PLOIDY IN TRIFOLIUM AMBIGUUM

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By

Lyndon M. Kannenberg

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Submitted to the School of Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements

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Department of Farm Crops

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ABSTRACT

Fourteen sources of Trifolium [illegible] L. (2n=), Kura clover, were cytologically examined for ploidy level. The most successful of the three methods tried was the counting of somatic chromosomes of root tips from greenhouse plants. By double-potting these plants, clean root tips could be obtained as they emerged into the void between the pots. Generally the sources were pure for a 2x, 4x, or 6x ploidy level, but variants were found in two predominantly hexaploid sources. These two sources had, respectively, a few tetraploid and pentaploid plants. Since no aneuploids were found it was hypothesized that these variants were FI's from interploidy matings involving different sources.

Plants grown in the field and greenhouse were examined for several morphological and physiological characteristics known to be associated with polyploids. The results indicated that under field conditions at East Lansing, polyploids of Kura clover have larger length/breadth ratios, increased leaf area, fewer but larger stomata, increased but later flowering, longer florets and pistils, larger pollen, larger and heavier seeds, increased rhizome production, and greater vigor. Although non-stainability of pollen tended to increase with ploidy level, some polyploid sources had low percentages of non-stained pollen. Winter losses were small and apparently not related to ploidy level. In the greenhouse, floret length, number of heads per plant, and number and size of stomata did not reflect the above trends at all ploidy levels. Overall vigor was also less. In any case, cytological examination is still necessary for verification of ploidy level.

Fertility studies indicated that interploidy fertility decreased. Interploidy fertility was also noted, especially in 4x X 6x crosses. Mura clover showed no self-compatibility at any ploidy level. In addition, self-incompatibility mechanisms appeared to exist within and between sources of all ploidy levels.

It is suggested that the strong asexual capacity of Mura clover permits the species to experiment with ploidy for maximum adaptation to a given environment. Furthermore the coexistence of 2x, 4x, and 6x types as well as the apparent lack of higher ploidy levels indicate the species may presently be undergoing important evolutionary changes. Differences in response of certain sources to environmental changes is discussed from the viewpoint of the severity of their previous area of adaptation.

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INTRODUCTION

Trifolium ambiguum M. Bieb. (x=8), variously termed Kura, honey, Caucasian, or Peilett's clover is a rhizomatous perennial. Its habitat ranges from river valleys to sub-alpine regions in southern Russia, Crimea, the Caucasus, and Asia Minor. In Russia, Kura clover is reported to be a valuable forage legume with wide adaptation (20). In the United States it has shown promise but has not achieved significant utilization (1),(25),(26),(27),(28).

Some desirable features of Kura clover include resistance to some serious clover diseases (24) as well as heavy production of nectar. One of the earliest studies of the species in the United States was based on the potential utility of the species as a honey plant (1), (25),(26),(27),(28). In addition, T. ambiguum is winter hardy, highly palatable, drought resistant, and yet capable of fair productivity on wet lands. A severe handicap to utilization has been the lack of effective nodulation by rhizobia, but Erdman and Means (5) have now isolated a satisfactory strain from a Turkish soil. Consequently, further evaluation and improvement of the species as a forage in the United States is warranted.

One of the important characteristics of the species is the existence of different ploidy levels including 2x, 4x, and 6x types. Mixtures of ploidy levels are possible, therefore, between and within

sources. Since intercrossing between ploidy levels may occur, fertility problems arising from mixtures are extremely important. For this reason cytological examination of individual plants to establish their chromosome numbers is an integral part of an improvement program with the species. Certain morphological and physiological characteristics are known to be associated with polyploids. An attempt was made to relate these characteristics to the chromosome numbers of plants from several sources of I. ambiguum. From such a study a simple key to a rapid determination of ploidy level might be constructed.

LITERATURE REVIEW

Although T. ambiguum has many desirable features, to-date little work has been done in the United States on this clover.

Characteristics of T. ambiguum

In 1941, a few seeds of T. ambiguum from the Caucasus were planted in the American Bee Journal test garden in Iowa. This garden was used for testing the honey producing qualities of different species. F. C. Pellett (25) (26) and M. Pellett (27) (28) reported that in this garden T. ambiguum had excellent vigor and rhizome development, an extensive deep root system, apparent drought resistance, resistance to heaving, high palatability, excellent flowering from 1 June - 20 July (in Iowa), high insect activity including honeybees, and good seed set, but trouble with shattering. Leaf stalks were about one foot high while flowering stems were between 2-3 feet. Four year old plants still showed good vigor. However, Pellett (27) noted that correspondents who received either seed or cuttings from the garden reported results ranging from equal success to complete failure. These results were apparently not correlated with soil type. Although the ploidy level was not given in the articles, the materials shown in accompanying photographs appeared to be hexaploid.

Rein (17) (18) used hexaploid T. ambiguum as well as other species of Trifolium in interspecific hybridization experiments. Under greenhouse

conditions he reported no flowering in T. ambiguum between Sept. 15, and March 1 despite numerous treatments involving day length, temperature, moisture, nitrogen level, and age of plants. At the 6x level, the clover appeared to be completely self-incompatible. Guravich (7) also reported high self-incompatibility and poor flowering under greenhouse conditions during the winter months.

In an Australian study of T. ambiguum which included 2x, 4x, and 6x ploidy levels, Hely (10) (12) described the clover as having great root storage capacity, drought resistance, tolerance of waterlogged conditions, palatability, and high honey production. Less than 1% self-fertility was reported. The main difficulty involved nodulation. For effective nodulation, massive innoculation was necessary. The 2x plants were found to be less susceptible to effective infection by nodulating bacteria. Hexaploid plants were most freely nodulated, but the tetraploids did nodulate early and effectively with the Turkish isolates.

In the Czechoslovakian climate, Vacek and Ded (31) have reported that T. ambiguum had wide ecological adaptation and produced a high quality forage.

Effects of polyploidy

The common species of Trifolium are normally of one ploidy level. If polyploidy is artificially induced many changes occur in the plant. T. ambiguum, although existing in nature at several ploidy levels, appears to reflect some of the characteristics of induced polyploids. Consequently, several features of artificial polyploidy were studied in this examination of ploidy in T. ambiguum.

In the survey by Mehta and Swaminathan (23), several phases of induced polyploidy in forage crops are discussed. In this study, polyploidy is reported to generally have the following effects on morphological characteristics: increased vigor and plant height, thicker stems, fewer branches and tillers, fewer leaves, fewer but bigger flowers, larger pollen, larger but fewer seeds. Cell size and volume is also increased. The most common criteria maintained for the detection of polyploidy included the above as well as leaf length/breadth ratio, stomatal frequency, and pollen fertility. None of these were reported to be accurate in all cases. Autotetraploids were said to have lower seed set. In Trifolium the oppositional incompatibility factor system was mentioned as having a bearing on fertility. This becomes more complex in tetraploids but may also facilitate self-compatibility at this level (22).

In white clover (T. repens), Hutton (15) found that autopolyploids had coarser stems and larger leaves. In addition they were less vigorous and persistent than diploids. Cyanogenetic activity was also intensified in autopolyploids.

Sjoseth (30) has reported tetraploid red clover (T. pratense) to be more sensitive to frost injury than its diploid progenitor. Laczynska-Hulewiczowa (21) noted the following effects in artificially induced tetraploid red clover: larger stomata and epidermal cells, higher phloem/xylem ratio in the stems, better yields (especially on dry and light soils), and more leafiness. In addition, the tetraploid was richer in protein and showed higher self-fertility. The 1000 seed weight was higher but seed production was lower. Lower seed set was

due in part to later flowering and ripening but longer corolla tubes of the tetraploids impaired pollination by bees. Pollen was normal. Interploidy crosses did not occur. Difficulty in pollination of tetraploid red clover has also been reported by Singefors (2). Evans (5) noted larger stomata and pollen in red clover, white clover, and lucerne.

MATERIALS AND METHODS

General

Plants of T. amabile from fourteen sources were examined in this study. Nine of these sources were received from F. W. Hely, Senior Research Officer, Division of Plant Industry, Canberra, A.C.T. Excerpts from correspondence with Hely (11) describe these materials and certain problems associated with their cultivation in Australia.

> "-----The C.P.I. number is the Commonwealth Plant Introduction Section's accession number which is retained for convenience, although the material may have undergone some modification during seed increase. However, each sample of seeds was obtained from plants grown in isolation. In this environment one might expect ultimate selection away from long day, winter-dormant plants towards less dormant or non-dormant short day types. There has not been sufficient time for any significant amount of this to occur.
>
> C.P.I. 2264. Apparently diploid. Received in 1931 from Botanic Gardens, Tiflis, Georgia, U.S.S.R. Remarks in accompanying letter: 'Native to the high fields and lower alpine slopes of the mountains of the Caucasus region and in Armenia. A valuable hay and pasture plant.' The same general remarks have been received with other introductions of T. amabile from Russia and obviously refer to this clover generally rather than to any particular introduction. One generation in Australia.
>
> C.P.I. 2771. Apparently diploid. From Botanic Gardens, Tiflis, Georgia, U.S.S.R., 1932. No remarks. Distinctly leafier, later-flowering and less likely to flower than C.P.I. 2264 in Canberra. One generation in Australia.
>
> C.P.I. 6874. Apparently tetraploid. From Botanical Institute, Tiflis, Georgia, U.S.S.R., 1937. Remarks: 'A perennial, rhizomatous plant which is reputed to be valuable for hay and pasture.' A rather poor type agronomically here but fairly good seed producer in some seasons. One generation in Australia.

C.P.I. 9'49. Apparently tetraploid. From Botanic Garden, Erevan, Armenia, U.S.S.R., 1[illegible]. No remarks. Good hardy type here and seed production fair in southernmost Australia. One generation in Australia.

C.P.I. 9161. Apparently hexaploid. From Department of New Cultures and Introductions, Institute of Plant Industry, Leningrad, U.S.S.R., 1939. Remarks from flora. One generation in Australia.

C.P.I. 10,863. Apparently hexaploid. From Institute of Plant Industry, Leningrad, U.S.S.R., 1947. Remarks: 'Native to high fields and lower alpine slopes in Caucasia and Armenia' - a general statement. One generation in Australia.

C.P.I. 18,119. Apparently hexaploid. From Botany Division, Department of Scientific and Industrial Research, New Zealand, 1939. Introduced there but origin obscure. At least one generation in New Zealand and two generations in Australia.

C.P.I. 23,198. Apparently hexaploid. From Grasslands Division, D.S.I.R., Palmerston North, New Zealand, 1944. Introduced there but origin obscure. At least one generation in New Zealand but none in Australia.

C.P.I. 23,408. Apparently hexaploid. From Seed Department, Rothamsted Experimental Station, Harpenden, England, 1947. Some doubt as to whether the original material was received at Rothamsted from Russia or Pellett Gardens - probably the latter, but this is quite different from material I obtained from Pellett Gardens. This is portion of a repeat introduction recently received here. I have not been able to get seed from this Rothamsted material grown in isolation under good conditions in Canberra and two sites in southern Victoria. Employing hand pollination we have not found any combination which produced more than an occasional seed. However, certain combination between plants of the Rothamsted material and your P.I. 108,699-C-39 have given quite good seed production."

Hely also commented that much of the F. ambigua material in circulation at present is probably undesirable inbred and suggests distinguishing between the ploidal groups and making polycrosses from plants selected from a number of introductions. Hely observed that a

high percentage of 6x plants gave ineffective nodulation with Dr. Erdman's Turkish isolates of Rhizobium trifolii as well as uncertain flowering and very poor seed production under the Australian environment. On the whole, massive inoculations were necessary to establish T. amoigum bacteria in competition with other strains of Rhizobium trifolii.

In later correspondence (12), it was noted that in Canberra the diploids (2264 and 2771) were better in seed production and had a number of effectively nodulated plants. These plants were small but denser and could easily out yield the polyploids. The tetraploid CPI 9949 was described as being strong-growing, leafy, shy flowering, and subject to early and effective nodulation. The other tetraploid, CPI 6884, was described as a poor agronomic type but a good seed producer. This also nodulated early and effectively with the Turkish isolates.

In addition to the Australian material the following sources were also studied. No description accompanied these sources.

FC 33109. Received from Dr. E. A. Hollowell, Head of Clover section Forage and Range Research Branch, Agricultural Research Service, U. S. D. A., Beltsville, Maryland.

Pellett Clover. Irradiated material received from Dr. M. J. Metzger Farm Crops Dept., Corvallis, Oregon.

PI 229665, PI 228370, PI 2296246. Received from Northeast Regional Plant Introduction Station, Geneva, N. Y. Iran is listed as the country of origin.

Early in the winter of 1957-58 part of the seed from each source, with the exception of the Iranian materials, was planted in flats in the greenhouse. The Iranian materials, PI 229625, PI 225270, and PI 2296246, were not received until the spring of 1958. Seeds not planted were held for later use since one of the techniques tried for determining chromosome number required germinating seed. This will be described later. At the completion of the aforementioned experiment in the spring of '58, the remaining seeds were sown in flats along with the newly arrived Iranian seeds. The same technique was used in the sowing of all seeds. Seeds were planted ¼ inch deep in regularly spaced rows in sand-filled flats. Full width partitions were used to prevent mixtures when two or more sources were planted in the same flat. From seedling emergence until the termination of the study nutrient solutions were applied to greenhouse plants at 10 day intervals. When the seedlings reached a suitable size, the more vigorous ones from each source were transplanted to four inch pots. Discard of the less vigorous types in each source may have eliminated some slower growing or slower germinating types. Aneuploids, triploids, pentaploids, etc., may fit into this category. A loam-peat potting mixture was used. Labeled 6-inch stakes were used to identify plants as to source and number. Each pot was then placed in another four-inch pot. The void between the pots allowed root tips emerging from the drainhole of the upper pot to be uncontaminated by foreign materials. Clean root tips were desirable for cytological investigations. No control was exerted over light and temperature until October, 1958.

By June, 1958 many plants from the earlier seedings had produced

rhizomes. A rhizome was removed from each of these plants and double-potted in four inch pots as described above. The remainder of each plant was then transplanted to the field nursery at East Lansing. In August all plants from the earlier seedings were transplanted to the field whether or not rhizomes had been produced. However, cuttings were made from plants which had initiated rhizomes after the earlier field transplanting. These cuttings were also kept in the greenhouse in labeled double pots. With the exception of a few seedlings from the Iranian sources, all of the later seedlings were maintained in the greenhouse. These comprised the bulk of the greenhouse plants. A few plants from each of the Iranian sources were transplanted to the field in order to have replication of each source in both the greenhouse and the field.

Plants in the field were space planted at two foot intervals in rows 3 feet apart. 15-15-15 fertilizer was applied in a ring around each plant at a rate of approximately 1000 lbs/acre. Identification of plants was accomplished by stamping the source and plant number on a metal tag. The tag was then stapled to a 3/4" x 2" x 8" wooden stake which was positioned by the plant. Only those plants which had cuttings in the greenhouse were individually identified in the field. The others were identified only by source. Since plants initially were placed in the field more or less according to the time of rhizome production the plants of most sources were represented in varying numbers at two or more places in the nursery.

From these greenhouse and field materials several studies were made. These included:

1. Determination of ploidy level for each source,
2. Studies of the effect of ploidy and source on various morphological and physiological characteristics including leaf size, stomata length and number, size of floral parts, pollen size and viability, fertility, seed size and weight, vigor, winter hardiness, growth habit, and flowering,
3. Fertility studies.

Comparisons could not be made between all plants represented in both the field and the greenhouse among all the sources because:

1. The Iranian source materials were not vegetatively propagated since rhizome initiation was not yet apparent at the time of transplanting. These seedlings were approximately six months younger than the transplants from the other sources.
2. Some sources had only a few plants producing rhizomes at the time of transplanting. Consequently, the number of plants represented both in the field and greenhouse was too small for adequate sampling.
3. Losses occurred in cuttings from both the greenhouse and field for a variety of reasons.
4. In the greenhouse erratic flowering further reduced the opportunities to compare field and greenhouse data from the same plants.

Determination of ploidy level

The ploidy levels of the various sources were not known with

the exception of the material received from Hely which had been tentative identified. In most sources, however, variation between plants within a source was such that it was suspected a range of ploidy levels might exist.

Generally Trifolium chromosomes are small and not amenable to ordinary smearing techniques. Three methods of cytological examination were investigated in an attempt to find a fast and accurate procedure to determine chromosome number:

1. Examination of root tips from germinated seeds,
2. Examination of pollen mother cells,
3. Examination of root tips from greenhouse plants.

Examination of root tips from germinated seeds

Seeds were scarified with sandpaper and treated with Arasan. Petri dishes labeled according to source were used as germinators. Each Petri dish was held at room temperature and under 24 hour illumination. The seeds were germinated on blotters saturated with 0.2% KNO3 solution. Identification of individual seeds was facilitated by the following steps. Holes $\frac{1}{8}$" in diameter were punched in a blotter and numbered consecutively with indelible ink. Stapling a nonperforated blotter to the blotter helped to hold the seeds in place. One seed was placed in each hole.

The stage of germination in which the first flush of mitotic division in the root tip occurred was not known. Therefore, samples of root tips were taken from the time the seed coats ruptured until the roots were approximately 2-4 mm long. The root tip from a particular plant was placed in a labeled 10 ml shell vial containing .004 M

3- hydroxyquinoline and held at approximately 4.5°C. After 5 hours the hydroxyquinoline was replaced with Newcomer's solution. The root tip was kept at 4.5°C. in Newcomer's solution until used. Standard procedures were used to prepare slides for study. Propionic carmine was used as the stain.

No divisions were found in root tips sampled as outlined above. To determine if mitotic divisions were under diurnal control, root tips were taken every half hour over a 24 hour period. In addition the maximum length of the root before sampling was increased to approximately 6 millimeters. Several stages of divisions were found among the larger root tips at varying times. Therefore diurnal control was not apparent.

This method of determination of chromosome number was discontinued for these reasons:

1. Plant recovery was too slow after the root tip had been removed,
2. The number of dividing cells per slide were too few for rapid determination of chromosome number,
3. Space limitations in the field and greenhouse were such that it was desirable to save only those plants which showed good vigor. This would have been quite difficult to determine in germinated seedlings.

