Comparative Cytogenetics in the Genus *Trifolium* Section *Trifolium* (Clover)

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ABSTRACT

Five species in genus *Trifolium* section *Trifolium* were investigated cytologically. The species investigated were *T. pratense*, *T. hirtum*, *T. incarnatum*, *T. alexandrinum* and *T. striatum*.

A new modified air-dried technique was used to prepare the chromosomes in order to overcome difficulties related to small chromosome size and also to produce metaphases suitable for fluorescence *in situ* hybridisation.

Chromosome numbers were confirmed for all species. *T. hirtum* was morphometrically analysed using the confocal microscope and Silicon Graphics image analysis software, C-banded, Q-banded and subjected to fluorescence *in situ* hybridisation (FISH). The FISH revealed a unique distribution pattern for 18s and 5s rDNA with the 5s and 18s signals present on the satellited chromosome pair only. For 5s rDNA, hybridisation sites were observed in three areas of the satellited chromosome, two of those sites were on either side of the 18s signal. Idiograms showing chromosome lengths and the position of C-bands were also produced. *T. pratense* was Q-banded and its chromosome number confirmed as 2n=2x=14. The chromosome number of *T. incarnatum* was confirmed as 2n=2x=14 rather than 2n=2x=16 as reported in some literature; the species was also C-banded. The chromosome number of *T. alexandrinum* was confirmed as 2n=2x=16. The chromosome number of *T. striatum* was confirmed as 2n=2x=14.

This is the first time any species in the genus *Trifolium* section *Trifolium* have been successfully C-banded, Q-banded, and subjected to fluorescence *in situ* hybridisation.

The information gained will go some way towards illuminating the evolutionary relationships between species in the section *Trifolium* and also in the genus *Trifolium*, whilst also giving support to breeding programs in place and those planned for the future.

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1.0 INTRODUCTION

The genus *Trifolium* L. (family Leguminosae), commonly known as clover, is comprised of approximately 250 species (Taylor, 1985). The genus is considered to have its main centre of origin in the Mediterranean (Pritchard, 1967, 1969; Taylor and Quesenberry, 1996); other centres of diversity include Europe, the montane and alpine zones of Africa and Central, South and North America (Zohary and Heller, 1984). Approximately one-third of *Trifolium* species are perennials with the rest being annuals. *Trifolium* leaves usually consist of three leaflets although a few have five leaflets. All *Trifolium* species possess the papilionaceous legume flower with ten stamens. All species require nodulation with strains of *Rhizobium* enabling the plants to fix nitrogen in the soil (Taylor, 1985).

Described as forage legumes, the genus has been cultivated in Europe as early as the 4th century A.D. (Zohary and Heller, 1984). More recently the genus has shown its usefulness as an animal feed in hay, pasture and silage with its high levels of protein and certain minerals (Taylor, 1985; Christou, 1994; Badr, 1995). *Trifolium* plays an important role in improving soil conditions through atmospheric nitrogen fixation, assisting in the improvement of soil tilth and water-holding capacity. Clover also plays a role in world honey production. It is estimated that eleven species in *Trifolium* are used to some extent in planted pastures. Of those eleven species, three are spread across three sections, four belong to the section *Lotoidea* that is the largest section of the genus, and four belong to section *Trifolium,* the second largest section of the genus, (Taylor and Quesenberry, 1996). Section *Trifolium* contains the species *T. pratense* (red clover)

which is the type species or lectotype of the genus as chosen by Zohary and Heller (1984). Red clover and other species in section *Trifolium* are widely used as pasture crops. In much of Eastern and central Europe, *T. pratense* is the leading legume in forage production and rates highly in the United States (Taylor and Smith, 1979).

Cytogenetic studies and cytotaxonomy lead to a better understanding of phylogenetic relationships and evolution of a genus. Karyotype characteristics are one of the important species-specific features of a eukaryote. Chromosome number, size and morphology of the chromosomes and molecular structure of the chromosomes are all karyotype characteristics. These aspects are extremely important when looking to improve a species through plant breeding techniques such as interspecific hybridisation. The closer a species is taxonomically, the more feasible the attempted hybridisation. Considering the agricultural importance of *Trifolium* it is apparent that the cytogenetics of the genus is lagging behind other commercially important species such as wheat (Gill *et al.*, 1991), barley (Marthe and Künzel, 1994), rye (Stößer *et al.*, 1993) rice (Ohmido and Fukui, 1995) and bananas (Osuji *et al.*, 1997) to name a few. The research does not advance far beyond chromosome counts that have been performed on approximately 180 out of the 250 species in the genus (Taylor, 1985). The accuracy of some of those counts is in question (Gillett, 1980; Taylor and Giri, 1984). In 1974, Gill and Kimber (as quoted by Gill, *et al.*, 1991) published research detailing C-banding in rye and wheat; to date only *Trifolium repens* L. (white clover) in the genus *Trifolium* subsection *Lotoidea* has been C-banded (Zhu *et al.*, 1996). No species in section *Trifolium* has been investigated further than a description of chromosome number, basic karyotype and idiogram. This lack of information may be related to the extensive problems in clover chromosome preparations due to their small size (Zohary and Heller, 1984). C-banding

and fluorescence banding are accepted techniques in animal and plant cytogenetics for the classification and characterisation of chromosomes and chromosome pairs. The advent of recombinant DNA technology has seen molecular cytogenetics revolutionised. Techniques such as fluorescence *in situ* hybridisation (FISH) where chromosome specific DNA sequences are hybridised *in situ* on metaphase chromosomes give us a better understanding of the molecular structure of the chromosomes as well as providing useful markers in order to identify specific chromosome pairs.

The objective of this study is to investigate cytogenetically five species in the genus *Trifolium* section *Trifolium.*. The species to be investigated are *Trifolium pratense, Trifolium hirtum, Trifolium incarnatum, Trifolium alexandrinum,* and *Trifolium striatum.* The first four were quoted by Taylor in 1996 as being used to some extent in planted pastures, the last species is also used but not to the same degree. In conducting a comparative study, proposals for evolutionary divergence and structural dynamics can be made, enhancing the understanding of the genus and sections, also expressing the relative distances between different species.

1.1 Aims

1. Confirm the chromosome number in certain species of the genus *Trifolium* section *Trifolium.*
2. Attempt to characterise the above chromosomes by chromosome banding techniques.
3. Perfect a chromosome preparation technique in order to perform *in situ* hybridisation on *Trifolium* chromosomes.
4. Identify marker chromosomes using fluorescence *in situ* hybridisation (FISH).

2.0 LITERATURE REVIEW

2.1 The genus *Trifolium* L. (clover)

A detailed overview of the taxonomic status of the genus *Trifolium* has been presented by Zohary and Heller (1984). According to most accepted classification, *Trifolium* has been placed in the tribe *Trifolieae* in the subfamily *Papilionoideae*, family *Leguminosae* (Gillett, 1980; Zohary and Heller, 1984). Many attempts have been made to classify the species within this genus into clearly defined taxonomic groups. Linnaeus (1753) divided the then known 41 species into five units, some of which were later accepted as sections (as quoted in Zohary, 1984). Presl (1830-1832) divided the whole genus into 9 genera, a move not generally followed at the time (as quoted in Gillet, 1980). However, the recent classification of the genus recognises 8 sections (Zohary and Heller, 1984), which are *Lotoidea*, *Paramesus*, *Mistyllus*, *Vesicaria*, *Chronosemium*, *Trifolium*, *Trichocephalum* and *Involucrarium*. Most of these sections have further been classified into subsections and series. These finer divisions are often contentious with different researchers recognising slightly different groupings at the subsectional level. At the variety and cultivar level a number of differences can be common with species and varieties being recognised by different names in different research works.

As was mentioned earlier, cytological investigations in the genus are limited. The first accurate recordings of chromosome numbers for *Trifolium* were published by both Bleier and Karpechenko in 1925 (Taylor, 1985). The basic chromosome numbers found in the genus *Trifolium* are x = 8, x = 7, x = 6 and x = 5. The basic number of x = 8 is found in approximately 80% of species with a known chromosome number and is present in all 8 sections. The basic number of x = 7 is found in approximately 28 species

in 5 sections. The basic number of x = 6 is found in approximately 5 species in 3 sections. The basic number of x = 5 is found in 6 species and 2 sections. Some of the records constituting these statistics are thought to be doubtful and in some cases two basic numbers have been recorded, usually x = 7 and x = 8 (Pritchard, 1969; Zohary and Heller, 1984; Taylor, 1985). It is suggested that the basic ancestral chromosome number is x = 8 and that the other basic numbers are derived, descending through an aneuploid series. The reduction in chromosome number appears to have occurred mainly in the Mediterranean species, which are thought to be more advanced (Pritchard, 1969; Zohary and Heller, 1984; Taylor, 1985). Approximately 54 species and subspecies in the genus are described kayotypically, such as chromosome lengths, position of centromeres, arm-length ratios and in a few instances distribution patterns for DNA and rDNA sequences localised by *in situ* hybridisation (Ansari *et al.*, In Press; Taylor, 1985; Zhu, *et al.*, 1996). Most of the analyses other than the molecular research were carried out before the 1980's. C-banding, the distribution pattern for a 350 bp tandemly repeated DNA sequence and the localisation of 18s and 5s rDNA sequences have been described for *T. repens* L., (Ansari, *et al.*, In Press; Zhu, *et al.*, 1996). 18s and 5s rDNA distribution patterns have also been described for *T. occidentale, T. uniflorum, T. nigrescens* ssp. *nigrescens, T. nigrescens* ssp *petrisavii, T. isthmocarpum, T. ambiguum* and *T. hybridum* (Ansari, *et al.*, In Press).

2.2 Section *Trifolium*

Among the 8 sections of the genus *Trifolium*, section *Trifolium* is the second largest, and includes 72 annual or perennial species, which are heterogenous in appearance. The section is divided into 17 subsections of the genus *Trifolium* (Zohary and Heller, 1984).

The systematic positions of the five researched species are as follows (Zohary and Heller, 1984).

Family: Leguminosae

Genus: *Trifolium*

Section: *Trifolium*

Subsection: *Trifolium*

Species: *T. pratense* L.

Subsection: *Stellata*

Species: *T. incarnatum* L.

Subsection: *Stenosemium*

Species: *T. striatum* L.

Subsection: *Lappacea*

Species: *T. hirtum* All.

Subsection: *Alexandrina*

Species: *T. alexandrinum* L.

The lectotype of the genus, red clover (*Trifolium pratense*) belongs to the section *Trifolium*, subsection *Trifolium*. Red clover is self-incompatible, cross-pollinating and diploid (2n=2x=14) (Meglic and Smith, 1992). Red clover and its relatives are internationally important legumes in many areas of pasture, soil and stock development particularly in temperate regions. Red clover can be grown in a variety of soil types, pH levels, and environmental conditions. It gives good yields in place of alfalfa which has problems in areas of high moisture and soil acidity (Kongkiatngam *et al.*, 1995). Although red clover has had long-standing acclaim in Australasia as well as internationally as a widely used pasture mix the species does have a setback in lacking

long-term persistence (Phillips *et al.*, 1982; Sawai *et al.*, 1995). *T. hirtum* (rose clover), appears to be somewhat related to *T. pratense* having produced a single weak hybrid plant in a cross with 4x red clover (Schwer and Cleveland, 1972b) and is of interest as a possible hybridisation partner. *T. hirtum* has the lowest basic number of x = 5. *T. alexandrinum* (Egyptian clover) is an annual non-reseeding, cool-season forage crop. Egyptian or Berseem clover is a valuable, highly nutritious forage crop in the United States and is widely cultivated in many other countries. The wild progenitors of *T. alexandrinum* are unknown. *T. alexandrinum* has the common basic number of x = 8 (Taylor, 1985). *T. incarnatum* (crimson clover) is a winter annual, important in the winter grazing programs of the southern United States. The species has been recorded with two differing chromosome counts of 2n = 2x = 14 and 2n = 2x = 16, basic numbers of x = 8 and x = 7 (Taylor, 1985). *T. incarnatum* has been used in hybridisation experiments with red clover but no successful hybrids were gained (Taylor and Quesenberry, 1996). *T. striatum* (striated or knotted clover) is a winter annual, reseeding forage crop. The species has the basic number of x = 7.

Out of 72 species included in the section *Trifolium*, chromosome counts are known from 52 (72%) of them. All the basic chromosome numbers (x = 8, 7, 6 and 5) of the genus *Trifolium* are represented in this section (Zohary and Heller, 1984; Taylor, 1985). 29 species are reported as having the most common basic chromosome number of x = 8, 16 species are reported as having the chromosome number x = 7, only 2 species are reported as having the chromosome number of x = 6 and 5 species are reported as having x = 5. Only one other section, *Trichocephalum*, carries the basic number of x = 5. In section *Trifolium* the basic number of x = 5 is spread over 3 subsections, with 2 species in subsection *Scabroidea*, 2 in subsection *Lappacea*, and 1 species in subsection

Augustifolia. Eight species belonging to section *Trifolium* are reported to be polyploid, out of which one appears to be exclusively polyploid (Zohary and Heller, 1984).

Approximately 11 species in the section are described karyotypically (Zohary and Heller, 1984; Taylor, 1985). All of the five species researched in this thesis have had their karyotypes described to some extent. *T. striatum* and *T. hirtum* (Fig. 2) were last analysed in the 1970's (Taylor, 1985), whereas *T. pratense* (Fig. 3), *T. alexandrinum* and *T. incarnatum* (Fig. 1) were all karyotypically described again by Badr, 1995. None of the species in section *Trifolium* have been researched beyond the basic karyotype of chromosome length, arm ratios and centromeric position.

