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Title:

Asymmetrical hybridization between *Trillium apetalon* and *T. tschonoskii* for the formation of a hybrid *T. miyabeanum* (Melanthiaceae)

A short running title: Asymmetrical hybridization in *Trillium*

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Author contributions

RO, SI and MO led the writing of the manuscript, designed the experiments, and collected and analyzed the data; TM and SK contributed designing the experiments and to data collection and analysis. All authors contributed critically to the drafts and gave final approval for publication.

Abstract

Trillium apetalon (4x) and *T. tschonoskii* (4x) hybridize commonly where both species grow sympatrically, leading to the formation of tetraploid *T. miybeanum* in Hokkaido, Japan. The present study aimed to determine which isolation factor is responsible for the frequency and asymmetry of hybrid *T. miyabeanum* formation in a sympatric population of *T. apetalon* and *T. tschonoskii*. We examined the contributions and strengths of four reproductive isolation barriers of *T. miyabeanum* formation: flowering phenology, breeding system, genetic isolation, and hybrid inviability. In addition, we also investigated the effect of flowering phenology on reproductive success (i.e., seed production and outcrossing rates) and outputs (i.e., ovule production) for *T. apetalon* and *T. tschonoskii*. We calculated the absolute contribution of each isolation barrier to the total reproductive isolation, and found that flowering phenology and differences in breeding systems between the two parental species were more effective when *T. apetalon* was the maternal parent. Furthermore, hybrids with *T. apetalon* as the maternal parent had lower viability than those of the reciprocal cross and did not reach the flowering stage. Especially, absolute contribution of premating isolation, especially by flowering phenology and breeding system, was higher than that of other isolation factors for both crossing directions. For the formation of *T. miyabeanum*, we concluded that asymmetry of hybridization between *T. apetalon* and *T. tschonoskii* would be

caused by strong premating isolations. The asymmetry of the isolating barriers may promote *T. tschonoskii* as the maternal parent of *T. miyabeanum*.

Key words: asymmetric hybridization, flowering phenology, reproductive isolation, *Trillium*

Introduction

Natural hybridization is a significant evolutionary process that may enhance speciation in plants (Stebbins 1950; Grant 1981; Ramsy and Schemske 1998; for recent reviews, see Alix et al. 2017; Goulet et al. 2017). On the other hand, the existence of reproductive isolation is one of the ways in which species are frequently distinguished and their existence is intimately related to the process of speciation (for reviews, see Sobel and Chen 2014; Baack et al. 2015). Reproductive isolation exists at different life history stages of plants, and is classified into two categories, i.e., prezygotic mechanisms (ecogeographic, temporal, and behavioral differences between species, e.g., Ishizaki et al. 2013; Melo et al. 2014) and postzygotic mechanism (hybrid inviability, hybrid sterility, and F_2 breakdown, e.g., Dell'Olvio et al. 2011; Abadie et al. 2012;

Lepais et al. 2013). Then, natural hybridization and reproductive isolation between plant species must be synchronously approached from multiple angles.

In the present study, we investigated the ecological and genetic speciation in the genus *Trillium*, especially focusing on reproductive isolation. The genus *Trillium* (Melanthiaceae), is one of the representative temperate woodland elements, which currently shows disjunct distribution between north America and eastern Asia. The genus includes approximately 45 species (Samejima and Samejima 1962, 1987; Freeman 1975), and ten of these are distributed in eastern Asia. Of the Asiatic species, two species are endemic to Taiwan (*T. taiwanense* Ying) and the Himalayas (*T. govanianum* Wallich et Royle), and all of the other eight species occur in Hokkaido, Japan. Based on extensive chromosome studies using cold-induced heterochromatin response on the Japanese *Trillium* species, it is known that the eight species consist of three species and five hybrids and/or hybrid derivatives, forming a polyploid series, 2x, 3x, 4x, and 6x (Haga 1951, 1956; Kurabayashi 1958). By contrast, all of the North American species are known to be diploid (Bailey 1951, 1954; Darlington and Shaw 1959).

Here, we focus on *T. miyabeanum* Tatewaki, which is a natural hybrid between *T. apetalon* Makino (4x) and *T. tschonoskii* Maximowicz (4x). *Trillium miyabeanum* occasionally occurs where both parental species grow sympatrically in Hokkaido, Japan

(Fig. 1), and at present only 12 localities have been recorded in Hokkaido (Samejima and Samejima 1962, 1987). Although both parental species are distributed in the southern part of Japan, *T. tchonoskii* mainly grows on mountain slopes or mixed forest in the subalpine zone. Therefore, there is a degree of geographical isolation between these two species in southern part of Japan. However, in Hokkaido, at higher latitude, *T. tschonoskii* sometimes occurs sympatrically with *T. apetalon* in lowland deciduous forests. Mitani (2005) reported that both parental species can act as either the female or male parent in breeding experiments although the flowering individuals of *T. miyabeanum* found in natural habitats have exclusively the same chloroplast and mitochondrial genome type as *T. tschonoskii*. It suggested that hybridization occurs asymmetrically (i.e., unidirectional) with *T. tschonoskii* as maternal species under the natural conditions.

Lowry et al. (2008) reported that premating isolations are less asymmetric than postmating isolations by comparing 19 pairs of plant taxa, and that the most asymmetrically acting isolations are pollen competition, F_1 inviability, and F_1 seed setting. In addition, various prezygotic isolations that may account for asymmetries in the seed and fruit set have been proposed including differences in style length and mating system (self-compatible versus self-incompatible) (for reviews, see Baack et al. 2015).

In the present study, we aimed to clarify which isolation factor is responsible for frequency and asymmetry of hybrid *T. miyabeanum* formation in sympatric populations of *T. apetalon* and *T. tschonoskii*. We examined four isolation factors of two parental species: flowering phenology, breeding system, and genetic isolation as prezygotic isolation, and hybrid inviability as postzygotic isolation. In addition, we investigated the effect of flowering phenology on reproductive success (i.e., seed productions and outcrossing rates) and outputs (i.e., ovule productions) for *T. apetalon* and *T. tschonoskii*.

Materials and Methods

Study species and study site

Japanese *Trillium* species are nonclonal and exclusively reproduce by seed production (Ohara and Kawano 1986). After seed gemination, it normally takes more than 10 years from seedling to mature flowering (FL) stage (Samejima and Samejima 1962; Ohara and Kawano 1986). *Trillium apetalon* (4x), *T. tschonoskii* (4x) and their hybrid, *T. miyabeanum* (4x), can be morphologically distinguished at the flowering stage by using floral characteristics (Fig. 1). *Trillium apetalon* has no petals with only reddish-purple sepals, and *T. tschonoskii* has white petals. *Trillium miyabeanum* has purple petals and has generally larger gross morphology than both parental species. However, it is

difficult to morphologically distinguish these species at non-flowering stages, i.e., seedling (SD), one-leaf stage (1L), and three-leaf stage (3L) stages (Ohara and Kawano 1986). Accordingly, genetic analyses as described below should be very efficient for identifying each species at vegetative growth stages.

