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Analysis of relative nuclear DNA content in carnation (*Dianthus caryophyllus*) accessions reveals ploidy levels by flow cytometry

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ABSTRACT

Carnation (*Dianthus caryophyllus* L.) is one of the fifth most important ornamental species worldwide. Many desirable plant characteristics such as big size flower, adaptation to stress, and intra or interspecific hybridization capability are dependent on plant ploidy level. We optimized a quick flow cytometry method for DNA content determination in carnation accession samples that allowed a systematic evaluation of ploidy levels. To verify the actual ploidy levels, we counted chromosome numbers in the root tips of representative cultivar for each ploidy level. The relative nuclear DNA content was distributed into four kinds of discontinuous groups: 1.32 to 1.95 pg (group 1), 2.03 to 2.72 pg (group 2), 2.98 to 4.65 pg (group 3) and 5.33 pg (group 4) which might correspond to the following ploidy levels; diploid, triploid, tetraploid and hexaploid. The results showed that out of 60 carnation accessions, 33 were diploid, 5 were triploid, 21 were tetraploid and 1 was hexaploid.

Key words: Carnation accessions, Chromosome counting, Flow cytometry, Ploidy level

Carnation (*Dianthus caryophyllus* L.), family Caryophyllaceae, is an important flower crop having great commercial value as a cut flower due to its excellent keeping quality, wide array of colour and forms (Sheela 2008). The genus *Dianthus* comprises more than 300 species, including carnation. *Dianthus* has a basic chromosome number of n = × = 15 (Carolin 1957), and varied level of ploidy were reported (Gatt *et al.* 1998 and Nimura *et al.* 2008). Breeders involved in crop improvement of carnation need to understand and address the issues arising out of unfavorable geo-climatic conditions for normal sexual reproduction, presence of different ploidy types, limitation of varieties with fertile and functional pollen and incompatibility. Most of these problems result in non-viable gametes, embryo abortion and in turn poor seed set. The knowledge of ploidy levels is useful for determining the potential cross compatibility and for drawing inferences about the origin of cultivars and lines. The success of such hybridizations depends on the ploidy levels of the parents used (Gatt *et al.* 1998, Andersson-Kotto and Gairdner 1931).

Ploidy level determination is usually achieved by direct counting of chromosomes or by flow cytometry methods (Galbraith *et al.* 1983 and De Laat *et al.* 1987). The karyotyping is a time consuming process that requires certain expertise in cytological techniques. Besides, chromosome counting is difficult in some species, such as *Dianthus* spp. with abundant and small size chromosomes (Carolin 1957 and Balao *et al.* 2009). On the contrary, flow cytometry method, based on the detection of the fluorescence emitted by compounds that specifically bind to DNA, is cheaper, faster and very effective in estimating ploidy levels in plants (Dolezel *et al.* 2007 and Dolezel and Bartos 2005). In the present study, we investigated the ploidy levels of 60 carnation accessions necessary for planning a successful hybridization programme which aimed at cultivar development for varied ornamental usage and trait specific utility using FCM.

MATERIALS AND METHODS

We analyzed 60 carnation accessions which were grown under polyhouse at ICAR-Indian Institute of Horticultural Research, Hessarghatta Lake Post, Bengaluru under standard growing conditions during 2013-14 to 2014-15. The 60 genotypes used for the study were IIHR-CG-199, IIHR-CG-200, IIHR-CG-201, IIHR-CG-202, IIHR-CG-203, IIHR-CG-204, IIHR-CG-205, IIHR-CG-206, IIHR-CG-207, IIHR-CG-208, IIHR-CG-209, IIHR-CG-210, IIHR-CG-211, IIHR-CG-

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212, IIHR-CG-213, IIHR-CG-214, IIHR-CG-215, IIHR-CG-216, IIHR-CG-217, IIHR-CG-218, IIHR-CG-219, IIHR-CG-220, IIHR-CG-221, IIHR-CG-222, IIHR-CG-223, IIHR-CG-224, IIHR-CG-225, IIHR-CG-226, IIHR-CG-227, IIHR-CG-228, IIHR-CG-229, IIHR-CG-230, IIHR-CG-231, IIHR-CG-232, IIHR-CG-233, IIHR-CG-234, IIHR-CG-235, IIHR-CG-236, IIHR-CG-237, IIHR-CG-238, IIHR-CG-239, IIHR-CG-240, IIHR-CG-241, IIHR-CG-242, IIHR-CG-243, IIHR-CG-244, IIHR-CG-245, IIHR-CG-246, IIHR-CG-247, IIHR-CG-248, IIHR-CG-249, IIHR-CG-250, IIHR-CG-251, IIHR-CG-252, IIHR-CG-253, IIHR-CG-257, IIHR-CG-258, IIHRCH 1, IIHRIS 1 and IIHRIS 2.

