Genome Size Variation and Basic Chromosome Number in Pearl Millet and Fourteen Related *Pennisetum* Species

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A systematic analysis of *Pennisetum* species has shown their cytological variability for basic chromosome number (x = 5, 7, 8, and 9), ploidy level, and chromosome size. We have identified the genome size and base composition of 15 species using flow cytometric procedure in order to test the relationship of these characters with the variation in basic chromosome number and to evaluate the ascendant dysploidy hypothesis from an ancestral x = 5 group. Ethidium bromide, bisbenzimide Hoechst 33342, and chromomycin dyes have been used for genome size, percentage of AT, and percentage of GC nucleotides determination, respectively. Results show that genome sizes are variable among the *Pennisetum* species. This variation appears to be negatively related to the basic chromosome number. On the contrary, genome size is not correlated with percentage of GC. This result shows that loss or gain of DNA that has occurred during evolution within this genus seems not to be especially associated with GC- or AT-rich sequences.

The genome size and chromosome size variation between species may be immense (Bennett and Leitch 1995; Bennett and Smith 1976; 1991; Bennett et al. 1982; Hinegardner 1976; Price 1976). In higher plants, the 2C value ranges at least from 0.33 pg in *Arabidopsis thaliana* ($2x = 2n = 10$) to 155 pg in *Viscum album* (C. Cantrel in Marie and Brown 1993). Only a minor fraction comprises sequences with coding function. The chromosomes of eucaryotes carry a major component of DNA that consists substantially of repetitive sequences that are not transcribed (Dover and Flavell 1982; Flavell 1980; Vedel and Delseny 1987). The amount of heterochromatin, frequently containing highly repeated GC-/or AT-rich sequences (John et al. 1985; Sentis et al. 1986), has been positively correlated with genome size in the *Crepis praemorsa* complex (Godelle et al. 1993), in the genus *Allium* (Narayan 1987), and in the genus *Zea* (Rayburn et al. 1985, 1989). These repeated sequences presumably have a functional activity. They are present in some specific domains such as telomeres and centromeres or interspersed in the genome (Bedbrook et al. 1980; Ganal et al. 1988; Lapitan et al. 1989). Their participation in organization of the three-dimensional matrix of the interphase nucleus has been described (Manuelidis 1982). They are also involved in processes like sister chromatid exchange, transposition, and Robertsonian fusion, and thereby may act on the long-term evolution of the species and upon speciation itself, acting as a barrier to exchange. Fixation of macromutations associated with chromosomal rearrangements, amplifications, deletions, and translocations has created structural and sequence composition differences between genomes of closely related species (Hulbert et al. 1990; Moore et al. 1995; Tanksley et al. 1988). Some quantitative changes affect chromosome pairing and chiasma formation at meiosis (Rees et al. 1982) and have often accompanied divergence and evolution of species. For example, changes in DNA repetitive sequences due to some macromutation events have been described between *Hordeum* and *Secale* (Flavell 1982). These changes may be linked to the divergence of these genera or may have occurred since.

The genus *Pennisetum* includes pearl millet (*P. glaucum*), an important crop widely cultivated in Africa and Asia, and its two wild relatives *P. violaceum* and *P. mollissimum*. These species are not reproductively isolated and are subunits of the same gene pool (Brunken 1977). Other species of this genus are prized for their fodder (*P. purpureum, P. ramosum*) or are used as ornamental plants (*P. villosum, P. setaceum*). This genus is a heterogeneous assemblage of species with different basic chromosome numbers (x = 5, 7, 8, and 9), ploidy levels (diploid to octoploid), reproductive behavior (sexual or apomictic), and life cycle (annual, biennial, or perennial). According to morphological characters, species of this genus are classified into five sections: *Penicillaria, Brevivalvula, Eu-pennisetum, Heterostachya,* and *Gymnothrix* (Stapf and Hubbard 1934). Some sections involve species with different ba-

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Table 1. *Pennisetum* species used in this study[a]

