Trifolium L.

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Trifolium L. is an economically important genus that is characterized by variable karyotypes relating to its ploidy level and basic chromosome numbers. The advent of genomic resources combined with molecular cytogenetics provides an opportunity to develop our understanding of plant genomes in general.

Keywords: Trifolium ; Chromosome

1. Introduction

The Fabaceae family (Leguminosae, legume or bean family) is the third-largest flowering plant family, after the Asteraceae and Orchidaceae families [1][2]. It is agronomically important, as it can form a symbiotic association with nitrogen-fixing bacteria. Several species from this family serve as genetic model organisms (e.g., *Medicago truncatula Gaertn.* , *Pisum sativum* L., and *Lotus japonicus* L.). With more than 250 species, the clover genus, *Trifolium* , is one of the largest genera in this family [1][2][3]. This herbaceous genus acquired its name in reference to the characteristic form of the leaf, usually consisting of three leaflets (trifoliolate), and includes both annual and perennial species occurring natively across a large range of biotopes from meadows and open woodlands to semi-deserts and mountain ridges in temperate and, to a lesser extent, subtropical regions. The genus's origin has been estimated to have occurred in the Early Miocene, 16–23 million years ago, and its center of origin was first assumed to be in California with its subsequent spread into Asia and hence to Europe and Africa [4]. Later, a new hypothesis was proposed of clovers originating in the Mediterranean region due to their species diversity, including the diversity in their chromosome numbers, and because the greatest occurrence of their endemic species is found in this area, with a secondary center of distribution in North America and East Africa [3][5][6][7]. By contrast, native clovers are absent from Australia and Southeast Asia.

Attempts have been made to divide this genus into natural groups. In the 19th century, Bossier [8] divided the genus into seven sections. A century later, eight subgenera were recognized and revised [1][9]. Better insight into phylogeny and the origin of the genus was facilitated by molecular analyses. These showed that *Trifolium* is a member of a large clade of legumes that lack one copy of the chloroplast inverted repeat [10][11], and a further molecular phylogenetic analysis of the internal transcribed spacer (ITS) and chloroplast genes provided evidence that most of these proposed sections are not monophyletic [12][13]. The most recent subgeneric classification, based on phylogenetic analyses of 218 species' ribosomal ITS and chloroplast trnL intron sequences, was proposed by Ellison et al. [3], who divided the genus into two subgenera, Chronosemium and Trifolium , with the further subdivision of *Trifolium* into eight sections— *Glycyrrhizum* (2 species), *Paramesus* (2 species), *Lupinaster* (3 species), *Trifolium* (73 species), *Trichocephalum* (9 species), *Vesicastrum* (54 species), *Trifoliastrum* (20 species), and *Involucrarium* (72 species). In 2014, *Trifolium* phylogenetic analyses were conducted, based on highly unusual *Trifolium* plastomes [14].

The economic importance of this genus lies in its agricultural utilization. Historically, clovers, and especially red clovers, have been cultivated in rotation with other crops to maintain soil fertility due to their ability to establish a mutualistic relationship with root-nodulating and nitrogen-fixing bacteria. Their value was later diminished by the advent of nitrogen fertilizers, but the global need for sustainable and conservation agriculture is bringing this historical approach back into focus. Nowadays, many *Trifolium* species are extensively cultivated as fodder plants (*Trifolium pratense* L., *Trifolium repens* L., *Trifolium hybridum* L., and *Trifolium resupinatum* L.), and also as green manure crops to enhance soil fertility and sustainability [15]. Further knowledge about the genomes of both wild and cultivated clovers and an understanding of their evolution will prove to be of great benefit in the future of clover breeding.

