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Description and inheritance of new genes in red clover

Abstract: Four traits found in red clover (*Trifolium pratense* L.) were each determined to be controlled by single, recessive genes in the homozygous state. Three morphological traits, split leaflet, round pollen, and purple-red flower color, should serve as useful genetic markers. The remaining gene affects meiosis, and results in univalents at diakinesis and the subsequent formation of $2n$ eggs. This has been useful in producing tetraploid red clover. The symbols *sl*, *rp*, and *sy*, have been proposed to designate, respectively, genes for split leaflet, round pollen, and synaptic mutant. As the purple-red flower color may be a rediscovery of an earlier gene, *p*, this symbol has been maintained to designate purple-red flower color.

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Red clover (*Trifolium pratense* L.) is a diploid ($2n = 2x = 14$) forage legume important in the temperate regions of the world. Tetraploid ($2n = 4x = 28$) forms also are cultivated in Europe.

Clover geneticists in the early part of the century were familiar with numerous genes. Williams[19] published a partial linkage map in 1937, but failed to provide references or explanations for the various gene symbols in his linkage map. This genetic material has since been lost. In recent years, only a few genetic markers have been described, and even fewer are currently available. Taylor and Smith[15] listed four genes that controlled morphological traits, four that conditioned for male sterility, four that affected nodulation, and nine that controlled disease reactions. Taylor[14] later released eleven genetic marker stocks of red clover. Four of these stocks have traits that are under polygenic control, making them of limited use to breeders or geneticists as genetic markers. Three of the remaining marker genes were dominant and the remaining four were recessive.

Four new traits were recently discovered in red clover. These are split leaflet, round pollen, synaptic mutant, and purple-red flower color. These are expected to serve as useful genetic markers. One of these traits, the synaptic mutant, leads to the formation of $2n$ (i.e., numerically unreduced) eggs and has facilitated both interspecific hybridizations[9] and the development of tetraploid red clover germplasm[10].

Materials and Methods

Split leaflet refers to a condition in which one or more of the leaflets of a trifoliolate leaf are split. The separation can begin anywhere along the margin, and continue, to varying degrees, towards the petiolule or the midvein (Figure 1). Although the split leaflet condition can resemble the multifoliolate condition if the split reaches the petiolule, it differs in that each leaflet, regardless of the degree of splitting, has one petiolule, whereas each leaflet in multifoliolate plants has its own petiolule. The entire pubescence of plants with split leaflet is tufted in appearance. This may be due to a pleiotropic effect or to close linkage of the split leaflets factor to another gene. However, no recombinants have been observed.

The original trait was found in a plant that was obtained by selfing a plant with normal phenotype, then backcrossing (BC) the I_1* to the parent to obtain an I_1BC_1 generation. The plant with the split leaflets (referred to as plant A) was crossed to a normal, unrelated plant. Backcrosses were then made, in reciprocal directions, by crossing the resulting F_1 (plant B) to the split leaflet (I_1BC_1) parent (plant A). The progeny were scored for phenotype. Thirteen of these new BC_1 plants, each of which had a normal pheno-

* In clover genetics, the symbol "I" is used instead of the symbol "S" to denote selfing or inbreeding. The symbol "S" is used to refer to the self-incompatibility alleles.

type, were then intercrossed among themselves inside an isolation cage using honey bees (*Apis mellifera* L.) as pollinators. All seed were planted in progeny rows and scored for phenotype in the greenhouse.

Segregation ratios for this and all traits were tested for goodness-of-fit by the chi-square test. Chi-square values for related families were added together when the homogeneity chi-square was not significant[12].

Round pollen refers to dry, unstained pollen that has retained the spherical shape of the microspore, as opposed to the oblong tricolporate morphology normally associated with mature red clover microgametophytes (Figure 2). Round pollen is easily detected by placing dry pollen on a microscope slide and observing it under low magnification. Round pollen has an exine that appears normal and may be monoporate. This trait is under sporophytic control, as all the pollen produced by a plant is either round or normal, but never a mixture of both. Plants with round pollen appear to have normal male fertility as evidenced by seed set when crossed to normal diploids. This trait originally appeared in five segregating progeny of a plant from the cultivar Florex. Chromosome counts in root tip squashes were used to verify the diploid nature of these plants. A plant (referred to as plant C) with round pollen was selected and crossed to a plant with normal pollen. Four of the F_1 plants obtained (referred to as plants D, E, F, and G) were paired and reciprocal crosses made within pairs to obtain the F_2 generation. Plants D and E also were backcrossed, in reciprocal directions, to plant C (the round pollen parent) to obtain the backcross generation.

