P. KUMAR, V.K. SINGHAL
Department of Botany, Punjabi University, Patiala-147 002, Punjab, India
E-mail: vksinghal53@gmail.com

CHROMOSOME NUMBER AND SECONDARY CHROMOSOMAL ASSOCIATIONS IN WILD POPULATIONS OF *GERANIUM PRATENSE* L. FROM THE COLD DESERTS OF LAHAUL-SPITI (INDIA)

In this work we studied the meiotic chromosome number and details of secondary chromosomal associations recorded for the first time in Geranium pratense L. from the alpine environments in the cold deserts of Lahaul-Spiti (India). All the presently studied individuals of the species existed at 4x level (x = 14). The present chromosome count of n = 28 in the species adds a new cytotype to the already existing diploid chromosome count of 2n = 28 from the Eastern Himalayas and outside of India. Out of the six accessions scored presently four showed normal meiotic course. However, two accessions investigated from Mud, 3800 m and Koksar, 3140 m depicted abnormal meiotic course due to the presence of multivalents and univalents, and secondary associations of bivalents/ chromosomes. The secondary chromosomal associations in the species existed among bivalents/chromosomes were noticed in the PMCs at prophase-I (diakinesis) and persisted till the separation of sister chromatids at M-II. The variation in the number of bivalents/chromosomes involved in the secondary associations at M-I (2–8) and A-I/M-II (2–12) has also been recorded. The occurrence of such secondary associations of bivalents/ chromosomes in G. pratense which existed at 4x level indicated the secondary polyploid nature of the species.

Introduction. Presence of bivalents/chromosomes in pairs or groups in close proximity having diffused connections is referred to as secondary associations or secondary pairing of chromosomes [1]. The general phenomenon of secondary association was first observed in *Oryza sativa* by Kuwada [2] followed by Ishikawa [3] in *Dahlia variabilis* and Marchal [4] in *Amblystegium*. Since then, the phenomenon had been encountered during meiosis in several plant species in tracing the basic chromosomes number and the polyploid status of species [5–21]. During the cytomorphological surveys to determine the chromosome number in selected angiosperms from alpine regions in cold deserts of Lahaul-Spiti (Himachal Pradesh, India) we have found the occurrence of secondary associations of chromosomes in two accessions of *Geranium pratense* L. *G. pratense* is a stout, erect perennial herb having short rootstocks covered with stipules of basal leaves and bluish-purple to bluish-violet flowers on long glandular-hairy peduncles. The species is commonly found growing in the subalpine and alpine meadows in the North West Himalayas between altitudes of 3000–4500 m. A black dye is made by boiling the roots of the species with a sulfurous raw mineral called «Naktsur». The roots are applied as poultice to bruises and local Vaidyas also use the plant for stomach trouble. In Lahaul Valley its flowers are offered during religious function to deities and in Spiti Valley powder made of whole plant are given twice a day to treat cough, jaundice and gastric disorders [22].

Lahaul-Spiti, a cold and desolate alpine region in northwest Himalayas is known for its seclusion, Buddhist culture, harsh climate, unexplored, formidable and breathtaking scenic beauty of the high mountains. It constitutes a part of Indian cold deserts which is situated between 31°44′57″ and 32°59′57″ N latitudes and between 76°29′46″ and 78°41′34″ E longitudes. The cold deserts of Lahaul-Spiti are characterized by the presence of low temperature (>–30 °C), a shorter vegetation period, more snow, dry arid weather, high velocity winds, low precipitation and harsher conditions owing to the rising number of weather related extreme events. Perennation of plants growing in such extreme environmental conditions depends upon their successful adaptation to these hostile habitats and ability to overcome frequent disturbances caused by climatic oscillations. Due

to these particular selective conditions the cold desert vegetation is remarkably different from that of the lowlands [23, 24]. Consequently, the flora of the area exhibited a number of ecological, morphological, physiological and reproductive adaptations, which make the species able to withstand the extreme harsh climatic conditions. Owing to such xeric and inhospitable ecological conditions, plants of the area adopted specific habits and tend to become prostrate, hairy, thick, hardy, sturdy, bushy, mat and cushion forming and spiny with long roots and small succulent or woolly leaves. Perennation through vegetative means (rootstocks, runners, bulbs, rhizomes, tubers, etc) is another remarkable feature of these plants which show their adaptability to these environmental conditions.