2. Chromosome Identification in *Trifolium*

In legumes with large chromosomes, such as *Pisum sativum* L. or *Vicia faba* L., individual chromosomes can be distinguished by ordinary karyotyping or banding methods [16][17], although the process is rather complicated in species with small chromosomes, such as *Trifolium*. Both repetitive and low- or single-copy sequences are important tools for

chromosome identification in cytogenetic studies. Usually, a mix of different probes is used, which can include localizing ribosomal DNA (rDNA) sites, telomeric probes, large plasmid, bacteriophage, or bacterial artificial chromosomes (BACs) containing specific single-copy or repetitive inserts. Based on 5S rDNA, 25S rDNA, and seven bacterial artificial chromosome probes containing microsatellite markers with a known position, a cytogenetic map has been constructed for red clover (**Figure 1**).

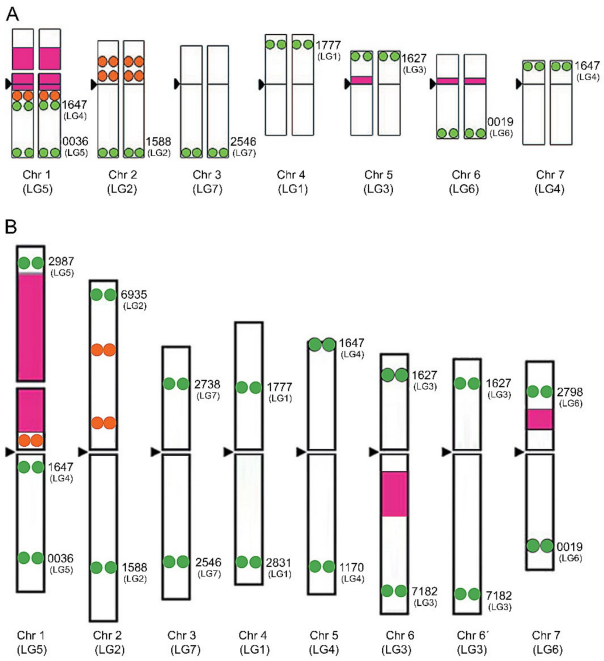

Figure 1. Cytogenetic map of *T. pratense* based on the hybridization pattern of probes derived from 5S rDNA (orange circles) and 26S rDNA (pink boxes) and localization of 7 (**A**) or 14 (**B**) BAC clones corresponding to chromosome-specific microsatellite markers (green circles) (adapted from Sato et al. [18] and Kataoka et al. [19].

The easy design and production of oligonucleotide libraries has presented new opportunities to plant cytogenetics. Recently, an oligonucleotide barcode system was developed to identify all cowpea and common bean chromosomes [20] [21]. Despite the availability of genome sequences for selected *Trifolium* spp., however, oligonucleotide libraries have not yet been exploited for *Trifolium* research.

3. Chromosomal Distribution of Ribosomal DNA Genes

Because ribosomal genes are among the best-researched regions of eukaryotic genomes, fluorescence in situ hybridization (FISH) analyses, using rDNA genes as probes, have been conducted in numerous plant species, including *Trifolium*. The 35S and 5S ribosomal genes are located independently in one or several loci as tandem repeats, ranging from hundreds to thousands of copies in higher vascular plant genomes [22]. While polycistronic gene 35S consists of 18S-5.8S-25S rDNA and occurs on chromosomal regions known as nucleolus organizer regions (NORs), the 5S rDNA gene is usually independent from NORs [23][24]. Ribosomal genes have undergone rapid evolution in their means of altering the number of copies and their localization on chromosomes [25][26][27][28]. Therefore, rDNA genes have been proven to act as excellent cytogenetic markers for karyotype analysis, and they have been widely used to examine and understand phylogenetic relationships, chromosomal organization, and evolution in many plant species.

Roa and Guerra [29][30] found that, in angiosperms rDNA sites, most often number one or two 45S and one 5S per haploid genome. The localization of 45S rDNA sites was observed preferentially on the short arm and in the terminal region of chromosomes in general, but genera with predominant proximal localization were found in some families, including Fabaceae (*Arachis*, *Lens*). On the other hand, 5S rDNA localization varies in different angiosperm families. In Fabaceae, 5S rDNA is preferentially found in the proximal region.

