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# Molecular Cytogenetic Organization of 5S and 18S-26S rDNA Loci in White Clover (*Trifolium repens* L.) and Related Species

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The genus *Trifolium* (Leguminosae), which includes several important forage legumes of temperate and subtropical regions, is characterized by small chromosomes of uniform size. Cytogenetic mapping of the two multigene families coding for 18S-5.8S-26S rRNA and 5S rRNA in white clover (*T. repens*) and seven closely related species/subspecies was carried out using fluorescence *in situ* hybridization (FISH). The 18S-26S rDNA, generally confined to nucleolus organizer regions (NORs), was consistently located proximally on the shorter arm of one or two metacentric or submetacentric chromosome pairs. DAPI-negative, diffused and highly stretched 18S-26S FISH signals, representing previously transcriptionally active NORs, were observed up to late metaphase. The flanking condensed ends of the diffused 18S-26S rDNA signals were DAPI-positive and represented perinucleolar knobs which are presumed to be transcriptionally inactive. In interphase cells, the decondensed NOR chromatin passed through the nucleolar domain. In one species (*T. isthmocarpum*), in addition to two NOR chromosome pairs, 18S-26S signals were also located on a third pair, with no apparent NOR association. The 5S rDNA signals were located proximally on a long arm in six species and were syntenic to the NORs in all these species except *T. ambiguum* and *T. nigrescens* ssp. *petrisavii*. In two species (*T. hybridum*, *T. isthmocarpum*), 5S sequences occurred in the chromosomal satellites aligned distally to the NOR sequences. The relative distribution patterns of the two types of tandemly repeated DNA sequences were species-specific except for *T. nigrescens* ssp. *petrisavii* and *T. ambiguum*, both of which showed identical hybridization patterns. The two types of rDNA sequences have provided molecular markers for individual identification of a set of clover chromosomes. Present findings support the allotetraploid origin of white clover (*T. repens*) and suggest that *T. nigrescens* ssp. *petrisavii* may be one of the present day diploid ancestors.



**Key words:** White clover, *Trifolium*, FISH mapping, 5S rDNA, 18S-26S rDNA, nucleolus organizer regions, genome evolution.

## INTRODUCTION

The clover genus *Trifolium* (Leguminosae: Papilionoideae) includes almost 250 species distributed throughout temperate and subtropical regions. Despite its large size and considerable agricultural importance, most cytogenetic investigations of *Trifolium* have been limited to orcein-stained chromosome counts with vague karyomorphological descriptions and basic meiotic studies. This is attributed to the small sizes of clover chromosomes and symmetrical karyotypes. The basic chromosome number ($x = 8$) is found in 80% of the 185 cytologically screened species and, therefore, is regarded as the primitive number. The reduced basic numbers 7, 6 and 5 are treated as derived. Less than 20% of examined species are either polyploid or display ploidy variation.

*Trifolium repens* L. (white clover), an important forage legume of moist temperate regions, has a chromosome number of $2n = 4x = 32$, and uniformly small biarmed chromosomes that are individually extremely difficult to identify. Because *T. repens* seldom shows multivalent formation during meiotic prophase I and exhibits disomic inheritance, it is considered to be allotetraploid in origin with two ancestral genomes (Williams, 1987).

The origin of white clover as a species and its phylogenetic relationships are unknown. Three species, *T. occidentale* ($2n = 2x = 16$). *T. nigrescens* ($2n = 2x = 16$) and *T. uniflorum* ($2n = 4x = 32$) are considered to be close relatives of white clover because they can be crossed relatively easily with white clover and form interspecific hybrids (Chen and Gibson, 1970*a*, *b*, 1971). Furthermore, the chromosomes of these species have varying degrees of pairing affinity with those of white clover during meiotic prophase I in the $F_1$ hybrids. Interspecific hybrids involving white clover can also be obtained with much greater difficulty using *T. isthmocarpum* ($2n = 2x = 16$), *T. hybridum* ($2n = 2x = 16$) and *T. ambiguum* ($2n = 2x = 16$) (see Cleveland, 1985; Williams, 1987). The affinity of meiotic pairing of these species with white clover, however, is small.

