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RESEARCH ARTICLE

Cytogenetic and molecular evidences revealing genomic changes after autopolyploidization: a case study of synthetic autotetraploid *Phlox drummondii* Hook

Tanvir H. Dar[1,3] · **Soom N. Raina**[2] · **Shailendra Goel**[1]

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Abstract Polyploidy is known to be common in plants; indeed most of the world's economically important crop plants are polyploids. Recent studies revealed extensive genomic changes in synthetic polyploids after genome doubling, although most of the information available is with regards to allopolyploids and little information have been generated in autopolyploids. In the present study, we used *Phlox drummondii* Hooker ($2n = 2x = 14$) as a model plant to observe genomic changes, if any, in synthetic autopolyploids. Colchitetraploids were produced and followed through different generations (C_0, C_1, C_2 and C_3). Male meiosis analysis showed differences between the frequency of both quadrivalents and bivalents from C_0 to C_2 generations. RAPD analysis revealed 2.8, 1.6, 2.1 and 3.2% polymorphism in C_0, C_1, C_2 and C_3 colchitetraploids respectively. The polymorphic fragments were further characterized after cloning. Dot blot assay was performed to confirm high copy/low copy nature of fragments showing variation. The analysis revealed changes in both repetitive and non-repetitive regions. Out of the six fragments only one fragment T01 was found to be of high copy, while four fragments were of the moderate copy and one fragment of the low copy nature.

✉ Tanvir H. Dar
tanvirulhasan@gmail.com

[1] Department of Botany, University of Delhi, Delhi 110007, India

[2] Amity Institute of Biotechnology, Amity University, Sector-125, Noida 201303, UP, India

[3] Present Address: Centre for Biodiversity Studies, School of Biosciences & Biotechnology, Baba Ghulam Shah Badshah University, Rajouri 185234, Jammu and Kashmir, India

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Introduction

Polyploidy has played an important role in the evolution of plants. Polyploids are mainly classified into autopolyploids and allopolyploids, depending on the nature of their formation. The doubling of a single species genome forms autopolyploids, whereas interspecific hybridization and subsequent doubling of genome form allopolyploids. Autopolyploids are characterized by multivalent formation and polysomic inheritance, while allopolyploids exhibit normal bivalent formation and disomic inheritance (Jackson 1982; Ramsey and Schemske 2002). Genome duplication has been shown to play a major role in the plant evolution. Adaptive changes, such as increased tolerance to extreme environmental conditions, effective means of vegetative reproduction, apomixes, pest resistance and variation in organ size, flowering time and biomass have facilitated the evolutionary success of polyploids, thereby making them a preferred choice for agricultural use (Stebbins 1950; Ramsey and Schemske 2002; Gaeta et al. 2007).

Recent studies have revealed that polyploidy genomes are highly dynamic and are associated with rapid structural and functional changes (Doyle et al. 2008; Soltis and Soltis 2009; Parisod et al. 2010; Yang et al. 2011; Chester et al. 2012; Dar et al. 2013). Song et al. (1995) reported changes in genomic organization of synthetic allotetraploid *Brassica* sp. and detected non-additive inheritance of genomic fragments. Wheat allotetraploids have also displayed genomic changes immediately after hybrid formation (Feldman et al. 1997; Ozkan et al. 2001; Town et al. 2006).

In contrast to *Brassica* sp and Wheat, genomic changes in cotton and *Spartina* polyploids occur at a very low frequency (Liu et al. 2001; Salmon et al. 2005). These studies suggest that cotton and *Spartina* have a high-level of tolerance for genome doubling and interspecific hybridization as compared to *Brassica* and wheat. As compared to changes observed in allotetraploids, negligible amount of changes were observed in synthetic autotetraploids. Allario et al. (2011) showed identical microsatellite profiles of diploid and tetraploid *Citrus limonia*. Similarly, Aversano et al. (2013) did not find any structural changes in *Solanum* wild species after autopolyploidization. Large-scale microarray studies in a range of plant species have confirmed that gene expression is radically changed by polyploidy (Wang et al. 2006; Stupar et al. 2007). Recently, Li et al. (2012) reported a small but stable increase in the expression of the cell cycle genes in autotetraploid seedlings of *Arabidopsis thaliana*.

Induction of chromosome doubling has both practical as well as theoretical importance in genetics and evolution. Analysis of meiotic chromosome association analysis in autopolyploids can give precise information on organization of chromosome pairing and synapsis during meiosis (Sybenga 1975). Extensive chromosomal variation was observed in *Tragopogon miscellus* after about 40 generations, suggesting prolonged chromosomal instability in natural populations after polyploidization (Chester et al. 2012).

