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Domenico Pignone
CNR Istituto del Germoplasma

Roberto Mezzanotte
University of Cagliari

Roberto Cremonini
University of Pisa

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Chromatin Characterization in *Dasyphyrum*

Domenico Pignone, Roberto Mezzanotte, Roberto Cremonini

CNR Istituto del Germoplasma, Bari, Italy
Istituto di Biologia Generale, Università di Cagliari, Italy
Dipartimento di Scienze Botaniche, Università di Pisa, Italy

ABSTRACT

An open pollinated natural population and an inbred line of *Dasyphyrum villosum* were cytologically examined. Nuclear DNA content, chromosomal distribution of the C-banded heterochromatin and the chromosomal site of action of restriction endonucleases were investigated. The results demonstrate that in *D. villosum* two classes of heterochromatin exist with different chromosomal location and reacting properties. One fraction of heterochromatin appears to be more affected by individual variation than the other. Preliminary examination of the chromosomal characteristics of *D. breviaristatum* indicate that the evolution of this latter species involved a more complicated process than the simple duplication of its chromosome number.

INTRODUCTION

The genus *Dasyphyrum* comprises two Mediterranean species: *D. villosum* (L.) Candargy (formerly *Haynaldia villosa* Schur), annual, $2n = 2x = 14$, widely spread in the coastal areas of the Mediterranean region, and *D. breviaristatum* (Lind. f.) Frederiksen (formerly *D. hordaceum* Candargy), perennial, $2n = 4x = 28$, whose range is limited to Morocco, Algeria and Greece (Frederiksen 1991).

Dasyphyrum villosum is considered an important source of genes for powdery mildew resistance, seed storage protein content and quality (De Pace et al. 1988). *Dasyphyrum breviaristatum* is thought to be an autotetraploid having the same genomes as *D. villosum* (Frederiksen 1991).

Natural populations of *D. villosum* produce two types of caryopses, yellow and brown, in the same ear. The inheritance of the seed color does not show any dominance effect, nor does it follow Mendelian segregation. Mature plants developed from the two types of seeds do not show evident morphological differences; both of them are able to produce ears with yellow and brown caryopses.

Analysis of interphase nuclei chromatin organization, by use of densitometric determination at different thresholds of optical density, and of chromosomal heterochromatin distribution by means of C-banding, fluorochromes Hoechst 33258, DAPI, CMA, and Ag-NOR staining, were applied for characterizing heterochromatin. To reach a better level of understanding, *in situ* restriction endonuclease digestion was performed on an inbred line of *D. villosum*. Finally a preliminary characterization of *D. breviaristatum* chromatin was started.

MATERIALS AND METHODS

A natural population of *D. villosum* was collected near Campobasso (central Italy); the caryopses of this population exhibited yellow and brown color. From a natural population collected near Bari (south Italy) an inbred line (GHA 01) was derived by selfing, in each generation, a plant derived from a single seed of a selfed ear of the former generation. The process was carried out for eight generations, with the residual heterozygosity estimated below 1%.

Seeds were germinated in Petri dishes in the dark at 21 °C. For cytophotometric analyses, root tips were fixed in ethanol-acetic acid (3:1, v/v); squashes were made after digestion in pectinase and staining with Feulgen. Squashes of *Vicia faba* were concurrently stained as internal standards. Absorption was measured using a Leitz MPV3 integrating microdensitometer. Feulgen DNA absorption of chromatin fractions with different condensation level was determined by measurements of one and the same nucleus, after selecting different thresholds of optical density in the instrument according to the method discussed in Cremonini et al. (1992). The instrument reads all parts of the nucleus where the optical density is greater than the preselected limit, regarding those below this limit as a clear field. The value of Feulgen absorption at 3 (arbitrary units) thresholds of optical density is the total value (100) of Feulgen absorption. Measurements carried

Table 1. Mean absorption, DNA amount and nuclear area of yellow and brown seeds of *D. villosum*

Caryopses	Absorption (a.u. \pm S.E.)	DNA content per (4C nucleus (pg))	Nucleus area ($\mu\text{m}^2 \pm$ S.E.)
Yellow	2615 \pm 32.4	23.7 \pm 0.3	330 \pm 4.7
Brown	2033 \pm 25.1	19.1 \pm 0.2	249 \pm 3.0

out on the same nucleus at different thresholds of optical density were expressed as a percentage of the initial value.

For chromosome banding, roots were excised and treated overnight with ice cold distilled water and fixed in ethanol-acetic acid (3:1, v/v). C-banding, Ag-NOR and fluorochromes staining were performed as described in Galasso and Pignone (1992)

In situ digestion with restriction endonucleases (RE-banding) was carried out according to Mezzanotte et al. (1983) using the following enzymes: Alu I, Dde I, Taq I, Dra I, Eco RI, and Hae III.