During the course of evolution of a genus, major reorganization of genomes, that can be detected at the molecular as well as chromosomal levels, may occur. Study of the physical organization of chromosomes of related

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species with respect to the distribution of specific DNA sequences provides insight into these molecular evolutionary changes. The fluorescence *in situ* hybridization (FISH) technique is a powerful tool for the regional mapping of DNA sequences onto chromosomes in interphase and metaphase cells. The genes for 18S, 5·8S and 26S rRNA occur as a large number of tandem repeats of a single transcription unit (18S-5·8S-26S rDNA). These sequences are located in the nucleolus organizer regions (NORs) of eukaryotic chromosomes. Sequences of 5S rDNA, coding for another type of rRNA, also occur as tandem repeats but these are often located independently of NORs in eukaryotes. The general distribution patterns and regional mapping of these two multigene families have revealed the evolutionary reorganization of genomes in several plant taxa (Badaeva, Friebe and Gill, 1996; de Bustos *et al*., 1996; Hanson *et al*., 1996; Thomas *et al*., 1996). Such studies have also helped in the understanding of transcriptional activity through the observation of condensation and decondensation patterns of chromatin in nucleoli (Leitch *et al*., 1992; Martel, Ricroch and Sarr, 1996; Shi, Zhu and Keim, 1996).

As an initial part of a programme to physically map *Trifolium* genomes, we have greatly improved cytogenetic preparations and have succeeded in developing an air-drying method of clover chromosome preparation which is ideally suited to various molecular cytogenetic techniques. Here we report for the first time the comparative cytogenetic mapping of 5S and 18S-26S rDNA sequences in eight species/subspecies of the genus *Trifolium* belonging to the section Lotoidea and closely related to white clover (*T. repens*). This study has provided molecular markers for identification of some individual clover chromosomes, which are otherwise indistinguishable. It also provides a better understanding of the expression pattern of rDNA sequences through the observation of the physical appearance of these regions during the cell cycle. Finally, on the basis of these results, an attempt is made to decipher the evolutionary reorganization of rDNA in clovers and to trace the phylogenetic relationships of white clover.

## MATERIALS AND METHODS

### *Plant material and chromosome preparation*

Seven species and two subspecies of the genus *Trifolium*, belonging to the section Lotoidea, were included in the present study. These were *T. repens* L. (C7544, C13176, Regal, English origin), *T. uniflorum* L. (AZ4192, AZ4194), *T. occidentale* Coombe. (AZ3277, AZ4190), *T. nigrescens* Viv. ssp. *nigrescens* (AZ2225, AZ2987), *T. nigrescens* Viv. ssp. *petrisavii* (Clem.) (AZ1308, AZ3296), *T. ambiguum* M. Bieb. (AZ1120, AZ858, S-4-74), *T. isthmocarpum* Brot. (AZ2933, AZ2934) and *T. hybridum* L. (S-19-31). Of the 18 accessions/cultivars used during the present study, 14 were selected from the Margot Forde Forage Germplasm Centre at AgResearch Grasslands, Palmerston North. Professor Norman Taylor, University of Kentucky (Lexington) and Dr Gary Pederson, Crop Science Research Laboratory (Mississippi State) supplied the remaining accessions.

Prometaphase and early metaphase chromosomes were obtained using a newly developed air drying technique. Actively growing root tips from seedlings or potted plants were pre-treated with 4 mM 8-hydroxyquinoline for 2 h at 20–25 °C and then 6 h at 4 °C. The root tips were fixed overnight in 3:1 methanol-acetic acid, washed in citrate buffer at pH 4·8 and then macerated in 2% (w/v) cellulase (1·6% cellulase Calbiochem + 0·4% cellulase 'Onozuka' R-10) in citrate buffer at pH 4·8 and 20% (v/v) pectinase (from *Aspergillus niger* in 40% glycerol, ICN # 156058) for 50 min at 37 °C. After rinsing and incubation in citrate buffer for 20 min at room temperature, the meristematic region was placed in a drop of 45% acetic acid on a clean slide. The root tip was tapped gently to release the free cells. Two drops of chilled (−20 °C) 3:1 methanol acetic acid fixative were placed on the droplet of 45% acetic acid and air- or flame-dried. One slide from each accession was stained with 5% Giemsa solution diluted with Sorensen's buffer at pH 6·8.

### *Probe DNA and labelling*

To localize the clusters of 18S-5·8S-26S rDNA, pTa71 (Gerlach and Bedbrook, 1979), a 9 kb *EcoRI* fragment from wheat containing the above rDNA repeats and pTr18S (GenBank accession # AF 071069), a 1·8 kb fragment from *T. repens* containing almost the entire 18S sequence were used. The 5S rDNA was localized using clone pTa794 (Gerlach and Dyer, 1980), a 410 bp BamHI fragment of the 5S sequence from wheat and pTr5S (GenBank accession # AF 072692), a 596 bp fragment from *T. repens*. The DNA probes were labelled with direct fluorochrome-labelled nucleotides Cy3-dCTP (Amersham), FluorX-dCTP (Amersham), Texas Red-5dCTP (NEN Life Science) or Fluorescein-12dCTP (DuPont NEN) by nick translation according to manufacturer's specifications. Figure 2C represents labelling with biotin-16dUTP (Boehringer Mannheim).

