GENOME AND POLYPLOIDY IN THE GENUS *TRILLIUM*. VI. HYBRIDISATION AND SPECIATION BY CHROMO- SOME DOUBLING IN NATURE *

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I. INTRODUCTION

THERE are two forms in *Trillium Hagei* Miyabe et Tatewaki, the sterile triploid and the chromosome-doubled fertile hexaploid. These two forms were concluded from chromosome evidence to be the hybrids between diploid *Trillium kamschaticum* Pallas and tetraploid *T. Tschonoskii* Maximowicz (Haga, 1937, 1951a, 1952; Haga and Kurabayashi, 1953). The triploids were found at almost all the localities where both parental types are growing close together. The chromosome constitution was revealed to be that expected of the F_1 without exception. This is obviously due to the frequent occurrence of natural hybridisation and the nearly complete defectiveness of the triploids in seed setting. On the other hand, the chromosome-doubled forms are completely fertile in seed setting. These facts suggest that hybridisation and chromosome doubling are at present occurring in nature. In other words, it is likely that a new hexaploid species is arising following the processes by which widespread older polyploid species of the genus originated.

The chromosomes of *T. kamschaticum* revealed profound structural diversification and heterozygosity in the patterns of the heterochromatic segments after cold-treatment (Haga and Kurabayashi, 1954). This finding suggests that chromosome variation might be present in the chromosome-doubled forms at the time of their emergence. This was proved to be the case, as has already been briefly reported (Haga, 1951b). This article attempts to relate these observations with the problem of speciation in the genus.

Cytological observations were carried out using the preparations of cold-treated chromosomes in young ovular tissues, using the method of Darlington and La Cour, 1940, with some modification in the method of cooling (Haga and Kurabayashi, 1953). This method differentiates the eu- and hetero-chromatic segments which are constant in number, size and position for a given chromosome, as was observed by Darlington and La Cour (1938, 1940, 1941), La Cour (1951), Haga (1944), Haga and Kurabayashi (1953, 1954).

Some minor controversies in respect of the constancy of the patterns are due in my opinion to the varying degrees of differentiation.

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This may range from the non-differentiated to the fully-differentiated state even in cells of the same cold-treated tissue (Kurabayashi, 1948, 1952), a fundamental fact which appears not to have been clear in some other studies (*cf.* Wilson and Boothroyd, 1940, 1944). Observations reported in this article were based on patterns observed in the final stage of differentiation. It should be noted that the heterochromatic segments are seen only after the above treatment.

All the text-figures are the classified alignments of chromosomes in single cells at metaphase.

FIG. 1.—Range of variation in the patterns of the cold-treated chromosomes of *T. kamtschaticum*, heterochromatic segments being shown in dotted line or in slit-like gap, euchromatic segments in solid black, and centromere diamond-shaped. Top, 8 types of chromosome D. Bottom, 8 types of chromosome E. $\times 1500$. (For the other chromosomes, A to C, see Haga and Kurabayashi, 1954.)

2. CHROMOSOME CONSTITUTION OF THE PARENTS

(a) *T. kamtschaticum*. Five chromosomes, A to E, constituting a gametic set of this species, revealed many types in respect of the patterns of the heterochromatic segments (*cf.* fig. 1). The types were indicated by numbering as, for example, A₁, A₂ and so forth in chromosome A. The homozygous presence, for example, of A₂ is shown as A-2.2 and a pair heterozygous for A₁ and A₂ as A-1.2. This method of description was adopted for all the five chromosomes, the numbering of the types being the same as that used in the previous paper (Haga and Kurabayashi, 1954).

Plants from homogeneous populations show one and the same

constitution, that is all five pairs are homozygous (fig. 2), whereas those from highly heterogeneous populations show constitutions differing from plant to plant, the plants being heterozygous for one to all five pairs (fig. 3).

FIG. 2.—*T. kamtschaticum* (2x) from Kusakai.
Homozygous plant, Ks-49-2. $\times 1500$.