Species	Chromosome number and ploidy[b]	Morphological section	Reproductive behavior[c]	Life cycle[d]	Source of plant material
P. ramosum	$2n = 2x = 10$	*Gymnothrix*	s, a	a, b	ORSTOM
P. violaceum	$2n = 2x = 14$	*Penicillaria*	s	a	Orsay
P. mollissimum	$2n = 2x = 14$	*Penicillaria*	s	a	Orsay
P. glaucum	$2n = 2x = 14$	*Penicillaria*	s	a	Orsay
P. schweinfurthii	$2n = 2x = 14$	*Heterostachya*	s	a	ORSTOM
P. purpureum	$2n = 4x = 28$	*Penicillaria*	s	p	ICRISAT
P. mezianum	$2n = 4x = 32$	*Gymnothrix*	a	p	ICRISAT
P. hohenackeri	$2n = 2x = 18$	*Gymnothrix*	s, a	p	ICRISAT
P. alopecuroides	$2n = 2x = 18$	*Gymnothrix*	s	?	ICRISAT
P. setaceum	$2n = 3x = 27$	*Eu-pennisetum*	a	p	ICRISAT
P. setaceum	$2n = 6x = 54$	*Eu-pennisetum*	a	p	ICRISAT
P. orientale	$2n = 4x = 36$	*Heterostachya*	a	p	ICRISAT
P. villosum	$2n = 4x = 36$	*Eu-pennisetum*	a	p	Belgium
P. polystachyon	$2n = 6x = 54$	*Brevivalvula*	a	a	ICRISAT
P. pedicellatum	$2n = 6x = 54$	*Brevivalvula*	a	p	ORSTOM
P. squamulatum	$2n = 6x = 54$	*Heterostachya*	a	p	ORSTOM

[a] Some characteristics of the 15 *Pennisetum* species used in this study

[b] The chromosome number was confirmed by cytogenetics with Feulgen-stained material and ploidy deduced with reference to the literature.

[c] s = sexual; a = apomictic.

[d] a = annual; b = biennial; p = perennial; ? = not available

sic chromosome numbers. There is also diversity in the size of the chromosomes, with the larger size for the lower numbers (Jauhar 1981). This diversity in size and chromosome number reflects chromosome repatterning during evolution within the genus. Based on cytogenetic data, a hypothesis on the evolution of the chromosome complement in *Pennisetum* from a basic number $x = 5$ has been proposed (Jauhar 1968, 1981; Rao et al. 1989). Changes in basic chromosome numbers during evolution could have been produced by irregular meiosis in hybrids with multivalent formation, consequently resulting in loss or gain in chromosomes (Jauhar 1993). When speciation is associated with modification in chromosomes size, differences in base pair composition may be detected if this modification has involved repeated sequences, particularly those that are GC or AT rich.

In order to test for these modifications, we present here data of a study of genome size and base composition using flow cytometric procedures on 15 *Pennisetum* species. These species were chosen to represent the different basic chromosome numbers ($x = 5$, 7, 8, and 9) and diverse ploidy levels (diploid, triploid, tetraploid, and hexaploid).

Materials and Methods

Plant Material

The species used in this study are listed in Table 1. Seeds obtained from collections of the botanic garden of Antwerpen (Belgium), University of Orsay (France), ORSTOM (France), and ICRISAT (India) were germinated and grown in a glasshouse. The chromosome number and ploidy level were cytologically confirmed by Feulgen procedure according to Khalfallah et al. (1993). Their morphological section, reproductive behavior, and life cycle are also indicated.

Nuclear Preparation and DNA Staining

Nuclei were isolated from the leaf tissue according to Galbraith et al. (1983) and stained for flow cytometry using a buffer of Marie and Brown (1993). A piece of leaf of *Medicago sativa* ssp. X varia cultivar Rambler A2 ($2n = 4x = 32$) as internal standard was chopped together with the *Pennisetum* leaf. This *Medicago* species has 2C = 3.47 pg with 38.7% GC (Blondon et al. 1994). Ethidium bromide (Sigma) was used at 50 μg/ml for genome size determination, after RNase incubation. Measurements with bisbenzimide Hoechst 33342 (5 μg/ml; Aldrich Chimie) and with chromomycin (50 μg/ml; Serva) were made to determine base composition. Each measurement was made on 2,000–3,000 nuclei using an EPICS V cytometer (Coultronics, France). Analyses were repeated at least twice for three to five plants per species (Table 2). The coefficients of variation observed for the *Pennisetum* peak within each histogram averaged 4.1%, with a median of 3.8% (data not shown).

DNA Amount and Base Composition

The fluorescent ratios obtained for each sample are

- R_{Eb} = intensity of the sample/intensity of the standard, with the ethidium bromide dye.
- R_{Ho} = intensity of the sample/intensity of the standard, with the Hoechst 33342 dye.
- R_{Ch} = intensity of the sample/intensity of the standard, with the chromomycin dye.

