Differential reactivity of chromosomes in *Trillium*

By

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(With 2 plates and 18 text-figures)

The differential reactivity of chromosomes in *Trillium Kamtschaticum* PALL. and its allied species affords suitable means for the analysis of the chromosomal variation in natural population of these plants (HAGA and KURABAYASHI '50). The idea that the differential segments of chromosomes consist of heterochromatin and the patterns in these segments are indicative of structural hybridity among the individuals of the same species has been introduced by DARLINGTON and LA COUR ('40, '41). In this connection, however, some other investigators pointed out that one must be very cautious before coming to a conclusion on the structural heterozygosity from the stand point of the difference in the patterns of the differential segments (KURABAYASHI '48, WILSON and BOOTHROYD '41, '44). In the present paper, some detailed studies are reported on the differential reactivity in chromosomes of these plants from this point of view.

Material and method

Corms of *Trillium* species were collected from various districts in Japan and transplanted in the "*Trillium* Garden" of our University. They were then brought into a green house kept at about 15°C–20°C and were placed there from mid-February to early or mid-March when the flowers begin to open.

Differential reactivity in chromosomes was examined exclusively with young ovular tissues of the flowers which had opened in the green house just before the low temperature treatment was begun. The low temperature treatment was carried out in an "igloo", in which both temperature and moisture were kept quite constant at 0°C and at 100 per cent from the beginning until the end of March when the snow began to melt. Seventy two hours' chilling in the igloo gave satisfactory differential reaction in *T. kamtschaticum* and 96 hours' chilling

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was proved to be suitable for the other species. Cytological preparations were made after FEULGEN's smear method: fix the tissue with LA COUR 2BE in cold place for 20–25 min., rinse in running water for 10 min., hydrolyse with 1 N HCl at 60°C for 18–22 min., rinse in running water for 5 min., stain with leuco fuchsin for 40–60 min., rinse in running water for 10 min., maserate in 45% acetic acid under pressure. The treatments of shorter duration were employed for *T. Kamtschaticum* and the longer one for the other species.

Observations

CONSTANCY IN THE REACTIVITY. The differential reaction becomes clear after 48 hours' chilling and attains to the final state after 72 hours in *T. Kamtschaticum*, and 96 hours in other species. This difference in the length of the cold treatment among these species may be due to (1) the difference in the velocity of cell division in each species and (2) the inherent properties, which will be described below, of the differential segments in each genom. After the reaction attains to the final state it remains fairly constant, as in the case of *Paris tetraphylla* (KURABAYASHI '48), for about 24 hours in the chilled condition, and then begins to become more or less altered due to the contraction of chromosomes. As for the alterations in the configuration of differential segments in various temperatures, detailed studies were made by the present writer with *Paris tetraphylla* ('48) and by WILSON and BOOTHROYD with *Trillium electum* ('41, '44). In the present material the mode of the progress of the reaction was the same as that in the above-mentioned plants. In the final state of the reaction, all the differential segments manifest the patterns characteristic for each individual, but the variations of the patterns among different cells of the same individual observed by WILSON and BOOTHROYD in the root tips of *T. electum* were not found in the present material except when the control of the conditions mentioned below is not proper (see the Figures in the Plates).

For the purpose of analysing the structural hybridity in these segments, the constancy of the reaction is the indispensable condition, and therefore close attention was paid to conditions, internal and external, influencing the morphological features of the differential segments, as will be mentioned below.

ALTERATION IN THE REACTIVITY. It must be stated first that there is some internal condition influencing the reaction. As mentioned

above the duration of low temperature treatment necessary for attaining the final state of the reaction differs among different genoms. This difference is a genetical one and cannot be controlled by external conditions. The genom K_1 proved to be of the strongest reactivity, the genom K_2 the next, and genom T the weakest one. These three genoms brought into the same nucleus of *T. Hagae* by hybridization also retain these differences in strength of reactivity. So it is clear that the reactivity of each genom is controlled directly by the property of the differential segments, probably by their chemical compositions.

In hexaploid *T. Hagae*, the mitotic process bing slower than in its triploid relative, all these three gemons show slower reactivity than those in the triploid nucleus. The velocity of cell division is thus one of the internal conditions affecting the differential reaction. Accordingly the reactivity of each species is controlled by both the property of the differential segments of its chromosome complements and the velocity of mitosis.

Each species has its own division velocity. Among the individuals of the same species, it is possible to change it by physiological conditions. Among them, the vigor of each individual and the age of its ovular tissue are the main internal conditions affecting the velocity. The ovular tissue of a vigorous individual which has blossomed in the day just before the low temperature treatment begins, shows quite constant reaction, when all the external conditions were adequately controlled. With ovular tissues of individuals of poor vigor, mitosis proceeds slowly and chromosome contraction advances so far that one cannot see the detailed structure of differential segments. Too early beginning of temperature treatment arrests the reaction before it attains to the final state and too late beginning results in uneven reaction.

