Journal of Genetics (2020)99:40
https://doi.org/10.1007/s12041-020-1179-x

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RESEARCH ARTICLE

Male and female meiosis evince differential patterns in chiasma formation: a case study of ornamental plant, *Delphinium ajacis* L.

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Received 9 August 2019; revised 10 December 2019; accepted 13 December 2019

Abstract. Chromosomal behaviour during megasporogenesis and microsporogenesis has been studied in ornamental *Delphinium ajacis* L. Meiosis in female sex cell initiates later than male. The floral buds which carry egg mother cell (EMC) at diplotene stage has pollen mother cells (PMCs) at tetrad stage of meiosis suggesting protandry. Although the 16 chromosomes formed regular eight bivalents in both the sex cells, they differed in overall chiasma frequency which was 32.95% higher in EMCs and found to be 18.52 ± 2.12 per cell. In PMCs, the average chiasma frequency recorded was 13.93 ± 1.40 per cell. Interestingly, this variation in chiasma frequency was largely confined to the two large bivalents which shared 42.61% chiasma per EMC. The use of Q–Q plot, Box plot and Whisker plot showed departure in the chiasma frequency distributions in EMCs and PMCs from the normal distribution pattern. The difference in chiasma frequency in the two sex cells was significant at all levels as indicated by the low *P* values of 3.094×10^{-11} obtained from nonparametric test, i.e. Wilcoxon rank-sum test. It is suggested that the two different mechanisms of recombination are operational in the two sex cells, and the sex differences of chiasma frequency could have arisen due to differential epigenetic modifications of the chromatin which pattern the double-strand breaks, and the position and frequency of crossing over visible as chiasmata.

Keywords. female meiosis; protandry; chiasmata; epigenetic modifications; *Delphinium ajacis*.

Introduction

The genus *Delphinium* (Ranunculaceae) makes itself visible across 370 species all over the world (Blanché 1991). Commonly named as 'larkspur', the species included in this genus are annuals, biennials and perennials, known for its colourful blossom that exist in a variety of hues of pink, white, scarlet, purple and blue, the latter being the most common. In India, *Delphinium* is represented by 27 species, one subspecies and one variety (Agnihotri *et al.* 2014) mainly confined to alpine ranges in the Himalayas with one species *Delphinium ajacis* L. (syn. *Consolida ajacis*, *C. ambigua*, *D. ambiguum*), a popular winter seasonal in gardens, being widely used as ornamental for its attractive spike like raceme. Several efforts have been made to create plants with large-sized blossoms of shades not existing naturally in *Delphinium* by induction of polyploidy (Singh 1991), hybridization (Royal Horticultural Society 1949; Legro 1961), mutation breeding and tissue culture (Honda and Tsutsui 1997; Honda *et al*. 2003; Kolar *et al*. 2015). The cytological studies carried out until date in *D. ajacis* have been restricted to the study of X-ray induced chromosomal rearrangements (Jain *et al*. 1963), karyotypes, meiotic behaviour of chromosomes in pollen mother cells (PMCs) (Singh and Roy 1983; Mehra and Remanandan 1972; Subramanian 1985; Kaur and Sidhu 2014) and study of chromosomes in endosperm (Mandal and Basu 1978) and colchitetraploids (Singh 1991). Thus, until now, no attempt has been made to study the behaviour of chromosomes during megasporogenesis. Comparative study of meiosis in PMCs and the egg mother cells (EMCs) is crucial to gain an insight into whether the behaviour of chromosomes with respect to the number and position of chiasmata in the two sex cells is synchronous or not. This information is useful for the breeders, aiming to create novel types with attractive blossom, and enhanced therapeutic and antimicrobial efficacy of various bioactive compounds identified in *Delphinium* (Pratap *et al*. 2016).

Published online: 14 May 2020

Materials and methods

For the study of male and female meiosis in *D. ajacis*, young unopened buds/inflorescences, collected from the botanical garden of Hindu College, University of Delhi, were used. These buds were fixed in 1:3 acetic alcohol for 24 h and was later transferred to 70% alcohol until further use. For staining anthers and ovaries, and to study meiotic behaviour of chromosomes in PMCs and EMCs, the method was same as followed earlier (Koul and Nagpal 2002, 2004).

