CHROMOSOME NUMBERS AND MORPHOLOGY IN TRIFOLIUM

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University of California Publications in Agricultural Sciences
Volume 2, No. 13, pp. 355–376, 4 figures in text
Issued May 12, 1928

University of California Press
Berkeley, California

Cambridge University Press
London, England

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INTRODUCTION

The genus *Trifolium* is an outstanding genus within the family Leguminosae. It contains a large number of species which show great morphological variation and a wide geographical distribution and includes several very important agricultural crops, such as *T. pratense*, *T. repens*, *T. hybridum*, *T. incarnatum*, and *T. alexandrinum*. Although considerable plant breeding work has been carried out, especially with *T. pratense*, no genetic analysis of any of these species has been made and the cytological investigations are of very recent date. The genetic analysis of other agricultural crop plants has rendered important service to the plant breeder, and there is every reason to assume that the same will be the case with the clovers in which there are a large number of "good" genetic characters. It is of importance that the chromosome situation in these species should be known before genetic investigations are started. The results of the cytological investigations have been encouraging to the geneticist and plant breeder as they show that in the most important

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agricultural plants the chromosome numbers are fairly low—7 and 8 haploid, according to which 7 and 8 linkage groups are to be expected.

A genetic and cytological investigation in *Trifolium* was started by the writer at the Division of Genetics of the Department of Agriculture, University of California, Berkeley, in July, 1926, and carried on until December, 1927. In this paper will be included only the results of the cytological investigations and the attempts at species crossing. I take great pleasure in thanking Professor E. B. Babcock for laboratory facilities and give my best thanks to all the members of the staff in the Division of Genetics for help and advice. I am greatly indebted to Professor P. B. Kennedy and Mrs. A. Frederick, of the Division of Agronomy, for the material and for help in identification of the species used. Acknowledgment is also given to the International Education Board for the fellowship granted to me.

MATERIAL AND METHODS

Most of the material was grown from seeds furnished by Professor Kennedy. The seeds of the American species had been obtained either from plants growing wild or from plants grown one generation in the greenhouse. Plants of these species have been compared with the specimens in the Herbarium of the University of California and in the collection of Professor Kennedy. In the nomenclature and grouping of these species I have followed McDermott (1910). The other species used were for the most part well-known cultivated species, with the exception of *Trifolium glomeratum* from Syzran, Russia, and *T. subterranum*, which was grown only to a seedling stage. These seeds had been obtained from the United States Department of Agriculture. The two strains of *T. repens* used were obtained from the following sources: (1) *T. repens* var. *sylvestre*, wild white clover, plants growing wild on the campus of the University of California; (2) *T. repens* var. *giganteum*, Ladino clover; Italian white clover; seeds from Vilmorin, France. Three strains of *T. pratense*, Italian, Late Swedish, and Canadian, were obtained from the Central Experiment Station, Ottawa, Canada.

The chromosomes were studied in somatic divisions in root tips; in two species the reduction division in the pollen mother cells was also investigated. For the root tips the fixative of S. G. Nawaschin (Karpechenko, 1927, p. 367) was always used. Buds for the study of pollen mother cells were fixed either in Flemming's medium or

Nawasehin's fixative. Most of the plates were stained with Haidenhain's iron-haematoxylin, a few with iodine-gentian-violet (Huskins, 1927). For *Trifolium* the following procedure was found to be the best: (1) Root tips: 70 per cent alcohol; iodine (5–10 min.); gentian violet (5–10 min.); iodine (30 sec.). (2) Pollen mother cells: 70 per cent alcohol; gentian-violet (5–10 min.); iodine (30 sec.).

Attempts were made to study the reduction division in pollen mother cells by the aceto-carmine method, but with no success. It is difficult to get the anthers out of the small buds and they are filled with inclusions (starch?) which apparently prevent the absorption of the fixative. The methods of emasculation and pollination will be described in the section on "Attempts at species crossing."

CHROMOSOME NUMERS AND MORPHOLOGY

Chromosome Numbers

Martin (1924) counted the chromosomes in *Trifolium pratense* and *T. repens* and found the number in both to be 12, haploid. Karpechenko (1925) examined the chromosomes in somatic cells—root tips—of twenty-four species and found the following series of diploid chromosome numbers:

Diploid number of chromosomes	14	16	32	48	about 80	about 130
Number of species	8	12	1	1	1	1

Bleier (1925) studied the reduction division in eighteen species and found the following series of haploid chromosome numbers:

Haploid number of chromosomes	7	8	9	14	16	48
Number of species	5	8	1(?)	2	—	2

I have obtained chromosome numbers in ten native American species with the following distribution in the groups given by McDermott (1910).

Section I. Tridentatae

	$2n$
T. obtusiflorum Hook., 2 strains	16
T. obtusiflorum var. *majus* (*T. majus* Greene)	16

Section II. Variegatae

T. variegatum Nutt.	16
T. wormskjoldii Lehm.	48(?)*

* But little material was available for fixation and the chromosomes were much crowded in the cells, so that the number could not be obtained with certainty. There are in figure 1*c* 47 bodies, one of which probably represents 2 chromosomes; 48 is very probably the correct number of chromosomes present.