### In situ *hybridization*

Three-to-five day old chromosome preparations were incubated in 10 $\mu$g ml$^{-1}$ DNase-free RNase for 45 min at 37 °C, fixed in 4% paraformaldehyde and then dehydrated at room temperature. Chromosomal denaturation was carried out in 70% formamide in 2xSSC (0·3 M sodium chloride, 0·03 M sodium citrate) for 2 min at 72 °C and quickly dehydrated through a −20 °C ethanol series and air dried. A 35 $\mu$l aliquot of heat denatured hybridization mixture containing approx. 60 ng of each labelled probe (18S-26S rDNA and 5S rDNA), 50% formamide, 20% dextran sulphate, 0·5% SDS (sodium dodecyl sulphate) and 2xSSC, was then applied to each slide and covered with a plastic coverslip. Hybridization was carried out at 37 °C in a humid chamber for 16 h. Post-hybridization washing of slides at 42 °C involved two changes of 2xSSC for 5 min, 50% formamide in 2xSSC for 10 min and two changes of 2xSSC for 5 min. After cooling of the jar for 10 min, slides were rinsed in 2xSSC and 4xSSC/Tween 20 at room temperature. The slides were counterstained with DAPI before mounting in Vectashield (Vector Laboratories).

Photomicrographs were directly taken on Fuji Super G Plus 400 film with Nikon Microphot-SA and Olympus BH-2 epifluorescence microscopes using standard filter sets for DAPI, propidium iodide, FITC and rhodamine excitation.

## RESULTS

Prometaphase to early metaphase cells were consistently obtained using the air-drying technique. Giemsa staining was used to screen for the presence of normal chromosome complements in all the accessions. Prometaphase cells from some of these are shown in Fig. 1. Chromosome counts of all these species (Fig. 3), determined after Giemsa staining and FISH analysis, confirmed earlier counts (Chen and Gibson, 1971; Cleveland, 1985). The mitotic preparations consistently showed the presence of Giemsa-positive prominent dot-like structures that proved, after FISH mapping with 18S-26S rDNA sequences, to be satellites of active NOR chromosomes.

*T. repens*, *T. uniflorum* and *T. ambiguum* were subjected to *in situ* mapping using the probes pTa71 (representing 18S, 5·8S and 26S rDNA sequences) as well as pTr18S (representing only 18S rDNA sequences). A comparison of hybridization patterns between the two probes revealed identical results in each species. Therefore, the term 18S-26S has been used to represent sequences detected by either of the above two probes. The two probes for 5S rDNA, *viz*., pTa794 and pTr5S also displayed identical FISH signals in each of the above three species.

FISH mapping results using single or double coloured labelled probes (18S-26S rDNA and 5S rDNA) are shown in Fig. 2 while their regional distribution is summarized in Fig. 3. In *T. repens*, DAPI stained early and mid-metaphases always showed a pair of satellites (Fig. 2A) without any visible connection to other chromosomes. After *in situ* hybridization with 18S-26S rDNA, one pair of chromosomes displayed a characteristic signal that corresponded to the secondary constriction (NOR) of the short arm of the NOR chromosome. The FISH signal was visualized in interphase to late metaphase cells as faint, decondensed and stretched chromatin fibrils with short but brighter and condensed chromosomal segments (Fig. 2B and C). The proximal flanking segment in the pericentromeric region was smaller while the distal one was slightly larger and constituted the proximal part of the DAPI and Giemsa positive satellite. In highly extended prometaphase cells, the proximal part (approximately half) of the satellite was labelled while the remaining distal part of the satellite did not show a 18S-26S hybridization signal (result not shown). The decondensed chromatin in the secondary constriction region represented previously transcriptionally active NORs which were located in the nucleolar domain of interphase cells. On the other hand, the proximal and distal condensed knobs of 18S-26S FISH signals remained outside the nucleolar domain. Due to the air-drying technique used here, the active NORs were invariably highly stretched in all the preparations. In highly condensed late metaphases, the NOR signal appeared as a compact interstitial block on the short arm of the sub-metacentric chromosome. In agreement with Zhu, Ellison and Rowland (1996), the NOR chromosome pair was the smallest of the *T. repens* karyotype.