It is known that both *T. apetalon* and *T. tschonoskii* are self-compatible plants and need pollinators for outcrossing, but these species fertilize most of their ovules by self-pollen (Ohara et al. 2001; Ohara and Kawano 2006; Ishizaki et al. 2013). The plants of each species flower in May, and the flowers remain at anthesis for about one week. Each flowering plant of *Trillium* species has one or several flowers, mainly one flower (Fig. 1). In our samplings and field experiments described below, all of the flowers or fruits we used were from single-flowered plants (i.e., one flower or fruit per individual). Ecological studies were conducted in deciduous forests near Lake Shikotsu (42°45'0" N, 141°20'0" E) in Chitose City, Hokkaido, Japan in 2009 and 2010. This is one of the major sites where it is possible to often encounter *T. miyabeanum* with its parental species (Fig. 1).

Hybridization rates

In order to estimate the rates of hybrid seed production in natural conditions, we collected about 20 fruits from each of *T. apetalon* and *T. tschonoskii* in July 2009 and

2010. Fruits were collected randomly throughout the study site. The genotype of the progeny was examined by using microsatellite markers. In previous studies, the seed output per plant of each *T. apetalon* and *T. tschonoskii* was approximately 124 and 85, respectively (Ohara et al. 2001; Ohara and Kawano 2006). For DNA extraction, eight seeds were randomly selected per fruit, and the embryo was removed from each seed and stored in a 1.5 ml sample tube at -80^{o}C for subsequent DNA extraction. Genomic DNA was extracted from frozen embryos using the CTAB extraction procedure (Stewart and Via 1993).

DNA was amplified with a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, California, USA) using two microsatellite markers: TC2 and TC44 (Kubota et al. 2006). In our investigation of hybrid frequencies at each life history stage described below, flowering individuals of *T. apetalon* and *T. tschonoskii* defined by their floral morphologies differed in the number and frequency of alleles at TC2 and TC44 (Table 1). There was no overlap in allele frequency distributions for the two species, which made the identification of species straightforward. Therefore, we could recognize the hybrid individuals which have the alleles of both parental species. All experimental procedures were carried out in accordance with Kubota et al. (2006). We calculated the rates of hybrid seed production per individuals [the number of seeds defied as hybrid per 8 seeds].

To evaluate the composition of each parental species and hybrid at each life history stage, SD, 1L, 3L and FL stages at the study site, we conducted a field sampling and DNA analysis. We established randomly ten plots (1m x 1m) at the study site and collected leaves (one leaf per individual) of all individual *Trillium* plants in the plots in 2009, and also recorded their life history stages. The leaves were silica-dried for subsequent DNA extraction. All individuals were analyzed by two microsatellite markers to detect their genotypes as described above. Individuals defined as hybrids were examined for their chloroplast DNA haplotypes to determine their maternal species, using universal primer ccmp4 (Weising and Gardner 1999). *Trillium apetalon* and *T. tschonoskii* differed in the number and frequency of alleles at chloroplast DNA marker (Kubota et al. 2006). There was no overlap in allele frequency distributions for the two species, which made identification of the maternal species straightforward. All voucher specimens (Ohara 20090286-20090305) are deposited in the herbarium at Hokkaido University Museum (SAPS).

Flowering phenology

We randomly selected and marked flowering plants of both *T. apetalon* (n=117) and *T. tschonoskii* (n=86) in May in 2010 and recorded the sequences of anther dehiscence every two to three days. We regarded the flowering date of individual plant as the day

when we first observed anther dehisced in the flower of each plant. Ovules of both parental species were fertilized immediately after anther dehiscence mainly by self-pollen (Mitani 2005). Therefore, we estimated the flowering period of each species as from the day when anther dehiscence began to the day when anthers of all flowers monitored dehisced. In July after the fruits ripened, we collected the fruits from the marked individuals.

To estimate ovule number, seed number, and seed setting rate per fruit, we counted the numbers of seeds and unfertilized ovules, and calculated the ovule number and the seed set rates per individual [the seed-ovule (S/O) ratio]. For the estimation of rates of hybrid seed production and outcrossing of each individual, eight seeds per fruit were randomly selected. The embryo was removed from each seed and stored in a sample tube (1.5 ml) as a tissue sample of each offspring. For estimating outcrossing rates, the seed coat was removed from one seed as a maternal tissue sample and stored in a sample tube. To identify the genotypes of seeds and their parents, DNA extraction and DNA amplification were conducted as described above using four microsatellite markers; two additional markers TC48 and TC77 (Kubota and Ohara 2009; Table 1), as well as TC2 and TC44, were used to increase resolution of genotyping. We calculated the rates of hybrid seed production per individual (number of seeds defined as hybrid per 8 seeds). In general, outcrossing means seed production from pollen donors

comprising other conspecific or allospecific individuals. However, here, outcrossing rates were determined as seed production from non-self pollen of conspecific individuals (ruling out seeds defined as hybrid). Estimation of outcrossing was carried out using MLTR 3.1 software, which is based on Ritland's mixed mating model (Ritland and Jain 1981; Ritland 2002). Spearman rank correlation test (r_s) was applied using JMP statistical software (version 8.0; SAS Institute) to evaluate the effect of flowering phenology.

Breeding system

To determine the breeding system (selfing or outcrossing) of the two parental species, we estimated outcrossing rates. Therefore, outcrossing rates were determined as seed production with pollen from other conspecific and interspecific individuals. To estimate outcrossing rates, ripened fruits of *T. apetalon* and *T. tschonoskii* were randomly collected in 2010. Estimation of outcrossing was performed as described above.

Genetic isolation

To characterize genetic isolation of the two parental species, *T. apetalon* and *T. tschonoskii*, two pollination experiments were conducted in 2010: (1) flowers were emasculated prior to anthesis, hand-pollinated using pollen from more than two plants

of the other species, and then bagged with cellophane bags to prevent pollen from the other individuals (i.e. interspecific pollination), and (2) before anthesis, flowers were emasculated and hand-pollinated using pollen from more than two distant plants of the same species as control (i.e. intraspecific pollination). For each experiment, 15 plants were used for both species. Anthers were collected from flowers before anther dehiscence, and then wrapped with medicine paper. After dehiscence, pollen was used to the experiment.

