HETEROCHROMATIN ORGANIZATION IN METAPHASE CHROMOSOMES AND INTERPHASE NUCLEI OF *DASYPYRUM BREVIARISTATUM* (LINDB F) FREDERIKSEN

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ABSTRACT

Metaphase chromosomes of *Dasypyrum breviaristatum* (Lindb f) Frederiksen, a tetraploid wild species, were differentially stained with C-banding and fluorochromes in order to acquire information on heterochromatin chromosomal distribution and composition. DNA content and relative amount of nuclear heterochromatin were determined by cytophotometric analysis after Feulgen reaction. The results were compared to those of *Dasypyrum villosum* (L.) P. Candargy, a diploid species of the same genus. The achieved information indicate that *D. breviaristatum* and *D. villosum* differ in the composition, organization and distribution of heterochromatin, and may suggest that the telomeric regions of the chromosomes of the two species are more differentiated than the centromeric ones, as a result of a long lasting divergence between the two species.

KEY WORDS: *Dasypyrum breviaristatum*, *Dasypyrum villosum*, heterochromatin distribution, chromatin organization, nuclear DNA content, phylogenetic relationships.

INTRODUCTION

The genus *Dasypyrum* comprises two wild growing species: the tetraploid perennial *D. breviaristatum* (Lindb f) Frederiksen [$2n=4x=28$; syn. *D. hordaceum* Candargy, *Haynaldia hordeacea* (Coss. et Dur.) Kackel], growing in limited inner mountain areas of Morocco, Algeria and Greece (Frederiksen 1991) and the diploid annual outcrosser *D. villosum* (L.) Candargy ($2n=2x=14$; synonyms *Secale villosum* L., *Haynaldia villosa* Schur) which is widespread in dry grassland of the Mediterranean region, SE Europe and NW Africa. The genus *Dasypyrum* is a potential donor of useful genes for powdery mildew and eyespot resistance, drought tolerance, seed storage protein content and quality to wheat (De Pace et al. 1988; Murray et al. 1994; Ricciardi et al. 1996; Chen et al. 1997) but their use may be limited by a reduced level of recombination between *Dasypyrum* and wheat chromosomes in hybrids.

Characterization of genome structure, organization and relationships is important for the exploitation of wild species germplasm in plant breeding using lines derived by wide hybridization. Studies at the cytogenetic level are particularly useful in reaching a better understanding of the genomes of species which are of interest to breeders but of yet unproven

value, and increase the possibility of introgressing chromosome segments into breeding lines (Gill and Sears 1993). The genome of *D. breviaristatum* is much less characterized than that of *D. villosum*, which in recent years has been the object of many studies.

The origin and genomic constitution of *D. breviaristatum* and its phylogenetic relationships with *D. villosum* have long been debated. Autopolyploid derivation (Sarkar 1957; Frederiksen 1991 and references therein) and allopolyploid origin (Linde-Laursen and Frederiksen 1991) have been suggested. Blanco et al. (1996) based on the study of DNA sequences of the nuclear and plastidial genome, isozyme analysis, interspecific hybridization, and chromosomal localization of structural genes were inclined to support the autopolyploid origin of *D. breviaristatum* with some reservations. Galasso et al. (1997) using Southern and *in situ* hybridization of total genomic and cloned DNA probes substantiated the assumption of an autotetraploid origin but pointed out that *D. breviaristatum* is unlikely to have directly derived from *D. villosum* by simple autopolyploidization.

The characterization of heterochromatin and its chromosomal distribution have often been used for taxonomical and phylogenetic studies in higher plants. For instance, C-banding

and other differential staining techniques are useful to localize and recognize different types of heterochromatin and the organization of the amount of condensed chromatin in the interphase nucleus has been used as a parameter for heterochromatin determination (Schweizer 1977; Havelange and Jeanny 1984; Pignone et al. 1995).

The aim of the present study is to contribute to the investigation on the phyletic relationships between the two species of *Dasyphyrum* using the structure and localization of chromatin in metaphase chromosomes and interphase nuclei as discriminating characters.

MATERIALS AND METHODS

Seeds of a natural populations of *D. breviaristatum*, (HH8130 Plant Germplasm Institute, University of Kyoto, Japan, and increased at the University of Bari) and *D. villosum* (collected near Campobasso, IT) were germinated in Petri dishes at $22 \pm 1^\circ\text{C}$ in the dark.