Review of literature of the work carried out on the plants of this region revealed that most of the previous studies undertaken by different workers either pertain to floristic and ethnobotanical surveys [25–30]. No systematic attempt has been made so far to explore the existence of cytomorphological diversity in the plant resources of Lahaul-Spiti. Even the basic information regarding the cytological status, meiotic behaviour particularly on male side and pollen/seed viability is not available for the species growing in the region. Thus data and information obtained from such cytomorphological surveys would be of colossal significance and certainly provide a helping hand in understating the cytological evolution of the species growing in the cold deserts. In view of this, cytomorphological surveys were undertaken to the area and results so obtained on various parameters of cytomorphological interest have already been published from this laboratory in some species [31–45].

Previous cytological studies on *G. pratense* either restricted to eastern Himalayas in India or outside of India and so far species has neither been counted chromosomally nor studied for cytological details from the Lahaul-Spiti. The present study was undertaken to determine the meiotic chromosome number, examining the behaviour and frequency of chromosomes involved in secondary associations at different meiotic stages, course of meiosis and pollen fertility.

Materials and methods. Materials for male meiotic studies were collected from six different accessions collected from alpine regions in the cold deserts of Lahaul-Spiti (India) in May-July, 2008–2009 (Table 1). The young developing floral buds from healthy plants were fixed in freshly prepared Carnoy's fixative (1 Glacial acetic acid : 3 Chloroform : 6 Ethanol, v:v:v) for 24 h and subsequently stored in 70 % ethanol in a refrigerator. Developing anthers from floral buds were squashed in 1 % acetocarmine and preparations were studied for chromosome counts, and detailed meiotic behavior in pollen mother cells (PMCs) at prophase-I, metaphase-I (M-I), anaphases-I/II (A-I/II), telophases-I/II (T-I/II) and sporad stage. A number of slides were prepared from different anthers/flowers for analysis of chromosomal associations.

Pollen fertility was estimated through stainability tests for which anthers from mature flowers

Table 1

Details of the different accessions of *G. pratense* collected from different localities of Lahaul-Spiti

Locality with altitude	Geographical co-ordinates	Accession number	Meiotic chromosome number ('n')	Ploidy level	Pollen fertility
		Lahaul Valley			
Kukumsheri, 2800 m	32°42′4″ N; 76°41′28″E	51164	28	4*x*	100
Kishori, 2900 m	32°41′1″ N; 76°41′48″E	51149	28	4*x*	100
Koksar, 3140 m	32°24′48″ N; 77°14′7″E	51472	28	4*x*	100
Keylong, 3340 m	32°34′14″ N; 77°2′1″E	51574	28	4*x*	100
		Spiti Valley			
Mud, 3800 m	31°57′32″ N; 78°1′52″E	51497	28	4*x*	85
Lossar, 4110 m	32°26′17″ N; 77°44′55″E	51572	28	4*x*	100

