Sequential Reduction of *Pennisetum squamulatum* Genome Complement in *P. glaucum* (2n=28)×*P. squamulatum* (2n=56) Hybrids and their Progenies Revealed its Octoploid Status


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**Summary**  
*P. squamulatum* Fresen. is an important *Pennisetum* species by virtue of its characters, such as, apomixis, perenniality and stress tolerance, which can be utilized for pearl millet improvement. This species is hitherto believed to be 2n=6x=54, however, recent reports of 2n=56 cytotypes rendered to recheck the ploidy status. In present study, meiotic system of F1 and advanced hybrids, such as F2, F1 sibs, and BC1, involving *P. squamulatum* (2n=56) and *P. glaucum* (2x and 4x) were investigated following a scheme to sequentially reduce *P. squamulatum* chromosome complement to half, in order to determine its ploidy. The cytological results indicating high bivalent frequencies in these generations confirmed octoploid (instead of 6x) nature of *P. squamulatum*, alongwith its basic chromosome number as 7 (similar to pearl millet) with its allo-octoploid (or auto-allo octoploid) status. On the basis of cytogenetical studies viz. crossability, DNA content, genetic relatedness and cytology of advanced hybrids, placement of *P. squamulatum* in secondary gene pool of pearl millet is supported.

**Key words**  
Apomixis, Gene pool, Interspecific hybridization, *Pennisetum*, Perenniality

Genus *Pennisetum* (Family Poaceae) comprises of many agriculturally important species. The genus is polybasic in nature, representing species with x=5, 7, 8 and 9 (Jauhar 1981). *P. glaucum* (pearl millet) is utilized for grain and fodder, whereas species such as *P. purpureum* and *P. pedicellatum* are utilized exclusively as fodder crops. *P. squamulatum* is another important species by virtue of its tolerance to abiotic and biotic stresses (www. fao.org/ag/AGA/AGAP/frg/afris/DATA/138.htm), perenniality and apomictic mode of reproduction. This species has been utilized in breeding programmes of pearl millet for improvement of fodder characters (trispecific hybrids involving *P. glaucum*, *P. purpureum* and *P. squamulatum*) (Rangasamy and Ponnaiya 1963) as well as transfer of apomixis (Dujardin and Hanna 1989). Owing to high polymorphism for traits between pearl millet and *P. squamulatum*, the interspecific hybrids may be an excellent resource for genetic studies including QTL analysis of important traits, as well as production of chromosome addition lines. However, for such genetic analysis, information regarding cytological (ploidy) status is a prerequisite.

*P. squamulatum* has been hitherto believed to be with 2n=6x=54 based on x=9 (Dujardin and Hanna 1983, Jauhar 1981, Raman *et al.* 1959). However, cytotypes with 2n=56 chromosomes have been recently reported, independently by our laboratory (Roy *et al.* 2003) as well as from elsewhere (Goel *et al.* 2003, Akiyama *et al.* 2006). This cytotype is recently been registered by Plant

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Germplasm Registration Committee of Indian Council of Agricultural Research, India (Roy et al. 2006). On the basis of cytological investigations, we had presumed \(2n=8x\) (\(x=7\)) status of the species “...it could be an octoploid \(2n=8x=56\) with a basic chromosome number \(x=7\). However, this presumption requires detailed genetic analysis” (Roy et al. 2003). Molecular cytogenetic analysis using rDNA hybridizations, Akiyama et al. (2006) also suspected \(8x\) (\(x=7\)) nature of \(2n=56\), based on their studies on five diverse \(P. squamulatum\) accessions, some of them previously marked with \(2n=54\) chromosomes, “...due to differences between accessions or inaccurate counts because of large chromosome number”.

Sporadic evidences for closeness of \(P. glaucum\) with \(P. squamulatum\) have been gathered by reports on crossability between the two species (Dujardin and Hanna 1983, Kaushal et al. 2007, Marchais and Tostain 1997), DNA content trends in \(Pennisetum\) species (Martel et al. 1997), isozyme studies (Kauhali et al. 1997), internal transcribed spacer (ITS) sequences (Martel et al. 2004), and crossability with other \(x=7\) species such as \(P. purpureum\) (Hanna 1990).

