INVESTIGATION OF THE POSSIBILITY OF INTROGRESSION FROM *Trifolium ambiguum* M. Bieb. INTO *T. repens L.*

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ABSTRACT

The objective of this project was to investigate the possibility of introgression of stress resistance traits from *T. ambiguum* (A) into *T. repens* (R) by interspecific hybridisation, using two approaches. The first used *T. occidentale* Coombe (O) as a genetic bridge because this species has chromosome pairing homology to both the two other species. The second approach attempted direct integration of genomes from the two species through ploidy manipulation.

For the first approach, four crossing strategies used *T. occidentale* as a genetic bridge. Each started with different multispecies hybrids with various genomic contributions from the three parental species. The second approach began with 5x and 7x *T. ambiguum* x *T. repens* hybrids (ARRRR and AAARRRR). All the initial hybrids were repeatedly selfed, inter-crossed and backcrossed with colour-marked white clover and advanced progenies with reasonable levels of fertility were obtained in every strategy. Advanced hybrids were selected on the basis of flow cytometric ploidy estimation, phenotypes and somatic chromosome counts and were characterised for chromosome pairing and introgression events using both conventional and molecular cytogenetics. The advanced hybrid progenies were also grown in a sandpit to determine the relative expressions of the parental traits.

Chromosome analyses showed evidence of chromosome elimination, chromosome addition/substitution, allosyndetic pairing involving A-derived chromosomes and inter-specific genomic recombination. GISH analysis revealed that the genetic bridge strategy 1, which started with RRAO, gave a plant with four apparently large A-R and A-O chromosomal exchanges along with an A chromosome addition and a O-R substitution. No apparent signs of introgression were detected by GISH in the other strategies but introgression could not be ruled out because the sample was small and the morphology of the hybrids tested in the sandpit showed the expression of characters from both the parental species. These plants need to be characterised by using more genetic markers. The advanced progeny in the strategy based on direct integration of AxR genomes showed a low level of inter-specific chromosome pairing consistent with an absence of, or very low level of, introgression. The introgression revealed by GISH in the advanced progeny of RRAO hybrids provides evidence that using *T. occidentale* as genetic bridge

has worked by disrupting the genomic integrity in *T. repens*. The material having introgression has many applications from white clover improvement point of view. While direct hybrids with A & R genomes did not lead to any apparent introgression.

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ABBREVIATIONS AND TERMINOLOGY

The following abbreviations and terminology were used:

µl	microlitre
33	designation for 4x hybrid between *6x T. ambiguum* and 2x *T. occidentale* (AAAO).
434-1	designation for 3x hybrid between 4x *T. ambiguum* and 2x *T. occidentale* (AAO).
AA	acetic acid
AFLP	amplified fragment length polymorphism
AMV	alfalfa mosaic virus
BAR	Bridging *Ambiguum Repens.*
BAR09	the first progeny of original BAR hybrids bred and grown in 2009.
BAR10	the second progeny of original BAR hybrids bred and grown in 2010.
BC	backcross
BL	a 4x hybrid between between 4x *T. ambiguum* and 4x *T. occidentale* (AAOO).
BN	a 4x hybrid between between 4x *T. ambiguum* and 4x *T. occidentale* (AAOO).
CBC	congruity backcross
cm	centimetre
CYVV	clover yellow vein virus
DAPI	4',6-diamidino-2-phenylindole.
DNA	deoxyribonucleic acid
EBN	endosperm balance number
F_1	first filial generation
F_2	second filial generation
FC	flow cytometry

FISH	fluorescence *in situ* hybridization
GISH	genomic *in situ* hybridization
H-435	hybrid 435 with genomic composition, A^TA^TRR (4x)
Hybrid 70	designation for 4x hybrid, A^DA^TRR with two *T. ambiguum* genomes coming from diploid and tetraploid sources.
IIIs	trivalents
IIs	bivalents
Is	univalent chromosomal associations during anaphase-I
ITS	internal transcribed spacer
IVs	quadrivalents
MPV	mid parental value
mRNA	messenger RNA
MSAP	methylation sensitive amplification polymorphism
N	nitrogen
ng	Nanograms
NOR	nucleolar organizer region
OP	open-pollinated
Ph1	pairing homoeologous 1
PI	Propidium iodide
PMCs	pollen mother cells
PSV	peanut stunt virus
rDNA	ribosomal DNA
RET	*T. repens* with tetraploid genomic composition.
RNA	ribonucleic acid
RO	a white clover genotype named as Red One
ROS	designation for a 4x hybrid, AARR.
SC	self-compatible

SDR	second division restitution
SI	self-incompatible
SSC	saline sodium citrate
v/v	volume/volume
Vs	pentavalents
w/v	weight/volume
WCMV	white clover mosaic virus

Terminology for identifying different genomes/sub-genomes in different species

A	one sub-genome (x=8) from *T. ambiguum* (origin unspecified)
A^D	one sub-genome (x=8) from 2x *T. ambiguum*
A^T	one sub-genome (x=8) from 4x *T. ambiguum*
A^H	one sub-genome (x=8) from 6x *T. ambiguum*
O	one sub-genome (x=8) from 2x *T. occidentale*
R	one sub-genome (x=8) from white clover
R^P	*T. pallescens*-derived subgenome of *T. repens*
R^O	*T. occidentale*-derived subgenome of *T. repens*

Partial and mixed sub-genomes are designated as follows:

(A)	a partial sub-genome (x=unspecified number, 1-7) from *T. ambiguum*
(A_4)	a partial sub-genome (x=4) from *T. ambiguum*
(R/A)	a mixed sub-genome (x=~8) containing both white clover and *T. ambiguum* chromosomes.
(A/O)	a mixed sub-genome (x=~8) containing both *T. occidentale* and *T. ambiguum* chromosomes
(R_4/A_4)	a mixed sub-genome (x=8) containing four white clover and four *T. ambiguum* chromosomes.
(AO)	partial sub-genomes (x = 1-7) from *T. ambiguum* and *T. occidentale*.
(RAO)	partial sub-genomes (x = 1-7) from all the three species, white clover, *T. ambiguum* and *T. occidentale.*

A^O	a recombinant chromosome having *T. ambiguum* centromere with arms introgressed from *T. occidentale*
O^A	a recombinant chromosome having *T. occidentale* centromere with arms introgressed from *T. ambiguum.*

CHAPTER 1

INTRODUCTION

White clover, *T. repens*, L., (2n = 4x = 32) (Britten, 1963), is agronomically the most important of the 250-300 species in the genus *Trifolium* (Williams, 1987a) which taxonomically belongs to the family Leguminosae (Gillett, 1985). It is a perennial species which is stoloniferous in growth habit (Thomas, 1987). Although being allopolyploid, it behaves as a regular diploid at meiosis (Atwood and Hill, 1940; Majumdar *et al*., 2004). Europe, North Africa and West Asia are considered as native areas of its distribution. The recent findings of Ellison *et al*. (2006) based on DNA sequence analysis of species in the genus support a Mediterranean origin, which is consistent with findings reported earlier (Kousnetzoff, 1926; Vavilov, 1951; Williams, 1987).

White clover is the legume of choice for grazed pastures in moist regions with temperate climates throughout the world and its good attributes i.e., the ability to spread laterally by rooted-stolons, high growth rate, good seed production, high establishment potential, reasonable grazing tolerance due to prostrate stems (stolons) trailing along the ground with roots from nodes, large quantities of atmospheric N fixation in symbiosis with the bacterium *Rhizobium leguminarosum var. trifolii* and high forage quality, contribute towards its wide scale acceptance (Williams, 1978; Williams and Verry, 1981; Meredith *et al*., 1995; Abberton *et al.,* 2002; Abberton and Marshall, 2005; Williams *et al*., 2006a; Williams *et al*., 2007). High forage quality; comparatively lesser fibre contents than grasses, higher ratio of carbohydrates and higher protein content with greater palatability in white clover (Ulyatt *et al*., 1977; Verbal communication by Dr. Warren Williams) lead to higher milk and meat production (Chapman *et al*., 1993; Davies and Hopkins, 1996). Atmospheric N fixation by white cover in symbiosis with *Rhizobium* contributes towards sward by not only using it itself but also by benefiting the companion grasses. In New Zealand, white clover is the most important legume, well suited to sheep and cattle grazing-based pastures; being well adapted to the temperate climate with enough rainfall evenly distributed throughout the year.

Despite being an important (economically and agronomically) pasture legume in many temperate parts of the world, white clover is vulnerable to a number of biotic and abiotic

stresses restricting its adaptive range and value in agriculture. It is intolerant of drought stress due to its shallow root system and inability to control water loss through transpiration (Brink and Pederson, 1998). This poor drought tolerance is one of the major problems associated with this species (Barbour *et al.*, 1995; Brink and Pederson, 1998). It also has limited potential to persist and remain in the field under intensive grazing (Knight, 1985; Van Keuren and Matches, 1988; Forde *et al*., 1989) because the stolons trailing along the surface of ground either get ripped off during grazing by animals or damaged under the hooves. Its persistence in the field is also affected by its susceptibility to many diseases and damage caused by viruses, and insects and nematodes (McLaughlin and Pederson, 1985; Alconero *et al*., 1986; Gaynor and Skipp, 1987; Latch and Skipp, 1987). Unfortunately, due to non-availability of enough genetic variation in white clover for resistance to different stresses, breeding efforts have not been very successful (Brink and Pederson, 1998; Abberton *et al*., 2002; Williams *et al*., 2007). This situation leaves us with the only option of inter-specific hybridization which can broaden the white clover gene pool available to the breeders and has been used for improving its varieties agronomically by bringing in the desirable variation from other related species. The breeding efforts using inter-specific hybridisation in *Trifolium* have been focussed on one hand to unravel the phylogenetic relationship among the species and on the other hand to improve genetically the agriculturally important species including white clover against different biotic and abiotic stresses (Abberton, 2007).

By introducing drought tolerance to white clover and improving its persistence in the sward through inter-specific hybridization, the adaptive range of white clover might be expanded to areas with low rainfall. The ultimate aim of white clover breeding is not yield maximization as in other crops but to make it more persistent in swards with a sustained contribution over a number of years by bringing about improvement in tolerance to different biotic and abiotic stresses (Abberton and Marshall, 2005).

T. ambiguum M. Bieb. (Caucasian or Kura clover) is a species of great interest for breeders because of its high level of persistence in the field, drought tolerance (Spencer *et al*., 1975; Marshall *et al*., 2001) and resistance to many viral diseases (Barnett and Gibson, 1975; Pederson and McLaughlin, 1989). It has a rhizome-based spreading system and a large tap root system and, relative to other species in the genus, *Trifolium*, it allocates a large proportion of biomass to the underground parts (Genrich *et al.,* 1998; Widdup *et al.,* 1998; Black *et al.,* 2006a). Rhizomes and thick taproots are desirable

attributes in *T. ambiguum* (Ford *et al*., 1989; Ferguson *et al*., 1990) which contribute to its resistance against drought (Spencer *et al*., 1975; Marshall *et al.*, 2001) and make it persistent under heavy grazing pressure (Bryant, 1974; Dear and Zorin, 1985; Daly and Mason, 1987; Woodman, 1993; Coolbear *et al*., 1994; Virgona and Dear, 1996; Marshall *et al*., 2001). Rhizomes can provide water to the plant during water stress (Marshall *et al*., 2001) and under very heavy grazing, the growing points on rhizomes, being under the soil surface, contribute to its persistence (Moorhead *et al.*, 1994; Allan and Keoghan, 1994) because, after severe defoliation due to intensive grazing, the clover re-sprouts from underground growing points on rhizomes (Sheaffer *et al*., 1992; Peterson *et al.,* 1994; Brummer and Moore, 2000; Abberton *et al*., 2002). It is also resistant to most viral, foliar, stem, and root diseases and nematodes that seriously affect white clover and so threaten its survival in the sward (Barnett and Gibson, 1975; Mercer, 1988; Pederson and Windham, 1989; Pederson and McLaughlin, 1989; Ferguson *et al*., 1990). There are, however, some weaknesses associated with this species; the important ones are slow establishment in the field, very low seed production (Bryant, 1974) and specific rhizobial strain requirements (Hely, 1957; Pryor *et al*., 1998).

The superior persistence, drought tolerance and resistance to many viruses, none of which exists in white clover, make *T. ambiguum* a potentially important source of desirable genes for white clover breeders. Interestingly, the strengths of Kura clover can improve all the weaknesses associated with *T. repens* and vice versa (Williams, 1987). Inter-specific hybridization is one of the ways of utilizing the good agronomic traits associated with *T. ambgiuum* for white clover improvement and which might lead to the development of white clover varieties with a nice blend of desirable attributes from both the species. But hybridization between the species in this genus is not easy due to strong post hybridization barriers and, consequently, natural hybrids are very rare in this genus and inter-specific hybridization seems to have played very little role in the evolution of the genus (Evans, 1976; Zohary and Heller, 1984; Ellison *et al*., 2006). Embryo rescue/ovule culture methods have been used to get over these issues and consequently some hybrids have been produced involving different species. The successful hybridization between white clover and *T. ambiguum* followed by the inter-specific recombination would allow the breeder to alter the genetic constitution of white clover through the introgression of desirable genes from *T. ambiguum*. Despite many desirable attributes in *T. ambiguum* for white clover improvement, it is advisable to investigate the

possibility of homoeologous chromosome pairing between the genomes of these two species and the exchange of chromosome segments (recombination) by testing different strategies before embarking on a comprehensive introgressive breeding programme.

1.1 Background of the project

Previously, several researchers reported hybrids between 4x *T. ambiguum* and white clover but they were cross and self sterile and produced no F_2 or backcross progeny (Chen and Gibson, 1972; Williams and White, 1976; Williams, 1978). The first partially fertile 4x hybrid designated as Hybrid 435 (briefly H-435) between tetraploid *T. ambiguum* (2n=4x=32, $A^TA^TA^TA^T$) and 4x *T. repens* (2n=4x=32, RRRR) was produced by Williams and Verry (1981) using embryo rescue. This hybrid was only cross fertile with *T. repens* and produced no BC progeny when crossed with *T. ambiguum*. It had the expected 2n chromosome complement of 32, 16 coming from each parent. These chromosomes associated predominantly as bivalents (IIs) (15.6 II/PMC) with very rare trivalents (III) and quadrivalents (IV) during meiotic metaphase. IIs showed presumably intra-genomic or autosyndetic chromosome pairing (pairing between the two basic genomes of the parental species) due to greater synaptic attraction of intra-specific homoeologous chromosomes as compared to that between inter-specific homoeologous chromosomes. IIIs and IVs, which occurred in H-435 in very low frequencies, and must involve both autosyndetic and allosyndetic pairing, indicated a very low level of recombination potential in this hybrid (Williams *et al*., 1982). Williams and Verry (1981) reported two backcross hybrids resulting from crosses between H-435 (A^TA^TRR) used as female with white clover (RRRR) contributing the pollen. One had the expected 32 chromosomes with probable genomic formula of ARRR. This hybrid had very disturbed meiosis, having up to 15 univalents (Is) with reasonable numbers of IIIs (up to 6) and very low frequency of IVs (up to 1) (Williams *et al.,* 1982). Presence of IIIs might show homologous pairing among the white clover chromosomes as well as homoeologous chromosome pairing. Ignoring the occurrence of illegitimate pairing, IVs must involve both autosyndetic and allosyndetic chromosome pairing. The other BC plant was near-5x with 42 chromosomes and was ambiguous as far as its origin is concerned, keeping in view the parental species 2n chromosome count in mind. This hybrid had mostly IIs and IIIs with a very low frequency of IVs and pentavalents (Vs).

Meredith *et al*. (1995) also produced a sterile 4x hybrid AARR with the expected chromosome number of 32, using ovule culture after crossing 4x *T. ambiguum* as female with *T. repens*. The majority of BC_1 plants to *T. repens* were 6x (AARRRR) due to the contribution of functional 2n gametes by the AARR hybrid used as female. In the BC_1 hybrids, chromosomes associated predominantly as IIs, indicating probable homologous or intra-genomic pairing. This increase in chromosome number due to 2n gamete contribution by the F_1 hybrid potentially frustrates the breeder's ability for introgressing desirable traits from *T. ambiguum* into *T. repens* directly because of the availability of a homologue for every chromosome in 6x BC_1 progeny. The BC_2 plants had 40 chromosomes with probable genomic formula of ARRRR and the meiotic analysis of these plants showed 16 IIs presumably formed by 32 white clover-derived chromosomes and eight Is derived from *T. ambiguum* showing, again, very little indication of inter-genomic pairing. Both the BC_1 and BC_2 had very low frequencies of multivalent chromosome association (IIIs & IVs) and these too might involve homologous or intra-genomic homoeologous chromosomes derived from white clover with few chances of allosyndesis.

Anderson *et al*. (1991) obtained 18 BC_1 plants by pollinating the partially fertile H-435 hybrid with white clover. Ten were 6x with 2n chromosome counts of 48, five were 4x with 2n of 32 and the rest were aneuploids. They observed large numbers of IIs in 4x BC_1 (presumed ARRR) which is not consistent with the findings obtained by Williams *et al*. (1982) in similarly derived 4x BC_1 plants (ARRR. The high frequency of IIs in the BC_1 hybrid (ARRR) reported by Anderson *et al*. (1991) suggested eight homologous IIs made by the gametic set of 16 white clover chromosomes and eight allosyndetic IIs involving the remaining eight white clover and eight *T. ambiguum* chromosomes. This evidence of inter-specific chromosome pairing offers some potential of genomic recombination, despite *T. ambiguum* being distantly related to *T. repens* (Anderson *et al*., 1991; Meredith *et al*., 1995).

Although *T. ambiguum* and *T. occidentale* have totally different phenotypes, ecological adaptations and geographical distributions but they have been crossed successfully (Williams *et al.,* 2011), producing partially fertile progeny. The successful hybridization, although difficult, is evidence of their recent evolution from a common parental source species. This close phylogenetic relation between them is also supported by DNA sequence similarities (Ellison *et al*., 2006) and the partial sharing of a centromeric repeat

DNA sequence (Ansari *et al*., 2004). Williams *et al.* (2011) reported a high frequency of chromosome pairing between *T. ambiguum* and *T. occidentale* chromosomes in 2x hybrids, presumably involving high levels of recombination. This high level of chromosome pairing was also consistent with a close phylogenetic relationship between these two species. This close genetic relationship might lead to recombination between the two species at a high level. On the other hand, Chen and Gibson (1970) reported a high frequency of homoeologous chromosome pairing in 3x hybrids between *T. repens* and *T.occidentale*. Together, these two phenomena show that *T. occidentale* is genetically similar to both *T. ambiguum* and *T. repens*. I therefore hypothesise that by combining *T. ambiguum* (A) and *T. occidentale* (O) genomes that recombinant chromosomes having *T. occidentale* centromeres with *T. ambiguum* introgression on the arms O^A or the other way round A^O would be obtained. Then crossing of these hybrids, having recombinant chromosomes, with *T. repens* might lead to introgression of *T. ambiguum* genomes into *T. repens* genomes as a result of *T. occidentale* chromosomes with *T. ambiguum* introgression might pair with *T. repens* chromosomes. So keeping in mind this close genetic relation of *T. occidentale* to both the species, the concept of using *T. occidentale* as a genetic bridge was developed. The hypothesis was that if we put all three species genomes together *T.occidentale* would work as a genetic bridge to transfer *T. ambiguum* genomes onto *T. repens*.

Based on the above research findings the following two approaches were developed for assessing introgression possibilities between the genomes of *T. ambiguum* and *T. repens*:

1.2 Approach 1

Use of *T. occidentale* as a genetic bridge between *T. repens* and *T. ambiguum.*

A range of BAR (**Bridging *Ambiguum Repens***) hybrids were developed by the Forage Improvement Programme, Grasslands Research Centre, AgResearch, involving *T. ambiguum* and *T. occidentale*. These included 2x *T. ambiguum* (A^DA^D) x 2x *T. occidentale* (OO), 4x *T. ambiguum* ($A^TA^TA^TA^T$) x colchicine doubled 4x *T. occidentale* (OOOO), 4x *T. ambiguum* ($A^TA^TA^TA^T$) x 2x *T. occidentale* (OO), and 6x *T. ambiguum* ($A^HA^HA^HA^HA^HA^H$) x 2x *T. occidentale* (OO). Strong evidence of genomic mixing between *T. ambiguum* and *T. occidentale* deduced from cytological studies of AxO hybrids (Williams *et al*., 2011) indicated that it should be possible to transfer *T. ambiguum* chromosome segments (chromatin) to *T. occidentale* centromeres leading to

recombinant chromosomes, O^A or vice versa. Furthermore, a high level of chromosome pairing and recombination between *T. occidentale* (2x) and *T. repens* (4x) genomes had also been demonstrated (Chen & Gibson 1970). Four strategies were developed based on the concept using *T. occidentale* as a genetic bridge. Of the four strategies, three involved mixing A and O genomes first and then combining with R genomes. The idea behind these strategies was that, if A and O genomes are combined first there might be more chances of getting recombinant chromosomes i.e. presumably having *T. occidentale* centromere with arms containing *T. ambiguum* segments i.e. O^A. Crossing these A x O hybrids with RRRR might then lead to pairing of these recombinant chromosomes with white clover chromosomes and so in this way transferring *T. ambiguum* genomes into white clover. The fourth strategy involved crossing A x R hybrids with *T. occidentale* to investigate the consequences when O genomes are added after A x R crossing.

1.2.1 Strategy 1

BAR parents

4x $A^TA^TA^TA^T$ (Turkish source) was crossed with 4x *T. occidentale* (OOOO) (OCT) to give 4x A^TA^TOO.

This was used to pollinate white clover (RRRR x A^TA^TOO = 4x RRA^TO)

A further backcross to white clover was then done (RRA^TO x RRRR = 4x $RRR(A_4O_4)$

The seed of the cross, RRA^TO (4x) x RRRR with the expected genomic formula of $RRR(A_4O_4)$ (4x) was provided by the Forage Improvement Program and was used as the starting material for this strategy.

In the RRA^TO hybrids**,** the A and O genomes have been isolated and during meiotic cell division they might pair and exchange chromosome segments and so gametes with recombinant chromosomes could be formed. There are several possible scenarios for chromosome pairing in RRAO hybrids. First, the synaptic attraction between R and R **(**two sub-genomes of white clover coming from different species) may be more as compared to those between R and A, R and O or A and O. In this case R will expectedly pair with R and A with O. This will produce gametes with recombinant chromosomes with an O centromere and arms with introgressed A segments (O^A) or vice versa (A^O). In

the following generation with genomic formula of RRR(A_4O_4), this recombinant chromosome might pair with a R chromosome and A derived genes could be transferred. Alternatively, in hybrid RRAO**,** the O genome might pair with one of the white clover genomes of *T. occidentale* origin making IIs or IIIs, and paving the way for RxO mixing. These RxO recombinant chromosomes in the following generations might pair with A chromosomes. To test the feasibility of this strategy, the BAR hybrids with genomic composition of RRR(A_4O_4) will be repeatedly selfed as well as backcrossed with white clover in order to give the isolated A genomes maximum opportunities to recombine with O chromosomes and, at the same time, regain the *T. repens* chromosomal composition presumably with *T. ambiguum* introgression. The advanced self and backcross progeny will be screened for genomic exchange using both conventional and molecular tools.

1.2.2 Strategy 2

BAR parents

4x *T. ambiguum* ($A^TA^TA^TA^T$) was crossed with 2x *T. occidentale* (OO) to produce a 3x hybrid A^TA^TO.

Similarly, 4x *T. ambiguum* ($A^TA^TA^TA^T$) was crossed with 4x T. *occidentale* (OOOO) to produce 4x A^TA^TOO.

These hybrids were intercrossed (A^TA^TO x A^TA^TOO) to produce 5x $A^TA^TA^TOO$.

Thirteen 5x A x O hybrids with genomic formula of AAAOO were provided by the Forage Improvement Programme. They might have had recombinant chromosomes because they had already gone through one meiotic cycle with A or O genomes in odd number in one of the parental hybrids, leading to homoeologous pairing. To test this hypothesis, and to achieve introgression, these plants will be repeatedly backcrossed with white clover in addition to selfing in between. The advanced selfed and backcross progeny will be studied for phenotypic and chromosomal evidence of introgression using both conventional and molecular cytogenetic approaches.

1.2.3 Strategy 3

BAR parents

6x *T. ambiguum* = ($A^HA^HA^HA^HA^HA^H$) was crossed with 2x *T. occidentale* = (OO)

($A^HA^HA^HA^HA^HA^H$ x OO) to produce a 4x hybrid AAAO (Hybrid 33)

Hybrid 33 was pollinated by *T. repens* (during open-pollination) (AAAO x RRRR) to give a near-6x hybrid AAAORR (33 OP-1)

33 OP-1 was backcrossed to *T. repens*, as female:

(33(OP)-1 x RRRR = ~6x AAAORR x RRRR = ~5x RRRA (A_4O_4)

The backcross hybrid was then backcrossed a second time to *T. repens*, this time as male:

RRRR x RRRA (A_4O_4) = ~4.5x RRR ($R_4A_6O_2$).

Five BAR hybrids with genomic compositions of RRRA (A_4O_4) (~5x) and nine RRR ($R_4A_6O_2$) (~4.5x), were provided by the forage improvement programme. This strategy is of special interest because these BAR hybrids combine A genomes from 6x *T. ambiguum* with white clover. Hexaploid *T. ambiguum* is agronomically superior to other ploidies. The aim was to achieve introgression and to bring the ploidy back to 4x by taking these hybrids through several meiotic cycles of repeated selfing, inter-crossing and backcrossing to white clover. The advanced progeny would then be tested for introgression events using both conventional and non-conventional approaches.

1.2.4 Strategy 4

A 4x hybrid between *T. ambiguum* and *T. repens* (AARR) was crossed with both 2x and 4x *T. occidentale* = (OO) and (OOOO). The resulting hybrids were 3x and 6x, respectively:

AARR x OO = ARO (3x)

AARR x OOOO = AARROO (6x)

Five BAR hybrids with genomic composition of ARO (3x) and seven AARROO (6x) were used to start this strategy. In this approach, instead of mixing A and O genomes first, followed by mixing with R genomes, A and R genomes were put together first and then these A x R hybrids were crossed with 2x and colchicine doubled 4x *T. occidentale*. The aim was to enhance the chances of homoeologous chromosome pairing by generating plants with odd numbers of parental genomes by repeatedly backcrossing the 3x and 6x multiple species hybrids with white clover. The crossing scheme is as given below. The advanced progeny will be evaluated for the presence and expression of *T.*

ambiguum genes/chromosomes using morphological phenotyping, conventional cytology and GISH/FISH.

ARO (3x) x RRRR = RRRAO (5x) x RRRR = RRR ($R_4A_4O_4$) x RRRR = RRR ($R_6A_2O_2$)
AARROO (6x) x RRRR= RRRAO (5x) x RRRR= RRR($R_4A_4O_4$) x RRRR= RRR($R_6A_2O_2$)

1.3 Approach 2

Direct integration of *T. ambiguum* and *T. repens* genomes through ploidy manipulation

This concept tests the hypothesis that it is possible to achieve direct genomic introgression of *T. ambiguum* into *T. repens* genomes or, alternatively, the creation of addition/substitution lines by generation of hybrid plants with odd numbers of A genomes.

1.3.1 Strategy 5.1

Before the start of this project, 12 5x BAR hybrids with genomic composition of ARRRR were available from the Forage Improvement Programme, Grasslands. They were made by backcrossing 6x A^DA^TRRRR hybrids to RRRR and had reasonably high pollen fertility. The logic behind using these hybrids was that if we isolate an A genome then there might be pairing with R chromosomes during the meiotic phase leading to introgression because there would be no A homologues to pair with. The first aim was to subject ARRRR plants to several meiotic generations by selfing, inter-crossing and backcrossing them to *T. repens* in order to give both the genomes maximum chances of chromosomal exchange. At the same time, the aim of back-crossing was to bring the ploidy level down to 4x with all the chromosomes coming from white clover but augmented by introgression of *T. ambiguum* chromosomal segments. Or by doing so we might get addition/substitution lines by selfing and backcrossing these 5x ARRRR plants to white clover. This strategy is important from another angle as well because the original BAR hybrids used here contained an A genome from 2x *T. ambiguum* (A^D) and this is the first time that A^D has been combined with white clover genomes. How the BAR parents were developed is given below:

BAR parents

4x *T. repens* = RRRR

2x *T. ambiguum* = A^DA^D

4x *T. ambiguum* = $A^TA^TA^TA^T$

$A^TA^TA^TA^T$ x RRRR

↓

(Hybrid 435) A^TA^TRR

(Colchicine doubling) ↓

(8x H-435) $A^TA^TA^TA^TRRRR$ x RRRR

↓

A^TA^TRRRR (6x) x $A^DA^D \rightarrow A^DA^TRR$ (4x Hybrid 70)

A^DA^TRR x RRRR = A^DA^TRRRR (6x)

RRRR x A^DA^TRRRR (6x) = ARRRR (5x)

In this strategy, these pentaploid (ARRRR**)** hybrids were selfed, inter-crossed and repeatedly crossed back with white clover (RRRR). The advanced progeny of these hybrids was subjected to conventional and non-conventional screening to see if there has been some sort of genomic mixing. Here only the crossing scheme is given below:

ARRRR (5x) x RRRR = RRRR(A_4) (4.5x)

RRRR (A_4) (4.5x) x RRRR = RRRR(A_2) (4.25x)

1.3.2 Strategy 5.2

BAR parents

4x *T. repens* = RRRR

4x *T. ambiguum* = $A^TA^TA^TA^T$

$A^TA^TA^TA^T$ (4x) x RRRR = A^TA^TRR (4x)

A^TA^TRR (4x) x A^DA^TRRRR (6x) = AAARRRR (7x)

Seven BAR hybrids with the probable genomic composition of AAARRRR (7x) were available and provided by the programme. These were made by crossing a 4x hybrid (A^TA^TRR) with A^DA^TRRRR (6x). The aim was to follow a scheme of crossing them back with white clover combined with selfing and inter-crossing in order to isolate *T. ambiguum* derived chromosomes and to pass the two genomes through several meiotic cycles. This will supposedly increase the chances of inter-genomic chromosome pairing

leading to recombination and at the same time, will restore the 4x ploidy level of white clover. The advanced progeny will be characterised for introgression using different approaches as mentioned for the other hybrids in other strategies. Only the crossing scheme is given below:

AAARRRR (7x) x RRRR = RRRRA(A_4) (5.5x)
RRRRA(A_4) (5.5x) x RRRR = RRRR(A_6) (4.75)

1.4 Summary of the aims of this thesis:

1. Hybridity of the advanced backcross derivatives with *T. repens* will be assessed by visible colour marker genes, flow cytometry based DNA contents, phenotypic analyses, somatic chromosome counts.
2. Recombination between *T. repens* and *T. ambiguum* chromosomes or *T. repens* chromosomes and *T. occidentale* chromosomes carrying *T. ambiguum* chromatin will be studied by detailed metaphase-I chromosome pairing configuration analysis, plant morphological data and by using molecular cytogenetic tools i.e., fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH).
3. Phenotypic characterization of the advanced progenies to detect the presence/absence of *T. ambiguum* traits will be done in replicated experiments in a sand-area so that plants can easily express root type and the plants can easily be harvested because some of the best of these are associated with root traits.
4. The proof of the concept will be the introgression of *T. ambiguum* traits into white clover-like plants at the 4x level.

CHAPTER 2
LITERATURE REVIEW

2.1.1 Wide crosses in germplasm improvement

The success of a breeding program depends on the continued supply of genetic variability with new beneficial traits (Poehlman, 1979). The use of genetic variation which exists in the primary gene pool, i.e. variation within the species, including land races, varieties and ecotypes, does not require special crossing techniques. However, often the required genetic variation exists in the secondary (closely related species) or tertiary (distantly related species) gene pools. Such genetic variation can be used either by direct domestication or by introducing it into cultivars through various conventional and non-conventional crossing techniques (Brar and Khush 1997; Zamir, 2001; Tan *et al*., 2004a; Dwivedi *et al.,* 2008). Where postzygotic hybridisation barriers exist due to endosperm degeneration, and the consequent embryo abortion, special crossing techniques such as embryo rescue and ovule culture are required.

Despite many problems associated with wide hybridization, this breeding method becomes necessary when genetic improvement is stifled by the lack of enough genetic variation within the species (Harlan and De Wet, 1971). The contribution of genetic resources through the improvement of crop responses to various biotic and abiotic stresses (Dwivedi *et al*., 2008) has led to a 30% increase in the yield of world crops and, interestingly, much of this improvement has come from the use of desirable genetic variation which exists in crop wild relatives. The yearly increase in crop yield due to the introgression of desirable genes from wild relatives is worth US$115 billion worldwide (Pimentel *et al*., 1997). Wild relatives are important because they harbour useful genes conferring high levels of resistance to environmental stresses (Harlan, 1976; Hoisington *et al*., 1999). A remarkable contribution of the wild relatives is the enhancement of the disease and pest resistance in many crops (Harlan, 1976; Goodman *et al.,* 1987; Lenne and Wood, 1991). Many examples can be cited where useful genes have been transferred into cultivated crops (Prakash *et al*., 2009) e.g., wheat (Bothmer *et al*., 1995), barley (Pickering and Johnston, 2005) and maize (Hoisington *et al.*, 1999) etc. Many wild relatives are also reported to have tolerance to abiotic stresses like drought, salinity, cold and heat (Dwivedi *et al*., 2008). However, gene introgression from wild relatives is not

necessarily easy because of cross-incompatibility; hybrid sterility and linkage drag (Zeven *et al*., 1983; Dwivedi *et al.*, 2008).

2.2.1 Introduction to and significance of white clover (*T. repens* L.)

White clover (*Trifolium repens* L.) is a tetraploid stoloniferous species (Britten, 1963; Thomas, 1987; Abberton & Marshall, 2005) and has been classified to the section *Lotoidea* of the genus *Trifolium (*Zohary and Heller, 1984), one of the largest genera of family Leguminosae. However, Ellison *et al*. (2006) have recently classified it to section *Trifoliastrum*. The genus contains 250-300 species distributed throughout the world in temperate and subtropical areas. *T. repens* is the most important temperate species (Williams, 1987a) and is the most widely used forage legume in cattle and sheep grazing-based temperate pastures around the world (Mather *et al*., 1995; Laidlaw & Teuber, 2001) usually mixed with perennial ryegrass *Lolium perenne* (Laidlaw and Teuber, 2001).

T. repens, although a temperate species, is found in areas ranging from the Arctic to the subtropics and has a wide altitudinal range (Williams, 1987a). The Mediterranean region, East Africa and North and South America are known as the centres of diversity of *Trifolium* (Vavilov, 1951; Zohary and Heller, 1984). Ellison *et al*. (2006), using a comprehensive phylogenetic analysis based on the nucleotide sequence of the ITS region of nucleolar organizer regions (NOR) of ribosomal DNA and *trnL* intron sequences of chloroplast DNA, supported the Mediterranean region as the centre of origin for clovers. For *T. repens*, the indigenous areas include the whole of Europe and central Asia west of Lake Baikal as well as areas in North Africa (Algeria, Morocco, Tunisia) (Williams, 1987a). It has also become naturalised in China, Mongolia, Korea, Japan, as well as in the Americas. It was also introduced into New Zealand by the early settlers where it has become the most important pasture legume; being well adapted to the well distributed rainfall and temperate climate.

The attributes making *T. repens* the legume of choice are its potential to spread laterally through rooted stolons, a high growth rate, good seed production, high establishment potential, reasonable grazing tolerance due to stolons anchoring to the ground by nodal rooting (Sanderson *et al*., 2003), high forage yield, palatability and high feed quality. The importance of clover in the forage is evident from the enhanced meat and milk production (Chapman *et al*., 1993; Davies and Hopkins, 1996) arising from not only

enhanced forage quality but also higher intake by the animals due to its palatability and high digestibility (Frame and Newbould, 1986). Another advantage is its ability to provide nutrition for the companion grass species by fixing large quantities of atmospheric N symbiotically with *Rhizobium trifolii* Dangeard (Williams, 1987; Anderson *et al*., 1991; Abberton *et al.,* 2002). N fixation by *T. repens* reduces the amount of artificial N fertilizer required (Frame and Newbould, 1986) and so lowers the cost of agriculture and the negative environmental impacts of these artificial fertilizers.

2.2.2 Cytogenetic description of *T. repens*

T. repens, although a tetraploid species (2n=4x=32) (Thomas, 1987), behaves as a diploid with regular bivalent pairing at meiosis (Atwood and Hill, 1940) and disomic patterns of inheritance of genetic markers (Atwood and Hill, 1940; Davies, 1970). The diploid-like behaviour of *T. repens* provides evidence for its being an amphidiploid (allotetraploid) (Atwood and Hill, 1940; Williams, 1987b). The high level of fertility also is strong evidence of *T.repens* being allotetraploid because an autotetraploid species might be less fertile due to irregular meiosis based on the high level of intergenomic homology (Majumdar *et al*., 2004). On the other hand, predominantly trivalent chromosome associations occur in the 3x F_1 hybrids of white clover with *T. nigrescens* (2x) and *T. occidentale* (2x) (Chen and Gibson, 1970a, b) and quadivalents occur in 4x F_1 hybrids between *T. ambiguum* and *T. repens* (Williams *et al*., 1982) and *T. repens* and *T. uniflorum* (Chen and Gibson, 1972). These results indicate that chromosome pairing occurs between the two gametic genomes of white clover, which could be consistent with an autotetraploid origin followed by evolution of a genetic mechanism ensuring perfect bivalent chromosome pairing within sub-genomes (Pandey *et al*., 1987). To explain the above results, this genetic system for suppressing homoeologous chromosome pairing would have to lose effectiveness after hybridization due to its hemizygous condition or disturbance of the genetic background.

Ansari *et al*., (1999) and Ellison *et al*., (2006), using molecular cytogenetic markers and sequence data of nuclear ITS and chloroplast *trnL* intron DNA, confirmed that *T. repens* is allopolyploid with divergent sub-genomes. An alternative explanation for the absence of homoeologous pairing in white clover is that put forward by Williams *et al*. (1982) that bivalent pairing in white clover is restricted to the homologues because of their greater synaptic attraction. In hybrid situations due to absence of homologues,

homoeologous pairing can occur between the gametic chromosome sets of white clover. Such pairing between the gametic chromosomes in white clover is consistent with a close phylogenetic relationship between the progenitor species (Chen and Gibson, 1970b).

Although the parental species of white clover are not yet known with certainty, *T. occidentale, T. nigrescens* and *T. uniflorum,* are phylogenetically very close to white clover, and are considered to be putative progenitor species (Chen and Gibson, 1971, 1972; Badr *et al*., 2002). Chen and Gibson (1972) suggested that *T. uniflorum* and *T. occidentale* (4x) might be the progenitor species because of the presence of IVs in 4x hybrids of white clover with *T. uniflorum* and 4x *T. occidentale.* Ellison *et al*. (2006), based on the DNA sequence similarities of the ITS region and the chloroplast *trnL* intron of more than 200 clover species, predicted that *T. pallescens* and *T. occidentale* were likely to be the mother and father of *T. repens*, respectively, because the chloroplast DNA of *T. pallescens* and the ITS of *T. occidentale* had strong sequence homology to the corresponding sequences in *T. repens*. Recently, Williams *et al.* (2012), based on the above DNA sequence analysis, created a diploid F_1 hybrid between *T. pallescens* and *T. occidentale* by embryo rescue. By using cyto-molecular markers, they found perfect bivalent pairing in the diploid F_1 hybrid along with formation of 2n gametes on a large scale. This provided evidence to support the hypothesis that *T. pallescens* and *T. occidentale* or some taxa phylogenetically very close to them were, respectively the mother and father of *T. repens*. The progenitor species of *T. repens*, when known, would be good sources of new variation for its improvement (Williams *et al*., 2012).

2.2.3 Why *T. repens* needs agronomic improvement

Lack of persistence of legumes, including *T. repens*, in grazed swards is the major factor limiting their use world-wide (Barnes *et al*., 1985; Knight, 1985; Forde *et al*., 1989). *T. repens*, although a perennial species in nature, is not very persistent in pasture due to being susceptible to a number of biotic and abiotic factors (Zohary, 1972). These factors include death of the taproot in the second year (Westbrook and Tesar, 1955; Brock and Tilbrook, 2000), low tolerance under drought stress (Brink and Pederson, 1998; Abberton *et al*., 2002), treading damage to stolons due to intensive grazing (Van Keuren and Matches, 1988; Forde *et al*., 1989; Ferguson *et al*., 1990), diseases caused by fungi and viruses (Barnett and Gibson, 1975; McLaughlin and Perderson, 1985; Alconero *et*

al., 1986; Pederson and McLaughlin*,* 1989; Taylor, 2008) and pest damage caused by nematodes and insects (Gaynor and Skipp, 1987; Mercer, 1988; Pederson and Windham, 1989). McLaughlin and Pederson (1985) and Pratt (1967) reported that high susceptibility to several viruses plays a major role in the USA in making *T. repens* less persistent. White clover mosaic virus (WCMV) is of special significance in the NZ environment in terms of its level of damage to white clover yield (Dudas *et al*., 1998).

The focus of germplasm improvement in most crops has been overcoming the impact of various biotic and abiotic stresses on performance (Abberton, 2007). Similarly, the ultimate objective of *T. repens* breeding is to enhance its persistence in the sward (Taylor, 2008) by improving its response to environmental (biotic and abiotic) stresses (Rhodes and Ortega, 1996; Abberton & Marshall, 2005). Breeding *T. repens* for higher yield has never been a primary objective like it is in other crops (Abberton and Mashall, 2005). Being an out-breeding (allogamous) species, *T. repens* populations are characterised by remarkable genetic diversity within very small areas (Williams, 1987; Caradus *at al*., 1989). However, the primary gene pool still lacks enough variation for stress tolerance, especially under prolonged drought, pest attacks and intensive grazing (Abberton & Marshall, 2005). The breeding efforts for improving stress resistances of white clover, although going on for 50 years (Abberton, 2007), have not been fully successful, probably due to lack of the necessary genetic variation in the species. Barbour *et al*., (1996) reported limited variation in white clover in response to drought. This lack of variation is probably due, in part, to its reproductive isolation due to being allotetraploid. Genetic variation for some biotic and abiotic stresses can be introduced by crossing white clover with related species carrying the required genetic variation (Barnett and Gibson, 1975; Abberton & Marshall, 2005) and so potentially broaden the genetic base, and leading ultimately to the evolution of new germplasm with the potential to adapt to different environmental stresses. The evolution of persistent varieties of *T. repens* under stressed conditions would ensure its reliable and sustained contribution to sward yields over long periods of time (Williams *et al.,* 2003b). Varieties with drought tolerance are very important in many parts of Australia and New Zealand and will probably become more important in future due to effects of climate change (Abberton and Mashall, 2005). However, some strong pre- and postzygotic barriers provide challenges to this approach (Chen and Gibson, 1970). Of these, the postzygotic barriers are more important in *Trifolium* (Řepkovā *et al*., 2006). The success of

hybridization between two species depends on both the genetic and structural relatedness of the genomes (Leflon *et al*., 2006). The level of homoeologous chromosomes pairing is also important because without a high frequency of homoeologous genetic exchange, inter-specific hybridization would be of no use. This depends on the extent of small genetic and structural differences in chromosomes accumulated over a period of time during their separate evolution in different taxa (Smith, 1968; Stebbins, 1971; Williams *et al.*, 1982).

2.3.1 T. *ambiguum* M. Bieb. as a novel source of variation for *T. repens*

Daly and Mason, (1987) described *T. ambiguum* M. Bieb., (Kura or Caucasian clover), as a long lived perennial herb with prostrate to erect stems, long trifoliate leaves and a large underground system of rhizomes and taproots. The root in Kura clover can go down to a depth of 60 cm (Speer and Allinson, 1985). The leaflets in *T. ambiguum* are often longer and narrower compared to the almost round leaflets of white clover (Williams and Hussain, 2008). The rhizomes branch out from the crown; eventually giving rise to daughter plants, both terminally and from the nodes (Bryant, 1974; Sheaffer and Martin, 1991). It is native to the southern part of Russia, the Caucasus region and Western Asia with its habitats ranging from valleys to subalpine regions (Zohary and Heller, 1984). *T. ambiguum* is adapted to a range of soil and climatic conditions from ill-drained lowlands to alpine-type meadows at 3200 m above sea level (Speer and Allinson, 1985; Taylor and Smith, 1998). It is one of the very few species in the genus *Trifolium* having three ploidy levels 2x (A^DA^D), 4x ($A^TA^TA^TA^T$) & 6x ($A^HA^HA^HA^HA^HA^H$) (Kannenberg and Elliott, 1962). DNA sequences have revealed that the genomes in the different *T. ambiguum* ploidies are quite divergent and so autopolyploidy cannot be assumed (Dr N.W. Ellison, unpublished data). Hexaploid *T. ambiguum* is considered agronomically superior to other ploidies because of its more vigorous vegetative growth (Kannenberg and Elliott, 1962; Taylor, 2008). The leaves in 2x and 6x versions are longer as compared to 4x version (Kannenberg and Elliott, 1962).

T. ambiguum is a species that is very fascinating for white clover breeders because it possesses a number of useful agronomic traits including the thick, deep taproot system and the rhizome-based spreading habit (Yamada and Fukuoka, 1986; Forde *et al*., 1989). The rhizomatous nature makes it useful not only for pasture but also for soil conservation (Bryant, 1974; Speer and Allinson, 1985). The rhizomatous root system

makes a sizable proportion of the total biomass in *T. ambiguum* (Spencer *et al*., 1975; Genrich *et al.,* 1998; Widdup *et al*., 1998: Black *et al.,* 2006a) and so it has higher underground: aerial biomass ratio compared to other clover species including *T. repens* (Spencer *et al*., 1975). It is a self-incompatible species with terminal flowering and up to 175 small white florets per inflorescence. It has low or highly variable seed production (Hampton *et al.,* 1990), possibly because extensive root growth and the development of crowns is a prerequisite for reproductive growth and stands may take several years to reach their maximum reproductive potential (Coolbear *et al*., 1994). Other useful agronomic attributes of *T. ambiguum* (Barnett & Gibson, 1975) include persistence in the field presumably due to its perennial deep thick rhizomatous taproot system (Spencer *et al*., 1975; Ferguson *et al*., 1990, Coolbear *et al*., 1994; Williams *et al*., 2007), water stress tolerance probably due to having thick, deep taproots (Caradus, 1977; Coolbear *et al*., 1994; Marshall *et al*., 2001) and resistance to various biotic stresses including several viruses which seriously affect white clover (Barnett and Gibson, 1975; Pederson and McLaughlin, 1989). Huge underground root system (roots and rhizomes) due to the allocation of a large proportion of assimilates (Genrich *et al.,* 1998: Widdup *et al*., 1998: Black *et al.,* 2006a) might provide water to the plant during water stress (Marshall *et al*., 2001). It can recover very quickly after drought stress (Dear and Zorin, 1985; Daly and Mason, 1987; Woodman, 1993). Spreading from the growing points which are located on rhizomes underground (Allan and Keoghan, 1994) makes *T. ambiguum* more tolerant to intensive grazing (Guy, 1996) than *T. repens* which spreads by stolons which get uprooted/damaged during intensive cattle or sheep grazing (Moorhead, *et al.*, 1994). Virgona and Dear (1996) also reported that *T. ambiguum* was more persistent in the face of heavy defoliation than *T. repens.* Pederson and McLaughlin (1989) reported not only high levels of resistance in *T. ambiguum* to all viruses affecting *T. repens* but also that this resistance was transmitted to hybrids with *T. repens*. Allinson *et al.* (1985), Scheaffer and Martin (1991), Schearffer *et al.* (1992) and Abberton *et al*., (2002) have shown that digestibility of *T. ambiguum* was equal to white clover and better than other forage legumes.

However, *T. ambiguum* also has some weak aspects (Williams and Verry, 1981) i.e., slow establishment threatening its survival due to competition from the companion grasses or drought (Hill and Mulcahy, 1995; Taylor, 2008), poor growth during winter (Williams, 1978; Hill and Hoveland, 1993; Hill and Mulcahy, 1995; Taylor and Smith,

1998), low seed production (Bryant, 1974) and very specific rhizobial-strain needs (Pryor, *et al*. 1998). The weaknesses of *T. ambiguum* are the main obstacles to its large scale adoption in pastoral agriculture (Hill and Mulcahy, 1993; Peterson *et al*., 1994; Widdup *et al*., 1996). Abberton (2007) reported that the slow establishment of *T. ambiguum* is due to the early development of the large rhizome and root system to which a large proportion of biomass is allocated (Genrich *et al*., 1998). However, despite some problems associated with *T. ambiguum*, it is being advocated quite widely in northern USA because of its comparatively better forage quality as comapared to other forage legumes except *T. repens* (Albrecht, 2002) and rhizomatous tap-root system which presumably contribute towards comparatively higher persistence under drought conditions and grazing tolerance (Dear and Zorin, 1985; Peterson *et al*., 1994; Williams *et al*., 2007; Warren Williams, verbal comm.).

The introgression of drought tolerance and persistence from *T. ambiguum* into white clover is one of the possible ways that has been suggested for the improvement of white clover (Williams, 1978; Williams and Verry, 1981; Williams *et al*., 1982; Abberton *et al*., 2003). Hybrids between *T. ambiguum* and white clover have the potential to increase the adaptive range of white clover without any compromise on its forage quality (Allinson *et al.,* 1985; Sheaffer *et al.,* 1992).

2.4.1 *T. occidentale* as a genetic bridge

T. occidentale Coombe (2n=2x=16) is a stoloniferous perennial clover which is indigenous to Portugal, Spain, England, France, Ireland, Wales and the Channel Islands where it is found in relatively dry coastal areas (Coombe, 1961; Coombe and Morisset, 1967). This species apparently resembles *T. repens*, although it is cytologically, ecologically, and geographically different and lacks the vegetative vigour of white clover. It has limited morphological variability (Coombe, 1961), although Williams *et al*. (2009) have shown that Spanish populations are more variable than those from further north. It has relatively short stems and very small leaves which make it suitable for dry, windy, coastal areas. It is also probably resistant to salty soils (Williams, 1987). *T. occidentale* is also a good source of resistance to several viruses (Gibson *et al*., 1971). Gibson and Chen, (1975) and Gibson *et al*. (1971) reported that *T. occidentale* can be used as a bridging species to facilitate hybridization between *T. repens* and *T. uniflorum.* The hybrids of *T. occidentale* with white clover (Gibson and Beinhart, 1969; Chen and

Gibson, 1970), *T. nigrescens* (Williams *et al*., 2008), *T. pallescens* (Williams *et al*., 2006, 2012) and *T. ambiguum* (Williams *et al*., 2011) have shown allosyndetic chromosome pairing which offers some possibility of introgression.

T. occidentale is phylogenetically very close to *T. repens* and is one of its probable progenitor species (Ellison *et al*., 2006). These two species have the same basic karyotypes, each having a pair of chromosomes carrying 18S-26S rDNA and 5S rDNA sequences in approximately the same chromosomal positions. Both species have an additional pair of chromosomes with 5S rDNA, but these are in slightly different physical positions. In *T. occidentale,* the 5S rDNA signals are comparatively smaller and are on the short arms of a chromosome pair as opposed to *T. repens* where these signals are bigger and are on the long arms (Ansari *et al*., 1999). All the chromosomes of these two species share a unique centromeric DNA repeat sequence, *TrR350,* which is also present on a single chromosome pair in 2x *T. ambiguum (*Ansari *et al.,* 2004). Despite apparent resemblance with white clover, *T.occidentale* differs in a few ways. It has hairy petioles and peduncles and the leaves are comparatively thicker and opaque with a shiny lower surface (Coombe, 1961). The genetic closeness of *T. occidentale* to *T. repens* (Gibson and Beinhart, 1969; Chen and Gibson, 1970; Ellison *et al*., 2006; Hand *et al*., 2008) and *T. ambiguum* (Williams *et al.,* 2011) suggests that it could be used as genetic bridge between these species (Williams *et al*., 2006a). Gene transfer using bridging species has also been reported in other crops (McCoy and Echt, 1993).

2.5.1 Wide hybridization in *Trifolium*

Twenty four *Trifolium* species are considered to be polyploid in origin and only five are of clear hybrid origin with different sub-genomes, as revealed by DNA sequence homology analysis (Ellison *et al*., 2006). The existence of such a small number of hybrid species is evidence of the existence of very strong barriers to inter-specific hybridization in the genus (Chen and Gibson, 1972; Williams, 1987; Ellison *et al*., 2006).

The purpose of wide hybridization in *Trifolium,* which has been going on for almost 50 years, has been to firstly discover the evolutionary relationship among the species and, secondly, to introduce useful genetic variation from the related species (secondary gene pool) into white clover for its agronomic improvement (Brewbaker and Keim, 1953; Abberton, 2007). White clover has, so far, been crossed with the closely related species, *Trifolium nigrescens* Viv (Brewbaker and Keim, 1953; Marshall *et al*., 1995), *T.*

ambiguum Bieb. (Williams, 1978; Williams and Verry, 1981; Yamada *et al*., 1989), *T. occidentale* D. Coombe (Gibson and Beinhart, 1969), *T. uniflorum* L. (Pandey *et al*., 1987), *T. hybridum* L. (Przywara *et al*., 1989) and *T. isthmocarpum* (Kruse, 1971). Although, success has been achieved in making hybrids and transferring desirable traits like persistence and drought tolerance into white clover, so far no commercial variety has been released based on these crosses. In the case of hybrids between *T. ambiguum* and *T. repens* the reason probably is the lack of a high level of homoeologous chromosome pairing, which precludes the possibility of inter-genomic recombination. Genetic recombination between homoeologous chromosomes is as important as making hybrids (Singh and Jauhar, 2006). This process helps to fix the transferred trait into the cultivated species with stable inheritance (Castillo *et al*., 2012). Repeated backcrossing of an F_1 hybrid to the cultivated parent and selection of the plants expressing characters of interest from the donor species can lead to the generation of genotypes with chromosome addition. The isolated alien chromosomes from the donor species might also pair with the homoeologous counterpart from the cultivated species and so recombination (genetic exchange) can be achieved (Meredith *et al*., 1995) which is a key step of inter-specific hybridization (Hussain and Williams, 1997a).

2.5.1.1 Hybrids between *T. nigrescens* and *T. occidentale*

Gibson and Beinhart (1969) reported for the first time that 4x *T. occidentale* could be crossed with 2x *T. nigrescens* if used as a male parent. The cross between these species at diploid level failed due to the early degeneration of embryos. The 3x F_1 hybrids had reasonably high numbers of trivalents (5.69) per microsporocyte showing a very close genetic relationship between the species, which was recently confirmed by Ellison *et al*. (2006). These 3x hybrids were nearly sterile but produced a few seeds on backcrossing only to *T. nigrescens*. Williams *et al*. (2008) reported the success of *T. nigrescens* x *T. occidentale* crosses without any special techniques at 2x ploidy level with the F_1 hybrids showing very regular meiosis (eight bivalents). However, the reciprocal cross at this ploidy level failed due to early embryo abortion. Williams *et al*. (2008) further reported that these 2x F_1 hybrids could be easily crossed back to *T. nigrescens,* again without any special techniques, and that introgression from *T. occidentale* to *T. nigrescens* occurred unidirectionally. The formation of eight IIs (showing allosyndetic chromosome pairing) in the 2x hybrids confirmed the findings of Gibson and Beinhart (1969) regarding the close phylogenetic relationship between these species. Williams *et al*. (2008) reported

that the morphology of hybrid between *T. nigrescens* and *T. occidentale* was more like *T. nigrescens* but the morphology of same hybrid with 4x *T. occidentale* was reported by Gibson and Beinhart (1969) to be more like *T. occidetanle*. *T. nigrescens* x *4x T. occidentale* hybrids were reported to be virus resistant by Pederson and McLaughlin (1989). The 2x F_1 hybrid between *T. nigrescens* and *T. occidentale* could be distinguished on the basis of chromosomal distribution of 5S rDNA and 18S-26S rDNA in the parental species. Both species have one pair of chromosomes with 5S rDNA on the long arm and the 18S-26S rDNA on the short arm. *T. occidentale* has an additional pair of 5S rDNA on the short arm of another chromosome pair. The F_1 was expected to have two chromosomes with both 5S r DNA and 18S-26S rDNA and one with 5S rDNA and this was confirmed (Ansari *et al*., 1999).

2.5.1.2 Hybrids between *T. ambiguum* and *T. occidentale*

The first partially fertile 2x hybrids between *T. ambiguum* and *T. occidentale* (AO) were reported by Williams *et al*. (2011). Morphologically these 2x hybrids had characters coming from both the parents (stolons form *T. occidentale* and rhizomes from *T. ambiguum*) plus some transgressive expression in some traits where the expression of the characters were outside the parental range. These hybrids exhibited predominantly bivalent chromosome associations showing allosyndetic pairing with rare or no univalents. This was evidence of a close genetic relationship between *T. ambiguum* and *T. occidentale*, showing their recent evolution from a common lineage (Williams *et al*., 2011). In addition to 2x hybrids between *T. ambiguum* and *T. occidentale,* 3x and 4x hybrids with partial fertility were also developed at Grasslands, AgResearch, New Zealand using different ploidy levels of two species (Warren Williams, unpublished data). They included hybrids between 4x *T. ambiguum* and 2x *T. occidentale* (AAO, 3x) designated as 434-1, 4x *T. ambiguum* and 4x *T. occidentale* (AAOO, 4x) designaed as BL or BN and 6x *T. ambiguum* and 2x *T. occidentale* (AAAO, 4x) designated as hybrid "33". The 4x hybrids (BL and BN) were used in the second strategy (Bridge breeding) of our project as one of the parents of 5x hybrids (AAAOO). While hybrid "33" was allowed to be open pollinated and the resulting progeny which was approximately 6x in ploidy (designated as 33 OP-1) was used as parental material in the third strategy (Bridge breeding). The genomic composition of 33 OP-1 was AAAORR because the unreduced (2n= ~ 4x) female gamete from hybrid "33" was pollinated by the normal

haploid gamete from *T. repens*. The origin of male gamete in 33 OP-1 hybrid was confirmed by using cyto-molecular analysis (Dr. Helal Ansari; unpublished data).

2.5.1.3 Hybrids between *T. repens* and *T. nigrescens*

T. nigrescens is known for its profuse flowering and resistance against clover cyst nematode. It was considered to be one of the progenitors of white clover (Williams, 1987a; Williams *et al.*, 1998; Badr *et al*., 2002), although the hybrid between *T. nigrescens* (2x) and *T. occidentale* (2x), which is considered contributor of the second genome to white clover, was more *T. nigrescens*-like rather than white clover (Williams *et al*., 2008). *T. nigrescens* has been successfully crossed with *T. repens* Viv. with contrasting objectives in different programmes. Marshall *et al*. (1995, 1998, 2002a, 2003a) aimed to improve seed production potential in white clover by introgressing the profuse flowering trait from *T. nigrescens*. Hussain *et al*. (1997a) aimed to enhance resistance to white clover cyst nematode. Hussain *et al.* (1997a) reported the development of a series of backcross hybrids from a single triploid (2n=3x=24) *Trifolium repens* x *T. nigrescens* F_1 hybrid. The 3x F_1 hybrid was sterile and did not produce any seed by backcrossing with any of the parents. Chromosome doubling to hexaploid resulted in a marked increase in pollen fertility from 9.9 % to almost 89.2 %. Then crossing this 6x hybrid with both parents and inter-crossing of these backcross derivatives resulted in a number of fertile hybrids with a range of ploidy levels (4x, 5x, 7x and some aneuploids). The presence of 7x BC_1F_1 plants in the progeny of a cross between *T. repens* and the 6x hybrid confirmed the production of 2n gametes in *T. repens* (Hussain and Williams, 1997b). Meiotic studies showed allosyndetic chromosome pairing in the F_1 and BC_1F_1, which indicated the possibility of genetic exchange between the two species. Hussain *et al*. (1997a) also reported the transfer of clover cyst nematode resistance to *T. repens* from *T. nigrescens*. These hybrids showed the same level of resistance to cyst nematode as did *T. nigrescens*. This nematode is very serious threat to white clover (Cook and Yeates, 1993; Mercer and Watson, 1996) and badly affects its yield (Yeates, 1977; Watson *et al.*, 1994). Marshall *et al*. (1995) reported that the BC_1 hybrids between *T. repens* x *T. nigrescens* were intermediate in reproductive morphology but further backcrossing using white clover as recurrent parent resulted in progeny which were morphologically more like white clover than *T. nigrescens* (Marshall *et al*., 1998; 2002a) but seed yield was still significantly higher than the white clover parent (Marshall *et al*., 1999; 2003a). Ferguson *et al*. (1990)

reported that nodal rooting in a hybrid between *T. repens* and *T. nigrescens* was confined to only initial 2-3 nodes of the a sort of semi-erect stem.