Two pairs of 5S signals were invariably observed in metaphase (Fig. 2B) as well as in interphase cells of *T. repens*. These were located in the pericentromeric regions of two different chromosome pairs. A major 5S signal was located proximally on the long arm of the NOR chromosome while a metacentric pair had an intermediate-sized 5S signal proximally on the longer arm. Unlike the 18S-26S signal, 5S

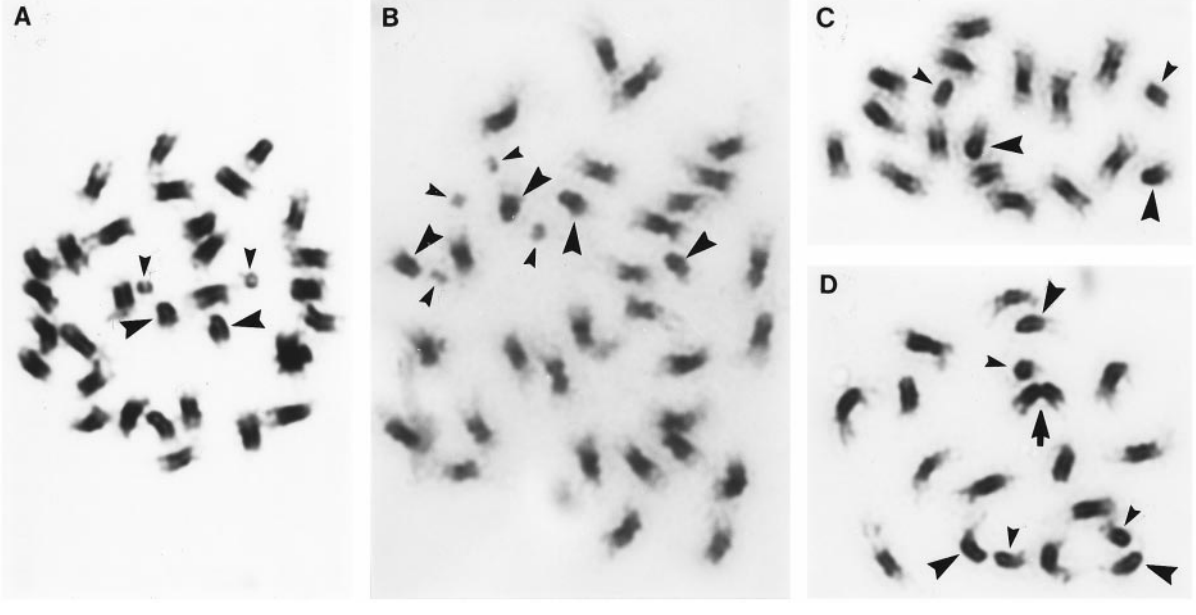



FIG. 1. Prometaphase cells from the root tips of *T. repens* (A), *T. uniflorum* (B), *T. hybridum* (C) and *T. isthmocarpum* (D). Large arrowheads indicate the long arms of the NOR-bearing chromosomes and small arrowheads indicate satellited knobs/short arms which are separated from the former due to the decondensation of NORs. In *T. isthmocarpum* (D), both the components of a fourth NOR-bearing chromosome (arrow) are very close. Difference in arm ratio of NOR-bearing chromosomes in *T. repens* and *T. uniflorum vs. T. hybridum* and *T. isthmocarpum* is evident.