Section III. Monantheae

Section IV. Cyathiferae

T. microcephalum Pursh.	16

Section V. Vesiculeae

T. fucatum Lindl.	16
T. fucatum var. *virescens* (*T. virescens* Greene)	16

Section VI. Bracteolateae

Section VII. Macreae

	$2n$
T. albopurpureum T. and G.	16
T. dichotomum H. and A.	32

Section VIII. Longifoleae

T. reflexum L.	16

Section IX. Ciliatae

T. ciliolatum Benth. (*T. ciliatum* Nutt.)	16

The other species counted are:

	$2n$		$2n$		n
T. pratense	14	Karpechenko	14	Bleier	7
T. incarnatum	14	Karpechenko	14	Bleier	7 and 8
T. repens, 2 varieties	32	Karpechenko	32	Bleier	14
T. hybridum	16	Karpechenko	16	Bleier	8
T. glomeratum	16			Bleier	7
T. minus(?)*	32			Bleier	14
T. subterranum	16				
T. alexandrinum	16				

* The identification of this species is not certain, as it was not observed in flower. *T. minus* ought to be studied anew to ascertain whether it has 28 chromosomes as found by Bleier or 32 as found by me. If 28 is correct, this represents the only double species of the 7 series.

The eighteen species counted by the writer form the following series of diploid chromosome numbers:

Diploid number of chromosomes	14	16	32	48
Number of species	2	12	3	1

In *Trifolium incarnatum*, Karpechenko found 14 somatic chromosomes and Bleier has plates with both 7 and 8 bivalents at heterotypic metaphase. I found the somatic number to be 14, which is probably correct for this species. In *T. repens*, Karpechenko found 32 diploid chromosomes and Bleier found 14 bivalents at first metaphase. Erith (1924) counted the chromosome numbers in three varieties of *T. repens*. On page 113, Erith states, "the two cultivated races of white clover have the same number of chromosomes as the small wild

species.'' On page 92 it is stated, ''The diploid number of chromosomes is sixteen.'' Figure 62*b* on the same page shows, however, a heterotypic metaphase with 16 bodies, and figure 62*d* a homotypic metaphase with 16 bodies. From these figures the conclusion must be drawn that the forms investigated by Erith had 32 and not 16 as the diploid number. I found the somatic number to be 32 in two varieties of this species. In *T. montanum*, Bleier found 9 bivalents at first metaphase; the count was not certain and as Karpechenko found the diploid number in *montanum* to be 16, it is probable that there is no species of *Trifolium* with 18 as the diploid number. There is now established the following series of haploid chromosome numbers in forty-three species of *Trifolium:*

Haploid number of chromosomes	7	8	14	16	24	about 48	about 130
Number of species	11	23	1	3	2	2	1

The basic numbers of this series are 7 and 8. The 7-series consists of single, double, and possibly higher multiple numbers; the 8-series of single, double, triple, and probably higher multiples. This is the terminology suggested by Belling (1927); the term single is used for the species with the basic number, and double and triple for species with two and three times this number, corresponding to the old terms tetraploid and hexaploid. As for the relation between chromosome numbers and the systematic classification of species, Karpechenko (1925) states: ''Hence it is evident that in the process of divergence of species of clover certain chromosome changes, undiscerned by observation, have greater significance, whereas the number of chromosomes plays a subordinate rôle.'' The species studied by the writer give evidence in the same direction. Widely different species, such as *Trifolium variegatum* and *T. reflexum* have the same number of chromosomes, while in one group are found species with 16 and 14 chromosomes. Among the American species studied there is no representative of the 7-series. These species form a regular multiple series, 8–16–24.

Variations in Chromosome Size in the Genus

The chromosomes in *Trifolium* are in general small. There is, however, a very large range of variation in length from about 1μ in *T. variegatum* (fig. 1*d*) to 4μ in *T. reflexum* (fig. 1*b*). There is a still greater difference in total chromosome volume, as illustrated by the complexes of *T. variegatum* (fig. 1*d*) and *T. dichotomum* (fig. 1*k*).

Fig. 1. Somatic metaphase figures from root tips of: *a*, *T. obtusiflorum*; *b*, *T. majus*; *c*, *T. wormskjoldii*; *d*, *T. variegatum*; *e*, *T. microcephalum*; *f*, *T. fucatum*; to the left a plate with 16 chromosomes, to the right a satellited pair from another plate; *g*, *T. virescens*; *h*, *T. albopurpurcum*; *i*, *T. ciliolatum*; *k*, *T. dichotomum*; *l*, *T. reflexum*. All drawings for this paper were made with the aid of a camera lucida with a Zeiss 18 compensating ocular and a Leitz apochromatic 2 mm. objective, N.A. 1.3; magnification 3650; figures not reduced; sections 7μ, stained with Haidenhain's haematoxylin.

The species can be grouped as follows according to chromosome size, the species in each group being arranged according to increasing size of the chromosomes:

SMALL	MEDIUM	LARGE
1. *T. variegatum*	5. *T. microcephalum*	18. *T. incarnatum*
2. *T. repens* var. *sylvestre*	6. *T. obtusiflorum*	19. *T. dichotomum*
3. *T. minus* (?)	7. *T. glomeratum*	20. *T. reflexum*
4. *T. wormskjoldii*	8. *T pratense*	
	9. *T. subterranum*	
	10. *T. albopurpureum*	
	11. *T. majus*	
	12. *T. alexandrinum*	
	13. *T. repens* var. *giganteum*	
	14. *T. ciliolatum*	
	15. *T. hybridum*	
	16. *T. virescens*	
	17. *T. fucatum*	

These groups are not sharply set apart; if all the chromosomes in all the complexes are arranged according to size they will form a continuous series, but if one looks at the chromosome complexes as such, the complexes in the small groups are distinctly smaller, and those in the larger group larger, than the complexes in the medium group. It is not contended that these groups have any phylogenetic significance, but they may serve to give a picture of the situation.