RESULTS AND DISCUSSION

DNA content

Yellow and brown seeds showed different nuclear

DNA content: 23.7 pg and 19.1 pg in 4C interphase nuclei respectively (Table 1). The data clearly demonstrated a significant amount of variation in the chromatin organization of the two types of caryopses. The Feulgen absorption of brown seeds is reduced to nothing at 21 thresholds of optical density and the Feulgen absorption of yellow seeds is reduced to nothing at higher thresholds of optical density of 24 (Figure 1).

A mathematical elaboration based on the Simpson's rule, allows one to determine the inflection point of the two curves (Figure 1). This point allows us to distinguish two areas in each curve, the first being the integral of the half-curve left of the inflection point and the other, the integral of the half-curve to the right of the same point. The integral calculation was carried out on the two semi-areas (Figure 2). While the values of the left areas are rather similar (454 and 448, yellow and brown respectively), the values of the right areas are different in the two types of

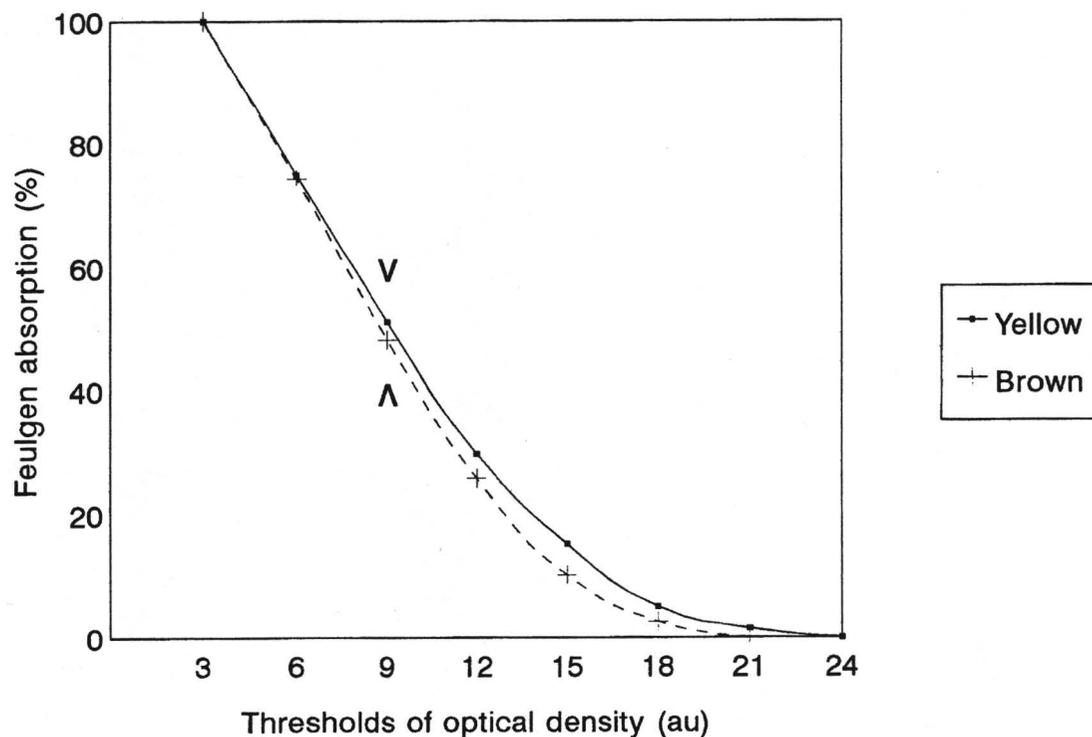


Figure 1 Absorption curves from nuclei of yellow and brown seeds of *D. villosum*; the inflection point is indicated (v)

