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Karyotype diversity and interspecific 4C DNA variation in *Bupleurum*

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Abstract

Investigation on karyotype, 4C nuclear DNA amount and interphase nuclear volume (INV) of different Himalayan *Bupleurum* species belonging to *Umbelliferae* revealed genetic differentiation. Numerical and structural alternation of chromosomes in interspecific level were manifested in their statistically significant altered species specific 4C nuclear DNA content. Somatic chromosome number ranged between 2n = 14 and 2n = 16. *B. himalayense* was reported for the first time having 2n = 16 chromosomes. Correlation coefficient among the various chromosomal and nuclear parameters showed no significant progressive or regressive interdependence except in between INV and nuclear DNA amount. Critical differences between 4C DNA content showed interspecific variation.

Introduction

Cytophotometric measurements of nuclear DNA content have been made to some extent at inter- and intraspecific level in the members of *Apiaceae* (Das and Mallick 1989a,b, 1993, Chattopadhyay and Sharma 1990, Das 1991). The importance of nuclear DNA study in different taxa in various facets of chromosomal research have gaining more and more importance (Price 1976, Sharma 1983). The idea behind the interspecific variation of 4C nuclear DNA content is the fluctuation of repetitive and non-repetitive sequences (Bennett *et al.* 1977, Rees and Narayan 1977) between the species as well as the cytogenetic alterations of genome such as, structural rearrangements of chromosomes and polyploidy. In order to ascertain precisely the importance of DNA in speciation of the different species of *Bupleurum* under the tribe *Ammineae* belonging to *Apiaceae*, a concrete understanding is necessary. So far the chromosomal studies reported in the species of *Bupleurum* shows 2n = 14 chromosomes (Delay 1947, Gardé and Malheiros-Gardé 1954) in *B. fruticosum.*

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Varied chromosome numbers have been published such as 2n = 16, 28, 32 (Suzuka 1950, Bell and Constance 1957, Cauwet 1971, Ahmad and Koul 1980) in *B. falcatum* and *B. candollei*, again reported differently *i.e.*, 2n = 14 (Ahmad and Koul 1980) and 2n = 16 chromosomes (Wanscher 1932). Somatic chromosome number 2n = 16 chromosomes have furthermore been lightened by Cauwet *et al.* (1980) and Ahmad and Koul (1980) in *B. longicaule*. However, detailed chromosomal data are also meagre in the above mentioned species. The 4C nuclear DNA content have not yet been studied in these species, too. In addition, the interphase nuclear volume (INV) has direct relation with DNA content per nucleus (Sparrow *et al.* 1975) in the interspecific level. Several reports confirmed the high correlation between INV and DNA content per nucleus (Avanzi *et al.* 1966, Yamaguchi and Tsunoda 1969, Katayama 1971, Das and Mallick 1989c) not only in species level but also in cultivar level. In *Bupleurum* no such report have yet been recorded. The present investigation mainly deals with the estimation of 4C nuclear DNA content in relation with their genomic variance, if any, and correlation between different cytological parameters to delineate the interspecific heterogeneity during evolution.

Materials and methods

Five species of *Bupleurum* namely *B. candollei, B. falcatum, B. fruticosum, B. longicauli* and *B. himalayense,* having mature seeds, were collected from Garwal Himalaya - the Kedarnath. The type specimens were kept in the Calcutta University herbarium and were identified properly from Indian Botanical Garden, Howrah. Dry seeds were grown in the experimental garden of the Department of Botany, University of Calcutta.

Young healthy root-tips were pretreated in saturated para-dichlorobenzene solution and aqueous aesculine mixture (1:1) for 3 h at 14 °C followed by 1:3 acetic acid:ethanol fixation. Chromosome staining was made in 2 % acetic orcein and 1M HCl mixture (9:1) for 4 h. Root-tips were squashed in 45 % acetic acid. Well scattered metaphase plates were selected for karyotype analysis. Total chromosome length were carried out by adding the length of all the chromosomes in the karyotype and total chromosome volume of a karyotype was calculated by applying the formula

$$\text{total chromosome volume} = \pi r^2 h$$

where r and h represented radius and length of the chromosome, respectively. Form percentage (F %) of individual chromosome was calculated by the formula

$$\frac{\text{length of short arm of chromosome}}{\text{total length of that chromosome}} \times 100$$

following the method of Levan *et al.* (1964). Total form percentage (TF %) was the average of sum total F % of a karyotype.