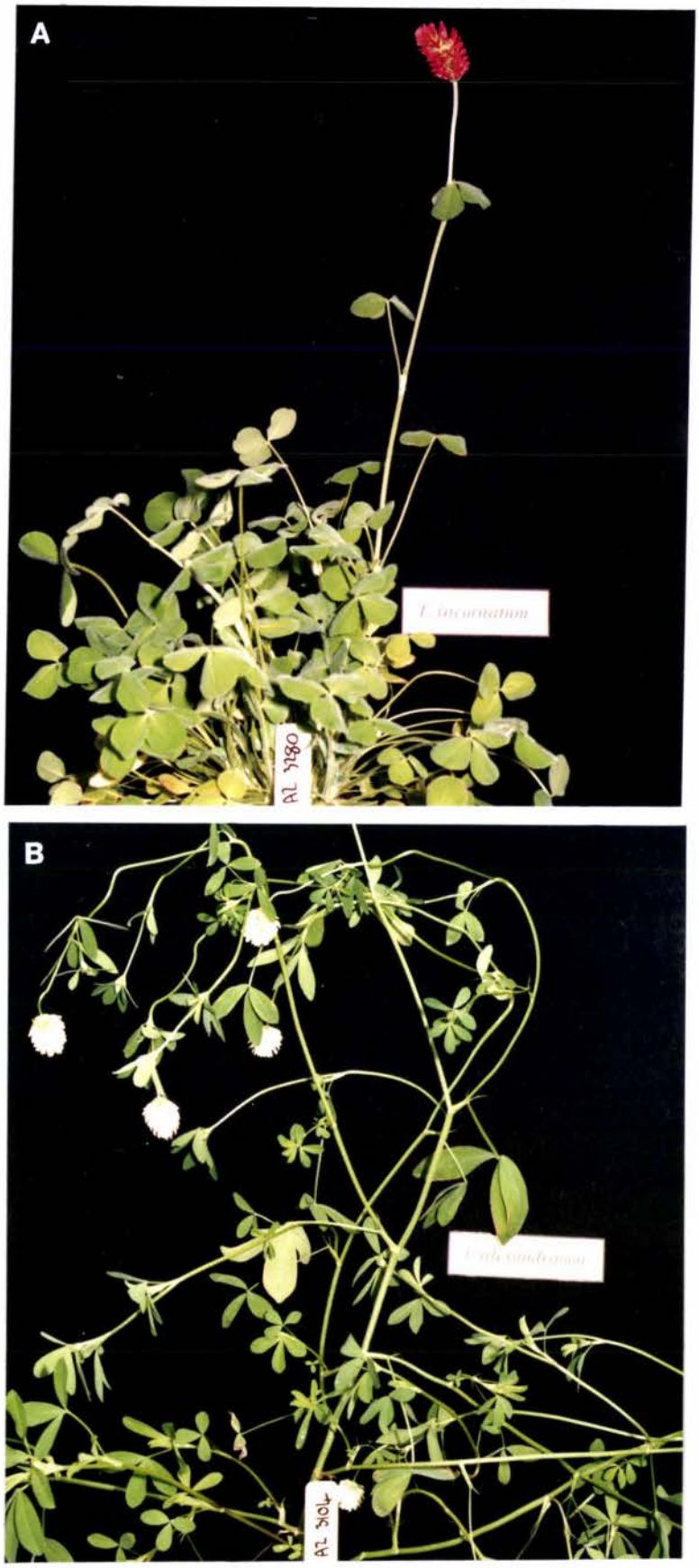

Fig. 1 Two of the species investigated were: **A**. *T. incarnatum* and **B**. *T. alexandrinum*

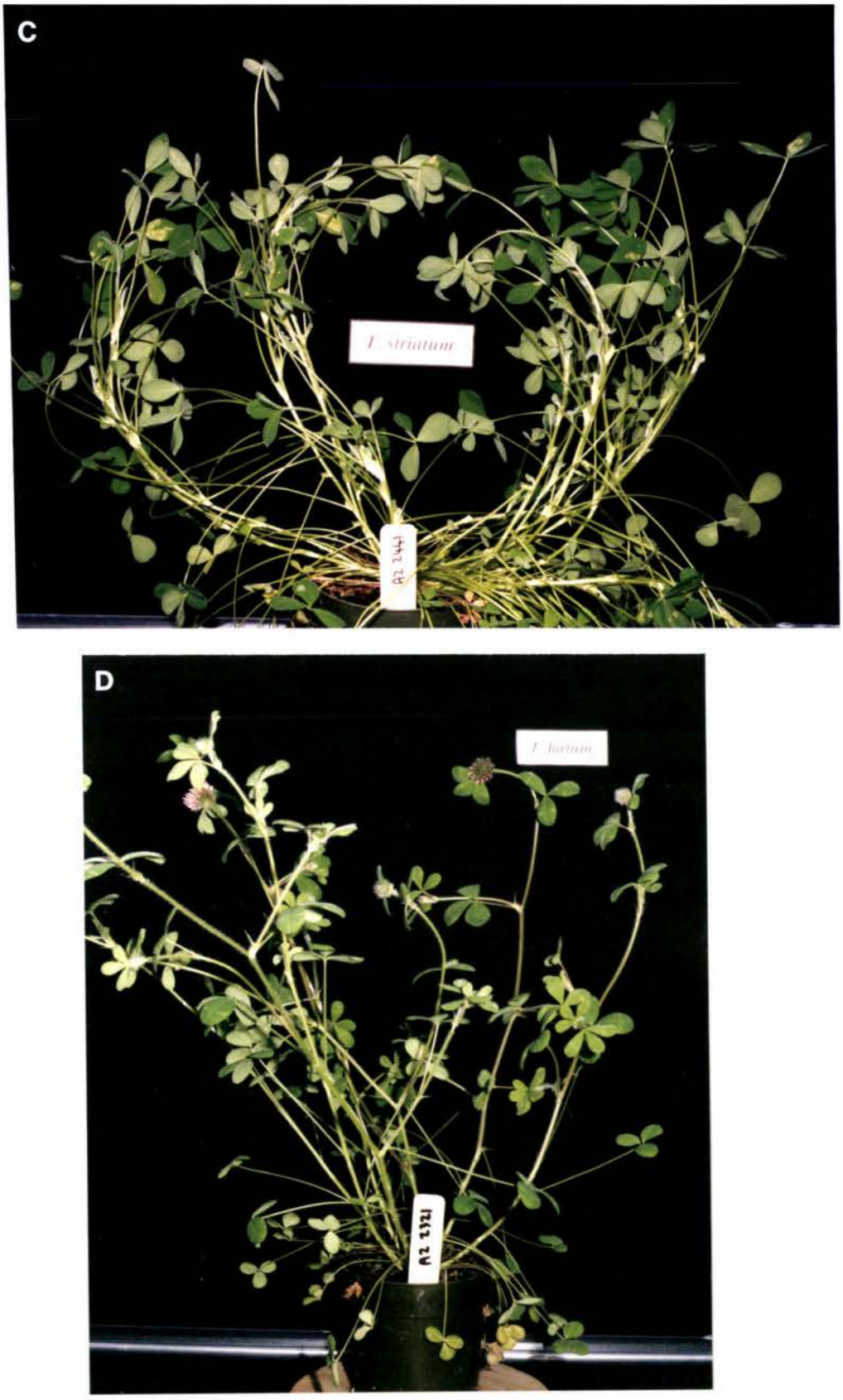

Fig. 2 Two other species investigated were: **C**. *T. striatum* and **D**. *T. hirtum*

Fig. 3 The fifth species investigated was *T. pratense*

2.3 Cytotaxonomy and Plant Breeding

Investigations into improving a plant species for agricultural purposes and research into that species taxonomy frequently run parallel or in conjunction with each other. Often the spur for investigating plant taxonomic relationships is a commercial one. Assessing the closeness of species can lead to the discovery of viable hybrid parents or even suppliers of desirable genetic material for insertion into commercially important species (Anderson *et al.*, 1972). Suggestions of progenitor species can lead to backcrossing and the exposure of favourable genetic traits for agriculture, such as persistence (Osuji, *et al.*, 1997). Species in a genus are often divided into subgroups or sections and subsections as is the case in the genus *Trifolium* (8 sections and 17 subsections, Zohary, 1984). Subdivisions are frequently suggested by looking at the morphology of the plants, habitat, seed phenotype, and persistence. It is thought that hybrid parents chosen from the same subsection are taxonomically close. Hybrid production itself is another area of taxonomic investigation; hybrid fertility, hybrid vigour, type of breeding barriers, pollen viability, seed set, and meiotic associations are all analysed. In hybridisation attempts, if incompatibility arises at the pollen germination level the species would genetically be further apart compared to incompatibility arising at the post-zygotic level (Schwer and Cleveland, 1972a; Phillips, *et al.*, 1982; Taylor and Giri, 1984; Phillips *et al.*, 1992; Yao *et al.*, 1994; Taylor and Quesenberry, 1996).

Cytotaxonomy comes in the middle of this investigative area: chromosome number, size, symmetry, satellites, banding, trisomy, translocations, inversions, DNA sequence location, the karyotype and the idiogram are all investigated (Pritchard, 1967, 1969; Anderson, *et al.*, 1972; Badaev *et al.*, 1985; Taylor and Chen, 1988; Badr, 1995; Friebe

et al., 1996; Osuji, *et al.*, 1997). The ability to identify individual chromosomes and examine any reorganisation, as well as correlate them with marker genes, is valuable for plant breeding. The molecular identity of plants has also been investigated in the form of isozyme analysis, screening of RAPDs and RFLPs, also analysis of seed proteins (Badr, 1995; Crea *et al.*, 1997; Qi *et al.*, 1997).

Senn in 1938 (as quoted by N. L. Taylor, 1985), made the first attempt to relate chromosome number and taxonomic position in *Leguminosae*. More recently Taylor and Smith, 1979 studied relationships among chromosome numbers and other characteristics of genetic systems for 200 species of *Trifolium* (Taylor, 1985). Goldblatt in 1981 (as quoted by Taylor, 1985) found anomalies with some of the chromosome counts in Senn's research, he also expanded on the influence of polyploidy and aneuploidy on the taxonomic evolution in *Trifolium.* Interspecific sexual cross-compatibility and chromosome pairing affinity in hybrids of red clover have been investigated in an attempt to relate the species taxonomically (Phillips *et al*, 1982, 1992; Taylor *et al,* 1996).

Plant breeding and the techniques involved in hybridisation have become important in attempting to overcome problems at the agricultural level in the use of the genus *Trifolium.* Such problems include bloating, dermatological conditions, estrogenic activity, toxicity due to cyanogenic glycosides, lack of persistence, plus disease and insect susceptibility (Schwer and Cleveland, 1972a; Taylor and Smith, 1979; Phillips, *et al.*, 1982, 1992; Christou, 1994; Marshall *et al.*, 1995; Sawai, *et al.*, 1995; Taylor and Quesenberry, 1996). Agricultural improvements in the genus *Trifolium* have mainly been achieved via schemes such as cultivar production through breeding of parents from

the same species with desirable traits and in the selection of ecotypes. The production of highly crossable polyploids (such as tetraploids) using chemicals such as colchicine and nitrous oxide, UV treatments, or by crosses using gametic non-reduction has helped to expose advantageous traits for breeding in the same species and also between genetically close species (Williams *et al.*, 1982; Taylor and Chen, 1988). Inter and intra specific hybrids in the genus are not common (Anderson *et al*, 1972). Some work has been done to achieve novel hybrids via embryo rescue techniques, and regeneration via tissue culture although very few species in the genus can be regenerated from *in vitro* culture (Mousset-Declas *et al.*, 1989; Christou, 1994). Further cytotaxonomic investigations would be of great assistance in the improvement of both the genus and section *Trifolium*

2.31 Some Techniques and Applications of Cytotaxonomy

Information gained about a species karyotype through the tools of cytotaxonomy such as morphometric analysis, banding and molecular techniques such as fluorescence *in situ* hybridisation (FISH), assists in the more accurate derivation of species and can lead to the likely origin of desirable attributes.

The chromosome number can often reveal a great deal about a species, such as ploidy level and type of ploidy, either alloploidy (extra chromosome sets originating from a different species) or autoploidy (extra chromosome sets originating from the same species).

The production of an idiogram is a useful tool in the cytotaxonomic characterisation of a species. An idiogram of a species chromosomes can assist in comparative work between

closely related species. It can also be utilised in the identification and investigation into addition or substitution lines, monosomy or polysomy (Jahier *et al.*, 1996). They also assist as a reference point in mutation studies (Nkongolo, 1996; Wang and Iwata, 1996; Ruas and Aguiar-Perecin, 1997). In essence, the idiogram gives a representation of each chromosome in a species genome along with the information used to identify that chromosome, such as the position of the centromere, length of the chromosome, position of any centromeric constriction(s) and any banding patterns observed (Nkongolo *et al*, 1995). Morphometric analysis involves the measuring of chromosome pairs in a genome to gain information needed to produce an idiogram. Cell states are variable at any given time. The cell cycle is dynamic, therefore a heterogenous cell population as found in a root-tip, contains mitotic stages with chromosomes in varying states of contraction. To compensate for differing states of chromosomal contraction, as well as the influence of antimitotic agents and any aberrations due to chromosome preparation, arm ratios are used to express measurements in lengths. The percent relative length ($\%L^R$) is the ratio of total chromosome length to average length of all the chromosomes in the metaphase plate. The centromeric index (I^C) is the ratio of one chromosome arm to the other, although conventionally it is the percentage of the long arm to the short arm (Jahier *et al*, 1996). The centromeric index is used to classify the chromosomes as either metacentric, submetacentric, subtelocentric or acrocentric (Stebbins, 1971). It is often difficult to identify homologous chromosome pairs in species with smaller chromosomes having similar centromere positions. The prior identification of pairs allows for a more accurate morphometric analysis hence a more informative idiogram of the species. The identification of chromosome pairs is often greatly assisted by the use of banding techniques.