Bleier (1925) discusses the question of chromosome size in relation to chromosome number, nuclear size, and plant size. His discussion is based on the size of the bivalent chromosomes at the heterotypic metaphase and on measurements of the nuclear diameter of the pollen mother cells at the synaptic stage. As is pointed out by him there is great variation in the size of the metaphase chromosomes within a species. The same was found in *T. alexandrinum* in which a large number of metaphase plates were studied. In the same way the chromosomes at the somatic metaphase show some variation within the species (see figs. 2*a* and 2*b* of *T. pratense*), but the variation is less than in the pollen mother cells. Bleier makes the following statements regarding chromosome size in *Trifolium:*

1. Species with the same number of chromosomes have chromosomes of different size.

2. The nuclear volume is not dependent upon the number of chromosomes, but on the mass of chromatic substance.

3. There is no correlation between chromosome number and plant size, but species with a larger nuclear volume have larger growth than species with small volumes.

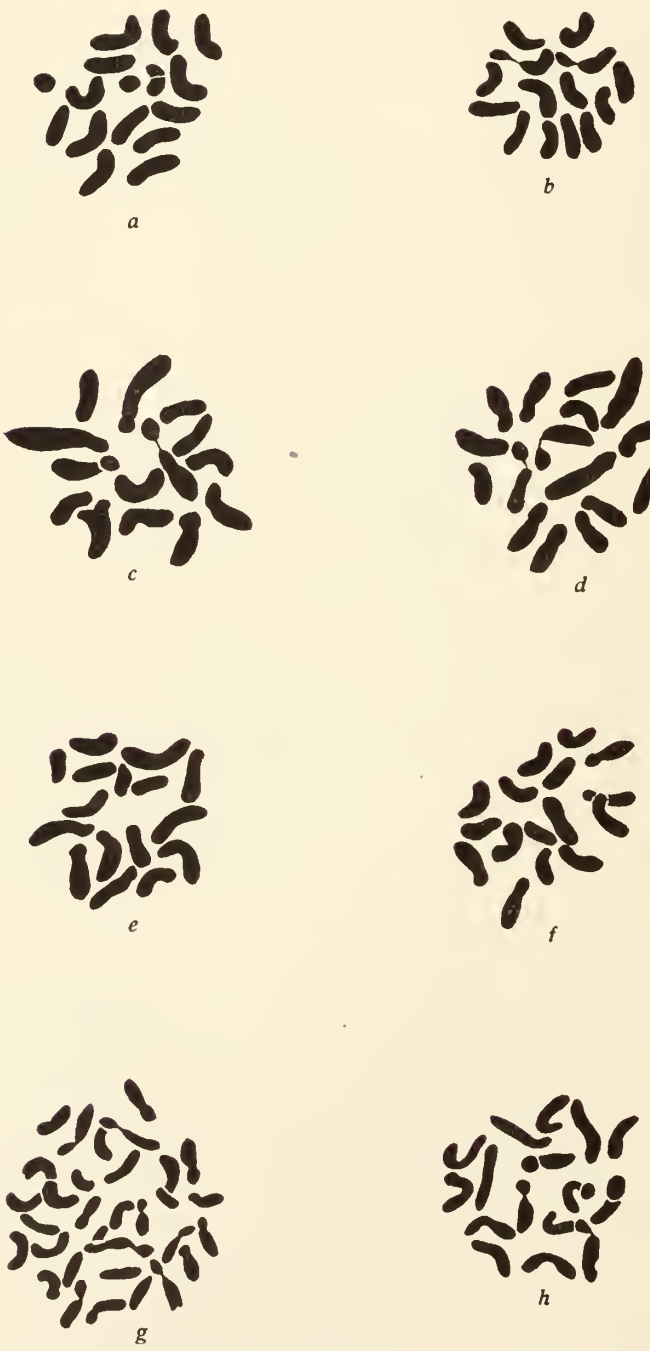

Fig. 2. Somatic metaphase figures from root tips of: *a*, *T. pratense*; *b*, *T. pratense*; *c*, *T. incarnatum*; *d*, *T. alexandrinum*; *e*, *T. hybridum*; *f*, *T. subterranum*; *g*, *T. minus*; *h*, *T. glomeratum*. Figure 2*b* was stained with gentian-violet, the others with Haidenhain's haematoxylin.

The first statement is well illustrated by a comparison of the chromosomes in *T. variegatum* (fig. 1*d*) and *T. reflexum* (fig. 1*l*). The third statement may hold as a general rule, but a rule to which there are many exceptions. *T. obtusiflorum* (fig. 1*a*) has 16 medium to small chromosomes, *T. reflexum*, 16 large, but the former has the largest plant size. That one must be careful in conclusions based on comparisons of chromosome size in species of the same genus is also brought out by the cases of intraspecific variability in shape and size of chromosomes discussed below.

