

The evolution of self-compatible and self-incompatible populations in a hermaphroditic perennial, *Trillium camschatcense* (Melanthiaceae)

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Abstract The evolution of selfing from outcrossing ancestors is known to have occurred repeatedly in angiosperms. Theoretical studies have argued that the transition from outcrossing to selfing is accomplished more easily than the reverse case, and phylogenetic analyses involving self-compatible (SC) and self-incompatible (SI) species has basically supported this assumption. The evolutionary direction of self-compatibility and self-incompatibility was examined in *Trillium camschatcense*, which contains geographically widespread SC populations, and restricted SI populations. Ecological surveys have revealed that the SC populations were suitable for outcrossing, and selfing in these populations did not confer any fitness advantage. Since reproductive fitness indicates the possibility of an evolutionary shift from self-compatibility to self-incompatibility, the phylogenetic relationships of SI and SC populations of *T. camschatcense* were investigated based on cpDNA variations and nuclear DNA microsatellite polymorphisms. Although phylogenetic analyses did not provide credible evidence to determine evolutionary direction, the SI populations turned out to be monophyletic with extremely low genetic differentiation. Based on these results, we proposed two possible scenarios for the evolutionary backgrounds of SI and SC populations in *T. camschatcense*. The plausibility of each scenario was evaluated

based on the reproductive and geographical features of the mating systems.

Keywords Evolutionary irreversibility · Intraspecific · Mating system evolution · Outcrossing · Phylogeny · Selfing

Introduction

The shift from outcrossing to selfing is one of the most common evolutionary transitions in the angiosperms (Stebbins 1974; Grant 1981; Barrett et al. 1989, 1996; Shimizu et al. 2004). Darwin (1876) pointed out that selfing would be evolutionary advantageous by ensuring seed production under mate- or pollinator-scarcity, and many empirical studies have investigated the reproductive assurance provided by selfing (Eckert and Schaefer 1998; Herlihy and Eckert 2002; Kalisz et al. 2004; Moeller and Geber 2005). Additionally, selfing should be advantageous over outcrossing in terms of gene transmission; selfing transmits two sets of genes to the offspring, whereas outcrossing can transmit only a single set (Fisher 1941). Thus, the evolution from selfing to outcrossing may occur only if the fitness of outcrossed progeny is more than twice that of selfed progeny (Darwin 1876; Charlesworth and Charlesworth 1987; Uyenoyama et al. 1993). However, theoretical studies have presumed that such an evolutionary shift might be unlikely to occur (Lloyd 1979; Charlesworth 1980; Feldman and Christiansen 1984; Lande and Schemske 1985). Although inbreeding depression (i.e., diminution of vigor and fertility of selfed offspring) can diminish the transmission advantage of selfing, continuous selfing may purge the genetic load by increasing the opportunity for selection to act against deleterious alleles.

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Once inbreeding depression is purged, a selfing population should never revert to outcrossing, which means that the evolution of selfing should be one-way (Lande and Schemske 1985). However, one should also note that purging could be difficult if inbreeding depression is maintained by mildly deleterious mutations, or genetic variance in quantitative characters, or if it involves high genomic mutation rate (Lande and Schemske 1985; Charlesworth et al. 1990).

Empirical studies on the evolutionary direction of mating systems, i.e., from outcrossing to selfing and vice versa, have focused mainly on comparisons of self-compatible (SC) and self-incompatible (SI) species within closely related species groups and SC and SI races within a species (Bateman 1955; Lloyd 1965; Brauner and Gottlieb 1987; Macnair et al. 1989; Wyatt et al. 1992). SC plants are capable of selfing, whereas in the case of SI plants, selfing is prevented by the ability to reject their own pollen and only outcrossing takes place. Phylogenetic analyses involving SC and SI species generally implicate SI species in the ancestral lineages (Goodwillie 1999; Kondo et al. 2002; Tao et al. 2007), and a review of seven phylogenetic studies also demonstrated that most studies are in accord with the above-mentioned theoretical hypotheses (Takebayashi and Morrell 2001). In addition, macrophylogenetic studies in Solanaceae have indicated clearly that the loss of self-incompatibility is essentially irreversible (Igic et al. 2003, 2006). However, the restoration of self-incompatibility has been confirmed by transgenic experiments in *Arabidopsis thaliana* (Nasrallah et al. 2004) and hybridizing experiments in *Lycopersicon hirsutum* (Rick and Chetelat 1991), indicating the possibility that the evolution of mating system could be reversible. Moreover, macrophylogenetic studies in Polemoniaceae (Barrett et al. 1996) and Asteraceae (Ferrer and Good-Avila 2007) have indicated rare cases in which SI species were derived from SC lineages. Therefore, broad surveys will be required before generalizing the one-way evolution hypothesis, i.e., from outcrossing to selfing and from SI to SC.

The present study focused on the evolutionary direction of self-compatibility and self-incompatibility in a hermaphroditic perennial, *Trillium camschatcense*. Because this species retains both SC and SI populations within a species (Ohara et al. 1996), the two mating systems can be expected to have differentiated relatively recently. Thus, the selective pressure that induced the differentiation could be currently acting, and the geographical distribution of the mating systems may reflect past evolutionary history, allowing both ecological and geographical approaches to be taken in investigating the evolutionary direction. Therefore, *T. camschatcense* should be a valuable species for studying the evolutionary direction of mating systems. In fact, in genetic analyses using microsatellite makers,

Kubota et al. (2008) demonstrated that selfing rates of the studied SC populations were extremely high (0.96 and 0.88) compared to that of the SI populations (0.04 and 0.01). Despite the high selfing rates, selfed progeny underwent severe inbreeding depression in both SC populations (0.93 and 0.91). Because pollination experiments did not detect any pollen limitation in SI populations, and the potential for sufficient cross-fertilization in SC populations existed, selfing in *T. camschatcense* does not appear to provide reproductive assurance. On the contrary, it appears to lead to seed discounting (Kubota et al. 2008). In other words, the SC populations were under a selective pressure, which should drive the evolution of predominant outcrossing. Although evolution from self-compatibility to self-incompatibility is highly unusual in the angiosperms, it is clear that both evolutionary directions must be considered in this species. Here, molecular phylogenetic analyses were conducted using cpDNA non-coding regions and nuclear DNA microsatellites to investigate the evolutionary direction between self-incompatibility and self-compatibility in *T. camschatcense*.