Examination of pollen mother cells (PMC)

During meiosis chromosome associations can be determined and counted at late Diakinesis and Metaphase 1. In T. ambiguum, dividing PMC's were found when the heads had just begun to emerge from their

protective sheaths in the axils of the flowering shoots. In this study one or two such heads were removed from each of several plants. Heads from a particular plant were placed in labeled 10 ml. shell vial containing Newcomer's solution and held at approximately 4.5°C. for at least 12 hours or until used. Since the florets of a clover head mature acropetally the area of the head in which the P.M.C's are dividing also moves from the bottom to the top of the head. This region must be determined by trial and error procedure although floret size is often an indication of the approximate region of the head in which divisions are taking place.

Anthers were removed from florets and squashed in a drop of propionic carmine stain. The preparation was then briefly observed under 150x to determine if the P.M.C's were undergoing meiosis. If meiotic stages were observed, a cover slip was put in place and the preparation was smeared. Once the area of the head was determined in which divisions were occurring all florets at this level were isolated and slides were prepared as needed. Often the anthers from two or three florets were combined in one slide. Although the ploidy level of several plants was determined using the P.M.C method, it was discontinued for the following reasons:

1. The process of finding the exact level of the head in which meiosis was taking place was too slow.
2. Multivalent configurations, especially in the tetraploid and hexaploid levels, made accurate counting very difficult.

Cytological examination of root tips of greenhouse plants

The most successful of the three methods for determining chromosome numbers was the study of mitotic divisions in the root tips of developing plants. Each plant in the greenhouse was double-potted in four inch pots. The void between the bottoms of the two pots allowed root tips to grow uncontaminated by foreign materials after emerging from the drainhole of the first pot. In this study root tips were taken at various times between 9 A.M. and 5 P.M. with no apparent differences in the number of dividing cells found. Upon removal, root tips were placed in a labeled 10 ml shell vial containing .002 m 8-hydroxyquinoline at approximately 4.5°C. After approximately 2½ hours, the hydroxyquinoline was replaced by Farmer's solution (3 parts Ethyl alcohol: 1 part glacial acetic acid). Several drops of aceto carmine were added to the fixative (9). After 12-24 hours the fixative was poured off and 1N HCL added for 15 minutes to facilitate maceration. Finally, the root tips were transferred to 70% ethyl alcohol until used. Best results were obtained when slides were prepared from root tips within four days after removal.

The aceto carmine added to the fixative served two purposes:

1. Since only the meristematic region of the root tip took up stain readily, the remainder of the root tip could be discarded when a slide was prepared.
2. Since materials treated with HCL do not stain readily with aceto carmine, pre-treatment with stain intensified the staining of the final product.

In each slide preparation, 2-5 root tips were squashed in

aceto carmine to which iron citrate had been added. The number of root tips used was dependent on their size. Best results were obtained when a hard even pressure was exerted on the cover slip during the smearing process. While pressure was maintained melted wax was applied to the perimeter of the cover slip. This helped to prevent bubbles due to the intake of air under the cover slip when pressure was released.

Leaf length/breadth ratios

Artificially doubled plants tend to have a larger leaf length/breadth ratios than their undoubled progenitors (29). Trifolium ambiguum was examined for this characteristic.

Eight plants from each source were sampled in the field and eleven plants from each source were sampled in the greenhouse. In either case sample size was based on the source with the smallest number of plants. Length and width measurements were made on each leaflet of the largest leaf of the plant. Generally the three leaflets from the largest leaf appeared more fully developed than those from smaller leaves. Length measurements were made in millimeters along the midrib of each leaflet from the base of the leaflet to its apex: width measurements were made in millimeters at the widest portion of the leaflet. To obtain the length/breadth ratio for the leaf, the sum of the lengths of the three leaflets was divided by the sum of the widths of the three leaflets. The ratios so obtained were used in calculating the mean length/breadth ratio for source and ploidy level in the field and greenhouse respectively.

Since some plants in the field had been vegetatively propagated

from duplicate plants in the greenhouse, correlation coefficients were calculated to determine environmental effects on length/breadth ratios.

Leaf length x breadth data

One of the characteristics often found in artificially induced polyploids is larger leaves (23). This characteristic was studied in T. ambiguum by using the same data compiled for each leaf in the determination of length/breadth ratios. In this study, however, the sum of the length of the three leaflets was multiplied, rather than divided, by the sum of the widths of the three leaflets. The length-breadth product so obtained indicates the relative size of the leaf. Average size was then calculated for each source and ploidy level in both the greenhouse and field.

Stomata length and number

In artificial polyploids the size of stomata increase and the number of stomata per unit area decrease as the ploidy level increases (23). To determine if such a trend existed in T. ambiguum, stomatal measurements and counts were made on fourteen plants from each of the three ploidy levels. Seven of the plants from each ploidy level were selected from those in the field and seven from those in the greenhouse. In each place at least one plant from each source was included. One of the largest leaflets was removed from each plant and transferred to ice water until used. Since it has been reported that the number and size of stomata vary according to the portion of the leaf studied, all studies were made at one region of the leaflet (6). The area examined was the lower epidermis at a point adjacent to the mid-rib and approximately

half-way up the long-axis of the leaflet.

The leaflet was taped to a glass slide by masking tape in which a diamond shaped hole approximately 5/16" long had been cut. The masking tape was placed so as to position the hole over the area to be studied. A drop of water placed under the leaflet prevented immediate wilting and emphasized the outline of the stomata. By placing a drop of immersion oil directly on the leaflet and using a 15 x 50 (oil immersion) magnification, the stomata could be accurately measured on a micrometer scale. Measurements were made in divisions. At this magnification each division was equal to 2 microns.

The average stomata length was determined from the measurements of ten stomata selected at random from those in the aforementioned area. Under the conditions of this experiment the stomata were closed when measured. Two replications of ten stomata each were measured for each leaflet removed from plants in the greenhouse. The small (0-1.2) microns deviation between replication averages indicated that one replication was adequate. These averages were then used in calculating the ploidy average for the field and greenhouse respectively.

The average number of stomata per unit area of each leaflet was determined by counting the number of stomata in the field of view at 750x using an American Optical 4T Microstar. The numbers found at two locations was averaged. From these averages the average number of stomata per unit area was calculated for each ploidy level in the field and greenhouse, respectively. In addition, an overall mean for number of stomata per unit area was determined by combining the data for field and greenhouse.

A spot check on the stomata of the upper epidermis indicated that although these were smaller and more numerous, the same trend of size and number occurred between ploidy levels.

Although the sample size in this investigation was quite small, the ranges of the results indicated that further sampling would have been of little value.

Floral initiation in the greenhouse

Difficulties in flower induction were reported between September and March in T. ambiguum under greenhouse conditions (17) (18). In early October 1959, plants in the greenhouse were subjected to a 17 hour day with a temperature range of 40°-50°F at night and 70°-75°F during the day. Normal day length was extended by the use of 150 watt reflector flood lamps spaced at 4 ft intervals over each bench. The lights were adjusted according to plant growth so as to give maximum light intensity. Temperatures adjustments were automatically controlled by outdoor light conditions. Nutrient solutions were applied at 10 day intervals. Under these conditions flowering was initiated. The number of plants showing their first flower was noted bimonthly. This study was terminated on May 15, 1959.

Average number of heads per plant in the field

Fewer flowers are reported on artificially induced polyploids than in their diploid parents (43). T. ambiguum was studied for this characteristic. The number of flower heads on several (6-9) plants from each source were counted at the peak of initial flower production in the field. From this data the average number of heads per plant was

calculated for each source and for each ploidy level. No allowances were made for rhizomes producing flowers since at the time of this study flowering on rhizomes was insignificant. Time was not sufficient to note any differences in the length or intensity of the flowering period. Because the number of heads was correlated with the size and vigor of the plant, some bias probably occurs in favor of those plants transplanted in June 1958 which at the time of the study were generally more vigorous than those transplanted in August 1958. However, some of the sources represented only in the August transplants may have flowered about the same even if transplanted earlier.

Average number of heads per plant in the greenhouse

Flowering was first noted in the greenhouse on November 15, 1958. Subsequently, mature heads were removed and their numbers recorded at two week intervals until June 1, 1959. By the latter date only a few plants were still flowering and the study was terminated. Data were compiled only on the basis of flowers produced per plant irrespective of rhizome development or length and intensity of the flowering period. The mean number of heads per plant was calculated for each source and ploidy level. Some bias is introduced in that one of the tetraploids and four of the hexaploids initiated flowering on comparatively few (4-7) plants.

Average number of florets per head

Artificially induced polyploids of clover reportedly have larger heads than diploids (23). Consequently, the number of florets per head would be expected to increase as the ploidy level increased.

The number of florets per head was determined by stripping off and counting the florets from fully matured heads. The mean number of florets per head was calculated for each plant. This average was then used in tabulating the average number of florets per head for both source and ploidy level. Stunted or damaged heads were not used as well as those which had florets removed in the course of fertility studies. The number of heads examined per plant was also dependent on the intensity of the flowering period and the time of flower initiation relative to the termination of the study. At the conclusion of this study several sources were still inadequately sampled because of erratic flowering. Four hexaploid sources, for example, had 5 or less plants sampled. Time did not allow adequate field sampling in the spring of '59. However, florets were counted on 6 field plants which, by virtue of vegetative cuttings, were also represented in the greenhouse. The number of florets per head in each case was within the range found for the greenhouse.

Floret and pistil length in the field and in the greenhouse

Induced tetraploids of clover have been reported to have larger floral parts than their undoubled progenitors (23). To examine the effect of polyploidy on the size of florets and length of pistils in T. amoenum, samples for measurements were taken at the field and the greenhouse. At both places the number sampled in each source was dependent upon the number of plants flowering and/or the number of plants in that source. When possible at least eight plants per source were studied.

Length measurements of florets were made on three fully opened florets. Pistil length was also determined from these same florets. In

the field the florets were generally taken from different heads. However, flowering in the greenhouse was erratic at the time of sampling and by necessity the three florets were generally removed from the same head. Before the pistil was extracted, each floret was measured in millimeters from the top of the receptacle to the tip of the standard. Variation in floret length was never more than one millimeter between and within heads from the same plant.

The plant averages for floret length were used in calculating mean floret length for source and for ploidy level in the greenhouse and field, respectively. In addition an overall average was obtained by combining the plant averages of floret length from both the greenhouse and the field. In the field the mean floret length for both source and ploidy level was calculated using data obtained from each source. In the greenhouse the mean was determined only for those sources that had five or more plants flowering at the time of sampling. Since only one of the seven hexaploid sources in the greenhouse was adequately sampled, the 6x mean floret length for ploidy level was not calculated.

The pistil was extracted from each floret and measured in millimeters from the base of the ovary to the uppermost part of the style. No allowance was made for the amount of curvature in the style. Variation in pistil length was never more than 0.5 mm between and within heads from the same floret; generally, the deviation was in the range of 0.0 mm to 0.3 mm. The number of plants sampled and the methods of calculation to determine the mean length of pistil coincide with those for length of floret measurements.

Generally the pistil was either shorter than or about the same

length of the longest stamen. However, some instances were noted in which the pistil was longer than any of the stamens. The effects on fertility, if any, were not noted. In the Iranian materials, the pistils were easier to extract than in the other sources. This indicated a weak vascular connection between the pistil and the receptacle. If this connection is comparatively weak, the nourishment required for complete seed development may be lacking. In addition, shattering would probably occur more readily.

Pollen diameter and stainability

Artificially induced polyploids tend to have larger pollen grains than their undoubled progenitors (6) (23). In addition a higher frequency of non-stainable pollen is reported. Non-stainable pollen grains are assumed to be non-viable.

Pollen diameter and stainability was studied for the 2x, 4x, and 6x ploidy levels in Trifolium ambiguum. Samples were initially taken from plants flowering in the greenhouse. Because of erratic flowering however, several sources were not adequately sampled. Therefore in order to make a more complete survey, a minimum of four samples from each source were taken in the field. Enough plants were sampled from each source so that a minimum of eight plants had been examined in the field and greenhouse combined. Each sample consisted of one floret removed from a plant. The floret was then placed on a moist blotter in a labeled Petri dish and refrigerated until used. Unless refrigerated, germination of pollen grains occurred. Germinated pollen grains were not only more difficult to measure but were also difficult to distinguish

from the non-stained pollen grains when the germinated grain did not remain intact. Florets from plants in the greenhouse were removed when fully opened; in the field, however, it was necessary to use florets at a state of development just prior to opening because of the problems of pollen eating insects and bee visitations.

Slides were prepared for pollen diameter measurements and pollen stainability counts as follows. Floret parts were stripped back to expose the stamens. If dehiscence had not occurred, the anthers were cut open. In either case the anthers were brushed on a clean glass slide in an area approximating that of a 22 mm square cover slip. This area was then ringed by a band of aceto carmine stain in order to prevent hollow pollen grains from floating off when the cover slip was dropped in place. Ten well-filled pollen grains (c) were randomly selected from different portions of the slide and their diameters measured on a micrometer scale under 64.5x. The measurements were recorded as divisions with each division equivalent to 2.4 microns at the aforementioned magnification. An average was obtained for the ten counts from each floret. These plant averages were then used to calculate the average for source and ploidy group in the field and greenhouse, respectively. In the field the mean pollen diameter for both source and ploidy level was calculated using data obtained from each source. In the greenhouse the mean pollen diameter was determined only for those sources that had four or more plants flowering at the time of sampling. Since only one of the seven hexaploid sources in the greenhouse was adequately sampled, the 6x mean pollen diameter for ploidy level was not calculated.

Utilizing the same slide that was prepared for pollen diameter

measurements, two counts of one hundred pollen grains each were made. One count was taken from the upper portion of the slide and the other from the lower portion. In most cases the two counts were spread less than 4 points. A third reading was included if the range was considered too large. Only those pollen grains which showed absolutely no visible staining were counted as non-stained pollen. Thus pollen grains which were obviously shrunken but somewhat stained were included as stainable pollen and therefore presumed to be viable pollen. The counts per slide were then averaged and converted to percent. Since the range within some sources was quite extreme, the data from the greenhouse and field were combined and not analyzed separately. The means for source and ploidy level were determined for the combined data.

Fertility

Seed set in T. amblyum had been reported as being relatively low (11). Therefore fertility studies were initiated on plants growing in the greenhouse. Although some crosses were achieved between and within all ploidy levels, the most intense study was at the diploid level because of recurrent flowering in the same plant: a factor which made several crosses possible. Four types of crosses were made:

1. Intersource crosses at the same ploidy level.
2. Intrasource crosses at the same ploidy level. The reader might note that 2 of the 14 sources were found to have two ploidy levels. No mixtures of ploidy level were found in the other 12 sources.
3. Interploidy crosses.
4. Selfing of plants from each ploidy level.

Some crosses were made by hand pollination and some through pollination by honeybees. Only fully opened unwithered florets were pollinated by hand. Presumably these would be receptive to pollen. A toothpick was used to effect pollination. Care was taken to ensure that the stigmatic surface had been rubbed. Florets not pollinated were removed. Hand crosses were made reciprocally between plants. Selfing was also accomplished by hand.

The use of active honeybees for crossing is desirable for the following reasons:

1. Multiplant crosses are readily performed,
2. Repeated visits by bees make effective pollination more likely,
3. Pollination data obtained from bee activity may be more useful in a practical way than data obtained through hand pollinations. For example, low seed set is reported in induced tetraploids of red clover presumably because the larger florets do not permit effective bee visitation (2) (21). This situation could be duplicated in T. amb<u>i</u>guum since plants of higher ploidy levels tend to have larger florets.

Initially a small colony of honeybees was kept in a 4' x 4' x 4' cage in the greenhouse. Under these conditions the bees failed to work the flowers. In mid-January the bee population was increased to two sections of a hive and a 4' x 8' x 3' cage was used. Excellent activity was apparent and several crosses were made. Each cross was kept in the cage for at least 3 days. A minimum of 24 hours was allowed

between crosses in order to ensure that viable pollen was not carried over to the next cross. In March a drop in bee activity again occurred and crossing was discontinued until April. At this time, the hive was moved outside and the bees were allowed to fly free for several days. The bees were then returned to the cage and crossing was continued until late May. Activity during this period appeared to be very good. Bee activity is apparently a function of daily environment. For example, in both the greenhouse and outside, bee visitations on flowers were greatly increased on sunny days when the temperature was warm. Therefore a certain bias is presented if a cross happened to be in the cage over a period of inclement weather. Generally a cross was left in the cage longer if such a condition existed.

Before placing plants in the bee cage, withered florets were extracted from the head. After taking the plants from cage, portions of the head which had unopened florets were removed. This was done to eliminate those florets which were probably not receptive. The flowering stems were tied to stakes in order to make the heads as accessible as possible. This was particularily necessary in those plants which tended toward a prostrate growth habit.

All heads of plants involved in either hand or bee pollinations were tagged with the appropriate crossing information. Data obtained were based on the number of seeds produced per number of florets involved in the cross. Since a pistil from a floret of T. ambigum generally had two ovules, it was theoretically possible that twice as many seeds as florets would be produced.

Seed size

Artificial polyploids usually have larger and heavier seeds than their undoubled progenitors (21) (23). Three ploidy levels in T. ambiguum were studied with respect to this characteristic. Seed size was measured in two ways:

1. The average weight of 100 seeds from each ploidy level without respect to source.
2. The percent of 1000 seeds from each ploidy level passing through each of a series of hand screens.

The above experiments were conducted using seed which was obtained from crosses within a ploidy level. The crosses were made on greenhouse materials.

The approximate total number of seeds from which the determinations were made were as follows:

2x - 5940
4x - 2060
6x - 1155

Average weight per 100 seeds for each ploidy level was determined from 5 replications of 100 seeds each.

Relative sizes of seeds from each ploidy level were obtained from the average of two replications of 1000 seeds each which were passed through a graduated series (1/12" - 1/21" diameter) of hand screens.

Vigor rating

On May 29, 1959 plants in the field were compared for vigor on a 0 to 10 scale. A 0 rating was given to plants which failed to survive while progressively higher ratings were given as comparative

vigor increased. The June, 1958 transplants were rated as a distinct group from the later 1958 transplants because of overall increased vigor among these earlier transplants.

Data for a given source were then combined regardless of transplant date. One average was determined which included those plants failing to survive and, in addition, another was determined which included only the surviving plants. At the time of this study the only losses were in the later transplants

Rhizome development

Rhizome development in polyploids is usually less extensive than among diploids (23). On May 29, 1959, the number of rhizomes on each plant in the field was counted. Only those rhizomes were included that resulted in the emergence of new shoots. No attempt was made to correct any discrepancies due to differences in time of transplanting. The average number of rhizomes for each source and ploidy level was determined.