(b) *T. Tschonoskii*. As to the patterns of the heterochromatic segments of the tetraploid parent only a single plant from Siraoi was studied and it was found to be homozygous for two sets of chromosomes

FIG. 3.—*T. kamtschaticum* (2x) from Siraoi. Plant heterozygous for
A, B and D, Sr-49-7. $\times 1500$.

which were distinguishable by the patterns of differentiation (fig. 4). As manifested in the meiotic chromosome pairing in the triploid hybrid, one of these two sets, K_2 , is intimately homologous with set K_1 of *T. kamtschaticum*, though these two sets are not morphologically identical in untreated as well as in cold-treated chromosomes.

The other set, T, of the tetraploid parent has also relations to the sets K_1 and K_2 , though the relation is distinctly not intimate in comparison with that between K_1 and K_2 (Haga, 1937, 1951a). However, it is impossible to sort out individually the members composing K_2 and T from the combined set of 20 chromosomes. Arbitrarily five pairs having a large number of heterochromatic segments were

FIG. 4.—*T. Tschonokii* (4x) from Siraoi, homozygous allotetraploid, K_2K_2 and TT being sorted out above and below. Chromosomes A to E are aligned from left to right in each of the four sets. $\times 1500$.

sorted out and designated as K_2 , and the remaining five pairs as T. Thus assortment of chromosomes by shape at mitosis into K_2 and T is less clear than the distinction manifested by the meiotic pairing. The patterns of differentiation of K_2 and T comprised in 59 triploid hybrids and in 2 chromosome-doubled ones were the same as one another and as those of *T. Tschonokii* (figs. 4, 5 and 7). Thus there is a high stability of the linear chromosome organisation of the tetraploid species.

FIG. 5.—*T. Hage* (3x) from Kusakai. Plant H-Ks-50-2. Top, K_1 , middle K_2 , bottom T. $\times 1500$.

FIG. 6.— K_1 from 3 triploid hybrids, K_2 and T which are the same as shown in fig. 5 are omitted. Top, plant H-Sr-48-1 from Siraoi. Middle, plant H-Ut-48-3 from Utoma. Bottom, plant H-Ty-48-1 from Toyoni. $\times 1500$.

3. CHROMOSOME CONSTITUTION OF THE HYBRIDS

As mentioned above, two sets of chromosomes K_2 and T in the hybrids revealed no variation. On the other hand, K_1 from *T. kamtschaticum* showed variations in the patterns as was to be predicted

TABLE 1
Chromosome constitution of K_1 in 59 triploid hybrids
in nine populations

(Figures under the chromosome symbol indicate the number of the chromosome types)

Locality	A	B	C	D	E	Freq.	Total
1. HOMOGENEOUS							
Kusakai . .	13	12	6	3	7	6	6
Hagino . .	13	2	6	3	2	11	11
Shadai . .	13	7	6	7	2	7	7
2. HETEROGENEOUS							
Siraoi . .	13	6	6	3	2	1	9
	13	6	6	7	2	1	
	14	6	6	7	2	7	
Utoma . .	5	2	3	5	1	1	9
	5	2	3	5	3	1	
	5	2	3	5	4	1	
	5	2	7	5	3	1	
	5	3	7	5	4	1	
	6	2	2	5	5	1	
	6	2	9	5	3	1	
	16	14	5	7	3	1	
	17	2	3	5	3	1	
Minami-Sat. .	10	2	3	5	1	1	5
	10	2	3	5	2	1	
	10	3	5	5	3	1	
	16	2	3	5	3	1	
	16	3	5	5	4	1	
Toyoni . .	5	1	3	5	3	1	10
	5	2	3	5	8	1	
	5	2	8	5	3	1	
	5	3	3	5	3	1	
	10	1	3	5	3	2	
	14	2	3	5	8	1	
	18	2	3	5	8	1	
	21	1	3	5	8	1	
	23	2	3	5	1	1	
3. SINGLE SPECIMENS							
Samani . .	2	2	6	6	3	1	1
Shoya . .	8	3	3	5	3	1	1

from the intraspecific chromosomal variations in *T. kamtschaticum* (figs. 5 and 6).

