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# A reconsideration of relationships among Japanese *Trillium* species based on karyology and AFLP data

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**Abstract.** To reexamine the relationships among the Japanese *Trillium* species that form a polyploid series, we performed principal-coordinates analysis (PCOA) based on proposed karyotypic compositions and on amplified fragment length polymorphism (AFLP) analysis. A hexaploid species, *T. smallii*, whose karyotypic composition had been hypothesized as $K_2K_2SSUU$, with hybridization between tetraploid *T. apetalon* (SSUU) and a presumed $K_2K_2$ diploid species, showed a genotype corresponding to *T. yezoense* ($K_1SU$). Accordingly, *T. smallii* appears to be an allopolyploid of *T. yezoense*, with the karyotypic composition $K_1K_1SSUU$. *Trillium channellii*, a recently described tetraploid species whose origin and genealogical position remains unclear, showed a genotype corresponding to $K_1K_1K_2T$. We conclude that *T. channellii* may be derived from hybridization of *T. camschatcense* ($K_1K_1$) as the seed parent and *T. tschonoskii* ($K_2K_2TT$) or hexaploid *T. hagae* ($K_1K_1K_2K_2TT$) as the pollen parent.

**Key words:** AFLP, allopolyploid, karyotype, natural hybridization, PCOA, polyploidization, *Trillium*.

Natural hybridization and subsequent polyploidization have been recognized as important factors in plant speciation (Stebbins 1950, Grant 1971, Rieseberg 1997, Soltis and Soltis 1999), and Masterson (1994) estimated that 70% of angiosperms have undergone one or more episodes of polyploidization. Natural hybridization, however, is nonrandomly distributed among plant taxa, but concentrated in certain families and genera suggesting that certain phylogenetic groups are predisposed for the formation and maintenance of hybrids (Ellstrand et al. 1996).

*Trillium* is a herbaceous member of the Melanthiaceae (APG II 2003), formerly placed in a family named Trilliaceae (*sensu* Dahlgren et al. 1985) along with *Paris*, *Daiswa*, and *Kinugasa*. The relations between *Trillium* with other three genera, *Paris*, *Daiswa*, and *Kinugasa*, have been studied by means of morphological and molecular data analysis, indicating the monophyly of *Trillium* (Farmer and Schilling 2002). The genus *Trillium* contains about 45 species, of which 11 occur in eastern Asia, and the remainder in North America (Freeman 1975, Samejima and Samejima 1987). Although the North American species are highly diverse in their gross morphology, comprising both pedicellate- (erect and declinate) and sessile-flowered groups, all of them are known to be diploid ($2n = 10$), except for the rare occasional triploid and supernumerary aneuploid (Bailey 1954, Darlington and Shaw 1959, Fedorov 1969). On the other hand, the

Asiatic species represent past hybridization and polyploidization events, and all but one species are polyploids. Therefore, elucidating the relationships of polyploids would be a necessary step toward considering the contrasting occurrence of polyploids between the continents.

Of the 11 Asiatic species, two species are endemic to the Himalayas (*T. govanianum* Wallich et Royle) and Taiwan (*T. taiwanense* Ying), and the remaining nine species are found in Hokkaido and Honshu, Japan. Extensive chromosome studies have confirmed that the Japanese *Trillium* species comprise polyploids ($2n = 10$, 15, 20 and 30) with a basic chromosome number five, designated as A, B, C, D, and E according to their length (Gotoh and Stow 1930, Haga 1934). The chromosome A, B, C, D, and E in Haga and Kurabayashi (1953) correspond to I, II, III, IV, and V in Warmke and Johansen (1935), and A, B, C, E, and D in Darlington and La Cour (1940) respectively. Comparing the differential chromosome segments after cold-treatment, each of these five chromosomes were further classified. A combination of the chromosome sets gave the karyotypic component $K_1$, $K_2$, T, S, U (Haga and Kurabayashi 1953; Haga 1937, 1951, 1956). Recently, a study using AT-specific fluorochrome revealed that the karyotypic component $K_2$ is more similar to T, rather than $K_1$ (Myakoshina et al. 2004). While they argued that the nomenclature of the five karyotypic component should be K, $T_1$, $T_2$, S, U instead of the conventional $K_1$, $K_2$, T, S, U, it is impregnable that there are five distinctive karyotypic component in the Japanese *Trillium*, and even allowing for the nomenclature, relationships among species are unchangeable.