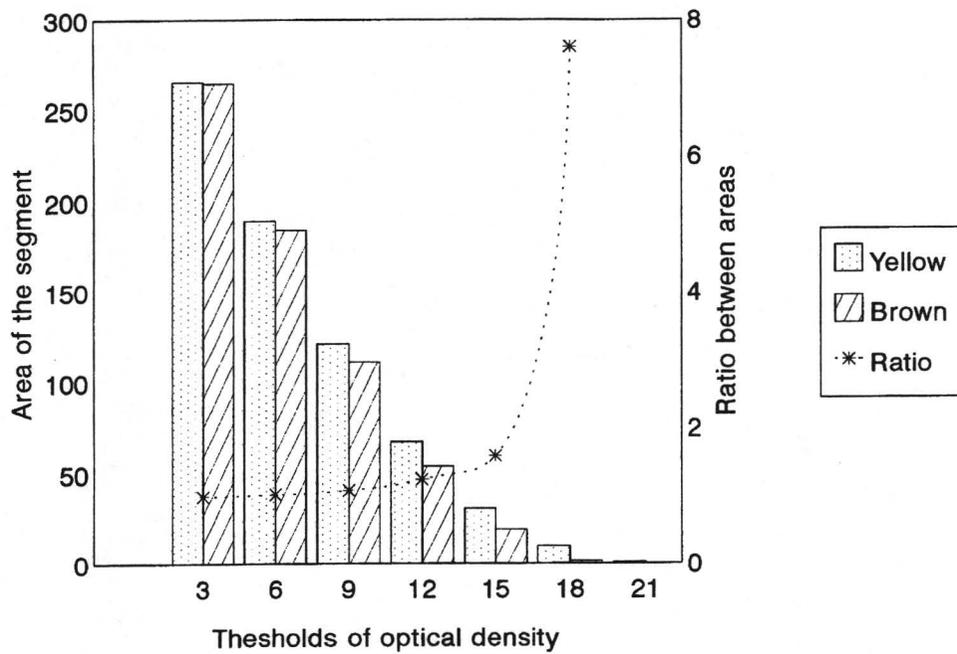


Figure 2 Areas of the absorption curves of yellow and brown seeds of *D. villosum*; the ratio between areas is shown with a dotted line

caryopses, 229 and 184 for yellow and brown, respectively. Moreover, it is interesting to point out that the ratio between these latter areas is 1.244, similar to that between the total DNA contents. The data indicate that the difference in DNA amount only regards the condensed fraction of the genome, that is the heterochromatin.

were carried out on meristematic cells in order to make photometric results comparable with those from chromosome analyses. The percentage of heterochromatin is higher in meristematic cells than in differentiated ones indicating underreplication of heterochromatin during differentiation (Bassi 1990 and references therein).

The analyses at different thresholds of optical density

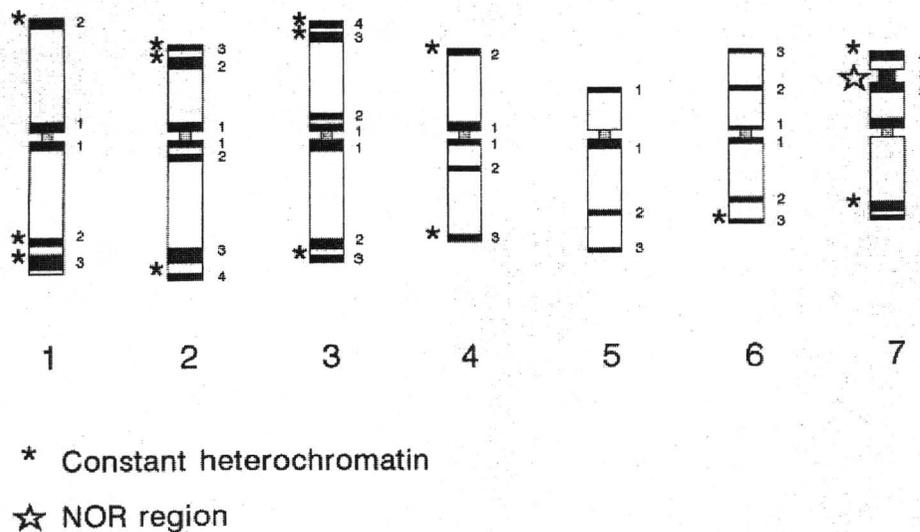


Figure 3 General karyotype of *D. villosum*; the more constant bands are indicated.

Heterochromatin detection and classification

The general karyotype of *D. villosum* (Figure 3) is similar to that reported by Friebe *et al.* (1987) and Linde-Laursen and Frederiksen (1991). The techniques of differential chromosome staining allowed the identification of each pair of chromosomes, although a high degree of variation was observed within homologous chromosomes of different individuals of the same population. The general karyotypes of the two types of caryopses did not change too much and only few differences could be evidenced. C-banding revealed a large amount of chromosomal heterochromatin as well as a high degree of variation. Hoechst 33258 and DAPI produced the same banding pattern. These fluorochromes induce occasional dots in the centromeric areas while revealing intensely stained bands in the telomeric regions, except on chromosome 6 (Figure 3). They allowed the identification of only a part of the C-bandable heterochromatin. This fraction was uniformly distributed and did not show any difference in yellow and brown caryopses. Finally only the NOR region was identified with both CMA and Ag-NOR staining.

It is worth noting that the polymorphism occurs on different bands in yellow and brown types. Bands that are stable in one type of caryopse may be polymorphic in the other. The polymorphism generally occurs on bands that are not evidenced with fluorochromes, with some exceptions. This might indicate that the different classes of heterochromatin are involved at a different level in the polymorphism.