C-banding is a useful tool in plant cytogenetics. The technique reveals information about the distribution of constitutive heterochromatin and in doing so it often identifies unique C-band patterns within a species relating to homologous pairs (Chen and Roath, 1995; Falistocco *et al.*, 1995; Sheikh and Kondo, 1995; Badaeva *et al.*, 1996; Ebert *et al.*, 1996; Joachimiak and Kula, 1996; Taketa and Kawahara, 1996). In many species heterochromatin occurs only at the centromere and/or telomere (Guerra, 1993; D'Emerico *et al.*, 1996; Bauchan and Hossain, 1997; Bickmore and Craig, 1997). The position of the accumulation of heterochromatin does appear to be precise within the chromosome of any particular species, although the quantity of heterochromatin between individuals in a species may vary as was found by Martin and Hesemann, 1988 in their research of an improved C-banding technique in rye. Heterochromatin often contains repeated sequences such as satellite DNA (Wagner *et al.*, 1993; Bickmore and Craig, 1997). The detection of heterochromatin using the technique of C-banding is often enhanced by the use of early metaphase chromosomes. It is pertinent to the current study, where an *in situ* hybridisation technique relevant to clover was developed, to mention the chemical process by which C-banding reveals constitutive heterochromatin. This has been outlined by Clark and Wall, 1996. The chemical process begins with the depurination of the chromosome without breaking the phosphate-sugar backbone. This is often achieved using HCl. The depurination step is stopped before the DNA is completely degraded. Subsequent alkali treatment (often with barium hydroxide) denatures the DNA (aiding solubilisation). Incubation in a warm salt wash (usually 2xSSC) breaks the sugar-phosphate backbone and DNA fragments pass into solution. Staining has been shown to be reliant on the retention of both the DNA and its proteins (yet to be isolated and characterised). The result is that the highly compacted

heterochromatin, which stains positively with Giemsa, is the only part of the chromosome that retains both components.

Q-banding was the first chromosome banding technique to be used. In 1968 Casperson first used Q-banding in plants (Caspersson *et al.*, 1968). In Q-banding, quinacrine dihydrochloride acts as an intercalating agent, preferentially fitting between the A and T residues while simultaneously enhancing the fluorescence in these positions, therefore revealing AT-rich segments on the chromosomes (Wagner *et al*, 1993; Bickmore and Craig, 1997). This technique also reveals unique banding patterns that can be related to homologous pairs (Rowland, 1981). There is evidence to suggest that the associated proteins may also be involved. The bonding is electrostatic rather than covalent and therefore can be easily reversed.

In situ hybridisation is a technique where DNA sequences are directly assigned to chromosomes. The technique was first demonstrated by Pardue and Gall in 1970 (as quoted in Wagner *et al*, 1993). These researchers used a radioactively labelled probe of mouse satellite DNA to directly hybridise with mouse chromosomes on a microscope slide. From this work it was discovered that satellite DNA mapped to the centromeric regions of mouse chromosomes. Fluorescence *in situ* hybridisation (FISH) is an advancement on this technique using a fluorescent rather than radioactive label for the specified probe (van Dekken, 1989). FISH is a useful tool for the identification of chromosomes by the use of specific DNA markers and for the differentiation of parental chromosomes in interspecific hybrids (Ansari, *et al.*, In Press; Bennett *et al.*, 1995; Riera-Lizarazu *et al.*, 1996; Fominaya *et al.*, 1997; Pederson and Langridge, 1997; Schuster *et al.*, 1997; Schwarzacher *et al.*, 1989).

3.0 MATERIALS AND METHODS

3.1 Plant material

Seeds were obtained from the Margot Forde Forage Germplasm Centre, AgResearch Grasslands, Palmerston North, New Zealand. Five species of the genus *Trifolium* belonging to section *Trifolium* were investigated. The species/cultivars, accession numbers and the origin of the seed collection are listed in Table 1.

Species	Accession Number	Origin of Seed (Company and Country)
Trifolium hirtum (Rose Clover)	AZ 2321	Seed Control Department, Agricultural Technical Services, Pretoria, SOUTH AFRICA
Trifolium hirtum c.v. Kondinin	AZ 898	CSIRO, Canberra, AUSTRALIA
Trifolium pratense c.v. Colenso (Red Clover)	F 2657	Grasslands, Pyne Gould Guiness, Christchurch, NZ
Trifolium pratense c.v. Hamua	F 2256	Grasslands, MAF, Palmerston North, NZ
Trifolium alexandrinum c.v. Meteor (Egyptian Clover)	AZ 310	P.H. Peterson Saatzucht, GERMANY
Trifolium incarnatum (Crimson Clover)	AZ 3280	Germplasm Centre, Aorangi, NZ
Trifolium striatum (Striated Clover)	AZ 2441	Grasslands, Palmerston North, NZ

Table 1. Species studied along with their accession numbers and the origin of seed collection.

3.2 Root-tip pretreatments

Scarified seeds were sown on moistened filter paper in plastic petri dishes, and germinated in a 25°C incubator or left at room temperature (ranging from 20-26°C). Once seedlings reached 5mm-15mm they were transferred onto filter paper soaked with an antimitotic agent. Three antimitotic agents were used, applying different treatment times and temperatures to find the agent that gave a high mitotic index with chromosomes predominantly in prometaphase. The antimitotic agents, concentrations, treatment times, temperature and specifics are listed in Table 2.

After the completion of pretreatments, the seedlings were rinsed in chilled distilled water and transferred to vials of freshly prepared methanol:glacial acetic acid (3:1) fixative (AnalaR BDH). To reduce dilution of the fixative with phenolic compounds released from the whole seedlings, the first aliquot of fixative was drawn off after a brief agitation, and a fresh aliquot added, then the vial capped and sealed with parafilm. The fixed tissue was left at room temperature in the dark for 24 hrs. After 24 hrs, the old fixative was decanted and fresh fixative added. The tissue was stored at 4°C for 5-7 days before being used for chromosome preparation.

Antimitotic Agent	Concentration	Treatment	Duration and Temperature
8-Quinolinol (Sigma and BDH)	4mM	root-tips transferred to treated filter paper and kept in darkness for the duration	2 hours room temperature (18-25°C), then 10 hours at 4°C
Colchicine (Sigma) (HPLC)	0.05%	root-tips transferred to treated filter paper and kept in darkness for the duration	3 hours room temperature (18-25°C) or alternatively 4 hours room temperature (18-25°C)
Tap water	–	root-tips transferred to vial of tap water in an ice-bucket stored in a cold room	24 hours, 0°C- 2°C

Table 2. Antimitotic agents, concentrations, treatment duration, and temperature.

3.3 Glass slide cleaning.

The following process was initiated to remove waxy layers on the slides which could have interfered with cytological work such as *in situ* hybridisation. The slides were soaked in Pyroneg detergent (Diversey, NZ Ltd) for 2hrs, then washed for four hours under running tap water. After which, each slide was rinsed in running RO (Reverse Osmosis purified tap water), then transferred to a jar containing 100% ethanol for storage until use.

3.4 Chromosome preparation

The Feulgen squash technique and a modified air-dried method developed at AgResearch (Ansari *et al*, In Press) were both used in chromosome preparations. The Feulgen technique was used for *Trifolium hirtum* in some preparations viewed with the confocal microscope. The air-dried method was used for all species. In previous protocols for chromosome preparation, plant tissue was often softened by hydrolysis

using 1-5N HCl at room temperature or 60°C. The air-dried technique was developed to avoid HCl hydrolysis, which alters the chromosome chemical structure, affecting the success of *in situ* hybridisation as probe binding is reduced.

3.41 Air-dried method

Five to seven day old fixed seedlings, stored at 4°C, were rinsed in 1x citrate buffer at room temperature (see Appendix 1.1). The proximal 5mm region of each root-tip was separated from the rest of the seedling and then washed 3x in citrate buffer for five min per wash. While rinsing was proceeding, a microcentrifuge tube of enzyme solution (see Appendix 4.2) was removed from -20°C storage and brought to room temperature. After the final buffer rinse, 6-8 drops (just enough to cover the root-tips) of enzyme solution was added, the vial capped and immediately incubated at 37°C for a selected length of maceration time, depending on the species (from 44 min 30 sec for *T. striatum* to 65 min for *T. hirtum*). Maceration time was highly critical as excessive treatment would also digest the chromosomes, hence the species-specific timing.

During incubation, fresh fixative was made and stored in a -20°C freezer. At the end of maceration, the enzyme solution was pipetted off and fresh citrate buffer added to rinse and stop the digestion process. The root-tips were incubated for 15 min in citrate buffer at room temperature.

Slides were removed from ethanol and dried with tissue paper. Using a pasteur pipette, a root-tip was placed on a slide along with a drop of buffer and viewed under a dissecting microscope. Using 0.1ml syringes with needles (Becton-Dickinson, 1ml 26_G 1/2), an attempt was made to remove any sheath material adhering to the root as this material

appeared to hamper obtaining a clear chromosome spread. The meristematic tissue was then cut from the main part of the root and the excess root tissue discarded. The buffer droplet was syringed off and the surplus buffer blotted with filter paper. A small drop of 45% acetic acid was added to the meristematic tissue and left for up to one and a half minutes to penetrate the tip and force the cells apart. The tip was then tapped and macerated with the point of a syringe needle to release the cells; debris was also removed. Fresh fixative removed from -20°C storage was applied to the slide as a single drop from a height of approximately 20cm, bursting cells and spreading chromosomes while dislodging adhering cytoplasmic material. The slide was air-dried and viewed under a microscope using phase contrast optics to obtain a general indication of the mitotic index, and clarity of chromosome preparation. If needed, minor changes in protocol such as additional maceration in 45% acetic acid were made at this point before continuing with the chromosome preparations.

3.42 Feulgen squash technique

Root-tips were rinsed in citrate buffer and then hydrolysed in a vial of 1N HCl in a beaker of water at 60°C for 10 minutes. Hydrolysis was stopped by pouring vial contents into a beaker of cold tap water. Root-tips were removed from the beaker to a vial of Schiff's reagent and stored in the dark for approximately 40-60 minutes until the root-tips stained purple. The root-tips were macerated on slides in a drop of 45% acetic acid, then 22x22 mm coverslips were placed on the tissue. With the help of blotting paper the tissue was squashed by pressing down on the coverslips using great force. Coverslips were removed after freezing the slide in liquid air. Slides were rinsed in 100% ethanol for 2 minutes before air-drying. These slides were then viewed using the confocal microscope.

A modification of the above method involved taking slides prepared with the air-dried method, hydrolysing them in 1N HCl in a Coplin jar in a waterbath at 60°C for 10 min, rinsing them under tap water, applying Schiff's to the slide surface, and incubating in the dark for 40-60 min. After incubation, the slides were rinsed with 45% acetic acid, air-dried and viewed with the confocal microscope.

3.5 Chromosome staining

Classical cytogenetic analysis included conventional Giemsa staining, C-banding, Q-banding and Feulgen staining. In the present work, molecular cytogenetics included the distribution pattern of two types of rDNA sequences in *T. hirtum* using fluorescence *in situ* hybridisation (FISH).

3.51 Conventional Giemsa staining

A number of slides from each air-dried preparation were subjected to conventional Giemsa staining. This was performed in order to determine chromosome number, degree of extension of the chromosomes (whether they were in early, middle or late metaphase) and chromosome morphology.

1.5ml of Giemsa stock (see Appendix 2.1) was mixed with 60ml Sorensen's buffer (see Appendix 1.2) at pH 6.8. The slides were stained for 18 minutes, rinsed in distilled water, air-dried and the preparations mounted in immersion oil; a coverslip was applied to the surface of the mountant. The slides were screened for clean metaphases with minimum overlaps. The co-ordinates for these metaphases were noted. Suitable metaphases were photographed by a camera mounted on the microscope (either Zeiss

Axioskop or Nikon Microphot-SA microscopes and Zeiss 35mm or Nikon NFX-35 cameras, using Kodak TechPan 2415 black and white 35mm film). Once the negatives were developed and ascertained for quality, the slides were destained.

Destaining involved soaking slides in a Coplin jar of xylene for two to three hours to loosen the coverslip, facilitating its removal. The slides were then rinsed in fresh xylene for three minutes, and air-dried. The slides were immersed in fixative (3:1 ethanol:acetic acid) and gently agitated for two minutes to remove the bulk of the Giemsa stain, and rinsed in fresh fixative, removing any residual stain. The slides were air-dried for two days before further manipulation was carried out. In this study the process of subjecting particular metaphases to a number of staining/destaining/banding regimes is described as consecutive staining.

3.52 C-banding

T. hirtum, T. incarnatum and *T. pratense* were C-banded. C-banding was also attempted on previously Giemsa stained, photomicrographed and destained metaphases of *T. pratense* and *T. hirtum.*

Fresh air-dried chromosome preparations were placed in 100% ethanol for 24 hours, after which the slides were air-dried for five minutes and then incubated in 0.2N HCl for 2.0-2.5 minutes at room temperature. The slides were rinsed three times in RO water and incubated in a freshly prepared, saturated solution of barium hydroxide (see A4.1) in a water bath at 50°C for 6.5-7.5 minutes. The slides were then rinsed three times in distilled water and incubated in 2 x SSC (see A1.3) for 1 hour at 60°C. Then the slides were stained (without rinsing) in fresh 2.5% Giemsa for 19 minutes. The stain intensity

was checked at this stage and the slides stained for longer if the chromosomes appeared too pale or rinsed in RO water to lighten the stain if the chromosomes appeared too dark. Metaphases that appeared to be banded were photomicrographed. Some preparations that had already been Giemsa stained, photomicrographed and destained were also C-banded, Giemsa stained and the same metaphases photomicrographed.

3.53 Quinacrine mustard staining

T.pratense and *T.hirtum* chromosomes were Q-banded. Chromosome preparations were made using the air-dried method. The preparations were initially screened under brightfield conditions for metaphases with extended chromosomes, the co-ordinates of which were noted. The chromosomes were stained for 40 sec in quinacrine mustard stain at room temperature (see A2.2, working solution). The slides were then dipped in fresh MilliQ, dipped in Sorensens buffer pH 6.8, once again dipped in fresh MilliQ and drained before mounting in Sorensens buffer. Q-banded metaphases were located using the co-ordinates noted earlier, and photomicrographs taken using a microscope fitted with epifluorescence equipment. The excitation filter used was 400-440 nm and the barrier filter used was 470 nm. Kodak TechPan 2415 black and white 35mm film was used. The slides were destained by rinsing in RO and air-dried for at least three days. Some preparations were treated consecutively; firstly stained with quinacrine mustard, then destained and stained with Giemsa, the same metaphases being photomicrographed in both treatments.

