

1 Comparisons among populations and individuals to evaluate pollen–pistil interaction
2 as a mechanism of reproductive interference in *Taraxacum*

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21

1 **Abstract** Reproductive interference (RI), an interspecific mating interaction that
2 reduces the fitness of at least one of the species involved, can lead to exclusive
3 distributions in closely related species. A hypothesis previously proposed is that RI in
4 plants may occur by ovule usurpation, in which pistils lack interspecific
5 incompatibility and mistakenly accept heterospecific pollen, thereby losing an
6 opportunity for conspecific pollen fertilization. However, few comparative studies
7 have evaluated the consistency of the inferred mechanism within and among
8 individuals and populations. We conducted hand-pollination experiments in six
9 populations of three native *Taraxacum* species that suffered from different levels of RI
10 from an alien congener, *T. officinale*, and compared pollen–pistil interactions among
11 populations. We also investigated the interactions for eight individual *T. japonicum*
12 plants whose response to heterospecific pollen deposition had been previously
13 measured. Our results revealed that pollen tubes often penetrated native ovaries
14 following heterospecific pollination in populations suffering from strong RI, whereas
15 they seldom did in populations suffering from marginal RI. However, the relative
16 frequency of the pollen tube penetration was not significantly related to the strength of
17 alien RI. Not all pistils on an individual plant showed the same pollen receptivity
18 following heterospecific pollination; rather, some accepted and some refused the pollen
19 tubes. The relationship between pollen tube penetration following heterospecific
20 pollination and the strength of the alien RI was also not significant among individuals.
21 Our present results generally support the ovule usurpation hypothesis, but suggest that

1 other factors, such as competition for pollinator services, variation in the effects of
2 heterospecific pollen donors, and condition of the native inflorescences, might also
3 affect the observed RI strength.

4

5 Keywords. Heterospecific pollen transfer, Pollen–pistil interaction, Reproductive
6 interference, *Taraxacum*, *Taraxacum officinale*

7

1 **Introduction**

2 Reproductive interference (RI) is a type of negative interaction between species that
3 can be of ecological and evolutionary importance in shaping ecological communities
4 (Kyogoku 2015). RI has been defined as any interspecific sexual interaction that
5 adversely affects the fitness of at least one of the species involved (Gröning and
6 Hochkirch 2008), usually by reducing the number of offspring, the fitness of the
7 parents, and/or the fitness of the offspring, through interspecific copulation in animals
8 or pollination in plants. RI has a frequency dependent effect on population dynamics: if
9 one of a pair of species interferes with the reproduction of the other species, the
10 number of viable and fertile offspring produced by the latter species is decreased and
11 the decrease is larger when members of the interfering species are more abundant
12 (Takakura et al. 2010). As a result, in the next generation, the latter species will suffer
13 more from RI because the relative frequency of the interfering species has been
14 increased. Owing to this positive feedback effect of RI, the interfering species will
15 displace the latter species within a relatively short period of time (Takakura et al. 2010).
16 RI is an important concept in ecology and population dynamics because it has the
17 potential to explain competitive exclusion, habitat segregation, and character
18 displacement among closely related plant species (e.g., Burgess and Husband 2006;
19 Eaton et al. 2012; Harder et al. 1992; Nishida et al. 2020; Takahashi et al. 2016;
20 Takakura and Fujii 2010; Takakura et al. 2010).

21 In plants, two major consequences of RI associated with heterospecific pollen

1 deposition have been recognized: reduction of seed set and the production of hybrid
2 offspring (e.g., Burgess and Husband 2006; Matsumoto et al. 2010). However, few
3 studies have investigated the process between heterospecific pollen deposition and the
4 seed set reduction or hybrid offspring production. According to Morales and Traveset
5 (2008), four mechanisms might explain how heterospecific pollen deposition causes
6 adverse effects: Stigma clogging (Galen and Gregory 1989), which is the physical
7 obstruction or saturation of stigmatic surfaces by heterospecific pollen; stigma closure
8 (Waser and Fugate 1986), which refers to the loss of pollen receptivity by the stigma
9 following heterospecific pollination; pollen allelopathy (Sukhada and Jayachandra
10 1980), the chemical inhibition of conspecific pollen germination or pollen tube growth
11 by heterospecific pollen; and, finally, ovule usurpation (Harder et al. 1992), which may
12 occur if the prior arrival of heterospecific pollen tubes at the ovules prevent the
13 later-arriving conspecific pollen tubes from penetrating the ovary. Studies have mainly
14 investigated these effects in a single pair of plant species in a single population of each
15 species at a time; the consistency of these effects among multiple taxa and multiple
16 populations has not been evaluated. In this study, we focused on ovule usurpation and
17 evaluated its association with RI in multiple species and populations of genus
18 *Taraxacum*.