In situ 4C DNA amount were measured following Feulgen cytophoto-densitometric method. Ten fixed root tips from each species were hydrolysed in 1M HCl for

10 min at 60 °C; washed in distilled water; stained in Schiff's reagent for 2 h at 14 °C. Each root-tip squash was made in 45 % acetic acid separately and ten scorings were taken from each slide. 4C nuclear DNA content was estimated from metaphase chromosomes using *Leitz MPV Wetzler Aristophot* with microspectrophotometer following the method of Sharma and Sharma (1980) applying monochromatic light of 550 nm. 4C DNA amounts obtained on the basis of absorbance, then converted to picograms (pg) by using Van't Hof's (1965) 4C nuclear DNA value 67.1 pg for *Allium cepa* as standard.

For scoring of INV, the root-tips of about 2.5 mm length were fixed in 1:3 acetic acid : ethanol for 24 h at 25 °C, hydrolysed in 1M HCl at 4 °C for 15 min. After a thorough washing root-tips were put into Schiff's reagent for 1 h at 20 °C and kept in dark for staining. Squash preparation was done in 45 % acetic acid. Ten randomly selected nuclei were scored from each root-tip. Sample size in each species was 20 root-tips per species. Under oil immersion objectives the mean of the two diameters of nuclei, obtained by measuring at right angles to each other; was used to calculate the volume using the formula

$$\text{volume} = 4/3\ \pi\ r^3$$

where r is the radius of the nucleus.

To investigate the significant differences of 4C nuclear DNA content, if any, in the species of *Bupleurum ANOVA* test (Sokal and Rohlf 1973) was performed which followed by Duncan's multiple range tests (Harter 1960). The correlation coefficient analysis followed by *t*-test was done in between different chromosomal and nuclear parameters to find out the relationships of genomic characteristics.

Results

Karyotype analysis: Somatic chromosome number 2n = 14 was noted in *B. fruticosum* where rest of the studied species showed 2n = 16 chromosomes. On the basis of size, structure and position of primary and secondary constrictions four types of chromosomes (Fig. 1) were obtained in the species of *Bupleurum.* Karyotype formula and number of nucleolar chromosome studies revealed clear interspecific differences in minute structural details of the chromosome morphology (Table 1, Figs. 2-6). Type A chromosomes were present in *B. fruticosum* and *B. longicaule* and B types were noted in the other three species as well as latter one also (Table 1, Fig. 7). An interesting feature was noticed in *B. himalayense* that the absence of C type *i.e.*, submedian chromosomes in the genome. Moreover, all the types of chromosomes were represented by *B. longicaule.* The total chromosome length varied from 18.14 μm in *B. himalayense* to 33.48 μm in *B. candollei.* Maximum total chromosome volume recorded was 14.44 μm^3 in *B. fruticosum* (Table 1), whereas 3.20 μm^3, minimum value, was noted in *B. himalayense.* The subsequent variation in the chromosome architecture of genome were also reflected on their average TF % value.

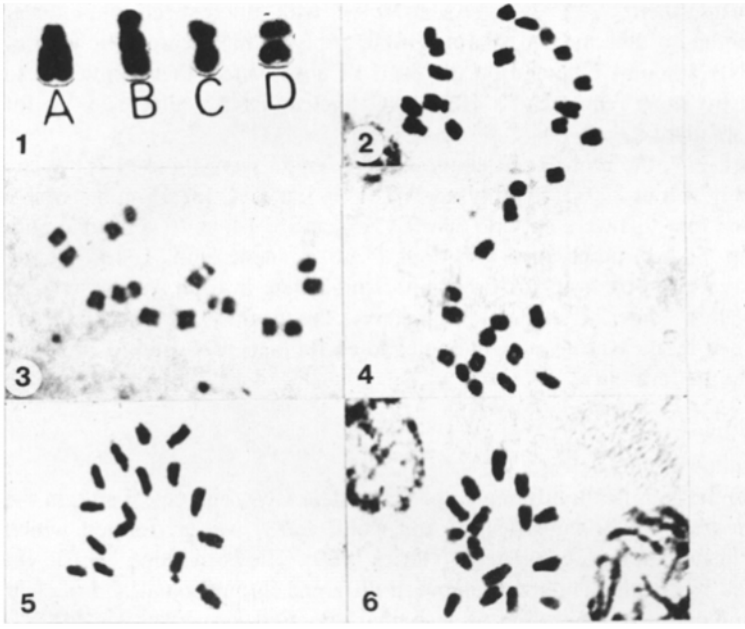

Fig. 1. Standard types of chromosomes present in different species of *Bupleurum*: A type - medium sized chromosomes with submedian and nearly terminal constrictions, satellite is present on proximal end of short arm; B type - medium sized chromosome having two constrictions, one nearly submedian and another nearly subterminal; C type - medium to short sized chromosomes with submedian primary constriction; D type - Comparatively short sized chromosomes with median primary constriction.
Figs. 2, 4, 5, 6. Somatic metaphase chromosomes 2n = 16 in *B. candollei, B. himalayense, B. falcatum, B. longicaule,* respectively × 2139.
Fig. 3. Karyotype showing diploid chromosome number 2n = 14 in *B. fruticosum* × 2139.