Allopolyploids have received more attention than autopolyploids due to immense economic value associated with allopolyploid species such as wheat, cotton, coffee, and tobacco. In contrast to allopolyploids, little is known about the genetic impact imposed by autopolyploidization. In view of these facts, in the present study *Phlox drummondii* Hooker ($2n = 2x = 14$), an ornamental plant species belonging to the family Polemoniaceae, was used as a model plant to investigate genomic changes, if any, in synthetic autopolyploid. The autopolyploids were raised in *P. drummondii* by giving colchicine treatment. Four different colchitetraploid (C_0, C_1, C_2 and C_3) generations were raised. Colchitetraploids produced were analyzed meiotically to confirm chromosomal associations. RAPD marker used showed variation between diploids and colchitetraploids. Bands showing variation were eluted, cloned and then sequenced, in order to characterize the nature of variation.

Materials and methods

Plant material

The seeds of diploid *P. drummondii* Hook. used in the present study were obtained from Sluis and Groot, Enkhuizen, Netherlands. Initially 20 diploid plants were grown in earthen pots in glass house. The seeds obtained from these plants were sown in pots and colchicine treatment was given to 3–4 days old seedlings. The seedlings were allowed to grow in glass house till maturity without any intervention under glass house conditions to prevent pollen contamination from outside sources. The seeds were collected from confirmed colchitetraploids (C_0) and sown in order to produce C_1 generation. In a similar fashion, C_2 and C_3 colchitetraploid generations were raised from seeds produced by C_1 and C_2 generations respectively.

Methods

Total DNA isolation

Total genomic DNA was extracted from leaf material according to the procedure of Murray and Thompson (1980).

Induction of polyploidy

For colchicinization cotton swab method was used. Sterilized cotton swabs, of suitable size, were placed on the emerging apical tips between two cotyledonary leaves of 3–4 day old seedlings. The aqueous solution of colchicine (Sigma, USA) in three concentrations (0.1, 0.15 and 0.2%) was added drop by drop with the help of Pasteur pipette, at some intervals, to the cotton swab. Care was taken that the thin film of colchicine solution between the shoot tip and the cotton was maintained throughout the treatment. The seedlings were subjected to this treatment early in the morning for 6 h duration till 1–3 days.

Male meiosis

Young flower buds of appropriate size were fixed in glacial acetic acid: ethanol (1:3) solution for at least 24 h. After fixation, the anthers were dissected out from the flower buds and squashed in 1% acetocarmine. At least 20 cells in each five different plants of C_1, C_2 and C_3 generations were analyzed at diakinesis, metaphase and anaphase stages. Suitable chromosome preparations were photographed from temporary slides using BH-2 microscope (Olympus, USA) in combination with digital DSC- W7 (Sony, Japan) photographic attachment.

Random amplified polymorphic DNA (RAPD) fingerprinting

RAPD analysis was performed using 10-mer primers of A, C, H, I, K R, and T series, obtained from Operon

Table 1 Induction of polyploidy by colchicine in *P. drummondii* Hook (2n = 2x = 14)

Concentration of colchicine	Duration of treatment in hours	No. of days	No. of seedlings treated	No. of seedlings survived	No. of colchitetraploids	Percentage of tetraploids
0.1	6	2	50	42	13	30.95
0.1	6	3	50	38	12	31.57
0.15	6	2	40	30	9	30.00
0.15	6	3	40	35	18	51.42
0.2	6	2	30	15	7	46.66
0.2	6	3	30	13	5	38.46
Total			240	173	64	38.17

Technologies Inc., California, USA. Amplification was carried out in 25 µl reaction volume, containing 2.5 µl of 10X assay buffer with 20 mM $MgCl_2$ (Intron Biotechnology, Korea), 0.24 mM dNTPs (Amersham Pharmacia Biotech, UK), 15 ng primer, 1U Taq DNA polymerase (Intron, Korea), and 20 ng template DNA. DNA amplification was performed in Veriti thermal cycler (Applied Biosystems, USA) programmed to 1 cycle of 4 min at 94 °C (denaturation), 1 min at the 37 °C (annealing) and 2 min at 72 °C (extension); followed by 44 cycles of 1 min at 94 °C, 1 min at 37 °C, and 2 min at 72 °C, and finally 1 cycle of 15 min at 72 °C. Using these conditions, 120 randomly selected decamer primers were tested, of these 25 primers were found to produce good amplification products. After completion of the PCR, 2.5 µl of 10X gel loading buffer (bromophenol blue 0.4%, xylene cyanol FF 0.4%, sucrose 66%) was added to the sample. The amplification products were size separated by electrophoresis on 1.2% agarose (Sigma, USA) gels containing 0.05 µg/ml ethidium bromide in 0.5X TBE buffer. Lamda DNA, digested with *Hind* III, served as a marker. After agarose gel electrophoresis, the gel was visualized and photographed on Gel documentation instrument (UVP, USA). The reproducibility of DNA profiles was tested by repeating the PCR amplification twice, for each of the primers used. Only the consistently reproducible bands were considered for analysis.