Synaptic mutant refers to a condition in which the chromosomes are unpaired at diakinesis (Figure 3), resulting in almost complete male sterility and greatly reduced female fertility. Normal female gametes have ($n = x = 7$) chromosomes. Chromosome numbers of progeny obtained when synaptic mutants are pollinated with pollen from euploid plants suggest that the female gametes formed by synaptic mutant plants can have 7, 8, 9, or 10 chromosomes. Consequently this mutation is useful to create trisomic plants. Synaptic mutant plants also produce 0–10 percent restitution eggs, making them a valuable source of $2n$ ($2n = 2x = 14$) eggs. Frequency of $2n$ egg formation was determined by the frequency of tetraploid progeny obtained following pollination of synaptic mutant plants with pollen from tetraploid ($2n = 4x = 28$) plants. Lack of chromosome pairing during meiosis has been described in red clover by Strzyzewska[13] and Whittington[17], but its inheritance has not been reported.

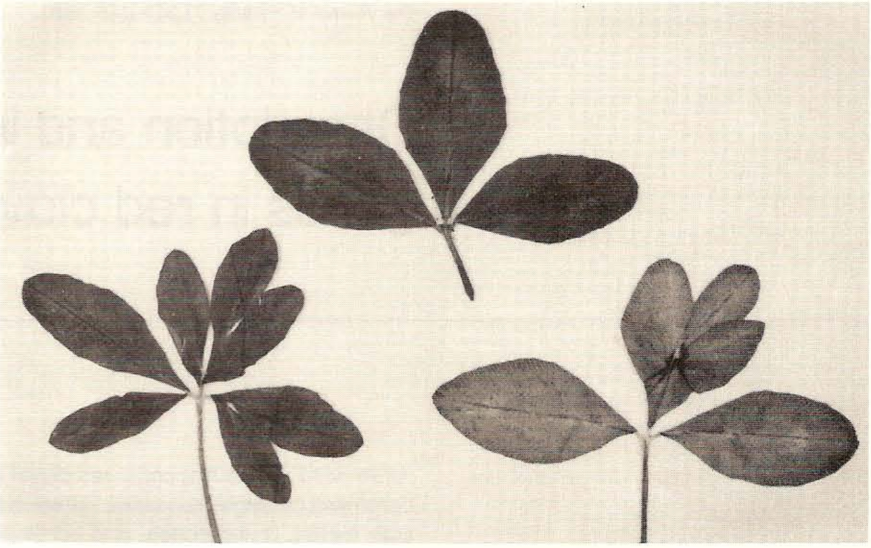

FIGURE 1 A normal trifoliolate leaf of red clover (center top), and two leaves (bottom left and right) with leaflets showing various degrees of splitting.

This mutant was found in a plant, C51, of cv. Chesapeake, and has been described previously by Parrott and Smith[8]. It was possible to obtain three F_1 seed from crosses between C51 and three normal plants. These F_1 plants are referred to as H, I, and J. The half-sib F_1 plants were then intercrossed by hand. Only the crosses H × I, I × H, and J × I produced sufficient F_2 seed for testing. The F_2 plants were scored for male sterility. Plants with no extractable, stainable pollen grains in the flowers were considered to be male sterile. Male-sterile plants were considered to be synaptic mutants.