19 RI from an alien species to a native species has been intensively studied in
20 *Taraxacum*, and some studies have shown that the strength of RI from an alien species
21 varies among native species (Table 1). For example, a native Japanese species *T.*

1 *japonicum* suffers greatly from RI by the alien species *T. officinale* in Kansai region
2 (Matsumoto et al. 2010; Takakura et al. 2009), whereas in the Tokai region, *T.*
3 *longeappendiculatum* is mainly not susceptible to RI from *T. officinale* (Nishida et al.
4 2012). Moreover, different populations of a native *Taraxacum* species differ in the
5 strength of the RI suffered from the alien species (Nishida et al. 2017). These
6 differences can be reflected in the population dynamics: native populations suffering
7 from strong RI by *T. officinale* have been reported to be largely displaced by the alien
8 species, whereas other native populations not suffering from RI or suffering from only
9 marginal RI may coexist with or even be dominant over *T. officinale* (Nishida et al.
10 2017).

11 In spite of such intensive studies on RI phenomena in *Taraxacum*, the mechanisms
12 of the RI have scarcely been investigated, especially those acting after heterospecific
13 pollen deposition (see Kandori et al. 2009 and Takemori et al. 2019 for mechanisms
14 acting before heterospecific pollen deposition). In this regard, ovule usurpation by
15 alien pollen has been demonstrated in only one population of *T. japonicum* (Nishida et
16 al. 2014), despite the wide reported variation in the strength of RI from *T. officinale* to
17 native *Taraxacum* populations (Nishida et al. 2017).

18 Therefore, to investigate pollen–pistil interactions and evaluate ovule usurpation as
19 a mechanism of RI, we conducted a comparative study of multiple populations of
20 native *Taraxacum* species. First, in a series of hand-pollination experiments, we
21 compared pollen tube growth in native flowers following conspecific pollination,

1 heterospecific pollination with *T. officinale* (alien) pollen, and mixed pollination with
2 native followed by alien pollen. If ovule usurpation functions as the primary
3 mechanism of RI, in populations more susceptible to RI by *T. officinale*, pollen tubes
4 should more frequently penetrate the ovaries of native species following heterospecific
5 pollen deposition in those populations than in less susceptible populations. If the
6 mechanism of RI involves an interaction between conspecific and heterospecific pollen
7 tubes, such as pollen allelopathy (Sukhada and Jayachandra 1980), then discrepant
8 phenomena might be observed, e.g., pollen tubes penetrate ovules following
9 conspecific pollination whereas they scarcely do so following mixed pollination.

10 In addition, we compared pollen–pistil interactions among native *T. japonicum*
11 individuals that differed in the degree to which they suffered from RI by the alien.
12 With this comparison, we aimed to clarify whether pistil receptivity to heterospecific
13 pollination was consistent among florets within individuals, and whether the
14 receptivity of the florets was associated with the strength of RI suffered by individuals
15 from the alien. If the receptivity is consistent within an individual, pistils of different
16 florets on an individual should show the same receptivity following heterospecific
17 pollination, whereas if it is not consistent, some pistils should accept and some should
18 refuse pollen tubes. Further, because the susceptibility to alien RI of each of the
19 examined individuals had been measured previously (Takemori et al. 2019), we were
20 able to evaluate the association between the receptivity and susceptibility to RI from
21 the alien species at the individual level.

1 In performing these experiments, our aim was to answer the following two
2 questions: Is ovule usurpation a universal mechanism of RI across these native
3 *Taraxacum* species? and Is individual-level variation in the intensity of RI caused by
4 the variation in pollen recognition ability?

5

6 **Materials and methods**

7

8 **Study species**

9

10 *Taraxacum japonicum*, *T. longeappendiculatum*, and *T. platycarpum* subsp. *hondoense*
11 are distributed almost entirely allopatrically in lowland regions of western to
12 east-central Japan (Fig. 1). These native dandelion species can be distinguished from
13 one another by certain quantitative characters, especially, the size and shape of the
14 bracts composing the involucre: The involucre of *T. japonicum* is 12–15 mm long; its
15 outer bracts cover two- to three-fifths of the inner bracts, and they have short (up to 1.5
16 mm long) corniculate appendages at their tips (Morita 2017). The involucre of *T.*
17 *longeappendiculatum* is 15–23 mm long; its outer bracts cover more than two-thirds of
18 the inner bracts, and they have large (2–6 mm long) corniculate appendages at their
19 tips (Morita 2017). The involucre of *T. platycarpum* subsp. *hondoense* is 15–23 mm
20 long; its outer bracts cover from half to two-thirds of the inner bracts, and they either
21 have no appendages or only short (up to 1.5 mm long) corniculate appendages at their

1 tips (Morita 2017). All studied populations of these native species are diploid ($2n = 16$)
2 (Hashimoto, unpublished), and diploid dandelions are known to reproduce sexually
3 and to be self-incompatible (Richard 1970).