For the isolation of nuclei, about 0.5 cm^2 of a mature leaf, which was chopped with a new razor blade in a petri dish in 0.25 ml nuclei-isolation buffer A of the Partec high resolution DNA kit (Partec, Munster) to which per ml 0.25 mg RNAse was added. After adding 0.75 ml propidium iodide (PI) solution (50 mg/l in water) the suspension with nuclei was filtered through a 30 µm mesh nylon filter. The fluorescence of the nuclei was measured using a Partec CA-II flow cytometer. DAPI (4, 6-Diamidino-2-Phenylindole Dihydrochloride) fluorescence intensities of the suspensions were measured using Partec Ploidy Analyser PA (Partec GmbH) to determine relative DNA fluorescence. Ploidy level was determined using variety Master as the most suitable external standard cultivar (Maria *et al.* 2013) and genome size was determined by comparing mean relative florescence of each sample with the 2C peak of unknown sample and an internal standard of known genome size. *Pisum sativum* L. Citrad with genome size of 9.09 pg (Eeckhaut *et al.* 2004, Vainola 2000, Dolezel and Bartos 2005, Dolezel *et al.* 2007) was used as an internal standard to calculate nuclear DNA content [2C DNA content of sample= 9.09 × (Mean fluorescence value of sample/mean fluorescence value of standard)]. The total number of base pair present in 2C nuclear DNA of each sample of carnation was calculated. The standard 1 pg of DNA contains 980 Mbp (Benett and Smith 1979). In a study by four laboratories (Dolezel *et al.* 1998) the nuclear DNA contents of *P. sativum* and *H. vulgare* were calculated to be 9.09 and 10.04 pg, respectively.

To corroborate and authenticate the results of flow cytometry, the actual ploidy level was ascertained through conventional cytology by counting chromosomes in root tip cells of four selected cultivars representing ploidy groups as distinguished by FCM. The root apices were collected from healthy plants, washed thoroughly with distilled water to remove soil particles and pre-treated with 0.003 M 8-Hydroxy quinoline for two hours at 14-16^oC. Then the pre-treated root tips fixed in Carnoy's fluid (6% absolute alcohol: 3% glacial acetic acid: 1% Chloroform) for 24 hours and were transferred to 70% ethanol and stored at -20^oC until needed and stored root tips were rinsed in distilled water and hydrolysed in water bath with 5 N HCL at room temperature for 30 min. Then the hydrolysed root tips was transferred to Schiff's reagent after rinsing in distilled water and stored in dark for one and half to two hours. The stained tips were squashed in a drop of 1% aceto-carmine and slides were sealed with gum mastic, and observed under the microscope on the same day or the next day. After scanning the slides the metaphase cells, where the chromosome was well spread was selected for counting chromosome number using Olympus research microscope model: Bx51. Three to five slides per accessions were prepared and several cells were observed to ascertain the chromosome number (Carolin 1957).

RESULTS AND DISCUSSION

Ploidy status by flow cytometry

The differences in ploidy level among the 60 carnation accessions studied are presented in Table 1.

Table 1 Nuclear DNA content (pg) and ploidy information of the studied carnation accessions as determined by flow cytometry

