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2 **Title:** The compounding effects of high pollen limitation, selfing rates and inbreeding depression
3 leaves a New Zealand tree with few viable offspring

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17 **Running title:** High pollen limitation, selfing rates and inbreeding depression

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19

1 **Abstract**

- 2 • *Background and Aims* Interactions between species are especially sensitive to
3 environmental changes. The interaction between plants and pollinators is of particular
4 interest given the potential current global decline in pollinators. Reduced pollinator
5 services can be compensated for in some plant species by self-pollination. However, if
6 inbreeding depression is high, selfed progeny could die prior to reaching adulthood
7 leading to cryptic recruitment failure.
- 8 • *Methods* To examine this scenario, we examined pollinator abundance, pollen limitation,
9 selfing rates, and inbreeding depression in populations of varying disturbance levels in
10 *Sophora microphylla* (Fabaceae), an endemic New Zealand tree species.
- 11 • *Key Results* We found high pollen limitation in all populations (average of 58% reduction
12 in seed production, 9 populations), high selfing rates (61% of offspring selfed, 6
13 populations), and high inbreeding depression (selfed offspring 86% less fit, 6
14 populations). Pollen limitation was associated with lower visitation rates by the two
15 endemic bird pollinators.
- 16 • *Conclusions* Our results suggest that for these populations, over half of the seeds
17 produced are genetically doomed. This reduction in the fitness of progeny due to reduced
18 pollinator service is likely important to population dynamics of New Zealand species and
19 worldwide.

20 **Key words:** cryptic recruitment failure, inbreeding depression, Kowhai, mating system,
21 Meliphagidae, pollen limitation, pollinator decline, reproductive ecology, *Sophora microphylla*

22

1 INTRODUCTION

2 Pollinators provide a key ecosystem service in both natural and agricultural ecosystems.
3 Over 87% of flowering plant species rely on biotic pollination to reproduce (Ollerton *et al.*,
4 2011). Similarly, although few crop species rely solely on biotic pollinators, one estimate
5 suggests that only 7% of crop species receive no benefit from pollinators (Klein *et al.*, 2007).
6 Interactions between species, such as those between plants and their pollinators, are especially
7 sensitive to environmental changes (Tylianakis *et al.*, 2008, Dunn *et al.*, 2009, Berg *et al.*, 2010,
8 Kiers *et al.*, 2010, Yang and Rudolf, 2010, Northfield and Ives, 2013). Current environmental
9 changes are resulting in negative effects on pollinator abundance, fuelling concerns of a global
10 pollination crisis and its effect on agricultural and ecological services (Biesmeijer *et al.*, 2006,
11 Hegland *et al.*, 2009, Potts *et al.*, 2010, Thomann *et al.*, 2013, but see Ghazoul, 2005). These
12 pollinator declines have the potential to negatively affect both the quantity (through pollen
13 limitation) and quality (through selfing and inbreeding depression) of offspring. While negative
14 effects of pollinator abundance on the quantity of progeny are well documented (Linhart and
15 Feinsinger, 1980, Spears, 1987, Steffan-Dewenter and Tschardt, 1999, Chittka and Schurkens,
16 2001, Quesada *et al.*, 2003), the effect on the quality is virtually unknown (but see Eckert *et al.*,
17 2010, Delmas *et al.*, 2015).

18 Reduced pollinator service can negatively affect the quality of offspring if plants must
19 supplement their pollen receipt with self-pollen, leading to an increase in selfing rates. Many
20 plant species can self-pollinate in the absence of pollinators (autonomous self-pollination),
21 termed reproductive assurance (Baker, 1955, Jain, 1976, Herlihy and Eckert, 2002, Busch and
22 Delph, 2012). Despite the fact that selfing usually results in less fit offspring due to inbreeding
23 depression, reproductive assurance is generally considered an adaptation to variable pollinator

1 abundance in species where all selfed offspring do not die (inbreeding depression less than 1;
2 Lloyd, 1992). However, consistently higher selfing rates due to low pollinator abundance could
3 negatively affect both short-term (decreased germination, growth, competitive ability) and long-
4 term processes (loss of genetic diversity, reduced gene flow; Stebbins, 1957, Takebayashi and
5 Morrell, 2001). Thus, understanding the impact of pollinator declines on plant population
6 processes requires information on the type of selfing that occurs, the amount of selfing and the
7 severity of inbreeding depression.

8 New Zealand is unfortunately an excellent location to examine the effect of human
9 disturbance on plant-pollinator interactions. Prior to human arrival, bird species were the primary
10 vertebrates with roles as pollinators, fruit dispersers, herbivores, and predators (Holdaway,
11 1989). Human colonization of the islands from ~1280 AD (Wilmshurst *et al.*, 2008) and the
12 resulting deforestation and introduction of mammal predators, led to the extinction of at least
13 41% of endemic bird species (Holdaway *et al.*, 2001). Range reductions of the remaining birds
14 have continued; from 1979 to 2004, 44% of forest bird species have experienced range
15 restrictions (Innes *et al.*, 2010). Because bird species were integral to ecosystem functioning, the
16 loss of so many species led to the disruption of many interactions, including pollination (Kelly *et*
17 *al.*, 2010). Understanding the impact of these changed interactions is the first step in conserving
18 the remaining species and their interactions.

19 Here we address the issue of pollinator abundance, selfing rates and inbreeding
20 depression using a widespread, self-compatible, canopy tree species, *Sophora microphylla*.
21 Plants flower spectacularly in the spring over a period of about one month during which a tree
22 can produce many thousands of yellow to gold flowers. Flowers are visited by a variety of
23 animals that are thought to vary in their effectiveness. The native honeyeaters are considered to

1 be the primary pollinators, particularly tui (*Prosthemadera novaeseelandiae*) and bellbirds
2 (*Anthornis melanura*) (Castro and Robertson, 1997, Anderson, 2003), both of which are
3 territorial (Craig *et al.*, 1981, Bergquist and Craig, 1988). Other floral visitors include introduced
4 insects such as bumblebees and honeybees (Anderson, 2003), the recently-arrived silvereye
5 (*Zosterops lateralis*), the house sparrow (*Passer domesticus*) and chaffinch (*Fringilla coelebs*)
6 (McCann, 1952) all of which are most likely less effective pollinators due to their size relative to
7 the floral tube and robbing behaviour (Anderson, 2003, pers. comm. AW Robertson). Although
8 *S. microphylla* is distributed throughout New Zealand, it prefers open habitats including steep
9 slopes along rivers, lake margins, dunes, and even pastures (Heenan *et al.*, 2001). Due to this
10 restricted range of appropriate habitats, few large populations occur in areas with high bird
11 abundance. Previous work has suggested that selfing can occur in the absence of pollinators
12 (Rattenbury, 1979) and that inbreeding depression is strong; selfed offspring were 96-99% less
13 fit than outcrossed offspring after 11 years of growth in a common-garden (Robertson *et al.*,
14 2011). The combined effect of populations occurring in marginal habitats, the ability to self-
15 pollinate, and strong inbreeding depression could lead to substantial negative effects of pollinator
16 declines on offspring quality.

