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Development of a ploidy series from a single interspecific *Trifolium repens* L. × *T. nigrescens* Viv. F_1 hybrid

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Abstract The objective of the current research was to generate a ploidy series of backcross progenies from a single triploid (2n = 3x = 24) *Trifolium repens* × *T. nigrescens* F_1 hybrid (3x H-6909-5). The 3x H-6909-5 plant was highly sterile and produced no seeds from approximately 3000 reciprocal backcrosses to both parental species. Chromosome doubling by an in vitro colchicine method resulted in a marked increase in fertility. Pollen stainability was increased from 9.9% in 3x H-6909-5 to an average of 89.2% (range 87.7–90.9%) in the three chromosome-doubled 6x H-6909-5 plants. Subsequent backcrosses of 6x H-6909-5 and interbreeding of backcross derivatives resulted in an array of fertile hybrids at 4x, 5x and 7x levels and some aneuploids. The occurrence of 7x BC_1F_1 progeny from the *T. repens* × 6x H-6909-5 (4x × 6x) cross is the first unequivocal evidence of functional female 2n gametes in white clover. Meiotic pairing in F_1 and BC_1F_1 progeny indicated the presence of allosyndetic pairing, suggesting that genetic exchange between the two species is possible.

Key words *Trifolium* spp. · Polyploidy · Interspecific hybrids · Cytogenetics

Introduction

Trifolium repens L. (white clover, 2n = 4x = 32) is one of the most important and widely used forage legumes in temperate regions of the world. White clover is a perennial species but stands often decline significantly in the second or third year of growth due to susceptibility to a number of stress factors including drought, viruses, nematodes and root-chewing insects (Williams 1987).

Interspecific hybridisation of *Trifolium* species has long been suggested as a means of improving commercial white clover cultivars. White clover has been successfully hybridised with three annual and four perennial *Trifolium* species (Williams 1987). Most of these crosses required embryo rescue, were obtained with difficulty, and the success rates were very low. Despite the production of interspecific hybrids between *T. repens* and other *Trifolium* species, their potential as useful genetic material for the improvement of standard white clover cultivars has not been exploited. The main obstacles to the use of existing interspecific hybrids have been listed by Hussain and Williams (1997 a).

T. nigrescens Viv. (2n = 2x = 16), is an extremely variable, non-stoloniferous free-seeding annual species occurring in natural pastures of the Mediterranean area (Williams 1987). It has been used before in interspecific crosses with *T. repens* (Brewbaker and Keim 1953; Keim 1953 a, b; Evans 1962 a; Trimble and Hovin 1960; Hovin 1962; Chen and Gibson 1970 b; Kazimierski and Kazimierska 1970; Williams et al. 1978; Marshall et al. 1995) but its potential as germ plasm for the improvement of white clover has not, to-date, been exploited. The species has been reported to be unpromising for interspecific hybridisation with white clover as it appeared to be highly susceptible to viruses (Gibson et al. 1971) and the hybrids were weakly perennial and showed low fertility. However *T. nigrescens* was later evaluated for resistance to nematodes and was found to be highly resistant to clover cyst nematode (*Heterodera trifolii* Goffart) (Mercer 1988) and southern root knot nematode [*Meloidogyne incognita* Kofoid & White (Chitwood)] (Pederson and Windham 1989).

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T. repens and *T. nigrescens* cross with some difficulty, although certain combinations of plants produce large numbers of hybrids (3x = 24) (Williams et al. 1978; Marshall et al. 1995). The cross can be more successful when *T. repens* is used as the female parent (Kazimierski and Kazimierska 1970; Hovin 1962). Genetic segregation and studies of chromosome pairing at meiosis in *T. repens* × *T. nigrescens* hybrids indicate some homology between the chromosomes of the two species (Brewbaker and Keim 1953; Hovin 1962; Chen and Gibson 1970 b).

The present project was initiated with a single triploid (2n = 3x = 24) *Trifolium repens* × *T. nigrescens* F_1 hybrid, designated 3x H-6909-5. The objective of this cross was to transfer clover cyst nematode resistance from *T. nigrescens* to *T. repens* (White and Mercer, unpublished work). This triploid F_1 hybrid (3x H-6909-5) was resistant to clover cyst nematode, but was highly sterile and did not produce seed after backcrossing to the parental species. The objectives of the current research were to generate a wide range of backcross progenies at various ploidy levels for future evaluation of economic parental characteristics, and to estimate the extent of chromosome homology between *T. repens* and *T. nigrescens* by studying the pollen stainability and cytogenetics of F_1 and first-backcross progeny.

Materials and methods

An individual triploid interspecific *T. repens* × *T. nigrescens* hybrid (3x H-6909-5) obtained through embryo culture was provided by Dr. Derek White (AgResearch Grasslands, Palmerston North, New Zealand). Three hexaploid clones of H-6909-5 (designated as CT-1, CT-14 and CT-28) were obtained by in vitro colchicine doubling (Hussain 1995).

In most subsequent crosses, one genotype of *T. repens* "Grasslands Crimson Charm" (CC-1) was used. CC-1 has one red and two white leaf-mark alleles in a heterozygous condition (*Vm*, *Vi*; *Rl*, *r*) and carries a multi-leaflet trait, probably also in heterozygous form (the expression of this character is variable). In some backcrosses three other genotypes of *T. repens* (cv Grasslands Huia) with no leaf markings were used. One genotype of *T. nigrescens* (Tn-167) from line Az 2225 was obtained from the Margot Forde Forage Germplasm Centre, AgResearch Grasslands, Palmerston North, New Zealand.

Pollination

Reciprocal backcrosses of 3x and 6x H-6909-5 to *T. repens* and *T. nigrescens* were made by hand on potted plants grown in an insect-proof glasshouse. Before pollination, flowers on the female parent were emasculated by the forceps technique of Williams (1954). Pod development was recorded as the total numbers of pods developed during the first 2 weeks after each crossing. Approximately 4–5 weeks after pollination, seeds were harvested from mature flower heads. Self-incompatibility of individual plants was assessed by gently rolling at least four bagged flower heads of each plant between the thumb and fingers daily for 3 days after bagging (Williams 1987). The backcrossing schemes and terminology used in the present study were adapted from Haghighi and Ascher (1988). The first backcross, termed BC_1F_1, involved the F_1 (3x or 6x) H-6909-5 as one parent and *T. repens* or *T. nigrescens* as the other parent. The second backcross, termed BC_2F_1, involved the same recurrent parental species. The F_1 (6x H-6909-5) hybrid backcrossed to each of the parental species in alternate generations was termed the congruity backcross (CBC). Progenies from $BC_1F_1 \times BC_1F_1$ intercrosses were termed BC_1F_2 .

Cytological techniques

For pollen stainability estimates, 2–3 anthers from glasshouse-grown plants were dehisced over a glass slide to which a drop of 2% acetocarmine was added. The material was then covered with a cover slip and after 5 min of staining, the percentage of plump, fully stained grains was determined. At least 1200 grains from six or more flowers and three or more inflorescences per plant were examined.

