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Impact of Interspecific Hybridization of *T. pratense* × *T. medium* and Backcrossing on Genetic Variability of Progeny

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Abstract

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Red clover (*Trifolium pratense* L.) is a high-quality fodder crop which has been hybridized successfully with its wild relative zigzag clover (*T. medium* L.). The aim of this study was to evaluate the genetic impact of interspecific hybridization and subsequent repeated backcrossing on the variability within hybrid progeny genomes. Nuclear DNA content of 800 and 753 hybrid plants from F_7/F_8 and F_8/F_9 generations, respectively, was measured by flow cytometry. Resulting values were converted to estimated chromosome counts, which were successfully validated on a sample of 28 plants by counting mitotic chromosomes. The two generations showed a similar distribution of various chromosome counts ranging from 22 to 47 chromosomes. In total, 24.0% and 34.3% of plants from the two generations had different numbers of chromosomes from their parental plants. Variability within the hybrid population was assessed by fluorescent *in situ* hybridization using rDNA probes. Individual plants had a pattern of 5S and 45S rDNA loci rather more similar to that of *T. pratense* than of *T. medium*. Numbers of chromosomes with clusters of 5S rDNA ranged from 6 to 14 while those of 45S rDNA varied between 4 and 13. Individual arrangements were almost unique, and some plants possessed also novel formations which were not present in any of the parental species, such as a cluster of 5S rDNA surrounded by 45S rDNA clusters or a 45S rDNA cluster surrounded by 5S rDNA clusters. This suggests complex rearrangements connected with post-hybridization stabilization of hybrid genomes.

Keywords: cytology; FISH; hybrids; rDNA; red clover; zigzag clover

Red clover (*Trifolium pratense* L.) is an important forage legume that is widely cultivated in most temperate regions within Europe and worldwide. Like other legume species, it has an ability to fix atmospheric nitrogen through symbiosis with bacteria of the genus *Rhizobium*. This significant attribute makes it highly important both economically and agriculturally. Red clover is widely sown as a companion crop and a green manure crop to increase soil fertility. Clovers are grown in pastures for grazing and harvested for hay as a staple feedstuff for cattle (Taylor & Quesenberry 1996).

Red clover breeding is focused mainly upon improving important traits like persistency, resistance to both biotic and abiotic factors, forage yield, or protein quality characteristics (Taylor 2008). Trends in breeding are shifting towards the utilization of modern methods based on genetic improvement such as marker-assisted selection, candidate gene identification, or genotyping of individual plants based on sequencing data from next generation sequencing (NGS; Řepková & Nedělník 2014). These methods can significantly shorten the time needed for selection and subsequent breeding of varieties with improved traits. Nevertheless, some traits are highly complex and cannot be easily changed even with contributions from the aforementioned molecular methods. Recent upgrades of molecular methods

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have enabled us to modify certain traits, such as disease resistance, by means of genetic transformation using *Agrobacterium tumefaciens* (Khanlou *et al*. 2011). To overcome traits such as low persistency, introduction of appropriate traits from closely related species by means of hybridization without genetic modification would still be preferred if viable progeny could be obtained.

Artificial interspecific hybridization of red clover offers a huge potential for the long-lasting introgression of new and useful traits. To date, *T. pratense* has been successfully crossed with five species: *T. sarosiense* Hazsl., *T. medium* L., *T. alpestre* L., *T. ambiguum* M. Bieb., and *T. diffusum* Ehrh. (reviewed by Abberton 2007). All published reports have mostly been limited to a comparison of the intermediate morphological appearance of the F_1 hybrids to both parents, and none of these has yet been able to obtain a stable and fertile hybrid progeny.