The number of EMCs available for study is less than the number of PMCs available. Realizing the fact that the present investigation involves the study of chiasma frequency distribution in PMCs and EMCs, the latter being very difficult to undertake owing to technical problems faced in handling the cell, nonparametric statistical test like Wilcoxon rank-sum test was applied to compare the outcome and also ascertain the equality of mean in the chiasma frequency of two independent samples of sex cells. Further, to provide a graphical view of the distribution pattern followed by chiasma frequency in the two samples of sex cells, quantile–quantile (QQ), Box plot and Whisker plots were also used.

Observations

For the unambiguous comparison of meiotic behaviour of chromosomes in the two sex mother cells, i.e. PMCs and EMCs, data was compiled only from those buds in which both male and female meiosis could be studied.

Study of each flower bud showed the presence of 15 anthers arranged in five bundles of three each in basipetal order with each anther existing at different stages of meiotic development. Each anther had filaments swollen at the base. The ovary showed marginal arrangement of ovules which appeared in rows (figure 1a), closely juxtaposed to each other. This arrangement of ovules made the detailed study of individual ovules difficult. Any attempt to separate the ovules would disintegrate the tissue. Further, a gentle tap under the coverslip would merge the nucellar tissue of all the ovules together making location of EMCs difficult (figure 1, b & d). Even the EMCs would appear broken with chromosomes scattered (figure 1c). Despite various technical complications, an attempt was made to study the details of male and female meiosis in five plants. In one plant, 26 EMCs and 60 PMCs were available for detailed chromosomal study; in the remaining four plants, although 50 PMCs per anther were available for detailed study, the number of EMCs available was very less, i.e. 5, 4, 4 and 3. Nevertheless, the meiotic observations made in all the five plants were more or less similar. While the characteristics of meiosis in PMCs and EMCs in the plant in which 26 EMCs were available is detailed under, the salient features of meiosis with respect to chiasma characteristics, observed in all other plants, is summed up in table 1.

The plants exhibited asynchrony in the meiotic progression in EMCs and PMCs with male sex cells progressing faster through meiotic stages reflecting protandry. In all the tested flower buds, the EMCs appeared at diplotene stage and the PMCs had entered into the tetrad stage of meiosis.

Male meiosis

Male meiotic studies revealed the presence of 16 chromosomes which resolved into eight regular bivalents in all the PMCs studied at diplotene and metaphase-I. A total of 60 cells were studied at diplotene for comparison with the female sex cells. This stage was preferred, as chiasmata are conspicuous and more importantly most of the cells observed during female meiosis were at diplotene stage. The eight bivalents were of varying lengths. While two bivalents were large sized, five were similar in size (figure 1, i–k), with a solitary small bivalent indicating asymmetric and advanced nature of karyotype. The chiasma distribution was random and average frequency per cell was 13.93 ± 1.40. Overall, a total of 836 chiasmata were observed of which 268 (32.05%) were terminalized (table 1). While the two large bivalents invariably showed 2–3 chiasmata (figure 1, i & j), with occasional cell showing one bivalent with four chiasmata, the rest of the six bivalents had a maximum of two chiasmata. The cells (50) observed at metaphase-I had bivalents arranged in a single row on the equatorial plate. Of the eight bivalents, six appeared as rod and two as ring (figure 1k). This was followed by regular disjunction of 8:8 chromosomes at anaphase-I (25 cells; figure 1l).

Female meiosis

In female meiosis studies, although a total of 2780 ovules were squashed, meiotic studies were possible only in 33 EMCs of which 26 existed at diplotene stage, three at metaphase-I and four at anaphase-I. The 26 EMCs studied at diplotene stage although showed meiosis almost similar to PMCs with 16 chromosomes resolving as eight bivalents with randomly distributed chiasma, they however, differed in having higher (32.95%) average chiasmata frequency that was 18.5 ± 2.12 per EMC. Overall, a total of 481 chiasma were studied of which 126 (26.19%) had terminalized (table 1). Interestingly, the differences in chiasma frequency were conspicuous in the two large bivalents which shared, on an average, 42.61% chiasma of the total scored in each cell with their number ranging per bivalent from 3 to 5. The remaining six bivalents invariably had 1–2 chiasmata. Since the two large bivalents appeared more or less similar in lengths, it was not possible to assign the variation in chiasma number to a specific bivalent or relate the noticed variation in chiasma frequency with their lengths (figure 1, e & f). However, in one of the EMCs, the apparently larger looking bivalent, interestingly, had less chiasmata (3) than the

