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Stigmatic Self-Incompatibility and Mating Patterns in *Trillium grandiflorum* and *Trillium erectum* (Melanthiaceae)

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Post-pollination processes governing mating patterns in *Trillium*, a well-known genus of insect-pollinated woodland herbs, are poorly understood. Mechanisms influencing outcrossing were investigated in *T. grandiflorum* and *T. erectum*, two widespread species native to eastern North America. In southern Ontario, Canada, the two species are often sympatric; they flower in early May, and are pollinated by different assemblages of insects. Controlled cross- and self-pollinations and structural observations of pollen germination and pollen tube growth were conducted to determine whether the two species possess a self-incompatibility (SI) system and, if so, the specific site(s) of self-rejection. Controlled pollinations indicated that both species set significantly more seeds from cross-pollination than self-pollination, implicating the action of SI. This was confirmed by structural studies which demonstrated that self-recognition and rejection reactions occurred on dry-type stigmatic papillae. Observations of pollen hydration revealed that self-rejection was rapid, being initiated within 10 min of pollination and prior to pollen tube emergence. Final self-rejection resulted in failure of pollen tube growth at the base of stigmatic papillae. SI was expressed more weakly in *T. erectum* and thereby resulted in considerable self-seed set in some individuals. Estimates of outcrossing rates using allozyme markers indicated that *T. erectum* displayed a mixed-mating system whereas *T. grandiflorum* was more highly outcrossed. Structural studies of pollen traits indicated that the two species differed with respect to the size of grains and their aggregation with implications for pollen dispersal and mating. The ecological and evolutionary implications of the variable expression of SI in *Trillium* are discussed.

Key words: *Trillium grandiflorum*, *Trillium erectum*, self-incompatibility, mating.

INTRODUCTION

Post-pollination control of offspring production in angiosperms is regulated, in part, by a cascade of cellular communications within and between carpellary tissues and male gametophytes (O'Neill, 1997; Franklin-Tong, 1999; Gaude and McCormick, 1999). Temporal and spatial control of the cellular phenomena varies depending on the quantity and quality of the pollen load. Interactions between compatible pollen and carpel tissues function to enable double-fertilization by ensuring retention, recognition and transmission of male gametophytes to female gametophytes. In contrast, the delivery of incompatible pollen to stigmas invokes a series of responses resulting in the recognition and rejection of self-pollen as a result of diverse self-incompatibility (SI) mechanisms. Most commonly, SI acts prezygotically over short cellular distances to prevent germination of self-pollen on the stigma surface or self-pollen tube growth in stylar and ovarian transmitting tissues (de Nettancourt, 1977, 1997, 2001; Kenrick *et al*., 1986; Chichiricco, 1993). Notably, prezygotic SI can also operate over longer distances whereby self-pollen or pollen tubes negatively affect ovule development and hence female receptivity prior to pollen tube entry into the ovary (Sage *et al*., 1999). Regardless of the site and mechanism of SI, SI reduces the harmful effects of selfing and inbreeding depression in plant populations and promotes outcrossing (de Nettancourt, 1977; Charlesworth and Charlesworth, 1987; Barrett, 1988).

While SI is often claimed to be present in numerous angiosperms, having been reported in roughly 19 orders, 71 families and 250 genera (Sears, 1937; East, 1940; Brewbaker, 1957; Arasu, 1968; de Nettancourt, 1977; Givnish, 1982; Charlesworth, 1985; Barrett, 1988, 1992), most reports of SI are based on the observation of reduced seed set following self-pollinations compared with cross-pollinations. However, without evidence of the site of inhibition of pollen germination and/or pollen tube growth, studies of seed set alone cannot rule out the possibility that differential seed abortion largely governs the female fertility of self- *vs*. cross-pollinations. Such effects commonly occur in outcrossing species and result from the abortion of selfed offspring homozygous for deleterious genes due to early-acting inbreeding depression (Charlesworth and Charlesworth, 1987; Barrett, 1988; Walsh and Charlesworth, 1992; Sage *et al*., 1994). Hence, in reality, SI has been definitively confirmed in far fewer taxa than is widely assumed. Moreover, in even fewer species is the genetic, molecular and cellular basis of self-rejection fully understood (Franklin *et al*., 1995). The absence of such information results in a limited understanding of the evolutionary relationships between various SI systems in flowering plant taxa.

The monocotyledonous genus *Trillium* (Melanthiaceae) is comprised of approximately 42 species distributed in temperate woodlands of Asia and western and eastern

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North America (Gray, 1846, 1860; Axelrod, 1966; Graham, 1972; Tiffney, 1985; Ohara, 1989). Members of the genus are also widely grown in temperate regions as spring ornamentals (Case and Case, 1997). Studies of the reproductive biology of *Trillium* species indicate a wide diversity of sexual systems influencing mating and fertility. Self-incompatibility has been reported for several species (Dyer, 1963; Broyles *et al*., 1997; Kalisz *et al*., 1999), but apomixis and self-compatibility have also been documented (Swamy, 1948; Nesom and La Duke, 1985; Barrett and Helenurm, 1987; Fukuda, 1987). All reports of SI in *Trillium* have been based either on studies of seed set following cross- and self-pollination or have been inferred based on the finding of high outcrossing rates estimated using genetic markers (e.g. Broyles *et al*., 1997; Kalisz *et al*., 1999). Confirmation of SI using structural studies on pollen-carpel interactions is absent from the literature and nothing appears to be known about the potential site of self-rejection in *Trillium* species.

The major objective of our study was to determine whether physiological SI occurs in *T. grandiflorum* and *T. erectum*. To address this issue, pollen-carpel interactions following controlled cross- and self-pollinations were characterized in both species at the microscopic level. The site and timing of self-recognition and rejection and the structural characteristics of cells involved in the SI reaction were determined. In addition, to provide the necessary ecological context for interpreting our structural studies, field studies were conducted to compare the flowering phenology, outcrossing rates and seed fertility of *T. grandiflorum* and *T. erectum* following cross- and self-pollination. Finally, pollen was examined to explore whether differences in the reproductive biology of the two species was reflected in contrasting features of their pollen.

We chose specifically to investigate *T. grandiflorum* and *T. erectum*, two wide-ranging North American taxa, for two major reasons. First, several contradictory reports exist concerning the reproductive systems of these two species. Several authors have described both species as self-compatible, predominantly autogamous or inbreeding (Fukuda and Grant, 1980; Ohara, 1989; Irwin, 2000). Alternatively, others have challenged these characterizations by providing evidence that the two species exhibit moderate to high outcrossing rates (Broyles *et al*., 1997; Kalisz *et al*., 1999). Clearly, additional information on the compatibility status of populations and their mating patterns is required. Second, in southern Ontario, Canada, where our study was conducted, *T. grandiflorum* and *T. erectum* occur sympatrically and differ in a variety of features that could potentially influence mating patterns and fertility. These include differences in flowering phenology, pollination biology, and population size and structure. Populations of *T. grandiflorum* are often very large and plants occur at high density, they flower later than those of *T. erectum*, possess larger, white flowers, and are pollinated primarily by bees, particularly *Bombus* spp. (Wright and Barrett, 1999; Irwin, 2000). In contrast, *T. erectum* commonly occurs in low density patches, has red flowers and is pollinated primarily by coleoptera and diptera. Because of these differences, we were particularly interested in exploring the extent to which the contrasting features of the reproductive biology of the two species might influence reproductive traits, especially those governing mating patterns.

MATERIALS AND METHODS

Study site

Field studies of *T. grandiflorum* and *T. erectum* were conducted at Joker's Hill Field Station in York RM, southern Ontario, Canada. At this site, both species are understorey herbs occurring in mixed deciduous forest, primarily composed of sugar maple, beech, oak and occasional hemlock and white pine. Populations of the two species grow sympatrically and plants used in this investigation were sampled from an area encompassing several hectares of forest. Some plants used in structural studies were excavated prior to anthesis (before emergence of petals from sepals) and transferred to the University of Toronto glasshouse facility and grown at 20 °C. In our study, floral anthesis was defined as the day on which stigmatic crests were fully elongated and recurved.

Flowering phenology

To quantify differences in flowering phenology between *T. grandiflorum* and *T. erectum*, flowering schedules of the two species were monitored in two consecutive years. In 1999, 127 *T. grandiflorum* and 88 *T. erectum* plants were marked before anthesis. Individuals were chosen along four transects that varied in length from 20 to 50 m. Starting on 30 April, the number of open flowers was recorded each morning until all marked plants were in bloom. Marked plants were monitored until all flowers had senesced. In 2000, 105 *T. grandiflorum* and 74 *T. erectum* plants that were marked the previous year produced flowers. Starting on 29 April, the number of open flowers was recorded each morning until all flowers had opened. A Kolmogorov-Smirnov two-sample test (Sokal and Rohlf, 1995) was used to examine differences in phenology between the species in 1999 and 2000 and to test for differences within species between years. To reduce Type I error we used a sequential Bonferroni correction (Rice, 1989) on the observed *P*-values.