Somatic chromosome counts were made from root-tip squashes by collecting root tips 1–2 cm long from mature plants early in the morning, pre-treating in 0.004 M 8-hydroxyquinoline for 5–7 h at 4°C and fixing in 3:1 95% ethanol:glacial acetic acid at room temperature. The material was then rinsed twice with distilled water and hydrolysed in 1 N HCl at 60°C for 10–12 min and stained in Feulgen stain for 15–30 min (Williams 1978). Stained root tips were squashed in 2% acetocarmine for chromosomal counts at metaphase. At least ten cells from five root tips were examined for each plant.

For meiotic chromosome configurations in pollen mother cells (PMCs), young inflorescences (about 2 mm in diameter and just emerged from the stipules) were fixed in Carnoy's fluid (6:3:1 95% ethanol:chloroform:glacial acetic acid) for 24 h at room temperature. Fixed flower buds were rinsed three times with 70% ethanol allowing at least 20 min for each change and stained in alcoholic hydrochloric acid-carmine stain (Snow 1963) for at least 72 h. After rinsing with 70% ethanol, the stained material was stored in 70% ethanol in the refrigerator until used. Anthers were squashed lightly with a flat needle in a drop of 1% acetocarmine and the slide warmed to just below boiling point of the liquid for about 30 s. The cover slip was then pressed between two folds of filter paper with progressively increasing pressure. Chromosomal associations were recorded at metaphase-I in 15–35 pollen mother cells from at least ten flower buds from each plant.

Results

First backcross (BC_1F_1)

The plants of both *T. repens* (CC-1) and *T. nigrescens* (Tn-167) used in backcrosses were not the original parents of 3x H-6909-5, but had the same chromosome numbers (2n = 4x = 32 and 2n = 2x = 16 respectively).

Reciprocal backcrosses of 3x H-6909-5 (Fig. 1 A) with *T. repens* (CC-1) and *T. nigrescens* (Tn-167) showed either extremely poor or no pod development and no seeds were obtained. On the other hand, reciprocal backcrosses involving 6x H-6909-5 (Fig. 1 B) showed significant pod development during the 1st and 2nd weeks after pollination but nevertheless very few seeds were obtained (Table 1).

The backcross hybrid obtained from using 6x H-6909-5 (plant CT-14) as the female parent after pollination with *T. nigrescens* (Tn-167) was tetraploid

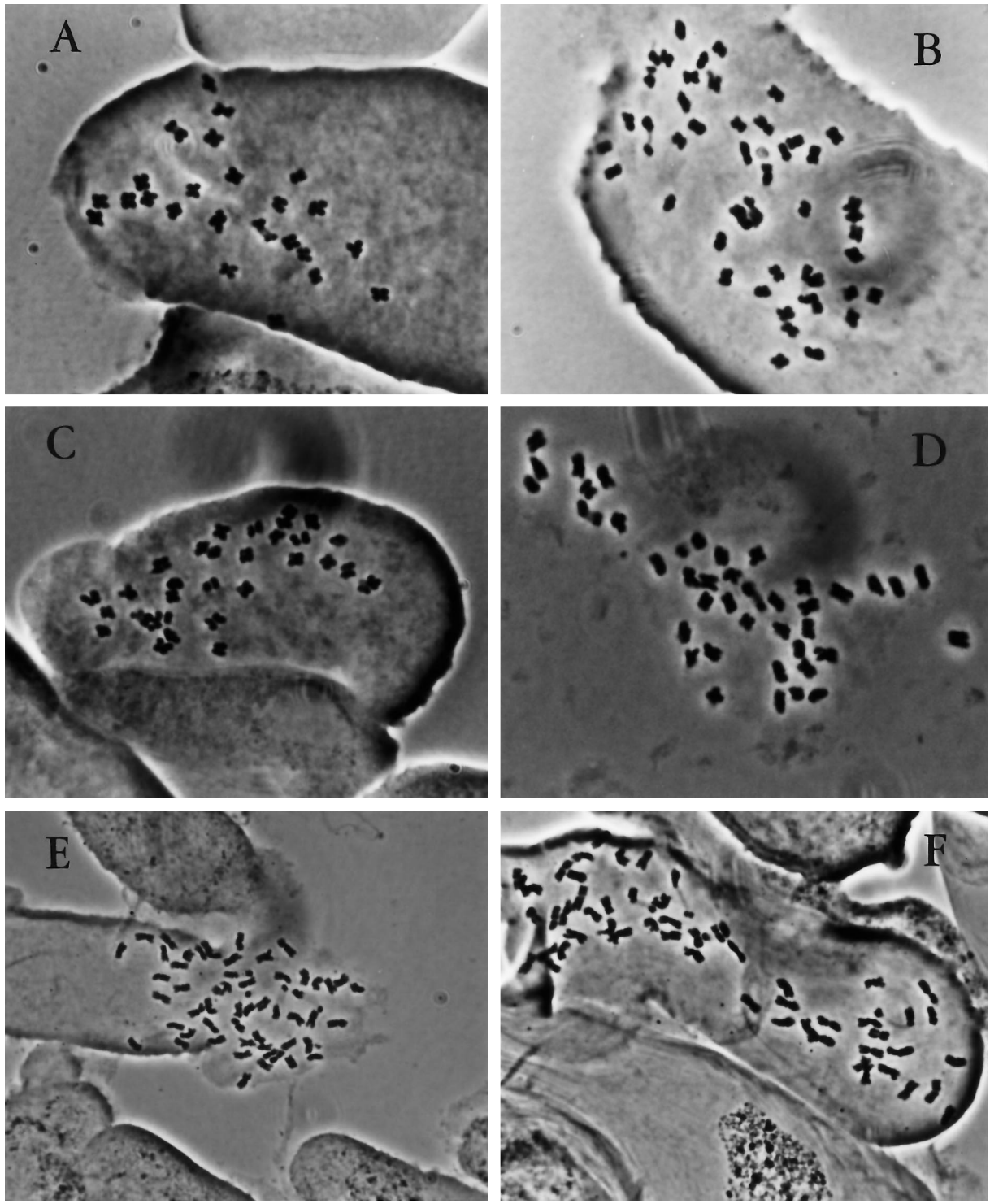


Fig. 1 Somatic chromosomes of (**A**) 3x H-6909-5 (*T. repens* × *T. nigrescens*) F_1 hybrid, 2n = 3x = 24, (**B**) 6x H-6909-5, 2n = 6x = 48, (**C**) 4x BC_1F_1 (6x H-6909-5 × *T. nigrescens*), 2n = 4x = 32, (**D**) 5x BC_1F_1 (6x H-6909-5 × *T. repens*), 2n = 5x = 40, (**E** and **F**) 7x BC_1F_1 (*T. repens* × 6x H-6909-5), 2n = 7x = 56. **A**–**D** × 1600, (**E**) × 1150, (F) × 1400