17 Using a combination of field observations, mating system analyses and population
18 genetics, we asked several questions: 1) Are populations pollinator and/or pollen-limited? 2)
19 Does reproductive assurance lead to an increase in selfing rate as pollinator abundance
20 decreases? and 3) Is inbreeding depression strong enough to negate the benefits of selfing?
21 Because *S. microphylla* is pollinated by a range of bird species and is widespread across New
22 Zealand, it serves as a useful indicator species for the health of plant-pollinator interactions. Any

1 changes in the quality of offspring for this species would likely be an underestimate of changes
2 experienced in rarer or more specialized taxa.

3

4 **MATERIALS AND METHODS**

5 *S. microphylla* populations were chosen throughout New Zealand with varying amounts
6 of human disturbance – from isolated trees in grazed pasture, to a conserved “mainland island”
7 whose purpose is to preserve the unique collection of plant species (Fig. 1; Supplemental Table
8 1). All populations have a disrupted avifauna compared to pre-human estimates as none of these
9 populations occur in predator-free areas (Diamond and Veitch, 1981). According to the New
10 Zealand Bird Atlas (Robertson, 2007) both tui and bellbirds occur in the areas around most of the
11 populations with the exceptions of: bellbirds are not found near Lake Waikare and tui are not
12 found near Waimakariri, Ahuriri or Rakaia Gorge.

13 Several closely related *Sophora* species co-occur near some of the populations chosen
14 (Supplemental Table 1) and are known to hybridize with *S. microphylla* (Heenan *et al.*, 2001).
15 Thus, some populations may include a mixture of pure parental species and hybrids. This was a
16 particular problem at Wenderholm, which was the only site with a mixture of *S. microphylla* and
17 *S. chathamica*, as we discovered after the 1999 pollination season (*S. chathamica* was not
18 formally described until 2001). In the 2000 season at Wenderholm on 10 *S. microphylla* trees we
19 hand-outcrossed ~10 flowers per plant with *S. microphylla* pollen from a different tree, and ~10
20 flowers with *S. chathamica* pollen. Fruit set for the *microphylla* x *chathamica* crosses (13.2%)
21 was only half that for *microphylla* x *microphylla* crosses (26.8%), and natural fruit set (11.6%)
22 was comparable to the *microphylla* x *chathamica* crosses, suggesting that low fruit set from
23 hybrid crosses may be an issue at Wenderholm. Species differ only slightly in floral morphology

1 (Heenan *et al.*, 2001), however, it is unknown if self-compatibility or selfing rates differ amongst
2 them.

3

4 *Pollinator observations*

5 To quantify bird visitation rates, 10 populations were chosen in which to observe the
6 types of birds visiting and length of these visits (Fig. 1; Supplemental Table 1). Within a
7 population several observation stations were chosen from which multiple trees could be observed
8 simultaneously. During observation periods, ranging from 5 to 20 minutes, the type of floral
9 visitor and the length of their foraging bout were recorded. To standardize across floral
10 abundance and observation lengths, for each observation period we calculated the visitation rate
11 as: seconds of visitation per 100 flowers per hour. Observations were conducted in 1999, 2000 or
12 2013 depending on the population (Supplemental Table 1).

13 Although observations differed in many ways (year, observers, length) we were
14 interested in statistically determining how populations differed in their pollination rates. To this
15 end, we used population means in a Kruskal-Wallis non-parametric test to determine if
16 population type (pasture or conserved, Supplemental Table 1) differed in the visitation rates of
17 legitimate pollinators (tui or bellbirds, hereafter “good”) or potentially illegitimate (all others,
18 hereafter “bad”) visitors or the total visitation rates. Additionally, we used Pearson’s correlation
19 to determine if population size affected any visitation rates (results were qualitatively the same if
20 using Spearman’s rank correlation or population size categories in an ANOVA).

21 To determine how much nectar is generally produced we measured nectar amounts and
22 sugar concentration on pollinator-excluded flowers in the Waimakariri population in 2003.
23 Flowers that were bagged prior to flower opening produced on average $13.9 \mu\text{L} \pm \text{s.e. } 2.19$ of

1 nectar (N = 59) with an average BRIX reading of $20.17\% \pm 1.74$ (N=13). This gives a sucrose
2 equivalence of 3.01 mg per flower (Bolten *et al.*, 1979), which is comparable to hummingbird-
3 pollinated flowers (13 species average = 11.32 μ L or 2.39 mg sucrose; Cruden, 1976).

4 5 *Manipulative crosses*

6 To determine the potential for autonomous self-pollination, pollen limitation and early
7 inbreeding depression, we performed several types of crosses in nine populations (Fig. 1;
8 Supplemental Table 1) in 1999 and/or 2000. On each of up to 11 plants in a population, flowers
9 were chosen for: hand-pollination with a mixture of outcrossed pollen from at least three donor
10 trees (mean = 10 flowers per tree); self-pollination (mean = 10); pollinator exclusion (mean =
11 17); or natural pollination (mean = 37). Flowers for the self-pollination and pollinator exclusion
12 treatments were placed into a mesh bag prior to opening and after treatment for approximately
13 one month to ensure pollinators did not visit receptive flowers. Fruit set and seeds per fruit were
14 determined after three months. In Lake Rotoroa, Waimakariri and Ahuriri the same individuals
15 were scored in two consecutive years. In 2000 no trees set fruit at Lake Rotoroa, probably due to
16 flooding, and these were excluded from the analysis.