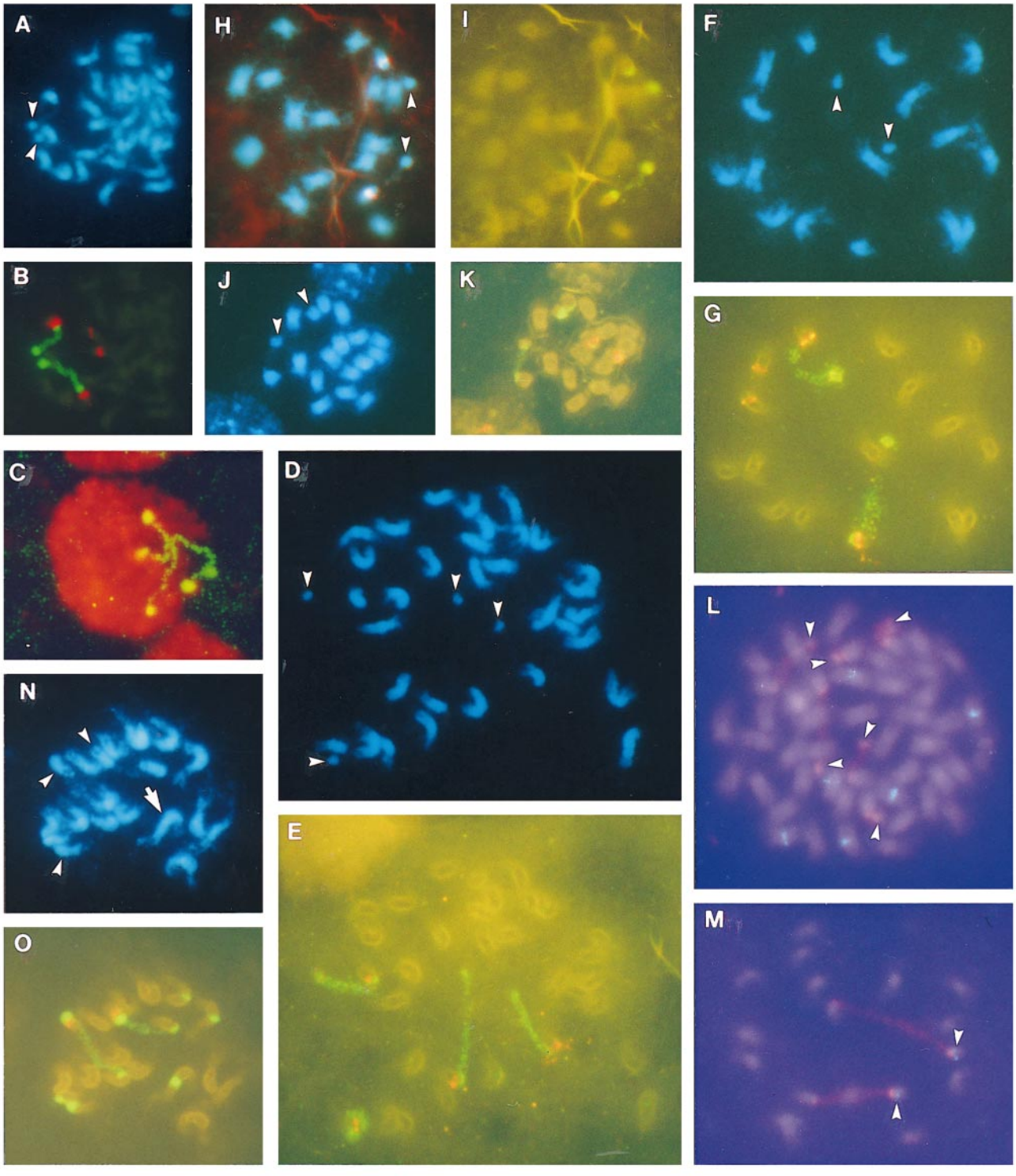
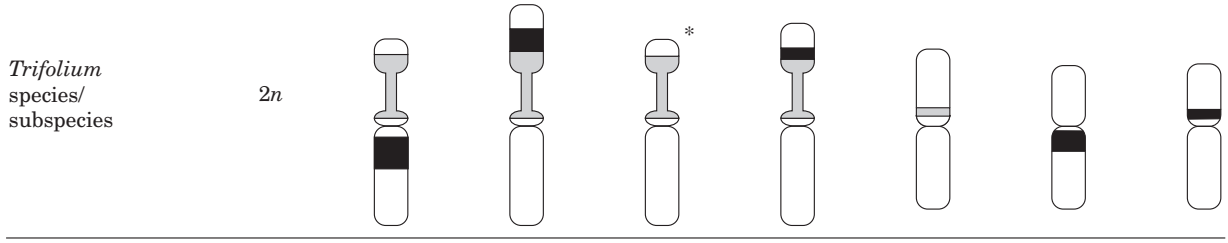



FIG. 2. Interphase and prometaphase cells from root tips of *T. repens* (A–C), *T. uniflorum* (D–E), *T. occidentale* (F–G), *T. nigrescens* ssp. *nigrescens* (H–I), *T. nigrescens* ssp. *petrisavii* (J–K), *T. ambiguum* (L), *T. hybridum* (M) and *T. isthmocarpum* (N–O) after *in situ* hybridization with 18S-26S or 18S rDNA and/or 5S rDNA probes and counter staining with 4′, 6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI). Arrowheads indicate the satellited knobs/short arms of NOR-bearing chromosomes. A, *T. repens*-counterstained with DAPI; B, same cell with FISH signals of 18S-26S (green- fluorescein) and 5S (red- Texas Red) rDNA probes; C, *T. repens*- PI-stained interphase cell with highly decondensed FISH signals of 18S-26S rDNA (biotinylated probe detected with fluorescein); D, *T. uniflorum*- DAPI staining; E, same cell with FISH signals of 18S (green- Fluor-X) and 5S (red- Cy-3) rDNA probes; F, *T. occidentale*-DAPI staining; G, same cell with FISH signals of 18S (green- Fluor-X) and 5S (red- Cy-3) rDNA probes; H, *T. nigrescens* ssp. *nigrescens*- DAPI-stained cell with FISH signals of 5S rDNA (red- Texas Red) probe; I, same cell with FISH signals of 18S rDNA (green- Fluorescein) probe; J, *T. nigrescens* ssp. *petrisavii*- DAPI-stained cell; K, same cell with FISH signals