Growth habit

Growth habit as used in this study refers to the degree of erectness of a plant. For example, some plants of T. ambiguum were virtually prostrate while others were quite erect. In contrast to upright plants, prostrate plants would presumably have relatively inaccessible as well as dirty flowers because of proximity to the soil. This would possibly discourage bee visitations and result in reduced seed set. Seed harvest would also be complicated in prostrate types. In addition, erect plants would presumably be more desirable in a hay or pasture program not only because of easier handling and

grazing. However, erect types would require more of a management program than prostrate types which could probably survive better under conditions of heavy grazing.

Plants in the field were rated 1 to 5 according to growth habit. A rating of 1 was assigned to prostrate plants while the most erect plants were given a rating of 5. A mean rating was determined for source and ploidy level.

Winter hardiness

In some clovers, tetraploids reportedly have less winter hardiness than their diploid parents (30). In October 1958, all living plants in the field were noted. The following May another count was made. Losses were attributed to lack of winter hardiness.

Source ratings

Fourteen sources of T. ambiguum were rated on a scale of 1 to 14 for several of the characteristics under study. The rating for a particular characteristic was dependent on the ranking of a source with respect to the other sources. A rating of 14 was most desirable for a characteristic under study while a rating of 1 was least desirable. Thus, the source with the most total points would be considered the most outstanding in this study. Conversely, the sources which had the least total points would be generally undesirable, at least on the basis of the characteristics examined. However, a specific plant in any source might vary sharply from the average of that source for any characteristic. While no question would probably arise on the desirability of such factors as high pollen fertility, good vigor, and extensive

rhizome development, the desirability of certain of the other characteristics might be open to question. For example, a plant having large leaves would presumably be the most adversely affected during a drought. Long pistil length might result in poor fertilization because of the added growth required by the germination tube of the pollen grain. Large florets, although presumably containing more nectar, might also be difficult for a bee to work, etc.

Ratings were made for sources from both greenhouse and field data. The combined data of field and greenhouse were used in both tables in the case of pistil length, pollen size, and pollen stainability since separate analysis for each place was not appropriate.

RESULTS

Cytological examination

Table 1 shows the results of cytological examinations. In cytological examination, an attempt was made to include the unusual types in each source as well as the more commonly appearing types. If the plants examined in a source were then found to have only one euploid level, it was assumed in further studies that all plants of that source were of the same ploidy level. Because of overlapping of chromosomes, the number of chromosome in a dividing cell often could only be approximated. Therefore, although only two aneuploids were definitely established, others may have existed.

4x plants were found in CPI-6161, primarily a 6x source. CPI 23403, another 6x source, was found to have some 5x plants. The remaining 12 sources appeared to be pure for a given ploidy level (see Figures 1-6).

However, it should be noted that because of lack of greenhouse space only the more vigorous seedlings in each source were maintained. Therefore, if irregular types were slower germinating or slower growing, they would have been discarded. This could possibly account for the lack of ploidy mixtures within a source.

Table 1. Results of cytological examinations of Kura clover plants.

Source	No. greenhouse plants	No. plants analyzed	Results of examination
CPI 2284	68	23	All 4x
CPI 2771	44	13	All 4x
CPI 6984	47	13	All 4x
CPI 9949	13	8	All 4x
PI 229625	95	26	All 4x (4-5 chromosome types)
PI 228370	109	29	All 4x
PI 229624b	50	15	All 4x
CPI 6161	11	10	8-6x, 2-4x
CPI 23408	38	19	4-5x, 15-6x
CPI 18115	18	6	All 6x
CPI 23158	47	10	All 6x
CPI 10803	12	3	All 6x
PC	53	6	All 6x
FC 33109	270	52	All 6x
Total	875	248	

Total % analyzed - 28%

Fig. 1. Somatic chromosomes of root tip of Kura clover plant.
2n = 16

Fig. 2. Diakinesis in PMC of Kura clover plant.
4n = 32

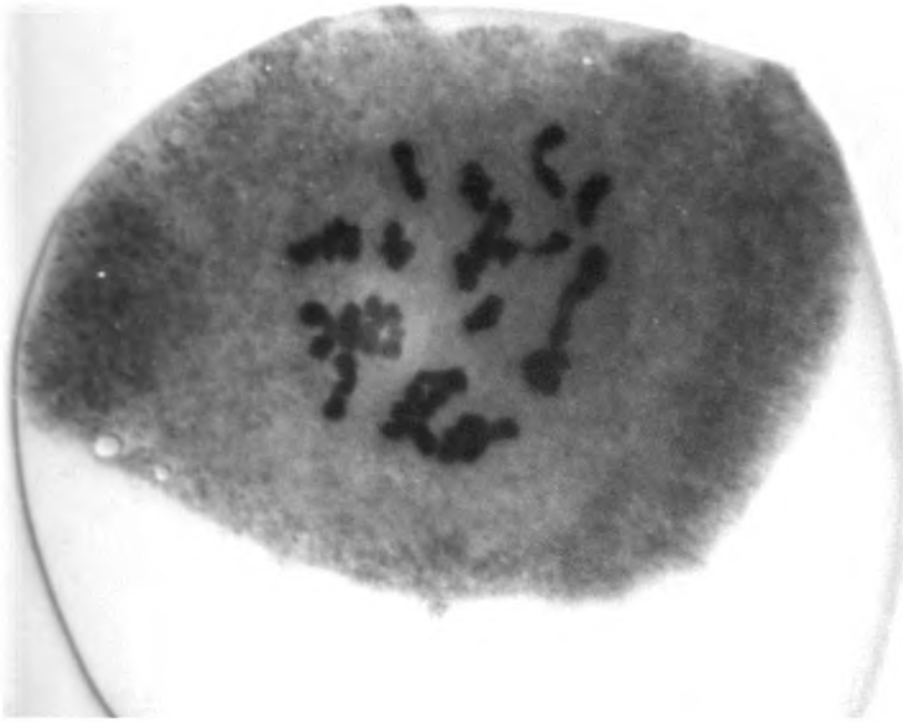

Fig. 3. Somatic chromosomes of root tip of Kura clover plant.
4n =-32

Fig. 4. Early Anaphase I in PMC of Kura clover plant.
6n = 48

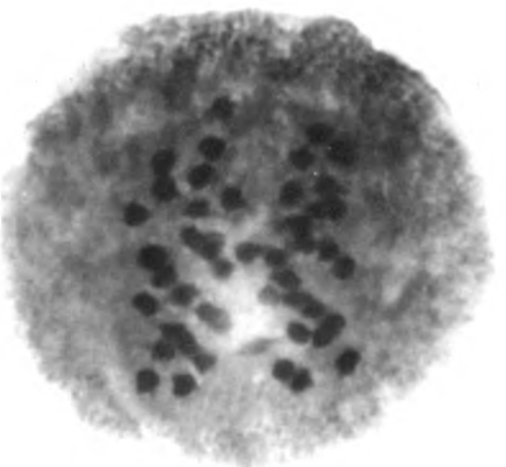

Fig. 5. Somatic chromosomes of root tip of Kura clover plant. 5n = 40 (verified in other cells)

Fig. 6. Somatic chromosomes of root tip of Kura clover plant. 6n = 48

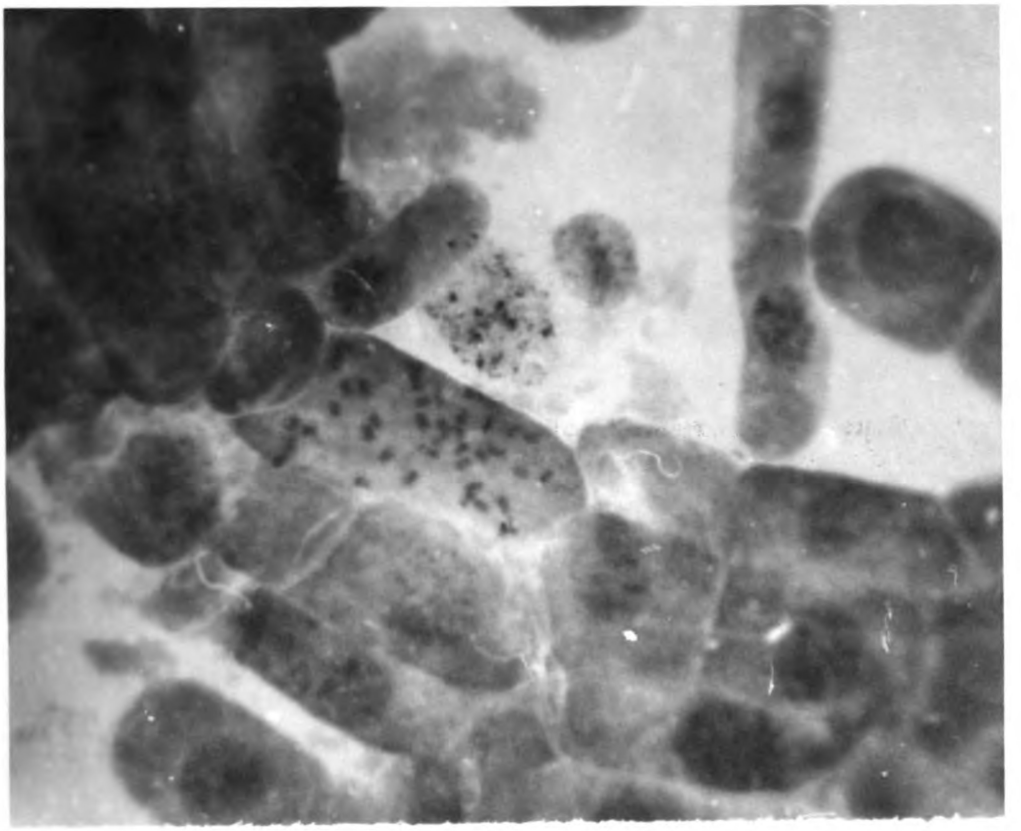

Table 2. Average length/breadth ratios of leaves from kura clover plants grown in the field, May 1959 (8 plants per source).

Ploidy level	Source	X	SD	Range	Ploidy X	Ploidy SD	Ploidy range
2x -	CPI 2264	1.66	0.19	1.41-2.04			
	CPI 2771	1.51	0.14	1.27-1.70	1.59	0.18	1.27-2.04
4x -	CPI 6584	2.29	0.23	1.99-2.68			
	CPI 9949	2.33	0.33	1.78-2.89			
	PI 229625	2.16	0.16	1.91-2.38			
	PI 228370	2.40	0.45	1.91-3.42			
	PI 229624b	2.13	0.21	1.90-2.37	2.26	0.30	1.78-3.42
6x -	CPI 6161	2.01	0.34	1.57-2.56			
	CPI 23408	2.05	0.22	1.65-2.52			
	CPI 13115	1.98	0.29	1.60-2.53			
	CPI 23158	2.01	0.33	1.35-2.33			
	CPI 10903	1.69	0.36	1.08-2.23			
	PC	2.23	0.30	1.77-2.81			
	PC 33109	2.23	0.21	1.86-2.50	2.03	0.33	1.08-2.81

Table 3. Average length/breadth ratios of leaves from Kura clover plants grown in the greenhouse, Winter 1958-59 (11 plants per source).

Ploidy level	Source	X	SD	Range	Ploidy X	Ploidy SD	Ploidy range
2x -	CPI 2264	1.64	0.16	1.44-1.95			
	CPI 2771	1.43	0.17	1.26-1.83	1.53	0.20	1.26-1.95
4x -	CPI 6884	2.03	0.43	1.58-3.08			
	CPI 9949	2.03	0.31	1.58-2.76			
	PI 249625	2.04	0.19	1.74-2.34			
	PI 228570	2.14	0.26	1.75-2.57			
	PI 249624b	2.07	0.17	1.81-2.31	2.05	0.28	1.58-3.08
6x -	CPI 6161	1.76	0.23	1.52-2.27			
	CPI 23408	2.40	0.43	1.93-3.07			
	CPI 18115	2.19	0.18	1.80-2.77			
	CPI 23158	2.30	0.23	1.88-2.71			
	CPI 10903	1.69	0.32	1.42-2.09			
	PC	2.30	0.35	1.92-2.71			
	PC 33109	2.34	0.43	1.86-2.76	2.14	0.39	1.42-3.07

Leaf length/breadth ratios

The average L/B ratios of the 4x and 6x sources were approximately the same (see Tables 2 and 3). The 2x sources have a lower average ratio, indicating a tendency toward more ovate leaf shapes (see Appendix A). However, the overlapping of the ploidy ranges indicated that L/B ratio is not a good criterion for distinguishing ploidy levels, even at the 2x level.

The intrasource correlation coefficients (Table 4) are low in most cases. However, two hexaploid sources, CPI 20803 and PC, have a high coefficient. In addition, each of these have similar averages and standard deviations in the greenhouse and field. The averages and standard deviations of the other hexaploid sources vary between these two places.

Leaf length-breadth product

From the data shown in Tables 5 and 6, the following was noted:

1. The mean leaf size in the field increased over that from the greenhouse in thirteen of the fourteen sources (Figures 7-10).
2. Average leaf size increased directly with ploidy level (see Appendix A). However, this tendency appeared primarily in the upper limits of each ploidy range. The lower limits overlapped to a large extent.

Table 4. Intrasource correlation coefficients for length/breadth ratios of leaves from cuttings of Kura clover grown in the field and greenhouse.

Ploidy level	Source	No. of Plants	Correlation Coefficient
2x -	CPI 2264	9	0.21
	CPI 2771	5	0.50
4x -	CPI 6384	9	-0.09
	CPI 9949	8	0.13
6x -	CPI 6161	9	0.20
	CPI 23403	10	0.42
	CPI 18115	9	-0.51
	CPI 23158	9	0.37
	CPI 10803	8	0.83
	PC	8	0.71

Table 5. Average length x breadth product of leaves from kura clover plants grown in the field, May 1959 (8 plants per source).

Ploidy level	Source	X̄	SD	Range	Ploidy X̄	Ploidy SD	Ploidy range
2x -	CPI 2264	5934	2954	4464-7240			
	CPI 2771	8251	2294	5010-12012	7092	2117	4464-12012
4x -	CPI 6834	9589	3221	6878-15682			
	CPI 9949	9315	1672	7774-12086			
	PI 229625	7244	2159	5029-10803			
	PI 228370	4578	1821	2210-7142			
	PI 229624b	5401	822	3977-6608	7225	2642	2210-15682
6x -	CPI 6161	11122	3664	5175-16008			
	CPI 23408	15787	3365	11250-18239			
	CPI 18115	15064	3869	4120-19768			
	CPI 23158	14613	5157	10579-26394			
	CPI 10803	16551	6459	10920-28427			
	FC	13545	6301	5108-28862			
	FC 33109	17001	7153	9281-29809	15055	5417	5175-29809

Table 6. Average length x breadth product of leaves from Kura clover plants grown in the greenhouse, Winter 1958-59 (11 plants per source).

Ploidy level	Source	X	SD	Range	Ploidy X	Ploidy SD	Ploidy range
2x -	CPI 2264	4743	1920	2044-8315			
	CPI 2771	6249	2503	3378-12616	5499	2311	2044-12616
4x -	CPI 6084	6547	2967	3563-13967			
	CPI 9949	7641	2739	4435-13214			
	PI 229625	3877	1047	1400-5900			
	PI 229670	4308	1339	2412-6547			
	PI 229624b	4827	2572	2228-11704	5542	2621	1400-13967
6x -	CPI 6161	5739	1954	3067-8901			
	CPI 23408	9956	3122	5166-15659			
	CPI 12115	9724	2507	6066-13748			
	CPI 23158	10196	2975	6500-19364			
	CPI 10803	6567	2158	3140-9162			
	PC	9383	3292	4704-16280			
	PC 33109	11072	5422	3759-22413	9020	3862	3067-22413

Figs. 7 to 10. Comparisons of largest leaves from plants represented in both the greenhouse and field by cuttings. Some bias had been introduced at the time of these photographs because of adverse environmental conditions in the greenhouse. However, the trend for larger leaves in the field was apparent (tables 5 and 6).