Based on these karyotypic components, the relationships among Japanese *Trillium* species have been proposed as shown in Fig. 1 (Kurabayashi 1958). The three progenitor species, which are the diploid *T. camschatcense* Ker Gawler ($2n = 10$) and the tetraploids *T. tschonoskii* Maximowicz ($2n = 20$) and *T. apetalon* Makino ($2n = 20$), hybridize with each other and produce three sterile hybrids: *T. hagae* Miyabe et Tatewaki ($2n = 15$), *T. miyabeanum* Tatewaki ($2n = 20$), and *T. yezoense* Tatewaki ($2n = 15$). In addition, hexaploid *T. hagae* Miyabe et Tatewaki ($2n = 30$) is formed by chromosome doubling of triploid *T. hagae* (Haga 1937, 1951, 1952, 1956, Haga and Kurabayashi 1953, Kurabayashi 1958, Haga et al. 1974). Natural hybridization of these species can be observed in Hokkaido and Honshu, Japan.

However, the origin of hexaploid species, *T. smallii* Maximowicz, has not been explained in terms of hybridization among the three progenitor species: karyotypic composition $K_2K_2$SSUU (Haga and Kurabayashi 1953) of *T. smallii* has been hypothesized to result from hybridization between *T. apetalon* (SSUU) and a hypothetical diploid species with a $K_2K_2$ karyotype (Fig. 1). In addition, neither a karyotypic composition nor a relationship with other hybrids has been identified for a recently described species, *T. channellii*. Fukuda et al. (1996) proposed three hypotheses about the origin and the karyotypic composition of this species: (1) an unreduced gamete from *T. camschatcense* ($K_1K_1$) combined with a normal gamete from *T. tschonoskii* ($K_2$T) would give the karyotypic composition $K_1K_1K_2$T, (2) a gamete from *T. camschatcense* ($K_1$) combined with one from hexaploid *T. hagae* ($K_1K_2$T) would give the same karyotypic composition $K_1K_1K_2$T, and (3) a gamete from *T. camschatcense* ($K_1$) combined with one from a previously unrecognized diploid with a karyotypic composition $K_2K_2$ or TT ($K_2$ or T) would create a diploid hybrid $K_1K_2$ or $K_1$T, which would subsequently become $K_1K_1K_2K_2$ or $K_1K_1$TT by means of somatic doubling.

Although classical methods (e.g. morphologic and karyotypic analyses) are suitable for distinguishing distantly related species, it is dubious for comparing closely related species. Recently, molecular DNA analysis made it possible to investigate the relationships of lower taxonomic levels more accurately. Several phylogenetic studies based on DNA sequence data have been conducted, and