4 The alien congener, *T. officinale*, is native to Europe but is now distributed
5 throughout Japan (Hoya 2010; Ogawa and Mototani 1985). This species is polyploid
6 and reproduces agamospermously (Morita 1980), but its pollen is sometimes carried by
7 pollinators to native dandelion flowers, which then produces hybrid offspring
8 (Shibaïke et al. 2002). Flowers of hybrid offspring may be morphologically
9 indistinguishable from genuine *T. officinale* flowers (Hoya 2010; Shibaïke 2005). In
10 fact, many of the putative alien dandelions in western and central Japan are actually
11 hybrids (Shibaïke et al. 2002), because, like *T. officinale*, the hybrids reproduce
12 agamospermously (Hoya 2010). For this reason, and also because all previous studies
13 on RI in *Taraxacum* have treated the hybrids as aliens (e.g., Matsumoto et al. 2010;
14 Nishida et al. 2012, 2014, 2017; Takakura et al. 2009), we treated hybrids as aliens in
15 this study. Native and alien *Taraxacum* species share pollinators: their flowers are
16 visited by a variety of small insects as well as by efficient honeybees, *Andrena* spp.,
17 and species of Syrphidae (Kandori et al. 2009; Takemori et al. 2019).

18

19 **Study populations**

20

21 To investigate pollen–pistil interactions in native populations suffering from alien RI

1 with different strengths, we studied six different populations (Fig. 1): three populations
2 of *T. japonicum*, one each at Okayama City (JOO; 34°41'15"N, 133°55'12"E) and
3 Takahashi City (JOT; 34°47'51"N, 133°39'13"E) in Okayama Prefecture, and one at
4 Naruto City (JTN; 34°12'18"N, 134°36'17"E) in Tokushima Prefecture; one population
5 of *T. longependiculatum* at Nagoya City (LAN; 35°9'14"N, 136°57'50"E) in Aichi
6 Prefecture; and two populations of *T. platycarpum*, one at Nagano City (PNN;
7 36°33'44"N, 138°10'19"E) and one at Omi Village (PNO; 36°26'42"N, 138°02'12"E)
8 in Nagano Prefecture. Besides these populations, in our comparison among populations,
9 we used data obtained by Nishida et al. (2014) from a population of *T. japonicum* at
10 Otsu City (JSO; 35°8'11"N, 135°52'47"E) in Shiga Prefecture, although we did not
11 investigate pollen–pistil interactions in this population in this study. In each of these
12 different regions, the strength of the RI from the alien suffered by the native plants of
13 each population is roughly associated with the current stage of displacement of the
14 native populations by the alien species: RI from the alien to the native species is strong
15 where alien individuals outnumber the native individuals, and relatively weak where it
16 does not (Nishida et al. 2017).

17

18 **Comparison of pollen–pistil interactions among the populations**

19

20 To evaluate whether ovule usurpation functions as a mechanism of RI in the native
21 *Taraxacum* populations, we conducted a series of hand-pollination experiments and

1 observed pollen behavior in pistils of the native species. In particular, we examined
2 whether, following heterospecific pollination, pollen tubes frequently penetrated native
3 ovaries in populations suffering from strong RI by the alien species.

4 The hand-pollination experiments were conducted in spring (April to June) in 2009
5 (LAN), 2010 (LAN), 2011 (JOO, JOT, JTN, LAN), 2012 (PNN, PNO), and 2014
6 (JOO), basically according to the methodology of Nishida et al. (2014). All *T.*
7 *japonicum* plants used for the experiments, except some plants of JOO, as well as some
8 *T. longeappendiculatum* plants (LAN), were transplanted to Nagoya University, where
9 they were grown in pots. Experiments with plants of LAN in 2009, of JOO in 2014,
10 and of both populations of *T. platycarpum* (PNO and PNN) were conducted in the field
11 using plants growing wild. We arbitrarily selected up to 12 individual flowering plants
12 from each population (the number of individuals depended on the flowering condition
13 of the population). Then, we arbitrarily selected at most three inflorescences on each
14 plant that were at the similar stage of anthesis, and assigned them to the following
15 three treatments (one inflorescence per each treatment): conspecific pollination
16 (pollination of the floret by a different individual of the same native species),
17 heterospecific pollination (pollination of the floret by an alien individual only), and
18 mixed pollination (sequential pollination of the floret by a different conspecific
19 individual and by an alien individual). In all treatments, we collected a few florets from
20 each pollen donor with tweezers, and then applied their pollen grains directly onto the
21 stigmas of the recipient florets. We did not count the number of pollen grains that