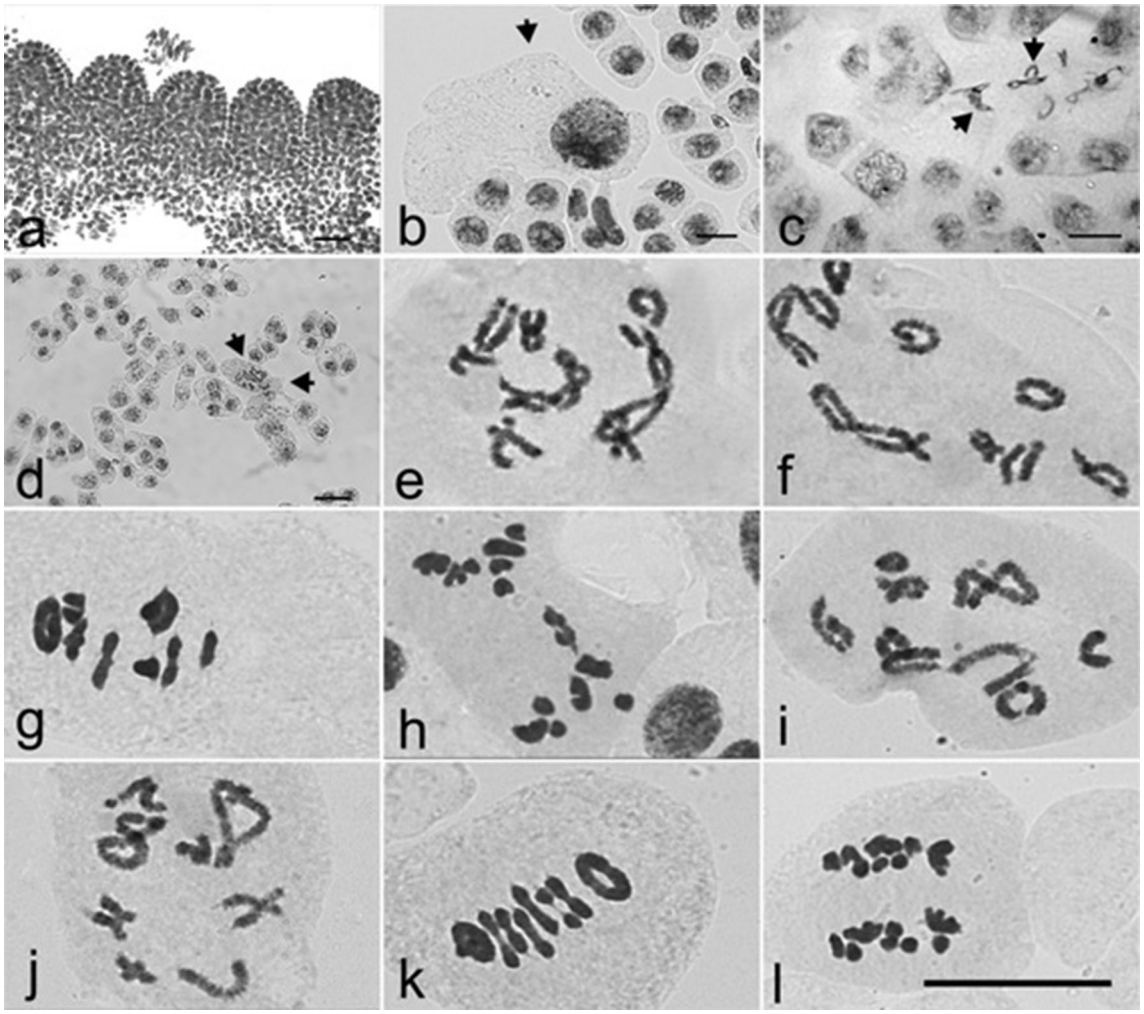

Figure 1. Male and female meiosis in *D. ajacis*. (a) Ovules arranged in a row; (b) an EMC at uninucleate stage; (c) scattered bivalents (at arrows) of a broken EMC within nucellar tissue; (d) intact EMC (at arrow) surrounded by nucellar tissue; (e, f) EMCs at diplotene stage; (g) EMC at metaphase-I; (h) EMC at anaphase-I; (i, j) PMCs at diplotene stage; (k) PMC at metaphase-I; (l). PMC at anaphase-I. Scale = 10 μm.

Table 1. Sex incidences of variation in chiasma frequency in five plants of *D. ajacis*.

Plant number	No. of cells studied EMCs/ PMCs	Total no. of chiasmata studied EMCs/PMCs	Mean chiasmata per cell EMCs/PMCs	Per cent chiasmata terminalized EMCs/PMCs	Per cent increase in chiasmata frequency in EMCs	Calculated *P* values Wilcoxon rank-sum test
1	26/60	481/836	18.52 ± 2.12/13.93 ± 1.40	26.19/32.05	32.95	3.094×10^{-11}
2	4/50	69/651	17.25 ± 0.83/13.02 ± 0.73	23.18/24.42	24.52	2.0749×10^{-4}
3	4/50	97/656	17 ± 0.71/12.58 ± 0.78	21.64/26.52	26	3.0005×10^{-4}
4	5/50	68/609	18.20 ± 0.75/13.12 ± 0.77	20.58/24.95	27.91	4.5195×10^{-5}
