

Discovery of male sterile plants and their contrasting occurrence between self-compatible and self-incompatible populations of the hermaphroditic perennial *Trillium camschatcense*

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Abstract

The sex type of *Trillium camschatcense* has been considered to be strictly monomorphic, composed of only hermaphroditic individuals. However, field observations have detected anther-degenerated (AD) plants coexisting with hermaphrodites. The present study aimed to elucidate whether AD plants could be regarded as females and, if so, how their loss of male function is compensated. Because *T. camschatcense* retains both self-compatible (SC) and self-incompatible (SI) populations, the frequencies of AD plants in multiple populations (22 SC and eight SI) were examined to predict the fate of these individuals under contrasting breeding systems. Morphological and genetic analyses in a SC population demonstrated that AD plants were completely male sterile, but female fertile. Although the quantity of seed produced was similar, hermaphrodites produced seeds predominantly via selfing, whereas seeds of AD plants were entirely outcrossed. Because inbreeding depression was severe, AD plants achieved a fitness advantage through inbreeding avoidance. However, the frequency of AD plants varied among SC populations (0–42%), suggesting that environmental variance can modify the relative fitness. Conversely, the frequency was suppressed among SI populations (0–2%). Because SI hermaphrodites are free from inbreeding, AD plants would not benefit from inbreeding avoidance and would find it difficult to invade a SI population.

Keywords: gynodioecy, inbreeding depression, male sterility, self-compatibility, self-incompatibility.

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Introduction

Flowering plants possess diverse sexual systems from monoecy, where both sexes are expressed within an individual (hermaphroditism), to dioecy, where each sex is expressed in distinct individuals. Gynodioecy, a sexual system in which both hermaphroditic and female individuals coexist in a population (Darwin 1877), has gained special interest because this sexual system is predicted as an intermediate step in the evolution from hermaphroditism to dioecy (reviewed in Geber *et al.* 1999). It has been inferred that approximately 5–10% of plant species

possess this sexual system (Charlesworth 2002). Female individuals in gynodioecious populations are characterized as male sterile mutants of the hermaphrodites. These mutants may invade and persist in a population if their loss of male reproductive success is compensated for via increased female reproduction (Darwin 1877). The extent of the required reproductive advantage depends on the genetic bases of the male sterility genes. If male sterility is caused by nuclear genes, females must present at least a twofold reproductive advantage compared with hermaphrodites. In contrast, if male sterility is determined by cytoplasmic genes, which is more common among angiosperms, only a slight advantage is required (Lewis 1941; Lloyd 1975). However, it may not be this simple because cytoplasmic male sterility is usually counteracted

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by nuclear restorer genes (Saumitou-Laprade *et al.* 1994). This 'masking' of female phenotypes by restorer genes seems to be common, given that most angiosperm species consist of only hermaphroditic individuals (Geber *et al.* 1999), despite the ubiquitous presence of cytoplasmic male sterility genes (Laser & Lersten 1972; Schnable & Wise 1998; Tiffin *et al.* 2001; Chase 2007). Nevertheless, a population with only hermaphroditic phenotypes does not necessarily indicate a lack of potential to produce male sterile mutants. For example, although natural populations of *Schiedea menziesii* consist of only hermaphrodites, females have been detected in the progenies raised from field-collected seeds (Rankin *et al.* 2002). Moreover, Case and Willis (2008) extracted and characterized a cytoplasmic male sterility gene from a completely hermaphroditic population of *Mimulus guttatus*. The scarcity of gynodioecy in angiosperms is probably not because of a lack of mutants, but rather because of the strict conditions required for the females to persist in a hermaphroditic population (Lloyd 1975).

Females can compensate their loss of male reproductive success through two basic reproductive advantages. One is through an increment of seed fertility by allocating the reserved resources to female reproduction, which otherwise would have been used to form and develop male reproductive organs (Lewis 1941). In contrast, predominant outcrossing in females may improve the genetic quality of the seeds if hermaphrodites suffer from inbreeding depression (Lloyd 1975). The latter advantage is the major hypothesis explaining the persistence of female individuals in gynodioecious populations (Charlesworth & Charlesworth 1978). Consistent with this presumption, many studies have demonstrated significant inbreeding depression among the progenies of hermaphrodites in various gynodioecious species (Maki 1993; Sakai *et al.* 1997; Thompson & Tarayre 2000; Emery & McCauley 2002; Medrano *et al.* 2005). Because this advantage is achieved only under certain levels of self-fertilization in hermaphrodites, gynodioecy is generally expected to evolve from self-compatible (SC) ancestors that suffer from self-fertilization and inbreeding depression (Charlesworth & Charlesworth 1979; Charlesworth & Ganders 1979).