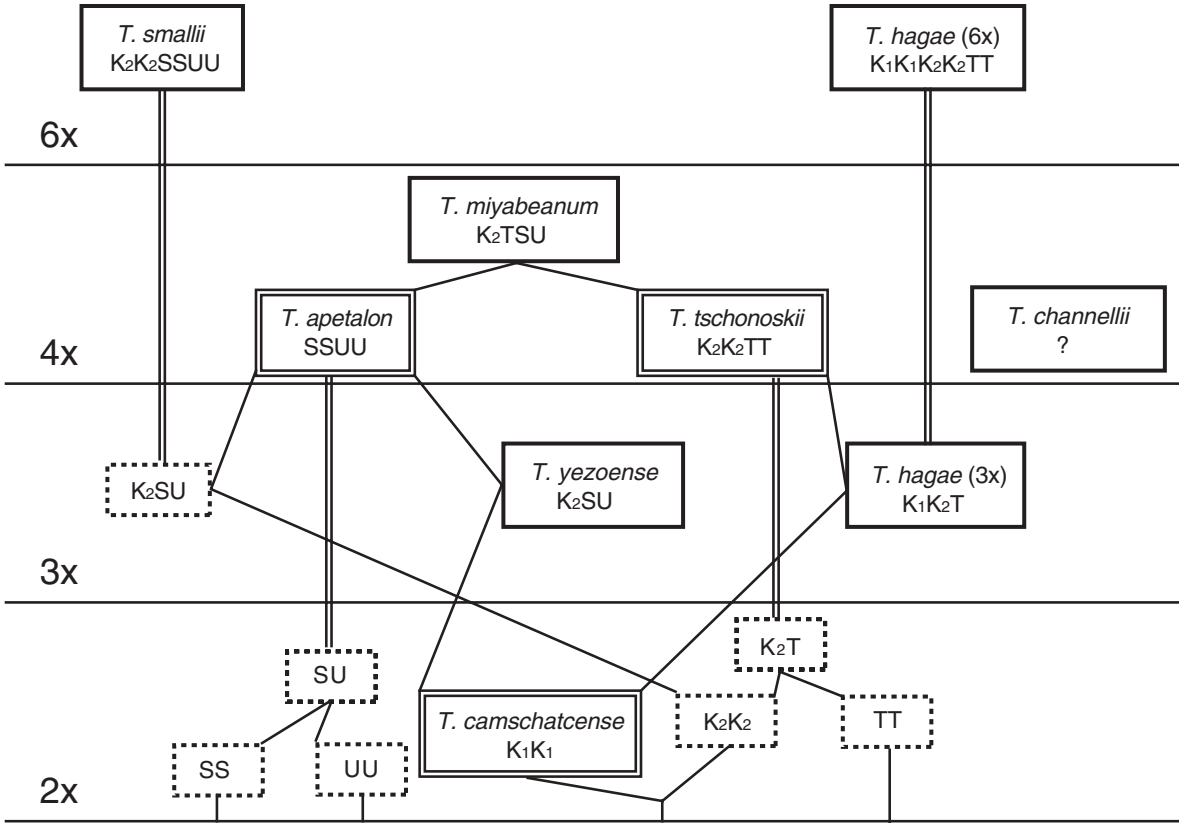


**Fig. 1.** Diagrammatic representation of speciation and ploidy level in the Japanese *Trillium* by Kurabayashi (1958). Extant species are in solid boxes, and unconfirmed species are in dotted boxes. Double lines at the top of the figure represent chromosome doubling

reviewed the relationship of Trilliaceae (Osaloo et al. 1999, Farmer and Schilling 2002, Punina et al. 2005). In addition, Li et al. (2005) employed amplified fragment length polymorphism markers (AFLP) to investigate the genetic diversity and genetic structure of a *T. tschonoskii* population in China, demonstrating the reliability of the tool. In the present study, we re-examined the relationships among Japanese *Trillium* species employing highly polymorphic DNA markers: AFLP. To our knowledge, this is the first paper to compare the conclusion based on karyotype analysis to modern AFLP analysis.

## Materials and methods

**Sampling.** Sixty-five samples of all Japanese *Trillium* species (except *T. yezoense*) were collected at the distance of ca. five to 10 meters with each other, at various localities in Hokkaido and northern Honshu (Shimokita) from April 2004 to May 2005 (Table 1). Because *T. yezoense* is rarely found under natural conditions, we used an individual cultivated in the "Trillium Garden" of the Botanical Gardens of Hokkaido University. The leaves were stored at –80 °C for subsequent DNA extraction. The voucher specimens (except *T. yezoense*) are deposited in the herbarium of the Hokkaido University Museum (SAPA).

**PCOA based on karyotypic composition.** We conducted principal-coordinates analysis (PCOA) based on the presence/absence data for five karyotypic components ($K_1$, $K_2$, T, S, U) (Haga and Kurabayashi 1953). For example, *T. tschonoskii* ($K_2K_2TT$) would be coded as: $K_1$, $K_2$, T, S, U = 0, 1, 1, 0, 0, whereas *T. miyabeanum* ($K_2TSU$) would be: $K_1$, $K_2$, T, S, U = 0, 1, 1, 1, 1. We used the three possible karyotypic composition for *T. channellii*: $K_1K_1K_2T$, $K_1K_1K_2K_2$, and $K_1K_1TT$. We