The only viable progeny resulted from hybrids between *T. pratense* cv. Tatra ($2n = 4x = 28$) and *T. medium* ($2n = 8x = 64$) obtained by embryo rescue (Řepková *et al*. 1991, 2006). The resulting F_7/F_8 and F_8/F_9 progeny was thoroughly inspected on the levels of morphological, agronomic and reproductive traits, and this work determined statistically significant differences between hybrids and *T. pratense* in nearly all of the analysed characteristics (Jakešová *et al*. 2011, 2014). However, genetic variability of the resulting progeny has never been further evaluated. The only examination was performed on the fifth hybrid generation by means of flow cytometry measurement, which showed DNA content corresponding to the range of 30–45 chromosomes (Řepková *et al*. 2003). Subsequent hybrid generations were not examined at all and their genetic states have so far remained unknown, even though the previous reports of clover hybrids had observed genomic instability among successive generations leading to the loss of chromosomes (Isobe *et al*. 2002).

In this paper, we present an evaluation of the genetic impact of interspecific hybridization (*T. pratense* × *T. medium*) and subsequent backcrossing on progeny variability in chromosome number and rDNA loci at the level of individual hybrid plants.

MATERIAL AND METHODS

Plant material. Plant material consisted of maternal plants of the *T. pratense* variety Tatra, paternal plant of *T. medium* clone 10/8, and interspecific hybrid progeny called JEH plants. JEH plants originated from interspecific hybridization of maternal tetraploid *T. pratense* variety Tatra ($2n = 4x = 28$, 1C = 418 Mbp) and paternal octoploid *T. medium* clone 10/8 ($2n = 8x = 64$, 1C = 3154 Mbp) (Řepková *et al*. 1991; Nedbálková *et al*. 1995). JEH plants chosen for our analyses were F_7/F_8 and F_8/F_9 generations labelled as JEH1V (sowing in 2007) and JEH1F (sowing in 2010), respectively. Seeds of the Tatra variety were obtained from the Gene Bank of the Crop Research Institute (Prague-Ruzyně, Czech Republic), accession 13T0200327. JEH plants as well as the paternal octoploid *T. medium* clone 10/8 plant were obtained from the breeding facility Dr. Hana Jakesova Clovers and Grass Plant Breeding (Hladké Životice, Czech Republic).

Flow cytometry. Approximately 50 mg of fresh, fully developed true leaf from 4-week-old plants was used for the sample preparation. One leaf per plant was cut and immediately mixed with 250 µl of nuclei isolation solution (0.1 M citric acid monohydrate, 0.5% Tween-20), mechanically chopped with a razor blade and stained with 500 µl of DAPI solution (15 µM 4',6-diamidino-2-phenylindole dihydrochloride, 0.4 M sodium phosphate dibasic dodecahydrate). The cellular debris was subsequently filtered by passing through a polyamide (uhelon) mesh (50 µm pore size). After 10 min, DNA content of the nuclei solution was measured by flow cytometry using a Partec Ploidy Analyser-I (Sysmex Partec, Görlitz, Germany). The measured values were then converted to hypothetical chromosome counts using the red clover Start variety as an internal standard reference with the known and validated chromosome count of $2n = 14$.

Chromosome count validation. Twenty freshly cut root tips were synchronized overnight on ice and stored in Carnoy's fixative at –20°C. Chromosome spreads were prepared after pretreatment with pectolytic enzyme mixture (0.3% pectolyase, 0.3% cellulase, and 0.3% cytohelicase in 1× citrate buffer) by the SteamDrop method according to Kirov *et al*. (2014) with a Double SteamDrop modification. Slides were counterstained with DAPI in Vectashield (Vector Laboratories, Burlingame, USA), then evaluated using an Olympus BX-51 fluorescence microscope and captured using Olympus DP72 CCD.