Structural and histochemical investigations of pollen-carpel interactions

Structural and histochemical features of pollen, the transmitting tissues encountered, and spatial and temporal aspects of cross- and self-pollen tube growth were assessed using fluorescence (FM), light (LM), and scanning electron (SEM) microscopy, as described previously by Pontieri and Sage (1999). Carpels and pollen from fresh and fixed unpollinated flowers of both species were sampled at anthesis and 24 h post-anthesis for histochemical and microscopic analysis. The size and clumping characteristics of fresh, unhydrated pollen were examined prior to histochemical analysis by tapping anthers to disperse pollen

onto a glass microscope slide. Cross- and self-pollinated flowers of *T. grandiflorum* were harvested for microscopy and histochemistry 5, 10, 30 and 50 min and 9, 24 and 48 h following pollination ($n = 6$ flowers per time per treatment for each microscopic analysis). Cross- and self-pollinated flowers of *T. erectum* were harvested for microscopy and histochemistry 24 h after pollination. All pollinations conducted in this experiment were undertaken in the glasshouse using a separate, single pollen donor for each cross pollination ($n = 6$ flowers per time per treatment for each microscopic analysis).

Spatial and temporal aspects of cross- and self-pollinations in *T. grandiflorum* and *T. erectum* were also analysed in two additional experiments. First, using intact flowering plants from the glasshouse, we determined whether differences were evident between the two pollination types in the timing of pollen hydration on stigmas. Unstained, living cross- and self-pollen grains were applied with an eyelash to stigmas of separate intact flowers (*T. grandiflorum*, $n = 21$ flowers; *T. erectum*, $n = 18$) while under the 20X objective of a Zeiss Axioplan fluorescence microscope equipped with an image analysis system (Northern Exposure; Empix Imaging, Inc., Missisauga, Ontario, Canada). Flowers remained attached to the stem and potted rhizome. Pollen grain diameter was measured upon application of pollen (time zero) and every 5 min thereafter until pollen grains germinated. Changes in pollen diameter over time for the two pollination treatments were compared using a two-way repeated measures ANOVA and Tukey multiple-comparison test. In a second experiment, conducted under field conditions, we examined whether self-pollen tubes penetrated ovaries and ovules at different times than cross-pollen tubes. Cross- or self-pollen was applied to stigmas of separate flowers of both species *in situ*. Flowers were harvested daily for 6 d ($n = 6$–8 d^{-1} per pollination treatment per species) and used for fluorescence microscopy to examine pollen tube growth following staining with the fluorochrome aniline blue (Martin, 1959). At each sampling period for each pollination treatment, the location of pollen tubes and the number of pollen tubes in the ovary and micropyles were determined.

Compatibility status under field conditions as determined from seed set data

Controlled crosses were performed to further assess the compatibility status of *T. grandiflorum* and *T. erectum* under field conditions in 1999. Plants produce a single flower and floral longevity ranges from 2–3 weeks depending on temperature. Four treatments were assigned randomly: (1) flowers were emasculated and bagged before anthesis to test for non-pseudogamous apomixis; (2) flowers were bagged and then self-pollinated approximately 6 d after anthesis; (3) flowers were emasculated, bagged and cross-pollinated with one anther from each of three donors approx. 6 d after anthesis; and (4) flowers remained unmanipulated to serve as open-pollinated controls. Fruits were collected in 70 % ethanol in early July 1999 just before fruit dehiscence. The number of fruits that developed was determined, as was the number of plump and aborted (small and shrunken) seeds, and potentially unfertilized ovules per fruit. The total number of ovules per fruit was calculated as the sum of seeds, aborted seeds and apparent unfertilized ovules.

To analyse differences in seed set per fruit between pollination treatments, an ANCOVA was performed on square-root transformed data of seeds per flower, with ovule number as a covariate. The square-root transformation of seed number best met the parametric assumptions of homoscedasticity and normality. Due to limited fruit set from treatments involving flowers that were emasculated and bagged, and those that were self-pollinated, only the open- and cross-pollinated treatments were included in the ANCOVA. Analyses of seed set were performed with JMP (version 3.2.2; SAS 1997).

Estimates of mating patterns

Starch gel electrophoresis was performed on seeds collected from open-pollinated plants in 1998 to estimate outcrossing rates of *T. grandiflorum* and *T. erectum*. The resulting gels were scored for genotype frequencies at allozyme loci. Seeds were ground in a dithiothreitol-BSA-Tween extraction buffer and the extract absorbed onto paper chromatography wicks. For *T. grandiflorum*, 11 % starch gels were run in histidine-citrate buffer (pH 6·1) gels at 45 mA and 190 V for 4 h; for *T. erectum*, 11 % starch gels were run in both histidine buffer as above and lithium-borate buffer at 50 mA and 200 V for 4 h (for details see Wendel and Weeden, 1991). For *T. grandiflorum*, variability was scored at PGI and PGM. For *T. erectum*, IDH and PGM from histidine gels and ADH and GDH from lithium-borate gels were scored. More than ten seeds per family were scored from 40 *T. grandiflorum* and 24 *T. erectum* families. Ritland's MLTR program (Ritland 1986, 1990) was used to estimate the multilocus outcrossing rate (t_m). The standard error of t_m was calculated as the standard deviation of 1000 bootstraps with the family as the unit of resampling. To determine whether outcrossing rates differed significantly between *T. grandiflorum* and *T. erectum*, randomly paired bootstrap estimates of t_m were compared. The contrast was considered significant if less than 2·5 % of the differences [i.e. (t_m of *T. grandiflorum*) minus (t_m of *T. erectum*)] were greater or less than zero (i.e. a two-tailed test with alpha = 5 %; see Eckert and Barrett, 1994 for details).

RESULTS

Flowering phenology

Flowering in *T. grandiflorum* and *T. erectum* occurred during late April to early-mid May with *T. erectum* commencing bloom and reaching peak flowering a few days before *T. grandiflorum* (Fig. 1). Flowering was remarkably synchronous, with all flowers of both species opening within an 8 d period in both years. There was a significant difference in the cumulative proportion of flowers in anthesis between species within years and within species between years (Kolmogorov-Smirnov two-sample

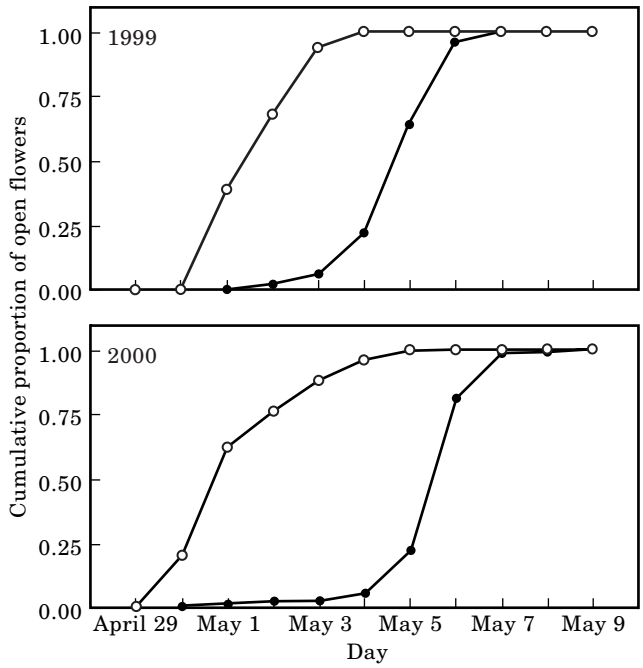


FIG. 1. Flowering phenology of *Trillium grandiflorum* (●) and *Trillium erectum* (○) at Joker's Hill, southern Ontario, Canada, during 1999 and 2000.

test; all $D \geqslant 0{\cdot}235$; $P < 0{\cdot}05$ for all tests with the sequential Bonferroni correction). In 1999, the average individual flower life from anther dehiscence to floral senescence was 16·2 d for *T. erectum* and 17·0 d for *T. grandiflorum*.

Structural features of pollen and the pollen tube pathway

The six anthers and three stigmatic crests of *T. grandiflorum* were in contact during anthesis (Fig. 2), whereas anthers of *T. erectum* were spatially separated from the stigmatic crests (Fig. 3). The spinose pollen of *T. grandiflorum* (Fig. 4) was significantly larger than the globose pollen of *T. erectum* (Fig. 5) in both the dry and hydrated state (*T. grandiflorum* diameter dry = 33·9 ± 0·34 μm; *T. erectum* diameter dry = 29·7 ± 0·3 μm; $F = 105{\cdot}99$, d.f. = 1, 221, $P < 0{\cdot}001$; *T. grandiflorum* diameter wet = 39·4 ± 0·5 μm; *T. erectum* diameter wet = 33·1 ± 0·3 μm; $F = 121{\cdot}65$, d.f. = 1, 110, $P < 0{\cdot}001$). Pollen from both species was covered with pollen coat (Fig. 6) that was histochemically positive for pectin, lipids and protein. There was noticeably less pollen coat in *T. erectum*, resulting in a dry, dusty pollen dispersed from anthers in small, non-sticky clumps. This contrasted with the dispersal of *T. grandiflorum* pollen that was packaged in large sticky aggregations. The number of pollen grains per clump ranged from two–25 in *T. grandiflorum* and from two–seven in *T. erectum*. The mean number of pollen grains per clump in *T. grandiflorum* was significantly greater than that of *T. erectum* (6·8 ± 5·0 and 2·8 ± 1·7, respectively; $H = 199{\cdot}96$, d.f. = 1, $P < 0{\cdot}001$).

