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Hybrids between Tetraploid Italian and Perennial Ryegrass

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Summary. Hybridization frequency was investigated between tetraploid perennial and Italian ryegrass (*Lolium perenne* × *multiflorum*) without emasculation by using genetic markers. The Italian phenotypes, fluorescent-roots and awned florets, were dominant. About 82 % of the plants in perennial × Italian and nearly 93 % of the plants in the reciprocal crosses were hybrids. The hybrids had a high multivalent frequency and involved homoeologous chromosome pairing. Aneuploids with 2n = 26, 27 and 29 chromosomes were present. The hybrids were highly fertile. The cytogenetic behaviour of these allopolyploids suggested that the genomes of the parental species have undergone little repatterning and have free genetic exchanges. The species maintained their self-incompatibility and cross-compatibility at the tetraploid level.

Introduction

Italian (*Lolium multiflorum* Lam.) and perennial (*L. perenne* L.) ryegrass are diploid (2n = 14) and self-incompatible species. A wide range in seed set may, however, be obtained by forced selfing, depending upon genotype and season (Jenkins 1959). The diploid species are cross-compatible and "hybrid ryegrass" varieties, based on segregating hybrid-populations, have been in commercial use for some time (Corkill 1964).

Hybridization between tetraploid Italian and tetraploid perennial ryegrass would be highly desirable for combining the valuable agronomic characters of the two species. Such hybrids have been obtained for producing improved ryegrass varieties (Schumann 1968; Stephens and Breese 1971). Italian ryegrass has fluorescent-roots, due to the presence of annuloline (Linehan and Mercer 1931, 1933; Axelrod and Belzille 1958), and has awned-florets. These genetic markers may be used in the detection of hybrids, for both characters are absent in some perennial strains (Nyquist 1963; Copeland and Hardin 1970). This report presents the results of cytogenetic studies on hybrids between tetraploid Italian and perennial ryegrasses.

Materials and Methods

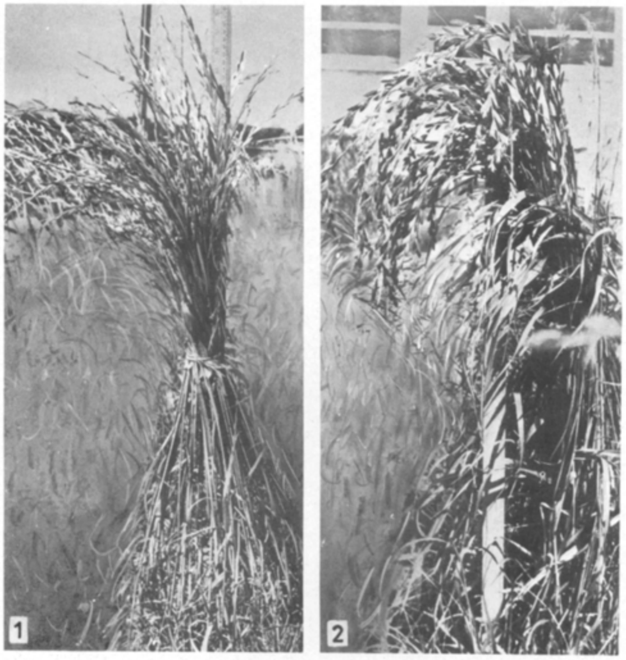
Second-generation tetraploids (2n = 28) and aneuploids (2n = 27, 29) of perennial ryegrass were crossed with advanced-generation tetraploid Italian ryegrass var. 'Tetrone' and Westerwolds var. 'Billion' (van der Have, Netherlands). Glasshouse grown plants in pots were transferred to a growth room (22-24°C, 16 h day length) a few days before anthesis. About 10 or more spikes each of an Italian and a perennial plant were bagged together, without emasculation. The glassine bags were plugged at the base with cotton-wool to prevent contamination, and shaken once or twice daily during anthesis. The spikes of different parental plants were separated 10-15 days after pollination and seeds were allowed to mature. Each group of spikes was harvested individually.

The seeds were germinated on ordinary blotting paper and ten-day old seedlings were scored for fluorescent-roots under Philips TL 40 'Black-light', 310-420 nm wave length. Non-fluorescent seedlings were rechecked after ten more days.

The seedlings were raised in peat pots for six months and then transplanted outdoors. Very few plants flowered during the first year, but nearly all flowered during summer of the following year, when they were scored for awning. Plants showing rudimentary awns were considered as possessing the character.