There is another internal condition which is controlled genetically, affecting the reactivity. It has been noted that there are various differential segments with varying degrees of reactivity in chromosomes of different genoms and among the same chromosome in the same genom (HAGA and KURABAYASHI '50, KURABAYASHI '48, WILSON and BOOTHROYD '41, '44). These qualitative differences in the reactivity may most probably be controlled genetically. Further, if DARLINGTON and LA COUR's assumption is correct that the differential reaction is conditioned by the change in nucleic acid metabolism in nucleus, it seems probable that there may be genic control of the reaction in quantitative

manners, because SCHULTZ and CASPERSSON ('40 a, '40 b) have shown that the quantitative change of nucleic acid metabolism is induced by the difference in genetic composition, especially that of heterochromatin. In this connection it seems worthy of mention that there are slight variations in the mode of the reaction among the individuals of *T. kamtschaticum*. The individuals collected from *Kuzakai* and *Shiraoi* show high homogeneity in the patterns of the differential segments and those from *Shizunai* and *Samani*, Hokkaido, show high heterogeneity in them. Thus the constancy of the differential reaction is much higher in the former specimens than in the latter. In the latter, slight difference in the mode of the reaction is seen (Plate XVIII, Figs. 1, 2). Such variations among individuals, however, do not alter the position, the size or the chromaticity of the differential segments. They concern with the spiralization system of chromonemata in the differential segments, which are apt to separate into the component chromonemata, resulting in the increase in the width of these segments (Plate XVIII, Fig. 2). Such a state of affairs, as extensively found previously in *Paris teteraphylla* in the final state of the reaction (KURABAYASHI '48) (Plate XVIII, Fig. 5) and also in the present material under some inadequate chilling treatments described below (see Plate XVIII, Figs. 3, 4), indicates the possibility of quantitative control of nucleic acid metabolism due to the composition of differential segments in chromosomes.

Next we shall see how the external conditions affect the reaction. The most necessary condition is temperature. For the sake of attaining the final state of the reaction marginal temperature condition, 0°C, must be kept. Any slight deviation from this temperature during the chilling brings about unfavorable results. Not only temperature but also moisture affects the differential reaction. In Plate XVIII, Figures 3 and 4, there are represented such examples from two plants. These plants were kept in the thermostat adjusted at about 0°C, the thermostat being placed in a cold room of temperature at about −10°C. The adjustment of temperature was inadequate and the air in the thermostat was much dehumidified.

The influence of the fixation and the staining method upon the differential segments cannot be overlooked here. It is not the present aim to examine whether the obtained configuration of the differential segments is an artifact or not, but to find out the way to get the constant reaction pattern. Empirically it was found that LA COUR 2BE is the best fixative. No other fixatives gave suitable figures for the

detailed analysis of the differential patterns. The time and the temperature during the hydrolysis with 1 N HCl are the most important factors in the course of FEULGEN's method. Insufficient hydrolysis makes maceration difficult and the staining of chromosomes remains incomplete. Excess treatment makes the hydrolysed products diffuse away into cytoplasm, damaging the detailed structure in chromosomes. A rapid rinse in running water after the staining gets rid of the overstaining of cytoplasm due to the excess of leuco fuchsin. The danger of overstaining of chromosomes is avoided by storing the slide for about one month before the observation. By doing so the red colour of the chromosome fades to some extent. The fading, however, does not proceed uniformly in chromosomes. The differential segments tend to lose the stain more rapidly than the other regions.

By keeping all these internal and external conditions constant, one can get characteristic and uniform reaction patterns for each individual.

CONFIGURATION OF DIFFERENTIAL SEGMENTS: It has been shown in the present and previous investigations that the configurations of differential segments in chilled condition are modified by the failure of spiralization due to the nucleic acid starvation in these segments. Then, how are the variations of configurations among different genoms, different chromosomes and different segments induced ?. Two possibilities may be considered, both being responsible for the difference in the strength of the affinity between the acids and the segments. The one is the qualitative difference among the segments. The strength of this reaction in each genom, as described above, may be determined by the inherent chemical quality.

The other concerns with the mode of the longitudinal rearrangements of the differential segments in chromonemata. As for the differentiation in chromosomes at issue, one may expect various degrees of alternative rearrangements of differential and nondifferential segments. The order of the magnitude of these alternations may affect the configuration of the differential segments. When the differential segments occupy enough large regions along chromonemata the failure of spiralization and loss of stainability in these regions become conspicuous, and the small euchromatic segments inserted there appear as deeply stained dots. Such is actually the situation observed in all large differential segments (see all Figures in this paper and those in HAGA and KURABAYASHI '50). In the reverse case, *i. e.*, when small differential segments are inserted in large non-differential regions, the former may