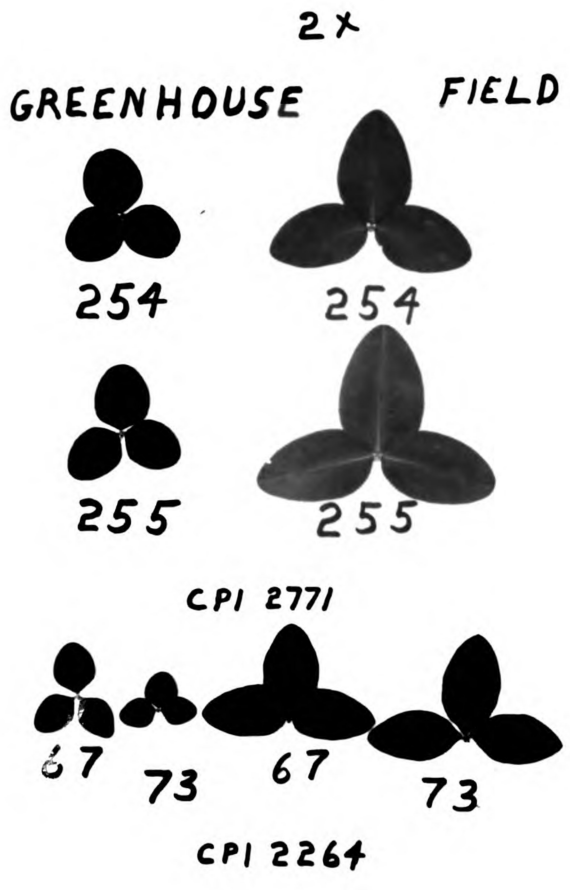
2X
GREENHOUSE
FIELD
254
254
255
255
CPI 2771
67
73
67
73
CPI 2264

Fig. 7

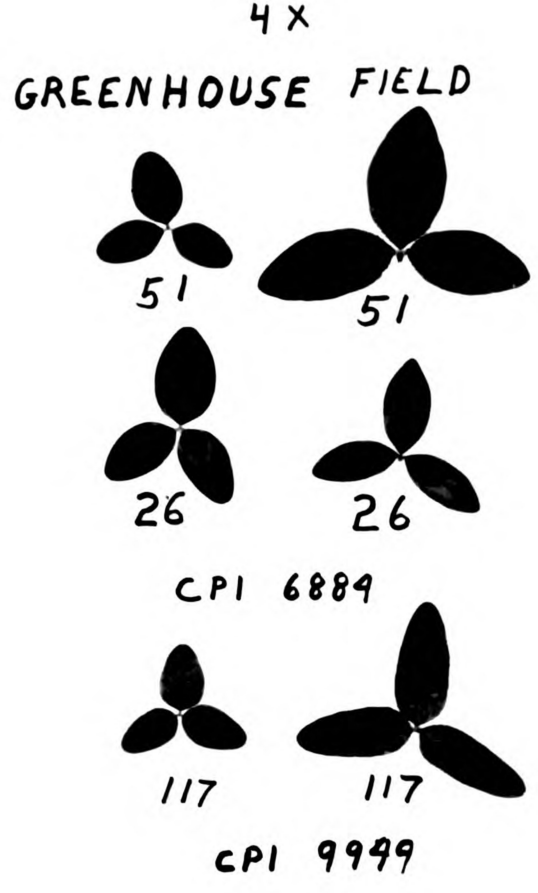
4X
GREENHOUSE
FIELD
51
51
26
26
CPI 6884
117
117
CPI 9999

Fig. 8

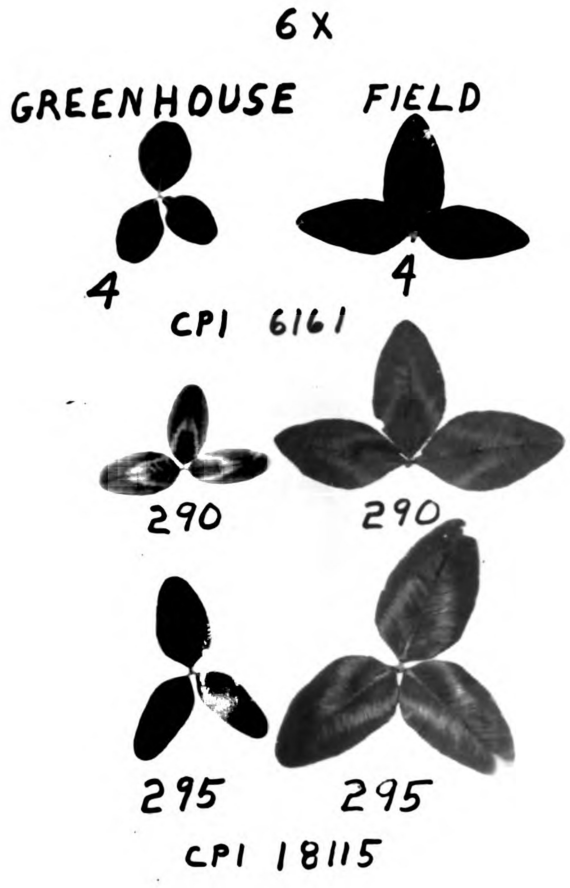
6 X
GREENHOUSE
FIELD
4
4
CPI 6161
290
290
295
295
CPI 18115

Fig. 9

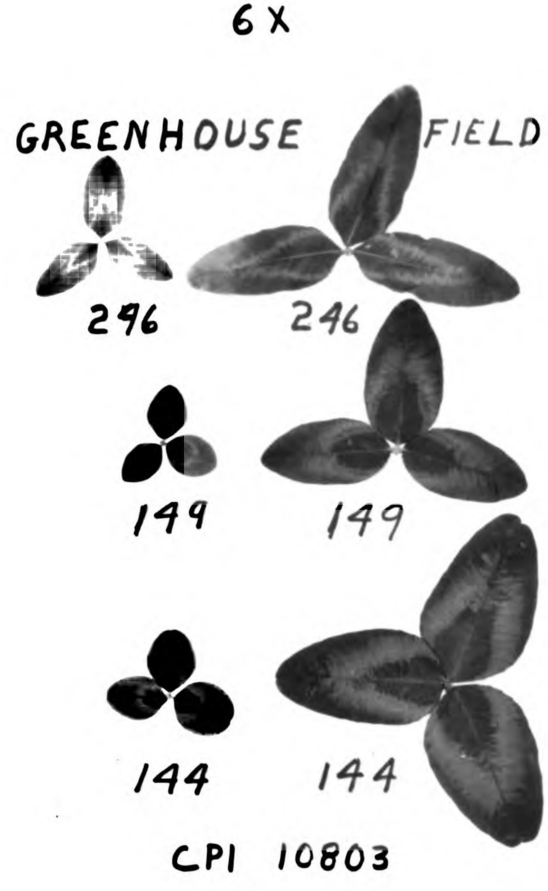
6 X
GREENHOUSE
FIELD
296
296
149
149
144
144
CPI 10803

Fig. 10

Stomata length and number

In the field, average stomata length appeared to increase directly with ploidy level (see Tables 7 and 8). However, as indicated from the ranges and standard deviations, the determination of ploidy level by this characteristic would be illogical. In the greenhouse there was no indication of variation in stomata length between ploidy levels. In either case, the size of the sample was not large enough to determine any definite trends related to ploidy level other than definite overlapping of range limits.

Under field conditions, the number of stomata per unit area appeared to decrease with increasing ploidy level (Table 9). In the greenhouse (Table 10) this relationship was found at the 6x level but not at the 4x level. In both cases the same remarks which were made for stomata length are applicable. Because of the small sample sizes no conclusions were drawn regarding discrepancies in the averages between the same ploidy levels in the greenhouse and the field.

Floral initiation in the greenhouse and field

In the greenhouse flower buds were first noted in mid-November, (see Table 11). As a group the diploids flowered earliest and heaviest. By January 1, for example 79% of the CPI-2264 and 65% of CPI-2771 had initiated flowering. The majority of plants in CPI-2264 was about three weeks earlier than those in CPI-2771. At the termination of the study on May 15, 1959, 98% of the diploid plants had flowered. Many of the diploids flowered intermittently over the entire period. Flowering among plants of tetraploid and hexaploid sources, however, was erratic with respect to date of initial floral production and

Table 7. Average length in microns of stomata from similar areas in the lower epidermis of mature leaves from Kura clover plants grown in the field, May 1959 (10 measurements per plant, 7 plants per ploidy level).

	2x	4x	6x
Ploidy ave.	26.4	29.4	31.6
Ploidy S.D.	1.[illegible]	[illegible].0	1.9
Ploidy range	25.0-28.8	24.6-32.2	29.4-34.4

Table 8. Average length in microns of stomata from similar areas in the lower epidermis of mature leaves from Kura clover plants grown in the greenhouse, April 1959 (2 measurements of 10 each per plant, 7 plants per ploidy level).

	2x	4x	6x
Ploidy ave.	26.0	26.0	27.6
Ploidy S.D.	4.6	1.6	2.3
Ploidy range	22.6-32.4	23.0-27.6	25.3-31.6

Table 9. Average number of stomata from similar areas in the lower epidermis of mature leaves from Kura clover plants grown in the field, May 1969 (2 counts per plant, 7 plants per ploidy level).

	2x	4x	6x
Ploidy ave.	16.4	12.9	10.0
Ploidy S.D.	2.77	3.19	2.00
Ploidy range	13-20	10-19	8-13

Table 10. Average number of stomata from similar areas in the lower epidermis of mature leaves from Kura clover plants grown in the greenhouse, April 1969 (2 counts per plant, 7 plants per ploidy level).

	2x	4x	6x
Ploidy ave.	16.0	18.0	13.3
Ploidy S.D.	4.2	5.6	4.2
Ploidy range	11-23	12-27	10-19

Table 11. Numbers of greenhouse plants of Kura clover initiating flowering during the winter of 1958-59 under conditions of a 17 hour day with day temperatures of 70°-75° F. and night temperatures of 40°-50° F. (bimonthly readings).

Ploidy level	Source	Nov. 15-31	Dec. 1-15	Dec. 16-31	Jan. 1-15	Jan. 16-31	Feb. 1-15	Feb. 16-28
2x	CPI 2264	4	23	18	5	3	..	1
	CPI 2771	..	..	27	3	4	1	2
	Total	4	23	45	8	7	1	3
4x	CPI 6934	..	1	..	2	2	..	..
	CPI 9949	..	..	3	..	1	1	..
	PI 219625	..	..	4	3	6	9	6
	PI 228370	..	..	..	8	9	7	6
	PI 229624b	..	..	..	..	3	3	..
	Total	..	1	7	13	21	20	12
6x	CPI 6161	2	..	1	1	..	..	..
	CPI 23408	..	..	4	3	1	1	1
	CPI 18115	..	1	..	1	..	..	1
	CPI 23153	..	2	..	..	..	1	..
	CPI 10803	..	..	1	4	..	..	..
	PC	1	1	9	1	..	1	..
	PC 33109	..	6	47	6	5	14	13
	Total	3	10	58	26	6	17	15
Grand Total		7	39	110	47	34	38	30

Table 11--Continued

Mar. 1-15	Mar. 16-31	Apr. 1-15	Apr. 16-30	May 1-15	Total no. flowering	Total no. in greenhouse	% plants flowering
2	1	..	..	1	65	68	93
1	2	..	..	..	40	44	91
3	3	..	..	..	103	112	92
..	3	..	..	..	8	47	17
..	1	..	..	..	6	13	46
3	9	..	2	2	49	95	52
10	8	..	2	..	50	109	46
3	1	3	1	3	17	50	34
16	22	3	5	5	130	314	41
..	..	..	..	..	4	11	36
2	..	1	1	..	19	58	33
..	1	..	..	..	4	18	22
..	1	..	..	1	5	27	19
..	..	..	..	..	5	12	42
..	..	1	..	..	10	53	19
21	29	6	5	4	156	270	58
23	31	8	6	5	203	449	45
42	56	11	11	11	436	875	50

number of plants flowering at any one time. At the termination of the study, 41% of the 4x and 4% of the 6x plants had flowered. At the tetraploid and hexaploid levels flower induction appeared to vary more between sources than between ploidy groups. Among all sources, flowering decreased after April 1. Artificial extension of the photoperiod was discontinued toward the end of March. This may have contributed to the decreased level of flowering.

In the field, the diploid sources reached the peak of initial flowering about May 20. The tetraploids were generally 3-4 days later with the exception of CPI-9949 which was an additional 1-2 days later. Hexaploids reached the peak of initial flowering approximately two weeks after the diploid plants. However, in each ploidy level plants were noted which flowered earlier or later than the average for that ploidy level. For example, two tetraploid and four hexaploid plants were flowering vigorously at the peak of initial flower production of the diploid plants. By mid-June all of the vigorous plants had initiated flowering. At this time, some flowering was occurring at all ploidy levels. It is therefore apparent that ample opportunity existed for interploidy crossing.

The following results are shown in Tables 12 and 13:

1. Flower production was much greater in the field.
2. Flower production varied more between sources than between ploidy levels in the field. In the greenhouse this was true of the 4x and 6x levels. Both 2x sources, however, averaged more flowering per plant than either of the polyploids.

Table 12. Average number of heads on Kura clover plants grown in the field, May 1959.

Ploidy level	Source	No. of plants	X	SD	Range	Ploidy X	Ploidy SD	Ploidy range
2x -	CPI 2264	7	53.3	30.9	12-101			
	CPI 2771	8	41.9	23.6	21-83	49.9	26.6	12-101
4x -	CPI 6884	8	85.9	54.9	26-184			
	CPI 9949	9	72.9	29.7	32-110			
	PI 229625	8	41.8	24.3	9-76			
	PI 229670	6	22.7	5.5	18-33			
	PI 229624b	8	41.5	37.9	10-124	55.0	40.3	9-184
6x -	CPI 6161	8	64.8	45.6	11-156			
	CPI 25408	9	93.4	73.7	24-245			
	CPI 18115	8	52.0	24.9	20-93			
	CPI 25158	8	47.9	17.4	29-84			
	CPI 10903	8	103.4	56.3	37-185			
	PC	8	48.9	32.5	19-112			
	PC 33109	8	95.5	46.7	42-160	72.6	49.7	11-245

Table 13. Average number of heads on Kura clover plants grown in the greenhouse, Winter 1958-59.

Ploidy level	Source	No. of plants	X	SD	Range	Ploidy X	Ploidy SD	Ploidy range
2x -	CPI 2264	77	9.7	6.60	2-24			
	CPI 2771	43	8.2	5.42	1-31	9.1	6.25	1-31
4x -	CPI 6884	10	3.6	2.31	1-8			
	CPI 9949	7	4.0	4.00	2-6			
	PI 229625	44	5.4	3.59	1-14			
	PI 228370	57	5.9	5.16	1-21			
	PI 229624b	18	7.1	5.02	1-23	5.6	4.45	1-23
6x -	CPI 6161	4	6.0	2.06	3-8			
	CPI 23408	13	4.2	1.91	2-8			
	CPI 18115	4	6.5	5.74	2-14			
	CPI 23158	5	6.0	3.39	1-10			
	CPI 10803	5	5.6	3.36	2-9			
	PC	9	5.7	4.42	2-17			
	PC 33109	157	6.9	4.83	1-24	6.5	4-56	1-24

3. In the fields as the ploidy level increased, the upper limits of flower production increased. The lower limits of the range remained approximately the same. No such trend was apparent in the greenhouse.

Average number of florets per head in the greenhouse

The average number of florets per head is approximately the same between sources. The apparent tendency of some sources toward larger number of florets per head may have been due to sampling (Table 14).

Floret length in the field and in the greenhouse

Tables 15 and 16 indicate that:

1. In the field average floret length tends to increase as the ploidy level increases. However, in the greenhouse, the 2x and 4x floret lengths are approximately the same, although the 6x florets tend to be longer.
2. At the 4x and 6x levels, the ranges in a given source show similar floret length for plants in the greenhouse and plants in the field. Under the greenhouse environment, however, 2x plants tend to produce larger florets than when under field environment.
3. The overlapping of ranges is such that floret length would generally not be an effective means of determining ploidy level. However, a plant with comparatively long florets might be strongly suspect of being a hexaploid.

Table 14. Average number of florets per head from hubam clover plants grown in the greenhouse, winter 1958-59.

Ploidy level	Source	No. of plants	X	SD	Range	Ploidy X	Ploidy SD	Ploidy range
2x -	CPI 2264	60	87	21.7	35-145			
	CPI 2771	33	96	18.9	54-139	90	21.1	35-145
4x -	CPI 6984	9	88	22.8	62-127			
	CPI 9949	6	123	29.0	92-172			
	PI 229625	27	75	16.0	47-117			
	PI 228370	34	71	17.3	43-120			
	PI 229624b	8	75	16.4	59-101	79	22.5	42-172
6x -	CPI 6161	3	121	16.7	113-133			
	CPI 23408	12	106	17.1	84-131			
	CPI 18115	3	67	31.9	43-103			
	CPI 23158	4	73	5.8	64-77			
	CPI 10803	5	105	13.3	91-119			
	PC	7	90	15.0	72-113			
	PC 33109	86	87	51.5	56-123	90	60.0	43-133

Table 15. Average length in millimeters of florets from Kura clover plants grown in the field, May 1959 (3 florets per plant).

Ploidy level	Source	No. of plants	X	SD	Range	Ploidy X	Ploidy SD	Ploidy range
2x -	CPI 2264	8	10.0	0.30	9.7-10.3			
	CPI 2771	8	11.1	0.55	10.0-12.0	10.6	0.72	9.7-12.0
4x -	CPI 6384	8	11.7	0.12	11.2-12.5			
	CPI 9949	8	11.4	0.53	10.5-12.0			
	PI 229625	8	12.4	0.79	11.0-13.3			
	PI 228370	5	11.2	0.80	10.5-12.5			
	PI 229624b	8	12.2	0.81	10.8-13.3	11.8	0.76	10.5-13.3
6x -	CPI 6161	8	12.7	3.95	10.7-14.5			
	CPI 23408	8	13.7	3.87	12.5-15.0			
	CPI 18115	8	13.1	0.62	12.5-14.2			
	CPI 23158	8	12.5	0.77	12.0-14.3			
	CPI 10403	8	13.0	0.64	11.8-14.5			
	PC	8	14.1	0.50	13.5-15.0			
	PC 33109	8	13.4	0.52	12.8-14.5	13.2	1.13	10.7-15.0

Table 16. Average length in millimeters of florets from Kura clover plants grown under greenhouse conditions, April 1959 (3 florets per plant).

Ploidy level	Source	No. of plants	X	SD	Range	Ploidy X	Ploidy SD	Ploidy range
2x -	CPI 2464	9	12.0	0.05	11.9-12.1			
	CPI 2771	6	12.3	0.47	12.0-13.2	12.1	0.33	11.9-13.2
4x -	CPI 6484	1	11.7					
	CPI 9949	1	12.7					
	PI 229625	9	11.5	1.30	9.2-13.0			
	PI 229370	7	12.1	0.61	11.2-13.0			
	PI 229624b	5	11.6	0.91	10.7-13.0	11.8	0.99	9.2-13.0
6x -	CPI 6161	1	11.7					
	CPI 23408	3	15.1		14.0-16.0			
	CPI 13115							
	CPI 23159							
	CPI 10803							
	PC	1	15.3					
	FC 33109	35	13.9	4.76	11.3-15.8			

Pistil length

The following information was obtained from studies on lengths of pistil (Tables 17 and 18).

1. Average pistil length was approximately the same for a given source both in the field and greenhouse. This indicated little effect by environment. It should be noted, however, that floret length was apparently affected by environment.
2. As ploidy level increased, average pistil length also tended to increase.
3. The lower limits of the 6x averages of pistil length were transcended in many instances by the 4x averages. However, the lower limits of the 4x and 6x averages were exceeded in only one instance by a 2x plant. This may be one of the more effective criteria for helping to establish ploidy level, at least at the 2x level.

Pollen diameter

Average pollen size tended to increase as ploidy level increased (Tables 19 and 20). However, despite the small sample size the lower limits of the range in each ploidy level were approximately the same. Pollen diameter apparently would not be an effective means of indicating ploidy level except for those plants which were at the upper limits of the 6x range.

Some tendency for smaller pollen in the greenhouse can be noted (Table 20). However this may well have been a function of sample size.

Table 17. Average length in millimeters of pistils from florets of Kura clover plants grown in the field, May 1959 (3 florets per plant).

