C-band Polymorphism in *Paris tetraphylla* Chromosomes in Four Populations of Kumamoto Prefecture

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The *Paris tetraphylla* (*Liliaceae*) is a perennial herb distributed widely in the Japanese Islands; Hokkaido, Honshu, Shikoku and Kyushu (Kitamura *et al.* 1987). This species usually grows in the broad-leaved deciduous forest of mountainous regions, but rarely in grasslands and so on. Four populations of the species have been found in Kumamoto Prefecture of Kyushu, *i.e.*, Miike (broad-leaved deciduous forest), Kunimi (broad-leaved deciduous forest), Kikuchi (evergreen-deciduous mixed forest), and Aso (grassland).

Recently, the species ($2n=2x=10$) was found to be chromosomally polymorphic as revealed by the patterns of C-banded segments (Miyamoto and Kurita 1990, Uchino and Wang 1996). The purpose of the present article is to report detailed features of chromosomal polymorphism due to the patterns of C-banded segments and to discuss relationships between chromosomal polymorphism and environmental conditions with special reference to vegetation in the four populations.

Materials and methods

The plants of *Paris tetraphylla* A. Gray (*Liliaceae*) were collected from the four natural populations in Kumamoto Prefecture of Kyushu; 50 plants from Miike (Mi), 50 plants from Kunimi (Kn), 50 plants from Kikuchi (Kk), and 9 plants from Aso (As). The Mi and Kn populations are located at the mountainous region of central Kyushu and are ca. 10 km distant from each other. The Kk and As populations are located at the Aso area and the distance between them is ca. 11 km. The As population is ca. 50 km distant from Kn population (Table 1, Fig. 1). The Mi and Kn populations were similar in environmental condition and vegetation, *i.e.*, *Leucosceptrum stellipilum* var. *tosaense*–*Fagus crenata*–Gesellschaft in Mi population and *Sapio japonici*–*Fagetum crenatae* in Kn population. The vegetations of Kk and As populations were evergreen-deciduous mixed forest and *Arundinaria pygmaeae*–*Miscanthum sinensis*, respectively, and also the populations differed in their environmental conditions (Table 1).

Young ovular tissues were used for the analyses of C-banded segments in chromosomes. Cytological preparations were made according to the method described in the previous paper (Uchino and Wang 1996). Briefly, the ovules were pretreated with a 0.002 mol aqueous solution of 8-hydroxyquinoline before fixation with Carnoy's solution. The ovules were macerated and then squashed in 45% acetic acid solution. After coverslips were removed, the slides were air dried. They were then immersed in a 4% Ba(OH)₂ solution, washed in distilled water and incubated in 2×SSC solution. The slides were rinsed in distilled water and stained with 1% Giemsa solution (PBS, pH 6.8). Finally the preparations were rinsed in distilled water, air dried and mounted.

Table 1. Location and vegetation of the four populations of *Paris tetraphylla* in Kumamoto Prefecture

