Adaptive significance of self-fertilization in a hermaphroditic perennial, *Trillium camschatcense* (Melanthiaceae)[1]

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The evolution of self-fertilization from primarily outcrossing ancestors is one of the most common evolutionary transitions in plants; however, the ecological mechanisms that maintain self-fertilization have remained controversial. Theoretical studies suggest that selfing is advantageous over outcrossing in terms of genetic transmission and assurance of seed production under pollen-limited circumstances. *Trillium camschatcense* is a herbaceous perennial distributed in Hokkaido and northern Honshu, Japan. Geographical variation in the breeding system (self-compatible, SC; or self-incompatible, SI) has been reported in populations in Hokkaido. Here, we used several SC and SI populations of *T. camschatcense* to investigate the adaptive significance and the evolutionary basis of self-fertilization. Pollination experiments and genetic analyses demonstrated that the potential availability of outcross pollen in SC populations was sufficient and that the number of pollen donors was equal to that of SI populations. However, despite the high availability of outcross pollen, the SC populations produced seeds predominantly by selfing and so underwent severe inbreeding depression. Although none of the suggested advantages for self-fertilization were supported by our analyses, we propose two possible scenarios for the evolution of self-fertilization in *T. camschatcense*.

Key words: biparental inbreeding; correlated paternity; inbreeding depression; mating system; pollen limitation; reproductive assurance; self-incompatibility.

Flowering plants have complex mating patterns and reproduce in an almost bewildering variety of ways because of their immobility and reliance on vectors for pollen transfer. The evolution of self-fertilization from primarily outcrossing ancestors is one of the most common evolutionary transitions in plants and has gained the attention of plant evolutionary biologists (e.g., Stebbins, 1974; Grant, 1981; Barrett et al., 1989, 1996; Shimizu et al., 2004). Breeding systems in the genus *Arabidopsis* have been studied in relation to self-incompatibility (Charlesworth and Charlesworth, 1995; Charlesworth et al., 2000, 2003a, b; Shimizu et al., 2004; Mable et al., 2005). The model species *Arabidopsis thaliana* is self-compatible (SC; Jones, 1971) and is considered to have evolved from a self-incompatible (SI) ancestor (Shimizu et al., 2004). There are many similar examples in the Brassicaceae and other families (Bateman, 1955; Lloyd, 1965; Brauner and Gottlieb, 1987; Macnair et al., 1989; Wyatt et al., 1992). The directionality of such changes has been demonstrated on the basis of phylogenetic analyses, which often imply that outcrossing systems are ancestral (Schoen et al., 1997; Goodwillie, 1999). Recent theoretical works on the shift from self-incompatibility to self-compatibility has established models that support these empirical results (Vallejo-Marín and Uyenoyama, 2004; Porcher and Lande, 2005a). Moreover, most genetic theories considering the direction of mating-system evolution suggest that the evolution of outcrossing from a selfing population is unlikely (Lloyd, 1979; Charlesworth, 1980; Feldman and Christiansen, 1984; Lande and Schemske, 1985).

Selfing has two primary advantages over outcrossing. First, it ensures seed production when the population lacks mates or pollinators, such as those at the edge of a species' range or among colonizing species. This advantage is termed "reproductive assurance" (Baker, 1955; Stebbins, 1957; Jain, 1976; Barrett and Shore, 1987; Schoen et al., 1996; Kalisz et al., 2004; Schueller, 2004; Busch, 2005; Herlihy and Eckert, 2005). Second, alleles promoting self-fertilization increase in frequency because individuals that carry them can serve as pollen donors for other individuals (outcrossing) and for themselves (selfing), giving a twofold "transmission advantage" to genes (Fisher, 1941).

In contrast, several aspects of plant reproduction, such as inbreeding depression (i.e., diminution of vigor and fertility of self-fertilized offspring), pollen discounting (i.e., reduction in opportunities of pollen export for outcrossing caused by self-fertilization), and seed discounting (i.e., reduced production of outcrossed seeds caused by preceding self-fertilization), oppose these advantages of selfing (Darwin, 1876; Charlesworth and Charlesworth, 1987; Uyenoyama et al., 1993). Inbreeding can take the form of uniparental inbreeding (selfing) or biparental inbreeding (mating between relatives). Biparental inbreeding is often caused by restricted pollen dispersal and modifies both the costs and benefits of selfing (Uyenoyama, 1986; Yahara, 1992). Moreover, reproductive assurance will be costly if selfing uses the ovules that would otherwise have been outcrossed, and selfed offspring are less viable due to inbreeding depression (seed discounting; Lloyd, 1992). Thus, to determine the accurate advantage of reproductive assurance, both seed discounting and inbreeding depression must be assessed (Herlihy and Eckert, 2002). Similarly, if selfing reduces the amount of pollen that would otherwise have been exported for outcrossing (pollen discounting; Nagylaki, 1976), the benefit from transmission advantage can be eroded. The cost of pollen discounting can increase under low pollen limitation or severe inbreeding depression (Harder and Wilson, 1998; Johnston, 1998; Porcher and Lande, 2005b). Clearly, the components of selfing (reproductive assurance, transmission advantage, inbreeding depression, seed or pollen discounting) are related to each

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other, and to understand the selective pressure for or against selfing demands comprehensive vision.