Trillium camschatcense (Melanthiaceae) is a hermaphroditic, perennial herb that mainly occurs in the understory of broad-leaved deciduous forests in Hokkaido and northern Honshu, Japan. In the present study, we report the discovery of anther-degenerated (AD) plants in a SC population of *T. camschatcense* and we examined their reproductive fitness relative to the hermaphrodites. First, the fundamental nature of these plants, including morphological variations, microscopic features of the male organ, genetic consistency of the phenotype and their frequency in the population, was investigated. In particu-

lar, their potential ability for male and female reproduction was tested. Because neither gynodioecy nor male sterile phenotypes have been reported within the genus, we carefully judged whether these plants could be regarded as functional females. Second, the outcrossing rate and seed fertility of the plants were compared with the rate and fertility of the hermaphrodites. We examined whether these plants retained any of the above-mentioned reproductive advantage. A previous study in this SC population revealed that the microsatellite-based selfing rate of the hermaphrodites was extremely high (0.881), despite severe inbreeding depression (0.914). However, because emasculated hermaphrodites produced abundant seeds, similar to artificially outcrossed individuals, the potential for sufficient outcrossing was high (Kubota *et al.* 2008). Therefore, if the AD plants are capable of producing abundant seeds exclusively by outcrossing, avoidance of inbreeding depression should provide a significant reproductive advantage for these individuals. Furthermore, if this reproductive advantage is to be achieved by the sacrifice of male function, the AD plants could be regarded as functional females.

Another interesting feature of *T. camschatcense* is the existence of self-incompatible (SI) populations. Although most populations possess self-compatibility, comprehensive surveys covering various populations in Hokkaido have detected self-incompatibility within restricted regions. Populations located in northern and southern Hokkaido are SC, potentially capable of both selfing and outcrossing, whereas eastern populations are SI and all seed production results from outcrossing (Ohara *et al.* 1996). Microsatellite-based analyses and pollination experiments have revealed that natural seed production in the SI populations results completely from outcrossing and pollen limitation was not detected during a 2-year survey (Kubota *et al.* 2008). In the present study, we examined the frequency of AD plants among 22 SC and eight SI populations of *T. camschatcense* to investigate whether the breeding system determined the existence of females. If inbreeding avoidance is the major factor allowing AD plants to invade the SC population, as expected, self-incompatibility would inhibit the plants from achieving a reproductive advantage. In other words, because both male sterility and self-incompatibility are reproductive strategies to avoid inbreeding depression, their simultaneous existence within a population would be unlikely. In accordance with this assumption, theories and previous observations generally support an exclusive relationship between self-incompatibility and male sterility (see Charlesworth 2001). However, substantive evidence has not been collected using empirical studies, particularly regarding the selective factor responsible for the relationship, because species suitable for such studies are extremely rare. Moreover, even in a SC population, inter-

population variation in the mating system of hermaphrodites (selfing rate and levels of inbreeding depression), the pollination ecology (pollinator activity and faunal composition), and many other ecological contexts can alter the relative fitness of female plants (reviewed in Ashman 2006). For example, if AD plants are unable to self-fertilize, pollen limitation could significantly reduce their fertility in a SC population. A study examining *Trillium grandiflorum*, which is in the same genus, clearly demonstrated that pollen limitation was negatively related to flowering density as a consequence of the Allee effect (Knight 2003). To test whether pollen limitation could affect the frequency of AD plants, the correlation between flowering density and frequency was analyzed among the 22 SC populations. Because a species that retains both SC and SI populations with a combination of male sterility has rarely been reported (but see Maki 1992), the present findings in *T. camschatcense* could provide new insights into the evolution of plant mating systems.

Materials and methods

Study species and study sites

The genus *Trillium* (Melanthiaceae; Angiosperm Phylogeny Group II 2003) contains approximately 45 species distributed in eastern Asia and North America. To date, all known *Trillium* species are hermaphroditic (Samejima & Samejima 1987), and either male sterility or gynodioecy have never been reported. *Trillium camschatcense* Ker Gawler is a hermaphroditic perennial that mainly occurs in the understory of broad-leaved deciduous forests in northern Honshu and Hokkaido, Japan (Samejima & Samejima 1962, 1987). The plants are non-clonal, long-lived and polycarpic (Ohara & Kawano 1986). Stems and leaves emerge from the underground rhizome during late April to early May. Reproductive individuals produce a single or sometimes a few nectarless flowers that arise from separate stems. Flowers are visited by generalist pollinators (e.g. Coleopteras and Dipteras) during the flowering period, which lasts for approximately 2 weeks (Ohara *et al.* 1991; Tomimatsu & Ohara 2003). Pollination experiments in Hokkaido have revealed that although most of the populations are SC, some populations in eastern Hokkaido are SI. Self-pollinated flowers of SI plants produce no seeds at all, but self-pollinated SC plants produce abundant seeds (Ohara *et al.* 1996). Details of self-incompatibility in the genus *Trillium* have been reported in the North American species *Trillium grandiflorum* and *Trillium erectum* (Sage *et al.* 2001). Although the genetic and physiological mechanisms remain unclear, self-rejection is known to occur at the stigma, associated with a dry-type stigma and bicellular pollen. We have also confirmed the same features in *T. camschatcense*, where

self-rejection is accomplished by inhibiting pollen tube growth at the stigma within 24 h after self-pollination (S. Kubota and M. Ohara, unpubl. data, 2009).