Fluorescent *in situ* hybridization (FISH) with rDNA probes. Slides with prepared chromosome spreads were subsequently used for FISH with rDNA probes. Probes for both 5S and 45S rDNA were derived from previously published *T. pratense*

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sequencing data (Ištvánek *et al*. 2014). Selected rDNA sequences were amplified using loci-specific primers (5S_F: GGTGCGATCATACCAGCACTAA, 5S_R: AGGTGCAACACAAGGACTTC, 45S_F: TTCCCACTGTCCCTGTCTACTAT, 45S_R: TTACGAGGTCCACCAACGGCA) by polymerase chain reaction (PCR) with a reaction mixture containing 1× GoTaq Reaction Buffer (Promega, Madison, USA), 0.2 mM dNTPs, 1 µm primers, 0.5 U Taq Polymerase (Promega) and 20 ng of gDNA. PCR products were separated by agarose electrophoresis, excised from the gel, purified using a PCR purification kit (Qiagen, Hilden, Germany), then quantified using a NanoDrop Spectrophotometer. Amplified probes were labelled by nick translation using Biotin or DIG Nick Translation Mix (Roche, Mannheim, Germany). Slides with chromosome spreads were treated with 100 ug/ml RNase A (Sigma, St. Louis, USA) in 2× SSC for 1 h at 37°C and 0.1 mg/ml pepsin in 10 mM HCl for 2 min at 37°C. Next, 100 ng of labelled denatured probes in hybridization buffer of 77% stringency were applied to suitable chromosome spreads, co-denatured at 80°C for 2 min, and left for hybridization overnight at 37°C in a humid box. Post-hybridization washing was carried out at 42°C with the following steps: 2× SSC twice for 5 min, 10% formamide/0.1× SSC twice for 5 min, 2× SSC for 5 min, and 4× SSC/0.05% Tween-20. Biotin or DIG-labelled probes were immunodetected with streptavidin-Cy3 (GE Healthcare, Buckinghamshire, United Kingdom; 1:1000 dilution) and anti-DIG-FITC (Roche; 1 : 200 dilution) antibodies.

RESULTS AND DISCUSSION

The main objective of this study was to assess the genetic impact of *T. pratense* × *T. medium* hybridization and subsequent backcrossing on variability among the hybrid progeny. Counting of mitotic or meiotic chromosomes of individual plants is usually the first step in a hybrid state evaluation, because the knowledge of exact chromosome count is necessary for follow-up breeding programmes. Recently, this task has been replaced by flow cytometry measurement, which has become an easy and reliable method for identification of plants with altered genome size. This technique is widely used today for identifying various hybrid progenies (Ochatt 2008). A total of 800 JEH 1V and 753 JEH1F plants were included into the measurement of nuclear DNA content by flow cytometry. The resulting values converted to chromosome counts ranged from the lowest value of 22 to the highest value of 47 chromosomes and with a similar distribution in both generations (Table 1). In contrast to the fifth generation (Řepková *et al*. 2003), the majority of plants today possess $2n = 28$ chromosomes. This shows a strong tendency to stabilize the unbalanced genomes into the maternal state, as was previously reported also in clover (Isobe *et al*. 2002) as well as in other crop plants (Renny-Byfield & Wendel 2014). Aside from plants with $2n = 28$ chromosomes, a significant proportion of differing chromosome counts dissimilar to both parents was recognized in both generations (24.0% in JEH1V and 34.3% in JEH1F). This suggests that the hybrid populations successfully maintain a stable proportion of genetically variable plants, even after several rounds of repeated backcrossing with a red clover variety for improving yield characteristics. Despite yield improvement, slightly lower fertility and survivability were observed in some plants with less than 25 chromosomes presumably because of a loss of a substantial proportion of the genome.

Nevertheless, validation of a representative sample should be conducted in order to assess the strength of the correlation between measured DNA content and number of chromosomes, because nuclear DNA content can be distorted due to a possible shift of GC content in the hybrid progeny (Doležel & Bartoš 2005). A representative proportion of 28 JEH plants from the two generations was randomly chosen for chromosome count validation. The tested sample included 5 and 23 plants from JEH1V and JEH1F generations, respectively. Plants were selected from

Table 1. Distribution of nuclear DNA content values of 800 individual JEH1V and 753 individual JEH1F plants as measured by flow cytometry (percentages are given in parentheses)