At fruiting in July, all fruits in the pollination treatments were collected. we calculated seed set rates. Data were analyzed for significance using the *t*-test ($P<0.05$) between two pollination experiments after arcsine transformation.

Hybrid inviability

Because individuals in the genus *Trillium* require at least 10 years from seedlings to become flowering plants (Ohara and Kawano 2006), it is difficult to study hybrid viability by monitoring the fates of individuals derived from hybridization in the field. Instead, we compared the viabilities of hybrids and parental species from seedling to flowering stage using a static approach. That is, we examined the composition of each parental species and hybrids at each growth stages (seedling, one-leaf, three-leaf and flowering stage). We collected the leaves from each individual within the ten plots

described above in 2010. The identification of each individual and the maternal species of hybrids using DNA analysis were conducted as described above. Viability index of each hybrid and parental species was calculated using following formula:

$$\text{Viability index} = \frac{\text{No. of individuals at flowering stage}}{\text{No. of individuals at non - flowering stage}}$$

The strength and absolute contribution of reproductive isolation

Total reproductive isolation (T) between *T. apetalon* and *T. tschonoskii* was estimated following the methods of Coyne and Orr (1989, 1997) and Ramsey et al. (2003), as a multiplicative function of individual components of reproductive isolation (RI) at sequential stages in the life history. The strength of RI for each isolation factor, generally varying between zero and one, is estimated independently (Table 2; cf. Husband and Sabara 2003; Ramsey et al. 2003; Martin and Willis 2007). Then, absolute contribution (AC_n) of the n-th component to reproductive isolation (RI_n) was calculated using following formula:

$$AC_1 = RI_1$$

$$AC_2 = RI_2(1 - AC_1)$$

$$AC_3 = RI_3[1 - (AC_1 + AC_2)]$$

And more generally:

$$AC_n = RI_n\left(1 - \sum_{i=1}^{n-1} AC_i\right)$$

It represents that a given reproductive barrier eliminates gene flow that has not already been prevented by previous stages of reproductive isolation. For *m* isolation barriers, total reproductive isolation (*T*), which varies from zero to one, is:

$$T = \sum_{i=1}^{m} AC_i$$

A third value is calculated to examine the relative influence of different barriers to total isolation. In accordance with Ramsey et al. (2003), we estimated the relative contribution (*RC*) of a reproductive barrier of the *n*-th component as:

$$RC_n = \frac{AC_n}{T}$$

As total isolation approaches one (i.e., isolation is complete), the relative contribution for any given reproductive barrier will approach the absolute contribution. This approach also accommodates scenarios in which hybridization and hybrids are favored at particular stages in the life history, as might be caused by disassortative mating in sympatry or hybrid vigor (Ramsey et al. 2003). Such a situation results in negative measures of reproductive isolation, and hence negative contributions that erase a portion of the total isolation achieved at prior stages in the life history (Ramsey et al. 2003).

Results

Hybridization rates

The rate of hybrid seed production per fruit of *T. apetalon* was 0.099 ± 0.033 (± SE, n=18) in 2009 and 0.099 ± 0.034 (n=11) in 2010 (Fig. 2). On the other hand, the rate of hybrid seed production per fruit of *T. tschonoskii* was 0.198 ± 0.056 (n=13) in 2009 and 0.231 ± 0.035 (n=23) in 2010. Although the difference in the rates of hybrid seed production between these two species in 2009 was not statistically significant (*t*-test: P=0.145), the rate of hybrid seed production was slightly higher in *T. tschonoskii*. In 2010, hybrid seed production of *T. apetalon* was significantly lower than that of *T. tschonoskii* (*t*-test: P<0.05).

Of 604 individuals examined in the study plots, 452 (74.8%) were *T. apetalon* and 116 (19.2%) were *T. tschonoskii* (Table 2), and only 36 (5.9%) hybrids were detected. Of these, hybrids produced from *T. apetalon* (HA) were fewer than those produced from *T. tschonoskii* (HT) in all life history stages. It is noteworthy that hybrids originating from *T. apetalon* as the maternal parent were not found at flowering stages. Thus, hybridization occurred asymmetrically with only *T. tschonoskii* being the maternal parent as found in the previous study (Mitani 2005).

Flowering phenology

Flowering periods for *T. apetalon* and *T. tschonoskii* were 12 days and 7 days, respectively (Fig. 3). *Trillium apetalon* initiated and terminated flowering earlier than *T.*

tschonoskii (Fig. 3). Although overlap of the flowering date observed was only a few days between the two species, the overlap was nearly a third of the flowering period for *T. apetalon*, while for *T. tschonoskii* it coincided with almost total period of flowering.

Seed setting rates were not affected by flowering phenology in both *T. apetalon* and *T. tschonoskii* (r_s =0.333, -0.317; *P*=0.266, 0.094). However, the seed number was positively correlated with flowering phenology in *T. apetalon* (r_s =0.716; *P*=0.0059; Fig. 4a). On the contrary, there was no significant relationship between flowering phenology and the seed number in *T. tschonoskii* (r_s =-0.056; *P*=0.761; Fig. 4b).

The ovule number was positively correlated with the flowering phenology in *T. apetalon*. (r_s =0.838; *P*=0.0002; Fig. 4c). On the contrary, there was no significant relationship between flowering phenology and the ovule number in *T. tschonoskii* (r_s=0.027; *P*=0.886; Fig. 4d).

Figure 4e and 4f show the relationships between flowering phenology and outcrossing rates for *T. apetalon* and *T. tschonoskii*. Although outcrossing rates were positively correlated with flowering phenology in *T. apetalon* (r_s =0.893; *P*=0.0005; Fig. 4e), those were not significantly correlated in *T. tschonoskii* (r_s =0.188; *P*=0.389; Fig. 4f).

There was no significant relationship between flowering phenology and the rate of hybrid seed production in *T. apetalon* (r_s =-0.1831; *P*=0.59; Fig. 5a). In *T. tschonoskii*,

rates of hybrid seed production were negatively correlated with flowering phenology (r_s =-0.515; P<0.05; Fig. 5b). $RI_{phenology}$ was 0.5 for *T. apetalon* and 0.142 for *T. tschonoskii*.

Breeding system and genetic isolation

As shown in Fig. 6, the outcrossing rate of *T. apetalon* (0.236 ± 0.06 (± SE, n=10)) was lower than that of *T. tschonoskii* (0.492 ± 0.048 (n=23)) (*t*-test: P<0.05). $RI_{breeding}$ was 0.639 for *T. apetalon* and 0.5 for *T. tschonoskii*.