For the field experiments and the sampling of materials, a SC population, Ogifushi (OGF), was selected because it contained a large number of AD plants (Table 1). This population was previously used to assess the reproductive advantage of selfing among hermaphroditic individuals (Kubota *et al.* 2008). In the present study, five 2 m × 2 m quadrats were set randomly where *T. camschatcense* dominated, with each quadrat set at least 5 m apart. In the same way, five 2 m × 2 m quadrats were set in the 22 SC and in the eight SI populations (Table 1) for field observations.

Field experiments

The reproductive organ of a normal hermaphroditic *T. camschatcense* individual is characterized by six stamens and a pistil that splits at the style into three stigmas. The stamens are composed of a short filament and a longer anther with two thecas combined via the connective. The stamen of *T. camschatcense* is bright yellow and is generally longer than the pistil. In population OGF, we confirmed that hermaphroditic individuals shared these characteristics. In contrast, shrunken and whitened anthers characterize the morphologies of AD plants. Although quantitative analyses were not carried out, other characteristics except the anthers coincided with those of hermaphrodites (Fig. 1a). To standardize the present comparative analyses, the AD plants were categorized into four phenotypic categories according to their morphology. The definitions for each category are as follows: AD 1, stamens longer than the pistil with shrunken and whitened anthers; AD 2, stamens equal to the pistil with shrunken and whitened anthers; AD 3, stamens shorter than the pistil with shrunken and whitened anthers; and AD 4, stamens shorter than the pistil with minimized anthers. Individuals other than these categories were defined as hermaphroditic (HMP; Fig. 1b).

In May (the flowering period) 2007, all flowering individuals within the five quadrats were labeled and their phenotypic categories were noted according to the above-mentioned definitions. The flowering density and the average proportions of the categories were obtained by combining the observations from the five quadrats. In addition to population OGF, the same observations were conducted at the 22 SC and eight SI populations without labeling each individual. The correlations between flowering density and the frequency of AD plants in the SC populations were analyzed using Spearman's rank correlations with JMP version 4.0 software (SAS Institute 2000).

To investigate the female function of each category, fruits from population OGF were collected in July and seed set was examined. It is important to note that all

Table 1 Flowering density and frequency of the anther-degenerated plants in populations of *Trillium camschatcense*

Abbreviation	Population name	Latitude (E)	Longitude (N)	Breeding system	Flowering density (per 4 m ²)	Proportion of each phenotypic category					Frequency of AD plants
						HMP	AD 1	AD 2	AD 3	AD 4	
ISK	Ishikari	141.36	43.18	SC	45.3	0.86	0.03	0.04	0.01	0.06	0.14
ENW	Eniwa	141.60	42.88	SC	27.2	0.85	0.07	0.06	0.02	—	0.15
HKD	Hakodate	140.70	41.76	SC	15.3	1.00	—	—	—	—	0.00
KNK	Kaminokuni	140.09	41.81	SC	9.3	0.97	—	0.03	—	—	0.03
STN	Setana	139.85	42.43	SC	35.5	0.92	0.01	0.06	—	0.01	0.08
MOR	Mori	140.54	42.12	SC	40.4	1.00	—	—	—	—	0.00
NNE	Nanae	140.69	41.89	SC	23.8	0.99	0.01	—	—	—	0.01
OMB	Oshamanbe	140.35	42.50	SC	50.2	0.95	0.04	0.01	—	—	0.05
KYW	Kyouwa	140.59	43.01	SC	31.6	0.89	0.05	0.05	0.01	—	0.11
OGF	Ogifushi	142.67	42.20	SC	29.4	0.58	0.15	0.14	0.08	0.05	0.42
HOU	Houei	142.67	42.22	SC	4.2	0.79	—	0.05	0.16	—	0.21
ERM	Erimo	143.23	42.02	SC	3.3	0.70	—	0.10	0.10	0.10	0.30
KSH	Kamishihoro	143.41	43.28	SC	25.2	0.72	0.05	0.13	0.09	0.01	0.28
EHK	Ehoku	141.80	45.38	SC	23.2	0.99	0.01	—	—	—	0.01
TTM	Toyotomi	141.82	45.09	SC	24.6	0.99	0.01	—	—	—	0.01
SKR	Sakkuru	142.27	44.69	SC	18.8	0.89	0.07	0.04	—	—	0.11
STM	Shintomi	142.14	44.18	SC	16.8	0.83	0.03	0.03	0.01	0.10	0.17
SRM	Saroma	143.73	43.99	SC	42.2	0.99	0.01	—	—	—	0.01
HTB	Hamatonbestu	142.35	45.12	SC	27.2	0.98	0.01	0.01	—	—	0.02
ESS	Esashi	142.57	44.89	SC	6.6	0.85	—	—	0.10	0.05	0.15
YBT	Yobito	144.15	43.94	SC	34.0	1.00	—	—	—	—	0.00
TKG	Teshikaga	144.32	43.56	SC	18.6	1.00	—	—	—	—	0.00
ONB	Onbetsu	143.92	42.90	SI	25.5	1.00	—	—	—	—	0.00
YDN	Yuudounuma	143.55	42.62	SI	22.2	1.00	—	—	—	—	0.00
SHR	Shimohororo	144.33	43.13	SI	12.6	1.00	—	—	—	—	0.00
MUS	Musa	144.43	42.99	SI	38.4	1.00	—	—	—	—	0.00
MAU	Mauta	142.38	42.33	SI	30.4	0.98	—	—	0.01	0.01	0.02
NKP	Niikappu	142.30	42.37	SI	44.2	1.00	—	—	—	—	0.00
URK	Urakawa	142.75	42.18	SI	25.8	0.98	—	0.01	—	0.01	0.02
KKW	Kiyokawa	143.10	42.77	SI	26.0	1.00	—	—	—	—	0.00

