a sexual, off-type, mutant plant was discovered growing in a seed production field in south Texas in 1958 (Bashaw, 1962). Prior to the discovery of this unique plant, all commercially available buffel grass cultivars were naturally occurring apomictic ecotypes that were selected and released because of their superior traits, usually greater vigor and higher forage yields (Loch, 1999; Hanselka et al., 2004). However, this sexual plant provided a means whereby it was
possible to breed new genotypes. The sexual plant was used as the female parent and was pollinated with pollen from superior apomictic accessions to produce a wide array of hybrids that segregated for numerous traits, including method of reproduction (Taliaferro and Bashaw, 1966). This breeding approach was successfully used to produce high yielding, apomictic hybrids with increased cold tolerance that were selected and released as improved cultivars (Bashaw, 1980). After the discovery of this sexual plant, other researchers determined that some other buffel grass genotypes were facultative apomicts (Bashaw and Johns, 1983; Bray, 1978; Sherwood et al., 1980; Visser et al., 2000). The expression of apomixis and sexuality in the facultative apomicts varies depending on the genotype, and those with a high level of sexuality can be used as the female parent in a hybridization program (Hussey et al., 1993).

Buffel grass has a base chromosome number of x=9, and its most commonly reported chromosome number is 36 (tetraploid) (Fisher et al., 1954; Hignight et al., 1991; Mehra et al., 1968; Ramaswamy et al., 1969; Snyder et al., 1955; Visser et al., 1998a,b,c). However, pentaploids (2n=5x=45) and hexaploids (2n=6x=54) have also been reported for the species, as well as a wide range of aneuploids (Fisher et al., 1954; Hignight et al., 1991; Khan and Evans, 1997; Mehra et al., 1968; Ramaswamy et al., 1969; Snyder et al., 1955; Visser et al., 1998a,b,c). Prior to the discovery and use of sexual buffel grass germplasm in breeding programs, the ploidy of germplasm used by breeders was not an issue because they only selected and released the most productive apomictic ecotypes with sufficient fertility to be propagated by seed. Once crosses between sexual and apomictic plants were made; however, knowledge of the ploidy, meiotic behavior, and fertility of the plants used in hybridization programs became more important because success depended upon using meiotically stable parental lines with compatible chromosome numbers. Little is known about the chromosome number of the buffel grass accessions in the USDA National Plant Germplasm System (NPGS) because the species has small chromosomes, that makes them difficult to count using traditional cytological methods. Rather than laboriously counting the chromosomes of each accession, an alternative approach is to use flow cytometry to determine total DNA content and hence ploidy level permitting the prediction of chromosome number of each accession. The objective of this investigation was to measure the nuclear DNA content of the buffel grass accessions in the collection using flow cytometry, allowing us to determine their ploidy. We also discuss possible ways in which ecotypes of this apomict taxon arose and evolved.

2. Materials and methods

2.1. Plant material

Seed of 568 individual buffel grass plant introductions were obtained from the USDA National Plant Germplasm System’s Plant Introduction Station at Griffin, GA. Seed of each accession was germinated and seedlings transplanted into a commercial potting soil in individual compartments 5 cm × 5 cm square and 6 cm deep in a 30 cm × 30 cm plastic potting flat. Each flat consisted of 36 individual 5 × 5 cm cells. After transplanting, the flats were placed in a warm greenhouse and regularly watered and fertilized to promote growth. When the seedlings had grown to a height of approximately 15 cm, they were clipped to induce tillering. After the seedlings produced three to five tillers, five seedlings of each accession were transplanted into a field nursery at the Texas A&M University Research Farm near College Station, TX. The soil was a Westwood silt loam (fine-silty, mixed, superactive, thermic Udifluventic Haplusterts). The rows in the nursery were 1 m apart and the distance between plants within each row was 0.91 m. These seedlings were fertilized and watered as needed and allowed to grow into mature plants.