Ploidy level	Source	No. of plants	X	SD	Range	Ploidy X	Ploidy SD	Ploidy range
2x -	CPI 2264	8	4.0	0.14	3.7-4.1			
	CPI 2771	8	4.3	0.33	4.1-5.0	4.1	0.29	3.7-5.0
4x -	CPI 6084	8	5.1	0.21	4.7-5.4			
	CPI 9949	8	4.9	0.37	4.4-5.6			
	PI 229625	8	5.3	0.26	5.0-5.8			
	PI 223370	5	5.2	0.26	4.9-5.6			
	PI 229624b	7	5.4	0.29	5.2-6.1	5.2	0.33	4.4-6.1
6x -	CPI 6161	8	5.8	0.40	5.3-6.5			
	CPI 23408	8	5.7	0.33	5.3-6.3			
	CPI 18115	8	6.1	0.37	5.3-6.5			
	CPI 23158	8	5.5	0.46	4.9-6.3			
	CPI 10303	8	5.7	0.43	5.0-6.3			
	PC	8	5.9	0.26	5.3-6.1			
	PC 33109	8	6.3	0.46	5.7-7.0	5.8	0.45	4.9-7.0

Table 13. Average length in millimeters of pistils from florets of Kura clover plants grown under greenhouse conditions, April 1959 (3 florets per plant).

Ploidy level	Source	No. of plants	X	SD	Range	Ploidy X	Ploidy SD	Ploidy range
2x -	CPI 2264	9	4.0	0				
	CPI 2771	6	4.1	0.14	4.0-4.4	4.0	0.11	4.0-4.4
4x -	CPI 6384	1	4.5					
	CPI 9949	1	5.1					
	PI 229625	9	5.4	0.40	4.9-6.0			
	PI 228370	7	5.5	0.34	5.0-6.0			
	PI 229624b	5	5.2	0.30	5.0-5.7	5.3	0.35	4.9-6.0
6x -	CPI 6161	1	6.0					
	CPI 23408	3	5.8		5.5-6.1			
	CPI 18115							
	CPI 23158							
	CPI 10803							
	PC	1	6.6					
	PC 33109	35	6.0	0.32	5.2-6.5			

Table 19. Average diameter in microns of pollen grains from Kura clover plants grown in the field, May 1959 (10 measurements per plant).

Ploidy level	Source	No. of plants	X	SD	Range	Ploidy X	Ploidy SD	Ploidy range
2x -	CPI 2264	9	32.2	2.33	30.0-36.0			
	CPI 2771	8	30.7	1.49	28.8-33.1	31.4	2.04	28.8-36.0
4x -	CPI 6884	8	33.1	1.39	31.4-35.3			
	CPI 9949	7	32.2	2.83	28.8-37.4			
	PI 229625	4	32.9	1.22	31.7-34.3			
	PI 228370	4	32.2	0.74	31.4-33.1			
	PI 229624b	4	34.1	2.57	31.9-37.4	32.9	1.94	28.8-37.4
5x -	CPI 23408	4	30.7	0.74	29.8-31.4			
6x -	CPI 6161	8	34.6	0.46	33.8-35.3			
	CPI 23408	4	32.4	0.50	30.2-33.8			
	CPI 18115	8	33.4	3.53	30.5-36.7			
	CPI 23158	8	34.1	1.20	33.1-36.5			
	CPI 10803	8	36.0	2.23	33.6-39.7			
	PC	8	37.0	3.24	32.4-42.7			
	FC 33109	4	31.4	1.27	30.0-32.9	34.1	2.81	30.0-42.7

Table 20. Average diameter in microns of pollen grains from Kura clover plants grown under greenhouse conditions, May 1959 (10 measurements per plant).

Ploidy level	Source	No. of plants	X	SD	Range	Ploidy X	Ploidy SD	Ploidy range
2x -	CPI 2264	6	29.5	0.53	28.6-30.0			
	CPI 2771	7	30.0	1.32	27.6-31.7	29.8	1.03	27.6-31.7
4x -	CPI 6334	1	34.6					
	CPI 9949	1	29.5					
	PI 229625	6	32.4	1.15	31.2-34.6			
	PI 228370	9	32.4	3.19	29.0-37.4			
	PI 229624b	6	31.9	2.59	28.3-35.3	32.4	2.45	28.3-35.3
6x -	CPI 6161	1	34.8					
	CPI 23403	4	29.8	2.93	26.9-32.9			
	CPI 13115							
	CPI 23158							
	CPI 10803							
	PC	1	33.6					
	FC 33109	36	34.6	2.23	30.0-39.8			26.9-39.8

The CPI-23408 pentaploid plants have average pollen diameters within the range of the CPI-23403 hexaploid plants. Pollen diameter apparently is not radically different between these two ploidy levels.

Pollen stainability

As shown in Table 21, a tendency exists for increased pollen non-stainability as ploidy level increases. This is indicated by the upper limits of the ploidy ranges. However, pollen infertility (as measured by non-stainable pollen) does not necessarily become greater with an increase in ploidy level. For example, two of the hexaploid sources and one of the tetraploid sources had less non-stainable pollen on the average than either of the diploid sources. Examination of the aforementioned table shows extreme variability in the ranges of most sources. Pollen stainability is apparently more a function of the genetics of an individual plant than of source or ploidy level.

Pollen non-stainability of the CPI-23408-5x plant is well within the range of the CPI-23408-6x plants. Thus, pollen stainability is not an adequate method to distinguish 5x types in this source.

Fertility studies

The following results are shown in Tables 22-25 and Appendix B:

1. Seed set tends to decrease as the ploidy level increases (Table 22).
2. No self fertility was observed in hand selfed plants

Table 21. Average percent of non-stained pollen from florets of Kura clover plants grown in the greenhouse and field respectively, May 1959 (Average of 2 counts of 100 each per plant).

Ploidy level	Source	No. of plants	X	SD	Range	Ploidy X	Ploidy SD	Ploidy range
2x -	CPI 2264	11	6.9	4.02	2.5-13.5			
	CPI 2771	15	5.3	7.48	0-23.5	6.4	6.21	0-23.5
4x -	CPI 6884	9	1.2	0.83	0.5-3.0			
	CPI 9949	8	27.1	14.04	4.0-49.0			
	PI 229625	8	9.5	5.89	1.0-20.5			
	PI 228370	10	6.8	8.83	0.5-27.5			
	PI 229624b	9	17.2	14.49	4.0-47.0	11.2	13.34	0.5-49.0
5x -	CPI 23408	4	17.9	11.87	6.5-34.5			
6x -	CPI 6161	12	4.7	4.47	1.0-15.0			
	CPI 23408	8	25.4	19.67	10.0-68.5			
	CPI 15115	8	18.1	18.52	6.5-61.0			
	CPI 23158	8	22.6	16.64	3.0-51.0			
	CPI 10803	8	2.6	2.81	0-7.5			
	PC	8	13.9	11.13	3.5-36.0			
	PC 33109	30	8.2	7.04	1.0-35.0	13.3	13.34	0-68.5

Table 22. Average seed yield per floret from crosses of T. ambiguum within and between ploidy levels.

Type of Cross	No. of plants	No. of florets	No. of seeds	Seeds/ florets
2x selfed	15	1020	0	0
2x X 2x multiplant intersource				
CPI 2264	15	3279	3318	1.01
CPI 2771	11	2158	2339	1.08
Combined	26	5437	5657	1.04
2x X 2x multiplant intrasource				
CPI 2264	17	1418	635	0.45
CPI 2771	8	1061	762	0.72
2x X 4x reciprocal	14	3794	20	0.005
2x X 6x reciprocal	20	3049	55	0.02
4x selfed	10	617	0	0
4x X 4x multiplant intersource				
CPI 6884	1	173	134	0.77
PI 229625	2	566	568	1.00
PI 228370	6	1957	1715	0.88
PI 229624b	1	405	365	0.90
Combined	10	3101	2782	0.90
4x X 6x reciprocal	14	1620	458	0.28
5x X 6x reciprocal	2	Approx. 50	19	0.38
6x selfed	16	935	0	0
6x X 6x multiplant intersource				
PC 33109	7	314	150	0.48
CPI 23408	1	60	27	0.45

regardless of ploidy level (Table 22). In addition, no seeds were found in non-pollinated heads examined during other phases of this study. Lewis (22) has shown that induced autotetraploidy often resulted in the reestablishment of self compatibility at the 4x level. T. ambiguum, however, showed no self compatibility at the 2x, 4x, 5x, and 6x ploidy level.

3. Intraploidy crosses involving two plants either from the same or different sources, generally had one of three levels of fertility. These levers were: (Tables 23-24 and Appendix B)
   1. High fertility in both parents,
   2. High fertility in one parent but low fertility in the other,
   3. Low fertility in both parents.
4. 2x X 4x and 2x X 6x crosses, generally had very low fertility (Table 22 and Appendix B, Tables 41 and 42). However in both cases, one cross was noted in which one parent set a number of apparently viable seeds.
5. 4x X 6x crosses gave an appreciable seed set in the majority of crosses (Table 22 and Appendix B, Table 46). Most crosses indicated relatively high or low fertility in both parents, but one cross had very high fertility in one parent and none in the other.
6. One cross was made between a 5n and a 6n plant (Table 22). 19 apparently viable seeds were set on the 5n

Table 22--Continued

Type of cross	No. of plants	No. of florets	No. of seeds	Seeds/ florets
CPI 23158 226	1	89	31	0.35
PC 411	1	55	35	0.64
Combined	10	518	243	0.47
6x X 6x multiplant intrasource				
FC 33109	9	1558	1045	0.67

Table 23. Fertility data from 2x X 2x crosses of T. [illegible] (see Appendix B, Tables 36-40).

Female parent			Male parent		Method of pollination	No. of florets	No. of seeds	Seeds/ florets
Source	Plant no.		Source	Plant no.				
CPI 2264	783	X	multiplant intersource		B*	246	261	1.06
"	"	X	multiplant intersource		B	65	49	0.75
"	"	X	CPI 2264	773	H**	44	23	0.52
"	"	X	CPI 2264	764	H	67	0	0
CPI 2264	764	X	multiplant intersource		B	149	144	0.97
"	"	X	multiplant intrasource		B	101	71	0.70
"	"	X	CPI 2264	783	H	44	2	0.05
"	"	X	CPI 2264	773	H	35	38	1.09
"	"	X	CPI 2264	749	H	16	9	0.56
CPI 2264	764	X	multiplant intrasource		B	54	35	0.65
"	"	X	CPI 2264	763	H	68	0	0
"	"	X	CPI 2264	786	H	46	0	0
"	"	X	CPI 2264	773	H	38	35	0.92
CPI 2264	773	X	multiplant intrasource		B	165	35	0.21
"	"	X	CPI 2264	749	H	31	0	0
CPI 2264	773	X	CPI 2264	773	H	35	49	1.40
CPI 2264	749	X	multiplant intersource		B	360	402	1.10
"	"	X	multiplant intrasource			143	46	0.32
"	"	X	CPI 2264	786	H	46	29	0.63
"	"	X	CPI 2264	761	H	49	22	0.45

Table 23--Continued

Female parent			Male parent		Method of pollination	No. of florets	No. of seeds	Seeds/ florets
Source	Plant no.		Source	Plant no.				
CPI 2264	749	X	CPI 2264	773	H	26	0	0
"	"	X	CPI 2264	781	H	18	0	0
CPI 2264	786	X	multiplant intrasource		B	50	21	0.42
"	"	X	CPI 2264	764	H	50	0	0
"	"	X	CPI 2264	749	H	43	0	0
CPI 2264	747	X	multiplant intrasource		B	196	2	0.01
"	"	X	CPI 2264	93	H	25	0	0
"	"	X	CPI 2264	760	H	22	0	0
CPI 2264	761	X	multiplant intrasource		B	60	47	0.78
"	"	X	CPI 2264	749	H	47	0	0
CPI 2264	760	X	multiplant intersource		B	58	22	0.38
"	"	X	CPI 2264	747	H	23	0	0
CPI 2771	813	X	multiplant intersource		B	193	193	1.00
"	"	X	multiplant intrasource		B	108	65	0.60
CPI 2771	807	X	multiplant intersource		B	234	273	1.17
"	"	X	CPI 2771	817	H	82	0	0
CPI 2771	799	X	multiplant intersource		B	134	141	1.05
"	"	X	multiplant intrasource		B	82	49	0.60
"	2	X	CPI 2771	794	H	24	0	0
CPI 2771	811	X	multiplant intersource		B	176	158	0.90

Table 23--Continued

Female parent			Male parent		Method of pollination	No. of florets	No. of seeds	Seeds/ florets
Source	Plant no.		Source	Plant no.				
CPI 2771	811	X	multiplant intrasource		B	122	92	0.75
CPI 2771	810	X	multiplant intrasource		B	43	31	0.72
CPI 2771	813	X	CPI 2771	798	H	48	23	0.74

B* - pollination by honey bees

H** - pollination by hand

Table 24. Fertility data from 4x X 4x crosses of T. ambiguum (see Appendix B, Table 45).

Female parent			Male parent		Method of pollination	No. of florets	No. of seeds	Seeds/ florets
Source	Plant no.		Source	Plant no.				
CPI 228370	662	X	CPI 226370	630	H*	18	4	0.22
"	"	X	"	599	H	49	20	0.41
"	"	X	"	628	H	35	38	1.09
CPI 228370	628	X	"	662	H	40	25	0.63
"	"	X	"	625	H	22	15	0.68

H* - pollination by hand

Table 25. Fertility data from 6x X 6x crosses of T. ambiguum (see Appendix B, Tables 47-50).

Female parent			Male parent		Method of pollination	No. of florets	No. of seeds	Seeds/ florets
Source	Plant no.		Source	Plant no.				
CPI 6161	7	X	PC	411	H*	28	27	0.96
"	7	X	CPI 23408	335	H	22	7	0.32
"	7	X	FC 33109	851	H	32	26	0.81
FC 33109	897	X	multiplant intersource		B**	51	31	0.61
"	"	X	FC 33109	962	H	59	15	0.25
FC 33109	851	X	CPI 6161	7	H	27	23	0.85
"	"	X	FC 33109	993	H	36	21	0.58
PC	411	X	multiplant intrasource		B	55	35	0.64
"	"	X	CPI 6161	7	H	29	12	0.41
FC 33109	860	X	FC 33109	982	H	32	11	0.34
"	"	X	FC 33109	869	B	179	47	0.26

H* - pollination by hand
B** - pollination by bees

plant. None were set on the 6n plant. Approximately 50 florets were involved in each plant but the exact number was lost.

7. The 2x multiplant intersource cross produced a higher average seed set per floret than either of the 2x multiplant intrasource crosses (Table 22 and Appendix B, Table 36, 37, and 38). This was true not only for the crosses as a whole but also for those individual plants represented in both the intersource and intrasource crosses.
8. The 6x multiplant intersource cross produced a lower average seed set per floret than the intrasource cross (Table 22 and Appendix B, Table 47 and 48).

Seed size

The following results were noted:

1. Seed weight increased significantly as the ploidy level became higher (Table 26).
2. Screens which removed the most tetraploid seed also removed some diploid and hexaploid seed (see Table 27). For example, although 61% of the 4x seed was retained on the 1/16-1/17 screens, these screens also retained 13% of the 2x and 10% of the 6x seed. Therefore, separation of mixtures of 2x, 4x, and 6x seeds on this basis would be impractical.
3. A 1/14 hand screen removed 40% of the 6x seed but none of

Table 26. Analysis of variance for average wt. in grams per 100 seeds of 2x, 4x, and 6x ploidy levels of Kura clover respectively.

Source	df	SS	M S	F
Total	14	.0630		
Reps.	4	.0001	.00003	0.5
Ploidy	2	.0624	.03120	520.0**
Error	8	.0005	.00006	

Average wt. in grams per 100 seeds:

2x - .1510

4x - .1701

6x - .2906

Standard deviation of ploidy average, .0035 gms.

Duncan's multiple range test indicates 2x 4x, 4x 6x, and 2x 6x at 1% level.

Table 27. Percent of Kura clover seed passing through hand screens.

Screens	2X	4X	6X
1/12	100	100	99.7
1/13	100	100	95.0
1/14	100	100	60.3
1/15	100	99.1	26.0
1/16	98.5	74.3	8.3
1/17	87.1	38.9	1.3
1/18	56.9	9.8	0.3
1/19	43.8	4.1	0.05
1/20	18.4	0.9	0.02
1/21	0.1	0	0

the 2x and 4x seed. A 1/15 screen removed 74% of the 6x seed, 1% of the 4x, and none of the 2x seed. Therefore, hexaploid seed might be effectively removed from mixtures of 2x, 4x, and 6x seeds by screening. The size of seeds from interploidy crosses, however, would depend on the ploidy level of the female parent. Thus, a seed developed on the 6x parent of a 4x X 6x cross would approximate 6x seed size. Since these studies were carried out on greenhouse materials extrapolations to field harvested seed may be unwarranted.

Vigor rating

The following results were noted: (Tables 28 and 29)

1. The range in vigor ratings is about the same over all ploidy level but there is an increase in average vigor as ploidy level increases.
2. CPI 10803, a hexaploid source, contained a large proportion of plants receiving a high vigor rating.
3. The diploid source, CPI-2771, was rated second in vigor while the other diploid, CPI-2264, had the lowest rating. This indicated that vigor is probably more strongly correlated with source than with ploidy level.

Rhizome development

Average rhizome production increased at the higher ploidy levels (see Table 30). This tendency was not found in all sources. For example, one hexaploid and two tetraploid sources produced fewer rhizomes

Table 28. Average of 0-10 ratings for vigor of Kura clover plants grown in the field, May 1959 (0 = dead, 10 = most vigorous).

Ploidy level	Source	Ave. rating	No. of Plants	Range	Ploidy x̄	Ploidy range
2x -	CPI 2264	4.3	30	1-7		
	CPI 2771	7.6	9	1-10	5.0	1-10
4x -	CPI 6684	4.8	27	1-9		
	CPI 9949	6.7	15	3-9		
	PI 229625	5.8	16	1-8		
	PI 229370	5.1	7	1-7		
	PI 229624b	5.6	16	1-8	5.5	1-9
6x -	CPI 6151	6.9	13	4-10		
	CPI 23408	7.1	78	1-10		
	CPI 18115	6.9	32	2-9		
	CPI 23158	6.0	32	1-9		
	CPI 10803	8.5	12	7-10		
	PC	7.1	76	1-10		
	PC 33109	6.4	55	1-10	6.9	1-10

Table 29. Average of 1-10 ratings for vigor of Kura clover plants grown in the field, May 1959 (1 = least vigorous, 10 = most vigorous).