5	3/50	52/656	17.33 ± 0.47/13.12 ± 0.79	23.07/27.59	24.31	1.4542×10^{-3}

smaller looking one which had five chiasmata (figure 1e). The number of chiasmata shared by the two large bivalents in EMCs are provided in table 2. Figure 2 sums up the chiasma frequency at diplotene stage in EMCs and PMCs. At metaphase-I, three cells were observed and each had three ring and five rod bivalents arranged on the equatorial plate (figure 1g). The anaphase-I segregation observed in four EMCs was regular (figure 1h).

To find whether the chiasma frequency data in the two sex mother cells follow a normal distribution pattern similar to the parent population from where the samples were drawn, the QQ plot (figure 3), Box plot and Whisker plot (figure 4)

Table 2. Frequency of EMCs showing varying chiasmata number in two large bivalents of *D. ajacis.*

No. of chiasmata per EMC	21	20	19	18	17	16	15	14
No. of chiasmata shared by two large bivalents (chiasmata in each bivalent)	9–10 (5,4;5,5)	8–9 (5,3;5,4)	8–9 (5,3;5,4)	7–8 (4,3;5,3)	7 (4,3)	7 (4,3)	6 (3,3)	6 (3,3)
Total EMCs studied	4 (3;1)	6 (4;2)	5 (4;1)	5 (2;3)	1	2	1	2