2.2. Ploidy determination

Two or three young leaf blades were removed from one plant of each accession and placed in a labeled, plastic zip lock bag placed on crushed ice in a Styrofoam cooler for further processing and analysis. DNA determinations were made on each individual accession. Common buffel grass (2n=4x=36) was used as an internal standard. Common (T-4464) is an apomictic cultivar was selected from PI 153671 and released by the USDA-Soil Conservation Service in 1949 (Alderson and Sharpe, 1994). Five or six leaf blades were also removed from a common buffel grass plant growing in a greenhouse and maintained similarly as the test plants. A piece of leaf approximately 1 cm$^2$ was cut from a leaf blade of an individual accession and placed in a 15-cm petri dish together with a similar size piece from the standard. A drop of a commercial buffer solution (Partec GmbH, Münster, Germany) was added to the dish, and both leaf samples were finely chopped together with a double-edged razor blade until the tissue was thoroughly macerated. Approximately 0.5 ml of the buffer solution was added to the chopped tissue in the petri dish. This solution was resuspended several times with a pipette and then poured into a 3.5 ml specimen tube through a 30-μm filter to remove the debris. The tubes were maintained in crushed ice for at least 5 min, after which 1.5 ml of a commercial Partec DAPI (4′-6-diamidino-2-phenylindole) staining buffer was added to the filtered buffer solution containing the nuclei and the solution was resuspended. The samples remained on ice in the dark for a minimum of 5 min before analysis. Samples were analyzed with a Partec CA II flow cytometer and a minimum of 600 nuclei were analyzed for each sample. The ploidy of each buffel grass accession was determined by comparing the G1 peak of each accession with the G1 peak of common buffel grass. A minimum of five samples were analyzed for each accession.

Common buffel grass is a tetraploid with 36 chromosomes and it was used as an internal standard for all buffel grass accessions analyzed. The ploidy of each accession was determined by comparing the G1 peak of each accession with the G1 peak of common buffel grass. Because DAPI binds preferentially with the A and T bases, it does not accurately quantify the amount of DNA in the nuclei but is an effective fluorochrome.
for determining ploidy level. Propidium iodide (PI) by comparison has no base preference and is a more accurate fluorochrome. The DNA content of common buffel grass was quantified using PI and the cultivar had a 2C DNA content of 3.08 pg (Burson et al., 2002). Common buffel grass was also standardized with the sorghum (Sorghum bicolor (L.) Moench) line ATx623 to estimate the base pair or nucleotide content of common buffel grass based upon the sorghum genome’s complete annotated (Paterson et al., 2009) and ATx623 has a well documented 2C DNA content of 1.74 pg (Price et al., 2005). The ratio of G1 peaks between common buffel grass and ATx623 was also determined in order to relate buffel grass accession values to sorghum. In an attempt to adjust the DNA content values to more closely represent the actual quantity of DNA in the nuclei of buffel grass accessions, we adjusted the values by analyzing common buffel grass and sorghum G1 peak ratios using PI and incorporating a DAPI: PI correction factor.

2.3. Chromosome counts

Chromosome number of selected accessions was determined by counting the somatic chromosomes in their root tips. The roots were collected between 08:00 and 10:00 from plants growing in pots in a greenhouse. Depending on the size of a root, the lower 2 to 6 mm of the root was removed and incubated in a concentrated solution of 1-bromonaphthalene for 2 h. Roots were stained using the Feulgen technique; they were hydrolyzed in 1 N HCl for 10 min at 60 °C and then stained in Schiff’s reagent. After the root tips became deep purple in color, the root cap was removed and the lower 0.05 to 0.15 mm of the root tip was excised and placed on a microscope slide in a drop of aceto-carmine stain. After the root tip was physically broken into small pieces, the large debris was removed and the remaining tissue was covered with a cover slip, heated, firmly pressed, and observed using phase contrast microscopy.

3. Results

3.1. DNA content

Based on 2C DNA content, the 568 buffel grass accessions analyzed separated into six different ploidy groups that correlated with four euploid numbers and two ranges of aneuploid numbers (Table 1). The euploid plants comprised 308 presumptive tetraploid accessions, 152 presumptive pentaploid accessions, 20 presumptive hexaploid accessions, and two presumptive septaploid accessions. The two aneuploid groupings included 83 accessions with 2C DNA amounts matching a chromosome number ranging from 37 to 44 chromosomes and 16 accessions with 2C DNA amounts matching 46 to 53 chromosomes (Table 1). Based on these findings, 54.2% of the accessions were presumptive tetraploids, 24.5% were presumptive pentaploids, 3.5% were presumptive hexaploids, 0.4% were presumptive septaploids, and 17.4% were presumptive aneuploids.