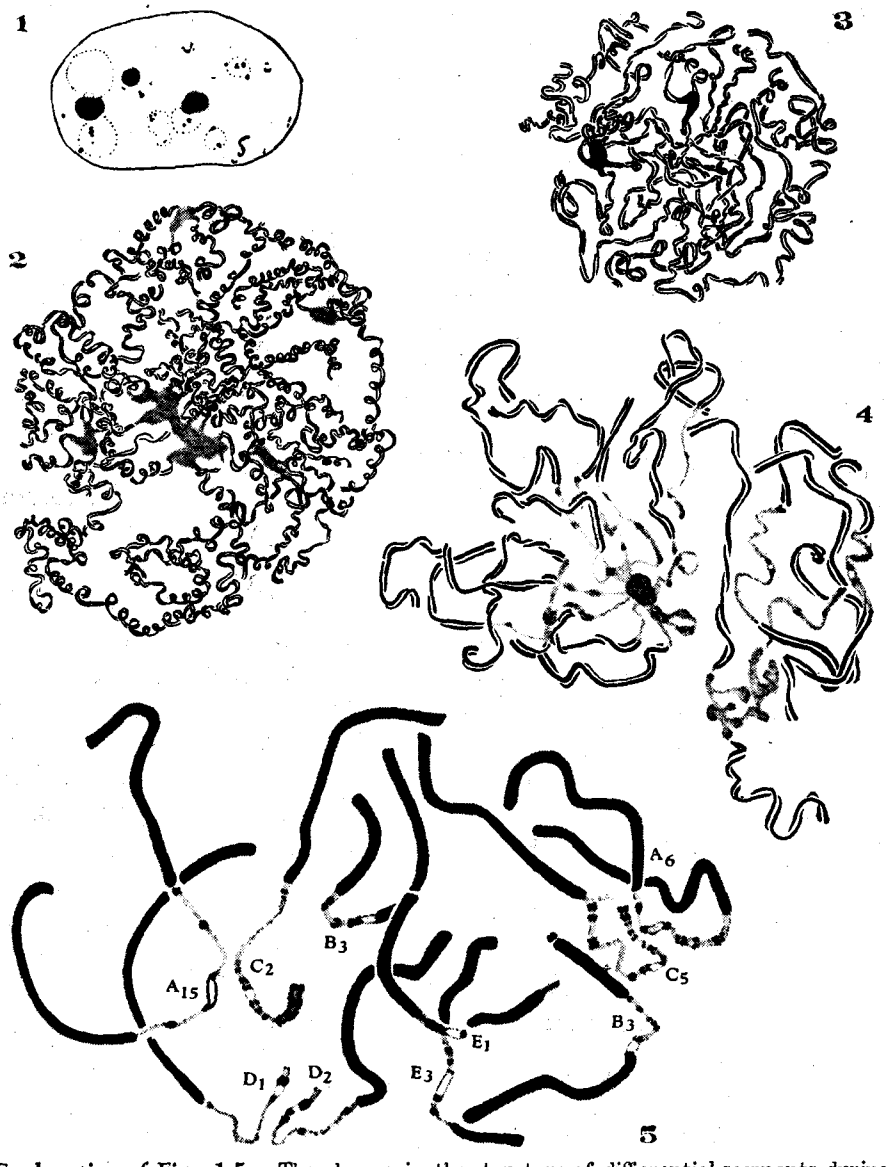
sometimes be consumed during the spiralization, into the latter as was found by WILSON and BOOTHROYD ('41, '44).*

The alternations of differential and non-differential segments of nearly equally small size may give rise to differential segments with intermediate stainability. Actually in the differential segments with intermediate stainability several small deeply stained granules can be seen embedded in faintly stained substance or matrix of chromosome (see the Figures in HAGA and KURABAYASHI '50). This faint coloration is not reduced after passing through several baths of sodium bisulphite (1% aq. sol.) before the washing of the material with running water. It has been shown by STEDMAN and STEDMAN ('47) that the mechanism of FEULGEN's stain consists in that the diffusible substances produced as the result of nucleic acid hydrolysis react with leuco fuchsin to become coloured compounds which have affinity with chromatin. The STEDMANS' opinion, being very unorthodox, was denied by many investigators (BRACHET '47, etc). However, as pointed out by DANIELLI ('47), the diffusion of the hydrolysis products has been empirically noticed during the preparation of this reaction. The faint coloration of the matrix mentioned above is due to this diffusion of the hydrolysis products from the non-differential granules, because this coloration becomes less faint after a little excess of hydrolysis which rather reduces the depth of colour of the non-differntial granules.

The considerations offered above are hypothetical bing supported by no direct chemical analysis. However they may be the most plausible explanation how the changes in the configuration of various differential segments are induced by the variation of the internal and external conditions.

THE CHANGE IN THE STRUCTURE OF DIFFERENTIAL SEGMENTS DURING NUCLEAR CYCLE: DARLINGTON and LA COUR ('40, '41) assumed that the differential segments in metaphase chromosomes condense into masses, chromocenters, in resting nuclei. This was also the case in the present material. In resting nuclei there are many chromocenters, large and small, and the number of the large masses in some nuclei roughly corresponds with that of the large differential segments in the metaphase chromosomes of the same plant. The re-

* The varying number of differential segments seen in the experiments carried out by them may be due to such disappearance owing to incomplete differentiation and abvanced chromosome contraction. In the present materials also chromosomes in root tips showed such conditions.