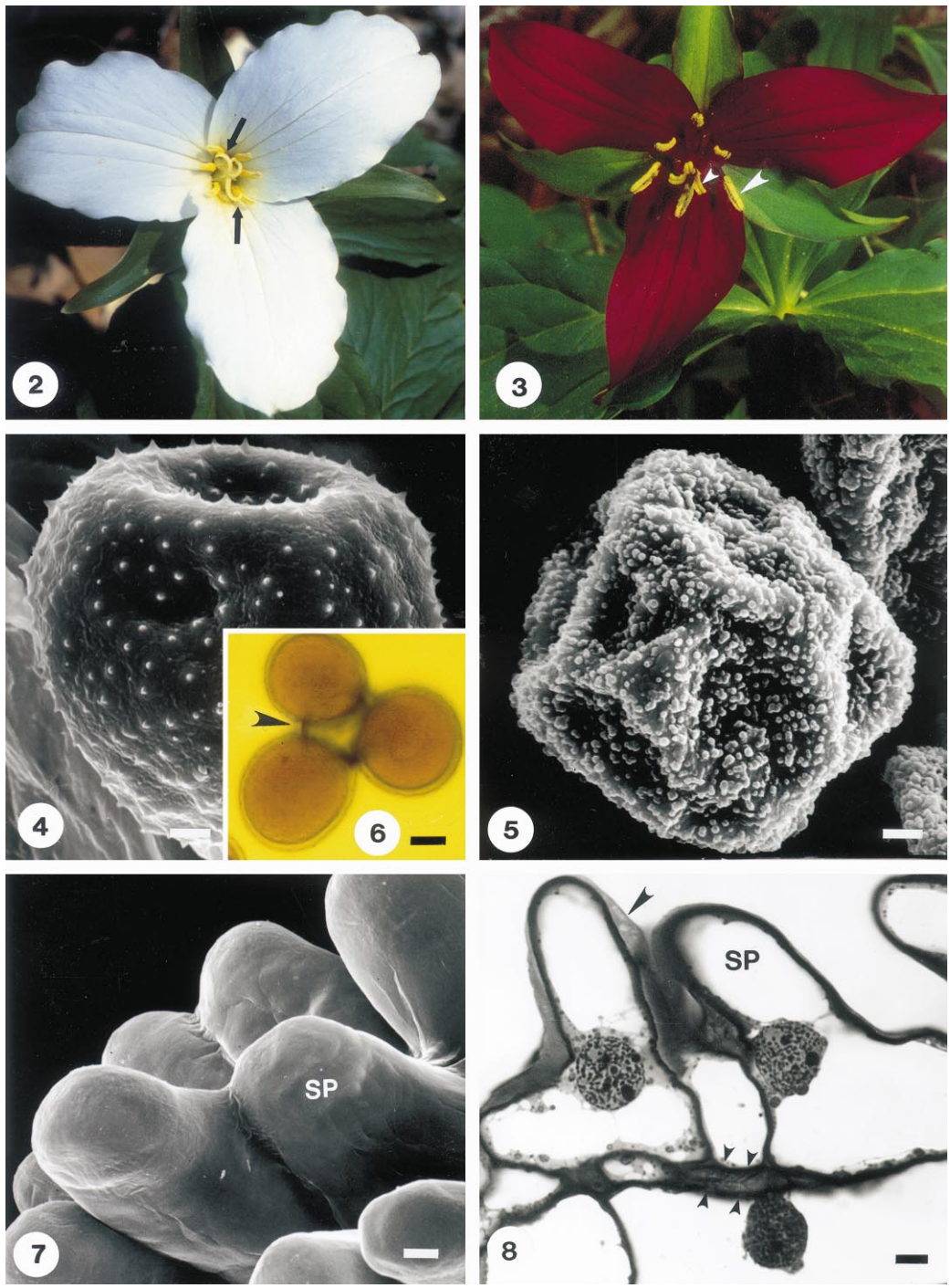
The unicellular, elongate stigmatic papillae comprising the stigmatic crest of both *T. grandiflorum* and *T. erectum* were of the dry type (Figs 7 and 8). Papillate stigmatic cells contained large vacuoles, prominent nuclei and numerous amyloplasts (Fig. 8). The thick proteinaceous and pectinaceous cell wall of stigmatic papillae was covered by a cuticle and was also associated with strong esterase activity, indicative of a pellicle (Mattson *et al.*, 1974). The broad, tabular abaxial base of a stigmatic papilla was subtended by a thickened extracellular matrix of similar histochemical composition to the tip of the papilla (Fig. 8). The one–two cell layers of ground tissue subtending stigmatic epidermal cells, epidermal cells of the hollow style, and placental transmitting epidermal cells had similar protoplast characteristics as stigmatic papillae at the light microscope level. Within the short hollow style, a secretion histochemically positive for proteins, lipids, carbohydrates and AG/AGPs subtended a thin uplifted cuticle. A similar, less abundant exudate was present adjacent to the placenta and in the micropyle. Mean ovule number per flower was 26 (±5) and 56 (±14·9) in *T. grandiflorum* and *T. erectum*, respectively.

Pollen-carpel interactions following cross- and self-pollinations

Timing of pollen hydration and germination. Cross- and self-pollen of both species hydrated readily following application to stigmas under experimental conditions (Figs 9 and 10). Pollen of *T. grandiflorum* hydrated more rapidly following pollination than pollen of *T. erectum* (Figs 11 and 12). Two-way repeated measures ANOVA indicated a significant difference between the size of hydrated cross- and self-pollen over time in both species (*T. grandiflorum*: cross *vs.* self, $F = 35{\cdot}74$, d.f. = 1, $P < 0{\cdot}001$; cross *vs.* self × time, $F = 16{\cdot}69$, d.f. = 7, $P < 0{\cdot}001$; *T. erectum*: cross *vs.* self, $F = 50{\cdot}51$, d.f. = 1, $P < 0{\cdot}001$; cross *vs.* self × time; $F = 25{\cdot}69$, d.f. = 5, $P < 0{\cdot}001$). Pairwise comparisons between the size of hydrated cross- and self-pollen indicated that only values at 10 min and beyond were significantly different ($P < 0{\cdot}001$) in both species. No differences were observed between the timing of cross- and self-pollen tube emergence from grains in either species. Cross- and self-pollen tubes germinated at 20–25 min and 30–35 min post-pollination in *T. grandiflorum* and *T. erectum*, respectively.

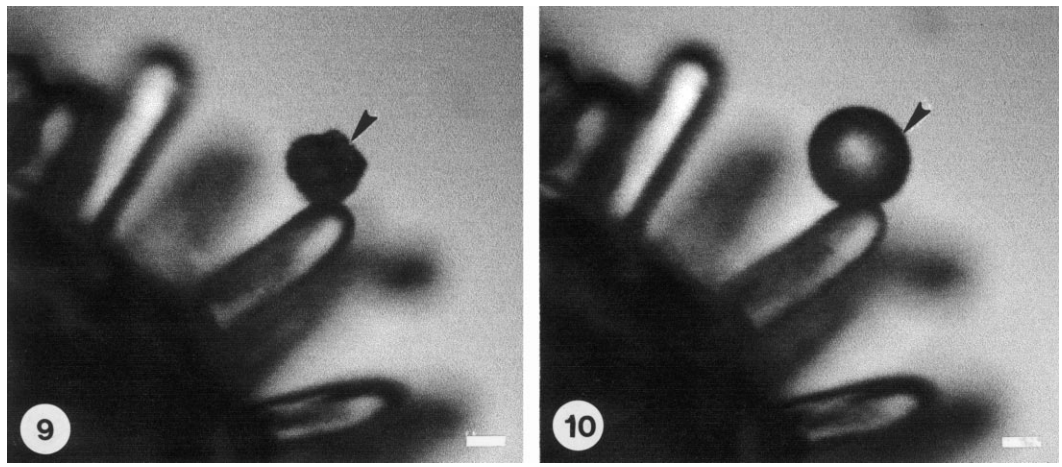
Structural, spatial and temporal aspects of pollen grain/pollen tube-carpel interactions. Structural and spatial aspects of pollen-carpel interactions following cross- and self-pollinations were similar in both *T. grandiflorum* and *T. erectum* during the first 24 h following pollinations in the glasshouse. Because of the similarities, a detailed description is provided for *T. grandiflorum* only.