Isolation and characterization of RAPD fragments

RAPD fragments showing variation between diploids and colchitetraploids were excised from the agarose gel by visualizing it under UV transilluminator (Pharmacia, USA) and purified using the SV wizard gel purification system (Promega, USA) according to manufacturer's instruction. The purified fragments were then cloned in pGEM-T easy vector (Promega, USA) according to manufacturer's instruction. The cloned fragments were sequenced at sequencing centre, University of Delhi, South Campus, Delhi. Sequenced data obtained were compared with the GenBank data base at NCBI using the BLASTN program. For each sample three colonies were sequenced.

Dot blot

The method used for obtaining Dot blot was carried out as described by Dar et al. (2013).

Results

Induction of polyploidy

Three different concentrations of colchicine were used for creating polyploids. Details of the experiment are given in (Table 1). In total, 240 plants were treated, out of which only 173 survived till maturity. Tetraploids were obtained in all three colchicine treatments, (0.10, 0.15, and 0.20%) although highest percentage of tetraploids was achieved with 0.15% colchicine treatment, with 6 h duration per day for 3 consecutive days.

All colchitetraploids were identified by stomatal size and eventually confirmed by meiotic analysis. The tetraploids showed initially slower growth as compared to diploids, but robust growth was achieved later. The leaves of the colchitetraploids were larger in size than the diploid (Fig. 1a, b). The number of stomata per unit area was less as compared to diploids (Fig. 1c, d). When compared to diploid parents, the flower size in colchitetraploids was larger and flowering was delayed. The pollen grains were also considerably larger in colchitetraploids (Fig. 1e, f).

Male meiosis

Male meiotic analysis was carried out to study the chromosomal associations and stability of colchitetraploids of *P. drummondii* through generations. At least 20 PMCs were analyzed at diakinesis/metaphase I for five different plants in each generation.