To reveal the evolutionary basis of self-fertilization, many researchers have examined the factors involved in the selection of selfing, that is, the costs (inbreeding depression and seed or pollen discounting) and the benefits (reproductive assurance and transmission advantage). However, most of these have focused on single SC species (Eckert and Schaefer, 1998; Herlihy and Eckert, 2002; Kalisz et al., 2004), often omitting some of the selective factors. Intraspecific comparisons of SC and SI populations within species, such as provided by *Leavenworthia crassa* and *L. alabamica* (Rollins, 1963; Lloyd, 1965; Solbrig, 1972; Solbrig and Rollins, 1977; Lyons and Antonovics, 1991; Busch, 2005), enable more direct investigations of ecological factors favoring the evolution of self-fertilization.

Trillium camschatcense is a hermaphroditic, perennial herb that grows mainly in the understory of the broad-leaved deciduous forests of Hokkaido and northern Honshu, Japan. Breeding experiments in the field in various populations in Hokkaido have detected geographical variation in the mating system among populations. Populations located in northern and southern Hokkaido are SC, potentially possessing a mixed system of outcrossing and selfing, whereas eastern populations are SI, and all seed production results from outcrossing (Ohara et al., 1996). Because *T. camschatcense* does not reproduce asexually, and most of the individuals possess one-flower per individual (Ohara and Kawano, 1986), geitonogamy can be ignored. Thus, evaluation of the effect from selfing is more simplistic, and along with the breeding system of this species (SC or SI), *T. camschatcense* is an ideal species to investigate the adaptive significance of self-fertilization.

The current study investigated the adaptive significance and the evolutionary basis of self-fertilization in *T. camschatcense*. We examined the costs (inbreeding depression, seed discounting, and pollen discounting) and benefits (reproductive assurance and transmission advantage) of selfing in two SC and three SI populations. We focused on the differences in availability and quality of outcross pollen and the magnitude of inbreeding depression among populations. First, we conducted breeding experiments (bagging, emasculation, and hand-pollination) in the field to quantify the pollen limitation and the degree of reproductive assurance in each population. Second, using microsatellite markers, we estimated the proportion of seeds produced via selfing and inbreeding depression of each population. These results were combined with the data from breeding experiments to estimate the extent of seed discounting. Pollen discounting was assessed indirectly by the degree of pollen limitation, proportion of selfing, and inbreeding depression. Third, by analyzing genotypes of the seeds from emasculated individuals from the SC populations, we estimated the magnitude of biparental inbreeding and the potential availability of pollen donors in each SC population. We then compared these to the parameters of the SI population, which is expected to be ancestral, to determine which selective factors may be responsible for the evolution of self-fertilization in *T. camschatcense*.

MATERIALS AND METHODS

Study species and study sites—*Trillium camschatcense* Ker Gawler (Melanthiaceae; APG II, 2003) grows mainly in the understory of broad-leaved deciduous forests of Hokkaido and northern Honshu, Japan (Samejima and Samejima, 1962, 1987). Plants are nonclonal, long-lived, and polycarpic (Ohara and Kawano, 1986). Stems and leaves emerge in late April to early May from an underground rhizome. In mid May, reproductive individuals produce single or sometimes a few flowers that arise from separate stems. Most reproductive plants have one flowering stem, but some have up to four. Flowers have no nectar, but three large white petals with a sweet scent attract some beetles (e.g., Nitidulidae and Melandryidae) and flies (e.g., Bibionidae and Scathophagidae) as potential pollinators (Ohara et al., 1991; Tomimatsu and Ohara, 2003).

Four geographical groups—North, East, South, and Ishikari—have been recognized in Hokkaido on the basis of chromosome and allozyme variations (Kurabayashi, 1957, 1958; Ohara et al., 1996; Fig. 1). All local populations of the North, South, and Ishikari groups are SC and have low genetic diversities. By contrast, most populations in the East group have higher genetic diversities and are SI, with the exception of some small populations in the Hidaka region. Self-pollinated flowers of SI plants produce no seeds at all, but self-pollinated SC plants produce abundant seeds (Ohara et al., 1996), indicating that self-incompatibility of *T. camschatcense* is a dichotomous trait (i.e., either strong or no ability to self-fertilize).