Two hundred F_1 plants showing vigorous growth and hybrid phenotypes were retained and inter-pollinated (Figs.1-2). Each plant was harvested singly. Two hundred F_2 seeds per F_1 plant were germinated and scored for fluorescence in the same manner as F_1 seed. The F_2 seedlings were space planted in the field and scored for awning in the following year.

Two spikes per F_1 plant were fixed in Carnoy 6:3:1 mordanted with ferric chloride. Smears of pollen mother cells were stained with acetic-carmine. Twenty cells at meiotic metaphase were studied for chromosome association. Pollen-stainability was determined as an index of fertility, using alcoholic-safranin.



Figs. 1-2. Tetraploid hybrids of ryegrass. 1. Perennial × Italian; 2. Italian × Perennial

Results

Detection of hybrids: The Italian-ryegrass characters of fluorescent-roots and awned-florets were dominant over non-fluorescent roots and awnless-florets of perennial ryegrass. The efficiency of hybrid detection was, therefore, far better with perennial than with Italian ryegrass as the seed-parent. F_1 seed germination was good, although it was slightly higher from crosses in which Italian ryegrass was the female parent (Table 1).

Perennial × Italian crosses: In these crosses, most hybrid-seedlings could be distinguished from the selfs by their fluorescent-roots. The frequency of the fluorescent-seedlings varied from 19 to 93 per cent (Table 1). On an average 53 % of the euploid progeny showed fluorescent roots and hence were of hybrid origin. Since the tetraploid parents produced seed on controlled selfing, either all or a proportion of the non-fluorescent seedlings could be selfs. A large number of non-fluorescent seedlings were, therefore, rejected.

Of the 38 non-fluorescent seedlings retained, however, 24 had awns and were hybrids (Table 2) indicating that the Italian pollen parent was heterozygous

Table 1. Seed germination and number of fluorescent seedlings in the F_1 reciprocal crosses between perennial and Italian ryegrass

		Perennial(♀) × Italian(♂)					Italian(♀) × Perennial(♂)				
		Germination(%)		*F(%)			Germination(%)		F(%)		
Number of crosses	2n×2n	Mean	Range	Mean	Range	Number of seedlings	Mean	Range	Mean	Range	Number of seedlings
8	28×28	73	40-94	53	19-88	446	91	78-98	88	78-100	524
3	27×28	74	59-89	67	64-72	152	98	93-100	86	92	188
1	29×28	74	-	93	-	74	-	-	-	-	-

*F = fluorescent

Table 2. Number of F_1 plants with fluorescent-roots and awned-florets in the reciprocal crosses between perennial and Italian ryegrass

		Perennial(♀)×Italian(♂)				Italian(♀)×Perennial(♂)			
Number of crosses	2n×2n	FA	Fa	fA	fa	FA	Fa	fA	fa
8	28×28	180	22	24	14	226	5	25	0
3	27×28	68	15	10	4	34	6	13	3
1	29×28	48	14	0	4	-	-	-	-

F = fluorescent, f = non-fluorescent, A = awned, a = awnless

Table 3. Segregation for the fluorescent-root phenotype in the F_2 of polyploid perennial × Italian hybrids

Plant No.[1]	2n	No. of seedlings		F_2 ratio	χ^2	expected	
		F	f			F_1 genotype ♀×♂	types of segregation ♀×♂
5F-19	28	166	4	35:1	0.114	AAaa×AAaa	A×A
5F- 9	28	157	3	"	0.485	" × "	"
1F- 9	28	180	8	20.8:1	0.049	" × "	B×B
5F-15	28	124	7	"	0.168	" × "	"
5F-25	28	176	9	"	0.032	" × "	"
5F- 1	28	170	15	11:1	0.013	Aaaa × "	A×A
7F- 4	28	120	13	"	0.075	" × "	"
5F- 6	28	151	24	7.7:1	0.855	" × "	B×B
3F-19	29	166	10	20.8:1	0.480	AAaa× "	"
Lp-selfed	28	0	180	-	-	aaaa ×aaaa	

[1] F-fluorescent, f = non-fluorescent; A = chromosomal, B = chromatid type segregation

for root-fluorescence. Were it assumed that only the non-fluorescent but awnless-plants originated through selfing, then the expected number of selfs in the original population of 446 seedlings would be about 78. Thus nearly 83 % of the tetraploid and 89 % of the aneuploid progeny were of hybrid origin. The efficiency of hybrid detection, therefore, improved considerably by using two instead of one genetic marker.