Preliminary information supporting \(x=7\) nature of \(P. squamulatum\) has, thus, been accumulating. However, one of the convincing approaches to confirm the ploidy status (6x or 8x?) should come from cytology of \(P. squamulatum\) hybrids and its advanced generations by sequentially reducing its chromosome number to half, specially involving a dissimilar genome species such as \(P. glaucum\), characterized by restricted pairing between chromosomes from two parental species, a principle generally followed in production of alien addition lines (Khush 1973). The pairing behaviour of chromosome complement derived from 6x vs. 8x species will differ in subsequent generations. The species, if 8x, is likely to produce gametes containing monoploid (or its multiples) till three advanced generations involving dissimilar genome crosses, i.e. 4x in \(F_1\), 2x in \(BC_1\) and 1x in \(BC_2\). Alternatively, the 6x species is expected to produce 3x gamete in \(F_1\) and aneuploids in subsequent generations. With this hypothesis, interspecific hybrids between \(P. glaucum\) and \(P. squamulatum\) were produced and advanced to \(F_2\), \(BC_1\) and \(BC_2\) generations, to confirm the ploidy status of \(P. squamulatum\).

Materials and methods

Experimental material included three accessions of \(P. squamulatum\) (viz. IG 98-360, IG 98-361 and PS24), two induced tetraploids of \(P. glaucum\) (IG 99-748 and Tift #643). Accessions PS24 and Tift #643 were provided by W. W. Hanna, U.S.D.A. Tifton, Georgia, while other \(P. squamulatum\) accessions were indigenous collections. Accession IG 99-748 was a colchicine induced tetraploid of pearl millet (from a male fertile line 81B procured from ICRISAT, India). Five generation of hybrids between these species were produced during 2002-06, represented by \(F_1\) (5 plants with diverse morphology) (Anonymous 2003), \(F_2\) (15 plants derived from two obligate sexual \(F_1\)s), sibs between \(F_1\)s (25 plants, designated as \(F_1\) sibs), \(BC_1\) (52 plants representing different parental combinations including reciprocal crosses), as well as hybrids (5 in number) between diploid pearl millet (male sterile accession 81A, procured from ICRISAT, India) and \(F_1\) of tetraploid pearl millet and \(P. squamulatum\) and were included in the investigation. Data from same parentage was pooled for subsequent analysis, represented as generation mean, since no significant difference in pairing behaviour was observed.

Hybridization was carried out by pollinating the receptive gynoecium, previously bagged at boot stage (prior to stigma emergence), with freshly dehisced pollen from suitable parent. Apomictic plant was always used as male parent. Apomictic (aposporous) plants were discriminated from sexual plants by 4-nucleated embryo sacs that lacked antipodals, following ovule-clearing method of Young et al. (1979) on whole mount of cleared gynoecium followed by observations under differential interference contrast (DIC) microscope.

True hybrids were discriminated on the basis of morphology, flow cytometry and cytology. Flow cytometric analysis was useful in hybrid identification where parents were polymorphic in
total DNA content, such as in crosses involving *P. glaucum* (2x, 4x), *P. squamulatum* and their F1 hybrids. Flow cytometry was performed on young and disease free leaves, chopped with fine razor blade and the isolated nuclei stained in DAPI (4′-6 diamidino-2 phenylindole) following Kaushal *et al.* (2007) and observations with Partec PA1 flow cytometer.

Cytological analysis was performed to observe extent of bivalent formation during microsporogenesis to estimate genome contributions in different generations. Young inflorescences at boot stage were fixed in 3:1 solution of ethyl alcohol and glacial acetic acid. Young anthers were squashed in 2% solution of acetocarmine and data on chromosome number and associations thereof was recorded at diakinesis stage of Meiosis I in temporary slides. Suitable/representative cells were photographed. Pollen stainability was calculated by dusting freshly dehisced anthers in 1:1 solution of glycerine/acetocarmine. Well-stained and filled grains were scored from a minimum of 25 microscopic fields.

**Results**

All the three accessions of *P. squamulatum* were apomictic and contained 2n=56 chromosomes. Bivalents were predominant, with a mean of 19.3 bivalents per pollen mother cell (PMC), thereby accounting for 68.9% chromosomes involved. Other configurations included uni-, tri-, tetra-, penta-, and hexavalents (Table 1). Pollen stainability varied from 71.0 to 74.5 percent, with an average of 72.3%. Two accessions of tetraploid pearl millet (2n=28) exhibited 8.6 bivalents and 75% mean pollen fertility, and were sexual in mode of reproduction.