Table 1 Pod development and number of seeds obtained after reciprocal first backcrosses of 3x and 6x H-6909-5 to *T. repens* and *T. nigrescens*

Cross	Ploidy level	No. pollinations	Pods developed		No. seeds obtained
			No.	(%)	
First backcross (BC_1F_1)					
3x H-6909-5 × CC-1	3x × 4x	800	19	2.4	0
CC-1 × 3x H-6909-5	4x × 3x	750	0	0.0	0
3x H-6909-5 × Tn-167	3x × 2x	800	29	3.6	0
Tn-167 × 3x H-6909-5	2x × 3x	600	2	0.3	0
6x H-6909-5 × Tn-167					
CT-1 × Tn-167	6x × 2x	190	23	12.1	0
CT-14 × Tn-167 (CBC_1)	6x × 2x	380	89	23.4	1 = 4x BC_1F_1
CT-28 × Tn-167	6x × 2x	230	46	20.0	0
Tn-167 × 6x H-6909-5					
Tn-167 × CT-1	2x × 6x	270	117	43.3	0
Tn-167 × CT-14	2x × 6x	500	227	45.4	0
Tn-167 × CT-28	2x × 6x	430	134	31.2	0
6x H-6909-5 × CC-1					
CT-1 × CC-1	6x × 4x	410	90	22.0	0
CT-14 × CC-1 (CBC_1)	6x × 4x	360	114	31.7	1 = 5x BC_1F_1
CT-28 × CC-1	6x × 4x	430	154	35.8	0
CC-1 × 6x H-6909-5					
CC-1 × CT-1	4x × 6x	200	83	41.5	0
CC-1 × CT-14	4x × 6x	260	92	35.4	3 = 7x BC_1F_1
CC-1 × CT-28	4x × 6x	240	117	48.8	0

Table 2 Pod development and number of seeds obtained after second backcrosses, congruity backcrosses, $BC_1F_1 \times BC_1F_1$ intercrosses and $BC_1F_1 \times F_1$

Cross	Ploidy level	No. pollinations	Pods developed		No. seeds obtained
			No.	(%)	
Second backcross (BC_2F_1)					
(CT-14 × CC-1) × Huia-1	5x × 4x	160	28	17.5	1
Huia-1x (CT-14 × CC-1)	4x × 5x	160	97	60.6	8
(CC-1 × CT-14)-1 × Huia-1	7x × 4x	180	43	23.9	3
Huia-1x (CC-1 × CT-14)-1	4x × 7x	240	29	12.1	1
Congruity backcross (*CBC*)					
CT-14 × Tn-167 (CBC_1)	6x × 2x	380	89	23.4	1
(CT-14 × Tn-167) × CC-1 (CBC_2)	4x × 4x	170	46	27.1	3
Huia-1x (CT-14 × Tn-167) (CBC_2)	4x × 4x	130	39	30.0	3
CT-14 × CC-1 (CBC_1)	6x × 4x	360	114	31.7	1
(CT-14 × CC-1) × Tn-167 (CBC_2)	5x × 2x	460	17	3.7	0
Tn-167 × (CT-14 × CC-1) (CBC_2)	2x × 5x	240	69	28.8	0
$BC_1F_1 \times BC_1F_1$ (*Intercross*)					
(CT-14 × Tn-167) × (CT-14 × CC-1)	4x × 5x	30	19	63.3	6
(CT-14 × CC-1) × (CT-14 × Tn-167)	5x × 4x	30	13	43.3	3
(CT-14 × Tn-167) × (CC-1 × CT-14)-1	4x × 7x	230	26	11.3	1
(CC-1 × CT-14)-1 × (CT-14 × Tn-167)	7x × 4x	145	21	14.5	1
(CT-14 × CC-1) × (CC-1 × CT-14)-1	5x × 7x	100	16	16.0	1
(CC-1 × CT-14)-2 × (CT-14 × CC-1)	7x × 5x	75	36	48.0	0
(CC-1 × CT-14)-1 × (CC-1 × CT-14)-2	7x × 7x	60	41	68.3	6
$BC_1F_1 \times F_1$ (6*x*)					
(CT-14 × Tn-167) × CT-28	4x × 6x	80	11	13.8	0
CT-28 × (CT-14 × Tn-167)	6x × 4x	130	90	69.2	10
(CT-14 × CC-1) × CT-28	5x × 6x	80	7	8.8	1
CT-28 × (CT-14 × CC-1)	6x × 5x	100	87	87.0	54
(CC-1 × CT-14)-1 × CT-28	7x × 6x	80	5	6.3	0
CT-28 × (CC-1 × CT-14)-1	6x × 7x	100	92	92.0	56
CT-1 × (CT-14 × CC-1)	6x × 5x	75	54	72.0	8
(CT-14 × CC-1) × CT-1	5x × 6x	60	17	28.3	0

($2n = 4x = 32$, Fig. 1 C). The plant was self-incompatible as it did not set any seed after selfing ten inflorescences and had 59.6% pollen stainability. This BC_1F_1 was vegetatively propagated from stem cuttings as it showed the annual growth habit of *T. nigrescens*. The hybrid flowered profusely throughout the summer and the parent plant died after the completion of flowering. This backcross had no root primordia at the nodes, and thus had no nodal rooting.

The backcross plant from 6x H-6909-5 (CT-14) × *T. repens* (CC-1), was pentaploid ($2n = 5x = 40$, Fig. 1 D) with a pollen stainability of 86.7%. The backcross origin of this pentaploid hybrid was confirmed by the presence of the *Vm* and *Vi* leaf marks and multi-leaflets derived from the male parent. The hybrid strongly resembled *T. repens* in morphology, having similar leaflet and inflorescence sizes, a perennial stoloniferous growth habit, and was very easily propagated from stolon cuttings as it had root primordia at each node and also frequent nodal rooting.

The three backcrossed plants from CC-1 × 6x H-6909-5 were all heptaploid ($2n = 7x = 56$, Fig. 1E, F) with pollen stainabilities from 72.7% to 74.3%. These three 7x plants strongly resembled the female *T. repens* parent in morphology, having true stoloniferous growth with root primordia at each node and frequent nodal rooting.