1 adhered to each stigma in the experiments because, in *Taraxacum*, pollen abundance
2 on the stigma does not correlate with the number of pollen tubes that penetrate the
3 pistil (Nishida et al. 2014). For conspecific pollination, we used pollen from different
4 individuals of the same population. For heterospecific pollination, we arbitrarily
5 selected *T. officinale* individuals growing around the experiment site, whose genomic
6 composition was not examined. For mixed pollination, we first pollinated the recipient
7 florets with conspecific pollen grains and then immediately pollinated the same florets
8 with alien pollen grains. For all pollination treatments, we used one donor individual
9 for each recipient individual, and we used three to five individual donors in total for
10 each population. Before each hand pollination, we confirmed that there were no pollen
11 grains on the adaxial side of the stigma. After the hand pollination, we confirmed
12 presence of pollen grains on the stigma, then covered the recipient inflorescence with a
13 polyester bag to prevent additional pollination. *Taraxacum* florets usually have
14 self-pollen (the recipient's own pollen) on the back side of their stigmas when they
15 flower, and these pollen grains may attach to the adaxial side of the stigma when the
16 inflorescences close in the evening. We could not prevent self-pollen from attaching to
17 the adaxial side of the stigma after we conducted the pollination treatments, because
18 removing the stamens before flowering would damage the florets severely. In the
19 heterospecific and mixed pollinations, therefore, the possibility that pollen tubes in the
20 pistils were actually produced by self-pollen through a mentor effect, as reported by
21 Morita et al. (1990), could not be ruled out.

1 At most 22 (usually about eight) recipient florets were collected from each
2 pollinated inflorescence with tweezers about 72 h after the pollination and fixed in a
3 formalin-acetic acid-alcohol (FAA) solution. 72 hours is usually a sufficient time
4 interval for pollen tubes reaching to ovules in *Taraxacum*, whereas fixation after longer
5 hours than this interval may increase a risk of breaking the pistil samples into two parts
6 (style and ovary parts) (Keisuke Hashimoto, Sachiko Nishida, and Akane Yamamoto,
7 personal observations). The pollen tubes were observed basically as described by
8 Nishida et al. (2014). The collected florets were removed from the FAA solution and
9 soaked in 90% ethanol for 20 min and in 70% ethanol for 20 min, followed by soaking
10 in 1 N NaOH solution overnight at room temperature. The next morning, the florets
11 were washed in distilled water and stained with 0.1% aniline blue dye dissolved in 0.1
12 N K₃PO₄ (pH 12.4) for at least 1 h. Then, after removal of the pappi and petals with
13 tweezers, the florets were mounted on slides in 10% glycerol and observed under a
14 fluorescence microscope (IX51; Olympus, Tokyo, Japan). Because pollen tubes in all
15 parts of the pistil are not stained by aniline blue solution (Nishida et al. 2014), if we
16 observed stained pollen tubes in the ovary of a native, we assumed that they had
17 penetrated the entire pistil, whether or not we were able to detect stained tubes in the
18 style.

19 We analyzed the presence or absence of pollen tubes in the ovary between
20 conspecific and heterospecific pollinations, and between conspecific and mixed
21 pollinations. We employed a generalized linear mixed models (GLMM, Wolfinger and

1 O'Connell 1993) with a binomial error structure and a logit link function for each of
2 the analyses. The response variable was the presence or absence of pollen tubes in the
3 ovary, and the explanatory variable was the treatment (conspecific pollination or
4 heterospecific pollination, conspecific pollination or mixed pollination). As a random
5 effect, for all populations except JOO and LAN, we incorporated the individual
6 recipient plant. For JOO and LAN, we used the individual recipient plant nested within
7 year as a random effect, because for these populations we used data from different
8 years. The analyses were carried out with R 3.0.3 software (R Core Team 2014). We
9 considered the effect of the treatment on the presence or absence of pollen tubes to be
10 significant if the *P* value was less than 0.05.