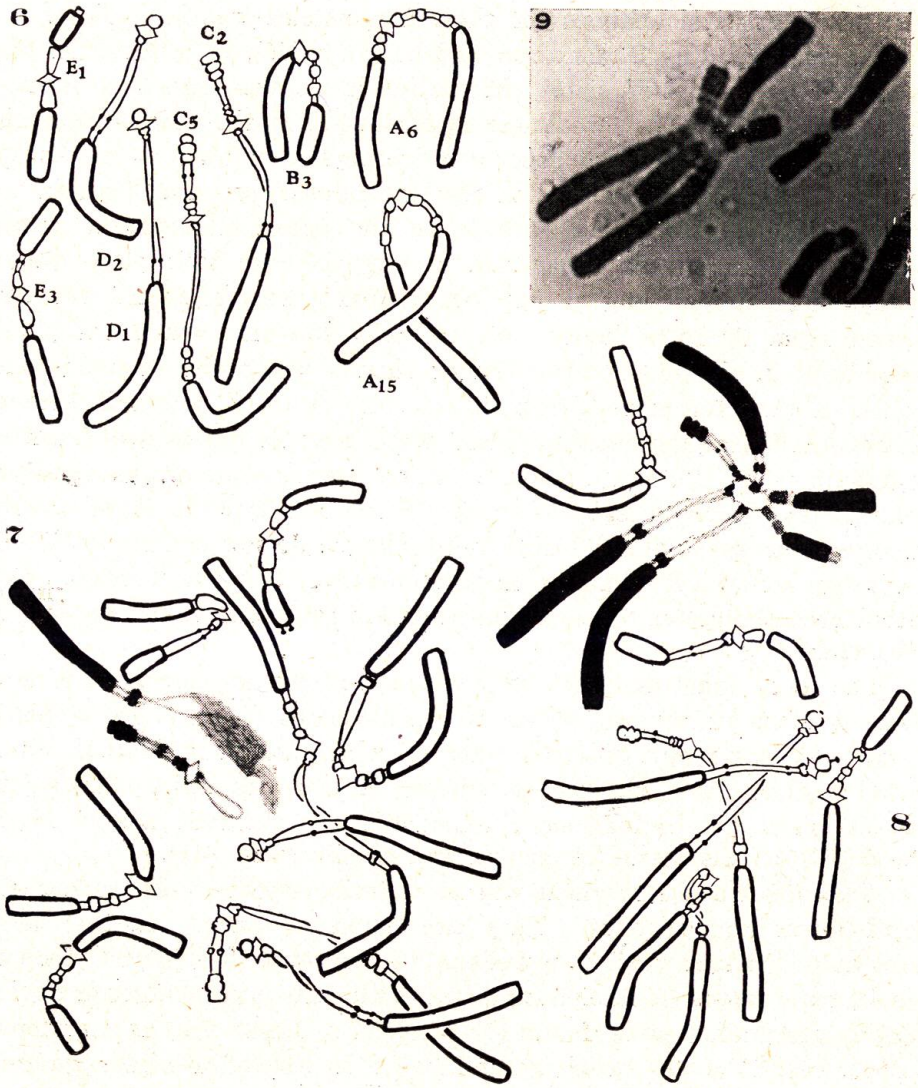
Explanation of Figs. 1–5. The change in the structure of differential segments during mitotic cycle in young ovular tissue of *T. kamtschaticum*. 1. resting nucleus kept at 0°C for 72 hours. Black circles are chromocenters. White circles are nucleoli. 2. mid-prophase nucleus kept at 0°C for 72 hours, showing chromocenters and chromonemata. 3. late prophase nucleus in moderate temperature. The black knots are the chromocenters. 4. still later prophase nucleus kept at 0°C for 72 hours showing the beginning of separation and unravelling of chromocenters into the differential segments. 5. metakinesis chromosomes kept at 0°C for 72 hours. × 1400.

duction in the number of these large masses, which is often met with, is due to the fusion of them (Fig. 1). In prophase the chromocenters become less condensed (Fig. 2). As the spiralization of chromonemata advances, it is seen that they are all branching out from the chromocenters which fuse into several large masses in a majority of the cases (Figs. 2, 3). In the beginning the chromaticity of the chromocenters is nearly the same, irrespective of the environmental temperature condition. Soon afterwards, in chilled condition, differential regions appear being unravelled from the chromocenters which become diffuse and lose stainability (Fig. 4). At moderate temperatures no such diffusion and fading is seen and the disappearance of the masses occurs at much earlier stage in prophase than it does in chilled condition (Fig. 3). In the latter condition they separate into individual differential regions finally, in the beginning of metakinesis. These separated differential regions then gradually begin to spiral into thin threads after the euchromatic regions have nearly completed spiralization (Fig. 4). The degree of the retardation in spiralization differs more or less in each differential region in the same nucleus (Fig. 4). All these findings mentioned above completely coincide with DARLINGTON and LA COUR's ('40) statement about the nucleic acid cycle in chromosomes.