Each accession analyzed, its country of origin, and predicted ploidy level/chromosome number are listed in Supplementary Table 1. This table also includes the somatic chromosome numbers of those accessions whose chromosomes were counted during this investigation as well as the chromosome numbers of accessions previously reported by Fisher et al. (1954) and Hignight et al. (1991).

3.2. Chromosome numbers

Chromosomes were counted in 39 accessions (Supplementary Table 1). In each instance the number counted matched the ploidy level predicted for that accession based on its 2C DNA content (Table 2; Supplementary Table 1). The chromosome number was counted for two of the predicted hexaploids (PIs 185562 and 365730) and one (PI 414500) septaploid. Both hexaploids had 54 chromosomes and the septaploid had 63 chromosomes (Table 2; Supplementary Table 1).

4. Discussion

Of the 568 accessions analyzed, 393 were collected in South Africa, mainly from the area considered the center of origin of the species. A total of 533 accessions were collected from what is considered to represent the natural distribution of the species which consists of Africa (485), Madagascar (1), Jordan (1), Pakistan (8), and India (38). Passport data in the USDA-ARS-NPGS’s Germplasm Resources Information Network (GRIN) online database indicates a majority of these accessions were collected in the wild. The collection site of a limited number of accessions was not documented. One accession (PI 308595) was from the Food and Agriculture Organization of the United Nations (FAO) collection in Rome, Italy. The remaining accessions were from Argentina (4), Australia (24), Mexico (1), Philippines (1), and the USA (6). Most were ecotypes introduced into these countries primary from Africa or India. Some, however, were apomictic ecotypes released as cultivars, and others were cultivars developed and released from breeding programs.

Most ploidy levels that we recorded corroborate other researchers’ findings from chromosome counts (Fisher et al., 1954; Hignight et al., 1991; Khan and Evans, 1997; Ramaswamy et al., 1969; Snyder et al., 1955; Visser et al., 1998a,b,c) that tetraploids, pentaploids, and hexaploids occur in the species as well as a range of aneuploids, but this is the first report of a septaploid (2n=7x=63) for the species. However, the frequency of the ploidy levels reported differs. Visser et al. (1998c) counted the chromosome number of 76 buffel grass

| No. accessions | 2C DNA content (pg) | Ploidy level | Predicted chromosome number (2n) |
|---------------|---------------------|--------------|----------------------------------|
|               | Range               | Mean         |                                  |
| 308           | 2.86–3.28           | 2.96         | Tetraploid 36                    |
| 83            | 3.43–3.78           | 3.69         | Aneuploid 37–44                  |
| 139           | 3.81–4.08           | 3.94         | Pentaploid 45                    |
| 16            | 4.11–4.37           | 4.25         | Aneuploid 46–53                  |
| 20            | 4.43–4.79           | 4.56         | Hexaploid 54                     |
| 2             | 5.56–5.68           | 5.62         | Septaploid 63                     |

Table 1: DNA content and predicted ploidy levels of buffel grass accessions.
Table 2
Comparison of ploidy levels of different buffel grass accessions as determined by nuclear DNA content vs. somatic chromosome counts.