Bold text indicates the population used for the microscopic and genetic analyses. AD, anther degenerated; HMP, hermaphrodite; SC, self-compatible; SI, self-incompatible.

fruits were pollinated under natural conditions. The numbers of 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 the total number of mature seeds produced per individual to the total number of ovules per individual) was calculated and arcsine transformed (Sokal & Rohlf 1995). Significant differences in total ovule numbers and in the proportion of seeds produced between the phenotypic categories were assessed using Tukey–Kramer's multiple comparison at $P=0.05$ with JMP version 4.0 software (SAS Institute 2000). In addition, for structural observation of the anthers and pollen, 10 flowers per phenotypic category were collected randomly from outside the established quadrats. Flowers just prior to anther dehiscence were chosen and stored in formalin-acetic-alcohol (FAA) solution. To dem-

onstrate the genetic consistency of the phenotypes, the phenotypic categories of all labeled individuals in population OGF were observed again in May 2008. We monitored any observational differences between the categories throughout the present study.

Microscopic analyses

The number of pollen per flower was estimated from the 10 FAA-fixed flowers collected from population OGF. The genus *Trillium* has three united carpels and six stamens. Of the six stamens, two adjacent stamens were selected from each flower. Each stamen was transferred into a 2.0-mL tube containing 1.5 mL of distilled water. Pollen grains were scraped off the anthers using pestles and then vortexed for suspension. The number of pollen grains included in 5 μ L of this solution was counted using a stereomicroscope and multiplied by 900 (multiplied by