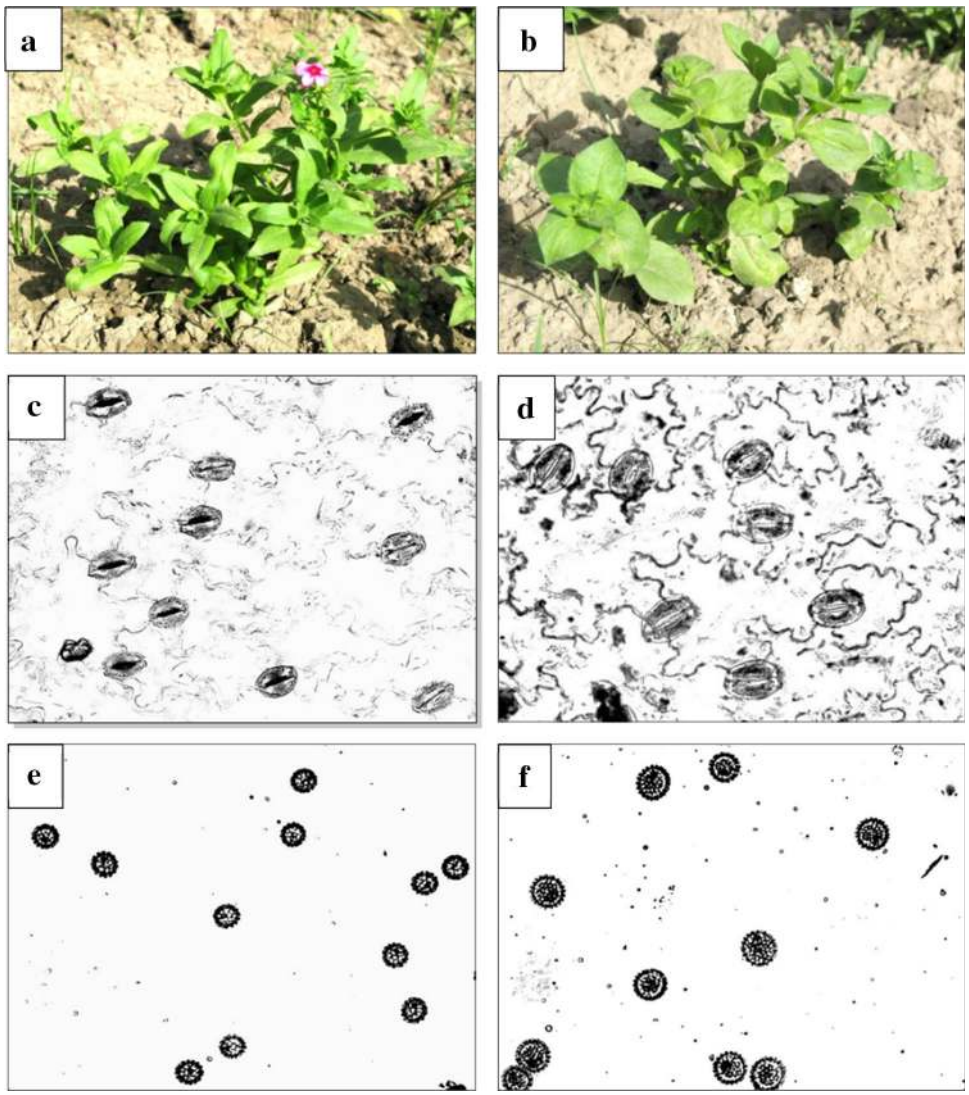

Fig. 1 Comparison of diploid (*left*) and colchitetraploid (*right*), plant morphology (**a**, **b**), stomata (**c**, **d**), pollen grains (**e**, **f**) of *P. drummondii*

Diploids

All the PMCs analyzed in 23 diploid plants exhibited 7II and did not show any variation in the type and number of chromosomal associations (Fig. 2c, d).

Colchitetraploids

PMCs were analyzed from colchitetraploids of C_0, C_1 and C_2 generations. In all, 20 PMCs from 5 different plants were analyzed for each generation. All the colchitetraploids investigated had zygotic number of $2n = 28$ although there were significant differences in number and type of chromosomal associations observed. Number of bivalents ranged from 2 to 10 in C_0 and C_1 generation with an average of 5.05 and 5.17 per cell respectively. This range increased to 2–14 in C_2 generation with an average of 5.35 per cell. All the bivalents were usually of ring type. Trivalents were only seen in C_0 generation ranging from 0 to 2 with an average of 0.04, although they were conspicuously absent from C_1 and C_2 generations.

Quadrivalents, showed an increasing trend from C_0 to C_2 generation ranging from 1 to 7 in C_0, 2–7 in C_1 and C_2 generation with an increasing average per cell from 4.05 in C_0, 4.23 in C_1 and 4.52 in C_2 generation. Contrary to bi- and quadrivalents, univalents exhibited a downward trend from C_0 to C_2 generation. The range of univalents was 0–8 in C_0, 0–6 in C_1 and 0–4 in C_2 with average per cell decreasing from 1.21 in C_0 to 0.94 in C_1 to 0.4 in C_2 generation (Table 2). Figure 2 shows representative images from C_0 (2e–g), C_1 (2h–j) and C_2 (2k–m) generations.

RAPD analysis

A set of 100 primers were tested on two diploid and two plants each from C_0, C_1, C_2 and C_3 generations of colchitetraploids. Of these primers, 23 primers produced clear, scorable amplification products. These 23 primers were then

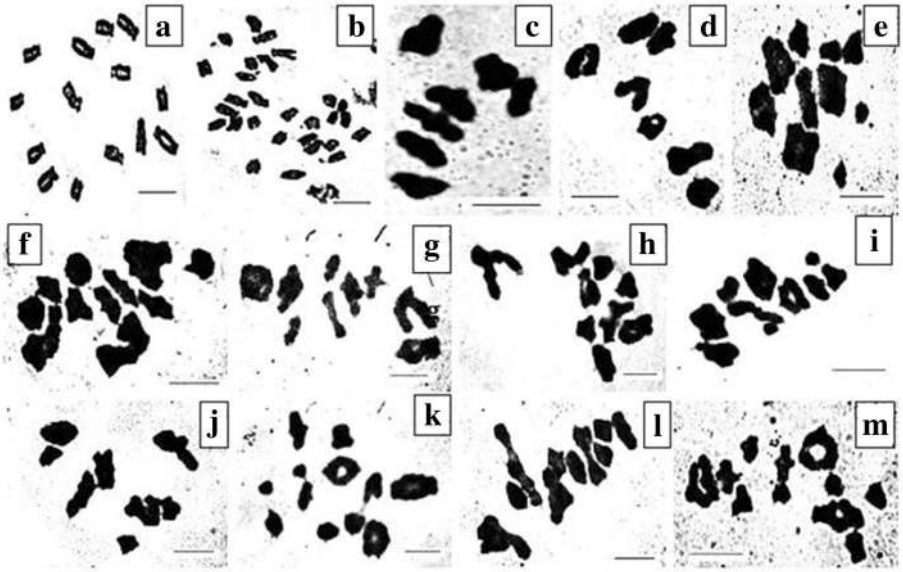

Fig. 2 Chromosomal associations of diploid and colchitetraploid *P. drummondii*. Root tip mitotic metaphase spread of diploid (**a**) and colchitetraploid (**b**). **c**, **d** Male meiosis in the diploid. **e**–**g** Male meiosis in the C_0 generation. **h**–**j** Male meiosis in the C_1 generation. **k**–**m** Male meiosis in the C_2 generation