Variations in Chromosome Size Within the Species

In *Trifolium repens,* two varieties were examined cytologically, *T. repens* var. *sylvestre,* wild white clover, and *T. repens* var. *giganteum,* Italian white clover or Lodi clover, three plants being studied in each variety. The three plants of *giganteum* all had chromosomes of about the same size (fig. 3*a*, which is a metaphase plate from a root tip of plant 13*a*). Of the three *sylvestre* plants, plant 1*a* showed very small chromosomes, 1*b* and 1*c* somewhat larger, but all considerably smaller than the chromosomes of 13*a* (fig. 3*b*, *c*, *d*). Karpechenko (1925) studied the somatic metaphase in *T. repens;* he makes no statement as to the variety used but his figure shows chromosomes of the same size as those found in *giganteum*. Bleier (1925) and Erith (1924) both studied pollen mother cells of *repens*. Bleier says nothing about which variety was studied, Erith states that she counted *giganteum, hollandicum,* and *sylvestre,* but does not say anything about differences in chromosome size, and it is not clear from which variety her figures are taken. However, when the bivalent chromosomes in her plates (1924, p. 92, × 1750) are compared with those of Bleier (1925, p. 618, × 2150) it is clear that the chromosomes pictured by him are at least three times as large as those of Erith.

This case is very interesting because *giganteum* with the large chromosomes is a giant variety, *sylvestre* a small variety. Erith (1924) has given detailed morphological descriptions of the two varieties which correspond to the plants used by the writer. The length and breadth of the terminal leaflet in several plants of each variety were measured and the measurements for the plants studied cytologically are given below. The figures represent the average of ten measurements.

Variety	Plant No.	Leaf size in mm. Length	Breadth
giganteum	13*a*	44.1	32.4
sylvestre	1*a*	17.5	13.8
	1*b*	12.7	10.2
	1*c*	11.4	10.5

In agreement with the results of Erith, I found no difference in flower size in the two varieties. Apparently the increase in size in *giganteum* is only in the vegetative parts. In accordance with this is the fact that the pollen is about the same size in the two varieties, whereas the cells of the roots are considerably larger in *giganteum*. The same was found to hold true for the stolons by Erith (1924, pp. 110–111) who states, "In older plants the stolons of *giganteum* have a diameter two to three times that of *sylvestre*, the larger dimensions of the former being due to a greater number of individually larger cells."

The origin of *giganteum* is not known, but very likely it arose from *sylvestre*. The genetic relations of the two varieties have not been determined, but some study has been given to chromosome size in F_1 hybrids. Plant 1*a* of *sylvestre* was crossed with plant 13*a* of *giganteum* with 1*a* as the mother plant. The F_1 plants are still too young to make possible any conclusion as to the behavior of plant size in this cross. Two somatic metaphases from F_1 are pictured in figure 3*e* and *f*. The chromosome size is intermediate, being nearer to that of the *giganteum* parent. This result suggests that the case may be one of Mendelian inheritance of chromosome size. Mendelian inheritance of a chromosomal character has been recorded by Lesley and Frost (1927) in *Matthiola*, in which they found that one Mendelian factor was responsible for the difference in shape of the metaphase chromosomes of the first meiotic division. Because of lack of material of the variety *sylvestre*, more work must be done to complete the study of chromosome size in *T. repens*. As this species is self-sterile, all the varieties are very heterozygous, and the plants used by the writer were very variable in morphological characters. One might expect, therefore, to find many chromosome sizes. It is hoped that it will be possible to follow up this problem by further study of the parent varieties, the F_1, and later generations. As the cytological work of the writer has been discontinued, at least for some time, it seems justifiable to give a preliminary account of it.

Chromosome Individuality

Karpechenko (1925) states that he finds no chromosome individuality in the species examined by him. In contrast to this the species reported upon here exhibit many differences in chromosome size and shape within the haploid sets. The most striking of these is the presence of satellites attached to the chromosomes. In five