Chromosome banding: actively growing roots were excised and treated for 24 h with ice cold distilled water, then fixed for 24 h in ethanol-acetic acid (3:1, v/v). Root tip meristems were squashed under coverslips in drop of 45% acetic acid and coverslips were removed by the dry-ice method. C-banding, and fluorochromes staining (DAPI, Chromomycin A3, and Hoechst 33258) were performed according to Giraldez et al. (1979), and Galasso and Pignone (1992), respectively. A Leitz Aristoplan microscope fitted with epiluminescence was used. The filter combination A was used to observe Hoechst 33258 (H 33258) and DAPI staining, and filter block E3 to observe Chromomycin A3 (CMA) fluorescence.

Pictures were taken on film or with a Sony XC 75 b/w CCD camera; digital images, with 512×512 pixel $\times$ 8 bit or $720 \times 512 \times 8$ resolution, were processed running the Leica QWIN software to obtain quantitative information; some images were subsequently improved with Adobe Photoshop 3.0, using algorithms applying to the whole image at the same time, prior to printing with a Mitsubishi CP-D1E photo printer.

Chromosomal C-banded heterochromatin (C-Het.) amount was determined on unprocessed digital images using the QWIN software, following the method described by Venora et al. (1995); this value represents the ratio between the chromosomal surfaces occupied by C-bands and the total surface of the chromosomes. The term surface is intended as the two-dimensional projection of chromosome image on the CCD plane. The value was calculated on 20 well spreaded metaphases per species.

Cytophotometric analyses: one cm long root tips were fixed in ethanol-acetic acid (3:1, v/v) and squashes were made in a drop of 45% acetic acid after treatment with a 5% aqueous solution of Pectinase (Sigma) for 1 h at 37°C , with the addition of 1 mM EDTA in order to neutralise the activity of possibly present DNase. After coverslip removal by the dry-ice method, squashes were hydrolysed in 1N HCl at 60°C for 7 min, stained with Feulgen reagent, subjected to three 10 min washes in SO_2 water, dehydrated and mounted in DPX (Fluka). Squashes of root tips of *Vicia faba* were concurrently stained for each group of slides and were used as internal standard; absorptions measured in *Vicia faba* preparations were also used to convert relative Feulgen arbitrary units into picograms of DNA. Feulgen DNA absorption in nuclei in post-synthetic condition (G_2 phase) was measured at 550 nm by a Leitz MPV3 integrating microdensitometer equipped

with Halogen-Bellaphot lamp (Osram) and HP computer. With the same instruments and at the same wavelength, the Feulgen DNA absorption of chromatin fractions with different condensation levels was measured in the same nucleus, after selecting different thresholds of optical density. A mathematical elaboration based on Simpson's rule allows the determination of the position of the inflexion point of the optimized curves obtained from the experimental data. This point makes it possible to discriminate two areas (A_1 and A_2) in each curve, and to determine the amount of nuclear heterochromatin (Cremonini et al. 1993).

RESULTS

The distribution of the heterochromatin in *D. breviaristatum* was mainly centromeric (Fig. 1a) while in *D. villosum* the more conspicuous bands are located at the telomeric regions (Fig. 1b). Visual examination of the chromosomal distribution of C-bands, therefore, evidenced little similarity between the karyotypes of the two *Dasyphyrum* species. Nevertheless, C-Het. values, i.e. the amount of C-banded heterochromatin determined by image analysis, were similar in the two species: 0.34 in *D. breviaristatum* and 0.31 in *D. villosum* (Table 1).

TABLE 1. Areas (A_1 and A_2 , arbitrary units), nuclear heterochromatin amount (Het.), and chromosomal C-banded heterochromatin (C-Het.) in *Dasyphyrum* species.

Species	A_1	A_2	Het.	C-Het.
<i>D. breviaristatum</i>	916	493	0.3499	0.34 ± 0.12
<i>D. villosum</i>	467	244	0.3432	0.31 ± 0.09

The absence of apparent similarity between the karyotypes of the two species is more evident after H33258 and DAPI staining. In fact, *D. breviaristatum* chromosomes showed no regions with bright fluorescence (Fig. 1c), contrary to *D. villosum* which showed bright blocks at the telomeric regions with both fluorochromes (Fig. 1d).