For field experiments and sampling of materials, we selected two SC (Ishikari and Ogifushi) and three SI populations (Niikappu, Mauta, and Urakawa) in the Ishikari and Hidaka regions of Hokkaido (Fig. 1, Table 1). To standardize sampling conditions, we set a 20 × 20 m quadrat in each population where *T. camschatcense* predominated.

Pollination experiments—To determine the extent of pollen limitation and reproductive assurance, we applied four pollination treatments to each population: (1) control—flowers were left intact to determine seed production under natural conditions; (2) emasculation—flowers were emasculated before anther dehiscence and left under open-pollinated conditions to test whether seed production is limited by the availability of outcross pollen; (3) outcrossing:—flowers were emasculated before anthesis and then hand-pollinated with pollen collected from distant flowers; and (4) selfing—flowers were bagged with nylon bags before anthesis, hand-pollinated with self-pollen, and bagged again to prevent pollination by other flowers to determine potential seed production through selfing. Only control and emasculation treatments were applied in 2005, and all treatments were performed in 2006. Each treatment was applied to 20–42 single-flowered plants randomly selected in each 20 × 20 m plot. Pollen used for outcrossing was collected from more than three individuals 3–20 m away from pollen recipients. Individual plants used for all experiments were single-flowered, and thus geitonogamy was excluded from the entire experimental system.

At fruiting in July, fruits were collected, and seed set was examined. The mature seeds, aborted (small and shrunken) seeds, and unfertilized ovules per fruit were counted using a stereomicroscope and summed to give the total number of ovules per individual. The proportion of seeds to total ovules produced (proportion of total number of mature seeds produced per individual to total number of ovules per individual) was calculated and arcsine transformed (Sokal and Rohlf, 1995). Because significant effects of year and population on seed production were detected, the effect of treatment was analyzed by one-way ANOVA separately for each population in each year. Significant differences were assessed with Tukey–Kramer multiple comparison at $P = 0.05$ with the program JMP version 4.0 (SAS Institute, 2000).

DNA analysis (sampling, extraction, and genotyping)—In May (flowering time) 2005, we collected the leaves of control (40 leaves per population) and emasculated (30 leaves per population) individuals from four of the five populations, excluding Urakawa where the treated plants were damaged by herbivores. To estimate the proportion of seeds produced via selfing and inbreeding depression under natural conditions, we selected 10 control fruits from each population and randomly selected 12 seeds from each fruit. To evaluate the potential availability of pollen donors in SC populations, we randomly selected 12 seeds from 10 fruits of emasculated plants. The embryo was taken from each seed and stored in a 1.5-mL sampling tube. All leaves and embryos were stored at −80°C for subsequent DNA extraction.

Genomic DNA was extracted from the frozen leaves and embryos by a 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 five microsatellite primer sets: TC2, TC15, TC44, TC48, and TC69 (Kubota et al., 2006). PCR was performed in a 10-μL reaction mixture containing 0.5 μL of template DNA, 0.2 mM each dNTP, 1× PCR buffer with 1.5 mM MgCl$_2$ (Applied Biosystems), 0.25 U of Ampli*Taq* Gold DNA polymerase (Applied Biosystems), and two primers: fluorescent-dye-labeled forward primer (0.5 μM; Applied Biosystems) and a reverse nontailed primer (0.5 μM). The PCR conditions were 9 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at the annealing temperature, and 1 min at 72°C; followed by 72°C for 7 min. The PCR products were determined on an ABI Prism 3100 automated sequencer with its GeneScan analysis software (Applied Biosystems).
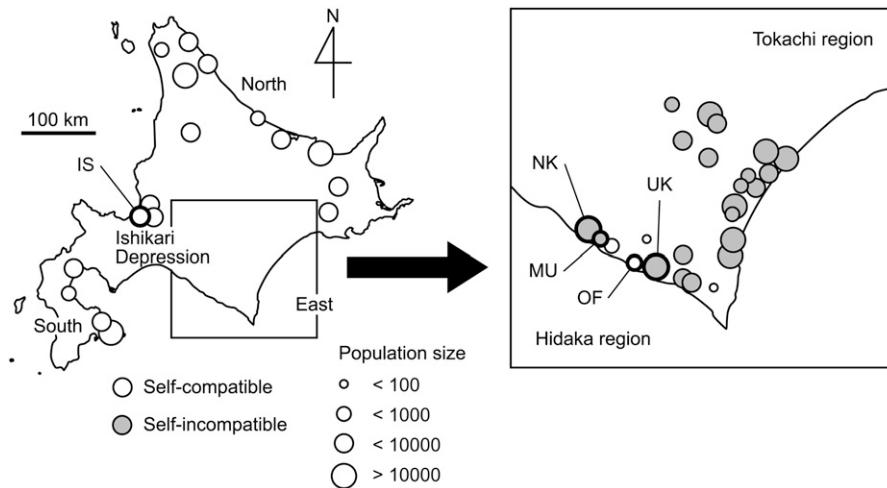
Fig. 1. Study sites and mating systems of *Trillium camschatcense* in Hokkaido (after Ohara et al., 1996). Name and location of each population are shown in Table 1. The designation for the geographical groups (North, East, South, and Ishikari Depression) is taken from the work of Kurabayashi (1957).