Since the aneuploids are often partially male-sterile, the expected recovery of hybrids from them as seed-parent would be higher than from normal tetraploids. The results obtained agreed with this expectation. Aneuploids may thus be a valuable tool for producing hybrids and new recombinants through chromosome substitution. The drawback, however, would be the presence of aneuploids in the subsequent generations.

Italian × perennial crosses: Unlike their reciprocal crosses, the hybrid seedlings could not be distinguished from selfs in such matings. About 88 % of the F_1 seedlings showed fluorescent-roots (Table 1), which is a dominant character of the Italian parent. All the non-fluorescent seedlings produced awned-florets at maturity and very few of the fluorescents were awnless (Table 2). Obviously, some Italian ryegrass plants were heterozygous for alleles determining either one or both of the phenotypes used as markers. Even the recombinants, such as non-fluorescent-awnless, could originate either by crossing or selfing of the heterozygous Italian parent. It was, therefore, necessary to study F_2 segregation to find whether an F_1 plant originated through crossing or selfing.

Segregation for root-fluorescence in F_2

The heterozygosity of the Italian ryegrass parents was confirmed by examining a population of seedlings from the variety 'Tetrone'. Of the 980 seedlings, 954 showed fluorescent-roots and 26 were non-fluorescent, suggesting a segregation ratio of 35:1 (χ^2 = 0.0571, p ⩾0.80), which would be expected from selfing or sibbing of genotype AAaa, when chromosome type of segregation prevails, i.e. there is no crossing-over between the locus and the centromere. A heterozygous tetraploid population in equilibrium, however, would be expected to show 15:1 ratio; the observed 35:1 ratio in 'Tetrone' cannot be explained unless a strong selection pressure had increased the frequency of dominant alleles within the population. Alternatively, Italian pollen parents were heterozygous and were of AAaa genotype. On back-crossing with perennial aaaa genotype, a 5:1 ratio would be expected in the B_1; this indeed appeared to be the case, since both awning (A) and fluorescent-character (F) showed 204A:36a, and 202F:38f, giving 5:1 ratio, χ^2 = 0.48 and 0.12 respectively. This would also explain 35:1 ratio in the variety 'Tetrone', which would be expected from sib-mating of AAaa genotype. A similar ratio (5:1) was confirmed for both awning and fluorescent characters in F_1 when aneuploid rather than tetraploid perennial ryegrass

Table 4. Segregation for the fluorescent-root phenotype in the F_2 of polyploid Italian × perennial hybrids

Plant No.[1]	2n	No. of seedlings[1]		F_2 ratio	χ^2	expected	
		F	f	F:f		F_1 genotype	type of segregation
4F-5	28	181	6	35:1	0.130	AAaa × AAaa	A × A
4F-31	28	175	8	20.8:1	0.019	" × "	B × B
4F-49	28	168	11	"	0.993	" × "	"
4F-36	28	164	6	"	0.435	" × "	"
4F-6	28	178	10	"	0.238	" × "	"
2F-1	28	140	22	7.7:1	0.694	Aaaa × "	B × B
4F-3	28	159	21	"	0.006	" × "	"
4F-4	28	171	19	"	0.417	" × "	"
4F-45	28	164	21	"	0.003	" × "	"
4F-50	28	154	23	"	0.391	" × "	"
4F-7	27	150	10	20.8:1	0.988	AAaa × AAaa	B × B
2F-5	29	336	22	"	1.988	" × "	"
2F-15	29	218	41	5:1	0.131	AAaa × aaaa	A × A
Tetrone (Lm)	28	954	26	35:1	0.001	AAaa × AAaa	A × A

[1] F-fluorescent, f = non-fluorescent; A = chromosomal, B = chromatid type segregation

Table 5. Segregation for awning in the F_2 of polyploid perennial × Italian hybrids