3.6 Chromosome measurement and morphometric analysis

Feulgen-stained root-tip squashes showing metaphase chromosomes were viewed with the confocal microscope. The fluorescent chromosome images were scanned using an Argon/Krypton laser, and initially saved to an OS9 computer hard drive then transferred to a Silicon Graphics computer containing an image analysis software package called ImageSpace, developed by Molecular Dynamics. Twelve metaphases with no overlapping chromosomes and of a similar extension were eventually scanned. The metaphases were numbered, each chromosome in a metaphase was assigned a code, and a mark indicating the position of the centromere was made.

The long and short arm of each chromosome was measured in μm using the 2 dimensional polygon tool (for curved chromosomes) and the straight line tool in the software. The mouse was used to indicate the start and end point of each measurement. Three measurements were made of each chromosome arm and the values averaged to reduce measurement error. Values for total length, percent relative length (total chromosome length x 100 ÷ total genome length) and centromeric index (length of short arm of a chromosome x 100 ÷ total chromosome length) were calculated for every chromosome. A combination of these calculations and photographic data were used to pair homologous chromosomes. Data from paired chromosomes were then averaged and the chromosomes ranked from largest to smallest in each metaphase. Finally data from all the metaphases were combined to give a measurement for each chromosome. Standard errors were calculated in the final data analysis for homologous pairs across the metaphases (Anderson, M. K. *et al*, 1972). The nomenclature used to describe the position of the centromere followed that of (Levan *et al.*, 1964).

3.7 Fluorescence *In Situ* Hybridisation (FISH)

FISH was performed on *T. hirtum* only. Slides were prepared using the modified air-dried method. The slides were screened in order to locate metaphase spreads that appeared free of cytoplasmic debris, with no overlaps and were situated around the middle area of the slide. The relevant area was marked with a diamond marker. The slides were dried for two hours at 37°C.

3.71 Probe labelling

The two rDNA probes were 5s and 18s ribosomal RNA sequences (accession numbers AF072692 and AF071069 respectively in the Genbank DNA sequence database). The probes were isolated from *T.repens*, amplified by PCR and then cloned in a plasmid vector by Dr Nick Ellison of AgResearch, Grasslands, Palmerston North, New Zealand. A nucleotide-fluorescent dye complex (supplied by Amersham) was used to directly label the two rDNA probes using a nick translation kit and following the manufacturer's instructions. The fluorescent dye Cy3 (red) was used in the label for the 5s probe and the fluorescent dye FluorX (green) was used in the label for the 18s probe.

3.72 *In situ* hybridisation

135 µl of 0.1µg/µl RNAse (see A3.1) was added to the slides, covered with plastic coverslips and incubated in a humid chamber (see A3.2) at 37°C for 50 minutes. The plastic coverslips were removed and the slides washed in a Coplin jar of 2xSSC (see A1.3) at room temperature three times at five minutes per wash. The chromosome preparations were then fixed in 4% paraformaldehyde for ten minutes at room

temperature and rinsed three times in 2xSSC for five minutes per rinse. The slides were then taken through a dehydration series of 70%, 90% and 100% AnalaR ethanol for three minutes each respectively. The slides were left to air dry for one and a half hours. The chromosomal DNA was denatured in 70% formamide-2xSSC (see A3.4) in a waterbath at 73°C for two minutes and ten seconds. The slides were then passed through another dehydration series at 0°C (the ethanol grades having been stored at -20°C), 70% ethanol for 10-15 seconds, then 70% ethanol for two minutes, then 90% and 100% ethanol for two minutes respectively. The slides were air-dried for 10 minutes. While the slides were air drying, the hybridisation mixture was prepared in a microcentrifuge tube (see A3.8). The mixture was mixed by hand and briefly vortexed. After microfuging for 30 seconds, the top of the microcentrifuge tube was pierced to prevent 'bumping' and the microcentrifuge tube was boiled for seven minutes, denaturing the mix. The mix was chilled for three minutes and briefly centrifuged to bring down any condensed water. The hybridisation mixture was applied to each slide in a quantity of 37-40µl, plastic coverslips were applied and the slides incubated in a humid chamber at 37°C for sixteen hours.

3.73 Post-hybridisation washing

After sixteen hours the slides were dipped in 2xSSC at 40°C in a waterbath, rinsed twice in 2xSSC for four minutes per rinse, washed in 50% formamide-2xSSC for six minutes (see A3.5), and finally washed twice in 2xSSC for four minutes per wash. The slides were left to come to room temperature, then fresh 2xSSC was added and the slides rinsed for a further ten minutes at room temperature.

3.74 Chromosome counterstaining and mounting

A DAPI counterstain was used as follows in order to identify metaphases for photographing without quenching their probe fluorescence signal. After ten minutes the slides were briefly rinsed in 4xSSC-Tween20 (see A3.6), drained and 245µl of 1µg/ml DAPI stain (see A2.3) was placed on each slide, covered with a plastic coverslip and stained for six minutes. After removing the coverslip, each slide was rinsed in 4xSSC-Tween 20, drained and mounted in Vectashield (a mountant that reduces fading of the fluorescence during storage, supplied by Vector Laboratories); the excess mountant was removed by pressing the coverslip. The slides were left for two hours at room temperature then transferred to a fridge at 4°C for storage. In order for the fluorescence to fully develop the slides were left for twenty-four hours before being viewed under the fluorescence microscope.

3.75 FISH fluorescence photomicography

The labelled metaphases were first located using the co-ordinates obtained earlier. They were initially viewed with DAPI fluorescence, using the filter set UV2A, with an excitation filter of 330-380 nm and a barrier filter of 420 nm. The filter set B-2A was then used, with an excitation filter of 450-490 nm and a barrier filter of 520 nm to view and photomicrograph the green Fluor-X label. Then the filter set was changed to G-2A, with an excitation filter of 510-560 nm and barrier filter of 590 nm for viewing and photographing the red Cy3 label. In culmination a double exposure was made of one metaphase using the two different filter sets for the two labels in order to obtain a photo with both labels visible.

3.76 Developing film

Kodak TechPan 2415 35mm black and white films, exposed for brightfield photomicography, were developed using Kodak Technidol liquid developer (Cat no. 1083849) at 21°C for 12 min and fixed in Ilford Hypam fixative (Ilford Hypam concentrate and tap water 1:4, stored at room temperature) for 6 min.

QFQ-stained metaphases were photographed with Kodak TechPan 2415 using fluorescence photomicography. Kodak D-19 developer was used to develop the film. 350 ml developer at 21°C was used to develop the film for 5 min, and Ilford Hypam fixative was used to fix the film for 6 min. Merit RC glossy graded paper and Ilford Ilfospeed glossy, medium weight paper was used to print the black and white photos. Photos of FISH were taken using Fuji G Plus 400 colour film and developed by a commercial processor. Confocal photos were taken with Kodak daylight colour film and commercially developed.

4.0 RESULTS

4.1 *T. hirtum*

Conventional Giemsa staining

More then 50 mitotic cells ranging from late prophase to late metaphase were studied. Late metaphase cells from 18 plants belonging to two accessions revealed the diploid chromosome number to be 2n=10. Prometaphase and early metaphase cells, however, showed 12 chromosomal bodies in conventional Giemsa stained preparations. The two additional chromosomal bodies proved to be the satellites of the chromosome pair carrying NORs (nucleolus organising regions) (Fig.4). The size of the secondary constriction on these chromosomes was significantly enhanced because of the highly decondensed and stretched chromatin fibrils of the NORs. The chromatin fibrils connecting the satellite and the main body of the NOR chromosome were visible in some metaphases. The satellited body was relatively larger than other species studied here.

Morphometric analysis

The results of the morphometric analysis of *T. hirtum* based on 12 prometaphase cells are presented in Table 3. SEM is the standard error of the mean and the chromosome classification is the description of the position of the centromere. All lengths are in μm. The satellite body is included in all calculations as part of the chromosome. The decondensed NOR region is not measured. In other words, the long arm of the satellited chromosome is that region of the chromosome from the centromere to the telomere of the satellite excluding the decondensed region.

The total haploid genome length of *T. hirtum* was found to be 17.50 µm. Chromosome 1, the largest chromosome, was 4.29 µm long and constituted 24.5% of the average haploid relative length (%L^R). There was a marked size difference between chromosome pairs 1 and 2 while chromosomes 2 to 5 decreased gradually in size. The smallest chromosome pair, chromosome 5 was 2.97 µm long and constituted 16.97% of the haploid relative length. The NOR was located slightly proximal to the middle of the long arm of chromosome 2. All the chromosomes of *T. hirtum* were biarmed and their centromeres were located in the median region. The chromosomes were classified according to the guidelines of Levan *et al* (1964) as seen in Table 4. An idiogram of *T. hirtum* was constructed using the data obtained from the morphometric analysis (Fig. 5).

C/some Number	AvT (µm) ± SEM	%L^R ± SEM	I^C ± SEM	C/some Classification
1	4.29 ± 0.17	24.50 ± 0.99	39.57 ± 1.18	m
2 (Satellite C/some)	3.51 ± 0.13	20.06 ± 0.71	41.09 ± 1.15	m
3	3.45 ± 0.17	19.73 ± 0.95	39.34 ± 1.18	m
4	3.28 ± 0.14	18.74 ± 0.83	42.29 ± 0.95	m
5	2.97 ± 0.15	16.97 ± 0.88	39.73 ± 1.41	m
Total haploid genome length =17.50 µm				

Table 3 Results of morphometric analysis. Average length (AvT), average percent relative length (%L^R), and centromeric index (I^c) in µm. The chromosome classification for each pair of homologous chromosomes of *T. hirtum* is also shown.

Label	I^c	Centromere Position
M	50.0	Median point
m	50.0-37.5	median region
sm	37.5-25	submedian region
st	25.0-12.5	subterminal region
t	12.5-0.0	terminal region
T	0.0	Terminal point

Table 4 Classification of chromosomes on the basis of centromeric position according to Levan *et al* (1964).

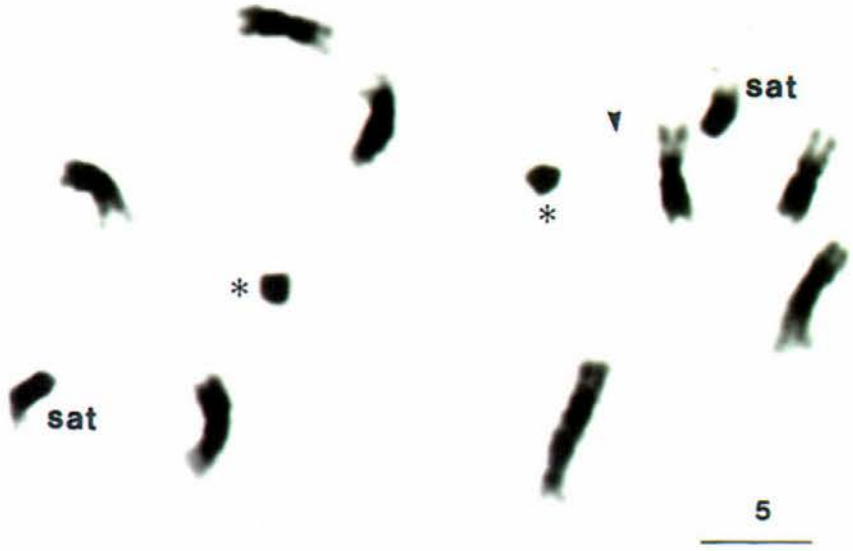

Fig. 4 Conventionally Giemsa stained prometaphase spread of *T. hirtum* showing satellite bodies ✱ and chromatin fibrils connecting a satellite to the main body of the NOR chromosome (arrowhead). Scale bar in μm.

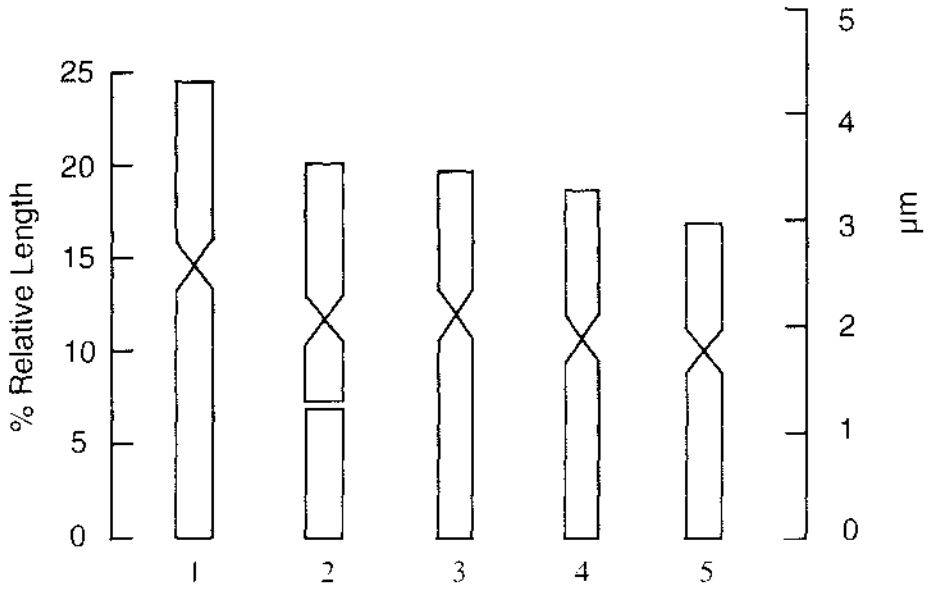

Fig 5 Idiogramof *T. hirtum* chromosomes. Size as a percentage of relative length is shown on the left-hand scale. Size in µm is shown on the right-hand scale. Chromosomes are ordered and numbered in decreasing length. Chromosome 2 carries a satellite on the long arm, with the secondary constriction indicated by a space.