used on 18 diploid, 30 C_0, 15 C_1, 15 C_2 and 15 C_3 colchitetraploid plants. A total of 144 amplification products were produced with an average frequency of 6.2 amplicons per primer (Table 3). Primer OPA-07 produced the maximum (9) and primers OPT-16 and OPH-19 produced minimum (3) number of amplicons. Only 7 primers exhibited polymorphism in colchitetraploids when compared to their diploid parents. None of these 7 primers produced more than one polymorphic fragment differentiating between diploids and colchitetraploids. These polymorphic amplicons ranged from 400 to 1014 bps in size (Table 4). These novel amplicons appeared randomly in colchitetraploids belonging to C_0, C_1, C_2 and C_3 generations.

The average polymorphism observed in C_0, C_1, C_2 and C_3 colchitetraploids was 2.8, 1.6, 2.1 and 3.2% respectively (Table 3). Polymorphic amplicons produced by primer OPA-07 and OPH-15 were present in all the four-colchitetraploid generations studied. The polymorphic amplicon produced by

Table 2 Average number and range of associations in C_0, C_1 and C_2 colchitetraploids of *P. drummondii*

Colchiploid generation	Plant no.	No. of cells analyzed	Quadrivalents		Trivalents		Bivalents		Univalents	
			Mean	Range	Mean	Range	Mean	Range	Mean	Range
C_0	C_{0-1}	20	4.60 ± 1.42	2–7	0.20 ± 0.63	0–2	4.20 ± 2.29	2–8	0.60 ± 0.96	0–2
	C_{0-2}	20	4.60 ± 1.07	3–6	_	_	4.10 ± 2.28	2–8	1.40 ± 1.64	2–4
	C_{0-3}	20	4.09 ± 1.30	3–6	_	_	5.09 ± 3.04	2–8	1.45 ± 3.58	0–2
	C_{0-4}	20	3.30 ± 1.41	2–6	_	_	6.70 ± 2.66	2–10	1.40 ± 2.31	0–4
	C_{0-5}	20	3.70 ± 1.15	1–5	_	_	5.20 ± 1.54	3–8	1.20 ± 2.50	2–8
Over all mean			4.05 ± 1.27		0.04 ± 0.12		5.05 ± 2.36		1.21 ± 2.19	
C_1	C_{1-1}	20	4.80 ± 1.31	3–6	_	_	4.20 ± 2.20	2–7	0.40 ± 0.84	0–2
	C_{1-2}	20	4.70 ± 1.15	3–7	_	_	5.18 ± 3.06	2–8	0.90 ± 1.37	0–4
	C_{1-3}	20	4.27 ± 1.34	3–6	_	_	4.40 ± 1.95	2–7	1.00 ± 3.31	2–6
	C_{1-4}	20	3.50 ± 1.26	4–6	_	_	6.60 ± 2.54	2–8	1.20 ± 2.10	0–2
	C_{1-5}	20	3.90 ± 1.19	2–7	_	_	5.50 ± 1.43	2–10	1.20 ± 2.52	2–4
Over all mean			4.23 ± 1.25	_			5.17 ± 2.23		0.94 ± 2.02	
C_2	C_{2-1}	20	5.08 ± 1.62	2–7	_	_	3.25 ± 2.70	2–8	0.83 ± 1.33	2–4
	C_{2-2}	20	4.09 ± 2.21	3–6	_	_	5.72 ± 4.38	2–14	0.18 ± 0.60	0–2
	C_{2-3}	20	5.00 ± 1.24	3–7	_	_	4.00 ± 2.49	2–8	_	_
	C_{2-4}	20	5.18 ± 0.80	4–7	_	_	3.45 ± 1.96	2–8	0.36 ± 0.80	0–2
	C_{2-5}	20	4.66 ± 1.00	3–7	_	_	4.33 ± 2.30	2–7	0.66 ± 0.98	0–2
Over all mean			4.52 ± 1.47				5.35 ± 2.76		0.40 ± 0.74	

Table 3 Summary of bands appearing in different colchitetraploid generations with RAPD