Estimates of selfing and inbreeding depression—Ten control fruits were used for multilocus estimation of seeds produced via outcrossing (t_m) in each population. The progeny genotypes were obtained from 12 seeds per fruit. Calculation was performed with the program MLTR 3.1, which is based on Ritland's mixed-mating model (Ritland and Jain, 1981; Ritland, 2002). For parameter computation, adult inbreeding was set to the value estimated from the genotypes of 32 leaves per population. Standard errors were estimated from 1000 bootstraps. The proportion of seeds produced through selfing ($s = 1 - t_m$) of each population was obtained from the estimated t_m.

The parental fixation index (F') was estimated from 32 leaf genotypes per population according to Weir and Cockerham's (1984) estimator of Wright's fixation index in the program FSTAT version 2.9 (Goudet, 2002). The seed fixation index (F'') was calculated in the same way from 120 seed genotypes per population. Statistical significance was calculated from 2000 randomized procedures. Inbreeding depression ($d = 1 - $ [fitness of selfed progeny/fitness of outcrossed progeny]) for survival from seed to reproductive maturity was calculated from the estimated selfing ($s = 1 - t_m$), parental fixation indices (F'), and seed fixation indices (F'') with Ritland's (1990) two-generation equilibrium estimator:

$$d_t = 1 - [(1 - s)F''/(F' - sF'')].$$

The same genotype data sets from the seeds (12 seeds per family, 10 family) were used to jointly estimate the parental fixation indices (F) and proportion of selfing ($s = 1 - t_m$) in MLTR 3.1. Inbreeding depression was also calculated using Ritland's (1990) single-generation estimator:

$$d_s = 1 - [2(1 - s)F/s(1 - F)].$$

Statistical significance of d_s was calculated from the 1000 bootstrap estimates of s and F.

Estimates of biparental inbreeding and effective number of pollen donors—To determine the magnitude of biparental inbreeding in each population, we selected 10 fruits of emasculated plants in SC populations (Ishikari and Ogifushi) and 10 control fruits in SI populations (Niikappu and Mauta). We used the fruits of emasculated plants in the SC populations because the estimated selfing (s) of control fruits was too high to allow us to analyze the outcrossing parameters. Therefore, the values given by these data sets are derived from artificial conditions in which the SC populations bred only by outcrossing. Because self-pollination and geitonogamy are eliminated from these data sets, the magnitude of biparental inbreeding can be estimated as the apparent selfing ($s = 1 - t_m$) (Eckert, 2000; Lu, 2000). The multilocus estimates of outcrossing (t_m), and the biparental inbreeding ($1 - t_m$) of each fruit were estimated from 12 seeds of each fruit by MLTR 3.1. Standard errors and statistical significance were estimated from 5000 bootstraps.

The same data sets (potential condition in SC populations and natural condition in SI populations) were used to evaluate the effective number of pollen donors as the reciprocal of correlated paternity (proportion of full-sibs among outcrossed sibling pairs). Correlated paternity was calculated by the kinship coefficient method (Hardy et al., 2004). Although there are several approaches to estimate correlated paternity (e.g., mixed-mating model by Ritland [1989]; TWOGENER approach by Austerlitz and Smouse [2001]; assignment procedure of sibling relationships by Hirao et al. [2006]), Hirao et al. (2006) compared these methods and demonstrated that the different approaches were equally reliable. Computations were carried out with the program SPAGeDi (Hardy and Vekemans, 2002) by encoding paternal gametic genotypes as diploid genotypes and designating them as homozygous when the paternal contribution was unambiguous and as heterozygous when the paternal contribution was ambiguous. For instance, if the seeds possess alleles A and B and its mother alleles B and C, it means that the pollen brought allele A, and thus the paternal gametic genotype would be considered as AA. However, if the mother possesses alleles A and B, the pollen could have brought allele A or allele B. In this case, the paternal gametic genotype would be defined as AB (see Hardy et al., 2004). We used J. Nason's multilocus kinship estimator, described by Loiselle et al. (1995), to calculate the pairwise kinship coefficient. The Steel–Dwass test was used to evaluate the differences among populations using the program R (R Development Core Team, 2005). The reciprocal of the kinship coefficient ($2F_s$) corresponds to the effective number of pollen donors (N_{ep}). All statistical significance levels were set at less than 5% ($P < 0.05$).