Materials and methods

Study species and study sites

Trillium camschatcense Ker Gawler (Melanthiaceae; APG II 2003) is a hermaphroditic perennial that is distributed from northern Honshu and Hokkaido, Japan, to further north in Sakhalin, the Kurile Islands, and the Kamtchatka Peninsula (Hultén 1927; Tatewaki 1957; Samejima and Samejima 1962, 1987). The plants are nonclonal, long-lived, and polycarpic (Ohara and Kawano 1986). Pollination experiments in Hokkaido have revealed that while most of the populations are SC, some populations in eastern Hokkaido are SI (Fig. 1). Self-pollinated flowers of SI plants produce no seeds at all, but self-pollinated SC plants produce abundant seeds (Ohara et al. 1996). In addition, the flowers of the SI populations are morphologically characterized by their larger petal size and longer anther compared to the flowers in other SC populations (Samejima 1958). So far, mixed population with both SC and SI plant has never been reported. Details of self-incompatibility in the *Trillium* genus have been reported in the North American species *T. grandiflorum* and *T. erectum* (Sage et al. 2001). Although the genetic and physiological mechanisms remain unclear, self-rejection is known to occur at the stigma, associated with 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 et al.

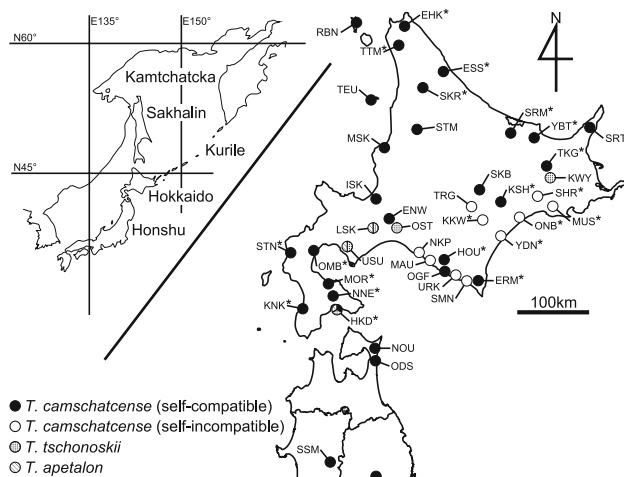


Fig. 1 Study sites of *Trillium* species used in this study. The name and location of each population are shown in Table 1. Asterisks indicate populations that were used for nuclear DNA microsatellite analyses

unpublished data). Self-rejection of *T. grandiflorum* and *T. erectum* exhibits some leakiness, leading to occasional selfing, whereas self-rejection of *T. camschatcense* is complete, which is consistent with former pollination experiments and genetic analyses in this species (Ohara et al. 1996; Kubota et al. 2008).

In May (flowering time) 2007, leaves of *T. camschatcense* were collected from 39 populations located in Hokkaido and northern Honshu, Japan (Fig. 1, Table 1). The self-compatibility or self-incompatibility of each population was determined either by previous hand-pollination experiments or by flower morphology. Leaves were collected randomly from 20 flowering individuals per population and silica-dried for subsequent DNA extraction. As an outgroup, leaves of five populations of *Trillium tschonoskii* and three populations of *Trillium apetalon* collected in 2003 were used. These are the closest relatives of *T. camschatcense* (Osaloo et al. 1999), and both species possess self-compatibility (Ohara and Kawano 1987). For all three species, one individual from each population was used for cpDNA analysis. For nuclear DNA microsatellite analysis, we used 21 populations of *T. camschatcense*. Five SI populations were chosen from their main distribution in eastern Hokkaido. For SC populations, 16 populations were chosen from throughout the species range in Hokkaido (Fig. 1). From each population, 20 individuals were used, except for populations HOU and ERM, where flowering individuals were scarce (15 and 6 individuals, respectively).

PCR amplification and sequencing of cpDNA

Genomic DNA was extracted from the silica-dried leaves by a CTAB extraction procedure (Stewart and Via 1993). Three noncoding regions of cpDNA, *psbD-trnT*, *ndhF-rpl32*, *rpl32-trnL*, were amplified using primer pairs *psbD* and *trnT*^(GGU)-R, *ndhF* and *rpl32*-R, and *rpl32*-F and *trnL*^(UAG), respectively, as designed by Shaw et al. (2007). Polymerase chain reaction (PCR) was performed in a 20- μ L reaction mixture containing 1.0 μ L template DNA, 0.2 mM each dNTP, 1 \times PCR buffer with 1.5 mM MgCl₂ (Applied Biosystems, Foster City, CA), 0.5 U *AmpliTaq* Gold DNA polymerase (Applied Biosystems), and 0.5 μ M of each primer. All PCR amplifications were performed using a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) using the “*rpl16*” cycling program of Shaw et al. (2005). The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA), and sequenced directly using a Big Dye terminator ready reaction mix (Applied Biosystems). Both forward and reverse strands of each cpDNA region were sequenced independently, and the sequences were determined on an ABI Prism 3100 automated sequencer (Applied Biosystems). The sequence of each cpDNA region will appear in GenBank under the accession numbers AB385700–AB385837 (Table 1).