17 From these results a variety of measures were calculated. Capacity for autonomous self-
18 pollination was determined by calculating the mean seeds per flower in the pollinator exclusion
19 treatment for each population. Pollen limitation was analysed by comparing the natural to hand-
20 outcrossed treatments for fruits per flower and seeds per flower. We chose not to include seeds
21 per fruit because fruits per flower is easier to compare to other studies, seeds per flower is a more
22 complete measure of reproductive success, and seeds per fruit was positively correlated with fruit
23 set (Pearson's $r = 0.192$, $P = 0.001$). We used a generalized linear model with specific

1 probability distributions (fruit set = binomial, seeds per flower = negative binomial) in proc
2 GENMOD in SAS v9.3. The population:year combination, pollen source (natural or outcrossed)
3 and their interaction were used as predictors. A significant interaction effect would indicate
4 differences among populations in the severity of pollen limitation. We also calculated the
5 population pollen limitation index (PLI) as 1-(natural/outcrossed) from the LS Means derived
6 from the above models (Larson and Barrett, 2000). To determine if pollen limitation was
7 correlated with pollinator visitation rates, for the populations that had both measures we used a
8 Spearman's rank correlation for both total pollinator visitation rates (all birds including
9 silvereyes) and "good" visitation rates using proc CORR in SAS v9.3. To determine if
10 population type (pasture or conserved) affected the PLI we used an ANOVA with the population
11 means as the dependant variable and population type as the predictor.

12 To compare pollen limitation to previous studies, a variety of other calculations were
13 made. Limitation indices have been tabulated in reviews using several different methods, which
14 we attempted to calculate using the most comparable data. For comparison with Ashman *et al.*
15 (2004), we averaged the standardized effect size ((outcrossed-natural)/s.d.) of seeds per flower
16 for each population. To compare with results from Knight *et al.* (2005) we averaged the log
17 response ratio (ln (outcrossed/natural)) of fruit set for each population. For comparison with
18 Newstrom and Robertson (2005) we averaged their measure of PLI (natural/outcrossed) of fruit
19 set or seeds per flower for each population.

20 Pre-dispersal inbreeding depression was similarly analysed by comparing the self-
21 pollination and outcross-pollination treatments using the same predictors and probability
22 distributions as above. Population pre-dispersal inbreeding depression (i.e., from pollination to
23 developed seed) was calculated as $\delta_{pre} = 1-(self/outcrossed)$ using the LS Means.

1

2 *Mating system and inbreeding depression*

3 For the mating system analysis, leaves and up to 10 mature fruits from up to 20 trees
4 from six populations (Fig. 1; Supplemental Table 1) were collected in the summers of 2012 and
5 2013. Tissue collection for DNA extraction from these seeds differed among populations due to
6 necessary changes in the germination method. Initially seeds from two populations (Ahuriri and
7 Kowhai Point) were scarified, their seed coats removed and then planted in soil. However, this
8 resulted in low germination (average of 55%), which could severely downwardly bias selfing
9 rate estimations if caused by early acting inbreeding depression. To determine if estimated
10 selfing rates differed between soil-germinated and petri-dish germinated seeds, we chose one of
11 these populations (Ahuriri) to compare selfing rates estimated from soil-germinated and petri-
12 dish germinated seeds. To reduce the effect of low germination on the selfing rates we chose to
13 germinate seeds in petri-dishes for the remaining four populations. For soil-grown plants we
14 collected a single leaf while for petri-dish germinated seeds we collected the shoot apex after the
15 root had begun growing. Sample sizes for each population and collection type can be found in
16 Table 2.

17 For the inbreeding depression analysis, we collected leaf tissue from up to 50 adults and
18 50 juveniles (when present), along with an estimation of their height under the assumption this
19 correlates with age. Leaf tissue was dried and stored in silica gel.

20 DNA was extracted using a modified CTAB protocol (Doyle and Doyle, 1987). For
21 Ahuriri and Kowhai Point, six loci from Van Etten *et al.* (2014) were amplified as described in
22 the paper (Sop-248, 802, 806, 807, 808, 825) with the exception of 40 cycles of PCR rather than
23 35. Samples were genotyped at Massey Genome Services (Massey University, New Zealand) on

1 an ABI3730 DNA Analyzer (Applied Biosystems, Carlsbad, California, USA). Genotypes were
2 scored using GeneMapper v 3.7 (Applied Biosystems). Comparisons of progeny and maternal
3 genotypes revealed four loci with null alleles (Sop-802, 806, 807, 808), especially in the Kowhai
4 Point population. Decreasing the annealing temperature from 53°C to 48°C reduced the
5 frequency of null alleles in loci Sop-806 and 808. For Sop-802 and 807 we redesigned the
6 primers from the original sequence (GenBank accessions KF672187 and KF672189,
7 respectively), which decreased the frequency of nulls. The new primers were Sop-802 forward 5'
8 to 3':
9 CACGACGTTGTAAAACGACAAGCTCTCAAGAGATCCTC; reverse:
10 GTTTCTTTCAGGTCGTGGTATGAGTC; Sop-807 forward:
11 CACGACGTTGTAAAACGACAATAGGTTGCTCTTGACCC; reverse:
12 GTTTCTTTCCTAAGTTGCATGCAGTGG. The PCR program was as described in Van Etten *et*
13 *al.* (2014) with a 53°C annealing temperature. For all other populations, we replaced the original
14 loci with the newly designed primers.

15 From the progeny genotypes we estimated selfing rates using both COLONY (Jones and
16 Wang, 2010) and MLTR (Ritland, 2002). COLONY uses maximum-likelihood to identify
17 putative parents and thereby the selfing rate (s) and inbreeding coefficient (F). We chose this
18 program in addition to the more common MLTR because COLONY can use loci with null alleles
19 and has been shown to be less biased than other similar programs (Wang *et al.*, 2012). We used
20 the following settings: monoecious species, inbreeding present, diploid, polygamy for males and
21 females, sibship complexity prior, full-likelihood method, medium length run, medium precision,
22 and no updating allele frequencies. Locus-specific error rates per population were estimated from
23 an initial run. Seeds from a mother were coded as a known maternal sibship with their known

1 maternal individuals. The estimated probability of the father being in the potential genotyped
2 pool was dependent on the population (Paengaroa 0.3, Vinegar Hill 0.3, Kowhai Point 0.3,
3 Waimakariri 0.7, Ahuriri 0.5, Rakaia Gorge 0.3). Average selfing rates and F values were
4 estimated for each population separately. Additionally, because Ahuriri had genotypes for a
5 reasonable number of progeny from both petri-dish and soil-germinated seeds, we estimated
6 selfing rates for each group separately. We compared selfing rates using the 95% confidence
7 intervals. To test if visitation rates correlated with selfing rates we used Pearson's correlation
8 between population mean visitation rates ("good", "bad", and total) and the populations' selfing
9 rate. Differences in selfing rates by population type were not tested because only one pasture
10 population had selfing rate data. To compare to previous research and to validate the COLONY
11 results we also ran the traditional MLTR analysis without the two loci with the most frequent
12 null alleles (Sop-806 and Sop-807). Default options were used except we increased the bootstrap
13 replicates to 1000, which were used to compute standard errors.

