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Relative DNA Content of Three Cytotypes of *Pohlia Nutants*

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Abstract

Relative DNA content of three cytotypes of the moss *Pohlia nutants* have been estimated, using DAPI staining technique. Evidence is presented and showing that the mean relative DNA contents increased from haploid to diploid and from diploid to triploid *P.nutants* cytotypes, but they differ significantly from an expected 1: 2: 3 ratio in haploid, diploid and triploid races. Polyploidy has played a main role in generating the cytotypes. It seems likely that the haploid, diploid and triploid races of *P. nutants* are of long standing autopolyploid.

الملخص

تم قياس محتوى DNA النسبي في ثلاثة أنواع خلويه لحزاز Pohlia nutants باستخدام طريقه الصبغ DAPI. أظهرت النتائج أن معدل محتويات DNA النسبي زاد في أنواع خلويه لحزاز P.nutants من أحادي الصيغة الصبغية الى ثنائي الصيغة الصبغية ومن ثنائي الصيغة الصبغية الى ثلاثي الصيغة الصبغية ولكنها أختلفت بشكل واضح عن النسبه المتوقعه 3:2:1 في ألأجناس أحادي الصيغة الصبغية و ثنائي الصيغة الصبغية و ثلاثي الصيغة الصبغية. لعب تعدد الصيغة الصبغية دوراً رئيساً في تكوين الأنواع الخلويه. من المحتمل على ما يبدو أن ألأجناس أحادي الصيغة الصبغية و ثنائي الصيغة الصبغية و ثلاثي الصيغة الصبغية لحزاز P.nutants تتصف بذاتية تعدد الصيغة الصبغية منذ القدم.

Keywords: DNA Content, DAPI Staining Technique, Mosses, *Pohlia nutants*.

1. Introduction

Genome size variation below the species level is attracting considerable interest among plant biosystematists and cytogeneticists. Measurements of the relative DNA content of nuclei from gametophytes from different populations may provide an efficient mean of differentiating populations where karyotype analysis is difficult or impracticable. The DNA content of nuclei of gametophytes may also provide a useful taxonomic criterion and may provide evidence of evolution of one taxon from another (Grellhuber and Obermayer, 1998).

The estimation of nucleic acid contents by chemical means requires a large amount of material and is relatively time consuming. The DNA content of single nuclei can be estimated by measuring the density of Feulgen staining. The light absorbed by the stained nucleus is proportional to the DNA content. A more sensitive measure can be made by using a fluorochrome that stains DNA in a quantitative manner. Such a fluorochrome is DAPI. Other methods depend upon the relationship between nuclear volume and DNA content. These relationships show that nuclear volume and interphase volume are directly proportional to DNA content per cell and per chromosomes, respectively. Therefore when the nuclear volume of meristematic cells is known, an estimate of DNA content can be made (Sparrow et al., 1972). This method appears to be inadequate specially when the interphase chromosome volume is obtained by dividing the average of the interphase nucleus by somatic chromosome number; and represents the volume occupied by the average chromosome at interphase, neglecting other nuclear components such as the nucleolus or volume changes during replication of chromosomes. Clearly, the accuracy of measurements with very small nuclei is questionable. To assess the DNA content in nuclei, Feulgen stain was used, but there are many difficulties , for example excess or too little stain affect the results as does the influence of the background. The fluorescence of the DAPI/DNA complex has been used as a quantitative estimate of the DNA (Brunk et al., 1979; Lin et al., 1977). Many workers are satisfied that DAPI can be successfully applied to measure DNA and detect intraspecific variation (Rayburn et al., 1989; Biradar and Rayburn, 1993). Moreover, the simplicity of the staining procedure coupled with the brightness of the DAPI/DNA complex provide a convenient technique of cell cycle studies and comparative estimates of DNA contents of nuclei.

So to obtain satisfactory results, the fluorochrome 4,6-diamidino-2-phenylindole (DAPI), which has been shown to bind specifically to DNA, will be applied.

To date, comparatively few reports on DNA content of bryophytes are available. Isolation of nuclear, chloroplast and mitochondrial DNA from the moss *Physcomilrella patens* has been reported (Marienfeld et al., 1989 and Knight, 1994). DNA content of a wild type strain and a somatic hybrid *Physcomitrella patens* was estimated, using flow cytophotometry (Reski et al., 1994). Relative changes in DNA content in the hornwort *Anthoceros punctatus*, using DAPI stain, have been demonstrated by Izumi and

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Ono (1994). Nuclear DNA contents of 17 species of bryophytes have been studied by Renzaglia *et al.* (1995).Thoni and Schnepf (1994) studied the nuclear DNA content in spore nuclei of *Funaria hygrometrica.* Moreover, numerous reports of intraspecific polyploidy in mosses were also published (Abderrahman and Smith, 1983; Abderrahman, 1998 and Abderrahman, 2004).