| *Trifolium* species/ subspecies | 2*n* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *repens* | 4*x* = 32 | 2 | | | | | 2 | |
| *occidentale* | 2*x* = 16 | 2 | | | | | | 2 |
| *uniflorum* | 4*x* = 32 | 4 | | | | | | |
| *n. nigrescens* | 2*x* = 16 | 2 | | | | | | |
| *n. petrisavii* | 2*x* = 16 | | | 2 | | | 2 | |
| *ambiguum* | 2*x* = 16 | | | 2 | | | 2 | |
| *hybridum* | 2*x* = 16 | | | | 2 | | | |
| *isthmocarpum* | 2*x* = 16 | | 2 | 2 | | 2 | | |

* Does not represent arm ratio for *T. isthmocarpum*

FIG. 3. Diagrammatic representation of chromosomal distribution of 18S-26S and 5S rDNA sequences in the genus *Trifolium*. ⚃, 18S-26S rDNA; ■, 5S rDNA.

rDNA was never found in a decondensed state either in prometaphase or interphase cells.

The hybridization patterns of the two classes of rDNA on the four NOR chromosomes of *T. uniflorum* (2*n* = 32) appeared to be identical to those of *T. repens* (Figs 2D, E, 3). The long arm had a major 5S rDNA signal proximally while 18S-26S rDNA occupied the secondary constriction area on the short arm of a small submetacentric pair. Both pairs displayed highly decondensed 18S-26S signals in prometaphase to metaphase cells. Unlike *T. repens*, 5S hybridization was not observed on any other chromosome in *T. uniflorum*.

A single pair of NOR chromosomes of *T. occidentale* (2*n* = 16) revealed the same hybridization pattern of 18S-26S and 5S sequences as described in the above two species. In addition a pair of metacentric chromosomes also showed a minor 5S signal proximally on the short arm in all the metaphase cells observed (Figs 2F, G, 3).

Initially, two accessions of *T. nigrescens* were used during the present study. The regional assignment of 5S rDNA relative to the 18S-26S rDNA was found to be different in the two accessions, leading to further morphological identification of these accessions. Accession AZ 2987 was identified as *T. nigrescens* ssp. *nigrescens* and accession AZ 1308 as *T. nigrescens* ssp. *petrisavii*. Both subspecies (2*n* = 16) had one pair of NOR chromosomes with 18S-26S signals on the secondary constrictions. In *T. nigrescens* ssp. *nigrescens*, the major 5S signal was syntenic to the 18S-26S signal on the opposite arm as was the case in *T. repens* (Figs 2H, I, 3). No other chromosome pair hybridized to 5S sequences in *T. nigrescens* ssp. *nigrescens*. In contrast, the NOR chromosomes of *T. nigrescens* ssp. *petrisavii* did not hybridize to the 5S rDNA probe. Instead, a metacentric pair displayed an intermediate-sized 5S signal proximally on the longer arm (Figs 2J, K, 3) which could be homologous to the *T. repens* metacent pair having a 5S

of 18S (green- fluorescein) and 5S (red- Texas Red) rDNA probes; L, *T. ambiguum*- DAPI-stained cell with FISH signals of 18S-26S (red- rhodamine) and 5S (blue- fluorescein) rDNA probes; M, *T. hybridum*- DAPI-stained cell with FISH signals of 18S-26S (red- rhodamine) and 5S (blue- fluorescein) rDNA probes; N, *T. isthmocarpum*- DAPI stained cell; O, same cell with FISH signals of 18S (green- Fluor-X) and 5S (red- Cy-3) rDNA probes. Due to the inactive NOR, the secondary constriction of a fourth NOR-bearing chromosome in *T. isthmocarpum* (N) is not visible (arrow).

signal. *T. ambiguum* ($2n = 16, 48$) also displayed essentially the same distribution pattern of the two rDNAs (Fig. 2L) as found in *T. nigrescens* ssp. *petrisavii*.

The only pair of NOR chromosomes of *T. hybridum* ($2n = 16$) showed a relatively larger satellited part bringing the arm ratio to nearly metacentric morphology (Figs 1C, 3). As expected, the 18S-26S signals spanned through the whole secondary constriction area on the short arm. Both the NORs were highly decondensed. Unlike *T. repens*, the long arm did not show 5S hybridization but a minor 5S signal was located in each satellite aligned to the distal flanking end of the 18S-26S signal (Figs 2M, 3). These rDNA probes did not hybridize to any other *T. hybridum* chromosomes.