14 To account for inbreeding depression before the genotyping stage (from fertilization
15 through seed development), we also calculated the zygotic selfing rate (s_z) using the equation
16 from Maki (1993). Because the inbreeding depression analysis above indicated a range of
17 inbreeding depression in fruit and seed set, we calculated s_z based on low (25th quartile $\delta_{pre} =$
18 0.361) and high (75th quartile $\delta_{pre} = 0.804$) estimates from that analysis. For the selfing rates from
19 post-germination seedlings, we used the estimates of inbreeding depression in germination from
20 Robertson *et al.* (2011) of low ($\delta_{germ} = 0$) and high ($\delta_{germ} = 0.571$) to calculate the combined
21 inbreeding depression as: $1 - [(1 - \delta_{pre}) * (1 - \delta_{germ})]$.

22 To estimate the inbreeding depression between seed and adult stages and to investigate its
23 timing, we calculated inbreeding coefficients for adults and juveniles. A decrease between life

1 stages suggests inbreeding depression has decreased the proportion of selfed progeny. Because
2 the data were known to have null alleles and few programs are available to account for this, we
3 used COLONY to calculate the F values for the juveniles and adults. From the adult F values and
4 selfing rates of progeny, we calculated the post-dispersal inbreeding depression using Ritland's
5 (1990) equation: $\delta_{\text{post}} = 1 - [2(1-s)F/s(1-F)]$. This estimate differs from the previous inbreeding
6 depression study (Robertson *et al.*, 2011) in that it is a cumulative estimate from germination to
7 adulthood under field conditions; the previous estimate was only from seed to 11-years in a
8 common-garden experiment, which may lead to an underestimate of inbreeding depression in
9 natural populations (Dudash, 1990, Husband and Schemske, 1996b). To compare with other
10 studies we also calculated observed and expected heterozygosity (H_o and H_e , respectively) and
11 F_{IS} of the adults without the loci with frequent null alleles (806 and 807) using GenAIEx v.6.501
12 (Peakall and Smouse, 2006, 2012).

13

14 **RESULTS**

15 *Pollinator observations*

16 A range of birds were observed visiting flowers, with tui, silvereyes, house sparrows
17 (*Passer domesticus* at Uni. Canterbury) or chaffinches (Taihape) being the most common
18 depending on the population (Fig. 2A). Other visitors included (with number of sightings):
19 blackbirds (*Turdus merula*; 4), starlings (*Sturnus vulgaris*; 3), kaka (*Nestor meridionalis*; 1),
20 yellowheads (*Mohoua ochrocephala*; 1) and New Zealand pigeons (*Hemiphaga*
21 *novaeseelandiae*; 1).

22 Visitor types and visitation rates varied among populations and years. Many of the
23 populations received visits from both “good” (tui or bellbirds) and “bad” pollinators (others).

1 However, the populations furthest south had very low “good” pollination rates. In fact, only 3 out
2 of 130 observation periods had any “good” pollinators in the Waimakariri, University of
3 Canterbury or Ahuriri populations. Visitation rates tended to differ with population type;
4 populations in pastures had slightly lower “good” visitation rates ($\chi^2_1=3.62$, $P=0.06$) but higher
5 “bad” visitation rates ($\chi^2_1=6.51$, $P=0.01$). However, within each population type was
6 considerable variation. For example, the highest “good” visitation rates were found at
7 Wenderholm (former pasture) in 1999 and Lake Rotoroa (conserved forest) in 2000. Population
8 size was not correlated with “good”, “bad” or total visitation rates ($P>0.4$). Additionally, in two
9 out of the three populations for which multiple years of data were collected, visitation rates
10 differed significantly between years (Lake Rotoroa: $\chi^2_1=5.93$, $P=0.01$; Waimakariri: $\chi^2_1=1.83$,
11 $P=0.2$; Ahuriri: $\chi^2_1=11.34$, $P=0.0008$).

12

13 *Pollen limitation index*

14 Overall, bagged flowers generally showed the lowest seed production, followed by
15 naturally pollinated and hand-pollinated selfed flowers, and hand-pollinated outcrossed flowers
16 producing the most seeds (Fig. 2B). Bagged flowers generally produced very few seeds, with the
17 highest average of 0.4 seeds per flower in the University of Canterbury population. Across
18 populations, naturally pollinated flowers produced 60% fewer seeds per flower than hand-
19 pollinated outcrossed flowers ($\text{natural}_{\text{lsmean}} = 0.738 \pm 0.088$ s.e., $\text{outcross}_{\text{lsmean}} = 1.854 \pm 0.149$; P
20 <0.0001 ; Table 1; Fig. 2B) partly due to decreased fruit set ($\text{natural}_{\text{lsmean}} = 0.199 \pm 0.007$,
21 $\text{outcross}_{\text{lsmean}} = 0.420 \pm 0.017$; $P <0.0001$).

22 Pollen limitation varied widely among populations (PLI fruits/flower range = 0.242 -
23 0.719, mean = 0.478; seeds/flower range = 0.303 - 0.807, mean = 0.575; Fig. 2C) and differed

1 significantly among sites for fruits per flower (population x pollen source $P < 0.0001$; Table 1)
2 but not seeds per flower ($P = 0.55$). Higher rates of “good” pollinators had a negative
3 relationship with pollen limitation after removing an outlier (Wenderholm 1999, see methods
4 and discussion) for both seeds/flower ($F_{1,8} = 7.91$, $P = 0.026$) and fruits/flower ($F_{1,8} = 10.66$, $P =$
5 0.014). There was no relationship between total visitation rate and the PLI ($F_{1,8} = 0.10$, $P = 0.76$
6 for seeds per flower) even with Wenderholm excluded. Population type did not significantly
7 affect pollen limitation, although pasture populations had slightly higher pollen limitation scores
8 ($F_{1,9} = 2.44$, $P = 0.15$; PLI seeds/flower means: pasture = 0.61 ± 0.05 s.e., conserved = 0.43 ± 0.06).