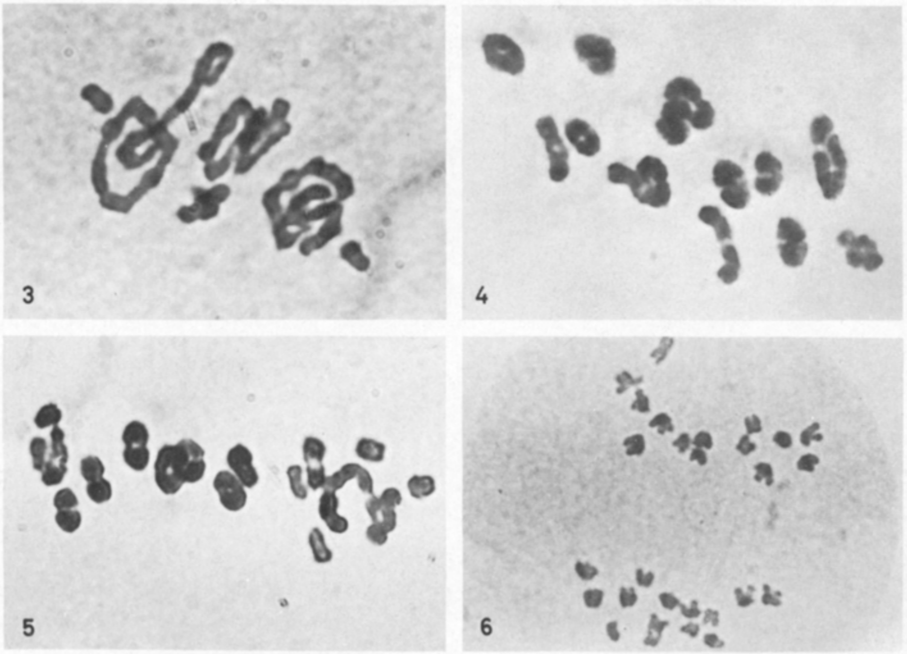
Plant No.	2n	No. of plants		F_2 ratio	χ^2	Expected F_1 genotype	Type of segregation
		+	a	+:a		♀×♂	♀×♂
5F-19	28	80	20	5:1	0.802	AAaa × aaaa	A × A
5F-9	"	105	20	5:1	0.070	"	"
1F-9	"	131	19	7.7:1	0.203	Aaaa × AAaa	B × B
5F-15	"	70	43	2.5:1	4.982	Aaaa × Aaaa	B × B
5F-25	"	81	44	2.5:1	2.699	"	"
5F-1	"	112	13	7.7:1	0.145	Aaaa × AAaa	B × B
7F-4	"	41	9	5:1	0.069	AAaa × aaaa	A × A
5F-6	"	116	8	20.8:1	0.986	AAaa × AAaa	B × B
3F-19	29	85	64	1:1	2.960	Aaaa × aaaa	A × A
Lp ⊕	28	0	185	-		aaaa × aaaa	-

+ = awned, a = awnless; A = chromosomal, B = chromatid type segregation

was used as the female parent (Table 2). Therefore, tetraploid hybrids between the perennial and Italian ryegrass under study represented test-crosses (aaaa) × AAAA, AAAa, AAaa, Aaaa and aaaa for the genetic markers. All the 22 F_1 plants except one showed segregation for fluorescence (Tables 3 and 4), which could be explained as modified ratios of 20.8:1, 11:1, 7.7:1, 5:1, etc., expected on mating F_1 female genotypes AAaa and Aaaa with F_1 AAaa and aaaa pollen parents, and whether the chromosome or chromatid (a cross-over between the locus and the centromere occurs) type of segregation prevailed. On this basis, of the 14 plants from Italian × perennial crosses studied for segregation, only one was not a hybrid; thus 93% of the Italian ryegrass progeny were hybrids. This may represent a slightly biased figure since a number of selfed plants may have been eliminated due to lack of vigour.

Segregation for Awning in F_2

The F_2 spaced plants showed segregation for awning. The obtained ratios could be explained on a basis similar to



Figs.3-6. Meiosis in hybrids of ryegrass. Fig.3. 2n = 28, 2 I + 1 II + 6 IV; Fig.4. 2n = 29, 9 II + 1 III + 2 IV; Fig.5. 2n = 29, 2 I + 7 II + 2 IV + 1 V; Fig.6. 2n = 28, anaphase I, 14:14 segregation

Table 6. Segregation for awning in the F_2 of polyploid Italian × perennial ryegrass hybrids