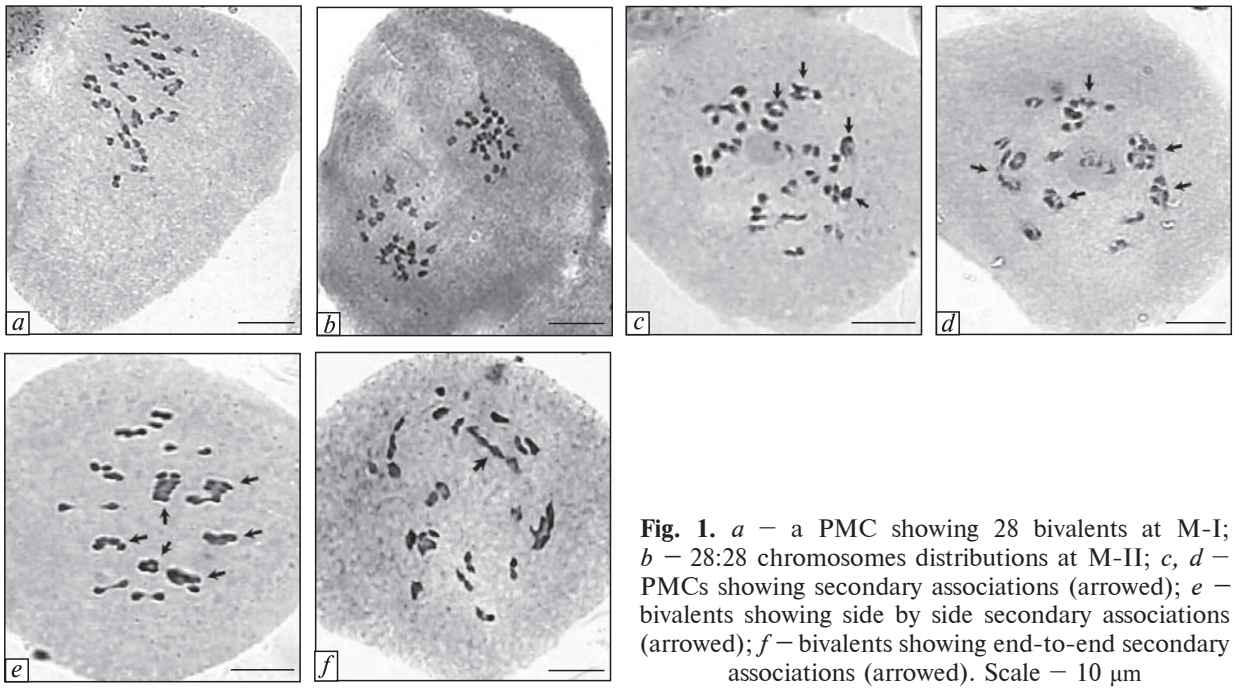

Fig. 1. *a* – a PMC showing 28 bivalents at M-I; *b* – 28:28 chromosomes distributions at M-II; *c, d* – PMCs showing secondary associations (arrowed); *e* – bivalents showing side by side secondary associations (arrowed); *f* – bivalents showing end-to-end secondary associations (arrowed). Scale – 10 μm

were squashed in glycerol-acetocarmine mixture (1:1) and 1 % aniline blue dye. Well-filled pollen grains with uniformly stained cytoplasm were scored as fertile/viable while shrivelled pollen with unstained or poorly stained cytoplasm were counted as sterile/unviable. Photomicrographs of PMCs showing chromosome counts and secondary chromosomal associations were taken from the temporary mounts with a digital imaging system of *Leica QWin*, version V 2.3 (Leica Microsystems, UK)

Results. Cytological studies in the species have been carried out presently in the six different accessions collected from the cold desert regions of Kukumsheri (2800 m), Kishori (2900 m), Koksar (3140 m) and Keylong (3340 m) in Lahaul Valley, and Mud (3800 m) and Lossar (4110 m) in Spiti Valley. The four accessions (Kukumsheri, Kishori, Keylong and Lossar) showed the presence of 28 bivalents at M-I (Fig. 1, *a*), and 28:28 chromosomes distribution at M-II (Fig. 1, *b*). Further meiotic course and sporad formation in these accessions were also normal resulting into cent per cent pollen fertility. However, the accessions scored from Mud in Spiti Valley and Koksar in Lahaul Valley depicted abnormal meiotic course due to the presence of multivalents and univalents, and secondary associations of bivalents/chromosomes. Data regarding the frequency of secondary chromosomal associations, and multivalents and univalents in the two accessions are provided in Tables 2, 3, 4 and 5. The detailed meiotic behaviour in both the accessions with secondary associations has been discussed separately here under.

Mud, 3800 m; Spiti Valley. Secondary associations of chromosomes in the accession were observed in the PMCs at diakinesis, M-I, A-I and M-II. Most of the bivalents/chromosomes were present in groups widely spaced from each other, and the number of bivalents/chromosomes per group ranges from 2 to 12 (Fig. 1, *c–f*, 2, *a–c*). The bivalents/chromosomes showing secondary associations existed in two forms, side by side (Fig. 1, *e*) and end-to-end (Fig. 1, *f*). The secondary associations were so intense that these can be observed even during the disjunction of chromosomes at A-I. Fig. 2, *b* showing association between the non-homologous separating chromosomes. The number of such groups in a PMC varied from 9–25 (diakinesis, M-I, Table 2) and 22–44 (A-I and M-II, Table 3). Besides, up to 3 multivalents and 2 univalents were also recorded in some PMCs (Fig. 2, *d*). In spite of