Generation	$2n < 28$	$2n = 28$	$2n = 29$ to 30	$2n = 31$ to 40	$2n = 41$ to 47
JEH1V	68 (8.5)	608 (76.0)	120 (15.0)	3 (0.4)	1 (0.1)
JEH1F	48 (6.4)	495 (65.7)	199 (26.4)	9 (1.2)	2 (0.3)

JEH1V – F_7/F_8; JEH1F – F_8/F_9; resulting values were converted to chromosome counts using an internal *Trifolium pratense* standard reference of $2n = 14$

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all across the chromosome count range except for the prevailing category with $2n = 28$. Half of the tested plants had only one prevailing chromosome count while the other half showed a chromosome mosaicism in the root apex. These plants had two or three prevailing chromosome counts of different proportion, and individual roots exhibited only one specific chromosome count. Estimated chromosome counts were successfully validated in all 28 analysed JEH plants. The highest deviation was observed in

Table 2. Summary of the validation of chromosome count estimated by flow cytometry for individual JEH plants

	Chromosome counts	
	estimated	prevailing (%)
JEH1V (F_7/F_8)		
JEH 26/7	43.4	43 (81.54), 42 (12.31)
JEH 34/3	29.4	29 (63.89), 28 (15.28), 30 (12.50)
JEH 34/14	30.3	30 (93.55)
JEH 35/6	26.8	27 (88.52)
JEH 35/17	29.3	29 (95.59)
JEH1F (F_8/F_9)		
JEH 1/9	30.5	30 (90.48)
JEH 3/3	26.6	27 (95.31)
JEH 3/8	29.0	29 (92.54)
JEH 5/2	30.0	29 (87.32), 30 (8.45)
JEH 7/4	30.6	30 (59.42), 31 (34.78)
JEH 7/9	29.9	29 (69.44), 30 (23.61)
JEH 9/6	30.0	30 (69.12), 29 (16.18), 31 (7.35)
JEH 11/8	29.9	30 (72.86), 31 (11.43), 29 (11.43)
JEH 13/9	29.9	30 (96.88)
JEH 18/1	26.4	27 (93.65)
JEH 18/10	31.1	31 (93.75)
JEH 19/10	29.6	32 (65.75), 30 (16.44), 31 (10.96)
JEH 20/2	30.8	31 (83.95), 30 (11.11)
JEH 21/8	26.9	27 (89.55)
JEH 27/20	30.4	30 (63.64), 29 (25.97)
JEH 34/12	29.2	30 (76.19), 29 (17.46)
JEH 35/7	43.2	44 (48.48), 24 (45.45)
JEH 42/2	29.0	29 (79.73), 28 (14.86)
JEH 60/4	29.8	30 (90.63)
JEH 62/8	30.5	30 (49.35), 31 (38.96)
JEH 74/7	26.8	27 (90.54)
JEH 79/1	26.6	27 (92.31)
JEH 79/3	29.0	29 (93.75)

Estimated chromosome counts were inferred from nuclear DNA content values measured by flow cytometry using an internal *Trifolium pratense* standard reference of $2n = 14$

Table 3. Comparison of the number of 5S and 45S rDNA loci among 28 tested JEH plants and in relation to parental *Trifolium pratense* and *T. medium*