10 *Pre-dispersal inbreeding depression*

11 Selfed flowers produced 68% fewer seeds per pollinated flower than outcrossed flowers
12 ($\text{self}_{\text{Ismean}} = 0.602 \pm 0.092$ s.e., $\text{outcross}_{\text{Ismean}} = 1.854 \pm 0.149$; $P < 0.0001$; Table 1). In part this
13 was due to selfed flowers having 51% lower fruit set than outcrossed flowers ($\text{self}_{\text{Ismean}} = 0.206 \pm$
14 0.014 s.e., $\text{outcross}_{\text{Ismean}} = 0.420 \pm 0.017$; $P < 0.0001$; Table 1). Populations differed in the
15 severity of inbreeding for fruit set (population x cross type $P < 0.0001$; Table 1; Fig. 2D) with
16 Wenderholm, Waimakariri 2000 and Uni. Canterbury being low and Lake Rotoroa and Ahuriri
17 2000 being higher.

19 *Selfing rates*

20 On average, 61% of genotyped seeds were estimated to result from selfing (Fig. 3A).
21 Selfing rates from seeds ranged from 40-76%, with high rates at Vinegar Hill and Paengaroa and
22 low rates at Rakaia Gorge. The selfing rates from soil-germinated seeds in the Ahuriri population
23 were the lowest (34%) and lower than the rates estimated from the petri-dish germinated seeds in

1 the same population (54%). Estimated zygotic selfing rates ranged from 45-94% depending on
2 the strength of inbreeding depression used in the calculation (Fig. 3A). Results from MLTR
3 (Table 2) were qualitatively similar to results from COLONY with a slightly lower average
4 selfing rate for seeds of 57%. Biparental inbreeding rates were low (mean = 0.06) as was the
5 correlation of paternity (mean = 0.134), suggesting that within a tree seeds rarely shared a father.
6 Visitation rates did not have a significant relationship with selfing rates (“good” $F_{1,3}=0.50$,
7 $P=0.53$; “bad” $F_{1,3}=0.01$, $P=0.93$; total $F_{1,3}=0.12$, $P=0.75$).

8

9 *Post-dispersal inbreeding coefficient*

10 The average inbreeding coefficient changed dramatically between life stages (Fig. 3B).
11 Adult inbreeding coefficients were very small in all populations, ranging from 0.04 to 0.17.
12 However, seeds and seedlings had high inbreeding coefficients, ranging from 0.21 to 0.61 (Fig.
13 3B). This large difference between the inbreeding coefficients of adults and seeds resulted in
14 estimates of inbreeding depression ranging from 0.79 to 0.97 (Table 2). The low inbreeding
15 coefficient for juveniles (average=0.19, Fig. 3B) suggests that most of this reduction occurs prior
16 to the juvenile stage.

17

18 **DISCUSSION**

19 We found that the abundance and type of pollinators differed by population, with some
20 indication that pasture populations had fewer “good” pollinators. Higher visitation rates of these
21 “good” pollinators were negatively correlated with pollen limitation, suggesting that both the
22 abundance and effectiveness of visitors limited seed production in many of the populations. We
23 found high rates of selfing, especially after correcting for early inbreeding depression. Little of

1 this seems to be accounted for through autonomous self-pollination suggesting that reproductive
2 assurance does little in this species to compensate for pollinator limitation. Despite high selfing
3 rates, adult trees were not highly inbred suggesting very strong inbreeding depression. Due to the
4 high selfing rates and high inbreeding depression, almost half of the seeds produced are
5 genetically doomed. This study highlights the importance of assessing both the quantity and
6 quality of offspring when determining population health.

7 8 *Pollen limitation*

9 We found variation among populations in the types of birds visiting and the abundance of
10 birds. Populations in pastures tended to have higher rates of “bad” pollinators, which ranged
11 from exotic silvereyes to native parrots, and lower rates of “good” pollinators. Not surprisingly,
12 types of visitors differed geographically. In particular, populations near Christchurch
13 (Waimakariri, University of Canterbury and Ahuriri) had effectively no “good” pollinators.
14 While seed production does occur in these populations, suggesting that “bad” visitors are doing
15 some pollination, our results suggest they are not as effective; visitation rates by the presumed
16 legitimate pollinators (the two honeyeaters, bellbirds and tui) were negatively related to the
17 degree of pollen limitation after removing Wenderholm (Wenderholm appeared to have fruit set
18 depressed by hybridization with *S. chathamica*) while it was not related to PLI for “bad”
19 visitation rates. This suggests that low pollination is due to local shortages of the two large, long-
20 tongued endemic birds that are best able to probe *S. microphylla* flowers, and that other native
21 and exotic birds are not adequate substitute pollinators.

22 In the populations studied, *S. microphylla* plants were more pollen limited than most
23 species globally (Ashman *et al.*, 2004, Knight *et al.*, 2005). We found a range of pollen

1 limitation with Lake Waikare being the lowest (~0.30 for seeds/flower) and Wenderholm being
2 the highest (~0.81, but note caution above about possible hybridization). The average pollen
3 limitation found in this study was much higher than for most plant species based on comparison
4 of our results to those in several reviews. The average standardized effect size (1.10, range: 0.38-
5 2.62) was higher than 90% of the studies reviewed by Ashman *et al.* (2004). Even the least
6 pollen-limited population (Lake Waikare) had greater pollen limitation than 58% of the studies.
7 Similarly, compared to the results from Knight *et al.* (2005; range: 0.58-0.87), our average log
8 response ratio (0.67, range: 0.27-1.26) was higher than 75% of the species. However,
9 comparisons to New Zealand species show *S. microphylla* to have about average pollen
10 limitation. Compared to results from Newstrom and Robertson (2005), our values of 0.53 (fruit
11 set) and 0.43 (seeds per flower) were slightly lower than other trees, but similar to the average
12 over all life forms (0.42). Similarly, comparisons in Kelly *et al.* (2010) indicated that *S.*
13 *microphylla* was in the middle of the range of PLIs for New Zealand, bird-pollinated species.
14 These results show that *S. microphylla* is more pollen limited than most plants worldwide, but
15 about average for plant species measured in New Zealand.

16

17 *Selfing*

18 We found high selfing rates, ranging from 34-76%. The rate increases to a range of 45-
19 94% when accounting for early selfed seed and fruit abortion prior to genotyping. Our average of
20 61% selfing in seeds is higher than ~60% of animal-pollinated species (Vogler and Kalisz,
21 2001). If using the estimated zygotic selfing rate, it is higher than 65-90% of the species.
22 Whether our rates are higher than most species in the New Zealand flora is more difficult to
23 determine as few studies have measured the selfing rates of species in New Zealand. A review of

1 New Zealand plant species suggests that ~21% of species are self-incompatible and ~21% are
2 apomictic or autonomously selfing, leaving 58% of the species as possibly mixed-mating
3 (Newstrom and Robertson, 2005). Despite this high percentage, general conclusions are difficult
4 to draw given that only three species have been examined (Barrell *et al.*, 1997, Schmidt-Adam *et*
5 *al.*, 2000, Jesson *et al.*, 2006, Schmidt-Adam *et al.*, 2009, Howell and Jesson, 2013). However,
6 considering that pollen limitation is higher in New Zealand species and many of the plants can
7 have mixed-mating systems, higher selfing rates in New Zealand compared to elsewhere might
8 be expected.