To date, the numbers and positions of rDNA loci on chromosomes have been reported for 42 *Trifolium* species (**Table 1**; adapted from Vozárová et al. [31]). Based on ancestral state reconstruction, Vozárová et al. [31] suggested the occurrence of one 5S and one 26S locus per haploid genome separately as an ancestral condition for the whole genus. The ancestral karyotype referencing the basic chromosome number and rDNA loci constitution may resemble the karyotype of *T. diffusum* (**Figure 2**).

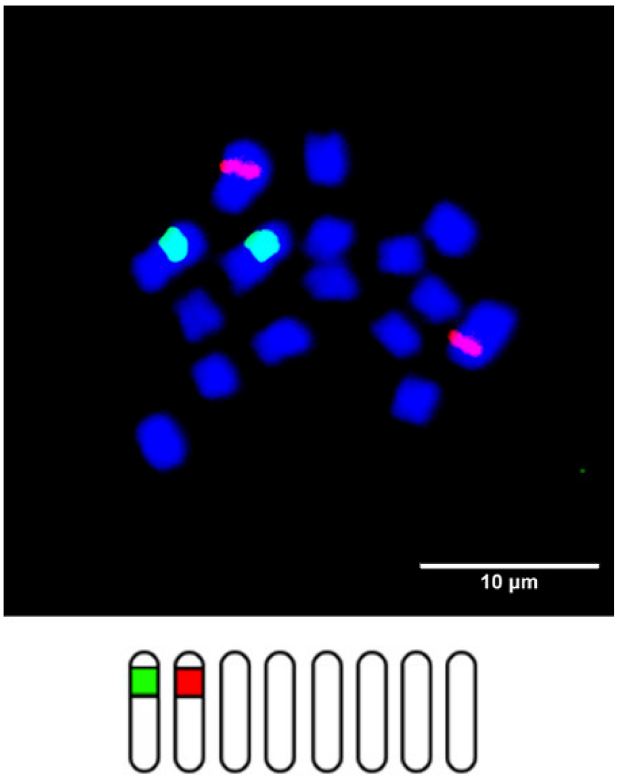

Figure 2. Fluorescence in situ hybridization image and schematic karyotype of *T. diffusum* with 5S (red) and 26S (green) rDNA probes suggested to represent the ancestral state in the *Trifolium* genus (adapted from Vozárová et al. [31]).

Table 1. Reported chromosome numbers 5S and 25S rRNA loci numbers in *Trifolium* species (adapted from Vozárová et al. [31]).

Subgenus/Section	*Trifolium* Species	2n	Loci Number per 2n		Reported in
			5S	25S	
CHRONOSEMIUM					
	T. aureum	**2x = 16**	**4**	**2**	**Vozárová et al. [31]**
		2x = 14	**4**	**2**	
	T. badium	**2x = 14**	**2** **2**	**4** **2**	
	T. campestre	**2x = 14**	**2**	**2**	**Ansari et al. [32]**
	T. micranthum	**2x = 16**	**2**	**2**	
	T. dubium	**4x = 30**	**4**	**4**	
TRIFOLIUM					

Subgenus/Section	*Trifolium* Species	2n	Loci Number per 2n		Reported in
			5S	25S	
TRIFOLIUM	*T. alpestre*	2x = 16	10	2	
			11	2	
	T. arvense	2x = 14	2	2	
	T. bocconei	2x = 12	2	2	
	T. cherleri	2x = 10	4	10	
	T. diffusum	2x = 16	2	2	
	T. hirtum	2x = 10	6	2	
	T. ligusticum	2x = 12	2	2	
		2x = 14	2	2	Vozárová et al. [31]
	T. pallidum	2x = 16	4	2	
	T. purpureum	2x = 14	2	2	
	T. rubens	2x = 16	4	2	
	T. squamosum	2x = 16	4	2	
	T. stellatum	2x = 12	4	2	
		2x = 14	4 (2w)	2	
	T. pannonicum	16x = 128	16	16	
	T. pratense	4x = 28	8	8	Dluhošová et al. [33]
		2x = 14	4	5	Sato et al. [18]
	T. medium	8x = 64	12	8	Dluhošová et al. [33]