Purple-red flower color. Plants with this trait produce flowers that range from an average of 5 RP 5/10 to 7.5 RP 4.5/8 on the Munsell[3] color charts. This can be described as a moderate purplish red[16]. One plant bearing moderate purple-red flowers was found in a population undergoing recurrent selection for branched root types. This plant was

Table I. Red clover backcross progenies and BC_1F_1 families segregating for split leaflet

	Observed values			
	normal	split	χ^2 (1 *df*)	*P*
Backcross*				
A × B	18	25	1.14	0.250–0.100
B × A	22	18	0.40	0.975–0.950
Total	40	43	1.54 (2 *df*)	0.500–0.250
Pooled			0.11 (1 *df*)	0.750–0.500
Family†				
1	14	8	1.16	0.500–0.250
2	44	11	0.73	0.900–0.750
3	16	12	4.76	0.050–0.025
4	25	4	1.94	0.250–0.100
5	8	1	0.93	0.500–0.250
6	17	8	0.65	0.500–0.250
7	39	12	0.05	0.900–0.750
8	4	3	1.19	0.500–0.250
9	10	4	0.10	0.900–0.750
10	41	13	0.02	0.900–0.750
11	17	6	0.14	0.750–0.500
12	59	23	0.41	0.750–0.500
Total	294	105	12.08 (12 *df*)	0.500–0.250
Pooled			0.37 (1 *df*)	0.950–0.900

* Backcross of the F_1 (B) to the split leaflet parent (A); expected 1:1 ratio
† Each family is the polycross progeny of normal BC_1 progeny; expected 3:1 ratio

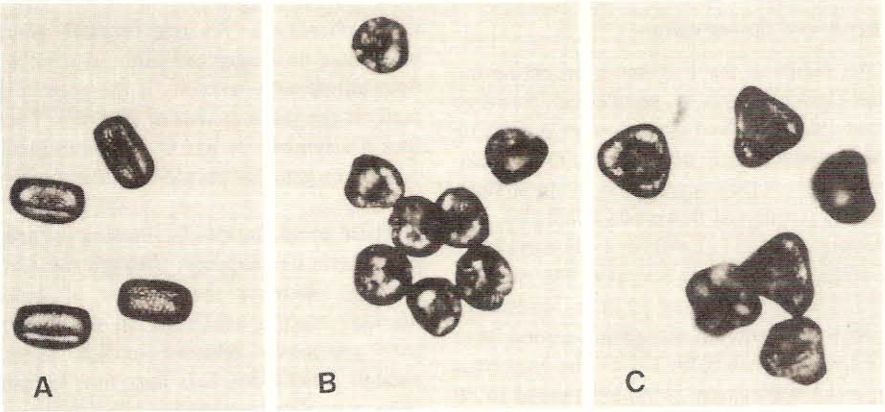

FIGURE 2 Photomicrographs of red clover pollen grains. *A*—normal haploid ($n = x = 7$). *B*—round haploid ($n = x = 7$). *C*—normal diploid ($n = 2x = 14$).

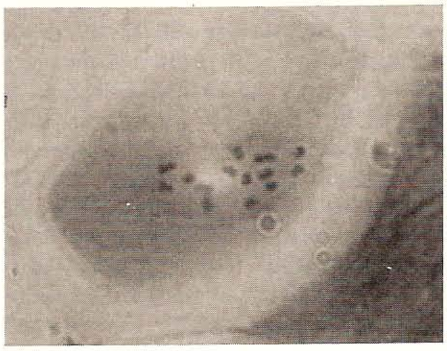

FIGURE 3 Photomicrograph of a red clover pollen mother cell with 14 univalents at diakinesis.

crossed to a plant with pink flowers. Two of the resulting F_1 plants (L and M) were intercrossed to obtain the F_2 generation. In addition, plant L was backcrossed to the purple-red-flowered parent (plant K).

Results and Discussion

Split leaflet

The F_1 between a split leaflet plant and a normal plant had a normal phenotype. Assuming that split leaflet is conditioned by a single recessive gene, plants obtained from a backcross to the original parent would be expected to segregate 1 normal:1 split leaflet. This backcross (Table I) gave an actual ratio of 18 normal:25 split leaflet ($\chi^2 = 1.14$, 1 *df*) and the reciprocal segregated 22 normal:split leaflet ($\chi^2 = 0.40$, 1 *df*). Thus neither segregation was significantly different from the expected 1:1 ratio. As the homogeneity chi-square (143, 1 *df*) also was not significant, the two backcrosses were pooled.

All plants with normal phenotypes, which were obtained by backcrossing the F_1 to the recessive parent, were expected to be heterozygotes. Their polycross progeny should segregate in a 3 normal:1 split leaflet ratio. Only one of the 12 families (family 3) had a chi-square value that indicated its segregation did not fit a 3:1 model (Table I). As the homogeneity chi-square value (11.56, 11 *df*) was not significant, the various families were pooled. Their combined segregation was 294 normal:105 split leaflet. The gene symbol *sl* is proposed for the split leaflet character.