Giemsa C-banding

The Giemsa C-banding method revealed the presence of a small amount of constitutive heterochromatin in the karyotype of *T. hirtum*. C-bands were confined to centromeric and/or pericentromeric regions (Figs 6 and 7). No telomeric C-bands were detected. A narrow C-band was observed at the centromeric region of the largest chromosome pair. The satellited chromosome showed relatively large amounts of constitutive heterochromatin. The NOR-bearing arm displayed a narrow C-band immediately

adjacent to the centromere and a C-band at the proximal end of the satellite. The non-satellited short arm had a broad C-band immediately flanking the centromere, which in extended preparations, could be distinguished as two bands (Fig.6). A broad C-band was found at the centromere and pericentromeric regions of the apparently third largest chromosome. The smallest two pairs of the karyotype showed a narrow C-band in the centromeric region. One of these pairs had an additional, pale, intercalary C-band on the short arm (Fig. 7). Because of the minor difference in size, it was difficult to assign chromosome pair numbers to the last three pairs of C-banded chromosomes, nevertheless approximate band positions are represented on the idiogram of *T. hirtum* (Fig. 8).

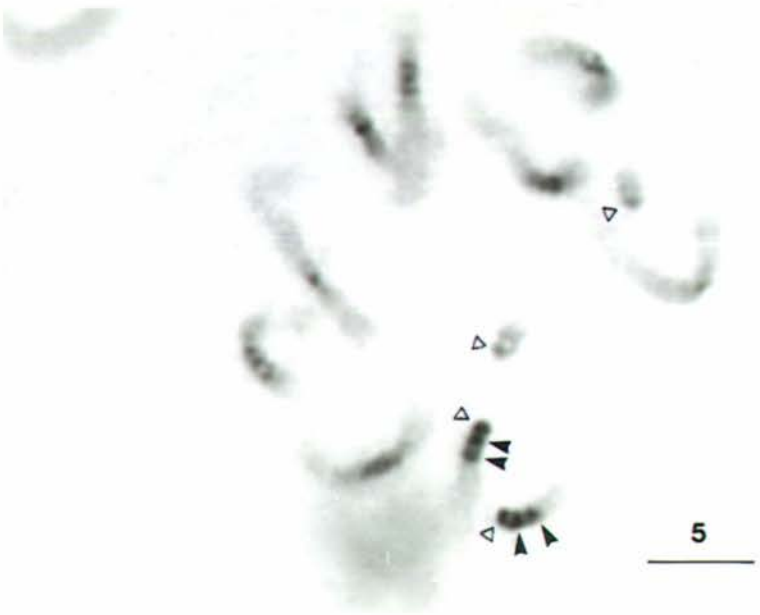

Fig. 6 C-banding of an extended metaphase spread of *T. hirtum*. Positions are indicated for a C-band on the long arm of the satellite chromosome adjacent to the centromere (open arrowhead), a C-band at the proximal end of the satellite (open arrowhead) and two C-bands on the short arm of the satellite chromosome adjacent to the centromere (closed arrowheads). Scale bar in μm.

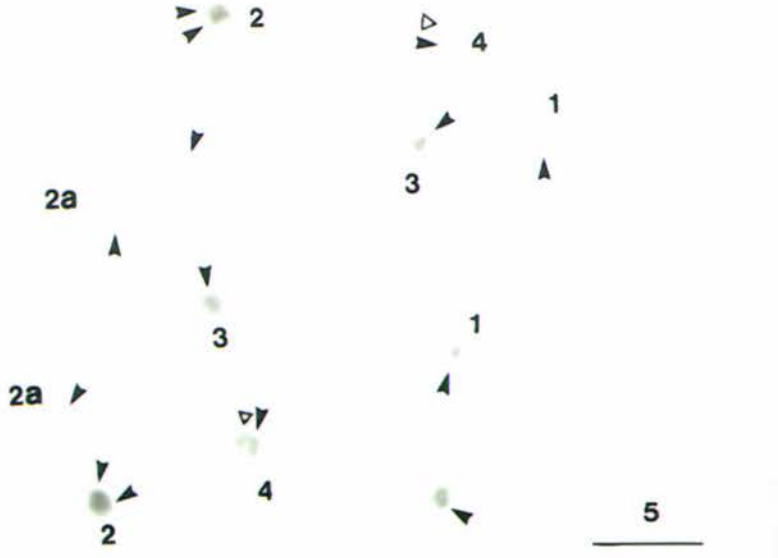

Fig. 7 A C-banded metaphase of *T. hirtum* showing C-bands at the centromeres of all chromosomes (closed arrowheads). (**1**) Indicates the largest chromosome pair with a narrow C-band at the centromere. (**2**) Indicates the satellited chromosome with C-bands on the long and short arms on either side of the centromere. (**2a**) Indicates the satellite body with a faint proximal C-band. (**3**) Indicates the apparently 3rd largest chromsome with a broad C-band at the centromere and pericentromeric regions. (**4**) Indicates a chromosome pair with an additional pale intercalary C-band (open arrowhead). Scale bar in μm.

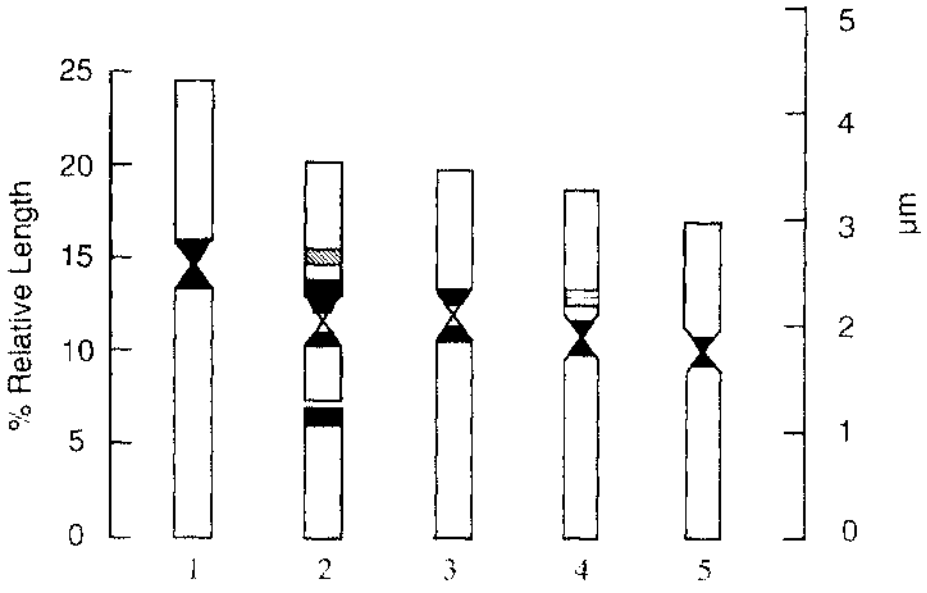

Fig 8 Idiogram of *T. hirtum* showing the distribution of heterochromatin in the form of C-bands. The secondary constriction on the long arm of chromosome two is indicated by a space. ■ Indicates the position of a C-band. ▨ Indicates the position of a C-band visible in only some extended preparations, ▭ Indicates the position of a pale intercalary band on the short arm of one of the two shortest pairs of chromosomes (pairs 4 and 5). The assignment of banding patterns to the three shortest chromosomes was not conclusive.

Q-banding

Differential staining of the chromosomes was observed at metaphase, although the resolution of these bands was clearer in prometaphase spreads. Prometaphase cells showed Q-bands on at least two pairs of chromosomes (Fig. 9). Both the satellited chromosomes displayed a prominent and bright Q-band adjacent to the centromere on the satellited arm but no bands were seen on the satellite itself. In all preparations the decondensed NOR region did not show quinacrine mustard staining. Another pair also showed a distinctive banding pattern with one Q-band located immediately adjacent to

the centromere and another band in the intercalary region of the same arm (Fig. 9, 'b'). Indistinct Q-bands were found at the centromeric regions on at least one other pair of chromosomes (Fig. 9, 'c').

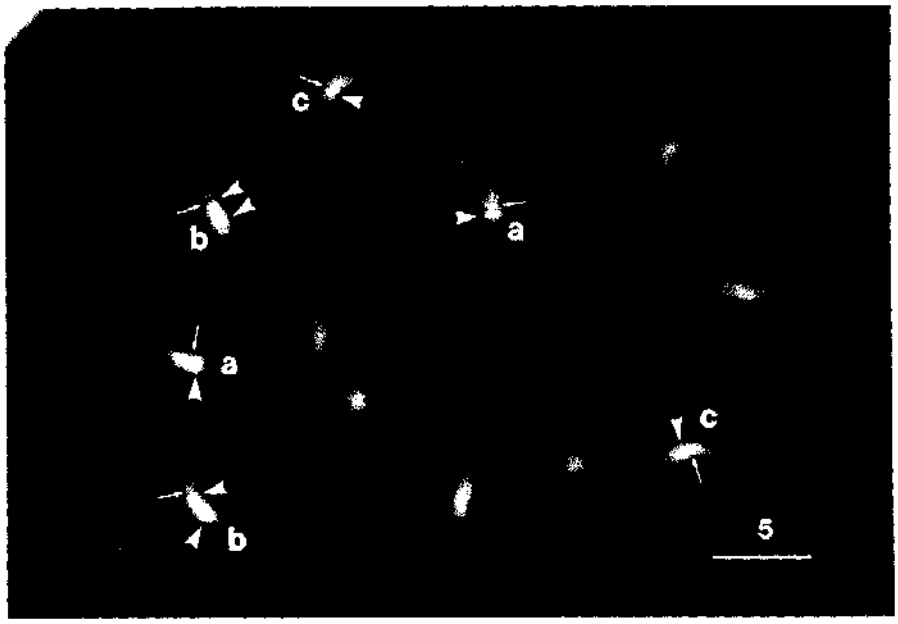

Fig. 9 A Q-banded prometaphase spread of *T. hirtum*. (**a**) Indicates the satellite chromosome with a bright Q-band adjacent to the centromere on the satellited arm (arrowhead). (**b**) Indicates a chromosome pair with two Q-bands, one adjacent to the centromere and one in the intercalary region of the same arm (arrowheads). (**c**) Indicates a chromosome pair with a faint Q-band at the centromeric region (arrowhead).

→ Indicates the position of centromeres on Q-banded chromosomes. Scale bar in μm.

FISH

DAPI fluorescence was used to identify metaphases (Fig. 10). The 18s probe signal was found on the satellited chromosome pair only. The signal was spread along the decondensed NOR region and also on small condensed regions of chromatin on either side of the decondensed area (Figs. 11).

The 5s probe signal was also found only on the satellited chromosome pair. The signal was present in three areas of the chromosome: (i) adjacent to the centromere on the short arm, (ii) all of the long arm from the centromere to the 18s signal on the proximal side of the NOR, and (iii) on the proximal side of the satellite, adjacent and distal to the 18s signal (Fig. 12). A double exposure showed the exact positions of the probes in relation to one another (Fig. 13). A drawing showing the positions of the signals on the satellite chromosome is shown in Fig. 14.

The interphase nucleus in Fig.15 shows two red/green complexes. In one complex, two red 5s dots ('a' in photo) are positioned close together, separated presumably by the centromere. The third red 5s dot ('b' in photo) is further away from the other two red dots and is likely to be the 5s signal on the satellite of the same chromosome. A fine green thread ('c' in photo), presumably the labelled decondensed NOR region, connects this third red dot to a green dot which is likely to be the 18s signal on the proximal side of the NOR. The 18s signal on the satellite body that is seen on metaphase chromosomes (Fig. 11) is not obvious in this particular nucleus. Interestingly, a noticeable unlabelled gap (between 'a' and 'c') is observed between the proximal 18s dot and the closer of the two red pericentromeric 5s signals, whereas in metaphase

spreads these two signals appear to be immediately adjacent to one another. The other red/green complex in the same nucleus would be the homologous chromosome.

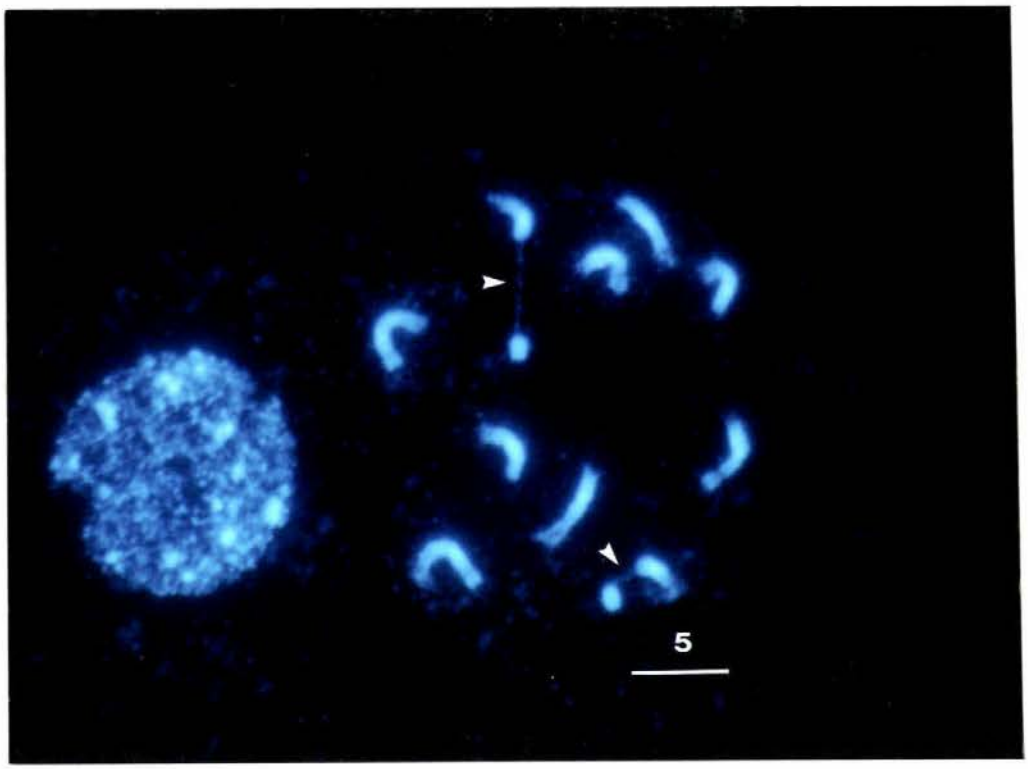

Fig. 10 Metaphase spreads of *T. hirtum* were stained with DAPI after FISH in order to identify the metaphases when viewed under a fluorescence microscope. Note the chromatin fibrils connecting the satellite to the main body of the satellite chromosome (arrowheads). Scale bar in μm.