9 There are several types of selfing including autonomous within-flower self-pollination,
10 facilitated within-flower self-pollination, and between-flower self-pollination (geitonogamy;
11 Lloyd and Schoen, 1992). Our results from the pollinator exclusion treatment suggest that
12 autonomous self-pollination is effectively zero, suggesting selfing does little to assure
13 reproduction in this species. Facilitated self-pollination within flowers should also be low due to
14 the presence of both temporal and spatial separation between male and female function.
15 Between-flower selfing, on the other hand, may be quite frequent due to trees having thousands
16 of flowers open at one time and pollinators visiting multiple flowers before leaving a tree. The
17 limited data we have on the number of flowers visited within trees from Wenderholm in 1996
18 (N=51 tui visits; average of 32.7 ± 8.4 s.e. flowers per tree) suggests that considerable
19 geitonogamy could be occurring. Comparing the selfing rates of emasculated (only between-
20 flower selfing) and non-emasculated flowers (within- and between- flower selfing) would
21 measure how much selfing is due to geitonogamy versus autogamy. Regardless of the type of
22 selfing, it appears that selfing is not a guaranteed way to reproduce in the absence of pollinators
23 in this species.

1 The absence of a negative correlation between selfing rates and visitation rates also
2 suggests that reproductive assurance is not occurring in this species. If plants supplement pollen
3 receipt through self-pollination when pollinators are rare there should be a negative correlation
4 between the selfing rate and visitation rates, which our data do not support. Other population
5 characteristics may be more important in determining the selfing rate in this species particularly
6 mate availability (Delmas *et al.*, 2015). Mate availability can potentially affect the selfing rate
7 via two mechanisms. First, pollinator behaviour may differ when resources are abundant.
8 Optimal foraging theory suggests that when resources are abundant pollinators should visit fewer
9 flowers per plant (Charnov, 1976), which would decrease geitonogamous pollen transfer.
10 Second, more pollen-producing individuals decreases the relative frequency of self-pollen in the
11 population, which should decrease self-pollination rates. Unfortunately, most populations of *S.*
12 *microphylla* are small (10-100 individuals), limiting the possibility of experimentally testing
13 these hypotheses. However, Rakaia Gorge, the largest of the populations we sampled, had the
14 lowest selfing rate, suggesting that population size may be important in determining selfing rates.

15

16 *Inbreeding depression*

17 Selfing could increase fitness if plants are pollen limited and inbreeding depression is not
18 too high (Lloyd, 1992). Unfortunately, in *S. microphylla* inbreeding depression is very strong,
19 leading to most selfed offspring not surviving to adulthood. Our hand pollinations show strong
20 inbreeding depression prior to seed dispersal and our comparison of seeds to adults shows
21 additional strong inbreeding depression after seed dispersal. Calculating a cumulative inbreeding
22 depression from the population averages yields $\delta = 1 - (1 - \delta_{\text{pre-dispersal}}) * (1 - \delta_{\text{post-dispersal}}) = 0.95$, i.e.,
23 selfed progeny have only 5% of the fitness of outcrossed progeny. This estimate has two caveats.

1 First, the decrease in seed production between outcrossed flowers and selfed flowers could be
2 due to late-acting self-incompatibility (LASI) rather than pre-dispersal inbreeding depression.
3 LASI could manifest at a variety of stages including pollen tube growth and successful
4 fertilization, differential provisioning of seeds or selective fruit abortion (Seavey and Bawa,
5 1986). While the mechanisms and evolutionary pressures may be very different between LASI
6 and early acting inbreeding depression, distinguishing which of the two is causing the difference
7 is not easy (Seavey and Bawa, 1986). Regardless of which factor is causing our results, it does
8 not change the result that selfed pollen does little to help with seed production.

9 The second caveat is that the method we used to estimate inbreeding depression in natural
10 populations relies on several assumptions that may be violated in our populations (Ritland,
11 1990). In particular, the method assumes a constant inbreeding coefficient of adults, i.e., that the
12 seedlings sampled will eventually reach the same F value as the adults sampled. This assumption
13 could be violated if inbreeding depression or selfing rates varied among generations. Given our
14 disturbed habitats it is possible that both of these violations could have occurred. For example,
15 the adults may have been produced when pollinators were more abundant leading to lower
16 selfing rates, which would explain their low F values rather than high inbreeding depression. In
17 this case, our inbreeding depression estimate would be upwardly biased. However, two results
18 suggest that while the estimate calculated here might not be precisely correct, inbreeding
19 depression is high in this species. First, a common garden experiment measuring inbreeding
20 depression for the first 11 years resulted in similarly high values (Robertson *et al.*, 2011).
21 Second, the juveniles in our study, which should be from a similar pollination and selective
22 environment to the seeds, show similar F values to adults, suggesting that the reduction in F

1 values is due to inbreeding depression and not changes in the selfing rates. Thus, we feel it is
2 safe to say that inbreeding depression is very strong in this species.

3 This strong inbreeding depression starts early and continues through the juvenile stage.
4 As found in a previous study (Robertson *et al.*, 2011), our hand-crosses showed inbreeding
5 depression started prior to seed dispersal. The dramatic selfing rate differences between the
6 Ahuriri seeds and seedlings show that inbreeding depression is strong even over this short
7 growth period. Strong, early-acting inbreeding depression is common in long-lived species
8 (Charlesworth and Charlesworth, 1987, Husband and Schemske, 1996a, Scofield and Schultz,
9 2006). Various reasons for this have been proposed including: small fitness differences between
10 selfed and outcrossed individuals accumulate over multiple reproductive bouts to higher lifetime
11 fitness; a longer pre-reproductive growth period increases cumulative inbreeding depression; and
12 selfing is more costly due to between-season seed discounting (reviewed in Petit and Hampe,
13 2006). This early inbreeding depression means that by the time juveniles are established, most of
14 the selfed individuals have died.