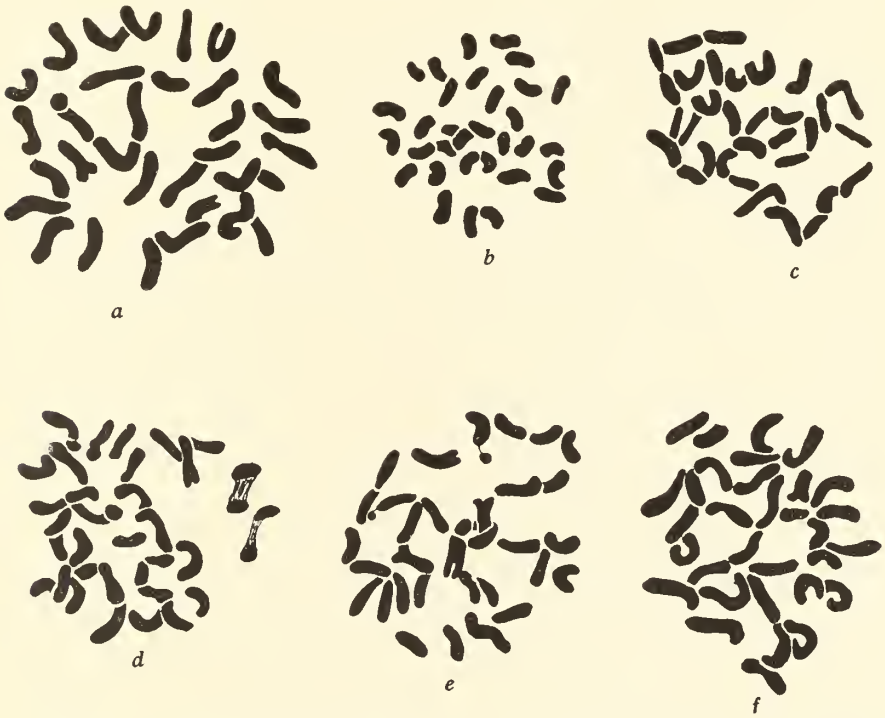

Fig. 3. Variations in chromosome size in *T. repens*. Somatic metaphase figures from root tips of: *a*, var. *giganteum*, plant no. 13*a*; *b*, var. *sylvestre*, plant no. 1*a*; *c* and *d*, of plants 1*b* and 1*c* of the same variety; *e*, and *f*, from F_1 of the cross 1*a* × 13*a*. Stained with Haidenhain's haematoxylin.

American species, representing three sections of the genus; and in four European species, also from three sections, there is 1 pair of satellited chromosomes. In one species, *Trifolium minus*, there are probably 3 pairs; in *T. repens* 1 pair of satellited chromosomes was seen in one plate only (fig. 3*a*). Although large, the satellites in *Trifolium* are often difficult to observe, because the chromosomes have a tendency to stick together end to end, and in the same way the satellites will stick to the end of the mother chromosome. This may

explain why Karpeehenko did not find any satellites in *T. pratense* and *T. incarnatum*, in each of whieh 1 pair of conspicuous satellites was found. Of the species in whieh no satellites were found, only one, *T. hybridum*, has been investigated thoroughly enough to state with eertainty that it does not have satellites.

The existence of satellites was first established by S. G. Nawaschin (1912) in *Galtonia*. Sinee that time they have been observed in many speeies and genera. The most outstanding works are those of M. Nawaschin (1925, 1926) on the genus *Crepis* and of Taylor (1924, 1925, 1926) on *Crepis*, *Gasteria*, *Allium*, and other genera. In the Leguminosae, satellites have been found in *Pisum*, *Lathyrus*, and *Vicia* (Nawaschin, 1925; Sveshnikova, 1927).

The satellites in *Trifolium* are large compared with those observed in most other species. *T. fucatum* (fig. 1*f*) seems to have smaller satellites, while *T. virescens* which is nearly related to *fucatum*, and perhaps should be regarded as a variety (fig. 1*g*) of this speeies, has large satellites. This may be a ease of the same nature as that reported by Nawasehin (1926) in *Crepis dioscoridis*, in which he found strains differing in satellite size. It eannot be stated with certainty that there is a real difference in satellite size in *fucatum* and *virescens*. There is some variation in satellite size within the strains and as the chromosomes of *fucatum* were mueh crowded on the plates only a few observations of satellites were made in this speeies. In *virescens*, however, many observations were made, but satellites as small as those observed in *fucatum* were never seen.

A peculiar feature of these satellites is that they sometimes seem to lie free on the metaphase plate without any visible eonnection with any of the chromosomes, as shown in the metaphase plate of *T. pratense* (fig. 2*a*). The free satellites often have a more elongated shape, resembling very much a pair of small ehromosomes. Anyone unfamiliar with the material would in sueh plates count 16 chromosomes in *pratense* and 18 in *alexandrinum*. In these two speeies the reduction divisions in the pollen mother cells were also studied. In *alexandrinum*, many plates of the first metaphase showed 8 bivalents (fig. 4*a*) and, in agreement with this, 8 chromosomes were found at second metaphase (fig. 4*b*). No trace of satellites was found at these stages. In *T. pratense*, both Bleier and Karpechenko found the haploid number to be 7 in the reduction divisions of pollen mother cells. Although only a little pollen mother-cell material of *T. pratense* was available, several good diakinesis plates showed 7 bivalents

(fig. 4*c*). As to the nature of the free satellites several interpretations can be given. It is possible that the fixation has failed to bring out the connecting thread which is really present; in this case the phenomenon has of course no significance. Against such an interpretation there is the fact that when the satellites appear to be free they are usually found far from any chromosome, on the outside of the plate, while the attached satellites usually lie in the middle of the plate and are connected with the chromosome by a short and rather thick thread. It may be, therefore, that the satellites sometimes become free in the living cell; in that case they may easily be lost in the mitotic division, giving rise to "sports" without the satellites.

Fig. 4. *a*, Heterotypic division in pollen mother cell of *T. alexandrinum*; *b*, homotypic division in same; *c*, diakinesis in pollen mother-cell of *T. pratense;* to the right is shown a bivalent chromosome with attached satellites from another diakinesis plate. Figure 4*a* was stained with Haidenhain's haematoxylin; figures 4*b* and *c*, with iodine-gentian-violet.

This suggests the possibility that Karpechenko worked with strains of *pratense* and *incarnatum* which lacked the satellites. This does not seem likely in the case of *pratense*, however, as satellites were found in four strains of this species. Without making any definite conclusions as to the nature of the free satellites, it seems useful to point to the situation as a possibility for geneticists and cytologists to bear in mind when working with these species.