2.5.1.4 Hybrids between *T. ambiguum* and *T. repens*

T. ambiguum is a part of "the white clover complex" which includes eight species closely related with white clover (Ellison *et al*., 2006). The close phylogenetic relationship among the species of this complex indicates a recent origin of these species from a common ancestor as reported by Ansari *et al*. (2004) and Williams *et al.,* (2011). But *T. ambiguum* is the most distantly related species to white clover in the complex (Ellison *et al*., 2006) and this is also supported by its contrasting morphological traits and eco-geographic adaptations. This relatively remote relationship between *T. ambiguum* and white clover is further corroborated by their inability to produce fertile progeny through artificial hybridization. Production of hybrids between them has always required embryo rescue/ovule culture because of endosperm failure at a very early stage (Williams and White, 1976; Anderson *et al*., 1991; Meredith *et al.,* 1995) and several intensive efforts made by the breeders have to-date produced very few fertile hybrids (Williams and Verry, 1981; Meredith *et al*., 1995).

The first F_1 hybrid (AARR) between *T. ambiguum* and *T. repens*, using embryo culture, was reported by Williams (1978) but this was sterile. Later, NZ and UK researchers succeeded in getting hybrids with reasonable fertility between these two species using a Turkish 4x *T. ambiguum* as female parent. Backcrosses to *T. repens* were also obtained from these hybrids (Williams and Verry, 1981; Williams *et al*., 1982; Anderson *et al*., 1991; Meredith *et al.,* (1995). The F_1 hybrid of *T. ambiguum and T. repens* (AARR) designated as Hybrid 435 (briefly H-435) was self-compatible while the parents were self-incompatible (Williams and Verry, 1981). Self-fertile hybrids from self-incompatible parents, (*T. uniflorum* and *T. repens*) were reported previously by Pandey (1957), who explained this condition on the assumption that the incompatibility gene (S) was located at different loci in the two species, either on homoeologous chromosomes but with the allosyndetic paring and considerable crossing over, or on different, independently assorting chromosomes. The S gene is a complex locus involved in many activities of reproductive physiology, and its effective functioning depends on a stable polygenic background (Pandey, 1968). The disturbed genetic background of a hybrid may impair S gene action and thus enhance self compatibility. Self compatible progeny

from self incompatible parents has also been described by Atwood and Brewbaker (1953) and Williams *et al*. (1982). Variation in the fertility level of H-435 was observed under different environments showing that meiotic stability is influenced by external factors as well (Williams and Verry, 1981; Anderson *et al*., 1991).

H-435 was reported to be more like *T. ambiguum* (the female parent) in some characters but exhibited intermediate morphology in others including stem habit, leaf shape, veining and texture and flower length etc. The F_2 plants were also more like *T. ambiguum* than white clover and had only a few nodal roots at base of the stems (Williams and Verry 1981). The best level of expression of *T. ambiguum* associated characters including rhizomes was reported in the BC_1 to white clover while BC_2 had the desired level of expression of white clover related traits but was genetically unstable due to being pentaploid (ARRRR) (Williams and Hussain, 2008). The low fertility and instability (both cytological and genetical) are the main hurdles in the direct use of such hybrids as cultivars (Zwierzykowski *et al*., 2011). Williams and Hussain (2008) reported a range of morphologies in the backcross derivatives of the cross (AAAARRRR x RRRR) and further reported that the *T. ambiguum* related traits including rhizomes faded as the number of backcrosses to white clover increased while pollen fertilities increased with further backcrossing and selfing. Similarly, Abberton *et al*. (1998) reported that, morphologically BC_3 hybrids were more similar to *T. repens* than to *T. ambiguum* but they also showed some small percentage of their total dry weight (3%) as rhizome, which confirmed the introgression of *T. abmiguum* into *T. repens*. Meredith *et al.* (1995) reported similar results that the BC_1 was morphologically closer to *T. repens* and the characters of *T. ambiguum* were not as visible as in the F_1 probably showing the impact of gene dosage from the parental species. Some BC_1 and BC_2 plants gave evidence of rhizomes because some of the shoots originated from growing points below the soil surface. In comparison to BC_1, BC_2 showed greater variability in the expression of characters of Caucasian clover and the possible explanation for this large variation in the expression of characters was the independent assortment of *T. ambiguum* chromosomes of the two genomes making bivalents in the meiosis of 6x BC_1F_1 and so allowing the allelic differences in the two genomes to show up. High frequencies of seedling abnormalities were reported by Meredith *et al*. (1995) and Williams and Hussain (2008) in the BC_1F_1 (AARRRR) which decreased with further backcrossing (Williams and Hussain 2008). Yamada and Fukuoka (1986) reported a 5x hybrid AAARR from the

cross of 6x *T. amiguum* with white clover with intermediate phenotype to the parents but with more resemblance to *T. ambiguum,* probably due to gene dosage effect. The characters with intermediate morphology included leaflet shape and rhizomatous character. But, unfortunately, this hybrid did not produce any viable pollen.

Marshall *et al*. (2001) reported a higher level of drought tolerance than white clover in BC_2 plants (*T. ambiguum x T. repens*) x *T. repens*) having rhizomes less than 5% of the total dry weight. Similarly higher drought tolerance was reported by others as well (Allinson *et al.,* 1985; Sheaffer and Martin 1991; Abberton *et al*., 2002). The level of N fixation in hybrids was not different from that in white clover (Abberton *et al*., 2001). Marshall *et al*. (2003a) reported higher root weight ratio to total biomass in the backcross hybrids between *T. ambiguum* x *T. repens* as compared to white clover. Isobe *et al*. (2002) produced the hybrids and their backcross progney between red clover and *T. medium* with the aim to improve the persistence level of red clover in the field by the incorporation of the rhizomatous trait from *T. medium*. They reported that the rhizomatous character did not go beyond BC_1 but persistency in the advanced BC_4 hybrids was higher as compared to the red clover. Rhizome development takes at least 18 months so to monitor their introgression into white clover, molecular marker based approaches would be more useful to track down the polymorphism due to the rhizome presence without waiting for the development of rhizomes (Abberton *et al*., 2003). The effect of plant age and environment on the expression of rhizomes has already been reported by Beuselinck *et al*. (2005) in lotus hybrids. Abberton *et al*. (2002) and Marshall *et al*. (2003a, 2004) investigated forage quality and other agronomic traits in backcross hybrids between white clover and Kura clover and found no significant differences between the backcross hybrids and white clover in yield, persistency, N fixation and dry matter digestibility except water soluble carbohydrates (WSC) and crude protein content. Higher levels of resistance against peanut stunt virus (PSV), clover yellow vein virus (CYVV), alfalfa mosaic virus (AMV) and southern root knot nematode (*Meloidogyne incognita*) were reported in hybrids between *T. ambiguum and T. repens* (Pederson and McLaughlin, 1989; Pederson and Windham, 1989).

Highly regular meiosis (15.6 IIs/PMC) in H-435 (AARR) was reported by Anderson *et al*. (1991) but whether these IIs involved auto- or allosyndetic chromosome paring was not known. No GISH/FISH experiment was conducted on this hybrid and

conventionally-stained chromosomes of these species are not distinguishable because they are karyotypically similar, being very small and bi-armed (Chen and Gibson, 1971; Williams *et al*., 1982; Ansari *et al*., 1999; Jeridi *et al*., 2011; Dr. Helal Ansari, personal communication). However, Giemsa-stained chromosomes of these two species can sometimes be differentiated during somatic metaphase because *T. ambiguum*-derived chromosomes are larger than *T. repens* chromosomes and often have more defined telomeric ends probably due to a differential rate of condensation. Common sense suggests that the predominantly bivalent pairing in the H-435 is likely to be within the sub-genomes of the parental species because of the availability of an intra-specific homologue/homoeologue for each chromosome. The intra-specific homoeologous chromosomes are more similar than the inter-specific homoeologues and so the affinity between the chromosomes is greater in the former case than in the latter. However, Williams *et al*. (1982) and Meredith *et al*. (1995) attributed the occurrence of occasional multivalents (IIIs & IVs) in H-435 (AARR) to the possibility of R/A allosyndetic chromosome pairing which might lead to genetic exchange between the two species. This is consistent with the predominantly multivalents in 3x and 4x hybrids of white clover with *T. nigrescens* (2x), and *T. occidentale* (2x and 4x) and supports the hypothesis of Williams *et al*. (1982) that, given no homologue, homoeologous pairing might occur in a hybrid situation. The findings of Anderson *et al*. (1991) of predominantly bivalent formation in the 4x BC_1F_1 hybrid resulting from the cross of H-435 (AARR) to *T. repens* (with expected genomic composition of ARRR) would indicate possible evidence for inter-specific (allosyndetic) chromosome pairing. But these findings do not agree with results obtained by Williams *et al*. (1982) and Meredith *et al*. (1995) and so the plants studied by Anderson *et al*. (1991) might have been self progeny of the H-435 (AARR). This is because, on one hand, crosses of AARR with white clover give mostly 6x (AARRRR) progeny and, on the other hand, such a high level of bivalent paring in a 4x BC_1F_1 (ARRR) hybrid would be unexpected keeping in view the remote genetic relationship between the parental species. Hussain and Williams (1997) suggested that it may be possible that, in the presence of homologues, pairing between homoeologues is suppressed due to preferential pairing between homologues as reported by Menzel (1964) and Stift *et al*. (2008). On the other hand, pairing between homoeologous chromosomes can be expected in a hybrid having odd numbers of parental genomes (Zhang *et al*., 2010).

H-435 was found to be cross-compatible only with white clover, and the resulting BC_1F_1 progeny were 6x (AARRRR) instead of 4x (ARRR), due to the preferential functioning of 2n gametes from the hybrid parent (Meredith *et al*., 1995). Theses 6x plants had reduced chances of homoeologous chromosome pairing because of the availability of homologues for every chromosome. In these circumstances, introgression and the production of novel genetic combinations would not occur as this requires homoeologous chromosome pairing (allosyndesis) (Williams *et al.,* 1982). Chromosome elimination leading to generation of aneuploids has been reported to occur either during gamete formation in the hybrid or after fertilization during embryogenesis (Williams and Verry, 1981; Tu *et al*., 2009). Chromosome breakage and loss have also been reported in hybrid or partial hybrid situations by Lukaszewski (2010) in wheat and by Wang *et al*. (2010) in sugarcane. The production of more 6x BC_1 plants than the expected 4x from the above cross shows that 2n gametes produced by the H-435 (AARR) were more functional than haploid gametes (Anderson *et al*., 1991; Meredith *et al*., 1995). Unreduced gametes, which result from irregularities during meiosis both in male and female (Miller, 1963; Veilleux, 1985) have been reported in white clover (Hussain and Williams, 1997b) and *T. alpestre* (Maizonnier, 1972). Production of 2n gametes at a very low rate (0.1-2%) is common (Ramsey, 2007) but the frequency is higher in hybrid situations (Ramsey and Schemske, 2002). Meredith *et al*. (1995) produced 5x BC_2 hybrids (ARRRR) using white clover as the recurrent parent and they reported the indication of inter-specific chromosome pairing due to the occurrence of multivalents. However, the nature of those multivalents was not confirmed, and they might have been among white clover chromosomes.

Hussain and Williams (1997a) attempted to overcome the crossing barriers by creating a fertile bridge between *T. ambiguum* and *T. repens*, to enable the transfer of desirable characters between the two species either way without requiring any further special techniques. They produced 6x BC_1F_2 plants by backcrossing colchicine doubled 8x H-435 hybrid (AAAARRRR) with *T.repens* (RRRR) followed by inter-crossing (selfing) to enhance fertility among the BC_1F_1 population. They showed a high frequency of multivalent formation during meiosis indicating both autosyndetic and allosyndetic pairing. One BC_1F_2 plant had a pollen stainability of 65.8% and was apparently cross compatible with hexaploid *T. ambiguum* as female and produced an apparent congruity

backcross (CBC_2). However, the CBC_2 was not verified and, later, Williams *et al*. (2006) found that the production of CBC_2 plants required embryo rescue.

Abberton *et al.* (2003) using the AFLP technique for assessing the introgression of the rhizomatous trait into white clover from *T. ambiguum,* analysed a large number of backcross plants and found polymorphic bands distinguishing between rhizomatous and non-rhizomatous bulks. They found a single band which was associated with the presence of the rhizomatous trait in the individual plants. This band was present in the BC_2, the BC_1 and *T. ambiguum* but not in white clover. Due to the aneuploid nature of BC_2 plants, this band would appear on a specific chromosome tightly linked to the locus controlling rhizome expression. Marker assisted selection using AFLP could therefore help accelerate the rapid incorporation of the rhizomatous trait into white clover.

Yamada *et al*. (1989) also produced 5x hybrids (AAARR) by crossing 6x *T.ambiguum* with *T. repens* using ovule culture but these 5x hybrids performed very poorly and had low fertility. Due to the complex genetic makeup of the progeny, their poor performance and fertility are understandable. However, the low fertility of a hybrid cannot be attributed always only to the meiotic irregularities because sometimes very regular meiosis is associated with very low fertility. Thus sterility might be caused by genetic and structural deficiencies in gametes arising from the independent assortment in a hybrid situation of chromosome pairs which normally stay together (Stebbins, 1971; Williams *et al*., 1982). Scewer and Cleveland (1972) reported intermediate phenotype in hybrids, *T. pratense* x *T. hirtum* and *T. pratense* x *T. pallidum. T. resupinatum* x *T. alexandrinum* were morphologically intermediate to the parents but they showed delayed flowering as compared to the parents (Kaushal *et al.* (2005). The hybrids between *T. alexandrinum* and *T. constantinopolitanum* also showed intermediate morphological features but with reduced fertility and early flowering as reported by Roy *et al*. (2004). Pandey *et al.* (1987) reported successful crosses between *T. repens* (2n=32) and *T. uniflorum* (2n=32). Backcrosses to both the parents were also produced. Hybrids showed intermediate morphology as was expected. One F_1 hybrid was self-compatible while the parents were self-incompatible. Pollen fertility was higher in the F_1 than that in F_2 and in backcrosses with *T. repens* than that in backcrosses to *T. uniflorum*. Malaviya *et al*. (2004) reported F_1 hybrids between *T. alexandrinum* and *T. apertum* with intermediate morphology but some of the traits showed transgressive expression.

2.5.2 Problems associated with wide crosses and their solution

The barriers to inter-specific hybridization can be broadly classified into pre- and post-fertilization barriers (Hovin, 1962b; Evans, 1962b; Chen and Gibson, 1972). For circumventing pre-fertilization barriers, various techniques have been used, including, (1) use of compatible pollen with inactivated nuclei mixed with the desired pollen (Taylor *et al*., 1980), (2) mixtures of compatible and incompatible pollen with subsequent identification of hybrids versus selfs in the progeny, (Brown and Adiwilaga, 1991), (3) cutting stylar tissue to remove the obstacles caused by the stigma and the inhibition factors present there (Ascher and Peloquin, 1968) and (4) application of chemicals like gibberellins or auxins and cytokinins to enhance pollen tube growth (Dionne, 1958; Alonso and Kimber, 1980; Sastri and Moss, 1982; Baker *et al.*, (1975). Mujeeb-Kazi and Rodriguez (1980) also reported the use of immunosuppressants for enhancing hybridization in cereals and legumes. Post-fertilization barriers result from differences in ploidy levels, chromosome loss/rearrangement, genic incompatibilities (Stebbins, 1958) cytoplasmic incompatibilities, physiological abnormality, seed dormancy and hybrid breakdown resulting from lethal or low plant vigour in the first or subsequent generations (Taylor *et al*., 1980; Williams, 1987; Repkova *et al*., 2006). Like other species, *T. repens* improvement through hybridization is predominantly hampered by strong postzygotic barriers (Chen and Gibson, 1970).

Chou and Gibson (1968) reported that in crosses of 2x and 4x *T. occidentale* with *T. nigrescens* pollen tubes penetrated the style and reached the ovule. So the failure of these crosses was due to postzygotic barriers caused by the lack of normal endosperm development. In the failed crosses, the endosperm first became abnormal and started degenerating and then the embryo collapsed. In *T. repens,* all ovules were fertilized within 24 hours of pollination when stigmas were pollinated with *T. repens* pollen (Chen and Gibson, 1972). But when *T. repens* was pollinated by other species, pollen germination took comparatively longer and the frequency of germination was lower. The pollen tubes in the inter-specific crosses were shorter than those in intra-specific pollination. However, pollen tubes of the species like *T. occidentale* and *T. nigrescens* grew more normally than *T. uniflorum* and *T. ambiguum* on the stigma of *T. repens* probably due to close genetic relationship. Pollen of autotetraploid *T. occidentale* appeared to germinate better on white clover stigmas than that of diploid *T. occidentale*. Pollen of the other species (*T. hybridum*, *T. uniflorum* and *T. ambiguum*) mostly swelled,

burst or coiled in the style or ovary of *T. repens* and very few pollen tubes reached the ovules. Roy *et al.* (2004) developed a hybrid, *T. alexandrinum* x *T. constantinopolitanum* and reported slow development of the embryo in the cross as compared to the intra-specific cross of *T. alexandrinum*.

After fertilization and gamete fusion in wide crosses, *in vitro* culture techniques are usually employed to recover young embryos before they abort due to endosperm degeneration (Brewbaker and Keim, 1953; Pandey, 1957; Evans, 1962; Williams, 1987c). Ovule culture is used in cases where embryos abort very early and, alternatively, sequential culturing of ovules and then embryos can be employed (Przywara *et al.*, 1989). Embryo rescue/ovule culture has been used by several researchers for developing hybrids between different *Trifolium species* (Williams and Verry, 1981; Yamada and Fukuoka, 1986; Pandey *et al.*, 1987; Ferguson *et al.*, 1990).

When the genotypes used in the cross are at the same ploidy level and have common genomes, then genetic recombination is a straightforward event. An important aspect of wide hybridization is the complexity created by different ploidy levels of the species used in the crossing scheme. Many cultivated crops are polyploids while their wild relatives are diploids and there is often reproductive isolation between the polyploid species and its progenitor or related species. Many crop species that were, until recently, considered typical diploids, are in fact ancient polyploids but behave cytologically like diploids (Leitch and Bennett, 1997; Wolfe, 2001). The narrow genetic base of these polyploids due to their reproductive isolation can present problems to plant breeders trying to cope with evolving biotic and abiotic stresses. The genomic imbalances in the hybrids between species with different ploidy levels can be overcome through various approaches. First is direct crossing followed by chromosome doubling in the hybrid by chemical treatment to restore fertility in the F_1. Later on, this plant is either backcrossed with the cultivated parent as a recurrent parent or selfed to generate spontaneous chromosome reduction to the ploidy level of the cultivated species. A second method is to first raise the ploidy level of the species having lower ploidy and then cross it with the other species which is usually the cultivated species. This approach is successful for crops which are autopolyploid, but for allopolyploid species, high sterility can occur in the F_1. Using bridging species at a lower ploidy level and then chromosome doubling can help circumvent sterility problems in the desired hybrid (Simpson, 1991). Some species can be manipulated to lower the chromosome number of the higher ploidy

species as reported by Voigt (1971), Burk *et al.* (1979) and Peloquin and Ortiz (1992). Ploidy manipulation with haploids, 2n gametes and use of wild species is an impressive and fascinating method which offers big opportunities for the improvement of crop germplasm. Another approach, re-synthesizing ploypoids for creating diversity and gene introgression, has also been used and has proved successful in wheat (Fernandes *et al*, 2000; del Blanco *et al*., 2001), and Brassicaceae (Lu *et al.*, 2001; Summers *et al.*, 2003; Pires *et al.*, 2004).

2.5.3 Endosperm balance number (EBN) and inter-specific crosses in *Trifolium*

Normal development of endosperm as a nutrition source is a pre-requisite for normal seed development to prevent early abortion of the embryo. Many hypotheses have been presented to explain the failure of normal endosperm development. Lin (1975) and Nishiyama and Inomata (1966) reported that the development of endosperm depended on a 2:1 ratio of maternal and paternal genomes within the endosperm regardless of the ploidy level or species differences. Later, Johnston et *al*. (1980) proposed the EBN hypothesis in which every species is given an "effective ploidy level" (EBN) with respect to endosperm functions by crossing it to a standard species. Normal endosperm development requires 2:1 maternal: paternal ratio of EBN values in the endosperm. Peloquin *et al*. (1982) reported that any deviation from 2:1 maternal paternal ratio in endosperm tissue would cause its degeneration and lead to the death of the embryo. EBN is not related only to the ploidy level of a species. Two species with the same ploidy level can have different EBNs and consequently be highly cross-incompatible and vice versa, as reported by Johnston and Hanneman (1980) and Johnston and Hanneman (1982).

Parrott and Smith (1986) suggested the use of EBN as a strategy to predict the outcome of complex crosses in *Trifolium.* This method can filter the gametes with certain genomic make up from hybrids with unbalanced genomes. Based on the easy crossability of *T. repens* (2n=2x=32) and *T. nigrescens* (2n=2x=16) and production of fertile progeny, both the species were given the same EBN of 4. This method was further developed by giving EBNs to gametes in other species, enabling breeders to predict which gametes would effect fertilization in crosses involving complex polyploid hybrids with uneven genomic constitution. *T. ambiguum* and *T. occidentale* have been given

EBN values of 0 and 2, respectively (A=0 & O=1) (Dr. Wajid Hussain, personal communication).

As endosperm development is a post-fertilization process, crosses in which other pre- or post-fertilization barriers exist, irrespective of the EBN, can fail to produce normal seed. Sometimes, genotypes with different EBN can produce seed and this is frequently due to production of 2n gametes by one parent (Parrot and Smith, 1986). Also, the system is not perfect i.e. it is leaky. Chromosome doubling is another way to get over the problem produced by different EBN of species by changing the EBN of one of the species in the cross to the same level as that of the other (Parrott and Smith, 1986). An example is the cross of *T. repens* (4x) x *T. occidentale* (2x), which did not cross at the natural ploidy level due to different EBNs but they can easily cross when *T. occidentale* is tetraploidized by colchicine application (Gibson and Beinhart, 1969), making their EBN values the same (although Gibson and Beinhart did not know about EBN at that time). EBN can also serve as a screen for n or 2n gametes in a cross, depending on the EBN of the parents (Carputo *et al*., 2003).

2.5.4 Meiotic abnormalities in hybrid situations

Meiotic abnormalities, including chromosome scattering throughout the cytoplasm, chromosomes lagging during anaphase-I and II leading to the formation of micronuclei and chromosome stickiness leading to formation of lumps etc., have been reported recently by Felismino *et al*. (2012). Miller (1963), Pandey *et al.* (1987), Zhang *et al*. (1999) and Sato *et al*. (2006) reported the effect of environment and genotype on the stability of meiotic process and inter-genomic pairing. Meiotic stability does not always lead to higher pollen fertility due to genic incompatibility (Felismino *et al*., 2012) but, on the other hand, Obute *et al*. (2006) reported that meiotic regularity is directly proportional to the pollen stainability.

The fate of unpaired chromosomes (univalents) during metaphase-1 can be either movement of the intact univalent to one pole, or precocious separation into sister chromatids and their consequent movement to opposite poles due to the bipolar spindle fibre attachment. Alternatively, if the sister centromeres remain fused together, anaphase bridges can occur, leading to misdivision or breakage across the pericentromeric regions of the chromosome. Lagging chromosomes often suffer breakage due to mis-division during meiosis and fail to become part of the daughter nuclei and so make micronuclei

(Ahuja *et al*., 2003; Gernand *et al*., 2006; Tu *et al*., 2009; Ishii *et al*., 2010) and sometime lagging chromosomes making bridges during anaphase-I lead to rearrangement of chromosomes after breakage (Ishii *et al*., 2010).

2.6.1 Use of cyto-molecular tools in the characterization of hybrids

Various morphological, cytological, cyto-molecular, and biochemical methods are commonly used for hybridity testing (e.g. Williams and Verry, 1981; Meredith *et al*., 1995; Roy *et al*., 2004; Tan *et al*., 2006; Tu *et al*., 2009). In *T. repens*, the white and red colour marks on leaves, controlled by dominant alleles (Brewbaker, 1955; Williams *et al*., 2008; Tashiro *et al*., 2010) have been used in identifying hybrids at an early stage, although their expression can be influenced by the environment (Tashiro *et al.,* 2010). Chromosome number and morphology can be also used for hybridity verification if the two parental species have different 2n chromosome numbers or the karyotypes of the two species are visually distinct and differentiable (Obute *et al*., 2006; Benavente *et al.,* 2008). The chromosomes of *T. ambiguum* and *T. repens* in a hybrid are not differentiated because of their karyotypic similarity especially during meiotic metaphase-I using conventional cytology (Chen and Gibson, 1971). However, in Giemsa-stained somatic metaphase spreads, *T. ambiguum* chromosomes can be differentiated from *T. repens* chromosomes, due to their larger size. Differentiation of chromosomes on the basis of size in hybrids between *T. nigrescens* and *T. occidentale* (Williams *et al*., 2008) and in different hybrids of cultivated rice (*Oryza sativa* L.) with different wild relatives (Tan *et al*., 2006) has been reported.

Recently there has been an enormous advancement in the techniques of monitoring alien genes/chromosomes in hybrids (Ceoloni *et al*., 1998). Genomic *in situ* hybridization (GISH) and fluorescent *in situ* hybridization (FISH) (Schwarzacher *et al*., 1989) are tools which are very helpful in the analysis of genomic composition, the nature of chromosome pairing during metaphase-I, presence of alien chromosomes, identification of specific chromosomes and exchange of chromatin involving transfer of chromosomal segments or genes in hybrids, backcross derivatives and polyploids (Thomas *et al.,* 1994; Pickering *et al*., 1997; Zhang *et al*., 2002; Devi *et al*., 2005; Tan *et al*., 2006; Yu *et al*., 2010; Zou *et al*., 2012; Jeridi *et al*., 2011). GISH is also an important tool for the study of evolutionary relationship among species (Tan *et al*.,

2006), which can help in planning future hybridization programmes for crop improvement (Meredith *et al*., 1995; Ansari *et al.,* 2008).

Fluorescently labelled 5S and 18S-5.8S-26S ribosomal RNA multigenic families used as probes have been used in many different crops for karyotypic analysis (Devi *et al*., 2005; Rosato *et al*., 2008) and it can be also helpful in identifying the parental genomes of putative hybrids because the numbers and chromosomal localizations of these sequences are often species specific (Ansari *et al*., 1999, 2008). Ansari *et al*. (2008) concluded that *T. dubium* is an allotetraploid species based on evidence derived from FISH using these rDNA sequences and GISH using genomic DNA from the putative progenitors as labelled probes. Variation in the signal size of 5S rDNA probably indicates variation of copy number (Li *et al*., 1997) and can also be used to differentiate 5S bearing chromosomes from *T. repens* and *T. occidentale* in a hybrid due to their different copy numbers (Ansari *et al*., 1999).

The labelled DNA which is used as a probe can also be the whole genomic DNA from one of the putative progenitors of a polyploid crop or one of the parents of a hybrid under study. The basic requisite for a GISH experiment is that the genome of the species used as the probe should be sufficiently divergent in its DNA sequence from the alternative genome that it will differentiate from (Markova and Vyskot, 2009). In cases of close genetic relationships between two species, differentiation can be improved by using excessive amounts of DNA of the species other than the one used as probe as blocker (Anamthawat-Jonsson *et al*., 1993; Devi *et al*. 2005). The blocking DNA hybridizes to the common DNA sequences of the two species, thus leaving only species-specific DNA sequences for the probe DNA to hybridize with. DNA probes are usually directly labelled by incorporating nucleotides having fluorescent dyes emitting different wavelengths which can be detected by fluorescence microscopy (Devi *et al*., 2005).

GISH using genomic DNA from two or three species as probes labelled with different fluorescent dyes can simultaneously differentiate each genome in natural or synthetic polyploids (Herrera *et al*., 2007). Meredith *et al*. (1995), using labelled genomic DNA of *T. ambiguum,* determined the composition of a BC_1 hybrid (AARRRR). Using the total genomic DNA of the putative progenitors species as labelled probes, the genomic compositions of the allotetraploid species, *T. dubium* and *T. repens* were determined (Ansari *et al*., 2008; Williams *et al*., 2012).

GISH can only unambiguously differentiate the genomic composition of hybrids and allopolyploid species if the genomes share 80% - 85% or less sequence homology (Schwarzacher *et al*., 1989; Tan *et al*., 2006). Anamthawat-Jonsson *et al*. (1993) reported that if GISH fails to differentiate one genome from the other, even in the presence of increased blocking DNA, it indicates that the two species share a higher level of sequence homology in their genomes. The signals resolution in GISH becomes very low in case of multi-colour GISH and in case of GISH on polyploid species, it is necessary to know at least one of the putative progenitors species (Devi *et al*., 2005). Sometimes, GISH is unable to visualise a very small piece of introgressed alien DNA (Humphreys and Pašakinskiene, 1996; Kosmala *et al*., 2007; Tu *et al*., 2009). Sometime it is a challenge to get a good meiotic chromosome spread which can be used successfully for FISH studies because cytoplasmic impurities can block the access of the probe DNA to the chromosomes and at the same time causes background noise (Jeridi *et al*., 2011). Sometimes, GISH hybridization signals are not evenly distributed and are confined to the pericentromeric regions as reported by Ansari *et al*. (2008). This phenomenon can be partially attributed to accumulation of repeat DNA sequences in the regions on or around the centromere (Lysak and lexer, 2006; Wang *et al*., 2010). Cross hybridization occurs in the regions of repeat sequences which are highly conserved between species and this is usually stronger as compared to the unique sequences (Ansari *et al*., 2008; Benavente *et al.,* 2008; Wang *et al*., 2010).

2.7.1 Genomic consequences of inter-specific hybridization and chromosome doubling

Hybridization followed by chromosome doubling brings about extensive genomic changes (Kovarik *et al*., 2005; Hegarty *et al.,* 2006; Rapp and Wendel, 2005) which occur either from changes in DNA sequences or epigenetic alterations (Salmon *et al*., 2005). Extensive research, using various genomic approaches, is investigating these genomic changes (Salmon *et al*., 2005) and the underlying mechanisms are still not clearly understood (Chen and Ni, 2006). These changes include translocation and activation of transposons, multiplication and/or deletion of parental DNA sequences, gene repression and silencing (changes in gene expression), DNA methylation, histone modifications, and tissue specific differential expression of some homoeologous genes (Song *et al*., 1995; Liu *et al*., 1998; Matzke *et al*., 1999; Chen and Ni, 2006). These changes might have important phenotypic consequences (Madlung *et al*., 2002; Baack

and Rieseberg, 2007) leading, for example, to the enhanced adaptive potential of the newly formed polyploids (Wendel and Doyle, 2004). McClintock (1984) termed the genomic changes associated with hybridization and chromosome doubling as '*genomic shock'* while Adams and Wendel (2005) used the term '*transriptomic shock'* for changes in the expression levels of genes. Wang *et al.* (2006) and Hegarty *et al*. (2006), studying genomic change in *Arabidopsis* and *Senecio* hybrids, attributed these changes to the event of inter-specific hybridization rather than polyploidization. On the other hand, Comai (2000) and Shaked *et al*. (2001) reported molecular evidence in support of the involvement of both hybridization and genome doubling in causing changes in DNA sequences and gene expression.

New hybrids/polyploids must establish a compatible relationship between an alien cytoplasm and nucleus and between two divergent nuclear genomes. This entails large scale genomic (genetic and epigenetic) changes. Chen (2007) reported that in polyploid hybrids many homoeologous genes may be co-expressed, but some duplicate genes are lost, mutate or diverge due to genetic change, while epigenetic changes reprogramme gene expression patterns. The impact of these genomic changes on hybrids can vary. The common phenomena are, elimination of non-coding repetitive sequences (e.g. in wheat, Shaked *et al*., 2001 and in *Tragopogon*, Tate *et al*., 2006), translocation and transposon activation (e.g. in *Brassica,* Song *et al*., 1995) and changes in duplicate gene expression (e.g. in *Arabidopsis* and cotton, Adams *et al*., 2003). Chen and Ni, (2006) reported that genomic changes observed in various allopolyploids may be aimed at overcoming meiotic abnormalities because they lead to diploidization. Examples include the evolutionary loss of NOR DNA from the chromosome pair contributed by one of the parental species in allotetraploid speices, as reported in *T.repens (*Ansari *et al*., 1999*), Glycine* (Joly *et al*., 2004) and *Medicago* (Rosato *et al*., 2008). Alternatively, condensation of the NOR sequences from one parent and de-condensation from the other parent in polyploid hybrids (nucleolar dominance, Pikaard, 1999) has also been reported in *T. dubium* (Ansari *et al*., 2008) and *Glycine* (Joly *et al*., 2004) and this phenomenon is reported to enhance diploidization in polyploids (Ansari *et al*., 1999, 2008) leading to higher meiotic stability (Weiss-Schneeweiss *et al*., 2007; Rosato *et al*., 2008). Williams *et al*. (2011) also reported the condensation of the *T. ambiguum*-derived NOR in a 3x AOO hybrid derived from backcrossing of an AO hybrid (2x *T. ambiguum* (AA) x 2x *T. occidentale* (OO)) with *T. occidentale* (OO), while the *T. occidentale* derived NORs

were de-condensed and so transcriptionally active. Wang *et al*. (2004) reported in *Arabidopsis* that the overall level of fertility in hybrids and allopolyploids improves after each generation of selfing, which suggests that genomic incompatibility is gradually resolved probably due to genomic changes on a wide scale, leading to diploidization. Epigenetic changes, including DNA methylation and protein (histone) modification are considered responsible for bringing about condensation (non transcription of genes) and de-condensation (transcriptional activity of genes) in the rDNA sequences (Appels *et al*., 1986; Suja *et al*., 1997; Volkov *et al*., 2007).

Hegarty *et al*. (2008) reported that hybridization in *Senecio* largely resulted in non additive changes in gene expression, leading to novel phenotype variation such as hybrid vigour. Shaked *et al.* (2001) while studying F_1 hybrids and their respective allopolyploids in wheat using amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995) and methylation sensitive amplification polymorphism (MSAP) (Xiong *et al*., 1999) reported that sequence elimination was the most frequent genomic rearrangement. The disappearance of bands in wheat was not associated with the appearance of new ones so this phenomenon could not be just associated with heterozygosity of parents or methylation changes (Song *et al*., 1995; Shaked *et al*., 2001; Ozkan *et al*., 2001). Xiong *et al*. (1999), using MSAP analysis, reported that most of the bands affected by methylation in a rice hybrid were from the genome of one parent. Similar findings were reported by Salmon *et al*. (2005) in *Spartina.* Methylation changes affected 4.1% of the parental fragments in a rice F_1 hybrid (Xiong *et al*., 1999), 6.9% in a wheat hybrid (Shaked *et al*., 2001), 8.3 % in a re-synthesized *Arabidopsis* allopolyploid (Madlung *et al.*, 2002) and with the highest being 30% of the parental fragments in *Spartina* (Salmon *et al*., 2005). These findings suggest that there may be differences between genomes in their ability to be modified in hybrid and allopolyploid backgrounds.

Contrary to the rapid genomic changes in *Brassica* and wheat allotetraploids (Song *et al.,* 1995; Shaked *et al*., 2001), synthetic cotton allotetraploids have shown few changes in genomic sequences. These findings show that as compared to wheat and *Brassica*, cotton genomes have greater tolerance of hybridization and chromosome doubling (Chen and Ni, 2006). Wang *et al*. (2006), while comparing *m*RNA abundance in an allopolyploid *Arabidopsis* with the mid-parental value of progenitors (MPV: an equal mixture of mRNA from the two parents), reported that 8% of the genes were non-additively expressed in the allopolyploids and, among these genes, around 68 % were

non-additively expressed between the two parents. This suggests that the genes which are differentially expressed in the progenitors are subjected to expression changes in the allopolyploids. The genes coming from *A. thaliana* were mostly repressed while those coming from *A. arenosa* were transcriptionally dominant in the allopolyploids. This was consistent with the phenotypic and nucleolar dominance of *A. arenosa* in the allotetraploids (Pikaard, 1999).

2.7.2 Gene dosages and expression levels

Guo *et al*. (1996) and Auger *et al*. (2005) reported that the expression level of different homoeologous genes in maize hybrids was affected by the genome dosage, indicating positive correlation of expression levels of genes in allopolyploids or hybrids with the dosage of the genes. Gene dosage effects on the phenotypic expression of characters were also reported by Anssour *et al*. (2009) and Stupar *et al*. (2007) in *Nicotiana* and potato respectively. In allopolyploids, both copies of the homoeologous genes may be expressed if the dosage effect is advantageous (Thomas *et al*., 2006) or one copy may be mutated to evolve a novel function (neo-functionalization) (Lynch *et al*., 2001) or both the copies may diverge their expression patterns in different organs via sub-functionlization (Lynch and Force, 2000). The expression changes are not confined to specific categories of genes and involve a wide variety of transcripts (Hegarty *et al*., 2006) and they generally belong to important biological pathways for metabolism, energy, cell defence, signalling, aging and plant hormonal regulation (Chen and Ni, 2006).

2.7.3 Advantages of genomic changes

Immediate changes in the expression of homoeologous genes after hybridization and polyploidization (including changes in DNA sequence, *cis* and *trans*-acting effects, chromatin modifications, RNA-mediated pathways, regulatory networks changes) create an inexhaustible genetic novelty and phenotypic variation on which natural selection works, leading to the adaptation of hybrids and polyploids to new environments (Grant, 1971; Stebbins, 1971; Masterson, 1994; Ramsey and Schemske, 1998; Chen and Ni, 2006; Hegarty *et al*., 2006; Chen, 2007). The available data suggest that the gene expression variation triggered by hybridization often exceeds the parental range (Birchler *et al.,* 2003). Lai *et al*. (2006) reported transgressive (up regulated) patterns of parental gene expression in the homoploid sunflower hybrid (*Helianthus deserticola*),

that was potentially important for drought tolerance, and so made this hybrid adaptable to extreme arid conditions in which neither of its parents, *H. annuus* and *H. petiolaris*, could survive. This shows that changes in gene expression in new hybrids and allopolyploids may help in their adaptation to a new environment that is not accessible to the parents. Similar findings were reported by Riddle and Birchler (2003) and Salmon *et al*. (2005). Moreover, polyploids can tolerate larger genetic changes due to compensation effect as compared to diploid species, which cannot tolerate huge genetic changes (Du *et al*., 2008).

2.8.1 Flow cytometric analysis of DNA content in hybrids

Currently the two methods that are used for DNA content/ploidy analysis are Feulgen densitometry and flow cytometry with the latter being more convenient, and so more popular. The DNA flow cytometry method involves the preparation of an aqueous suspension of intact nuclei from young leaves (Dolezel *et al*., 1998) whose DNA is stained with a DNA-specific fluorochrome, either *Propidium iodide* (PI) which intercalates into double-stranded DNA or 4', 6- diamidino-2-phenylindole (DAPI) which binds at AT-rich regions of DNA. The amount of fluorescent light emitted by each nucleus, usually displayed in histogram form, is quantified and shows the DNA content in the sample (Dolezel and Bartos, 2005). As flow cytometry analyses relative fluorescence intensity and so relative DNA content, the genome size of an unknown sample can be determined only after a comparison with nuclei of reference standards with known DNA size (Emshwiller, 2002; Dolezel and Bartos, 2005). Flow cytometry has been used frequently for the analysis of ploidy level in various crops (Lysak and Dolezel, 1998; Brummer *et al*., 1999) because the knowledge of the ploidy level of a crop and its wild relatives is crucial for their potential use in future breeding programmes (Emshwiller, 2002). However, the use of this technique for the determination of hybridity is only appropriate when species used in a cross differ in their cell DNA content (Marasek *et al*., 2006). Tan *et al*. (2006) while measuring the size of nuclear genomes in different diploid *Oryza* species through flow cytometry and image analysis demonstrated that the total chromosome length was well correlated with the nuclear DNA content.

Plant nuclear genomic DNA content varies enormously (Bennetzen *et al*., 2005) and in angiosperms, interspecific DNA content variation can be more than 800-fold (*Fritillaria*

assyriaca, 1C= 127.4 pg, as compared to *Arabidopsis thaliana*, 1C= 0.165 pg) (Bennett and Leitch, 1997; Bennett *et al*., 2000). Sometimes, the DNA content within the same species is surprisingly divergent (Evans *et al*., 1966; Bennett and Leitch, 1995, 1997; Rayburn *et al*., 1997). The existence and extent of this intra-specific variation in DNA content is difficult to explain (Greilhuber and Obermayer, 1997). Price and Johnston (1996) reported quantitative changes in genomic DNA in an organism in response to environmental or developmental stimuli and this concept was called "the plastic genome".

No significant intraspecific DNA content variation was reported among different varieties of *Allium cepa* L. collected from different geographical locations (Bennett *et al*., 2000), in different cultivars of *Hordeum vulgare* L. and *Vicia faba* L. (Bennett and Smith, 1976), or among cultivars of *Pisum sativum* (Greilhuber and Ebert, 1994) . However, Besnard *et al*. (2008), using flow cytometry, reported intra-specific variation in genome size in the Olive complex (*Olea europaea*). Greilhuber (2005) reported that DNA content variation of small magnitude may be technique related. However, Piegu *et al*. (2006) attributed minor differences in genome size existing between different geographic populations of *Oryza australiensis*, a wild relative of cultivated rice, to the presence of a variable content of repeated elements such as tandem repeats or transposable elements. Kubis *et al*. (1998) and Sanmiguel and Bennetzen (1998) reported that molecular mechanisms are now known which can generate variation in genome size. Because of intraspecific variation in DNA content, the error control in sample preparation and analysis becomes more important (Dolezel *et al*., 1998; Dolezel and Bartos, 2005).

2.9.1 Control of chromosome pairing and the role of the wheat *Ph1* gene

Multivalent formation at metaphase-I in polyploids can lead to irregular disjunction of chromosomes and the production of aneuploid gametes leading to sterility (Naranjo and Corredor, 2004). According to Majumdar *et al*. (2004) when two genomes in an allopolyploid are divergent, that is, when they have little homology, the pairing of chromosomes in meiosis will be regular and only bivalents will be formed at metaphase-I. Such an allopolyploid will be fertile and stable both genetically and cytologically (Stift *et al*., 2008). Contrary to this situation, if the two genomes are very closely related, then

multivalent formation will be frequent, and this will usually lead to partially fertile hybrids with cytological and genetic instability (Ramsey and Schemske, 2002).

Bread wheat, *Triticum aestivum* (AABBDD) and durum wheat, *Triticum durum* (AABB) have genomes which are genetically related (homoeologous). Consequently, the loss of chromosome segments or whole chromosomes, as in monosomics and nullisomics, can be tolerated (Naranjo and Palla, 1982) and this has allowed the detection of genetic systems controlling chromosome pairing (Okamoto, 1957; Riley and Chapman, 1958). The donor of the A genome is *Triticum urartu* Tumanian (Dvořāk *et al*., 1993). The other two genomes, B and D, were derived from *Aegilops speltoides* Tausch (Sarkar and Stebbins, 1956; Wang *et al*., 1997; Dvořāk, 1998) and *Aegilops tauschii* Coss (McFadden and Sears, 1946), respectively. So in wheat, in addition to homologous pairing, there is a strong possibility of homoeologous pairing. But, unexpectedly, wheat makes only bivalents involving homologues (Sears, 1976; Al-Kaff *et al.,* 2008). This is evidence that it has evolved a genetic system restricting chromosome pairing to between homologues only (Nicolas *et al.*, 2009). A gene designated as *Ph1,* located on the long arm of chromosome 5 of the B genome, is a locus which plays a major role in ensuring diploid-like behaviour in wheat by suppressing pairing between homoeologous chromosomes (Riley and Chapman, 1958; Sears and Okamoto, 1958; Chen *at al*., 1991; Naranjo and Corredor, 2004). Rajhathy and Thomas (1972) and Jauhar (1977) have reported a genetic control mechanism for chromosome pairing in allohexaploid oat as well. *Ph1* ensures genomic integrity and cytogenetical and reproductive stability in wheat (Jauhar, 2006) but, on the other hand, it minimises the chances of inter-genomic recombination (Sears 1976; Gillies, 1987; Martinez-perez and Moore, 2008). Miller *et al*. (1994) reported the presence of genetic systems in other related species which suppressed *Ph1* and could be used to promote homoeologus chromosome pairing when introduced into inter-specific hybrids (Sears, 1976; Jauhar, 1992). For example, Sears (1976), Jauhar (1991b) and Chen *el al.* (1991) reported that *Agropyron cristatum* (4x) had a genetic system that inactivated *Ph1* in its hybrids with wheat and so allowed a high frequency of homoeologous pairing. Alternatively, homoeologous pairing can be achieved if *ph1b/ph2b* mutants (Sears, 1976) or a 5B deficient genetic stock (Jauhar and Almouslem, 1998) are used.

2.9.2 Mechanism of action of the *Ph1* gene

The mode of action of the *Ph1* locus is still uncertain and will remain so until the isolation and the determination of its product (Mikhailova *et al*., 1998). Riley (1960) suggested that *Ph1* reduced the long-range pairing forces that brought chromosomes together in meiotic prophase. Since the attractive forces between homoeologues could be supposedly lesser than those between homologues, the reduced pairing forces were assumed to be no longer able to bring homoeologues together, although still able to unite homologues. The most plausible explanation for the mechanism of action of *Ph1* gene was given by Feldman (1966). He obtained plants which were tri-isosomic for 5BL, which therefore had six doses of *Ph1*. These plants were partially asynaptic, demonstrating that the *Ph1* locus can suppress pairing not only between homoeologues but also between homologues. He also reported that in somatic cells the chromosomes did not lie at random as was believed, but the homologues were already lying side by side and therefore did not have to find each other from a distance. Schwarzacher *et al*. (1989) also reported the presence of different parental genomes in separate domains inside the nuclear membrane. In the absence of *Ph1*, homoeologues also associated somatically and competed with homologues in pairing; but when *Ph1* was present, somatic association was suppressed to the extent that homoeologues no longer lay together but homologues still did. With six doses of *Ph1*, even homologues no longer associated somatically; with the result that all the chromosomes were distributed randomly (Sears, 1976) allowing the homoeologues to pair as well.

Aragon *et al*. (1997) and Prieto *et al*. (2004, 2005) reported that *Ph1* is associated with initiation and co-ordination of chromatin remodelling during the onset of meiosis which enable the homologues to pair. *Ph1* controls this chromatin remodelling at the onset of meiosis (Colas *et al*., 2008). *Ph1* is involved in the initiation of this remodelling, and in co-ordinating the remodelling of chromatin on both homologues so that they are in the same conformation at the onset of pairing (Prieto *et al*., 2004). In the absence of *Ph1*, the chromatin remodelling can be initiated asynchronously and prematurely so that homologues are in different conformation states at the onset of meiosis (Prieto *et al*., 2004). Thus a chromosome would be just as likely to pair with a related chromosome (homoeologue) as with its true homologue (Colas *et al*., 2008).

Martinez-Perez *et al*. (2000) in his hypothesis of correction reported that the presence of *Ph1* enables discrimination and separation of non-homologous (homoeologous) centromeres which pair in its absence. They also postulated that pairing correction, which is under the control of *Ph1* locus, might be connected with different rates of condensation of homologous and homoeologous chromosomes. Pairing between homologues within the chromosome cluster can be more stable then pairing between homoeologues. Thus homologous pairing initiated at one chromosome end and confirmed at the multicentromere structure can proceed and be completed. By contrast, synapsis initiated between homoeologues and interpreted as wrong at the centromeres is immediately corrected. Jauhar (1992) reported that it is not yet known that how much sequence divergence is required between the homoeologous chromosomes for *PhI* suppressor to operate on.

Riley and Law (1965) reported that *Ph1* suppresses homoeologous pairing in a polyploid situation having both homologous and homoeologous chromosomes, but in a specific situation where only homoeologues are present it cannot prevent homoeologous pairing. In wheat hybrids where the opportunity for homologue pairing does not exist, homoeologues synapse and in that case the presence or absence of *Ph1* does not matter (Gillies, 1987).

2.9.3 The significance of *Ph1* in cytogenetics, breeding and evolution

Genetic pairing regulation has important implications in cytogenetics, evolution and plant breeding (Jauhar, 1975c). Prevention of homoeologous chromosome pairing in polyploids can ensure diploid-like behaviour which leads to good fertility (Jauhar, 2003b). The *Ph1* locus is hemizygous-effective in *durum* and bread wheat (Jauhar *et al*., 1999) but the equivalent locus is hemizygous-ineffective in hexaploid tall fescue (Jauhar, 1975a, b). The hemizygous ineffectiveness of *Ph1* in hexaploid tall fescue and other polyploids allows gene flow between species (Jauhar 1975d) which is also important from a crop breeding point of view.

2.9.4 Lack of chromosomes pairing leads to sterility

Inter-specific hybrids are sometimes viable but sterile; the sterility may result from genic and/or chromosomal effects (Stebbins, 1958). Sterility of the latter type occurs when genomes are so diverged that chromosomes from different species fail to pair and

recombine, leading to abnormal assortment at meiosis (Canady *et al*., 2006; Rick, 1951; Deverna *et al*., 1990). Canady *et al*. (2006) reported that homoeologous chromosome pairing is antagonized by homologous association as well as sequence divergence. Evidence from bacteria (Shen and Huang, 1986), Yeast (Datta *et al.,* 1996) and *Arabidopsis* (Li *et al*., 2006), has clearly demonstrated that recombination is strongly dependent on the degree of sequence similarity and can be inhibited by as little as a single nucleotide mismatch.

CHAPTER 3
MATERIALS AND METHODS

This research project was carried out in the Forage Improvement Section, Grasslands Research Centre, AgResearch, Palmerston North which provided the starting materials. These consisted of a diverse group of BAR (**B**ridge ***A****mbiguum* and ***R****epens*) hybrids (having a range of genomic contributions from *T. repens*, *T. ambiguum* and *T.occidentale*) and a range of white clover genotypes with coloured leaf markings. The original BAR hybrids used as starting material are given in the Tables 3.1, 3.2, 3.3. 3.4 and 3.5 along with their pedigrees, pollen fertility (%) and estimated ploidy levels (based on flow cytometry). Potted BAR hybrids were placed in an insect-free unheated glasshouse with temperature ranging from 15-28^{O}C under natural photoperiod.

3.1 Crossing Techniques

The first breeding cycle was initiated in December, 2008 and continued until the end of January, 2009. During this breeding cycle, as described by Williams and Verry (1981), five to 20 inflorescences at a suitable stage for each BAR hybrid were emasculated using fine forceps to remove the corolla and stamens. Pollination was then done manually on the same day with pollen freshly collected from different *T. repens* genotypes having dominant red colour leaf markings. This was done by removing the corolla from the flower being used as pollen donor parent and rubbing the anthers very gently on the exposed stigma of the female parent using fine forceps. This was done between 8-11am and was repeated on the following two consecutive days usually at the same time. Reciprocal crosses were also made. Selfing was done between 8-11 am by rolling gently 5-20 inflorescences per BAR hybrid between thumb and fingers and this practice was repeated on the same heads for 2-3 consecutive days. The same crossing techniques were used for selfing, inter-crossing and backcrossing BAR09 and BAR10 generation hybrids during the subsequent two breeding cycles of 2009-10 and 2010-11. Progeny seeds were harvested four to five weeks after pollination, usually after the peduncles turned yellowish. The flower heads were allowed to dry and then threshed between two pieces of corrugated rubber. Data on seeds per inflorescence, total number of seeds, and ratio of shrunken and normal seeds were recorded.

3.2 Self-compatibility (SC) testing techniques

Self-compatibility of all plants used as female parents was determined by using the simple techniques suggested by Williams (1987b). Five to ten inflorescences at a stage with at least 50% of florets fully open in each plant were rolled gently between thumb and fingers and this practice was repeated two to three days consecutively on the same heads.

3.3 Pollen stainability (%)

Pollen stainability (pollen fertility) of the original BAR hybrids and their derived progenies was estimated by counting the proportion (%) of full-sized and fully stained pollen grains in 2% (w/v) aceto-carmine at 100x magnification. For this purpose at least 300 pollen grains were scored from at least three florets from each hybrid plant.

3.4 Seed germination and initial screening of the progeny

In May, 2009, five to 30 progeny seeds (depending on the availability of seed) from each cross or selfing of the original BAR hybrids carried out during the first breeding cycle (December 2008 to January 2009) were scarified manually by gently rubbing them between a hard surface and P 120 sandpaper. Scarified seeds were put on moist filter paper in Petri dishes at room temperature for germination. After germination, the progeny of around 1,000 seedlings with two fully expanded green cotyledons were shifted to plastic pots 8cm in diameter containing potting mix (peat and sand in equal ratio) in a greenhouse under natural daylight without heating. Plants of this generation were labelled as BAR09 because they were bred and grown during 2009. After three weeks, preliminary observations on seedling characters were recorded on all of the progeny plants i.e. albinism, presence or absence of dominant red leaf marker from the male parent and stem type. After one month, the seedlings with 2-3 fully developed trifolioliate leaves were shifted to bigger pots with the same potting mix. The plants were watered as needed and fed once fortnightly in solution form with a commercially available complete nutritional supplement called "Yates Thrive®" (containing (w/w) 27 % nitrogen, 5.5 % water soluble phosphorus and 9.0 % potassium as nitrates plus other minor elements). One hundred and forty plants from the BAR09 generation were selected on the basis of preliminary morphological data as mentioned earlier for their flow cytometry based DNA content analysis, using *T. repens* as the reference standard.

At maturity, selected plants from this generation were used for a second breeding cycle from November 2009 to-January 2010. Further selection of 140 BAR09 plants for the next generation of breeding was based on flow cytometry based ploidy estimates, presence/absence of colour leaf alleles, morphology showing the combination of characters from both the species and vigour of the plants. Similarly, the seed obtained from the second breeding cycle of 2009-10 were germinated in May, 2010 and the progeny labelled as BAR10 hybrids. Selection among BAR10 progeny was done according to above mentioned criteria. These selected plants were used for the third cycle of breeding which was carried out in January, 2011. In this breeding cycle, a range of white clover genotypes with distinctive white leaf markings controlled by co-dominant alleles was used for hybridity verification in the progeny.

3.5 Flow Cytometry-based ploidy analysis

Samples of three young leaves were collected from all the plants, initially selected to be hybrids on the basis of plant vigour and leaf colour marker, and sent to Plant and Food Research, Lincoln, New Zealand, for DNA content and ploidy level analysis. The analysis was done using the method described by Otto (1990) and the detailed procedure was given in our paper on AO hybrids (Williams *et al*., 2011). But, briefly, a small quantity of leaf tissue was chopped finely in 400 *ul* of extraction buffer (2.0% (w/v) citric acid, 0.5% (v/v) Tween) and filtered through 30-um mesh (Partec filter). Before flow cytometry, 1.6 ml of staining solution was added (2 mg/ml DAPI in saturated dibasic sodium phosphate to give a final concentration of DAPI 2.5 ug/ml). To provide an unbiased relative measure of DNA content, all samples were co-chopped with leaf tissue of *Bellis perennis* as an internal standard and only readings with a co-efficient of variation of <3% were used to minimize experimental error. DAPI fluorescence was measured in a Partec PALL Flow cytometer with relative fluorescence compared with karyotyped control plants. Data were analysed using Flowmax software. Selection of plants for further analysis and use in subsequent breeding cycles was done on the basis of flow cytometry results.

3.6 Somatic chromosome preparation

For chromosome preparations, 2-3 month-old potted plants were used that had been shifted to 10 cm diameter pots and root-trimmed 8-10 days previously. Actively growing healthy-looking root tips (2 cm) were obtained either in the morning (7-9am) or evening

(5-6pm) (Williams 1978). These were thoroughly washed with distilled water under a stereomicroscope using a soft brush to remove dirt. The root tips were pre-treated with 0.003 M 8-hydroxyquinoline in petri dishes on filter papers at room temperature at around 22^{O}C for 2 hours in the dark and then in a refrigerator at 4^{O}C for 6-7 hrs. They were then washed three times in distilled water before they were fixed in 3: 1 (methanol: glacial acetic acid). The fixative was replaced twice at 10 minute intervals and then the vials with root tips in the freshly changed fixative were left overnight at room temperature. Next morning, again the fixative was changed using a freshly prepared mixture. Thereafter, the vials were sealed and placed in fridge at 4^{O}C until used. These root tips were used for somatic chromosome preparations using conventional Giemsa staining and for fluorescence *in situ* hybridization. The chromosome preparation procedure, was the flame drying technique (with some modifications), described by Ansari *et al*. (1999). Briefly, after removing the root cap, the translucent meristimatic part of each root tip was cut into 2-3 pieces of approximately 1-2 mm length. These were washed in citrate buffer (pH 4.8) followed by maceration in an enzyme cocktail having 2% (w/v) cellulase (1.6 % cellulase Calbiochem + 0.4% cellulase 'Onozuka' R 10) and 20% (v/v) pectinase (from *Aspergillus niger* in 40% glycerol, ICN # 156058) at 37^{O}C for 44 minutes in a water bath. After rinsing three times followed by incubation in the citrate buffer for at least 20 minutes, two pieces of macerated root tips were placed on a grease-free clean slide with a few drops of citrate buffer. The pieces were then dissected under a stereomicroscope to separate the core meristematic region from the peripheral tissue. Excess citrate buffer along with debris was removed very gently. The meristematic tissues were then broken into small pieces using the tip of a needle to form a droplet of cell suspension. A couple of drops of 48% acetic acid (AA) were then added to this cell suspension and the slide was left undisturbed for 2 minutes. Thereafter, a few drops of chilled fixatives (3:1, methanol : glacial acetic acid) stored at -22^{o}C were gently placed on the droplet of the cell suspension. The slide was brought to flame and the burning alcohol was immediately extinguished by giving a jerk to the slide so as to avoid overheating. Each slide was marked properly and screened under phase contrast optics for useful cells. Slides having at least 20 good quality relevant cells were used for fluorescence *in situ* hybridization.

3.7 Meiotic chromosome pairing analysis (conventional cytology)

Flower buds were collected in the morning (8-10am) or evening (5-7pm) when they were approximately 1/3 emerged from the calyx and fixed in Carnoy's solution, 6:3:1 (95 % ethanol/chloroform/glacial acetic acid, v/v/v) overnight at room temperature. After thorough washing in 70% ethanol to completely remove traces of chloroform, they were shifted to alcoholic hydrochloric acid carmine (Snow's solution, Snow, 1963) for 4-5 days. The floral buds were then washed thoroughly in 70% ethanol three to four times and stored in 70% ethanol in the refrigerator for later use. At the time of use, the anthers from a single floret at the relevant meiotic stage were squashed in 2% acetocarmine on grease-free slides and a cover slip was added after removing the anther debris. After adding a two drops of 48% acetic acid on the side of the cover slip, the slides were heated moderately on a flame and then slowly and gradually the cover slip was pressed with progressively increasing pressure between the two folds of filter paper so as to remove the acetocarmine dye and cell debris and bring the chromosomes to the same plane with a good spread. Slides were sealed with nail polish and studied under a compound microscope using phase contrast optics. Analysis was made of meiotic chromosome configurations during diakinesis/metaphase-I and of disjunction during anaphase I & II in pollen mother cells (PMCs). At least 40 PMCs from each genotype were used for recording the numbers of univalents (I), bivalents (II), trivalents (III), quadrivalents (IV) and pentavalents (V) per cell during metaphase-I, and for calculating the average frequencies. Data on chromosome disjunction during anaphase-I were also recorded.