15

16 *Conservation implications*

17 Declines in bird pollinators caused by human impacts appear to have reduced seed
18 quantity in *S. microphylla* and, due to the high levels of selfing and the strong inbreeding
19 depression, most of the seeds produced are effectively useless. If on average over half of the
20 seeds produced are selfed and about 86% of the selfed seeds die before adulthood, nearly half of
21 the seeds produced in a population are doomed. We can incorporate these values into a measure
22 of the effective pollen limitation, i.e., the reduction of fitness due to too little pollen from non-
23 self sources, as $[1 - ((1 - \text{PLI}) - (1 - \text{PLI}) * s * \delta)]$. Using the average PLI (0.57), average selfing rate

1 (0.61) and average inbreeding depression (0.86), the average effective pollen limitation index
2 increases to 0.80. Described another way, seed fitness could increase on average 80% if flowers
3 were entirely outcrossed.

4 Because *S. microphylla* is a relatively common species with abundant nectar production
5 and large floral displays that receive much pollinator attention, we suspect that bird declines will
6 have had an even greater effect on other New Zealand species. Other less-rewarding or less-
7 apparent species may have even higher selfing rates, especially if they can autonomously self-
8 pollinate or occur at low adult densities. In addition, inbreeding depression in New Zealand trees
9 is probably high, given that it is a trait common to long-lived species (Charlesworth and
10 Charlesworth, 1987, Husband and Schemske, 1996a, Scofield and Schultz, 2006). Therefore, we
11 suggest that high selfing rates and high inbreeding depression could lead to many offspring being
12 low quality in New Zealand.

13 More broadly, our results suggest that measures of seed production or seedling densities
14 may be a gross overestimate of the effective offspring production. This could lead to cryptic
15 recruitment failure, i.e., a decline in successful reproduction despite high progeny production.
16 Given the global extent of pollinator declines, cryptic recruitment failure may be widespread.
17 Future studies measuring the impact of pollinator declines should consider both offspring
18 quantity and quality when determining population health and making conservation
19 recommendations.

20

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4

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9

10

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37

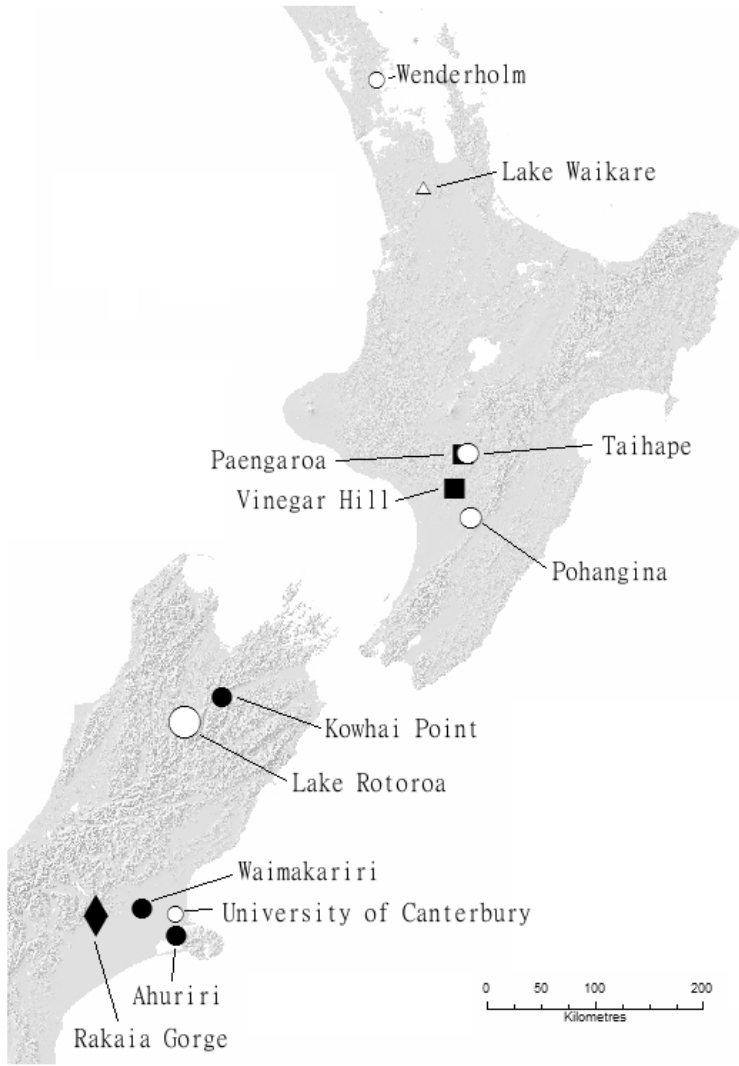
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1 **FIGURE CAPTIONS**

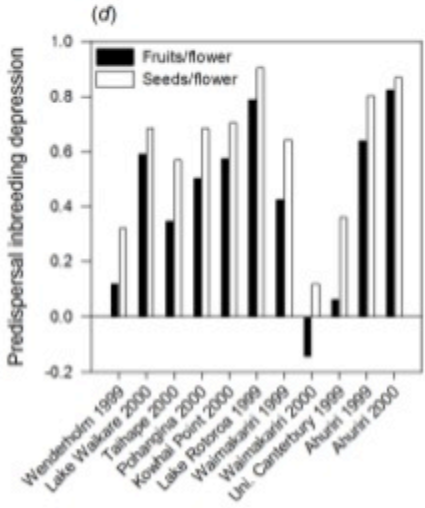
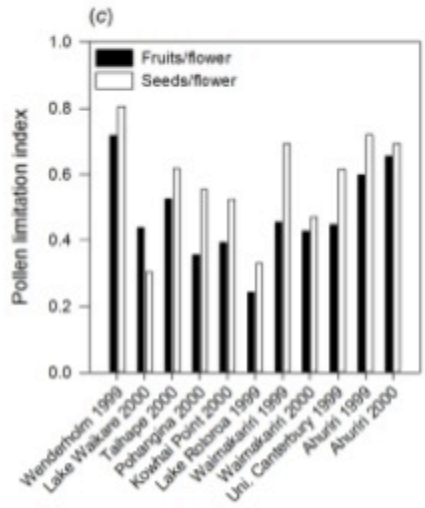
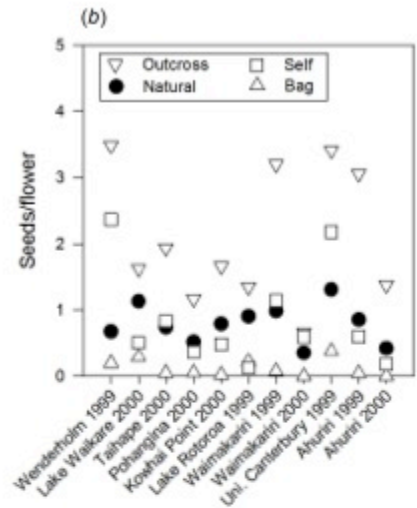
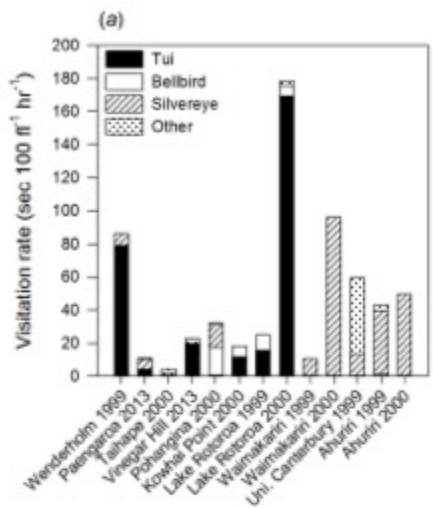
2 **Figure 1.** Map of locations of each population with the symbol type indicating pollinator
3 abundance measures sampled (circle=both observations and pollinations, square=observations,
4 triangle=pollinations, diamond=none), the symbol fill indicating if the mating system was
5 measured (black=measured, white=not measured), and symbol size indicating approximate
6 population size. See Supplemental Table 1 for sample sizes for each type of measurement and
7 additional population information.