Chromosome constitutions of K_1 in 59 triploid hybrids are presented in table 1, the constitutions of K_2 and T being omitted. At Kusakai, all 50 plants of *T. kamtschaticum* showed one and the same homozygous

FIG. 7.—*T. Hage* (6x) homozygous allohexaploid from Sizunai. $\times 1500$.

constitution ; A-13·13, B-12·12, C-6·6, D-3·3, and E-7·7 (fig. 2). The constitutions of K_1 of all six hybrids from this locality were revealed to be the same as the gametic set of *T. kamtschaticum* at this locality ; A-13, B-12, C-6, D-3 and E-7 (fig. 5). This is a natural consequence of the complete homozygosity of the diploid parent population at this locality. On the other hand, at localities where

TABLE 2
*Frequencies in T. kamtschaticum populations of the chromosome types
comprised in the triploid hybrids*

Population *	Chromosome type and its actual frequency ()				
Kusakai . .	A13 (100)	B12 (100)	C6 (100)	D3 (100)	E7 (100)
Siraoi . .	A13 (44) A14 (56)	B6 (60) B7 (18)	C6 (100) ...	D3 (27) D7 (73)	E2 (100) ...
Nisikioka **	A38 (30)	B2 (30)	C6 (30)	D7 (30)	E3 (30)
Samani . .	A2 (14) A5 (19) A6 (7) A8 (3) A16 (12) A17 (2)	B2 (63) B3 (33) B14 (0)	C2 (8) C3 (31) C5 (22) C6 (16) C7 (2) C9 (1)	D5 (26) D6 (39) D7 (14)	E1 (9) E3 (67) E4 (17) E5 (1)
Toyoni **	A5 (11) A10 (4) A14 (1) A18 (8) A21 (5) A23 (10)	B1 (23) B2 (100) B3 (59)	C3 (155) C8 (0)	D5 (34)	E1 (12) E2 (0) E8 (5) E1 (12) E2 (0)
Minami-Sat **	A10 (6) A16 (4)	B2 (23) B3 (13)	C3 (33) C5 (1)	D5 (34) ...	E3 (20) E4 (13)

* Shadai is located close to Nisikioka, and Samani to Utoma. The number of plants observed is as follows : Kusakai = 50, Siraoi = 50, Nisikioka = 15, Samani = 50, Toyoni = 86, and Minami-Satunai = 36.

The number of chromosomes observed for each of the chromosomes, A to E, is twice the number of plants observed. (ex Haga and Kurabayashi, 1954, and unpublished data (**)) accumulated by the *Trillium* Research Group of the Hokkaido University).

T. kamtschaticum populations are large, dense and highly heterozygous, the constitution of K_1 in the hybrids varies from plant to plant even within a population, that is, at Utoma, Minami-Satunai and Toyoni (fig. 6 and table 1). Thus it is evident that the hybrids with different K_1 constitutions were raised through matings of the *T. kamtschaticum* gametes of various constitutions with those of *T. Tschonokii*. Further, we can attribute the uniformity of the chromosome complements of the hybrids in certain populations to the complete or very high homozygosity of the *T. kamtschaticum* population. Such cases are found

at Hagino and Shadai in addition to the case found at Kusakai (table 1). Generally speaking, K_1 comprised in the hybrids is found to consist of chromosomes which are frequent in the *T. kamtschaticum* population at the locality where the hybrids were sampled (tables 1 and 2). In other words, hybridisation takes place where both the parents are growing close together, and the hybrids grow up to maturity in the localities native to their parents (fig. 8).

FIG. 8.—A map of the northern districts of Japan, showing localities where the hybrids *T. Hage* (3x) were found growing, except at Ino (2) and Tanabu (3) where no hybrid was recorded.

Chromosome constitutions of the doubled hybrids were analysed in two plants, one from Sizunai and the other from Utoma (fig. 8). These two plants were homozygous for all three sets K_1 , K_2 and T (fig. 7). However, these two doubled plants were different in respect to the constitution of K_1 as shown below.