Second backcross (BC_2F_1)

Nine BC_2F_1 seeds were obtained from reciprocal backcrosses between the pentaploid BC_1F_1 (CT-14 × CC-1) and *T. repens* (Huia-1) and four fully developed BC_2F_1 seeds were harvested from reciprocal backcrosses between the heptaploid BC_1F_1 [(CC-1 × CT-14)-1] and *T. repens* (Huia-1) (Table 2). *T. repens* Huia-1 did not carry any leaf mark and so the *Vm* and *Vi* leaf marks of the pentaploid BC_1F_1, the *Vi*-; *Rl*-leaf marks of the heptaploid BC_1F_1 and the multi-leaflet character of both BC_1F_1s were used to confirm the second backcross progeny in crosses involving BC_1F_1s as the male and Huia-1 as the female parents. One BC_2F_1 seed from each cross using the BC_1F_1s as the male parents was germinated and the backcross origins of these two

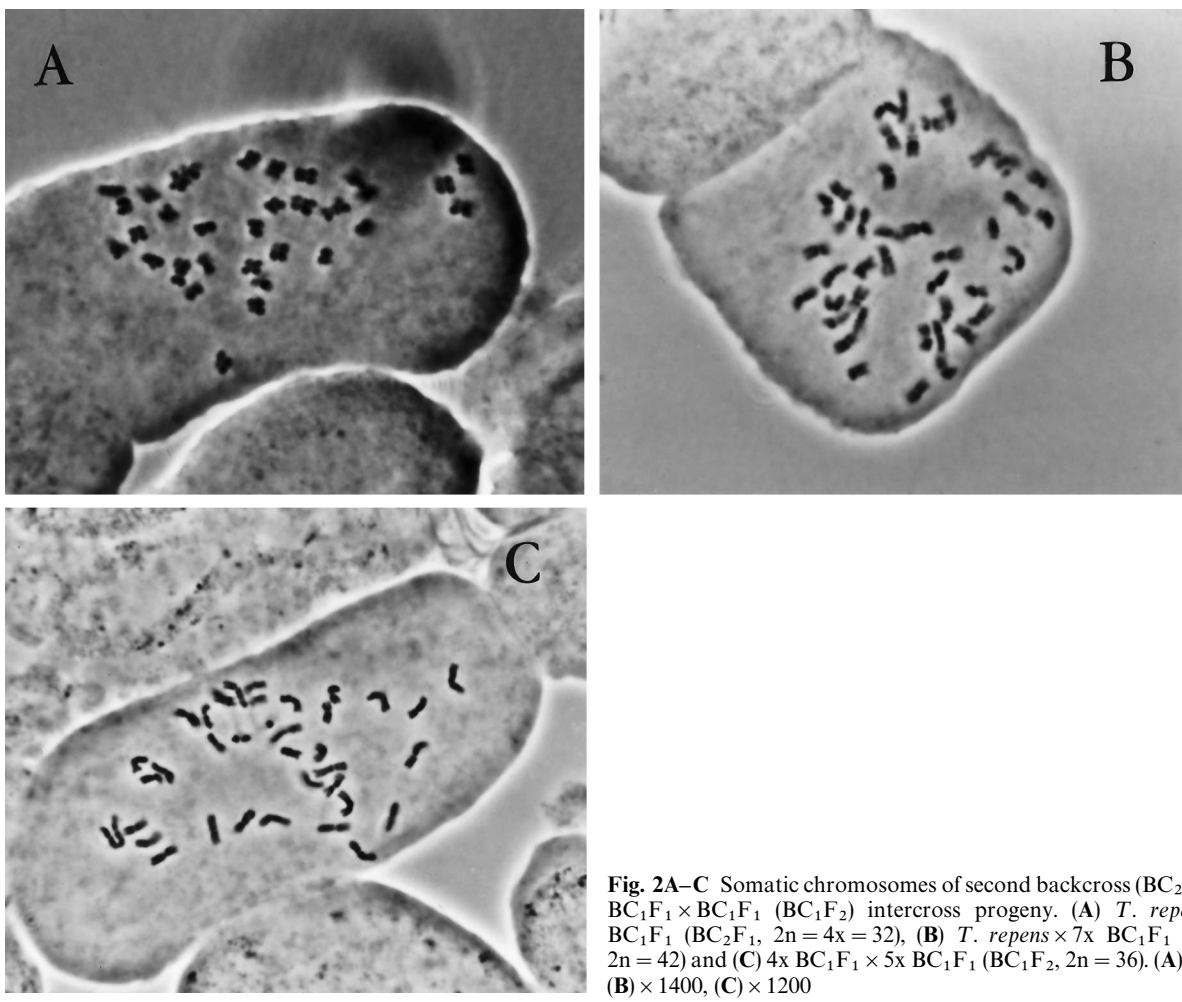


Fig. 2A–C Somatic chromosomes of second backcross (BC_2F_1) and $BC_1F_1 \times BC_1F_1$ (BC_1F_2) intercross progeny. (**A**) *T. repens* × 5x BC_1F_1 (BC_2F_1, $2n = 4x = 32$), (**B**) *T. repens* × 7x BC_1F_1 (BC_2F_1 $2n = 42$) and (**C**) 4x BC_1F_1 × 5x BC_1F_1 (BC_1F_2, $2n = 36$). (**A**) × 1600, (**B**) × 1400, (**C**) × 1200

plants were confirmed by the transmission of the markers. The BC_2F_1 plant obtained using the 5x BC_1F_1 as the male was $2n = 4x = 32$ (Fig. 2A). The other BC_2F_1 plant obtained from the Huia-1 × 7x BC_1F_1 backcross was an aneuploid with $2n = 42$ (Fig. 2 B).

Congruity backcross (CBC_2)

The tetraploid BC_1F_1 (CT-14 × Tn-167) was not backcrossed with *T. nigrescens* for a second generation as it already showed strong similarity to the *T. nigrescens* parent. Instead it was crossed reciprocally to *T. repens* (Huia-1 used as the female and CC-1 as the male) to obtain CBC_2 progeny (Table 2). One of the CBC_2 seeds from the cross 4x BC_1F_1 × CC-1 was germinated. The resultant plant carried the *Vm* leaf mark derived from the male *T. repens* parent (CC-1), thus confirming its hybridity.

Another congruity second backcross (CBC_2) was attempted between the 5x BC_1F_1 (CT-14 × CC-1) and *T. nigrescens* (Tn-167), but no seeds were obtained from 700 reciprocal crosses (Table 2).

$BC_1F_1 \times BC_1F_1$ intercross (BC_1F_2)

The tetraploid, pentaploid and two heptaploid BC_1F_1s were reciprocally crossed amongst themselves. Although the numbers of these intercrosses were comparatively small (Table 2), at least one seed was obtained from each cross, with the exception of 7x (CC-1 × CT-14) -2 × 5x (CT-14 × CC-1). The most successful intercrosses were (1) between the tetraploid (female) and pentaploid (male) BC_1F_1s and (2) between the two heptaploid BC_1F_1s where six well-developed seeds were obtained from relatively few pollinations (Table 2). Two plants from the intercross 4x BC_1F_1 × 5x BC_1F_1 exhibited multiple leaflet or leaf mark traits inherited from the male parent, confirming their intercross origin. One of these plants was evaluated cytologically and was an aneuploid with $2n = 36$ (Fig. 2 C).