The DNA amount of each sample is taken from R_{Eb} and the known DNA amount of the standard:

$$Q_{\text{DNA sample}} = R_{Eb} \times Q_{\text{DNA standard}} \quad (1)$$

An estimation of the mean DNA content per chromosome for each species (= DNA content in 2C nucleus (pg)/total chromosome number) has been calculated. This parameter is useful when comparing the

Table 2. DNA amount and base composition by flow cytometry with *Pennisetum* species

Species	2C DNA (pg)	Replications (n)	Standard error (pg)	DNA per haploid genome (pg)	Mean DNA per chromosome (pg)	GC (%)	Replications (n)	Estimated standard deviation[a] (%GC)
P. ramosum	4.04	4	0.02	2.02	0.404	43.7	10	0.16
P. violaceum	4.52	8	0.03	2.26	0.323	47.2	5	0.64
P. mollissimum	4.51	9	0.12	2.25	0.322	46.5	6	0.76
P. glaucum	4.71	11	0.05	2.36	0.336	44.9	13	0.61
P. schweinfurthii	4.97	11	0.13	2.49	0.355	44.0	13	0.48
P. purpureum	4.59	6	0.08	1.15	0.164	44.3	7	0.27
P. mezianum	3.01	8	0.09	0.75	0.094	43.0	6	0.17
P. hohenackeri	1.69	12	0.06	0.85	0.094	42.7	10	0.19
P. alopecuroides	1.90	13	0.10	0.95	0.106	43.5	12	0.18
P. setaceum (3×)	2.78	8	0.04	0.93	0.103	43.6	10	0.27
P. setaceum (6×)	5.28	2	0.00	0.88	0.098	43.9	2	0.06
P. orientale	3.77	15	0.10	0.94	0.104	44 6	19	0.60
P. villosum	3.47	7	0.00	0.87	0.097	43.6	11	0.73
P. polystachyon	5.66	9	0.08	0.94	0.104	44.7	15	0.35
P. pedicellatum	5.61	11	0.26	0.94	0.104	43.7	9	0.53
P. squamulatum	9.56	5	0.43	1.59	0.177	44.1	9	0.65

[a] See Methods for calculation of standard deviation of mean percent GC.

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genome size at the chromosome level between species.

The base composition is calculated from the ratios and the known base composition of the standard. We have used the simplified formulae defined by Godelle et al. (1993), taking into account that a fluorochrome can only bind and fluorescence efficiently at a series of several bases of the same type:

$$\%AT_{sample} = \%AT_{standard} \times (R_{Ho}/R_{Eb})^{1/5} \quad (2)$$

$$\%GC_{sample} = \%GC_{standard} \times (R_{Ch}/R_{Eb})^{1/3} \quad (3)$$

To estimate a standard deviation (SD_{GC}) of the mean percent GC, we have used the standard error and mean of R_{Ch} (namely σ and $\bar{\chi}$) and taken into account the cube root function of Equation 3:

$$SD_{GC} = \%\ GC \times [(1 + (\sigma/\bar{\chi})^{1/3} - 1] \quad (4)$$

Results and Discussion

Genome Size

The results of DNA content (Table 2) reveal three main features. First, the DNA amount per basic chromosome set is similar for the cultivated species (*P. glaucum*) and its two wild relatives (*P. violaceum* and *P. mollissimum*) belonging to the primary gene pool, according to the Harlan and de Wet (1971) classification. These species with $x = 7$ have haploid genome size ranging from 2.25–2.36 pg (Table 2). This result is consistent with the notion that *P. glaucum* derived from *P. violaceum sensu lato*, and accordingly is conspecific with its wild and weedy relatives (Brunken 1977). Previous cytogenetic investigations of cultivated and wild stocks of this gene pool have shown the high similarity between their karyotypes (Khalfallah et al. 1993). In situ hybridization analysis has shown the similar localization of rDNA among members of the primary gene pool (Martel et al. 1996). Apparently the cultivated species and the wild forms from which it has evolved (Pernès 1983), although ecologically isolated by man's agricultural activities, have conserved a high level of similarity of their genomes.