In some of the diakinesis plates of *pratense* one bivalent was seen with a pair of small bodies attached like a pair of satellites (fig. 4*c*). The observation was made near the close of the work and no time was available to follow it up by a further study of the reduction divisions. It seems very probable, however, that this bivalent corresponds to the one pair of satellited chromosomes to be seen in figures of somatic metaphase. No instance is known to the writer in which satellites have been observed attached to bivalent chromosomes in the reduction division. The observation suggests that the maturation division in species with somatic satellited chromosomes should be studied, with

the particular aim of tracing the satellites through the meiotic stages. If this could be done it would add materially to the genetic significance of satellites, as it would show that they are not only a peculiar structure of the somatic metaphase chromosomes, but are also a part of the chromosome which is permanently differentiated out from the rest of the chromosome.

In many species of plants it has been found that certain pairs of the somatic metaphase chromosomes have definite and constant constrictions. In *Trifolium* the constrictions are not easily observed on account of the small size of the chromosomes. Some constricted pairs were established in several species, but an intensive study would probably reveal more constricted chromosomes. The constrictions are all subterminal.

In general there is not a great variation in chromosome size within the haploid sets in *Trifolium*. Some species, however, show conspicuous size differences, such as *T. alexandrinum* (fig. 2*d*), *T. incarnatum* (fig. 2*c*), *T. hybridum* (fig. 2*e*), and *T. reflexum* (fig. 1*l*). The chromosome morphology of the species was studied with two main objectives in view:

1. In order to be able to distinguish each member or at least the groups of a haploid set. This is usually done in combination with a genetic analysis, by which method it is possible to assign a particular gene to a particular chromosome.

2. In order to compare the chromosome complexes in species of the same genus and by this method to study their relationship and origin. It has now come to be used also in practical plant taxonomy to determine, in cases of doubt, whether nearly related forms should be ranked as distinct species. The first problem has been the chief concern of the present study of chromosome morphology in species which seemed the most promising from a genetic standpoint. In these species the following features of chromosome individuality have been revealed:

T. pratense:

1 pair of satellited chromosomes, 6 pairs of about equal size, without visible constrictions.

T. incarnatum:

1 pair of satellited chromosomes.
1 pair of large, constricted chromosomes.
2 pairs of medium, constricted chromosomes.
3 pairs of medium chromosomes without visible constrictions.
1 pair of small, constricted chromosomes.

T. alexandrinum:

1 pair of satellited chromosomes.
1 pair of large, constricted chromosomes.
3 pairs of medium, constricted chromosomes.
2 pairs of medium chromosomes without visible constrictions.
1 pair of small, constricted chromosomes.

The complexes in the last two species are similar but *alexandrinum* has one more pair of medium sized chromosomes.

T. hybridum:

6 pairs of large chromosomes, at least three pairs with constrictions.
1 pair of very small chromosomes.
1 pair of small, constricted chromosomes.

The smallest pair of chromosomes in *hybridum* is of the same size as the satellites of *pratense*, and the plates of *hybridum* resemble very much the plates of *pratense* in which the satellites have no visible connection with the chromosomes.

In *T. repens*, Bleier found in the first metaphase of the reduction division 4 small and 10 large bivalents. The somatic plates studied also indicate that there is one group of small and one of large chromosomes, but it is very difficult to get 32 chromosomes, all lying flat on the plate, so that nothing can be said with certainty as to the number of chromosomes in each group. It may be of interest to note that in the two nearly related species, *hybridum* and *repens*, we find in the former 2 pairs of small chromosomes and in the latter probably 4 pairs. In one plate of *repens* (fig. 3*e*) 1 pair of satellited chromosomes was seen, so it is probable that *repens* has satellited chromosomes. As *hybridum* has no satellites this would mean that *repens* has not simply twice the complex of *hybridium*.

Outstanding in their chromosome morphology are also *T. minus* and *T. reflexum*. It is interesting that the double species, *T. minus* (32 diploid), has probably 3 pairs of satellited chromosomes (fig. 2*g*) while no single species has been found with more than one pair of satellites.

T. reflexum (fig. 1*l*) exhibits a chromosome complex different from all other investigated species, with 5 pairs of large constricted chromosomes and 3 pairs of smaller chromosomes.

ATTEMPTS AT SPECIES CROSSING

The cases of recorded species hybrids in *Trifolium* are listed by Karpechenko (1925) and Bleier (1925). In all cases one of the parents was a species with a high chromosome number, *T. pannonicum* (130) or *T. medium* (80). Hitherto, however, no report has been given of an F_1 hybrid which has been cytologically investigated. Crosses were attempted between nine species in eighteen different combinations. The material was the same as that used for cytological investigations. The methods used were mainly two:

1. The heads were enclosed in paper bags before any flower had opened. Emasculation was performed when the head was about half developed. The flowers which were either too old or too young were cut away and in the rest of the flowers the anthers were removed with a forceps. This operation is fairly easy in the species with large flowers, but difficult in the small-flowered ones. The flowers were mostly pollinated immediately after emasculation, in some cases the next day. All the instruments used were washed in alcohol frequently during the work and only very few cases of selfing occurred.

2. Using plants of self-sterile species as mother plants, the pollen from other species was applied without emasculation to the stigma in flowers which had been bagged before opening.