**Table 1.** Location of the study sites, and the number of individuals collected

| Population | Latitude(N) | Longitude(E) | *T. camschatcense* | *T. tschonoskii* | *T. apetalon* | *T. hagae* (3×) | *T. hagae* (6×) | *T. miyabeanum* | *T. yezoense* | *T. smallii* | *T. channellii* | Total individuals |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Shimokita | 41°20' | 141°22' | - | - | 2 | - | - | - | - | - | - | 2 |
| Mt. Hakodate | 41°45' | 140°42' | 2 | 1 | 3 | - | - | - | - | 3 | - | 9 |
| Mt. Usu | 42°32' | 140°52' | - | 2 | 2 | - | - | - | - | 3 | - | 7 |
| Lake Shikotsu | 42°47' | 141°33' | - | 2 | 2 | 2 | - | 7 | - | - | - | 13 |
| Osatsu | 42°52' | 141°38 | 2 | 2 | - | 3 | - | - | - | - | - | 7 |
| Mashike | 43°51' | 141°31' | 2 | - | - | - | - | - | - | 2 | - | 4 |
| Mituishi | 42°14' | 142°33' | - | - | - | 3 | - | - | - | - | - | 3 |
| Kawayu | 43°38' | 144°26' | 2 | 2 | - | - | - | - | - | - | 8 | 12 |
| Lake Abashiri | 43°57' | 144°13' | 1 | - | - | - | 6 | - | - | - | - | 7 |
| Botanic Garden | 43°03' | 141°20' | - | - | - | - | - | - | 1 | - | - | 1 |
| Total | | | 9 | 9 | 9 | 8 | 6 | 7 | 1 | 8 | 8 | 65 |

estimated similarity as $S_{ij} = 2N_{ij}/(N_i + N_j)$, where $N_{ij}$ is the number of karyotypic components in common between species $i$ and $j$, and $N_i$ and $N_j$ are the number of karyotypic components found in species $i$ and $j$, respectively (Dice 1945). We then transformed the similarity values into dissimilarity values using the formula $D_{ij} = 1 - S_{ij}$, and subjected these results to PCOA. All calculations were performed using version 4.0 of the R software (Casgrain and Legendre 1999).

**DNA extraction and AFLP analysis.** We isolated total genomic DNA from ca. 50 mg of tissue from each of the 65 frozen leaves using a CTAB (Cetyl trimethyl ammonium bromide) miniprep procedure (Stewart and Via 1993). We performed AFLP according to Vos et al. (1995) with some modifications. Genomic DNA (~0.1 μg per sample) was digested with the restriction enzymes *Eco*RI and *Mse*I at 37 °C for 1.5 h. We then ligated double-stranded adaptors to the ends of the digested DNA fragments at 20 °C overnight. The products were amplified using two steps: pre-amplification using primers with one additional (selective) base, and selective amplification using primers with four selective bases. Pre-amplification were conducted with the *Mse*I-C in combinations with *Eco*RI-A primers. Selective amplifications were conducted with the *Mse*I-CAGC primer in combination with *Eco*RI-ACGA (VIC) or -AGCC (NED) primers. The AFLP Amplification Core Mix (Applied Biosystems, Foster City, California, USA) and the GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) were used for both amplifications. AFLP fragments were detected using an ABI Prism 3100 automated sequencer and GENESCAN v3.7.1 analysis software (both from Applied Biosystems). PCOA analysis was conducted based on the presence/absence data for the AFLP segments (bands) as described above.

## Results

**PCOA of karyotypic compositions.** The PCOA results based on the karyotypic compositions (Haga and Kurabayashi 1953) demonstrated the relationships among Japanese *Trillium* species (Fig. 2). The three progenitor species (*T. camschatcense*, *T. tschonoskii*, and *T. apetalon*) were positioned at approximately equal distances from the origin, forming an equilateral triangle. In addition, the hybrids of these species (*T. hagae* [3x, 6x], *T. miyabeanum*, and *T. yezoense*) were positioned at intermediate points between the positions of their putative parental species (Fig. 2).