TABLE 1. *Trillium camschatcense* study sites in Japan. See Fig. 1 for locations. Average number of flowering plants was calculated by randomly placing a 5 × 5 m quadrat five times within each population.

Population	Abbreviation	Latitude (N)	Longitude (E)	Mating system	Population size (m²)	Flowering density (/25 m²)
Ishikari	IS	43°18′	141°36′	SC	3000	133.4
Ogifushi	OF	42°20′	142°68′	SC	2000	54.4
Niikappu	NK	42°37′	142°30′	SI	30 000	94.8
Mauta	MU	42°33′	142°38′	SI	1000	56.0
Urakawa	UK	42°18′	142°75′	SI	40 000	79.8

Notes: SC, self-compatible; SI, self-incompatible.

RESULTS

Pollination experiments—The results of pollination experiments at each site are presented in Fig. 2. Population UK in 2005 is not included because the plants were damaged by herbivores. *Trillium camschatcense* had an average of 212.2 ± 8.6 ($\pm$SE) ovules per flower. The average proportion of seeds to total ovules produced by open-pollinated (control) plants varied among populations from 46.5% (NK) to 70.9% (OF) in 2005 and from 47.8% (NK) to 74.2% (OF) in 2006.

The average proportion of seeds produced in open-pollinated plants did not differ significantly from those of outcrossed plants in all five populations (Fig. 2). In the SC populations (IS and OF), self-pollinated plants produced mature seeds, and their average proportion of produced seeds did not differ significantly from those of outcrossed plants. However, self-pollinated plants in the SI populations (NK, MU, and UK) produced no seeds at all, confirming the strict self-incompatibility reported by Ohara et al. (1996).

Plants emasculated before anthesis produced mature seeds in all populations examined. More importantly, the average proportion of produced seeds in emasculated plants did not differ significantly from that of open-pollinated plants, except at IS in 2005 and UK in 2006 (Fig. 2). Because UK plants have no selfing ability, the reduced proportion of produced seeds of emasculated plants in this population (54.2% vs. 37.3%) might have been caused by accidental damage to the flowers during the emasculation treatment.

Estimates of selfing and inbreeding depression—The multilocus estimate of outcrossing (t_m) and selfing (s) of open-pollinated plants are shown in Table 2. Population UK is not included because of herbivore damage. The mating system of *T. camschatcense* was either predominant selfing in the SC populations or predominant outcrossing in the SI populations. The estimated t_m of SC populations was low, but that of SI populations was extremely high (Table 2).

Parental fixation index (F'), seed fixation index (F''), and inbreeding depression from two-generation estimator (d_t) are also shown in Table 2. All fixation indices of SC populations were relatively high and significantly different from zero ($P < 0.001$). In these populations, however, F'' was higher than F', leading to extremely high values of inbreeding depression (d_t). Although fixation indices of SI populations showed positive deviations ($P < 0.05$), except for F' in NK, all values were very low, and the estimation of inbreeding depression (d_t) was impossible. However, similar results for inbreeding depression were obtained from the single generation estimator (d_s). Inbreeding depression (d_s) for the SC populations was 0.979 in IS and 0.935 in OF; values significantly greater than zero ($P < 0.001$ in IS, and $P < 0.001$ in OF). Due to low F values, estimates of d_s in SI populations indicated abnormal values exceeding one (2.607 in NK, and 4.098 in MU). However, the values for both populations were significantly greater than zero ($P = 0.029$ in NK, and $P = 0.049$ in OF).

Biparental inbreeding and effective number of pollen donors—The magnitude of biparental inbreeding ($1 - t_m$), correlated paternity ($2F_S$), and effective number of pollen donors (N_{ep}) are shown in Table 3. Because the samples used for the analysis in SC populations came from emasculated plants (i.e., all seeds derived from outcrossing), the values given here estimate the potential capacity of outcrossing, instead of that under natural conditions. Biparental inbreeding, which is the degree of mating between close relatives, differed significantly from zero in three of four populations, except for population MU ($P = 0.351$).