| PI number | 2C DNA content (pg) | Predicted ploidy level from DNA content | Actual 2n chromosome number | Actual ploidy level |
|-----------|---------------------|----------------------------------------|-----------------------------|---------------------|
| 161633    | 2.96                | 4x                                     | 36                          | 4x                  |
| 216374    | 2.96                | 4x                                     | 36                          | 4x                  |
| 271208    | 2.96                | 4x                                     | 36                          | 4x                  |
| 365694    | 2.96                | 4x                                     | 36                          | 2n                  |
| 414461    | 2.96                | 4x                                     | 36                          | 2n                  |
| 414471    | 2.96                | 4x                                     | 4x                          | 2n                  |
| 409694    | 4.01                | 6x                                     | 54                          | 6x                  |
| 409695    | 4.05                | 6x                                     | 54                          | 6x                  |
| 226090    | 4.08                | 6x                                     | 54                          | 6x                  |
| 409287    | 4.10                | 6x                                     | 54                          | 6x                  |
| 409401    | 4.12                | 6x                                     | 54                          | 6x                  |
| 414534    | 4.11                | 6x                                     | 54                          | 6x                  |
| 409426    | 4.12                | 6x                                     | 54                          | 6x                  |
| 409675    | 4.12                | 6x                                     | 54                          | 6x                  |
| 164414    | 4.14                | 6x                                     | 54                          | 6x                  |
| 414473    | 4.15                | 6x                                     | 54                          | 6x                  |
| 409193    | 4.17                | 6x                                     | 54                          | 6x                  |
| 414446    | 4.18                | 6x                                     | 54                          | 6x                  |
| 217951    | 4.21                | 6x                                     | 54                          | 6x                  |
| 409720    | 4.22                | 6x                                     | 54                          | 6x                  |
| 409573    | 4.28                | 6x                                     | 54                          | 6x                  |
| 210693    | 4.38                | Aneuploid                              | 43                          | Aneuploid           |
| 271207    | 4.61                | Aneuploid                              | 43                          | Aneuploid           |
| 409708    | 4.69                | Aneuploid                              | 43                          | Aneuploid           |
| 284837    | 4.69                | Aneuploid                              | 43                          | Aneuploid           |
| 414485    | 4.72                | Aneuploid                              | 43                          | Aneuploid           |
| 365663    | 5.84                | 5x                                     | 45                          | 5x                  |
| 409439    | 5.84                | 5x                                     | 45                          | 5x                  |
| 409571    | 5.84                | 5x                                     | 45                          | 5x                  |
| 409557    | 5.87                | 5x                                     | 45                          | 5x                  |
| 203632    | 5.93                | 5x                                     | 45                          | 5x                  |
| 409223    | 4.17                | 5x                                     | 45                          | 5x                  |
| 409703    | 4.21                | 5x                                     | 45                          | 5x                  |
| 409704    | 4.22                | 5x                                     | 45                          | 5x                  |
| 414515    | 4.29                | Aneuploid                              | 51                          | Aneuploid           |
| 365730    | 4.35                | Aneuploid                              | 53                          | Aneuploid           |
| 414506    | 4.35                | Aneuploid                              | 53                          | Aneuploid           |
| 185562    | 4.43                | 6x                                     | 54                          | 6x                  |
| 299537    | 4.47                | 6x                                     | 54                          | 6x                  |
| 409500    | 5.68                | 7x                                     | 63                          | 7x                  |

Ploidy levels are reported as 4n, 5n, or 6n. The number of accessions collected in the area considered the species’ center of origin and reported 82.9% were tetraploids, 9.2% were pentaploids, 6.6% were hexaploids, and 1.3% were aneuploids. Our findings differed substantially for the percent of ploidy levels in that we found a lower frequency of tetraploids (54.2% vs. 82.9%) but a higher frequency of both pentaploids (24.5% vs. 9.2%) and aneuploids (17.4 vs. 1.3%). These findings further confirm the genetic and cytological diversity that exists in this highly apomictic taxon.

Findings from earlier cytological studies support our ploidy extrapolations from analyses of DNA content. Fisher et al. (1954) determined the chromosome number of five accessions analyzed in this study, reporting that PIs 161633, 171944, and 164414 were tetraploids with 36 chromosomes, PI 156546 was an aneuploid with 40 chromosomes, and PI 185562 was a hexaploid with 54 chromosomes. The flow cytometry findings in all of these accessions are consistent with the ploidy levels reported by Fisher et al. (1954). We re-counted the chromosomes in PIs 161633, 164414, and 185562 and confirmed the numbers reported by Fisher et al. (1954). This finding incidentally validates the reliability of the NPGS in maintaining germplasm. Hignight et al. (1991) reported chromosome numbers for five additional accessions (PIs 409506, 409287, 409338, 414485, and 409557) that we analyzed. Predictions from DNA content matched the reported ploidy in four instances but not in the fifth, PI 414485, which Hignight et al. (1991) reported as a pentaploid with 2n=5x=45 chromosomes but which flow cytometry data indicated was an aneuploid in the 37 to 44 chromosome range. We counted its chromosomes and determined it had 43 chromosomes, thus agreeing with the DNA analyses. Comparing DNA quantification findings with published chromosome numbers for several of the same accessions confirms the reliability of flow cytometry data in accurately predicting ploidy levels in buffel grass accessions. This observation is strengthened by correlation between our counts of chromosome numbers in 39 accessions and their DNA content.