	Generation	Chromosome count	No. of chromosomes with rDNA loci		
			both 5S and 45S	only 5S	only 45S
T. pratense	♀	28	4	4	4
T. medium	♂	64	0	12	8
JEH 3/3	F_8/F_9	27	4	3	3
JEH 3/8	F_8/F_9	27	5	4	3
JEH 18/1	F_8/F_9	27	3	4	4
JEH 21/8	F_8/F_9	27	4	4	2
JEH 35/6	F_7/F_8	27	3	5	1
JEH 74/7	F_8/F_9	27	3	3	1
JEH 79/1	F_8/F_9	27	4	4	1
JEH 5/2	F_8/F_9	29	5	4	2
JEH 7/9	F_8/F_9	29	4	5	3
JEH 34/3	F_7/F_8	29	4	5	2
JEH 35/17	F_7/F_8	29	4	4	3
JEH 42/2	F_8/F_9	29	6	4	0
JEH 79/3	F_8/F_9	29	5	5	2
JEH 7/4	F_8/F_9	30	4	5	1
JEH 9/6	F_8/F_9	30	4	4	3
JEH 1/9	F_8/F_9	30	4	5	4
JEH 11/8	F_8/F_9	30	4	4	4
JEH 13/9	F_8/F_9	30	4	4	3
JEH 27/20	F_8/F_9	30	4	4	2
JEH 34/12	F_8/F_9	30	4	4	4
JEH 34/14	F_7/F_8	30	4	5	3
JEH 60/4	F_8/F_9	30	4	4	3
JEH 62/8	F_8/F_9	30	4	5	3
JEH 18/10	F_8/F_9	31	5	3	3
JEH 20/2	F_8/F_9	31	6	4	4
JEH 19/10	F_8/F_9	32	5	5	4
JEH 26/7	F_7/F_8	43	7	6	4
JEH 35/7	F_8/F_9	44	9	5	5

The number of chromosomes with particular rDNA loci was assessed according to fluorescent *in situ* hybridization (FISH) with 5S and 45S rDNA probes

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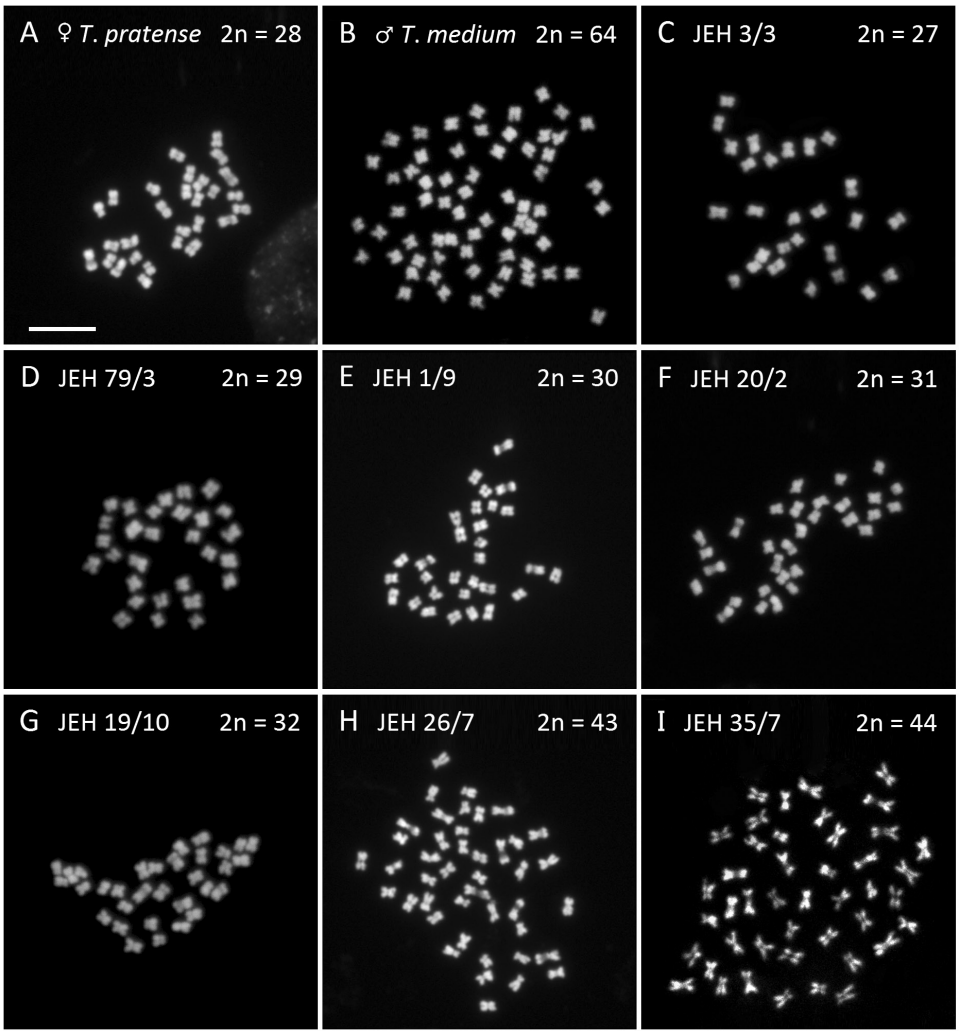