One curious figure was observed at metaphase of one individual in which about three-quarters of the differential region of one long arm of chromosome C had failed in the spiralization and stayed in prophasic condition (Fig. 7). There was also a chromosome breakage at the proximal end of that affected region, the remaining proximal one quarter of the differential segment having spiralled in time. The cause of such a curious phenomenon is unknown at present, but it confirms the chromocenter origin of differential segments.

From metaphase to anaphase some contraction in length is seen in differential segments but their spiralization yet remains incomplete. The alteration in the configuration of the differential segments mentioned before (Plate XVIII, Figs. 2–4) may be due to the degree of incompleteness in spiralization, resulting in the extreme case in wide separation of their constituent chromonemata, which normally occurrs in the differential segment of *Paris tetraphylla* in the final state of the reaction (Plate XVIII, Fig. 5 and Figures in KURABAYASHI '48).

MITOTIC ABNORMALITIES DUE TO LOW TEMPERATURE: In the course of these observations several abnormalities which seem to have been produced as a result of the low temperature treatment were



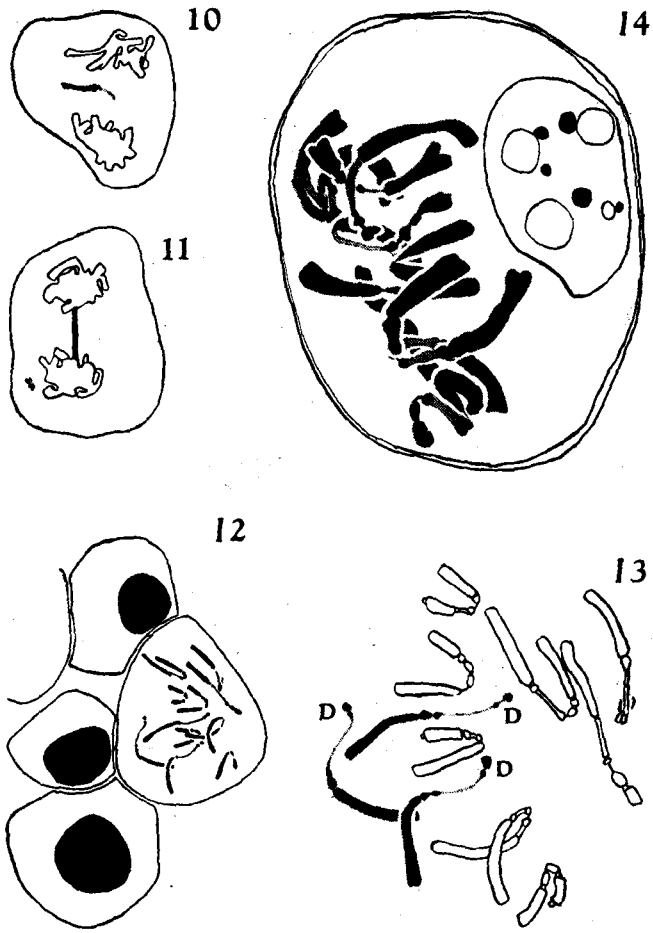
Explanation of Figs. 6–9. 7. Exceptional failure of unravelling and spiralization in a metaphase chromosome. The schematic representation of the differential patterns of this plant is presented in Figure. 6. 8, 9. The associations of kinetochore regions of different chromosomes at metaphase. All these Figures were obtained from materials kept at 0°C for 72 hours. × 1500.

observed in the ovular tissue of *T. kamtschaticum*. They were so rare that any statistical analysis for their frequencies was impossible.

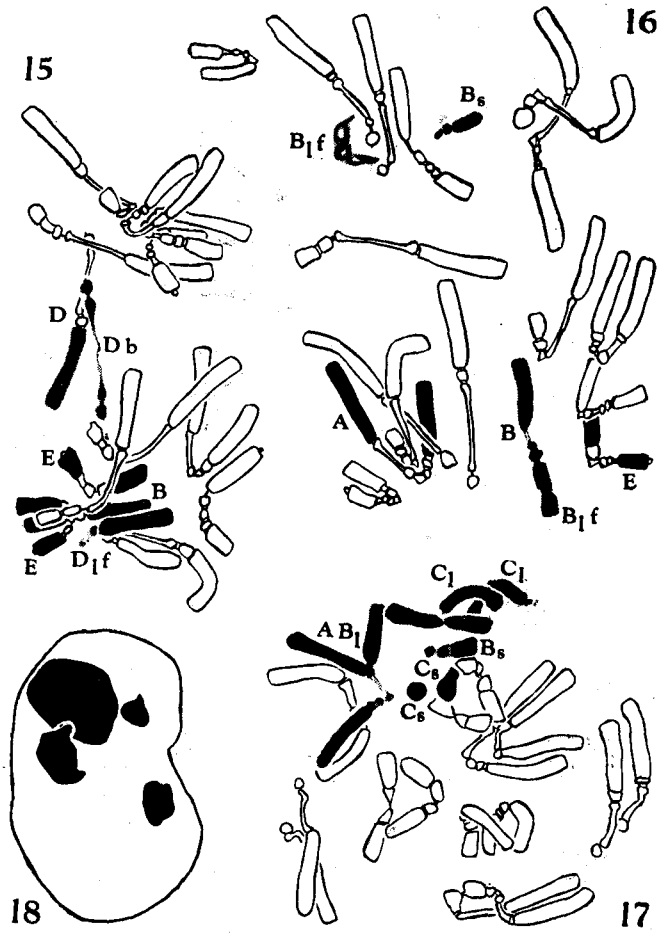
As was found by DARLINGTON and LA COUR ('40), in differential segments the repulsion of sister chromatids is weaker than that in non-differential ones. That weakness is evidenced in the present material by the greater number of relational coillings remaining at metaphase in the former segments than in the latter ones (see the Figs. in the Plates). However, owing perhaps to the proximal situation of the segments the anaphase separation of chromosomes took place almost always normally so long as the low temperature treatment was continued from 72 to 96 hours. Occasionally and only when the plants were kept in chilled condition longer than a week, the breakage and fusion of chromatids resulting in the formation of dicentric bridges and acentric fragments at anaphase were seen in the long differential segments (Figs. 10, 11). Differing from the analogous abnormalities taking place in the distally locating differential segments found by the previous workers, these intercalary breakages do not contribute to the heritable structural changes in chromosomes but they should give lethal recombinations, because euchromatic segments must be lost in the latter case.