Adhesion of cross- and self-pollen to stigmatic papillae in *T. grandiflorum* was effected within 5 min of pollination (Figs 13 and 14) and germination ensued within 20–25 min (Figs 15 and 16). Germinating pollen tubes penetrated and grew beneath the cuticle within an expanded fibrillar extracellular matrix adjacent to the papilla cell wall (Figs 17 and 18). Following growth to the base of the papilla tip, cross-pollen tubes took one of two possible pathways in the transmitting tissue of the stigmatic crest. They either grew intercellularly between the expanded



FIGS 2–8. Micrographs illustrating floral, pollen and stigma morphology and anatomy. Fig. 2. *T. grandiflorum*. Arrows denote points of contact between elongate stigmatic crest and dehisced anther. Fig. 3. *T. erectum*. Anthers (large arrowhead) do not contact stigmatic crests (small arrowhead). Fig. 4. Spinose pollen of *T. grandiflorum*. Bar = 3·3 μm. Fig. 5. Globose pollen of *T. erectum*. Bar = 2·5 μm. Fig. 6. Pollen coat (arrowhead) adhering pollen grains of *T. grandiflorum*. Bar = 14·6 μm. Fig. 7. Scanning electron micrograph of dry stigmatic papillae of *T. grandiflorum*. Unpollinated. Bar = 8·8 μm. Fig. 8. Light micrograph of dry stigmatic papillae of *T. grandiflorum*. Arrowhead denotes cuticle. Double arrowheads denote expanded extracellular matrix between stigmatic papillae and substigmatic ground tissue. Unpollinated. Bar = 8·8 μm. SP, Stigmatic papilla.

tabular base of two papillae and subsequently within the extracellular matrix between papillae and the ground transmitting cells, on route intercellularly to the hollow style (Fig. 19). Or, they grew to the adaxial papilla cell wall surface beneath the cuticle on route towards the inner margins of the stigmatic crest without growing intercellularly (Fig. 20). The extracellular matrix subtending the stigmatic cuticle in the region of the crest cleft was greatly



FIGS 9 and 10. Digital micrographs illustrating hydration of *T. erectum* pollen grain (arrow) after application to stigma with eyelash. Fig. 9. Immediately after pollen grain arrival prior to hydration. Fig. 10. Hydrated pollen grain 40 min after pollination. Bar = 12 μm.

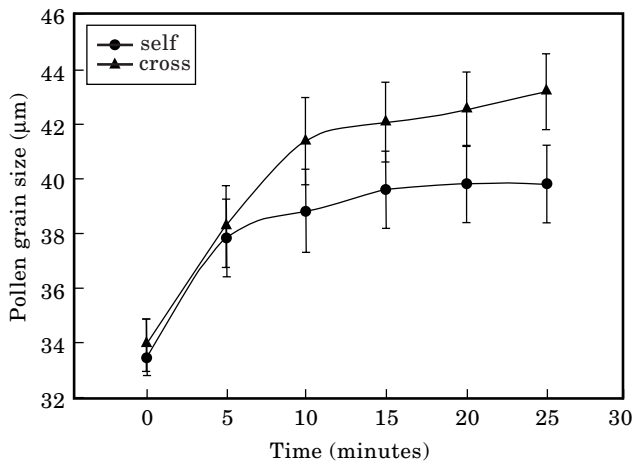


FIG. 11. Hydration of cross- and self-pollen of *Trillium grandiflorum* during the first 25 min following application of pollen to stigmas. Two-way repeated measures ANOVA indicated that hydration of cross- and self-pollen over time was significantly different (cross *vs.* self: $F = 35{\cdot}74$, d.f. = 1, $P < 0{\cdot}001$; cross *vs.* self × time: $F = 16{\cdot}69$, d.f. = 7, $P < 0{\cdot}001$). Tukey's pairwise multiple comparison procedures indicated that only values at 10 min and beyond were significantly different ($P < 0{\cdot}001$).

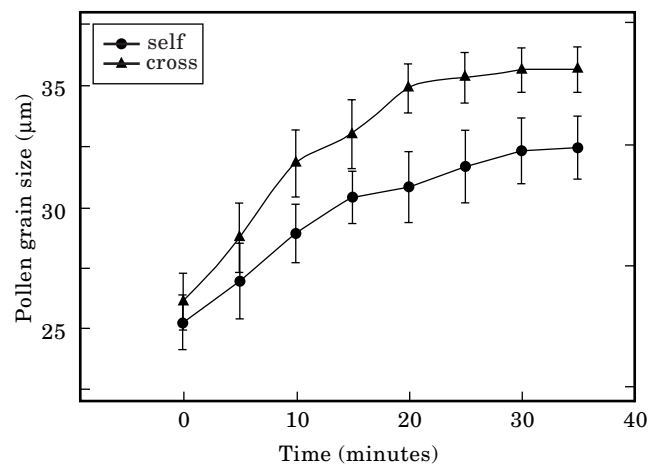


FIG. 12. Hydration of cross- and self-pollen of *Trillium erectum* during the first 35 min following application of pollen to stigmas. Two-way repeated measures indicated that hydration of cross- and self-pollen over time was significantly different (cross *vs.* self: $F = 50{\cdot}51$, d.f. = 1, $P < 0{\cdot}001$; cross *vs.* self × time: $F = 25{\cdot}69$, d.f. = 5, $P < 0{\cdot}001$). Tukey's pairwise multiple comparison procedures indicated that only values at 10 min and beyond were significantly different ($P < 0{\cdot}001$).

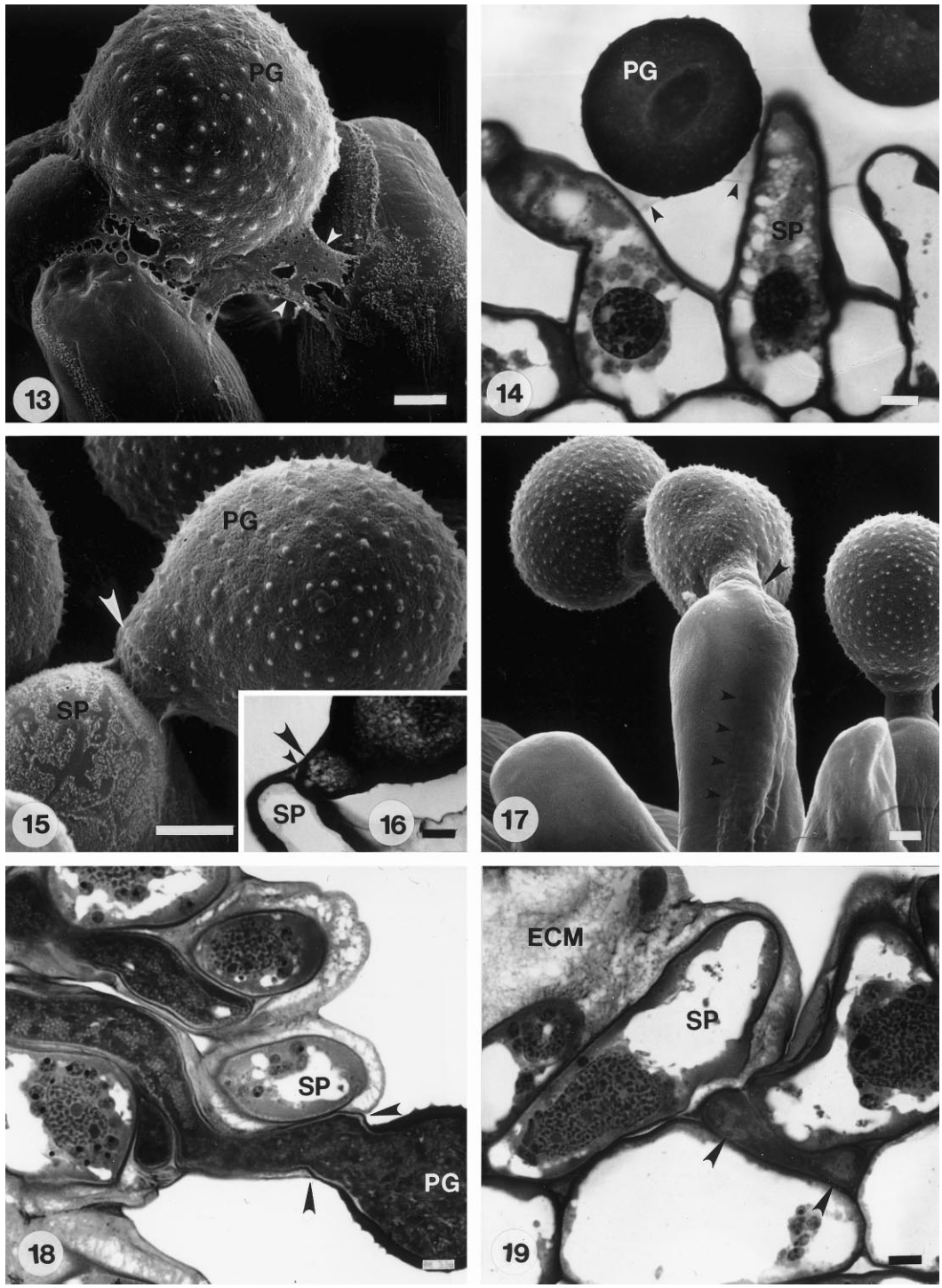
expanded by 30 min following cross-pollination prior to pollen tube arrival in the crest cleft (Fig. 21). In contrast to cross-pollen tube growth, self-pollen tube growth was terminated at the base of a papilla tip prior to intercellular growth (Figs 22 and 23). Growth of self-pollen tubes to the base of the papillate tip of a stigmatic cell was completed within 50 min following self-pollination. Self-pollen tube growth was terminated by this time except in 'leaky' interactions (see below).