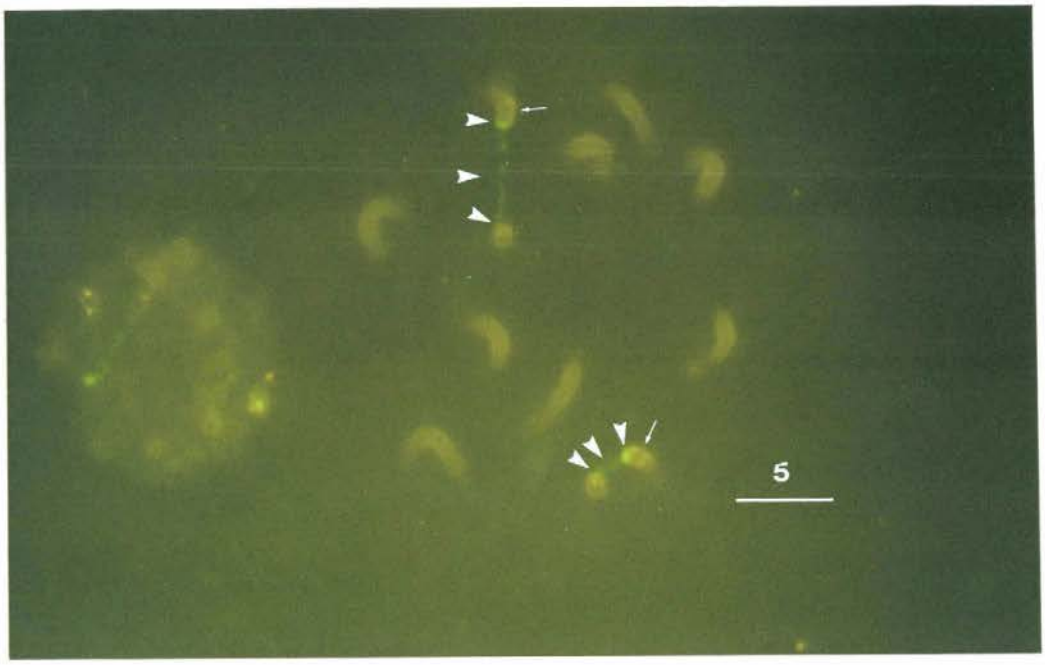

Fig. 11 Metaphase spread of *T. hirtum* after FISH. The position of the 18s signal (green) is shown spread along the decondensed NOR region and on small condensed regions of chromatin on the satellite and long arm of the satellite chromosome (arrowheads). An arrow indicates the position of the centromere. Scale bar in μm.

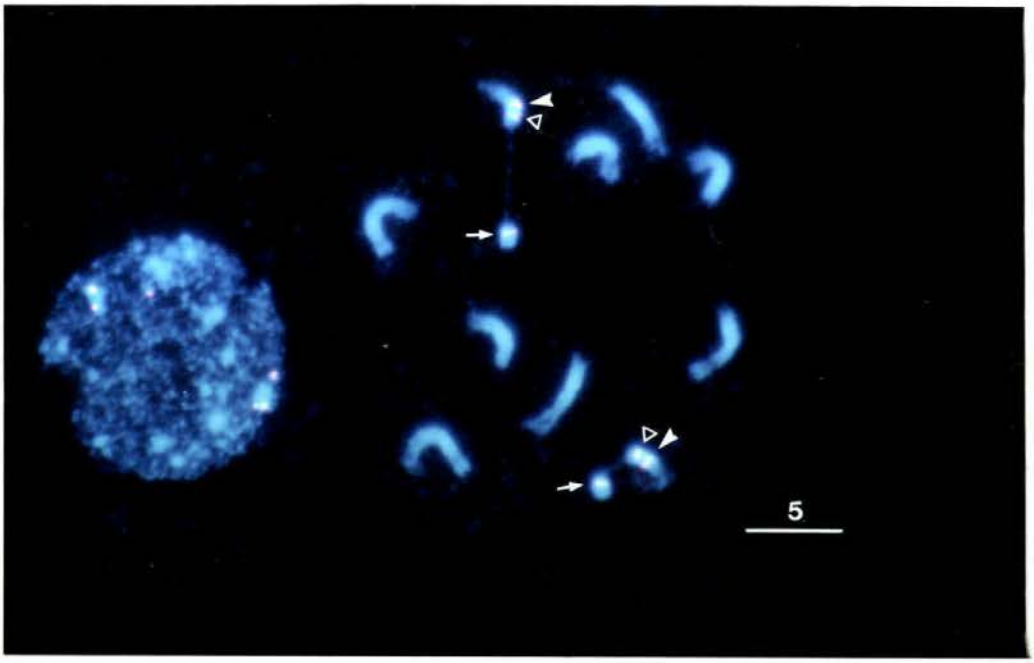

Fig. 12 The same metaphase spread of *T. hirtum* in Figs. 10-11 is shown here with the position of the 5s signal indicated in pink. The signal is seen adjacent to the centromere on the short arm (closed arrowhead), adjacent to the centromere on the long arm (open arrowhead) and on the satellite (long arrow). Scale bar in μm.

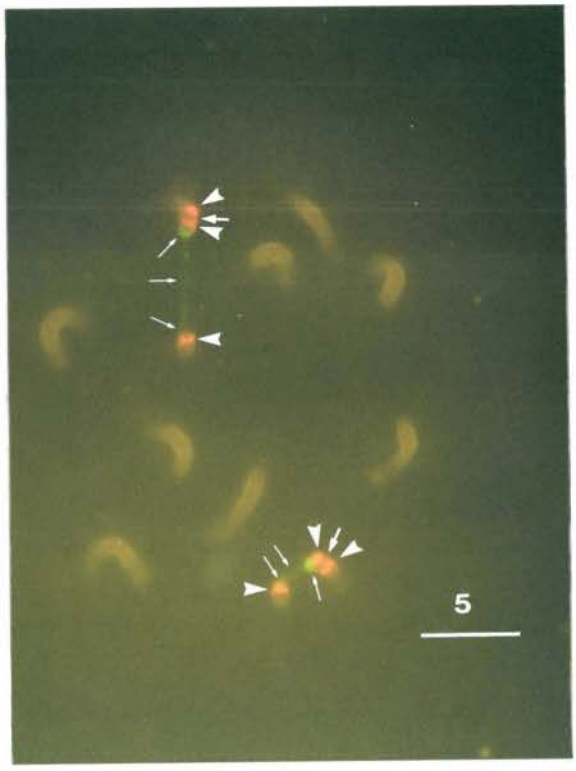

Fig. 13 A double exposure on the same metaphase of *T. hirutm* as in Figs. 10-12 indicates the positions of the 5s and 18s signals in relation to one another. The 5s signal (red) is seen adjacent to the 18s signal (green) on both the long arm and satellite (closed arrowheads). Long arrows indicate the 18s signal. A thick arrow indicates the position of the centromere. Scale bar in μm.

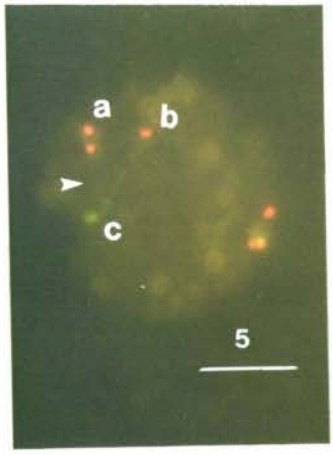

Fig. 14 An interphase nucleus of *T. hirtum* after FISH. The red/green complex indicates the positions of the 5s and 18s signals. (**a**) Indicates the position of the 5s signal presumably on the main body of the satellite chromosome, separated by the centromere. (**b**) Indicates the third position of the 5s signal most likely on the satellite body. **c** shows the 18s signal spread along a fine thread, presumably the decondensed NOR region. The thread connects to a green dot likely to be the 18s signal on the proximal side of the NOR as seen in Fig. 11. Note the unlabelled gap between (**a**) and (**c**) (arrowhead), in Fig. 12 these signals are adjacent to one another. Scale bar in μm.

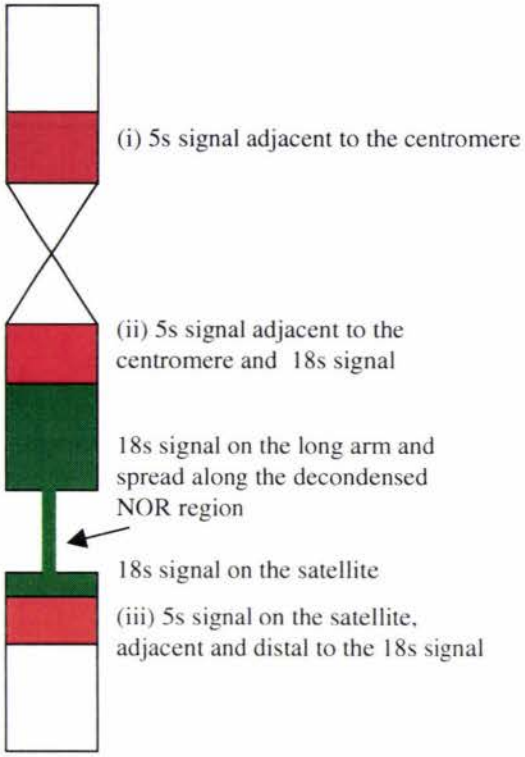

Fig. 15 Distribution pattern for 5s ■ and 18s ■ rDNA. Arm lengths and signal sizes are not to scale

4.2 *T. pratense*

Conventional Giemsa staining

An analysis of over 50 metaphases from 2 accessions confirmed the chromosome number to be 2n = 14. Only a homologous pair of satellited chromosomes could be distinguished with certainty by conventional Giemsa staining (Fig. 16). A peculiar yet common feature of *T. pratense* metaphase preparations is the differential appearance of the homologous pair of satellited chromosomes. In one chromosome the NOR and the satellite body are extended, whilst in the other the NOR and satellite body are usually contracted (Fig. 16).

Q-banding

Q-banding on prometaphase chromosomes revealed broad imprecise fluorescent regions on most of the chromosomes (Figs. 17 and 18), although the clarity and intensity of the bands varied between complements. In the satellited pair of chromosomes, the highly contracted satellite appeared to be brightly stained over its entire length. In some preparations the decondensed NOR region stained very faintly with quinacrine (Fig. 17). In both of these same satellited chromosomes, broad fluorescence was also seen on either side of the centromere. In clear preparations the broad fluorescent region on the non-satellited arm appeared to comprise two Q-bands (Fig. 18).

All the other Q-banded chromosomes also showed imprecise broad fluorescent regions around their centromeres. Some of the chromosomes showed intercalary bands, but difficulty in identifying the centromeres and telomeres with surety meant the precise

position of these bands could not be determined. Nor could the chromosomes themselves be identified with certainty.

Quinacrine stained preparations were destained then stained consecutively with Giemsa (Fig.19). Those regions that fluoresced brightly with quinacrine subsequently stained positive with Giemsa. In addition, certain other chromosomal features could now be seen more clearly. For example, the decondensed NOR region could now be seen as two fine threads connecting the satellite to the main body of the chromosome.

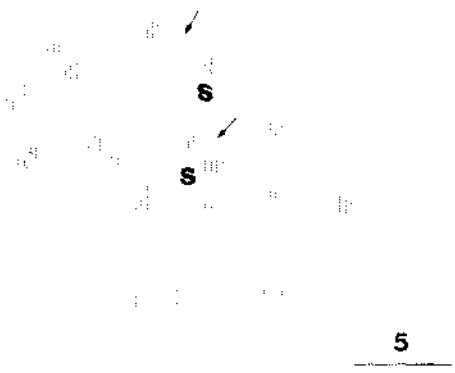

Fig. 16 Conventional Giemsa stained metaphase plate of *T. pratense*. A homologous pair of satellited chromosomes is shown (s). Note the differential contraction of the NOR region (arrows). Scale bar in µm.

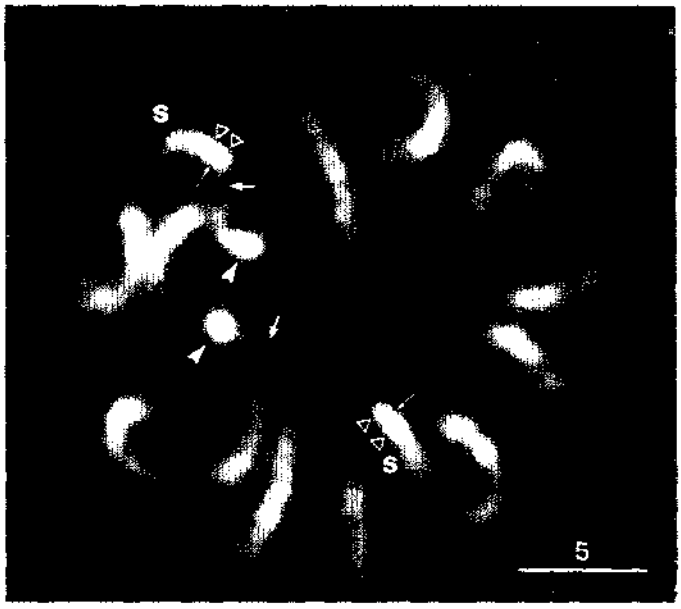

Fig. 17 Q-banded prometaphase plate of *T. pratense*. The satellites appear brightly stained over their entire length (arrowheads). In this preparation the decondensed NOR region is faintly stained (arrow). On the main part of the satellited chromosome (s), broad fluorescence can be seen on either side of the centromere (open arrowheads). A fine arrow indicates the position of the centromere on the satellited chromosomes. Scale bar in µm.

Fig. 18 Q-banded prometaphase plate of *T. pratense* showing two Q-bands on the non-satellited arm of the satellite chromosome (arrows), these can only be defined on one of the satellited pair. Arrowheads indicate the brightly stained satellites. Q-bands on other chromosomes are indicated by fine arrows. Scale bar in μm.