It is now well established that there are three cytotypes of the moss *Pohilia nutants* with (n=11, 22 and 33) respectively (Smith, 1978; Fritsch, 1982, 1991). They are indistinguishable morphologically. In view of their morphological similarity, it was decided to investigate possible cytological differences, and to this end relative DNA contents of the cytotypes will be investigated using DAPI staining technique.

2. Materials and Methods

Previous studies indicate that factors such as latitude, altitude, and time of the day may affect mitotic activity and cell cycle duration, thus having impact on the c-value and intraspecific genome size. So, materials were collected from different localities in Jordan by ourselves (Table 1) and kept under uniform conditions for at least four weeks in polythene bags at room temperature out of direct sunlight in the laboratory. Gametophyte shoot apices were fixed in a glacial acetic acid and ethyl alcohol (1:3) solution for 2h. Cells, then were transferred into Feulgen stain for cytological studies. Chromosome number of each sample will be determined i.e. haploid diploid and triploid cytotypes.

Gametophyte cells from each cytotype samples were measured, using DAPI staining technique described by Lin et al. (1977) and Brunk et al. (1979). Materials were fixed in 5% gluteraldehyde EM in Tris buffer, pH 7 for 5 min. The stain was made up as stock solution of 100 µg/ml in buffer containing 100m M NaCl, 10 mM EDTA and 10m M Tris, pH 7.

Shoot apices stained with DAPI were squashed on slide, and a microscope fitted with an incident U.V. light source measured fluorescence of nuclei, and photomultiplier coupled microscope to a pen recorder. Filters were used giving a final wavelength of 350 nm. The DAPI / DNA complex fluoresces at 450 nm. As the variation in fluoresces is proportional to the mean value in the first peak G1 of the cytotypes, transformation of data by means of Log_{10} will be used to overcome this proportional variation. Measurements of fluorescence were transformed by Log_{10}, and are expressed as arbitrary units.

A one-way analysis of variance was carried out. Tukey's test was applied, and found to be suitable for this sort of data. Tukey's interval estimate was also applied to calculate the 95% confidence intervals for the differences between means of pairs of groups of samples (Neter and Wasserman, 1974). The statistic q' was calculated by:

$$q' = q_{v.r}\sqrt{\frac{MSE}{2}\left(\frac{1}{n1}+\frac{1}{n2}\right)} \qquad (1)$$

Where $q_{v.r}$, was derived from tables for v= number of groups (i.e. cytotypes) and r= number of degrees of freedom of error term in the analysis of variance. MSE is the mean square error from the analysis of variance Both nl and n2 are sample sizes of the two groups. 95% confidence intervals were then calculated as:

$$(\overline{x}_i - \overline{x}_j) - q' \leq (\mu_i - \mu_j) \leq q' + (\overline{x}_i - \overline{x}_j) \qquad (2)$$

Where x_i, x_j were the means of Log_{10} transform data of the two groups and (µi- µj) was the expected difference *Pohilia nutants* between gatherings means. The DNA content in haploid diploid and triploid was assumed to be in the ratio 1: 2:3.

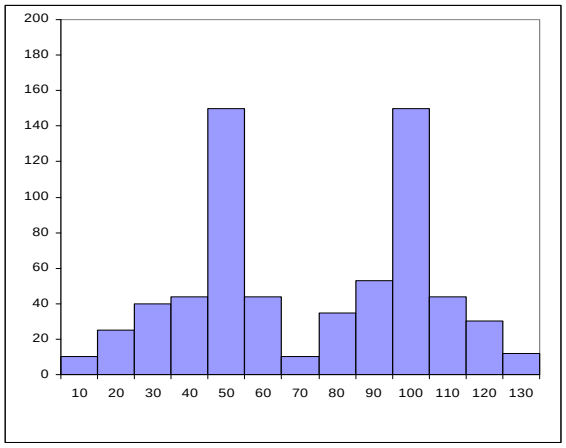
3. Results

As may be seen from Figs. 1a, 1b, and 1c there are biomodality in DNA content within each category of *Pohlia nutants* corresponding to G1 and S, G2 and M phases of the cell cycle. Estimates of DNA content for G1 nuclei in arbitrary units of the three cytotypes of *Pohlia nutants* showed that the mean value of the first peak was estimated to be 49 ± 2.70 in the haploid, the mean value of the diploid cytotype was estimated to be 86 ± 1.95, and the mean value of the triploid cytotype was estimated to be 120 ± 2.20 (Table 2).