'Leaky' self-pollen tube growth

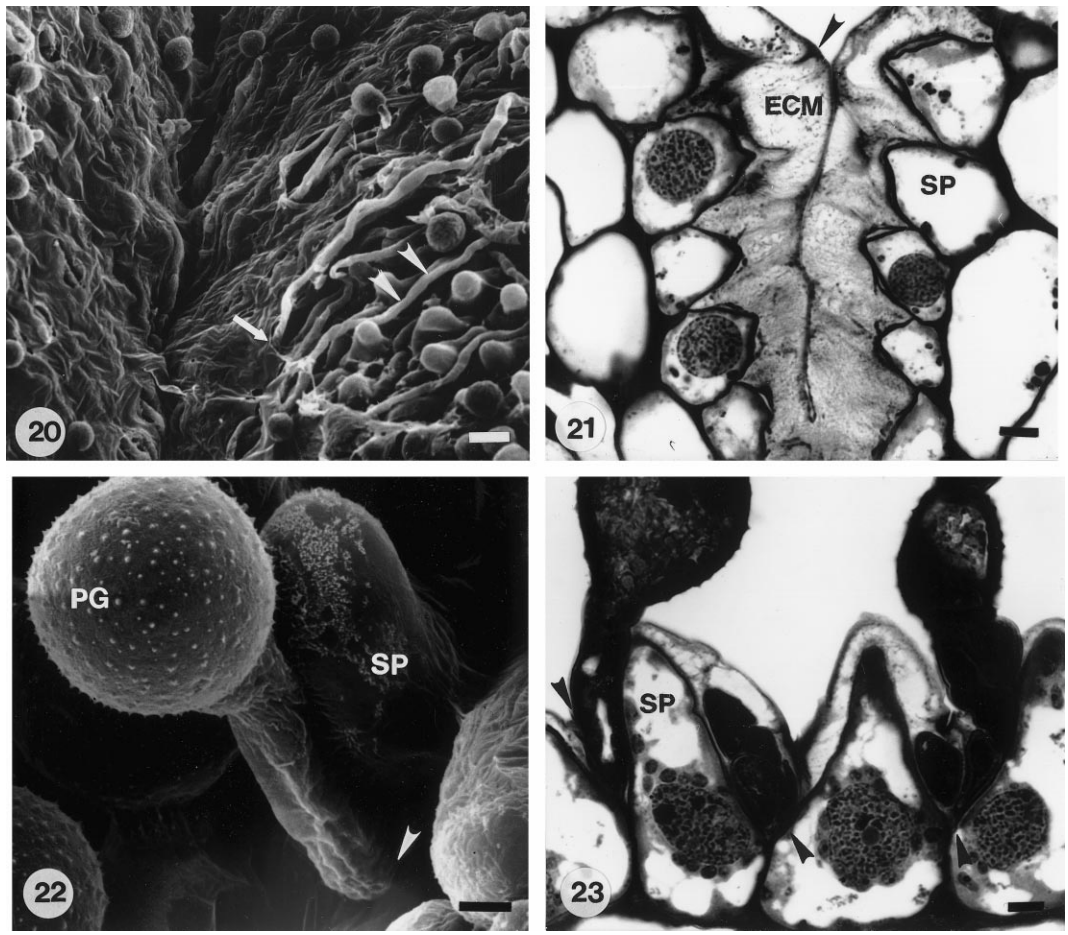
Observations of pollen tube growth over 5 d following *in situ* cross- and self-pollination of *T. grandiflorum* and *T. erectum* indicated that in most flowers, self-pollen tubes ceased growth at the stigmatic surface. However, in a few flowers of both species, self-pollen tubes were observed growing into the ovaries and ovules. In each case, however, self-pollen tube growth lagged well behind that of cross-pollen tube growth. In *T. grandiflorum*, cross-pollen tubes entered ovaries and ovules by 48 h following cross-pollination; self-pollen tubes entered ovaries after 72 h in the occasional instances where this was observed. Parallel events occurred more slowly in *T. erectum* with cross-pollen tubes entering the ovary and ovules by 72 h and self-pollen tube entry occurring by 5 d.

Compatibility status as determined from seed set data under field conditions

In conjunction with the structural studies described above, results from experimental hand-pollinations on



FIGS 13–19. Light and scanning electron micrographs of cross- and self-pollinated stigmas of *T. grandiflorum*. Figs 13 and 14. Pollen grain adhesion to stigmatic papillae. Arrowheads denote pollen coat. 5 min post-pollination. Fig. 13. Cross-pollination. Fig. 14. Self-pollination. Figs 15 and 16. Pollen tube emergence (large arrowhead). 20 min post-pollination. Fig. 15. Cross-pollination. Fig. 16. Self-pollination. Small arrowhead denotes papilla cuticle. Figs 17 and 18. Growth of cross-pollen tubes within the extracellular matrix underneath the papillae cuticle. Large arrowheads denote point of pollen tube entry beneath the cuticle and into the expanded extracellular matrix. 50 min post-pollination. Fig. 17. Small arrowheads demark pollen tube. Fig. 19. Intercellular growth of cross-pollen tube in substigmatic tissue (arrowheads). Bars = 8·8 μm. ECM, Extracellular matrix; PG, pollen grain; SP, stigmatic papilla.



FIGS 20–23. Light and scanning electron micrographs of cross- and self-pollinated stigmas of *T. grandiflorum*. Fig. 20. Pathway of cross-pollen tubes (arrow) that do not grow intercellularly but track the adaxial surface of stigmatic papilla beneath the cuticle in an expanded extracellular matrix. 3 h post-pollination. Fig. 21. Expanded extracellular matrix of stigmatic papillae. 30 min post-pollination. Figs 22 and 23. Cessation of self-pollen tube growth at the base of stigmatic papilla (arrowheads). Bars = 8·8 μm. ECM, extracellular matrix; PG, pollen grain; SP, stigmatic papilla.

both species conducted under field conditions confirmed the presence of a physiological SI system. Levels of fruit and seed set per fruit were substantially lower in self- *vs*. cross-pollination (Fig. 24). Self-pollination of *T. grandiflorum* and *T. erectum* resulted in a 98 % and 95 % reduction in number of seeds per pollination, respectively, in comparison with cross-pollination. Emasculated and bagged flowers of *T. grandiflorum* and *T. erectum* produced very few fruits (two and one fruits, respectively) indicating that non-pseudogamous apomixis is unlikely to be present in either species. These three fruits probably arose from pollen contamination. While there was a trend towards lower seed set per fruit in open-pollinated flowers compared to cross-pollinated flowers in both species (Fig. 24), the differences were not statistically significant (*T. grandiflorum*: ANCOVA $F_{1,35} = 0{\cdot}77$, $P = 0{\cdot}40$; *T. erectum*: ANCOVA $F_{1,26} = 1{\cdot}4$, $P = 0{\cdot}24$). A significant proportion of the variation in seed set per fruit was explained by variation in ovule number (*T. grandiflorum*: $F_{1,35} = 101{\cdot}2$, $P < 0{\cdot}001$; *T. erectum*: $F_{1,26} = 25{\cdot}2$, $P < 0{\cdot}001$), but not by the interaction of ovule number and treatment (*T. grandiflorum*: $F_{1,35} = 0{\cdot}4$; $P = 0{\cdot}54$; *T. erectum*: $F_{1,26} = 3{\cdot}7$, $P = 0{\cdot}06$).

Mating-system estimates

Based on allozyme analysis of open-pollinated seeds collected in 1998, a significantly greater proportion of *T. grandiflorum vs. T. erectum* seeds were outcrossed ($P = 0{\cdot}017$). Estimates of the mean outcrossing rate and standard error ($t_m \pm$ s.e.) of populations of *T. grandiflorum* and *T. erectum* in 1998 were $0{\cdot}681 \pm 0{\cdot}085$ and $0{\cdot}529 \pm 0{\cdot}072$, respectively.