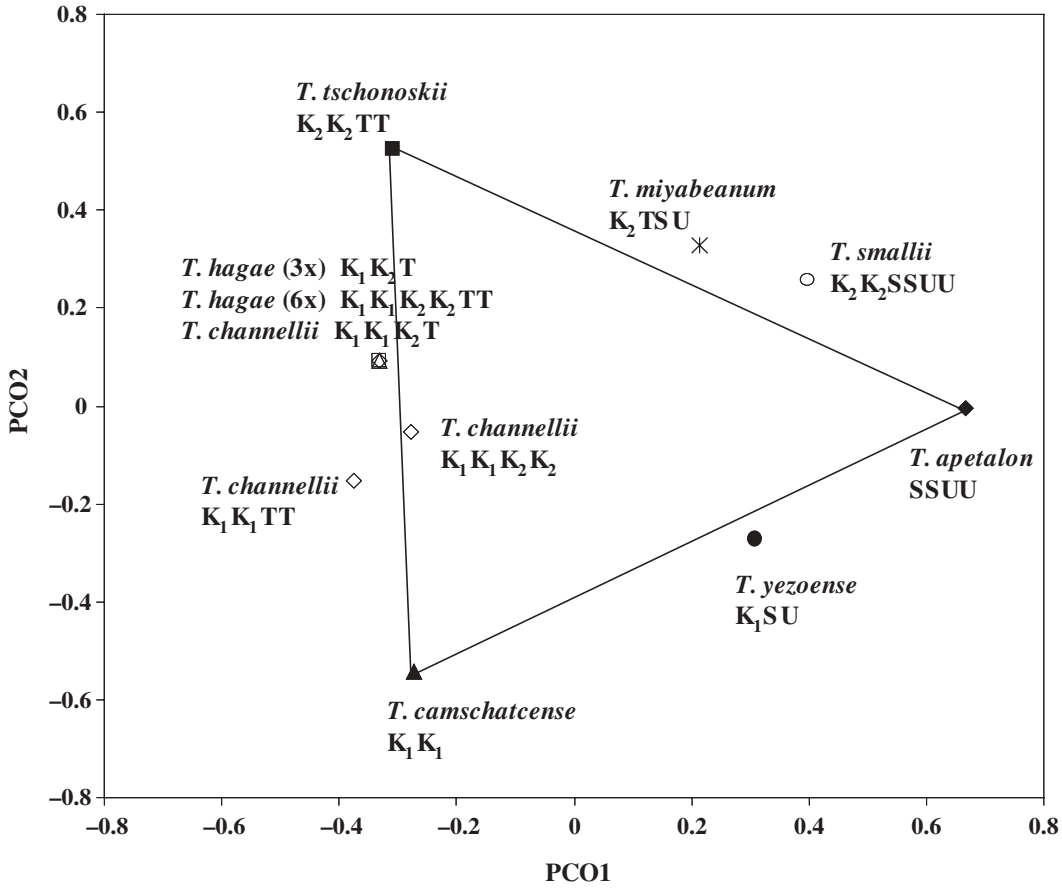


**Fig. 2.** PCOA plots of the first and second axes based on the karyotypic compositions of each species. The proportions of total variance along the two axes were 35.0% for PCO1 and 23.5% for PCO2

The hexaploid *T. smallii*, whose karyotypic composition was hypothesized as $K_2K_2SSUU$ (Haga and Kurabayashi 1953), was located between *T. tschonoskii* and *T. apetalon* (Fig. 2). Three possible karyotypic compositions had been proposed for *T. channellii* (Fukuda et al. 1996): $K_1K_1K_2T$, $K_1K_1K_2K_2$, and $K_1K_1TT$. PCOA based on the proposed karyotypic compositions revealed that all of these groups were located at intermediate points between *T. tschonoskii* and *T. camschatcense*, but that the positions of the three karyotypic types differed (Fig. 2).

**PCOA of AFLP.** We identified 142 polymorphic bands for the Japanese *Trillium* species using two primer pairs. The AFLP genotypes of the three progenitor species (*T. camschatcense*, *T. tschonoskii*, and *T. apetalon*) were clearly distinguished with each other, and again formed an approximately equilateral triangle (Fig. 3). The relationships among the three progenitors and their hybrids (*T. hagae*, *T. miyabeanum*, and *T. yezoense*) were consistent with those based on the karyotypic compositions (Fig. 2), with the exception of *T. smallii*. The position of *T. smallii* obtained by PCOA of AFLP bands differed from that of the hypothesized karyotypic compositions (Haga and Kurabayashi 1953). PCOA of AFLP indicates that *T. smallii* is located between *T. apetalon* and *T. camschatcense* (Fig. 3), whereas PCOA of karyotypic compositions resolves *T. smallii* between *T. tschonoskii* and *T. apetalon* (Fig. 2). A tetraploid species, *T. channellii*, whose origin is not yet known, is located between