Cross	Number of flowers crossed
1. *T. pratense* × *T. incarnatum*	950
2. *T. pratense* × *T. repens*	327
3. *T. pratense* × *T. hybridum*	284
4. *T. pratense* × *T. fucatum*	61
5. *T. pratense* × *T. virescens*	154
6. *T. pratense* × *T. obtusiflorum*	32
7. *T. repens* × *T. hybridum*	129
8. *T. repens* × *T. incarnatum*	20
9. *T. incarnatum* × *T. alexandrinum*	186
10. *T. incarnatum* × *T. reflexum*	26
11. *T. incarnatum* × *T. obtusiflorum*	290
12. *T. incarnatum* × *T. virescens*	92
13. *T. virescens* × *T. fucatum*	33
14. *T. virescens* × *T. obtusiflorum*	6
15. *T. virescens* × *T. reflexum*	—
16. *T. obtusiflorum* × *T. fucatum*	6
17. *T. obtusiflorum* × *T. reflexum*	15
18. *T. reflexum* × *T. ciliolatum*	11

With both methods the results were completely negative; a few seeds obtained by either method proved to be due to selfing. Outside of these there seemed to be no seed development at all. Below are given the combinations which were tried and the number of flowers crossed in each combination. All the crosses were made reciprocally except in 4, 8, 10, 14, 15, and 18.

In crosses 1, 2, 3, 5, 7, 9, and 11 the number of trials is large enough to allow the statement that hybrids between these species are not easily obtained.

In *pratense, repens, hybridum,* and *virescens* intraspecific crosses were made and seeds easily obtained, so the negative results are not due to faulty technique. *T. fucatum* and *T. virescens* are two very nearly related species or varieties of the same species which did not seem to cross. The number of flowers crossed is not large, but when crossing plants within *virescens* seeds were easily obtained. These results do not, of course, allow the conclusion that hybrids cannot be obtained between these species, but they suggest, in agreement with the results of other investigators, that interspecific hybrids are difficult to secure.

In case of the crosses *T. pratense* × *T. repens,* and *T. hybridum,* respectively, it was attempted, using the method described by Martin (1913), to study the behavior of the pollen of *repens* and *hybridum* on the stigma of *pratense.* Flowers of *pratense* were emasculated, pollinated immediately, and the stigmas picked out for observation after 18, 24, 48, and 72 hours. The stigmas were mounted on a slide in aceto-carmine and a slight pressure was exerted on the coverglass to flatten the stigma. The pollen both of *repens* and *hybridum* germinated readily on the stigma of *pratense,* but it was not found possible to follow the pollen tube growth through the style by Martin's method. Nothing, therefore, was ascertained as to what happened to the pollen tubes. It may be that the situation is the same as in self-sterile species of *Trifolium* in which, when selfed, the pollen will germinate, but the pollen tube growth is too slow to reach the ovary.

EVOLUTION OF THE CHROMOSOME COMPLEXES IN TRIFOLIUM

The chromosome complexes in many genera are now studied with the aim of tracing the relationship between the species and of finding the way in which the evolution of the species has proceeded. Attempts are also made to base the classification of species on chromosome morphology. For *Vicia* Sveshnikova (1927) has worked out a key based on chromosome morphology and finds that it corresponds very nearly to the key worked out by Ascherson based on external morphology. In *Trifolium* it is evident that there is no such parallelism in the differentiation of the chromosome complexes and of the external morphology of the species. Species which are far removed taxonomically and very different in their morphology have very similar chromosome complexes; for instance, the European species, *T. glomeratum*, and the Californian species, *T. obtusiflorum*. On the other hand, we find nearly related species with very different chromosome complexes. The wild red clover, *T. pratense*, is very similar to *T. medium*, but the former has 14 and the latter about 130 chromosomes. Furthermore, though the number is the same, the shape and the size of the chromosomes may be different. *T. pratense* and *T. incarnatum* are placed in the same subsection of the section, *Eulagopus*, but the chromosome complexes are very unlike. In *T. alexandrinum* and *T. incarnatum* we have two species differing in external morphology and in chromosome number (16 and 14) but very similar as regards the shape and size of the chromosomes, *alexandrinum* having an extra pair of medium sized chromosomes. The situation in *Trifolium* suggests that it will not be easy on the basis of chromosome morphology to trace the mutual relationship and origin of the species in this genus. The basis for such a study must be the possibility of establishing certain types of chromosomes, which can be identified in related species. Nawaschin's (1925) work on the genus *Crepis* is of this type. In ten species with 3, 4, and 5 pairs of chromosomes he established five types of chromosomes, one of which was a satellited chromosome. In the summary Nawaschin states, "Es wurde von mir festgestellt dass dieselbe homologischen Typen und Formen der Chromosomen in den Chromosomsätzen aller untersuchten Arten hervortreten." In *Trifolium* ten species at least have

1 pair of satellited chromosomes; but considering the fact that species from very different sections have the satellites and that, on the other hand, species with and without satellites occur in the same section, this feature does not help much in establishing any relationship between the species. It does not seem safe, either, to take chromosome size in general as an evidence of relationship, when we remember that the one species, *T. repens*, includes in itself almost the total variability in chromosome size in the genus. It is apparently only in the narrowest taxonomic groups that there is a similarity in chromosome morphology which points to common descent, and it is probably here that the study of the chromosomes may be of help to the taxonomist. Some facts pointing to this conclusion may be mentioned. *T. variegatum* in the section Variegatae has 16 very small chromosomes. In the same section is *T. wormskjoldii* with 48 equally small chromosomes. This suggests that these two species, in regard to their chromosomes, are more nearly related than the Californian clovers of other sections. The two nearly related forms, *T. fucatum* and *T. virescens*, have almost identical chromosome complexes. The chromosome sizes of the two related species, *T. hybridum* (16 diploid) and *T. repens* (32 diploid), indicate that the latter may have a complex which is twice that of the former.