Population	Location and vegetation
Miike (Mi)	Yatsushiro-gun, Izumi-mura, Mt. Shiratori-yama. Altitude: 1,550–1,600 m (mountainous region slope) Vegetation: <i>Leucosceptrum stellipilum</i> var. <i>tosaense</i> – <i>Fagus crenata</i> –Gesellschaft Growing plants: <i>Pterocarya rhoifolia</i> , <i>Fagus crenata</i> , <i>Elatostema umbellatum</i> , <i>Laportea bulbifera</i> , <i>Persicaria debilis</i> , <i>Paeonia japonica</i> , <i>Hydrangea macrophylla</i> var. <i>acuminata</i> , <i>Kirengeshoma palmata</i> , <i>Acer rufinerve</i> , <i>Euonymus fortunei</i> vari. <i>radicans</i> , <i>Viola shikokiana</i> , <i>Leucosceptrum stellipilum</i> var. <i>tosaense</i> , etc.
Kunimi (Kn)	Yatsushiro-gun, Izumi-mura, Mt. Kunimi-dake. Altitude: 1,550–1,600 m (mountainous region slope) Vegetation: <i>Sapio japonici</i> – <i>Fagetum crenatae</i> Growing plants: <i>Fagus crenata</i> , <i>Lindera sericea</i> , <i>Parabenzoin trilobum</i> , <i>Stewartia monodelpha</i> , <i>Hydrangea luteovenosa</i> , <i>Sapium japonicum</i> , <i>Acer sieboldianum</i> , <i>Enkianthus cernus</i> f. <i>nipponicus</i> , <i>Rhododendron degranianum</i> ssp. <i>metternichii</i> , <i>R. reticulatum</i> , <i>Styrax shiraianus</i> , <i>Symplocos coreana</i> , <i>Sasamorpha borealis</i> , etc.
Kikuchi (Kk)	Kikuchi-city, Fukaba, Kikuchi-suigen. Altitude: ca. 600 m (forest along mountain stream) Vegetation: Evergreen–deciduous mixed forest Growing plants: <i>Abies firma</i> , <i>Torreya nucifera</i> , <i>Quercus salicina</i> , <i>Q. sessilifolia</i> , <i>Celtis jessoensis</i> , <i>Zelkova serrata</i> , <i>Cercidiphyllum japonicum</i> , <i>Camellia japonica</i> , <i>Acer mono</i> , <i>A. palmatum</i> , <i>A. sieboldianum</i> , <i>Cornus controversa</i> , <i>Pterostyrax corymbosus</i> , etc.
Aso (As)	Aso-gun, Minamioguni-machi, Myogatani. Altitude: ca. 800 m (grassland) Vegetation: <i>Arundinario pygmaeae</i> – <i>Miscanthetum sinensis</i> Growing plants: <i>Osmunda japonica</i> , <i>Pteridium aquilinum</i> var. <i>latiusculum</i> , <i>Ranunculus japonicus</i> , <i>Potentilla fragarioides</i> var. <i>major</i> , <i>P. freyniana</i> , <i>Sanguisorba officinalis</i> , <i>Lespedeza bicolor</i> , <i>Viola mandshurica</i> , <i>Gallium verum</i> f. <i>nikkoense</i> , <i>Achillea alpina</i> ssp. <i>subcartilaginea</i> , <i>Arundinella hirta</i> , <i>Imperata cylindrica</i> var. <i>koenigii</i> , <i>Miscanthus sinensis</i> , <i>Pleiblastus chino</i> var. <i>viridis</i> , <i>Zoysia japonica</i> , etc.

Results

1. Classification of chromosome types

The *Paris tetraphylla* has a diploid chromosome number of $2n = 10$. The five chromosomes constituting a genomic set were designated according to length as A, B, C, D and E, A being the longest and E the shortest (Haga 1934, Uchino and Wang 1996). Among them, chromosome A was metacentric and B, C and E were submetacentric, but D was subtelocentric. Chromosome D usually has a small satellite attached to the distal end of the short arm, but the appearance of the satellite and length of stalk were variable. In addition, telocentric small supernumerary chromosomes were found occasionally in some plants.

Positive segments (C-bands) for preferential Giemsa staining were found in some regions of all chromosomes A to E (Fig. 2). The C-banded pattern was polymorphic in each of chromosomes A to E. According to the location along the chromosomes, five classes of C-bands were distinguished: satellite, telomeric, centromeric, pericentric and intercalary bands. If the size of bands are regarded as criteria, three kinds of bands were recognized: thick, thin and granular bands. However, small granular bands were unstable in the occurrence on chromosomes. The number of different types due to the patterns of C-banded chromosomes was 5 in each of chromosomes A, B and E, 3 in C, and 4 in D (Fig. 3). The different types were

indicated by numbering, *e.g.*, A1–A5 in chromosome A, B1–B5 in B, *etc.*

Chromosome A (A1–A5): A1 and A2 have a intercalary band near the centromeric region in one arm. A1 and A2 differ from each other in the other arm. A1 has no C-band but A2 has a thick intercalary band near the centromeric region. Three types, A3 to A5, all have two intercalary bands in one arm but differ from each other in the other arm. A3, A4 and A5 have no band, one thick intercalary band and two intercalary bands, respectively. In addition, a granular centromeric band is present at all types and small granular bands are on the telomeric and intercalary regions of both arms of all types.

Chromosome B (B1–B5): B1 and B2 have a intercalary band near the centromeric region in the long arm but differ from each other in the absence or presence of a intercalary band near the centromeric region in the short arm. B3 and B4 have two intercalary bands in the long arm but also differ from each other in the absence or presence of a intercalary band in the short arm. B5 has two thick and a granular bands only in the

Fig. 1. Location of the four populations (●) of *Paris tetraphylla* in Kumamoto Prefecture.