PCR amplification and genotyping of microsatellites

DNA was amplified with a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) using seven nuclear DNA microsatellite primer sets: TC2, TC15, TC44, TC48, TC69 (Kubota et al. 2006), and two newly designed primer sets TC77 and TC96 (Table S1). PCR was performed in a 10- μ L reaction mixture containing 0.5 μ L template DNA, 0.2 mM each dNTP, 1 \times PCR buffer with 1.5 mM MgCl₂ (Applied Biosystems), 0.25 U *AmpliTaq* Gold DNA polymerase (Applied Biosystems), and two primers: fluorescent-dye-labeled forward primer (0.5 μ M; Applied Biosystems) and a reverse nontailed primer (0.5 μ M). 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 analyzed on an ABI Prism 3100 automated sequencer using GeneScan analysis software (Applied Biosystems).

Phylogenetic analyses

Sequence data of cpDNA were aligned manually using ProSeq version 2.95 (<http://www.biosciences.bham.ac.uk/labs/filatov/proseq.html>). Phylogenetic trees were constructed based on neighbor-joining (NJ), maximum parsimony (MP), and

Table 1 Study sites of *Trillium* species and GenBank accession numbers used for phylogenetic analyses

Abbreviation	Population name	Latitude (E)	Longitude (N)	Sampled species	Breeding system	GenBank accession numbers		
						<i>psbD-trnT</i>	<i>ndhF-rpl32</i>	<i>rpl32-trnL</i>
MSK	Mashike	141.58	43.87	<i>T. camschatcense</i>	SC	AB385700	AB385747	AB385794
ISK	Ishikari	141.36	43.18	<i>T. camschatcense</i>	SC ^a	AB385701	AB385748	AB385795
ENW	Eniwa	141.60	42.88	<i>T. camschatcense</i>	SC ^a	AB385702	AB385749	AB385796
HKD	Hakodate	140.70	41.76	<i>T. camschatcense</i>	SC ^a	AB385703	AB385750	AB385797
				<i>T. tschonokii</i>	SC	AB385742	AB385789	AB385836
				<i>T. apetalon</i>	SC	AB385746	AB385793	AB385840
KNK	Kaminokuni	140.09	41.81	<i>T. camschatcense</i>	SC	AB385704	AB385751	AB385798
STN	Setana	139.85	42.43	<i>T. camschatcense</i>	SC	AB385705	AB385752	AB385799
MOR	Mori	140.54	42.12	<i>T. camschatcense</i>	SC	AB385706	AB385753	AB385800
NNE	Nanae	140.69	41.89	<i>T. camschatcense</i>	SC ^a	AB385707	AB385754	AB385801
OMB	Oshamanbe	140.35	42.50	<i>T. camschatcense</i>	SC ^a	AB385708	AB385755	AB385802
ODS	Odanosawa	141.39	41.23	<i>T. camschatcense</i>	SC ^a	AB385709	AB385756	AB385803
NOU	Noushi	141.36	41.31	<i>T. camschatcense</i>	SC ^a	AB385710	AB385757	AB385804
KZK	Kuzakai	141.35	39.64	<i>T. camschatcense</i>	SC ^a	AB385711	AB385758	AB385805
SSM	Sashimaki	140.69	39.67	<i>T. camschatcense</i>	SC ^a	AB385712	AB385759	AB385806
OGF	Ogifushi	142.67	42.20	<i>T. camschatcense</i>	SC ^a	AB385713	AB385760	AB385807
HOU	Houei	142.67	42.22	<i>T. camschatcense</i>	SC ^a	AB385714	AB385761	AB385808
ERM	Erimo	143.23	42.02	<i>T. camschatcense</i>	SC ^a	AB385715	AB385762	AB385809
KSH	Kamishihoro	143.41	43.28	<i>T. camschatcense</i>	SC	AB385716	AB385763	AB385810
SKB	Shikaribetsu	143.10	43.27	<i>T. camschatcense</i>	SC	AB385717	AB385764	AB385811
TEU	Teuri	141.33	44.43	<i>T. camschatcense</i>	SC	AB385718	AB385765	AB385812
RBN	Rebun	141.02	45.40	<i>T. camschatcense</i>	SC	AB385719	AB385766	AB385813
EHK	Ehoku	141.80	45.38	<i>T. camschatcense</i>	SC	AB385720	AB385767	AB385814
TTM	Toyotomi	141.82	45.09	<i>T. camschatcense</i>	SC ^a	AB385721	AB385768	AB385815
SKR	Sakkuru	142.27	44.69	<i>T. camschatcense</i>	SC ^a	AB385722	AB385769	AB385816
STM	Shintomi	142.14	44.18	<i>T. camschatcense</i>	SC ^a	AB385723	AB385770	AB385817
SRM	Saroma	143.73	43.99	<i>T. camschatcense</i>	SC ^a	AB385724	AB385771	AB385818
ESS	Esashi	142.35	45.12	<i>T. camschatcense</i>	SC ^a	AB385725	AB385772	AB385819
YBT	Yobito	144.15	43.94	<i>T. camschatcense</i>	SC ^a	AB385726	AB385773	AB385820
TKG	Teshikaga	144.32	43.56	<i>T. camschatcense</i>	SC ^a	AB385727	AB385774	AB385821
SRT	Shiretoko	145.20	44.16	<i>T. camschatcense</i>	SC	AB385728	AB385775	AB385822
ONB	Onbetsu	143.92	42.90	<i>T. camschatcense</i>	SI	AB385729	AB385776	AB385823
YDN	Yuudounuma	143.55	42.62	<i>T. camschatcense</i>	SI*	AB385730	AB385777	AB385824
SHR	Shimohororo	144.33	43.13	<i>T. camschatcense</i>	SI*	AB385731	AB385778	AB385825
MUS	Musa	144.43	42.99	<i>T. camschatcense</i>	SI	AB385732	AB385779	AB385826
MAU	Mauta	142.38	42.33	<i>T. camschatcense</i>	SI ^a	AB385733	AB385780	AB385827
NKP	Niikappu	142.30	42.37	<i>T. camschatcense</i>	SI ^a	AB385734	AB385781	AB385828
SMN	Samani	142.93	42.14	<i>T. camschatcense</i>	SI ^a	AB385735	AB385782	AB385829
URK	Urakawa	142.75	42.18	<i>T. camschatcense</i>	SI ^a	AB385736	AB385783	AB385830
KKW	Kiyokawa	143.10	42.77	<i>T. camschatcense</i>	SI ^a	AB385737	AB385784	AB385831
TRG	Tsurugi	142.89	42.85	<i>T. camschatcense</i>	SI	AB385738	AB385785	AB385832
LSK	Lake Shikotsu	141.44	42.77	<i>T. tschonokii</i>	SC	AB385739	AB385786	AB385833
				<i>T. apetalon</i>	SC	AB385744	AB385791	AB385838
OST	Osatsu	141.66	42.86	<i>T. tschonokii</i>	SC	AB385740	AB385787	AB385834
USU	Usu	140.82	42.54	<i>T. tschonokii</i>	SC	AB385741	AB385788	AB385835
				<i>T. apetalon</i>	SC	AB385745	AB385792	AB385839
KWY	Kawayu	144.45	43.62	<i>T. tschonokii</i>	SC	AB385743	AB385790	AB385837