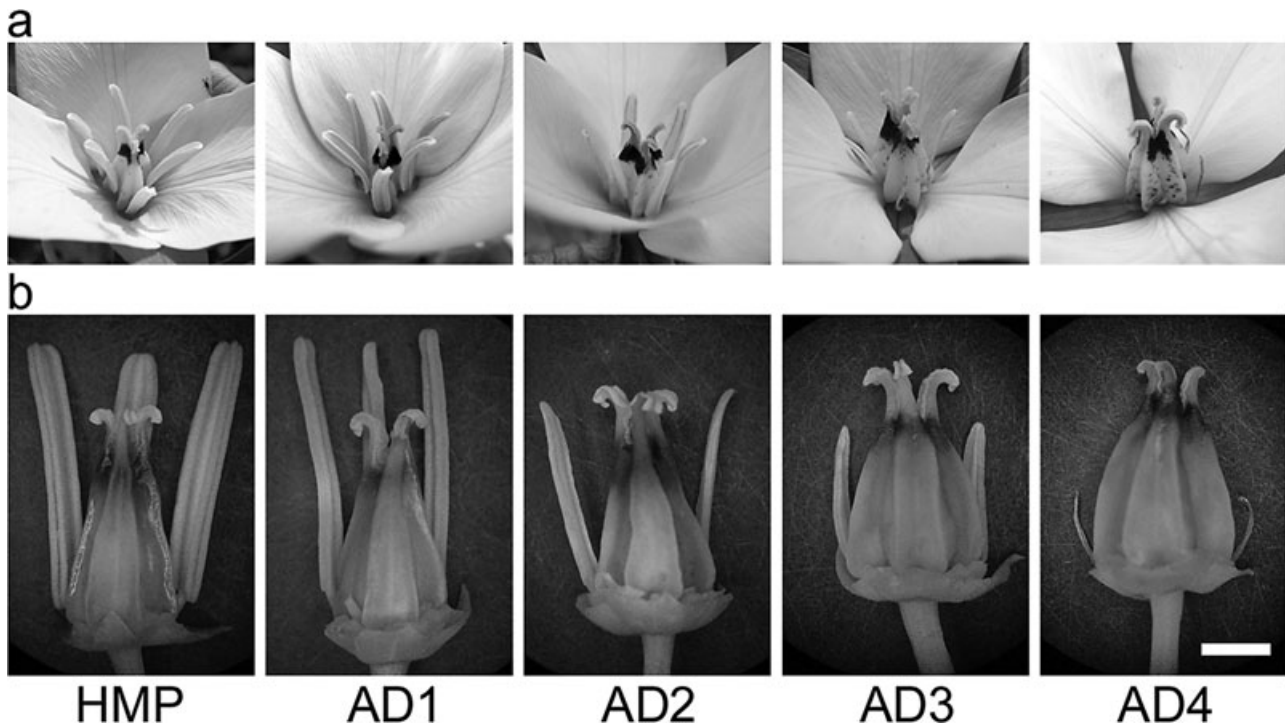


Fig. 1 Morphologies of each phenotypic category in the Ogifushi population. (a) Representative individuals for each phenotypic category found in the field. (b) Relationship between the length of the stamens and pistils observed using a stereomicroscope. Note that the sepals, petals and three stamens on the front side were removed to improve visualization. Scale bar = 5 mm.

Table 2 Characteristics (mean \pm standard error) of male and female function for each phenotypic category

Phenotypic category	Pollen number	Ovule number	Proportion of seeds produced	N_f	N_s	Multilocus outcrossing rate
HMP	557328 \pm 62611	212.88 \pm 12.93	0.685 \pm 0.036	9	99	0.149 \pm 0.054
AD 1	150588 \pm 60439	148.67 \pm 21.11	0.741 \pm 0.044	6	66	0.897 \pm 0.041
AD 2	2124 \pm 519	156.00 \pm 21.11	0.628 \pm 0.070	6	66	0.996 \pm 0.016
AD 3	1836 \pm 527	156.40 \pm 23.12	0.701 \pm 0.032	5	55	0.858 \pm 0.073
AD 4	252 \pm 142	185.50 \pm 25.85	0.662 \pm 0.036	4	44	0.996 \pm 0.011

AD, anther degenerated; HMP, hermaphrodite; N_f , number of fruits used for the genetic analysis; N_s , number of seeds used for the genetic analysis.

300 to obtain the total pollen number in the 1.5 mL solution, and again multiplied by three to obtain the total pollen number of the six stamens) to obtain the total number of pollen grains per flower. For each flower, the pollen count was repeated five times and the average number of pollen grains per flower was combined for each phenotypic category. In addition, these pollen grains were stained with hematoxylin and their structural features were examined using light microscopy.

Genetic analysis

To estimate the proportion of seeds produced via outcrossing under natural conditions, 11 seeds from each

fruit collected from the OGF population were selected randomly for DNA analysis. Because most of the labeled fruits were lost as a result of herbivore activity, all collected fruits were used for the analysis (see Table 2 for fruit numbers). The embryo was taken from each seed and stored in a 1.5-mL sample tube. In addition, the seed coat was detached from one seed per fruit as a maternal tissue and also stored in a 1.5-mL sample tube. All tubes were stored at -80°C prior to DNA extraction.

Genomic DNA was extracted from the frozen embryos and seed coats using a cetyltrimethyl ammonium bromide (CTAB) extraction procedure (Stewart & Via 1993). The DNA was amplified using a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA,

USA) with seven microsatellite primer pairs: TC2, TC15, TC44, TC48, TC69, TC77 and TC96 (Kubota *et al.* 2006; Kubota & Ohara 2009). A polymerase chain reaction (PCR) was carried out in a 10- μ L reaction mixture containing 0.5 μ L of template DNA, 0.2 mmol/L of each dNTP, 1 \times PCR buffer with 1.5 mmol/L MgCl₂ (Applied Biosystems), 0.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems) and two primers: fluorescent-dye-labeled forward primer (0.5 μ mol/L; Applied Biosystems) and a reverse non-tailed primer (0.5 μ mol/L). 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 fragment lengths of the PCR products were analyzed using an ABI Prism 3100 automated sequencer with GeneScan analysis software (Applied Biosystems).

Estimates of outcrossing were carried out with MLTR 3.1 software, which is based on Ritland's mixed mating model (Ritland & Jain 1981; Ritland 2002). Default values were used for parameter computation. Standard errors were estimated from 5000 bootstraps.