Figure 7 illustrates the results of hand-pollination experiments. Seed setting rate of intraspecific pollination for *T. apetalon* was 0.831 ± 0.024 (± SE, n=12) and that of interspecific pollination for this species was 0.693 ± 0.083 (n=13). The difference in seed setting rates between the two pollination experiments for *T. apetalon* was not statistically significant (*t*-test: P=0.169). On the other hand, seed setting rate of intraspecific pollination for *T. tschonoskii* was 0.875 ± 0.017 (n=9) and that of interspecific pollination for this species was 0.481 ± 0.152 (n=13). Seed setting rates of interspecific pollination for *T. tschonoskii* were lower than that of intraspecific pollination for this species (*t*-test: P<0.05). $RI_{genetic}$ was 0.166 for *T. apetalon* and 0.45 for *T. tschonoskii*.

Hybrid inviability

Viability index of hybrid and pure offspring produced by *T. apetalon* were 0 (0/10) and 0.081 (34/418), respectively, representing that isolating barrier due to hybrid inviability is complete. On the other hand, viability index of hybrid and pure offspring produced by *T. tschonoskii* were 0.3 (6/20) and 0.126 (13/103), respectively. $RI_{inviability}$ was 1 (complete) for *T. apetalon* and -1.307 (negative effect) for *T. tschonoskii*.

The strength and absolute contribution of reproductive isolation

Trillium miyabeanum is a sterile hybrid (Kurabayashi 1958). Therefore, reproductive isolation caused by hybrid sterility was estimated as 1 (complete) for *T. tschonoskii*, and 0 for *T. apetalon* because no hybrids derived from *T. apetalon* were observed in flowering stage (Table 3).

We summarized the components of reproductive isolation for *T. apetalon* and *T. tschonoskii* (Fig. 8). Total reproductive isolation for each species was both 1 (complete). Reproductive isolation strength (RI_n) of *T. apetalon* was higher than that of *T. tschonoskii* in three factors, flowering phenology, breeding system and hybrid inviability (Fig. 8a). On the other hand, $RI_{genetic}$ of *T. tschonoskii* was higher than that of *T. apetalon*.

We calculated cumulative absolute contributions (AC_n) to total reproductive isolation and revealed that reproductive isolation was estimated to be nearly complete at the prezygotic stages, 0.849 for *T. apetalon* and 0.764 for *T. tschonoskii* (Fig. 8b). The isolation with highest absolute contribution in *T. apetalon* was flowering phenology. On the other hand, the factor with highest contribution for the reproductive isolation of *T. tschonoskii* was the breeding system.

Discussion

Our results show that reproductive isolation of *T. apetalon* was higher than that of *T. tschonoskii* in three factors, flowering phenology, breeding system and hybrid inviability. The isolation with highest absolute contribution in *T. apetalon* was flowering phenology. On the other hand, the highest absolute contribution in *T. tschonoskii* was observed at isolation of the breeding system. Here, we discuss first the effect of flowering phenology on outcrossing of parental species, and then the effect of each isolation on asymmetrical hybridization.

The effect of flowering phenology on outcrossing of parental species

Flowering phenology varies among the species and even within the species growing at the same habitat. Such variation should result in differences in reproductive success

among the plants. It is interesting to recall here that the large ovule number was detected in late flowering individuals for *T. apetalon* (Fig. 4c). Although we did not measure the size of flowering plant, late flowering individuals may be larger in plant size with many ovules. Large plant may need more time to grow and to flower after shoot sprout. In addition, the seed number and outcrossing rate were also positively correlated with flowering phenology in *T. apetalon* (Fig. 4a, e). These results mean that late flowering individuals receive more pollen from both self and other individuals than early flowering individuals. Therefore, even if late flowering individuals produced many ovules, these individuals may produce many seeds by receiving enough pollen. In previous studies, reproductive success (i.e., the number of seeds and fruits produced) has been suggested to depend on flowering phenology (Tarayre et al. 2007; Thomson 2010; Rodríguez-Pérez and Traveset 2016; Waters et al. 2020). In addition, the number of selfed and outcrossed seeds produced also depend on its flowering phenology in self-compatible plants (Morinaga et al. 2003). Some studies have indicated various reasons (e.g., plant size and pollinator visitation) for the correlations between reproductive success and flowering phenology (Widen 1991; Ollerton and Lack 1998; Kelly and Levin 2000). Although, in the present study, we cannot identify the reasons for correlations between reproductive success (i.e., the seed number and outcrossing rates) and flowering phenology for *T. apetalon*, it may be related with the density of

flowering individuals in the population. Because flowering density would become higher in late flowering period, the late flowering individuals will be able to receive more pollen from other individuals than early flowering individuals. Further investigation of the effect of flowering phenology on seed production and outcrossing should be addressed.

Flowering season of *T. tschonoskii* was shorter than that of *T. apetalon* (Fig. 3). This result may indicate that *T. tschonoskii* flowers synchronously among individuals in the same habitat. In this study, outcrossing rate of *T. tschonoskii* tended to be higher than that of *T. apetalon* throughout the flowering period (Fig. 4e, f), suggesting that number of incoming pollens from other individuals for *T. tschonoskii* would be larger than that for *T. apetalon* because of synchronously flowering. Therefore, reproductive output and success of *T. tschonoskii* may not be affected by flowering phenology unlike *T. apetalon* (Fig. 4b, d, f).

The effect of each isolation factor on asymmetrical hybridization

Flowering phenology

It has been demonstrated that reproductive asynchrony, or temporal isolation, is an important barrier between plant species (Vallejo-Marín et al. 2016; Yan et al. 2017; Pieper et al. 2017; Hornych et al.2019). *Trillium apetalon* flowers the earliest among

Trillium species in Japan, and this would increase the probability of *T. apetalon* of being the paternal species in the hybridization of Japanese *Trillium* species. In addition, *T. tchonoskii* with white petals more frequently visited by various insect groups belonging to Diptera, Coleoptera, Hemiptera than *T. apetalon* with no petals (Mitani 2005). The high diversity of pollinators can result in greater diversity in their physical and behavioral characteristics, which can lead to a greater chance of transporting pollen among plant individuals. In fact, the production of hybrid seed of *T. apetalon* was low in both overlapping period and non-overlapping period with flowering of *T. tschonoskii* (Fig. 5a). In contrast, hybrid seed productions of *T. tschonoskii* were high in overlapping period with flowering of *T. apetalon* (Fig. 5b).

Breeding system

It has been reported that isolation of breeding system caused by divergent outcrossing rates is important for reproductive isolation in plants (Widmer et al. 2009; Baack et al. 2015; Lafon-Placette et al. 2017; Liao et al. 2019). In the present study, outcrossing rates that were estimated directly from progeny genotype showed that *T. tschonoskii* cross-fertilizes more than *T. apetalon*. According to Ferguson et al. (1999), the outcrossers may be the maternal parent for a larger proportion of hybrids than selfers, because they highly depend on incoming pollen from other individuals than selfers.