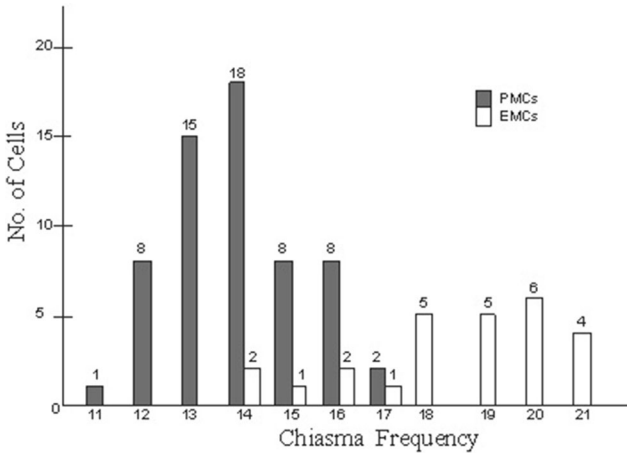

Figure 2. Chiasma frequency in EMCs and PMCs of *D. ajacis.*

were used. The box plot shows the data partially symmetric indicating this the samples might have come from a normal population. However, to ascertain that Shapiro–Wilk normality test was employed. The R-output of the test contains test statistics W and its *P* value (table 3). Since the calculated *P* value in both the samples was very low at 0.05 significance level, it is clear that the two populations did not follow a normal distribution pattern and that there was a departure from the normal pattern. The QQ plot (figure 3) also supported the departure in the distribution pattern from the normal. Therefore, to find whether the chiasma frequency data in the two sex cells varied significantly or not, non-parametric test like Wilcoxon rank-sum test was used. The very low *P*-value of 3.094×10^{-11} obtained after applying the test suggest the chiasma frequency differences in the two sex cells to be highly significant at all levels. Very low *P* values were also obtained for the remaining four plants (table 1).

Discussion

The cytogenetic information available for male and female meiosis, albeit limited owing to the technical difficulties, has clearly shown asynchrony in the behaviour of chromosomes with respect to the chiasma distribution and frequency (Koul *et al*. 2000; Drouaud *et al*. 2007). While high chiasma frequency has been recorded for EMCs of some angiosperms

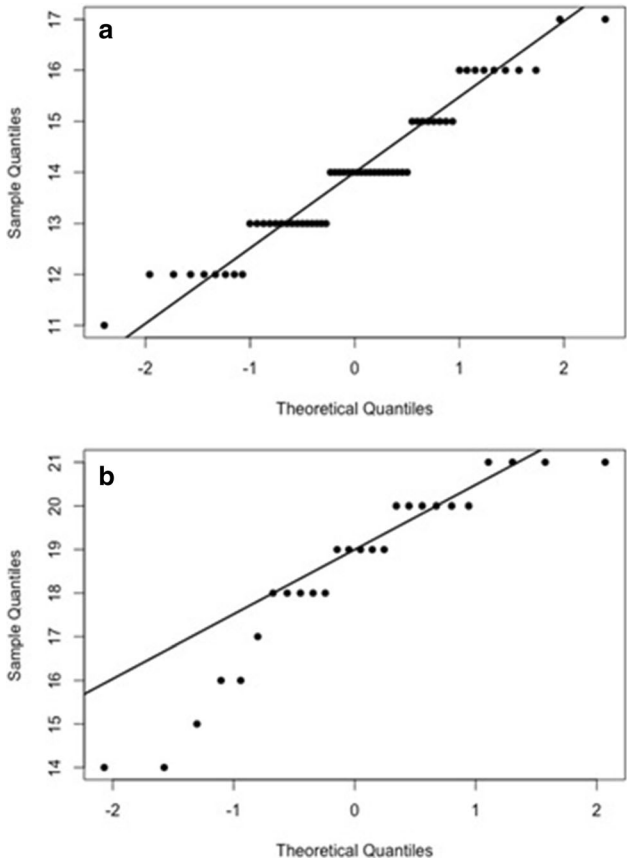

Figure 3. Q–Q plot of chiasma frequency distribution in PMCs (a) and EMCs (b).

(Fogwill 1958; Ved Brat 1966; Vosa 1972; Kearsey *et al*. 1995; Koul and Raina 1996; Drouaud *et al*. 2007), other PMCs still showed a high chiasma frequency (Håkansson and Levan 1957; Ved Brat 1966; Gohil and Kaul 1980, 1981; Koul *et al*. 1995; Koul and Raina 1996). Moreover, within the same genus various species have shown inconsistency in the behaviour of chromosomes (Gohil and Kaul 1980; Koul *et al*. 1999, 2000) implicating that the differences are not sex-specific but species-specific. Overt cases of sex differences have also been reported in various species of *Fritillaria* where chiasmate versus achiasmate meiosis have been observed in EMCs and PMCs, respectively (Noda 1975). While in *Zea mays* the chiasmate versus desynaptic meiosis has been recorded in EMCs and