11 We used the results of these statistical analyses to interpret these data, but to present
12 the data in a more intuitive format, we depicted the percentages of florets with pollen
13 tube penetration of the ovary in each treatment and population as bar graphs, and we
14 also plotted the relative frequency of alien pollen tube penetration and the strength of
15 alien RI in each population on a scatter diagram. To construct the bar charts, we
16 calculated the proportion of florets in which pollen tubes were observed in the ovary in
17 each treatment in each population. To construct the scatter diagram, we calculated the
18 relative penetration frequency (RPF) of alien pollen tubes, which we defined as the
19 ratio of the number of florets with pollen tubes in the ovary following heterospecific
20 pollination (HF) to the number following conspecific pollination (CF): $RPF = HF/CF$.
21 Values for the strength of alien RI in each population were the estimated coefficients

1 for the effect of the relative abundance of alien individuals on seed set by native plants
2 in the field, which were obtained from the literature (Nishida et al. 2017; Takakura et al.
3 2009). Because no data on the strength of alien RI in the field were available for JTN,
4 that population is not shown on the scatter diagram. Instead, we plotted data for a *T.*
5 *japonicum* population in Otsu, Shiga Prefecture (JSO), obtained by Nishida et al.
6 (2014) on the scatter diagram, and we also depicted data from that population on the
7 bar graph. We used Kendall's rank correlation to evaluate the relationship between
8 RPF and RI. We considered the relationship to be significant if the *P* value was less
9 than 0.05.

10

11 **Comparison of pollen–pistil interactions among individual plants**

12

13 We conducted a detailed study of the *T. japonicum* population at JOO in which we
14 observed pollen tube behavior and compared the results with the strength of alien RI in
15 individual plants. Pollen tube behavior was observed as described in the previous
16 section. We examined pollen tube behavior in the same individuals from which
17 Takemori et al. (2019) had obtained seed set data following conspecific pollination
18 (pollination with pollen of another *T. japonicum* individual) and mixed pollination
19 (pollination with pollen of both another *T. japonicum* individual and an alien individual).
20 Both the pollen tube data and seed set data were collected in 2014. Although
21 hand-pollination treatments were performed in more than 20 individuals, we report

1 results for only eight individuals here; data from individuals in which pollen–pistil
2 interactions could not be observed, and from those in which seed set was unusually low
3 (less than 50%) following conspecific pollination, were excluded.

4 To construct a scattered diagram, we calculated the relative penetration frequency of
5 the alien pollen (RPF) from our results and compared them to the strength of alien RI
6 determined by analyzing the data reported by Takemori et al. (2019). RPF was
7 obtained by the same methodology used for the comparison among populations (see
8 the previous section for details). The strength of alien RI in each individual was the
9 estimated effect size of the mixed pollination, which indicated the extent of the
10 reduction in the seed set following the mixed pollination compared to following the
11 conspecific pollination. We used this estimated effect size because it was impossible to
12 obtain estimated coefficient of relative abundance of the alien individuals for each of
13 the native individuals, since they grew in wild field and number of surrounding alien
14 individuals could not be changed. To obtain the values, we analyzed the data by using a
15 GLM (Generalized Linear Model; Nelder and Wedderburn 1972) with a binomial error
16 structure and a logit link function. The response variable in the analysis was
17 development of the ovules, and the explanatory variable was the treatment (pollination
18 with conspecific pollen only or mixed pollen). We carried out the analysis with R 3.0.3
19 software (R Core Team 2014). We used Kendall’s rank correlation to evaluate the
20 relationship between RPF and alien RI strength, and considered the relationship to be
21 significant if the *P* value was less than 0.05.

1

2 **Results**

3

4 **Comparison of pollen–pistil interactions among populations**

5

6 No occurrence of unusual pollen tube growth behavior, such as irregular callose
7 deposition or elongation in the reverse direction, as seen in *Nicotiana* (Kuboyama et al.
8 1994), was observed following conspecific pollination, heterospecific pollination, or
9 mixed pollination. Neither any greatly discrepant results were visually observed
10 between following the conspecific and mixed pollination, which may have suggested
11 some negative effect of the heterospecific pollen on the conspecific pollen. The
12 proportion of florets with pollen tube penetration differed among treatments, species,
13 and populations (Fig. 2, Table 2). The proportions varied greatly among populations
14 following heterospecific pollination, whereas following conspecific pollination and
15 mixed pollination, the proportion was more or less similar among populations within
16 each species. The proportion in *T. longeappendiculatum* at LAN differed from that
17 reported previously (Nishida et al. 2014): compared to the results of Nishida et al.
18 (2014), the proportion in our results was higher following heterospecific pollination
19 but lower following conspecific or mixed pollination. The proportion was much lower
20 in *T. platycarpum* than in the other species following all pollination treatments,
21 presumably because pollen tube growth was slower under the cooler prevailing

1 temperatures in the *T. platycarpum* distribution range.

2 The proportion of florets with pollen tube penetration was significantly lower
3 following heterospecific pollination, compared to that following conspecific
4 pollination, at JOO, JOT, JTN, LAN, and PNN, populations in which, according to
5 Nishida et al. (2017), RI from the alien to the native was marginal or insignificant
6 (Table 2). The magnitude of the difference was small, however, at JOO compared to
7 the differences in the other populations. The proportion was lower, but not significantly,
8 following heterospecific pollination at PNO, as well as at JSO (reported by Nishida et
9 al. 2014), where, according to Nishida et al. (2017), RI from the alien to the native
10 species was significant and severe (Table 2).