3.8 Enzyme macerated meiotic chromosome preparation

Floral buds for meiotic chromosome preparations were collected either in the morning or evening, as previously described, and fixed in ethanol: glacial acetic acid (3:1, v/v). The floral buds were left in fixative overnight at room temperature after two changes at 10 minute intervals. After refreshing the fixative once again next morning with a freshly prepared mixture, the floral buds were stored at -22 ^{O}C for later use. Whirls of florets with PMCs at metaphase-I and anaphase-1 were carefully located along the axis of the inflorescences by taking florets and checking them under a phase contrast microscope using acetocarmine squash preparations. Anthers from florets with PMCs at an appropriate stage were first treated with an enzyme cocktail (2% (w/v) cellulase (1.6 %

cellulase Calbiochem + 0.4% cellulase 'Onozuka' R 10) and 20% (v/v) pectinase (from *Aspergillus niger* in 40% glycerol, ICN # 156058) at 37°C for 45 minutes. Then after three or four rinses in citrate buffer, around 10 enzyme treated anthers were crushed in a citrate buffer droplet on a grease-free slide to get the PMCs out. Two drops of 60 % acetic acid were added after removing the anther sacs and other supporting tissues with fine forceps. The slide with PMCs suspended in acetic acid was left for 2 minutes. A cover slip was added and, after adding a few more drops of 60 % acetic acid around the cover slip, it was gradually pressed. The slide was then dipped slowly in liquid nitrogen for freezing. The cover slip was then removed very carefully with the help of a blade. The slide was dipped twice into 70 % ethanol and then air dried. The quality of the slides was assessed under phase contrast optics and the best slides with at least 15 relevant cells were used for chromosome pairing analysis using genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH).

3.9 Giemsa staining.

Four slides for each selected BAR09 and BAR10 hybrid were stained conventionally in 5% Giemsa solution diluted with Sorensen's buffer at pH 6.8 for 18 minutes. After that the slides were washed gently with tap water and air dried. The slides were then mounted with oil and, at least 40 metaphase cells were photographed at 60x oil immersion objectives and studied for somatic chromosome counts and morphology in each BAR09 and BAR10 hybrid.

3.10 Molecular cytogenetic analysis of hybrids

3.10.1 DNA probes and labelling for FISH and GISH

Two DNA probes were used, i.e. total genomic DNA of *T. ambiguum* labelled with Fluor-X-dCTP (Amersham Pharmacia) (nucleotides attached with fluorescein) (green) and Cy3-dCTP labelled pTr5S (Amersham Pharmacia) (red) (Gen-Bank accession AF 072692), a 596-bp long fragment from *T. repens* representing a part of the 5S ribosomal RNA gene family. For each double colour GISH experiment, the probes were individually labelled with direct fluorochrome-labelled nucleotides Cy3-dCTP or FluorX-dCTP (Amersham Pharmacia) by nick translation according to the manufacturer's specifications. The total genomic DNA from *T. repens* and *T. occidentale* was isolated from young leaves using a modified CTAB protocol of Doyle

and Doyle (1987) and was used as competitor or blocking DNA after shearing it into small fragments of size 100 – 300bp by heating.

3.10.2 Fluorescence *in situ* hybridization

Combined GISH and FISH experiments were carried out, using Cy3-dCTP labelled pTr5S and Fluor-X-dCTP labelled genomic DNA from *T. ambiguum* as probes, on the mitotic metaphase chromosome preparations of the selected BAR09 and BAR10 hybrids. The procedure for hybridization, post hybridization stringent washing and counter staining with 4', 6-diaminodino-2-phenylindole (DAPI) was followed as described by Ansari *et al*. (1999) with some minor modifications. For this experiment, already screened slides that were a few days old (two slides per genotype) with comparatively high mitotic index were used. They were treated with 0.1 *ug/u*l RNase in 2x SSC for 55 minutes at 37^{O}C in a humid chamber. Thereafter, the slides were washed three times for one, five, & four minutes respectively in 2x SSC, fixed in 4% paraformaldehyde for 10 minutes at room temperature and dehydrated through a series of alcohol (ethanol) concentrations followed by air drying. Before hybridization, chromatin denaturation was carried out at 72^{o}C in 70% formamide-2xSSC (0.3 M sodium chloride, 0.03 M sodium citrate) for two minutes and the material quickly dehydrated through an ice-cold ethanol series (70%, 90% & 100%) and then air dried for 10-20 minutes at room temperature.

A 20 *u*l aliquot of hybridization mixture containing 2.8 and 0.91 *ng/u*l of each probe (genomic DNA of *T. ambiguum* and 5S rRNA), 50 % formamide, 20% dextran sulphate, 0.5% SDS in 2x SSC and 10 and 40 times excess of sheared unlabelled DNA of *T. occidentale* and *T. repens* respectively, was heat denatured for 10 minutes at 90 ^{O}C. After chilling on ice for a few minutes the mixture was applied to an already marked area on each slide and then coverslips made from polythene disposable waste bags were added. One slide from each hybrid underwent another denaturation in a thermocycler (Techne PHC-3) for two minutes at 72^{O}C after which the temperature gradually came down to 37 ^{O}C and then the hybridization was allowed to go on at 37 ^{O}C for 30 minutes before the slides were shifted to a humid chamber. The other slide from each hybrid was transferred directly to the humid chamber set at 37 ^{O}C without putting in the thermocycler for a second denaturation. The hybridization was carried out overnight (almost 20 hours) in the humid chamber at 37 ^{O}C. Next morning the cover slips were removed carefully from the slides and then post hybridization washing was done at 42^{0} C

using two changes of 2xSSC for 5 minutes, one change of 50 % (v/v) formamide in 2xSSC for 10 minutes and then again two changes of 2xSSC for 5 minutes. After cooling of the jar for 10 minutes, slides were rinsed in 2xSSC and 4xSSC/Tween 20 at room temperature. The chromosomes were counterstained in 4',6-diaminodino-2-phenylindole (DAPI) and then mounted in Vectashield (Vector Laboratories, USA) under a glass coverslip. Slides were then studied under a Nikon Microphot-SA epifluorescence microscope. The images were taken using an AxioCam MRm CCD camera (Carl Zeiss, Germany) attached to the microscope and processed with ISIS imaging software (MetaSystems, Germany). The individual photographs were later customized for best contrast and brightness by using Adobe Photoshop software.

The procedures for hybridization, post hybridization stringent washing and counter staining with DAPI in GISH/FISH on mieotic chromosome spreads followed the protocol of Ansari *et al*. (1999) except for one additional step of pepsin treatment. After RNase treatment, the slides were incubated in 10 *u*M HCl at room temperature. Thereafter, 180 *ul* of working solution of pepsin (5*ug* of pepsin/*ul* of 10 Mm of HCL) was added to each slide and covered with a plastic coverslip. Slides were incubated at 37^{O}C for 30 minutes in the humid chamber so as to digest and remove the proteinaceous background caused by the cytoplasm density of PMCs. After pepsin treatment, the slides were twice washed with 2xSSC involving two changes of 2xSSC for five and four minutes respectively. The rest of the process was carried out as given for GISH/FISH on mitotic chromosome preparations.

At least 40 PMCs were analysed from each selected BAR hybrid to examine, in particular, the behaviour of *T. ambiguum*-derived chromosomes.

3.11 Morphological characterization of BAR09 and BAR10 hybrids

For morphological characterization of BAR09 and BAR10 hybrids in the field, experiments containing selected progeny plants along with the original BAR hybrids and genetically colour marked white clovers (as controls) were planted on 5-12 May, 2010 and 06-08 June, 2011. For these experiments, clonal cuttings were obtained from the plants selected to be included in the experiment. Care was taken that the cuttings were of the same size and physical condition. Each cutting consisted of 3-4 cm of stem with one active growing point and one main root with three fully opened trifoliolate leaves. Initially these were planted in plastic pots of 8 cm diameter filled with 1:1 sand and peat

potting mix and placed to establish in a glasshouse with natural day length and a temperature range of 15-25 ^{O}C. The pots were watered as needed and a complete soluble fertilizer (Yates Thrive®) was applied fortnightly to maintain soil fertility. After one month they were shifted to a sandpit with a depth of 45 cm where the experiment was laid out in a randomized complete block design (RCBD) with four replicates. Each plant was allocated an area of 0.36 m^2. They were watered daily, usually in the evening, and fed with a complete soluble commercially available plant nutrient supplement (Yates Thrive®) on a weekly basis at the rate of 250 ml per plant. Morphological observations on experiments containing BAR09 hybrids were recorded on the above-ground qualitative and quantitative traits in January, 2011. During May, 2011, this experiment was harvested destructively by digging up the plants and data were recorded on above and under-ground components of the plants. Similarly, data on different morphological characteristics of BAR10 progenies were recorded during January and February, 2012. Following destructive harvest in January, 2012, plant components were oven-dried at 80 ^{O}C for 48 hours and then the dry weights of above-ground parts, root system and total biomass were recorded using an electronic scale. The traits on which data were collected are given below:

Stem type (whether stoloniferous or not) (SToNST), i.e. rooting at the nodes or not

Stolon number per plant (SN)

Average stolon length based on five randomly selected stolons (cm) (ASL)

Stem anchorage on the scale of 0-10 (0=no nodal rooting at all, 10 nodal rooting from every node as in white clover)

Number of inflorescences per plant (NI)

Growth habit, whether determinate or indeterminate (GH)

Florets/inflorescence (head) (F/H)

Average peduncle length based on five randomly selected peduncles (cm) (APL)

Leaflet shape (length/width ratio) (LS)

Average petiole length (cm) (APL)

Average stolon thickness (cm) (ST)

Flowering, whether axillary, terminal or both (FATC)

Seeds/Inflorescence (head) (S/H)

Main root thickness immediately under soil surface (cm) (MRT)

Main nodal root thickness adjacent to the point of attachment (cm) (MNRT)

Above ground dry weight (gm) (AGDW)

Root dry weight (gm) (RDW)

Total biomass (gm) (TBM)

Root weight % of the total biomass (RW%TBM)

3.12 Analysis of variance, ANOVA

Analysis of variance (ANOVA) was carried out by using Genstat 12th edition (Payne *et al*., 2010).

Table 3.1 Original 4x hybrids with genomic formula RRAO used in strategy-1 based on using *T. occidentale* as a genetic bridge with estimated ploidies according to flow cytometry.

Plant	Pedigree	Expected chromosome composition	Ploidy (x)
119	(RO x BL)-1	RRRR x AAOO= RRAO	3.9
122	(RO x BL)-5	RRAO	3.9
123	(RO x BL)-6	RRAO	3.8
124	(RO x BL)-7	RRAO	3.6
125	(RO x BL)-8	RRAO	3.9
136	(RO x BL)-19	RRAO	3.9

RO: Stands for Red One, a coloured white clover (RRRR).

Table 3.2 Original 5x hybrids with genomic formula AAAOO used in strategy-2 based on using*T.occidentale* as a genetic bridge with pollen fertility and flow cytometric ploidy estimates.

BAR #	Pedigree	Expected genomic composition	Pollen Fertility (%)	Ploidy (x)
100	(434-1 x BN)-1	AAO x AAOO= AAAOO	83	5.2
101	(434-1 x BN)-2	AAO x AAOO= AAAOO	DNF	5.0
102	(434-1 x BN)-3	AAO x AAOO= AAAOO	64	4.9
103	(434-1 x BN)-4	AAO x AAOO= AAAOO	89	4.9
104	(434-1 x BN)-5	AAO x AAOO= AAAOO	63	5.0
105	(434-1 x BN)-6	AAO x AAOO= AAAOO	DNF	4.9
106	(434-1 x BN)-7	AAO x AAOO= AAAOO	68	4.8
107	(434-1 x BN)-8	AAO x AAOO= AAAOO	89	4.8
108	(434-1 x BN)-9	AAO x AAOO= AAAOO	82	4.9
109	(434-1 x BN)-10	AAO x AAOO= AAAOO	64	4.9
110	(434-1 x BL)-2	AAO x AAOO= AAAOO	46	4.9
111	(434-1 x BL)-3	AAO x AAOO= AAAOO	64	4.8
112	(434-1 x BL)-5	AAO x AAOO= AAAOO	21	4.6
115	(434-1 OP-4 x KOPCRU-1)-1	AAAAOO x RRRR=AAORR	0	5.6

DNF. Did not flower

Table 3.3 Original near-5x RRRA(A_4O_4) and 4.5x RRR($A_6R_4O_2$) hybrids used in strategy-3 based on using *T. occidentale* as a genetic bridge with male fertility and flow cytometry based ploidy estimates.

BAR #	Pedigree	Expected genomic composition	Pollen Fertility (%)	Ploidy (x)
58	(33 OP-1 x (PxB-1))-12	AAAORR x RRRR= RRRA(A_4O_4)	DNF	5.3
59	(33 OP-1 x (PxB-1))-13	AAAORR x RRRR= RRRA(A_4O_4)	41	5.0
60	(33 OP-1 x (PxB-1))-14	AAAORR x RRRR= RRRA(A_4O_4)	DNF	5.2
61	(33 OP-1 x (PxB-1))-15	AAAORR x RRRR= RRRA(A_4O_4)	24	5.2
62	(33 OP-1 x (PxB-1))-17	AAAORR x RRRR= RRRA(A_4O_4)	55	4.9
64	(33 OP-1 x (PxB-1))-20	AAAORR x RRRR= RRRA(A_4O_4)	27	5.1
66	(Kopu II R3-2 x (33 OP-I x (PxB)-1))-1	RRRR x RRA(A_4O_4)=RRR($A_6R_4O_2$)	33	4.5
67	(Kopu II R3-2 x (33 OP-I x (PxB)-1))-2	RRRR x RRA(A_4O_4)=RRR($A_6R_4O_2$)	69	4.8
68	(Kopu II R3-2 x (33 OP-I x (PxB)-1))-3	RRRR x RRA(A_4O_4)=RRR($A_6R_4O_2$)	DNF	4.5
69	(Kopu II R3-2 x (33 OP-I x (PxB)-1))-4	RRRR x RRA(A_4O_4)=RRR($A_6R_4O_2$)	33	5.0
70	(Kopu II R3-2 x (33 OP-I x (PxB)-1))-5	RRRR x RRA(A_4O_4)=RRR($A_6R_4O_2$)	33	5.0
71	(Kopu II R3-2 x (33 OP-I x (PxB)-1))-6	RRRR x RRA(A_4O_4)=RRR($A_6R_4O_2$)	34	4.6
72	(Kopu II R3-2 x (33 OP-I x (PxB)-1))-7	RRRR x RRA(A_4O_4)=RRR($A_6R_4O_2$)	30	4.7
73	(Kopu II R3-2 x (33 OP-I x (PxB)-1))-8	RRRR x RRA(A_4O_4)=RRR($A_6R_4O_2$)	62	5.1
74	(Kopu II R3-2 x (33 OP-I x (PxB)-1))-9	RRRR x RRA(A_4O_4)=RRR($A_6R_4O_2$)	100	3.6
75	(Kopu II R3-2 x (33 OP-I x (PxB)-1))-10	RRRR x RRA(A_4O_4)=RRR($A_6R_4O_2$)	29	4.5

Kopu II: a cultivar of white clover

(PxB)-1: a coloured white clover genotype

Table 3.4 Original 3x (ARO) and 6x (AARROO) hybrids used in strategy-4 based on using *T. occidentale* as a genetic bridge with other details as mentioned in Table 3.3.

BAR #	Pedigree	Expected genomic composition	Pollen Fertility (%)	Ploidy (x)
BAR 5	ROSx (OCD-48-17)-2	AARR x OO= AARROO	<1	6.0
BAR 6	ROS x (OCD-48-17)-3	AARR x OO = ARO	0	3.0
BAR 7	ROS x (OCD-48-17)-4	AARR x OO = ARO	<1	3.1
BAR 8	ROS x (OCD-48-17)-7	AARR x OO = ARO	8	3.1
BAR 9	ROS x (OCD-48-17)-8	AARR x OO = ARO	22	3.1
BAR 10	ROS x (OCD-48-17)-9	AARR x OO = ARO	6	3.0
BAR 12	ROS x (OCT-48-617)-1	AARR x OOOO= AARROO	18	5.9
BAR 13	ROS x (OCT-48-617)-2	AARR x OOOO= AARROO	34	5.9
BAR 14	ROS x (OCT-48-617)-3	AARR x OOOO= AARROO	19	6.0
BAR 15	ROS x (OCT-48-617)-4	AARR x OOOO= AARROO	34	5.8
BAR 16	ROS x (OCT-48-617)-5	AARR x OOOO= AARROO	12	5.8
BAR 17	ROS x (OCT-48-617)-6	AARR x OOOO= AARROO	7	5.9

OCD & OCT represents 2x and chromosome doubled 4x versions of *T. occidentale* respectively.
ROS = a 4x hybrid (AAOO) from cross *T. ambiguum (4x) x T. occidentale (4x)*

Table 3.5 Original 5x RRRRA and 7x AAARRRR hybrids (with pollen fertility and ploidy estimates based on flow cytometry) used in strategy-5 based on the direct integration of A and R genomes with ploidy manipulation and not using *T. occidentale* as a genetic bridge.

BAR #	Pedigree	Expected genomic composition	Pollen Fertility (%)	Ploidy (x)
22	(Trophy-2 x (70xCrau 38))-1	RRRR x (AARRRR) = ARRRR	DNF	5.4
23	(Trophy-2 x (70xCrau 38))-2	ARRRR	70	5.0
24	(Trophy-2 x (70xCrau 38))-3	ARRRR	DNF	5.2
25	(Trophy-3 x (70xCrau 38))-1	ARRRR	DNF	5.2
26	(Trophy-3 x (70xCrau 38))-2	ARRRR	DNF	5.1
27	(Trophy-3 x (70xCrau 38))-3	ARRRR	DNF	5.2
29	(Kopu II-35 x (70xCrau 38))-1	ARRRR	DNF	5.1
30	(Kopu II-35 x (70xCrau 38))-2	ARRRR	DNF	4.8
31	(Kopu II-35 x (70xCrau 38))-3	ARRRR	90	5.2
32	(Kopu II-R3-3 x (70xCrau 38))-1	ARRRR	DNF	5.3
33	(Kopu II-R3-3 x (70xCrau 38))-2	ARRRR	84	5.0
34	(Kopu II-R3-3 x (70xCrau 38))-3	ARRRR	DNF	5.0
42	(ROS x (70xCrau 38))-1	AARR x AARRRR = AAARRRR	47	7.1
43	(ROS x (70xCrau 38))-2	AAARRRR	61	6.8
44	(ROS x (70xCrau 38))-3	AAARRRR	69	6.8
45	(ROS x (70xCrau 38))-4	AAARRRR	32	6.5
46	(ROS x (70xCrau 38))-5	AAARRRR	19	4.7
47	(ROS x (70xCrau 38))-6	AAARRRR	47	6.9
48	(ROS x (70xCrau 38))-8	AAARRRR	76	6.7
49	(ROS x (70xCrau 38))-9	AAARRRR	76	6.8

DNF: Did not flower

Trophy and Crau are white clover cultivars

CHAPTER 4
RESULTS

4.1 Strategy 1: (using *T. occidentale* as a genetic bridge to combine 4x *T. ambiguum* and *T. repens* genomes)

The aim of this strategy was to incorporate genomic segments from 4x *T. ambiguum* into *T. repens* either by introgression or by chromosome additions or substitutions. This method will simultaneously also introduce genome components from *T. occidentale* which might act as a genetic bridge for introgression.

The strategy was based on 4x BAR hybrids derived from the cross between a red leaved white clover plant, Red One (designated as RO) and a 4x AAOO hybrid (designated as BL) which was obtained through embryo rescue as explained earlier.

4.1.1 Hybrids from the cross, RO (RRRR) x BL (AAOO) - RRAO (4x)

Seeds from the cross, RRAO x RRRR were provided by the Forage Improvement Programme. This seed was germinated in May, 2009 and the resulting progeny, after preliminary selection based on morphology, were used in further breeding. The details of these BAR hybrids are given in Table 4.1.1. Because the A sub-genomes in plant BL were derived from tetraploid *T. ambiguum*, the A refers to A^T throughout in this section. On the basis of the flow cytometric ploidies deviating from 4x, aneuploid 2n chromosome numbers and in some cases the leaf colour marks along with lower pollen stainabilities as compared to white clover which were observed in the progeny of the RRAO x RRRR crosses (Table 4.1.2), we assumed that the original cross between RO x BL (RRRR x AAOO) had been successful and the progeny were RRAO and not the self progeny of white clover (RRRR) (Table 4.1.1).

4.1.2 Progeny of the original BAR hybrids (BAR09 hybrids)

4.1.2.1 Progeny of cross, RRAO (4x) x RRRR and selfing of RRAO (4x) hybrids

One hundred seeds obtained from the crosses, RRAO x RRRR and selfing of RRAO plants were germinated and the resulting seedlings were transplanted to pots. This progeny set had pollen stainability ranging from 30% in BAR09-128 to 66 % in BAR09-130 (Table 4.1.2). The expected genomic composition of these plants was RRR(A_4O_4).

Table 4.1.1 Pedigrees of six original 4x BAR hybrids, derived from the cross RRRR x AAOO = RRAO, with flow cytometric ploidy estimations and details of further crosses.

Hybrid	Pedigree	Expected genomic composition	Estimated Ploidy (x)*	Crosses	Expected genomic composition
BAR-119	(RO x BL)-1	RRRR x AAOO= RRAO	3.9	x C2418-2(6)-1, (P/B)-17, (P/B)-5	RRAO x RRRR= RRR(A_4O_4)
BAR-122	(RO x BL)-5	RRRR x AAOO= RRAO	--	Selfed	RRAO x RRAO = RRAO
BAR-123	(RO x BL)-6	RRRR x AAOO= RRAO	3.8	x (P/B)-17	RRAO x RRRR= RRR(A_4O_4)
BAR-124	(RO x BL)-7	RRRR x AAOO= RRAO	3.6	x (P/B)-5 ♀	RRRR x RRAO=RRR(A_4O_4)
BAR-125	(RO x BL)-8	RRRR x AAOO= RRAO	3.9	x (P/B)-17	RRAO x RRRR= RRR(A_4O_4)
BAR-136	(RO x BL)-19	RRRR x AAOO= RRAO	3.9	x (P/B)-17	RRAO x RRRR= RRR(A_4O_4)

* Flow cytometry based

Table 4.1.2 Progeny of RRAO hybrids (Table 2) with pedigrees, expected genomic composition, estimated ploidies (flow cytometry), expected and actual chromosome numbers, pollen fertility, details of further crosses and breeding system (SC, self-compatible, SI, self- incompatible).

BAR09 No.	Pedigree	Expected genomic composition	Ploidy (x)	Exp. 2n chrom. #	Actual 2n chrom. #	Pollen fertility (%)	Crossed with	Total crosses	Total seeds	Breeding system
BAR09-120	((P/B)-5 x (RO x BL)-6)-1	RRRR x RRAO = RRR(A_4O_4)	4.4	~32	33	43	Kopu II-906♀	150	21	SC
BAR09-128	((RO x BL)-1 x (P/B)-5)-1C	RRAO x RRRR= RRR(A_4O4)	3.8	~32	31	30	Kopu II-901 ♀	150	207	SC
BAR09-130	((RO x BL)-1 x (P/B)-5)-2B	RRAO x RRRR= RRR(A_4O_4)	3.8	~32	31	67	Kopu II-910 ♀	150	199	SC
BAR09-131	((RO x BL)-1 x C2418-2(6))-1	RRAO x RRRR= RRR(A_4O_4)	4.5	~32	34	58	Kopu II-903 ♀	100	46	SC
BAR09-132	((RO x BL)-19 x (P/B)-17)-1	RRAO x RRRR= RRR(A_4O_4)	4.2	~32	33	66	Kopu II-905 ♀	100	59	SC
BAR09-133	((RO x BL)-1 x (RO x BL)-1)-5	RRAO x RRAO = RRAO	5.0	~32	35	43	Kopu II-918 ♀	100	7	SI

(2n=32), having three full sub-genomes from white clover and partial genomes from each of *T.ambiguum* and *T.occidentale*. One of the *T. repens*-derived genomes is expectedly a mixture of chromosomes derived from both the sub-genomes of white clover with unspecified numbers. The somatic chromosome number in these hybrids was around 32 except for BAR09-133 which had 35 chromosomes. The aneuploid 2n chromosome counts were compatible with variable flow cytometric ploidy estimates of BAR09 plants (Table 4.1.2). Based on our results, *T. ambiguum* chromosomes have higher flow cytometry (FC) values due to having more DNA content than the chromosomes from *T. repens* and *T. occidetnale* (1A genome = ~ 1.4 R genomes and 1O genome = ~1.1R genomes), so sometime genetically complex hybrids with more chromosomes from *T. repens* and *T. occidentale* than *T. ambiguum* would have relatively lesser FC value or vice versa. As these plants had leaf colour marker genes, they were used as male parents to pollinate a totally green white clover (Kopu-II, a commercial white clover variety). All these selected BAR09 plants produced seed when crossed with green white clover (Table 4.1.2). All except BAR09-133 also proved to be self-compatible, setting large quantities of seed on selfing. The self seed, due to being large in number, was not counted.

4.1.2.2 Chromosome pairing analysis in BAR09-120

One BAR09 plant (BAR09-120) was analysed for its meiotic chromosome behaviour (Table 4.1.3). This hybrid had an expected genomic formula of RRR(A_4O_4) (2n~32). It proved to be aneuploid with a somatic chromosome number of 33 and so its actual genomic composition was RRR(A_4O_4)+1 (Figure 4.1.1a). Thirty five pollen mother cells (PMCs) were analysed for meiotic chromosomal pairing behaviour of this hybrid. In addition to Is, IIs, IIIs and IVs, pentavalents (Vs) were also observed in a very low frequency (Table 4.1.3, Figure 4.1.1b, c, d). Trivalents (IIIs) might have involved both homologous and homoeologous chromosome pairing within and among white clover sub-genomes, but IVs and Vs must have involved inter-specific chromosome pairing among all the three species, if there has been no illegitimate pairing. The chances of getting recombinant chromosomes were high in this hybrid which had a strong chance of chromosomal exchange between A and O sub-genomes during gamete formation in the hybrid parent, RRAO.

Table 4.1.3 Meiotic chromosome associations at diakinesis/metaphase-I in PMCs of BAR09-120 (2n=33).

Plant number	Pedigree	Expected genomic composition	No. of PMCs scored	Mean frequency (range) of meiotic configurations					
				I	II	III	IV	V	Pollen fertility (%)
BAR09-120 (2n=33)	((P/B)-5 x (RO x BL)-6) -1	RRRR x RRAO = RRR(A_4O_4)	35	2.5 (0-7)	9.2 (5-15)	2.0 (0-5)	1.3 (0-4)	0.1 (0-1)	43

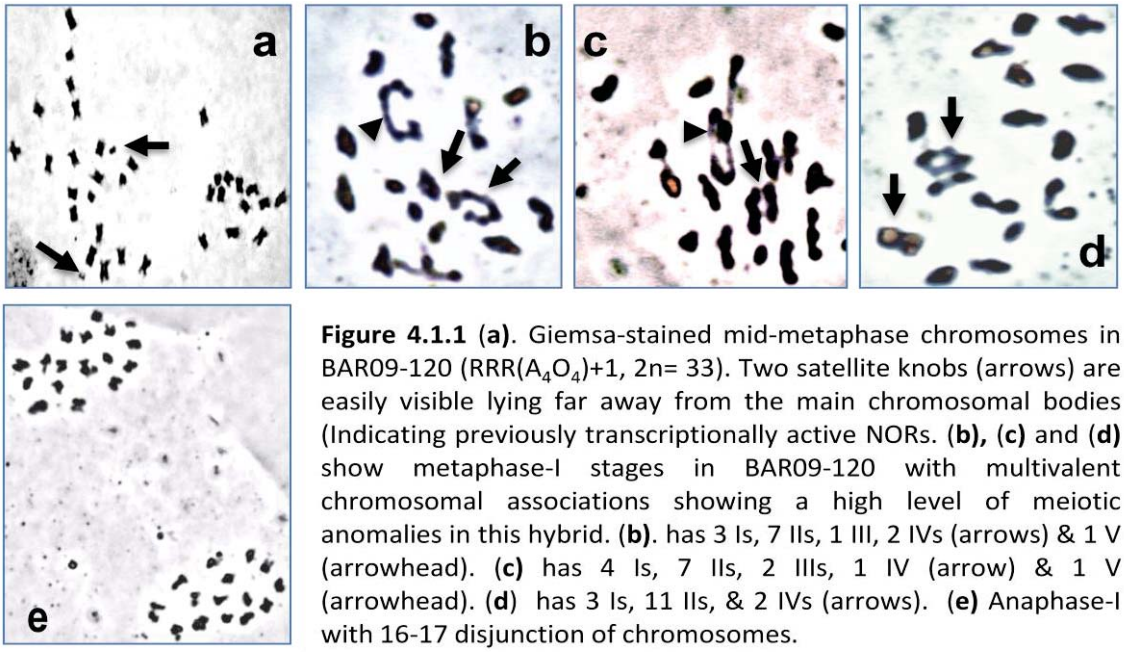

Figure 4.1.1 (**a**). Giemsa-stained mid-metaphase chromosomes in BAR09-120 (RRR(A_4O_4)+1, 2n= 33). Two satellite knobs (arrows) are easily visible lying far away from the main chromosomal bodies (Indicating previously transcriptionally active NORs. (**b**), (**c**) and (**d**) show metaphase-I stages in BAR09-120 with multivalent chromosomal associations showing a high level of meiotic anomalies in this hybrid. (**b**). has 3 Is, 7 IIs, 1 III, 2 IVs (arrows) & 1 V (arrowhead). (**c**) has 4 Is, 7 IIs, 2 IIIs, 1 IV (arrow) & 1 V (arrowhead). (**d**) has 3 Is, 11 IIs, & 2 IVs (arrows). (**e**) Anaphase-I with 16-17 disjunction of chromosomes.

4.1.3 Self and cross progeny of the BAR09 hybrids (BAR10 hybrids)

4.1.3.1 Cross and self progeny of RRR(A_4O_4) plants

BAR09 hybrids with genomic formula of RRR(A_4O_4) were selfed and, because of their self compatibility, crossed as male parents with self-incompatible totally green white clover (RRRR). The resulting seed produced a progeny group of 63 plants. On the basis of leaf colour markers, seven plants were selected (Table 4.1.4). The expected genomic formula in BAR10-126, BAR10-129 and BAR10-131 was RRR($R_4A_2O_2$) with expected 2n chromosomes numbers of 32-33, 31-32 and 31-32 respectively (Table 4.1.4). BAR10-136 resulted from the cross of BAR09-133 (with genomic composition of RRAO+3 – Table 4.1.2) as a male parent with green white clover plant, Kopu II-918. The female gamete from white clover in this case was fertilized by a male gamete from BAR09-133 having 19 chromosomes (n=2x+3). BAR10-137, BAR10-138 & BAR10-140 were the self progeny of BAR09-128, BAR09-131 & BAR09-132 and had 2n chromosome numbers of 32, 34 & 32 respectively. The actual chromosome numbers in these hybrids agreed reasonably well with the modified expected genomic constitutions (Table 4.1.4).

Male fertility in all these hybrids was above 50% except for BAR10-136 (32%) (Table 4.1.4). Flow cytometry based ploidy levels were compatible with the actual somatic chromosome counts. The Giemsa-stained mitotic chromosome complement of BAR10-126 is given in Figure 4.1.2a. These plants have had two opportunities for genomic mixing, in the first generation most probably between A & O genomes and in the second generation among R, A

and O genomes and so are potentially very important from a recombination view point. BAR10-126 was used for further cyto-molecular analysis.

4.1.3.2 Conventional chromosome pairing analysis in BAR10-126 (2n = 33)

The meiotic analysis results for BAR10-126 (RRR($R_4A_2O_2$)+1) was based on the study of 47 PMCs. Chromosomal associations ranged from Is through to IVs. (Table 4.1.5, Figure 4.1.2b, c). Lagging chromosomes, sometimes precociously splitting into sister chromatids during anaphase-I, were frequently observed (Figure 4.1.2d, e). In the normal PMCs the most frequent chromosome disjunction at anaphase-I was 16-17 (Figure 4.1.2f).

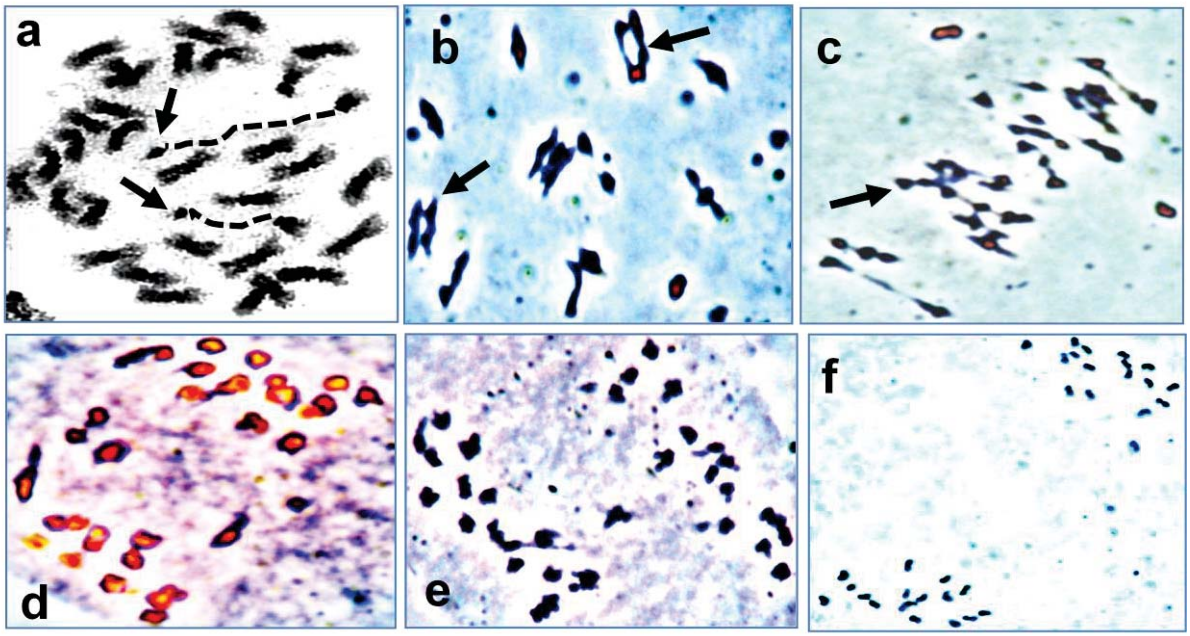

Figure 4.1.2 (a). Giemsa-stained early metaphase chromosomes in BAR10-126 (RRR($R_4A_2O_2$)+1, 2n=33) which was the progeny of BAR09-120 (RRR(A_4O_4)+1, 2n=33) crossed with white clover (RRRR). It has two satellite knobs (arrows) connected with the main chromosomal bodies through highly de-condensed (transcriptionally active) NOR DNA which is visible as shades (broken lines). **(b)** & **(c)** Two metaphase-I stages in BAR10-126 showing ring and chain IVs (arrows) in addition to predominant IIs and a few Is. (**d**). Anaphase-I with three lagging chromosomes one of which is probably a univalent precociously splitting into chromatids. (**e**) Anaphase-I with only one lagging chromosome and 16 chromosomes at each pole. (**f**). Anaphase-I showing 16-17 chromosome disjunction.

Table 4.1.4 Selected progeny of the BAR09 hybrids with expected genomic formulae RRR(A_4O_4) (4x) or RRAO (Table 3), with estimated ploidy (flow cytometry), expected and actual chromosome numbers and pollen fertility.

Hybrid	Pedigree	Expected genomic composition	Pollen fertility (%)	Ploidy (x)	Exp. 2n chrom. #	Actual 2n chrom. #
BAR10-126	(Kopu II-906 x BAR09-120)-1	RRRR x RRR(A_4O_4)+1= RRR($R_4A_2O_2$)+(0-1)	51	--	32-33	33
BAR10-129	(Kopu II-901 x BAR09-128)-10	RRRR x RRR(A_4O_4)-1= RRR($R_4A_2O_2$)-(0-1)	94	4.1	31-32	32
BAR10-131	(Kopu II-910 x BAR09-130)-4	RRRR x RRR(A_4O_4)-1 = RRR($R_4A_2O_2$)-(0-1)	70	4.2	31-32	32
BAR10-136	(Kopu II-918 x BAR09-133)-5	RRRR x (RRAO)+3 = RRR(A_4O_4)+(0-3)	32	4.6	32-35	35
BAR10-137	(BAR09-128-Selfed)-1	RRR(A_4O_4)-1xRRR(A_4O_4)-1 = RRR(A_4O_4)-(0-2)	62	4.3	30-32	32
BAR10-138	(BAR09-131-Selfed)-8	RRR(A_4O_4)+2xRRR(A_4O_4) +2= RRR(A_4O_4)+2	61	4.4	34	34
BAR10-140	(BAR09-132-Selfed)-3	RRR(A_4O_4)+1 x RRR(A_4O_4)+1 = RRR(A_4O_4)+(0-2)	52	4.2	32-34	32

Table 4.1.5 Meiotic chromosome associations at diakinesis/metaphase-I in PMCs of BAR10-126 (2n=33)

Hybrid name	Pedigree/ identification	Exp. genomic composition	No. of PMCs scored	**Mean frequency (range) of meiotic configurations**					
				I	II	III	IV	V	Pollen fertility (%)
BAR10-126	(Kopu II-906 x BAR09-120)-1	RRRR x RRR(A_4O_4)+1= RRR($R_4A_2O_2$)+(0-1)	47	3.5 (0-9)	12 (6-15)	1.1 (0-4)	0.6 (0-2)	0	51

4.1.4 Molecular cytogenetic analyses of BAR10-126

Investigations using GISH/FISH were carried out for analysis of the genomic composition of BAR10-126, as well as the chromosome pairing and separation during anaphase-I. For molecular cytogenetic analyses of meiotic and mitotic preparations, two types of DNA probes were used i.e., total genomic DNA of *T. ambiguum* labelled with Fluor-X-dCTP (green) and Cy3-dCTP labelled 5S rDNA (red). The GISH and FISH experiments gave stronger fluorescence signals with more even distribution along the whole chromosome length when the chromosomes preparation underwent two cycles of DNA de-naturation.

Two chromosomes having satellites were apparent in the Giemsa- and DAPI-stained mitotic preparations (Figure 4.1.2a and Figure 4.1.3a). A single chromosome derived from *T. ambiguum* was present and clearly identifiable by its larger in size (Figure 4.1.3a). GISH using labelled genomic DNA from *T. ambiguum* (green) as probe painted only this chromosome thus indicating that only one chromosome in this hybrid came from *T. ambiguum* (Figure 4.1.3b). This hybrid gave six signals when probed with 5S rDNA (red) (Figure 4.1.3b, c). Two 5S signals were on a pair of chromosomes bearing de-condensed NOR sequences, and were thus identifiable as being from *T. repens* or *T. occidentale*. Fortuitously, the *T. ambiguum*-derived chromosome was a marker chromosome with a 5S rDNA signal on the long arm (Figure 4.1.3b, c). Two of the remaining three 5S signals, were on white clover derived chromosomes while the third was very small and identified as being on a *T. occidentale* derived chromosome. The presence of A and O-derived chromosomes and the formation of multivalents in the PMCs of BAR10-126 not only shows these chromosomes are taking part in pairing but it also indicates there can be some possibility of chromosome substitution along with addition. GISH (Figure 4.1.3b, arrows) consistently revealed small green signals on two chromosomes in telomeric regions and on another two interstitially, indicating possible chromosomal exchange and incorporation of *T. ambiguum* DNA into *T. repens* or *T. occidentale*-derived chromosomes. The *T. occidentale* derived chromosome with small 5S signal has also has green signals on both the chromatids which were consistently observed (Figure 4.1.3b).

GISH analysis in BAR10-126 was also carried out on a meiotic chromosome preparation using two probes mentioned in the preceding section. In total, 26 PMCs were analysed for chromosome pairing. In 13 PMCs (50 %), the *T. ambiguum*-derived chromosome did not pair with any other chromosome (Figure 4.1.4c, d). In the remaining 50 % of the PMCs, the *T.*

ambiguum-derived chromosome did pair during metaphase-I either as IIs or IIIs with homoeologous chromosomes from either white clover or *T. occidentale.* In nine of these 13 PMCs, the *T. ambiguum* chromosome associated as IIs but, in four, it paired with other homoeologous chromosomes to form IIIs (e.g. Figure 4.1.4a, b). Precocious chromatid separation of the *T. ambiguum* univalent followed by chromatid movement to opposite poles was observed very frequently (Figure 4.1.4e, f). In many cases, these chromatids lagged behind and did not become part of the tetrads and so the *T. ambiguum*-derived chromosome was eliminated during meiosis, making micronuclei (Figure 4.1.4g). In BAR10-126, the *T. ambiguum* chromosome had a 5S red signal and so, after splitting, two chromatids with signals could be seen moving to opposite poles, and sometimes lagging behind (Fig. 4.1.4f).

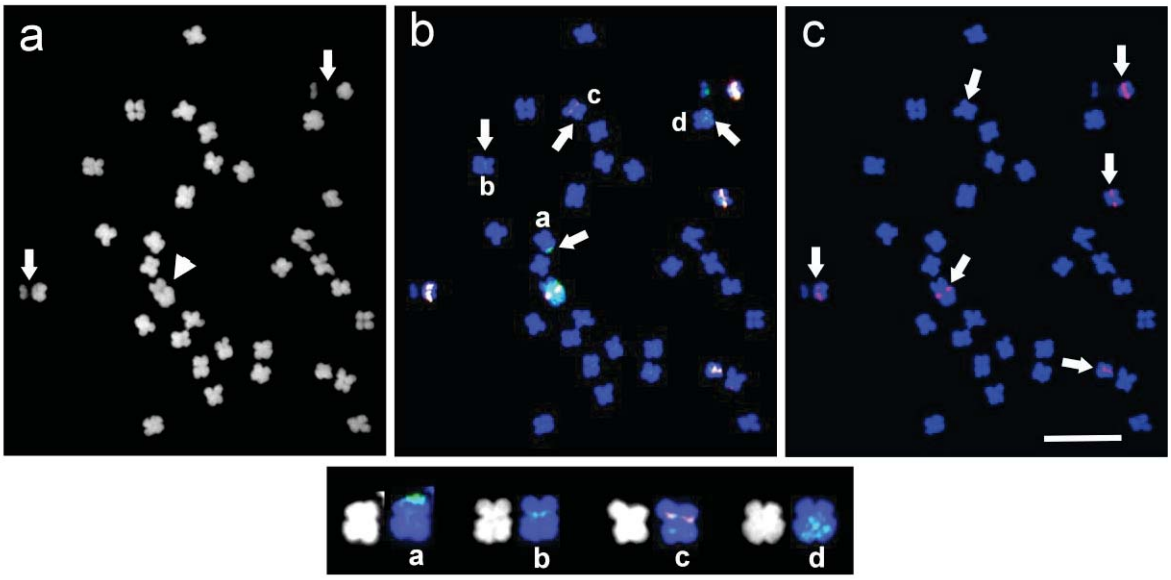

Figure 4.1.3 (**a**). DAPI-stained (grey scale) metaphase chromosomes in BAR10-126 (RRR($R_4A_2O_2$)+1, 2n=33). Two NORs are visible (arrows) which are not very stretched due to the highly condensed nature of chromosomes being in late metaphase. A *T. ambiguum*-derived chromosome is identifiable even in the DAPI-stained chromosome preparation because of its comparatively larger size (arrowhead). (**b)**. The same cell probed with total genomic DNA of *T. ambiguum* labelled with Fluor-X-dCTP (green) and Cy3-dCTP labelled pTr5S (red). One *T. ambiguum* chromosome was painted as a result of hybridization confirming the bigger chromosome was from *T. ambiguum*. Green signals were consistently observed on four non-*T. ambiguum* chromosomes on both chromatids (on two chromosomes telomerically and two interstitially) and are evidence of inter-genomic recombination (arrows). The chromosomes with introgression (a-d) are also shown in an enlarged shape. (**c)**. Six 5S red signals in the same cell probed with Cy3-dCTP labelled pTr5S (red). Two were on the NOR bearing chromosomes coming either from *T. repens* or *T. occidentale*. One was on the *T. ambiguum*-derived chromosome. Of the remaining three signals, two were on *T. repens*-derived non-NOR chromosomes while the remaining one, with very low copy number, might be on a chromosome from *T. occidentale* which has got introgression too. Scale bar = 10µm.

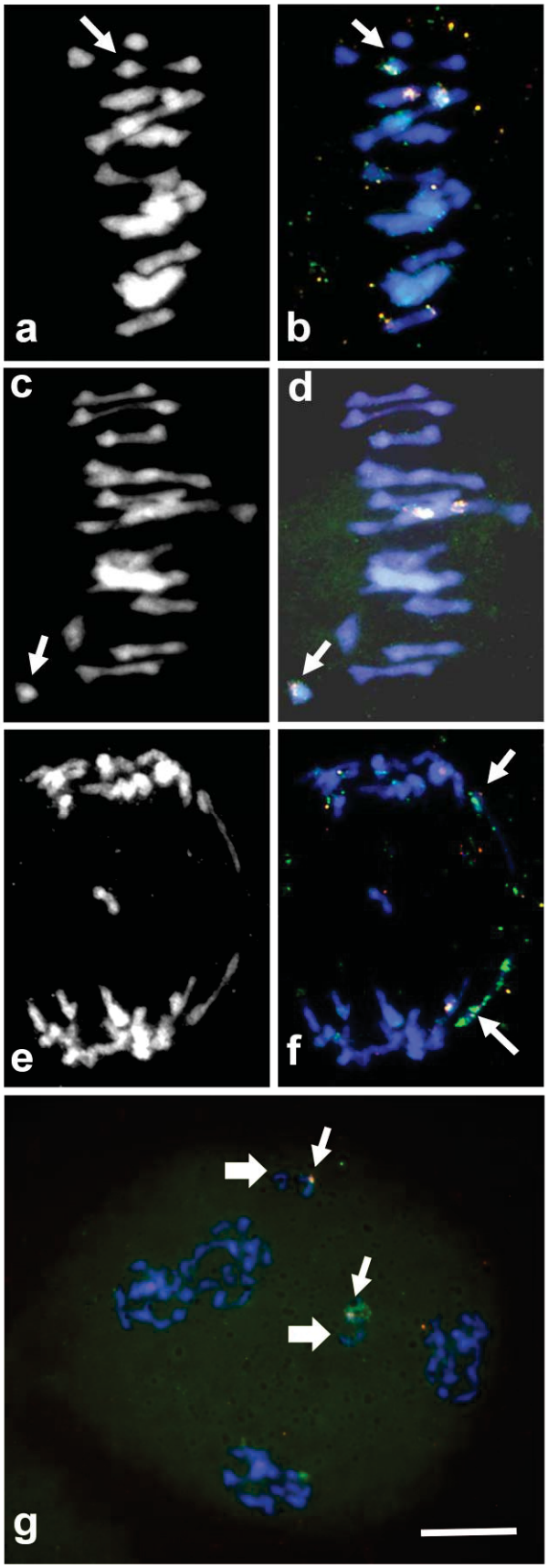

Figure 4.1.4 GISH/FISH was carried out on the meiotic chromosomes of BAR10-126 (RRR($R_4A_2O_2$+1, 2n=33)) using the same probes as in Fig. 3 to follow the behaviour of the *T. ambiguum* chromosome during metaphase-I**. (a)**, (**b);** The same cell showing that, the *T. ambiguum*-derived chromosome has a 5S signal (red) and is making a bivalent with another chromosome (arrows). (**c),** (**d);** the *T. ambiguum*-derived chromosome is behaving as a univalent (arrows). (**e),** (**f**), The *T. ambiguum* chromosome preciously split into two chromatids with partial movement to the opposite poles. In (**f**) Chromatids have the 5S red signals split into two. (**g**) a telophase-II in BAR10-126 with 2 chromosomes (one A-derived and one more chromosome with unspecified origin either from O or R genomes) after having split into chromatids during anaphase-I lagged behind and did not become part of the tetrads so made micronuclei. They are shown by arrows (A-derived micronuclei) and bold arrows (micronuclei derived from the unspecified chromosomes). Bar = 10µm.

4.1.5 Morphological description

4.1.5.1 Morphological characterization of self and cross progeny of RRAO hybrids

The aim of these sand-pit experiments was to see the relative expression of inherited parental species specific characters especially focussing on *T. ambiguum*-associated traits. Five BAR09 hybrids (Table 4.1.6.1) were evaluated in the first experiment along with (RO x BL)-19 and (P/B)-17 (white clover) as control parents. The expected genomic composition of these backcross hybrids was RRR(A_4O_4). However, BAR09-133 was a self-progeny of hybrid RO x BL (RRAO) with an expected genomic composition of RRAO, but with additional opportunity for inter-genomic recombination. All the hybrids had stolons except BAR09-133 which was semi-stoloniferous having nodal roots from the basal two to three nodes and then the stems were erect or semi-erect without nodal roots (Table 4.1.6.1). BAR09-128 had the highest number of stolons with the longest length as compared to the other hybrids and control parents. The extent of nodal rooting (anchorage) ranged from 0.8 in BAR09-133 to 9.3 in BAR09-128 followed by BAR09-120 (8.5) and BAR09-132 (8.0). Significantly higher numbers of inflorescences (604.8) were recorded in BAR09-128 followed by BAR09-132 (425.2) as compared to the parents. The growth habit was determinate *(T. ambiguum*-like) in BAR09-120, BAR09-133 & (RO x BL)-19 while the rest had indeterminate growth habits (white clover-like) (Table 4.1.6.1). Terminal growth was highly reduced in BAR09-120, BAR09-133 & (RO/BL)-19. All the hybrids were female-fertile except BAR09-133 which did not set seed after open pollination (Table 4.1.6.2). Root weight % of the total biomass was significantly higher in two BAR09 hybrids (BAR09-120 & BAR09-133) as compared to the white clover parent while BAR09-128, BAR09-130 and BAR09-132 had almost the same root weight ratio to total biomass as white clover (Table 4.1.6.2).

4.1.5.2 Morphological description of the self and cross progeny of RRR(A_4O_4)

BAR10 progeny, derived from selfing and backcrossing of BAR09 hybrids to Kopu II (white clover) as female parent, were studied for phenotypic analysis in a second sand-pit experiment. Here, BAR10-126, BAR10-129, BAR10-131, BAR10-136, BAR10-137 and BAR10-140 were compared with white clovers (Kopu II & (P/B1 x P/B2)-2). The expression of *T. ambiguum*-associated characters was comparatively of lower intensity in this generation than the previous one (BAR09). With a few exceptions, the hybrids were more similar to white clover than to *T. ambiguum* in many of the studied traits (Table 4.1.7.1, Table 4.1.7.2).

Except for BAR10-136, the hybrids formed stolons with indeterminate apical growth and axillary flowering as in white clover. However, the level of nodal rooting (anchorage) was lower than white clover, ranging from 4 in BAR10-126 to 8 in BAR10-129 (Table 4.1.7.1). The only BAR10 hybrid which was non-stoloniferous and had determinate growth with a combination of both axillary and terminal flowering was BAR10-136 (*T. ambiguum*-like) (Table 4.1.7.1). The root to total biomass ratio was again a trait which showed the impact of *T.ambiguum* but the difference was of much lower range than in the previous generation (BAR09 hybrids). All the hybrids had significantly higher root to total biological yield ratio than the white clover commercial variety, Kopu II, except BAR10-137 where the difference was not significant (Table 4.1.7.2). BAR10-136 and BAR10-140 were also significantly better than the other white clover control, (P/B1 x P/B2)-2.

Table 4.1.6.1. Mean morphological data of the above-ground traits of the advanced progeny of the cross between RRAO and white clover (RRRR).

Hybrids	Stolon length (cm)	Stolon number	Stem anchorage (0-10)	Inflorescence number	Growth habit	Peduncle length (cm)	Florets/ head	Leaflet length/ width ratio	Petiole length (cm)	Flowering; terminal, axillary or combination
BAR09-120	24.4	27.7	8.5	148.0	determinate	17.4	67.4	1.32	13.5	combination
BAR09-128	67.3	71.3	9.3	604.8	combination	20	49.9	1.45	11.6	combination
BAR09-130	58.6	41.5	7.5	176.0	combination	16.1	45.3	1.57	9.2	axillary
BAR09-132	53.4	68.8	8.0	425.2	combination	19.3	59.4	1.25	11.2	combination
BAR09-133	6.0	13.2	0.8	46.8	determinate	10.0	34.5	1.88	4.4	combination
(RO x BL)-19	15.9	20.0	7.3	78.2	determinate	12	77.7	1.53	4.4	combination
(P/B)-17	29.8	35.0	10	83.2	indeterminate	13.6	64.6	1.44	9.7	axillary
CV%	**11.2**	**15.0**	**13.2**	**23.5**		**14.8**	**8.0**	**6.40**	**31.4**	
SEM	**4.1**	**6.3**	**1**	**52.5**		**2.3**	**4.6**	**0.09**	**2.9**	
LSD	**6.1**	**9.3**	**1.4**	**78.0**		**3.4**	**6.8**	**0.14**	**4.3**	
Prob. (5%)	***	***	***			***	***	***	***	

Table 4.1.6.2 Mean morphological data of the above and under-ground traits of the advanced progeny of the cross between RRAO and white clover (RRRR).

Hybrids	Stem thickness (mm)	Seeds /head	Main root thickness (mm)	Main nodal root thickness (mm)	Dry weight top (DWT) (g)	Dry weight root (DWR) (g)	Total Biomass (TBM) (g)	Root weight % of the TBM
BAR09-120	2.43	32.2	4.75	2.02	8.6	5.3	13.8	38.1
BAR09-128	3.12	30.7	6.30	2.14	121.0	44.0	165.0	26.6
BAR09-130	2.83	12.4	6.93	2.48	59.1	18.9	78.1	23.2
BAR09-132	3.32	10.8	6.90	1.84	71.1	22.5	93.6	24.8
BAR09-133	1.99	0.0	7.33	1.94	2.0	2.5	4.5	54.5
(RO x BL)-19	2.51	3.7	6.12	1.95	3.9	5.7	9.6	49.2
(P/B)-17	2.27	37.2	5.77	1.25	26.2	7.3	33.5	23.3
CV%	**11.70**	**46.7**	**22.40**	**19.70**	**56.5**	**54.0**	**54.1**	**27.8**
SEM	**0.30**	**12.6**	**2.18**	**0.57**	**35.0**	**12.2**	**45.7**	**14.1**
LSD	**0.46**	**8.5**	**0.70**	**0.27**	**11.8**	**4.1**	**15.4**	**4.8**
Prob. (5%)	***	***	*	*	***	***	***	***

Table 4.1.7.1. Mean morphological data of the above- ground traits of the progeny of the cross between RRR(A_4O_4), 4x) and white clover (RRRR).

Hybrids	Stolon Length (cm)	Stolon number	Stem Anchorage (0-10)	Inflorescence No. ‡	Growth habit	Peduncle length (cm)	Flowering-terminal, axillary or combination	Florets/head	Leaflet length/width ratio
BAR10-126	18.2	24.3	4.0	3.9 (50.4)	indeterminate	11.3	axillary	43.1	1.25
BAR10-129	25.0	39.8	8.0	4.4 (79.8)	indeterminate	15.4	axillary	53.1	1.32
BAR10-131	9.5	21.0	5.3	2.9 (17.6)	indeterminate	9.1	axillary	55.6	1.31
BAR10-136	NS	NS	0.0	1.2 (3.4)	determinate	6.2	combination	25.3	1.08
BAR10-137	22.2	27.6	6.5	3.7 (41.3)	indeterminate	10.7	axillary	48.9	1.39
BAR10-140	7.9	9.1	6.7	4.0 (51.9)	indeterminate	10.7	axillary	48.2	1.22
Kopu II	48.8	84.5	10.0	6.0 (407.5)	indeterminate	14.7	axillary	51.4	1.30
(P/B1 x P/B2)-2	16.2	20.1	10.0	2.7 (14.4)	indeterminate	9.0	axillary	48.6	1.19
LSD	**12.0**	**18.1**	**2.3**	**1.1**		**4.23**		**13.9**	**0.20**
CV%	**36.1**	**33.8**	**23.9**	**20.0**		**25.80**		**19.3**	**10.50**
SEM	**7.9**	**11.8**	**1.5**	**0.7**		**1.98**		**9.1**	**0.09**
Prob. (5%)	***	***	***	***		**		*	*

‡ Data were log transformed. Values in parenthesis show back-transformed data

Table 4.1.7.2. Mean morphological data of the above and under-ground traits of the progeny of the cross between RRR(A_4O_4), 4x) and white clover (RRRR).

Hybrids	Stem thickness (mm)	Petiole length (cm)	Seeds/head ‡	Main root thickness (mm)	Dry weight top (g) ‡	Dry weight root (g)‡	Total biomass (TBM) (g) ‡	Root weight % of the TBM
BAR10-126	3.32	6.6	1.9 (6.75)	2.41	2.1 (7.85)	1.3 (3.67)	2.5 (11.59)	32.0
BAR10-129	2.38	9.5	4.3 (74.44)	2.65	3.2 (24.78)	2.2 (9.12)	3.5 (34.12)	27.1
BAR10-131	2.24	7.0	3.1 (22.42)	2.07	1.8 (5.87)	1.0 (2.64)	2.2 (8.67)	31.3
BAR10-136	2.35	3.5	0.2 (1.22)	1.98	0.2 (1.25)	0.1 (0.9)	0.8 (2.16)	41.9
BAR10-137	2.41	7.6	3.4 (29.08)	2.25	2.4 (10.8)	0.8 (2.20)	2.6 (13.07)	17.0
BAR10-140	2.15	5.8	2.9 (17.29)	1.34	1.1 (0.35)	1.1 (0.33)	0.4 (0.68)	48.9
Kopu II	3.51	11.8	3.9 (48.91)	2.93	5.3 (204.38)	3.2 (25.50)	5.4 230.40)	11.3
(P/B1 x P/B2)-2	1.92	5.0	3.2 (23.81)	2.20	1.3 (3.63)	0.5 (1.60)	1.7 (5.26)	30.7
LSD	**0.38**	**2.9**	**1.1**	**0.73**	**1.0**	**1.0**	**1.0**	**8.0**
CV%	**9.80**	**27.3**	**24.7**	**21.80**	**36.1**	**71.4**	**27.5**	**18.2**
SEM	**0.18**	**1.4**	**0.5**	**0.34**	**0.5**	**0.5**	**0.5**	**3.9**
Prob. (5%)	***	***	***	**	***	***	***	***

‡ Data were log transformed. Values in parenthesis show back-transformed data

4.2 Strategy 2: (using *T. occidentale* as genetic bridge to combine 4x *T. ambiguum* genomes with *T. repens*)

This strategy is based on the nearly 5x BAR hybrids with genomic constitution of AAAOO derived from the cross, AAO (434-1, 3x) x AAOO (BN or BL, 4x). In this cross, hybrid 434-1 used as female parent contributed unreduced gametes which were fertilized by the normal haploid (n=2x) pollen from the male parents (BN or BL, AAOO). This strategy is important from a recombination point of view because the hybrid 434-1 had an unpaired *T. occidentale* genome and gametes made by this plant, although unreduced might have some recombinant chromosomes, with a *T. occidentale* centromere and arms introgressed from *T. ambiguum,* or vice versa i.e. O^A or A^O. Backcrossing these 5x BAR hybrids with white clover repeatedly might lead to the introgression of *T. ambiguum* chromosomal segments into white clover genomic background or to some chromosome addition/substitution lines.