The use of 18S-26S sequences as a probe for FISH in *T. isthmocarpum* ($2n = 16$) confirmed two pairs of metacentric NOR chromosomes (Figs 2N, O, 3). Because the two arms were almost equal in size in these two NOR chromosome pairs and two to three NORs were always decondensed, it was sometimes difficult to ascertain the diploid count in Giemsa preparations (Fig. 1D). A third metacentric pair had a relatively small 18S-26S FISH signal in the pericentromeric region on the short arm. The signal on this chromosome was never found to be decondensed even in the interphase cells. One pair of major 5S signals was aligned distal to the 18S-26S sequences on the short arm of one pair of NOR chromosomes (Figs 2O, 3). The other NOR chromosome pair did not have 5S sequences. Consequently, three pairs of *T. isthmocarpum* chromosomes could be distinguished using double coloured FISH.

## DISCUSSION

### *Distribution of rDNA*

The positions of both 18S-26S and 5S sequences in different accessions were fully consistent for each taxa. Thus, with the exception of *T. hybridum* for which only one accession was analysed, the present results were replicated within species. The locations of 18S-26S sequences appeared to be highly conserved, being always located interstitially in the pericentromeric region on the short arm of a submetacentric or metacentric chromosome. The 5S loci were always interstitial and were pericentromeric in six of the eight species/subspecies (Fig. 3). The highly conserved interstitial location of rDNA loci, as observed here, is consistent with the suggestion of Hanson *et al.* (1996) that an interstitial location may provide greater karyomorphological stability during evolution. The pericentromeric location of the two multigene families probably represents the primitive character of clover molecular karyology. On the other hand, in two exceptional species (*T. hybridum*, *T. isthmocarpum*), 5S rDNA was located distal to 18S-26S sequences on the satellited chromosomal part. Furthermore, in both species, the satellited chromosomal part was significantly larger than normal. These observations are consistent with a translocation of the 5S locus and subtending chromosomal material from the pericentromeric region to a distal position on the short arm.

The 18S-26S sequences were predominantly in the secondary constrictions which form nucleolar organizer regions (NORs). An exception was *T. isthmocarpum* where, in addition to two pairs of NOR-bearing chromosomes, a very small 18S-26S signal was located proximally on a third pair of metacentric chromosomes. This was never found to form NORs. On the other hand, 5S loci have been assigned to three different locations relative to the NORs and could be syntenic to the NOR signal and/or located on another biarmed chromosome. A 5S site syntenic to the NOR signal was located either pericentromerically on the long arm (e.g. *T. repens*, *T. uniflorum*, *T. occidentale*, *T. n. nigrescens*) or on the NOR-bearing short arm distal and aligned to the NOR sequences (*T. isthmocarpum* and *T. hybridum*) (Fig. 3). The localization of 5S sequences in the satellited region indicates clearly that plant chromosome satellites may contain important coding regions.

Variation in the size of the hybridization signal has been inferred to reflect a difference in copy number of the tandemly repeated units (Li *et al.*, 1997; Murata, Heslop-Harrison and Motoyoshi, 1997). Copy number of rDNA has not been determined in clovers. However, the present study has shown that 5S occurred in three sizes while 18S-26S appeared in two sizes, the prominent one forming typical NORs while the smaller one, found only in *T. isthmocarpum*, did not form a secondary constriction.

### *Organization of NORs*

Decondensation of NOR sequences during the interphase stage has been correlated with transcriptional activity (Appels, Moran and Gustafson, 1986). It was proposed that the appearance of DAPI negative secondary constrictions at metaphase reflects the remnants of decondensed ribosomal chromatin that was transcriptionally active in the preceding cell cycle at NORs (Suja *et al.*, 1997). In clovers, decondensation of 18S-26S rDNA hybridization sites was visualized in the form of stretched or diffused chromatin in interphase cells indicating the transcriptional activity of these sequences. It is notable that the decondensed NORs of clover chromosomes, unlike other plant species, were present in almost every cell up to late metaphase (Fig. 2). Such decondensation so late in the cell cycle is not normal for plant chromosomes. This new observation may be a consequence of the improved air drying technique used, whereby mechanical stretching of the decondensed chromatin fibrils at metaphase was enhanced relative to other techniques.

It is generally accepted that the whole array of chromatin in NORs of eukaryotes is not decondensed at a given time (Leitch *et al.*, 1992; Maluszynska and Heslop-Harrison, 1993; Suja *et al.*, 1997; Montijn *et al.*, 1998). Working on chromatin packaging in mammalian NOR chromosomes by the application of confocal and immunofluorescence microscopy, Suja *et al.* (1997) proposed that the proteinaceous chromosomal scaffold in the NORs is longitudinally uncoiled except for the flanking regions. Decondensed rDNA exists in the uncoiled scaffold area. In clovers, the reticulate network of 18S-26S FISH signals (DAPI-negative) with their flanking condensed parts (DAPI-positive), observed up to midmetaphase was consistent with the proposal of Suja *et al.* (1997). The condensed flanking parts were equivalent to the perinucleolar knobs observed in

*Petunia hybrida* which represented the transcriptionally inactive parts of the 18S-26S chromatin (Montijn *et al*., 1998). A spatial relationship of the active rDNA has also been observed in clovers. The decondensed parts of the rDNA fibrils were localized in the nucleolar domain of the nucleus.