Plant No.	2n	No. of plants F_2 ratio			χ^2	Expected F_1 genotype	Type of segregation
		+	a	+:a		♀×♂	♀×♂
4F-5	28	145	5	35:1	0.171	AAaa×AAaa	A×A
4F-31	"	130	20	7.7:1	0.499	Aaaa ×AAaa	B×B
4F-49	"	100	25	5:1	1.014	AAaa×aaaa	A×A
4F-36	"	140	10	11:1	0.545	Aaaa ×AAaa	A×A
4F-6	"	88	11	7.7:1	0.014	Aaaa ×AAaa	B×B
2F-1	"	83	16	5:1	0.018	AAaa×aaaa	A×A
4F-3	"	100	25	5:1	1.014	"	
4F-4	"	131	19	7.7:1	0.203	Aaaa ×AAaa	B×B
4F-45	"	120	5	20.8:1	0.099	AAaa×AAaa	B×B
4F-50	"	69	5	11:1	0.238	Aaaa ×AAaa	A×A
4F-7	27	113	11	11:1	0.044	Aaaa ×AAaa	A×A
2F-5	29	125	22	5:1	0.306	AAaa×aaaa	A×A
2F-15	29	83	15	5:1	0.137	AAaa×aaaa	A×A
Lm ⊕	-	147	3	35:1	0.333	AAaa×AAaa	A×A
Lp ⊕	-	70	5	35:1	4.215	AAaa×AAaa	A×A

+ = awned, a = awnless; A = chromosomal and B = chromatid type of segregation

that for the root-fluorescence, confirming the hybrid nature of F_1 and homoeologous chromosome pairing. The parental population of 'Tetrone' segregated 35:1 and that of perennial ryegrass did not show segregation for awning (Tables 5 and 6).

Meiotic Behaviour of the Hybrids

The chromosome association in pollen number cells at first metaphase is given in Tables 7 and 8. The mean chromosome association in the tetraploid

Table 7. Mean chromosome association at first metaphase in pollen mother cells of polyploid hybrid ryegrass

Plant No.		Mean association/cell			
		I	II	III	IV
Perennial × Italian					
2n = 28	1F-9	1.0	5.1	0.2	4.4
	3F-7	0.5	7.4	-	3.2
	3F-22*	0.6	6.8	0.1	3.4
	5F-1	0.8	5.5	0.5	3.7
	5F-6	0.6	8.3	0.2	2.6
	5F-9	0.4	5.6	0.1	4.1
	5F-15	0.7	5.8	0.2	3.8
	5F-19	0.6	6.1	0.3	3.6
	5F-25	0.2	7.0	0.1	3.5
	5F-26	0.5	7.1	0.2	3.3
2n = 29	3F-19	0.6	4.7	0.3	4.5+0.5V

* heterozygous for one reciprocal translocation, one heptavalent present

Table 8. Mean chromosome association at first metaphase in pollen mother cells of polyploid hybrid ryegrass

Plant No.		Mean association/cell			
		I	II	III	IV
Italian × perennial					
2n = 28	2F-1	0.3	6.8	0.02	3.4
	2F-49	1.1	5.7	0.60	3.5
	4F-3	0.5	6.2	0.15	3.7
	4F-4	0.8	7.3	0.05	3.2
	4F-5	0.9	5.9	0.15	3.8
	4F-6	0.2	7.6	0.10	3.1
	4F-31	0.4	3.8	0.15	4.9
	4F-39	0.5	5.1	0.35	4.1
	4F-40	0.4	5.5	0.20	4.0
	4F-45	0.3	3.1	0.15	4.9
	4F-46	0.7	6.3	0.30	3.5
	4F-47	1.1	5.9	0.45	3.5
	4F-50	0.9	5.9	0.35	3.6
2n = 27	4F-7	1.3	5.7	0.75	3.1
2n = 29	2F-5	0.9	6.1	0.60	2.9+0.6V
	2F-15	0.6	5.6	0.65	3.2+0.5V
	2F-9	0.1	5.8	0.45	3.3+0.4V

Table 9. Pollen fertility of F_1 between tetraploid Italian and perennial ryegrass

Hybrid* ♀×♂	No. of plants	Fertility (%)				
		80	81-90	91-95	96-100	Mean
Lm × Lp	103	6	29	53	15	91.7
Lp × Lm	100	11	23	49	17	90.1

* Lm - Italian, Lp - perennial

Table 10. Seed set in F_1 between tetraploid Italian and perennial ryegrass

Hybrid*	No. of plants	Seed g/plant								
		0-5	6-10	11-15	16-20	21-25	26-30	31-35	>36	Mean
Lm × Lp	104	9	15	21	24	20	8	4	3	17.7
Lp × Lm	100	4	26	42	15	7	3	2	1	14.3

* Lm - Italian, Lp - perennial

perennial × Italian hybrids was 0.6 I + 6.5 II + 0.2 III + 3.5 IV and that in the Italian × perennial hybrids was 0.6 I + 5.8 II + 0.2 III + 3.8 IV. In all hybrids, most pollen mother cells showed 3 to 4 quadrivalents per cell, which represents about 50 % of the genome associated into multivalents (trivalents plus quadrivalents) (Fig.3). A few aneuploids with 2n = 26,27 and 29 chromosomes were also present (Figs.4-5).