SC self-compatible, SI self-incompatible

^a Populations in which the breeding system was confirmed by previous hand-pollination experiments (Ohara et al. 1996)

Bayesian methods. Microsatellites (poly A and poly AT) were excluded while constructing these trees. All indels, other than microsatellites, composed of more than 5 bp were treated as a single character resulting from one mutational event. Because p -distance is generally the most reliable measure for constructing NJ trees of closely related sequences (Takahashi and Nei 2000), uncorrected p -distances were used to reconstruct a tree using the NJ method (Saitou and Nei 1987) in PAUP* 4.0 (Swofford 2002). In addition, the program Modeltest 3.7 (Posada and Crandall 1998) was used to search for the best-fit model for the evolution of these haplotypes. Note that p -distance is not included as a candidate model in this program. In accordance with the results of this program, a NJ tree based on a Jukes-Cantor model was also constructed in PAUP*. Support for branches was estimated with 1,000 bootstrap replicates. The MP analysis was also conducted in PAUP*, using TBR branch swapping options with bootstrap values based on 1,000 replicates. A Jukes-Cantor model was also employed to conduct a Bayesian analysis with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Four chains of Metropolis-coupled Markov Chain Monte Carlo (MC3) process were run for 5,000,000 generations with trees being sampled every 1,000 generations. Default parameters for cold and heated chain were used. Convergence of the runs was confirmed when the average standard deviation of split frequencies was <0.01 . The first 1,250 sampled trees (25%) were discarded and the remaining trees used to construct a majority-rule consensus tree.

Relationship among the cpDNA haplotypes, including microsatellite variance, was investigated in a median-joining network with maximum parsimony post-processing (Bandelt et al. 1999; Polzin and Daneschmand 2003), using the program NETWORK 4.5.0.0 (<http://www.fluxus-engineering.com>). All mutation data (base substitutions, indels, and microsatellites) were included to construct the network with the epsilon value at zero. Since the network with all mutations equally weighted was complicated due to superfluous parsimonious connections, the nucleotide weightings were changed as recommended by Bandelt et al. (1999). The changed weights were as follows: base substitutions = 10, indels = 30, microsatellites = 1. A species-specific large deletion (230 bp) found in all individuals of *T. camschatcense* was especially assigned a weight value of 50. These changes improved the clarity of the network remarkably.

Genetic differentiation among the 21 populations (16 for SC and five for SI) of *T. camschatcense* was measured based on seven nuclear DNA microsatellite loci. Genetic distance was calculated with the infinite allele model (IAM) and the stepwise mutation model (SMM). Weir and Cockerham's F_{ST} values were calculated for the former, and Slatkin's R_{ST} values (Slatkin 1995) for the latter with

the program Microsat (<http://hpgl.stanford.edu/projects/microsat/>). One thousand distance matrices were obtained by bootstrapping over loci, and an unrooted extended majority-rule consensus tree was constructed based on the NJ method with the program PHYLIP (Felsenstein 1989).