The F1 hybrids between *P. glaucum* and *P. squamulatum* were easily identified by visual morphological observations, being intermediate to parents and characterized by narrower leaves, multiple tillering, shedding spikelets and short involucral pedicles. Ratio of sporophytic (2n) DNA content between *P. squamulatum* (2n=56) and *P. glaucum* (2n=28) was 1.4, and thus F1 could also be identified exhibiting peak at intermediate place between parents in flow cytometric histograms (Fig. 1). Ovule clearing of F1 plants identified two plants as obligate sexual, two as obligate apomicts and one as facultative apomict. Apospory in *Pennisetum* is governed by Panicum type of embryo sac (ES) characterized by 4 nucleated ES that lack antipodals and contain single polar nucleus alongwith egg cell and synergids, in contrast to sexual plants that contained 8 nucleate ES (2 syn-
ergids, 1 egg cell, 2 polar nuclei and 3 antipodals) (Fig. 2). F₂ plants were raised from self-pollinated seeds from sexual F₁ hybrids, previously bagged at appropriate stage to avoid cross-pollination. F₁ sibs were obtained by crossing sexual and apomictic F₁s. Protogyny in F₁s was helpful in avoiding chance selfed-seeds, which was further taken care by repeated pollination of females (F₁s) at various stages of gynoecium emergence. Similarly, BC₁ plants were produced using sexual plants as females in crosses pearl millet × F₁s, or its reciprocal. True BC₁ plants were identified by morphology as well as chromosome counts (2n=35). Successful hybrids were also produced between diploid (2n=14) pearl millet (accession 81B) and a sexual F₁ hybrid (2n=42) from tetraploid pearl millet and P. squamulatum cross. The hybrid could be identified on the basis of morphology (preponderance of characters from male parent), DNA content utilizing flow cytometry (Fig. 1) and chromosome number (2n=28).

Fig. 1. Flow cytometer histograms for characterization of hybrids. X axis: relative fluorescence, Y axis: number of cells. a: Hybrids (2n=42, peak 2) between pearl millet (4x) (peak 1) and P. squamulatum (2n=56) (peak 3), b: Hybrids (2n=28, peak 2) between diploid pearl millet (2n=14, peak 1), and F₁ (2n=42) of pearl millet (4x) and P. squamulatum (peak 3).

Fig. 2. Embryo sac (ES) structures in cleared ovules as observed under DIC microscope, following whole ovule mount. Left: Sexual ES, Right: Aposporous ES, e: egg cell, p: polars, a: antipodals. Note two polys and antipodals in sexual ES, while one polar and absence of antipodals in aposporous ES.
Cytogenetic analysis of five F1 hybrids between *P. glaucum* (2n=28, represented by 28G) and *P. squamulatum* (2n=56, represented as 56S) revealed their 2n/H11005 42 chromosome complement (i.e. 14G/H11001 28S). No significant difference was observed between hybrids for chromosomal pairing behaviour and hence, data was pooled. Cytological features of parents and interspecific hybrids are represented in Fig. 3. Amongst these, 90.5 percent of chromosomes were involved in bivalent configurations that were higher than either of the parents. The pooled average association of all the F1 hybrids was observed to be 0.3 IV/H11001 0.14III/H11001 19.0II/H11001 2.7I. Similarly, average chromosome configuration...

Fig. 3. Chromosomal behaviour during microsporogenesis and pollen fertility in *P. squamulatum* (2n=56) (a–d), *P. glaucum* (2n=28) (e–h), F1 (2n=42) between *P. squamulatum* and *P. glaucum* (i–l), BC1 (2n=35) (m–p), hybrid (2n=28) between diploid pearl millet and F1 (q–t). First two columns showed Diakinesis, and last column showed pollen fertility in respective plants. a: (20II/H11001 4IV), b: (26II/H11001 1IV), c: metaphase I (1I/H11001 13II/H11001 6IV/H11001 1V), e: (7II/H11001 2III/H11001 2IV), f: (1I/H11001 4II/H11001 1III/H11001 4IV), g: anaphase (14:14), i: (21I), j: (2IV/H11001 17II), k: (dyad with 21 chromosomes each), m: (6I/H11001 13II/H11001 1III), n: (2I/H11001 12II/H11001 3III), o: tripolar meiosis with unequal chromosome distribution, q: (1I/H11001 12II/H11001 1III), r: (2I/H11001 10II/H11001 2III), s: telophase I with 14 chromosomes at each pole.