S. no.	Primers	C_0			C_1			C_2			C_3		
		T	NP	P (%)	T	NP	P (%)	T	NP	P (%)	T	NP	P (%)
1	OPC-11	8	0	0.0	8	0	0.0	8	1	12.5	8	1	12.5
2	OPA-07	9	1	11.1	9	1	11.1	9	1	11.1	9	1	11.1
3	OPH-05	4	0	0.0	4	0	0.0	4	0	0.0	4	1	25.0
4	OPC-16	7	1	14.2	7	0	0.0	7	0	0.0	7	0	0.0
5	OPC-15	8	1	12.5	8	0	0.0	8	0	0.0	8	1	12.5
6	OPH-15	8	1	12.5	8	1	12.5	8	1	12.5	8	1	12.5
7	OPR-09	7	1	14.2	7	1	14.2	7	1	14.2	7	0	0.0
8	OPA-10	8	0	0.0	8	0	0.0	8	0	0.0	8	0	0.0
9	OPT-08	8	0	0.0	8	0	0.0	8	0	0.0	8	0	0.0
10	OPA-03	6	0	0.0	6	0	0.0	6	0	0.0	6	0	0.0
11	OPT-02	8	0	0.0	8	0	0.0	8	0	0.0	8	0	0.0
12	OPT-16	3	0	0.0	3	0	0.0	3	0	0.0	3	0	0.0
13	OPA-09	7	0	0.0	7	0	0.0	7	0	0.0	7	0	0.0
14	OPR-02	4	0	0.0	4	0	0.0	4	0	0.0	4	0	0.0
15	OPI-07	5	0	0.0	5	0	0.0	5	0	0.0	5	0	0.0
16	OPH-19	3	0	0.0	3	0	0.0	3	0	0.0	3	0	0.0
17	OPT-20	6	0	0.0	6	0	0.0	6	0	0.0	6	0	0.0
18	OPC-08	8	0	0.0	8	0	0.0	8	0	0.0	8	0	0.0
19	OPK-14	7	0	0.0	7	0	0.0	7	0	0.0	7	0	0.0
20	OPK-19	5	0	0.0	5	0	0.0	5	0	0.0	5	0	0.0
21	OPK-07	4	0	0.0	4	0	0.0	4	0	0.0	4	0	0.0
22	OPK-06	7	0	0.0	7	0	0.0	7	0	0.0	7	0	0.0
23	OPC-14	4	0	0.0	4	0	0.0	4	0	0.0	4	0	0.0
	Mean	6.2	0.2	2.8	6.2	0.1	1.6	6.2	0.1	2.1	6.2	0.2	3.2

T total number of bands; *NP* number of polymorphic bands; *P* percentage polymorphism

Table 4 DNA fragments showing variation in diploids (2x) and different colchitetraploid generations (C_0, C_1, C_2 and C_3) of *P. drummondii* by RAPD

Fragment	Primer used	Band size (bp)	Samples showing variation				
			2x	C_0	C_1	C_2	C_3
TD01	OPC-11	1014	–	–	–	$C_2$13[a], $C_2$15	$C_3$2, $C_3$3, $C_3$14
TD02	OPA-07	651	–	$C_0$1, $C_0$8, $C_0$12, $C_0$22, $C_0$26	$C_1$9	$C_2$12	$C_3$1, $C_3$2, $C_3$13, $C_3$14
TD03	OPH-05	497	–	–	–	–	$C_3$2, $C_3$8, $C_3$10, $C_3$11, $C_3$13, $C_3$14, $C_3$15
TD04	OPC-16	680	–	$C_0$11, $C_0$13	–	–	–
TD05	OPC-15	587	–	$C_0$23, $C_0$26	–	–	$C_3$3, $C_3$13
TD06	OPH-15	446	–	$C_0$12, $C_0$22	$C_1$10	$C_2$13	$C_3$2, $C_3$3, $C_3$10, $C_3$11, $C_3$12,$C_3$13, $C_3$14, $C_3$15
TD07	OPR-09	400	–	$C_0$2, $C_0$4, $C_0$10, $C_0$26, $C_0$27	$C_1$7, $C_1$13	$C_2$9	–

There were 18 plants in 2x, 30 in C_0, 15 in C_1, 15 in C_2 and 15 in C_3 generation, numbered accordingly 1–30

[a] Represents plant number showing variation

primer OPR-09 was present in C_0, C_1 and C_2 generation but could not be identified in plants of C_3 generation. Polymorphic amplicon produced by primer OPC-11 was absent from initial generations of C_0 and C_1 and was present in C_2 and C_3 colchitetraploid generation. Similarly, polymorphic amplicon by primer OPH-05 was absent from C_0, C_1 and C_2 generations and was only present in C_3 colchitetraploid generation. The polymorphic amplicon by primer OPC-16

was only present in C_0 generation of colchitetraploids and was absent from subsequent generation. Most unique polymorphism was shown by OPC-15, which was present in C_0 generation but disappeared from C_1 and C_2 colchitetraploid generation and reappeared in C_3 generation (Table 4).

Characterization of RAPD fragments showing variation

Out of seven fragments showing variation, six were eluted and cloned. The cloned fragments were sequenced and compared to NCBI GenBank database by using BLASTN programme. Only four (T01, T02, T04 and T05) of the six fragments showed significant similarity to known sequences. Rest two (T03 and T06) did not show any significant similarity (Table 5).