Hypothesis testing

Although ancestral state reconstructions provide useful information with which to understand the evolutionary history of dichotomous traits, erroneous conclusions are frequently obtained, especially when the transition rate to and from a certain trait is not equal (Maddison 1990; Takebayashi and Morrell 2001; Igic et al. 2003, 2006). To avoid this problem, Takebayashi and Morrell (2001) suggested that hypothesis testing without ancestral reconstructions with a likelihood ratio test (LRT) approach may be appropriate for determining the evolutionary direction between selfing and outcrossing. Following their methods, we tested the adequacy of the general hypotheses, which assume that selfing is more often gained than lost, and that the evolutionary transition is irreversible. The likelihood of a full model (both transition rate for “SI to SC” and “SC to SI” are estimated) was compared to the likelihood of a constrained model (model A: rate for “SC to SI” and “SI to SC” are set equal) or a null model (model B: rate for “SC to SI” is set to zero; model C: rate for “SI to SC” is set to zero). If the likelihood of the constrained or null model is significantly lower than that of the full model, we can reject the corresponding transition hypothesis. We judged that the full model fits significantly better to the tree topology and the character status if the difference between log-likelihood exceeds 2.0. This criterion, suggested by Mooers and Schluter (1999), is a conservative value that corresponds to a significance level of $\alpha = 0.045$ in a χ^2 approximation with one degree of freedom. The majority-rule consensus tree from Bayesian analysis and the character status, either SC or SI, were used for maximum likelihood analysis in MultiState as implemented in the program BayesTraits (Pagel 2004).

Results

Phylogenetic analysis of cpDNA haplotypes

The lengths of the aligned sequence of three non-coding cpDNA regions, *psbD-trnT*, *ndhF-rpl32*, and *rpl32-trnL*, were 785, 784, and 676 bp, respectively. The total numbers of polymorphic characters in the three cpDNA regions were 13 substitutions, seven indels, and four microsatellites. Polymorphic characters among populations of *Trillium camschatcense* were eight, two, and four, respectively.

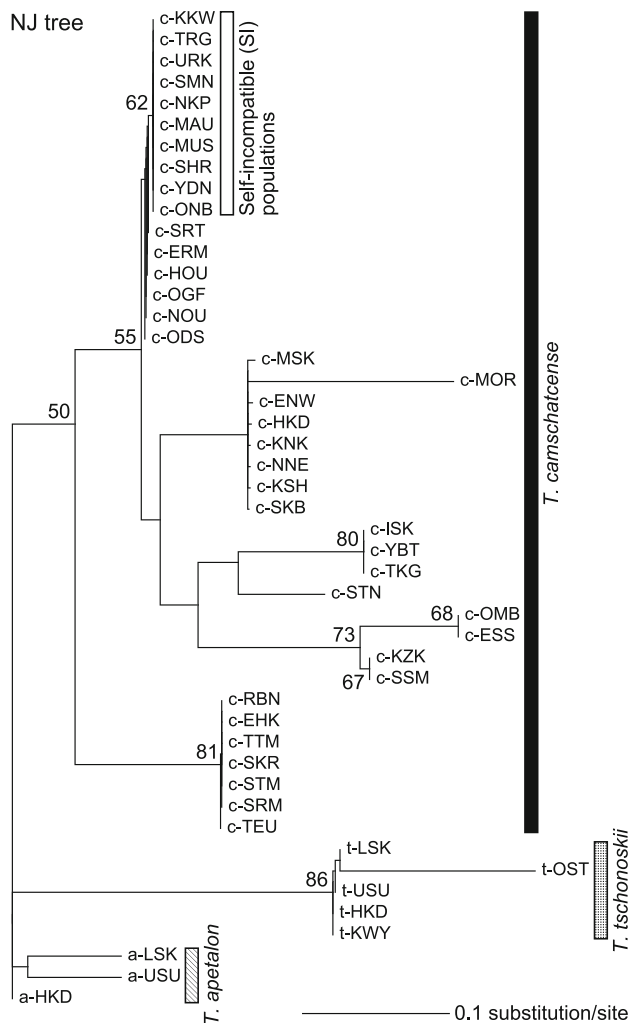


Fig. 2 Phylogenetic relationship of self-compatible (SC) and self-incompatible (SI) populations of *Trillium camschatcense* based on NJ analysis of three cpDNA non-coding regions. The genetic distance between populations was calculated based on uncorrelated p -distance. Bootstrap values are given above the branches of clades. The name and location of each population are given in Table 1

Figure 2 represents the p -distance-based NJ tree. Because the topology of the NJ tree from the Jukes-Cantor model and the MP tree were identical to the p -distance-based NJ tree, these former trees are not shown in the present study. Two major clades were distinguished in *T. camschatcense*, the first including seven northern SC populations with a bootstrap value of 81%, and the second including the remaining SC and SI populations with a bootstrap value of 55%. All SI populations were aggregated in a monophyletic clade with a bootstrap value of 62%, which branched from this second clade. A majority-rule consensus Bayesian tree based on the Jukes-Cantor model is shown in Fig. 3. Clustering of the populations was nearly the same with the NJ tree, except that most of