8
9 **Figure 2.** (A) Visitation rate by bird type, (B) seed set by pollination treatment type, (C) pollen
10 limitation for fruit set (black bars) and seed set (white bars), and (D) early inbreeding depression
11 for fruit set (black bars) and seed set (white bars) for each population:year.

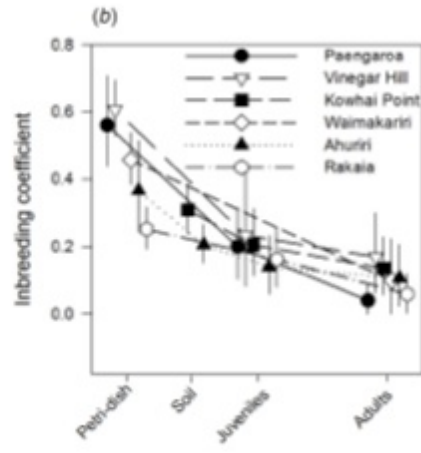
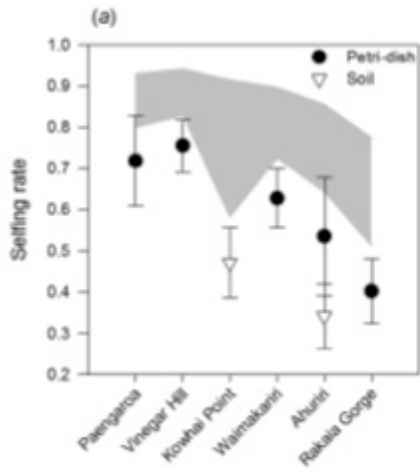
12
13 **Figure 3.** (A) Selfing rates from petri-dish germinated (circles) and soil-germinated (triangles)
14 seeds and back-calculated zygotic selfing rates (grey area; see text for calculation). (B)
15 Inbreeding coefficient with 95% confidence intervals by stage.



1



1



1

2

1 **Table 1.** ANOVA results (χ^2 values) from pollination treatments from 11 population:year
 2 combinations.

	Distribution	Population: Year (d.f.=10)	Treatment (1)	Interaction (10)
<i>Pollen limitation (natural versus outcross hand-pollinated)</i>				
Fruits/flower	Binomial	330.83***	164.28***	39.87***
Seeds/ flower	Negative binomial	28.51**	42.21***	8.77
<i>Early Inbreeding depression (self- versus outcross hand-pollinated)</i>				
Fruits/ flower	Binomial	265.83***	90.89***	47.41***
Seeds/ flower	Negative binomial	65.26***	48.30***	14.25

3 *P<0.05; **P<0.01; ***P<0.0001.

4 **Table 2.** Population genetic parameters (s.e.) of adult plants (without null allele loci), MLTR mating system parameters (s.e.) (without null allele
5 loci) for seeds (petri-dish germinated if not otherwise specified), and post-dispersal inbreeding depression for each population calculated from
6 COLONY results.

Population genetics				MLTR						
Population	H _o	H _e	F _{IS}	N _{fam}	N _{prog}	S _m	b=S _s -S _m	r _{pm}	r _s	δ _{post}
Paengaroa	0.800 (0.065)	0.830 (0.05)	0.038 (0.038)	8	73	0.673 (0.099)	0.057 (0.024)	-0.022 (0.301)	0.897 (0.064)	0.967
Vinegar Hill	0.715 (0.045)	0.824 (0.043)	0.132 (0.032)	21	202	0.766 (0.060)	0.050 (0.018)	0.164 (0.108)	0.836 (0.079)	0.867
Kowhai Point (soil- germinated)	0.727 (0.067)	0.819 (0.037)	0.108 (0.084)	23	144	0.495 (0.063)	0.04 (0.025)	0.106 (0.140)	0.867 (0.080)	0.646
Waimakariri	0.738 (0.029)	0.820 (0.002)	0.100 (0.036)	20	200	0.628 (0.066)	0.088 (0.027)	0.286 (0.148)	0.708 (0.120)	0.868
Ahuriri	0.785 (0.059)	0.832 (0.032)	0.052 (0.077)							

Petri-dish germinated				18	59	0.431 (0.172)	0.168 (0.055)	0.188 (0.226)	0.525 (0.213)	0.792
Soil- germinated				26	169	0.346 (0.064)	0.107 (0.018)	0.054 (0.035)	0.654 (0.108)	0.539
Rakaia Gorge	0.828 (0.026)	0.861 (0.019)	0.037 (0.04)	18	176	0.335 (0.062)	-0.007 (0.019)	0.082 (0.022)	0.925 (0.055)	0.815
Average	0.766 (0.020)	0.831 (0.013)	0.078 (0.021)							

7 H_o = observed heterozygosity; H_e = expected heterozygosity; F_{IS} = Wright's Inbreeding Coefficient; N_{fam} = number of families; N_{prog} = number of
8 progeny; s_m = multilocus selfing rate; s_s = single locus selfing rate; b = biparental inbreeding; r_{pm} = multilocus correlation of paternity; r_s =
9 correlation of selfing among loci; δ_{post} = inbreeding depression from seed or seedling to adult stage calculated per Ritland (1990).