$BC_1F_1 \times F_1$ (6x H-6909-5)

Initially all the colchicine-derived hexaploid plants (CT-1, CT-14 and CT-28) of H-6909-5 were used in backcrosses with *T. repens* (CC-1) and *T. nigrescens* (Tn-167). However only plant CT-14 was cross-fertile in producing BC_1F_1 seeds. Later, plant CT-28 was reciprocally crossed with 4x, 5x and 7x BC_1F_1s to examine its male and female fertility. The results of these crosses indicated that plant CT-28 was more female-fertile than male-fertile (Table 2). Plant CT-1 was not used in crosses with all three BC_1F_1s but produced eight seeds from 70 florets pollinated with 5x BC_1F_1 (CT-14 × CC-1) pollen, while reciprocal crosses were unsuccessful. Seeds from these crosses have not yet been germinated.

Table 3 Somatic chromosome number, meiotic configurations of pollen mother cells (PMCs) and pollen stainability of *T. repens*, *T. nigrescens*, the F_1 triploid hybrid (3x H-6909-5), the colchicine-induced hexaploid hybrid (6x H-6909-5) and backcrosses of 6x H-6909-5 to *T. repens* and *T. nigrescens*

Genotype/cross	Somatic chromosome no.	Total PMCs scored	Meiotic configuration at metaphase-I in pollen mother cells								Pollen stainability (%)
			I		II		III		IV		
			Mean	Range	Mean	Range	Mean	Range	Mean	Range	
T. repens (CC-1)	2n = 4x = 32	15	0.00	(0–0)	16.00	(16–16)	–	–	–	–	90.5
T. nigrescens (Az 2225-167)	2n = 2x = 16	20	0.00	(0–0)	8.00	(8–8)	–	–	–	–	93.6
(*T. repens* × *T. nigrescens*)											
3x H-6909-5	2n = 3x = 24	25	3.60	(1–6)	6.36	(3–8)	2.56	(1–5)	–	–	9.9
6x H-6909-5											
Plant no. CT-1	2n = 6x = 48	20	0.72	(0–2)	18.00	(14–23)	0.36	(0–1)	2.55	(0–5)	88.8
Plant no. CT-14	2n = 6x = 48	25	0.83	(0–3)	20.04	(15–24)	0.39	(0–2)	1.48	(0–4)	90.9
Plant no. CT-28	2n = 6x = 48	30	0.67	(0–5)	18.43	(14–22)	0.65	(0–1)	2.13	(0–4)	87.9
6x H-6909-5 × *T. nigrescens*	2n = 4x = 32	25	1.24	(0–2)	8.64	(6–14)	0.76	(0–2)	2.80	(0–5)	59.6
6x H-6909-5 × *T. repens*	2n = 5x = 40	35	3.67	(1–6)	10.71	(8–18)	1.09	(0–5)	2.91	(0–5)	86.7
T. repens × 6x H-6909-5											
Plant no. 1	2n = 7x = 56	15	6.00	(3–11)	9.08	(7–14)	3.84	(1–5)	5.08	(1–9)	72.7
Plant no. 2	2n = 7x = 56	–	–	–	–	–	–	–	–	–	71.8

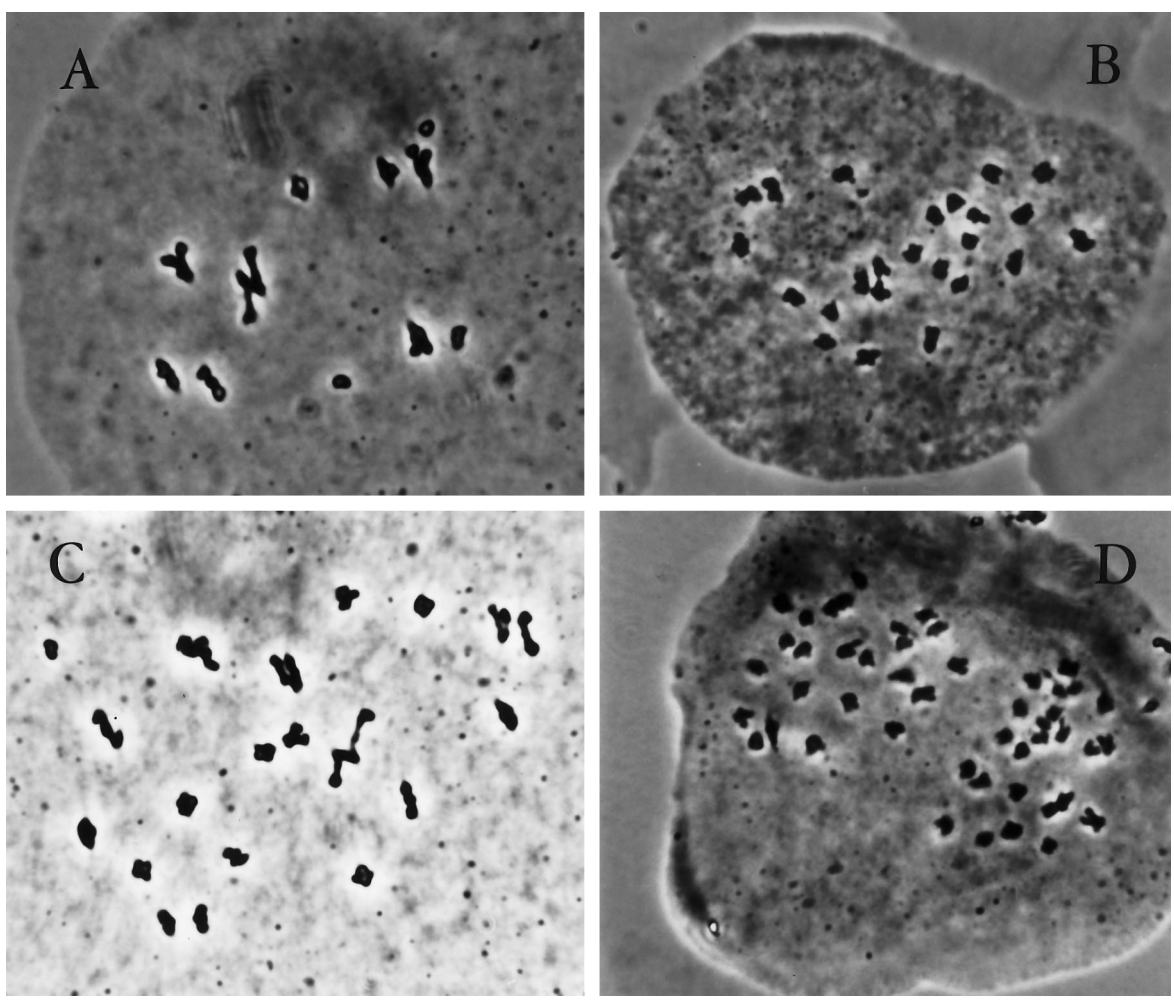
Meiotic configurations in H-6909-5 and BC_1F_1 progeny