The second feature is the similar DNA amount per chromosome set of species with the basic chromosome number $x = 9$ (Table 2), independent of their ploidy level (from 0.85–0.95 pg), with the exception of *P. squamulatum* from the section *Heterostachya*. These DNA amounts ranked among the lowest observed in the genus *Pennisetum* and concern mainly species with a perennial life cycle. In contrast to some observations, low nuclear DNA content is not associated with short life cycles, as Arumuganathan and Earle (1991) have also observed from a study of DNA content in over 100 important plant species. Among the five analyzed individuals of the triploid *P. setaceum* species, one hexaploid plant with a double genome size has been observed. Occurrence of hexaploid progenies in this triploid species has been previously observed (Simpson and Bashaw 1969). This probably results from spontaneous chromosome doubling. Genome size similarity among diploid species of this group may express their evolutionary proximity, even though they are well differentiated from a morphological point of view. The polyploid species, which seem to have maintained constant chromosome size, result from the multiplication of the basic chromosome number either by autopolyploidy or by allopolyploidy after hybridization between diploid species of the same group. Previous studies of some species of this group have noted similarities between their genomes. Hrishi (1952) observed the karyotype of six species with $x = 9$ and showed their almost identical chromosome sizes. The species of the *Brevivalvula* section form a complex where hybridization can occur (Clayton 1972) and where isoenzyme analysis shows close relationships (Lagudah and Hanna 1989).

The third feature concerns the five species with very different genome sizes contrasting with the two groups mentioned above. *P. ramosum*, the only species belonging to the $x = 5$ group, possesses a lower haploid genome size (2.02 pg) than the $x = 7$ group. Cytological observations of this species, with the lowest chromosome number of the genus *Pennisetum*, have shown that its chromosome sizes are the largest reported within this genus (Jauhar 1981; Rangaswamy 1972). Indeed, our estimation of the mean DNA amount per chromosome gave the highest value for *P. ramosum*. If the chromosome repatterning has occurred from the $x = 5$ to the $x = 7$ group, as hypothesized by Jauhar (1968), it has apparently increased the total genome size by increasing the basic chromosome number, but with a decrease of the mean chromosome sizes.

The tetraploid *P. mezianum* is the only species belonging to the $x = 8$ group and has a haploid genome size of 0.75 pg, which represents the lowest haploid genome size of the genus *Pennisetum*. The mean DNA amount per chromosome is similar for this species and those belonging to the $x = 9$ group. Such a result can be explained by many evolutionary events, among them duplication or loss of a chromosome pair according to the basic number of the ancestor. Considering that species with basic chromosome number $x = 9$ are predominant in the genus *Pennisetum*, we can suggested that $x = 8$ is probably a derivative number.

P. schweinfurthii, a diploid belonging to the $x = 7$ group, possesses the largest genome size of the genus (2.49 pg), significantly larger than species of the primary gene pool with the same chromosome number. This species is not closely related to pearl millet and possesses a strong reproductive barrier with it (Hanna and Dujardin 1986). This feature is consistent with the different chromosome organization observed between them (Martel et al. 1996). The karyotypes of these two species differ notably in the relative size of chromosome pair II, which is larger in *P. schweinfurthii* (Martel et al. 1996). This difference may explain in part the genome size distinction observed.

P. purpureum is an allotetraploid belonging to the $x = 7$ group. It represents the secondary gene pool of the pearl millet. It possesses one genome largely homologous with pearl millet and another of an unknown donor (Jauhar 1981). Reproductive barriers between *P. purpureum* and *P. glaucum* are strong, but some triploid hybrids that are partially male and female sterile occur in natural conditions. Meiotic analysis of these hybrids reveals formation of seven bivalents, where a maximum of five are the heteromorphic association of chromosomes belonging to each parent, and seven univalent chromosomes (Jauhar 1981). Despite differences in chromosome number, *P. purpureum* and *P. glaucum* have almost equal genome size (4.59 pg and 4.71 pg, respectively). These data lead us to reconsider hypotheses on genome constitution of *P. purpureum* as discussed above. Using molecular cytogenetic techniques like genomic in situ hybridization (GISH), it will be possible to test for the allotetraploid or diploid origin of *P. purpureum*. Indeed, this species may have been originated by mechanisms producing amphiploids via unreduced gametes followed by a decrease in the chromosome size. On the other hand, *P. purpureum* caryotype may have originated from fragmentation of large chromosomes of a hypothetical diploid ancestor with $2n = 14$ chromosomes. For this purpose total genomic DNA of *P. glaucum* will be probed

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on *P. purpureum* chromosomes. This technique is usually applied to detect parental genomes of a polyploid species (Bailey et al. 1993; Schwarzacher et al. 1989) and thus possibly can, in this case, confirm the tetraploid nature of *P. purpureum*.