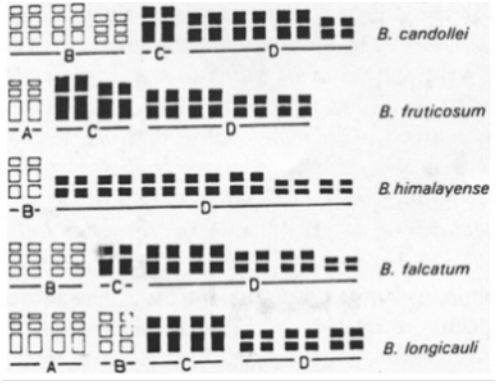

Fig. 7. Idiograms of different species of *Bupleurum*.

Table 1. 4C DNA amount in five species of *Bupleurum* along with different cytological parameters.

Species	Number of somatic chromosome (2n)	Number of secondary constricted chromosome	Karyotype formula	Total chromosome length ± S.E. [μm]	Total chromosome volume ± S.E. [μm³]	INV ± S.E. [μm3]	4C DNA amount ± S.E. [pg]	Total F [%]
B. candollei	16	6	$B_6C_2D_8$	33.48 ± 0.03	7.63 ± 0.09	202.15 ± 3.52	11.572 ± 0.003	36.95
B. fruticosum	14	2	$A_2C_4D_8$	25.78 ± 0.26	14.44 ± 0.18	165.12 ± 1.91	10.125 ± 0.002	37.04
B. himalayense	16	2	B_2D_{14}	18.14 ± 0.11	3.20 ± 0.04	189.70 ± 2.09	11.225 ± 0.001	47.13
B. falcatum	16	4	$B_4C_2D_{10}$	21.70 ± 0.06	6.26 ± 0.24	197.55 ± 2.44	11.418 ± 0.002	41.47
B. longicaule	16	6	$A_4B_2C_4D_6$	25.88 ± 0.07	5.30 ± 0.26	216.14 ± 3.72	12.276 ± 0.004	35.50

Table 2. *ANOVA* of the 4C DNA amounts within the species of *Bupleurum*.

Source of variation	DF	SS	MS	F
Between species	4	24.240	6.06	445.588**
Within species	45	0.611	0.0136	
Total	49			

** - significant at $P \geq 0.01$

INV and nuclear DNA studies: Interphase nuclear volumes (INV) were changed according to the species identity. The maximum value 216.14 μm^3 was recorded in *B. longicaule*. The frequency polygon of INV in different species showed differential frequency distribution around the mean keeping a constant sharp peak on its mean value (Fig. 8). 4C nuclear DNA amounts were also significantly different from species to species. It ranged from 10.125 pg in *B. fruticosum* to 12.276 pg in *B. longicaule.* Applying *ANOVA* test (Table 2) it was noted that the variation in 4C nuclear DNA content was very significantly high. The critical difference (CD) values at 1 % and 5 % levels were 0.146 and 0.112 respectively. The CD values between the means of 4C DNA following Duncan's multiple range tests showed significant differences between the species. No such definite correlations were obtained from the *r* values (Table 3) of different cytological parameters regarding genome behaviour. Although some significant negative correlation were obtained among the chromosome volume, INV and 4C DNA but these were not significant when values were considered per chromosome viz., $r = -0.384$ and -0.010 in between chromosome volume and INV and chromosome volume and 4C DNA respectively. Obtained *t*-values calculated from *r* values showed only significant positive correlation between the INV and 4C DNA amount within the species.

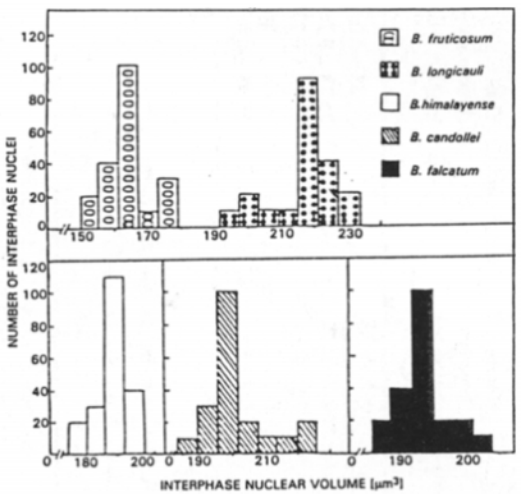

Fig. 8. Comparative frequency distribution of interphase nuclear volume of different species of *Bupleurum*.