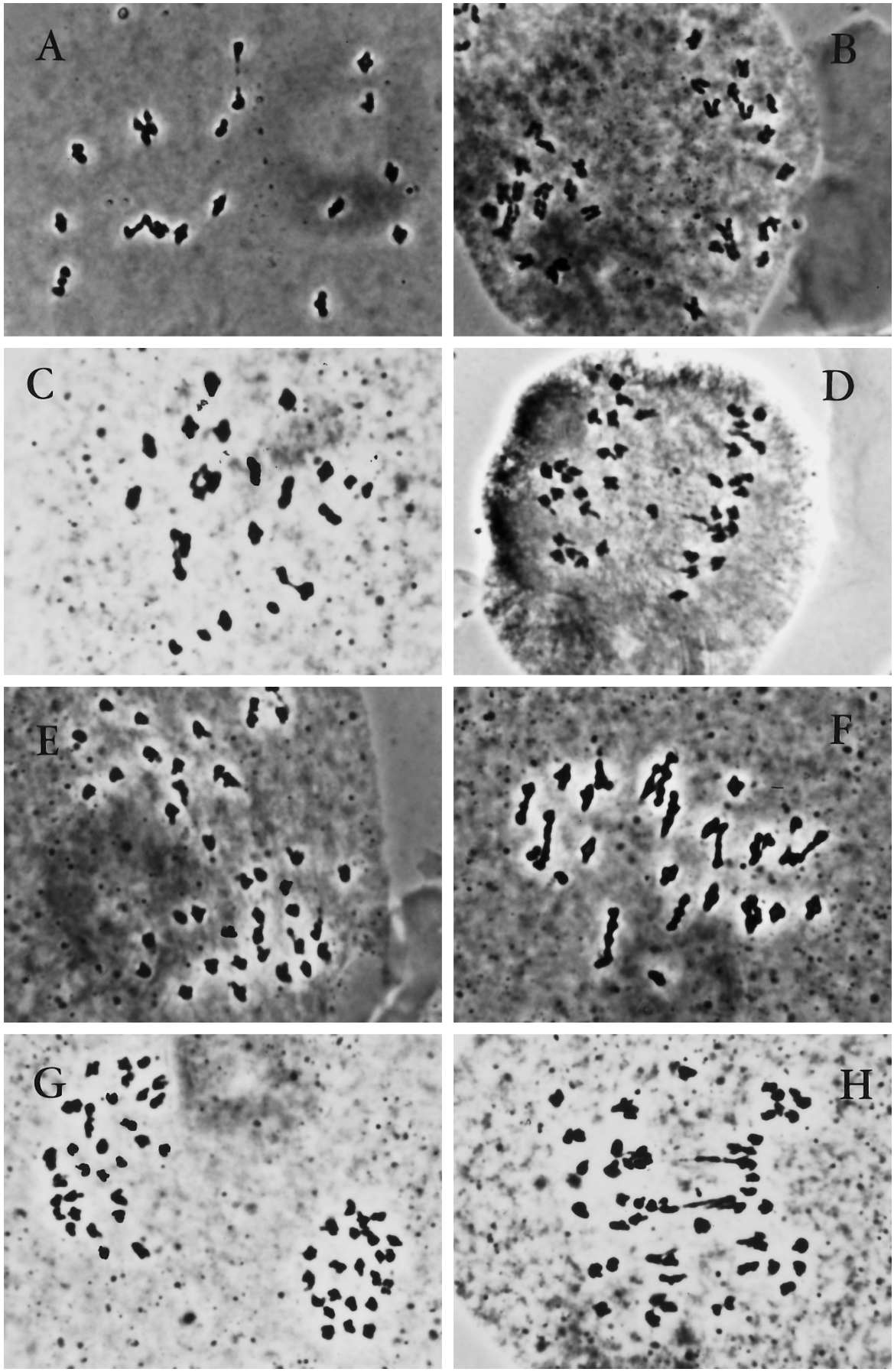
The means and ranges of meiotic chromosome associations at metaphase-I in pollen mother cells (PMCs) of *T. repens* (CC-1), *T. nigrescens* (Tn-167), 3x and 6x H-6909-5 F_1 hybrids, and the 4x, 5x and 7x BC_1F_1s are presented in Table 3.

Meiosis was highly regular in *T. repens* (CC-1) and *T. nigrescens* (Az 2225–167) but the hybrids and backcrosses all showed irregular chromosome pairing (Table 3, Figs. 3, 4). In 3x H-6909-5 all four PMCs observed at anaphase-I showed 12–12 chromosome disjunction (Fig. 3 B) and 24–24 chromosome disjunction was observed in at least four PMCs at anaphase-I for each 6x H-6909-5 plant (Fig. 3 D).

In the 4x BC_1F_1, 22 cells showed 16–16 disjunction (Fig. 4 B) and 16 quadrats at telophase-II had 16 chromosomes, indicating that the gross meiotic abnormality (univalents and multivalents) had no effect on the meiotic products. In the 5x BC_1F_1, 16 PMCs were found to have 20–20 disjunction of the chromosomes at anaphase-I (Fig. 4 D) while three showed an approximate 24–16 disjunction (Fig. 4 E). These results, combined with the results of crossing 5x BC_1F_1 with *T. repens* and 4x BC_1F_1, suggested that 5x BC_1F_1 produced three different types of gametes, i.e. 2x, 3x and aneuploid with $n = 20$. In some of the PMCs 1–2 lagging chromosomes were also observed at anaphase-I (Fig. 4 D, E). At anaphase-I in the 7x BC_1F_1, one PMC was found with an approximate 24–32 disjunction (Fig. 4 G) while four PMCs were found with a 28–28 disjunction. Two PMCs were observed with 2–4 lagging chromosomes (Fig. 4 H).

Fig. 3 Meiotic configurations in (**A**) 3x H-6909-5 (*T. repens* × *T. nigrescens*) F_1 hybrid with 3 I + 6 II + 3 III at metaphase-I, (**B**) 3x H-6909-5 showing 12–12 chromosome disjunction at anaphase-I. (**C**) 6x H-6909-5 with 2 I + 14 II + 2 III + 3 IV at metaphase-I and (**D**) 6x H-6909-5 showing 24–24 chromosome disjunction at anaphase-I. × 1600




A
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Discussion

Backcrosses of H-6909-5 to *T. repens* and *T. nigrescens*

The male and female sterility of the triploid F_1 hybrid (3x H-6909-5) was consistent with the results of Trimble and Hovin (1960) but contrary to those reported by Hovin (1962) and Marshall et al. (1995). Differences in cross compatibility due to the genotypes of individual plants or strains that are crossed were reported by Evans (1962 b), Hovin (1962) and Marshall et al. (1995). It is therefore possible that the failure to generate backcross progeny between 3x H-6909-5 and *T. repens* in the present study compared the success from a similar backcross reported by other authors might be due to the different genotypes involved in the crosses.