Therefore, *T. tschonoskii* may be a maternal parent for a larger proportion of *T. miyabeanum* than *T. apetalon*. *Trillium apetalon* has no petals and *T. tschonoskii* has three white petals. Therefore, frequency and diversity of the pollinator visitation to *T. apetalon* may be lower than that to *T. tschonoskii*. This difference in pollinator visitation may cause differences in outcrossing rates. In fact, Mitani (2005) has suggested the flower visitor frequency of *T. apetalon* was lower than that of *T. tschonoskii*. Not only frequency but also diversity of pollinators may influence outcrossing rate because various pollinators may vary their behavior which can lead greater chance to move pollen between individuals. However, we did not examine details of pollinator fidelity, and further investigations on the breeding system will be necessary.

Genetic isolation

In this study, high crossability between *T. apetalon* was also confirmed (Fig. 7). In *T. tschonoskii*, it was shown that seed production from interspecific pollination was slightly lower than that of intraspecific pollination. However, in the present study, competition between conspecific and non-conspecific pollen in fertilizing ovules has not been investigated. In the study of a hybrid derivative species, *T. hagae* Miyabe et Tatewaki (3x), formation (Ishizaki et al. 2013), mixed pollination (application of mixed

pollen of *T. camschatcense* Ker Gawler and *T. tschonoskii* in a 50:50 proportion) on both *T. camschatcense* and *T. tschonoskii* stigmas produced few hybrids. It has been suggested that hybrid production was limited by the potential conspecific pollen precedence of both species.

Reproductive isolation caused by genetic isolation ($RI_{genetic}$) for *T. tschonoskii* (0.450) was stronger than that for *T. apetalon* (0.166). Although this result meant that the probability of *T. apetalon* being the maternal species was high, this would not affect asymmetric hybridization.

Hybrid inviability

In this study, we found strict asymmetry in hybrid viability. We did not examine seed germination, and the viability of parental species and hybrids was not followed from seed to flowering stages. However, it was found that hybrids derived from *T. tschonoskii* as the maternal parent survived to the flowering stages at a higher rate. On the other hand, hybrids produced by *T. apetalon* as the maternal parent were absent at the flowering stage of *T. miyabeanum*. These asymmetric results on hybrid viability were the same as *T. hagae* (3x), a natural hybrid between *T. camschatcense* and *T. tschonoskii* (Ishizaki et al. 2013). According to Ishizaki et al. (2013), hybrids derived from *T. camschatcense* as the maternal parent seem to be able to survive to flowering

stage more than *T. tschonoskii*. On the other hand, hybrids produced by *T. tschonoskii* were absent at the flowering stage.

Such asymmetry in hybrid formation seems to be related to nuclear-cytotype interaction. Although the details of the genetic background are not known, several types of nuclear-cytotype interactions, such as divergence of nuclear and cytoplasmic genes coding for proteins that interact in photosynthesis or respiration (Levin 1978; Wu et al. 1999) and transposable element, are known to be related to asymmetry in postzygotic incompatibilities (reviewed in Tiffin et al. 2001; Vallejo-Marín et al. 2016).

Asymmetric hybridization

In the present study, we found that both prezygotic and postzygotic isolations occurred substantially between *T. apetalon* and *T. tschonoskii* in a natural population. However, our results revealed strong, nearly complete reproductive isolation between these species, with prezygotic barriers being much more important in preventing current gene flow than postzygotic barriers.

Our results also showed that stronger barriers in flowering in phenology, breeding system, and hybrid inviability in *T. apetalon* facilitated asymmetric hybridization. In particular, absolute contributions of premating isolation (i.e., flowering phenology and breeding system) of *T. apetalon* were higher than genetic isolation and postzygotic

isolation, indicating that premating isolation plays a role in preventing pollen movement between different species. On the other hand, the absolute contribution of premating isolation of *T. tschonoskii* was not high. Therefore, *T. tschonoskii* may tend to receive pollen of *T. apetalon* and be the mother to a larger proportion of hybrids than *T. apetalon*. These differences in absolute contribute to premating isolation between the two parental species particularly facilitated asymmetric hybridization（Vallejo-Marín et al. 2016; Yan et al. 2017; Pieper et al. 2017; Hornych et al. 2019).

In the genus *Trillium*, it has been also suggested that temporal isolation due to differences in the timing of anther dehiscence may be a reproductive barrier to gene flow via pollen between *Trillium* species (Mitani 2005; Ishizaki et al. 2013). In particular, Ishizaki et al. (2013) has suggested that the timing of anther dehiscence is also an important factor for asymmetric hybridization in the hybrid *T. hagae* (3x). It was confirmed that anthers in *T. camschatcense* dehisced after the anthesis, whereas the anther dehiscence in *T. tschonoskii* occurred before anthesis, and most of the stigmatic area should be covered by self-pollen earlier than *T. camschatcense*. Therefore, hybridization between *T. camschatcense* and *T. tschonoskii* also occurs asymmetrically in natural habitats with only *T. camschatcense* being the maternal parent. However, in our observations, anther dehiscence and anthesis in *T. apetalon* and *T. tschonoskii* occurred at almost the same time. Therefore, differences in the timing of flowering,

such as *T. apetalon* flowering earlier than *T. tschonoskii*, may contribute further to this isolation.

Our results showed that premating reproductive isolation and hybrid inviability contribute mainly to prevent hybrid formation and to the occurrence of asymmetric hybridization. However, these premating factors would depend on climate conditions and pollinator varieties and movements, which may vary between populations. Therefore, there may be a population where premating barriers cannot act strong, resulting in frequent hybridization. Further studies comparing the frequency of hybridization among multiple populations will be required to reveal environmental factors that facilitate hybridization and the process of speciation in the genus *Trillium*.

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Figure legends

Figure 1: Photograph showing the three *Trillium* species that occur sympatrically at the study site. Left, *T. apetalon*; middle, *T. miyabeanum*; right, *T. tschonoskii*.

Figure 2: Hybridization rates of *T. apetalon* and *T. tschonoskii* in open pollinated progeny in 2009 and 2010. *P* value indicates that the differences were significant by *t*-test ($P<0.05$). NS, not significant. Bars represent standard errors.

Figure 3: Flowering phenology of *T. apetalon* and *T. tschonoskii* in the study site in 2010.

Figure 4: The effect of flowering phenology on reproductive success and outputs for *T. apetalon* and *T. tschonoskii* in 2010. (a) and (b), relationship between seed number and flowering phenology; (c) and (d), relationship between ovule number and flowering phenology; (e) and (f), relationship between outcrossing rates and flowering phenology. Black circles, *T. apetalon*; white circles, *T. tschonoskii*. *P* value indicates that the differences were significant using Spearman rank correlation test ($P<0.05$). NS, not significant.