Sizunai	A-5.5	B-4.4	C-6.6	D-5.5	E-2.2
Utoma	A-5.5	B-2.2	C-6.6	D-5.5	E-3.3

All the chromosome types were those found to be highly frequent in *T. kamtschaticum* populations at Sizunai and Samani, the latter locality being close to Utoma. At Utoma *T. kamtschaticum* and *T. Tschonoskii* grow in abundance but unfortunately these parental

plants at this locality have not been analysed cytologically as yet. The chromosome doubling manifested in the hexaploid may have occurred in one of the following three ways; firstly, by the union of unreduced gametes produced in F_1 sterile triploid hybrids, secondly, by the union of unreduced gametes produced separately in both parents, and thirdly, by chromosome-doubling in the F_1 fertilised egg cells. The first possibility seems nearly negligible as is evident in the meiotic chromosome behaviour in F_1 triploid hybrids (*cf.* Haga, 1951; Haga and Takizawa, 1953). The second alternative is not utterly impossible, since unreduced pollen grains are produced with a frequency as high as 17 per cent. in *T. kamtschaticum* under extreme conditions (Haga and Kayano, in press). But it is not likely since no hybrid was of the composition $K_1K_1K_2T$ or $K_1K_2K_2TT$, which, if the second process is really occurring, will be of more frequent occurrence than the hybrids $K_1K_1K_2K_2TT$. Thus chromosome-doubling in the fertilised F_1 egg cell seems the most probable. This process further explains why the two sets K_1K_1 are homozygous in the doubled hybrids, despite the fact that they were sampled in the highly heterozygous *T. kamtschaticum* populations.

4. POLYPLOIDY

Speciation by polyploidisation takes place by means of the following three steps in a serial succession. First, genetic differentiation of the genomes which are to be combined in a new polyploid species, second, hybridisation of parental plants having differentiated genomes, and, third, chromosome-doubling which makes the hybrids constant through generations. Three genomes, K_1 , K_2 and T , are those differentiated from each other, K_1 being intimately homologous with K_2 , and T homologous with both, though the latter relation is not intimate. The triploid hybrids, K_1K_2T , show meiotic pairing $5_{11}+5_1$ and nearly complete sterility in seed setting, no F_2 or back-cross progeny being found in nature. As the chromosome-doubled hybrid, $K_1K_1K_2K_2TT$, forms 15_{11} by the differential affinity between the three genomes, the hybrids are perfectly fertile and capable of producing offspring.

Experimental crosses between the parental plants are easily attained irrespective of the direction of crossing, and as many good seeds are produced as are borne on the parent plants. Unfortunately, artificial germination of the seeds produced either by the hybrids or by the parental plants has not been successful. However, a method of germination by Barton (1944) was successful.

In nature, F_1 hybrids are found at almost every locality where both parental species are found growing together (fig. 8). The frequency of these F_1 hybrids will depend on various ecological factors: on numerical proportions and densities of the two parental species, and time relation in flowering of the parental plants. For example, even within the species *T. kamtschaticum* the average date of flowering

in the *Trillium* Reservatory Garden of the Hokkaido University, Sapporo, varied among the plants sampled from different localities. In 1953 it varied from May 4th to 5th in plants from Saruru (close to Shoya, cf. 11 in map) to May 13th to 14th in plants from Tomikawa close to Biratori, cf. 7 in map) (cf. Hiraizumi, 1954). Thus the frequency of hybridisation will vary at different localities and in different niches.

TABLE 3
Frequencies of parental plants and their hybrids in a summer-green forest at Siraoi (ex Haga 1942).

Year	1937	1939	1940	1941	Total	Per cent.
<i>T. kamtschaticum</i>	216	150	941	706	2016	56.5
<i>T. Hagae</i> (3x)	34	18	74	43	169	4.7
<i>T. Tschonoskii</i>	224	185	494	481	1380	38.7
Total	474	349	1509	1233	3565	99.9

With the assistance of several students, collections of *Trillium* plants were carried out at Siraoi in successive years 1937-41 (except 1938) with no preference for particular species or hybrids. The purpose of the collecting in these years was to stock the *Trillium* Reservatory Garden, therefore the records are not critical from an ecological point of view. However it will be helpful to see how frequently hybridisation is occurring at this locality. According to the records, the occurrence of F_1 hybrids is 4.7 per cent. of the total number of plants (table 3).