Accession number	Ploidy level	2C DNA content (pg)	Mbp (Mean)
IIHR-CG-200	2x	1.59	140
IIHR-CG-201	2x	1.43	126
IIHR-CG-204	2x	1.38	121
IIHR-CG-205	2x	1.42	125
IIHR-CG-208	2x	1.53	135
IIHR-CG-209	2x	1.39	122
IIHR-CG-213	2x	1.90	168
IIHR-CG-215	2x	1.46	129
IIHR-CG-217	2x	1.54	136
IIHR-CG-220	2x	1.92	169
IIHR-CG-221	2x	1.84	162
IIHR-CG-222	2x	1.95	172
IIHR-CG-223	2x	1.92	169
IIHR-CG-224	2x	1.82	160
IIHR-CG-226	2x	1.67	147
IIHR-CG-228	2x	1.56	138
IIHR-CG-230	2x	1.88	166
IIHR-CG-231	2x	1.47	130
IIHR-CG-232	2x	1.74	153
IIHR-CG-233	2x	1.78	157
IIHR-CG-234	2x	1.55	137
IIHR-CG-237	2x	1.57	139
IIHR-CG-239	2x	1.75	154
IIHR-CG-240	2x	1.39	122
IIHR-CG-243	2x	1.32	116
IIHR-CG-244	2x	1.66	146
IIHR-CG-246	2x	1.56	138
IIHR-CG-247	2x	1.44	127
IIHR-CG-248	2x	1.48	131
IIHR-CG-249	2x	1.65	145
IIHR-CG-250	2x	1.50	132
IIHR-CG-252	2x	1.73	152
IIHR-CG-258	2x	1.38	121
IIHR-CG-206	3x	2.03	179

Contd.

Table 1 (*Concluded*)

Accession number	Ploidy level	2C DNA content (pg)	Mbp (Mean)
IIHR-CG-207	3x	2.66	234
IIHR-CG-211	3x	2.06	182
IIHR-CG-219	3x	2.43	214
IIHR-CG-238	3x	2.72	239
IIHR-CG-203	4x	3.91	344
IIHR-CG-199	4x	2.98	263
IIHR-CG-210	4x	3.65	321
IIHR-CG-218	4x	4.06	358
IIHR-CG-235	4x	4.20	370
IIHR-CG-202	4x	4.31	380
IIHR-CG-212	4x	3.76	331
IIHR-CG-251	4x	3.98	350
IIHR-CG-227	4x	4.41	388
IIHR-CG-214	4x	4.55	401
IIHR-CG -225	4x	3.99	351
IIHR-CG-253	4x	2.98	263
IIHR-CG-241	4x	4.54	400
IIHRCH 1	4x	4.65	409
IIHRIS 1	4x	4.57	402
IIHRIS 2	4x	3.09	272
IIHR-CG-245	4x	3.87	340
IIHR-CG-242	4x	4.23	373
IIHR-CG-229	4x	4.27	376
IIHR-CG-216	4x	3.76	331
IIHR-CG-236	4x	4.29	378
IIHR-CG-257	6x	5.33	469

1 picogram (pg) = 965 million base pairs (Mbp)

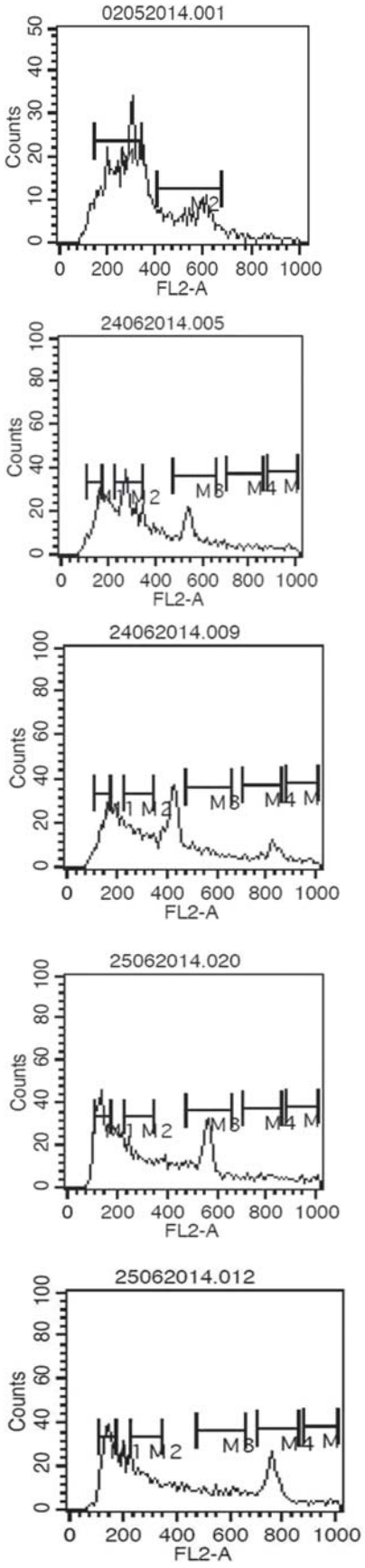
The genotype Master was selected as the most suitable internal calibration standard cultivar (control) for ploidy determination in carnation. Accessions with relative florescence of 150 to 300 range were selected as diploid (2x). Out of 60 carnation accessions, 33 were diploid (2x) with relative florescence of 150 to 300 range. These results are in accordance with results obtained by Yagi *et al.* (2009); Maria *et al.* (2013) and Bonos *et al.* (2002). Whereas, five accessions determined to be triploid (3x) ranged from 300 to 450, viz. IIHR-CG-206; IIHR-CG-207; IIHR-CG-211; IIHR-CG-219 and IIHR-CG-238. Similarly 21 accessions were tetraploid (4x) with relative florescence of 450 to 600, viz. IIHR-CG-203; IIHR-CG-199; IIHR-CG-210; IIHR-CG-218; IIHR-CG-235; IIHR-CG-202; IIHR-CG-212; IIHR-CG-251; IIHR-CG-227; IIHR-CG-214; IIHR-CG-225; IIHR-CG-253; IIHR-CG-241; IIHRCH 1; IIHRIS 1; IIHRIS 2; IIHR-CG-245; IIHR-CG-242; IIHR-CG-229; IIHR-CG-216 and IIHR-CG-236. Similarly, the 5 accessions of group 2 (triploid) and 21 accessions of groups 3 (tetraploid) had almost 2.5 and 3 times the value of Master was considered to be triploid and tetraploid accession. One accession IIHR-CG-257 was hexaploid (6x) with relative florescence of more the 900 (Fig 1) which had almost the value of *D. broteri* (Maria *et al.* 2013).