Figure 5: Relationship between hybridization rates and flowering phenology for *T. apetalon* (a) and *T. tschonoskii* (b) in 2010. *P* value indicates that the differences were significant using Spearman rank correlation test ($P<0.05$). NS, not significant.

Figure 6: Outcrossing rates of *T. apetalon* and *T. tschonoskii* in open pollinated progenies in 2010. *P* value indicates that the differences were significant using *t*-test ($P<0.05$). Bars represent standard errors.

Figure 7: Results of a hand pollination experiment. *P* value indicates that the differences were significant using *t*-test ($P<0.05$). NS, not significant. Bars represent standard errors. Intra, intraspecific pollination; Inter, interspecific pollination.

Figure 8: Components of reproductive isolation and contributions to total isolation were calculated separately for *T. apetalon* and *T. tschonoskii*. (a), The strength of individual reproductive isolation (*RI*); (b), Absolute contribution to total reproductive isolation (*AC*; columns) and cumulative AC (lines).

Table 1 Allele frequency at four microsatellite markers for *T. apetalon* and *T. tschonoskii*

Locus	Allele frequency (bp)	
	T. apetalon	*T. tschonoskii*
TC2	185-186	174-176
TC44	211-223	209
TC48	134-169	136-176
TC77	106-143	110-123

Allele frequency was indicated by size range.

Table 2 Equations used to quantify reproductive isolation (*RI*) of each isolation factor

Isolation factor	Equation for calculation of *RI*
Flowering phenology	$RIphenology = 1 - \left(\frac{\text{No. of days co-flowering}}{\text{Total no. of days flowering}}\right)$
Breeding system	$RIbreeding = 1 - \left(\frac{\text{Total no. of cross fertilized offsprings}}{\text{Total no. of offsprings}}\right)$
Genetic isolation	$RIgenetic = 1 - \left(\frac{\text{Seed set rates of interspecific pollination}}{\text{Seed set rates of intraspecific pollination}}\right)$
Hybrid inviability	$RIinviability = 1 - \left(\frac{\text{Viability index of hybrids}}{\text{Viability index of parents}}\right)$

Table 3 Summary of survey of two parental species and hybrid individuals in the study plots in 2009

Life history stages	Number of individuals			
	T. apetalon	*T. tschonoskii*	Hybrid (HA)	Hybrid (HT)
SD	324	88	7	10
1L	47	7	1	5
3L	47	8	2	5
FL	34	13	0	6
Total	452	116	10	26

SD, seedling; 1L, one-leaf; 3L, 3-leaf; FL, flowering. Hybrids derived from *T. apetalon* and *T. tschonoskii* as maternal parent are expressed by Hybrid (HA) and Hybrid (HT), respectively.



Fig. 1

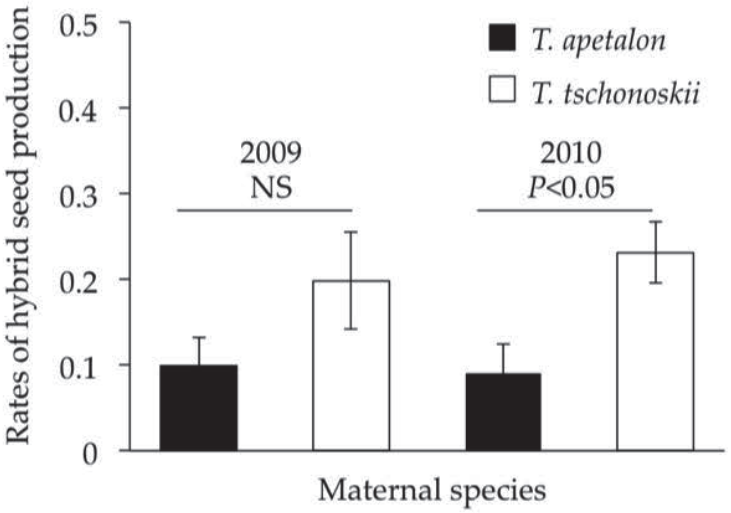

Rates of hybrid seed production
0.5
0.4
0.3
0.2
0.1
0
T. apetalon
T. tschonoskii
2009
NS
2010
P<0.05
Maternal species

Fig. 2

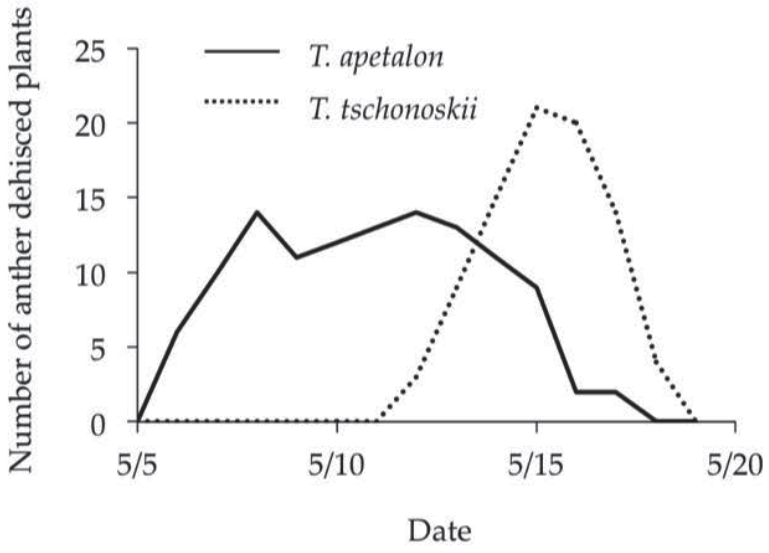

Number of anther dehisced plants
25
20
15
10
5
0
T. apetalon
T. tschonoskii
5/5
5/10
5/15
5/20
Date