Figure 1. Various chromosome counts present in individual hybrid JEH plants and their comparison with both parental species; validated chromosome counts of maternal *Trifolium pratense* (A), paternal *T. medium* (B), and hybrid progeny (C–I); chromosomes were counterstained with DAPI; scale bar 10 μm

plant JEH 35/7, with $2n = 44$ instead of the estimated 43 chromosomes. In the cases of 5 mosaic plants, however, the chromosome counts estimated by flow cytometry were not those most prevalent in these plants. This might possibly have been avoided if the flow cytometry sample had been pooled from several individual leaves as recommended (Doležel & Bartoš 2005; Wang *et al.* 2015). Aneuploid or mosaic plants were all healthy and vigorous and could not be distinguished morphologically from the rest of the plants. Results of prevailing chromosome counts for individual JEH plants as well as the differences from the estimated values are summarized in Table 2. Representation of all various chromosome counts from selected JEH plants as well as both parental plants is shown in Figure 1.

The hybridization of 5S and 45S rDNA probes was used to identify the main events responsible for changes in chromosome counts, especially for comparing the number of rDNA loci with maternal *T. pratense*. As was previously reported, rDNA clusters are typical breakpoints involved in chromosome rearrangements of several organisms (Bustamante *et al.* 2014) including hybrids (Kovařík *et al.* 2008). Variability within the 28 individual JEH plants was assessed using FISH on the level of different number and localization of both 5S and 45S rDNA. Hybridization of rDNA probes to maternal *T. pratense* shows 8 clusters of 5S rDNA repeats and 8 clusters of 45S rDNA repeats, which is in concordance with previous reports (Kataoka *et al.* 2012). Hybridization to paternal *T. medium* shows 12 clusters of 5S

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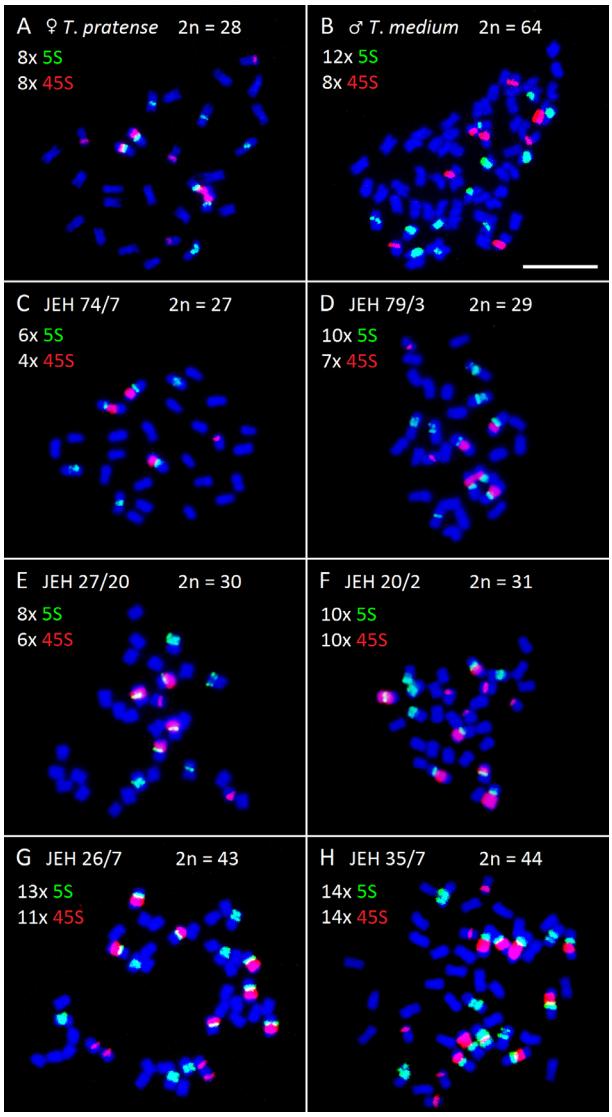