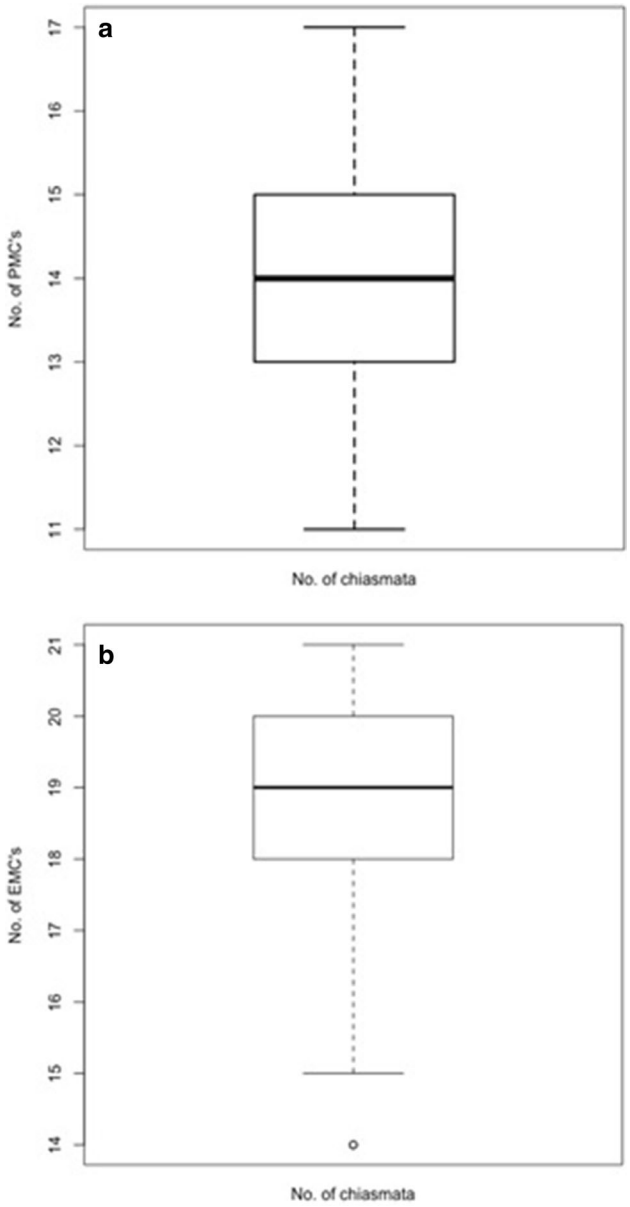

Figure 4. Box and Whisker plots of chiasma frequency distribution in (a) PMCs and (b) EMCs.

Table 3. R-output of Shapiro–Wilk normality test employed to study the chiasma distribution pattern of EMCs and PMCs in *D. ajacis.*

Data	W statistics	*P* value
PMCs	0.94002	0.005426
EMCs	0.89695	0.01339

PMCs, respectively (Nelson and Clary 1952), a reverse situation existed in *Lupinus albus* (Koul *et al.* 2002) and *Solanum tuberosum* (Iwanaga and Peloquin 1979); in *Allium tuberosum* (Gohil and Kaul 1981) and *Allium roylei* (Sharma and Gohil 2011), an increased chromosome number was observed in EMCs. Although heterochiasmy has been a common feature in the two sex cells in most of the species studied, synchronous chromosome behaviour in the two sexes has also been recorded for a few species (Brock 1954; Bennett *et al.* 1973; Davies and Jones 1974; Kelly *et al.* 1997; Koul *et al.* 2000; Drouaud *et al.* 2007).