Ploidy level	Source	Ave. rating	No. of Plants	Range	Ploidy X	Ploidy range
2x -	CPI 2264	3.0	47	0-7		
	CPI 2771	7.6	9	1-10	3.8	0-10
4x -	CPI 6984	3.1	44	0-9		
	CPI 9949	5.6	13	0-9		
	PI 229625	5.4	17	0-8		
	PI 226370	4.1	10	0-7		
	PI 229624b	4.7	18	0-8	4.2	0-9
6x -	CPI 6161	5.6	16	0-10		
	CPI 23403	6.9	81	0-10		
	CPI 18115	5.5	39	0-9		
	CPI 23158	4.9	39	0-9		
	CPI 10803	8.5	12	7-10		
	PC	6.4	87	0-10		
	PC 33109	5.8	62	0-10	6.2	0-10

Table 30. Average number of rhizomes produced by Kura clover plants grown in the field, May 1959.

Ploidy level	Source	X	No. of plants	Range	Ploidy X	Ploidy range
2x -	CPI 2264	0.36	30	0-4		
	CPI 2771	1.44	9	0-5	0.6	0-5
4x -	CPI 6284	1.52	27	0-10		
	CPI 9949	1.85	15	0-8		
	PI 229625	0.63	16	0-3		
	PI 228370	0.29	7	0-2		
	PI 229624b	1.81	16	0-11	1.5	0-11
6x -	CPI 6161	3.46	13	0-10		
	CPI 23408	3.01	78	0-15		
	CPI 13115	2.34	32	0-12		
	CPI 23158	2.97	32	0-25		
	CPI 10803	7.91	12	0-24		
	PC	2.34	78	0-12		
	PC 33109	1.39	55	0-15	2.7	0-25

on the average than did one of the diploid sources. As the ploidy level increased, there was a tendency for a higher level of rhizome production by some plants; yet some plants of each ploidy level produced no rhizomes. Rhizome production is apparently a function of the genetics of a given plant under a certain environment as well as a function of source and ploidy level.

It was noted that rhizome development was not necessarily correlated with the overall vigor of the plant. For example, some plants which produced several rhizomes had sparse vegetation while others with several rhizomes had dense vegetation.

Growth habit

Despite the small sample size in some instances, each source had a wide range of ratings with the exception of two of the tetraploids, PI 228370 and CPI 9949 (see Table 31). This indicates that growth habit is apparently more a function of the genetics of an individual plant rather than a function of source or ploidy level. However, definite differences do occur between source averages. This preponderance of one or another type might indicate better survival of that type under the environment in which the source arose.

Winter hardiness

All plants transplanted from the greenhouse to the field nursery prior to August survived the unusually severe winter of 1958-59. Of the later transplants, 88% survived (Table 32). All but the three Iranian tetraploid sources were represented in both transplantings; the Iranian materials were only among the second transplants.

Table 31. Average of 1-5 rating for degree of erectness of Kura clover plants grown in the field, May 1959 (1 = prostrate, 5 = erect).

Ploidy level	Source	X	No. of plants	Range	Ploidy X	Ploidy range
2x -	CPI 2264	3.22	27	1-5		
	CPI 2771	2.89	8	1-4	3.14	1-5
4x -	CPI 6384	4.50	24	1-5		
	CPI 9949	4.08	12	3-5		
	PI 229625	3.14	14	1-5		
	PI 228370	3.71	7	3-4		
	PI 229624b	2.69	13	1-5	3.74	1-5
6x -	CPI 6161	1.80	10	1-4		
	CPI 23408	2.85	77	1-5		
	CPI 18115	3.93	29	1-5		
	CPI 23158	3.57	12	105		
	CPI 10803	3.25	23	1-5		
	PC	3.19	51	1-5		
	PC 33109	2.49	67	1-5	3.05	1-5

Table 32. Survival of plants of Kura clover during the winter of 1958-59.

Ploidy level	Source	No. of plants Oct.1958	No. of plants May 1959	% Winter survival
2x -	CPI 2264	39	28	72
	CPI 2771	9	9	100
4x -	CPI 6834	21	17	81
	CPI 9949	10	8	80
	PI 229625	13	15	83
	PI 223370	10	7	70
	PI 229624b	20	16	80
6x -	CPI 6161	9	8	89
	CPI 23408	55	53	96
	CPI 18115	31	28	90
	CPI 23158	13	8	62
	CPI 10803	2	2	100
	PC	79	78	99
	PC 33109	38	35	82
Total		396	354	88%

Under the conditions at East Lansing, there was no apparent correlation between ploidy level and winter hardiness. For example, the three sources suffering the most winter loss included a diploid, tetraploid, and hexaploid source. The results did indicate, however, that some sources had less winter hardiness than others. This would be expected if the sources had arisen under varying environments or had been propagated for several years under less extreme conditions than in their place of origin.

Source ratings

As a group the hexaploid sources are rated highest in both the field and the greenhouse with the exception of CPI 23408 which is rated lower in the greenhouse (see Tables 33, 34 and 35). Ratings in the field and greenhouse both rank the Iranian tetraploids and the diploid, CPI 2264, low. The direct use of these last-mentioned sources is questionable. The field ratings were the most useful from a practical viewpoint but the general agreement between ratings indicates that greenhouse data are important (Figures 11-13).

Diseases and insects

In the greenhouse two abnormal situations involving flowering were noted. In one, certain florets developed a condition in which the anthers turned black. Usually only one or two florets per head were infected but occasionally a few more showed this condition. The pistil was apparently unaffected. Overall seed set would not be lowered appreciably unless the condition became more intense.

Table 33. Rankings for various characteristics of sources of Kura clover grown under field conditions.

Ploidy level	Source	Ave. no. heads per plant	Ave. floret length	Ave. pistil length	Ave. pollen size	Ave. % pollen stainability
2x -	CPI 2264	8	1	1	2.5	9
	CPI 2771	4	2	2	1	11
4x -	CPI 6834	11	5	4	8	14
	CPI 9949	10	4	3	4	1
	PI 229625	3	7.5	6.5	6.5	7
	PI 228370	1	3	6.5	5	10
	PI 229624b	2	6	5	6.5	5
6x	CPI 6161	9	11	10	12	12
	CPI 23408	12	13	10	2.5	2
	CPI 18115	7	10	13.5	9	4
	CPI 23158	5	7.5	8	10.5	3
	CPI 10803	14	9	10	13	13
	PC	6	14	12	14	6
	PC 33109	13	12	13.5	10.5	8

Table 33--Continued

Ave. length x breadth	Ave. vigor rating 0-10	Ave. vigor rating 1-10	Ave. no. of rhizomes	Ave. erectness rating	Total
3	1	1	2	8	36.5
5	13	13	4	5	60.0
7	2	2	6	14	75.0
6	8.5	8	8	13	65.5
4	6	5	3	6	54.5
1	3	3	1	11	44.5
2	4	4	7	3	44.5
8	8.5	9.5	13	1	94.0
13	12	11.5	12	4	92.0
9	7	9.5	9.5	12	79.5
10	5	6	11	10	76.0
11	14	14	14	9	121.
12	11	11.5	9.5	7	103.
14	10	7	5	2	95.0

Table 34. Rankings for various characteristics of sources of Kura clover grown under greenhouse conditions.

Ploidy level	Source	Ave. no. heads per plant	Ave. no. florets for head	Ave. pistil length	Ave. pollen size	Ave. % pollen stainability	Ave. length x breadth	Total
2x -	CPI 2264	14	6.5	1	2.5	9	2	35.0
	CPI 2771	13	10	2	1	11	6	43.0
4x -	CPI 6384	1	8	4	8	14	7	42.0
	CPI 9949	2	14	3	4	1	9	33.0
	PI 229625	4	4.5	6.5	6.5	7	1	29.5
	PI 228370	7	2	6.5	5	10	3	33.5
	PI 229624b	12	4.5	5.0	6.5	5	4	37.0
6x -	CPI 6161	8.5	13	10.0	12	12	5	60.5
	CPI 23408	3	12	10.0	2.5	2	12	41.5
	CPI 18115	10	1	13.5	9	4	10	47.5
	CPI 23158	8.5	3	8.0	10.5	3	13	46.0
	CPI 10803	5	11	10	13	13	8	60.0
	PC	6	9	12	14	6	11	58.0
	PC 33109	11	6.5	13.5	10.5	8	14	63.5

Table 35. Overall ranking of sources of kura clover based on totals from Table 33 and Table 34.

Field			Greenhouse		
Source	Ploidy level	Total points	Source	Ploidy level	Total points
CPI 10803	6x	121	PC 33109	6x	63.5
PC	6x	103	CPI 6161	6x	60.5
PC 33109	6x	95	CPI 10803	6x	60.0
CPI 6161	6x	94	PC	6x	58.0
CPI 23403	6x	92	CPI 18115	6x	47.5
CPI 18115	6x	79.5	CPI 23158	6x	46.0
CPI 23158	6x	76.0	CPI 2771	2x	43.0
CPI 6884	4x	73.0	CPI 6884	4x	42.0
CPI 9949	4x	65.5	CPI 23403	6x	41.5
CPI 2771	2x	60.0	PI 229624b	4x	37.0
PI 229625	4x	54.5	CPI 2264	2x	35.0
PI 228370	4x	44.5	PI 228370	4x	33.5
PI 229624b	4x	44.5	CPI 9949	4x	33.0
CPI 2264	2x	36.5	PI 229625	4x	29.5

Fig. 11. 2x plants of Kura clover during winter of '58-'59. 4x plants can be seen on extreme right. Bee cage used in this study is in background.

Fig. 12. 2x plant of Kura clover on right. 6x plants on left. Winter '58-'59.

Fig. 13. 6x plant of T. ambiguum. Winter '58-'59

Fig. 14. Vigorous 2n plant of Kura clover grown in the field. June, 1959

Fig. 15. Vigorous 4n plant of Kura clover grown in the field. June, 1959

Fig. 16. Vigorous 6n plant of Kura clover grown in the field. June, 1959

Fig. 17. Same 6n plant as in Fig. 16.

Fig. 18. Leaves from 2n, 4n, and 6n plants of Kura clover grown in the field. June, 1959

The other abnormal condition resulted in the failure of an entire head or part of a head to develop and mature normally. The number of heads affected as well as the extent of the abnormality on an individual head appeared to vary from plant to plant. In its most severe manifestation the number of florets lost to this malady would lower potential seed set appreciably. This condition was not apparent under field environment.

Under field and greenhouse conditions, most plants appeared to be free of virus infection. However, two plants in the field apparently succumbed to virus while a few appeared to be infected to varying degrees.

In the field, there was serious infestation of an insect which destroyed the upper part of the pistil. Although some plants appeared to be relatively unaffected, it was difficult in others to find an undamaged floret. If this same condition persisted proportionately under normal field operations, this factor would probably be extremely important in total seed set.

DISCUSSION

Ploidy levels

Although cytological examination revealed only one ploidy level in most sources, two predominantly hexaploid sources contained a few tetraploids and pentaploids, respectively. In one source, CPI 6161, 4x plants were found while in the other, CPI 23408 5x plants were revealed. In CPI 6161, a 4x X 6x cross might be expected, but no 5x plants were found in this source. In CPI 23408, the 5x plants presumably arose from 4x X 6x crosses but no 4x types were noted in this source. In addition, aneuploids would be expected in CPI 23408 because of 5x X 6x crosses; no definite aneuploids were found.

As previously suggested, irregular types might have existed in larger numbers and in other sources and might have been discarded because of low vigor or slower germination. Another possibility is that these plants resulted from seed mixtures between sources of different ploidy levels. But from the information at hand, the most plausible explanation would seem to be that these irregular types arouse from intersource crossings between different ploidy levels. Thus the 4x types of CPI 6161 might arise from a cross between a 2x source and hexaploid CPI 6161 plants. The 5x type might have resulted from a cross between a 4x source and hexaploid CPI 23408 plants. The

fact that aneuploids were not found suggests that these irregular types were F1's. Even at relatively low frequencies, interploidy mixtures would have a marked depressing effect on fertility.

Because of its strong asexual capacity, Kura clover is not subject to selection solely on the basis of a strong sexual mechanism. This permits experimentations with ploidy in extending the area of adaptation of the species. However, no polyploids greater than 6x have been found in this study. In addition none of the literature reviewed mentions higher ploidy levels in T. ambiguum. In other crops there is often maximum plant response within a certain ploidy range. The amount of response decreases according to how far a given ploidy level is above or below this optimum range. On this basis, even if the hexaploids of Kura clover represent an optimum ploidy level, at least an octaploid might be expected, but as noted above, none have yet been reported. The species may be presently undergoing critical evolutionary stages from which a more stable polyploid level may emerge. At these higher levels plants might show even greater vigor than the hexaploids.

For several characteristics examined in this study, ranges increased directly with ploidy level. This increased variability in polyploids might be an effect of the high degree of recombination possible in polyploids over a long period of time.

Leaf length/breadth ratios

In two hexaploid sources, CPI 10803 and PC, there were high correlation coefficients for length/breadth ratios in the field and

the greenhouse. In addition, each had similar averages and standard deviations in both places. Average ratios and standard deviations of the other hexaploid sources varied between field and greenhouse. In the field, plants of CPI 10803 and PC showed higher variability than all but one of the other hexaploid sources. In the greenhouse, however, the variability of these two hexaploid sources was about average for all hexaploids. The standard deviations of the length-breadth product indicated a similar pattern of variability.

If subjected to stress in a given environment over long periods surviving genotypes may produce a similar phenotype. When taken from this critical environment, genes which had been previously masked might then express themselves and new phenotypes would arise (3) (4). The changes expressed in the new environment would vary between plants and would depend on such factors as the effect of environmental change on the expression of genotype, the number of genes controlling a given trait, the number of chromosomes on which these genes are located and the amount of recombination between these genes.

Organisms which have not been exposed to critical environments may lack the genetic plasticity necessary to produce different phenotypes under comparatively minor environmental changes. On the other hand, such plasticity may be present to a high degree in organisms from areas of environmental stress. According to this hypothesis CPI 10803 and PC might have been grown in a favorable environment for hexaploid T. amoiguum while the other hexaploid sources might have come from more critical areas. The difference in magnitude of standard

deviations obtained from the same plants of a given source grown in different environments are, at best, indicative of phenotypic plasticity. The plasticity may be more or less directly related to environmental stresses of the area of adaptation. According to this hypothesis, source CPI 23158 would have arisen under the most critical environment.

Leaf length x breadth product

Leaf size appears to be a function of source as well as ploidy level. Apparently the environment under which a particular clone arose strongly influences size of leaf. Natural selection under some environments has probably been instrumental in removal of certain of the large leaf types expected in higher ploidy levels. For example, plants with large leaf areas and, consequently, large transpiration surfaces would presumably be selected against under drought conditions.

Pollen non-stainability

In discussing non-stainable pollen it is assumed that the percent of non-stainable pollen is directly related to the percent non-fertile pollen. If the above hypothesis is at least reasonably accurate, the low percent non-stainability found in many plants at the 2x, 4x, and 6x ploidy levels presumably indicates regular meiosis in these plants. A tendency toward more regular meiosis has been reported in induced polyploids after a few generations (23). This apparent tendency toward natural selection of fertile types is probably complicated in T. ambiguum by its strongly rhizomatous habit which would favor vigorous

clones regardless of any meiotic difficulties.

CPI 10803, which hypothetically may have come from a non-critical environment, had a very low percentage of non-stainable pollen. However, CPI 23158, which hypothetically was adapted to a critical environment, had a high percentage of non-stainable pollen. This might be expected if natural selection in fringe areas of adaptation was such that these perennial plants were selected more on their ability to survive as individual plants rather than their ability to function as a strongly sexual organism. In a non-critical environment, on the other hand, the ability to produce large quantities of seed might be advantageous. The more recombination types produced by an individual the greater are the opportunities for types more adapted than their parents. The heavy flowering and strongly rhizomatous habit of CPI 10803 lends support to this hypothesis. Conversely, CPI 23158 had fewer flowers on the average than the other hexaploid sources, but average rhizome production in this source was about the same as that of the other hexaploid sources with the exception of CPI 10803. However, all plants in the field were included in the study on number of rhizomes. Thus while CPI 10803 was represented primarily in the vigorous first transplants, the other sources were more or less represented in both the earlier and the less vigorous later transplants. In addition, the rapidity and extensiveness of rhizome development might vary according to environment. Therefore in its area of adaptation a plant might react entirely different than in the environments in which this study was carried out.

Fertility in general

Conclusions drawn from the results of fertility studies are based on the assumption that pollinations were equally effective in all crosses. If this assumption is generally valid, definite incompatibility mechanisms must exist in T. ambiguum. Three levels of fertility were noted in the various crosses:

1. High fertility in both parents,
2. High fertility in one parent, but low fertility in the other,
3. Low fertility in both parents.

The Nicotiana type of incompatibility mechanism might help explain the above phenomena. This type of incompatibility has been reported in many species of the Trifolium and does produce the effects noted above. Other incompatibility mechanisms would also be applicable to the above phenomena. The complexity of the mechanism would most probably increase with an increase in ploidy level.

The matings in this study have shown that fertility exists within and between all ploidy levels. Hagberg (6) noted a marked reduction in seed set when 1-2% diploid clover was grown with tetraploid clover. 4% diploid clover halved the seed yield of the tetraploid. If such a similar reaction occurred in interploidy mixtures in T. ambiguum, it would be especially important to maintain isolation of the ploidy groups. If the 2x, 4x, and 6x ploidy levels are not isolated from one another, it is possible that 2x, 3x, 4x, 5x, and 6x seeds would be harvested in the same lot. The 3x and 5x would not breed true to ploidy level. The 4x plants produced from a 2x X 6x

mating would also fail to breed true. Fertility would be quite low in any one of the above three cases. Subsequent generations would have a higher degree of aneuploidy due to such matings as 3x X 4x, 5x X 6x, etc. This again would decrease overall fertility.

In summary, three problems appear to affect fertility in T. ambiguum.

1. Different ploidy levels,
2. Chromosome aberrations, especially in the higher ploidy level. This includes chromosome aberrations arising from fragmentation of individual chromosomes as well as differences in whole chromosome numbers. The whole chromosome type of aberration for example, would result from crosses involving aneuploids.
3. Incompatibility systems.

The latter two are probably reasons for the lower seed set as ploidy level increases.