Of the 208 tetraploid cells examined at anaphase I, only 43 % showed 14:14 chromosome segregation (Fig.6); about 22 % of the cells showed 13:14 plus 1 laggard and 11 % exhibited 13:15; the remaining showed segregations such as 13:13, 12:14, 12:12 with 2 to 4 univalent laggards. A few cells had a dicentric bridge and fragment, indicating a paracentric inversion.

Fertility of the Hybrids

The hybrids, irrespective of their seed-parent, had high pollen fertility (Table 9). The seed set of hybrid plants varied widely. Hybrids from the Italian mother-parent had a slightly higher seed set than those from the perennial-parent (Table 10). To some extent this may reflect the effect of the seed parent. Seed of the tetraploid Italian ryegrass is larger than that of the perennial ryegrass.

Discussion

The dominance of the genetic markers, root-fluorescence and awning, in the reciprocal crosses between tetraploid perennial and Italian ryegrass showed that the distinction between seedlings of hybrid and selfed origin, obtained by mutual bagging of unemasculated florets, was possible only with the perennial ryegrass as the seed parent. Further, the efficiency of detecting hybrids was better with two markers instead of one, particularly when one of the parents (in this case Italian ryegrass) was heterozygous for the genetic markers. The hybrids from perennial (female) ×Italian (male) would combine cytoplasmic-perennial and nuclear-Italian characters, if a suitable method is not available to detect hybrids in the reciprocal crosses. A dominant genetic-marker in the perennial ryegrass would be of great practical value in hybrid detection when Italian ryegrass is the seed parent.

The F_2 segregation of fluorescent-root and awning showed that approximately 93 % of the F_1 plants were hybrids when Italian ryegrass was seed parent, whereas 83 to 89 % of the F_1 plants were hybrids from the reciprocal crosses. Although a suitable method to produce hybrids on a mass scale must be developed, the recovery of 80-90 % of the hybrids is highly efficient for exploiting hybrid vigour on a commercial scale. The high hybrid frequency also confirmed that the species maintained their self-incompatibility and cross-compatibility at the tetraploid level. Enforced selfing of tetraploids, however, did produce seed, which is also feasible in the diploids (Jenkins 1959).

The ratios for the fluorescent-root and awning in the individual F_2 progenies strongly suggested that both chromatid and chromosome type segregation occurred. Even if the F_2 individuals examined were considered too few to have confidence in the arbitrary ratios proposed, the over-all segregation of the fluorescent-root for the whole F_2 population would be 15:1 for perennial × Italian and 11:1 for the reciprocal crosses, the recessive gene-frequency being far in excess of the expected 35:1 on the basis of chromosome type segregation from a duplex ($\underline{AAaa}$) F_1 random mating.

The multivalent frequency in the F_1 plants was extremely high: 3 to 4 quadrivalents per cell were common in most microsporocytes. Since multivalents in these hybrids must arise from homoeologous rather than homologous chromosome associations, the frequency of recombinants through chromatid type segregation would be high, which was suggested by the segregation ratios of the two marker genes. Even with a limited number of exchanges between the homoeologous chromosomes, random segregation of multivalent-forming chromosomes would result in genomes with substituted chromosomes, producing a wide genetic variation.

In spite of the high multivalent frequency, these allopolyploids were highly fertile. Obviously, the genomes of the two parental species have a high degree of homology; indeed very little chromosomal repatterning has accompanied their differentiation, which showed up as occasional inversions and rare translocations in the present study. Thus, for all practical purpose, these allopolyploids resembled autotetraploids in their breeding and genetic behaviour. From a breeding view-point, their high seed set and the anticipated genetic variation in the subsequent generations makes these hybrids extremely valuable as a source of selecting the desired types.

Acknowledgement

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