Staining with CMA showed bright blocks at the secondary constrictions of the Sat-chromosomes in both species: *D. breviaristatum* showed three pairs of CMA-bright sites (Fig. 1e), while *D. villosum* only one pair (Fig. 1f). Additionally, in the tetraploid species a certain level of polymorphism was observed, since some nonsatellited chromosome showed 1 or 2 additional CMA-bright sites at their telometric region.

When the C-banded karyotype of *D. breviaristatum* was analysed to determine the internal homology groups, it was not possible to split it up into two similar sets as one would expect in a pure autotetraploid species (Fig. 1a and 1b). Chromosome pairs were rather easily formed, but only few showed a possible resemblance to other pairs.

Cytophotometric determination carried out in *D. breviaristatum* and *D. villosum* showed that the mean nuclear DNA content was 42.05 pg/4C nucleus in *D. breviaristatum*, and 24.17 pg/4C in *D. villosum* (Table 2). No significant difference in the nuclear DNA content of early prophase exists between primary and secondary root tips or between apical and root meristems of the same seedlings (data not shown). In order to assess the amount of nuclear heterochromatin in the two species, measurements were taken following the technique of Cremonini et al. (1993) at different thresholds of op-

Fig. 1. Mitotic metaphases of *Dasypyrum breviaristatum* (a, c, e) and *D. villosum* (b, d, f) after C-banding (a and b), DAPI (c and d), and Chromomycin A3 (e and f). Stars indicate CMA bright sites: two of them in *D. breviaristatum* (e) are not associated to secondary constrictions.

tical density. The residual Feulgen absorption at inflexion point of the optimized curves obtained from cytophotometric data marks the nuclear heterochromatin. According to Simpson's elaboration, it is possible to calculate the two areas separated by the inflexion point: A_1 and A_2 accounting respectively for less dense and denser nuclear structures (Fig. 2). The amount of nuclear heterochromatin (*Het.*) is expressed by the ratio:

$$Het. = \frac{A_2}{A_1 + A_2}$$

It is interesting to notice that the two species show quite different absorption curves, but similar ratios between the two areas, i.e., 0.3499 for *D. breviaristatum* and 0.3432 for *D. villosum* (Table 1). This indicates that both species possess approximately 35% of heterochromatin in interphase nuclei.

TABLE 2. Feulgen absorption, nuclear DNA content of early prophase nuclei (4C), and inflection point of absorption curves in the root meristems of *D. villosum*, *D. breviaristatum* and *Vicia faba* samples. Feulgen absorption is the mean of 100 determinations (20 nuclei for each of five seedlings); a.u. = arbitrary units.

Species	Feulgen absorption (a.u., mean $\pm$ S.E.)	Nuclear DNA content (pg, mean $\pm$ S.E.)	Inflection point a.u.
<i>D. breviaristatum</i>	4558.3 $\pm$ 31.6	42.05 $\pm$ 0.18	15
<i>D. villosum</i>	2628.4 $\pm$ 24.2	24.17 $\pm$ 0.11	9
<i>Vicia faba</i>	5695.8 $\pm$ 27.8	53.12*	18

*after Ceccarelli et al. (1995)

Fig. 2. Absorption curves of *Dasypyrum villosum* (top) and *Dasypyrum breviaristatum* (bottom). The inflexion point is indicated with the symbol V; the optimized areas A₁ and A₂ represent the amounts of lowly and highly condensed chromatin fractions (Cremonini et al. 1993).

DISCUSSION

The present study demonstrates that *D. breviaristatum* and *D. villosum* possess different chromatin organization. This assumption is based on evidence of chromosomal heterochromatin distribution and reacting properties: the two species of *Dasypyrum* do not show similar heterochromatin composition/organization in telomeric/subtelomeric regions, while a greater similarity is observed in the fraction of heterochromatin with centromeric distribution. In fact, *D. breviaristatum* heterochromatin is mostly located at centromeric and/or intercalary domains and does not show bright fluorescence when stained with DAPI or H33258, while *D. villosum* heterochromatin is organized in large telomeric blocks showing bright fluorescence when stained with DAPI or H33258.