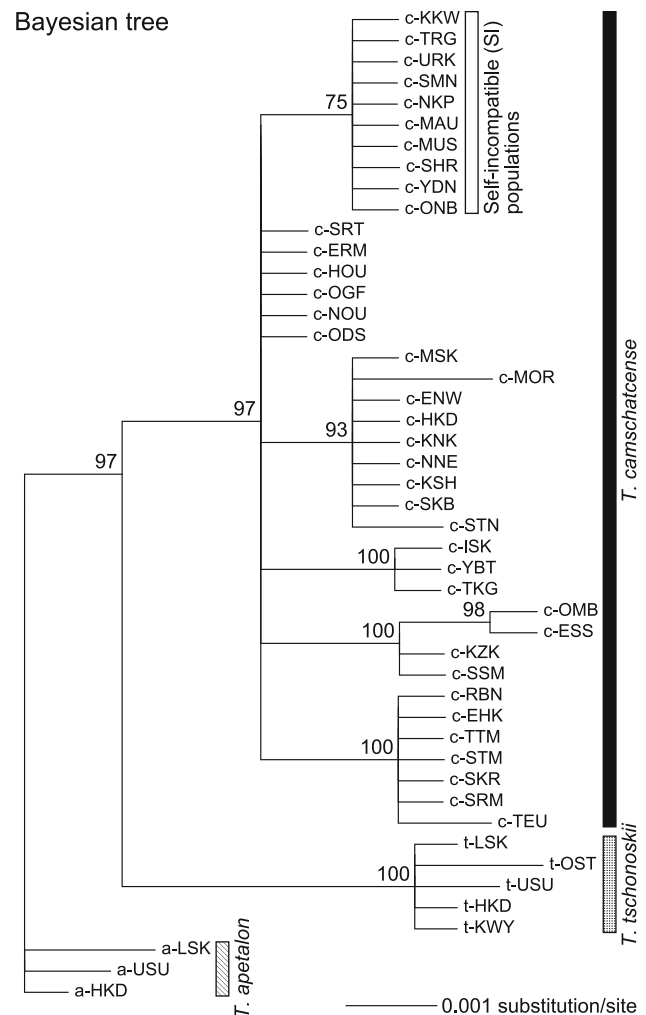
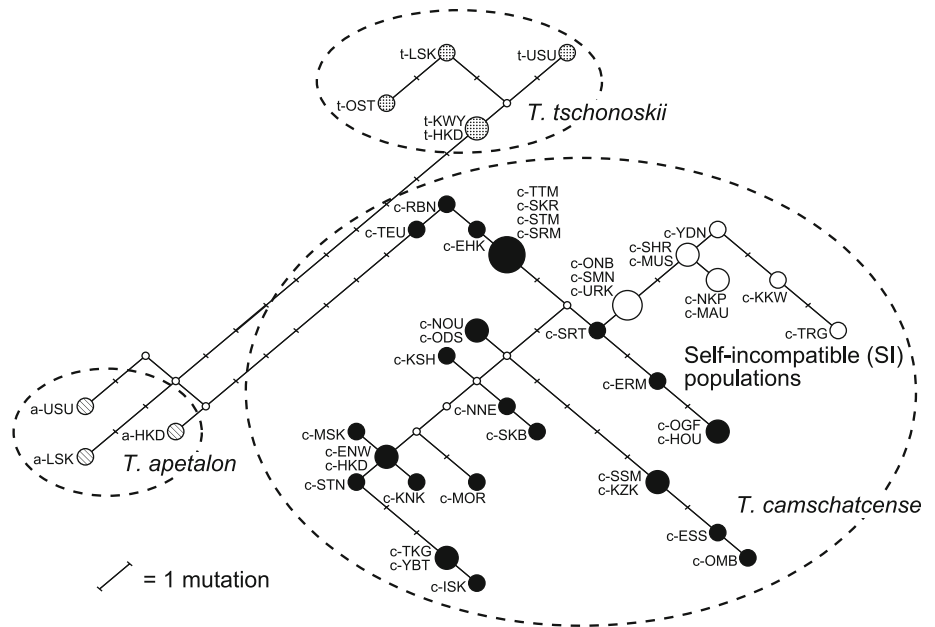


Fig. 3 Majority-rule consensus tree of SC and SI populations of *T. camschatcense* based on Bayesian analysis of three cpDNA non-coding regions. The genetic distance between populations was calculated based on Jukes-Cantor model. Posterior probabilities (%) are given above the branches of clades. The name and location of each population are given in Table 1

the clades in *T. camschatcense* were branching from a polytomy at the base of this species. Note that, because microsatellite polymorphisms were excluded from constructing both NJ and Bayesian tree, cpDNA haplotypes among the SI populations were completely identical. All polymorphic data were used in constructing the median-joining network (Fig. 4). In *T. camschatcense*, there were 21 distinct haplotypes among the SC populations, and 6 among the SI populations. As mentioned, the six haplotypes found among the SI populations differed only in their cpDNA microsatellite locus. The network indicated identical results to the NJ tree, where seven northern SC populations formed the base of the *T. camschatcense* tree, and SI populations were clustered at the terminus of the branch.

Fig. 4 Median-joining network of SC and SI populations of *T. camschatcense* based on maximum parsimony analysis of three cpDNA non-coding regions. Node sizes are proportional to haplotype frequencies. The name and location of each population are given in Table 1



Genetic relationships based on nuclear DNA microsatellites

The relationship between populations of *T. camschatcense* was examined using the pairwise genetic distances F_{ST} and R_{ST} . Mean values for pairwise F_{ST} and R_{ST} among all populations were 0.53 ± 0.02 and 0.35 ± 0.01 ($\pm SE$), respectively. Pairwise values for F_{ST} and R_{ST} among the SC populations were 0.65 ± 0.03 and 0.33 ± 0.02 , whereas the corresponding values were 0.10 ± 0.01 and 0.06 ± 0.01 among the SI populations. The two NJ trees constructed with distinct genetic distances were identical, thus we show only the dendrogram based on R_{ST} (Fig. 5). All SI populations formed a monophyletic clade with bootstrap support of 66%. Note that the evolutionary direction between SC and SI cannot be inferred since this tree is unrooted.

Hypothesis testing

Log-likelihood for the full model (unconstrained transition rates), and models A (transitions rate for both directions are set equal), B (rate for “SC to SI” is set to zero), and C (rate for “SI to SC” is set to zero) were -4.59 , -4.52 , -12.61 , and -4.59 , respectively. The full model was judged to fit significantly better when the difference between log-likelihood exceeded 2.0. Thus, among the three proposed models, only model B, which presumes that self-compatibility does not shift to self-incompatibility, was rejected. Estimated transition rates under the full model suggested that the shift from self-incompatibility to self-compatibility does not occur (0.00), whereas the reverse shifts were favorable (27.91).