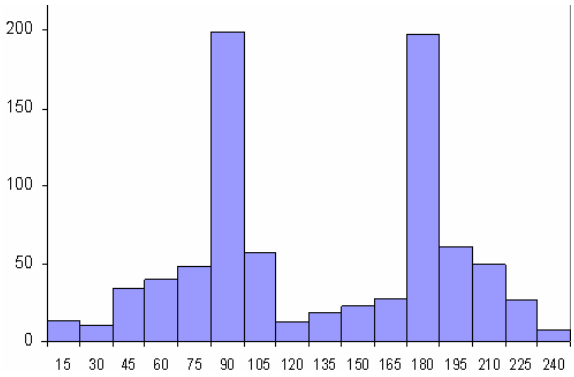
Differences between diploids and haploids and triploid and diploid were compared using Tukey's interval estimates (Neter and Wesserman , 1974). The mean relative contents increased from haploid to diploid and from diploid to triploid cytotypes, as expected. These results are outside the 95% confidence intervals (i.e the differences in means between diploids and haploids and those between triploids and diploids are outside the 95% confidence intervals). Therefore, the increase in the DNA content was not proportional to the increase in chromosome number in the three cytotypes of *Pohlia nutants.*

Table 1. Localities and habitat of *Pohlia nutant* cytotypes.

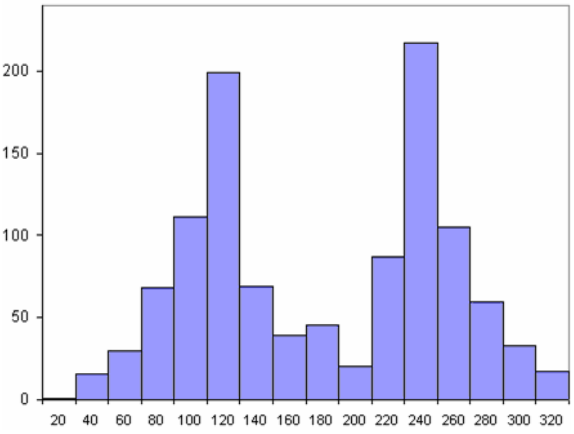
Locality	Habitat	Cytotype
Salt: 10 Km. West of Amman	Wet soil	Haploid and diploid
Om-Qais: 10 Km. North of Irbid	Shady moist soil	Diploid and triploid
Sweileh: 6 Km. North west of Amman	Shaded walls	Haploid and diploid
Ain AL-Basha: 20 Km. North of Amman	Damp soil	Haploid and triploid
Wadi AL-Sair: 20 Km. North west of Amman	Damp soil	Diploid and triploid
Fuhais: 10 Km. west of Amman	Wet soil	Haploid and triploid
Jubaiha: University of Jordan campus, Amman	Wet shaded	Haploid and triploid



(a)



(b)



(c)

Figure 1. Histogram of DNA quantities in Pohlia nutants: a, haploid; b, diploid and c, triploid cytotypes. (Vertical axis-number of readings; horizontal axis-arbitrary units)

Table 2. Mean DNA content of G1 nuclei expressed as arbitrary units of the three cytotypes of *Pohlia nutants*.

Cytotype	Mean DNA content expressed in arbitrary units
Haploid (n=11)	49 ± 2.70
Diploid (n= 22)	86 ± 1.95
Triploid (n= 33)	120 ± 2.20

4. Discussion

There are only few publications on DNA amounts of mosses; the only relevant publications dealing with absolute DNA contents of Bryatae are those of Abderrahman and Smith (1983), Reski et al. (1994), Renzaglia et al. (1995), Zouhair and Lecocq (1998), Lamparter et al. (1998), Temsch et al. (1998, 1999), Volglmayr (2000) and Abderrahman (1998, 2004).

Although the DAPI technique provides a quick and reliable measure of DNA content, the results from different organisms are not necessarily comparable, as DAPI results vary depending upon DNA base constitution which may differ from one group or organism to another (Schweizer and Nagl, 1976). Thus, it is only possible to provide relative DNA contents of the three cytotypes rather than estimate absolute quantities by comparison of DAPI data from an organism of known DNA content such as *Drosophila*.