Fig. 3

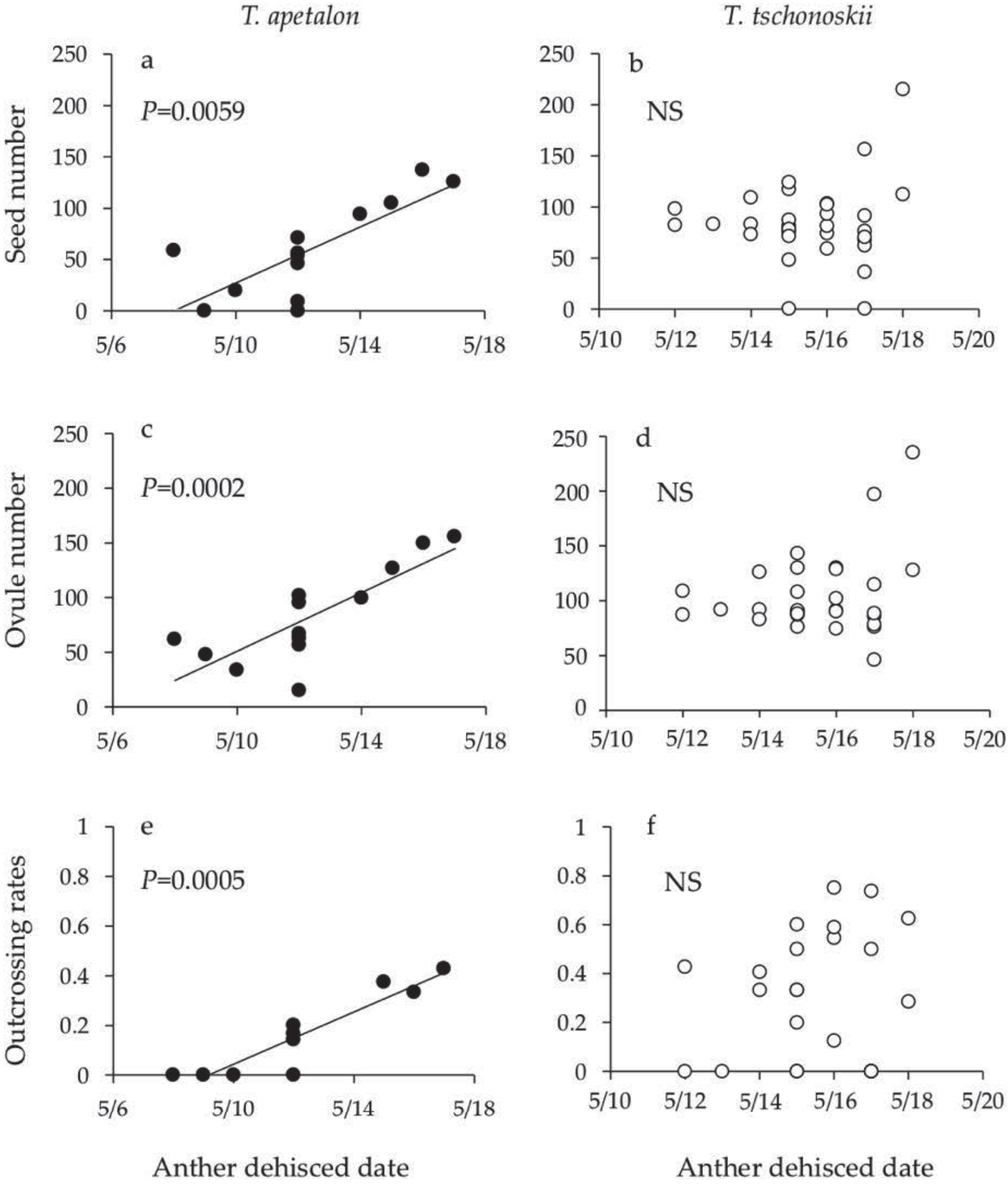

T. apetalon
T. tschonoskii
Seed number
Ovule number
Outcrossing rates
a
P=0.0059
b
NS
c
P=0.0002
d
NS
e
P=0.0005
f
NS
250
200
150
100
50
0
1
0.8
0.6
0.4
0.2
5/6
5/10
5/14
5/18
5/12
5/16
5/20
Anther dehisced date

Fig. 4

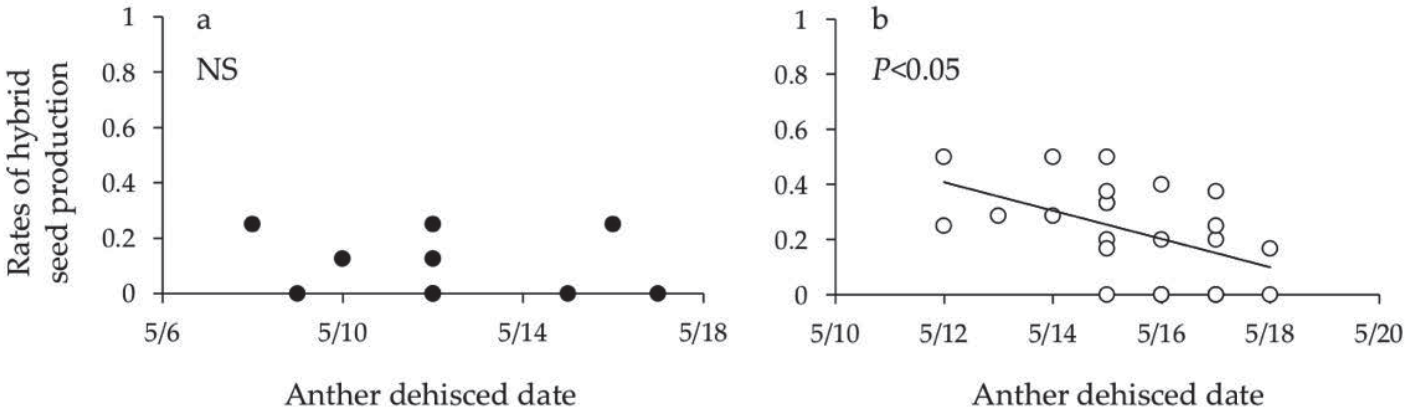

a
NS
Rates of hybrid seed production
1
0.8
0.6
0.4
0.2
0
5/6
5/10
5/14
5/18
Anther dehisced date
b
P<0.05
1
0.8
0.6
0.4
0.2
0
5/10
5/12
5/14
5/16
5/18
5/20
Anther dehisced date

Fig. 5

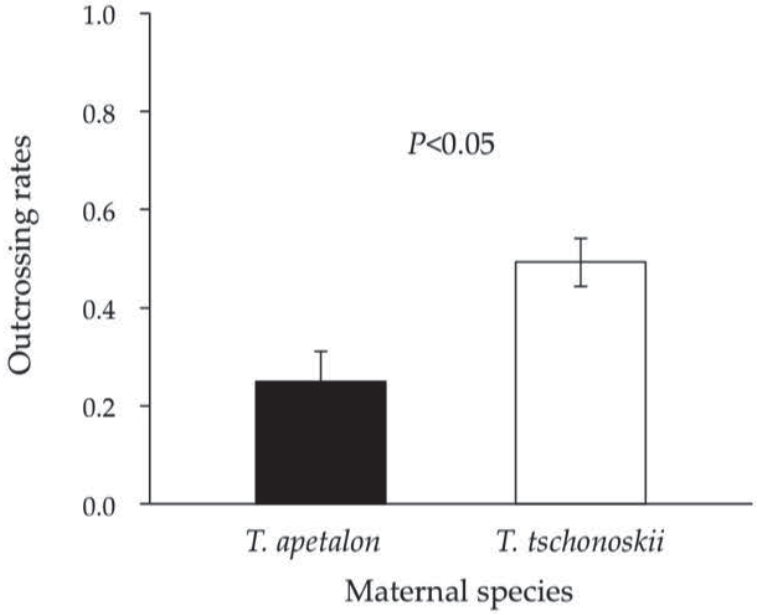

1.0
0.8
0.6
0.4
0.2
0.0
Outcrossing rates
P<0.05
T. apetalon
T. tschonoskii
Maternal species