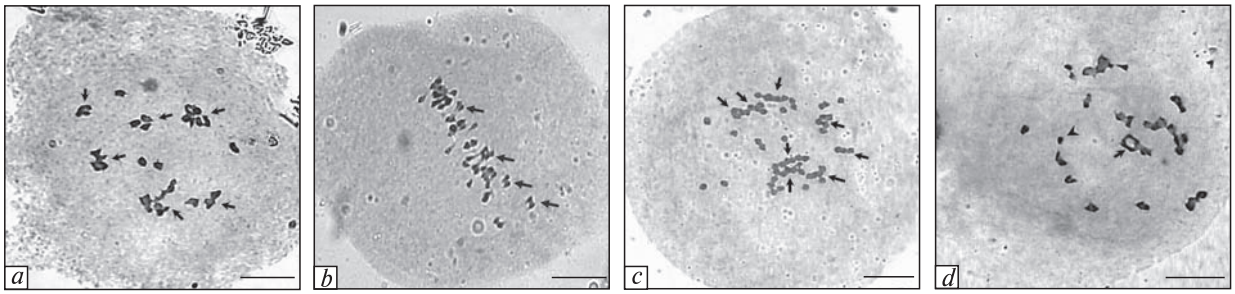

Fig. 2. *a* – varying number of bivalents involved in secondary associations arrowed and present in different groups; *b* – chromosomes showing association even during disjunction at early A-I (arrowed); *c* – secondary associations at M-II.; *d* – a multivalent (arrowed) and univalent (arrowhead). Scale – 10 μm

normal segregation of chromosomes at anaphases some pollen sterility (15 %) was resulted.

Koksar, 3140 m; Lahaul Valley. In this accession also secondary associations were observed at diakinesis, M-I, A-I and M-II. Of the total 18 analyzable PMCs, 10 PMCs (55.56 %) showed secondary associations. The number of bivalents/ chromosomes organized in a group due to secondary associations in a PMC during diakinesis, M-I, A-I and M-II varied from 2–8. The number of such groups in a PMC varied from 12–28 (diakinesis, M-I, Table 4) and 28–33 (A-I and M-II, Table 5). Some univalents and multivalents were also recorded in this accession. Normal segregation of chromosomes at anaphases resulted into 100 % pollen fertility.

Discussion. *Chromosomes number*. Based on $x = 14$, one of the basic chromosome number suggested for the genus *Geranium* the presently studied individuals of the species existed at $4x$

Table 2

Secondary chromosomal associations at MI in the Mud accession of *Geranium pratense*

PMCs		Secondary associations among bivalents at MI in different groups (I–VI*)					No. of chromosome groups per PMC	Chromosomal associations (multivalents & univalents)	
		VI	IV	III	II	I		IV	I
1		–	1	1	6	9	17	–	–
1		1	2	–	2	10	15	–	–
1		2	–	–	3	10	15	–	–
1		1	1	1	3	3	9	3	–
1		1	1	1	4	4	11	1	2
1		1	–	–	6	10	17	–	–
1		0	1	1	4	13	19	–	–
1		0	1	1	7	7	16	–	–
1		–	1	–	1	23	23	–	–
1		0	2	2	3	8	15	–	–
Total no. of PMCs analyzed	10								
Range of groups		1–2	1–2	1–2	1–6	3–24	9–25	1–3	0–2
Total no. of bivalents	280	36	40	21	80	94			
% age of chromosomes involved in association		12.86	14.29	7.5	28.57	33.57		2.86	0.35

* Roman numerals depict the number of bivalents present in a group.

Table 3

Secondary chromosomal associations at AI and MII in the Mud accession of *Geranium pratense*

PMCs		Secondary associations among chromosomes at MI and AI in different groups (I–XII*)								No. of chromosome groups per PMC
		XII	VIII	VI	V	IV	III	II	I	
1		–	–	1	1	4	1	7	12	26
1		–	–	–	1	–	1	14	20	26
1		1	1	–	1	1	1	7	10	22
1		–	–	–	–	–	–	12	32	44
1		–	–	–	–	–	1	14	25	40
Total no. of PMCs analyzed	5									
Range of groups		0–1	0–1	0–1	0–1	1–4	0–1	7–14	10–32	22–44
Total no. of chromosomes	280	12	8	6	15	20	12	108	99	
% age of chromosomes involved in association		4.32	2.86	2.14	5.34	7.12	4.32	38.56	35.34	

* Roman numerals depict the number of chromosomes present in a group.