There are numerous examples of intraspecific polyploidy in bryophytes (Smith, 1978; Fritsch, 1982, 1991). Among 289 accessions of 138 different moss taxa, Volglmayr (2000) found only three species of intraspecific polyploidy. These are *Aerichum undulatum*, *Fontinalis antipyretica* and *Amblystegium serpens*. The most prominent of these is *A. undulatum* with a DNA content ratio (max. /min.) of 2.7 suggesting triploidy.

In the present study, biomodality in DNA content within each cytotype corresponding to G1 and S, G2 and M phases of the cell cycle was present (Fig. 1. a, b and c).On the other hand , one single peak of fluorescence was generated by Reski et al. (1994) in studying four *Physcomiterella patents* genotypes, suggesting an arrest in the cell cycle during day time.

If, now appear to be the case, the chromosome races of *Pohlia nutants* (n=11, 22 and 33) series (Smith, 1978; Fritsch, 1982, 1991), it might be expected that DNA quantities in the three cytotypes would be present in a 1:2:3 ratio.

Reference to Table 2, it is clear that the mean relative DNA contents increased from haploid to diploid and from diploid to triploid as would be expected. Applying Tukey's interval estimate showed that the ratio between haploid and diploid is significantly less than 1: 2, and that between diploid and triploid is also significantly less than 2:3. Therefore, the increase in the DNA content is not proportional to the increase in chromosome number. It would appear that there are differences in DNA content between haploid and diploid and diploid and triploid plants. These results are in accord with the findings of Abderrahman and Smith (1983) studying chromosome length and relative DNA content of three cytotypes of *Atrichum undulatum* in which the three cytotypes differed significantly from an expected 1: 2: 3 ratio in haploid, diploid and triploid races. These results are also consistent with those obtained by Abderrahman (1998) studying two types of *Funaria hygrometrica* in which the mean relative DNA contents increased from haploid to diploid plants, but they also differ from an expected 1: 2 ratio. The presence of positive correlation between nuclear DNA content and chromosome number was also reported by Lobachevska and Demkiv (1990) in their comparative study *of Plagiothecium platyphyllum* and *Brachythecium*

velutinum. . Moreover, these results are also consistent with those reported by Abderrahman (2004) in a comparative investigation into nuclear DNA content of the moss *physcometruim pyriforme.* In contrast, the mean of DNA content in haploid and diploid *Sphagnum* (peat moss) were close to the expected 1:2 ratio, namely 1: 2.049 (Temsch et al., 1999).

It is evident that C-value variation within mosses is remarkably small, when compared with angiosperms (Bennet et al., 1998). As mosses can be supposed to be a very old group of plants (Kenrick and Crane, 1997), with the main clade already differentiated in the Palaezoic (Stewart and Rothwell, 1993). This constancy in C-values evidently needs an explanation, especially if compared with the phylogenetically young angiosperms with their vast heterochromatin and repetitive DNA accumulation. This implies the presence of a strong selection pressure towards the maintenance of small DNA amounts, which could be correlated with basic feature of moss biology. A possible mechanism which could contribute to maintenance of small C-values in mosses which has been recently recorded for *Physcomiterella patens* following transformation experiments (see Reski, 1998).

Although there have been numerous cytological observations of *Pohlia nutants* (see Smith, 1978 and Fritch, 1982, 1991) there have been no reports of cytological abnormalities suggestive of recent autopolyploidy. However, autopolyploidy is of frequent occurrence in mosses and there may be some mechanism promoting normal meiosis in recent autopolyploids. Chromosomal divergence between the three cytotypes, at least in the plants were studied, suggests that they are of long standing. In view of the morphological uniformity of the cytotypes (Smith, 1978) and on the basis of our observations, it is suggested that the haploid, diploid and triploid races of *P. nutants* are of long standing autopolyploid origin. As we have three cytotypes of *Pohlia nutants,* the possible role of aneuploidy has been ruled out.

There is abundant evidence of aneuploidy in bryophytes which may affect genome size. Some instances of aneuploidy in mosses are the result of variation in numbers of m-chromosomes. The occurance and distribution of m-chromosomes in mosses is not strong(Smith,1978). Moreover, *Pohlia nutants* posses three cytotypes, and there is no report in the literature to support the presence of aneuploidy in this plant . Thus, it seems more likely that the role of aneuploidy in *Pohlia nutants* is ruled out.

It is known that estimating genome size using DAPI as the flourochrome ”Further research, including more samples of. *Pohlia nutants* and the use of flow cytometry and molecular techniques, is required to elucidate causes of this variation in genome size.

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