Fertility at the 6x level

The 6x multiplant intersource cross produced a lower average seed set per floret than the intrasource cross. This was contrary to the results from the 2x multiplant crosses. Some of this deviation in trend might be explained by the following:

1) Bee activity might have been less during the 6x intersource cross;
2) The reciprocal crosses involving PI 93109 indicate that incompatibility mechanisms do exist between some of the plants from this source. It is possible that the plants

of PC 33109 in the intrasource cross were more mutually compatible than the plants of this source in the intersource cross. This may account for the low seed set on some of the PC 33109's in the latter cross. Since different plants were in each cross, it was not possible to correlate seed set and type of cross in the same plant;

3) Seven plants of PC 33109 were in the intersource cross but only one plant from each of three other sources was involved. One of these, CPI 23408-321, was later found to have approximately 70% non-fertile pollen (based on pollen stainability). Therefore, even if these plants were mutually compatible the opportunity for the introduction of compatible types from other sources is relatively low. However, reciprocal intersource crosses at the 6x level have indicated that incompatability mechanisms exist between sources as well as within sources. This could have further complicated the 6x intersource cross.

Seed set, in the aforementioned plant with 70% non-stained pollen grains, however, was about average for that particular multiplant cross. Despite the high percentage of apparently infertile pollen, a large proportion of eggs must have been fertile. In most plants which have normal meiosis, three of the four nuclei formed from the megaspore mother cell degenerate. The remaining megaspore, usually the one furthest from the micropylar end, then undergoes

division to form the female gamete. However, if meiotic difficulties occur, the most normal megaspore, regardless of position, survives. Of course it is possible that no normal female gamete would be formed. Since T. ambiguum usually has two ovules per pistil, one chance in eight exists that a normal or near normal egg will be developed in each floret.

Comparison of polyploidy in T. ambiguum and induced polyploidy in general

In this study there was general agreement between the effects of induced polyploidy and the effects of polyploidy in T. ambiguum. However, some discrepancies did exist. While induced polyploids are reported to have fewer flowers and rhizomes, rhizome and flower production in the field increased directly with ploidy level in T. ambiguum. In addition, there was no decrease in the number of florets per head in Kura clover as ploidy level increased although decreases are reported in induced polyploids. These changes are probably a result of recombination and natural selection over an extended period of time.

SUMMARY AND CONCLUSIONS

Trifolium amoenum M. B. (x=8) commonly exists in diploid, tetraploid, and hexaploid forms. Plants from fourteen sources were examined for ploidy level. Most sources were apparently pure for a given ploidy level, but two predominantly hexaploid sources had, respectively, a few tetraploid and pentaploid plants. Since no aneuploids were found in these sources, it was hypothesized that these variants were FI's from interploidy matings involving different sources.

Several morphological and physiological characteristics of plants in the greenhouse and field were examined in an attempt to determine possible relationships between ploidy levels and these characteristics. Generally, the following changes were noted in field plants as ploidy level increased: larger leaf length/breadth ratios (no change was noted between 4x and 6x averages), increased but later flowering, longer florets and pistils, larger pollen, larger and heavier seeds, increased rhizome production, and greater vigor. Although non-stainability of pollen tended to increase with ploidy level, some polyploid sources had low percentages of non-stainable pollen. Winter losses were small and apparently not related to ploidy level. Disease and insect problems generally appeared to be of little consequence except that many florets in the field were noted which had the upper portion of the pistils destroyed or severely

damaged by insects. If this situation prevailed in commercial production, a high seed loss might occur.

In the greenhouse, floret length, number of heads per plant, and number and size of stomata did not reflect the above trends at all ploidy levels. Overall vigor was also less in the greenhouse.

High intrasource correlation coefficients for leaf length/breadth ratios of plants grown in the field and greenhouse were found for only two sources. These two sources also had similar intrasource averages and standard deviations in the field and greenhouse. An hypothesis for the above phenomena was formulated and discussed.

Cytological examination was the only efficient criterion found for identification of ploidy level of any given plant. However, under the environmental conditions prevalent in this study, plants which exceeded one or more of the following limits could tentatively be regarded as hexaploids: length x width product greater than 16000 sq. mm., floret length greater than 14 mm., pistil length in excess of 6.0 mm., and pollen diameter greater than 38 microns. Plants having a pistil length of less than 4.5 mm. would probably be diploids. This included the bulk of the diploids examined in this study and appeared to be an important criterion. Hexaploid seed can apparently be efficiently removed from mixtures with 2x and/or 4x seeds by selection of proper screen size.

Crosses were made within and between ploidy levels. Intraploidy fertility appeared to decrease as the ploidy level increased. Some effective crosses were obtained from all interploidy crosses; however, the 4x X 6x crosses produced seed rather consistently. No

selfcompatibility was noted. In addition, self-incompatibility mechanisms also appeared to exist within and between sources of all ploidy levels. Fertility data emphasized the importance of maintaining effective isolation of ploidy levels if regular types are to be maintained.

It is suggested that Kura clover's strong asexual capacity enables the species to experiment with ploidy for maximum adaptation to a given environment. Because of coexistence of 2n, 4x, and 6x types as well as the apparent lack of higher ploidy levels, it is further hypothesized that the species may presently be undergoing important evolutionary changes.

In Michigan, Kura clover has a high potential as a more or less permanent forage crop on soils with drainage problems, but in any given area only one ploidy level should be grown. Under the environment at East Lansing the hexaploid sources showed the most promise. However, certain plants at the diploid and tetraploid levels had good vigor. At all ploidy levels, ruthless selection would be necessary before a breeding program was initiated. Seed yield would be related not only to ploidy level but also to the diversity of germ plasm within a ploidy level. Thus, a variety involving materials from several sources of the same ploidy level would probably outyield a variety involving only one or two sources because of the concentration of incompatible types in the latter. This is an important consideration at the diploid and tetraploid level since only a few plants were found which appeared to be desirable agronomic types.

The problem of sexual reproduction in kura clover could be bypassed by utilization of asexual propagation. Desirable clones could be propagated indefinitely without the inherent variability in progeny resulting from sexual propagation. The highly vigorous, extensively rhizomatous hexaploid types would be especially desirable in such a program. Asexual propagation has been successfully utilized in the propagation of Bermuda grass (Cynodon dactylon).

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APPENDIX A

LEAF SHAPES OF GREENHOUSE PLANTS OF KURA CLOVER

Figures 19 and 20 - 2x plants

Figures 21 to 25 inclusive and number 15 of Fig. 26 - 4x plants

Figures 26 to 32 inclusive - 6x plants

Figures 33 to 36 inclusive - abnormal leaf types

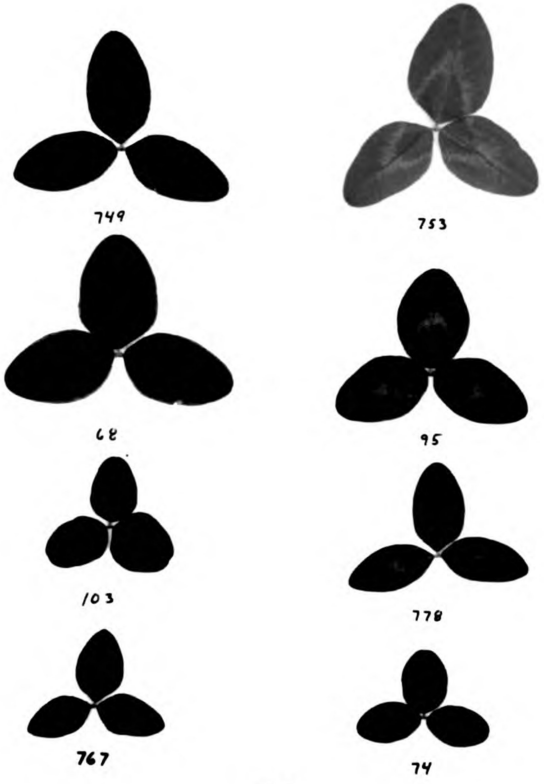

749
753
89
95
103
778
767
74
CPI
2264

Fig. 19

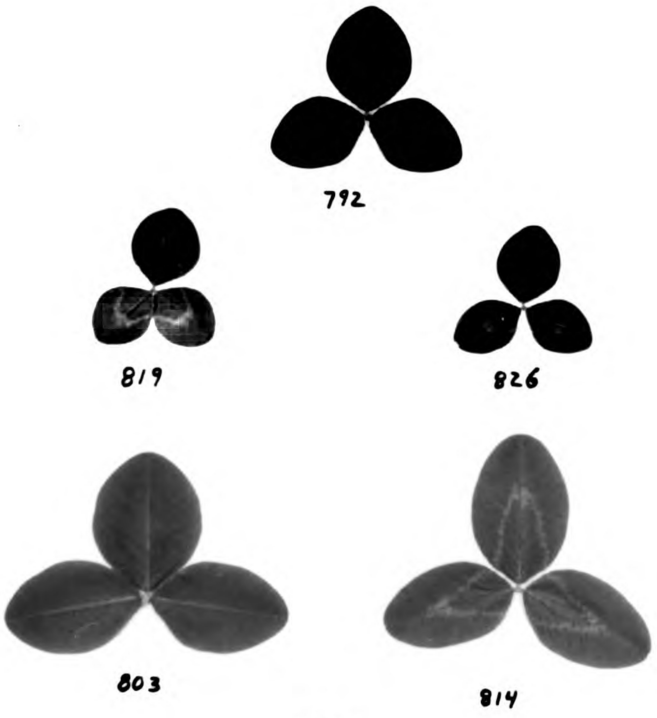
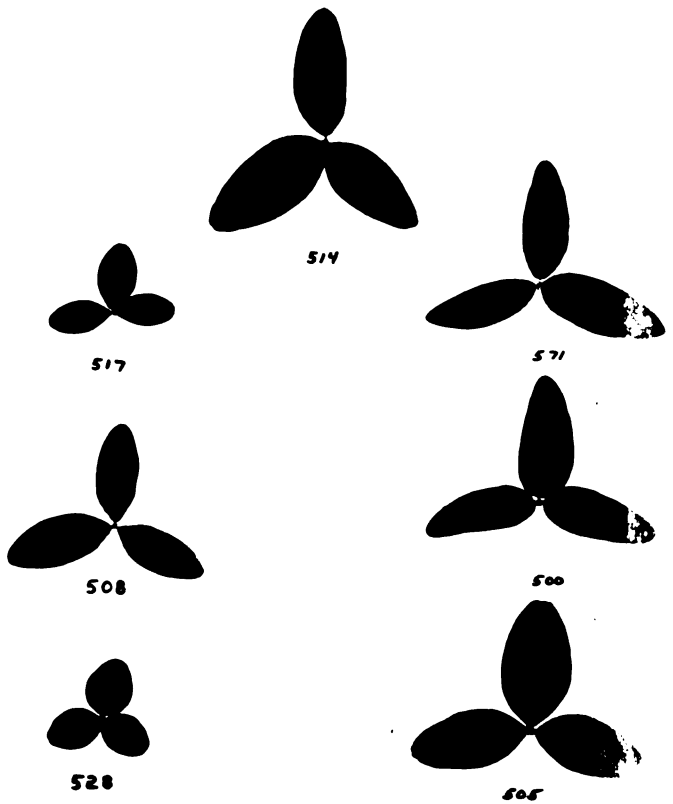
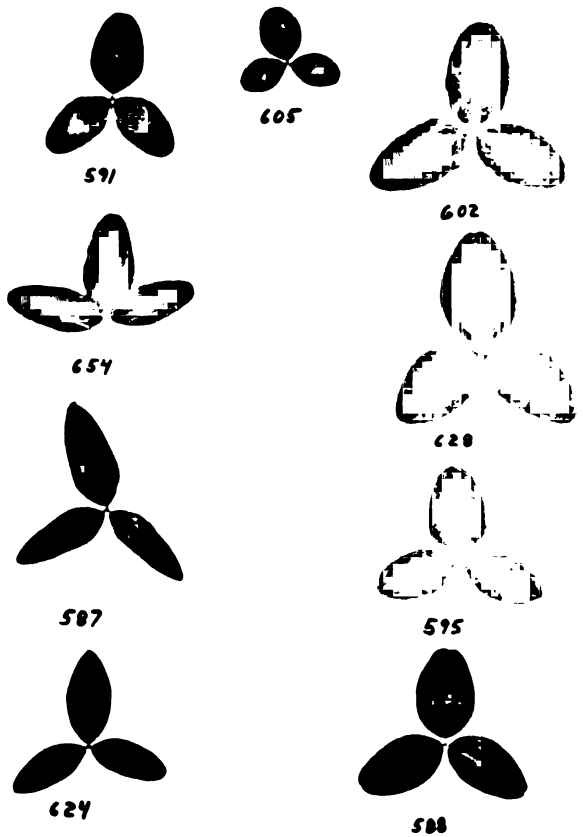

792
819
826
803
814
CPI
2771

Fig. 20



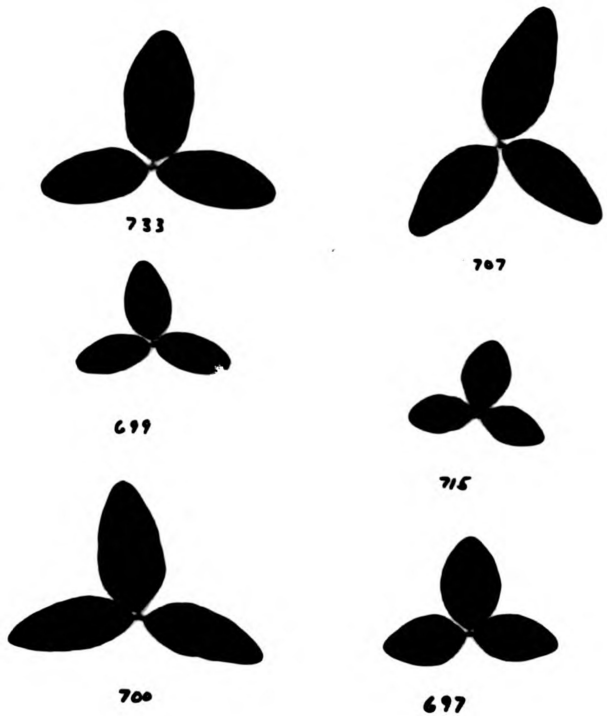
229625

Fig. 23



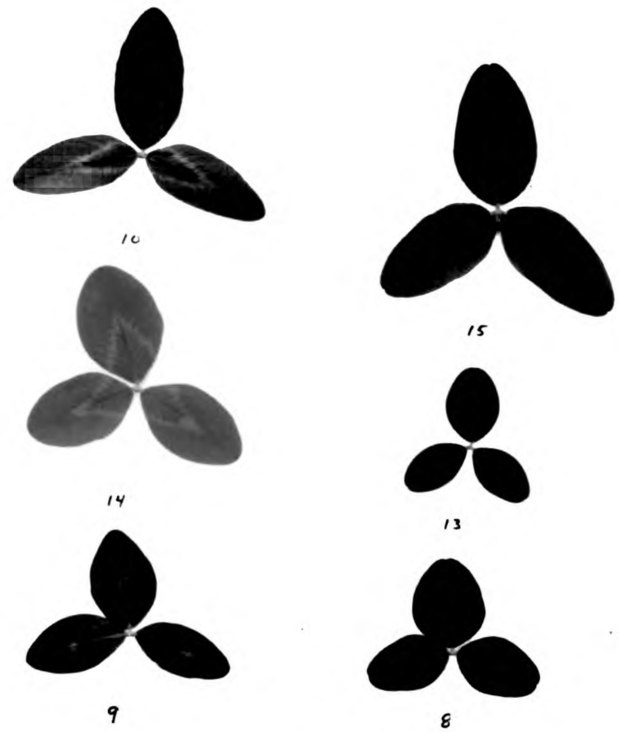
228370

Fig. 24



2296246

Fig. 25



CPI
C161

Fig. 26

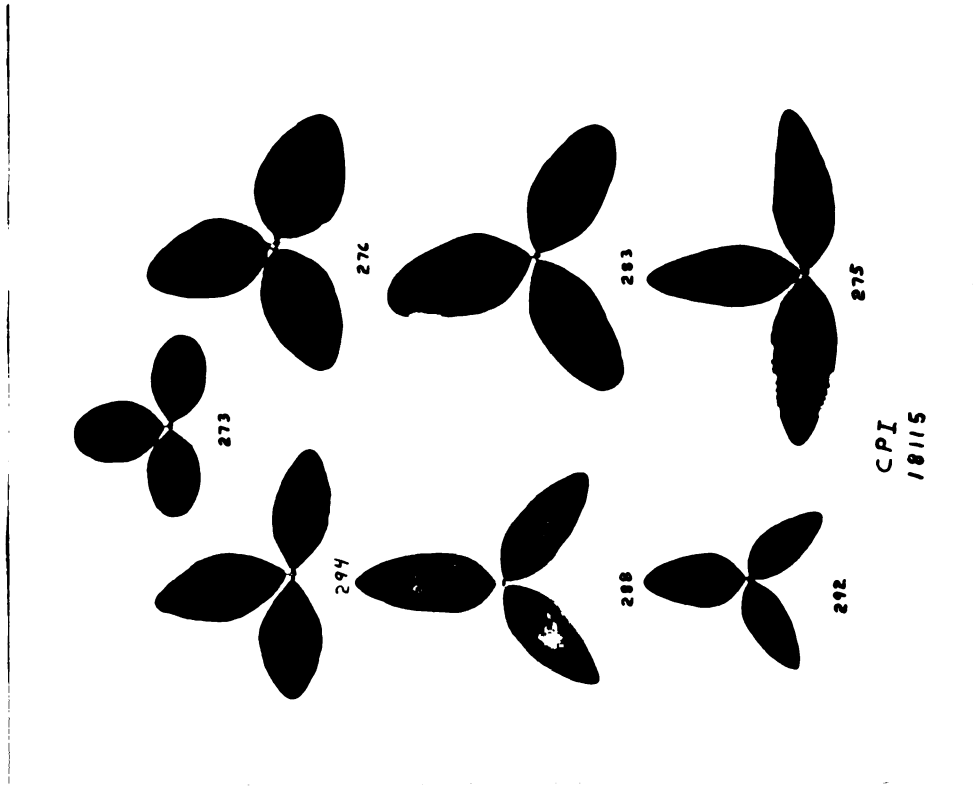
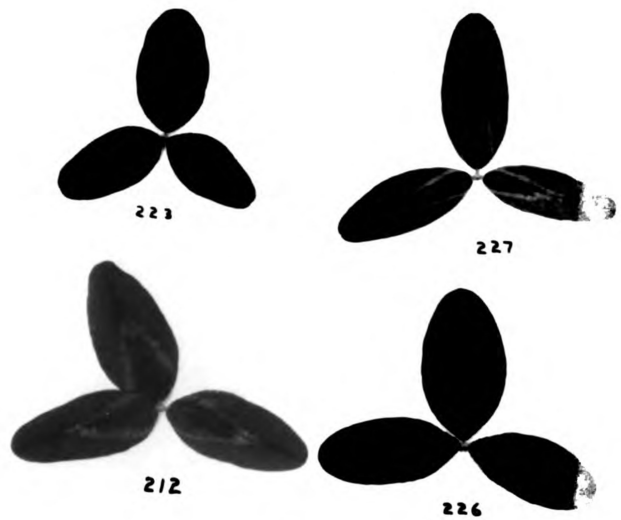