Fragments were also analyzed for their repetitive nature using dot blot (Fig. 3b). Fragment T01 was found to be of high copy while fragment T06 was of low copy. Fragments T02, T03, T04 and T05 were of moderate copy (Table 5; Fig. 3b).

Discussion

In the present study, colchitetraploids were generated and analyzed in *P. drummondii*. These colchitetraploids were followed through multiple generations (C_0, C_1, C_2 and C_3). Different markers, viz. cytological and DNA-based, were used to analyze variation, if any, between diploids and colchitetraploids. A significantly higher percentage of colchitetraploids was obtained in the present study when compared to earlier reports in *P. drummondii* (Dhillon 1970; Raghuvanshi and Pathak 1975; Rao et al. 1982; Verma and Raina 1991). Better success rate achieved in present investigation is due to proper monitoring and continuous flow of aqueous colchicine solution into apical buds. A slower rate of growth and development was observed at initial stages of colchitetraploid plants, substantiated by earlier reports (Zadoo et al. 1975; Srivastav and Raina 1982, 1987; Dibyendu 2010). The slow rate of growth has been linked with reduced rate of cell division due to longer mitotic cycle (Bennett 1985), lower amount

Table 5 Sequence analysis of RAPD fragments

Fragment	Size (bp)	Gene bank accession no.	Sequence homology	Query coverage (%)	Identity (%)	E-value	High copy/Low copy based on dot blot
T01	1014	AY053576.1	*Populus trichocarpa* cytochrome oxidase Subunit I gene, partial CDS	47	99	0.0	High copy
T02	651	XR002215763.1	*Ananas comosus* predicted uncharacterized ncRNA	20	73	2e-10	Moderate copy
T03	497	LM484600.1	*Parascaris equarum* genome assembly	7	89	0.71	Moderate copy
T04	680	GU3511514.1	*Sphenoclea zeylanica* NADH dehydrogenase, Subunit 5 gene, partial CDS	53	96	3e-166	Moderate copy
T05	587	EF442678.1	*Phlox pilosa* subsp. *pilosa* clone M115-M116 microsatellite sequence	35	88	1e-67	Moderate copy
T06	446	XM017389038.1	*Dacus carota* uncharacterized mRNA	13	80	0.18	Low copy

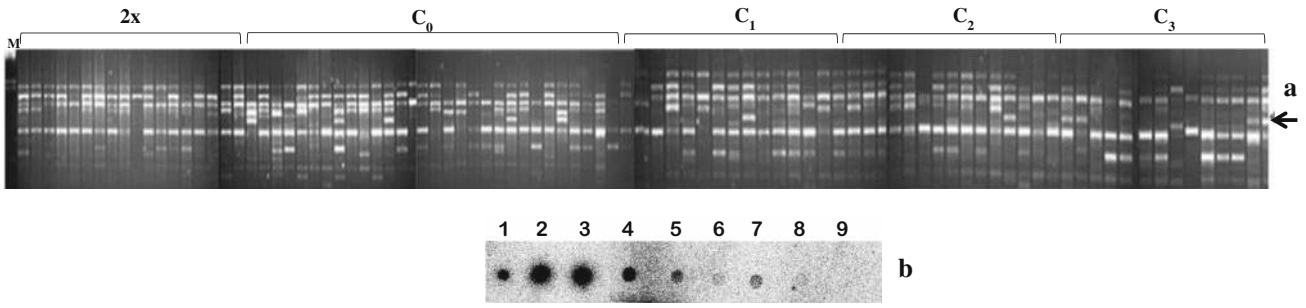

Fig. 3 **a** Gel electrophoresis of amplification products obtained with RAPD primer OPA-07 in the diploid and C_0, C_1, C_2 and C_3 colchitetraploids of *P. drummondii* plants. Lane M- λ phage DNA digested with Hind III (Marker), lane 1–18 (2x), lane 19–48 (C_0), lane 49–63 (C_1), lane 64–78 (C_2), lane 79–94 (C_3). **b** Dot blot analysis of DNA fragments showing variation in diploid and colchitetraploids of *P. drummondii*. Serial numbers 1–3 control ribosomal DNA 30, 50 and 100 ngs respectively. Serial numbers 4–9 RAPD fragments (T01, T02, T03, T04, T05, T06) showing variation. The blot was probed with P^{32} labelled genomic DNA from colchitetraploid *P. drummondii*

of growth hormones (Avery and Pottorf 1945; Larsen and Mintung 1950), and slower rate of metabolic activities (Chen and Tang 1945). However, there are a few reports where autopolyploids have been found to have faster growth and development at initial stages of the growth period (Mehta and Swaminathan 1957; Pal and Khoshoo 1968, 1977; Arora 1975; Bewal et al. 2009).