TABLE 4
Chromosome types of K_1 which have reached fixation

Population	Chromosome types					Species	n
Kusakai	A13	B12	C6	D3	E7	<i>T. kamtschaticum</i>	50
	A13	B12	C6	D3	E7	<i>T. Hagae</i> (3x)	6
Hagino	A13	B2	C6	D3	E2	<i>T. Hagae</i> (3x)	11
Siraoi	...	...	C6	...	E2	<i>T. kamtschaticum</i>	50
	...	B6 *	C6	...	E2	<i>T. Hagae</i> (3x)	9
Shadai	A13	B7	C6	D7	E2	<i>T. Hagae</i> (3x)	7
Nisikioka	A38	B2	C6	D7	E3	<i>T. kamtschaticum</i>	15

* Cf. table 2, Siraoi.

5. POPULATION STRUCTURE

Chromosome pairs of *T. kamtschaticum* are homozygous in small isolated populations, individual plants being composed of the same homozygous five pairs of chromosomes. This circumstance is manifested directly by the analysis of *T. kamtschaticum* populations and indirectly by the surveys of the constitutions of K_1 in the hybrids. According to these lines of evidence four populations of *T. kamtschaticum* seemed to have reached fixation in all five chromosome pairs (table 4).

Here it is important to note that chromosome constitutions of homozygous or nearly homozygous *T. kamtschaticum* populations are different from one another even though they are located close together, that is, populations at Shadai, Nisikioka, Siraoi and Hagino. Indeed, Shadai and Nisikioka are separated by only a few hundred metres; Shadai is located about 4 kilometres east of Siraoi and Hagino is located about 8 kilometres west of the latter locality (fig. 8). Intervening areas between these localities are occupied by dense forests, by meadows, or by agricultural land where *Trillium* plants are now entirely absent or almost so. The fixation of a definite chromosome constitution at these localities is considered to have been brought about by the cooperation of genetic drift and severe selection in small populations (Haga and Kurabayashi, 1954). Then chromosome-doubled hybrids, raised in isolated *T. kamtschaticum* populations such as are described above, would be different in their genetic constitutions from the very time of origin.

Large *T. kamtschaticum* populations, on the other hand, or those that have recently become small and discontinuous, are highly heterozygous for many chromosome types. In some extreme cases, every plant of a population differs from all the other members of the population in its chromosome constitution (Haga and Kurabayashi, 1954). It is not surprising therefore that the doubled hybrids appearing among such populations of *T. kamtschaticum* vary from plant to plant. In other words, the genetic variations in the allopolyploid species originate from the genetic variations already present within, as well as between, the populations of the lower-ploid progenitors. Of course, the variation will proceed further and seems to accumulate from the time of origin of the polyploid species (*cf.* Haga, 1940; Stebbins, 1950).

6. SUMMARY

1. The genome of the diploid *T. kamtschaticum*, K_1 , shows great variation. Many different types of chromosomes are distinguished in all five members, A to E, by the patterns of heterochromatic segments showing nucleic acid starvation. Populations of this species are either (i) completely homozygous for a certain definite type in all of the five chromosomes; or (ii) heterozygous for many types in some or all of the five chromosomes.

2. The two genomes of the tetraploid *T. Tschonoskii*, K_2 and T, were distinguishable in all five chromosome types but they revealed no heterozygosity or even variation between themselves which was studied in detail by the patterns of heterochromatic segments in the cold-treated chromosomes.

3. Natural hybrids *T. Hagae* ($3x = 15$, K_1K_2T and $6x = 30$, $K_1K_1K_2K_2TT$) between *T. kamtschaticum* and *T. Tschonoskii* are found at nearly all the localities where the parental species are growing together.

4. Triploid hybrids from a homozygous *T. kamschaticum* population possess genome K_1 identical with that of the homozygous diploid progenitor. Those from the heterozygous population show K_1 constitutions differing from plant to plant in accordance with the heterozygosity of the population of the diploid parent.

5. Generally, the K_1 chromosome types of the triploid hybrids were the same ones found in the neighbouring *T. kamschaticum* populations.

6. Both the chromosome-doubled hybrids sampled in two highly heterozygous *T. kamschaticum* populations proved to be homozygous for K_1K_1 . But the K_1 constitutions of these two plants were different. Chromosome doubling has thus taken place after fertilisation in the F_1 and has been followed by inbreeding. In this way new constant hexaploid plants are arising at present in nature and the genetic variations may be involved in allopolyploid species from the time that they arise.

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* Cf. English summaries in *Heredity*, 4, 391.

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