11 Figure 3 showed that there was a roughly positive relationship between the strength
12 of alien RI and RPF, but the relationship was not significant (Kendall's rank correlation,
13 $\tau = -0.414$, $P = 0.251$).

14

15 **Comparison of pollen–pistil interactions among the individuals**

16

17 Following both conspecific and heterospecific pollination (CF and HF columns in
18 Table 3), the proportion of florets in which pollen tubes penetrated the ovary varied
19 greatly among the eight individual *T. japonicum* plants analyzed. In these individuals,
20 the strength of alien RI was not significantly related to RPF (Fig. 4; Kendall's rank
21 correlation, $\tau = 0.071$, $P = 0.905$). Individuals #3, #5, and #6 had relatively low RPF

1 values, which indicates that pollen tubes did not often penetrate the ovaries following
2 heterospecific pollination, and significantly negative effects of alien RI were not
3 reported in these individuals (Table 3, Fig. 4). In contrast, individuals #1, #7, and #8
4 had relatively high RPF values, and the effects of alien RI were significant and severe
5 in these individuals (Table 3, Fig. 4). In individual #2, though RPF was high, a
6 negative effect of alien RI was not recognized (Table 3, Fig. 4). In individual #4, no
7 pollen tubes penetrated the ovaries following heterospecific pollination, but the
8 negative effect of alien RI was the strongest in this individual among the eight
9 individuals analyzed (Fig. 4): in fact, pollen tubes penetrated the ovary of only a
10 quarter of the pistils of #4, even following conspecific pollination (Table 3).

11

12 **Discussion**

13 Our results indicate that ovule usurpation following alien pollen deposition may
14 cause a reduction of seed set in native *Taraxacum* species, but it cannot fully explain
15 the strength of RI from the alien to the native. Deposition of alien pollen did not appear
16 to damage the native pistils, nor did the alien pollen interact negatively with the native
17 pollen tubes, as far as based on visual judgement. The proportion of pollen tubes
18 penetrating the native ovaries following heterospecific pollination was significantly
19 lower than that following conspecific pollination in populations that were
20 unsusceptible to alien RI, but not in those populations vulnerable to alien RI (Table 2,
21 Fig. 3). These observed phenomena suggest that the alien pollen does not damage

1 native pistils, nor does it alter the interaction of native pistils with conspecific pollen;
2 rather it usurps the native ovules, or facilitates usurpation of the ovules by self-pollen.
3 Our study could not discriminate whether the penetrating pollen tubes were produced
4 by alien pollen or self-pollen (recipient pollen), because to avoid damaging the
5 recipients, we did not emasculate the florets. Therefore, it is possible that it was not the
6 alien pollen tubes but the recipient's self-pollen that usurped the ovules through a
7 mentor effect (Morita et al. 1990). Usurpation of the ovules by either alien pollen or
8 self-pollen should most often result in seed development failure, but rarely in the
9 production of hybrid or self-fertilized seeds. Any of these outcomes might negatively
10 affect population dynamics of the native.

11 Nishida et al. (2014) inferred that a lack of interspecific pollen–pistil incompatibility
12 in native *Taraxacum* pistils and usurpation of the native ovules by alien pollen might
13 be the direct cause of RI from the alien to the native. Our results partly support this
14 inference, but not convincingly, considering that a significant association between a
15 lack of interspecific incompatibility and the strength of the RI was not found (Fig. 3).
16 A possible reason for our failure to find a significant association between them is that
17 factors other than the pollen–pistil interaction determined the strength of the alien RI.
18 Two methods have been used to measure the strength of RI: 1) analysis of the
19 association between the relative abundance of the counterpart species (candidate
20 species for exerting RI on the focal species) and reproductive success (i.e., seed set) of
21 the focal species; and 2) measurement of the relative reduction of reproductive success

1 between mixed hand-pollination (hand-pollination with pollen of the focal species and
2 that of the counterpart species) and conspecific hand-pollination (e.g., Matsumoto et al.
3 2010; Takakura et al. 2009). In our comparison among populations in this study, we
4 used the first method to measure RI strength, because reproductive success after hand
5 pollination might differ from that following natural pollination (e.g., Chautá-Mellizo et
6 al. 2012). RI strength measured by the first method, however, might reflect not only RI
7 occurring after heterospecific pollen deposition but also that caused by other factors,
8 including ones acting before pollen deposition. For example, population JOO appeared
9 to suffer relatively weak RI from the alien (Fig. 3), even though a high percentage of
10 florets in this population showed pollen tube penetration following heterospecific
11 pollination. At JOO, pollinators have been reported to visit native flowers more
12 frequently than alien flowers (Takemori et al. 2019). This pollinator behavior might
13 have resulted in more conspecific pollen than alien pollen being deposited on the
14 pistils of the native flowers, thereby mitigating the adverse effects of heterospecific
15 pollen deposition and causing the alien RI strength to be less severe compared to what
16 it would have been had pollinators displayed no preference for native flowers. The
17 wide variation in the RI strength (measured by the second method) recognized in the
18 comparison among individuals within the JOO population (vertical axis of Fig. 4)
19 might also suggest a possible importance of the other factors ruling the RI strength for
20 the population. Differences in the heterospecific pollen effect on reproductive success
21 of the native species might be also derived from differences of the alien species,