340
318
321
357
315
330
379
CPI
23408

Fig. 27

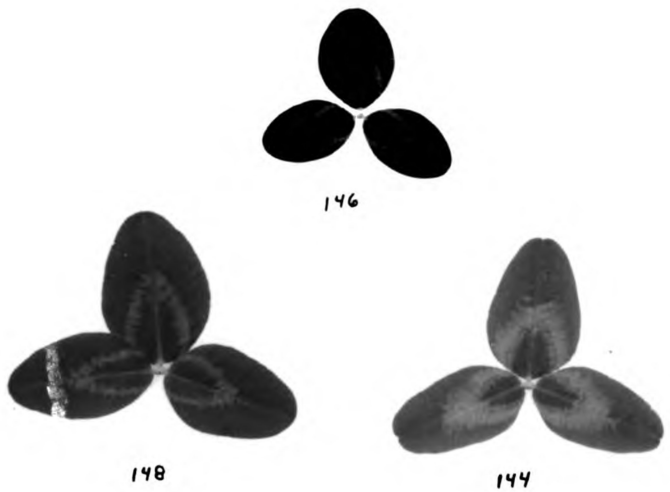
273
276
294
283
288
275
292
CPI
18115

Fig. 28



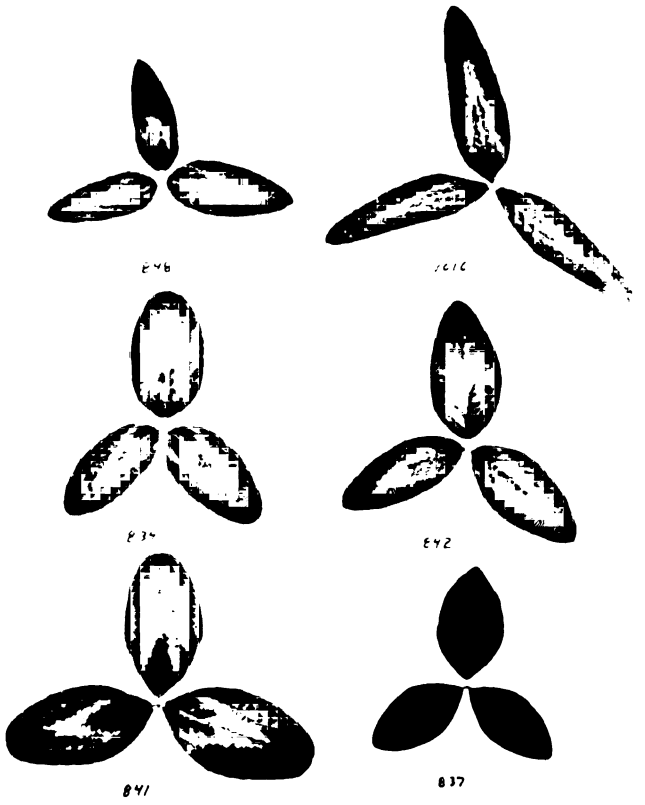
CPI
23158

Fig. 29



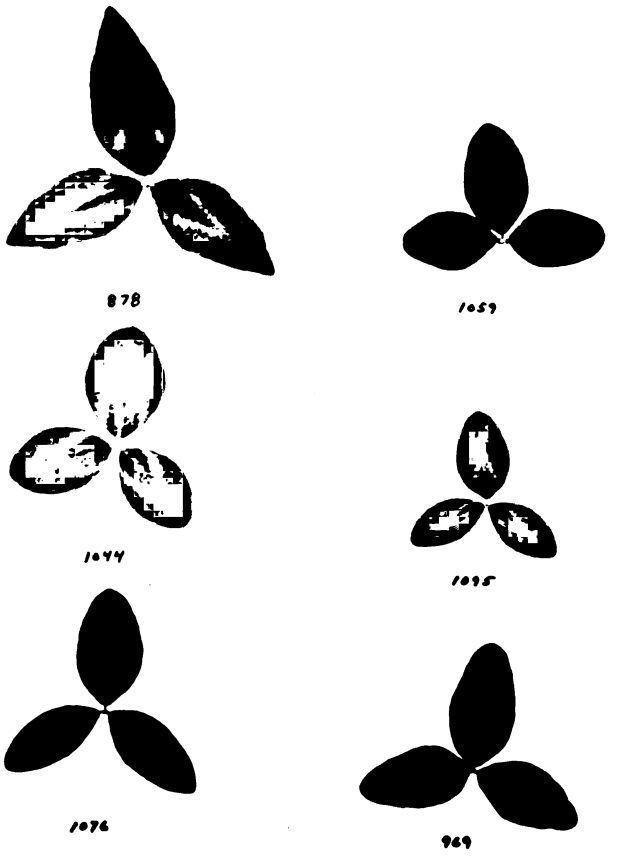
CPI
10803

Fig. 30



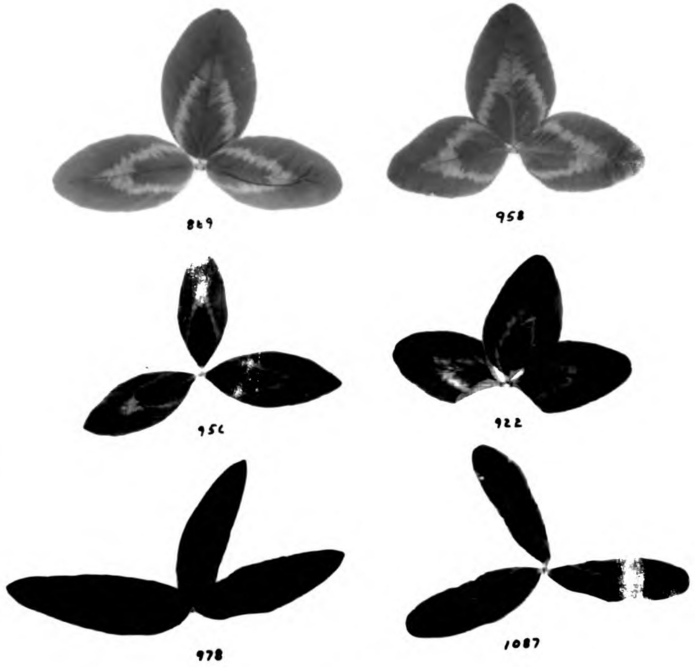
FC 33109

Fig. 31



FC 33109

Fig. 32



FC 33109

Fig. 33

Fig. 34

752

CPI
2264

Fig. 35

223

223

CPI
23158

Fig. 36

APPENDIX B

FERTILITY DATA

Tables 36 to 50 inclusive

B - pollination by honeybees

H - pollination by hand

TABLE 36

Fertility data from a 2x X 2x multiplant intersource cross

Source	Plant No.		No. of Florets	No. of Seeds	Seeds/Floret
CPI 2264	763	B	246	261	1.06
"	778		152	173	1.14
"	788		146	142	0.97
"	785		61	50	0.82
"	780		52	72	1.38
"	771		112	97	0.87
"	762		154	169	1.10
"	750		353	435	1.23
"	92		339	327	0.96
"	749		366	402	1.10
"	775		80	58	0.73
"	86		84	108	1.29
"	95		327	310	0.95
"	97		514	468	0.91
"	85		293	246	0.84
CPI 2771	819		189	172	0.91
"	808		254	291	1.15
"	813		193	193	1.00
"	793		167	198	1.19
"	792		174	219	1.26
"	802		330	303	0.92
"	809		189	260	1.38
"	807		234	273	1.17
"	799		134	141	1.05
"	795		118	131	1.11
"	811		176	158	0.90

	Total Florets	Total Seeds	Overall Average
CPI 2264	3279	3318	1.01
CPI 2771	2158	2339	1.08

TABLE 37

Fertility data from a 2x X 2x multiplant cross within source CPI 2264

Plant No.		No. of Florets	No. of Seeds	Seeds/Floret
763	B	65	49	0.75
96		111	63	0.57
747		196	2	0.01
748		31	7	0.23
749		143	46	0.32
790		74	33	0.45
104		52	14	0.27
764		54	35	0.65
761		60	47	0.78
91		54	38	0.70
773		165	35	0.21
782		58	46	0.79
760		58	22	0.38
776		83	78	0.94
788		101	71	0.70
79		63	28	0.44
786		50	21	0.42
		Total Florets	Total Seeds	Overall Average
		1418	1635	0.45

TABLE 38

Fertility data from a 2x X 2x multiplant cross within source CPI 2771.

Plant No.		No. of Florets	No. of Seeds	Seeds/Floret
814	B	320	195	0.61
799		82	49	0.60
821		46	43	0.93
810		43	31	0.72
804		191	127	0.66
813		108	65	0.60
811		122	92	0.75
815		149	160	1.07
		Total Florets	Total Seeds	Overall Average
		1061	762	0.72

TABLE 39

Fertility data from 2x X 2x reciprocal crosses within source CPI 2264

Plant No.		No. of Florets	No. of Seeds	Seeds/Floret
747	H	14	0	0
93		25	0	0
749	H	22	0	0
760		23	0	0
786	H	50	0	0
764		48	0	0
786	H	43	0	0
749		46	29	0.63
749	H	49	22	0.45
761		47	0	0
749	H	26	0	0
773		31	0	0
749	H	18	0	0
788		16	9	0.56
773	H	—	—	
764		38	35	0.92
773	H	35	49	1.40
788		35	38	
788	H	44	2	0.05
763		44	23	0.52
763	H	67	0	0
764		68	0	0

TABLE 40

Fertility data from 2x X 2x reciprocal crosses within source CPI 2771

Plant No.		No. of Florets	No. of Seeds	Seeds/Floret
817	H	76	0	0
807		82	0	0
799	H	24	0	0
794		—	--	--
805	H	29	12	0.41
821		30	12	0.40
798	H	37	13	0.35
810		38	28	0.74

TABLE 41

Fertility data from 2x X 4x reciprocal crosses

Source	Plant No.		No. of Florets	No. of Seeds	Seeds/Floret
CPI 2264	93	H	24	0	0
CPI 6161	1		25	0	0
CPI 2771	817	H	74	0	0
PI 229625	557		73	14	0.19
CPI 2771	806	H	61	0	0
CPI 6884	29		63	0	0
CPI 2264	766	B	580	0	0
PI 228370	654		150	0	0
CPI 2264	773	B	451	0	0
PI 229624b	722		275	0	0
CPI 2771	814	B	609	2	0.003
PI 228370	632		448	1	0.002
CPI 2264	747	B	533	3	0.005
PI 228370	626		428	0	0

TABLE 42

Fertility data from 2x X 6x reciprocal crosses

Source	Plant No.		No. of Florets	No. of Seeds	Seeds/Floret
CPI 2264	770	H	50	0	0
CPI 23158	244		50	0	0
CPI 2264	73	H	60	0	0
PC 33109	873		60	2	0.03
CPI 2771	792	H	44	28	0.64
PC 33109	890		44	0	0
CPI 2264	759	H	26	0	0
CPI 6161	5		--	--	--
CPI 2771	827	B	328	0	0
CPI 6161	10		173	2	0.01
CPI 2771	795	B	363	0	0
PC 33109	859		202	0	0
CPI 2771	831	B	--	--	--
CPI 23158	238		193	0	0
CPI 2264	773	B	296	10	0.03
PC 33109	1091		79	0	0
CPI 2264	74	B	353	0	0
PC 33109	851		239	9	0.04
CPI 2264	747	B	382	2	0.005
PC 33109	833		107	2	0.02
			Total Florets	Total Seeds	Overall Average
			3049	55	0.02

TABLE 43

Fertility data from a 4x X 4x multiplant intersource cross

Source	Plant No.		No. of Florets	No. of Seeds	Seeds/Floret
PI 229625	516	B	375	367	0.98
"	557		191	201	1.05
PI 228370	685		359	296	0.82
"	687		412	348	0.84
"	638		157	171	1.09
"	616		278	325	1.17
"	654		334	271	0.81
"	679		417	304	0.73
PI 229624b	711		405	365	0.90
CPI 6884	23		173	134	0.77
			Total Florets	Total Seeds	Overall Average
			3101	2782	0.90

TABLE 44

Fertility data from 4x X 4x intersource reciprocal crosses

Source	Plant No.		No. of Florets	No. of Seeds	Seeds/Floret
PI 229625	532	B	428	0	0
CPI 9949	129		164	0	0
PI 229625	505	B	331	215	0.65
PI 229624b	697		359	230	0.64
PI 229625	490	H	53	9	0.17
PI 228370	672		53	50	0.94

TABLE 45

Fertility data from 4x X 4x intrasource reciprocal crosses

Source	Plant No.		No. of Florets	No. of Seeds	Seeds/Floret
CPI 6884	29	M	49	39	0.80
"	60		49	31	0.63
PI 229625	571	M	43	28	0.65
"	490		46	3	0.07
PI 229625	540	M	32	20	0.63
"	575		40	37	0.93
PI 229625	565	M	49	35	0.71
"	507		48	36	0.75
PI 228370	662	M	18	4	0.22
"	680		18	14	0.78
PI 228370	662	M	49	20	0.41
"	599		50	36	0.72
PI 228370	662	M	35	38	1.09
"	628		40	25	0.63
PI 228370	628	M	22	15	0.68
"	625		--	--	--
PI 228370	613	M	28	0	0
"	657		27	0	0
PI 229624b	733	M	21	18	0.86
"	708		32	22	0.69
PI 229624b	722	M	37	17	0.46
"	724		44	25	0.57

TABLE 46

Fertility data from 4x X 6x reciprocal crosses

Source	Plant No.		No. of Florets	No. of Seeds	Seeds/Floret
CPI 6884	60	H	35	26	0.74
FC 33109	1083		42	0	0
CPI 6884	65	H	63	16	0.25
CPI 18115	276		58	6	0.10
CPI 6161	1	H	75	38	0.51
FC 33109	1033		75	44	0.59
PI 228370	672	H	47	18	0.38
FC 33109	1081		50	5	0.10
PI 229625	507	B	101	4	0.04
CPI 23408	335		105	4	0.04
PI 229625	579	B	350	57	0.16
CPI 18115	273		414	136	0.33
PI 229624b	724	B	205	104	0.51
FC 33109	860		--	--	--
			Total Florets	Total Seeds	Overage Average
			1620	458	0.28

TABLE 47

Fertility data from a 6x X 6x multiplant intrasource cross involving source PC 33109

Plant No.		No. of Florets	No. of Seeds	Seeds/Floret
956	B	197	170	0.86
1050		122	108	0.89
965		175	58	0.33
836		179	128	0.72
880		200	107	0.54
862		140	96	0.69
1006		164	96	0.59
1079		122	97	0.80
870		259	185	0.71
		Total Florets	Total Seeds	Overall Average
		1558	1045	0.67

TABLE 48

Fertility data from a 6x X 6x multiplant intersource cross

Source	Plant No.		No. of Florets	No. of Seeds	Seeds/Floret
PC 33109	835	B	41	20	0.49
"	172		42	27	0.64
"	1055		48	29	0.60
"	897		51	31	0.61
"	1059		47	12	0.26
"	1067		37	27	0.73
"	1004		48	4	0.08
CPI 23408	321		60	27	0.45
CPI 23158	226		89	31	0.35
PS	411		55	35	0.64
			Total Florets	Total Seeds	Overall Average
			518	243	0.47

TABLE 49

Fertility data from 6x X 6x reciprocal crosses within PC 33109

Plant No.		No. of Florets	No. of Seeds	Seeds/Floret
897	M	59	15	0.25
962		58	32	0.55
1050	M	37	—	
1033		37	0	0
851	H	36	21	0.58
993		34	29	0.85
1022	H	19	3	0.16
1087		20	7	0.35
982	H	32	12	0.38
860		32	11	0.34
860	B	179	47	0.26
869		268	17	0.06

TABLE 50

Fertility data from 6x X 6x intersource reciprocal crosses

Source	Plant No.		No. of Florets	No. of Seeds	Seeds/Floret
FC 33109	889	M	45	21	0.47
CPI 18115	276		43	3	0.07
FC 33109	873	M	46	22	0.48
CPI 10803	149		47	0	0
FC 33109	890	M	39	0	0
CPI 23158	244		38	0	0
CPI 6161	7	M	28	27	0.96
PC	411		29	12	0.41
CPI 6161	6	M	22	7	0.32
CPI 23408	335		24	3	0.13
CPI 6161	7	M	32	26	0.81
FC 33109	851		27	23	0.85
CPI 6161	5	M	35	31	0.89
CPI 23408	365		41	33	0.80

[redacted]

by

[redacted]

AN ABSTRACT

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

Approved [redacted]

[illegible] of [illegible] to [illegible]
[illegible] clover, with a wide [illegible] for plant, but in the [illegible]
[illegible] of two more [illegible] and the [illegible]
[illegible] of [illegible] from greenhouse plants. By [illegible]
these plants, clover [illegible] could be [illegible] as they [illegible] into
the void between the [illegible]. Generally the [illegible] were [illegible] for 4 to
6, or the [illegible] level, but variants were found in two [illegible]
[illegible]. These two [illegible], respectively, a low [illegible]
[illegible] and pentaploid plants. Since no [illegible] were found in [illegible]
[illegible] that these variants were [illegible] from [illegible]
[illegible] different sources.

Plants grown in the field and greenhouse were examined for several morphological and physiological characteristics known to be associated with polyploidy. The results indicated that under field conditions at East Lansing, polyploids of [illegible] clover had [illegible] length/breadth ratios, increased leaf area, fewer but larger [illegible], increased but later flowering, longer florets and pistils, [illegible] pollen, larger and heavier seeds, increased [illegible] production, and greater vigor. Although non-stainability of pollen [illegible] to increase with ploidy level, some polyploid sources had low percentages of non-stained pollen. Winter losses were small and apparently not related to ploidy level. In the greenhouse, floret length, number of heads per plant, and number and size of stolons did not fall in the above trends at all ploidy levels. Overall vigor was also [illegible]. In any case, cytological examination is still necessary for verification of ploidy level.