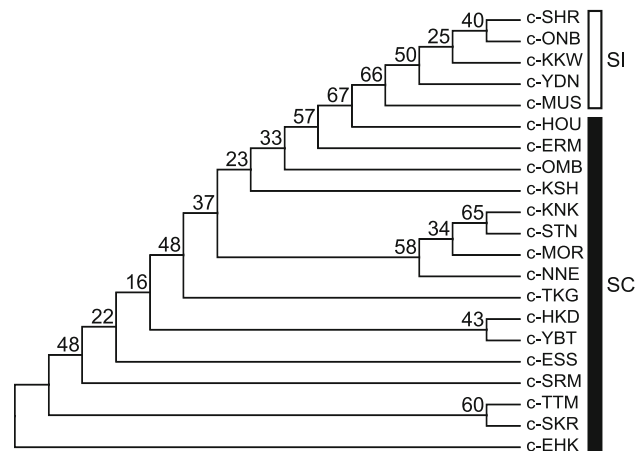


Fig. 5 Extended majority-rule consensus tree of SC and SI populations of *T. camschatcense* based on R_{ST} values of nuclear DNA microsatellite polymorphisms. Bootstrap values are given above the branches of clades

Discussion

Phylogenetic relationship of SC and SI populations in *T. camschatcense*

The NJ tree based on cpDNA variation (Fig. 2) and the median-joining network (Fig. 4) indicated a monophyletic derivation of SI populations from SC populations, which is generally predicted as an unlikely event in the angiosperms (Lloyd 1979; Charlesworth 1980; Feldman and Christiansen 1984; Lande and Schemske 1985). However, bootstrap support in the NJ tree was low, and the Bayesian tree using the same datasets (Fig. 3) resulted in a large polytomy.

Thus, neither phylogenetic tree provided any evidence for the evolutionary direction. Variance of cpDNA among the SI populations was extremely small, limited to microsatellite variations, whereas SC populations showed higher variability, including base substitutions, indels, and microsatellite variations. Along with the low genetic differentiation of cpDNA, F_{ST} and R_{ST} values based on nuclear DNA microsatellite variations also both indicated that genetic distances among the SI populations are smaller than among the SC populations. Indeed, the contrasting mating system (highly selfing in the SC populations and completely outcrossing in the SI populations; Kubota et al. 2008) can explain this difference. Selfing generally accelerates genetic differentiation of a population compared to outcrossing (Charlesworth and Charlesworth 1995; Hamrick and Godt 1996). However, despite their high genetic differentiation, none of the SC populations intruded into the clade of SI populations in any of the phylogenetic analyses. Thus, although the evolutionary direction remains inconclusive, the data indicate that the SI populations in *T. camschatcense* are monophyletic with low genetic differentiation.

The results from the hypotheses testing, which examined the likelihood of each evolutionary direction, were clear. The hypothesis presuming that self-compatibility does not shift to self-incompatibility was rejected, and transition rates indicated that shift towards self-incompatibility was rather frequent (0.00 for SI to SC vs 27.91 for SC to SI). These results oppose the common evolutionary trend that assumes frequent shift from outcrossing to selfing (Lloyd 1979; Charlesworth 1980; Feldman and Christiansen 1984; Lande and Schemske 1985). Moreover, the one-way evolutionary theory from outcrossing to selfing (Lande and Schemske 1985) was completely rejected. However, conclusions should be drawn carefully, because results from the LRT approach depend on the phylogenetic tree used. In the constructed Bayesian tree, SI populations were clustered in a single clade (Fig. 3). Regardless of the evolutionary direction, the topology indicates that transition of the mating system in *T. camschatcense* has occurred only once. In general, phylogenetic studies involve multiple SC and SI species from a relatively wide range of taxa, and mating systems are usually dispersed in several clusters, indicating multiple independent transitions (Goodwillie 1999; Igic et al. 2003; Ferrer and Good-Avila 2007; Shimizu et al. 2008). Thus, uncommon results from the LRT analyses may have roots in the unique topology found in *T. camschatcense*. In addition, the essential difficulty of interpreting transition rate estimates must be considered, because a sample size sufficient to conduct an accurate estimation is often unavailable (Moore and Schluter 1999; Takebayashi and Morrell 2001).

Although there was a marginal indication in the present study of an evolutionary direction from self-compatibility to self-incompatibility in *T. camschatcense*, such a scenario has been extremely unusual in the angiosperms. Thus, phylogenetic approaches may be inadequate to convincingly determine the evolutionary direction in this species. However, the fact that the SI populations are monophyletic with low genetic differentiation indicates that there are only two possible scenarios, either from self-incompatibility to self-compatibility, or self-compatibility to self-incompatibility. Next, we investigate both evolutionary directions by taking reproductive features and geographic backgrounds into account.

From self-incompatibility to self-compatibility

If SC populations were derived from SI populations, the high inbreeding depression found in SC populations (Kubota et al. 2008) may indicate the difficulty of purging the genetic load, or simply that the period since the transition is too short to have accomplished the purification. Additionally, the lower genetic differentiation among the SI populations indicates a recent bottleneck event. Because the SC populations are distributed widely in Japan, whereas the SI populations are restricted to eastern Hokkaido (Fig. 1), there are two additional pathways that could explain this geographical pattern.