Fig. 6

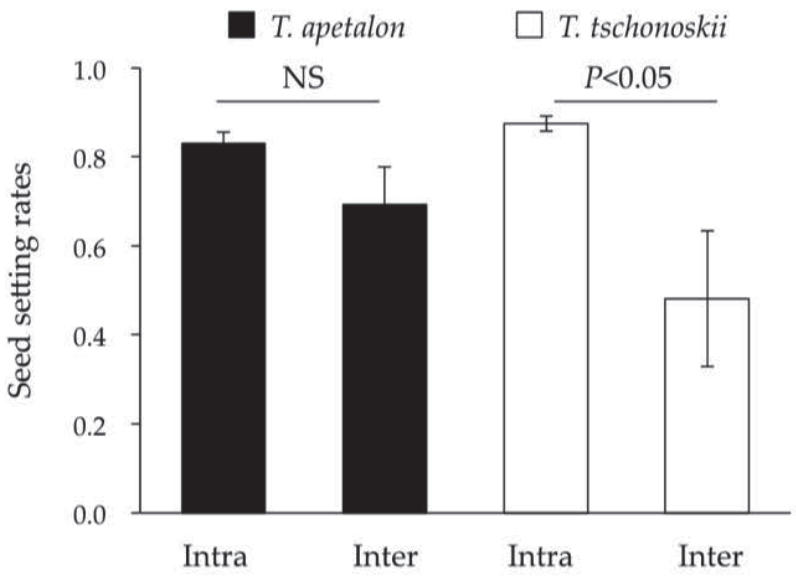

T. apetalon
T. tschonoskii
NS
P<0.05
Seed setting rates
1.0
0.8
0.6
0.4
0.2
0.0
Intra
Inter
Intra
Inter

Fig. 7

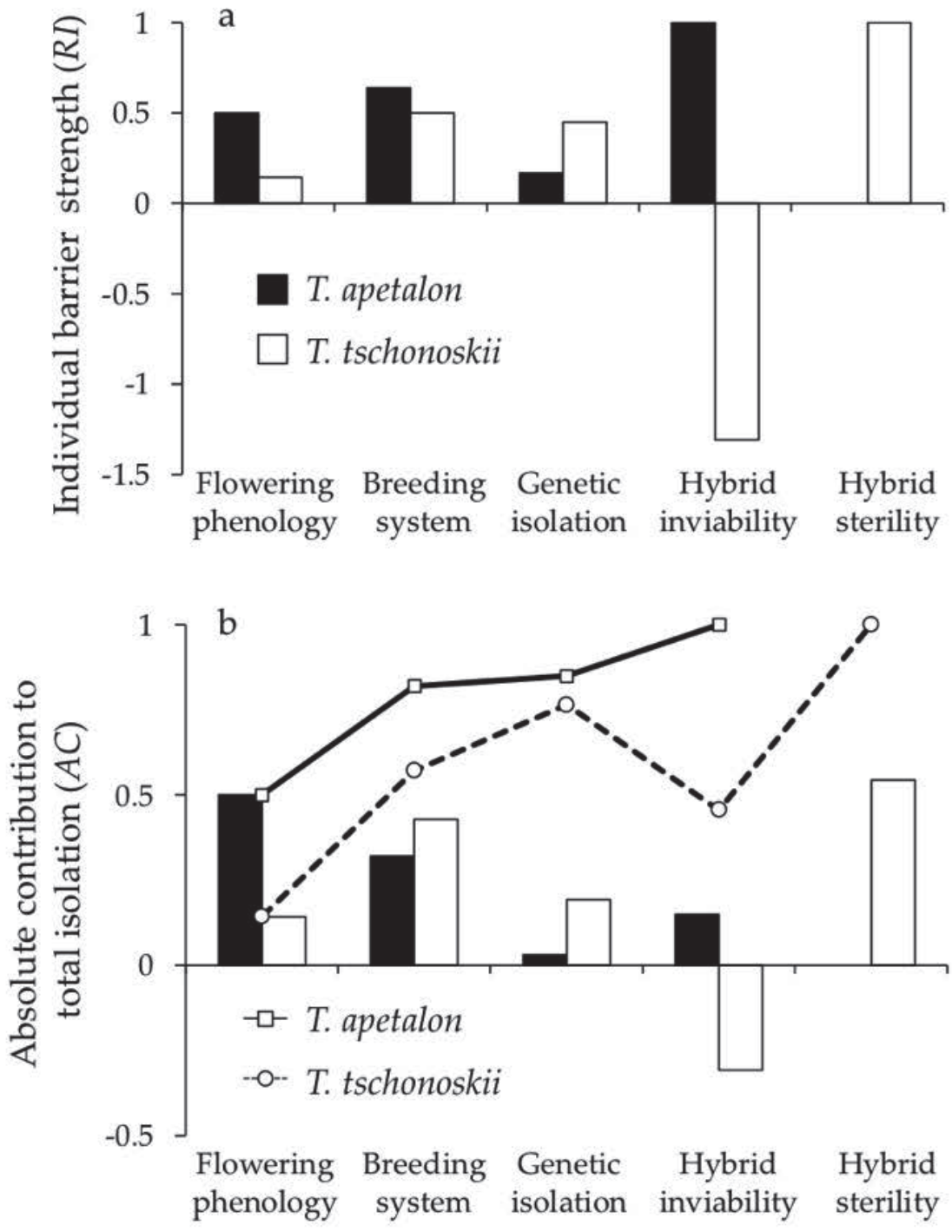

a
Individual barrier strength (RI)
1
0.5
0
-0.5
-1
-1.5
T. apetalon
T. tschonoskii
Flowering phenology
Breeding system
Genetic isolation
Hybrid inviability
Hybrid sterility
b
Absolute contribution to total isolation (AC)
1
0.5
0
-0.5
T. apetalon
T. tschonoskii
Flowering phenology
Breeding system
Genetic isolation
Hybrid inviability
Hybrid sterility

Fig. 8