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

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Tatyana Thelma Bucknell

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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).