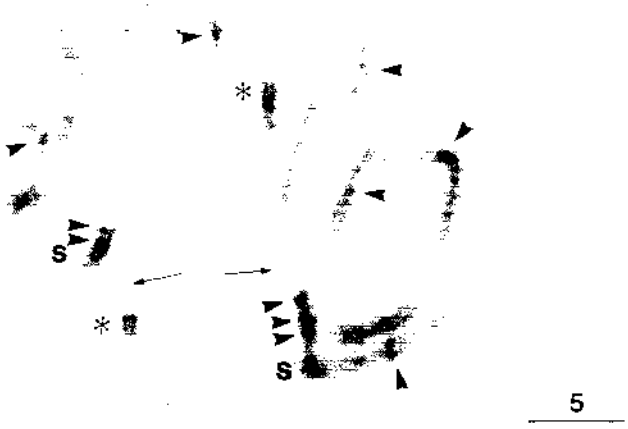

Fig. 19 The Q-banded metaphase in Fig. 18 was destained then consecutively stained with Giemsa. Regions previously stained brightly with quinacrine subsequently stained brightly with Giemsa (arrowheads). The decondensed NOR region can now be seen as two fine threads (thin arrows).
(s) Indicates the satellited chromosomes. ✱ Indicates the satellite body now positively stained with Giemsa. Scale bar in μm.

4.3 *T. incarnatum*

Conventional Giemsa staining

A detailed examination of over 50 metaphases from 1 accession confirmed the chromosome number as 2x = 14. Conventional Giemsa staining of *T. incarnatum* metaphase spreads showed differential staining of the chromosome arms. All chromosomes stained intensely around their centromeres (Fig. 20), although the longest pair showed additional uniform dark staining along their length except for the telomeres which stained pale. One pair of satellited chromosomes can also be distinguished; chromatin fibrils connecting the satellite to the main body of the chromosome were seen in some complements, although this feature was more obvious in C-banded preparations.

C-banding

A C-band was found consistently at the proximal end of the satellite body. C-bands were also present on either side of the centromere of the satellited chromosome. As noted previously, the chromatin fibrils connecting the satellite to the main body of the chromosome were more obvious in C-banded preparations. The size of the satellite appears quite large. All other chromosomes, except for the largest pair, showed a thin C-band at the centromere only. The longest chromosome pair remained relatively darkly stained, with no clearly defined C-banding pattern (Fig. 21).

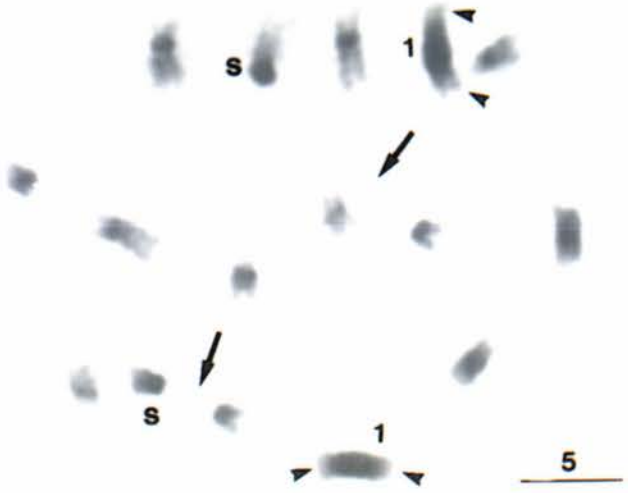

Fig. 20 Conventionally Giemsa stained metaphase plate of *T. incarantum*. Note the differential staining of the chromosome arms. The longest chromosome pair (**1**) are stained darkly except for the telomeres (arrowheads). In the satellite chromosomes (**s**), chromatin fibrils can be seen connecting the satellite to the main body of the chromosome (arrows). Scale bar in μm.

Fig. 21 C-banded prometaphase plate of *T. incarnatum*. The largest chromosome pair remained relatively darkly stained (**1**). The satellited chromosome pair (**s**) showed C-bands on either side of the centromere as well as on the proximal end of the satellite (fine arrows). All the other chromosomes showed a thin C-band at the centromere (arrowheads). Note the chromatin fibrils connecting the relatively large satellite to the main body of the satellited chromosome (thick arrows). Scale bar in μm.

4.4 *T. alexandrinum*

Conventional Giemsa staining

An analysis of over 50 metaphases from 1 accession confirmed the chromosome number to be 2x = 16. In Giemsa stained preparations the most clearly distinguishable chromosomes were one satellited pair together with the longest pair of chromosomes (Fig.22). Heteropycnotic staining of the chromosomes was seen, with the longest chromosome pair and the satellited pair stained uniformly except for the telomeres. The other chromosome pairs were more darkly stained around the centromere than the rest of the chromosome arms. The chromatin fibrils connecting the satellites to the main part of the chromsomes were quite obvious.

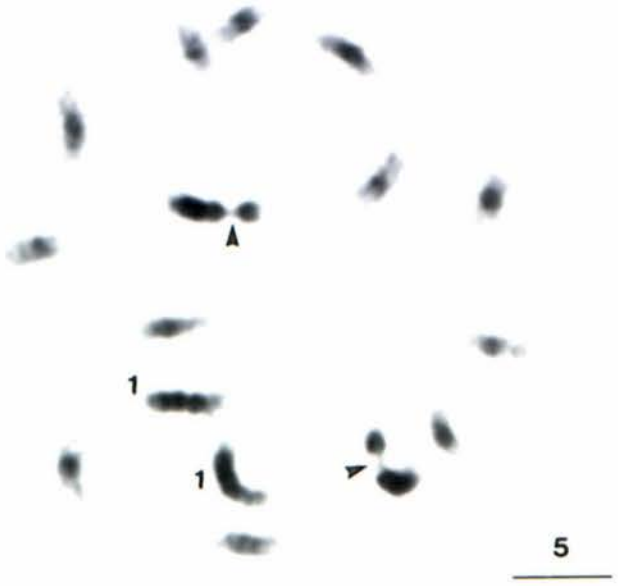

Fig. 22 Conventionally Giemsa stained metaphase plate of *T.alexandrinum*. Note the differentially stained chromosome arms. The longest chromosome pair (**1**) and the satellited pair are clearly distinguishable. Chromatin fibrils can be seen connecting the satellite to the main body of the satellited chromosome (arrowheads). Scale bar in μm.

4.5 *T. striatum*

Conventional Giemsa staining

An analysis of 25 metaphases from 1 accession confirmed the chromosome number to be 2x = 14 (Fig. 23). The most noticeable feature of this karyotype compared to the other species studied was the presence of a comparatively small satellite. As with *T. alexandrinum* and *T. incarnatum* the centromeres stained differentially darker than the chromosome arms, except for one pair which stained uniformly dark along their entire length except for their telomeres which stained pale.

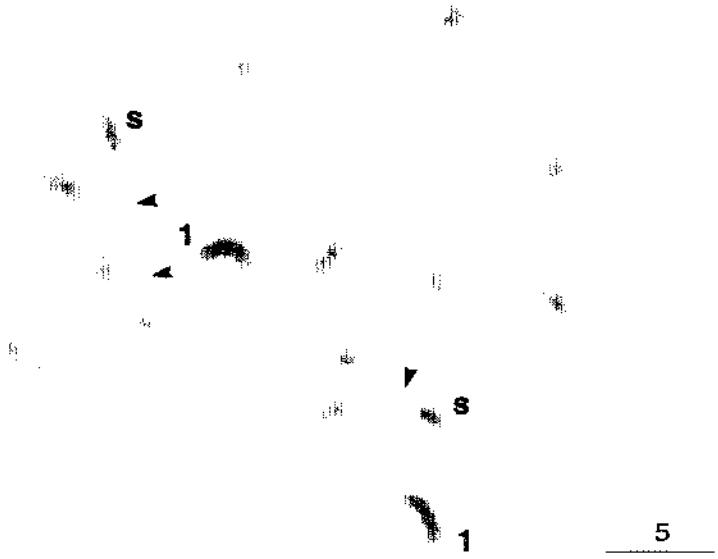

Fig. 23 Conventionally Giemsa stained metaphase plate of *T. striatum*. One pair of chromosomes stained darkly (**1**) while the others are differentially stained. (**s**) Indicates the satellited chromosome pair. Note the chromatin fibrils connecting the comparatively small satellite body to the main body of the satelllited chromosome (arrowheads). Scale bar in µm.

5.0 DISCUSSION

5.1 Methodology

The main thrust in this section of the thesis was to perfect a technique for preparing *Trifolium* chromosomes suitable for *in situ* hybridization. For standard chromosome preparation, plant tissue is usually softened by hydrolysis, commonly using 1N to 5N HCl at room temperature or 60°C (Jahier, 1996), followed by staining with a chromosome dye such as Feulgen. HCl hydrolysis is often combined with a squash technique to disperse the tissue, release the chromosomes and adhere them to the slide (Jahier, 1996). HCl hydrolysis, however, detrimentally and permanently alters the chemical structure of the chromosomes for *in situ* hybridization; this alteration reduces the probe's binding affinity to the chromosomes (Leitch *et al.*, 1994). Therefore an alternative method of chromosome preparation was sought for the present study. Although time-consuming, this was satisfactorily achieved in the current study using a modified air-dried technique. Apart from *T. hirtum*, the air-dried technique proved to be an inadequate method for morphometric analysis. The Feulgen technique was attempted repeatedly for *T. pratense* and *T. alexandrinum* metaphase spreads prepared by the air-dried method, but mysteriously without sufficient results for morphometric analysis by confocal microscopy.

In highly contracted chromosomes, techniques such as C-banding and FISH do not show clear resolution, especially when the chromosomes being analysed are small as in the genus *Trifolium*. The air-dried technique refined in the current study gives a range of chromosome extensions without any acid hydrolysis. *T. hirtum* and to a lesser extent *T. pratense* responded well to the air-dried technique, giving a range of chromosome

extensions from prometaphase to late metaphase. *T. pratense* proved slightly difficult to handle as the extended chromosomes produced tended to be diffuse and partially degraded. Time constraints prevented a further refining of the enzyme treatment times. The degradation resulted in a loss of definition of the centromere, making karyotypic analysis difficult. The satellite chromosomes were also difficult to identify in highly contracted preparations.

The air-dried technique initially resulted in poorly defined chromosomes in both *T. striatum* and *T. alexandrinum.* Further manipulations with the anti-mitotic agents and enzyme pretreatment times resulted in a clearer representation of the chromosomes. Differential staining, however, led to difficulties with interpreting chromosome morphology, especially the position of the centromere and the location of the chromosome ends. *T. incarnatum* responded well to the antimitotic agent hydroxyquinoline and to the air-dried preparations, except for the largest chromosomes which were extremely darkly stained with conventional Giemsa and C-banding techniques. In such preparations the centromere could not be identified. A change to ice-water as an antimitotic agent improved the appearance of the largest chromosomes and assisted in gaining a clearer representation of C-banded chromosomes.

During the current research it became apparent that not all species could be manipulated to the same extent. A standard protocol for all the species investigated was not possible, resulting in much fine-tuning of methodologies. The response to antimitotic agents differed greatly, as with the enzyme treatment times and banding procedures.

5.2 Results

5.21 Chromosome counts

Chromosome numbers can differ between accessions and also between samples of the same species from different regions or countries. Inaccuracies in the labelling of seed collections have also been known to occur. In considering these aspects, it is important to confirm the suggested chromosome number when performing cytological work (Taylor, 1985). The chromosome number for *T. incarnatum* was first described in 1925 as being 2n = 2x = 14 by Karpechenko, but in the same year reference was made to the chromosome number being 2n = 2x = 16 by Bleier (Talyor, 1985). Taylor (1985) suggested that this may be due to the satellited chromosomes and their satellite bodies not being identified as part of one chromosome. The satellite body appeared as large as the smallest chromosome in the genome of *T. incarnatum* in this research (Figs. 20-21). Some techniques for preparing chromosomes for microscope viewing can affect the attachment of the satellite body to the main part of the chromosome, in some cases completely dissociating the satellite body and giving no indication of previous attachment. As can be seen in Figs. 20 and 21, the tenuous connection of the satellite body to the main chromosome body is clear, thus giving a chromosome count of 2n=2x=14. The chromosome number was first described for *T. striatum* in 1939 by Wulff (as quoted in Taylor 1985), and is confirmed here as being 2n = 2x = 14. The chromosome number was first described for *T. pratense* by Bleier in 1925 (as quoted in Taylor, 1985) and is confirmed here as being 2n = 2x = 14. Red clover is the only species in section *Trifolium* subsection *Trifolium* with x = 7, other species in this subsection carry x = 8. In hybridisation experiments it was found that red clover is possibly closely related to species in subsections *Intermedia* and *Alpestria*, all species in

these subsections have x = 8 as their basic chromosome number. Hybridisation attempts with *T. incarnatum* have not been successful even though they have the same chromosome number. In looking at the evolution of the red clover karyotype previous research suggests speciation may have occurred through the loss of one chromosome pair (Taylor and Smith, 1979; Taylor and Quesenberry, 1996). The chromosome number was first described for *T. alexandrinum* in 1928 by Wexelson (as quoted in Taylor, 1985) and is confirmed here as being 2n = 2x = 16. The chromosome number for *T. hirtum* was first described in 1963 by Britten and is confirmed here as being 2n = 2x = 10. Only five other species in the whole genus have this chromosome number, four of those species are in the section *Trifolium*, one other species (*T. cherleri*) is in the same subsection.

5.22 C-banding

Certain species did not show C-bands, even after a number of changes to the protocol. The size of clover chromosomes may have some effect on the ability to clearly visualise bands as suggested by Zohary (1984). The added length and non-separation found in prometaphase chromosomes appeared to assist in visualising the presence, number and position of bands on the chromosomes, hence the seeking of preparations yielding a high proportion of prometaphase chromosomes.

T.hirtum showed a positive result for C-banding. Differing patterns of heterochromatin distribution could be identified, as can be seen in Figs. 7 and 8. The C-bands on the largest pair of chromosomes and on the satellited chromosomes were distinct from the other three pairs of chromosomes. Banding on the other chromosomes was difficult to differentiate, possibly due to the small size of the chromosomes, similarity in band

pattern and the quality of the C-bands. On this basis, it was not possible to separate all of the chromosomes into homologous pairs. Measurements of the banded chromosomes would have assisted immensely with the differentiation of homologous pairs, but the resolution of chromosome ends was unsatisfactory.