The determination of nuclear DNA absorption curves has been discussed in previous papers (Cremonini and Funari 1992; Cremonini et al. 1993) and it has been hypothesized that the residual Feulgen absorption at the inflexion point represents the nuclear heterochromatin (Cremonini et al. 1993), that is that fraction of the chromatin which stays condensed during interphase. The surface of two areas subtended by the two half-curves represent the relative amounts of euchromatin and heterochromatin and may therefore be used to compute the amount of nuclear heterochromatin. Only interphase nuclei

from the meristematic portion of the root were analysed since the percentage of heterochromatin is reported to be constant and higher in meristematic than in differentiated cells (Bassi 1990 and references therein).

It is worth noticing that chromosomal and nuclear heterochromatin values are rather congruent in each species, even though the measurements are done at different organization levels of the genome (Venora et al. 1995). It is also interesting to notice that the values are similar between the two species. This might imply that some subtelomeric structures have been totally or partially lost during the evolution of *D. breviaristatum* and have been replaced by other structures absent or underrepresented in *D. villosum*; the circumstance that centromeric heterochromatin of both species shows similar properties, may depend on the fact that the centromeric regions of both species share a high degree of homology. Galasso et al. (1997) showed evidence that highly repeated DNAs from the centromeric regions of the two *Dasypyrum* species show a high degree of similarity, while sequences in the telomeric regions appear to be rather species-specific.

In the tetraploid species it is difficult to order the karyotype in seven groups of four more or less homologous chromosomes, even though some similarity exists and all chromosomes pairs appear to possess similar general heterochromatin properties. Taken as a whole, these indications might suggest that, as hypothesized by Blanco et al. (1996), *D. breviaristatum* is an ancient autotetraploid species which has considerably diverged from *D. villosum* after polyploidization; this divergence is more manifest in the telomeric regions of the chromosomes. Additionally, chromosomal rearrangements might have accumulated among the two genomic sets increasing the diploidization of the karyotype; this process might have been favoured by the reduction of meiotic disturbance affecting pure autopolyploid plants. Since the diploidization process of the *D. breviaristatum* genome appears quite advanced, it can then be assumed that the time scale separating the two species of *Dasypyrum* is quite large (Barton and Rouhani 1991). Since not all systems, to which the experimental parameters relate, evolve at the same rate, this time gap might explain the apparent incongruence of experimental data present in the literature (Hansen 1997) regarding the origin of *D. breviaristatum*.

It has been recently demonstrated that at least in many Triticeae species the subtelomeric/telomeric regions are enriched in coding sequences, while the centromeric ones are quite poor of "genes" (Pedersen et al. 1995; Castilho et al. 1996). The observed differentiation in the telomeric regions might imply that the two species of *Dasypyrum* could possess different useful traits. Until now, no genetic analysis has been conducted in *D. breviaristatum* since it was considered a mere autotetraploid form of *D. villosum*. The growing evidence of differentiation between the two species should be considered by breeders in programming wide hybridization plans.

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ORGANIZACJA HETEROCHROMATYNY W CHROMOSOMACH METAFAZOWYCH I JĄDRACH INTERFAZOWYCH U *DASYPYRUM BREVIARISTATUM* (LINDB F)

STRESZCZENIE

Celem badań była analiza składu, ilości i rozmieszczenia heterochromatyny u dzikiego tetraploidalnego gatunku *Dasypyrum breviaristatum* (Lindb f) Frederiksen. Zastosowano barwienie metodą prążków C i fluorochromami oraz cytofotometryczne pomiary względnej zawartości DNA w heterochromatynie. Porównanie z gatunkiem diploidalnym *D. villosum* (L.) P. Candargy wykazało, że *D. breviaristatum* i *D. villosum* różnią się składem, organizacją i rozmieszczeniem heterochromatyny. Uzyskane wyniki mogą wskazywać, że obszary telomerowe badanych gatunków są bardziej zróżnicowane niż obszary centromerowe w wyniku długotrwałej dywergencji między tymi gatunkami.

SŁOWA KLUCZOWE: rozmieszczenie heterochromatyny, organizacja chromatyny, zawartość jądrowego DNA, związki filogenetyczne, *Dasypyrum villosum*.