4.2.1 Plants derived from crosses, 434-1 (AAO) x BN or BL (AAOO) - AAAOO (5x) and AAORR (5x)

In this strategy, 12 BAR hybrids with genomic formula, AAAOO (5x) and one plant with genomic formula, AAORR were used as starting material (Table 4.2.1). BAR-115 was different genomically and in its breeding history from the rest of the BAR hybrids used in this strategy. Its genomic composition was AAORR and was the progeny of the cross 434-1 OP-4 (AAAAOO) with white clover (RRRR). In all cases, the A genomes were derived from 4x *T. ambiguum* (cv. Treeline and Turkish sources). All hybrids proved to be near pentaploid (5x) by flow cytometry analysis and had reasonably high pollen fertility (%) except BAR-112 (21 %) and BAR-115 (0 %) (Table 4.2.1). All these hybrids were crossed with white clover and selfed using over 10,000 total crosses. All the hybrids produced seed after pollination with colour-marked *T. repens*. The most seed (47) was produced by BAR-110 followed by BAR-103 (40) and BAR-108 (38). All the hybrids except BAR-109, BAR-112 and BAR-115 produced a few seeds on selfing ranging from 0.2 to 1.4 seeds per head (Table 4.2.1).

Table 4.2.1 Pedigrees of 13 original 5x BAR hybrids, derived from the cross of 434-1 (AAO, 3x) with BN (AAOO, 4x) and BL (AAOO, 4x) with pollen fertility, flow cytometric ploidy estimates and details of further crosses.

Plant #	Pedigree	Exp. genomic composition	Pollen Fertility (%)	Ploidy level (x)	Crossed with	No. of crosses	Total Seeds	Breeding system (self seed/head)
BAR-100	(434-1 x BN)-1	AAO x AAOO = AAAOO	83	5.2	(P/B1xP/B2)-1, Scarlet-1, C21557-808	500	36	SC (0.8)
BAR-101	(434-1 x BN)-2	AAAOO	DNF	5.0				
BAR-102	(434-1 x BN)-3	AAAOO	64	4.9	(P/B1xP/B2)-1, Scarlet-1, C21557-808	500	26	SC (0.4)
BAR-103	(434-1 x BN)-4	AAAOO	89	4.9	(P/B1xP/B2)-1, Scarlet-1, C21557-808	350	40	SC (0.6)
BAR-104	(434-1 x BN)-5	AAAOO	63	5.0	(P/B1xP/B2)-1, Scarlet-1, C21557-808	400	15	SC (0.2)
BAR-106	(434-1 x BN)-7	AAAOO	68	4.8	(P/B1xP/B2)-1, Scarlet-1, C21557-808	500	14	SC (0.5)
BAR-107	(434-1 x BN)-8	AAAOO	89	4.8	(P/B1xP/B2)-1, Scarlet-1, C21557-808	700	29	SC (1)
BAR-108	(434-1 x BN)-9	AAAOO	82	4.9	(P/B1xP/B2)-1, Scarlet-1	400	38	SC (1.2)
BAR-109	(434-1 x BN)-10	AAAOO	64	4.9	Scarlet-1, C21557-808	300	9	SI
BAR-110	(434-1 x BL)-2	AAO x AAOO = AAAOO	46	4.9	(P/B)-17, C21557-808	500	47	SC (1.3)
BAR-111	(434-1 x BL)-3	AAAOO	64	4.8	(P/B)-17)	550	26	SC (1.4)
BAR-112	(434-1 x BL)-5	AAAOO	21	4.6	(P/B)-17), C21557-808	480	11	SI
BAR-115	(434-1 OP-4 x Kopcru-1)-1	AAAAOO x RRRR=AAORR	00	5.6	C21557-808, (P/B1xP/B2)-1, (P/B)-17), Scarlet-1	300	13	SI

DNF: Did not flower

4.2.2 Progeny of the original BAR hybrids (BAR09 hybrids)

4.2.2.1 Progeny of the crosses, AAAOO (5x) x RRRR and AAORR (5x) x RRRR

Seed from the crosses AAAOO x RRRR, AAORR x RRRR and selfing of AAAOO hybrids gave rise to 242 plants. Seven plants were selected for further cytological and morphological analysis on the basis of their intermediate morphological traits, presence of paternal leaf colour markings and flow cytometry based DNA content analysis (Table 4.2.2). The expected genomic make-up of these plants resulting from the cross, AAAOO (5x) x RRRR was RRAO(A_4) (2n=~4.5x=~36). However, in all cases, the actual chromosome number was 33 or 34. Figure 4.2.1a shows 2n=34 for BAR09-98 with three NOR carrying chromosomes. Two of the NORs in BAR09-98 came from *T. repens* and *T. occidentale* while the third one, bigger in size, came from *T. ambiguum*. Similarly, the plants derived from selfing (BAR09-108 & 110) had lower than the expected 40 chromosomes (34, 36). BAR09-114 resulting from the cross of BAR -115 (AAORR) with white clover had an actual chromosome number of 36 equivalent to the expected somatic chromosome count (RRRA(O_4). Pollen fertilities of the cross progeny of 5x BAR hybrids with *T. repens* were very low (<15%). However, the self-progeny (BAR09-108, BAR09-110) had > 60% pollen fertility while BAR09-114 had 37 % pollen fertility. All the plants produced seed on crossing with colour marked white clover but only BAR09-108 and BAR09-110 set seed on selfing. BAR09-114, although apparently self-incompatible, was crossed as pollen parent with a normal green white clover genotype, Triffid-905 because one of its parents (434-1, AAO) was self-compatible (Table 4.2.2).

4.2.2.2 Chromosome pairing analysis in BAR09-97, BAR09-98, BAR09-106 & BAR09-110

BAR09-97 and BAR09-98 resulted from the cross of BAR-102 (AAAOO, 5x) with coloured white clovers, (P/B1xP/B2)-1 and -2 respectively and their expected genomic composition was RRAO(A_4). BAR09-106, which had the same expected genomic constitution as BAR09-97 and BAR09-98, resulted from pollination of BAR-108 (AAAOO, 5x) with the same coloured white clover, (P/B1xP/B2)-1. BAR09-110 was the self-progeny of BAR-110 (AAAOO, 5x). The somatic chromosome numbers in these hybrids were confirmed to be 33 (BAR09-97), 34 (BAR09-98), 34 (BAR09-106) & 36 (BAR09-110) and so were 2-4 chromosomes less than the expected numbers, indicating

Table 4.2.2 Pedigrees, pollen fertility and cytogenetic data along with further crossing details of the progeny of 5x BAR hybrids AAAOO (Table 9), following selfing and crossing with different coloured marked *T.repens* genotypes.

BAR09 No	Pedigree	Expected genomic composition	Pollen fertility (%)	Ploidy level (x)	Exp. chrom. #	Actual chrom. #	Crossed with	Total crosses	Total seeds	Breeding system (Self seed/head)
BAR09-97	(BAR-102 x (P/B1xP/B2)-1)-1	AAAOO x RRRR = RRAO(A_4)	10	4.8	36	33	(P/B1xP/B2) -1& -2	300	13	SI
BAR09-98	(BAR-102 x (P/B1xP/B2)-1)-2	AAAOO x RRRR= RRAO(A_4)	14	4.9	36	34	(P/B1xP/B2) - 2	600	70	SI
BAR09-100	(BAR-103 x Scarlet-1)-1	AAAOO x RRRR= RRAO(A_4)	10	5	36	34	(P/B)-17), (P/B1xP/B2) -2, C21557-808	650	44	SI
BAR09-106	(BAR-108 x (P/B1xP/B2)-1)-1	AAAOO x RRRR = RRAO(A_4)	6	4.7	36	34	(P/B1xP/B2) -2	300	49	SI
BAR09-108	(BAR-108-Selfed)-4	AAAOO x AAAOO = AAAOO	67	5.8	40	34	(P/B1xP/B2) -1	150	23	SC (33)
BAR09-110	(BAR-110-Selfed)-4	AAAOO x AAAOO = AAAOO	62	5.7	40	36	(P/B1xP/B2) -2	400	18	SC (2.2)
BAR09-114	(BAR-115 x Scarlet-1)-3	RRAAO x RRRR = RRRA(O_4)	37	5	36	36	Triffid ♀, Kopu II♀	200	64	SI

Table 4.2.3 Meiotic chromosome associations at diakinesis/metaphase-I in PMCs of selected progeny plants of 5x hybrids, AAAOO.

Hybrid name	Pedigree	Exp. genomic composition	No. of PMCs scored	Mean frequency (range) of chromosomal configurations					
				I	II	III	IV	V	Pollen fertility (%)
BAR09-97 (2n=33)	(BAR-102 x (P/B1xP/B2) -1)-1	AAAOO x RRRR= RRAO(A_4)	46	3.4 (0-11)	7.2 (2-14)	2.9 (0-7)	1.7 (0-2)	0	10
BAR09-98 (2n=34)	(BAR-102 x (P/B1xP/B2) -1)-2	AAAOO x RRRR= RRAO(A_4)	43	4.2 (1-11)	6.2 (2-11)	3.6 (1-7)	1.6 (0-4)	0	14
BAR09-106 (2n=34)	(BAR-108 x (P/B1xP/B2) -1)-1	AAAOO x RRRR= RRAO(A_4)	31	6.1 (2-11)	7.9 (2-14)	2.9 (1-6)	0.8 (0-2)	0	6
BAR09-110 (2n=36)	(BAR-110-Selfed)-4	AAAOO x AAAOO= AAAOO	43	2.6 (0-6)	7.5 (2-16)	3.6 (0-8)	1.9 (0-6)	0	62

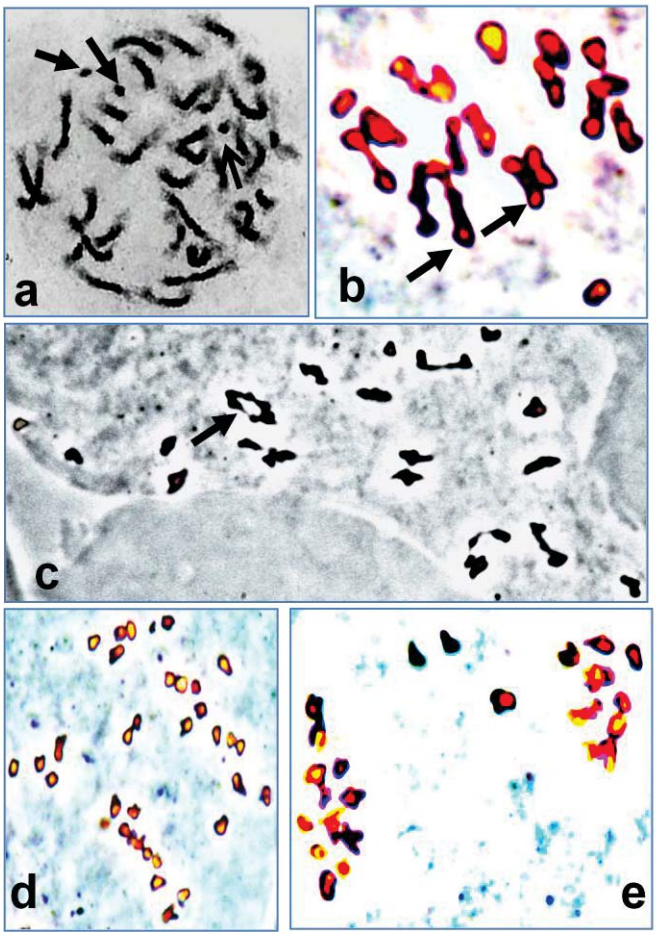

Figure 4.2.1 (**a**). Giemsa-stained early-to- mid metaphase chromosomes in BAR09-98 (RRAO(A_4)-2, 2n=34). Three satellite knobs are visible (arrows); two are small and similar in size while the third is larger and is possibly from *T.ambiguum*. The *T. ambiguum*-derived chromosomes in this image are not clearly recognizable from their size due to the highly de-condensed condition of the chromosomes. (**b**). Metaphase-I in BAR09-98, with 2 IVs (arrows) and 2 Is along with IIs and IIIs. The IVs are heteromorphic in appearance showing the possibility of homoeologous chromosomes pairing. (**c**) Metaphase-I in the same hybrid with a single ring IV (arrow) and 8 Is. (**d**). Anaphase-I in BAR09-98 with three lagging chromosomes. (**e**) One of the lagging chromosomes has precociously split into sister chromatids (top centre).

regular and frequent chromosome elimination (Table 4.2.2). The high frequencies of Is and multivalent (III & IV) chromosomal associations indicated highly disturbed meiosis leading to very low pollen fertility in the cross progeny of the 5x AAAOO hybrids (Table 4.2.3). Contrary to the above situation, the self plant, BAR09-110, had reasonably high male fertility (62 % stainable pollen, Tables 4.2.2, 4.2.3).

Multivalents (IIIs and IVs) indicated inter-specific chromosome pairing in these plants (Table 4.2.3). Quadrivalents in BAR09-97, BAR09-98 (Figure 4.2.1b, c) and BAR09-106 suggested the likelihood of inter-specific pairing among the chromosomes from all three species. Lagging chromosomes were observed in all four plants in this group. These frequently split during anaphase-I into chromatids which then moved to opposite poles, sometimes with partial lagging. Figures 4.2.1d, e show lagging chromosomes precociously splitting into sister chromatids during anaphase-I in BAR09-98.

4.2.3 Self and cross progeny of the BAR09 hybrids (BAR10 hybrids)

4.2.3.1 The self and cross progeny of RRAO(A_4) (~ 4.5x), AAAOO and RRRA(O_4)

All the selected plants of the BAR09 generation were selfed, inter-crossed and backcrossed with white clover. The progeny obtained from the harvested seed consisted of 98 plants and was designated as the BAR10 generation. Out of this progeny group, five plants were selected on the basis of intermediate morphology combining traits from both *T. ambiguum* and *T. repens*, leaf colour markers, and flow cytometry determined DNA contents (Table 4.2.4). BAR10-111 & BAR10-118 resulted respectively from the crosses of BAR09-98 and BAR09-106 as female parents pollinated by white clover. The expected genomic formulae in BAR10-111 and BAR10-118 would be RRR(A_6O_4)-1 with both having expected somatic chromosome counts of 33-34. However, while BAR10-118 had 33 chromosomes, the 2n chromosome number in BAR10-111 was 35 (Figure 4.2.2a). The expected genomic make up in BAR10-119, which was the cross progeny of BAR09-108, (AAAOO)-6, 2n=34) with white clover, was RRAO(A_4)-3 with an expected 2n chromosome number of 33-34. The actual somatic chromosome number of BAR10-119 was 34, within the expected range.

BAR10-120 was the self progeny of BAR09-108 (2n=34) and proved to have the same chromosome number as in BAR09-108. A self incompatible green leaved white clover (Kopu II) was used as female parent in crosses with BAR09-114, which had a coloured leaf marker. Seed set on the self-incompatible green white clover would indicate the likelihood of cross seed, and the presence of the leaf colour marker in the resulting progeny would confirm hybridity. BAR10-124 resulted from the cross of Kopu II (white clover cultivar) pollinated with BAR09-114 had a colour leaf marker and so it had probably genomic structure of RRR($R_4A_4O_2$). The 2n chromosome score in this hybrid was 33 which was one less than the expected number.

The pollen fertility in this group of BAR10 hybrids was highly variable, ranging from 16% in BAR10-118 to 90% in BAR10-119 (Table 4.2.4). Based on the breeding history and genomic composition, this group can be divided into four groups. BAR10-111 and 118 had similar genomic composition due to the same breeding history involving no selfings, although the 2n chromosome count was slightly different. Due to having similar genomic composition, their pollen fertilities lay in a close range. BAR10-119 which

Table 4.2.4 Selected progeny of the BAR09 hybrids with expected genomic formula RRAO(A_4) or RRRA(O_4) (Table 10), with estimated ploidy (flow cytometry), expected and actual chromosome numbers and pollen fertility.

Hybrid	Pedigree/identification	Expected genomic composition	Pollen fertility (%)	Ploidy (x)	Exp. 2n Chrom. #	Actual 2n chrom. #
BAR10-111	(BAR09-98 x (P/B1xP/B2)-2) - 2	RRAO(A_4)-2 x RRRR = (RRR(A_6O_4))-1	19	4.62	33-34	35
BAR10-118	(BAR09-106 x (P/B1xP/B2)-2) -5	RRAO(A_4)-2 x RRRR = (RRR(A_6O_4))-1	16	4.55	33-34	33
BAR10-119	(BAR09-108 x (P/B1xP/B2)-2) -1	(AAAOO)-6 x RRRR = (RRAO(A_4))-3	90	5.11	33-34	34
BAR10-120	(BAR09-108-Selfed)-2	(AAAOO)-6 x (AAAOO)- 6 = (AAAOO)-6	--	5.36	34	34
BAR10-124	KopuII-901 x BAR09-114-5	RRRR x RRRA(O_4) = RRR($R_4A_4O_2$)	50	4.22	34	33

Table 4.2.5 Meiotic chromosome associations at diakinesis/metaphase-I in PMCs of BAR10-111 and BAR10-124.

Hybrid name	Pedigree	Expected genomic composition	No. of PMCs scored	Mean frequency (range) of meiotic configurations					
				I	II	III	IV	V	Pollen fertility (%)
BAR10-111 (2n=35)	(BAR09-98 x (P/B1xP/B2)-2)-2	RRAO(A_4)-2 x RRRR= (RRR(A_6O_4))-1	68	6.7 (1-13)	10 (2-14)	1.7 (0-6)	0.8 (0-4)	0	19
BAR10-124 (2n=33)	(Kopu II-901 x BAR09-114)-5	RRRR x RRRA(O_4) = RRR($R_4A_4O_2$)	58	1.2 (0-3)	12.9 (8-16)	0.5 (0-3)	1 (0-4)	0.1 (0-2)	50

involved one selfing received two full genomes from white clover, one full genome each from *T. ambiguum* and *T. occidentale* and one partial genome from *T. ambigumm*. A high level of male fertility (90 % stainable pollen) provided strong evidence of both intra- and inter-genomic chromosome pairing. BAR10-120 did not flower, while BAR10-124 had comparatively higher pollen fertility (50%) than BAR10-111 and -118 probably due to having a higher number of white clover derived chromosomes.

4.2.3.2 Meiotic chromosome analysis in BAR10-111

Sixty eight PMCs were analysed for BAR10-111 which was from the cross of BAR09-98 with white clover. The expected genomic composition of BAR09-98 was RRAO (A_4) but its actual chromosome count was 34 (RRAO(A_4))-2. The expected chromosome number of BAR10-111 was 33 with genomic composition of RRR(A_6O_4)-1. However, the somatic chromosome count in the BAR10-111 was actually 35 (Table 4.2.4, Figure 4.2.2a), indicating that this plant had received an aneuploid gamete with 19 chromosomes (n=2x+3) from BAR09-98. The meiotic analysis of BAR10-111 (Figure 4.2.2b, c, d) showed very disturbed metaphase-I and anaphase-I stages. Although chromosomal associations ranged from Is to IVs (Figure 4.2.2b), this plant had a high number of Is (6.65/PMC) (Table 4.2.5, Figure 4.2.2b) many of which lagged behind in anaphase-I (Figure 4.2.2c, d). These Is are probably the 6-7 *T. ambiguum*-derived chromosomes. Probably because of the highly disturbed meiosis (e.g. Figure 4.2.2d), the pollen fertility in this plant was the lowest (19%) among the analysed hybrids (Table 4.2.5). The most frequently observed chromosomal disjunction during anaphase-1was 17-18 (Figure 4.2.2e). The IV chromosomal associations in these plants provided strong evidence of inter-specific chromosome pairing.

4.2.3.3 Meiotic chromosome analysis in BAR10-124

BAR10-124 (with the expected genomic composition of RRR($R_4A_4O_2$) but with 33 chromosomes), had fewer Is (1.21/PMC), more IIs (12.86/PMC) and consequently due to higher meiotic regularity had higher pollen fertility (Table 4.2.5) as compared to BAR10-111. In case of BAR10-124, the IIIs and IVs might represent homoeologous chromosome pairing within white clover.

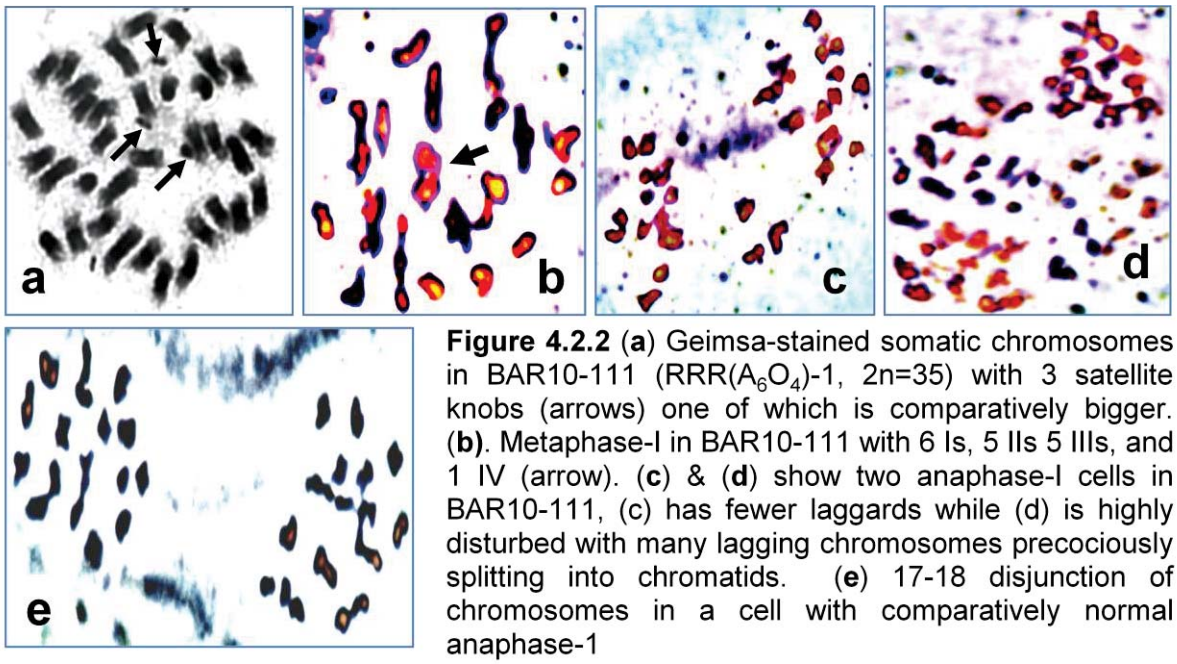

Figure 4.2.2 (**a**) Geimsa-stained somatic chromosomes in BAR10-111 (RRR(A_6O_4)-1, 2n=35) with 3 satellite knobs (arrows) one of which is comparatively bigger. (**b**). Metaphase-I in BAR10-111 with 6 Is, 5 IIs 5 IIIs, and 1 IV (arrow). (**c**) & (**d**) show two anaphase-I cells in BAR10-111, (c) has fewer laggards while (d) is highly disturbed with many lagging chromosomes precociously splitting into chromatids. (**e**) 17-18 disjunction of chromosomes in a cell with comparatively normal anaphase-1

4.2.4 Molecular cytogenetic analysis of BAR10-111

DAPI-stained (gray scale) metaphase chromosome preparations confirmed that the somatic chromosome number in BAR10-111 was 35 (Figure 4.2.3a). Three NOR bearing chromosomes were observed in metaphase cells. Two of the satellite knobs were comparatively smaller and were lying very far from their main chromosomal bodies. The DNA in these two NORs was highly de-condensed, appearing as shadowy connections between the satellite knobs and main chromosomes. One satellite knob was bigger and connected with the main chromosomal body through a partially de-condensed NOR appearing as a secondary constriction (Figure 4.2.3a).

When labelled genomic DNA from *T. ambiguum* was used as probe on the metaphase chromosome preparations of BAR10-111, six chromosomes were highlighted (Figure 4.2.3b). Interestingly, all of the largest chromosomes were confirmed to be from *T. ambiguum* (compare Figure 4.2.3a and b). Of the three NORs, the most condensed one was on the *T. ambiguum*-derived chromosome (painted in Figure 4.2.3b). FISH with 5S rDNA gave four signals (Figure 4.2.3c). Two large 5S signals were on the longer arms of a pair of chromosomes carrying highly de-condensed NORs with satellite knobs lying very far off. These NOR bearing chromosomes belong to either white clover or *T. occidentale*. One 5S signal was on a chromosome which was painted green during GISH, and so came from *T. ambiguum.* The other 5S carrying chromosome had a large signal and probably came from white clover rather than *T. occidentale* (In *T. occidentale* the equivalent signal is very small

and is hardly visible). Green signals are visible on chromosomes other than those coming from *T. ambiguum* but due to their non- consistency and sometime non-presence on both the chromatids of chromosomes, they cannot be regarded as evidence of genomic recombination (Figure 4.2.3b).

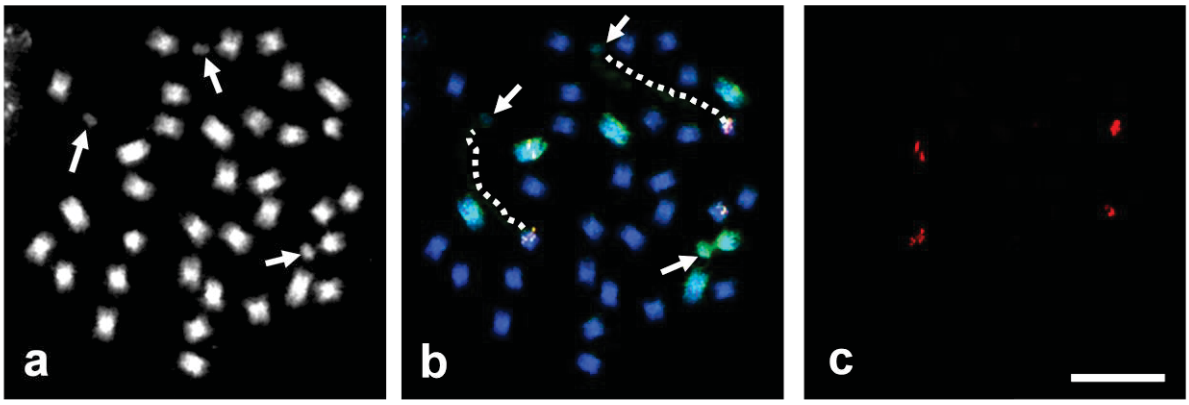

Figure 4.2.3 GISH-FISH on the mitotic chromosomes of BAR10-111 (RRR(A_6O_4)-1, 2n=35) using genomic DNA of *T. ambiguum* labelled with Fluor-X-dCTP (green) and Cy3-dCTP labelled pTr5S (red) as probes. (**a**). Giemsa-stained somatic spread . Three chromosomes had NORs (arrows), of which two were highly stretched and far away from the main chromosomal bodies without any visible connection. (**b**). GISH-FISH on the same cell painted six *T. ambiguum*-derived chromosomes. GISH confirmed that the chromosome having the comparatively condensed NOR came from *T. ambiguum*. The satellite knobs are connected with their main chromosomal bodies through highly de-condensed rDNA fibres (shown by dotted lines) which were painted green (due to cross hybridization of the highly conserved rDNA sequences). (**c**) In total, four 5S signals were highlighted by the 5S probe (red) of which two were on the NOR bearing chromosomes. Of the remaining two 5S signals, one was on a *T. ambiguum*-derived chromosome while the other was on a non-NOR bearing *T. repens* derived chromosome. Green signals are visible on chromosomes other than those coming from *T. ambiguum* but they were not consistent and sometimes they were not on both the chromatids. This does not provide strong evidence of introgression. Cases of consistency and presence on both chromatids were mostly confined to the centromeric regions and might be examples of cross hybridization due to presence of large blocks of centromeric/pericentromeric repeat sequences. Scale bar = 10 µm.

4.2.5 Phenotypic characterization of hybrids

4.2.5.1 Self and cross progeny of 5x AAAOO with white clover

The BAR09 group had seven BAR09 hybrids (Table 14a). BAR-101 & 115 and two white clover genotypes ((P/B1xP/B2)-1 and C21557) were used as controls. This group of BAR hybrids was morphologically intermediate between white clover and *T. ambiguum*, but more like *T. ambiguum* due to probably having a high number of *T. ambiguum*-derived chromosomes. The morphological descriptions of these hybrids are given in Tables 4.2.6.1

and 4.2.6.2. Despite high numbers of chromosomes from *T. ambiguum*, all the hybrids were stoloniferous (white clover and *T. occidentale* -like) in character. The highest stolon length and stolon number were recorded in BAR09-106 with 32.6 and 51.3 respectively, which were significantly higher than the white clover controls. Some nodal rooting (a *T. occidentale*/white clover trait) was observed in all the hybrids. The lowest nodal rooting score (1.8) was observed in BAR09-110 while the best anchorage (9.3) was observed in BAR09-100, followed by BAR09-97 (8.0). BAR09-108 and 110 had no white clover genomes in them so the sotoniferous stem with nodal rooting, although of low level, especially in the latter, must have come from stoloniferous *T. occidentale*. Except for BAR09-100, the hybrids showed higher (some of them were non-significant) numbers of inflorescences as compared to the average of white clover parents (34.5). Terminal growth was highly reduced in all the hybrids which apparently inherited terminal flowering from *T. ambiguum*. The most florets per head were observed in BAR09-114 (60.3) which was non-significantly higher than the average value (54) of the two white clover controls ((P/B1xP/B2)-1 and C21557). The differentiation on the basis of leaflet shape was not prominent and none of the hybrids had significantly longer leaflets (*T. ambiguum* trait) than the white clover parent, (P/B1xP/B2)-1) (Table 4.2.6.1). All the hybrids had apparent terminal flowering along with axillary flowering (combination) as compared to the exclusively axillary flowering pattern of white clover (Table 4.2.6.2). The morphological differentiation on the basis of root weight ratio to the total biomass was very prominent (Table 4.2.6.2). Except for BAR09-106 and BAR09-114, all the hybrids had significantly higher root weight % of total biomass (*T. ambiguum*-like) as compared to the two white clover genotypes. BAR09-106 and -114 had non-significantly higher root weight ratio to the total biomass as compared to one of the white clover controls (C21557). The root weight ratio in these hybrids was significantly higher than the second white clover control parent, (P/B1xP/B2)-1. The highest root weight ratio to total biomass was in BAR09-100 (54.9) followed by BAR09-108 (53.3) and BAR09-110 (51.1) (Table 4.2.6.2). The comparatively higher root weight ratio in BAR09-108 and -110 is understandable because they were the self progenies of the original 5x AAAOO plants having more *T. ambiguum* derived chromosomes. But the situation in BAR09-100 was different, having the same breeding history as BAR09-97, -98 and -106 but a comparatively higher root ratio (Table 4.2.6.2).

4.2.5.2 Self and cross progeny of RRAO(A_4) with white clover

Unlike the BAR09 hybrids, the next generation (BAR10) hybrids (resulting from the selfing and crossing of RRAO(A_4) hybrids with white clover were morphologically more like white clover than *T. ambiguum*. Results for the BAR10 hybrids along with the control parents are given in Tables 4.2.7.1 and 4.2.7.2. All the hybrids were stoloniferous with indeterminate growth habit and had axillary flowering (white clover-like). Except for BAR10-118, the nodal root anchorage was less than in the previous generation which was opposite to expectations. The ratio of dry root weight to total biomass was significantly higher in all the BAR10 hybrids as compared to the white clover cultivar, Kopu II. This ratio was also higher in all the hybrids as compared to the (P/B1xP/B2)-2 control, but the difference was statistically significant only for BAR10-118 (Table 4.2.7.2).

Table 4.2.6.1 Mean morphological data of the above -ground traits of the progeny of the crosses involving 5x AAAOO hybrids and white clover RRRR).

Hybrids	Stolon Length (mm)	Stolon number	Stem anchorage (0-10)	Inflorescence No. ‡	Growth habit	Peduncle length (cm)	Florets/ head	Leaflet length/width ratio	Petiole length (cm)	Stem thickness (cm)
BAR09-97	17.4	23.5	8.3	3.7 (42.1)	determinate	7.2	58.3	1.4	4.4	2.68
BAR09-98	13.6	16.5	5.0	4.0 (56.8)	determinate	10.1	59.1	1.4	4.5	1.88
BAR09-100	1.9	8.0	9.3	2.1 (8.1)	determinate	9.2	47.2	1.1	4.6	1.63
BAR09-106	32.6	51.3	5.0	5.5 (252.1)	determinate	12.0	52.5	1.5	4.5	2.78
BAR09-108	7.3	14.3	5.8	3.9 (48.9)	determinate	4.9	32.5	1.4	1.3	1.58
BAR09-110	6.2	9.0	1.8	3.7 (40.0)	determinate	5.3	30.3	1.3	2.2	1.74
BAR09-114	27.6	30.5	8.0	4.6 (94.6)	determinate	10.4	60.3	1.4	3.6	3.38
BAR-101	9.3	23.5	5.5	3.8 (45.2)	determinate	5.7	43.2	1.3	4.0	1.92
BAR-115	15.3	14.5	3.3	3.8 (43.8)	determinate	7.3	52.9	1.3	2.4	2.82
(P/B1 x P/B2)-1	18.6	25.8	10.0	3.7 (41.3)	indeterminate	8.8	58.7	1.4	6.3	2.00
C21557-Brick	13.5	24.3	10.0	3.3 (27.7)	indeterminate	13.2	49.3	1.2	7.0	2.30
CV%	**23.9**	**27.7**	**20.3**	**10.3**		**13.7**	**12.5**	**7.9**	**18.4**	**8.40**
LSD	**5.1**	**8.9**	**1.9**	**0.6**		**1.7**	**8.9**	**0.2**	**1.1**	**0.27**
SEM	**2.5**	**4.3**	**0.9**	**0.3**		**0.8**	**4.4**	**0.1**	**0.5**	**0.13**
Prob. (5%)	***	***	***	***		***	***	***	***	***

‡ Data were log transformed. Values in parenthesis show back-transformed data

Table 4.2.6.2. Mean morphological data of the above and below-ground traits of the progeny of the cross involving 5x AAAOO hybrids and white clover (RRRR).

HYBRIDS	Flowering-terminal, axillary or combination	Seed/head	Main root thickness (mm)	Main nodal root thickness (mm)	Dry weight top (g)	Dry weight root (g)	Total biomass (TBM) (g)	Root weight % of TBM
BAR09-97	combination	0	5.31	1.56	8.2	5.2	13.4	40.9
BAR09-98	combination	0.2	5.92	1.21	4.9	4.5	7.1	48.3
BAR09-100	combination	0	3.98	1.31	2.1	2.6	4.7	54.9
BAR09-106	combination	0.3	7.19	1.86	16.5	8	24.5	35.3
BAR09-108	combination	1.2	3.66	0.5	2.0	2.2	4.2	53.3
BAR09-110	combination	0.5	4.6	0.72	1.9	2.0	3.8	51.1
BAR09-114	combination	0	6.17	1.83	8.8	5.5	14.3	37.8
BAR-101	combination	0.3	6.21	1.22	3.6	3.5	7.1	48.9
BAR-115	combination	0	4.83	1.19	2.3	2.7	3.8	49.8
(P/B1 x P/B2)-1	axillary	23.4	3.1	1.4	8.7	2.2	10.9	19.6
C21557-Brick	axillary	20.3	4.52	1.63	5.2	2.1	7.2	28.5
CV%		**106.8**	**38.4**	**31**	**53.4**	**30.2**	**40.9**	**54.8**
LSD		**6.4**	**2.81**	**0.6**	**4.5**	**1.6**	**5.4**	**10.7**
SEM		**4.5**	**0.96**	**0.2**	**1.6**	**0.6**	**1.9**	**3.7**
Prob. (5%)		***	**	**	***	***	***	***

Table 4.2.7.1 Mean morphological data of the above-ground traits of the advanced progeny of the cross involving RRAO(A) hybrids and white clover (RRRR).

Hybrids	Stolon number	Stolon Length (SL) ‡	Stem anchorage (0-10)	Inflorescence No. ‡	Growth habit	Peduncle length (cm)	Flowering-terminal, axillary or combination	Florets/head
BAR10-111	4.3	2.0 (7.6)	2.3	2.1 (7.9)	indeterminate	9.4	axillary	38
BAR10-118	11.8	2.6 (13.3)	7.8	3.5 (32.8)	indeterminate	7.3	axillary	30.3
BAR10-124	12	2.6 (12.9)	3.8	2.4 (10.7)	indeterminate	8.3	axillary	37
Kopu II	30.5	4.6 (102.5)	10	6.2 (512.9)	indeterminate	16.4	axillary	49.2
(P/B1 x P/B2)-2	9.2	2.1 (8.2)	10	1.9 (6.4)	indeterminate	5.3	axillary	30.3
LSD	**8.1**	**0.7**	**1.1**	**1.4**		**3.3**		**17.2**
CV%	**38.8**	**15.1**	**10.2**	**0.7**		**23.1**		**30.2**
SEM	**3.7**	**0.3**	**0.5**	**28.5**		**1.5**		**7.0**
Prob. (5%)	***	***	***	***		***		*

‡ Data were log transformed
Values in parenthesis show back-transformed data

Table 4.2.7.2. Mean morphological data of the above and under-ground traits of the advanced progeny of the cross involving RRAO(A) hybrids and white clover (RRRR).

Hybrids	Leaflet length/width ratio	Stem thickness (mm)	Petiole length (cm)‡	Seed/head ‡	Main nodal root thickness (mm)	Dry weight top (g) ‡	Dry weight root (g)‡	Total biomass (TBM) (g)‡	Root weight % of TBM
BAR10-111	1.2	1.9	1.0 (2.8)	0.3 (1.4)	1.6	0.6 (1.8)	_0.1 (0.9)	1.0 (2.8)	33.6
BAR10-118	1.3	1.9	1.1 (2.9)	1.8 (5.8)	1.9	1.8 (6.1)	1.6 (4.8)	2.4 (10.9)	44.3
BAR10-124	1.3	2.5	0.9 (2.6)	2.4 (10.8)	2.2	1.4 (3.90)	0.6 (1.8	1.8 (5.9)	32.4
Kopu II	1.2	3.5	2.3 (10.2)	4.6 (97.5)	2.8	5.7 (308)	3.4 (30.3)	5.8 (343.8)	9.9
(P/B1 x P/B2)-2	1.5	2.0	1.0 (2.8)	3.5 (34.7)	1.2	2.2 (8.7)	1.1 (3.1)	2.5 (11.8)	26.4
LSD	**0.2**	**0.7**	**0.4**	**0.8**	**0.6**	**0.6**	**0.5**	**0.5**	**9.7**
CV%	**7.9**	**19**	**22.6**	**18.6**	**19.7**	**16.7**	**25.9**	**12**	**21.5**
SEM	**0.1**	**0.3**	**0.4**	**0.3**	**0.3**	**0.3**	**0.2**	**0.2**	**4.5**
Prob. (5%)	**	***	***	***	***	***	***	***	***

‡ Data were log transformed
Values in parenthesis show back-transformed data

4.3 Strategy 3: (using *T. occidentale* as genetic bridge to combine 6x *T. ambiguum* genomes with *T. repens*)

This strategy is different and more important than the previously given ones; firstly, the A genomes in this hybrid 33 OP-1 based strategy came from the 6x *T. ambiguum* which is considered agronomically superior to the other ploidies and, secondly, this is the first time that A genomes from 6x *T. ambgiuum* have been combined with *T. repens* genomes. The original BAR hybrid was 33 OP-1 (AAAORR, 2n=~6x) which resulted from hybrid "33" (AAAO, 2n=32) after it was allowed to be open pollinated. Hybrid "33" contributed a 2n gamete and through GISH, the male parent was confirmed to be white clover contributing a normal haploid (n) gamete (RR). The BAR hybrids used in the current project were obtained by crossing 33 OP-1 (AAAORR, 2n=~6x) once or twice back to different *T. repens* genotypes and so they were either expectedly RRRA(A_4O_4) (2n=~5x) or RRR($R_4A_6O_2$) (2n=~4.5x).

4.3.1 Hybrids derived from Hybrid 33 OP-1 – RRRA(A_4O_4) (~5x) and RRR($R_4A_6O_2$) (~4.5x)

There were originally 13 BAR hybrids available which were confirmed by the flow cytometry based DNA content analysis to be 4.5x to 5x in ploidy (Tables 4.3.1.1, 4.3.1.2). These 13 "33 OP-1" derived BAR hybrids belong to the same strategy but BAR-59 to 64 were different from BAR-66 to 75 because the latter set was a generation more advanced than the former set because it has been crossed back with white clover one more time. That is why the ploidy level in the latter set was lower as compared to the previous one, with a few exceptions. Pollen fertility in this group ranged from 24% in BAR-61 to 69% in BAR-67. All these hybrids (Tables 4.3.1.1 and 4.3.1.2) were crossed with leaf colour marked white clover. In total, around 9,000 crosses were made. Most produced reasonable quantities of seed when pollinated with white clover. However, BAR-60 did not flower and BAR-75 did not set any seed on crossing with white clover. BAR-74 was excluded from further breeding because its 100 % pollen fertility, apparent 4x ploidy level and high seed-set after crossing with white clover indicated that it was white clover. Most of these BAR hybrids produced seed on selfing. The highest numbers of self seed were produced by BAR-59 and BAR-67 with 2.1 and 2.2 seeds per head respectively.

Table 4.3.1.1 Pedigrees, pollen fertilities, flow cytometric derived ploidy estimates and further crossing details of the original 5x BAR hybrids with genomic formulae RRRA(A_4O_4) derived from Hybrid 33 OP-1 (AAAORR).

BAR #	Pedigree	Expected genomic composition	Pollen Fertility (%)	Ploidy level (x)	Crossed with	No. of crosses	Total seeds	Breeding System (Self seed/head
59	(33 OP-1 x (P/B)-1)-13	AAAORR x RRRR= RRRA(A_4O_4)	41	5.0	(P/B)-17, (P/B1xP/B2)-1	400	21	SC (2.1)
60	(33 OP-1 x (P/B)-1)-14	AAAORR x RRRR= RRRA(A_4O_4)	DNF	5.2				
61	(33OP-1 x (P/B)-1)-15	AAAORR x RRRR= RRRA(A_4O_4)	24	5.2	(P/B)-17, Scarlet-1, (P/B1xP/B2)-1	500	92	SC (1.4)
64	(33 OP-1) x (P/B)-1)-20	AAAORR x RRRR= RRRA(A_4O_4)	27	5.1	(P/B)-17	300	4	SI

DNF Did not flower

Table 4.3.1.2 Pedigrees, pollen fertilities, flow cytometric derived ploidy estimates and further crossing details of the original 4.5x BAR hybrids with expected genomic formula RRR($R_4A_6O_2$) derived from Hybrid 33 OP-1 (AAAORR).

BAR #	Pedigree	Expected genomic composition	Pollen Fertility (%)	Ploidy level (x)	Crossed with	No. of crosses	Total seeds	Breeding System (Self seed/head)
66	(Kopu II R3-2 x (33 OP-1 x (P/B)-1))-1	RRRR x RRRA(A_4O_4)=RRR($R_4A_6O_2$)	33	4.5	(P/B)-17, Scarlet-1	350	78	SC (2)
67	(Kopu II R3-2 x (33 OP-1 x (P/B)-1))-2	RRRR x RRRA(A_4O_4)=RRR($R_4A_6O_2$)	69	4.8	(P/B)-17, Scarlet-1	600	24	SC (2.2)
69	(Kopu II R3-2 x (33 OP-1 x (P/B)-1))-4	RRRR x RRRA(A_4O_4)=RRR($R_4A_6O_2$)	33	5.0	(P/B)-17, (P/B1xP/B2)-1	700	23	SI
70	(Kopu II R3-2 x (33 OP-1x (P/B)-1))-5	RRRR x RRRA(A_4O_4)=RRR($R_4A_6O_2$)	33	5.0	(P/B1xP/B2)-1, (P/B)-17, Scarlet-1	500	50	SI
71	(Kopu II R3-2 x (33 OP-1x (P/B)-1))-6	RRRR x RRRA(A_4O_4)= RRR($R_4A_6O_2$)	34	4.6	(P/B1xP/B2)-1, Scarlet-1	500	55	SC (1.6)
72	(Kopu II R3-2 x (33 OP-1 x (P/B)-1))-7	RRRR x RRRA(A_4O_4)=RRR($R_4A_6O_2$)	30	4.7	(P/B)-17, Scarlet-1, C21557-801	600	16	SI
73	(Kopu II R3-2 x (33 OP-1x (P/B)-1))-8	RRRR x RRRA(A_4O_4)=RRR($R_4A_6O_2$)	62	5.1	(P/B)-17, Scarlet-1, C21557-801	500	40	SC (1)
74	(Kopu II R3-2 x (33 OP-1x (P/B)-1))-9	RRRR x RRRA(A_4O_4)=RRR($R_4A_6O_2$)	100	3.6	(P/B)-17	500	200	SC (0.9)
75	(Kopu II R3-2 x (33 OP-1x (P/B)-1))-10	RRRR x RRRA(A_4O_4)=RRR($R_4A_6O_2$)	29	4.5	(P/B1xP/B2)-17	400	0	SI

4.3.2 Progeny of the original BAR hybrids (BAR09 hybrids)

4.3.2.1 Progeny of RRRA(A_4O_4) (5x) and RRR($A_6R_4O_2$) (4.5x) with *T.repens*

Eight plants were selected on the basis of phenotype and ploidy level from 272 plants obtained from the hybrids with genomic compositions of RRRA(A_4O_4) and RRR($A_6R_4O_2$) (Tables 4.3.1.1, 4.3.1.2) after selfing and crossing with *T.repens*. The details of the selected plants are given in Table 4.3.2. All the selected BAR09 hybrids in this group (Table 4.3.2) were aneuploids with somatic chromosome number ranging from 31 in BAR09-67 to 38 in BAR09-57. Figure 4.3.1a shows the karyotype of BAR09-63 (2n = 33). The expected genomic formulae of the progeny from the crosses, RRRA(A_4O_4) x RRRR and RRR($A_6R_4O_2$) x RRRR were RRR($A_6R_4O_2$) (2n=~36) and RRR($R_6A_3O_1$) (2n=~34) respectively. The actual somatic chromosome counts were lower by one to three chromosomes than the expected number except in BAR09-62, BAR09-65 & BAR09-75 where the expected and actual chromosome counts matched. All the hybrids proved to be cross-fertile and produced large numbers of seeds when pollinated with white clover. BAR09-54, BAR09-56, BAR09-62 and BAR09-75 did not produce any seed after self-pollination while BAR09-57, BAR09-63, BAR09-65 and BAR09-67 were self-compatible (SC) producing reasonable quantities of self seed. The highest count was for BAR09-63 (60 self seeds per head) (Table 4.3.2).

4.3.2.2 Meiotic chromosome pairing analysis in BAR09-62, BAR09-63 and BAR09-65

BAR09-62 (RRR($R_4A_6O_2$)) and BAR09-63 (RRR($R_6A_3O_1$)) were the cross progenies of original hybrids, BAR-64 and BAR-66 respectively with coloured leaf white clovers. BAR09-65 was the self progeny of BAR-66 (RRR($A_6R_40_2$). These three hybrids were aneuploids with somatic chromosome numbers of 2n=36 in BAR09-62, 2n=33 in BAR09-63 and 2n=36 in BAR09-65. The three different genomes (R, A and O) in these hybrids have gone together through 4-5 meiotic cycles thus giving them several chances of recombination. For meiotic chromosome pairing behaviour analysis, 63, 61 and 60 PMCs were screened in BAR09-62, BAR09-63 and BAR09-65, respectively (Table 4.3.3).

Chromosome stickiness was very frequently observed in BAR09-62 and BAR09-63 (Figure 4.3.1d) while cytomixis was observed in BAR09-62 and BAR09-65. Second division restitution (SDR) was also observed in BAR09-62. Lagging chromosomes

Table 4.3.2 Selected progeny plants of original BAR hybrids, RRRA(A_4O_4) and RRR($R_4A_6O_2$) (Table 16a, & b) with pedigrees, expected genomic composition, estimated ploidies (flow cytometry), expected and actual chromosome numbers, pollen fertility, details of further crosses and breeding system (SC, self-compatible, SI, self-incompatible).

BAR09 No	Pedigree	Exp. genomic composition	Pollen fertility (%)	Ploidy (x)	Exp. Chrom. #	Actual chrom. #	Crossed with	Total crosses	Total seeds	Breeding system (self seed/head)
BAR09-54	(BAR-59 x (P/B)-17)-1	RRRA(A_4O_4) x RRRR= RRR($R_4A_6O_2$)	18	4.6	36	35	(P/B1xP/B2)-2, C21557-801	400	85	SI
BAR09-56	(BAR-59 x (P/B)-17)-8	RRRA(A_4O_4) x RRRR= RRR($R_4A_6O_2$)	27	4.7	36	--	(P/B1xP/B2)-2	200	65	SI
BAR09-57	(BAR-59-Selfed)-1	RRRA(A_4O_4) x RRRA(A_4O_4)= RRRA(A_4O_4)	77	5.4	40	38	(P/B)-17	100	94	SC (38)
BAR09-62	(BAR-64 x Scarlet-1)-3	RRRA(A_4O_4) x RRRR= RRR($R_4A_6O_2$)	45	5.2	36	36	C21557-815	200	19	SI
BAR09-63	(BAR-66 x (P/B)-17)-7	RRR($R_4A_6O_2$) x RRRR= RRR($R_6A_3O_1$)	75	4.3	34	33	(P/B)-17	150	340	SC (60)
BAR09-65	(BAR-66-Selfed)-2	RRR($R_4A_6O_2$) x RRR($R_4A_6O_2$)= RRR($R_4A_6O_2$)	72	5	36	36	(P/B)-17	150	49	SC (19)
BAR09-67	(BAR-67 x Scarlet-1)-3	RRR($R_4A_6O_2$) x RRRR= RRR($R_6A_3O_1$)	56	4	34	31	C21557-808, -801	300	111	SC (9)
BAR09-75	(BAR-71 x (P/B1x P/B 2)-1)-2	RRR($R_4A_6O_2$) x RRRR= RRR($R_6A_3O_1$)	79	4.4	34	34	(P/B1xP/B2)-2	150	201	SI

Table 4.3.3. Meiotic chromosome associations at diakinesis/metaphase-I in PMCs of BAR09-62, BAR09-63 and BAR09-65.

Hybrid name	Pedigree	Expected genomic composition	No. of PMCs scored	Mean frequency (range) of chromosomal configurations					
				I	II	III	IV	V	Pollen fertility (%)
BAR09-62 (2n=36)	(BAR-64 x Scarlet-1)-3	RRRA(A_4O_4) x RRRR= RRR($R_4A_6O_2$)	63	5.4 (1-11)	9.7 (4-15)	2.4 (0-8)	1.0 (0-4)	0	45
BAR09-63 (2n=33)	(BAR-66 x (P/B)-17)-7	RRR($R_4A_6O_2$) x RRRR= RRR($R_6A_3O_1$)	61	1.5 (0-8)	12.6 (6-16)	1.1 (0-4)	0.8 (0-4)	0	75
BAR09-65 (2n=36)	(BAR-66-Selfed)-2	RRR($R_4A_6O_2$) x RRR($R_4A_6O_2$) = RRR($R_4A_6O_2$)	60	6.8 (1-16)	10.1 (4-15)	2.0 (0-6)	0.7 (0-3)	0	72

during anaphase-I were observed in all three hybrids (Fig. 4.3.1f). The chromosome association (I, II, III & IV) frequency varied among these hybrids. The number of Is was approximately proportional to the predicted number of *T. ambiguum*-derived chromosomes (Tables 4.3.1.1, 4.3.1.2, 4.3.2, 4.3.3). More Is were observed in BAR09-62 (5.4 /PMC) (Figure 4.3.1b) and BAR09-65 (6.8 /PMC) probably due to these having more *T. ambiguum* and *T. occidentale* derived chromosomes (Table 4.3.3). By contrast, comparatively more IIs were observed in BAR09-63 (12.6/PMC), probably because it had relatively more *T. repens*-derived chromosomes (approximately 30) than the other hybrids (Figure 4.3.1c). IIIs and IVs were also observed in significant frequencies in all three hybrids (Table 4.3.3, Figure 4.3.1b, c, e). IIIs could involve a combination of homologous white clover pairing with homoeologous chromosome coming from the other white clover sub-genome. IVs might represent either RRRR or RRRO or RROA chromosomal associations. Conceivably, any RRAO chromosomal associations could have A and O recombinant chromosomes from the previous generations (Table 4.3.3).

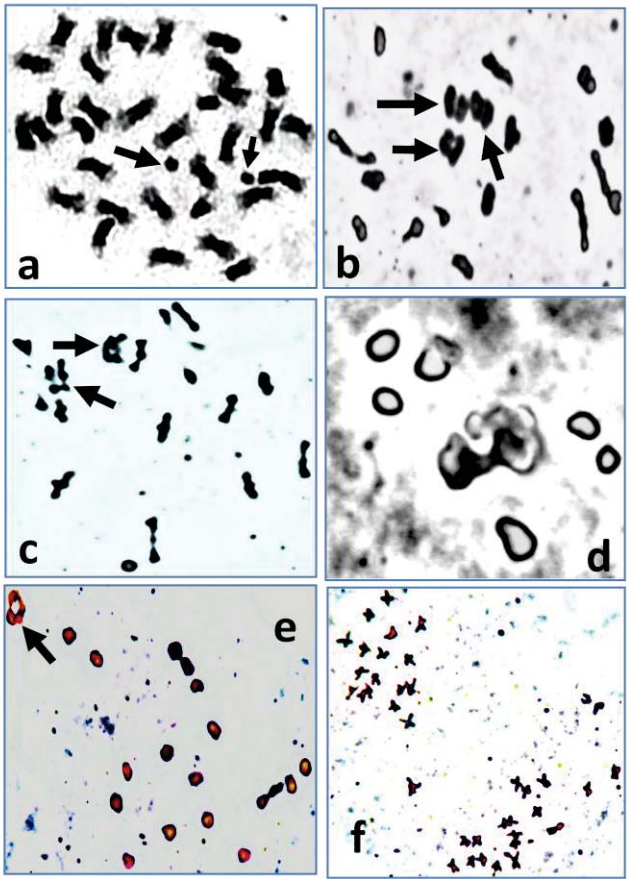

Figure 4.3.1 (**a**) Giemsa-stained somatic chromosomes in BAR09-63 (RRR($R_6A_3O_1$)-1, 2n=33). Two satellite knobs are seen lying away from the main chromosomal bodies (arrows). (**b**). Metaphase-I in BAR09-62 (RRR($A_6R_4O_2$), 2n=36) with 4 Is, 7 IIs, 2 IIIs and 3 IVs (arrows). (**c**). Metaphase-I in BAR09-63 with 1 I, 12 IIs and 2 IVs (arrows). The only univalent might be a *T. ambiguum*-derived chromosome because later, through GISH, it was confirmed that BAR09-63 had one chromosome from this species (Figures 11 and 12). (**d**) An abnormal situation during metaphase-I in BAR09-63 where chromosomes stuck to each other in lumps. This phenomenon of stickiness was observed in many hybrids. (**e**) Metaphase-I in BAR09-65 (RRR($A_6R_4O_2$) 2n=36) showing predominantly IIs and one IV (arrow). (**f**) Anaphase-I in BAR09-65 with a single laggard chromosome.

4.3.3 Self and cross progeny of the BAR09 hybrids (BAR10 hybrids)

4.3.3.1 Progeny of RRR($R_4A_6O_2$) ~ 4.5x and RRR($R_6A_3O_1$) ~ 4.25x

The BAR10 generation was obtained by selfing, inter-crossing and backcrossing BAR09 hybrids with the hypothetical genomic composition of RRR($R_4A_6O_2$)~4.5x and RRR($R_6A_3O_1$)~4.25x with white clover. Five to thirty seeds (depending on the availability of seed) per family were used and 170 progeny seedlings were obtained. Plant morphology, leaf colour, pollen fertility and flow cytometry ploidy estimates were used as criteria for the selection of plants for further cyto-morphological analysis. Ten hybrids were selected from this group of BAR10 hybrids and the details, including the expected genomic compositions, are given in Table 4.3.4. The somatic chromosome counts in BAR10-81 and BAR10-93 were 33 and 34 respectively (Figures 4.3.2a, 4.3.3a). The pollen stainability ranged from 37% in BAR10-76 to 88% in BAR10-88. Flow cytometry based ploidy levels matched well with the actual somatic chromosome counts. These plants given in Table 4.3.4 have been crossed with white clover four to five times and so the 2n chromosome number has dropped to near 4x, with some exceptions where there have been some selfing/inter-crossing and less back-crossing with white clover (one and two selfings in case of BAR10-72 and BAR10-76 respectively). Hypothetically BAR10-81 (with genomic formula RRR($R_7A_{1-2}O_{0-1}$)) would be expected to have 32-33 chromosomes with over 31 chromosomes from *T. repens* and 1-2 and 0-1 chromosomes respectively with *T. ambiguum* and *T. occidentale* origin. These BAR10 hybrids (Table 4.3.4) have gone through 5-6 generations with odd numbers of chromosomes from all the species (especially *T. ambiguum* and *T. occidentale*). Keeping in view the number of meiotic cycles these plants have gone through and the high numbers of multivalent chromosome associations, there have been many chances for introgression (recombination) among the sub-genomes. Potentially, therefore, these progenies are likely to show introgression, if it has occurred. For this reason, some of them were further characterised using molecular cytogenetic techniques (FISH and GISH) for introgression analysis.

4.3.3.2 Meiotic chromosome analysis in BAR10-80, BAR10-81 & BAR10-93

BAR10-80, BAR10-81 & BAR10-93 were selected from the BAR10 generation of this strategy. The expected genomic compositions of these hybrids were RRR($R_6A_3O_1$), RRR($R_7A_{1-2}O_{0-1}$) & RRR($R_4A_6O_4$), except that the hybrid parent of BAR10-93 (2n=34)

Table 4.3.4 Selected progeny plants of the BAR09 hybrids with expected genomic formula RRR($R_4A_6O_2$) and RRR($R_6A_3O_1$) (Table 17), with ploidy estimates (flow cytometry), expected and actual chromosome numbers and pollen fertility.

Hybrid	Cross/identification	Expected genomic composition	Pollen fertility %	Ploidy (x)	Exp. 2n chrom #	Actual 2n chrom. #
BAR10-72	(BAR09-57 x (P/B)-17)-1	(RRRA(A_4O_4))-2 x RRRR = (RRR($R_4A_6O_2$))-1	50	4.5	35	36
BAR10-76	(BAR09-57-Selfed)-1	(RRRA(A_4O_4))-2 x RRRA(A_4O_4)-2= (RRRA(A_4O_4))-2	37	5.23	38	39
BAR10-80	(BAR09-62 x C21557-815)-6	RRR($R_4A_6O_2$) x RRRR= RRR($R_6A_3O_1$)	79	4.31	34	34
BAR10-81	(BAR09-63 x (P/B)-17)-1	(RRR($R_6A_3O_1$))-1 x RRRR = (RRR($R_7A_{1-2}O_{0-1}$))-(0-1)	82	4.22	32-33	33
BAR10-86	(BAR09-63-Sefled)-1	(RRR($R_6A_3O_1$))-1 x (RRR($R_6A_3O_1$))-1= (RRR($R_6A_3O_1$))-(0-2)	85	--	32-34	32
BAR10-88	(BAR09-63 x BAR09-67)-4	(RRR($R_6A_3O_1$))-1x (RRR($R_6A_3O_1$))-3= (RRR($R_6A_3O_1$))-2	98	--	32	32
BAR10-90	(BAR09-65 x (P/B)-17)-1	RRR($R_4A_6O_2$) x RRRR = RRR($R_6A_3O_1$)	93	4.02	34	32
BAR10-93	(BAR09-65-Selfed)-1	RRR($R_4A_6O_2$) x RRR($R_4A_6O_2$)= RRR($R_4A_6O_2$)	74	4.34	36	34
BAR10-100	(BAR09-67-Selfed) - 1	(RRR($R_6A_3O_1$))-3 x (RRR($R_6A_3O_1$))-3 = (RRR($R_6A_3O_1$))-3	88	--	30-32	32
BAR10-105	(BAR09-75 x (P/B1xP/B2) -2)-10	RRR($R_6A_3O_1$) x RRRR = RRR($R_7A_{1-2}O_{0-1}$)	92	4.1	33	32

Table 4.3.5. Meiotic chromosome associations at diakinesis/metaphase-I in PMCs of BAR10-80, BAR10-81 & BAR10-93.