It is also interesting that on metaphase chromosomes banding techniques do not bring out striking evidence of variation in the level of heterochromatinization between yellow and brown types. The variation observed does not account for a 20% difference in heterochromatin content as cytophotometrically determined.

It has to be considered that the binding of Giemsa to C-bands is non-stoichiometric, therefore bands with similar appearance could possess different amounts of heterochromatic DNA, moreover, C-banding and cytophotometry consider cells in different functional stages. Nuclear heterochromatin represents unexpressed DNA and a difference in heterochromatin amount reflects differences in genome expression, whereas chromosomal heterochromatin represents an alternative level of packaging DNA into chromosomes. The fact that densitometric curves do not differ in the euchromatic fraction show that there is no difference in genome expression between brown and yellow types and, therefore, all the extra DNA in yellow type is unexpressed DNA.

RE-banding

In situ digestion with restriction endonucleases followed by staining with Giemsa or with DNA-specific fluorochromes may be an intermediate approach between

chromosome banding and the molecular level, capable of investigating the DNA base composition and the chromatin organization of specific chromosomal regions.

RE-banding revealed different patterns in relation to the enzyme used; some produced positive bands (Alu I, Dde I, Taq I, Hae III) other negative bands (Dra I, Eco RI). It is interesting to note that each enzyme had a characteristic pattern which differed from the other enzymes for the same bands. The action of REs does not appear to be in relation to the DNA base composition alone, as it can be demonstrated by the staining of digested chromosomes with DAPI. All areas that after RE digestion followed by Giemsa staining appeared as gaps showed a dull appearance also with DAPI; these areas are located centromerically and interstitially. Conversely, telomeric bands, which in undigested preparations often display DAPI bands, show a different reaction according to the enzyme used. They show a brilliant fluorescence after digestion with Alu I, Dde I, Taq I, and Hae III, but an indifferent reaction after digestion with Eco RI and Dra I. Taking into account the action of REs on the above mentioned heterochromatin classes, it is possible to detect different subclasses of heterochromatin in *D. villosum*.

C^+ /DAPI⁺ (class 1): This AT-rich heterochromatin, localized mostly in telomeric areas, generally shows RE⁺ reaction. RE⁻ reaction in class 1 heterochromatin is present only in a few centromeric areas and never in telomeric ones. This implies that: a) centromeric and telomeric areas possess two different sub-classes of heterochromatin, as is also suggested by their appearance as large fluorescent blocks at the telomeres as opposed to very thin bands at the centromeres, b) centromeres contain high frequency specific RE base sequences, and c) chromatin organization is a critical factor in permitting or prohibiting RE action, as hypothesized above.

C^+ only and no reaction with fluorochromes (class 2): the RE⁺ reaction indicates that this heterochromatin does not seem to be enriched in AT- or GC- base pairs nor does it contain appreciable amounts of specific DNA base sequences. By contrast class 2 areas showing RE⁻ reaction would contain, possibly with a high interspersed frequency, the base sequence target of specific REs. Even in this class of heterochromatin it is noteworthy that RE⁻ reaction is present only at centromeres and never at telomeres, which are again shown to be very resistant structures.

Finally, the NOR region positive to silver staining (CMA⁺ only) is localized on chromosome 7. This area, affected to a limited extent by all REs, is cleaved by Hae III, which produces a marked RE⁻ reaction. The cleavage and extraction of GC-rich DNA (CMA⁺, Sumner 1990 and references therein) from this area by Hae III (restriction target GGCC) is not surprising considering that in this case the NOR does not show a C^+ reaction, thus indicating that this chromatin possesses a further organization level.

D. breviaristatum

Our C-banding results are similar to those reported by Linde-Laursen and Frederiksen (1991). C-banding produces a rather complex pattern: bands are generally distributed at centromeric and interstitial positions; few and thin bands are seen at telomeric positions. The very high level of polymorphism observed within some chromosome pairs is clearly the result of the strict allogamic reproductive habit of this species. Preliminary attempts to produce fluorochrome staining did not produce good results and indicate the absence of the bright DAPI⁺ telomeric blocks seen in *D. villosum*. This might

appear in contrast with the hypothesis that *D. breviaristatum* is an autotetraploid species possessing the same genome as *D. villosum*. Nevertheless one has to consider that; a) the examined population might be poor in that class of heterochromatin, b) during the establishment process, following the evolution of the new polyploid, that class of heterochromatin has been specifically lost or restructured as a consequence of a phenomenon similar to amphiplasty, and c) differences in chromatin organization and distribution may be in relation to the perennial habit of this species. Studies are in progress to better clarify this point.

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