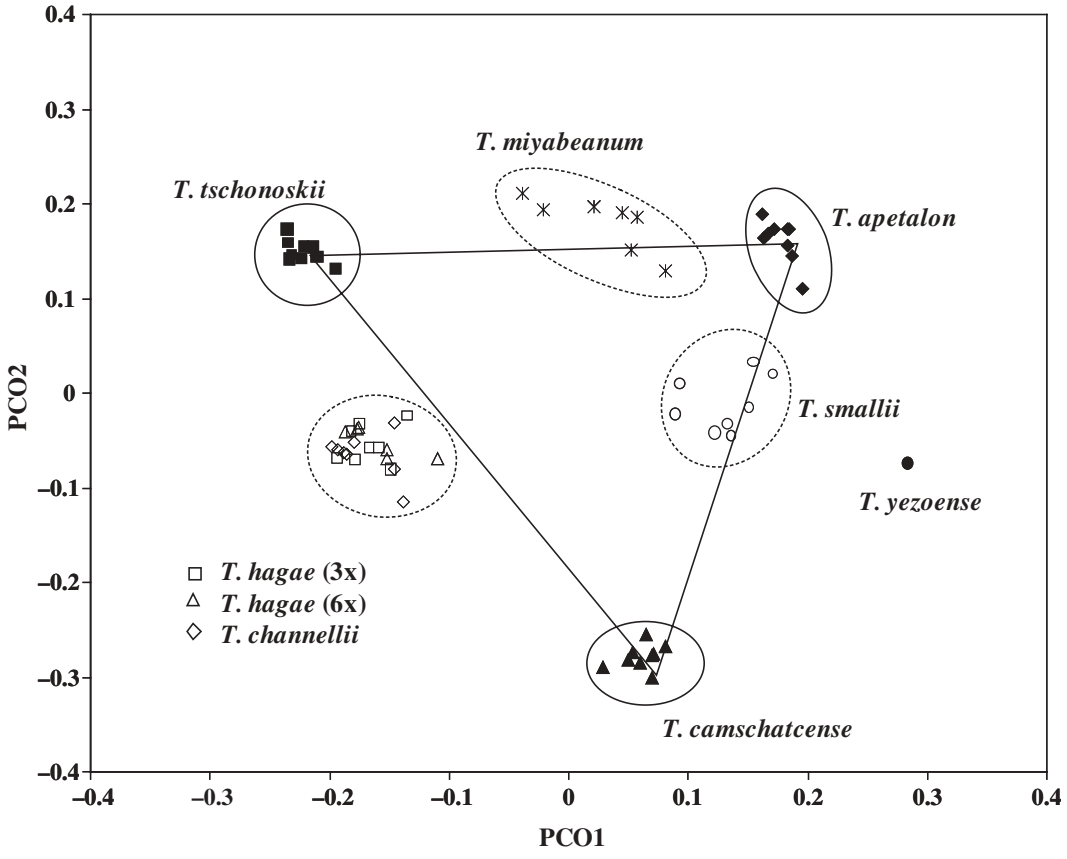


**Fig. 3.** PCOA plots of the first and second axis based on AFLP fingerprints of each individuals. The proportion of total variance along the two axes was 11.5% for PCO1 and 9.1% for PCO2

*T. camschatcense* (2x) and *T. tschonoskii* (4x), and every genotype of *T. channellii* that we examined overlapped with the positions of *T. hagae* (both 3x and 6x) (Fig. 3).

## Discussion

**The origin of *Trillium smallii*.** Our AFLP study supports the relationships among the three progenitor species and their hybrids (*T. hagae*, *T. miyabeanum* and *T. yezoense*) proposed by karyotypic compositions. The karyotypic composition of *T. smallii* has long been considered to be $K_2K_2SSUU$ (Haga and Kurabayashi 1953). If this hypothesis is appropriate, *T. smallii* should be positioned between *T. tschonoskii* ($K_2K_2TT$) and *T. apetalon* (SSUU), as shown by PCOA (Fig. 2). In our AFLP study, however, *T. smallii* was located between *T. camschatcense* ($K_1K_1$) and *T. apetalon* (SSUU) (Fig. 3). In addition, the fragment sizes of cpDNA SSR (atpF intron) (Weising and Gardner, 1999) clearly discriminated three progenitors: *T. tschonoskii*-type (193, 194 bp), *T. apetalon*-type (195, 196 bp) and *T. camschatcense*-type (197, 198, 200, 201, 202, 203, 204, 205 bp) (S. Kubota, unpubl. data). The cpDNA haplotype of *T. smallii* are 197, 203, 204 bp, identical with that observed in *T. camschatcense* (S. Kubota et al., unpubl. data), which is also inconsistent with the hypothesis of Haga and Kurabayashi (1953).