The average correlated paternity varied from 0.235 in MU to 0.333 in OF, but did not differ significantly among the populations according to the Steel–Dwass test. The effective number of pollen donors, which is the reciprocal of correlated paternity, varied from 3.00 in OF to 4.25 in MU.

DISCUSSION

Pollen availability in SI and SC populations—In general, SI plants have a higher pollen limitation than SC plants because of the lack of reproductive assurance. In a survey of studies of 46 SI and 117 SC species, SI species had significantly greater pollen
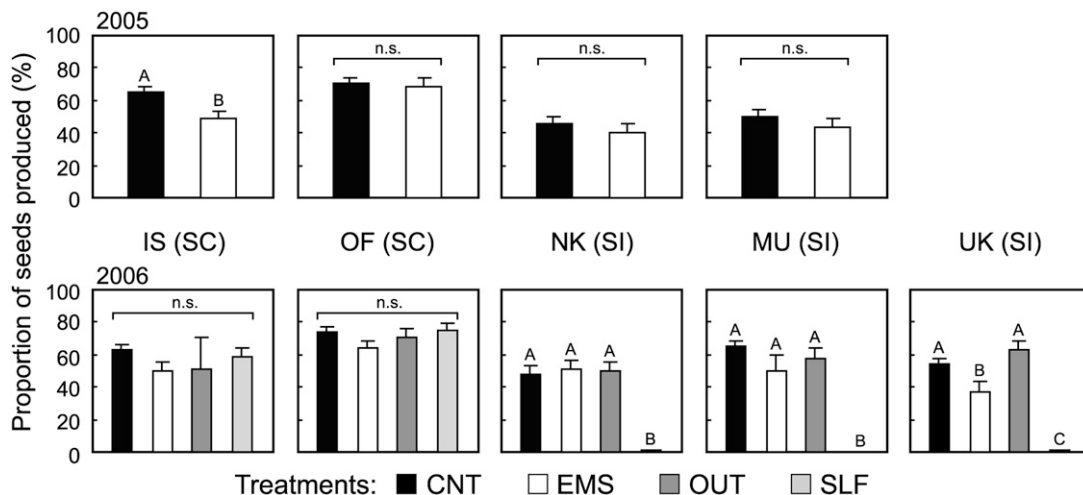

Fig. 2.   Mean proportion of produced seeds to total ovules in various pollination treatments conducted with each population of *Trillium camschatcense* in 2005 (upper row) and 2006 (lower row). Abbreviation and location of each population are shown in Table 1. Bars represent standard errors of the mean. Different letters indicate significant difference within treatments, and n.s. indicates that the differences were not significant (Tukey–Kramer test, $P < 0.05$). CNT, control; EMS, emasculation; OUT, outcrossing; SLF, selfing.

Table 2. Estimates of multilocus outcrossing (t_m), selfing (s), parental fixation index (F'), seed fixation index (F''), and inbreeding depression from two-generation estimator (d_t) of open-pollinated plants in each population of *Trillium camschatcense*. *P*-values for *F* indicate estimates that were significantly greater than zero.

Population[a]	Mating system	N_f	N_s	t_m (mean ± SE)	s $(1 - t_m)$	Parental F'	Seed stage F''	d_t
IS	SC	10 (control)	120	0.044 ± 0.034	0.956	0.483 ($P < 0.001$)	0.768 ($P < 0.001$)	0.931
OF	SC	10 (control)	120	0.119 ± 0.068	0.881	0.273 ($P < 0.001$)	0.620 ($P < 0.001$)	0.914
NK	SI	10 (control)	120	0.956 ± 0.052	0.044	−0.002 ($P = 0.496$)	0.055 ($P = 0.011$)	–
MU	SI	10 (control)	120	0.987 ± 0.111	0.013	0.087 ($P = 0.020$)	0.054 ($P = 0.009$)	–

[a] Name and location of populations are shown in Table 1. SC, self-compatible; SI, self-incompatible; N_f, number of fruits (treatment); N_s, number of seeds.

limitation than SC species (Burd, 1994). Similarly, Larson and Barrett (2000) found that the degree of pollen limitation in 102 SC species was half that observed in 66 SI species.

However, pollen availability in SI populations of *T. camschatcense* was equivalent to that in SC populations. Pollination experiments demonstrated that the proportion of seeds produced in open-pollinated individuals did not significantly differ from those of artificially outcrossed individuals in all five populations (Fig. 2). More importantly, the proportions of produced seeds in emasculated plants of the SC populations were not significantly different from those of control individuals, except population IS in 2005 (Fig. 2), indicating that SC populations of *T. camschatcense* seem to be highly capable of outcrossing.