The next abnormality to be mentioned is the association of kinetochore regions of several different chromosomes (Figs. 8, 9). This is apparently the result of the failure of separation of individual differential regions from the chromocenters in late prophase. Analogous association of non-homologous kinetochores was observed also at MI in the PMCs of this plant (MATSUURA and KURABAYASHI '51).

The abnormalities which will be mentioned below are rather exceptional or unusual ones. They are found exclusively in the plants kept at 0°C longer than two weeks. In one plant kept at 0°C for two weeks some telophase nuclei connected with chromatin bridges or having lagging fragments were found (Figs. 10, 11). These are, as mentioned before, results of the breakage and fusion in the differential segments. Another type of abnormality found in the material treated longer than two weeks is the failure of synchronization of mitotic process between the two daughter nuclei and, in the extreme case, among the chromosomes in the same nucleus. In Figures 12 and 13, one of the daughter cells, containing a supernumerary D chromosome, stays in anaphase condition, while its hypoploid sister nucleus already enters the resting stage and its cell size is much less than the former. In the binucleated



Explanation of Figs. 10–18. Mitotic abnormalities in chilled condition. 10. a lagging fragment. 11. a bridge. 12. failure of synchronization between two daughter cells. (10–12 ×350). 13. one of the daughter cells in the right side in Figure 12 drawn at higher magnification showing one extra chromosome D. 14. failure of synchronization between the two daughter nuclei in the same cell. 15. anaphase with a bridge (Db) and a fragment (D_1f) at the differential segment of chromosome D. Five chromosomes (B, D, D_1f, E and E) have failed in anaphase separation. 16. anaphase with one translocation (B_1f) and two breakages, one at the differential and one at the non-differential segment of chromosome B (B_1f). The latter is quite exceptional being produced by the



fusion between the small terminal differential segment at the short arm of chromosome B travelling towards the lower pole in the figure and the distal end of the long arm of chromosome B which is expanded to be broken. Such a case was met with only once during the course of this chilling experiment. Chromosomes A and E have failed in anaphase separation. 17. early anaphase having two degenerating fragments (Cs), one chromatid dislocation (AB) and three chromatid breakages (Bs, C_1 and C_1). 18. polynucleate cell. (13-18 $\times$1000). The materials of Figures 10-16 were kept at 0°C for two weeks and those in Figures 17 and 18 for twenty days.

cell shown in Figure 14, one of the nuclei attains to metaphase, probably, of the next division with a complete set of diploid chromosomes, leaving its sister nucleus in resting stage. In Figures 15 and 16, cells are shown which contain not only bridges and fragments, but also several chromosomes failing in anaphase separation due perhaps to the absence of reproduction of daughter chromatids at the preceding prophase. Further, as seen in Figure 17, something like the degeneration of chromatin substance occurs in several chromosomes. At the same time anaphase separation of chromosomes becomes so irregular that polynucleated cells are produced (Fig. 18).

During such long low temperature treatment the vigor of the plants did not decline at all and the flowers remained in full blossom.

Conclusion and summary

In the present investigation attempts were made to find out the way to make the differential reaction in chromosomes constant, through the examination of the conditions, internal and external, modifying the reaction. With the excellent material, young ovular tissue, with the splendid thermostat, igloo, and with the carefully defined technique, the final state of the reaction could be maintained constant in all the cells of one and the same individual allowing an estimation of the genic composition in the natural population of *T. kamtschaticum* and its allied species on the basis of the structural changes of the differential segments in chromosomes.