1 condition of the native individuals, and/or extent of the native florets maturity, as
2 mentioned by Kyogoku (2021). Thus, to clarify the association between a lack of the
3 interspecific incompatibility and RI strength, more studies are needed, especially more
4 field studies on effects of the factors other than pollen–pistil interactions, such as
5 competition for pollinator services, on RI strength, and more standardized
6 experimental studies to compare actual effects of the alien pollen on reproductive
7 success of the native species.

8 In addition to the possibility that other factors affect RI strength, the wide variation
9 we recognized in the proportion of the pollen tube penetration might also make it
10 difficult to detect a clear association between the pollen–pistil interaction and the
11 strength of RI. As the long error bars in Fig. 2 show, the proportion of the florets in
12 which a pollen tube penetrated the ovary varied greatly within each population,
13 especially following heterospecific pollination. Moreover, because the proportion at
14 LAN in this study differed from that reported by Nishida et al. (2014), the results
15 obtained might depend on the year, although the general tendency, for the proportion of
16 florets showing pollen tube penetration of the ovary to be smaller following
17 heterospecific pollination than following mixed or conspecific pollination, might not
18 change. More data, from more individuals and from more populations, especially from
19 populations vulnerable to RI from the alien, may help us obtain a clearer image of the
20 association between pollen–pistil interaction and RI strength. It is also true, however,
21 that finding such vulnerable populations in nature is hard (only one of the six

1 populations in our experiment and one from the previous literature were significantly
2 vulnerable), because strong RI might result in the swift displacement of a vulnerable
3 native species by the alien species (Takakura et al. 2012). Research on RI strength in a
4 wide variety of populations is needed to further our understanding of RI mechanisms,
5 and also for conservation of the native species.

6 The results of our comparison among individuals demonstrated that not all pistils on
7 an individual plant showed the same receptivity to alien pollen; rather, some accepted
8 and some refused alien pollen tubes (Table 3). Therefore, recognition of pollen identity
9 by native pistils is not consistent among florets on an individual plant. To explain the
10 observed inconsistency in receptivity on an individual plants, the mechanisms by which
11 the pistil recognizes the pollen must be explored. In *Arabidopsis*, whose reproductive
12 biology has been much studied, only one such mechanism has been identified so far.
13 Fujii et al. (2019) demonstrated that pistils of plants carrying the *SPR11* gene reject
14 pollen of distantly related species. Interestingly, they found a certain amount of
15 variation in the number of pollen tubes accepted by the pistils even in plants carrying
16 this gene (Fujii et al. 2019, their Fig. 5a). Furthermore, the pistils did not reject all
17 pollen; they allowed penetration of a few pollen tubes of even distantly related species.
18 These results indicate that the pollen recognition system conferred by this gene is not an
19 all-or-nothing system; rather, as in our results, some variation remains in pistil
20 receptivity. Further study on detailed mechanisms of interspecific incompatibility in not
21 only such model plants as *Arabidopsis* but also in non-model plants would provide

1 insights into the variation in pistil receptivity we found in *Taraxacum*.

2 The relationship between pollen tube penetration following heterospecific pollination
3 and the strength of alien RI was also not statistically significant among individuals. In
4 particular, the results obtained for individuals #2 and #4 appear to make the correlation
5 weak (Fig. 4).

6 Although among the eight individuals, individual #2 allowed pollen tube penetration
7 following heterospecific pollination relatively more frequently it nevertheless appeared
8 not to suffer from alien RI (Fig. 4). We can offer no plausible explanation for this result.
9 The combination of high acceptance of heterospecific pollen and high seed set might
10 imply hybridization, but we found no hybrid offspring among the seedlings obtained
11 from this individual following mixed pollination (based on data reported by Takemori et
12 al. 2019). It is possible that the result for individual #2 simply represents a sampling
13 error, or an unidentified factor may have enhanced the penetration rate of heterospecific
14 pollen. Further study is needed to explore the latter possibility.