Table 4

Secondary chromosomal associations at diakinesis and MI in the Koksar accessions of *Geranium pratense*

PMCs		Secondary associations among bivalents at diakinesis and MI in different groups (I–VIII*)							No. of chromosome groups per PMC	Chromosomal associations (multivalents & univalents)	
		VIII	VII	V	IV	III	II	I		IV	I
1		1	–	–	1	3	–	7	12	–	–
2		–	–	–	–	–	3	22	25	–	–
5		–	–	–	–	–	–	28	28	–	–
1		–	–	–	1	–	3	18	22	–	–
1		–	–	1	2	–	5	5	13	–	–
1		–	1	–	1	2	3	6	12	–	–
1		–	–	–	2	1	6	5	14	–	–
1		–	–	–	–	–	1	23	24	1	2
1		–	–	–	–	–	2	24	26	–	–
1		–	–	–	–	–	4	18	22	1	–
Total no. of PMCs analyzed	15										
Range of groups		0–1	0–1	0–1	1–2	1–3	2–6	1–28	12–28		
Total no. of bivalents	420	8	6	5	28	18	60	290			
% age of chromosomes involved in association		1.87	1.43	1.19	6.67	4.30	14.30	69.05		0.95	0.24

*Roman numerals depict the number of bivalents present in a group.

level. The present chromosome count of $n = 28$ in the species adds a new cytotype to the already existing diploid chromosome count of $2n = 28$ from the Eastern Himalayas [46, 47] and outside of India [48, 63]. The species is also known to have intraspecific aneuploid cytotype ($2n = 24$) from outside of India [64, 65]. Besides, some diploid populations of the species studied by Belyaeva and Siplivinskii [66] depicted the presence of up to 1B chromosome. It thus indicates that the species exhibited considerable amount of chromosomal variation involving aneuploidy and polyploidy.

Secondary chromosomal associations. The secondary chromosomal associations in the species existed among bivalents at diakinesis/M-I and between chromosomes at A-I/M-II. These secondary associations which were visible from prophase-I (diakinesis) persisted until the separation of sister chromatids at M-II. All the analyzable PMCs in the accession collected from Mud (Spiti Valley) showed the presence of secondary associations whereas the accession scored from Koksar (Lahaul Valley) showed secondary associations in 55.56 % PMCs. The number of bivalents/chromosomes in a group organized due to secondary associations in PMCs during M-I and A-I/M-II in the two accessions varies from 2–8 and 2–12, respectively. Darlington [67], Lawrence [68] and Malgwi et al. [13] were the first to relate this phenomenon of side by side association of bivalents in groups of two, three or more, to the homology existing between these bivalents. Primary pairing at zygotene and chiasma formation between more than two homologous chromosomes were known to result in multivalents. This type of pairing is distinct from the secondary pairing in which bivalents are loosely associated with each other without the existence of chiasmata. According to these authors the associations between pairs starts only at prometaphase and M-I and continues into the later meiotic stages. They suggested that chromosomes which have secondary association are homologous or partially so, and reflect the polyploid origin of the species exhibiting this phenomenon. However, Brown [69] attributed the phenomenon of secondary associations at M-I in *Luzula* as an artifact of the squash technique. Stebbins [70] recognizes that secondary associations serve as an indication of the polyploid origin of a species or genus, but he cautioned against elaborate phylogenetic conclusions based on such evidence. One more reason put forth by Jelenkovic et al. [11] states that secondary associations may be due to the presence of heterochromatin region in the genome which facilitates the association between nonhomologous parts. The occurrence of such secondary associations of bivalents/chromosomes in *G. pratense* which existed at $4x$ level indicated the secondary polyploid nature. The species might have undergone cytological diploidization in the course of evolution to achieve its near diploid like meiotic behaviour as has been suggested earlier for *Ocimum* [18, 19] and *Uraria picta* [21].

Table 5

Secondary chromosomal associations at AI and MII in the Koksar accession of *Geranium pratense*

PMCs		Secondary associations among chromosomes at AI and MII (at both poles) in different groups (1–IV*)				No. of chromosome groups per PMC
		IV	III	II	I	
1		3	2	11	16	33
3		–	–	–	56	28
Total no. PMCs analyzed	4					
Range of groups		0–3	0–2	0–11	16–28	28–33
Total no. of chromosomes	224	12	6	22	184	
% age of chromosomes involved in association		5.36	2.68	9.82	82.14	

*Roman numerals depict the number of chromosomes present in a group.