Cytogenetic analysis of five F1 hybrids between *P. glaucum* (2n=28, represented by 28G) and *P. squamulatum* (2n=56, represented as 56S) revealed their 2n=42 chromosome complement (i.e. 14G+28S). No significant difference was observed between hybrids for chromosomal pairing behaviour and hence, data was pooled. Cytological features of parents and interspecific hybrids are represented in Fig. 3. Amongst these, 90.5 percent of chromosomes were involved in bivalent configurations that were higher than either of the parents. The pooled average association of all the F1 hybrids was observed to be 0.3IV+0.14III+19.0II+2.7I. Similarly, average chromosome configura-
tion in F₂ hybrids represented 19.5 bivalents (i.e. 93% chromosomes involved in bivalent formation). Furthermore, in BC₁ hybrids (F₁×P. glaucum (4x), and its reciprocal cross), also the frequency of bivalents was higher (12.1) than multivalent and univalent associations (Table 1). Pollen fertility of F₁ plants exhibited a mean of 69% stainable pollen, while F₂ and F₁ sibs exhibited higher pollen stainability (77 and 90%, respectively). Male fertility of BC₁ plants was highly reduced (11.9%), while the crosses involving diploid pearl millet and F₁ were completely male sterile.

Hybrids between F₁ (2n=42, 14G+28S) (of tetraploid pearl millet and P. squamulatum) and male sterile diploid P. glaucum (2n=14, 14G) contained 2n=28 (14G+14S) chromosomes, as expected, with squamulatum chromosomes derived from the male parent, and 7 glaucum chromosomes each from male and female parent. The chromosomal association in these hybrids (2n=28) was also predominantly bivalents (average 12.11 bivalents) with 86.5% chromosomes involved in bivalent formation. It was, thus, invariably observed that bivalents were the predominant association in parental as well as hybrids and their advanced generations.

Discussion

The genus *Pennisetum* is polybasic in nature, represented by different species based on x=5, 7, 8 and 9. The earlier reports of 2n=54 in *P. squamulatum* (Jauhar 1981, Raman *et al.* 1959, Dujardin and Hanna 1983) led the erstwhile workers to suggest that the species in question might be an allo-hexaploid based on x=9. However, Sindhe (1976) observed 2n=56, in this species for the first time, and proposed the species to be with 2n=54+2B. The studies of Roy *et al.* (2003) and Akiyama *et al.* (2006), who reported the chromosome number of 2n=56, did not see any reason to classify two of the chromosomes to as Bs. The present work on three accessions, Roy *et al.* (2003) observations on single accession and Akiyama *et al.* (2006) report on five accessions confirms the existence of 2n=56 in *P. squamulatum*. In addition, one more *P. squamulatum* accession procured from ICRISAT (viz. IP 21948), was also identified to contain 2n=56 in our lab based on flow cytometry observations (data not shown).

With the establishment of the chromosome number of 2n=56 in *P. squamulatum* a valid question arises as to whether the accessions of *P. squamulatum* with 2n=56 should be classified as euploid, based on x=7 (2n=8x=56) or aneuploid based on x=9 (2n=6x+2). Cytological studies on F₁ hybrids involving *P. glaucum* (4x) and *P. squamulatum* 2n=56 (8x?) supported the view on this species being octoploid based on x=7 (Kaushal *et al.* 2007). Had the species been a hexaploid, the contribution of *P. squamulatum* in its F₁ hybrid with 4x, *P. glaucum*, would be a triploid gamete that will not show a near normal meiotic system with predominant bivalent associations and higher fertility, as observed by the present workers. In association with the diploid gamete from *P. glaucum* the resultant hybrid was a hexaploid based on x=7 (2n=6x=42). Presuming that 14 chromosomes, contributed by *P. glaucum*, pair as 7 bivalents in F₁ hybrids due to preferential mode of pairing, it can be deduced that out of 28 chromosomes contributed by *P. squamulatum*, 20–22 pair as bivalents. The BC₁ with tetraploid *P. glaucum* would be represented by 5x=35 with 21 glaucum (represented as 21G) and 14 squamulatum (14S) chromosomes. A maximum of 14 bivalents are expected in these plants (7 each involving glaucum and squamulatum chromosomes). Present results supported this as 12.07 bivalents were obtained.