Hybrid name	Pedigree	Exp. genomic composition	No. of PMCs scored	Mean frequency (range) of meiotic configurations					
				I	II	III	IV	V	Pollen fertility (%)
BAR10-80 (2n=34)	(BAR09-62 x C21557-815)-6	RRR($R_4A_6O_2$) x RRRR= RRR($R_6A_3O_1$)	60	2.1 (0-6)	13.1 (6-16)	0.8 (0-7)	0.8 (0-3)	0	97
BAR10-81 (2n=33)	(BAR09-63 x (P/B)-17)-1	(RRR($R_6A_3O_1$))-1x RRRR= (RRR($R_7A_{1-2}O_{0-1}$))-(0-1)	75	1.4 (0-5)	13.2 (8-16)	0.6 (0-4)	0.8 (0-3)	0.1 (0-1)	82
BAR10-93 (2n=34)	(BAR09-65-Selfed)-1	RRR($R_4A_6O_2$) x RRR($R_4A_6O_2$)= RRR($R_4A_6O_2$)	83	1.9 (0-6)	12.4 (6-16)	1.1 (0-5)	1.1 (0-5)	0	74

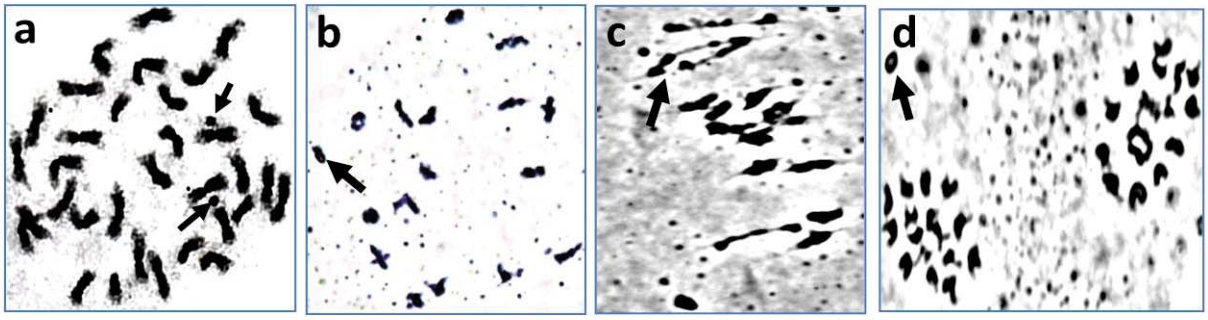

Figure 4.3.2 (**a**) Giemsa-stained somatic chromosomes in BAR10-81 (RRR($R_7A_{1-2}O_{0-1}$), 2n=33) having two satellite knobs (arrows) lying away from the main chromosomes. (**b**), (**c**); Two PMCs in BAR10-81 at metaphase-I in which the first has near-regular meiosis with 16 IIs and 1 I (arrow) and the second has a ring IV (arrow). (**e**) Anaphase-I in BAR10-81 with a laggard (arrow) and 16 chromosomes at each pole.

which has lost two chromosome (Table 4.3.5). The somatic chromosome counts in BAR10-80, BAR10-81 and BAR10-93 were 34, 33 and 34, respectively (Table 4.3.4). The additional numbers of chromosomes above 32 (possibly *T. ambiguum* chromosomes) ranged from 1 in BAR10-81 to 2 in BAR10-93. All these hybrids showed comparatively low levels of meiotic abnormality (non-bivalents) with more balanced disjunction of chromosomes during anaphase-1 (Figures 4.3.2b, c, d, 4.3.3b, c, d). This regularity in meiosis was reflected in the high pollen fertilities (Table 4.3.5). Nevertheless, meiotic disturbances at a low level were observed, e.g. lagging chromosomes (Figures 4.3.2d, 4.3.3e) and second division restitution.

In BAR10-80, chromosomal configurations ranging from Is to IVs were observed. The multivalent formations might have shown both homo- and homoeologous chromosome pairing within white clover. But, because the number of Is was less than the expected number of *T. ambiguum* derived chromosomes, there was also a possibility that they involved interspecific chromosome pairing. The most frequent chromosomal disjunction observed in this hybrid during anaphase-I was 16-18.

A similar situation regarding chromosome pairing was observed in BAR10-81. But, in addition to IIIs and IVs, a very low average frequency of Vs (0.1/PMC) was also recorded by this hybrid. These Vs might involve four chromosomes from white clover plus a homoeologous chromosome from *T. occidentale* or *T. ambiguum,* or a recombinant chromosome between A and O sub-genomes (Table 4.3.5). The most frequent chromosomal disjunctions observed in BAR10-81 during anaphase-I was 16-17, while the most common chromosomal associations in BAR10-81 were 16 IIs and one I.

Cytomixis was frequently observed in BAR10-93. The most common chromosomal association pattern in BAR10-93 was two Is, 12 IIs and 2 IVs. Figures 4.3.3b, c show PMCs in BAR10-93 with two I, 16 II, and one I, 13 II, one III and one IV, respectively. The most frequent chromosomal disjunction observed in BAR10-93 was 17-17 (Figure 4.3.3d).

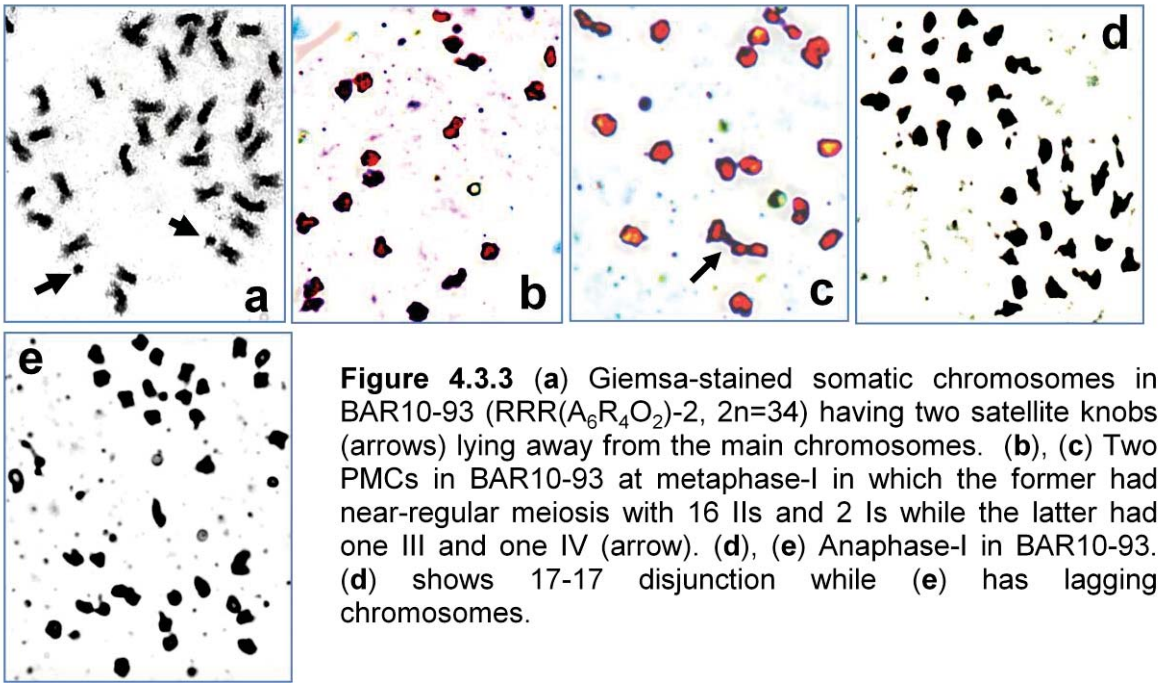

Figure 4.3.3 (**a**) Giemsa-stained somatic chromosomes in BAR10-93 (RRR($A_6R_4O_2$)-2, 2n=34) having two satellite knobs (arrows) lying away from the main chromosomes. (**b**), (**c**) Two PMCs in BAR10-93 at metaphase-I in which the former had near-regular meiosis with 16 IIs and 2 Is while the latter had one III and one IV (arrow). (**d**), (**e**) Anaphase-I in BAR10-93. (**d**) shows 17-17 disjunction while (**e**) has lagging chromosomes.

4.3.4 Molecular cytogenetic analysis of BAR09-63, BAR10-81 and BAR10-93

4.3.4.1 Meiotic chromosome pairing analysis in BAR09-63

GISH and FISH experiments were carried out to analyse the chromosome pairing in metaphase-I cells of BAR09-63 (2n=33). Two probes were used for this purpose i.e., fluorescently labelled total genomic DNA of *T. ambiguum* (green) and a part of 5S rDNA (red). BAR09-63 had one *T. ambiguum* chromosome confirmed by GISH analysis of somatic chromosomes (to be discussed later). In 31 % of the studied PMCs (8 of 26), the *T. ambiguum*-derived chromosome associated with other homoeologous chromosomes either in a bivalent (4 PMCs) or a trivalent (4 PMCs) (Figures 4.3.4a, b). In the remaining 69 % of PMCs, the *T. ambiguum*-derived chromosome behaved as a univalent (Figures 4.3.4c, d). Precocious chromatid separation of the *T. ambiguum* chromosome was observed very frequently, followed by the movement of each chromatid toward opposite poles. In many cases, these chromatids lagged behind and did not become part of the tetrads, and so the *T. ambiguum* derived chromosome was eliminated during meiosis (Figure 4.3.4e).

4.3.4.2 Genomic composition analysis in BAR09-63, BAR10-81 and BAR10-93

BAR09-63, which was from the cross BAR-66 x RRRR, had an expected genomic composition of RRR($R_6A_3O_1$), but had lost one chromosome, giving 2n=33 (Table 4.3.2, Fig. 4.3.1a). GISH, using genomic DNA of *T. ambiguum* labelled with Fluor-X-dCTP (green) and Cy3-dCTP labelled pTr5S, (red), painted one *T. ambiguum*-derived chromosome in this hybrid (Fig. 4.3.5b). BAR09-63 gave four 5S rDNA signals in response to the second probe (Cy3-dCTP labelled pTr5S, (red)), two were located on the longer arms of a pair of NOR-chromosomes (Fig. 4.3.5b, c) and two on another pair of chromosomes. Two satellite knobs and highly de-condensed NORs were observed in this hybrid. The high intensity of these 5S signals indicated that these chromosomes were

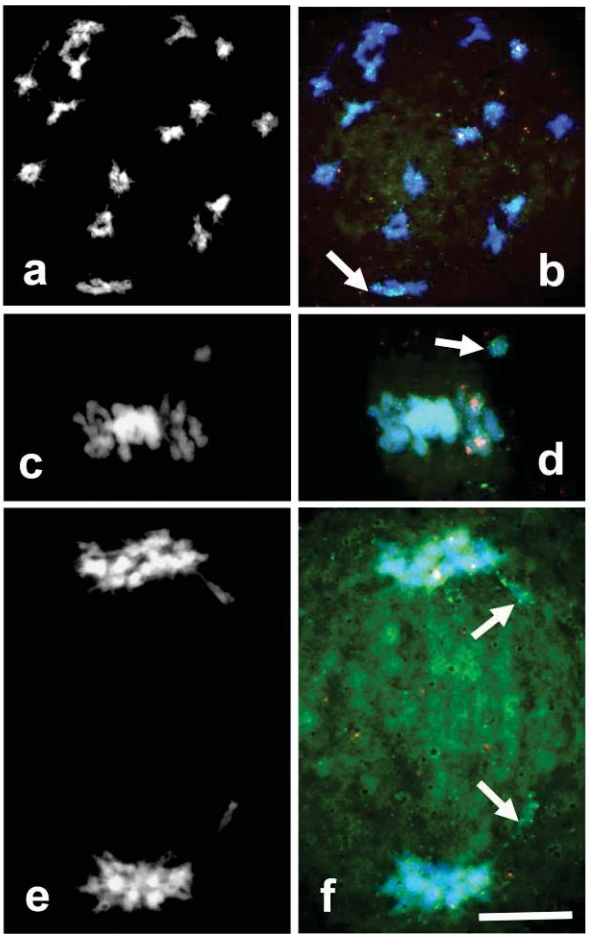

Figure 4.3.4 GISH-FISH on meiotic chromosomes spread in BAR09-63 using gDNA of *T. ambiguum* labelled with Fluor-X-dCTP (green) and Cy3-dCTP labelled pTr5S (red) as probes. (**a**). DAPI-stained diakinesis chromosomes in BAR09-63 with 15 IIs, 1 III. (**b**). GISH-FISH on the same cell, showing a T. *ambiguum* chromosome pairing with other chromosomes as a trivalent (arrow). (**c**), (**d**) show the same cell. In (**d**), after GISH/FISH followed by counterstaining with DAPI, shows the *T. ambiguum* derived-chromosome is not taking part in pairing (arrow). (**e**), (**f**) show the chromosome from *T.ambiguum* has precociously split into chromatids during anaphase-I and the partially lagging chromatids are moving to opposite poles (arrows). Bar = 10 µm.

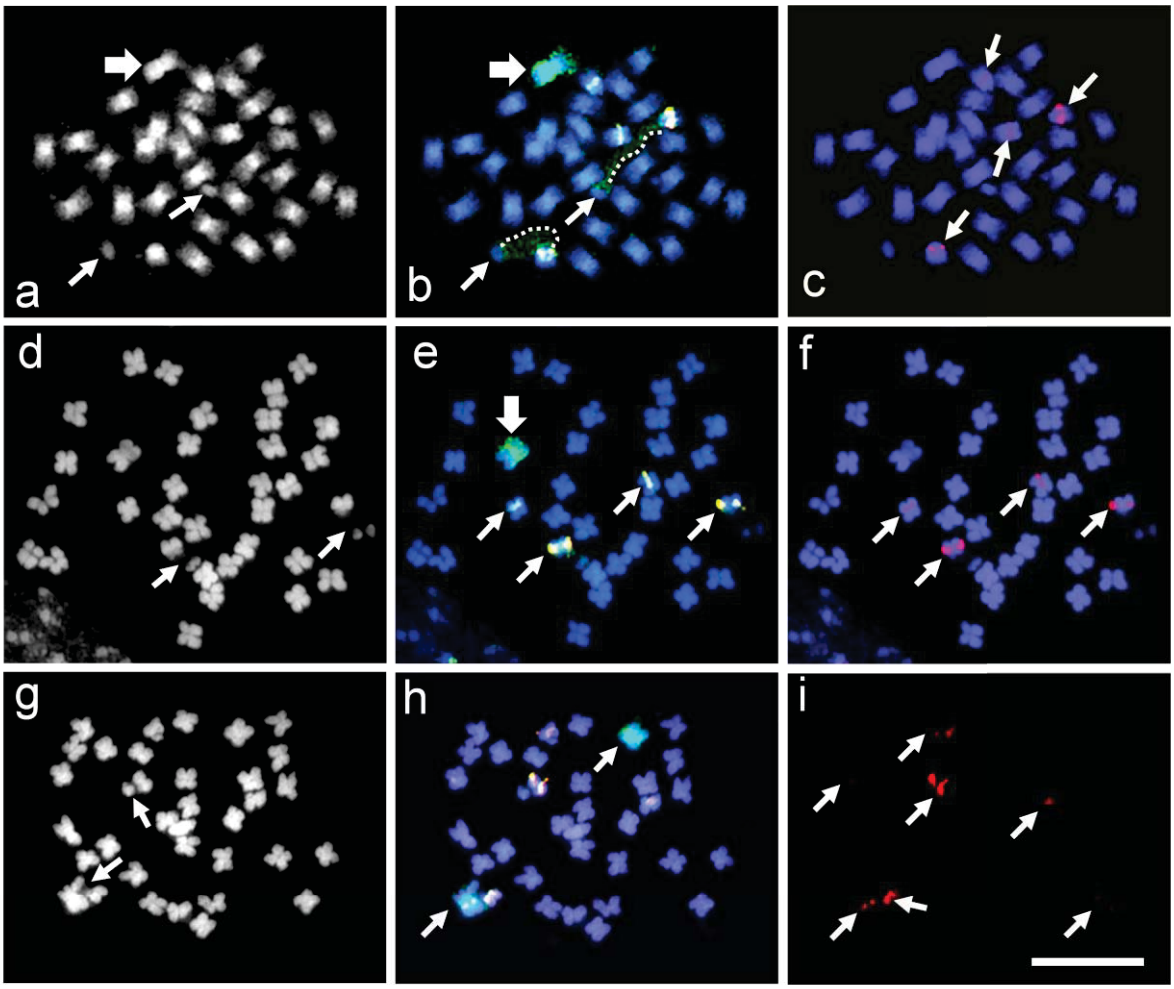

Figure 4.3.5 GISH-FISH on somatic chromosome preparations of BAR09-63 (a-c), BAR10-81 (d-f) and BAR10-93 (g-i). (**a)**. DAPI-stained somatic chromosomes in BAR09-63 (RRR($R_6A_3O_1$)-1, 2n= 33) showing two satellites (small arrows) and one comparatively larger chromosome (bold arrow). (**b**) The same cell as in (**a)** after GISH/FISH probed with genomic DNA of *T. ambiguum* labelled with Fluor-X-dCTP (green) and Cy3-dCTP labelled pTr5S, (red). GISH painted the one larger chromosome green (bold arrow). Highly de-condensed NOR DNA (connecting satellites with respective main chromosomal bodies) was also painted green due to cross-hybridization. (**c**) FISH with Cy3-dCTP labelled pTr5S, (red) on the same cell gave four 5S signals (arrows in **b** and **c**). Two were on a pair of chromosomes having highly stretched NORs, and two were located separately on non-NOR bearing chromosomes from white clover. (**d).** DAPI-stained metaphase chromosomes in BAR10-81, 2n=33. Two NOR bearing chromosomes are visible (arrows). (**e)**. GISH-FISH with the same probes as mentioned above on the same cell as in **d** detected one chromosome from *T. ambiguum* (bold arrow) and four 5S r DNA signals (arrows). (**f)**. FISH only using Cy3-dCTP labelled pTr5S as probe on the same cell gave four 5S signals. Two signals were on the NOR bearing chromosomes coming either from white clover or *T. occidentale*. The other two, based on size, came from *T. repens. (***g).** DAPI-stained somatic chromosomes in BAR10-93, showing 34 chromosomes with a pair having satellite knobs. (**h)**. The same cell as in (**g)** after GISH-FISH using the same two probes highlighted two chromosomes from *T. ambguum,* one with a 5S signal. **(i)** The 5S probe revealed seven 5S signals (arrows), of which two were on the NOR bearing chromosomes and two were on non_NOR chromosomes from white clover. The two smallest ones came from *T. occidentale* and the last was on one of the two *T. ambiguum* chromosomes. BAR = 10µm.

from white clover (Fig. 4.3.5c). The hybridization on the *T. ambiguum* was evenly distributed throughout the chromosome lengths and there were no apparent signs of inter-genomic recombination (introgression) on any chromosome.

BAR10-81 resulted from the cross of BAR09-63 with white clover to give an expectation of approximately RRR($R_7A_{1-2}O_{0-1}$), 2n=32-33) and an actual number of 2n=33 (Table 4.3.4, Figure 4.3.2a). GISH on BAR10-81 revealed one *T. ambiguum* chromosome (Figures 4.3.5e). A similar situation was observed in BAR10-81 as in BAR09-63 regarding probing with 5S rDNA but the size of one of the 5S rDNA signals on a non-NOR chromosome was very small, possibly indicating that that this chromosome had been inherited by BAR10-81 from the *T. occidentale* grandparent (Figure 4.3.5e, f).

BAR10-93 was unique in being derived by two generations of self-pollination from BAR-66 [RRR($R_4A_6O_2$), 2n=4.5x=36)], including the selfing of BAR09-65 (confirmed 2n=4.5x=36). It had 2n=34 chromosomes, and thus had lost two chromosomes (Table 4.3.4, Figure 4.3.3a). Two satellite knobs and highly de-condensed NORs were observed in BAR10-93 as in BAR09-63 and BAR10-81. Of the 34 chromosomes, two were painted by GISH and so were derived from *T. ambiguum,* with one having a 5S signal (Fig. 4.3.5h). This hybrid had seven 5S signals, with two of these on the NOR-carrying chromosomes, and so coming from either *T. repens* or *T. occidentale*. Of the remaining five 5S signals, two were carried by white clover-derived chromosomes, two (being very small) by *T. occidentale* derived chromosomes and one by a *T. ambiguum* derived chromosome (Figure 4.3.5h, i). In none of the three plants were any apparent signs of the exchange of inter-specific chromosomal segments observed. However, the presence of two *T. ambiguum* and two *T. occidentale* chromosomes indicated that some chromosome substitution of A for R and O for R had apparently occurred.

4.3.5. Phenotypic studies of BAR09 and BAR10 hybrids

4.3.5.1 Self and cross progeny of 5x RRRA(A_4O_4) and 4.5x RRR(R_4 A_6O_2)

This experiment included eight BAR09 hybrids resulting from the selfing and crossing of 5x (RRRA(A_4O_4)) and 4.5x (RRR($A_6R_4O_2$)) plants, and these are listed in Tables 4.3.6.1, 4.3.6.2. Two original hybrids (BAR-60 and BAR-66) and two white clover genotypes ((P/B)-17 and C21557-801) were used as control parents. The mean data on the studied characters are given in Tables 4.3.6.1, 4.3.6.2. All the traits recorded significant differences among the hybrids. All eight BAR09 hybrids along with the original BAR hybrids inherited

stoloniferous growth from white clover and/or *T. occidentale*, and all had some nodal rooting. The level of nodal rooting (anchorage) in the BAR09 hybrids varied from weak (2.25) in BAR09-57 to strong (8.75) in BAR09-62 and BAR09-75. Five of the BAR09 hybrids had significantly higher stolon numbers than the white clover controls. BAR09-63 recorded transgressively higher stolon number and length among all the hybrids and control parents. BAR 09-63, BAR09-67 and BAR 09-75 recorded significantly higher numbers of inflorescences per plant than the better white clover control. BAR09 hybrids with higher numbers of *T. ambiguum* chromosomes (Table 4.3.2, i.e., BAR09-54, BAR09-56, BAR09-57 and BAR09-62) had highly reduced terminal growth and apparent terminal flowering (*T. ambiguum*-like phenotype). The number of florets/ head in the BAR09 hybrids was also significantly higher than the white clover parents. Leaflet shape was intermediate, showing the influence of all parental species (Table 4.3.6.1). All the hybrids were female fertile, setting some seed on open pollination. BAR09-63 set the most seed and this was significantly higher than all the other hybrids and controls except (P/B)-17. Several of the hybrids recorded thicker main nodal roots (although some of them were non-significantly thicker) as compared to the control genotypes. However, BAR09-54 and 56 had, respectively, the same or thinner main nodal roots as compared to the white clover controls. As a result of comparatively thicker and longer nodal roots, all except three BAR09 hybrids (BAR09-63, 67, 75) had significantly higher root weight % of the total biomass (Table 4.3.6.2). No rhizomes were observed in any of the BAR09 hybrids or the original BAR hybrids. Anchorage scores of less than 10, highly reduced terminal growth, relatively longer leaflets, near-terminal flowering and higher root weight % of the total biomass showed the expression of *T. ambiguum* derived traits (Tables 4.3.6.1, 4.3.6.2).

4.3.5.2 Self and cross progeny of RRR($R_4A_6O_2$) and RRR($R_6A_3O_1$)

Nine progeny of the above plants after selfing/backcrossing with white clover (BAR10 hybrids) were evaluated morphologically. These are listed in Tables 4.3.7.1, 4.3.7.2. BAR-66 and two white clover genotypes (Kopu II and (P/B1xP/B2)-2 were used as controls. Although, all traits studied recorded highly significant variations, at the same time they indicated a progressive fading of the *T. ambiguum*-specific morphology with

Table 4.3.6.1 Mean data of different above -ground morphological characters of the advanced generation of the cross involving 6x 33-OP-1 (AAAORR) and white clover (RRRR).

HYBRIDS	Stolon number	Stolon Length (cm)	Stem anchorage (0-10)	Inflorescence No.	Growth habit	Peduncle length (cm)	Florets/head	Leaflet length/width ratio	Petiole length (cm)
BAR09-54	40.2	21.2	4.3	89.5	determinate	15.6	64.3	1.5	6.4
BAR09-56	3.6	1.5	4.5	3.3	determinate	7.2	44.8	1.4	3.4
BAR09-57	16.7	11.2	2.3	32.5	determinate	14.5	61.6	1.6	3.2
BAR09-62	15.9	18.0	8.8	54.5	determinate	11.8	62	1.6	5.5
BAR09-63	54.5	40.5	8.5	150.5	combination	18.8	78.4	1.5	9.5
BAR09-65	50.3	21.3	6.8	73.5	indeterminate	13	89	1.8	7.1
BAR09-67	49.1	23.5	8.5	186.2	combination	14.2	62.1	1.3	4.9
BAR09-75	34.9	34.3	8.8	161.8	combination	19.8	73.6	1.7	7.8
BAR-60	39.7	33.5	5.3	90.8	determinate	15.6	65.4	1.9	8.3
BAR-66	34.7	26.8	9	50	indeterminate	15.4	83.6	1.4	7.2
(P/B)-17	24.7	22	10	73.3	indeterminate	12.3	55.5	1.4	6.5
C21557-801	12.3	11	10	12.8	indeterminate	12.9	53.6	1.3	6.5
CV%	**16**	**25.2**	**24.8**	**33.8**		**10.3**	**9.5**	**7.8**	**16.7**
LSD	**7.2**	**8.0**	**2.6**	**39.6**		**2.1**	**9.0**	**0.2**	**1.5**
SEM	**3.6**	**3.9**	**1.3**	**19.5**		**1.0**	**4.4**	**0.1**	**0.7**
Prob. (5%)	***	***	***	***		***	***	***	***

Table 4.3.6.2 Mean data of different above and under-ground morphological characters of the advanced generation of the cross involving 6x 33-OP-1 (AAAORR) and white clover (RRRR).

HYBRIDS	Flowering-terminal, axillary or combination	Stem thickness (mm)	Seed/head	Main Root Thickness (mm)	Main nodal root thickness (mm)	Dry weight top (g	Dry weight root (g)	Total biomass (TBM) (g)	Root weight % of TBM
BAR09-54	combination	3.4	14.8	5.6	1.5	6.3	4.0	10.3	40.0
BAR09-56	combination	2.3	7.5	1.7	0.4	1.6	2.1	3.4	54.8
BAR09-57	combination	3.5	14.8	5.7	2.3	4.2	2.4	6.5	37.2
BAR09-62	combination	2.3	3.1	3.9	2	4.4	2.4	6.8	37.7
BAR09-63	combination	3.9	45.2	5.7	2.5	21.3	8.2	29.5	27.7
BAR09-65	axillary	3.5	19.2	5.1	2.6	4.9	1.4	6.1	31.8
BAR09-67	combination	3.0	14.0	3.4	1.8	11.8	3.7	15.6	22.0
BAR09-75	combination	3.7	25.3	5.1	1.9	8.8	4.5	10.8	28.4
BAR-60	combination	2.8	9.9	10.1	2.7	19.8	13.39	33.3	40.8
BAR-66	axillary	3.6	4.5	9.2	2.2	16.2	8.5	24.7	35.1
(P/B)-17	axillary	2.5	41.5	2.5	1.3	4.4	0.9	5.3	21.4
C21557-801	axillary	2.6	28.7	3	1.5	10.3	2.9	13.2	22
CV%		**8.9**	**53.4**	**40.7**	**17.5**	**50.4**	**67**	**71.5**	**35.8**
LSD		**0.4**	**14.6**	**3.2**	**0.5**	**10.3**	**4.4**	**14.3**	**8.7**
SEM		**0.2**	**10.1**	**1.0**	**0.2**	**3.6**	**2.2**	**4.9**	**3.0**
Prob. (5%)		***	***	***	***	***	***	**	***

Table 4.3.7.1. Mean data of different above-ground morphological characters of the advanced generation of the cross RRR(RAO) x white clover (RRRR).

Hybrids	Stolon number	Stolon Length (cm) ‡	Stem anchorage (0-10)	Inflorescence No. ‡	Growth habit	Peduncle length (cm)	Flowering-terminal, axillary or combination	Florets/ head
BAR10-72	10.2	3.3 (27.1)	7	3.3 (26.9)	indeterminate	11.8	axillary	58.5
BAR10-80	3.6	1.5 (4.3)	4.7	2.5 (12.1)	determinate	8.9	combination	52.4
BAR10-81	4.5	2.4 (10.5)	4.5	2.2 (8.9)	indeterminate	12.1	axillary	48.3
BAR10-86	22.2	2.7 (14.7)	8.3	2.0 (7.5)	indeterminate	12	axillary	56.7
BAR10-88	38	3.8 (45.2)	7.8	5.1 (165.7)	indeterminate	14.8	axillary	70.2
BAR10-90	14.8	3.4 (28.5)	8.5	4.1 (62.8)	indeterminate	16.3	axillary	55.8
BAR10-93	5.6	2.7 (15.0)	5.1	0.8 (2.2)	indeterminate	6.7	axillary	25.2
BAR10-100	5.7	2.2 (9.3)	8	2.2 (8.6)	indeterminate	7.4	axillary	37.3
BAR10-105	29	3.3 (28.2)	8.8	5.0 (144.0)	indeterminate	16.1	axillary	47
BAR-66	8.2	2.2 (8.7)	8	0.1 (1.0)	indeterminate	4.2	axillary	14.1
Kopu II	44.5	4.6 (96.5)	10	6.3 (533.8)	indeterminate	16.2	axillary	63.3
(P/B1 x P/B2)-2	10.5	2.2 (9.1)	10	2.8 (16.6)	indeterminate	7.6	axillary	53.3
L.S.D	**11.1**	**1.3**	**1.5**	**1.4**		**5.2**		**16.9**
CV%	**44.8**	**18.9**	**13.2**	**28.7**		**23**		**24**
SEM	**7.7**	**0.9**	**1.02**	**1.0**		**3.6**		**8.2**
Prob. (5%)	***	***	***	***		***		***

‡ Data were log transformed
Values in parenthesis show back-transformed data

Table 4.3.7.2 Mean data of different above & underground morphological characters of the advanced generation of the cross, RRR(RAO) x white clover (RRRR).

Hybrids	Leaflet length/width ratio	Stem thickness (mm)	Petiole length (cm)	Seed/head	Main nodal root thickness (mm)	Dry weight top (g) ‡	Dry weight root (g)‡	Total biomass (TBM) (g)‡	Root weight % of TBM
BAR10-72	1.5	3.6	3.7	42.2	3.0	1.7 (5.7)	0.7 (1.9)	2.0 (7.7)	26.3
BAR10-80	1.5	2.8	3.9	3	1.4	0.9 (0.4)	1.4 (0.3)	0.4 (0.7)	36.9
BAR10-81	1	2.5	4.8	12.5	2.9	0.4 (1.5)	0.7 (0.5)	0.7 (2.0)	27.6
BAR10-86	1.6	3.0	9.1	22.8	4.0	2.5 (12.6)	1.6 (4.9)	2.9 (17.6)	28.4
BAR10-88	1.4	3.2	9.1	75.8	4.1	4.2 (63.4)	3.0 (19.8)	4.4 (84.8)	24.6
BAR10-90	1.6	3.2	9.1	73.8	3.2	2.9 (17.2)	1.7 (5.3)	3.1 (22.7)	23.7
BAR10-93	1.5	2.8	4.0	85.9	2.4	0.2 (1.2)	0.6 (0.6)	0.6 (1.8)	32.7
BAR10-100	1.3	2.5	4.4	72.7	1.7	0.6 (1.8)	0.1 (0.9)	1.0 (2.7)	34.6
BAR10-105	1.4	2.6	10.7	70.5	3.2	3.5 (31.7)	2.4 (11.3)	3.8 (43.4)	26.5
BAR-66	1.2	2.5	2.9	0	1.3	0.8 (2.2)	0.3 (1.4)	1.3 (3.6)	39.0
Kopu II	1.2	3.6	7.9	64.5	2.7	5.3 (196.9)	3.3 (25.9)	5.4 (223.6)	11.9
(P/B1 x P/B2)-2	1.2	2.1	5.1	30.2	1.9	0.4 (1.5)	_0.4 (0.7)	0.8 (2.2)	30.5
P≤0.05	***	***	***	***	***	***	***	***	***
L.S.D	**0.3**	**0.6**	**3.2**	**34**	**1.2**	**1.4**	**0.9**	**1.3**	**9.7**
CV%	**12.6**	**14.0**	**34.1**	**46**	**30.6**	**46.2**	**26.3**	**38.1**	**24.0**
S.E.M	**0.2**	**0.4**	**2.2**	**16.4**	**0.8**	**0.9**	**0.6**	**0.9**	**6.7**

‡ Data were log transformed
Values in parenthesis show back-transformed data

increasing numbers of backcrosses with white clover (Tables 4.3.7.1, 4.3.7.2). With few exceptions, the BAR10 hybrids had stolons with strong nodal rooting and indeterminate terminal growth with axillary flowering, as in white clover. BAR10-80 had a more determinate growth habit with both axillary and terminal flowering, showing a combination of *T. ambiguum* and white clover traits. Numbers of florets per head in all the BAR10 hybrids except BAR10-93 were significantly higher than the original BAR hybrid, BAR-66, while BAR10-88 also had significantly more florets per head than the (P/B1xP/B2)-2 (white clover control). BAR10-100 had significantly fewer florets per head than the Kopu II control, while BAR10-93 had the lowest number of florets per head and was significantly lower than both the white clover genotypes. Except for BAR10-81, in which the leaflets were almost round, all the BAR10 hybrids recorded higher leaflet length: width ratios than white clover. BAR10-88, BAR10-90, BAR10- 93, BAR10-100 & BAR10-105 produced significantly more seed on open pollination than the poorer white clover control, but not significantly more than the Kopu II control. BAR-66 did not set any seed. The seed sets in BAR10-80, BAR10-81 and BAR10-86 were lower than both white clovers, while in BAR10-72 it was lower than only Kopu II. The dry root weight ratio to total biomass was significantly higher in all the BAR10 hybrids as compared to Kopu II but none differed significantly from the (P/B1xP/B2)-2, white clover control (Table 4.3.7.2).

4.4 Strategy 4: (inserting *T. occidentale* as a genetic bridge)

4.4.1 Hybrids derived from ROS (A^TA^TRR) x *T. occidentale* - ARO (3x) & AARROO (6x)

This is the first bridging based strategy which was started with a 4x hybrid combining A and R genomes directly and the bridging species, *T. occidentale* was added later on. All the previously given strategies were started with hybrids combining A and O genomes first. Eleven BAR hybrids with genomic compositions of ARO (3x) or AARROO (6x) were available at the start of this project. Flow cytometry analysis indicated that five were near triploid (3x) and seven were near hexaploid (6x) (Table 4.4.1). Triploid hybrids, ARO could be very important from a recombination view point because none of genomes/chromosomes have homologues available, so the chances of allosyndetic chromosome pairing during meiosis are potentially very high and this might lead to the formation of gametes with recombinant chromosomes.

The five 3x plants (ARO), BAR-6 to BAR-10, resulted from the cross, ROS (A^TA^TRR, 4x) x 2x *T. occidentale* (OO). Both the parents contributed normal haploid (n) gametes. Seven plants (BAR-12 to BAR-17) were 6x (AARROO) and resulted from the cross of 4x ROS (A^TA^TRR) with artificially chromosome doubled *T. occidentale* (OOOO) contributing the pollen. ROS contributed functional 2n gametes in this cross.

The pollen stainability in these plants was very low and ranged from 0 % in BAR 6 to 34 % in BAR 15. Generally, the male fertility in the 3x hybrids (ARO) was very low (<10%) except BAR-9 (Table 4.4.1) as compared to the hexaploid ones (AARROO) where the percentage of stainable pollen was > 10%, except BAR-17. The relatively higher pollen fertility in the hexaplpoid hybrid might be due to the availability of closely related counterpart for every chromosome leading to normal meiotic processes. Over 8,000 pollinations were made with these hybrids. The crossing details, along with male fertility (% stainable pollen), flow cytometry based ploidy estimates and expected genomic structures are given in Table 4.4.1. The seed set following crossing with white clover ranged from zero in BAR-7 and BAR-10 to 63 and 57 seeds (7.9 and 8.8 seeds per 100 florets) respectively in BAR-13 and BAR-17. All the BAR hybrids proved to be self-incompatible (SI), i.e. producing 0-1 seeds per 100 florets on selfing.

Table 4.4.1 Pedigrees of 3x (ARO) and 6x (AARROO) BAR hybrids derived from the cross of ROS (AARR, 4x) with *T. occidentale* (OCD = 2x and OCT= colchicine doubled 4x) with pollen fertility, flow cytometric ploidy estimates and details of further crosses.

BAR #	Pedigree	Expected genomic composition	Pollen Fertility (%)	Ploidy (x)	Crossed with	Total crosses	Total seed	Breeding system (Self seed/head)
BAR-6	(ROS x OCD-48-17)-3	AARR x OO = ARO	00	3.0	(P/B1xP/B2)-1, Scarlet-1	250	3	SI
BAR-7	(ROS x OCD-48-17)-4	AARR x OO = ARO	<1	3.1	(P/B1xP/B2)-1	300	NS	SI
BAR-8	(ROS x OCD-48-17)-7	AARR x OO = ARO	8	3.1	(P/B)-17, Scarlet-1, (P/B1xP/B2)-1	950	8	SI
BAR-9	(ROS x OCD-48-17)-8	AARR x OO = ARO	22	3.1	(P/B)-17, Scarlet-1, (P/B1xP/B2)-1	1450	1	SI
BAR-10	(ROS x OCD-48-17)-9	AARR x OO = ARO	6	3.0	(P/B)-17, Scarlet-1, (P/B1xP/B2)-1	650	NS	SI
BAR-12	(ROS x OCT-48-617)-1	AARR x OOOO= AARR'OO	18	5.9	Scarlet-1	70.	1	SI
BAR-13	(ROS x OCT-48-617)-2	AARR x OOOO= AARROO	34	5.9	Scarlet-1, (P/B1xP/B2)-1 & -2, Kopu II-1	800	63	SI
BAR-14	(ROS x OCT-48-617)-3	AARR x OOOO= AARROO	19	6.0	(P/B1xP/B2)-1, Scarlet-1	400	25	SI
BAR-15	(ROS x OCT-48-617)-4	AARR x OOOO= AARROO	34	5.8	(P/B)-17, Scarlet-1, (P/B1xP/B2)-1 & -2	1700	41	SI
BAR-16	(ROS x OCT-48-617)-5	AARR x OOOO= AARROO	12	5.8	(P/B)-17, (P/B1xP/B2)-1 & -2	500	21	SI
BAR-17	(ROS x OCT-48-617)-6	AARR x OOOO= AARROO	7	5.9	(P/B)-17, (P/B1xP/B2)-1 & -2	650	57	SC (0.2)

4.4.2 Progeny of the original BAR hybrids (BAR09 hybrids)

4.4.2.1 BAR09 progeny of the crosses, ARO (3x) x RRRR & AARROO (6x) x RRRR

The germination of the seed from the direct and reciprocal crosses (ARO x RRRR and AARROO x RRRR) and selfing of ARO (3x) and AARROO (6x) gave 88 progeny plants. This group had only one self plant (BAR09-15, from the selfing of BAR-17). Five plants were selected for further cyto-morphological analysis on the basis of intermediate phenotype, leaf colouring and ploidy level. The pedigrees of these five BAR09 hybrids, expected genomic composition, flow cytometry based ploidy level, somatic chromosome counts, crossing/selfing and quantities of seed obtained are given in Table 4.4.2. Pollen stainability in these hybrids ranged from 4% in BAR09-2 to 56% in BAR09-15. The expected genomic composition of these plants was RRRAO. Flow cytometry indicated that BAR09-3, BAR09-5 and BAR09-10 were 5x and this was later confirmed by somatic chromosome counts. For BAR09-2, the flow cytometry result (3.7x) did not match the actual chromosome count (40). The contribution of 2n gametes from the BAR hybrid (ARO, 3x) used as the female parent of BAR09-2 and BAR09-3 led to the 5x ploidy level. BAR09-15 derived from selfing of BAR-17 (AARROO), should have been AARROO, but had an actual chromosome count of 35. Almost all these plants turned out to be apparently self-incompatible (SI), although BAR09-15 set four seeds on selfing. All of these BAR09 hybrids produced reasonable quantities of seed when crossed with coloured leaf white clover (Table 4.4.2).

4.4.2.2 Meiotic analysis of BAR09-3

The meiotic analysis of BAR09-3 (RRRAO, 2n=40), which resulted from the cross of the original hybrid BAR-8 (ARO, 3x) and a colour marked white clover (RRRR), is given in Table 4.4.3. The large numbers of univalent and multivalent chromosome formations indicated that meiosis in this hybrid was highly disturbed (Figure 4.4.1a). Trivalents may have involved the pairing of a homologous pair from white clover with a homoeologous chromosome coming either from the third sub-genome of white clover or from *T. occidentale*. Alternatively, they might have involved pairing of a *T. ambiguum* chromosome with one *T. occidentale* chromosome and one *T. repens* chromosome. Presence of IVs with mean frequency of 2.1/PMC might indicate both intra- and inter-subgenomic chromosome pairing involving white clover and *T. occidentale* chromosomes or even chromosomes from

Table 4.4.2 Progeny of ARO (3x) and AARROO (6x) hybrids (Table 23) with pedigrees, expected genomic composition, estimated ploidies (flow cytometry), expected and actual chromosome numbers, pollen fertility, details of further crosses and breeding system (SC, self-compatible, SI, self-incompatible).

BAR09 No	Pedigree	Exp. genomic composition	Pollen fertility (%)	Ploidy (x)	Exp. 2n chrom. #	Actual 2n chrom. #	Crossed with	Crosses	Total seeds	Breeding system (Self
BAR09-2	(BAR-8 x (P/B1 x P/B2)-1)-1	ARO x RRRR = RRRAO	4	3.7	40	40	(P/B1 x P/B2)-2	300	23	SI
BAR09-3	(BAR-8 x (P/B1 x P/B2)-1)-6	ARO x RRRR = RRRAO	13	5.4	40	40	(P/B1 x P/B2)-2	300	63	SI
BAR09-5	(BAR- 13 x Scarlet-1)-9	AARROO x RRRR= RRRAO	17	5.6	40	40	(P/B)-17, (P/B1 x P/B2)-2	550	9	SI
BAR09-10	((P/B1 x P/B2) -1 x BAR-15)-3	RRRR x AARROO=RRRAO	17	5.5	40	39	(P/B1 x P/B2)-1 & 2	300	36	SI
BAR09-15	(BAR-17-Selfed) -8	AARROO x AARROO=AARROO	56	4.4	48	35	(P/B1 x P/B2)-1	250	24	SC (1.3)

Table 4.4.3. Meiotic chromosome associations at diakinesis/metaphase-I in PMCs of BAR09-3 (RRRAO, 2n=40).

Hybrid name	Pedigree	Expected genomic composition	No. of PMCs analysed	Mean frequency (Range) of meiotic configurations					
				I	II	III	IV	V	Pollen fertility (%)
BAR09-3 (2n=40)	(BAR-8 x (P/B1 x P/B2)-1)-6	ARO x RRRR= RRRAO	59	5.5 (0-12)	7.1 (0-15)	4 (0-9)	2.1 (0-7)	0	13

all three species. Figure 4.4.1b shows the level of meiotic abnormality in BAR09-3. Lagging chromosomes were observed at both anaphase-I and II. 20-20 chromosome disjunction at anaphase-I was observed in very few cells. Second division restitution (SDR) leading to triads was also observed. Lagging Is splitting precociously into chromatids were also observed during anaphase-I followed by movement of these toward opposite poles and partially lagging behind (Figure 4.4.1c). PMCs with such highly disturbed anaphase-I would lead to highly unbalanced gametes which would abort prematurely and be non-functional. The highly disturbed nature of meiosis in BAR09-3 was consistent with its low pollen fertility (13%) as compared to other hybrids with more regular meiosis.

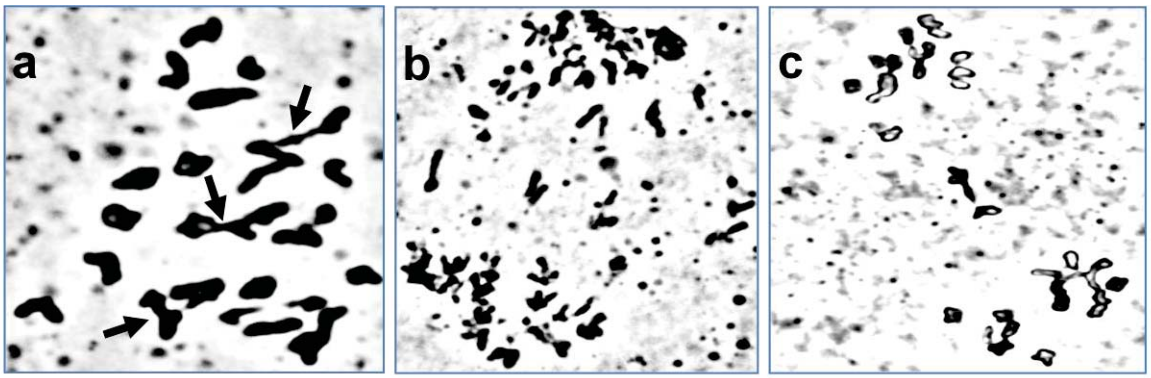

Figure 4.4.1 (**a**) Metaphase-I in BAR09-3 (RRRAO, 2n=40) with Is, IIs, IIIs and IVs (arrows). (**b**) Highly disturbed anaphase-I in BAR09-3 with several chromosomes lagging behind and precociously splitting into chromatids. (**c**) A lagging univalent has precociously split into chromatids which are moving towards opposite poles.

4.4.3 Self and cross progeny of the BAR09 hybrids (BAR10 hybrids)

4.4.3.1 Progeny of RRRAO x RRRR- RRR($R_4A_4O_4$)

Sixty progeny plants were derived from 5x BAR09 hybrids (RRRAO) by selfing, inter-crossing and back-crossing with white clover. Six plants were selected for further cyto-molecular analyses on the basis of their intermediate phenotype and flow cytometry based ploidies. They were BAR10-1, BAR10-12, BAR10-16, BAR10-17, BAR10-22 and BAR10-24 (Table 4.4.4). The hypothetical genomic composition in BAR10-1, BAR10-12, BAR10-16 and BAR10-22 which resulted from crosses of BAR09-2, BAR09-3, BAR09-5 and BAR09-10 with white clover was RRR($R_4A_4O_4$) with expected somatic chromosome numbers of around 36 in each. BAR10-17 resulted from cross, BAR09-5 (RRRAO, 2n=40) x BAR09-15 ((AARROO)-13, 2n=35). The expected

Table 4.4.4. Selected progeny of the BAR09 hybrids with genomic formula RRRAO (Table 24) with estimated ploidy (flow cytometry), expected and actual chromosome numbers and pollen fertility.

Hybrid	Pedigree/identification	Expected genomic composition	Pollen fertility %	Ploidy (x)	Exp. 2n chrom. #	Actual 2n chrom. #
BAR10-1	(BAR09-2 x (P/B1 x P/B2)-2)-1	RRRAO x RRRR= RRR($R_4A_4O_4$)	33	4.8	36	35
BAR10-12	(BAR09-3 x (P/B1 x P/B2)-2)-15	RRRAO x RRRR= RRR($R_4A_4O_4$)	0	4.1	36	33
BAR10-16	(BAR09-5 x (P/B)-17)-3	RRRAO x RRRR= RRR($R_4A_4O_4$)	0	4.6	36	35
BAR10-17	(BAR09-5 x BAR09-15)-1	RRRAO x (AARROO)-13=(RRAO($A_4R_4O_4$))-(6-7)	14	--	37-38	38
BAR10-22	(BAR09-10 x (P/B1 x P/B2)-2)-6	(RRRAO)-1 x RRRR= (RRR($R_4A_4O_4$))-(0-1)	56	4.2	35-36	33
BAR10-24	(BAR09-15 x (P/B1 x P/B2)-1)-1	(AARROO)-13 x RRRR= (RRRAO)-(6-7)	97	4.2	33-34	34

Table 4.4.5 Meiotic chromosome associations at diakinesis/metaphase-I in PMCs of hybrids BAR10-1 (2n=35) & BAR10-12 (2n=33).

Hybrid name	Pedigree	Expected genomic composition	No. of PMCs analysed	Mean frequency (Range) of meiotic configurations					
				I	II	III	IV	V	Pollen fertility (%)
BAR10-1 (2n=35)	(BAR09-2 x (P/B1 x P/B2)-1)-1	RRRAO x RRRR= RRR($R_4A_4O_4$)	62	3 (0-7)	8 (1-14)	2.1 (0-6)	2.4 (0-6)	0.3 (0-1)	33
BAR10-22 (2n=33)	(BAR09-10 x (P/B1 x P/B2)-2)-15	(RRRAO)-1 x RRRR= (RRR($R_4A_4O_4$))-(0-1)	66	2.2 (0-8)	8.4 (3-15)	2.6 (0-6)	1.4 (0-4)	0.1 (0-2)	56

somatic chromosome count in BAR10-17 was 37-38. BAR10-24, which was the progeny of BAR09-15 (2n=35) used as female parent with coloured white clover, had an expected genomic formula of (RRRAO)-7 with 2n chromosome number of 33-34.

The frequency of stainable pollen in this group of hybrids ranged from 0 (male sterile) in BAR10-12 and BAR10-16 to 97% in BAR10-24. The male fertility in BAR10-24 was unexpectedly high. The actual somatic chromosome counts were frequently lower than the numbers estimated from the parental counts, and were generally in line with flow cytometry-based estimates (Table 4.4.4). These results show that the 2n chromosome numbers dropped between the original BAR parent and BAR10 generations, partly due to crossing with white clover which had a lower ploidy level than the hybrids and partly due to chromosome elimination during meiosis in the BAR09 hybrids (Tables 4.4.1, 4.4.2, 4.4.4). A somatic cell of BAR10-22 is shown in Figure 4.4.2a.

4.4.3.2 Meiosis in BAR10-1 and BAR10-22

BAR10-1 was the progeny of the cross, BAR09-2 (2n=40) x (P/B1xP/B2)-2 (RRRAO x RRRR) and was selected for meiotic chromosome pairing analysis. The expected genomic composition in BAR10-1 was RRR($R_4A_4O_4$) and the actual somatic chromosome count was 35 (one less than expected). BAR10-1 had significant frequencies of univalent and multivalent configurations, including a very low average frequency of pentavalent chromosomal associations (Vs) showing high potential for inter-specific chromosome pairing (Table 4.4.5, Figure 4.4.2b).

BAR10-22 resulted from the cross, BAR09-10 (2n=39) x (P/B1xP/B2)-2 (RRRAO (-1) x RRRR). The hybrid parent of BAR10-22 (BAR09-10, RRRAO) had one less chromosome (2n=39) than the expected number. Therefore, the somatic chromosome number in BAR10-22 should have been 35-36 but was actually 33 (Table 4.4.4). This indicated loss of chromosomes during meiosis in BAR09-10. BAR10-22 had a larger average frequency of IIs and lower number of Is than BAR10-1, and the male fertility was comparatively higher (Table 4.4.5, Figure 4.4.2c). Frequent lagging chromosomes precociously splitting into chromatids during anaphase-I were observed in BAR10-22, along with cytomixis (Figure 4.4.2d).

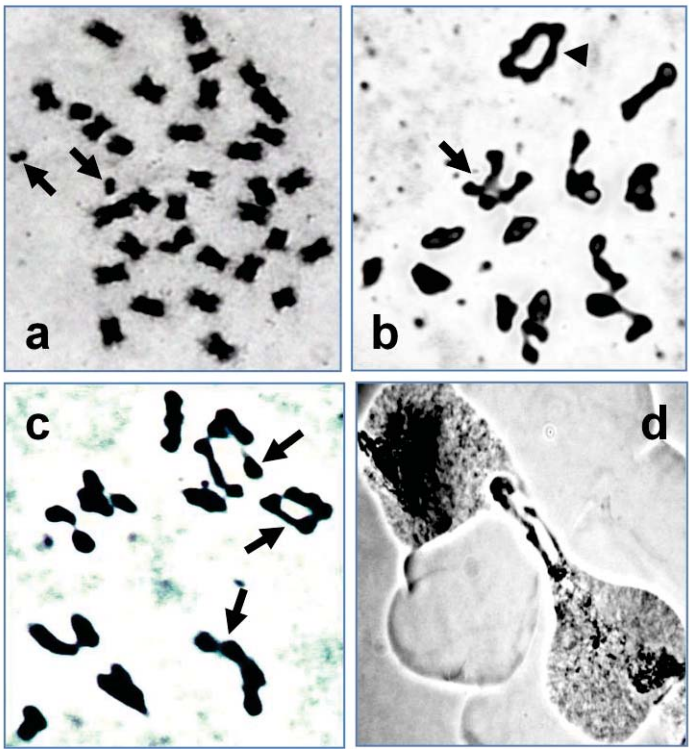

Figure4.4.2 (**a**) Giemsa-stained somatic chromosomes in BAR10-22 (2n=33) with two chromosomes having satellite knobs (arrows). (**b**) Metaphase-I in BAR10-1 (RRR($R_4A_4O_4$)-1, 2n=35), showing 2 Is, 6 IIs, 4 IIIs, I IVs (arrow) & I V (arrowhead). (**c**) Metaphase-I in BAR10-22 ((RRR($R_4A_4O_4$))-3, 2n=33) with 3 IVs (arrows), 4 IIIs, 4 IIs & 1 I. Of the three IVs, one was a chain IV and two were ring IVs. (**d**) Cytomixis in BAR10-22. The nuclear material from one PMC is moving to another PMC through a cytoplasmic bridge. This phenomenon was observed in other BAR hybrids.

4.4.4 Molecular cytogenetic analysis of BAR10-22

Based on Giemsa-stained mitotic chromosome preparations obtained from root tips, the somatic chromosome number in BAR10-22 was 33 (Figure 4.4.2a) instead of the expected number of ~36. It had a pair of chromosomes with satellite knobs and highly de-condensed nucleolar organizer regions (NORs). A single chromosome from *T. ambiguum* could be identified in DAPI-stained chromosome preparation on the basis of size, being clearly larger and with more defined telomeric ends than those coming from white clover and *T. occidentale* (Figures 4.4.2a, 4.4.3a).

GISH and FISH using labelled total genomic DNA of *T. ambiguum* and a 5S r DNA probe painted a single non-marker *T. ambiguum* chromosome in the metaphase chromosome spreads (Figure 4.4.3b). The painted chromosome was markedly larger than the other chromosomes and corresponded with the large chromosome identified in Figure 4.4.3a. The 5S rDNA probe gave five red signals. Two 5S signals were on the longer arms of a pair of chromosomes having highly de-condensed NORs connecting the main chromosomal bodies with satellited knobs (Fig. 4.4.3b, c). The remaining three 5S signals were smaller and, because similar 5S signals in *T. occidentale* are hardly visible, could be on white clover-derived chromosomes (Fig. 4.4.3c). GISH did not give any evidence of inter-genomic recombination in this hybrid.

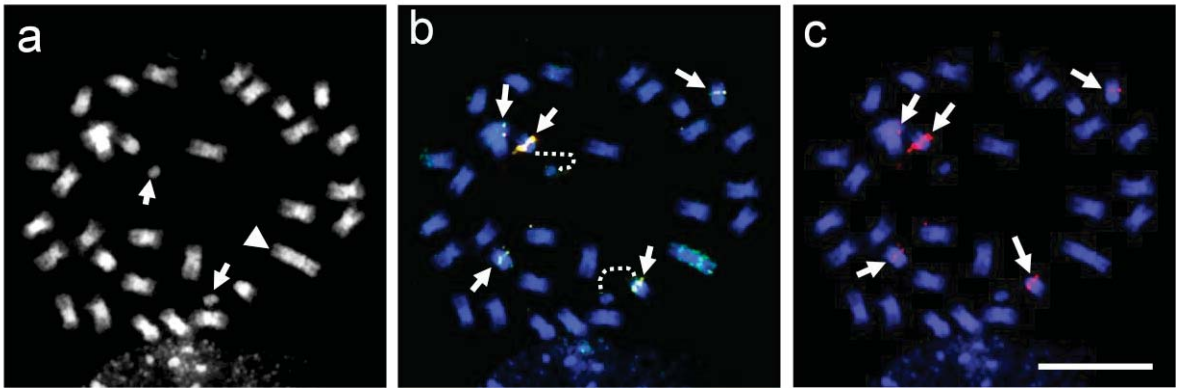

Figure 4.4.3 GISH and FISH on metaphase chromosomes of BAR10-22. (**a**) DAPI-stained metaphase chromosomes in BAR10-22 (RRR($R_4A_4O_4$))-3 showing two satellite knobs (arrows) and one comparatively larger chromosome (arrowhead). (**b**) GISH and FISH on the same cell as in (**a**) by using genomic DNA of *T. ambiguum* labelled with Fluor-X-dCTP (green) and Cy3-dCTP labelled pTr5S (red) as probes. The *T. ambiguum* DNA probe highlighted one chromosome which was already marked as comparatively bigger than *T. repens* chromosomes (arrowhead in **a**). pTr5S gave five red signals (arrows). The largest two were on a pair of NOR bearing chromosomes on the longer arms close to the centromeric constriction. (**c**) The same cell with five red signals after FISH but with a clearer situation. Three 5S signals were located on non-NOR-bearing chromosomes. The chromosome with the smallest signal is likely to have come from *T. occidentale* (bold arrow). BAR = 10 µm.

4.4.5 Phenotypic description of selected hybrids

4.4.5.1 Progeny of ARO (3x) & AARROO (6x) hybrids

Five hybrids were selected for morphological description along with two original BAR hybrids (BAR-8, BAR-15) and two white clover genotypes (Scarlet-1 and (P/B1xP/B2)-1) as control parents. The hypothetical genomic composition of each hybrid is given in Table 4.4.2. All the evaluated BAR09 hybrids were 5x except BAR09-15 which had 35 chromosomes.

The hybrids inherited morphological traits from both parents and all traits showed highly significant variances (Tables 4.4.6.1, 4.4.6.2). All the BAR hybrids including the original plants and progeny from both selfing and backcrossing to white clover were stoloniferous (a white clover/*T. occidentale* derived character). Anchorage of the stem was assessed on the scale, 0 (no nodal rooting, a *T. ambiguum* trait) to 10 (nodal rooting from every node, a white clover trait) and, like white clover, all BAR09 hybrids showed nodal rooting along the stolons. None of the hybrids had upright stems as in *T. ambiguum*. All the hybrids showed high flower production and this character showed transgressive expression. Terminal growth was highly reduced in all the hybrids (a *T. ambiguum*-derived trait) as compared to white

clover in which terminal growth continued. Leaflets were more like *T. ambiguum* (elongated leaflets) than in white clover (leaflet almost round). In addition to axillary flowering, the hybrids appeared to have terminal flowering. OP seed production/head was significantly lower in the BAR09 hybrids than in the white clovers. However, except for BAR09-5, the BAR09 hybrids showed better seed yield per head than the original BAR hybrids. The root weight % of the total biomass was significantly higher in all the BAR and BAR09 hybrids as compared to the white clover parent, except for BAR09-5 and BAR09-10 in which root weight % of the total biomass was higher but not significantly different from the white clover parents. This suggested the inheritance of the *T. ambiguum* derived shoot/root ratio. However, no rhizomes were observed in any of the BAR09 hybrids (Table 4.4.6.2).