Estimation of nuclear DNA contents by flow cytometry

Using a quick and reliable flow cytometry protocol, the frequency distribution of the relative nuclear DNA contents clearly classified the accessions into four groups (Table 1). The genome size was determined by comparing mean relative florescence of each sample with the 2C peak of unknown sample and an internal standard of known genome size. *Pisum sativum* Citrad was used as an internal standard. The DNA content was estimated in 60 carnation accessions. The nuclear DNA content analysis showed that the DNA contents for the diploid carnation accessions ranged from 1.32 pg in IIHR-CG-243 to 1.95 pg in IIHR-CG-222 (group 1). The nuclear DNA content for the triploid carnation accessions varied from 2.03 pg in IIHR-CG-206 to 2.72 pg in IIHR-CG-238 (group 2). For tetraploid carnation accessions IIHR-CG-199 had the lowest DNA content of 2.98 pg while, IIHRCH 1 had the highest DNA content of 4.65 pg (group 3). The nuclear DNA content of hexaploid carnation was 5.33 pg in IIHR-CG-257 (group 4), which had almost the value of *D. broteri* (Maria *et al.* 2013) for the same species (5-5.61 pg) considered to be hexaploid. However, estimates for DNA content were found to vary depending on the fluorescence used (Dolezel and Bartos 2005). For a quick determination of ploidy in different cultivars of a single species, Dolezel *et al.* (2007) suggested the use of an external standard from the same species. Maria *et al.* (2013) suggested that cultivar, Master was selected as the most suitable external standard cultivar for ploidy determination in cultivated carnation due to its commercial availability and wide use in breeding programs.

Elucidation of representative genotypes of chromosome number using conventional cytological technique

Many species in the subfamily Caryophylloideae have diploid and tetraploid cytotypes. The present data document polyploidy of *Dianthus*, with four ploidy levels (2x, 3x, 4x and 6x). The complexity of this series was comparable to those reported in *Dianthus broteri* (Balao *et al.* 2009, Weiss *et al.* 2002). Elucidation and validation of flow cytometry information on ploidy levels was done by using conventional cytological technique taking root tip of four carnation accessions each one representing different ploidy levels. Accession IIHR-CG-204 was found diploid cultivar by counting chromosomes in the root tips in metaphase at magnification of 100X. This result is in agreement with the results of Carolin (1957), Yagi *et al.* (2007) and Maria *et al.* (2013). Galbally and Galbally (1997) reported that majority of available cultivars of carnation in Australia and Europe are diploid. The accession IIHR-CG-207 had 2n=3x=45 was triploid; IIHR-CG-227 had 2n=4x=60 was tetraploid and similar findings was reported by Ion *et al.* (2003). Whereas, accession IIHR-CG-257 had 2n=6x=90 was hexaploid and similar results were obtained by Balao *et al.* (2009). Finally IIHR-CG-204, IIHR-CG-207, IIHR-CG-227 and IIHR-CG-257 were thus confirmed to be diploid (2x), triploid (3*x*), tetraploid (4x) and hexaploid (6x) and the ploidy levels of the accession analyzed by flow cytometry and conventional