DISCUSSION

The principal finding of this study is that both *T. grandiflorum* and *T. erectum* exhibit a physiological SI system that significantly reduces levels of seed set following self-pollination. Recognition and rejection of self-pollen tubes occurs on the stigma surface in association with a

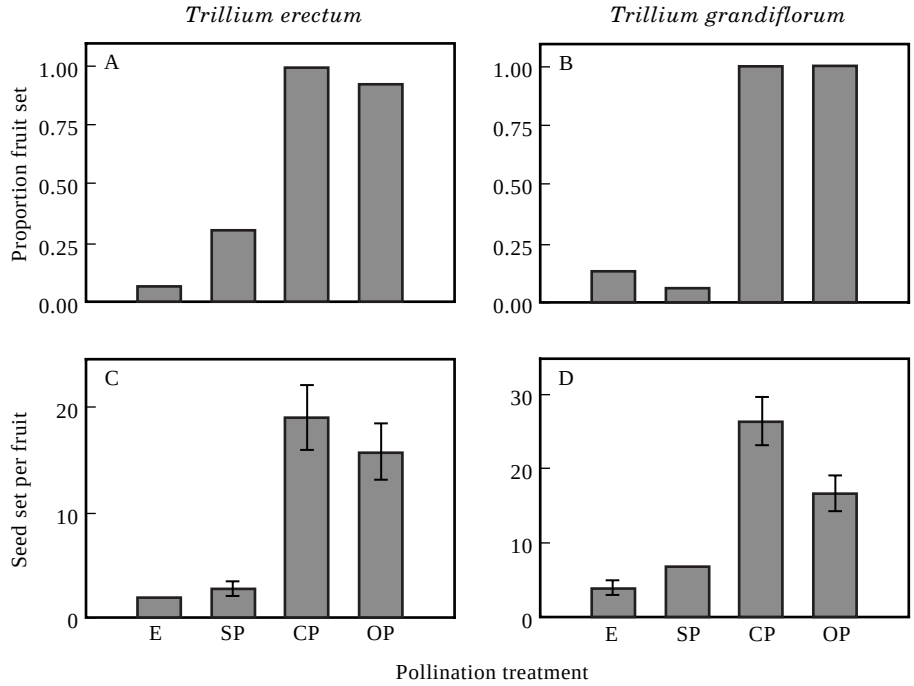


FIG. 24. The effect of experimental hand-pollinations on fruit and seed set per fruit in field populations of *Trillium erectum* (A, C) and *Trillium grandiflorum* (B, D). Four treatments were conducted on each species: E, Emasculated and bagged; SP, self-pollinated; CP, cross-pollinated; OP, open-pollinated. Sample sizes (flowers) for each treatment are: percent fruit set—$n = 14, 16, 15, 19$ for *T. erectum* and $n = 16, 16, 20, 19$ for *T. grandiflorum* and seed set (± 1 s.d.)—$n = 1, 5, 13, 17$ for *T. erectum* and $n = 2, 1, 20, 19$ for *T. grandiflorum*.

dry-type stigma. However, the expression of SI is not complete, and some pollen tubes entered into ovules and effected double-fertilization. Low levels of seed set were obtained with some experimental self-pollinations and our estimates of mating patterns in field populations indicated significant amounts of selfing, especially in *T. erectum*. We begin the discussion by placing our results on SI in *Trillium* in the context of what is known about SI in flowering plants. We then address the implications of leaky SI for the reproductive ecology and evolution of the species studied here. Finally, we discuss the potential functional significance of differences in reproductive traits that were revealed by our study.

Relationship of SI in Trillium *to other taxa*

Within the monocotyledons, SI is suspected to occur in at least 27 families and is known to operate at the stigma, or in the style or ovary, depending on the species (Sage *et al.*, 2000). The most common type of SI in the monocotyledons is gametophytic SI (GSI), whereby the SI phenotype of pollen is determined by its own haploid genotype. Virtually all detailed studies of SI in the monocotyledons have involved species with GSI. To date, no family of monocotyledons has been reported with homomorphic sporophytic SI (SSI) whereby the pollen SI phenotype is determined by the diploid genotype of the pollen parent (de Nettancourt, 1977, 1997, 2001). Sporophytic SI in the monocotyledons has been reported in association with heteromorphic incompatibility, but this is only known to occur in a single family, the Pontederiaceae (Barrett and Cruzan, 1993).

The Melanthiaceae represent the fifth monocotyledonous family in which stigmatic SI has been documented and the eighth family within the angiosperms that possesses SI in association with a dry-type stigma and bicellular pollen. These results are significant because most dry stigmatic SI systems were traditionally thought to be associated with tricellular pollen grains. Bicellular pollen grains were generally believed to be correlated with wet stigmas and stylar SI. Stigmatic SI in association with bicellular pollen and a dry stigma was considered anomalous, an observation confirmed as erroneous by Franklin *et al.* (1995) and the present study. Since SI in association with bicellular pollen grains has only been demonstrated to be under gametophytic control amongst all flowering plant taxa, and homomorphic SSI has not been reported in the monocotyledons, it seems reasonable to suggest that SI in *Trillium* may be under gametophytic control. However, given the diversity of SI systems now known to occur in flowering plants (de Nettancourt, 1977, 1997, 2001), this hypothesis clearly needs empirical corroboration through the appropriate genetic studies.

Self-rejection, the consequence of self-recognition, is rapid in *T. grandiflorum* and *T. erectum*, occurring within 10 min of pollination. Both species show differential hydration of cross- and self-pollen at this time. The final stage of rejection, cessation of self-pollen tube growth, is completed within 50 min in *T. grandiflorum*. Although pollen of *T. erectum* takes longer to germinate, cessation of

self-pollen tube growth probably occurs soon thereafter. Self-incompatibility directly affecting pollen tube growth after or during germination on the stigma, as reported here, has been documented in the Papaveraceae (Franklin-Tong *et al*., 1994), Onagraceae (Hecht, 1964), Poaceae (Heslop-Harrison, 1982), Commelinaceae (Owens, 1981), and Asteraceae (Knox, 1973, Hiscock, 2000). Even though pollen germination ensues, initiation of SI reactions, and hence self-recognition, is rapid in the Papaveraceae and Poaceae, occurring within seconds after being challenged with *S*-gene products (Heslop-Harrison, 1982; Snowman *et al*., 2000). Self-recognition in both taxa probably functions via signal-transduction mechanisms (de Nettancourt, 1997, 2001; Snowman *et al*., 2000). The action of SI through signal-transduction in some dry-stigmatic species is in contrast to SI operating in the style via S-RNAses as occurs in the Solanaceae, Rosaceae and Scrophulariaceae (de Nettancourt, 2001). Although the present investigation does not address the cellular mechanisms of SI in *T. grandiflorum* and *T. erectum*, we do provide the temporal and structural framework necessary for future studies to examine SI in both species in a more detailed manner. Such studies are essential to gain an improved understanding of the evolutionary relationships of SI in flowering plants.

Leaky self-incompatibility

Rejection of self-pollen through the action of stigmatic SI is not absolute in all individuals of *T. grandiflorum* and *T. erectum*. Structural studies revealed that a small number of self-pollen tubes penetrated ovules and low levels of self seed resulted from experimental hand self-pollinations. In fact, significantly higher levels of selfing were estimated using allozyme markers than might have been suggested by the observations of pollen tube growth and seed set following self-pollinations. In open-pollinated families of *T. grandiflorum* and *T. erectum* at Joker's Hill, nearly a third, or half, respectively, of all ovules in each species were self-fertilized under natural conditions. This clearly demonstrates that the SI system of these species is weakly expressed under field conditions, at least in some individuals. Hence, accurate characterization of SI in a population will depend to some extent on how many individuals with incomplete expression of SI are included in the sample under study.

Incomplete or 'leaky' SI is likely to occur in other populations of *T. grandiflorum* and *T. erectum*, and probably accounts for contradictory descriptions in the literature concerning the compatibility status and mating systems of the two species. For example, Irwin (2000) recently reported that *T. grandiflorum* and *T. erectum* were self-compatible, based on controlled cross- and self-pollinations of populations from Vermont, USA, although she noted that self-pollinations produced significantly fewer fruits and seeds compared with cross-pollinations. An alternative way to interpret Irwin's results would be to suggest that the respective populations of *T. grandiflorum* and *T. erectum* exhibit weak SI.

A more direct approach to determine the mating system of a species is to estimate natural levels of outcrossing and selfing using genetic markers. Broyles *et al*. (1997) estimated outcrossing rates for a population of both species from upper New York State, USA, and reported values (*T. grandiflorum*: $t_m = 0{\cdot}76$; *T. erectum*: $t_m = 0{\cdot}42$) similar to those reported in the present study. In contrast, Kalisz *et al*. (1999) reported a value of $t_m = 1{\cdot}05$ for a population of *T. grandiflorum* in Michigan, USA, and concluded that SI was strongly expressed. However, controlled self-pollinations in the Michigan population did reveal a few individuals that set seed. Hence the wide diversity of opinion concerning the mating systems of *T. grandiflorum* and *T. erectum* (and see also Fukuda and Grant, 1980; Ohara, 1989) most likely results from differences among populations in the strength of SI and differing opinions on how best to describe populations with weak SI. Further studies of variation in the mating patterns of both species would be useful to determine if leaky SI has a significant geographical component.