*Trillium smallii* is morphologically similar to the sterile triploid *T. yezoense* ($K_1SU$) in the petal color (reddish-purple), petal number (fluctuating from one to three), and leaf shape (depressedly obovate or rhomboid). The key morphological characteristic that distinguishes

these two species is fruit shape: ovoid in *T. smallii* vs. conical-ovoid in *T. yezoense* (Samejima and Samejima 1962, 1987), while *T. smallii* can reproduce sexually via seeds, and triploid *T. yezoense* cannot. This is similar to the comparison between triploid and hexaploid *T. hagae*. The triploid *T. hagae* is sterile, whereas the hexaploid is fertile and produces seeds. These species are morphologically similar, and the key distinguishing morphological characteristic is also the fruit shape: the triploid fruits are conical-ovoid whereas the hexaploid fruits are ovoid (Samejima and Samejima 1962, 1987). The hexaploid *T. hagae* is considered to have evolved from the sterile triploid by means of chromosome doubling, thereby acquiring the ability to reproduce sexually (Fig. 1, Haga 1951, 1952; Haga and Kurabayashi 1953; Haga et al. 1974).

Thus, based on the results of our AFLP analysis, as well as on the cpDNA haplotypes and morphological similarities, we conclude that the karyotypic composition of *T. smallii* is not $K_2K_2SSUU$ as proposed by Haga and Kurabayashi (1953), but rather $K_1K_1SSUU$ resulting from polyploidization of *T. yezoense* ($K_1SU$). *Trillium smallii* would be an allopolyploid of *T. yezoense*. Past studies distinguished karyotypic components $K_1$ and $K_2$ by monochromic staining patterns of the chromosome segments induced by cold-treatment. As mentioned in Introduction, Myakoshina et al. (2004) proposed that karyotypic component $K_2$ is more similar to T rather than $K_1$, by means of fluorescence nucleotide base-specific staining technique. Thus, the misidentification of *T. smallii* as $K_2K_2SSUU$ (Haga and Kurabayashi 1953), would attribute to a dubiousness of monochromic staining technique.

**The origin of *Trillium channellii*.** PCOA based on the AFLP bands revealed that the genotypes of *T. channellii* are indistinguishable from those of *T. hagae* (Fig. 3). In *T. channellii*, three plausible karyotypic compositions were proposed by Fukuda et al. (1996): $K_1K_1K_2T$, $K_1K_1K_2K_2$, and $K_1K_1TT$. Although these proposed karyotypic compositions are positioned between the same two progenitor species (*T. tschonoskii* and *T. camschatcense*), only the $K_1K_1K_2T$ karyotype should overlap that of *T. hagae*, as shown in Figs. 2 and 3. Thus, we conclude that *T. channellii* has this karyotypic composition.

As explained in the Introduction, Fukuda et al. (1996) proposed three hypotheses for the genesis of *T. channellii*. However, *T. channellii* did not originate from the third hypothesis (that a gamete from *T. camschatcense* [$K_1$] and one from a previously unrecognized diploid with a karyotypic composition $K_2K_2$ or TT [$K_2$ or T] would create a diploid hybrid $K_1K_2$ or $K_1T$ followed by somatic doubling), because that would produce karyotypic compositions of $K_1K_1K_2K_2$ or $K_1K_1TT$. In addition, the cpDNA SSR haplotypes of *T. channellii* (197 and 198 bp) are identical to those of *T. camschatcense*, suggesting that *T. channellii* has *T. camschatcense* as its maternal parent (S Kubota, unpubl. data). The karyotypic composition of $K_1K_1K_2T$ could then originate from two different processes: (1) crossing between an unreduced gamete from *T. camschatcense* ($K_1K_1$) and a normal gamete from *T. tschonoskii* ($K_2T$), or (2) crossing of normal gametes from *T. camschatcense* ($K_1$) and hexaploid *T. hagae* ($K_1K_2T$). Our data does not clarify which processes could be responsible for the origin of *T. channellii*. Since both scenarios will end up in the same genome, i.e. karyotypic composition $K_1K_1K_2T$, other approaches would be essential to resolve the evolutionary processes of *T. channellii*.

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