4.4.5.2 Self and cross progeny of RRRAO (5x) hybrids

Five BAR10 hybrids, representing the second self and backcross progenies, were selected for studying the relative expression of parental characters. These were BAR10-1, BAR10-12, BAR10-16, BAR10-17 and BAR10-24. The original BAR hybrid, BAR-8 and two white clover genotypes i.e., Kopu II (white clover cultivar) and (P/B1xP/B2)-2 were used as control parents. The expected genomic compositions of all the hybrids are given in Table 4.4.4.

The differences among the tested hybrids and controls were highly significant for each trait (Tables 4.4.7.1, 4.4.7.2). The hybrids in this generation were generally more similar to white clover than to *T. ambiguum*. All the hybrids were stoloniferous. For stolon number, stolon length, stem anchorage, peduncle length, florets per head, petiole length and stem thickness, the hybrids, with few exceptions, had intermediate morphology but skewed more towards white clover. All the hybrids except BAR10-17 and -24 showed highly reduced terminal growth which suggested the presence and expression of *T. ambiguum* derived genes. As a result, the flowering in these hybrids appeared to have (*T. ambiguum*-like) terminal flowering in addition to axillary flowering. The character showing the most predominant effect of *T. ambiguum* parentage was the dry root weight ratio to total biomass. All the hybrids had significantly higher root weight ratio to the total biological yield as compared to the Kopu II control. In comparison with (P/B1xP/B2)-2, the root weight ratio to total biomass in the hybrids was also higher except for non-significant differences in BAR10-16 and BAR10-24.

Table 4.4.6.1. Mean data of above-ground morphological data of the advanced progeny of the crosses of 3x ARO and 6x AARROO hybrids with white clover (RRRR).

HYBRIDS	Stolon number	Stolon Length (cm)	Stem anchorage (0-10)	Inflorescence No.	Growth habit	Peduncle length ((cm)	Florets/head	Leaflet length/width ratio	Petiole length (cm)
BAR09-2	18.6	30.0	8.8	88	determinate	11.2	55.7	1.5	4.1
BAR09-3	28.0	39.7	8.3	153.2	determinate	13.6	67.1	1.6	4.8
BAR09-5	25.1	8.0	5.0	44.8	determinate	12.2	52.5	1.8	5.0
BAR09-10	25.8	33.5	9.0	141.8	determinate	14.5	65.1	1.4	4.4
BAR09-15	21.6	16.6	9.5	12.8	indeterminate	11.4	27.7	1.4	5.3
BAR-8	12.0	24.0	5.5	105.2	determinate	7.7	54.7	1.5	3.7
BAR-15	14.1	20.5	8.0	83.2	determinate	7.4	47.6	1.4	3.1
Scarlet-1	2.5	12.5	10.0	11.5	indeterminate	12.4	49.4	1.3	6.4
(P/B1 x P/B2)-1	18.8	26.3	10.0	38.4	indeterminate	8.9	62.5	1.4	6.2
CV%	**17.3**	**23.4**	**9.3**	**29.4**		**10**	**6.8**	**5.7**	**11.7**
LSD	**4.9**	**8.0**	**1.1**	**32.4**		**1.6**	**5.3**	**0.1**	**0.8**
SEM	**2.4**	**3.9**	**0.5**	**15.7**		**0.8**	**2.6**	**0.1**	**0.4**
Prob. (5%)	***	***	***	***		***	***	***	***

Table 4.4.6.2 Mean data of above and under-ground morphological data of the advanced progeny of the crosses of 3x ARO and 6x AARROO hybrids with white clover (RRRR).

HYBRIDS	Stem thickness (mm)	Flowering-terminal, axillary or combination	Seed/head	Main Root Thickness (mm)	Main nodal root thickness (mm)	Dry weight top (g)	Dry weight root (g)	Total biomass (TBM) (g)	Root weight % of TBM
BAR09-2	2.24	combination	4.2	5.23	2.05	6.5	4.5	11.0	41.6
BAR09-3	2.98	combination	3.6	7.82	1.89	24.8	11.7	36.6	33.1
BAR09-5	3.15	combination	0.1	3.59	1.81	7.5	2.9	10.3	28.4
BAR09-10	2.38	combination	12.3	7.46	1.8	20.2	6.4	26.6	26.8
BAR09-15	2.01	axillary	1.6	3.61	1.52	2.2	1.9	4.1	47.5
BAR-8	2.21	combination	0	7.35	1.7	4.1	2.9	7.0	41.0
BAR-15	1.96	combination	0.3	3.02	1.66	2.8	2.7	5.5	48.8
Scarlet-1	2.28	axillary	26.5	2.93	1.09	3.0	1.0	4.0	22.5
(P/B1 x P/B2)-1	2.18	axillary	31.2	3.93	1.81	7.0	2.5	9.5	30.6
CV%	**7.6**		**51.5**	**22.7**	**21.9**	**75.5**	**45.2**	**65.2**	**15.2**
LSD	**0.26**		**6.7**	**1.66**	**0.54**	**9.6**	**2.7**	**12.1**	**7.9**
SEM	**0.12**		**4.6**	**0.57**	**0.18**	**3.3**	**0.9**	**4.2**	**2.7**
Prob. (5%)	***		***	***	***	***	***	***	***

Table 4.4.7.1 Mean data of above-ground morphological traits of the advanced progeny of the cross of RRRAO (5x) with white clover (RRRR).

Hybrids	Stolon number ‡	Stolon Length (cm) ‡	Stem anchorage (0-10)	Inflorescence No. ‡	Growth habit	Peduncle length (cm)	Flowering-terminal, axillary or combination	Florets/head
BAR10-1	2.1 (8.4)	2.6 (13.1)	5.8	2.8 (16.3)	determinate	8.7	combination	38.5
BAR10-12	2.0 (7.2)	2.3 (10.1)	4.8	3.0 (20.7)	determinate	8.3	combination	49.2
BAR10-16	2.0 (7.7)	2.1 (8.3)	4	2.3 (9.9)	determinate	12.6	combination	38
BAR10-17	2.0 (7.2)	2.2 (9.2)	8.8	1.5 (4.3)	indeterminate	10.2	axillary	29
BAR10-24	2.7 (15.0)	3.0 (19.5)	9.5	2.7 (15.0)	indeterminate	9.7	axillary	46.8
BAR-8	1.3 (3.7)	1.6 (4.9)	4.8	1.0 (2.8)	determinate	4.7	combination	31.5
Kopu II	3.4 (29.7)	4.6 (102.5)	10	6.2 (47)	indeterminate	14.5	axillary	63.8
(P/B1 x P/B2)-2	2.5 (12.7)	2.4 (11.5)	10	3.2 (1.6)	indeterminate	6.5	axillary	55.8
LSD	**1.0**	**0.4**	**2.3**	**1.3**		**2.4**		**12.1**
CV%	**30.3**	**10.1**	**21.3**	**28.9**		**17.8**		**18.6**
SEM	**0.5**	**0.2**	**1.1**	**0.9**		**1.6**		**5.8**
Pobab. (5%)	***	***	***	***		***		***

‡ Data were log transformed
Values in parenthesis show back-transformed data

Table 4.4.7.2 Mean data of above- & under-ground morphological traits of the advanced progeny of the cross of RRRAO (5x) with white clover (RRRR).

Hybrids	Leaflet length/width ratio	Stem thickness (mm)	Petiole length (cm)	Seed/head ‡	Main nodal root thickness (mm)	Dry weight top (g) ‡	Dry weight root (g) ‡	Total biomass (TBM) (g) ‡	Root weight % of TBM
BAR10-1	1.33	2.1	3.8	2.0 (7.17)	2.49	1.0 (2.61)	0.5 (1.7)	1.5 (4.35)	39.7
BAR10-12	1.4	2.37	3.5	2.8 (17.12)	2.85	0.5 (1.62)	0.1 (1.12)	1.01 (2.75)	41.1
BAR10-16	1.5	2.45	4.0	2.8 (17.12)	3.08	1.1 (3.10)	0.3 (1.32)	1.5 (4.44)	30.1
BAR10-17	1.33	2.76	4.4	0.5 (1.63)	2.09	0.1 (1.09)	_0.02 (0.98)	0.8 (2.14)	47.2
BAR10-24	1.25	2.32	4.6	4.0 (56.83)	2.97	2.7 (15.33)	1.9 (6.89)	3.1 (22.42)	31.2
BAR-8	1.38	1.73	2.3	2.8 (17.12)	1.48	_0.7 (0.48)	_0.7 (0.52)	0.01 (1.01)	52.0
Kopu II	1.06	3.24	8.4	3.7 (38.47)	2.47	5.6 (262.43)	3.2 (24.53)	5.7 (290.1)	9.6
(P/B1 x P/B2)-2	1.32	1.98	4.3	4.1 (57.97)	1.78	1.6 (4.85)	0.5 (1.65)	1.9 (6.62)	26.1
LSD		**0.54**	**1.2**	**0.9**	**0.77**	**1.5**	**1.4**	**1.4**	**13.1**
CV%		**15.5**	**18.0**	**20.4**	**21.8**	**66.9**	**126.5**	**47**	**25.7**
SEM		**0.26**	**0.6**	**0.4**	**0.37**	**0.7**	**0.7**	**0.7**	**6.3**
Prob. (5%)	**NS**	***	***	***	***	***	***	***	***

‡ Data were log transformed
Values in parenthesis show back-transformed data

4.5 Strategy 5: (direct integration of R & A genomes through ploidy manipulation)

4.5.1 Hybrids from (A^DA^TRR, Hybrid-70) x (RRRR) - RRRRA (5x) and AAARRRR (7x)

This strategy was based on direct integration of A and R genomes without using any bridge species. Secondly, this is the first time that, in addition to A genomes from a tetraploid source, an A genome from diploid *T. ambiguum* has been combined in a hybrid with white clover for potential introgression. The rationale behind this strategy was that isolated *T. ambiguum*-derived genomes/chromosomes having no homologues might pair with white clover derived homoeologous counterparts and lead to genomic exchange between the two species. Alternatively, some addition/substitutions lines might occur.

Two types of plants were used, i.e. 5x BAR hybrids (RRRRA) and 7x BAR hybrids (AAARRRR). Pentaploid plants resulted from the cross of white clover (RRRR) with 6x plants (A^DA^TRRRR). These plants were the progeny of hybrid 70 (A^DA^TRR) x RRRR, with hybrid 70 contributing 2n gametes. The 7x plants resulted from the cross of ROS (A^TA^TRR, 4x) with 6x plants (A^DA^TRRRR) with ROS contributing 2n functional gametes. The pedigrees of the 5x BAR hybrids (RRRRA) and 7x BAR hybrids (AAARRRR) used in this strategy are given in Table 4.5.1 along with expected genomic compositions, their pollen fertilities, ploidy levels estimated from flow cytometry and crossing/selfing details. Flow cytometry results approximately confirmed the expected ploidy levels, except for BAR-46 which was near-5x instead of 7x. Pollen fertility (%) in these hybrids was high especially in 5x RRRRA hybrids, where it was over 70 %, while in 7x AAARRRR hybrids it was more variable ranging from 19% in BAR-46 to 76% in BAR-48 and BAR-49 (Table 4.5.1). All these plants were crossed as female parents with colour marked white clover. Around 6500 crosses were made and all BAR hybrids except BAR-46 were cross-fertile and set seed. The seed set ranged from 1 seed (0.25 per 100 florets) in BAR-45 to 92 seeds (30.7 per 100 florets) in BAR-42. Five to 20 inflorescences were rubbed in order to get self seed. Most BAR plants produced no self seed but six produced low numbers, ranging from 1 in BAR-42 (0.1 self seed/head) to 17 in BAR-33 (2.8 self seed per head).

Table 4.5.1. Pedigrees of 14 nearly 5x (ARRRR) and 7x (AAARRRR) hybrids with pollen fertility, flow cytometric ploidy estimates and details of further crosses.

Plant #	Pedigree	Exp. genomic composition	Pollen Fertility (%)	Ploidy (x)	Crossed with	No. of crosses	Total seeds obt.	Breeding System (Self seed/head)
BAR-23	(Trophy-2 x (70xCrau 38))-2	RRRR x AARRRR = ARRRR	70	5.0	C21557-808	750	58	SC (1.4)
BAR-27	(Trophy-3 x (70xCrau 38))-3	ARRRR	--	5.2	Scarlet-1	200	16	SI
BAR-29	(Kopu II-35 x (70xCrau 38))-1	ARRRR	--	5.1	C21557-815	300	28	SI
BAR-31	(Kopu II-35 x (70xCrau 38))-3	ARRRR	90	5.2	Scarlet-1, C21557-808, 801	500	29	SC (1.9)
BAR-32	(Kopu II-R3-3 x (70xCrau 38))-1	ARRRR		5.3	Scarlet-1	500	11	SI
BAR-33	(Kopu II-R3-3 x (70xCrau 38))-2	ARRRR	84	5.0	C21557-808, (P/B1 x P/B2)-2, Scarlet-1	650	73	SC (2.8)
BAR-42	(ROS x (70xCrau 38))-1	AARR x AARRRR= AAARRRR	47	7.1	(P/B1 x P/B2)-1, Scarlet-1	300	92	SC (0.1)
BAR-43	(ROS x (70xCrau 38))-2	AAARRRR	60	6.8	C21557-808, Scarlet-1, (P/B1 x P/B2)-1 & -13	550	46	SI
BAR-44	(ROS x (70xCrau 38))-3	AAARRRR	69	6.8	C21557-808, (P/B1 x P/B2)-2, Scarlet-1	550	81	SC (0.4)
BAR-45	(ROS x (70xCrau 38))-4	AAARRRR	32	6.5	C21557-808, (P/B1 x P/B2)-1, P/B17, Scarlet-1	400	1	SI
BAR-46	(ROS x (70xCrau 38))-5	AAARRRR	19	4.7	Scarlet-1	350	0	SI
BAR-47	(ROS x (70xCrau 38))-6	AAARRRR	47	6.9	C21557-808, (P/B1 x P/B2)-2, Scarlet-1	400	59	SI
BAR-48	(ROS x (70xCrau 38))-8	AAARRRR	76	6.7	C21557-808, Scarlet-1, (P/B1 x P/B2)-1 & -13	400	52	SC (1.1)
BAR-49	(ROS x (70xCrau 38))-9	AAARRRR	76	6.8	Scarlet-1, (P/B1 x P/B2)-1 & -13	600	36	SI

4.5.2 Progeny of the original BAR hybrids (BAR09 hybrids)

4.5.2.1 Self and cross progeny of (5x) BAR hybrids, ARRRR with RRRR

The seed from selfing and pollination of 5x ARRRR plants with colour marked white clover was germinated and gave 304 progeny plants. Seven plants (Table 4.5.2) with appropriate flow cytometry results and with phenotypes combining both *T. repens and T.ambiguum* derived characters were selected for further cytological analysis and use in the breeding programme. Of the selected plants, BAR09-16, BAR09-17, BAR09-24 and BAR09-25 resulted from the crossing of 5x ARRRR plants with white clover. The expected genomic formula of these hybrids was RRRR(A_4). BAR09-19 was the self progeny of BAR-23 (RRRRA) while BAR09-27 and BAR09-28 were produced from the self seed of BAR-31 (RRRRA), all having the expected genomic formulae of RRRRA. No selection was made in the progeny of cross, 7x AAARRRR x RRRR. Most of the seedlings obtained from these crosses were weak with many physio-morphological abnormalities and died in the early stage of life. The surviving plants did not flower.

The pollen fertility in the selected hybrids was comparatively high and ranged from 59% in BAR09-16 to 98% in BAR09-25. These hybrids all proved to be aneuploids with chromosome numbers ranging from 34 in BAR09-24 and BAR09-25 to 39 in BAR09-28 (Table 4.5.2). The somatic chromosome scores in these hybrids were less than the expected number, except BAR09-16. The 2n=36 metaphase chromosome complement in BAR09-16 is shown in Fig. 4.5.1a. The *T. ambiguum*-derived chromosomes have been isolated from their homologous partners and so might pair with their homoeologous counterparts from white clover during gamete formation. This could lead to recombination of chromosome segments of the two species. All the hybrids produced seed after pollination by coloured leaf white clovers. BAR09-17, 27 and 28 proved to be self-compatible (SC) (Table 4.5.2).

4.5.2.2 Meiotic chromosome pairing analysis in BAR09-16, -19 & -24

The details of chromosome pairing behaviour in these hybrids have been given in Table 4.5.3 including somatic chromosome number, score of PMCs studied, average frequency of different chromosomal configurations and pollen stainability. All the hybrids showed irregularities in their meiotic behaviour and chromosome associations ranging from Is through to IVs were observed in all plants. Lagging chromosomes during meiotic

Table 4.5.2 Selected progeny plants of original hybrids RRRRA (5x) (Table 30) with their expected genome composition, pollen fertility, ploidy estimates (flow cytometry), expected and actual somatic chromosomes count and details of further crosses.

BAR #	Pedigree	Exp. genomic composition	Pollen fertility (%)	Ploidy level (x)	Exp. chrom. #	Actual chrom. #	Crossed with	Total crosses	Total seeds	Breed. System (Self seed/head)
BAR09-16	(BAR-23 x C21557-808)-1	ARRRR x RRRR= RRRR(A_4)	59	4.7	36	36	(P/B1 x P/B2)-1 & -2	250	113	SI
BAR09-17	(BAR-23 x C21557-808)-2	ARRRR x RRRR= RRRR(A_4)	80	4.9	36	38	(P/B1 x P/B2)-1 & -2	500	101	SC (10.2)
BAR09-19	(BAR-23-Selfed)-2	ARRRR x ARRRR= ARRRR	68	4.7	40	35	(P/B1 x P/B2)-2	250	81	SI
BAR09-24	(BAR-29 x C21557-815)-2	ARRRR x RRRR= RRRR(A_4)	85	4.6	36	34	C21557-815	250	200	SI
BAR09-25	(BAR-31 x Scarlet-1)-1	ARRRR x RRRR= RRRR(A_4)	98	4.8	36	34	C21557-815, P/B17	350	157	SI
BAR09-27	(BAR-31-Selfed)-2	ARRRR x ARRRR= ARRRR	75	5.1	40	-	(P/B1 x P/B2)-2	250	47	SC (6)
BAR09-28	(BAR-31-Selfed)-5	ARRRR x ARRRR= ARRRR	95	5.4	40	39	(P/B1 x P/B2)-1	250	117	SC (16.2)

Table 4.5.3 Meiotic chromosome associations at diakinesis/metaphase-I in PMCs of BAR09-16, BAR09-19 & BAR09-24.

Plant name	Pedigree	Expected genomic composition	No. of PMCs analysed	Mean frequency (Range) of meiotic configurations					
				I	II	III	IV	V	Pollen fertility (%)
BAR09-16 (2n=36)	(BAR-23 x C21557-808)-1	ARRRR x RRRR= RRRR(A_4)	42	2.7 (0-7)	12.9 (5-17)	2.0 (0-8)	0.4 (0-2)	0	60
BAR09-19 (2n=35)	(BAR-23-Selfed)-2	ARRRR x ARRRR= ARRRR	47	3.2 (0-11)	12.1 (6-16)	1.6 (0-5)	0.7 (0-3)	0	68
BAR09-24 (2n=34)	(BAR-29 x C21557-815)-2	ARRRR x RRRR= RRRR(A_4)	43	2.1 (0-5)	10.5 (4-16)	2.1 (0-5)	1.2 (0-3)	0	85

anaphase stages were also found in all the hybrids studied. Meiotic restitution and cytomixis, both leading to abnormal gametic chromosome numbers, were frequently observed.

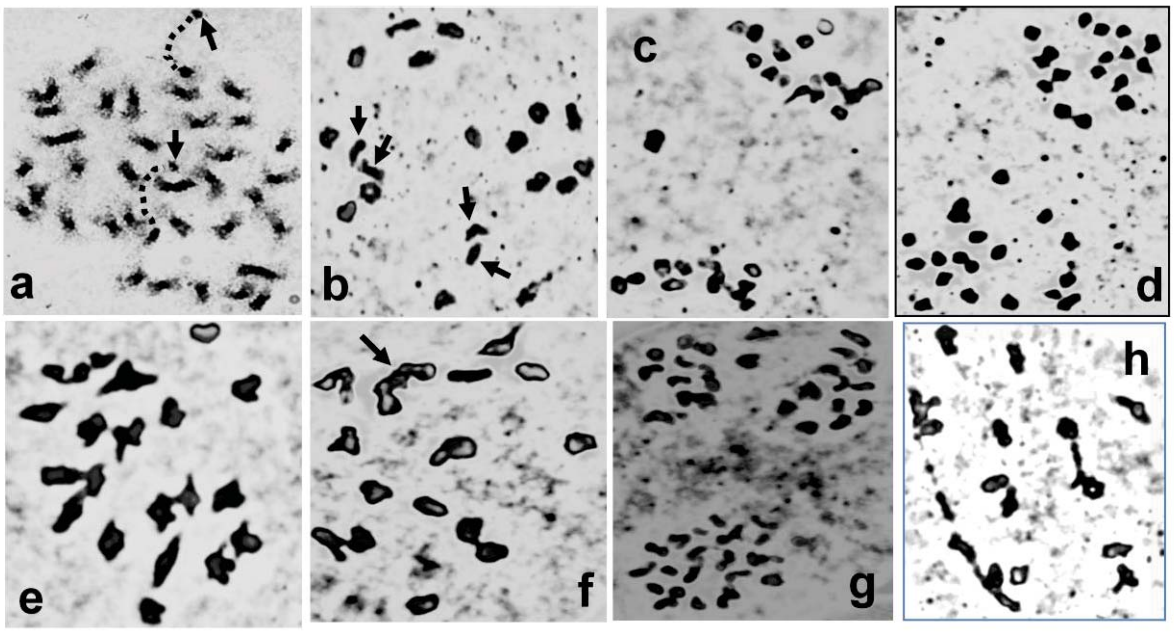

Figure 4.5.1 (a-d) BAR09-16 (RRRR(A_4), 2n=36) (**a**). Giemsa-stained somatic chromosomes showing 36 chromosomes in early metaphase. Two satellites (arrows) are visibly connected with main chromosomes through highly de-condensed rDNA. (**b**). Diakinesis/metaphase-I with 16 IIs and 4 Is (arrows). (**c**) Anaphase-I with a lagging chromosome. (**d**) Telophase-I with 17-19 disjunction. 17-19 disjunctions were frequently observed in BAR09-16. (**e-h**): BAR09-19 (RRRRA-5, 2n=35. (**e**) Shows predominantly bivalent chromosome pairing (16 IIs & 3 Is). (**f**) IIIs and a V (arrow) can also be seen. (**g**) Second division restitution (SDR) in BAR09-19 which was frequently observed in these hybrids. (**h**) Showing unusual situation probably univalents making bridges between IIs and IIIs. It might be due to segmental homology.

BAR09-16 (2n=36), BAR09-24 (2n=34) resulted from the direct cross of 5x RRRRA hybrids with white clover and BAR09-19 (2n=35) was a self progeny of BAR-23, (RRRRA, 5x). It is assumed that, respectively, these had respectively 4, 2 and 3 chromosomes from *T. ambiguum*. The meiosis in these hybrids was comparatively more diploid-like with high numbers of bivalent chromosome associations, averaging 10.5-12.9 per cell (Table 4.5.3). Figure 4.5.1b shows 16 II and 4 I in BAR09-16. Due to higher bivalent chromosome formation, the pollen fertility of this group of hybrids was high (around 60% or higher, Table 4.5.2). The average frequencies of univalent chromosomes in BAR09-16, BAR09-19 and BAR09-24 were 2.7, 3.1 and 2.1. These were most probably *T. ambiguum* derived chromosomes. Multivalent (III and IV) chromosome associations probably involved both homologous and homoeologous chromosomes of white clover. In BAR09-19, a very low frequency of pentavalents (0.02) was also observed. The data indicated the possibility of some inter-specific homoeologous chromosome pairing especially in BAR09-16 due to the

fact that the average frequency of Is was lower than the number of *T. ambiguum* chromosomes present (Table 4.5.2). Because the *T. ambiguum* derived chromosomes were isolated in these hybrids, the multivalents, IVs and especially Vs in BAR09-19, might also have involved chromosomes from both *T. ambiguum* and white clover. During anaphase-I, 17-19 and 16-19 chromosomes disjunctions were observed to be very common in BAR09-16 (Figure 4.5.1d) and BAR09-19 while lagging chromosomes were also observed in BAR09-16 (Figure 4.5.1c), BAR09-19 and BAR09-24. Figures 4.5.1e, f show contrasting metaphase-I cells in BAR09-19, the former having predominantly bivalent chromosomal associations while the latter shows some multivalents as well. Cytomixis and second division restitution were frequently observed in BAR09-19. Figure 4.5.1g shows SDR in BAR09-19.

4.5.3 Progeny of BAR09 hybrids (BAR 10 hybrids)

4.5.3.1 Progeny of cross RRRR(A_4) x RRRR - RRRR(A_2)

The seed obtained from the selfing, inter-crossing and backcrossing of selected BAR09 hybrids (Table 4.5.2) with white clover were germinated in May, 2010, using five to 30 seeds per cross. The resulting progeny set consisted of 130 plants. Eight plants were selected for further cytogenetic and morphological analysis on the basis of morphological evidence showing the presence of *T. ambiguum* genes and flow cytometry based ploidy estimates (Table 4.5.4). BAR10-39, BAR10-59 and BAR10-63 with expected genomic compositions of (RRRR(A_4))+2, RRRRA and (RRRRA)-2 resulted from selfing of BAR09-17 (2n=38), BAR09-27 (2n=unspecified) and BAR09-28 (2n=39) respectively. BAR10-32 was the progeny of the cross between BAR09-16 (RRRR(A_4), 2n=36) and BAR09-20 (RRRRA, 2n=40). BAR09-20 was the self progeny of original hybrid, BAR-23 (RRRRA). The remaining four BAR10 hybrids were obtained from pollination of the BAR09 hybrids with genomic formula of RRRR(A) by white clover. The pollen fertility (%) in these hybrids was high (Table 4.5.4), probably because all white clover chromosomes had homologues and only a few *T. ambiguum* derived chromosomes were unpaired. The numbers of *T. ambiguum* chromosomes ranged from probably none in BAR10-44 to four in BAR10-32, 39, 62 and 63. The actual chromosome numbers were less than the expected numbers in all hybrids except BAR10-49 where the numbers matched (Table 4.5.4).

Table 4.5.4 Selected progeny of the BAR09 hybrids with expected genomic formula RRRR(A_4) (4.5x) and RRRRA (5x) (Table 31), with estimated ploidy estimates (flow cytometry), expected and actual chromosome numbers and pollen fertility.

Hybrid	Pedigree	Expected genomic composition	Pollen fertility %	Ploidy (x)	Exp. 2n chrom #	Actual 2n chrom. #
BAR10-32	(BAR09-16 x BAR09-20) - 3	RRRR(A_4) x RRRRA = RRRR(A_2)	87	4.6	38	36
BAR10-39	(BAR09-17-Selfed)-1	(RRRR(A_4))+2 x (RRRR(A_4))+2 = (RRRR(A_4))+2	59	4.6	38	36
BAR10-44	(BAR09-19 x (P/B1xP/B2)-2)-7	(ARRRR)-5 x RRRR = (RRRR(A_4))-(2-3)	56	4	33-34	32
BAR10-49	(BAR09-24 x C21557-815)-10	(RRRR(A_4))-2 x RRRR = (RRRR(A_2))-1	94	4.2	33	33
BAR10-58	(BAR09-27 x (P/B1xP/B2)-1)-10	ARRRR x RRRR = RRRR(A_4)	73	4.3	--	35
BAR10-59	(BAR09-27-Selfed)-1	RRRRA x RRRRA = RRRRA	96	4.3	--	35
BAR10-62	(BAR09-28 x (P/B1xP/B2)-1)-3	(ARRRR)-1 x RRRR = (RRRR(A_4))-(0-1)	76	4.7	35-36	36
BAR10-63	(BAR09-28-Selfed)-3	(RRRRA)-1 x (RRRRA)-1 = (RRRRA)-(0-2)	97	4.5	38-40	36

Table 4.5.5 Meiotic chromosome associations at diakinesis/metaphase-I in PMCs of BAR10-39, BAR10-49, BAR10-58, BAR10-59 & BAR10-63.

Hybrid name	Pedigree	Expected genomic composition	No. of PMCs analysed	Mean frequency (Range of meiotic configurations					
				I	II	III	IV	V	Pollen fertility (%)
BAR10-39 (2n=35)	(BAR09-17 - Selfed)-1	(RRRR(A_4)) +2 x (RRRR(A_4))+2= (RRRR(A_4))+2	60	1.8 (0-6)	7.7 (1-13)	3.5 (0-9)	1.9 (0-5)	0.02 (0-1)	59
BAR10-49 (2n=33)	(BAR09-24 x C21557-815)-10	(RRRR(A_4))-2 x RRRR= (RRRR(A_2))-1	55	0.8 (0-3)	14.6 (7-16)	0.5 (0-6)	0.4 (0-3)	0.02 (0-1)	94
BAR10-58 (2n=35)	(BAR09-27 x (P/B1XP/B2) -1)-10	ARRRR x RRRR= RRRR(A_4)	41	2.9 (0-5)	14.2 (8-17)	0.7 (0-5)	0.3 (0-3)	0.1 (0-1)	75
BAR10-59 (2n=35)	(BAR09-27 - Selfed)-1	ARRRR x ARRRR= ARRRR	54	1.4 (0-4)	12.9 (2-17)	1.1 (0-5)	1.1 (0-4)	0.04 (0-1)	94
BAR10-63 (2n=36)	(BAR09-28 - Selfed)-3	(ARRRR)-1 x (ARRRR)-1= (RRRRA)-(0-2)	41	2.6 (1-5)	10.3 (5-15)	2.6 (0-6)	1.3 (0-4)	0	76

The actual somatic chromosome counts in these eight selected BAR10 hybrids ranged from 32 in BAR10-44 to 36 in BAR10-62 and BAR10-63. BAR10-32 had 36 chromosomes, of which one was apparently telocentric (Figure 4.5.2a). BAR10-44 had 32 chromosomes, which suggests that all the *T. ambiguum* derived chromosomes were eliminated totally from this hybrid during gamete formation in the parent, BAR09-19 (2n=35, Table 4.5.2) although some introgression from the A genome could not be ruled out. But any chromosome substitution of A for R or introgression was, however, not supported by the flow cytometric ploidy estimates (Table 4.5.4). The somatic chromosome numbers in BAR10-39, BAR10-44, BAR10-59 and BAR10-63 had dropped due to chromosome elimination during meiosis in the parental BAR hybrids. All the hybrids in this group possessed *T. ambiguum* chromosomes in isolated form except, perhaps, BAR10-44 which might not have any *T. ambiguum*-derived chromosomes and would be excluded from further analysis. These hybrids have gone through two meiotic cycles with isolated *T. ambiguum* chromosomes so had two chances to undergo inter-genomic mixing.

4.5.3.2 Meiotic analysis of selected BAR10 hybrids

Selected plants from the BAR10 generation were analysed for their meiotic chromosome behaviour during diakinesis/metaphase-I. BAR10-39, BAR10-49, BAR10-58, BAR10-59 and BAR10-63 were analysed for chromosome pairing. The results, along with the somatic chromosomes numbers, and numbers of PMCs studied are given in Table 4.5.5. All the hybrids in this group have probably one full white clover chromosome compliment plus some *T. ambiguum* chromosomes probably ranging from 1 in BAR10-49 to 4 in BAR10-63. All the hybrids showed I, II, III, IV and V chromosome associations in varying average frequencies except BAR10-63 which did not have any pentavalents. A metaphase-I image of BAR10-49 showing 16 IIs and 1 I is given in Figure 4.5.2b. The most frequent chromosome disjunction in BAR10-39, BAR10-58 and BAR10-59 was 17-18 in the PMCs having normal anaphase-I without any lagging chromosomes. In BAR10-49 and BAR10-63 the chromosomal disjunctions during anaphase-I were respectively 16-17 and 18-18. Figure 4.5.2c shows 16-17 disjunction in BAR10-49. Many meiotic abnormalities, including chromosome lagging during anaphase-I and II, SDR and unbalanced disjunction of chromosomes leading to aneuploid gametes were observed. Figure 4.5.2d shows second division restitution in BAR10-59.

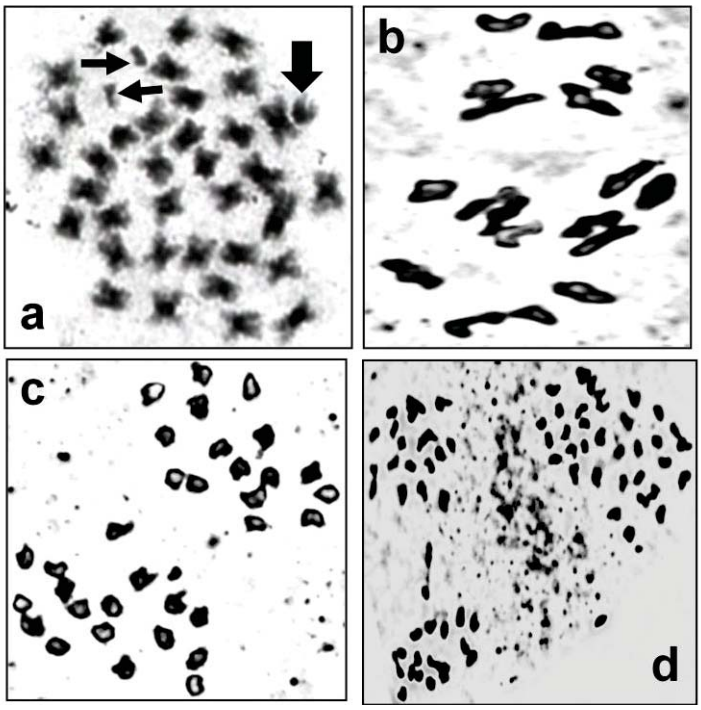

Figure 4.5.2 (**a**) Giemsa-stained chromosomes in BAR10-32 (RRRR(A), 2n=36) showing two satellite knobs (arrows). One broken chromosome was also present having a centromere at one end (large arrow). (**b**) Metaphase-I in BAR10-49 with exp. genomic composition of RRRR(A_2)-1 shows a near-normal meiosis with 16 IIs and 1 I. (**c**) Anaphase-I stage in BAR10-49 with 16-17 disjunction. (**d**) Second division restitution (SDR) in BAR10-59 (RRRRA-5, 2n=35).

BAR10-49, BAR10-58, BAR10-59 and BAR10-63 had comparatively high average frequencies of IIs per PMC and also high pollen stainabilities. With few exceptions, the frequency of Is was apparently related to the assumed number *T. ambiguum*-derived chromosomes present in these hybrids (Table 4.5.5). Multivalent (III, IV) chromosome associations might show within species intra-genomic chromosome pairing but the Vs must involve both intra- and inter-specific homoeologous chromosome pairing (Table 4.5.5). These Vs, although in low average frequencies, present some evidence of inter-specific chromosome pairing which might lead to genomic remixing between white clover and *T. ambiguum*.

4.5.4 Molecular cytogenetic analysis of BAR09-16 and BAR10-32.

The Giemsa-stained chromosome preparations of metaphase showed that both BAR09-16 and BAR10-32 had somatic chromosome compliments of 36 (Figures 4.5.1a, 4.5.2a). BAR09-16 resulted from the cross of original hybrid, BAR-23 (RRRRA, 5x) with RRRR (white clover, 4x) and had the expected genomic composition of RRRR(A_4). BAR10-32 was the progeny of the inter-crossing of BAR09-16 (RRRR(A_4)) and BAR09-20 (RRRRA). One of the chromosomes in BAR10-32 was telocentric, like a half chromosome with a centromere at one end (Figure 4.5.2a). Both BAR09-16 and BAR10-32 carried a pair of chromosomes having NORs with highly transcriptionally active 18S rDNA (Figures 4.5.1a, 4.5.2a). The

metaphase chromosome preparations in these two hybrids were probed with fluorescently labelled genomic DNA of *T. ambiguum* (green) and 5S rDNA (red).

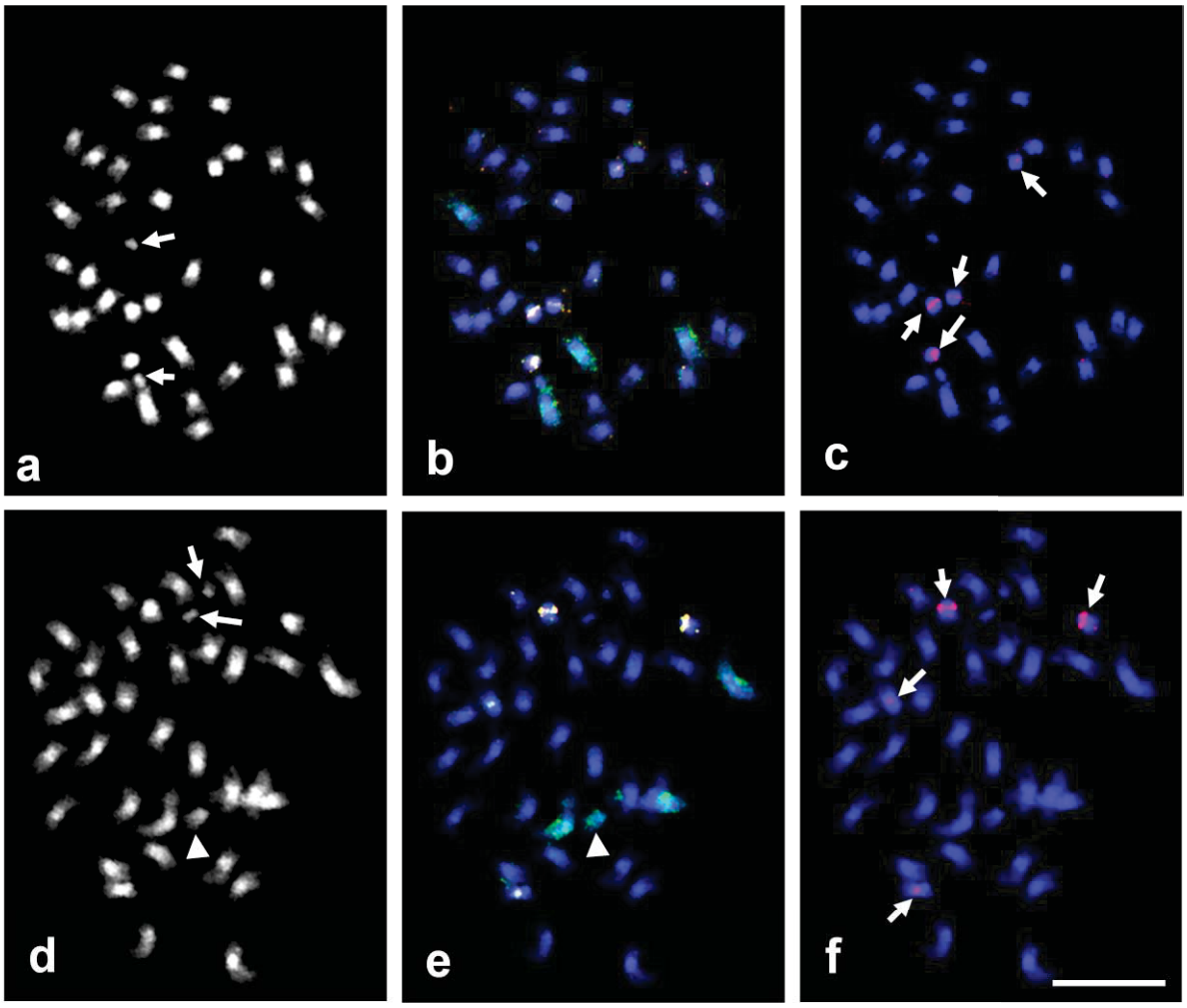

Figure 4.5.3 (**a**) DAPI-stained metaphase chromosomes in BAR09-16 (RRRR(A_4), 2n=36) with two satellite bearing chromosomes (arrows) with highly stretched NORs. (**b, c)** are the results of GISH and FISH and FISH only on the same cell. GISH highlighted four *T. ambiguum* chromosomes (green). FISH gave four 5S signals, (arrows) two on NOR bearing chromosomes while the other two were on other *T. repens* derived-chromosomes. (**d**), DAPI-stained chromosomes in BAR10-32 (RRRR(A), 2n=36), with two satellites (arrows). (**e**) GISH on the same cell as (**d**) highlighted four chromosomes from *T. ambguum* (green) of which one was apparently telocentric (a half chromosome with a centromere (arrowhead in **d** and **e**). (**f**) FISH on the same cell using 5S rDNA as probe resulted in four 5S signals (arrows), of which two were on the NOR bearing chromosomes from *T. repens* and two were on non-NOR *T. repens* chromosomes. No signs of genomic mixing were apparent in either hybrid. BAR=10 µm.

GISH on BAR09-16, using genomic DNA of *T. ambiguum* as a probe, highlighted four chromosomes from *T. ambiguum* (Figure 4.5.3b). None of these chromosomes carried 5S signals. Probing with 5S rDNA gave four red signals (Figure 4.5.3c). Two of these signals were on the longer arms of a pair of submetacentric chromosomes which carried 18S-5.8S-26S r DNA regions in highly stretched form on the shorter arms. Another pair of almost

metacentric chromosomes carried small 5S signals separately on the longer arms. These signals had smaller copy number than those on the NOR-chromosome pair and were consistent with an origin from white clover. There were some green signals on the non-*T. ambiguum* chromosomes but they cannot be taken as evidence of recombination because they were not consistently seen on both chromatids.

GISH revealed that BAR10-32 consistently had in each somatic cell three intact *T. ambiguum* chromosomes and one half chromosome with a centromere at one end (Figure 4.5.3e). This plant had four 5S signals with similar chromosomal organization as in BAR09-16 (Figure 4.5.3f). No evidence of interspecific genomic exchange was observed in these plants. The GISH and FISH results confirmed that BAR09-16 and BAR10-32 had one full chromosome compliment from white clover plus four and 3½ chromosomes, respectively, from *T. ambiguum*.

4.5.5 Morphological characterization

4.5.5.1 Self and cross progeny of 5x RRRRA hybrids

Seven BAR09 hybrids resulting from selfing and backcrossing of 5x RRRRA hybrids with white clover were evaluated in a sand-pit experiment, with BAR-23 (5x RRRRA) and white clover (C21557-815) as control parents. These are listed in Tables 4.5.6.1, 4.5.6.2 with mean data for the above- and under-ground morphological characters. All the studied traits recorded significant differences.

The phenotypes showed a combination of characteristics coming from both parents (white clover and *T. ambiguum*). All the hybrids were stoloniferous and had nodal rooting, as in white clover. The nodal rooting in the hybrids was almost as strong as in white clover, except for BAR09-27 and -28 in which it was significantly weaker (Table 4.5.6.1). The terminal growth of stems appeared to be highly reduced in all the hybrids except BAR09-25 which showed indeterminate terminal growth like white clover. Leaflet lengths were significantly longer in all the BAR09-hybrids than in the white clover parent, indicating the inheritance of this trait from *T.ambiguum*. BAR-23, the original ARRRR hybrid used as control, did not have significantly longer leaflets than white clover (Table 4.5.6.1). The number of florets/head was significantly higher in all the hybrids as compared to the white clover control. Due to highly reduced terminal growth, the BAR09 hybrids, other than BAR09-25, appeared to have both axillary and terminal flowering. The data for BAR09-25 makes it look

like a white clover plant, although it had 34 chromosomes. Except for BAR09-27, all the BAR09 hybrids were female fertile. Four of the hybrids (BAR-23, -17, -19 and -27) had higher root dry weight % than white clover. (Table 4.5.6.2). This difference in the root weight % of the total biomass was due to long and thicker nodal roots and not to the presence of rhizomes. Combinations of white clover-like morphology, sometimes *T. ambiguum*-like phenotypes and a range of intermediates showed the presence and expression of traits from both parent species.

4.5.5.2 Self and cross progeny of RRRR(A)

BAR10 progeny derived from the above hybrids by selfing and backcrossing with white clover were also evaluated in a sandpit for their morphological characterization. Six BAR10 hybrids, (BAR10-32, BAR10-39, BAR10-44, BAR10-49, BAR10-58 and BAR10-62) were included in this study with BAR-23 and two parental white clover genotypes (Kopu II and (P/BxP/B2)-2) as controls (Table 4.5.7.1). All the BAR 10 hybrids had stolons with nodal rooting, indeterminate apical growth, and axillary flowering (traits inherited from white clover). Stolon number in the hybrids was generally low as compared to the parents but only three hybrids (BAR10-32, 39 and 49) had significantly fewer stolons as compared to (P/BxP/B2)-2. Inflorescence numbers in the hybrids were significantly lower than the controls, except for BAR10-62 in which the number of inflorescences was within the control range. Although most of the other traits studied showed statistically significant differences, there were no clear differentiations between the hybrids and control parents on the basis of parental characters (Table 4.5.7.1). Dry root weight % was again the main morphological trait showing the influence of *T. ambiguum* parentage on the phenotypes of the hybrids. BAR10-44 and BAR 23 had a significantly higher root weight ratio to total biological yield than (P/BxP/B2)-2, while all the hybrids had significantly higher values for this trait than the Kopu II control (Table 4.5.7.2).

Table 4.5.6.1. Data on the morphological characteristics of the self and cross progeny of 5x BAR hybrids (RRRRA) with white clover (RRRR).

HYBRIDS	Stolon number	Stolon Length (cm)	Stem anchorage (0-10)	Inflorescence No.	Growth habit	Peduncle length (cm)	Florets/head	Leaflet length/width ratio	Petiole length (cm)
BAR09-16	26.6	18.3	9.3	99.2	combination	11.6	63.2	1.42	5.6
BAR09-17	25.0	35.5	10	107.8	combination	11.5	63.6	1.36	4.9
BAR09-19	18.0	26	10	59.2	combination	9.6	67.5	1.37	6
BAR09-24	27.3	25.8	8.3	74	combination	20.7	82.9	1.7	10.1
BAR09-25	54.2	41.5	8.5	191	indeterminate	16.7	68.9	1.6	7.1
BAR09-27	25.7	6.5	4.8	35.2	determinate	8.8	66.6	1.6	4.2
BAR09-28	40.0	20.3	5	145.2	combination	13.4	78.8	1.4	3.8
BAR-23	32.2	31	9.8	114.2	combination	12.2	76.9	1.3	4.8
C21557-815	10.3	17	10	10.5	indeterminate	12.2	53.8	1.2	7.3
CV%	**12.1**	**20.2**	**15**	**24.8**		**13.9**	**7.8**	**5.4**	**17.2**
SEM	**3.5**	**7**	**1.3**	**23.0**		**1.8**	**5.3**	**0.07**	**0.7**
LSD	**5.0**	**7.2**	**1.8**	**33.4**		**2.6**	**7.7**	**0.11**	**1.5**
Prob. (5%)	***	***	***	***		***	***	***	**

Table 4.5.6.2. Data on different above and underground morphological characteristics of the self and cross progeny of 5x BAR hybrids (RRRRA) with white clover (RRRR).

HYBRIDS	Stem thickness (mm)	Flowering-terminal, axillary or combination	Seed/head	Main Root Thickness (mm)	Main nodal root thickness (mm)	Dry weight top (g)	Dry weight root (g)	Total biomass (TBM) (g)	Root weight % of TBM
BAR09-16	3.6	combination	32.2	5.2	2.3	22	13	35	33.5
BAR09-17	2.7	combination	24.3	5.8	1.9	15.4	9.2	24.6	37.4
BAR09-19	2.5	combination	10.8	4.1	2	5.6	4.7	10.3	47.8
BAR09-24	3.4	combination	32.4	6.2	2.8	25.8	13.2	39	33.4
BAR09-25	3.4	axillary	56.3	4.7	1.9	55.6	27.3	82.8	31.8
BAR09-27	3	combination	0	3.8	2.4	3.3	2.3	5.6	44.2
BAR09-28	3.4	combination	9.1	6.7	2.2	16.5	4.9	21.4	22.9
BAR-23	2.7	combination	23.3	4.8	2.9	16.2	11	27.1	41.4
C21557-815	2.4	axillary	35.1	5.5	1.7	8.5	2.8	11.3	27.4
CV%	**9.5**		**48.2**	**27**	**30.6**	**57.5**	**78.3**	**64**	**17.8**
SEM	**0.28**		**11.98**	**0.7**	**0.34**	**5.4**	**3.9**	**9.1**	**3.2**
LSD	**0.41**		**17.49**	**2.1**	**0.99**	**15.8**	**11.2**	**26.7**	**9.3**
Prob. (5%)	***		***	**	**	***	***	***	***

Table 4.5.7.1 Mean data of above-ground morphological characters of the progeny of the cross between 4.5x hybrids RRRR(A_4) & white clover.

Hybrids	Stolon number	Stolon Length (cm)	Stem anchorage (0-10)	Inflorescence No. ‡	Growth habit	Peduncle length (cm)	Flowering-terminal, axillary or combination	Florets/ head	Leaflet length/width ratio
BAR10-32	10.8	9.9	7.3	2.3 (0.1)	indeterminate	6	axillary	34	1.1
BAR10-39	12.2	26.9	7	0.3 (0.7)	indeterminate	11.6	axillary	54.5	1.1
BAR10-44	22.5	26	9	0.4 (1.5)	indeterminate	12.1	axillary	50.8	1.3
BAR10-49	15	20.4	6.3	1.2 (0.3)	indeterminate	14.7	axillary	53.2	1.3
BAR10-58	22.2	49.1	4.5	0.2 (1.2)	indeterminate	14.4	axillary	54.5	1.4
BAR10-62	23	38.5	7.5	4.6 (100.5)	indeterminate	15.2	axillary	72.5	1.3
BAR-23	24.8	31	9.3	4.8 (115.6)	indeterminate	8.7	axillary	52	1.3
Kopu II	38.8	100.3	10	6.2 (502.7)	indeterminate	14	axillary	53.8	1.2
(P/B1 x P/B2)-2	27.5	15.3	10	3.4 (28.8)	indeterminate	5.5	axillary	47	1.2
L.S.D	**9.5**	**18.7**	**1.3**	**1.0**		**2.8**		**16.0**	**0.2**
CV%	**27.7**	**36.2**	**11.7**	**16.6**		**16.8**		**20.9**	**9.7**
SEM	**4.6**	**9.0**	**0.7**	**0.5**		**1.4**		**7.8**	**0.1**
Prob. (5%)	***	***	***	***		***		***	***

‡ Data were log transformed
Values in parenthesis show back-transformed data

Table 4.5.7.2 Mean data of above and under-ground morphological characters of the progeny of the cross between 4.5x hybrids RRRR(A) & white clover (RRRR).

Hybrids	Stem thickness (mm)	Petiole length (cm)	Seed/head ‡	Main nodal root thickness (mm)	Dry weight top (g) ‡	Dry weight root (g) ‡	Total biomass (TBM) (g) ‡	Root weight % of TBM
BAR10-32	2.8	3.6	3.2 (25.5)	1.5	0.7 (2.0)	_0.01 (1.0)	1.1 (3)	33.4
BAR10-39	2.9	4.6	3.4 (28.8)	2.6	1.0 (2.7)	0.1 (1.1)	1.3 (3.8)	29.3
BAR10-44	2.5	5.5	1.8 (5.8)	2.5	2.5 (12.4)	2.0 (7.6)	3 (20.1)	38.1
BAR10-49	2.7	5.7	3.2 (23.8)	3.0	1.7 (5.3)	0.9 (2.4)	2.1 (7.8)	32.0
BAR10-58	2.7	5.4	3.5 (32.5)	3.3	2.6 (14.0)	1.5 (4.6)	2.9 (18.7)	24.9
BAR10-62	2.7	5.6	3.7 (42.1)	2.4	3.0 (19.5)	1.9 (6.9)	3.3 (26.3)	26.3
BAR-23	3.1	5.1	3.2 (25.0)	2.1	2.9 (18.9)	2.5 (12.1)	3.4 (31.2)	38.8
Kopu II	3.3	7.9	3.8 (46.5)	2.4	5.6 (259.8)	3.2 (24.8)	5.7 (284.2)	9.0
(P/B1 x P/B2)-2	2.1	3.9	3.3 (27.9)	1.7	1.5 (4.4)	0.4 (1.5)	1.8 (6.0)	25.8
L.S.D	**0.5**	**1.5**	**1.4**	**1.0**	**1.1**	**1.0**	**1.1**	**8.1**
CV%	**11.4**	**19.2**	**28.1**	**27.4**	**31.8**	**49.9**	**26.6**	**19.4**
SE	**0.24**	**0.7**	**0.6**	**0.5**	**0.5**	**0.5**	**0.5**	**3.9**
Prob. (5%)	***	***	*	***	***	***	***	***

‡ Data were log transformed
Values in parenthesis show back-transformed data

CHAPTER 5
DISCUSSION

Introgression of alien genetic variation from secondary gene pools into cultivated crops is not always very easy. *T. ambiguum*, despite its huge potential importance for agronomic improvement of *T. repens*, is the most distantly related species to it among the closely related species making up the "white clover complex" (Ellison *et al*., 2006). This genetic remoteness is evident from the presence of strong reproductive barriers leading to considerable difficulty in making hybrids between these two species. Due to this genetic remoteness, breeding efforts to achieve introgression of useful genes from *T. ambiguum* into *T. repens* have not been successful. Keeping that difficulty in mind, a different way was followed in the current research project. Actually, two approaches were investigated focussing on the possibility of achieving introgression from *T. ambiguum* into *T. repens*. The first approach, consisted of four strategies, used an indirect method utilizing *T. occidentale* as a genetic bridge. The hypothesis of using *T. occidentale* as genetic bridge between *T. repens* and *T. ambiguum* was based on the findings of Gibson and Beinhart (1969) and Chen and Gibson (1970) showing close genetic relationship of *T. occidentale* with both *T. ambiguum* and *T. repens*. *T. occidentale* has recently been confirmed to be phylogeneically very close to white clover (Ellison *et al*., 2006, Hand *et al*., 2008, Williams *et al.,* 2012) and to *T. ambiguum* (Williams *et al.,* 2011). So this close genetic relationship can potentially be used for transferring traits across the two species using *T. occidentale* as a genetic bridge (Williams *et al*., 2006a). The second concept involved direct introgression of genes from *T. ambiguum* into *T. repens* through ploidy manipulation.

This is first time multispecies hybrids combining genomes from all three species i.e. *T. ambiguum* (A), *T. repens* (R) and *T. occidentale* (O) have been produced and tested for their potential in breeding improved cultivars of *T. repens*. All the strategy crosses worked producing progenies with reasonable levels of fertility and a strong evidence of inter-specific chromosome pairing with one case of introgression in one strategy was confirmed by GISH. Repeated back crossing to white clover, gave a large number of progenies in all the strategies which were near -32 chromosomes with *T. ambiguum* traits. The advanced backcrossed derivatives are apparently addition/substitution lines but their breeding values are not yet confirmed because they need more study for assessing their stability.

5.1 Original BAR hybrids

All the initial BAR hybrids of *T. ambiguum* with *T. repens* and *T. occidentale* used in five strategies were developed through the application of embryo rescue (Williams, 1978; Williams and Verry, 1981). They included A^TA^TRR (4x, designated as H-435) (Williams and Verry, 1981), A^DA^TRR (4x, designated as hybrid 70) (Williams *et al*., 2006), A^TA^TRR (designated as ROS), $A^TA^TA^TORR$ (6x, designated as hybrid "33 OP-1"), A^TA^TO (3x, designated as 434-1) and 4x hybrids having genomic composition of A^TA^TOO designated as BL and BN. H-435 and ROS were the same in genomic composition but were bred by crossing different plants of the same parental species, 4x *T. ambiguum* ($A^TA^TA^TA^T$) and *T. repens* (RRRR). Moreover, ROS inherited a diffuse red leaf allele from the male parent (*T. repens*) and so had pink flowers while H-435 was totally green. Hybrid 70 (A^DA^TRR) was different from the H-435 and ROS (A^TA^TRR) in two ways. First, it had A genomes from both 2x and 4x ploidy versions of *T. ambiguum*. It is interestingly the first time that A^D had been combined with *T. repens* genomes. Second, its breeding history was longer and more complex.

The BAR hybrids used in this study were derived from the original hybrids mentioned above and were divided into five categories depending on their genomic composition and pedigrees. Each category was treated as a different breeding strategy to combine the genomes of *T. repens* and *T. ambiguum*. These BAR hybrids are given in Tables 3.1, 3.2, 3.3, 3.4 and 3.5.

1. Multi-species 4x hybrids RRAO from cross, RRRR x AAOO.
2. Pentaploid hybrids AAAOO obtained from the cross, AAO x AAOO.
3. Hybrids derived from AAAORR (6x), (designated as "33 OP-1") after crossing with white clover once or twice and with genomic composition of RRRA(A_4O_4) and RRR($R_4A_6O_2$) respectively.
4. Multi-species tri-genomic ARO (3x) or hexa-genomic AARROO (6x) hybrids resulted from crosses, AARR (ROS) x OO (2x) and AARR (ROS) x OOOO (4x) respectively.
5. Direct hybrids between *T. repens* and *T. ambiguum* with genomic formula of RRRRA (5x) and AAARRRR (7x).

5.2 Strategy based on 4x BAR hybrids, RRAO

These 4x hybrids (RRAO) were the progeny of a single white clover plant (RRRR, Red One (RO)) pollinated with hybrid BL (AAOO) (Table 4.1.1). At first, these crosses did not seem to have worked because the resulting progeny were very similar morphologically to white clover. Also, under the EBN hypothesis where A has been given EBN value = 0, (Hussain and Williams, personal communication), the cross between RRRR and AAOO should not work if both parents contribute normal (n) gametes due to the different EBN values. Under the EBN hypothesis, the cross would work if white clover (RRRR) contributes normal gametes while the hybrid parent, BL contributes 2n gamete. In that case both the gametes would have the same EBN values (2), ensuring the required 2:1 maternal: paternal EBN ratio in the endosperm for the normal development of seed and so the success of a cross (Nishiyama and Inomata, 1966; Johnston et *al*., 1980; Hussain and Williams, 2007). Although the EBN indicated the likelihood of 6x progeny, the flow cytometry results confirmed them to be 4x plants with white clover-like morphology. This led to the conclusion that they were actually the self progeny of white clover. However, their low pollen fertility and, later on, GISH indicated their hybridity. The ploidy of these hybrids was 4x, as in the parents, indicating that both parents contributed haploid gametes. The success of this RRRR x AAOO cross with normal haploid gametes having different EBN can only be expected as rare exceptions, as reported by Parrot and Smith (1986), because embryo rescue was used later to repeat this cross. So the endosperm failure is normal and the success of this cross in the first place was exceptional.