The functioning and expression of SI is dependent on numerous intrinsic and extrinsic factors. These include: the types of *S*-alleles and their genetic backgrounds, mutations at *S*-alleles, temperature and humidity conditions, floral age, and the presence of cross (mentor)-pollen (reviewed in de Nettancourt, 1977; Barrett, 1988; Stephenson *et al*., 2000). The mechanisms responsible for leaky SI in both *Trillium* species observed in the present study are not known. The occurrence of sporadic self-fertile individuals within populations, and the variation among populations in the strength of SI, suggest that leaky SI is an intrinsic feature of the SI system of *Trillium* and probably has some genetic basis. However, the significantly higher rates of selfing that we observed *in situ* in open-pollinated flowers compared with those predicted from the single donor hand self-pollinations could also indicate that the potency of SI may be subject to the quality of pollen loads. Under field conditions, where pollen loads may contain a mixture of outcross- and self-pollen, it is possible that increased rates of selfing could occur because of the presence of cross-pollen. Such 'cryptic self-fertility' has been documented elsewhere in species that display strong SI following self-pollination only (Bertin and Sullivan, 1988). The germination and subsequent growth of cross-pollen tubes in carpellary tissues can apparently provide the necessary stimuli to enable self-pollen tubes to obtain a greater share of fertilizations than they could attain alone. Marker gene studies of mixed pollen loads would be necessary to determine if such a mechanism exists in *Trillium*.

Pollen traits and their influence on dispersal and mating

Mating and fertility in angiosperms is not only regulated by post-pollination interactions between pollen and carpellary tissues, but also by traits affecting pollen dispersal (Kress, 1981; Stanton *et al*., 1992; Stephenson *et al*., 1992; Harder and Barrett, 1996; Harder, 2000). Our observations indicate that pollen from both *Trillium* species is released from anthers in clumps. Significantly, the larger spinose pollen of *T. grandiflorum* is dispersed in aggregates containing a larger number of grains than the smaller globose pollen of *T. erectum*. To what extent these differences reflect the contrasting pollination syndromes of the two species is not

known. The larger number of pollen grains per clump in *T. grandiflorum* is probably due to the more copious pollen coat present on the grains of *T. grandiflorum* compared to those of *T. erectum*. Pollen coat, or tryphine, is essential for pollen adhesion, recognition and germination on stigmatic papillae of flowering plants (Wolters-Arts *et al*., 1998; Dickinson *et al*., 2000). However, it has also been proposed that pollen coat may be important for efficient pollen dispersal (Endress, 1994). Modifications of pollen coat that result in the clustering of pollen grains to one another can increase the amount of pollen removed from anthers by pollinators and deposited on stigmas. Since *T. grandiflorum* and *T. erectum* often experience low pollinator-visitation rates (Lubbers and Lechowicz, 1989; Broyles *et al*., 1997; Irwin, 2000), and can often be pollen limited (Smith, 1998), pollen clumping may function to reduce pollen limitation of reproductive success in the presence of infrequent pollinator service.

Pollen clumping may also influence mating patterns and male parentage in *T. grandiflorum* and *T. erectum*. Studies of correlated mating in both species indicate that the probability of outcrossed progeny being full-sibs is high (Broyles *et al*., 1997). These authors reported that the effective number of male parents per fruit was 1.12 in *T. erectum* and 1·01 in *T. grandiflorum* (and see also Kalisz *et al*., 1999). Deposition of a single pollen load from one male parent probably accounts for these patterns and is consistent with the observation of low pollinator visitation rates to the two species. High correlated mating is expected in species whose pollen is dispersed in packages or pollinia (e.g. legumes, orchids and milkweeds). However, our study suggests that the extent of this phenomenon in species with well developed pollen coats would repay further investigation. It seems probable that the high correlated mating reported in *T. grandiflorum* and *T. erectum* arises because low visitation rates combined with pollen clumping reduce the likelihood of multiple paternity within the multiovulate fruits of the two species.

CONCLUSIONS

Populations of *T. grandiflorum* and *T. erectum* flower in early spring when pollinator service is notoriously unreliable (Schemske *et al*., 1978; Baker *et al*., 2000). Both species come into flower rapidly in early May, with all plants entering anthesis during a short period of time. This flowering strategy increases the likelihood that most individuals will receive at least one pollinator visit. Nevertheless, the higher selfing rates in *T. erectum* compared to those in *T. grandiflorum* may be associated with limited pollinator visitation, earlier flowering and low population density. These aspects of the biology of this species would be predicted to be associated with selfing as a mechanism of reproductive assurance (Lloyd, 1980). Higher outcrossing in *T. grandiflorum* is no doubt favoured by the near synchronous, mass flowering of its larger populations and an SI system that is usually stronger in expression. Studies on sex allocation and pollen-ovule ratios of the two species at Joker's Hill (Wright and Barrett, 1999) are consistent with the differences in outcrossing rate revealed by our study. However, the sporadic occurrence of individuals of *T. grandiflorum* with weak SI may provide opportunities for the selection of increased selfing under conditions where pollinators are unreliable and fertility is compromised. While the function of SI in flowering plants is undoubtedly associated with reducing inbreeding depression, the maintenance of variation in SI expression, as occurs in *Trillium*, may reflect a flexible evolutionary response to the uncertain pollination conditions that characterize early spring conditions in many temperate plant communities.

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LITERATURE CITED

Arasu NN. 1968. Self-incompatibility in angiosperms: a review. *Genetica* **39**: 1–24.

Axelrod DI. 1966. Origin of deciduous and evergreen habitat in temperate forests. *Evolution* **20**: 1–15.

Baker AM, Barrett SCH, Thompson JD. 2000. Variation in pollen limitation in the early flowering Mediterranean geophyte *Narcissus assoanus* (Amaryllidaceae). *Oecologia* **124**: 529–535.

Barrett SCH. 1988. The evolution, maintenance, and loss of self-incompatibility systems. In: Lovett Doust J, Lovett Doust L, eds. *Plant reproductive ecology patterns and strategies*. Oxford: Oxford University Press, 98–124.

Barrett SCH, ed 1992. *Evolution and function of heterostyly*. Berlin: Springer Verlag.

Barrett SCH, Cruzan MB. 1993. Incompatibility in heterostylous plants. In: Williams EG, Knox RB, Clarke AE, eds. *Genetic control of self-incompatibility and reproductive development in flowering plants*. Dordrecht: Kluwer Academic Publishers, 189–219.

Barrett SCH, Helenurm K. 1987. The reproductive biology of boreal forest herbs I. Breeding systems and pollination. *Canadian Journal of Botany* **65**: 2036–2046.

Bertin R, Sullivan M. 1988. Pollen interference and cryptic self-fertility in *Campsis radicans*. *American Journal of Botany* **75**: 1140–1147.

Brewbaker JL. 1957. Pollen cytology and self-incompatibility systems in plants. *Journal of Heredity* **48**: 217–277.

Broyles SB, Sherman-Broyles S, Rogati P. 1997. Evidence of outcrossing in *Trillium erectum* and *Trillium grandiflorum* (Liliaceae). *Journal of Heredity* **88**: 325–329.

Case FW Jr., Case RB. 1997. *Trilliums*. Portland, Oregon: Timber Press Inc.

Charlesworth D. 1985. Distribution of dioecy and self-incompatibility in angiosperms. In: Greenwood PJ, Slatkin M, eds. *Evolution: essays in honour of John Maynard Smith*. Cambridge: Cambridge University Press, 237–268.

Charlesworth D, Charlesworth B. 1987. Inbreeding depression and its evolutionary consequences. *Annual Review of Ecology and Systematics* **18**: 237–268.

Chichiricco G. 1993. Pregamic and postgamic self-incompatibility systems in *Crocus* (Iridaceae). *Plant Systematics and Evolution* **185**: 219–228.

De Nettancourt D. 1977. *Incompatibility in angiosperms*. New York: Springer-Verlag.

De Nettancourt D. 1997. Incompatibility in angiosperms. *Sexual Plant Reproduction* **10**: 185–199.

De Nettancourt D. 2001. *Incompatibility and incongruity in wild and cultivated plants*. New York: Springer-Verlag.

Dickinson HG, Elleman CJ, Doughty J. 2000. Pollen coatings—chimaeric genetics and new functions. *Sexual Plant Reproduction* **12**: 302–309.

Dyer AF. 1963. Endosperm development after controlled pollination within and between species of *Trillium* and *Paris*. *Chromosoma* **14**: 549–567.

East EM. 1940. The distribution of self-sterility in flowering plants. *Proceedings of the American Philosophical Society* **82**: 449–518.

Eckert CG, Barrett SCH. 1994. Post-pollination mechanisms and the maintenance of outcrossing in self-compatible, tristylous *Decodon verticillataus* (Lythraceae). *Heredity* **72**: 396–411.

Endress P. 1994. *Diversity and evolutionary biology of tropical flowers*. Cambridge: Cambridge University Press.