Brewbaker and Keim (1953) showed that a hexaploid F_1 hybrid from 4x *T. nigrescens* × 8x *T. repens* was cross-sterile as male to the undoubled parental species. (crosses as female were not reported). In the present work, the CT-14 plant of 6x H-6909-5 was both male- and female-fertile at low frequency in crosses with *T. repens*. No seed was produced by crossing 6x H-6909-5 as male with 2n *T. nigrescens*, while only one seed was harvested from 6x H-6909-5 (CT-14) as the female parent pollinated with *T. nigrescens* (Tn-167). Differences in the results of the present experiment and those of Brewbaker and Keim (1953) may again be related to the use of different genotypes in these backcrosses, or to the large number of pollinations made in the present study.

The occurrence of heptaploids from a 4x-6x cross can only be explained by the union of n (= 3x = 24) pollen from 6x H-6909-5 (CT-14) with 2n (= 4x = 32) eggs from white clover (CC-1). This was the first evidence of functional 2n gametes in *T. repens*, as discussed in another paper (Hussain and Williams 1997 b).

From the observations recorded for F_1 (both 3x and 6x) and BC_1F_1 progeny it was evident that morphological features of the parental species in F_1s and BC_1F_1s were expressed according to the parental genomic ratios. The 3x and 6x F_1 hybrids having *T. repens* and *T. nigrescens* genomes in the ratio of 2 : 1 had an intermediate expression of parental morphology. These observations contrast with those of Brewbaker and Keim (1953) where the hexaploid hybrids, obtained after crossing 8x *T. repens* and 4x *T. nigrescens*, showed greater similarity to the *T. nigrescens* parent. In the present study, the tetraploid BC_1F_1 (CT-14 × Tn-167), with a parental genomic ratio of 1 : 1, had more similarity to *T. nigrescens* although the hybrid was more easily propagated from stem cuttings. In contrast to the tetraploid BC_1F_1, the pentaploid and heptaploid BC_1F_1s with parental genomic ratios of 4 or 6 *repens* : 1 *nigrescens* exhibited the true stoloniferous perennial growth habit of *T. repens*. A CBC_2 plant obtained after crossing the 4x BC_1F_1 (CT-14 × Tn-167) with *T. repens* was more like *T. repens*. This plant carries three genomes of *T. repens* and one of *T. nigrescens*, and shows that a genomic ratio of 3 : 1 is adequate to recover the perennial habit. These observations indicate that the recovery of strong perennial stoloniferous backcrosses with frequent nodal rooting depends on a high ratio of *T. repens* to *T. nigrescens* genomes.

Meiotic configurations in 3x and 6x H-6909-5 and first-backcross progeny

The regular bivalent pairing in *T. repens* with a somatic chromosome number of 2n = 4x = 32 observed by Atwood and Hill (1940) and the disomic inheritance of genetic markers (Davies 1970) indicated a diploid behaviour of the species. However, the two homoeologous genomes with a basic set of x = 8 chromosomes were subsequently found to have the potential to pair with each other after interspecific hybridisation with *T. nigrescens* (Chen and Gibson 1970 a, b).

The cytological observations recorded for the 3x and 6x F_1 hybrid (H-6909-5) and BC_1F_1 progeny provided further supporting evidence for (1) pairing between homoeologous chromosomes of *T. repens* (autosyndesis) following interspecific hybridisation and (2) pairing between the chromosomes of *T. repens* and *T. nigrescens* (allosyndesis).

Results obtained for meiotic configurations in triploid H-6909-5 in the present investigation contrast with those reported by Hovin (1962) but are consistent with the results of Chen and Gibson (1970 b). Hovin (1962) reported predominantly bivalent formation with an average of 9.6 and a range of 8–11 bivalents in 14 PMCs of a triploid *T. repens* × *T. nigrescens* hybrid. The presence of more than eight bivalents in a triploid F_1 hybrid between *T. repens* and *T. nigrescens* would suggest that, apart from autosyndetic or allosyndetic pairing, association of non-homologous chromosomes within the genomes of both species had occurred. However, as is evident from the present investigation (Table 3) and the data of Chen and Gibson (1970 b),

Fig. 4A–H Meiotic configurations in first-backcross (BC_1F_1) progeny from crossing 6x H-6909-5 F_1 with both parental species. (**A**) 4x BC_1F_1 (6x H-6909-5 × *T. nigrescens*) with 2 I + 11 II + 2 IV at metaphase-I, (**B**) 4x BC_1F_1 showing 16–16 chromosome disjunction at anaphase-I, (**C**) 5x BC_1F_1 (6x H-6909-5 × *T. repens*) with 5 I + 12 II + 1 III + 2 IV at metaphase-I, (**D**) 5x BC_1F_1 with approximately 20–20 chromosome disjunction at anaphase-I, (**E**) 5x BC_1F_1 with approximately 16–24 chromosome disjunction at anaphase-I, (**F**) 7x BC_1F_1 (*T. repens* × 6x H-6909-5) with 3 I + 11 II + 1 III + 7 IV at metaphase-I, (**G**) 7x BC_1F_1 with approximately 24–32 chromosome disjunction at anaphase-I and (**H**) 7x BC_1F_1 with approximately 28–28 chromosome disjunction at anaphase-I and lagging chromosomes. × 1600

very strict bivalent pairing has been observed for the parental species. In the present investigation up to eight (with an average of 6.36) bivalents were recorded for the triploid H-6909-5 indicating both auto- and allo-syndetic pairing of the parental genomes, but no non-homologous pairing.

The three chromosome-doubled hexaploid plants of H-6909-5 (CT-1, CT-14 and CT-28) showed very similar meiotic configurations. Comparatively greater numbers of bivalents (more than 18 on average) in the three hexaploid plants of H-6909-5 suggested that homologous chromosomes of each species even in hybrids had more pairing affinity and so auto- or allo-syndetic pairings were reduced. This has been shown by the less than one univalent and trivalent in 6x H-6909-5, although quadrivalents in 6x H-6909-5 were as frequent as trivalents in 3x H-6909-5 and demonstrate the occurrence of some auto- and allo-syndesis.

The meiotic configurations in the tetraploid BC_1F_1 (CBC_1) also suggested both auto- and allo-syndetic pairing. This BC_1F_1 presumably carries two homoeologous genomes of *T. repens* and two homologous genomes of *T. nigrescens*. Assuming again that non-homologous chromosomes within or between the parental genomes do not pair, the formation of up to five quadrivalents and two trivalents in the PMCs of this 4x BC_1F_1 is a strong indication of allo-syndetic pairing between *T. repens* and *T. nigrescens* chromosomes.

The occurrence of an average of 3.66 univalents in the pentaploid BC_1F_1 with four genomes of *T. repens* and one genome of *T. nigrescens* is consistent with the results obtained for the 3x H-6909-5. The increase in the number of univalents and trivalents in the pentaploid BC_1F_1 suggests that presumed homologous chromosome pairing between *T. nigrescens* genomes in 6x H-6909-5 might have been replaced by allo-syndetic pairing in the pentaploid BC_1F_1.