Fig. 2. C-banded metaphase in an ovular tissue of *Paris tetraphylla* ($2n=2x=10$). Bar indicates $10\ \mu\text{m}$. Chromosome constitution of the plant is A-2·4, B-2·3, C-1·1, D-1·2, E-2·4.

Fig. 3. Schematic representation of chromosome types due to the patterns of C-bands in *Paris tetraphylla*.

long arm. In addition, a granular centromeric band is present in all types and small granular bands are on the telomeric and intercalary regions of both arms of all types.

Chromosome C (C1–C3): C1 and C2 have a intercalary band near the centromeric region in the long arm. C1 has a thin pericentric and a large distal bands in the short arm. C2, however, has a intercalary band in addition to their bands in the short arm. C3 shows the same pattern as C2 in the short arm but has two intercalary bands in the long arm. In addition, a granular centromeric, a granular telomeric, as well as intercalary bands are present on the long arm of all types.

Chromosome D (D1–D4): D1 and D2 are similar in a thick intercalary band in the long arm and in the C-band positive short arm. D2, however, has a C-band positive satellite. D3 differs from D2 in a thick pericentric band in the long arm. D4 has a band near the centromeric region and a thick intercalary band in the long arm. The short arm of D4 has a C-band positive region and a large satellite which has a band near the secondary constriction. No granular centromeric bands were found but telomeric bands are present on the long arm of all types.

Chromosome E (E1–E4): E1 has a granular centromeric band, a granular intercalary as well as telomeric bands in both long and short arms. E2 differs from E1 with respect to an intercalary band in the long arm. E3 has a intercalary band in the short arm in addition to the

bands of E2. E4 and E5 have two intercalary bands in the long arm. E4 has no band but E5 a intercalary band in the long arm. In addition, a granular centromeric band is present in all types and small granular bands are on the telomeric and intercalary regions of both arms of all types.

Supernumerary chromosome: This was a small telocentric chromosome and its entire arm was C-banding positive.

2. Frequency of different chromosome types in the four populations

The chromosomal polymorphism due to the patterns of C-bands was found in all of the chromosomes A to E from the four populations (Table 2, Fig. 4).

Chromosome A: Four, three, four and three chromosome types were observed in Mi, Kn, Kk and As populations, respectively. A1 was observed in three populations except for As population. Two types of A2 and A4 were found in all populations. A3, however, was distinctive in Kk population and A5 was observed in Mi and As populations. The most

Table 2. Frequency of different types of chromosomes A to E in four populations of *Paris tetraphylla* in Kumamoto Prefecture*

Chromosome type	Population				Total
	Mi	Kn	Kk	As	
A1	34	40	6	—	80
A2	40	33	29	4	106
A3	—	—	9	—	9
A4	21	27	56	10	114
A5	5	—	—	4	9
Total	100	100	100	18	318
B1	24	44	4	2	74
B2	14	17	—	—	31
B3	62	39	44	4	149
B4	—	—	1	3	4
B5	—	—	51	9	60
Total	100	100	100	18	318
C1	60	65	42	5	172
C2	24	33	58	7	122
C3	16	2	—	6	24
Total	100	100	100	18	318
D1	1	20	—	—	21
D2	50	76	69	7	202
D3	45	4	31	11	91
D4	4	—	—	—	4
Total	100	100	100	18	318
E1	2	7	—	8	17
E2	54	53	33	3	143
E3	13	11	1	6	31
E4	31	29	66	1	127
E5**	—	—	+	—	—
Total	100	100	100	18	318

*Supernumerary chromosomes were found in thirteen plants among 50 plants from the Kn and all nine plants from the As populations. **E5 is the type found in plants from Kk population by the other study (Uchino *et al.* unpubl.).

Fig. 4. Pie chart of the frequency (%) of different types of chromosomes A to E in *Paris tetraphylla*.

frequent types were A2, A1, A4 and A4 in Mi, Kn, Kk and As populations, respectively. The next frequent types, and also, were A1 in Mi and A2 in Kn, Kk and As populations, respectively. The Mi and Kn populations, thus, were similar in the frequency of chromosome types, except for A5, but different from the other populations. The Kk and As populations were similar in the frequency of A2 and A4 but different in those of A1, A3 and A5.