These 4x hybrids (RRAO) were reciprocally crossed with *T. repens* in 2009, and the resulting generation was designated BAR09. Considering the RRAO plants as tri-specific F_1s, the BAR09 progeny were actually BC_1F_1. The initial selection in the BAR09 progeny was based on the morphological evidence of *T. ambiguum* traits. Secondary selection was based on ploidy levels determined through flow cytometry and pollen fertility (%). Final selections were made on the basis of somatic chromosome numbers. It is to be noted here that due to different DNA contents in nuclei of *T. ambiguum* and *T. repens* as has been reported in other species by Bennett and Leitch, (1997) Greilhuber, (1998) and Bennett *et al*., (2000), it is relatively easy to differentiate hybrids from their parents based on the their DNA contents (Marasek *et al*., 2006). Pollen fertility is another very easy indicator of hybridity because the pollen fertility in a hybrid having A, R and O should always be less than that in white clover (usually >90 %) (Atwood and Hill, 1940). Five selected BC_1F_1 BAR09 hybrids were

BAR09-120, BAR09-128, BAR09-130, BAR09-131, BAR09-132 while BAR09-133 was an F_2 produced by selfing (Table 4.1.2). The genome composition of the BC_1F_1 hybrids was expected to average RRR(A_4O_4). The selected hybrids were all aneuploids with 2n numbers from 31 to 35. This aneuploidy shows that the meiosis in the parental hybrids (RRAO) was not regular and, due to anomalies including univalent and multivalent chromosome associations, followed by unbalanced disjunction during anaphase-1, had produced either n+ or n- gametes as reported by Ferguson *et al.* (1990) in *Trifolium* hybrids and Felismino *et al*. (2012) in *Brachiaria* hybrids to combine with the normal haploid gametes from the white clover. This aneuploid condition of these BAR09 hybrids was further evidence that the BAR parents (Table 4.1.1) were not the self progeny of white clover but were actually hybrid in nature. BAR09-133, the self progeny of hybrid (RO x BL)-1 had a comparatively higher somatic chromosome number (2n=4x+3). This suggests that both gametes probably had n+ chromosomes, again consistent with a disturbed meiosis in the parent plant (RRAO). Aneuploidy resulting from aneuploid gametes has been reported by Anderson *et al*. (1991) in BC_1 progeny of hybrid AARR with white clover used as recurrent parent. These BAR09 progeny (BC_1F_1 and F_2,Table 4.1.2) showed reasonable levels of male fertility (> 40 %) which is consistent with a significant frequency of homoeologous chromosome pairing involving both intra-specific homoeologous chromosome pairing (autosyndesis, R^PR^O) and as well as inter-specific chromosomes pairing (allosyndesis, AO or may be R^OO) which reinforces the hypothesis given by Williams *et al*. (1982). This says that, in the absence of homologues, homoeologous chromosome pairing might happen in a hybrid situation. The genomic formula of amphidiploid white clover can be suggested to be $R^PR^PR^OR^O$ with R^PR^P and R^OR^O being the two full chromosome complements coming respectively from the probable female and male parents, 2x *T. pallescens* (PP) and 2x *T. occidentale* (OO) (Ellison *et al*., 2006; Williams *et al.*, 2012). Pairing between the gametic chromosome sets of white clover has already been reported from the presence of IIIs in 3x F_1 hybrids of white clover with *T. nigrescens* (2x) and *T. occidentale* (2x) by Chen and Gibson (1970a, b), from the presence of IVs in 4x F_1 hybrids between *T. ambiguum* and *T. repens* by Williams *et al*. (1982) and in 4x hybrids between *T. repens* & *T. uniflorum* by Chen and Gibson (1972). Pairing between the gametic sets does not happen in white clover itself, which is consistent with the hypothesis mentioned above that the presence of homologues discourages chromosome pairing between homoeologues.

All the selected BAR09 hybrids in this generation (Table 4.1.2) were self-compatible (SC) so during the next breeding cycle they were used as male parents to pollinate an elite green leaved, self-incompatible (SI) white clover, Kopu II. As the BAR09 hybrids had dominant leaf colour alleles and the female parent was self incompatible, the selection of BAR10 hybrid progeny was not difficult. In addition to dominant leaf colour marker alleles, pollen fertility levels, genome sizes, and morphologies were also considered in the selection of seven desirable BAR10 (BC_2F_1) hybrids for further analysis (Table 4.1.4). Hybrids BAR10-129, BAR10-131, BAR10-137 & BAR10-140 were exactly 4x (2n=32) while the rest were aneuploids as in the BAR09 generation with 2n of 33 in BAR10-126, 35 in BAR10-136 and 34 in BAR10-138 (Table 4.1.4) showing that the gametes from the hybrids had 2x+ chromosomal constitution.

Selected plants from both generations in this strategy were subjected to chromosome pairing analysis to check whether pairing was intra-specific homoeologous (autosyndesis) or inter-specific homoeologus (allosyndesis). The terms autosyndesis and allosyndesis have been adopted from Williams *et al.*, (1982). Although not very good terms in our case, but throughout the document they have been used and pairing between R^P and R^O would represent autosyndetic pairing because of their common recent source (white clover) and allosyndetic pairing would indicate all other chromosome pairings i.e., AR, AO and RO due to their different recent sources. During meiosis in RRAO, the situation can be complex because all four genomes are homoeologous. The two white clover genomes (R^PR^O), although coming from *T. repens* were homoeologous to each other, with one from each of the probable ancestral species, *T. pallescens* (PP) and *T. occidentale* (OO) (Williams *et al*., 2012), but these possibly had a less sequence divergence than the A and O sources. So, it was expected that original BAR hybrids (RRAO) might have predominantly bivalent pairing involving autosyndesis (pairing between respective R^P and R^O chromosomes) and allosyndesis (pairing between A & O or even R^O & O). Although R^O and O chromosomes have the same origin (*T. occidentale*) but the intensity of pairing between them would depend on the degree of small genetic and structural changes accrued in them over a long period of time during their separate evolution (Smith, 1968; Stebbins, 1971). This is further discussed below.

5.2.1 Meiosis in BAR09-120 (2n=4x=33)

This plant was selected on the basis of its somatic chromosome number, phenotype and flow cytometric ploidy estimate which indicated that it had *T. ambiguum*-derived traits. The aim of this analysis was to see if there was any indication of homoeologous chromosome pairing involving A-derived chromosomes leading to inter-genomic recombination.

BAR09-120 was the progeny of cross, $R^PR^PR^OR^O$ x ($R^PR^PR^OR^O$ x AAOO). The superscripts on Rs in the genomic formula of genetically allotetraploid *T. repens* show the two different sub-genomes contributed by *T. pallescens* (P) and *T. occidentale* (O), the possible progenitor species (Williams *et al.,* 2012). $R^PR^PR^OR^O$ x AAOO had the expected genomic composition of R^PR^OAO (4x). Then the expected genomic composition of BAR09-120 ($R^PR^PR^OR^O$ x R^PR^OAO) can be approximated as $R^PR^OR^{P/O}(A_4/O_4)$. This formula is based on the well-established fact that chromosome pairing in white clover was exclusively within sub-genomes (R^PR^P) and (R^OR^O), giving regular disomic (diploid-like) inheritance (Atwood and Hill, 1940). Pairing in R^PR^OAO was possibly between R^P and R^O and A and O, as pairing between the gametic chromosomes of white clover has been reported in hybrids, as mentioned earlier. In a genetically complex hybrid like R^PR^OAO, in which every genome is homoeologous to the others, common sense suggests that genetically and structurally more related chromosomes will preferentially pair, even if they are homoeologous (Chen and Gibson, 1970a, b; Atwood and Hill, 1940). In a hybrid with genomic formula R^PR^OAO, R^O, although having originated from the ancestral *T. occidentale* (O), has subsequently evolved separately within *T. repens* under different environmental pressures, and must be different from modern *T. occidentale*-derived chromosomes (O). With current knowledge, it is uncertain whether R^O will pair more preferentially with R^P or O. New knowledge e. g. DNA sequence similarities might be needed to enable us to better understand pairing preferences. Assuming intra-specific homoeologous pairing between the gametic chromosomes of white clover in R^PR^OAO and their independent assortment, BAR09-120 was expected to have two genomes from white clover one each originating from *T. occidentale* and *T. pallescens*. A third genome from white clover would be expected to be a mixture of chromosomes derived from both the progenitor species. Finally, it would be expected to have two partial genomes derived from *T. ambiguum* and *T. occidentale*. In a hybrid with genomic composition of $R^PR^OR^{P/O}(A_4/O_4)$, a maximum eight IIs can be formed between homologous *T. repens* chromosomes, assuming the mixed genome from *T. repens* had four chromosomes each from the two sub-genomes. Even with both homologous and homoeologus chromosome pairing

within white clover, there could only be up to eight IIs. Bivalents beyond eight could be either $R^{O}O$ or AR. The average frequency of IIs was 9.44, ranging up to 15. This shows that some bivalents involved interspecific homoeologous pairings, i.e., $R^{O}O$, $R^{P}O$, RA or AO. The least likely combination was pairing between A and O because most of the A and O chromosomes were probably from different linkage groups. If both homologous and homoeologous chromosome pairing occurred in BAR09-120 solely among the white clover-derived chromosomes then there would be up to 8 IIs with 17 Is or 8 IIIs and 9 Is. Deviation from this would represent inter-specific chromosome pairing. However, the actual situation was totally different, having less than the expected number of Is (2.5) and IIIs (2.0) and with some IVs (1.3) and Vs (0.1) (Table 4.1.3). This showed that meiosis was very disturbed and the pairing of chromosomes involved considerable inter-specific chromosome pairing

The Is might be from *T. ambiguum* keeping in view its more remote genetic/phylogenetic distance from white clover and *T. occidentale* (Ellison *et al.,* 2006). The number of *T. ambiguum* chromosomes in this hybrid was expectedly four or five (depending on what the extra chromosome was), but the average number of Is was 2.5. This might be evidence of *T. ambiguum* chromosome pairing during meiosis or possibly that some of the expected A chromosomes had actually been lost. The IIIs might have been within white clover involving homologous and intra-specific homoeologous R chromosomes (Williams *et al*., 1982). The IVs might have been RRRO but, given that there were 9.2 IIs, the likelihood of combinations of $R^{O}R^{O}AO$, $R^{O}R^{P}AO$, $R^{P}R^{P}OA$ are higher, assuming no illegitimate pairing. A IV consisting of all *T. repens* chromosomes was not possible because there could be only one of each chromosome in the $R^{P/O}$ set, assuming the random segregation of supposedly eight IIs ($R^{P}R^{O}$) in $R^{P}R^{O}AO$. Pentavalents were also observed in this plant, and, assuming that these Vs having no illegitimate pairing, involved chromosomes of the same linkage group from all the genomes present in the genomic formula, is evidence of participation of *T. ambiguum* chromosome in allosyndetic chromosome pairing.

5.2.2 Meiosis in BAR10-126 (2n=33)

This hybrid was the progeny of BAR09-120 (2n=33), which was used as the pollen parent onto white clover (Kopu II). Its expected composition genomic composition was approximately $R^{P}R^{O}R^{P/O}(R_4{}^{P/O}A_2O_2)$+(0-1). It had only one *T. ambiguum* chromosome confirmed through GISH/FISH (discussed later), one or two less than the expected number depending on what the extra chromosome was in BAR09-120. This hybrid is genomically so

complex that it is not easy to establish the nature of the likely chromosomal associations. The average frequency of IIs (12) might represent intra-genomic pairing within white clover genomes but the range (up to 15) showed the possibility of inter-genomic pairing within white clover and as well as the possible involvement of A and O chromosomes (Table 4.1.5). The IIIs (1.1) could be possibly within white clover or they could be $R^{O}R^{O}O$. But so far as IVs are concerned, they might be composed of chromosomes from all four genomes in this hybrid, or, possibly, they were totally within white clover or were $R^{O}R^{O}R^{O}O$. To further analyse this issue, GISH/FISH was carried out on meiotic chromosome preparations of BAR10-126 to see whether the *T. ambiguum* chromosome was pairing or not. Due to cytoplasmic impurities hampering the hybridization of probe DNA, the result was not very good, but such problems have also been reported by others as well (Jeridi *et al*., 2011). The *T. ambiguum*-derived chromosome in BAR10-126 was, fortuitously, a marker chromosome (sub-metacentric) with a 5S rRNA locus on the longer arm so it was very easy to monitor its behaviour during meiosis using GISH/FISH. Fluorescence studies confirmed that *T. ambiguum*-derived chromosome was pairing either as IIs or IIIs with other chromosomes in 50% of the studied PMCs. However, whether this pairing was with *T. occidentale* or *T. repens*-derived chromosomes is unknown. Also unknown is whether the inter-specific pairing would lead to some useful level of recombination although it is assumed that it might, keeping in view such a high level of inter-specific pairing. The GISH/FISH results were consistent with the findings of Anderson *et al*. (1991) and Meredith *et al*. (1995) which inferred A/R pairing in *T. ambiguum* x *T. repens* hybrids, although their findings were based only on conventional meiotic analysis. The A-containing IIs might have been AO or AR. and the IIIs may have been ROA. It might be expected that the *T. ambiguum*-derived chromosome with the 5S rDNA would pair with its homoeologous counterparts from either *T. occidentale* or *T. repens* with 5S signals. But as can be seen in Figure 4.1.4b, this chromosome paired with a chromosome without a 5S signal, suggesting either that its homoeologous counterpart in O or R might not have a 5S rRNA locus, or it has paired with a different linkage group. Whatever the case may be, such pairing outside the linkage group (illegitimate pairing) is rare, and has been reported by Ansari *et al*. (2012) in other AAAO (4x) hybrids. In Figures 4.1.4c and d, the lone *T. ambiguum* chromosome behaved as a univalent. Figures 4.1.4e and f show the I precociously splitting into sister chromatids and then partially lagging as they moved toward the opposite poles. Lukaszewski (2010) reported similar findings while studying the fate of Is in a wheat line with the chromosome 2B centromere introgressed from rye and a complete rye chromosome 2R. These Is in wheat

either migrated as intact chromosomes to one pole or split longitudinally into sister chromatids which moved to opposite poles because of bipolar attachment of spindle fibres to sister centromeres. Alternatively, mis-division and breakage occurred at the centromeres, resulting in telocentrics that moved to the poles. The predominant precocious separation during metaphase-I of Is into sister chromatids followed by their movement to opposite poles was also reported in 4x AAAO hybrids, from the cross, 6x *T. ambiguum* (AAAAAA) x *T. occidentale* (OO) (Ansari *et al.,* 2012). The fate of the lagging univalent *T. ambiguum* chromosome could frequently be seen in the form of micronuclei (Figure 4.1.4g) as they did not become incorporated into the tetrads. Similar findings for the fate of Is during meiosis were reported by Gernand *et al*. (2006), Tu *et al*. (2009), and Ishii *et al*. (2010). This was consistent with the apparent rapid elimination of *T. ambiguum* chromosome in various BAR hybrids during their breeding history (Tables 4.1.4, 4.3.2, 4.3.4, 4.4.4). Lagging chromosomes at anaphase-I, sometimes associated with anaphase bridges, is a common phenomenon in genetically complex hybrids with many chromosome configurations deviating from the normal (Gernand *et al*., 2006; Tu *et al*., 2009; Ishii *et al*., 2010). Some level of *T. ambiguum* chromosome pairing with O or R chromosomes in the hybrid situation is important, as otherwise they would probably be rapidly eliminated in the early generations.

5.2.3 Molecular cytogenetic analysis of BAR10-126

This plant was very important for potential introgression, having gone through three meiotic cycles with chances of inter-genomic chromosomal exchange. So BAR10-126 was subjected to GISH/FISH studies to see if there has been any genomic mixing. GISH highlighted only one A chromosome with a 5S signal, instead of the expected two or three, which is consistent with the rapid elimination of A chromosomes (Figure 4.1.3a, b, c). The successful differentiation of *T. ambiguum* chromosome by GISH in BAR10-126 shows the high level of sequence divergence in this species relative to *T. repens* and *T. occidentale* and is consistent with the findings of Ellison *et al.* (2006) showing the relatively distant phylogenetic relationship based on DNA sequences. BAR10-126 consistently showed green signals on both of the chromatids of four non-*T. ambiguum* chromosomes, strongly suggestive of introgression. The two distally located green signals are relatively larger as compared to the other two interstitially located signals (Figure 4.1.3b). They have very defined boundaries regardless of their locations. One of the chromosomes carrying interstitial introgression from A is probably from *T. occidentale* based on its smaller 5S signal but whether it is just an addition or probably substituting for A or R is not certain. The two chromosomes with

terminal segments of introgression could show evidence of an early recombination event that has become "homozygous" due to selfing or non-disjunction at some point, although the breeding history of the plant does not support the idea that they might be homologous chromosomes. Moreover, the chromosome arms carrying terminal introgressions appear to have different lengths. The idea that these green signals indicated the possibility of the presence of previously unseen segments of 18S rDNA, can be refuted by the absence or low intensity of 18S signals using this probe on the NOR bearing chromosomes. This suggested that this probe was not efficiently detecting 18s rDNA and so it is unlikely that these other signals were 18S. This unlikely possibility can be tested by further sequential GISH and, although this was attempted, the hybridization was not very successful, but the alternative explanation remains to be eliminated. The apparent amount of this introgression was very impressive and shows the potential of getting potentially useful recombination by using this strategy.

The strategy can be further extended by creating RRAO hybrids using different parental genotypes with odd genomes from the A and O parental species. Larger sample of plants should be subjected to molecular screening and, in cases of consistent evidence of introgression, then the advanced backcross derivatives should be further backcrossed with white clover. After getting back to exactly 4x ploidy level, the plants with introgression should be studied morphologically in a careful way. If found having morphological evidence of A-derived traits of interest, they should be selfed/inter-crossed to make the introgressed alien DNA homozygous. This would be big breakthrough for white clover improvement. In addition, a monosomic A addition line, in addition to its usefulness for introgression, could be used for mapping genes and markers on particular alien chromosomes introduced from the donor species (Tu *et al*., 2009). The chromosome addition could be stabilized by making it homologous through selfing and its cytological behaviour could be studied. If it is stably inherited and if it carries genes for traits of agronomic interest then it can be promoted as a new genotype. However, making a cultivar out of such plant with cross pollination is a challenge for breeders because of its reproductive instability.

5.2.4 Morphology of RRR(A_4O_4) (BAR09 hybrids) and RRR($R_4A_2O_2$) (BAR10 hybrids)

The BAR09 hybrids received a partial genome from *T. ambiguum*. All the plants had the expected genomic composition of RRR(A_4O_4) except BAR09-133 which was derived by

selfing from the primary RRAO hybrid. (RO x BL)-19, BAR09-120 and BAR09-133 showed strong *T. ambiguum*-like traits (Table 4.1.6.1, 4.1.6.2) and were not like white clover. (RO x BL)-19 and BAR09-133 had received comparatively more *T. ambiguum*-derived chromosomes (one full sub-genome) and so their morphological similarity to *T. ambiguum* was understandable and possibly due the dominance of some of the *T. ambiguum* specific traits. Similarly, BAR09-120, which was expected to have four or more *T. ambiguum* chromosomes, had a strong likeness to *T. ambiguum* in growth habit, flowering pattern and root morphology. Generally the phenotype would be expected to be more like *T. ambiguum* in those plants having larger numbers of *T. ambiguum* genomes. But these plants had only one full or partial A genome, and so the resemblance of some hybrids more to *T. ambiguum* than to white clover might be due to dominance rather than gene dosage effects. All the plants had the combination of stoloniferous traits from white clover and heavier root systems from *T. ambiguum* which had been the aim of the inter-specific hybridisation between white clover and *T. ambiguum*. Inheritance of characters from both these parents in interspecific hybrids has been reported by others (Scewer and Cleveland, 1972; Marshall *et al*., 1995, 2003; Williams *et al*., 2011). Rhizome development was not observed in any of the present hybrids and the reason may be they did not stay long enough in the field, as rhizome development requires 18 months to appear (Abberton *et al*., 2003). In birdsfoot trefoil, Beuselinck *et al*. (2005) reported the effects of environment on the expression of the rhizomatous trait, in addition to plant age. Abberton *et al*. (1998) and Meredith *et al.* (1995) respectively reported the presence of rhizomes up to BC_2 and BC_3 in hybrids between *T. ambiguum* and *T.repens* using the latter as recurrent parent. On the other hand, Isobe *et al*. (2002) reported that rhizome development did not go beyond BC_1 generation in hybrids between red clover and *T. medium* using red clover as recurrent parent. Although, rhizome development was not observed, the higher root weight ratio to the total biomass might make these advanced BC hybrids more drought tolerant (Marshall *et al*., 2001) and persistent in the field (Isobe *et al*., 2002). According to Isobe *et al*. (2002), although rhizome development was not observed beyond BC_1 in hybrids between red clover and *T. medium,* the generations beyond BC1 had relatively heavier root systems and proved to be more persistent than red clover.

In the BC_2F_1 plants (BAR10, Table 4.1.4), the morphology was, as expected, more like white clover (Table 4.1.7.1, 4.1.7.2). This was consistent with the concept that repeated crossing back of these hybrids with white clover would decrease the numbers of *T. ambiguum*

chromosomes and so the intensity of expression of *T. ambiguum* associated traits as reported by Williams and Hussain (2008) and Meredith *et al*. (1995). But on the other hand, the lower level of anchorage as compared to white clover and reduced apical growth provide evidence for the presence of *T. ambiguum* traits. BAR10-126, which had an extra A chromosome and evidence of introgression had some phenotypic evidence of *T. ambiguum* characters in shape of lower than white clover anchorage, highly reduced apical growth and higher root weight % of the total biomass as compared to white clover controls.

5.3 Strategy based on 5x BAR hybrids having 3 As and 2 Os (AAAOO)

The 5x hybrids (AAAOO), used in this second strategy based on using *T. occidentale* as a genetic bridge, were obtained from the cross of 3x hybrid 434-1 (AAO) as female parent with 4x hybrids BL and BN (AAOO) as pollen parents.

The 3x 434-1 contributed 2n gametes when crossed with BL & BN which contributed haploid gametes (AO). Using EBNs of A=0 and O=1, only 2n gametes from 434-1 would be functional. The resulting 5x BAR hybrids could have recombinant chromosomes because of the chances of homoeologous chromosome pairing in hybrid 434-1 (AAO). In such situations the chromosomes from the isolated O genome could pair with their homoeologous counterpart chromosomes from the other species despite a higher level of sequence divergence (Zhang *et al*., 2010). On the other hand, if AA were homologous genomes then, as reported by Hussain and Williams (1997a), preferential pairing between homologues might discourage A/O pairing. The occurrence of fewer than 8 IIs and low average frequencies of IIIs and IVs showed that the A genomes in the 3x AAO hybrid were not homologous. The occurrence of IIIs and IVs provided evidence of inter-specific chromosome pairing with high possibility of illegitimate synapsis especially in the IVs (Dr. Helal Ansari, unpublished work). In the 4x hybrids (AAOO), meiosis might be very regular with amphidiploid chromosome pairing making mostly intra-species IIs and giving minimal recombination between *T. ambiguum* and *T. occidentale* genomes. However, the AAOO hybrids were not amphidiploids in their chromosome pairing (Dr Wajid Hussain, unpublished data) which indicated that the two A genomes might not be homologous as mentioned before (Dr. Nick Ellison, unpublished data).

The 5x plants (with genomic composition of AAAOO) used as starting material in this strategy were highly male fertile as was evident from their pollen stainability given in Table 4.2.1. The reasonably high level of fertility was very encouraging for using these hybrids in

further breeding. Only BAR-112 had significantly lower stainable pollen percentage (21%). High pollen stainability in these plants may be the evidence of a high frequency of bivalent pairing. Keeping the genomic composition of these plants in mind, these IIs might be mostly AA and OO with some IIIs (AAA, AOO or AAO) and Is (A).

Hybrid 434-1 (AAO, 3x) was allowed to be open pollinated and the progeny were apparently self seeds with genomic composition of AAAAOO showing both the gametes had 2n chromosomes. After crossing this progeny with elite *T. repens*, KOPCRU-1 (RRRR), plants were obtained with a predicted genomic composition of something like AAORR (~5x). The production of 2n gametes is usually very low in plant species (Ramsey, 2007) but in hybrids the production rate of 2n gametes can be comparatively higher (Ramsey and Schemske, 2002). BAR-115 (AAORR) was male sterile but it was genetically more complex, having genomes from three species with none of the chromosomes having homologues and different from the rest of BAR hybrids in its breeding history (Table 4.2.1). Being genetically complex means cytologically complex, with many meiotic anomalies, probably causing sterility (Obute *et al*. 2006). Although male sterile, BAR-115 produced some seed after having been pollinated by coloured white clover, showing that it had some female fertility. The level of meiotic anomalies in megasporogenesis is probably lower as compared to the abnormalities in the male gamete formation (microsporogenesis). It has been reported that another *Trifolium* hybrid that was apparently male sterile produced seed after cross pollination (Meredith *et al*., 1995). The reasons may be lesser susceptibility of megasporogenesis to the environmental conditions as compared to the microsporogenesis.

The higher level of pollen fertility in the 5x (AAAOO) hybrids, associated with the likelihood that inter-genomic recombination might have produced recombinant chromosomes with centromeres from *T. occidentale* and arms with introgression from *T. ambiguum* or vice versa (O^A or A^O), offers considerable promise for further breeding. Accordingly, these plants were repeatedly crossed back with white clover having dominant leaf colour alleles, and although the success of the cross, AAAOO x RRRR was unlikely under the EBN formula, some progeny were obtained. The EBN system is not perfect and a low level of success can occur in rare cases even if the parents have different EBNs (Parrot and Smith 1986). Most of the plants were self compatible possibly because one of the parents (BL or BN) was self compatible (SC).

The (AAAOO, 5x) hybrids were totally green so the selection in the first progeny (BAR09) after backcrossing them with coloured *T. repens* was based on the presence/absence of leaf colour alleles from white clover, DNA contents, pollen fertility, somatic chromosome counts and morphology. Interestingly, the pollen fertilities in the BAR09 progeny were very low as compared to the parental hybrids (Table 4.2.2), especially in hybrids BAR09-97, BAR09-98, BAR09-100 and BAR09-106 resulting from the crosses of 5x AAAOO with white clover. Low levels of pollen stainability in the progeny with expected genomic composition of RRAO(A_4) were understandable because only a few *T. ambiguum* chromosomes had homologues. R and R were homoeologous because they came from the separate sub-genomes of white clover. The number of Is and multivalents in BAR09-97, BAR09-98, BAR09-100 and BAR09-106 with expected genomic formula $R^PR^OAO(A)$ might be higher than in the original BAR hybrids with genomic composition of AAAOO (although we did characterise these 5x AAAOO hybrids cytologically). So due to the more regular meiosis in AAAOO hybrids, their pollen stainability was higher as compared to the BAR09-97, 98, 100 & 106 which were genetically more complex with very few chromosomes having homologues. The higher male fertility observed in the self progenies, BAR09-108 and 110, was consistent with the finding that selfing tends to increase fertility by elimination of non pairing chromosomes (Williams and Hussain 2008). BAR09-114 (RRRA(O_4) had more white clover chromosomes than the other hybrids. In addition to pairing between R and R, pairing could also be expected between R and O (because *T. occidentale* was a contributor of one of the sub genomes of white clover) or between A and O which have been reported to pair perfectly in 2x hybrids (AO) between 2x *T. ambiguum* x 2x *T. occidentale* (Williams *et al*. 2011). However, the source of the A genome in AAO was different (4x *T. ambiguum*) than the A in the 2x hybrids (2x *T. ambiguum*) studied by Williams *et al*. (2011).

Somatic chromosome numbers in these hybrids, except BAR09-114, had dropped further below the expected level, indicating that anomalies during meiosis in the parental BAR hybrids had led to chromosome elimination (Table 4.2.2). Chromosome elimination as a result of meiotic disturbances has been reported by Ferguson *et al.* (1990), Gernand *et al*. (2006) and Ishii *et al*. (2010). Most of the original hybrids were self compatible but, after backcrossing with white clover, they lost their self compatibility (compare Tables 4.2.1 and 4.2.2). BAR09-108 and BAR09-110 retained self compatibility because they were self progeny of the original BAR hybrids, and selfing probably did not markedly disturb their genomic composition. The loss of self compatibility in the other BAR09 hybrids with

genomic composition of RRAO(A_4) might have been due to the introduction of functional *S* alleles from white clover.

The BAR10 progeny of AAAOO hybrids, obtained after backcrossing to white clover a second time were summarised in Table 4.2.4. Five plants were selected for study on the basis of morphology, DNA contents, pollen stainability and somatic chromosome number. The DNA content differences among the three genomes (A, R and O) made flow cytometry based hybrid testing very easy as reported by Marasek *et al*. (2006). The genomic composition of these plants was expected to be RRR(A_6O_4). The male fertility in this generation was higher than the previous one, possibly because there was more regular meiosis in these plants due to higher numbers of white clover-derived chromosomes (compare Tables 4.2.3 and 4.2.5). BAR10-111 had 2n = 35 and is a case of chromosome gain as against the trend of chromosome loss observed earlier. It resulted from the cross, BAR09-98 (2n=34) x white clover (RRAO(A_4)-2 x RRRR). BAR10-111 seems to have resulted from an aneuploid gamete (egg) from BAR09-98 with 19 chromosomes (2x+3). Plants belonging to the BAR10 generation have had two chances of recombination between A and O. Some of the plants were further analysed using conventional and molecular cytogenetic tools to see if there had been any chromosomal exchange leading to recombinant chromosomes (A^O, O^A) or any chromosome addition/ substitution.

The reasonable level of pollen fertility in these hybrids was possible due to both intra-specific (R^PR^O) as well as inter-specific homoeologous (AO) chromosomes pairing, leading to a reasonably high frequency of IIs (Table 4.2.5). The high level of male fertility (almost equal to white clover) in BAR10-119 with genomic composition of RRAO(A_4)-3 suggested that chromosome pairing between white clover gametic genomes, allosyndetic chromosome pairing between A and O and balanced disjunction of chromosomes had occurred. The less than expected chromosome number in this hybrid showed that probably many of A-derived chromosomes, having no pairing partners, were eliminated during gamete formation in the BAR parent (Table 4.2.4). Flow cytometry results showed that all of these plants had more than a 4x ploidy level (Table 4.2.4). Although this was confirmed by somatic chromosome counts, the 2n chromosome numbers in these hybrids did not match with flow cytometry estimates (compare FC results of BAR10-111, 118, 119 and 120, Table 4.2.4). The reason might be the presence of variable numbers of chromosomes from all three species, especially *T. ambiguum*. As mentioned earlier, these species vary in their nuclear DNA contents

(Bennett and Leitch, 1997; Greilhuber, 1998; Bennett *et al*., 2000; Bennetzen *et al*., 2005) in the order given as *T.ambiguum >T. occidentale > T. repens*.

5.3.1 Meiosis in BAR09 hybrids derived from cross, AAAOO (5x) x RRRR

Four plants were selected from the self and cross progeny of 5x (AAAOO) hybrids for studying meiotic behaviour of chromosome derived from different species (Table 4.2.3). BAR09-97, BAR09-98 and BAR09-106 were the progeny of crosses, (5x AAAOO x RRRR) with the same expected genomic composition (RRAO(A_4) 2n=36) although the chromosome number in BAR09-97 was three less than the expected chromosome number and BAR09-98 and 106 had two chromosomes fewer than expected. Due to the similar expected genetic compositions of these plants, only one hybrid, BAR09-98 (RRAO(A_4)-2, 2n=34) will be discussed from the point of view of chromosome pairing behaviour. BAR09-110, being the self progeny of BAR-110 (AAAOO, 5x) with 2n=5x-4=36 will be discussed with special focus on the pairing between the A and O genomes.

5.3.1.1 Meiosis in BAR09-98

If we assume perfect pairing between the gametic chromosomes (R^P R^O) of white clover, then there should have been at least 8 IIs in BAR09-98 (R^PR^OAO(A_4)-2. However, the mean number was only 6.21, which could mean that this pairing may have been predominant, as revealed by Chen and Gibson (1970), but was not perfect. Although BAR09-98 was expected to have eight or more *T. ambiguum*-derived chromosomes, the average number of Is (4.2) was less, suggesting that A chromosomes were taking part in chromosome pairing with O or R^O or R^P. The upper range of bivalent frequencies (up to 11) showed that, in addition to possibly a very few homologous bivalents (AA) and autosyndetic R bivalents (R^PR^O), allosyndetic pairing was also taking place between A and O or R^O and O or A and R. IIIs could have been AAO or RAO or RRO, while IVs definitely must have involved all the genomes (AAOR or RROA). In Figure 4.2.1b, which shows the metaphase-I stage in BAR09-98, the heteromorphic IVs (arrows) indicated their homoeologous nature. Due to the high possibility of inter-specific chromosomes pairing, this plant was used in further breeding and cytological analysis (see BAR10-111 below)

5.3.1.2 Meiosis in BAR09-110

In BAR09-110 (AAAOO-4), the pairing within species was not perfect because if perfect bivalent formation between AA and OO is assumed, then there should be, on average, 16 IIs.

The data in Table 4.2.3 show only 7.5 IIs, but ranging up to 16. This suggests perhaps that the OO pairing was consistent, but AA pairing was inconsistent, but sometimes fully pairing and giving up to 16, and explaining why this hybrid had a higher male fertility level than BAR09-98 (RRAO(A_4)-2. Trivalents in this hybrid might have indicated pairing among the three A genomes, but IVs were definitely from inter-specific chromosome pairing.

5.3.1.3 Meiosis in BAR10-111

As previously stated, BAR10-111 (2n=35) was the progeny of BAR09-98 (2x=34) in which chromosome pairing was discussed earlier. The expected genomic composition of BAR10-111 was $R^PR^OR^{P/O}(A_6O_4)$-1, i.e. three genomes from white clover and one partial genome from each of *T. ambiguum* and *T. occidentale*. But the somatic chromosome count was 35 indicating that it received a gamete from BAR09-98 with n=19 instead of the expected n=17. In this hybrid, out of the three white clover genomes, one was of *T. occidentale* origin, one of *T. pallescens* origin and the third could be either a mixture of the two or totally one or the other due to random assortment of R^O and R^P chromosomes in BAR09-98 (Williams *et al*., 2012). It is to be noted that in the third genome, although it could be mixture, all the chromosomes should belong to different linkage groups. The average number of Is, IIs, IIIs and IVs were respectively 6.7, 10, 1.7 and 0.8 (Table 4.2.5). We know that BAR10-111 was expected to have six *T. ambiguum* chromosomes, so these Is might represent these. However, the range of Is (1-13) does not support this, and rather indicated their participation in pairing. Based on chromosome pairing within the three white clover genomes (homologous or homoeologous or both types of pairing), BAR10-111could have a maximum eight IIs, but the average number was higher (10 IIs). Thus there was probably also some inter-specific homoeologous bivalent formation (R^OO, R^PO, RA or AO). If we assume intra-specific homoeologous *T. repens* chromosome pairing was occurring, making some of the bivalents like R^PR^O, then the question arises as to whether *T.occidentale*-derived *T. repens* chromosomes (R^O) were genetically closer to *T. pallescens*-derived *T. repens* chromosomes (R^P) or to *T.occidentale*-derived chromosomes (O). It is difficult to establish at this point. The likelihood of bivalent pairing between A and O was not very high because most would have belonged to different linkage groups. The multivalents (IIIs and IVs) suggest that they are made up of homoeologous chromosomes coming from all the three different species. The large number of Is reflects the level of meiotic irregularities in this hybrid (Figure 4.2.2b-d) which was probably the reason for the very low pollen fertility in this hybrid. There is also a possibility that some of the IIs involved pairing of recombinant chromosome (having a

centromere from *T. occidentale* and arms introgressed from *T. ambiguum* (O^A) with its homoeologuos counterpart from white clover. Trivalents (IIIs) might have been R^OR^PO but IVs could have been R^OR^POA. So far as Is were concerned in this hybrid, they might be composed of both *T. ambiguum* and *T. occidentale* derived chromosomes if R^O was pairing with R^P instead of pairing with O chromosomes. In such situations IIs, IIIs and IVs have been observed where, putting homologous and non-homoeologous pairing to one side, even chromosomes belonging to different linkage groups have paired with each other (illegitimate pairing) (Dr Helal Ansari, verbal communication). Further analyses are not currently possible as FISH markers for each and every chromosome are not yet available to identify them in meiotic chromosomes preparations.

5.3.1.4 Meiosis in BAR10-124

BAR10-124 (RRR($R_4A_4O_2$)-1 was the advanced progeny of RRAAO and considering this hybrid as F_1, then BAR10-124 is BC_2F_1. Although having a different breeding history, BAR10-124 is similar to BAR10-93 (discussed later) in its genomic composition and so has a similar meiotic chromosome pairing pattern. The frequency of Is (Table 4.2.5) in this hybrid was lower than the expected number of *T. ambiguum* chromosomes, suggesting their involvement in chromosome pairing. The other possibility is that some of the expected number of *T. ambiguum* chromosomes had been eliminated. Moreover, IIs (12.9) represents both intra- and inter-genomic pairing within white clover genomes. IIIs and IVs can be RRR or RRO and RROA respectively. The presence of Vs, although very low in frequency, represents the involvement of all genomes in the pairing process e.g. RRROA. Multivalents, especially IVs and Vs indicates the possibility of illegitimate pairing as well. Due to comparatively higher level of IIs as compared to BAR10-111, BAR10-124 has got higher pollen fertility which shows direct relationship of meiotic regularity with fertility as reported by Obute *et al*. (2006).

5.3.2 Genomic composition analysis of BAR10-111

Based on the breeding history and cytological evidence of interspecific chromosome pairing, BAR10-111 was selected for GISH analysis which painted six chromosomes from *T. ambiguum* (Figure 4.2.3b) which was consistent with expectations. One of these chromosomes had a 5S signal located close to the centromere on the longer arm. One A chromosome showed a NOR induced secondary constriction that was relatively more condensed than the highly stretched NORs on the corresponding chromosomes coming either

from *T. repens* or *T. occidentale*. This was consistent with the transcriptional silencing of the NOR from *T. ambiguum*. That raises a very important question as to whether such silencing applies to all the A-derived genes. It had become clear in the morphological characterization of these hybrids (discussed later) that this was not case. But again it is not clear whether the *T. ambiguum*-associated genes which are expressing are on the A chromosomes or they have been introgressed into white clover chromatin. Moreover, as was shown in Giemsa-stained mitotic chromosome preparations (Figures 4.2.1a, 4.2.2a), the *T. ambiguum*-derived chromosomes are identifiable by their comparatively bigger satellites which are lying at a distance from the main chromosomal bodies, indicating that the NORs are also highly stretched and are transcriptionally active. So perhaps these results are explained by the observation of different metaphase stages and differential/asynchronous cycles of condensations of the chromosomes belonging to the two different species genomes rather than the phenomenon of nucleolar dominance where the NOR in a hybrid situation coming from one parental species is silenced while the other stays transcriptionally active (Pikaard, 1999). A similar situation was reported by Williams *et al*. (2011) in a AOO hybrid where the NOR on the A chromosome was comparatively less de-condensed. The condensation and de-condensation in the rDNA is reportedly brought about by the epigenetic changes in DNA and histones and is related to the transcriptional activity (Appels *et al*., 1986; Suja *et al*., 1997; Volkov *et al*., 2007). Moreover such structural changes in chromosomes due to condensation of NOR DNA have been reported to be very important for diploidization of meiosis in polyploid hybrids (Liu *et al*., 1998).

Some very small green signals were observed on non-*T. ambiguum* chromosomes but mostly they were not on both the chromatids. However, some appeared to be on both the chromatids and might possibly be introgression. Alternatively, they might represent cross hybridization due to repeat sequences. Cross hybridization especially in centromeric/pericentromeric regions due to the accumulation of repeat sequences (Helal *et al*., 2008; Wang *et al*., 2010; Benavente *et al.,* 2008) and background noise due to cytoplasmic impurities restricting the access of the probe to the DNA (Jeridi *et al*., 2001), are the main problems associated with GISH and FISH studies. The meiotic chromosome pairing analysis in the BAR parent of this plant clearly indicated the involvement of *T. ambiguum* chromosomes in multivalent formations, which was a pre-requisite for re-combination. The lack of introgression signals in this plant might be due to the fact that the introgressed segments were very small and beyond

the scope of GISH (Tu *et al*., 2009; Kosmala *et al*., 2007; Humphreys and Pašakinskiene, 1996) or that pairing does not always lead to exchange of chromatin.

5.3.3 Morphology of hybrids derived from cross, AAAOO x RRRR – RRAO(A_4)

The BAR09 hybrids, RRAO(A_4) were aneuploid with higher numbers of *T. ambiguum* chromosomes than previously described hybrids. The morphology of these hybrids for some characters was intermediate between *T. repens* and *T. ambiguum* while, in others, it was more *T. ambiguum*-like especially in the determinate apical growth (Yamada and Fukuoka, 1986; Williams and Verry, 1981). All the hybrids appeared to be stoloniferous but the amount of nodal rooting varied considerably and was approximately inversely proportional to the number of *T. ambiguum*-derived chromosomes in some hybrids (Table 4.2.6.1). BAR09-106, although having the same pedigree as BAR09-97 and BAR09-98, behaved differently, showing transgressive expression of some characters such as significantly higher numbers of longer stolons. Morphologically this plant looked like white clover but the low level of stem anchorage, reduced apical growth and apparent terminal flowering made it look intermediate although more similar to white clover than *T. ambiguum*. The differentiation on the basis of leaflet shape was not very clear. The 4x version of *T. ambiguum* has sometimes leaflets which are almost round, as in white clover (Kannenberg and Elliott, 1962). But the 4x *T. ambiguum* used in our study has conspicuously elongated leaflets. Very low seed set reflected the highly disturbed meiosis leading to very low male and female fertility. The most important character making these hybrids look like *T. ambiguum* was their root morphology. The root system, although showing no signs of rhizomes, was longer and thicker than that of white clover and so the ratio of root dry weight to the total biological mass was much higher, showing the influence of *T. ambiguum* genomes on the root morphology (Table 4.2.6.2).

5.3.4 Morphology of hybrids derived from cross, RRAO(A_4) x RRRR – RRR(A_6O_2)

The morphology of the BAR10 generation (RRR(A_6O_4)) was less similar to *T. ambiguum* and more similar to white clover (Tables 4.2.7.1, 4.2.7.2). The number of *T. ambiguum* chromosomes in this generation hybrids was less (up to six) as compared to the previous one (up to 12). Thus the intensity of *T. ambiguum* related characters faded in this generation due to the gene dosage effects. These plants were like white clover in all characters except for the highly reduced apical growth, reduced stolon anchorage level, low seed set and comparatively higher root weight ratio of the total biomass. BAR10-111 was one of this

generation and can be considered as BC_1F_1 (assuming that its hybrid parent, BAR09-98 (RRAO(A_4)-2 was a tri-specific F_1). The number of *T. ambiguum* chromosomes in this plant was confirmed by GISH to be six, as expected. The stoloniferous stem type with nodal rooting from the first 2-3 nodes coupled with highly reduced apical growth, low seed set and higher than white clover root to total biological mass showed the influences of both white clover and *T. ambiguum* genes on the morphology of this plant. A low level of nodal rooting up to the third node was reported by Ferguson *et al*. (1990) in hybrids between *T.repens* and *T. nigrescens* and, because of the non stoloniferous nature of *T. nigrescens*, these hybrids were comparable to ours. BAR10-111could be seen as necessary intermediate (not an end point of selection) and after its repeated backcrossing to white clover, we might be able to select plants in its progeny which are similar to white clover, except for introgressed root system and drought resistance from *T. ambiguum*.

5.4 Strategy using hybrid "33 OP-1, AAAORR'" as starting material

AAAO (4x), designated as hybrid "33" was the progeny of 6x *T. ambiguum* ($A^HA^HA^HA^HA^HA^H$) crossed with 2x *T. occidentale* (OO). Under the EBN hypothesis this cross should not be successful due to parents having different EBNs and so embryo rescue was used to get progeny from this cross. Hybrid 33 was allowed to be open pollinated. The resulting progeny plant 33 OP-1 was a little over 6x (2n=51) with an unknown pollen parent. This mystery was solved by fluorescent *in situ* hybridization (Dr Helal Ansari, unpublished data) which revealed that *T. repens* (RRRR) had contributed a normal haploid male gamete (RR) while hybrid 33 had contributed a female gamete with near-2n chromosome constitution. Under the EBN hypothesis the cross, AAAO (EBN=1) x RRRR (EBN=4) should not be successful if they both contributed normal haploid gametes or even if AAAO contributed a 2n gamete. If AAAO contribute a 2n gamete its EBN=1 because the A genome has been assigned EBN=0 and the O genome has EBN=1. On the other hand, a haploid (n) gamete coming from white clover would have EBN=2 because each R genome has EBN=1. Perhaps the three extra chromosomes in 33 OP-1 have come from *T. occidentale* and carry EBN loci, they have changed the EBN genetics of the 33 OP-1 2n gamete, leading to seed production in this cross. Alternatively, as the EBN system is leaky and due to genomic balance disturbances in hybrid situations (Warren Williams, verbal comm.), some seed is produced on a small scale as possibly happened in this case.

This breeding strategy using 33 OP 1 (AAAORR, 6x) is potentially very important from two points of view. Firstly, the *T. ambiguum* source in these hybrids was hexaploid which is considered to be agronomically superior to the 2x and 4x *T. ambiguum* due to its growth vigour (Taylor, 2008; Kannenberg and Elliott, 1962). Secondly the advanced backcross and self derivatives of 33 OP-1 (AAAORR) after repeated crossing with white clover and selfing have gone through four or five meiotic cycles with odd genomes from the three species. So these hybrids not only have superior *T. ambiguum* genomes but they also have had very high chances of homoeologous chromosome pairing which might have led to the introgression of *T. ambiguum* genomes into white clover. The BAR hybrids which were used as starting materials for the current project were either the progeny of 33 OP-1 having been crossed back with white clover once (BAR-59 to BAR64) or twice (BAR-66 to BAR-75). Their expected genomic compositions were approximately RRRA(A_4O_4) (2n=40) and RRR($R_4A_6O_2$) (2n=36) (Tables 4.3.1.1, 4.3.1.2).

Pollen fertility in these hybrids was, with a few exceptions, mostly below 50% which was consistent with their hybrid nature, which was further confirmed by the flow cytometry results. These hybrids were genetically complex and the low level of pollen fertility resulted from a high frequency of anomalies at meiosis. BAR-74 had unexpectedly high male fertility (100% stainable pollen) and the ploidy level was close to 4 so this plant was suspected to be white clover and was excluded from further breeding. This group of hybrids, with the exception of BAR75, proved to be cross fertile with white clover and produced reasonable quantities of seed. Seed set on selfing was also observed in all the hybrids except BAR-75. Cross and self incompatibility of BAR-75 showed that it was female sterile (Tables 4.3.1.2). The reasonably high level of pollen fertility in these hybrids along with their cross compatibility with *T. repens* was very important for genome recombination.

5.4.1 First self and backcross progeny of hybrids, RRRA(A_4O_4) or RRR($R_4A_6O_2$)

Hybrids with approximate genomic compositions of RRRA(A_4O_4) and RRR($R_4A_6O_2$) were used as starting material. The first progenies of these hybrids after sefling and backcrossing with white clover were designated as BAR09 hybrids because it was done in 2009. Because the parental BAR hybrids already had co-dominantly expressed leaf colour alleles, the selection of hybrids in this first self and cross progeny of these plants was based on the DNA contents and phenotypes. The selected plants are listed in Table 4.3.2. The pollen fertilities in BAR09-54, BAR09-56, BAR09-57 and BAR09-67 were lower than in the parental BAR

hybrids (BAR-59, BAR-67) possibly because these BAR09 hybrids were derived from genetically complex gametes with high numbers of A and O derived chromosomes, leading to higher meiotic disturbances and consequently lower fertilities. On the other hand, BAR09-63, BAR09-65 and BAR09-75 had higher male fertilities than the parental BAR hybrids, which is consistent with their being a later generation, and so having comparatively more R-derived chromosomes (compare Tables 4.3.1.1, 4.3.1.2, with Table 4.3.2). BAR09-63, RRR($R_6A_3O_1$)-1 should theoretically have had three *T. ambiguum* chromosomes but FISH has shown it had only one, suggesting rapid elimination of *T. ambiguum* chromosomes. This could be due to *T. ambiguum* chromosomes mostly behaving as Is and leading to their elimination, as reported by Tu *et al*. (2009) and Ahuja *et al*. (2003) in different hybrids. Theoretically, BAR09-63 had at least 30 chromosomes from *T. repens* and so the higher pollen fertility is understandably due to a more regular meiosis, leading to higher number of seeds per head which is in line with findings of Obute *et al*. (2006). With some exceptions, the hybrids with lower male fertilities produced fewer seeds (Table 4.3.2).

5.4.2 Second self and backcross progeny of hybrids, RRRA(A_4O_4) or RRR($R_4A_6O_2$)

The second progeny (BAR10) of RRRA(A_4O_4) or RRR($R_4A_6O_2$) after having been selfed, inter-crossed or backcrossed with white clover a second time, are summarised in Table 4.3.4. After repeated backcrossing to white clover, the ploidy level in most of the plants had come down to almost 4x, probably for two reasons: chromosome elimination and the lower ploidy level of white clover used as recurrent parent. Many of these BAR10 plants had approximately 30 chromosomes from *T. repens*. This high number of *T. repens* chromosomes was reflected by higher male fertilities, similar to, or approaching that in white clover. BAR10-72 and 76 were exceptions with higher numbers of *T. ambiguum* chromosomes, because their pedigrees included selfing and less crossing with white clover. BAR10-72 was the progeny of the original BAR hybrid, BAR-59 (RRRA(A_4O_4), 5x) after one selfing and one backcrossing with white clover while BAR10-76 was the progeny of the same plant, BAR-59 (5x), after two selfings.

If we consider AAAORR (33 OP-1) as a tri-specific F_1, then the breeding generations of these BAR10 hybrids were BC_3F_1 (BAR10-80), BC_2F_3 (BAR10-93) or BC_4F_1 (BAR10-81). In other words they had been through 4-6 meiotic cycles with odd genomes/chromosomes from the three species (A, R and O). Three plants were selected from this strategy for GISH

and FISH experiments. These were BAR09-63 (BC_3F_1), BAR10-81 (BC_4F_1) and BAR10-93 (BC_2F_3) with expected genomic compositions of (RRR($R_6A_3O_1$)-1, (RRR($R_7A_{1.5}O_{0.5}$) and (RRR($R_4A_6O_2$)-2 respectively. BAR10-93 was included in the GISH screening because it had two selfings in its breeding history and selfing was expected to considerably increase the chances of recombination.

5.4.3 Chromosome pairing in BAR09-62, BAR09-63 & BAR09-65

If we consider 33-OP-1 (AAAORR) as tri-specific F_1, then BAR09-62, BAR09-63 & BAR09-65 are respectively BC_2F_1, BC_3F_1 and BC_2F_2 in their breeding status and they each had, respectively, four, five and five chances of inter-genomic mixing. The detailed expected genomic formulae were $R^PR^OR^{P/O}(R_4{}^{P/O}A_6/O_2)$ in BAR09-62 and BAR09-65 and $R^PR^OR^{P/O}(R_6{}^{P/O}A_3/O_1)$ in BAR09-63 which will make their meiotic pairing analysis a bit easier. They were expected to have six, six and three chromosomes from *T. ambiguum* in the order given earlier. BAR09-63 turned out to have just one *T. ambiguum* chromosome instead of three (GISH results, to be discussed later), again indicating rapid elimination of A-derived chromosomes during meiosis in the BAR hybrids. All three hybrids had the full range of metaphase-I chromosome formations (Is-IVs, Figure 4.3.1b, c, e). This showed the involvement of chromosomes from different species in the meiotic process, leading to deviation from the diploid-like meiosis of white clover. Although it was difficult to establish their identities during metaphase-I, due to the highly condensed nature of the chromosomes, the heteromorphic shapes of IIIs and IVs in BAR09-62, BAR09-63 and BAR09-65 suggested that they contained chromosomes from all the species (Figure 4.3.1b, c, e). Meiotic anomalies like chromosome stickiness and lagging chromosome led to lower pollen fertilities in these hybrids (Figures 4.3.1d, f & Table 4.3.3). Interestingly, the number of IIs was approximately proportional to pollen fertility (Table 4.3.3), which is consistent with the results of Obute *et al*. (2006) who reported a direct positive relationship between meiotic regularity and pollen fertility.

From Table 4.3.3, it is also evident that the number of Is was proportional to the number of *T. ambiguum* chromosomes in these three BAR hybrids. However, the ranges of IIs showed the liklihood of *T. ambiguum*-derived chromosomes involvement in meiotic pairing. On the other hand, the average frequencies of IIs, and the presence of multivalents, including both IIIs and IVs indicates the possibility of interspecies chromosome pairing. If these three hybrids had the expected numbers of 28, 30 and 28 chromosomes respectively from *T. repens* then,

assuming both intra-specific homologous and homoeologous chromosome pairing, they possibly could have up to 12, 14 and 12 IIs, respectively. But the situation on the ground was different, and they had lower average frequencies of IIs (Table 4.3.3). This showed that some of the *T. repens* derived chromosomes were taking part in multivalents either autosyndetically or allosyndetically. In addition, some of the R chromosomes after homologous and some level of intra-specific homoeologous pairing would not have R pairing partners because the left over white clover chromosomes would belong to different linkage groups. We can deduce from the expected genomic composition of these plants that IIIs probably involved pairing between a bivalent of *T. repens*-derived homoeologous chromosomes and their homoeologous counterpart from *T. occidentale* and was supported by the average frequencies of IIIs in these hybrids (Table 4.3.3). It is difficult to establish the nature of IVs. They might contain two homologous white clover-derived bivalents paired with each other homoeologously or they may have involved three chromosomes from *T. repens* pairing homoeologously with *T. occidentale* or they may be something like a RROA type IVs. Keeping in view the four-five generation breeding history of these plants, the O or A chromosomes might be recombinant with O centromeres with arms from *T. ambiguum* (O^A) or A centromeres and arms introgressed from O (A^O). Due to many chances of recombination between different genomes, introgression was likely in these plants. Out of these advanced BAR hybrid derivatives, BAR09-63 was subjected to further investigation using molecular cytogenetic tools to get more information about whether bridging had worked and recombination had taken place. Recent findings (Dr. Helal Ansari, unpublished data) showed that inter-genomic pairing between A and O genomes was of a lower level in a AAAO hybrid as compared to that reported in 2x AO hybrids by Williams *et al.* (2011). This suggests greater DNA sequence homology between respective chromosomes of 2x *T. ambiguum* and 2x *T. occidentale* than that between 6x *T.ambiguum* and 2x *T. occidentale.* This is consistent with the likelihood that the various *T. ambiguum* ploidies vary in genomic content and are probably a mix of allo- and auto-ploids (Warren Williams, unpublished data).

Pairing of *T. ambiguum* chromosome as IIs and IIIs in BAR09-63 was confirmed by the GISH on a metaphase-I chromosome preparation (Figure 4.3.4a, b). No IVs were observed with a *T. ambiguum* chromosome in BAR09-63. The *T. ambiguum* chromosome paired either as IIs or IIIs. It could not be established which other chromosomes it paired with because GISH could not distinguish between *T. repens* and *T. occidentale* chromosomes. In almost 70 % of the cells in this hybrid, the *T. ambiguum* chromosome behaved as a univalent (Figure

4.3.4c, d) and so 30% of PMCs showed *T. ambiguum* chromosome associated with other chromosomes which provides an excellent probability of recombination/introgression in breeding context. In this hybrid many PMCs had a regular anaphase-I with 16 IIs +1 I leading to 16-17 disjunctions. But, as is seen in Figure 4.3.4e, the *T. ambiguum* chromosome sometimes split into two chromatids which were pulled in opposite directions. These frequently lagged behind and were excluded from the daughter nuclei making future pollen grains, as also observed by workers in other hybrids (Ferguson *et al.,* 1990; Ishii *et al*., 2010; Felismino *et al*., 2012). In this way many of the progeny plants would not have the *T. ambiguum*-derived chromosome.

The percentage of PMCs with *T. ambiguum* chromosome pairing with inter-specific homoeologous chromosomes was higher in BAR10-126 (discussed earlier) as compared to that in BAR09-63. The reason may be the differential sources of *T. ambiguum* in these two hybrids. The source of *T. ambiguum* in BAR10-126 was 4x *T. ambiguum* which might be genetically closer to *T. occidentale* and so to white clover. In the case of BAR09-63, the *T.ambiguum* chromosome came from 6x *T. ambiguum* which might be genetically more distant from *T. occidentale*. A and O can perfectly pair as is reported by Williams *et al.* (2011) in a 2x hybrid between 2x *T. ambiguum* and *T. occidentale* (AO) while pairing between A and O in hybrid "33", AAAO, resulting from the cross, (6x AAAAAA x 2x OO) was very low (Dr. Helal Ansari, unpublished data). This might be evidence for evolutionary differences among the A genomes coming from the different ploidies. Or the difference can be due to presence or absence of O-derived homoeologous counterpart for A chromosome. In case of the availability of its homoeologous counterpart from *T. occidentale* the chances of its making IIs and IIIs might be more than in the situation where this homoeologous counterpart is not available.

5.4.4 Meiotic analysis of BAR10-80, BAR10-81 & BAR10-93

If we consider 33 OP-1 as a tri-specific F_1, then BAR10-80, 81 and 93 were BC_3F_1, BC_4F_1 and BC_2F_3 respectively (Table 4.3.4). BAR10-80 in its breeding position was just like BAR09-63 (discussed in the previous section) with almost same meiotic pairing results (compare tables 4.3.3 and 4.3.5). In this section we will discuss BAR10-81 and BAR10-93. These hybrids received one and two *T. ambiguum* chromosomes, respectively, based on GISH results which will be discussed later. The average frequencies of Is suggests that the *T. ambiguum*-derived chromosomes may not have been pairing, but the ranges show they

frequently paired. BAR10-81 and BAR10-93 were expected to have 31 and 28 chromosomes from white clover and so, assuming both the homologous and homoeologous pairing between white clover sub-genomes, 15 and 12 bivalents would have been expected. The average frequencies of IIs were less, suggesting that either there was not a homologous or homoeologous counterpart for every white clover-derived chromosome or only homologous pairing within white clover occurred leading to IIs. Alternatively, Homologous or homoeologous white clover IIs either paired with O chromosomes, making IIIs, or with both A and O chromosomes making IVs.

Due to the large numbers of white clover derived chromosomes, the frequency of IIs was very high, leading to very high male fertility in BAR10-81 and BAR10-93. The comparatively lower male fertility in BAR10-93 as compared to BAR10-81 might have been due to the presence of two *T. ambiguum* chromosomes in BAR10-93 as compared to one in BAR10-81. Based on the remote genetic relationship of the A genome with R genomes, it could be expected that larger numbers of A chromosomes would lead to higher level of meiotic anomalies due to lower participation in meiotic pairing. BAR10-81 showed a very low frequency of Vs as well which suggests the occurrence of some allosyndetic chromosome pairing. This pairing should be something like RRRRO or RRRRA. Because of the complex genomic composition of these hybrids with two genomes from white clover and one each from *T. ambiguum* and *T. occidentale* and the lack of chromosome-specific molecular markers, it is difficult to come to solid conclusions on the exact nature of chromosome pairing in these hybrids. Keeping in view the number of meiotic cycles (5-6) that these hybrids had gone through to maximise the chances of inter-genomic exchanges, some of the hybrids from this strategy were subjected to FISH/GISH analyses to get an insight into the actual situation.

5.4.5 GISH/FISH on hybrid “33, AAAO” derived advanced progeny

Three hybrids i.e. BAR09-63, BAR09-81 and BAR09-93 were investigated using GISH and FISH.

5.4.5.1 BAR09-63

The expected genomic composition of BAR09-63 was RRR($R_6A_3O_1$) but GISH revealed that it had only one *T. ambiguum*-derived chromosome (Figure 4.3.5). As its somatic chromosome number was 33 (one less than the expected number), it had either more than one

chromosome from *T. occidentale* or more than 30 chromosomes from white clover. Probing with 5S r DNA, BAR09-63 gave four signals, two bigger on NOR bearing chromosomes and the remaining two relatively smaller on a pair of non-NOR chromosomes. The origin of NOR bearing chromosomes remains unknown because, with current markers, they are same in white clover and *T. occidentale* (Ansari *et al*., 1999). BAR09-63, although having been through five meiotic cycles with odd chromosomes from all the three species, GISH did not show any evidence of genomic exchange (introgression). The meiotic chromosome pairing analysis showed the participation of *T. ambiguum* chromosomes either as IIs or IIIs. But lack of signs of recombination of any sort might suggest that pairing does not lead to genomic exchange as well. Alternatively, the introgressed pieces might be too small to be detected by GISH. Although there was no visible introgression, a fertile backcross population in itself is a significant achievement which might lead to introgression by further crossing/selfing or by using different genotypes of both the parental species.