Results

A total of 147 flowering individuals were found in the five quadrats established in population OGF, with an average flowering density of 29.4/4 m². The proportions for each phenotypic category were: 0.58, 0.15, 0.14, 0.08 and 0.05 for HMP, AD 1, AD 2, AD 3 and AD 4, respectively (Table 1). In summary, 42% of the individuals possessed some degeneration at their anthers. Genetic consistency was found among the categories, except for a few individuals of AD 1, AD 2 and AD 3, which shifted between the observations taken in 2007 and 2008. Because the relative length of the stamens and the pistil was used to distinguish the categories (Fig. 1b), slight changes in these traits between years could have altered the categories. Convincingly, all individuals in the HMP and AD 4 categories, which had clear-cut definitions in addition to stamen–pistil length, showed complete consistency. Figure 2 presents the proportion of HMP individuals versus AD plants in the 22 SC and eight SI populations. Anther-degenerated plants were found in various populations; however, most populations contained very small numbers of these plants. The frequencies of AD plants in the SC populations varied from 0 to 0.42, whereas the frequencies in the SI populations were suppressed to very low values, ranging from 0 to 0.02 (Table 1). Although flowering density in the SC populations also varied among populations, there was no significant correlation between flowering density and the frequency of AD plants (Spearman's rank correlation coefficient = -0.21; $P = 0.35$).

During observations of population OGF, we noticed that none of the AD plants experienced anther dehiscence

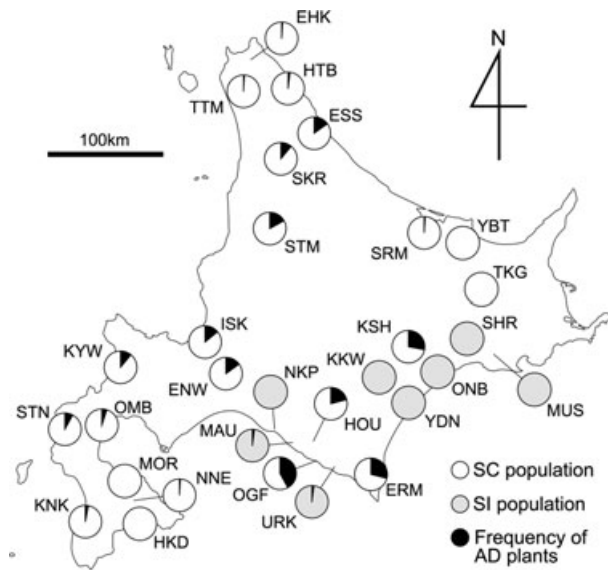


Fig. 2 Frequency of anther-degenerated (AD) plants in self-compatible (SC; white) and self-incompatible (SI; gray) populations. The names and locations of each population are shown in Table 1.

under natural conditions. This was also confirmed in the laboratory, where anthers of HMP underwent dehiscence after a drying period, whereas all anthers of the AD category plants did not dehisce even with sufficient drying. Structural features of the pollen grains also differed between the HMP and AD categories. Because the features among AD categories were identical, only the pollen grains of HMP and AD 1 are shown (Fig. 3). Pollen grains of HMP were powdery and disassociated from each other. In contrast, strong binding between pollen grains was found in all AD plants, and pollen grains removed from the anthers were united as a single cluster. The binding of pollen grains was remarkable and remained even after substantial vortex mixing. Most of the pollen grains could not be further subdivided from quartets. Although it is not clear, abnormal development during the pollen tetrad phase could be responsible for this clustering. The shape of the hydrated pollen grains of AD plants was rather awkward compared with the circular pollen grains of HMP. The number of pollen grains decreased greatly among the phenotypic categories, with AD 4 bearing less than 0.05% of the number of pollen grains that were found in HMP (Table 2).

Details of female reproduction are also shown in Table 2. Although the total number of ovules varied among the phenotypic categories from 148.67 (AD 1) to 212.88 (HMP), significant differences were not observed between any of the categories. However, there was an overall trend; AD categories retaining fewer ovules than HMP and there was a negative correlation between pollen

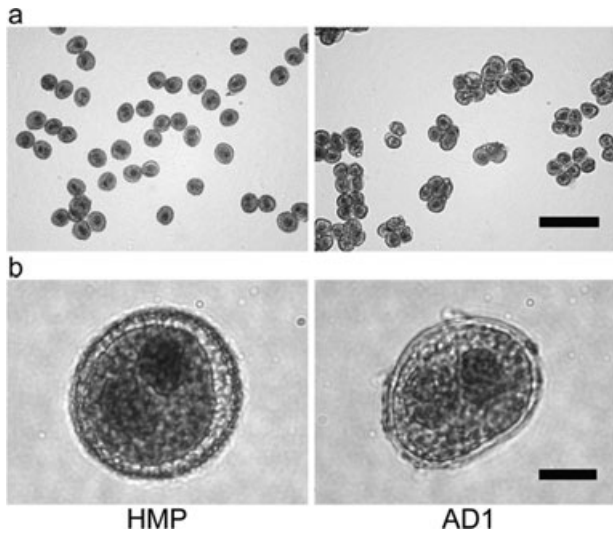


Fig. 3 Pollen grains of hermaphroditic (HMP) (left) and anther-degenerated (AD 1) (right) plants observed using a light microscope. (a) Powdery pollen grains of HMP (left) and clustered pollen grains of AD 1 forming quartets (right). Scale bar = 100 μm . (b) Isolated pollen grain of HMP (left) and AD 1 (right). Scale bar = 10 μm .

and ovule numbers within the AD categories. The average proportion of seeds produced under natural conditions did not differ significantly among the phenotypic categories. The multilocus estimate of outcrossing was low in HMP (0.149), as expected from our previous study (Kubota *et al.* 2008). Conversely, outcrossing rates of all AD categories were extremely high, ranging from 0.858 (AD 3) to 0.996 (AD 2).