High pollen fertility in hybrids was also an outcome of the balanced gamete formation by virtue of occurrence of higher bivalent frequency. Parents, F₁s, F₂s and F₁ sibs exhibited high pollen fertility except reduced pollen fertility in BC₁ plants (2n=35) that was attributed to unbalanced pearl millet chromosome complement, *i.e.* with 21G (=3x), which might segregate as 1–2x during microsporogenesis. Meiosis was found disturbed among these BC₁ plants and numerous meiotic abnormalities were observed (Fig. 3). Disturbed meiosis, leading to male and/or female sterility, in interspecific hybrids involving dissimilar genome parents is of common occurrence in grasses (Khush
allopolyploid origin of the species. However, the existence of a few multivalent configurations in
of two genomes. Together with cytological evidence by present workers, there is no doubt about the
18S-5.8S-26S rDNA chromosomes showed strong centromeric signals also supported the existence
centromeric signals, implying that at least two genomes in the said species. Half of the number of
and its F₂ hybrids with
squamulatum
reported triploid pearl millet (2n=3x=21) was also highly reduced (Jauhar 1981). Similarly,
male sterility in hybrids (2n=28, 14G+14S) between diploid pearl millet and F₁ might be due to
cytoplasmic male sterility of the female parent (81A), for which fertility restorers might be absent
in the male parent. Most convincing evidence had come from cytological analysis of these plants.
They showed up to 14 bivalents, with an average of 12.11 bivalents. This configuration wouldn’t
have been possible, had P. squamulatum been at x=9 level. At x=9 for P. squamulatum, low bivalents
and high univalents were expected, which was not the case in present study, as we obtained
only 3.62 univalents. In similar crosses, Dujardin and Hanna (1985) observed up to 10 bivalents
with as low as 2 univalents at metaphase I. However, mean bivalents frequency was 5.22, much
lower than expected with x=9 presumption. Genotypic differences of the parental accessions,
and/or factors such as early disjunction might be one of the reasons. Similarly, a polyhaploid from
F₁ hybrid between tetraploid pearl millet and P. squamulatum represented by 2n=21 (7G+14S),
showed up to 7 bivalents with a mode of 4 bivalents (Dujardin and Hanna 1986). These configurations
support our view, since such a higher frequency of bivalents is expected only if squamulatum
chromosomes were present in diploid dose (in this generation), thereby suggesting 8x nature of the
original P. squamulatum parent.

The very existence of predominant bivalent configurations together with negligible multivalent
and univalent configurations suggest that P. squamulatum is a segmental allo-octoploid with at least
four homeologous genomes involved in its origin, showing preferential/differential pairing depend-
ing upon the number of sets available in parents (8 sets), F₁ hybrids (4 sets) and backcross with
tetraploid or diploid glaucum (2 sets) (Fig. 3).

The conclusions based on cytological studies are aptly substantiated by previous studies
(Akiyama et al. 2006, Goel et al. 2003). The former workers observed 2n=56, in P. squamulatum
accession PS26, out of which eight chromosomes had 18S-5.8S-26S rDNA loci. Furthermore,
Akiyama et al. (2006) observed groups of chromosomes with weak and strong intensity of FISH
centromeric signals, implying that at least two genomes in the said species. Half of the number of
18S-5.8S-26S rDNA chromosomes showed strong centromeric signals also supported the existence
of two genomes. Together with cytological evidence by present workers, there is no doubt about the
allopolyploid origin of the species. However, the existence of a few multivalent configurations in P.
squamulatum and its F₂ hybrids with P. glaucum can be attributed to the segmental allopolyploid
nature of P. squamulatum.

The present studies on crossability of P. squamulatum with P. glaucum together with earlier re-
ports (Dujardin and Hanna 1983, Kaushal et al. 2007, Marchais and Tostain 1997), it is clear that P.
squamulatum hybridizes easily with P. glaucum when the chromosome number of latter is doubled
(2n=4x=28). The success of the cross is an ample indication that the two species are close enough
and only barriers to crossability is the difference in ploidy level between the two species, and not
the basic chromosome number (accounting for Endosperm Balance Number (EBN) considerations).
This, however, is not true with other (x=9) species like P. pedicellatum and P. polystachyon which
do not cross easily (?) with P. glaucum tetraploid as female parent. The only report of species with
x=9 which crosses easily with P. glaucum (diploid) is diploid P. orientale (2n=18) (Patil and Singh
1964, Zadoo and Singh 1986). The haploid complement of P. orientale as observed in F₁ and B₃ ill
hybrids with P. glaucum exhibits the formation of 1 or 2 intraspecific bivalents associations, which
gives an indication that x=9 of P. orientale may be probably derived from x=7.

Thus, based on the crossability and interfertility of P. glaucum and P. squamulatum, followed
by near normal meiotic system, the close relationship of the two species is implicit. It is thus imper-
vative to redesignate P. squamulatum, till now placed in tertiary gene pool, into secondary gene pool,
together with other x=7 species P. purpureum, rather than club it with P. pedicellatum, and P. poly-
stachyon (both based on x=9) of tertiary gene pool.
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References