5.4.5.2 BAR10-81

BAR10-81(2n=33) was BC_4F_1 and the progeny of BAR09-63 used as the female parent in a cross with white clover. Its expected genomic composition was RRR($R_7A_{1-2}O_{0-1}$). The aim of the project was to repeatedly backcross the original BAR hybrids to *T. repens* to bring back the ploidy level to 4x with all the chromosomes from white clover with possibly some recombinant chromosomes having introgression from *T. ambiguum*. The GISH analysis of this plant showed only one A-derived chromosome, as was expected (Figure 4.3.5e). The NORs in this hybrid were not as de-condensed as in BAR09-63 and they look like slightly stretched secondary constrictions. The probable reason for this was the very late somatic metaphase stage of the cell, where the chromosomes were extremely condensed and were splitting into chromatids (early anaphase). The hybridization on the *T. ambiguum* chromosome in BAR10-81 was not uniform, being stronger in the peri-centromeric region and gradually fading towards the telomeres. The reason may be the higher intensity of repeat sequences around the centromere. During this hybridization it was observed that hybridization on chromosome preparations that had gone through two cycles of de-naturation gave comparatively stronger and more uniform signals (compare figures 4.3.5b, e, h). This has also been observed in other hybrids (Dr. Helal Ansari, unpublished data). Again it is not known whether the NOR bearing chromosomes came from *T. occidentale* or *T. repens* because NOR bearing chromosomes in these two species are similar (Ansari *et al*., 1999). This hybrid has gone through six meiotic cycles with odd genomes/chromosomes from all

three species. Again, contrary to expectations, no introgression was observed by GISH analysis (Figure 4.3.5e). May be homoeologous pairing and recombination between chromosomes from species genetically very distantly related does not occur at a high enough frequency. Or, the introgression might involve very small segments of chromatin which might be out of the scope of the GISH detection capability. In addition to that, the sample of plants which was selected for genomic composition analysis was very small.

5.4.5.3 GISH on BAR10-93

It is known that including one or two selfing generations in a breeding scheme can increase the chances of recombination between homoeologous chromosomes many-fold. For this reason BAR10-93 was selected for introgression analysis. This hybrid was BC_2F_3 with the expected genomic composition of RRR($R_4A_6O_4$), being the progeny of the BC_2F_1 plant BAR-66 (RRR($R_4A_6O_2$)) after two further generations of selfing. The somatic chromosome count in BAR10-93 was 34 which was two chromosomes less than the expected 2n=36. Contrary to expectation, the number of A chromosomes turned out to be two. This was further evidence of A chromosome elimination, presumably due to pairing anomalies during meiosis. The hybridization signals on both the A chromosomes were uniform and very strong which might be due to the double de-naturation, as mentioned earlier (Figure 4.3.5h). One of the A chromosomes was a marker chromosome having a 5S signal on the longer arm located close to the centromere (Ansari, *et al*., 1999). This hybrid had seven 5S signals with six collectively from *T. repens* and *T. occidentale* (Figure 4.3.5i). Two of them were comparatively larger and on the NOR bearing chromosomes and so it is difficult to attribute their origin to white clover or *T. occidetnale*. On basis of signal size it can be safely argued that two of the remaining barely visible 5S signals were from *T. occidentale*, while the remaining two have come from *T. repens*. The large number of 5S signals is further evidence of genetic complexity of this hybrid. Despite keeping A, O and R genomes together for six generations (including two selfings) and mostly in odd numbers (which was likely to maximise homoeologous pairing), there was no indication of recombination. Nevertheless, it is encouraging that these hybrids had reasonable male and female fertility, which might lead to their utility in some other way.

5.4.6 Morphology of RRRA(A_4O_4) (5x), RRR($R_4A_6O_2$) (4.5x) and RRR($R_6A_3O_1$) (4.25x)

These hybrids received chromosomes from the agronomically superior 6x *T. ambiguum* and so were different from other BAR hybrids and potentially more valuable than hybrids that had received genomes from lower ploidies of *T. ambiguum*. They are aneuploids in their composition. In the BAR09 generation, the hybrids were morphologically like *T. repens* but skewed towards *T. ambiguum* (Tables 4.3.6.1, 4.3.6.2). In this work, the coloured marked white clover parents used were agronomically very weak and so they were not an ideal comparison. But on the basis of their contrasting characters as compared with *T. ambiguum* and being the parents of the hybrids, they were useful for morphological comparison. Stem anchorage in these hybrids showed some expression of *T.ambiguum* traits. White clover puts down roots from every node of the stem (stolon) moving horizontally along the ground (Thomas, 1987; Abberton & Marshall, 2005) while *T. ambiguum* is a non-stoloniferous species having semi-erect or erect stems (Daly and Mason, 1987). The nodal rooting of all the hybrids was intermediate showing additive inheritance of this trait. All the hybrids were apparently stoloniferous like white clover, but the stoloniferous character was only partially expressed because the nodal rooting in hybrids did not go to the last node of the trailing stems. Generally, there was a strong relationship between the number of A-derived chromosomes and A-associated characters in the hybrids. BAR09-54, 56 and 57 having more A-derived chromosomes, looked more similar to *T. ambiguum* than *T. repens*. BAR09-63 (2n=33) had only one chromosomes from *T. ambiguum* and was morphologically more similar to white clover, and thus had a relatively low root weight % of the total biomass. Similarly BAR09-67 appeared to have no or one chromosome of *T. ambiguum* and so it was also morphologically closer to white clover than to *T. ambiguum*.

The second generation of these hybrids (BAR10), although showing some intermediate morphology in a few characters, was closer to white clover (Tables 4.3.7.1, 4.3.7.2). The plants were stoloniferous with higher levels of anchorage (more nodal roots, white clover-like trait) than the previous generation. With a few exceptions, the growth was indeterminate, as in white clover. Leaflets, although still longer than the white clover, were less so, and seed set per head had gone up toward that in white clover. However, root traits still apparently showed the expression of *T. ambiguum*-like morphology. In comparison to Kopu II (white clover), root weights were higher than the above ground parts in all the hybrids. The comparison with coloured white clover might not be a good comparison because of its weak

above-ground growth and, as a result, its root weight % to total biomass was unusually high compared to normal growing white clover like Kopu II. Consequentially, some of the hybrids had non-significantly different root weight % to total biomass from coloured white clover. The morphology results showed that, with repeated backcrossing to white clover, the *T. ambiguum* like morphology was slowly diminishing. This was the consequence of decreasing numbers of *T.ambiguum* chromosomes with every backcrossing and selfing.

5.5 Strategy based on ARO (3x) and AARROO (6x)

Triploid hybrids with genomic composition of ARO were obtained by crossing the 4x hybrid, ROS (A^TA^TRR) with 2x *T. occidentale* (OO) (Table 4.4.1). Fusion of normal haploid (AR) gametes from ROS and 2x *T. occidentale* with the same EBN (1) resulted in these 3x hybrids.

The 6x BAR hybrids with genomic formula, AARROO resulted from the cross of ROS (A^TA^TRR) pollinated with artificially tetraploidized *T. occidentale* (OOOO). In this case ROS, used as the female parent, contributed 2n gametes while artificially chromosome doubled *T. occidentale* (OOOO) contributed normal haploid (n) pollen (Table 4.4.1). Diploid gametes from ROS (AARR) would have the same EBN value (2) as the haploid gametes (OO) produced by the 4x *T. occidentale,* i.e. *T. occidentale* gametes with genomic composition of OO would tend to screen for 2n gametes from ROS with the same EBN values. Except for occasional "leakage", other combinations would fail to produce normal seed after fertilization due to endosperm abortion.

The triploid and hexaploid conditions of all the original hybrids included in this strategy were confirmed by flow cytometry. The three genomes in the ARO hybrids were all from different species so none of the chromosome had homologues to pair with. The consequent low level of meiotic regularity in these 3x plants was reflected in very low pollen fertility and consequently very low seed production (Table 4.4.1). Because all the chromosomes in every set were homoeologous, metaphase-I in these plants might either be expected to range from up to 24 Is to up to eight IIIs. They were not characterized cytologically in this study so what actually happened is not known. Nevertheless, these 3x hybrids were potentially very significant if there was even a low frequency of homoeologous chromosome pairing because they might produce gametes with recombinant chromosomes. The resulting progeny from crossing the 3x plants with white clover were 5x, which indicated the contribution of unreduced gametes by these 3x plants. Nevertheless there was a possibility of recombinant chromosomes in these progeny hybrids.

The pollen fertility in the 6x hybrids (AARROO) was comparatively higher, almost certainly because every chromosome had either a homologue (OO) or at least an intra-specific homoeologue (AA, RR). AA genomes are considered here as potentially homoeologous based on our results, those reported by Williams *et al*., (2011) and Dr. Nick Ellison's unpublished data. However, the Turkish source (4x *T. ambiguum*) was used to make ROS and we have no evidence that this is allotetraploid. Intra-specific homoeologues are likely to have greater synaptic attraction than inter-specific homoeologues due to higher sequence similarity, as suggested by Williams *et al*. (1982). Thus the meiotic pairing in these 6x AARROO plants would be more regular, giving higher pollen fertility and seed production (Table 4.4.1) as compared to the 3x ARO hybrids. All these 6x hybrids produced seed in one direction when crossed as female parents with white clover, but the reciprocal cross did not set any seed, possibly because of the weak quality of the stainable pollen in these hybrids which, although stained, were smaller in size and not very spherical. This is consistent with more normal meiosis in megasporogenesis than in microsporogenesis. The number of seed was very low in the 3x hybrids when pollinated with white clover, suggesting that female fertility was very low. By contrast, the 6x hybrids produced larger quantities of seed after crossing them as female parents with white clover.

5.5.1 Characterization of the first self and backcross progeny of ARO and AARROO hybrids

In the first progeny (BAR09) that resulted from crossing and selfing of the above mentioned 3x and 6x BAR hybrids, five plants were selected for further characterization (Table 4.4.2). This selection was based on their intermediate morphology, presence of colour leaf makers and flow cytometry based ploidy estimations (suggesting the presence of *T. ambiguum* chromosomes). The somatic chromosome counts and flow cytometry based ploidy estimates matched, except for BAR09-2. Flow cytometry based ploidy estimation in BAR09-2 was 3.7x while the plant proved to have 40 chromosomes (5x), suggesting a possible flow cytometry sampling error. The somatic chromosome counts showed that BAR09-2 and BAR09-3 had the same chromosome number and genomic composition (2n=5x=40, RRRAO) as they resulted from the same cross and a 2n gamete contribution from the female parent, BAR-8 (ARO, 3x). Apparently only diploid (2n) gametes contributed by BAR-8 (ARO) were functional consistent with EBN expectations. All other gametes with unbalanced genomic compositions possibly did effect fertilization but the embryos failed to develop due to either embryo and/or endosperm failure.

BAR09-5 resulted from the cross of BAR-13 (AARROO, 6x) with coloured white clover, Scarlet-1. This hybrid was also pentaploid (2n=5x=40, RRRAO) like BAR09-2 and BAR09-3 but the parents contributed haploid gametes. BAR09-10 (2n=5x=39) resulted from the pollination of coloured white clover (P/B1 x P/B2)-1 by BAR-15 (AARROO) with both again contributing n gametes. BAR09-15 was the self progeny of BAR-17 (AARROO, 6x) and resulted from the only self seed set on BAR-17. The somatic chromosome count in BAR09-15 was 35 and markedly lower than the parental BAR plant which had 48 chromosomes. This shows substantial chromosomal elimination, presumably arising from major meiotic disturbances in BAR-17. It is not known what species-specific chromosomes were eliminated during both mega- and microsporogenesis in BAR-17. Alternatively, the possibility of sampling error or stray seed cannot be ruled out. All the 5x (RRRAO) selected plants resulting from pollination of 3x BAR hybrids by white clover produced reasonably good quantities of seed, suggesting greater meiotic regularity as compared to the previous generation (ARO, 3x). The seed-set produced by 5x hybrids (RRRAO) resulting from the 6x BAR hybrids was comparatively lower than in the 6x parental BAR hybrids (compare Tables 4.4.1 and 4.4.2), which suggests that the meiotic irregularities in these 5x BAR09 hybrids were at a higher level than in the parental 6x BAR hybrids. All of the BAR09 hybrids proved to be self incompatible like the previous generation, except BAR09-15 which produced 1.3 seeds per head on selfing, almost equal to plants regarded as being self incompatible. Where reduced fertility is also involved, it is very difficult to come to a certain conclusion as to whether a low or zero seed set is due to actual SI or to low fertility. In this whole group of hybrids, BAR09-2 and BAR09-3 had high chances of genomic recombination between the A, R and O genomes on the female side during meiosis in BAR-8 (ARO) and so they are potentially very important from a recombination point of view. During meiosis-I, the genomes may have paired because there was no homologue for any of the chromosomes and in such situation the chances of pairing between homoeologues increases (Williams *et al*., 1982). We got low level chromosome pairing between the gametic sets of white clover which indicates the possibility that white clover has a *Ph1*-like gene, as in bread wheat (Riley and Chapman, 1958; Chen *at al*., 1991; Naranjo and Corredor, 2004) restricting the pairing to only between homologues. However, in a hybrid like ARO, having only one white clover genome, this gene might not be present or it might be hemizygous-ineffective as in tall fescue (Jauhar, 1975a, b). The pairing between the gametic chromosomes in white clover in some hybrid situations (Chen and Gibson, 1970; Our own results) is consistent with either explanation. Alternatively, it might not function properly in a situation as in hybrid ARO

where every chromosome is homoeologous to every other one with no chance of homologous pairing. Gillies (1987) reported that *Ph1* did not stay effective where large numbers of chromosomes were homoeologous to each other with very little chances of homologous pairing.

5.5.2 Characterization of the second self and backcross progeny of ARO and AARROO hybrids

The selected plants from second self and backcross progeny of ARO (3x) and AARROO (6x) hybrids are listed in Table 4.4.4. Six plants were selected on the basis of showing the presence of *T. ambiguum* specific morphological traits and flow cytometry based DNA content estimations. The ploidy level, as is evident from Table 4.4.4, had come down to close to 4x and the actual somatic chromosome numbers were lower than expected from the parental numbers. BAR10-12 had 33 chromosomes while its female parent BAR09-3 was pentaploid with 2n=5x=40, giving an expected number of 36. This was consistent with highly disturbed meiosis leading to chromosomal elimination on a large scale in the BAR09 hybrid parent as reported by Ahuja *et al*. (2003) and Tu *et al*. (2009). BAR10-1, BAR10-12, BAR10-16 and BAR10-22 were each expected to have 28 chromosomes from *T. repens*, and the pollen fertility in this generation was comparatively higher than the previous generation, possibly due to increased bivalent formation among the higher numbers of homologous *T. repens* chromosomes. The 0% pollen stainabilities in BAR10-12 and BAR10-16 were exceptions and these might be explained by genic factors rather chromosome pairing problems as reported by Stebbins (1958) because BAR10-12 and 16 had similar genomic compositions to BAR10-1 which was reasonably fertile having >30 % stainable pollen. The unexpectedly high male fertility in BAR10-24 was mysterious, but may be this hybrid have had only white clover and *T. occidentale* chromosomes due to elimination of all *T. ambiguum* chromosomes in the meiotic cycle of the BAR parent of BAR09-15 which had 35 chromosomes instead of the expected 48. Or may be it had very few *T.ambiguum* chromosomes which were not influencing the normal meiosis. BAR10-1 and BAR10-12 had undergone two meiotic cycles, with maximum chances of genomic mixing while the rest of the plants have gone though one meiotic cycle with sure chances of inter-genomic recombination. Some of these plants with maximum chances of inter-specific recombination were further studied using conventional and molecular cytogenetic tools.

5.5.3 Chromosome pairing analysis in BAR09-3 (RRRAO, 5x)

Only BAR09-3 (RRRAO, 5x) was selected from the BAR09 generation from this strategy. BAR09-3 was the progeny of hybrid BAR-8 (ARO, 3x) pollinated by white clover (P/B1 x P/B2)-1. BAR-8 contributed 2n gametes which were fertilized by the haploid pollen (n) from white clover. This plant was very important from a chromosome pairing point of view. It received a 2n (ARO) gamete from BAR-8, with high chances of inter-specific chromosome pairing leading to recombination due to the absence of homologues. The male and female fertility (although low, Table 4.4.1) of BAR-8 was strong evidence of some level of allosyndetic chromosome pairing between A, R and O genomes. So the 2n gametes from ARO might have recombinant chromosomes most probably R/O or A/O, or there is the possibility of recombinant chromosomes having centromeres from *T. occidentale* and arms introgressed from *T. ambiguum* and *T. repens*.

The expected genomic formula of BAR09-3 shows that it has three genomes from *T. repens*, one each from *T. ambiguum* and *T. occidentale*. The average frequency of different chromosomal association during metaphase -I showed that it had all types of chromosomal formations ranging from Is to IVs. The ratios of Is and multivalents was very high and this high level of meiotic anomalies was reflected in its low level of male fertility (Table 4.4.3).

The average frequencies of Is, IIs, IIIs and IVs showed the high possibility of inter-specific homoeologous chromosome pairing involving *T.ambiguum*-derived chromosomes. It almost certainly had eight chromosomes from *T. ambiguum* but the average number of Is per PMC was 5.5 (Table 4.4.3) which indicates that some A chromosomes must have been participating in pairing. Whether the A-derived chromosomes associated as IIs, IIIs or IVs is not known because of the kayotypic similarities of chromosome in *Trifolium* and lack of suitable markers (Chen and Gibson, 1971; Williams, 1987; Benavente *et al.,* 2008). If we go a little further into the chromosomal composition of this plant it becomes clear. As we know, white clover is allopolyploid having two sub-genomes one probably from *T. pallescens* (mother) and the other one from *T. occidentale* (father) (Ellison *et al*., 2006; Williams *et al*., 2012). Then the theoretical genomic composition of this plant would be like $R^{P}R^{O}R^{P/O}AO$. If the *T. pallescens* and *T. occidentale*-derived eight chromosomes of the mixed white clover genome paired with their respective homologues in the other two white clover derived genomes making eight bivalents, then we would be left with 4 R^{P}, 4 R^{O}, 8 A and 8 O chromosomes. The chromosomes belonging to R^{P} and R^{O} were from different linkage groups

so they should not pair. Alternatively, among the white clover chromosomes, a maximum of eight IIs can be expected, even if within species homoeologous pairing is also occurring. The lower number of IIs than the expected number shows some white clover chromosomes were making multivalents as well. Among the remaining chromosomes, it is not known whether *T. occidentale* chromosomes would pair with *T. ambiguum* and *T. occidentale* derived chromosomes of white clover to make IIIs. An alternative would be that the *T. occidentale* chromosomes would pair with the intra-specific homoeologous chromosome pair (R^PR^O) from white clover to make IIIs. It is difficult to establish because we do not know whether R^O is genetically closer to O or R^P. Also, genetic distance does not necessarily mean that R^O will always pair with R^P and not O or vice versa (Warren Williams, verbal comm.). By contrast, the IVs (Figure 4.4.1a) definitely involved homoeologous chromosomes from all three species. The deduction is that A chromosomes, other than those behaving as Is, were pairing either as IIIs (ROA) or IVs (RROA), if the possibility of illegitimate pairing is ignored. The meiosis was very disturbed in this plant (Figure 4.4.1b) but comparatively normal PMCs with very few laggards were also observed (Figure 4.4.1c). In BAR09-3, O might also pair with A making IIs as was reported by Williams *et al.* (2011) in AO (2x) hybrids, where perfect pairing was observed between the genomes of the two species. This showed very close pairing affinity between 2x *T. ambiguum* and 2x *T. occidentale,* supporting the close phylogenetic relationship between them. Chromosome pairing is also affected by environmental conditions so sometime it is misleading to base phylogenetic relationship on pairing level during meiosis. Also, we do not know about the sequence homology level between 4x *T. ambiguum* and 2x *T. occidentale* because the source of A genomes in BAR09-3 was 4x *T. ambiguum*. The DNA sequence varies in different ploidy levels of *T. ambiguum* (Nick Ellison, unpublished work).

5.5.4 Meiotic analysis of BAR10-1(RRR($R_4A_4O_4$)) and BAR10-22 (RRR($R_4A_4O_4$))

BAR10-1 and BAR10-22, although having slightly different breeding histories, had similar expected genomic compositions. However, BAR10-22 had only one chromosome from *T. ambiguum* confirmed through GISH (discussed later) while BAR10-1 probably had 3-4 chromosomes from the same species. The pedigree difference was that BAR10-1 resulted from a cross of BAR09-2 (RRRAO) with white clover and this plant was itself the progeny of the cross of the original 3x BAR hybrid, BAR-8 (ARO) with white clover where BAR-8 contributed a 2n gamete. BAR10-22 resulted from the original 6x hybrid, BAR-15 (AARROO) after two backcrosses with white clover. Thus both had the same expected

genomic composition of $R^{P}R^{O}R^{P/O}(R_4{}^{P/O}A_4O_4)$ but with one and 2-3 chromosomes less than expected, respectively (Table 4.4.5). BAR10-1 had two chances of inter-genomic recombination in its breeding history as compared to BAR10-22 having had only one sure chance. The genomic composition of these hybrids was so complicated that it is not easy to reach definite conclusions regarding chromosome pairing to explain the average frequencies of different chromosomal configurations given in the Table 4.4.5. In these two hybrids, if we consider only homologous chromosome pairing within white clover genomes then there could be up to 12 IIs. If we assume both homologous and homoeologous pairing intra-specifically among white clover chromosomes, again there could be up to12 IIs. The average frequency of IIs was around 8, but it shows that some white clover-derived IIs are taking part in multivalent formation as well (IIIs and IVs) (Table 4.4.5). The multivalents expectedly involved allosyndetic pairing ($R^{O}R^{O}O$ or $R^{P}R^{P}O$ or RRAO). Based on its chromosome count and DNA content, BAR10-1 had more *T. ambiguum* chromosomes than BAR10-22 and the ratio of univalent chromosomes was also slightly larger in BAR10-1, suggesting that *T. ambiguum* chromosomes were probably behaving as Is. The pollen fertility in BAR10-1 was lower than in BAR10-22, consistent with the concept that plants having higher numbers of *T. ambiguum* chromosomes; also have more meiotic anomalies leading to lower fertility. Pentavalents were also observed in BAR10-1 and BAR10-22, which shows that, assuming no accidental illegitimate pairing of different linkage groups, they might have involved chromosomes from two or all three species. For example, they might be RRRRO or RRROA. However, it must be noted that apparent illegitimate pairing has been recorded in 4x hybrids of genomic constitution (AAAO) designated as hybrid "33" (Dr. Helal Ansari, unpublished data). Flow cytometry results showed that A chromosomes were larger in size than chromosomes from *T. occidentale* and *T. occidentale* chromosomes were larger than those of white clover. BAR09-3 and BAR09-5 were actually 5x on the basis of somatic chromosome counts but their flow cytometry based DNA contents using 4x white clover as the reference standard were more than 5x because of the higher DNA contents in A and O derived chromosomes (Table 4.4.2). Making use of this size difference, it might be possible to differentiate heterogeneous multivalents from those having only intra-specific chromosomes. But unfortunately due to extreme condensation during metaphase-I, it was not easy to distinguish between intra-specific and inter-specific multivalent chromosome associations. Nevertheless, it was evident from the chromosome pairing data that there was inter-specific homoeologous chromosome pairing in addition to pairing between white clover chromosomes intra-specifically.

5.5.5 Molecular cytogenetic analysis of BAR10-22

The GISH/FISH on BAR10-22 revealed that the actual situation was different from what was expected. As BAR10-22 resulted from the cross, RRRAO (5x) x RRRR (4x), the expected chromosome number was 36 with genomic formula of RRR($R_4A_4O_4$). But the actual somatic chromosomes score turned out to be 33. Instead of four *T.ambiguum*-derived chromosomes, GISH indicated only one chromosome from *T. ambiguum*. During metaphase-I, the chromosomes in *Trifolium* are not differentiable morphologically. However, during mitotic metaphase, *T.ambiguum* chromosomes could usually be identified in Giemsa or DAPI-stained preparations due to their larger size and well defined telomeric ends (Figure 4.4.2a, Figure 4.4.3a). The source of *T. ambiguum* chromosome in this hybrid was 4x *T. ambiguum*. The conventional cytology showed IIIs, IVs and Vs in BAR10-22 and it was expected that these multivalent chromosome formations must involve *T. ambiguum* chromosomes. However, GISH did not give any indication of recombination involving *T. ambiguum* chromatin. This might suggest that pairing did not always lead to chromatin exchange, perhaps because recombination requires a high level of sequence homology and even a single nucleotide mismatch can affect its happening (Shen and Huang, 1996; Datta *et al.,* 1996; Li *et al*., 2006). Alternatively, perhaps the multivalent pairing involved only *T. repens* chromosome associations or *T. occidentale* chromosomes in association with *T. repens* derived IIs, IIIs and IVs. Another possibility was that the introgression involved exchange of chromosomal segments which were too small to be highlighted by GISH. Such results were obtained by Tu *et al*. (2009) in intertribal partial hybrids between *Brassica rapa* and *Isatis indigotica*, Kosmala *et al*. (2007) and Humphreys and Pašakinskiene (1996) in *Festuca arundinacea* x *Lolium multiflorum* hybrids.

5.5.6 Phenotypic characterization of hybrids from crosses, ARO x RRRR & AARROO x RRRR – RRRAO (5x)

The white clover plants used as crossing parents had leaf colour markers and were not agronomically vigorous. Because of their poor above ground growth, they had higher root weight to total biomass ratios than other white clovers. However, as mentioned earlier, they were the parents, they were appropriate as controls for comparison of the hybrids with white clover and *T. ambiguum*. The morphology of hybrids belonging to the BAR09 generation (RRRAO, 5x) showed the presence of characters from both *T. repens/T. occidentale* and *T. ambiguum* (Tables 4.4.6.1, 4.4.6.2). All the hybrids including both the BAR09 and the

original hybrids showed stoloniferous stem types, although the anchorage of stems in the BAR09 plants was not as high as in white clover, consistent with their hybrid nature. All hybrids except BAR09-15 showed determinate or highly reduced apical growth. This phenotype again is evidence of expression of *T. ambiguum* traits whereas white clover has indeterminate apical growth. The indeterminate terminal growth in BAR09-15 might have been due to the loss of *T. ambiguum*-derived chromosomes from this hybrid. Its parental hybrid was BAR-15 with genomic composition of AARROO (6x) but its self progeny, BAR09-15 had only 35 chromosomes, showing chromosome loss on a large scale and possibly including all or most of the *T. ambiguum*-derived chromosomes. The hybrids also had axillary flowering but, due to highly reduced apical growth, they appeared to also have terminal flowering as in *T. ambiguum*. In *T. ambiguum* each shoot terminates in a floral bud. This combination of flowering indicates the presence and expression of genes from both the parental species. The most important morphological traits differentiating *T. ambiguum* from white clover was the perennial long thick tap root system with rhizomes which collectively make up to 3/4 of the total biomass (Spencer *et al*., 1975). Due to the presence of *T. ambiguum* chromosome in these hybrids, the dry root weight % of the total biomass was much higher than the white clover parents. Exceptions were BAR09-5 and BAR09-10. As mentioned earlier, the coloured marked white clovers used as controls in the morphological characterization were also the parents used in crossing. These had unusually high root weight % of the total biomass for white clover due to their poor above-ground growth but, nevertheless most of the hybrids were still significantly different from them (Table 4.4.6.2). Other morphological traits, including stolon length and number, inflorescence number, peduncle length, florets per head, leaflet shape, petiole length and stem thickness showed mixed tendencies, having intermediate morphology in some hybrids like BAR09-2 and transgressive expression in others, as in BAR09-3, where the expression of the character was outside the parental range for that trait. The leaflet shape in the 4x *T. ambiguum* used in this study was very narrow and long but the leaflets in these hybrids were not significantly longer than the average value of white clover parents for this character except four hybrids i.e. BAR09-2, BAR09-3, BAR09-5 and BAR-8 (Table 4.4.6.1). The reason may be the gene(s) responsible for leaflet shape might have been silenced in the hybrids or might be recessive to the white clover/*T.occidentale* derived genes. Seed set in hybrids was significantly lower than in the white clover parents. This was consistent with a low level of female fertility (which was also indicated by the low male fertility), probably due to highly disturbed meiosis.

5.5.7 Phenotypic description of second self and cross progeny of RRRAO (5x) – RRR($R_4A_4O_4$)

These hybrids were one generation further on from the previously evaluated hybrids. They had more white clover-derived chromosomes than the previous generation and were thus expected to be more like white clover. All the hybrids were stoloniferous as was expected because, on average, they had 28 *T. repens* chromosomes in their somatic chromosome complement as compared to expected four chromosomes from *T. ambiguum*. Despite a low number of chromosomes from *T. ambiguum*, the phenotypes, with few exceptions, still showed the impact of *T. ambiguum* parentage in the shape of fewer and shorter stolons, anchorage scores of less than 10 and determinate growth habit (Table 4.4.7.1). BAR10-22 which had only one *T. ambiguum* chromosome was included in this experiment but died before data collection was complete. But even with the presence of only a single *T. ambiguum* chromosome, this hybrid had very short stolons with apical flowers, both *T. ambiguum* characters. Possibly, this hybrid had, in addition to the single *T. ambiguum*-derived chromosome, some *T. ambiguum* chromatin introgressed into white clover/*T. occidentale* chromosomes, although not highlighted by GISH studies. As compared to Kopu II (commercial variety of white clover), the performance of these hybrids was poor partly because of their highly complex genetic nature and partly because of the repeated use of poor white clover genotypes as coloured marked male parents. Nevertheles, from a breeding point of view, these hybrids are very important. Seed set per head in this generation was far better than the previous generation, probably because in one more cycle of backcrossing to white clover, the meiosis has become more normal leading to a higher level of fertile gametes. The presence and expression of the *T. ambiguum* chromosomes/genes was also evident in the shape of higher ratio of root weight to total biomass. This ratio was very low in Kopu II and extremely significantly lower than all the hybrids (Table 4.4.7.2). As before, the coloured white clover also had a significantly lower ratio than the hybrids, except for BAR10-16 and BAR10-24. Whether the *T. ambiguum* genes were on the *T. ambiguum* chromosomes or introgressed into white clover chromosomes, their expression in the hybrid environment is encouraging. This shows that unlike the *T. ambiguum* NOR genes, which are suspected to have been transcriptionally inactivated in some hybrids, the morphological trait genes from *T. ambiguum* were expressed and contributed to the intermediate morphology. The highly condensed state of NORs on *T. ambiguum* derived chromosomes was later on turned out to be due to differential condensation cycle as previously discussed and was not due to the

nucleolar dominance phenomenon as reported in different hybrids by Joly *et al*. (2004) and Pikaard (1999).

5.6 Strategy based on the direct integration of A & R genomes-ARRRR (5x) and AAARRRR (7x) hybrids

At the start of this project, six hybrids were available with genomic composition of RRRRA (5x) (Table 4.5.1). They were obtained by crossing Trophy-2 or Kopu-II (commercial cultivars of white clover) as female parents with pollen from a 6x hybrid with genomic composition of A^DA^TRRRR and their ploidy level was confirmed by flow cytometry based DNA content analysis. The 6x hybrids (A^DA^TRRRR) used as parents of these 5x BAR hybrids, were actually the progeny of the cross, A^DA^TRR (hybrid 70) x RRRR (Crau-38, a commercial white clover cultivar). Hybrid 70 had two genomes from *T. ambiguum,* one from a diploid cultivar (A^D) and the other from a tetraploid accession (A^T). Hybrid 70 contributed 2n gametes as the female parent. Similar results regarding 2n gamete contributions from 4x hybrids between white clover and *T. ambiguum* were reported by Anderson *et al*. (1991) and Meredith *et al*. (1995). The 5x hybrids (ARRRR) were very important because the single A genome in these hybrids was a mixture of chromosomes derived from both 2x and 4x *T. ambiguum* forms. In addition, eight other hybrids were available with 7x ploidy level (Table 4.5.1). These 7x BAR hybrids had the genomic composition of AAARRRR and were obtained by crossing ROS (A^TA^TRR) as female parent with the 6x BAR hybrids, A^DA^TRRRR. Here, too the 4x hybrid, A^TA^TRR contributed 2n functional gametes as the female parent. This shows that 4x hybrids between *T. ambiguum* and *T. repens* (AARR) would always contribute functional 2n gametes if crossed with RRRR or AARRRR which is also in line with the EBN hypothesis. RRRR has been given an EBN of 4 while AAAA has been assigned 0 EBN. A haploid gamete (RR) from *T. repens* would have 2 EBN while a 2n gamete from hybrid AARR would have the same EBN value and so this cross would succeed, ensuring 2:1 maternal paternal EBN ratio in the endosperm tissues and thus its proper development. Normal haploid gametes (n) from the hybrids with genomic constitution of AARR would only rarely produce a seed if crossed with white clover or AARRRR due to their EBN value differences.

The 5x ARRRR hybrids had reasonably high pollen fertility which was unexpected keeping in view the distant genetic relationship between white clover and *T. ambiguum*. It was probably because they had one full chromosome complement from white clover, and so the

presence of one full genome from *T. ambiguum* caused only some reduction in pollen fertility. The reduced pollen fertility anyhow showed that meiotic abnormalities were caused by the *T. ambiguum*-derived genome probably because, even if they were not taking part in chromosome pairing, and behaved as Is, they might have disrupted the normal pairing in the white clover chromosomes. Alternatively perhaps, due to being isolated, these A derived chromosomes might have participated in pairing processes to make multivalents. The pollen fertility level in the 7x AAARRRR hybrids, was generally lower than in the 5x hybrids just described above (Table 4.5.1). This suggests a higher level of meiotic abnormalities in these hybrids than in the 5x hybrids, which is understandable due to the higher number of A chromosomes and that these came from 2x and 4x forms of *T. ambiguum* which are likely to be homoeologous to each other. The pollen fertility in these 7x hybrids was still at a reasonable level, suggesting that *T. ambiguum*-derived chromosomes had paired more or less intra-specifically or allosyndetically with their respective homoeologous counterparts from white clover to form multivalents.

5.6.1 The first self and backcross progeny of ARRRR and AAARRRR hybrids

The seed from the selfing and backcrossing to white clover of 5x BAR hybrids (ARRRR) was healthy and large and germinated within three days, producing strong seedlings. On the other hand, the seed from selfing and crossing of the 7x hybrids (AAARRRR) was small and apparently shrunken and took longer (more than 10 days) to geminate into seedlings with major physio-morphological abnormalities. Some of the seed did not germinate and died due to black mould development after having been in the petri dishes for a long time. Similar abnormalities have been described by Meredith *et al*. (1995) and Williams and Verry (1981) in BC hybrids, *T. ambiguum* x *T. repens* using *T. repens* as the recurrent parent.

Out of the self and cross progeny of the 5x hybrids (ARRRR) with white clover carrying co-dominant leaf colour makings, some initial selections were made on the basis of morphology and flow cytometry based DNA estimations for further characterization. The seven selected plants (Table 4.5.2) were aneuploids with somatic chromosome numbers ranging from 34 in BAR09-24 and BAR09-25 to 39 in BAR09-28. They were each expected to have one full chromosome complement from white clover and a partial genome from *T. ambiguum* to give an expected genomic formula of RRRR(A). BAR09-16, BAR09-17, BAR09- 24 and BAR09-25 were the cross progeny of different original hybrids pollinated with white clover. BAR09-19, BAR09-27 and BAR09-28 were self progeny of original BAR hybrids. Selfing

was included because it was expected that inclusion of selfing in the breeding scheme would enhance the chances of genomic recombination (by giving an additional meiosis with a full A genome present). The 2x chromosomes in some of these hybrids were less than expected, showing chromosome loss during meiosis in the BAR parents. The aim of this study was to keep crossing these original BAR hybrids with white clover on one hand to isolate *T. ambiguum*-derived chromosomes from their homologous counterparts to increase the chances of inter-specific chromosomal exchange and, on the other hand to bring the ploidy level down back to 4x. The 2n chromosome number in all the hybrids given in Table 4.5.2 was less than expected, except for two: BAR09-16 had the expected number and BAR09-17 gained two chromosomes. Chromosome elimination/gain may result from chromosome pairing deviating from the normal disomic pattern, leading to chromosome lagging and unbalanced segregation during anaphase-1(Mochida *et al.,* 2004). BAR09-19, the self progeny of a 5x ARRRR hybrid, BAR-23, had 35 chromosomes, indicating elimination of A-derived chromosomes, perhaps during both mega and microsporogenesis. With the decrease in the number of chromosomes from *T. ambiguum*, the pollen fertility increased and so did the seed set after crossing with white clover (Table 4.5.1). Apparently the hybrids with decreased numbers of *T. ambiguum*-derived chromosome became more normal at meiosis, producing more balanced gametes.

5.6.2 The second self and backcross progeny of 5x hybrids, ARRRR

The BAR09 hybrids listed in Table 4.5.2 were again selfed, inter-crossed and backcrossed with white clover. Eight BAR10 progeny plants were selected on the basis of morphology, male fertility and flow cytometry (Table 4.5.4). With few exceptions, flow cytometry results matched the actual somatic chromosome numbers (Table 4.5.4). In these hybrids the somatic chromosome number had further reduced as compared to the previous generation (Table 4.5.2), ranging from 32 in BAR10-44 to 36 in BAR10-32, BAR10-39, BAR10-62 and BAR10-63. The actual chromosome numbers in these hybrids were either lower or higher than the expected numbers because of unequal assortment during meiosis in BAR09 hybrids leading to aneuploid gamete formation (n=2x±). The expected numbers of *T. ambiguum* chromosomes in these hybrids ranged from 0 in BAR10-44 to 4 in BAR10-32, BAR10-39, BAR10-62 and BAR10-63 based on the actual somatic chromosome counts (Table 4.5.4). However, it can be only assumed that it was the *T. ambiguum* chromosomes that were being eliminated here. Similarly, it is assumed that the stainable pollen (%) in these hybrids was generally higher as compared to the BAR09 generation showing greater meiotic normality as

compared to BAR09 hybrids due to higher number of R-derived chromosomes. The pollen fertility in BAR10-44 (56%) was lower than white clover, possibly suggesting that multivalent pairing during megasporogenesis in the female parent, BAR09-19 (2n=35), may have led to elimination of some *T. repens* chromosomes as well as *T. ambiguum* chromosomes.

5.6.3 Meiosis in the hybrids of first self and cross progeny of 5x hybrids (ARRRR)

Three hybrids were included in the meiotic chromosome pairing analysis from BAR09 generation of this strategy. They were BAR09-16, BAR09-19 and BAR09-24. The details of these hybrids are given in Table 4.5.2. They were either selfs of the original 5x BAR hybrids (BAR09-19) or backcross progeny to white clover (BAR09-16, BAR09-24). The 5x parental hybrids (ARRRR) had two full sub genomes of white clover with one genome from *T. ambiguum* which was probably a mixture of chromosomes from 2x and 4x *T. ambiguum* sources (can be designated A^{DT}). BAR09-16 probably had four chromosomes from *T. ambiguum*, BAR09-19 three and BAR09-24 two. The sources of these partial genomes of *T. ambiguum* are not known, and could be 2x, 4x or a mix of both as mentioned above.

The progeny of the cross $AR^{P}R^{P}R^{O}R^{O}$ x $R^{P}R^{P}R^{O}R^{O}$**,** was expected to have a genomic composition like $R^{P}R^{P}R^{O}R^{O}(A_4)$. In hybrid BAR09-16 with one full chromosome compliment from white clover plus four chromosomes from *T. ambiguum*, assuming no inter-specific (allosyndetic) chromosomal pairing, we would expect perfect pairing of the white clover sub-genomes ($R^{o}R^{o}$ and $R^{p}R^{p}$**)** making 16 IIs and the four *T. ambiguum* chromosomes would behave as Is (Figure 4.5.1b). But the average frequency of Is based on the analysis of 42 PMCs was less than 4 (2.7) and, at the same time, the mean number of IIs was less than 16 (12.93). This showed that some allosyndetic chromosome pairing was also happening, and this was further evident from the occurrence of some multivalents (IIIs & IVs) (Table 4.5.3). Many PMCs having no Is or only one or two were observed. Also, in some PMCs strange situations were observed, e.g. chromosomes making bridges between two IIs or two IIIs or one II and one III, etc (Figure 4.5.1h). Similarly in BAR09-19 (RRRR(A), 2n=35) which was a self progeny of BAR-23 (ARRRR) and probably had three chromosomes from *T. ambiguum,* PMCs were found with 16 IIs plus 3 Is. This indicated perfect bivalent formation within the sub-genomes of white clover and the three *T. ambiguum* chromosomes behaving as Is (Figure 4.5.1e). The latter sometimes lagged behind during anaphase-I and II. On the other hand, as is shown in Figure 4.5.1f, sometimes there was only one univalent and several

multivalents could be seen, including one pentavalent. These multivalent chromosomal formations reinforce the findings reported by Anderson *et al*. (1991) and Meredith *et al.* (1995) that allosyndetic chromosome pairing occurred in AxR hybrids which might lead to inter-specific genetic exchange (Williams *et al*., 1982). Fewer Is than the expected number of A-derived chromosomes is strong evidence that *T. ambiguum*-derived chromosomes were pairing with *T. repens* chromosomes, but at a low level. The low male fertility levels in BAR09-16 and 19 (60 & 68% respectively) were much lower than in white clover, which is consistent with the possibility of inter-specific multivalent chromosome pairing. In BAR09-24, the average number of Is was proportional to the expected number of *T. ambiguum* chromosomes, but the range of IIs and the presence of IIIs and IVs showed that they may also be involved in inter-specific homoeologous chromosome pairing (allosyndesis).

5.6.4 Meiotic chromosome behaviour in the BAR10 progeny of 5x hybrids, ARRRR

In the BAR10 generation derived from the 5x hybrids (ARRRR), five hybrids were studied for their metaphase-I chromosome pairing. They were BAR10-39, BAR10-49, BAR10-58, BAR10-59 and BAR10-63 (Table 4.5.5). Considering the original BAR hybrid "70" with genomic composition of $A^DA^TR^OR^P$ as an F_1, then these were BC_3F_2, BC_4F_1, BC_3F_2, BC_2F_3 and BC_2F_3, respectively. If there had been no pairing between A and R chromosomes throughout their breeding then these hybrids, being at such advanced stages, would probably have few or even no A chromosomes due to chromosome elimination. The average frequencies of Is in these plants, although less than the expected numbers of *T.ambiguum* chromosomes, were approximately proportional to the expected numbers of *T.ambiguum* chromosomes, suggesting that Is were mostly derived from the *T.ambiguum* genome. However, some *T. ambiguum* chromosomes were definitely involved in allosyndetic chromosome pairing. These hybrids (Table 4.5.5) were assumed to have one full white clover chromosome complement, based on the assumption of perfect intra-genomic pairing in white clover and maintenance of genomic integrity. These should form 16 IIs, but the actual average frequency was lower (7.7 IIs in BAR10-39 to 14.6 IIs in BAR10-49). This probably indicates that some homoeologous chromosome pairing was going on among the white clover sub-genomes to make multivalents. BAR10-39 had a very low level of IIs and the high number of IIIs and IVs which showed not only homoeologous pairing within white clover but also the liklihood of allosyndetic chromosome pairing involving *T.ambiguum* chromosomes. Most of these hybrids also showed very low frequencies of Vs, which was evidence of inter-specific chromosome pairing between white clover and *T.ambiguum* chromosomes,

disregarding the possible occurrence of illegitimate pairing between chromosomes belonging to different linkage groups. The pollen fertility in these hybrids was variable ranging from 59 to 94% (Table 4.5.5). Although relatively high, on average, these were lower than pure white clover, probably indicating the participation of *T.ambiguum* chromosomes making multivalents in meiosis. Keeping in view some possibility of inter-specific chromosome pairing in these hybrids, directly integrating the genomes of white clover and *T. ambiguum*, some were subjected to GISH studies to see if this was observable.

5.6.5 Molecular cytogenetic analysis of BAR09-16 and BAR10-32

These two plants were selected from the strategy based on the direct integration of R and A genomes. The hypothesis was if we generate a plant with a situation having odd genomes/chromosomes from the parental species, then the chromosomes from the species lacking homologues might pair allosyndetically with the chromosomes from other species. BAR09-16 should have had four *T. ambiguum* chromosomes because of its expected genomic formula RRRR(A_4) and the actual somatic chromosome count was 36. That expectation was met because GISH with *T. ambiguum* genomic DNA painted four chromosomes green (Figure 4.5.3b). The clear differentiation of A and R chromosomes in this hybrid by GISH further confirmed the distant phylogenetic relationship between these two species as suggested by Rosato *et al*. (2008). There were four 5S signals, two on NOR bearing chromosomes while the remaining two were on a separate pair of non-NOR chromosomes. The chromosome pairing analysis in this hybrid showed that it averaged only 2.71 Is per PMC and there were IIIs and IVs as well. The average frequency being less than the actual number of *T. ambiguum* chromosomes and the presence of multivalent association (IIIs and IVs) was a strong indication of inter-specific chromosome pairing. Nevertheless, GISH results showed no cross hybridization and no green signals were observed on chromosomes other than those coming from *T. ambiguum* despite many generations of backcrossing (Figure 4.5.3b). The possible reasons are as mentioned before: either allosyndentic pairing had not led to recombination or any introgressed DNA sequences were too small to be detected by GISH. Also the small sample size because only one plant receiving a hybrid gamete was studied and so the chance of showing no recombination is high for sampling reasons. Given the high frequencies of PMCs with no multivalents, the gamete this hybrid received had a high chance of coming from one of those PMCs. So the chance of showing no recombination is high for sampling reasons. Of course, having 2 or 3 generations of recombination increases the probability of detection.

BAR10-32 RRRR(A) was the progeny of an inter-cross between BA09-16 (RRRR(A_4), 2n=36) and BAR09-20 (RRRRA, 2n=40) and can be designated as a BC_3F_2. The chromosome number in BAR10-32 should have been 38 but this expectation was not met because it was 36. When BAR10-32 was subjected to GISH using genomic DNA of *T. ambiguum* as probe, three intact chromosomes and one half chromosome were highlighted. The half chromosome was also identified in the Giemsa and DAPI-stained chromosome preparations (Figure 4.5.2a, 4.5.3d). Initially it was considered to be a complete telocentric chromosome which was unusual for the *Trifolium* genus which has either metacentric or sub-metacentric chromosomes (Williams, 1987). But later, it was confirmed by GISH that it was a broken chromosome apparently having a partial or complete centromere at one end. It cannot be said that whether this telocentric chromosome was contributed by BAR09-16 or BAR09-20. It has been reported that univalent chromosomes sometimes give rise to two telocentrics when there is bipolar attachment of spindle fibres to the centromere while it is fused as single unit. Pulling pressure by spindle fibres from both sides causes breakage across the centromere during anaphase-I and the consequent movement to the opposite poles is sometimes incomplete, causing lagging (Darlington, 1939; Lukaszewski, 2010). Nothing can be said with certainty about the fate of this telocentric chromosome segment in the next meiotic cycle but such broken chromosomes with a centromere at one end often pass on to the next generation. The survival chances of such broken chromosomes are dependent on the amount of chromatin flanking the centromere (Lukaszewski 2010). FISH highlighted four 5S loci, which showed that this hybrid had a full chromosome complement from white clover as in BAR09-16. Due to the breeding history of BAR10-32 involving backcrosses to *T. repens* plus selfing as well, the probability of recombination between the two species was maximised. However, no green signals were observed on the *T. repens* chromosomes, suggesting that although A and R chromosomes had apparently paired homoeologously in the BAR parent, the level of this pairing was very low due to probably the huge genomic divergence and did not lead to any useful level of recombination. Or may be that A and R chromosomes only pair in some PMCs and not others – increasing the chances of missing theses events by small sampling. Thirdly, there might be selection against gametes carrying recombined chromosomes. Alternatively, as mentioned earlier, the introgressed DNA being so small could not be detected by fluorescence *in situ* hybridization.

5.6.6 Morphology of hybrids derived from ARRRR x RRRR – RRRR(A)

The aim of the morphological analyses of the different backcross derived hybrids was to determine the relative expression of parental species-specific characters in them and to see if they were agronomically superior for direct use in the field.

BAR09 hybrids (RRRR(A)) were derived from either selfing or backcrossing 5x hybrids RRRRA with white clover. They were aneuploid with one full chromosome complement from white clover and a partial genome from *T. ambiguum*. The number of *T. ambiguum* chromosomes varied highly, ranging from two in BAR09-24 and 25 to seven in BAR09-28. Those hybrids which resulted from the crossing of 5x hybrids RRRRA with white clover had lower ploidy while those from selfing the 5x hybrids had comparatively higher ploidy. However, except for BAR09-16 and BAR09-17, the observed chromosome numbers were below the expected chromosome numbers, showing a high frequency of chromosome elimination. The number of chromosomes from *T. ambiguum* in BAR09-16 was confirmed to be four by GISH studies. The morphologies in these hybrids were intermediate (Tables 4.5.6.1, 4.5.6.2), which confirms not only the presence of chromosomes from both species but also the expression of traits from both species.

The stoloniferous stem (white clover-like) trait in all the hybrids (Table 4.5.6.1) was evidence of the expression of white clover genes. Stolon length and number showed intermediate morphology, with few exceptions. The level of stem anchorage was less in hybrids than in white clover because the nodal rooting did go to the last node as in white clover. This showed the hybrid nature of the progeny and at the same time the effect and expression of A-derived genes on their morphology. Stem anchorage was measured on a scale from 0 (no nodal roots in *T. ambiguum*) to 10 in white clover with nodal roots at every node. All the hybrids put down nodal roots but they were not usually at all nodes and not up to the youngest node, as in white clover. Similar patterns of nodal rooting from the initial 2-3 nodes of the semi erect stems were reported by Williams and Verry (1981) in 4x F_1 AARR hybrids and by Ferguson *et al*. (1990) in *T. repens* x *T. nigrescens* hybrids. The level of anchorage appeared to be generally related with the number of *T. ambiguum* chromosomes in the hybrids. With the exception of BAR09-17 and the control BAR parent (BAR-23), the higher the number of *T. ambiguum* chromosomes the lower the level of anchorage. Stem apical growth was stopped in the hybrids and the last inflorescences appeared to be terminal. By contrast, in the white clover parents the terminal buds continued to grow vegetatively and shoots did not terminate

in flower buds. Stolon lengths in the white clover parents were shorter than the hybrids. However, the white clover parents with leaf colour markings used in this experiment were very weak and did not grow very well (Table 4.5.6.1, compare stolon length in BAR09 hybrids with the coloured white clover control, C21557-815). Despite shorter stolon lengths than the BAR09 hybrids (except BAR09-27), white clover retained slow apical growth while the hybrids had terminal flowering. Numbers of florets per head were intermediate except for BAR09-24 and 28 which showed transgressive expression; numbers of florets per head in these plants outperformed both parents i.e. BAR-23 and white clover. The most obvious character which distinguished *T. ambiguum* from white clover was leaf shape. Although the leaflets were longer in all the hybrids than the white clover parent, no impact of the number of *T. ambiguum* chromosomes was evident. The thickness of the main root in the hybrids was either smaller or non significantly thicker than that in the white clover parents. However, it was observed that the main root in white clover abruptly tapered ending in fibrous roots while the main root in the hybrids was longer and tapered very gradually. Longer main roots with thicker and longer nodal roots, as compared to white clover, led to higher root to total biomass ratio in all the hybrids (with one exception) (Table 4.5.6.2). These hybrids showed the most sought after combination of stoloniferous growth and spreading system from white clover with the large and thick tap root system from *T. ambiguum*. Comparatively thicker and longer root systems in these hybrids might make them more persistent, as suggested by Isobe *et al*. (2002) for red clover hybrids.

The second generation of hybrids (BAR10) obtained from the cross, RRRR(A) x RRRR showed morphology which was more similar to white clover than the previous generation (Tables 4.5.7.1, 4.5.7.2) and this is in agreement with the results reported by Abberton *et al.* (1998) and Williams and Hussain (2008). These hybrids were aneuploids and the number of *T. ambiguum* chromosomes in this generation was lower, consistent with fading of the *T.ambiguum* specific characters in this generation. These hybrids were stoloniferous with indeterminate growth and axillary flowering as in white clover. The leaf shape was close to that of white clover. However, the fewer stolons per plant, lower level of stem anchorage than white clover and higher root to total biomass ratio showed the presence and expression of *T. ambiguum* genes. The number of *T. ambiguum*-derived chromosomes in these hybrids ranged possibly from zero in BAR10-44 to four in BAR10-62 and 63. The chromosome number in BAR10-44 was 32 but the anchorage was less than white clover and significantly higher dry root % of the total biomass as compared to the both white clover controls (Table

4.5.7.2) showed the presence of *T. ambiguum* traits. Perhaps this plant had some *T. ambiguum* chromatin introgressed into white clover chromosomes. All the hybrids had a larger root system than white clover. Kopu II which was included for comparison purpose and had the least % dry weight of its total biomass in its root system. BAR10-58, although expectedly having three chromosomes from *T. ambiguum,* had almost equal root weight to total biomass ratio to its colour marked white clover parent. It may be the *T. ambiguum* chromosomes it had might not have had strong loci responsible for the root associated characters. The moderate level of fertility and the expression of *T. ambiguum* associated phenotypes are encouraging and promising features of these hybrids.

CONCLUSIONS

All the BAR hybrids used in both the direct and indirect strategies were successfully selfed and backcrossed with white clover genotypes and large breeding populations were obtained with reasonable levels of fertility. The cross and self-compatibility of these advanced hybrids is very important for their further use in different breeding strategies.

The somatic chromosome analyses in the advanced progeny hybrids gave evidence of chromosome loss, with a few cases of chromosome gain. The chromosomes belonging to A genomes were progressively eliminated.

Meiosis was highly disturbed in the genetically complex initial hybrids but, with repeated selfing/backcrossing, meiotic chromosome pairing became progressively more normal (diploid-like) and consequently the male and female fertility and seed set improved. This was because of the elimination of non-pairing chromosomes.

Meiotic anomalies were recorded in the self and backcross derivatives of the initial hybrids. These included univalent and multivalent chromosome formations observed at metaphase, anaphase bridges, lagging chromosomes and chromosome breakage leading to the formation of micronuclei. However, these abnormalities diminished with repeated backcrossing and the hybrids with higher numbers of *T. repens* chromosomes were more diploid-like in their meiotic behaviour.

Meiotic pairing analyses based on both conventional and molecular cytogenetic methods gave evidence of allosyndetic chromosome pairing involving A-derived chromosomes in the advanced progeny in all the genetic bridge strategies. The genetic bridging species approach has apparently worked by disrupting the genomic integrity of *T. repens* due to its close genetic homology with it as well *T. ambiguum*. As a result, in addition to chromosome addition, chromosome substitution and recombination was confirmed by GISH in one strategy, and could not be ruled out in others as well.

In the hybrids integrating AxR genomes directly, white clover has apparently maintained its genomic integrity. Although a low level of allsyndetic chromosome pairing was observed in the advanced progeny, the chromosome pairing was probably mainly confined to the subgenomes of *T. repens*.

The results showed pairing, although not perfect, between the gametic chromosomes of *T. repens* (R^PR^O, autosyndesis) as well as interspecific chromosome pairing (R-pairing with O and/or A as bivalents or multivalens, allosyndesis) which provides evidence that it does not have a pairing control genetic system like the *Ph1* locus in wheat that disrupts interspecific pairing. Alternatively, if a genetic control allele exists, it might be ineffective in hemizygous condition in hybrid situations and works only where the full white clover genome is present irrespective of the fact whether it is hybrid or *T. repens*.

Molecular cytogenetics gave evidence of an impressive level of interspecific recombination (introgression) in one of the genetic bridge strategies which started with an RRAO hybrid. One plant which received a hybrid gamete apparently had four chromosomes with large sections of *T. ambiguum* DNA introgressed into a white clover background. This shows that inserting the genome of the genetic bridge species, *T. occidentale*, into a hybrid with A and R genomes can lead to a high level of interspecific chromosome pairing and, consequently, introgression.

Although no signs of introgression were apparent in the remaining genetic bridge strategies, the possibility of introgression cannot be ruled out. Either the introgressed DNA was too small to be detected by GISH or the introgression events might have missed because of the small sample size and the time limitation.

In direct hybrids having only A and R genomes and where white clover maintained its genetic integrity, there was a low level of interspecific chromosome pairing. Consequently, the possibility of introgression was very low, as evident from the GISH results. However, new knowledge (new markers) are needed for characterising introgression, as well as the use of larger samples for GISH studies.

Morphological characterization of hybrids showed that A-derived genes (whether on A chromosomes or introgressed into white clover) expressed in white clover genetic background contributing to the phenotype. Most hybrids recorded higher root percentage of the total biomass which is one of the objectives of white clover breeding using this wide hybridisation method.

It looks as though wide hybridisation can be successful in transferring traits from A to R, especially when O is used as a bridge. The direct hybrids having A& R genomes, because of

the low level of interspecific chromosome pairing and no apparent introgression, can be used as such, using no introgression breeding, if they do agronomically well in the field.

The "genetic bridge" concept

Distantly related wild species usually harbour beneficial genes conferring resistance to different diseases and other abiotic stresses. However, transfer of genes from a wild donor species to a crop species by wide hybridisation is often difficult due to the remote genetic relationship between the species. In the case of direct crossing, this leads to a low frequency of inter-specific chromosome pairing which precludes the possibility of any genetic exchange. Sometimes a third species can be used as a "genetic bridge" to facilitate wide crossing. Use of a species as a genetic bridge is based on its close genetic homology to both the recipient and the donor species thus facilitating the transfer of genes across species. The genetic bridge concept is not new and has been successfully used previously in one way or other in various crops e.g. cotton, wheat, maize, *Brassica* and alfalfa. The way it was used in cotton, maize and alfalfa specifically resembles the approach which was used in the current project. In cotton, Bi *et al*. (1999) used the diploid species, *Gossypium thurberi* Torado (2n=2x=26) and *G. raimondii* Ulbrich (2n=2x=26) as genetic bridges between cultivated cotton (*G. hirsutum,* 2n=4x=52) and *G. sturtianum* Willis (2n=2x=26). The latter species has the desirable trait of low seed gossypol and is taxonomically one of the most distantly related species to cultivated cotton. Eubanks (2006) used *Zea diploperennis*, a diploid perennial wild relative of cultivated maize as a genetic bridge for introgressing biotic and abiotic stress tolerance from eastern gamagrass (*Tripsacum dactyloides*) into cultivated maize. *Zea diploperennis* makes fertile hybrids with both maize and eastern gamagrass. Similarly, McCoy and Echt (1993) used *Medicago rupestris* as a bridging species between cultivated alfalfa (*M. sativa*) and two wild species (*M. daghestanica* and *M. pironae*). This approach has worked in the current project where four of the five breeding strategies for introgressing *T. ambiguum* chromatin into white clover were based on the use of *T. occidentale* as a genetic

bridge between *T. repens* and *T. ambiguum. T. occidentale* is one of the putative parents of white clover and is also related to the donor species (*T. ambiguum*), as is evident from their slight cross compatibility, and high level of inter-specific chromosome pairing (Williams *et al*, 2011). As a general principle, use of either a progenitor species or any other species which is genetically compatible with both the donor and the recipient species should be tried as a genetic bridge for transferring genes for stress resistance from distantly related species in the secondary gene pool to the cultivated species.

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