The different ploidy levels and high number of aneuploid plants encountered in this germplasm collection suggest that apomixis has played an important role in the origin and preservation of chromosomal anomalies in buffel grass. Fertilization of unreduced gametes – B III hybridization – increases the chromosome number of plants at the full genome level and is thought to be the primary mechanism for polyploidization in plant species (Harlan and De Wet, 1975). Since the female gametophytes of apomicts have an unreduced number of chromosomes and most apomictic grasses require pollination/fertilization for endospore development and seed set via pseudogamy, the chances for this process to occur in apomicts are increased. Such 2n+n fertilization events frequently occur during controlled hybridization between buffel grass and related Pennisetum species (Bashaw and Hignight, 1990; Hussey et al., 1993). Early pollination tends to increase the frequency of 2n+n fertilization in apomictic grasses, including buffel grass (Martinez et al., 1994; Burson et al., 2002). Since buffel grass is protogynous, the chances for early pollination and 2n+n fertilization are increased (Burson et al., 2002). Under such conditions, pentaploid, hexaploid, and septaploid buffel grass types would arise over time from 2n+n fertilization. The pentaploid (2n=5x=45) buffel grass accessions (PI 409—) collected in South Africa in 1976 are excellent examples of how B III hybridization can increase the ploidy level and create new ecotypes. These plants were morphologically distinct, more cold tolerant, and cytologically unique. During meiosis, the extra 9 chromosomes did not exhibit any pairing affinity with any of the 36 buffel grass chromosomes (Bashaw and Hignight, 1990; Bashaw and Johns, 1983; Hignight et al., 1991), indicating that these pentaploids (36+9=45) originated from the fertilization of an unreduced egg of an apomictic tetraploid buffel grass plant by a haploid sperm from an unidentified cold tolerant diploid species that was not closely related to buffel grass (Bashaw and Johns, 1983). Even though meiosis in these pentaploid B III hybrids is irregular, the plants maintain the 45 chromosomes from accessions.
Aneuploidy in buffel grass arises from a combination of events, including pollen with unbalanced chromosome numbers, hybridization, and apomixis. Tetraploid buffel grass is a segmental allopolyploid and its chromosomes usually associate during metaphase I of meiosis as 14 bivalents and 2 quadrivalents with occasional lagging chromosomes at anaphase I (Fisher et al., 1954; Visser et al., 1998a). Some of the lagging chromosomes are not incorporated into developing tetrads, resulting in aneuploid pollen. Since apomixis and sexuality both occur in buffel grass, sexual plants can be pollinated by either sexual or apomictic genotypes. When sexual and apomictic buffel grass genotypes hybridize, the offspring segregate for mode of reproduction. If a sexual plant is pollinated with aneuploid pollen from an apomictic plant and is fertilized by an aneuploid sperm cell, the resulting progeny would be an aneuploid that could reproduce by apomixis. If this plant reproduces only by apomixis, its aneuploid chromosome number would be maintained and perpetuated. This sequence of events explains the large number of aneuploid plants that were found in the germplasm collection.

The findings from this investigation reveal that the accessions in the buffel grass collection consist of a wide range of cytotypes, including polyploids and aneuploids with varying chromosome numbers. This demonstrates the amount of chromosomal diversity that occurs within the species and this collection. Grass breeders who use the buffel grass germplasm in their breeding programs should ensure that they know the chromosome number of the material requested. The predicted ploidy levels in Supplementary Table 1 will provide that information to the breeders.

Supplementary materials related to this article can be found online at doi:10.1016/j.sajb.2011.12.003.

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