Discussion

A careful investigation on the karyotype analysis of the five species of *Bupleurum* revealed interspecific chromosome number variation as well as structural alteration

of somatic chromosomes. Although the diploid chromosome number 2n = 14 and 16 were noted in the different species (Table 1, Figs. 2-6) but overall homogeneity in the chromosome types grouped them together. Notwithstandingly, the chromosome number of *B. himalayense* 2n = 16 was reported for the first time. In fact, D type of chromosomes were common in all the species keeping with constant presence of secondary constricted chromosomes either A or B in various doses (Fig. 7). An interesting finding was that the presence of all the chromosome types in *B. longicaule* but droping of A type was noted in *B. candollei, B. falcatum* and *B. himalayense.* Furthermore, C type along with A type and only B type were absent in *B. himalayense* and *B. fruticosum,* respectively. All these facts suggest that *B. himalayense* is an older species than *B. longicaule* and so it have relatively homogenous and genetically conserved genomic status in comparison with evolved advanced species with chromosomal structural alterations. The variation of total form percentage (TF %) was reflected in species differentiation, too. The range varied from 35.50 % to 47.13 % (Table 1) which might be due to gradual shifting of chromosomal types in the genome during speciation. Evidently, detailed structural alteration of chromosome morphology among the species might be due to duplication of chromosomes or translocation between the chromosomes with or without secondary constrictions at a very early stage of evolution (Sharma and Mukhopadhyay 1984, Das 1991, Das and Mallick 1991,1993).

Table 3. Correlation coefficient (r) values of different genomic parameters and corresponding t values in the species of *Bupleurum*

Cytological parameters	r-values	t-values
Chromosome length *vs.* chromosome volume	0.390	0.865 NS
Chromosome length *vs.* INV	0.194	-0.957 NS
Chromosome length *vs.* 4C DNA amount	0.133	0.247 NS
Chromosome volume *vs.* INV	-0.726	-0.957 NS
Chromosome volume *vs.* 4C DNA amount	-0.757	-0.989 NS
INV *vs.* 4C DNA amount	0.996	27.277 **

NS = non-significant; ** = significant at 0.5 % level
t- values at 5 % level 2.353; Df = 3; t- values at 1 % level 4.541; t- values at 0.5 % level 5.841

Total chromosome length also varied remarkably from 18.14 μm to 33.48 μm. Variation in chromosome volumes were very significantly noticeable (Table 1) in between species. The lowest value 3.20 μm^3 was recorded in *B. himalayense*. Total chromosome volume and chromosome length though not apparently correlated but positively correlated when average chromosome lengths and volumes were considered (r = 0.576). Perhaps species specific chromosome coiling to a great extent

controlled by the predetermined genetic factors (Das and Mallick 1991). Evidently differences in chromosome length or chromosome volume might be due to differential condensation and spiralization of chromosome arms.

Significant interspecific variation of 4C nuclear DNA amounts were obtained following *ANOVA* in the species of *Bupleurum* (Tables 1, 2). The 4C content though reported for the first time in these species but such type of interspecific variation were reported by the previous workers (Price *et al.* 1980, Mukherjee and Sharma 1986, Das and Mallick 1989a,b,c, Chattopadhyay and Sharma 1990, Das 1991). The maximum amount of nuclear DNA was noted 12.27 pg in *B. longicaule* having 2n = 16 chromosomes. Again, *B. fruticosum* showed 2n = 14 chromosomes having comparatively low amount *i.e.*, 10.125 pg of DNA in the genome. Duncan's multiple range tests showed significant difference in the 4C DNA content in the species leading to the genetic difference. Proportional increase of INV with 4C DNA was also reflected in their high positive correlation values (Table 3). Calculating *t*-values from their corresponding *r*-values in between different parameters confirmed that only nuclear DNA have some direct influence on INV (Table 3). Perhaps, the maximum correlation of these genomic characteristics (t = 27.277) leads to differential genetic interaction (Yamaguchi and Tsunoda 1969, Das and Mallick 1989c). All the other cytological parameters did not show any significant correlation whether it was positive or negative. However, such type of fluctuations of DNA amount might be dependent to some extent on the amount of repetitive DNA sequences (Price *et al.* 1980, Mukhopadhyay and Sharma 1987, Das and Mallick 1989b, Das 1991) in the genome. The variability could attributed to loss or addition of high repeats which were adapted species specifically for the stable structural gene function during micro- or macro-evolution maintaining genomic heritability.

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