Subgenus/Section	*Trifolium* Species	2n	Loci Number per 2n		Reported in
			5S	25S	
TRICHOCEPHALUM	*T. subterraneum subsp. subterraneum*	2x = 16	2	4 (2w)	Vozárová et al. [31]
	T. subterraneumsubsp. subterraneum	2x = 16	2	2	
	T. subterraneum subsp. brachycalycinum	2x = 16	2	4 (2w)	Falistocco et al. [34]
	T. israeliticum	2x = 12	10	4	
VESICASTRUM	*T. fragiferum*	2x = 16	2	2	
	T. resupinatum	2x = 16	2	2	Vozárová et al. [31]
		2x = 14	2	2	
	T. spumosum	2x = 16	2 4	2 2	
TRIFOLIASTRUM	*T. glomeratum*	2x = 16	2	2	Vozárová et al. [31]
	T. montanum	2x = 16	2	2	
	T. occidentale	2x = 16	4	2	Ansari [35]
	T. pallescens	2x = 16	2	2	Vozárová et al. [31]
	T. thalii	2x = 16	2	2	

Subgenus/Section	*Trifolium* Species	2n	Loci Number per 2n		Reported in
			5S	25S	
	T. repens	4x = 32	4	2	
	T. uniflorum	4x = 32	4	4	
	T. nigrescens subsp. nigrescens	2x = 16	2	2	
	T. nigrescens subsp. petrisavii	2x = 16	2	2	Ansari [35]
	T. ambiguum	2x = 16	2	2	
	T. hybridum	2x = 16	2	2	
	T. isthmocarpum	2x = 16	2	6	
INVOLUCRARIUM	*T. chilense*	2x = 16	4	2	
	T. microdon	2x = 16	2	2	Vozárová et al. [31]
	T. microcephalum	2x = 16	16 16	16 2	
PARAMESUS			4	2	
	T. glanduliferum	2x = 16	5 (1w)	2	Vozárová et al. [31]
			4	2	
	T. strictum	2x= 14	2	2	
LUPINASTER	*T. lupinaster*	4x = 28	8	4	Vozárová et al. [31]
		4x = 32	8	4	

4. Conclusions and Future Prospects

Improving clover productivity as a means of boosting yields and nitrogen fixation efficiency is therefore a central focus of plant breeders today. Information is mainly limited to the related legume model species, however, and unraveling the genome organization and understanding the evolution of clover are essential for greater breeding efficiency.

Advances in clover research within the genomics era have assisted in the development of an impressive array of genomic resources, including complete genome sequences of some clovers and related legumes. As high-throughput sequencing has revolutionized genome sequencing with its ultralow cost and overwhelmingly large data output, more and more new plant species sequences, as well as species' resequences, supported by a large range of bioinformatic tools, provide us

with more data applicable for more efficient breeding strategies. The combination of genomic and bioinformatic data with molecular cytogenetics may provide a more developed understanding of plant genomes in general.

Ribosomal DNA and other repetitive sequences have been widely used as plant cytogenetic markers, and recently, the development of large-DNA clones carrying target sequences, such as BACs, has facilitated the easier localization of low- or single-copy DNA sequences [36][37][38][39][40][41][42]. BAC-FISH has been applied successfully in clover karyotype characterization, and cross-species BAC-FISH has helped to identify chromosome structure and rearrangements in clover relatives such as the common and lima bean [43]. However, the extension of BAC-FISH to the BAC-painting of large chromosome regions is suitable only for species with small genomes and low proportions of repetitive fractions, and it has not been successfully established beyond crucifers [44][45], with the singular exception of Brachypodium [46].

The remarkable progress in plant genome research relating to reference sequences production and artificial DNA synthesis has provided an alternative chromosome painting technique. In silico designed and artificially synthesized oligonucleotide pools have already been applied successfully in various plant species to characterize chromosomal rearrangements [47][48][49][50][51][52][53][54]. The availability of the Trifolium genome and reference sequences means that the adoption of oligo painting within this genus, and the legume family more generally, is both possible and to be expected.

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