Although studied in only 15 PMCs, the meiotic configurations of one of the 7x BC_1F_1 plants also showed both auto- and allo-syndesis. This backcross carried only one genome of *T. nigrescens* with six genomes of *T. repens*. The occurrence of up to 11 (with an average of 6.00) univalents and nine (with an average of 5.08) quadrivalents demonstrates the probable occurrence of allo-syndetic pairing. The higher pollen stainability of 5x and 7x BC_1F_1s than the 4x BC_1F_1 suggested that gross meiotic abnormalities and odd ploidy levels did not greatly reduce the fertility of the plants.

Potential uses of BC_1F_1 progeny

The three different categories of BC_1F_1, i.e. 4x, 5x and 7x, have not yet been grown in replicated trials for an evaluation of agronomic characters or clover cyst nematode resistance. Instead these BC_1F_1s have so far provided useful genetic material at three different ploidy levels for further backcrosses.

From the meiotic data at anaphase-I of the 5x and 7x BC_1F_1, 20–20 and 28–28 chromosome disjunctions respectively might yield aneuploid gametes, which in crosses with parental species would presumably produce aneuploid progenies. One BC_2F_1 plant (*T. repens* × 7x BC_1F_1) and another plant from a 4x BC_1F_1 × 5x BC_1F_1 intercross were evaluated cytologically and were aneuploids with $2n = 42$ and 36 respectively. The aneuploid gametes in these crosses are most likely to have been contributed by the 5x and 7x BC_1F_1s, as 4x BC_1F_1 and *T. repens* formed euploid gametes. Aneuploid production is also expected from other $BC_1F_1 \times BC_1F_1$ intercrosses and $BC_1F_1 \times$ 6x H-6909-5 crosses (Table 2). Ten seeds of different $BC_1F_1 \times BC_1F_1$ and $BC_1F_1 \times$ 6x H-6909-5 crosses have been germinated and grown successfully. The aneuploids with different chromosome numbers will provide useful material for in situ DNA hybridisation to identify chromosomal exchange between the parental genomes and, potentially, the association of specific characters with certain chromosomes.

The second congruity backcross (CBC_2) obtained after crossing the 4x BC_1F_1 plant with *T. repens* produced six seeds. The one plant so far grown from these seeds is a tetraploid, as both of its parents produced euploid gametes with $n = 2x = 16$. This CBC_2 plant theoretically carries three genomes of *T. repens* and one genome of *T. nigrescens* in contrast to the tetraploid BC_1F_1 (CBC_1) with two genomes from each species. A backcross progeny with a 3:1 combination of parental genomes of these two species has not been reported before. The meiotic behaviour of these CBC_2 plants will provide additional information on the homology of chromosomes between these two species. All the BC_1F_1 progeny and their intercrossed progeny will be evaluated for clover cyst nematode resistance at later stages.

References

Atwood SS, Hill HD (1940) The regularity of meiosis in microsporocytes of *Trifolium repens*. Am J Bot 27:730–735

Brewbaker JL, Keim WF (1953) A fertile interspecific hybrid in *Trifolium* (4n *T. repens* L. × 4n *T. nigrescens* Viv.). Am Nat 87:323–326

Chen CC, Gibson PB (1970 a) Meiosis in two species of *Trifolium* and their hybrids. Crop Sci 10:188–189

Chen CC, Gibson PB (1970 b) Chromosome pairing in two interspecific hybrids of *Trifolium*. Can J Genet Cytol 12:790–794

Davies WE (1970) White clover breeding. British Grasslands Society Occasional Symposium 6:99–102

Evans AM (1962 a) Species hybridisation in *Trifolium*. I. Methods of overcoming species incompatibility. Euphytica 11:164–176

Evans AM (1962 b) Species hybridisation in *Trifolium*. II. Investigating the pre-fertilisation barriers to compatibility. Euphytica 11:256–262

Gibson PB, Chen CC, Gillingham JT, Barnett OW (1971) Interspecific hybridization of *Trifolium uniflorum* L. Crop Sci 11:895–899

Haghighi KR, Ascher PD (1988) Fertile, intermediate hybrids between *Phaseolus vulgaris* and *P. acutifolius* from congruity backcrossing. Sex Plant Rep 1 : 51–58

Hovin AW (1962) Interspecific hybridization between *Trifolium repens* L. and *T. nigrescens* Viv. and analysis of hybrid meiosis. Crop Sci 2 : 251–254

Hussain SW (1995) Effect of ploidy on interspecific hybridisation between *Trifolium repens* L. and related species. PhD thesis, Massey University, Palmerston North, New Zealand

Hussain SW, Williams WM (1997 a) Development of a fertile genetic bridge between *Trifolium ambiguum* M.Bieb. and *T. repens* L. Theor Appl Genet (in press)

Hussain SW, Williams WM (1997 b) Evidence of functional unreduced gametes in *Trifolium repens* L. Euphytica (in press)

Kazimierski T, Kazimierska EM (1970) Investigations of hybrids of the genus *Trifolium* L. III. Morphological traits and cytogenetics of the hybrid *T. repens* L. × *T. nigrescens* Viv. (in Polish). Acta Soc Bot Poloniae 39 : 565–591

Keim W F (1953 a) An embryo culture technique for forage legumes. Agron J 45 : 509–510

Keim WF (1953 b) Interspecific hybridization in *Trifolium* utilizing embryo-culture techniques. Agron J 45 : 601–606

Marshall AH, Michaelson-Yeates TPT, Aluka P, Meredith M (1995) Reproductive characters of interspecific hybrids between *T. repens* L. and *T. nigrescens* Viv. Heredity 74 : 136–145

Mercer CF (1988) Reaction of some species of *Trifolium* to *Meloidogyne hapla* and *Heterodera trifolii*. Proc 5th Aust Conf Grasslands Invertebrate Ecol, Melbourne, Australia, pp 275–280

Pederson GA, Windham GL (1989) Resistance to *Meloidogyne incognita* in *Trifolium* interspecific hybrids and species related to white clover. Plant Dis 73 : 567–569

Snow R (1963) Alcoholic hydrochloric acid-carmine as a stain for chromosomes in squash preparations. Stain Technol 38 : 9–13

Trimble JP, Hovin AW (1960) Interspecific hybridisation of certain *Trifolium* species. Agron J 52 : 485

Williams EG (1978) A hybrid between *Trifolium repens* and *T. ambiguum* obtained with the aid of embryo culture. NZ J Bot 16 : 499–506

Williams EG, Williams WM, White DWR, Pandey KK (1978) Interspecific hybridisation in *Trifolium*. Progress Report. Clover and Special Purpose Legumes Research 11 : 52–57

Williams W (1954) An emasculation technique for certain species of *Trifolium*. Agron J 46 : 182–184

Williams WM (1987) Genetics and breeding. In: Baker MJ, Williams WM (eds) White clover. C.A.B. International Wallingford, Oxon, pp 343–419