Our present observation of higher chiasma frequency in female sex cell in *D. ajacis* is significant in view of the fact that the variation in chiasma frequency was unequivocally restricted to two large bivalents only. Prior to this chromosome specific variation in crossover number, manifested as changed chiasma frequency, has been reported in *Arabidopsis thaliana* where this variation was confined to chromosome number 4 in male meiocytes which showed an increased chiasma frequency than the female sex (Vizir and Korol 1990; Armstrong and Jones 2001; Drouaud *et al.* 2007). Although the recorded difference in crossover number along the fourth chromosome in *A. thaliana* has been attributed to varying level of interference (Drouaud *et al.* 2007), in humans, mice, locust and the plant species *Crepis capillaris* increased crossover/chiasmata frequency has been attributed to varying lengths of synaptonemal complex (SC) (Quevedo *et al.* 1997; Codina-Pascual *et al.* 2006; Drouaud *et al.* 2007). In fact, a positive correlation has been observed between the increased crossover/chiasma number and the length of SC in these organisms (Quevedo *et al.* 1997; Codina-Pascual *et al.* 2006; Drouaud *et al.* 2007). These differences have existed from cell to cell within the same organism (Padmore *et al.* 1991; Quevedo *et al.* 1997) and even between different sexes (Bojko 1985; Wallace and Hulten 1985; Jones and Croft 1989; Quevedo *et al.* 1997). Keeping these insights in mind, one may be tempted to tentatively ascribe the observed sex differences in chiasma frequency in two large bivalents of *D. ajacis* to chiasma interference and/or varying SC characteristics. However, it would be precarious to pinpoint any specific factor responsible for such variations. This is, particularly, on account of the growing body of information available on molecular mechanism regulating crossing over and chiasma distribution, which suggest the state of chromatin and epigenetic modifications, prior to the initiation of recombination, to be playing a pivotal role in shaping or patterning the crossover (He *et al.* 2017; Choi *et al.* 2018; Wang and Copenhaver 2018). Recombination event during meiosis is preceded by some key changes in the chromosomal behaviour within the cell. Homologue search followed by synapsis of chromosomes and formation of double strand breaks (DSBs) at various sites within the pairing homologues provide the perfect platform for chromosomal recombination instrumental in generating genetically diverse gametes which subsequent upon random fertilization yield plants bestowed with qualities to invade new habitats with varying environment. In this whole scheme, the state of chromatin and the position of

DSBs at the time when cell enters in cell division mode are very crucial. Recognized as recombination hot spots, the sites of DSB have a unique GC-rich signature sequence that is accessed by special set of proteins, SPO11 (Yelina *et al*. 2012, 2015; He *et al*. 2017; Yamada *et al*. 2017; Choi *et al*. 2018; Wang and Copenhaver 2018), which introduces the DSBs in the pairing chromosomes that mature into crossover manifested as chiasmata. Crossovers not only reshuffle the genes in various combinations but its physically visible form, i.e. chiasmata also facilitates in faithful segregation of chromosomes at anaphase-I of meiosis. However, whether the proteins have free access to these hot spots depends on the compaction state of chromatin. Epigenetic changes, which include methylation of DNA particularly at sites rich in repeat sequences and carrying cytosine have been pivotal in positioning the crossing over (Derreumaux *et al*. 2001; Yamada *et al*. 2017; He *et al*. 2017; Choi *et al*. 2018). Methyl groups being bulky and hydrophobic in nature once attached to DNA alter base stacking ability of DNA which induces configurational changes in the chromatin that either make hot spots inaccessible to the protein complex responsible for inducing DSB or alter the functionality of such protein complexes that forestall any possibility of crossover or chiasmata formation (Maloisel and Rossignol 1998; Derreumaux *et al*. 2001; Norberg and Vihinen 2001; Gorelick 2003; Choi 2017; Wang and Copenhaver 2018).

Based on our present state of knowledge, although the actual causal factor responsible for heterochiasmy in two sex cells have remained elusive, it is ostensibly clear, keeping in view all the insights advanced thus far, that a web of factors operate synergistically in patterning the crossover/chiasma distribution within a cell. Any alteration probably in the chemical and molecular environment prevalent within the cell, perhaps, lead to disproportionate epigenetic modifications and/or SC growth between the pairing homologues that got manifested in contrasted behaviour of chromosomes with respect to chiasma frequency in the two sex cells. The resulting differential recombination pattern in the male and female meiocytes while ensuring gene reshuffling in one sex plays to stabilize the genetic polymorphism on the other side by preventing some gene groups from getting reshuffled. Such variations in the recombination pattern are significant as it may influence the pattern of genome evolution. It is surmized if the causal factors responsible for differential behaviour of chromosomes in two sex cells is identified it may open new vistas for genetic manipulations that would be pivotal in refining breeding strategies.

Acknowledgements

KKK thanks Sanjeev Dutt Sharma, the Librarian, Hindu College, for providing access to the journals beyond reach. The technical help rendered by Santosh Kumar and Ravi Kumar is also thankfully acknowledged.

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Corresponding editor: H. A. Ranganath