The situation in *Trifolium* is interesting because it seems to demonstrate another type of differentiation of the chromosome complexes than is found in many other genera studied. The genera which have been most intensively investigated cytologically are those in which interspecific hybridization has been carried out. There has been, then, a preference for genera in which interspecific hybrids are fairly easily obtained, and in which such hybrids are common in nature. This has led some investigators to emphasize hybridization as the only factor in species differentiation, and it may perhaps not be out of the way to hold forth that there may be other ways of evolution of species. It seems only fair to do so in connection with this study in *Trifolium*, because all evidence suggests that hybridization has not played a dominant rôle in the differentiation of this genus. Hybrids are very rare in nature, if, indeed, ever observed, and no hybrids have been obtained in experiments. Taking into account only the external morphology of the chromosomes, in *Trifolium* no certain instance of "homologous" chromosomes in different species is known, whether in the form of one single chromosome, a group of chromosomes, or a complete haploid set. The existence of a satellited chromosome pair

in many species cannot, in the opinion of the writer, be taken as evidence in this direction. The fact that the satellited chromosome pair varies in size in the different species according to the general size of the chromosome complex should make one cautious in drawing any such conclusions and this is still clearer when the distribution of the satellited chromosomes is taken into account. In the section Euamoria we find *T. hybridum* without satellites, and *T. glomeratum* with satellites which resemble the satellites in *T. obtusiflorum* from a very different section. We are not at all justified in concluding that *glomeratum* and *obtusiflorum* have obtained their chromosome complex from a common source. In genera in which interspecific hybridization is common there have been found not only polyploid series of chromosome numbers, but all intermediate numbers as well. A typical genus of this kind is *Viola* (Clausen, 1927), which in the section Melanium has the following haploid numbers of chromosomes: 7, 10, 11, 12, 13, 17, 18, 20, 24, 30.

In *Trifolium* simple polyploid series without intermediate numbers are found; $2n = 16, 32, 48$; and 14, 28, (?).

It may then perhaps be justifiable to give a suggestion as to the evolution of the chromosome complexes in *Trifolium*. This genus presents a very clear demonstration of parallel variation, i.e., we find in species belonging to very different sections that evolution has proceeded along parallel lines. It seems better in accordance with the facts to ascribe this parallel variation to parallel independent mutations than to a common descent. This is supported by the fact that we find in many species similar variations from the wild type. In species from different sections is found the mutant form characterized by the absence of leaf spots. In *T. pratense* is found a variation from the normal red flower color to white; in *T. repens* a variation from white to red, but the red and white flower color in *pratense* and in *repens* is a genetically different character. The chromosome complexes show the same picture as the morphological characters. The presence of one pair of satellited chromosomes should be due, then, to independent parallel mutations and not to the fact that they have been derived from a common source. This suggestion as to the way in which the chromosome complexes in *Trifolium* have been differentiated is supported also by the variation in chromosome size described in *T. repens*, which is just an example of that kind of variation which the hypothesis supposes to take place. The great variability in chromosome morphology in *Trifolium* is held to be due,

then, to mutational changes in species isolated by interspecific sterility. It is not contended that crosses have not taken place in this genus, but it is held that the species have been thus isolated for a long time and that many mutations have occurred.

SUMMARY

1. Somatic chromosome numbers have been obtained in eighteen species of *Trifolium*, twelve of which had not been counted before; the reduction division was studied in two species.

2. The ten American species studied have the diploid numbers 16, 32, 48. No representative is found of the 7-series which is found in European species of *Trifolium*.

3. The chromosomes of *Trifolium* are in general small, but they exhibit a great variation in size, both as to single chromosomes and to total amount of chromatin.

4. In *T. repens* L. the two varieties *giganteum* and *sylvestre* proved to have chromosomes of different size. *Giganteum* is a giant variety and has large chromosomes; *sylvestre* is a small variety and has small chromosomes. F_1 plants between these two varieties showed chromosomes of intermediate size.

5. Ten species from several sections of the genus have been shown to have 1 pair of satellited chromosomes and one species probably has 3 such pairs.

6. The satellites are in some plates without visible connection with any chromosome and appear like an extra pair of small chromosomes. In a few diakinesis plates of *T. pratense* were observed bodies which must be interpreted as 1 pair of satellites attached to a bivalent chromosome.

7. On the basis of satellites, constrictions, and chromosome size, a scheme of chromosome morphology has been given for some of the species.

8. Species crosses were attempted between nine species in eighteen different combinations, but with completely negative results.

9. The suggestion is made that the diversity of chromosome complexes in *Trifolium* is a result of mutational changes in species which have become isolated by intersterility rather than the result of hybridization.

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