Franklin FCH, Lawrence MJ, Franklin-Tong VE. 1995. Cell and molecular biology of self-incompatibility in flowering plants. *International Review of Cytology* **158**: 1–64.

Franklin-Tong VE. 1999. Signaling in pollination. *Current Opinions in Plant Biology* **2**: 490–495.

Franklin-Tong VE, Lawrence MJ, Franklin FCH. 1994. The molecular and cellular biology of gametophytic self-incompatibility in *Papaver rhoeas*. In: Williams EG, Knox RB, Clarke AE, eds. *Genetic control of self-incompatibility and reproductive development in flowering plants*. Netherlands: Kluwer Academic Publishers, 42–64.

Fukuda I. 1987. Mode of speciation in the *Trillium*-chromosome evolution and differentiation of the reproductive system. *American Journal of Botany* **74**: 641–642.

Fukuda I, Grant WF. 1980. Chromosome variation and evolution in *Trillium grandiflorum*. *Canadian Journal of Genetics and Cytology* **22**: 81–91.

Gaude T, McCormick S. 1999. Signaling in pollen-pistil interactions. *Cell and Developmental Biology* **10**: 139–147.

Givnish TJ. 1982. Outcrossing versus ecological constraints in the evolution of dioecy. *American Naturalist* **119**: 849–865.

Graham A. 1972. *Floristics and paleofloristics of Asia and eastern North America*. Amsterdam: Elsevier Publications Co.

Gray A. 1846. Analogy between the flora of Japan and that of the United States. *American Journal of Science and Arts II* **2**: 135–136.

Gray A. 1860. Illustrations of botany of Japan and its relation to that of central and northern Asia, Europe, and North America. *Proceedings of American Arts and Science* **4**: 131–135.

Harder LD. 2000. Pollen dispersal and the floral diversity of monocotyledons. In: Wilson KL, Morrison DA, eds. *Monocots: systematics and evolution*. Melbourne: CSIRO, 243–257.

Harder LD, Barrett SCH. 1996. Pollen dispersal and mating patterns in animal-pollinated plants. In: Lloyd DG, Barrett SCH, eds. *Floral biology: studies on floral evolution in animal-pollinated plants*. New York: Chapman and Hall, 140–190.

Hecht A. 1964. Partial inactivation of an incompatibility substance in the stigmas and styles of *Oenothera*. In: Linskens HF, ed. *Pollen physiology and fertilization*. Amsterdam: North-Holland, 237–243.

Heslop-Harrison J. 1982. Pollen-stigma interactions and cross-incompatibility in the grasses. *Science* **215**: 1358–1364.

Hiscock SJ. 2000. Self-incompatibility in *Senecio squalidus* L. (Asteraceae). *Annals of Botany* **85**: 181–190.

Irwin RE. 2000. Morphological variation and female reproductive success in two sympatric *Trillium* species: Evidence for phenotypic selection in *Trillium erectum* and *Trillium grandiflorum* (Liliaceae). *American Journal of Botany* **87**: 205–214.

Kalisz S, Hanzawa FM, Tonsor SJ, Thiede DA, Voigt S. 1999. Ant-mediated seed dispersal alters pattern of relatedness in a population of *Trillium grandiflorum*. *Ecology* **80**: 2620–2634.

Kenrick J, Kaul V, Williams EG. 1986. Self-incompatibility in *Acacia retinodes*: site of pollen-tube arrest is the nucellus. *Planta* **169**: 245–250.

Knox RB. 1973. Pollen wall proteins: pollen-stigma interactions in ragweed and cosmos (Compositae). *Journal of Cell Science* **12**: 421–443.

Kress WJ. 1981. Sibling competition and evolution of pollen unit, ovule number, and pollen vector in angiosperms. *Systematic Botany* **6**: 101–112.

Lloyd DG. 1980. Demographic factors and mating patterns in angiosperms. In: Solbrig OT, ed. *Demography and evolution in plant populations*. Oxford, UK: Blackwell, 67–88.

Lubbers AE, Lechowicz MJ. 1989. Effects of leaf removal on reproduction vs. below ground storage in *Trillium grandiflorum*. *Ecology* **70**: 85–96.

Martin FW. 1959. Staining and observing pollen tubes in the style by means of fluorescence. *Stain Technology* **24**: 125–128.

Mattson O, Knox RB, Heslop-Harrison J, Heslop-Harrison Y. 1974. Protein pellicle of stigmatic papillae as a probable recognition site. *Nature* **247**: 298–300.

Nesom GL, La Duke JC. 1985. Biology of *Trillium nivale* (Liliaceae). *Canadian Journal of Botany* **63**: 7–14.

Ohara M. 1989. Life history evolution in the genus *Trillium*. *Plant Species Biology* **4**: 1–28.

O'Neill S. 1997. Pollination regulation of flower development. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**: 547–574.

Owens SJ. 1981. Self-incompatibility in the Commelinaceae. *Annals of Botany* **47**: 567–581.

Pontieri V, Sage TL. 1999. Evidence for stigmatic self-incompatibility, pollination induced ovule enlargement, and transmitting tissue exudates in the paleoherb, *Saururus cernuus* L. (Saururaceae). *Annals of Botany* **84**: 507–519.

Rice WR. 1989. Analyzing tables of statistical tests. *Evolution* **43**: 223–225.

Ritland K. 1986. Joint maximum likelihood estimation of genetic and mating structure using open-pollinated progenies. *Biometrics* **42**: 25–43.

Ritland K. 1990. A series of FORTRAN computer programs for estimating plant mating systems. *Journal of Heredity* **81**: 235–237.

Sage TL, Bertin R, Williams EG. 1994. Ovarian and other late-acting self-incompatibility systems. In: Williams EG, Knox RB, Clarke AE, eds. *Genetic control of self-incompatibility and reproductive development in flowering plants*. Dordrecht: Kluwer Academic, 116–140.

Sage TL, Pontieri V, Christopher R. 2000. Incompatibility and mate recognition in monocotyledons. In: Wilson KL, Morrison DA, eds. *Monocots: systematics and evolution*. Melbourne: CSIRO, 270–276.

Sage TL, Strumas F, Cole WW, Barrett SCH. 1999. Differential ovule development following self- and cross-pollination: the basis of self-sterility in *Narcissus triandrus* (Amaryllidaceae). *American Journal of Botany* **86**: 855–870.

SAS.. 1997. *JMP (R) user's guide, version 3.2.1*. Cary, NC: SAS Institute.

Sears ER. 1937. Self-sterility in plants. *Genetics* **22**: 130–181.

Schemske DW, Willson MF, Melampy MN, Miller VJ, Verner L, Schemske KM, Best LB. 1978. Flowering ecology of some spring woodland herbs. *Ecology* **59**: 351–366.

Smith DM. 1998. *Habitat fragmentation and the reproductive success of* Trillium grandiflorum *in southern Ontario*. MSc Thesis, Ontario, Canada: Trent University.

Snowman BN, Geitmann A, Clarke SR, Staiger CJ, Franklin FCH, Emons AMC, Franklin-Tong VE. 2000. Signalling and the cytoskeleton of pollen tubes in *Papaver rhoeas*. *Annals of Botany* **85**: 49–57.

Sokal RR, Rohlf FJ. 1995. *Biometry: The principles and practice of statistics in biological research, 3rd edn*. New York: W. H. Freeman and Company.

Stanton ML, Ashman T, Galloway LF, Young HJ. 1992. Estimating male fitness of plants in natural populations. In: Wyatt R, ed. *Ecology and evolution of plant reproduction: new approaches*. New York: Chapman & Hall, 62–90.

Stephenson AG, Good SV, Vogler DW. 2000. Interrelationships among inbreeding depression, plasticity in the self-incompatibility system, and the breeding system of *Campanula rapunculoides* L. (Campanulaceae). *Annals of Botany* **85**: 211–219.

Stephenson AG, Lau T, Quesada M, Winsor JA. 1992. Factors that affect pollen performance. In: Wyatt R, ed. *Ecology and evolution of plant reproduction: new approaches*. New York: Chapman & Hall, 119–136.

Swamy BGL. 1948. On the post-fertilization development of *Trillium undulatum*. *Cellule* **52**: 7–14.

Tiffney BH. 1985. Perspective on the origin of the floristic similarity between eastern Asia and eastern North America. *Journal of the Arnold Arboretum* **66**: 73–94.

Walsh NE, Charlesworth D. 1992. Evolutionary interpretations of differences in pollen tube growth rates. *Quarterly Review of Biology* **67**: 19–37.

Wendel JF, Weeden NF. 1991. Visualization and interpretation of plant isozymes. In: Soltis DE, Soltis PS, eds. *Isozymes in plant biology*. Portland, USA: Dioscorides Press, 5–45.

Wolters-Arts M, Lush WM, Mariani C. 1998. Lipids are required for directional pollen-tube growth. *Science* **392**: 818–821.

Wright SI, Barrett SCH. 1999. Size-dependent gender modification in a hermaphroditic perennial herb. *Proceedings of the Royal Society of London Series B-Biological Sciences* **266**: 225–232.