Round pollen

All F_1 plants had normal pollen, indicating the trait was recessive. Assuming monofactorial control, F_2 progeny from intercrossing F_1 plants should segregate in a ratio of three normal pollen-producing plants to one round pollen-producing plant. Progeny resulting from backcrossing F_1 plants to the mutant parent (plant C) should segregate one normal to one round pollen-producing plant. The cross D × E produced 25 normal and 8 round pollen producing plants, the same ratio that would be expected under a single recessive gene model (Table II). Its reciprocal cross (E × D) produced progeny that segregated 14 normal:1 round, compared to the expected 11 normal:4 round. The chi-square value for the latter cross (2.68, 1 *df*) still indicated an acceptable fit to a 3:1 ratio. The homogeneity chi-square was not significant so both were combined, giving 39 normal:9 round, compared with the expected 36 normal:12 round.

The cross F × G produced F_2 progeny that segregated 41 normal:10 round pollen-producing plants as compared to the expected 38 normal:13 round. The chi-square (0.55, 1 *df*) for these segregates indicates a good fit to a 3:1 model. However, its reciprocal (G × F) had 59 normal:7 round segregation that did not fit a 3:1 model ($\chi^2 = 7.29$, 1 *df*).

Segregation of plants from both backcrosses and their reciprocals gave good fits to the expected 1 normal:1 round segregation, the largest chi-square being only 1.39 (1 *df*) (Table II). As the homogeneity chi-square (0.39, 3 *df*) was not significant, all the segregates were pooled, giving a final chi-square of 1.56 (1 *df*), indicating an acceptable fit to the expected 1:1 ratio. The gene symbol *rp* has been proposed for round pollen.

Table II. Red clover F_2 and backcross progenies segregating for round pollen

	Observed values			
	normal	round	χ^2 (1 *df*)	*P*
Cross*				
D × E	25	8	0.01	0.900–0.750
E × D	14	1	2.68	0.250–0.100
F × G	41	10	0.95	0.500–0.250
G × F	59	7	7.29	0.010–0.005
Backcross†				
C × D	18	20	0.11	0.750–0.500
D × C	18	21	0.23	0.750–0.500
C × E	19	22	0.22	0.750–0.500
E × C	19	27	1.39	0.250–0.100
Total BC	74	90	1.95 (4 *df*)	0.750–0.500
Pooled			1.56 (1 *df*)	0.250–0.100

* D, E, F, and G were normal F_1 progeny from the cross of C (round pollen) × normal; expected 3:1 ratio
† C is a round pollen producing parent and D and E are normal F_1 progeny from C × normal; expected 1:1 ratio

Synaptic mutant

Since all F_1 plants had normal fertility this trait was hypothesized to be recessive. If it were conditioned by a single gene, then the F_2 would be expected to segregate in a 3 normal:1 sterile ratio. All three crosses, including the one reciprocal (I × H), gave an excellent fit to a 3:1 ratio, the highest chi-square being only 0.11 (1 *df*) (Table III). As the homogeneity chi-square (0.05, 2 *df*) was not significant, data from all the crosses were pooled to obtain a final ratio of 90 normal:34 sterile plants, compared to the expected 93 normal:31 sterile plants. Due to the near sterility imposed by the synaptic condition, it was impossible to derive a population from backcrosses to the recessive parent that would have been large enough to adequately test a segregation ratio. However, there is enough information to support the hypothesis that this synaptic mutant is conditioned by a recessive gene in the homozygous state at a single locus. In order to incorporate the synaptic mutant characteristic into adapted germplasm, selected synaptic mutant plants were crossed to adapted diploid plants. The resulting F_1 plants were intercrossed. A sample of the F_2 generation segregated 88 normal to 24 synaptic mutants, a good fit to the expected 3 normal:1 synaptic mutant ($\chi^2 = 0.76$, 1 *df*; $0.500 > P > 0.250$). This is not surprising, as in their review article, Koduro and Rao[2] found that meiotic mutants are almost exclusively monofactorial and recessive in expression. The gene symbol *sy* is proposed for the gene that conditions for this character in red clover.