Many data have been accumulated to show that the differential segments in chromosomes which yield negative FEULGEN's reaction in low temperature are the inert heterochromatin, typically the one composing the chromocenter in salivary gland nuclei of *Drosophila*, and consist of polygene systems inducing quantitative variation to the phenotypes of organisms (DARLINGTON and LA COUR. '40, '41; KOLLER '46; DARLINGTON '47; DARLINGTON and MATHER '49 pp. 145–152, etc.). Whether these assumptions be applicable to the differential segments of the present material is the problem having essential importance for our work on population genetics because the chemical and genetical character of these segments determines the latitude of the structural changes in them, which have been actually found in the course of our analysis (HAGA and KURABAYASHI '50). Their cytological behaviour described above agreed quite well with that observed by previous workers.

Further cytological investigations on this problem are now under progress. It is hoped that some physiological assay of this reaction may be performed.

The detailed observation of these segments elucidates well the longitudinal differentiation in chromonemata and the mode of spiralization of them into chromosomes. Attachment of FEULGEN-positive nucleic acids to chromonemata seems to be essntial for the formation of regular spiral. The starvation of the acids causes not only the failure of spiralization but also, in some conditions, the separation of the componental chromonemata representing pseudo-polytene chromosomes in somatic metaphase.

The prolonged chilling of the plants caused several abnormalities in the mode of the synchronization of cell division and chromosome reproduction. They are worthy of note, having resulted probably from the extreme low supply of materials available for the fundamental synthesis in self-propagation of chromatin.

Finally the writer wishes to express his cordial thanks to Professor HAJIME MATSUURA and Professor TSUTOMU HAGA, under whose kind guidance and criticism the present investigation was carried out.

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Explanation of plates

Plate XVII.

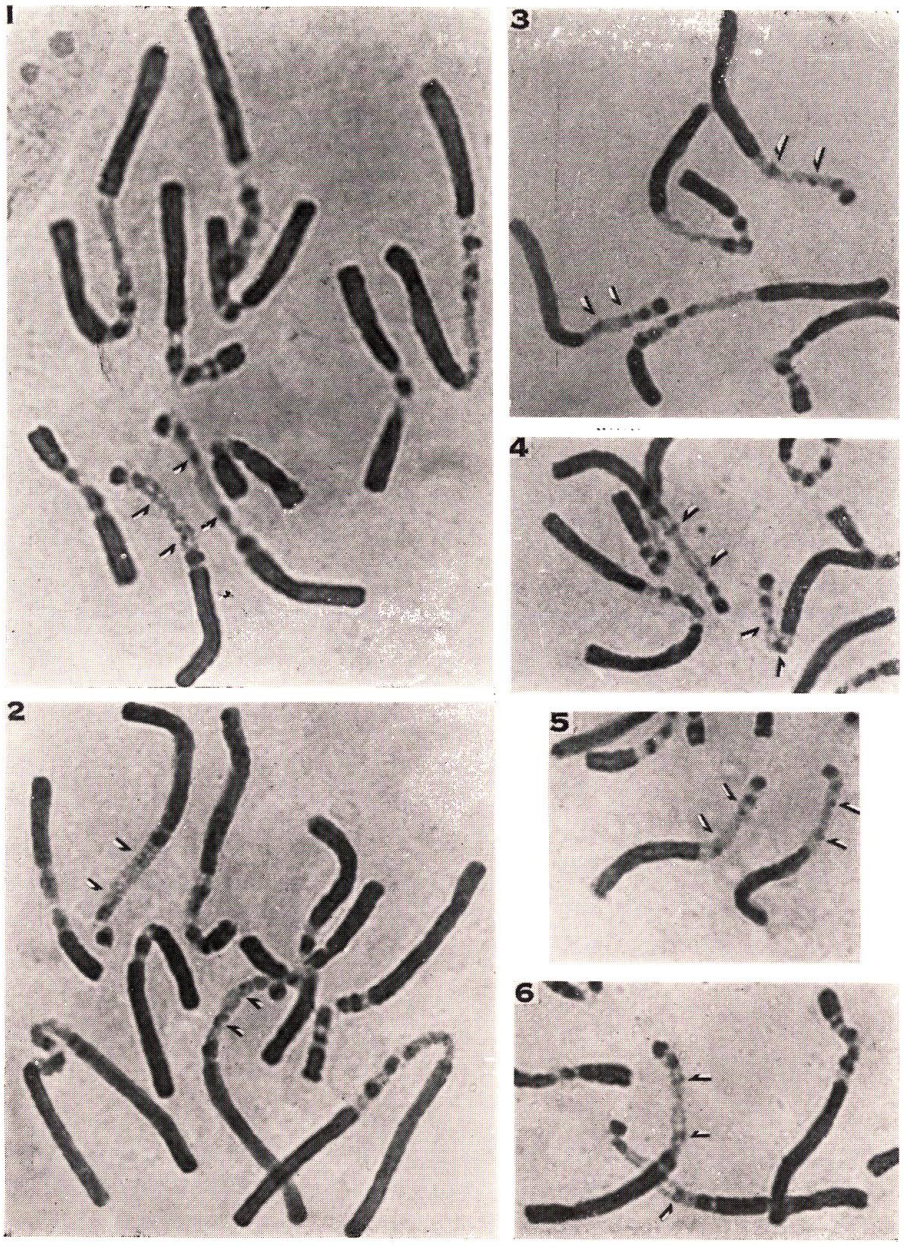
Figs. 1–6. Metaphase chromosemes in young ovular tissue of *T. kamtschaticum* kept in the igloo for 72 hours. The chromosomes were taken from different plants gathered from *Shiraoi* population. The arrows indicate the two small non-differential segments inserted in the large differential segment. × 1340

Plate XVIII.