Discussion

Feminization as a consequence of anther degeneration

Studies on *T. camschatcense* started to flourish in the 1930s because of its large chromosomes, which are suitable for karyotypic analyses (Gotoh & Stow 1930; Haga 1934, 1937). Since then, continuous studies on *T. camschatcense* have elucidated various aspects of its life history (reviewed in Ohara & Kawano 2005). The recent development of molecular markers has enabled further investigation, particularly into its reproductive ecology (Kubota *et al.* 2006). The unexpected combination of high selfing, severe inbreeding depression and sufficient pollen supply in the SC populations has indicated a possibility of the existence of obligate outcrossers in these populations (Kubota *et al.* 2008). Taking the above results into account, a careful investigation of the mating system may come up with a completely new finding in *T. camschatcense*.

As expected, anther degeneration in *T. camschatcense* was accompanied by decreased pollen numbers, abnor-

mal development of the pollen grains and a lack of anther dehiscence. In particular, the last phenomenon prohibited pollen emission, which led to a complete loss of male function. Because this inability of anther dehiscence was found throughout the AD plants, these individuals were completely male sterile, regardless of the phenotypes. In contrast, reproduction via female function seemed highly capable. All AD categories held abundant ovules and the outcrossing rates were extremely high. Because these outcrossing rates were similar to the rates estimated from emasculated hermaphrodites in the same population (0.937; Kubota *et al.* 2008), we conclude that all AD plants are obligate outcrossers. It remains unclear whether phenotypic categories, such as AD 1, AD 2 and AD 3, can be assumed to be an 'intermediate' step from hermaphroditism (HMP) to complete feminization (AD 4) because we could not detect significant differences in reproduction among the AD categories. Two years of observations in the field demonstrated the genetic consistency of anther degeneration. We assume that the yearly changes found in some of the AD categories resulted from slight changes in the relative length of the stamens and pistil. Support for anther degeneration as a genetically determined phenotype was also obtained from an observation that transplanted AD plants possessed anther-degenerated flowers every year over a 7-year period (M. Ohara, unpubl. data, 2009). Interestingly, among the 28 observed populations in Hokkaido, AD plants were found in 17 of them. Thus, anther degeneration seems to be somewhat general in this species. In this connection, we must refer to the triploid and hexaploid *T. hague*, which is a natural hybrid between diploid *T. camschatcense* and tetraploid *T. tschonoskii* and its polyploid form. Both triploid and hexaploid *T. hague* have stamens shorter than pistils and resemble the AD plants. Although few of the populations used in the present study contained this hybrid (with a very small number), AD plants and *T. hague* could be easily distinguished according to their morphological characteristics. Even if contamination had occurred, which was not the case in the present study, microsatellite markers can identify each species (S. Ishizaki and M. Ohara, unpubl. data, 2009). From this evidence, anther degeneration in *T. camschatcense* can be regarded as a feminization and AD plants, regardless of their categories, should be considered to be functional females. Therefore, facultative gynodioecy must be recognized as a component of the sexual system for *T. camschatcense*.

Reproductive advantage of anther-degenerated plants

Now that the AD plants can be considered as females, we must infer how they compensate their loss of male reproduction. Because the frequency of AD plants in the OGF population was relatively high (0.42), we can expect that

some type of reproductive advantage enabled these plants to invade and persist in this population. Compensation can take two forms. One is through an increase in seed fertility by reallocating additional resources to female reproduction, and the other is by improving the genetic quality of the seeds (Lewis 1941; Lloyd 1975). Because anther degeneration greatly decreased the pollen numbers of its host, we expected that a reallocation of the reserved resources was functioning in *T. camschatcense*. For example, AD plants could retain higher ovule numbers or increased seed sets compared with the hermaphrodites. Although a negative correlation between pollen and ovule number, which points to the possibility of resource allocation, was marginally indicated among the four AD categories, the trend was not statistically supported. In any case, all AD categories failed to exceed the total ovule number of HMP. In contrast, high proportions of the ovules were fertilized uniformly in all five categories. Pollen supplementation via artificial outcrossing in the same population indicated a similar value (0.705; Kubota *et al.* 2008). Thus, the proportion of seeds produced is at saturation, and all categories appear to be free from pollen limitation in this population. In summary, the quantity of seed produced in the AD plants was similar or slightly smaller than the number produced in the hermaphrodites, which means that compensation through resource allocation is not likely in *T. camschatcense*.