Table III. Red clover F_2 progenies segregating for lack of meiotic chromosome pairing (scored as male sterility)

Cross*	Obs. values normal	sterile	χ^2 (1 *df*)	*P*
H × I	29	10	0.01	>0.975
I × H	30	10	0.00	1.000
J × I	35	13	0.11	0.750–0.500
Total	94	33	0.11 (3*df*)	0.995–0.990
Pooled			0.07 (1*df*)	0.900–0.750

* H, I, and J are F_1 progeny between C51 and three plants with normal male fertility; expected 3:1 ratio

Table IV. Red clover F_2 and backcross progenies segregating for purple-red flower color

	Obs. values normal	purple-red	χ^2 (1 *df*)	*P*
Cross*	49	16	0.01	0.950–0.900
M × L	39	16	0.49	0.500–0.250
Total	88	32	0.50 (2 *df*)	0.500–0.250
Backcross†				
K × L	13	10	0.39	0.570–0.500

* L and M are F_1 progeny from the cross of K (purple-red) to unrelated pink-flowered plant; expected 3:1 ratio

† L is F_1 progeny from cross of k (purple-red) to unrelated pink-flowered plant; expected 1:1 ratio

Purple-red flower color

All plants of the F_1 generation between a pink-flowered and a purple-red flowered plant (plant K) had pink flowers indicating the recessive nature of this trait. The F_2 generation (L × M) segregated 49 pink-flowered:16 purple-red flowered (Table IV). The chi-square value ($\chi^2 = 0.01$, 1 *df*) is evidence of an excellent fit to a 3:1 ratio. The reciprocal (M × L) segregated 39 pink flowered:16 purple-red flowered, also giving a good fit to a 3:1 ratio ($\chi^2 = 0.49$, 1 *df*). The backcross (K × L) segregated 13 pink-flowered to 10 purple-red flowered. This segregation was not significantly different from the expected 1:1 ratio ($\chi^2 = 0.39$, 1 *df*). The F_2 and BC segregations indicate that purple-red flower color is controlled by a single recessive gene in the homozygous condition. Red clover flowers range from pink to purple-red in color. Pink flower color has been attributed to a single, recessive gene, *b*, by Nijdam[4], or *cl* by Williams[18]. Nijdam[6] later determined that pink flower color was controlled by either of two genes, *b* or *b′*, such that either bbB′– or B–b′b′ resulted in pink flower color. Williams[18] also described a gene, *ca*, that produced faint pink flower color, and Nijdam[5] described another gene, *e*, that produced a blue flower color. Purple-red flower color is reported to be dominant to pink by Nijdam[6], who attributed it to the E–B–b′b′ or E–bbB′– gene combination. Nijdam[5], described his flower colors using Oberthur's[7] *Répertoire de Couleurs*, and provided color plates of water color illustrations of the various flower colors. Picard[11] found a plant with purple-red flowers, which he claimed were identical to the purple-red in Nijdam's color reproductions. However, Picard[11] attributed purple-red flower color to a recessive gene, *p*. Taylor[14] has described a crimson flower color in red clover, which is due to the action of at least four genes. Cornelius and Taylor[1] obtained purple-flowered red clover through recurrent selection. They described it in terms of the Munsell Color Fan. Their descriptions were converted into the Munsell number 4RP 2.6/12 by using a conversion chart provided by the Munsell Corporation.

The Munsell number of 5 RP 5/10 was assigned to the purple-red color in Nijdam's[5] color plate, and to the standard in the *Répertoire de Couleurs*. This is essentially the same number assigned to the purple-red flower color in this study. Our observations agree with Picard[11], who reported purple-red flower color was due to a single, recessive gene. This does not, however, preclude the possibility that Nijdam[6] was working with an entirely different gene that resulted in the same phenotype. As the original genetic stocks are no longer available, it cannot be determined with certainty if the gene in this study is the same as that of Picard[11]. Therefore his symbol, *p*, has been maintained to denote the gene for purple-red flower color in red clover.

These genes, besides facilitating tetraploid germplasm development through the use of $2n$ eggs, increase the number of genetic markers readily available. In combination with previously released marker stocks[14] enough genetic markers may now be available to start reconstructing the linkage map for red clover.

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