Histogram Statistics

File: 02052014.001 Sample ID: Master Ctrl
Tube: Untitled Acquisition Date: 02-May-14
Gate: G1 Gated Events: 3984
Total Events: 10000 X Parameter: FL2-A (Linear)

Marker	Left	Right	Events	% Gated	% Tatal	Mean	CV
All	0,	1023	3984	100.00	39.84	335.14	43.04
M1	146,	342	2484	62.35	24.84	260.94	19.74
M2	400,	669	827	20.76	8.27	532.39	14.16

File: 24062014.005 Sample ID: IIHR-CG-204
Tube: Untitled Acquisition Date: 24-Jun-14

Marker	Left	Right	Events	% Gated	% Tatal	Mean	CV
All	0,	1023	5707	100.00	57.07	344.75	51.78
M1	107,	179	836	14.65	8.36	154.09	11.95
M2	234,	350	1953	32.47	18.53	285.22	10.95
M3	482,	661	925	16.21	9.25	552.47	7.71
M4	712,	870	182	3.19	1.82	783.73	6.07
M5	882,	1014	77	1.35	0.77	939.12	3.51

File: 24062014.009 Sample ID: IIHR-CG-211
Tube: Untitled Acquisition Date: 24-Jun-14

Marker	Left	Right	Events	% Gated	% Tatal	Mean	CV
All	0,	1023	5944	100.00	59.44	367.14	52.38
M1	107,	179	900	15.14	9.00	152.82	12.31
M2	234,	350	1237	20.81	12.37	286.80	11.98
M3	482,	661	585	9.84	5.85	556.50	9.08
M4	712,	870	407	6.85	4.07	805.97	4.86
M5	882,	1014	70	1.18	0.70	934.10	3.45

File: 25062014.020 Sample ID: IIHR-CG-227
Tube: Untitled Acquisition Date: 25-Jun-14

Marker	Left	Right	Events	% Gated	% Tatal	Mean	CV
All	0,	1023	6784	100.00	67.84	342.16	60.25
M1	107,	179	1870	27.56	18.70	140.03	14.50
M2	234,	350	1116	16.45	11.16	285.98	11.60
M3	482,	661	1430	21.08	14.30	555.07	6.59
M4	712,	870	248	3.66	2.48	788.40	5.78
M5	882,	1014	128	1.89	1.28	940.70	3.63

File: 25062014.012 Sample ID: IIHR-CG-257
Tube: Untitled Acquisition Date: 25-Jun-14

Marker	Left	Right	Events	% Gated	% Tatal	Mean	CV
All	0,	1023	6471	100.00	64.71	388.07	61.07
M1	107,	179	1517	23.44	15.17	146.57	12.48
M2	234,	350	1199	18.53	11.99	285.91	11.60
M3	482,	661	737	11.39	7.37	570.41	9.27
M4	712,	870	1029	15.90	10.29	764.39	4.27
M5	882,	1014	107	1.65	1.07	941.08	3.57

Fig 1 Histogram of fluorescence intensity carnation accessions nuclei stained with propidium iodide. Results represent measurements of diploid (2n=2x), triploid, (2n=3x), tetraploid (2n=4x) and hexaploid (2n=90) when used as cv. Master as external standard for ploidy.

cytological technique were found to be the same. Balao *et al.* (2009) detected extensive variation in chromosomes numbers (2n = 2x = 30, 2n = 4x = 60, 2n = 6x = 90 and 2n = 12x =180) and the dodecaploid cytotype was reported for the first time in this *Dianthus* genus. Among the previous studies on *Dianthus* sect. *Plumaria*, Weiss *et al*. (2002) identified tetraploid and hexaploid cytotypes for *D. nardiformis* Jan ka. Further Ion *et al*. (2003) reported diploid and tetraploid cytotypes for *D. spiculifolius* Schur. The results of the experiments will be utilised in planning and execution of systematic hybridization programme aimed at incorporation/development of trait specific genotypes for commercial use.

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