Figure 2. Summary of various numbers and arrangements of 5S and 45S rDNA clusters present in selected JEH plants compared to parental *Trifolium pratense* and *T. medium*; hybridization pattern of 5S rDNA (green) and 45S rDNA (red) in maternal *T. pratense* (A), paternal *T. medium* (B), and selected hybrid plants (D–H); scale bar 10 μm

rDNA loci and 8 clusters of 45S rDNA on separate chromosomes, and this has never been reported previously. The number and overall arrangement of both rDNA clusters in JEH plants were rather more similar to those of *T. pratense* than of *T. medium* due to the presence of chromosomes with 45S rDNA loci alongside that of 5S rDNA (Figure 2). Individual arrangements were more or less unique for each JEH plant, and differences were observed even between JEH plants with the same chromosome count. The most conserved patterns were observed within the group of $2n = 30$, where all plants possessed 4 chromosomes bearing both 5S and 45S rDNA clusters (Table 3). The overall numbers of chromosomes with clusters of 5S rDNA ranged from 6 to 14 while those for 45S rDNA varied between 4 and 14. This demonstrates a high level of genetic variability within the hybrid population as a whole. Moreover, new chromosome-scale rearrangements of 5S and 45S rDNA loci distinct from arrangements present in maternal *T. pratense* or paternal *T. medium* were observed in some JEH plants. Plant JEH 20/2 had a cluster of 5S rDNA surrounded by 45S rDNA clusters from both sides, and plants JEH 35/7 and 42/2 had a 45S rDNA cluster surrounded by 5S rDNA clusters from both sides (Figure 3). None of these formations has been observed to date in any other *Trifolium* species (Ansari *et al.* 1999, 2008; Williams *et al.* 2001; Kataoka *et al.* 2012; Falistocco *et al.* 2013).

All these findings, and particularly both high variability within the number of chromosomes and unique patterns of rDNA clusters with novel rearrangements suggest individual, plant-specific, complex rearrangements during the post-hybridization balancing of novel genomes which are still maintained even after several rounds of backcrossing with *T. pratense*. Such a high level of genetic variability will be very beneficial for future breeding.

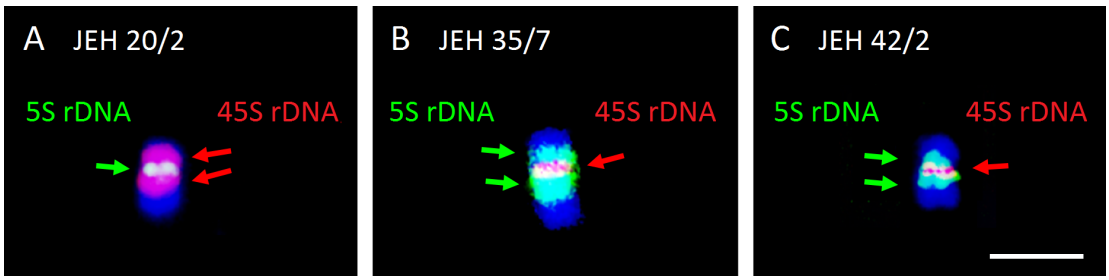

Figure 3. Detail of new rearrangements observed in JEH 20/2 (A), JEH 35/7 (B), and JEH 42/2 (C); JEH 20/2 (A) possesses a new rearrangement of single 5S rDNA cluster (green) surrounded by two 45S rDNA clusters (red) while JEH 35/7 (B) and JEH 42/2 (C) possess an inversed rearrangement; scale bar 5 μm

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