The C-banding pattern revealed in *T. incarnatum* was similar to the pattern of heteropycnotic staining with Giemsa. The longest pair of chromosomes were still darkly stained but their morphology was more apparent in C-banded preparations.

Few species in the genus *Trifolium* have been analysed using C-banding. Zhu *et al* (1988) analysed *T. repens* chromosomes by C-banding. C-bands in *T. repens* were identified around the centromeres of eight chromosome pairs, while the other eight pairs completely lacked bands, this is in contrast to both *T. incarnatum* and *T. hirtum*, where C-bands were identified on all the chromosome pairs. The satellited pair were not banded in *T. repens*, whereas once again both *T. hirtum* and *T. incarnatum* showed clear banding patterns on their satellited pair including the proximal region of the satellite body. In common with all three species, there were no terminal C-bands. Interstitial bands were identified in both *T. repens* and *T. hirtum*.

5.23 Quinacrine mustard staining

This is the first time any species in the genus *Trifolium* has been Q-banded. Q-bands were present on at least three pairs of *T. hirtum* chromosomes. One pair of *T. hirtum* chromosomes had two clear Q-bands, one at the centromere and one intercalary band on the same arm. In *T. hirtum* only the distal region of the long arm of the satellite chromosome is Q-banded whereas in *T. pratense* the satellite body is brightly Q-banded.

Q-bands are also present on either side of the centromere on the satellited chromosome in *T. pratense.* Although Q-bands were obviously present in *T. pratense*, their imprecise appearance meant that the Q-banding technique proved not to be a useful cytotaxonomic tool in this species. The lack of definition of the Q-bands in *T. pratense* was possibly related to the chromosomes degrading during the preparation of metaphase spreads. Contracted chromosomes of *T. pratense* showed such intense fluorescence that the Q-bands were indistinguishable.

5.24 Description of karyotypes

Pritchard first described the karyotype and idiogram of *T. hirtum* in 1967. (Angulo *et al.*, 1972a) in their paper written in Spanish, also described the karyotype of *T. hirtum*. This was the last time the karyotype of *T. hirtum* was described in any form. The current study in this thesis has clarified the karyotype further. Not only has the karyotype and idiogram been described but also additional information obtained through C-banding, Q-banding and FISH has been discovered. Metaphase photographs, karyotypes and idiograms of *T. striatum, T. incarnatum,* and *T. pratense* were first illustrated by (Angulo *et al.*, 1972b). In 1977, Hussein *et al.*, provided a karyotypic description of *T. alexandrinum* which included a metaphase plate photograph, and idiogram. As a new chromosome preparation technique was being perfected in the current study, some species do not have as full a description here as they have had in previous research, but the information gained on the response of the species to new techniques and manipulations is just as valuable. Chromosome numbers were confirmed for all species, which is an important process in taxonomic research as anomalies in the recording of chromosome numbers can be quite common (Zohary, 1984; Taylor, 1985). Preparation and staining for morphometric analysis had to be of a level where the chromosome ends

and the centromere could be well defined. The confocal microscope was used to introduce a new level of accuracy in chromosome measurement. With this technology fluorescent chromosomes were required, therefore the Feulgen technique was applied.

5.25 Fluorescent *in situ* hybridisation (FISH)

The technique of FISH was used as another tool to investigate the chromosomes of the selected species of *Trifolium*. Few species in the genus *Trifolium* have been analysed using FISH. Zhu *et al* (1988) analysed *T. repens* using *in situ* hybridisation with a digoxygenin-11-dUTP labelled probe. Ansari *et al* (In Press) investigated the distribution of 5s and 18s-26s rDNA in white clover and and some related species. Given the time constraints, the present study focused on only one species, *T. hirtum*, for *in situ* hybridisation. The aim of employing this technique was to discover whether the distribution of the two probes (18s and 5s rDNA isolated from *Trifolium repens* courtesy of Dr Nick Ellison) would allow differentiation of some of the pairs of homologous chromosomes. The result was surprising in relation to the aim of identifying individual chromosomes as *both* probes hybridised to the previously clearly identified satellited chromosomes. 18s rDNA does contain sequences for the NOR, so can be expected to hybridise to the satellited chromosomes. What is of interest is the pattern of distribution of the 5s rDNA signal. In *T. hirtum* it is distributed on the long and short arm of the satellited chromosome on either side of the 18s rDNA signal. This is unusual in other *Trifolium* species analysed in this manner (H Ansari *et al*, In Press) where the probe for 5s rDNA hybridises to either non-satellited chromosomes or to only one site on the satellited chromosomes. In both African cultivated rice (Ohmido and Fukui, 1995), and apple (Schuster, *et al.*, 1997), the probe for 5s rDNA is present on a separate pair of non-satellited chromosomes. Another interesting aspect is the 5s loci in

T. hirtum is a 'triple' site, consisting of two hybridisation sites separated by the 18s loci and one site separated by the centromere. (Osuji *et al.*, 1998) found a 'double' 5s location in *Musa acuminata* 'Calcutta 4' (2n=2x=22, AA genomes) and a pair of 'double' 5s sites in the Plantain cultivar 'Agbagba' (2n=3x=33, AAB genomes). Osuji notes that 'double' sites have been shown in *Hordeum* species analysed by de Bustos *et al,* 1996 and also recognised by Taketa, Harrison and Heslop-Harrison in personal communications. The significance of these 'double' sites is as yet unknown and the 'triple' site appears quite unique. 5s transcripts are combined with the 45s primary transcript in the nucleolus. The 5s transcripts are imported into the nucleolus after transcription from the 5s genes organised as tandem arrays elsewhere in the nucleus. In the case of *T. hirtum* the 5s genes are surrounding genes associated with the nucleolus. 5s rRNA is one of the ribosomal components and the genes encoding the 5s rRNA form a multigene family in almost all eukaryotic organisms. Generally in plants the 5s rRNA genes are organised in tandem arrays, forming clusters on a few chromosomes (Murata *et al.*, 1997). 18/25s rDNA signal was observed on 4 pairs of chromosomes in apple (Schuster *et al*, 1997), and 17s-5.8s-25s rDNA signal observed on the satellited chromosomes in African cultivated rice (Ohmido *et al*, 1995). Investigations into the number and size of rRNA gene sites by in situ hybridisation have contributed insight into the genomes of various plants (Nenno *et al.*, 1994; Galasso *et al.*, 1995; Zhu and Gardiner, 1995; Montijn *et al.*, 1998; Osuji, *et al.*, 1998). Differences in copy number and distribution of these genes in closely related plant species can highlight the species evolutionary relationship (Cuellar *et al.*, 1996; Cerbah *et al.*, 1998).

6.0 CONCLUSION

Trifolium section *Trifolium* contains species of international importance as forage legumes. To further improve these species, knowledge of their cytotaxonomy is desireable. It was noted that cytological investigations into the section were limited. The aims of the present study were to confirm chromosome numbers in selected species in the section *Trifolium*, employ banding techniques to characterise the chromosomes of those species, develop a chromosome preparation technique more suited for *in situ* hybridisation and to perform *in situ* hybridisation on the selected species.

All the five species, *T. hirtum*, *T. incarnatum*, *T. pratense*, *T. alexandrinum* and *T. striatum* had their chromosome numbers confirmed. C- banding patterns were analysed for both *T. hirtum* and *T. incarnatum.* In comparison to C-banding patterns in *T. repens* there was a great deal of similarity between the two species. These C-banding patterns appear to add support for the current taxonomic positions of these species in relation to one another. Further development of a C-banding protocol for section *Trifolium* species would enable more in-depth analyses to take place. *T. hirtum* and *T. pratense* were Q-banded for the first time, going some way towards characterising chromosome pairs. Through the development of a new air-drying technique for chromosome preparations, *T. hirtum* was analysed using fluorescence *in situ* hybridisation for the first time, revealing a unique 'triple' 5s rDNA distribution pattern. The information gained will go some way towards illuminating the evolutionary relationships between species in the section *Trifolium* and also in the genus *Trifolium*, whilst also giving support to breeding programs in place and those planned for the future.

APPENDICES

Appendix 1: Buffers

A1.1 1x Citrate Buffer

0.4203 gm citric acid and 0.8823 gm trisodium citrate were dissolved in 500 ml Milli-Q water, brought to pH 4.8 with dilute HCl , then stored at 4°C.

A1.2 Sorensens Buffer

A M/15 potassium di-hydrogen orthophosphate KH_2PO_4 solution in Milli-Q water, and a M/15 di-sodium orthophosphate anhydrous Na_2HPO_4 solution in Milli-Q water, were mixed to obtain a pH of 6.8 and stored at room temperature.

A1.3 2xSSC

This solution is equivalent to a 0.3 M NaCl and 0.03 M Tri sodium citrate solution. 17.53 g of NaCl was dissolved in 800 ml of RO, then 8.82 g of Tri sodium citrate was added, the pH was adjusted to7.0 with a few drops of 5N HCl and the volume raised to 1 litre. The solution was stored at room temperature.

A1.4 20xSSC

175.3 g NaCl was dissolved in a beaker of RO, then 88.2 g tri sodium citrate was added, then the pH adjusted to 7.0 with HCl and made up to 1 litre. The solution was at room temperature.

A1.5 4xSSC

200 ml of 20xSSC was added to a beaker, then 700 ml RO was added, pH adjusted to 7.0, then the volume made up to 1 litre with RO. The solution was at room temperature.

A1.6 McIlvaine's buffer

Solution **A** = 0.1 M citric acid (2.104 g/100ml RO)

Solution **B** = 0.2 M $Na_2HPO_4.7H_2O$ (7.1625 g/100 ml RO)

Solution **C** = 0.005 M $MgCl_2$ (0.1017 g/100 ml RO)

18 ml of **A** was added to 82 ml of **B**, then mixed 1:1 with **C**.

Appendix 2: Stains

A2.1 Giemsa stain

0.5g Giemsa powder (Merk Diagnostica, Azur-eosin-methylene blue, C.I.Nr.45380 39%, C.I.Nr.52015 + Azure 51%) was dissolved in 33 ml of glycerol (AnalaR), and incubated at 60°C with intermittent shaking. After for 3-4 days of incubation, 33 ml of AnalaR methanol was added at room temperature. The stain was left to mature for three weeks at room temperature, then filtered and stored in a brown bottle at room temperature.

A2.2 Quinacrine mustard stain (Sigma)

0.2% stock: 0.01 g of Quinacrine mustard powder was dissolved in 5 ml of RO, then aliquoted into 1 ml microcentrifuge tubes wrapped in foil and stored at 4°C.

0.002% Working solution: 0.5 ml of the 0.2% stock was added to 50 ml of RO and stored in a foil wrapped bottle or tube at 4°C.

A2.3 DAPI stain (Sigma, 4', 6-Diamidino-2-Phenylindole)

2 µg/ml DAPI powder was added to McIlvaine's buffer and stored at -20°C. The working solution was a 1+14 dilution of the stock solution with McIlvaine's buffer.

Appendix 3: Fluorescence *in situ* hybridisation solutions and equipment

A3.1 RNAse stock (Sigma R4875) not guaranteed DNAse free.

To first obtain DNAse-free RNAse, 10 mg of RNAse was dissolved in 10 ml MilliQ water in a sealed flask. The flask was suspended in boiling water for 20 minutes, left to cool and 0.5 ml aliquots stored at -20°C. A 0.1µg/µl working solution was made by diluting a 1µg/µl stock solution of RNAse with 2xSSC. The solution was stored at -20°C.

A3.2 Humid chamber

The humid chamber consisted of a plastic box with glass rods glued to the base for the slides to rest on, and filter paper strips soaked in 2xSSC sitting on the bottom of the box to increase the humidity inside the box once the lid was put on.

A3.3 Deionised formamide

In a fume hood, 5 g of ion exchange resin (duolite BDH) per 100 mls of formamide (BDH molecular biology grade) was added to a beaker, and stirred for 30 min to 2 hr at room temperature. The solution was then filtered twice through two layers of Whatman No 1 filter paper and stored in a brown bottle at -20°C.

A3.4 70% formamide-2xSSC

42 ml deionoised formamide was added to a Coplin jar, along with 6 ml 20xSSC, and 12 ml MilliQ water, then mixed on a magnetic stirrer, and pH adjusted to 7.0 with 1N HCl.

A3.5 50% formamide-2xSSC

350 ml deionised formamide mixed with 70 ml 20xSSC and made up to 700 ml with MilliQ water. Adjusted the pH to 7.0 with HCl.

A3.6 4xSSC-Tween20

500 µl of Tween20 added to 1 litre of 4xSSC. The bottle was inverted to mix. The solution was prepared fresh.

A3.7 Carrier DNA

The carrier DNA was sonicated Salmon sperm at 10 µg/µl.

A3.8 The hybridisation mixture

The hybridisation mixture consisted of: H_2O 31.4 µl, formamide 24 µl, 20xSSC 120 µl, 50% dextran sulphate 46.3 µl, 20% SDS 1.2µl, Carrier DNA (10µg/µl) 1.2µl, Probe (I) (18s rDNA-Fluor-x) 8.0µl, Probe (II) (5s rDNA-Cy3) 8.0µl.

Appendix 4: Miscellaneous

A4.1 Saturated barium hydroxide solution, Ba $(OH)_2.8H_2O$, BDH AnalaR

In a beaker with 100 ml of Milli-Q water at 60°C, approximately 5.5-6 gm barium hydroxide was added and stirred until it dissolved, then coarsely filtered into a Coplin jar and kept at 52°C in a waterbath until used.

A4.2 Enzyme Solution

Made according to (Schwarzacher, *et al.*, 1989) with modifications. In citrate buffer (section A1.1), a 2% w/v cellulase mixture of 1.6% w/v Calbiochem and 0.4% w/v 'Onozuka' R-10 Serva cellulase and 20% v/v pectinase from *Aspergillus niger* in 40% glycerol solution from either Sigma or ICN, was mixed to the desired volume and stored at –20°C

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