One scenario to explain the possible loss of self-incompatibility could have occurred if the SI populations formerly existed in a wider geographical range with higher levels of genetic variation. Then, independent transitions to self-compatibility may have occurred throughout the range in response to some environmental change, such as the loss or reduction of pollinators. A reduction or loss of pollinators may drive the SI populations to extinction or lead to the loss of self-incompatibility, because pollinator scarcity directly affects sexual reproduction in SI individuals, but not in individuals capable of autonomous self-pollination (Ohara et al. 1996; Kubota et al. 2008). This effect is expected to be exacerbated in species exhibiting specialist pollinators because the loss of only one pollinator could easily lead to a complete reproductive failure (Bond 1994). However, since *T. camschatcense* utilize a relatively wide range of insect species, such as beetles (e.g., Nitidulidae and Melandryidae) and flies (e.g., Bibionidae and Scathophagidae) (Ohara et al. 1991; Tomimatsu and Ohara 2003) for pollen transfer, loss of pollinators is probably unlikely. In fact, pollination experiments in the SI populations did not detect any pollen limitation (Kubota et al. 2008). Needless to say, we are not aware of any recent event that would have massively eliminated a wide range of insect species.

Another possibility is that the SI populations were originally distributed only in eastern Hokkaido, and a single transition to self-compatibility took place in this region. The SC populations may then have expanded their range. Because our previous study in the SC populations detected potential as successful colonists (Kubota et al. 2008), selfing should have been advantageous during such an expansion. Meanwhile, the SI populations remained in the same region and underwent a bottleneck. If this scenario were true, then *T. camschatcense* must have its origin in eastern Hokkaido. However, it is well known that many angiosperms found in Japan are immigrants from the Asian continent (Hotta 1974). Since all three *Trillium* species examined in this study are found throughout the eastern part of the continent, including Sakhalin to the north of Hokkaido (Samejima and Samejima 1962), it would make more sense to consider that *T. camschatcense* also migrated via the land bridge between the continent and Hokkaido during a glacial period. Thus, eastern Hokkaido should be considered as the eastern edge of the species range, rather than its origin. Moreover, if self-compatibility were the result of a single transition, the SC populations should have equal or lower genetic variation compared to the SI populations. This conflicts with the present results, which detected higher genetic variation among the SC populations compared to that among SI populations. Additionally, as mentioned above, the genetic bottleneck found among the SI populations is difficult to explain under this scenario.

From self-compatibility to self-incompatibility

If self-incompatibility derived from the SC population, the monophyly and low genetic diversity among existing SI populations indicate a recent single evolutionary transition. Our previous study demonstrated that the studied SC populations are under selective pressure that would select for the evolution of predominant outcrossing (Kubota et al. 2008). Thus, at least a selective potential for the evolution of self-incompatibility seems to exist in SC populations. In addition, the geographical distribution of the mating system can also be explained from the phylogenetic results. The NJ tree (Fig. 2) and the median-joining network (Fig. 4) revealed that the northern SC populations, such as RBN, EHK, and TTM, are ancestral to the Japanese *T. camschatcense*. As mentioned, the species may have migrated to Japan via a northern land bridge during glacial periods. The geographical spread of Japanese *T. camschatcense* can be predicted to have originated in northern Hokkaido, then advancing southward. The mating system in the initial phases of migration would have been SC. It is generally accepted that selfing should be adaptive during the

founding of new populations (Baker 1955), and indeed we have confirmed the high selfing ability of SC individuals of *T. camschatcense* (Kubota et al. 2008). Then, self-incompatibility might have evolved in SC populations located in eastern Hokkaido. The low genetic diversity among the SI populations indicates that these populations are congenetic, which have been established as distinct populations relatively recently. Because mating system in the continental *T. camschatcense* is not known, we are unaware whether the transition to self-incompatibility in the Japanese *T. camschatcense* is a de novo origination or the restoration of an ancestral system. Shimizu et al. (2004) demonstrated clearly that the loss of self-incompatibility in *Arabidopsis thaliana* was associated with the post-Pleistocene expansion from glacial refugia. Additionally, our previous study demonstrated that SC populations have potential as successful colonists (Kubota et al. 2008). If massive migration is likely to be associated with the ability to self-fertilize, early populations that successfully migrated to Japan would already have been SC. Obviously, the mating system and phylogenetic relationships of the continental *T. camschatcense* must be investigated to shed light on this issue.

Although phylogenetic, ecological, and geographical features of *T. camschatcense* seem to support the evolution from self-compatibility to self-incompatibility, such evidence may not be enough to support a convincing hypothesis. This type of evolution has also been opposed based on Dollo's law, where a complex character (self-incompatibility system) is more easily lost than gained (Gould 1970; Igic et al. 2006). However, it is hard to say if this theory fits in *T. camschatcense* because mechanisms of self-incompatibility in this species are poorly understood. Perhaps an alternative way to evaluate the validity of the unusual evolution suggested in *T. camschatcense* is to compare the evolutionary dynamics of self-incompatibility with its congeners. We have confirmed that the self-incompatibility system of *T. camschatcense* possess similar features to that of the North American *Trillium* species, *T. grandiflorum* and *T. erectum* (Sage et al. 2001; S. Kubota et al. unpublished data). Interestingly, these two species retain populations with variation in their ability to self-fertilize—a consequence of leaky self-incompatibility (Broyles et al. 1997; Kalisz et al. 1999; Irwin 2000; Sage et al. 2001). Although the presence of leakiness evokes a breakdown of self-incompatibility, the evolutionary direction of the mating system has not been investigated in either species. The investigative approach taken in *T. camschatcense* would help clarify whether this leaky self-incompatibility is an intermediate step between SI and SC, or SC and SI. Findings in the North American species may provide useful information to judge the plausibility of the evolution from self-compatibility to self-incompatibility in *T. camschatcense*.

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