The quality of the received outcross pollen was evaluated by estimating the magnitude of biparental inbreeding using open-pollinated plants of SI populations and emasculated plants of SC populations (Table 3). Both SC populations indicated a significant level of biparental inbreeding. Because self-pollination and geitonogamy are eliminated from this experiment, biparental inbreeding found in these populations may reflect mating between close relatives. However, the values were relatively small (0.108 in IS, and 0.063 in OF), and the effect from biparental inbreeding should be even smaller under exclusive self-pollination in the SC populations. Additionally, the correlated paternity showed no significant differences among populations, indicating comparable outcross pollen quality in all populations (Table 3). The effective number of pollen donors within a fruit ranged from 3.00 (OF) to 4.25 (MU), which was relatively high compared with values estimated in eastern North American *Trillium* species: *T. grandiflorum* = 1.01, *T. erectum* = 1.12 (Broyles et al., 1997), and *T. cuneatum* = 1.22–1.66 (Gonzales et al., 2006). The high value for the effective number of pollen donors may reflect the high outcrossing ability of *T. camschatcense*.

Adaptive significance of self-fertilization in Trillium camschatcense—Lloyd and Schoen (1992) recognized three timings of self-pollination: "prior," "delayed," and "competing."

Because the pistil of *T. camschatcense* becomes receptive to pollen slightly before anther dehiscence (Ihara, 1981), the mode of self-pollination would be "competing" to "slightly delayed." Therefore, self-fertilization in *T. camschatcense* can lead to either seed discounting or reproductive assurance, depending on pollen availability.

An allele that causes selfing can increase its own transmission by up to 50% in an outcrossing population (Fisher, 1941). Because the inbreeding depression in SC populations of *T. camschatcense* was larger than 0.5 (0.931 in IS and 0.914 in OF; Table 2), the gene transmission advantage of selfing might be eroded by the low survival of selfed offspring. Although reproductive assurance enables the evolution of self-compatibility even under high inbreeding depression, it is clear that the quantity and quality of outcross pollen availability in both SC populations is sufficient: there seems to be no fitness gain from reproductive assurance.

It is noteworthy that although the estimated selfing (s) in SC populations was extremely high (0.956 in IS and 0.881 in OF; Table 2), in spite of extensive inbreeding depression (0.931 and 0.914, respectively; Table 2), the potential for sufficient outcrossing was also high. These results indicate that the plants in SC populations are losing opportunities for outcrossing owing to competitive self-fertilization, which results in severe seed discounting. Additionally, considering the mode of selfing in *T. camschatcense* ("competing" to "slightly delayed"), the extensive selfing found in SC populations should result from the massive amount of self pollen landing on the stigma, which overwhelms the sufficient supply of outcross pollen. Massive investment of pollen to selfing could diminish the amount of exported pollen, and thus, SC populations may also suffer severe pollen discounting. Although direct measurement for pollen discounting is necessary, as far as our study is concerned, none of the fitness gain proposed for selfing (i.e., reproductive assurance and gene transmission advantage) was detected, but the fitness costs (i.e., inbreeding depression, seed discounting, and pollen discounting) were highly apparent.

Table 3. Biparental inbreeding and correlated paternity (mean ± SE) in each population of *Trillium camschatcense*. Because the effective number of pollen donors (N_{ep}) is the reciprocal of $2F_S$, standard errors are not included. *P*-values for biparental inbreeding indicate estimates that were significantly greater than zero.

Population[a]	Mating system	N_f	N_s	t_m (mean ± SE)	Biparental inbreeding $(1 - t_m)$	$2F_S$	N_{ep}
IS	SC	10 (emasc.)	108	0.892 ± 0.077	0.108 ($P = 0.015$)	0.319 ± 0.076	3.14
OF	SC	10 (emasc.)	120	0.937 ± 0.060	0.063 ($P = 0.031$)	0.333 ± 0.077	3.00
NK	SI	10 (control)	120	0.956 ± 0.052	0.044 ($P = 0.010$)	0.242 ± 0.057	4.13
MU	SI	10 (control)	120	0.987 ± 0.111	0.013 ($P = 0.351$)	0.235 ± 0.052	4.25

[a] Name and location of populations are shown in Table 1. SC, self-compatible; SI, self-incompatible; N_f, number of fruits (treatment); N_s, number of seeds; $2F_S$, pairwise kinship coefficient between pollen gametes within progeny arrays; N_{ep}, effective number of pollen donors.