Chromosome B: Two out of five types, B1 and B3, were common in all populations. B2, however, was found in Mi and Kn populations and B4 and B5 were in Kk and As populations. The most frequent type was B3 in Mi, B1 in Kn, B5 in Kk and As populations. Consequently, the Mi and Kn populations were slightly different in the frequency of chromosome types. The remaining two populations were different from the former populations but similar in the frequency of chromosome types each other; the most frequent type being B5 and the next type B3 in both populations.

Chromosome C: C1 and C2 were found in all populations but C3 not in Kk population. C1

was the most frequent in Mi and Kn populations and C2 in Kk and As populations. The Mi and Kn populations were similar in the frequency of chromosome types. The Kk and As populations were similar in the most frequent type but different in the absence of C3 in the Kk population.

Chromosome D: Four, three, two and two chromosome types were observed in Mi, Kn, Kk and As populations, respectively. D1 was observed in Mi and Kn populations and next most frequently in Kn population. D2 and D3 were common in all populations and D2 was the most frequent type in Mi, Kn and Kk populations, but its frequency differed among them. D3 was the most frequent in As population and the next most frequent in Mi and Kk populations. The As population, thus, differed from the other populations. D4 was found only in Mi population and, therefore, Mi population was distinguished from the other populations.

Chromosome E: Out of five types, E1 was found in Mi, Kn and As populations and was the most frequent in As population. Three types of E2, E3 and E4 were common in all populations. E2 was the most frequent in Mi and Kn populations and E4 in Kk population. E5 was found in the plants from Kk population by the other study (Uchino *et al.* unpubl.). The Mi and Kn populations were very similar in the frequency of chromosome types. The Kk population was different from the other populations in the most frequent E4, the next most frequent E2, and the absence of E1. The As population was also different from the other populations in the most frequent E1 and the next most frequent E3.

Supernumerary chromosome: Supernumerary chromosome was found in thirteen plants among 50 plants from Kn and all nine plants from As populations. Out of thirteen plants from Kn population, 6, 2, 2 and 1 plants carried 1, 3, 4 and 5 supernumeraries, respectively. The number of supernumeraries was stable among cells of each plant. However, intra-individual variation of the number of supernumeraries was observed in the remaining two plants. In the first plant, 79 cells (69.9%) showed $2n$, 12 cells (10.6%) $2n+1$ supernumeraries, 20 cells (17.7%) $2n+2$ supernumeraries, 2 cells (1.8%) $2n+3$ supernumeraries. In the second plant, the number of supernumeraries varied as follows: 0 supernumeraries (3 cells), 1 supernumeraries (3 cells), 3 supernumeraries (2 cells). Out of nine plants from As population, 8 and 1 plants carried 1 and 2 supernumeraries, respectively. The number of supernumeraries was stable among cells of each plant.

Discussion

The number of different types due to the patterns of C-banded chromosomes was 5 in each of chromosomes A, B and E, 3 in C, and 4 in D from a total of 159 plants from the four populations (Fig. 3). Miyamoto and Kurita (1990) analyzed the 692 plants from 38 localities of Hokkaido to Yakushima and found 3 types in chromosome A, 5 in B, 4 in C, 2 in D and 4 in E. In comparing these results, though the numbering systems are different from each other, 12 types of A3, A4, A5, B3, B4, B5, C3, D1, D3, D4, E4 and E5 were newly found in this study. The difference in the number of chromosome types between the two data sets might be due to the difference of materials, ovules and root-tips, and/or analyzed populations. *Paris tetraphylla*, in any case, was chromosomally highly polymorphic as to the patterns of C-banded segments; 6 types in chromosome A, 8 in B, 5 in C and D, and 6 in E in a total of the two data (Fig. 3, Miyamoto and Kurita 1990).

In comparison to the frequency of different chromosome types among populations (Table 2, Fig. 4), as already mentioned, Mi and Kn populations were similar in chromosome A, except for A5. Each of Kk and As populations was different from the other populations. In chromosome B, Mi and Kn populations were slightly different in the frequency of chromosome types. The remaining two populations were different from the former populations but similar