In contrast, substantial differences existed between AD plants and hermaphrodites with respect to the quality of the seeds produced. The multilocus outcrossing rate of the hermaphrodites was similar to that recorded in our previous study (0.119; Kubota *et al.* 2008), suggesting that extensive selfing is an inherent feature in *T. camschatcense*. Because inbreeding depression in this population was severe (0.914), despite the sufficient outcross pollen supply, hermaphrodites were losing opportunities for outcrossing because of competitive selfing (Kubota *et al.* 2008). Meanwhile, the loss of male function enabled the AD plants to conduct exclusive outcrossing and avoid inbreeding depression. Theory predicts that females can persist under high selfing rates and severe inbreeding depression of hermaphrodites, regardless of the genetic mechanisms for male sterility (Charlesworth & Charlesworth 1978). Although there are alternative ways to compensate for the loss of male function (e.g. extension of lifespan, increment of tolerance and effective seed dispersal), inbreeding avoidance appears to play a major role in maintaining the AD plants in *T. camschatcense*.

Anther degeneration in self-compatible and self-incompatible populations

If inbreeding avoidance is the major reproductive advantage of AD plants, the breeding system of the hermaph-

rodites should strongly affect the ability of these plants to invade and persist within a population. Because *T. camschatcense* retains both SC and SI populations (Ohara *et al.* 1996), the fate of an AD plant should differ between these contrasting breeding systems.

Gynodioecy as a consequence of male sterility is predicted to evolve from SC hermaphroditic species (Charlesworth & Charlesworth 1979; Charlesworth & Ganders 1979) and, indeed, hermaphrodites in most gynodioecious species possess self-compatibility. A study of *Chinographis japonica* var. *japonica* revealed that gynodioecy was found only in highly selfing SC populations, whereas outcrossing SI populations were completely hermaphroditic (Maki 1992). Consistent with this, high frequencies of AD plants were found in only the SC populations of *T. camschatcense*. As shown in the previous section, a combination of high selfing, severe inbreeding depression and sufficient pollen supply can maintain AD plants at a high frequency. However, inter-population variation of female frequency is common in many gynodioecious species (e.g. Sun & Ganders 1986; Delph & Carroll 2001; Asikainen & Mutikainen 2003; Murayama *et al.* 2004), suggesting that variations in environmental aspects can alter the relative fitness of the hermaphrodites and females (reviewed in Ashman 2006). For example, the scarcity of pollen donors can diminish the reproductive success of females more severely than hermaphrodites (Maurice & Fleming 1995). Not surprisingly, variation in the frequency of AD plants was also detected among the 22 SC populations, and no AD plants were found in five of these populations. In the present study, however, we could not detect any correlation between flowering density and the frequency of AD plants. The problem is that the present conditions of the populations may not necessarily reflect the past condition when the frequency of AD plants was determined. Because individuals of *T. camschatcense* have an extremely long lifespan (Ohara & Kawano 1986), a rapid decrease in population size may proceed without changing the frequency of AD plants. A direct investigation by pollination experiments in each population will be necessary to clarify these problems. In addition, there are many other factors (e.g. levels of inbreeding depression, resource availability and pollinator fauna or activity) that can alter the relative fitness of AD plants, and further investigations are needed to assess these various ecological contexts. Consequently, although self-compatibility appears to be a necessary condition for maintaining AD plants, there may be other ecological factors that modify their relative fitness.

Because hermaphrodites in SI populations are free from inbreeding depression (Ohara *et al.* 1996; Kubota *et al.* 2008), inbreeding avoidance would not serve as an advantage in these populations. Consistent with this assumption, the frequencies of AD plants among all eight SI populations were suppressed to very low values (< 0.02).

The finding of very few AD plants in these populations (two individuals at both MAU and URK) may indicate that the scarcity of these plants is not because of the lack of emergence, but rather the inability of the plants to spread in a SI population. Once emerged, the long lifespan of *T. camschatcense* could enable the AD plant to persist for at least several decades. We presume that the frequency of occasional emergence of AD plants is not very different between the breeding systems; however, successful invasion cannot take place under self-incompatibility. Baker (1959) was the first to point out the mutually exclusive relationship of self-incompatibility and sexual dimorphism. Simulations have predicted that gametophytic self-incompatibility is easily lost in a gynodioecious population compared with a hermaphroditic population (Ehlers & Schierup 2008). Empirical support for the prediction has been achieved by comparative analyses of multiple taxa with different mating systems. For example, a study in the North American species *Lycium* (Solanaceae) indicated a breakdown in the self-incompatibility system in all three gynodioecious species, whereas the closely related hermaphroditic species maintained self-incompatibility (Miller & Venable 2002). However, the consequence of the combination of male sterility and self-incompatibility has not been investigated directly because such an event is rarely found in flowering plants (but see Maki 1992; Dinnézt 1997). Thus, the present finding in *T. camschatcense* could be the first to provide an opportunity to compare the fate of male sterility under self-compatibility and self-incompatibility. In addition, investigations into the genetic background of anther degeneration and self-incompatibility could further clarify the evolutionary dynamics of the mating system in this species.

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