Figs. 1 and 2. Metaphase chromosomes chilled in the igloo with different differential patterns. They were taken from two plants of *T. kamtschaticum* gathered from *Samani* population. The arrows indicate the regions with separated chromonemata.

Figs. 3 and 4. Metaphase chromosomes of *T. kamtschaticum* (3) and 6X *T. Hagae* (4) chilled in unfavorable condition. The arrows indicate the regions in which the failure of spiralization and separation of chromonemata are conspicuous.

Fig. 5. Metaphase chromosomes in young ovular tissue of *Paris tetraphylla* kept at 0°C for 72 hours. The arrow indicates the differential segment which shows complete absence in spiralization and extreme separation of chromonemata. Such are the usual conditions in this plant in the final state of differential reaction. × 1340



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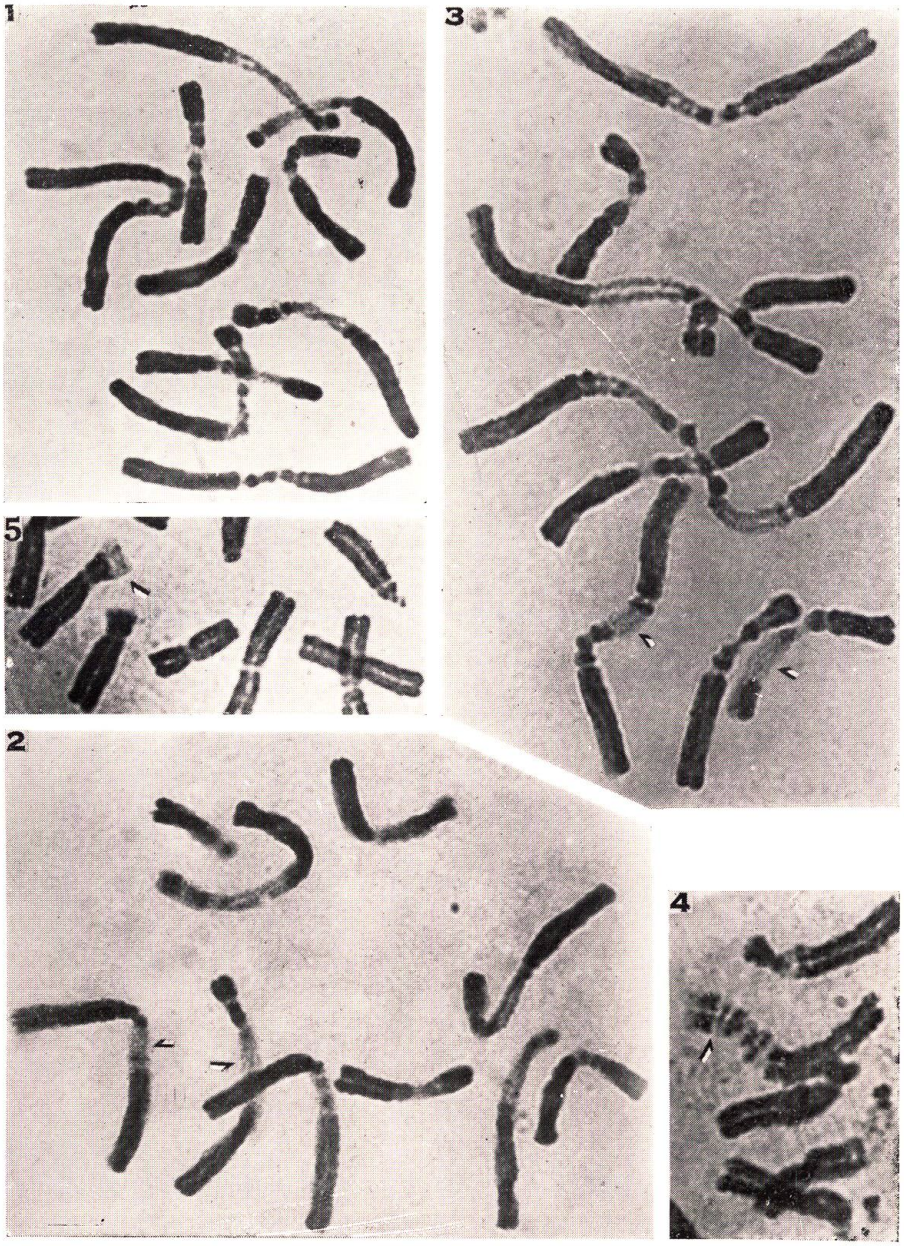

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