Maintenance of high inbreeding depression in selfing populations—If inbreeding depression is caused by deleterious recessive alleles, theory predicts that continuous selfing in a population will cause deleterious recessives to become homozygous, providing the opportunity for "purging" of genetic load by natural selection (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987; Charlesworth et al., 1990). Consistent with this prediction, a study in *Leavenworthia alabamica*, which also retains both SC and SI populations, confirmed low inbreeding depression in the highly selfing SC populations (Busch, 2005), and similar examples are shown in many other species (reviewed in Crnokrak and Barrett, 2002).

In contrast, our study in *T. camschatcense* revealed an unexpectedly high inbreeding depression in the selfing populations. A very similar phenomenon has been reported in *Aquilegia canadensis* (Herlihy and Eckert, 2002) and *Juncus atratus* (Michalski and Durka, 2007), which similarly detected high inbreeding depression despite sufficient selfing. The finding of many selfing species that maintain high inbreeding depression requires explanation (Husband and Schemske, 1996; Goodwillie et al., 2005).

Genetic theories suggest that if the inbreeding depression is maintained by mildly deleterious mutations, or genetic variance in quantitative characters, or if it involves high genomic mutation rate, purging would be difficult (Lande and Schemske, 1985; Charlesworth et al., 1990). Lande et al. (1994) suggested that under high genomic mutation rate, selective interference between recessive deleterious loci can provide severe inbreeding depression in a selfing population. Life history may also explain the maintenance of high inbreeding depression in populations of *T. camschatcense*. The opportunity to produce recessive deleterious alleles through mutation increases with the species lifespan (Klekowski, 1988). Morgan (2001) proposed a model, which predicted that substantial mutation would contribute to the maintenance of high inbreeding depression in perennials. *Trillium camschatcense* is a perennial with extremely long life span, living for more than several decades (Ohara and Kawano, 1986); *Aquilegia canadensis* and *Juncus atratus*, which like *T. camschatcense* have high selfing with high inbreeding depression, are also perennials, although *A. canadensis* is a short-lived perennial (Herlihy and Eckert, 2004). In contrast, *L. alabamica*, which is highly selfing and has low inbreeding depression, is an annual. These theories, however, cannot completely explain the joint occurrence of high inbreeding depression and of high selfing, and further empirical and theoretical work is necessary.

Evolution of self-compatibility in Trillium camschatcense—Our empirical results suggest that the shift from self-incompatibility to self-compatibility is unlikely in this species. So how can we explain the evolution of self-compatibility in *T. camschatcense*? Our results suggest two plausible scenarios.

First, the advantage from reproductive assurance does not have to be present every year in every population for self-fertilization to evolve. The unpredictable receipt of outcross pollen may also produce conditions that favor the evolution of self-fertilization through reproductive assurance (Morgan and Wilson, 2005; but see also, Cheptou and Schoen, 2007). Additionally, plant species capable of selfing are more likely to be successful colonists than are SI species (Baker, 1955). The founding of a population by too few individuals to permit mates or pollinators would favor the evolution of self-fertilization through reproductive assurance.

Second, the advantage of self-fertilization may be strongly influenced by plant density or population size (Baker, 1955, 1967). Large population size would enhance outcrossing and therefore negate the advantage of reproductive assurance. In a survey of populations of *T. camschatcense* in Hokkaido, Ohara et al. (1996) found that SI populations tend to be large with high density, whereas SC populations were often small and fragmented. They also detected greater pollen limitation in the small SC populations, relative to those of large populations. Because all the populations used in the current study were relatively large with high flowering density (Table 1), high outcrossing ability may result from sufficient population size. In addition, recent studies suggested that the magnitude of inbreeding depression is affected by environmental variation: a high density of flowering individuals and abundant seed production would increase competition among progeny, resulting in greater inbreeding depression (Cheptou and Dieckmann, 2002; Armbruster and Reed, 2005). Thus, the intrinsic inbreeding depression of *T. camschatcense* could be much lower, and the severe inbreeding depression detected in the current study may reflect environmental conditions.

Although many theoretical and phylogenetic studies support the assumption that the loss of self-incompatibility is an unlikely event, restoration of self-incompatibility has been indicated by transgenic experiments in *A. thaliana* (Nasrallah et al., 2004) and hybridizing experiments in *Lycopersicon hirsutum* (Rick and Chetelat, 1991). Additionally, Ferrer and Good-Avila (2007) found phylogenetic evidence that the loss of self-incompatibility is reversible in members of the Asteraceae. To determine whether self-fertilization is derived in *T. camschatcense*, we need further investigations on variant populations of this species and phylogeographic analyses.

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