An increase in stomatal size, pollen size and flower size was observed in colchitetraploids which is also in agreement with earlier reports (Srivastava and Srivastava 2002; Thao et al. 2003; Joshi and Verma 2004; Oliveira et al. 2004; Omidbaigi et al. 2010; Lavania et al. 2012). These three features have often been used as reliable indicators of ploidy level in a number of species (Blakeslee and Avery 1937; Beck et al. 2003; Gu et al. 2005; Dibyendu 2010). Increased size of flower has earlier been reported in *P. drummondii* (Rao et al. 1982; Verma and Raina 1991). Increase in the size of stomata, pollen grains and flowers are due to increased cell size which might be a direct consequence of increased DNA content (Randolph 1941). On the basis of results obtained with respect to morphological traits, it is clear that doubling of chromosome number in the diploid *P. drummondii* has proved to be of tremendous importance for the enhancement of its ornamental value.

Meiotic analysis did not show any drastic differences between the frequency of both quadrivalents and bivalents from C_0 to C_2 generations. The average frequency of bivalents was found to be higher than that of quadrivalents in all the generations (Table 2). In another study on *P. drummondii*, Raghuvanshi and Pathak (1975) reported increase in the mean number of quadrivalents from C_1 to C_2 generations. These results, however, contradict various reports in some other plant species (Morrison and Rajathy 1960; Scahnk and Knowles 1961; Sohoo et al. 1970; Kravchenko 1995; Dibyendu 2010), which showed a reduction in the frequency of quadrivalents in succeeding generations of autopolyploid lines. For example, in *Cicer arietinum* L. C_2 generation of autotetraploids depicted a decreased multivalent frequency than in C_1 generation (Sohoo et al. 1970). High bivalent frequency in all generations, as compared to that of quadrivalents, in the present study might be due to the presence of localized chiasmata (Srivastav and Raina 1982, 1987). According to Jackson (1982), a few multivalents and more bivalents in autopolyploids might be due to the presence of ph-like (pairing homoeologous-like) genes, conferring allopolyploid-like behavior through preferential chromosome pairing. High frequency of bivalents in C_2 generation, as compared to C_1 generation of colchitetraploids, was reported in Soybeans by Kumar and Rai (2007). Dikshit and Kumar (2007) also reported increased bivalent frequency in tetraploid ornamental plant, *Impatiens balsamina* L. After genome doubling in polyploids, multivalent formation generally causes meiotic irregularities, which result in their reduced fertility as compared to diploids. No significant changes observed in the frequency of quadrivalents and bivalents from C_0 to C_2 indicate that colchitetraploids of *P. drummondii* are stable at chromosomal level and does not suffer from sterility problems which is further supported by high seed set observed in tetraploids. In most of the autopolyploids, however, fertility does not reduce (Ramsey and Schemske 2002).

RAPD analysis revealed an average of 2.8, 1.6, 2.1 and 3.2% novel band polymorphism in C_0, C_1, C_2 and C_3, respectively. The RAPD targets are mainly repetitive sequences and genomic changes are considered to be frequent in the repetitive sequences (Paun et al. 2007; Hegarty and Hiscock 2008). Out of the six RAPD amplicons analyzed, five were of high or moderate copy number corroborating the view that RAPD primers target repeat sequences (Table 4). Our results are in agreement with those of Martelotto et al. (2007) who using RAPD technique, reported genomic changes in autopolyploid *Paspalum notatum*. Similar genomic changes have been observed in the synthetic allotetraploids also (Song et al. 1995; Feldman et al. 1997; Ozkan et al. 2001; Salmon et al. 2005; Gaeta et al. 2007; Chaudhary et al. 2009). These studies have reported rapid genomic changes in the synthetic allotetraploids, including loss/gain of parental fragments, gene expression changes, rapid and programmed sequence elimination of low copy sequences, rapid gene loss, changes in DNA methylation, transposon activation, and various other DNA rearrangements. Genomic changes observed in RAPD may be due to change in the primer binding sites. This suggests that after polyploid formation, genetic modifications do occur in the genome. Sequence analysis revealed genomic changes in both the repetitive and non-repetitive sequences (Table 5). Dot blot assay identified only one fragment (T01) to be of high copy, while four fragments were of the moderate copy and one fragment of the low copy. This corroborates the view that in RAPD, moderate repetitive sequences are frequently targeted as compared to low and high copy sequences (Martelotto et al. 2007).

In autopolyploids, same genome get doubled hence large scale changes are not observed as observed in allotetraploids due to the interaction of two different genomes. Present study concludes that genomic changes occur after autopolyploidization but these changes do not affect the genome on a large scale. Due to this cytogenetical analysis did not show any significant changes between diploids and colchitetraploids. This is due to the reason that in autopolyploid same genome gets doubled and their might be less chances of changes at chromosomal level.

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