Due to variation in the number of bivalents/chromosomes associations at M-I (2–8) and A-I/M-II (2–12), nothing can be concluded regarding the basic chromosome number in the genus or family. However, the presence of multivalents in the meiocytes suggests species to be an autopolyploid.

Bhattacharya and Datta [21] cautioned against deriving any conclusion regarding the auto- or allopolyploid origin of a species solely based on cytological data. In such conclusions cytological observation must be coupled with allozyme data and locus specific molecular markers using FISH (fluorescent *in situ* hybridization) to make precised decision [71, 72].

Some sterility (15 %) in the accession from Koksar could not be attributed to secondary associations as the other accession which also showed the occurrence of the secondary associations did not show any pollen sterility. And also the secondary associations did not interfere with the segregation of chromosomes at anaphases. A number of factors have been suggested for causing pollen sterility in angiospermic plants include ultrastructural changes in pollen, malfunctioning of the taptum or hypertrophy of tapetal cells resulting in the crushing of PMCs or pollen grains and some recessive genes in the chromosomes or a gene in the organelle other than the nucleus [73].

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ХРОМОСОМНЫЕ ЧИСЛА И ВТОРИЧНЫЕ ХРОМОСОМНЫЕ АССОЦИАЦИИ В ДИКИХ ПОПУЛЯЦИЯХ *GERANIUM PRATENSE* L. ХОЛОДНЫХ ПУСТЫНЬ LAHAUL-SPITI (ИНДИЯ)

Впервые изучены мейотическое число хромосом и особенности вторичных хромосомных ассоциаций растений *Geranium pratense* L., растущих на альпийских высотах холодных пустынь Lahaul-Spiti (Индия). Все изученные особи были 4*x* (*x* = = 14). Хромосомный набор *n* = 28 у этого вида добавляет новый цитотип к уже существующему диплоидному хромосомному набору 2*n* = 28 из Восточных Гималаев и за пределами Индии. Из шести изученных образцов у четырех установлен нормальный ход мейоза. В то же время два образца из районов Mud, 3800 м, и Koksar, 3140 м демонстрировали аномальное течение мейоза из-за наличия мульти- и унивалентов и вторичные ассоциации биваленты/хромосомы. Вторичные хромосомные ассоциации были замечены в профазе I (диакинез) и существовали до разделения сестринских хроматид на стадии M-II. Отмечена также изменчивость числа бивалентов/хромосом, вовлеченных во вторичные ассоциации на стадиях M-I (2–8) и A-I/M-II (2–12). Наличие у *G. pratense* таких вторичных ассоциаций, которые существуют на уровне 4*x*, указывает на вторичную полиплоидную природу этого вида.

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ХРОМОСОМНІ ЧИСЛА І ВТОРИННІ ХРОМОСОМНІ АСОЦІАЦІЇ В ДИКИХ ПОПУЛЯЦІЙ *GERANIUM PRATENSE* L. ХОЛОДНИХ ПУСТЕЛЬ LAHAUL-SPITI (ІНДІЯ)

Вперше вивчено мейотичне число хромосом і особливості вторинних хромосомних асоціацій рослин *Geranium pratense* L., що ростуть на альпійських висотах холодних пустель Lahaul-Spiti (Індія). Всі досліджені особини були 4*x* (*x* = 14). Хромосомний набір *n* = 28 у цього виду додає новий цитотип до вже існуючого диплоїдного хромосомного набору 2*n* = 28 зі Східних Гімалаїв та за межами Індії. З шести вивчених зразків у чотирьох був нормальний хід мейозу. У той же час два зразки з районів Mud, 3800 м, і Koksar, 3140 м демонстрували аномальний перебіг мейозу через наявність мульти- та унівалентів і вторинні асоціації біваленти/хромосоми. Вторинні хромосомні асоціації спостерігались в профазі I (діакінез) і існували до поділу сестринських хроматид на стадії M-II. Зазначено також мінливість числа бівалентів/хромосом, залучених у вторинні асоціації на стадіях MI (2–8) і AI/M-II (2–12). Наявність у *G. pratense* таких вторинних асоціацій, які існують на рівні 4*x*, вказує на вторинну поліплоїдну природу цього виду.

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