to each other in the frequency of chromosome types. By the most frequent types, C1 and C2, the four populations could be divided into the two groups. The Mi and Kn populations were similar in the frequency of chromosome types. The Kk and As populations were similar in the most frequent type, C2, but different in the absence of C3 in Kk population. In chromosome D, Mi population was distinguished from the other populations in the occurrence of D4. The Kn and Kk populations were different in the next most frequent types. The As population differed in having the most frequent D3 of all the populations. In the frequency of different types of chromosome E, Mi and Kn populations were very similar. The Kk and As populations were different from the other populations in the most frequent, E4 and E1, and the next most frequent types, E2 and E3. Consequently, Mi and Kn populations were more or less similar in the frequency of different types of chromosomes A to E. On the other hand, Kk and As populations were different from the other populations. These facts are in line with the difference of environmental condition and vegetation among populations. The environmental condition and vegetation were similar in Mi and Kn populations, *i.e.*, *Leucosceptrum stellipilum* var. *tosaense*-*Fagus crenata*-Gesellschaft in Mi population and *Sapio japonici*-*Fagetum crenatae* in Kn population. The Kk and As populations, however, were different from the other populations in the environmental condition including vegetation, respectively.

The observations that the heterochromatic chromosome segments are genetically inert have been made on several species (Brown 1966, Sieger *et al.* 1970, *etc.*). On the other hand, there are many examples of the genetic activity of the heterochromatin (Lima-de-Faria 1986). For example, the genetic activity within a heterochromatic region was provided by the delimitation of *nv* gene to the long arm of chromosome 9 in *Lycopersicon esculentum* (Khush *et al.* 1964). Ordinary Menderian genes, further, have been found within the C-heterochromatin of *Drosophila melanogaster* (Hilliker and Sharp 1988). It might be, therefore, reasonable for the consideration that the genetic activity exists within C-heterochromatic chromosome segments of *Paris tetraphylla*. Thus, the frequency of various chromosome types due to C-bands might be different according to the adaptability for environmental conditions including vegetation among the populations. Miyamoto and Kurita (1990) found in this species that several chromosome types were localized in specific geographic regions. In *Trillium kamtschaticum* being the relative species of *Paris*, also, various chromosome types revealed by the cold-sensitive heterochromatin differentiate geographically in their occurrence (Haga and Kurabayashi 1954, Fukuda and Kozuka 1958, Kurabayashi 1958, Haga 1974, *etc.*). Moreover, the total number of C-bands in chromosomes of *Chrysanthemum indicum* correlates with the warmth index (Taniguchi 1987). These facts, thus, might support our consideration.

The supernumerary chromosome found in this study was coincident with ones of the previous reports on the morphology and C-banding positive nature (Haga 1961, Kayano 1961, Miyamoto and Kurita 1990). The intra-individual variation of the number of supernumeraries was already found (Haga *op cit.*) and was presumed to be caused by the lagging and unequal distribution of sister chromatids of supernumerary chromosome at the mitotic anaphase (Kayano *op cit.*). The supernumeraries might be considered to have no relation with the adaptability for the environmental condition. The reason is as follows: The chromosome occurred in both Kn and As populations but not in Mi population, though environmental conditions of Kn and Mi populations were similar to each other but that of As population different from the other populations. Moreover, the individuals carrying 0, 1, 3, 4 and 5 supernumeraries were mingled in Kn population. The presence or absence of supernumeraries in Kn and Mi populations implies that the founders were different in their populations despite their near location. It is also true for Kk and As populations. These might support a speculation that different chromosome types due to C-bands distribute according to the adaptability for environmental conditions including vegetation among the populations.

Summary

The C-band polymorphism of chromosomes in *Paris tetraphylla* ($2n=2x=10$) was confirmed in the plants from the four populations, *i.e.*, Miike (Mi), Kunimi (Kn), Kikuchi (Kk) and Aso (As), in Kumamoto Prefecture of Kyushu. The five chromosomes, A, B, C, D and E, constituting a genomic set were polymorphic for the C-banded patterns, respectively. The number of different types due to the patterns of C-banded chromosomes was 5 in each of chromosomes A, B and E, 3 in C, and 4 in D. In addition, small telocentric supernumerary chromosome was C-banding positive in its entire arm.

The patterns of occurrence of various types of chromosomes A to E were more or less similar between Mi and Kn populations, there being similar environmental conditions, including vegetation. On the other hand, Kk and As populations were different from the other populations in the patterns of occurrence of various chromosome types and in their environmental conditions including vegetation. Therefore, it might be considered that different chromosome types due to the patterns of C-bands distribute according to the adaptability to environmental conditions including vegetation among the populations. This speculation might be supported by the occurrence of supernumerary chromosomes in both Kn and As populations but absence in both Mi and Kk population.

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