Contrary to the decondensation of NORs, chromatin regions containing 5S sequences have been reported to remain compact during the interphase stage in several plant species (Martel *et al*., 1996; Shi *et al*., 1996). Decondensed 5S hybridization signals were never observed in the interphase cells of any clover species studied here. Dense 5S bodies were generally located outside the nucleolar domain. Martel *et al*. (1996) were of the opinion that the decondensed 5S regions may be of such a small size that these could not be detected through conventional microscopy.

### *Phylogenetic considerations*

The chromosomal assignments of the two tandemly repeated rDNA sequences, summarized in Fig. 3, revealed that the distribution patterns were species-specific except that *T. nigrescens* ssp. *petrisavii* and *T. ambiguum* displayed identical mapping results. *T. occidentale* and the two subspecies of *T. nigrescens* show significantly different distribution patterns of 5S and 18S-26S sequences. It is interesting to note, however, that all three taxa are essentially indistinguishable on the basis of conventional karyology (present study; Chen and Gibson, 1971).

The presence of two pairs of NOR-bearing chromosomes in tetraploid *T. uniflorum* and identical distribution patterns of major 5S and 18S sequences on these chromosomes strongly support the opinion of Evans (1996) that *T. uniflorum* is an autotetraploid species. Moreover, the active state of 18S-26S chromatin fibrils in all four NORs at a given time reflects the absence of nucleolar dominance in *T. uniflorum* and hence further supports this view. However, the tetraploid species *T. repens* has only one NOR-bearing pair and the same distribution patterns of 5S and 18S-26S sequences as *T. uniflorum*. In addition, there is an intermediate-sized 5S signal on a single metacentric pair. These facts indicate an allotetraploid origin of *T. repens*, which is also supported by the disomic nature of isozyme inheritance in this species (Williams, Mason and Williamson, 1998) and the lack of multivalent formation during meiosis. The progenitor species of white clover have not yet been established. Based on crossability and reproductive data, *T. occidentale*, *T. uniflorum*, *T. nigrescens* ssp. *nigrescens*, and *T. nigrescens* ssp. *petrisavii*, were regarded as the closest relatives of white clover (Chen and Gibson, 1971, 1972; Cleveland, 1985). All these taxa, along with white clover, belong to subsection Lotoidea (Zohary and Heller, 1984). A metacentric chromosome with an intermediate-sized 5S signal as part of one diploid genome could have been contributed by *T. nigrescens* ssp. *petrisavii* that cross hybridizes with white clover (unpubl. res.). On the basis of the distribution patterns of 5S and 18S-26S sequences on the only pair of white clover satellited chromosomes, *T. occidentale*, *T. uniflorum* or *T. nigrescens* ssp. *nigrescens* might be possible donors for the other genome of white clover. This proposal is in agreement with Chen and Gibson (1972) who suggested that white clover may share a common genome with *T. uniflorum* and *T. occidentale* on account of the presence of quadrivalents observed in hybrids of white clover with these species. Each diploid ancestral genome of white clover must have brought at least one pair of NOR-bearing chromosomes. The presence of only two 18S-26S signals in white clover can be explained on the basis of evolutionary loss of NOR sequences from the second satellited chromosome pair contributed through the genome of *T. nigrescens* ssp. *petrisavii*. Such evolutionary reorganization in the form of partial or complete loss of a rDNA locus, which may be the result of competition between two ancestral genomes, has been reported in several plant genera with allopolyploid species including *Scilla* (Araki, 1985; Vaughan *et al*., 1993), *Milium* (Bennett and Thomas, 1991) and *Brassica* (Maluszynska and Heslop-Harrison, 1993; Snowdon, Kohler and Kohler, 1997). These genomic changes might have occurred during or after the process of polyploidization.

An approach involving genomic *in situ* hybridization (GISH) using one or two putative parents as probes will help in distinguishing the two subgenomes of white clover and is being pursued in our laboratory.

The regional mapping of the two multigene families of the present study has demonstrated clearly that genomic reorganization has occurred during speciation in *Trifolium*. The two classes of rDNA also provide molecular markers for the identification of sets of individual chromosomes in interspecific hybrids of clovers.

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