The hexaploid *P. squamulatum* belongs to the $x = 9$ group but possesses a larger basic chromosome set (1.59 pg). This species may have evolved since its divergence by increasing its genome size by molecular events like amplification.

Interspecific hybrids between the pearl millet and the wild species belonging to the morphological sections *Gymnothrix, Heterostachya, Eu-pennisetum,* and *Brevivalvula* have been investigated in order to combine the forage quality of the cultivated and the perennial habit of the wild species to fix hybrid vigor. Hybrids have been obtained between pearl millet and the triploid *P. setaceum* (Hanna 1979), the tetraploid *P. orientale* (Hanna and Dujardin 1982), and the diploid *P. schweinfurthii* (Hanna and Dujardin 1986), but these hybrids were largely sterile. Apomictic and fertile hybrids have been produced between the hexaploid *P. squamulatum* and pearl millet (Dujardin and Hanna 1983), and cytological studies of their meiosis have shown the limited homology existing between the two genomes. The three species *P. squamulatum, P. orientale,* and *P. schweinfurthii* belong to the *Heterostachya* section which seems to be closer to the members of the primary gene pool belonging to the *Penicillaria* section, according to their crossability, than members of the other morphological sections. Among the $x = 9$ group, *P. squamulatum* is exceptional both for its DNA amount and also for having a better crossability with *P. glaucum*. A tentative explanation could be offered, assuming that the reduction of genome size from the group $x = 7$ to $x = 9$ concerns essentially sequences involved in homologous pairing during meiosis. Of all the $x = 9$ members, *P. squamulatum* could have conserved the higher repertoire of this kind of sequence. Some experiments, like analysis of the genetic system that controls meiotic pairing and chiasma formation, like the "pairing homoeologous" system (*Ph*) in wheat (see Jauhar 1991), might be undertaken among species of the primary gene pool and the $x = 9$ group, including *P. squamulatum,* in order to test this hypothesis.

Base Pair Composition

Values of percent GC calculated with R_{Ch} and R_{Eb} according to Equation 3 are presented in Table 2. Addition of the estimated percent AT obtained with Hoechst 33342 (data not shown) gave a sum of [%GC + %AT] ranging from 96.6–98.9%. This deviation from 100% may be explained by the use of a simplified equation that assumed that fluorescence of the DNA-dye complexes occurred only with a succession of 5 AT or 3 GC for the Hoechst or chromomycin fluorochromes, respectively. More precisely, a series of two and three G or C bases allows binding of chromomycin, so the exponent in Equation 3 may be changed from 3 to x, with $2 < x < 3$ (Godelle et al. 1993). Rectification of the [%AT + %GC] sum may be obtained with the use of a rectified exponent, although this has not been systematically necessary with other genera (Brown S, personal communication). The average standard deviation for these measures of percent GC was 0.42 units GC.

The relationship between the genome size and base composition has been tested. The two wild species of the primary gene pool (*P. mollissimum* and *P. violaceum*) with high haploid genome size are relatively GC rich (46.5% and 47.2%, respectively) and species with low haploid genome size have lower GC composition, like *P. hohenackeri* (42.7%). This range of values is exceptional for a single genus; compare the *Crepis praemorsa* complex (Godelle et al. 1993), the genus *Medicago* (Blondon et al. 1994), or the *Hypochoeris* complex, where some differences in the base composition have been observed ranging from 39.6–41.9% GC (Cerbah et al. 1995). The differences observed in the base composition among the *Pennisetum* species are not informative about the sequences or genome parts that may have changed during evolution and chromosome repatterning. A positive correlation between haploid genome size and percent GC is not always observed (for example, *P. schweinfurthii* with the higher haploid genome size, 2.49 pg, possesses 44% GC). This indicates low phylogenetic value of such data in the genus *Pennisetum*.

Conclusion

The genome sizes are variable among the *Pennisetum* species (Table 2). The relationship between basic chromosome number and haploid genome size is positive when chromosome number increases from five to seven, excluding *P. purpureum,* and negative when it increases from seven to eight or nine. In the case of Jauhar's hypothesis (1968, 1981) on the evolution of the *Pennisetum* species from the low basic number ($x = 5$) to the higher number ($x = 7$, 8, and 9), this result implies a general loss of genetic material when the basic chromosome number increases, with a transitional gain for the $x = 7$ group, excluding *P. purpureum*. Mean DNA amount per chromosome, calculated for these species, shows that the chromosome repatterning that has occurred during evolution within the genus has involved important changes in chromosomes size and hence loss or gain of DNA sequences.

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