15 Although individual #4 did not allow any pollen tubes to penetrate the ovary
16 following heterospecific pollination, it appeared to suffer from severe alien RI. The high
17 RI strength must reflect its relatively low seed set following mixed pollination. In this
18 individual, the frequency with which conspecific pollen tubes penetrated the ovary was
19 also low (25% of the ovaries examined). Taken together, these results for this individual
20 suggest that its recognition range for conspecific pollen may have been narrow; thus, its
21 seed set was reduced not only because heterospecific pollen was correctly rejected but

1 also because conspecific pollen was wrongly rejected when its flowers received mixed
2 pollen.

3 In Fig. 4, the values on the vertical axis drop abruptly when the RPF reaches around
4 0.5 (individuals #1, #7, and #8). This plunge likely indicates that the negative effect of
5 RI can overwhelm the reproductive success of individuals when the frequency at which
6 inappropriate pollen (heterospecific pollen or self-pollen) is accepted exceeds a certain
7 threshold. An entirely withered capitulate inflorescence, apparently due to unsuccessful
8 pollination among many florets in the inflorescence, is sometimes observed on
9 *Taraxacum* (Nishida, personal observation). Cumulative failure of fertilization after
10 usurpation of the pistils by inappropriate pollen may cause entirely withered
11 inflorescences and lead to excessive deterioration of reproductive success in the genus.
12 The evidence provided by our results is insufficient for plausible or constructive
13 discussion of this issue. Although it is a difficult task to examine pollen–pistil
14 interaction and seed set in the same individual in the same season (we obtained a full
15 dataset from only 8 of the 20 individuals examined), further experimental studies are
16 needed to clarify individual differences in the strength of alien RI and the acceptance of
17 alien pollen, and their effects on reproductive success.

18 In conclusion, the results of our study suggest that in *Taraxacum*, RI does not occur
19 only through ovule usurpation but that other factors, such as competition for pollinator
20 services, may also cause RI. Variation in the effects of heterospecific pollen donors and
21 condition of the native inflorescences may also determine the RI strength. In addition,

1 our results revealed wide variation in pollen–pistil interactions not only among
2 populations but also among and within individuals. A promising approach for clarifying
3 the cause of this variation would be to measure the relative contributions to RI strength
4 of factors that act at different reproductive stages, and then to compare them among
5 different populations and among individuals with a standardized methodology.
6 Establishing the fundamental mechanism underlying interspecific pollen recognition by
7 pistils would also reveal details of within-individual variation in the response to alien
8 pollen and susceptibility to RI, but such mechanisms have yet to be explored in
9 non-model taxa such as *Taraxacum*.

10

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9 **Conflict of interest** The authors declare that they have no conflict of interests.

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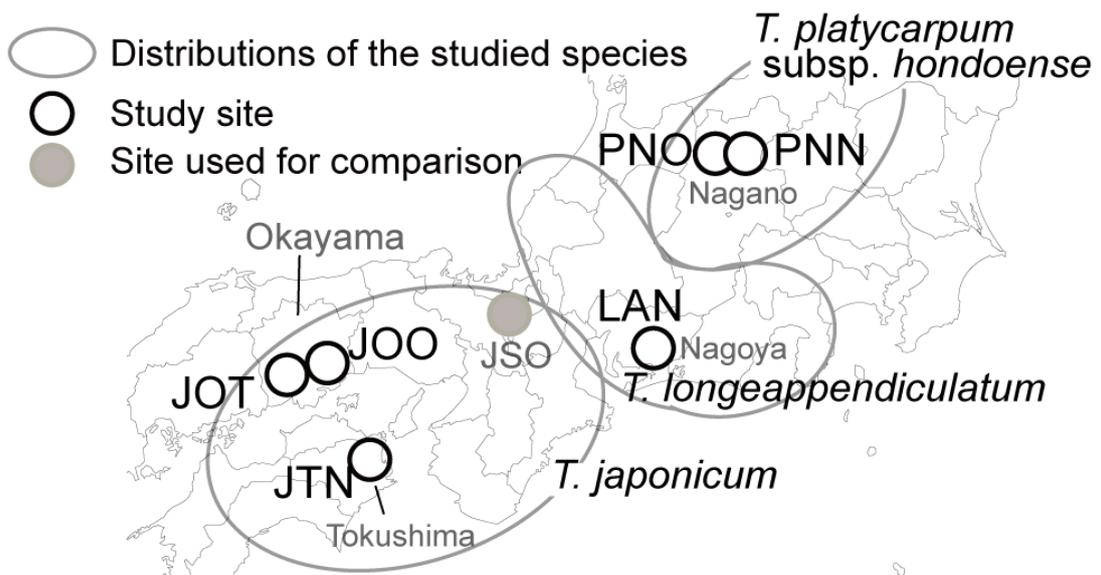
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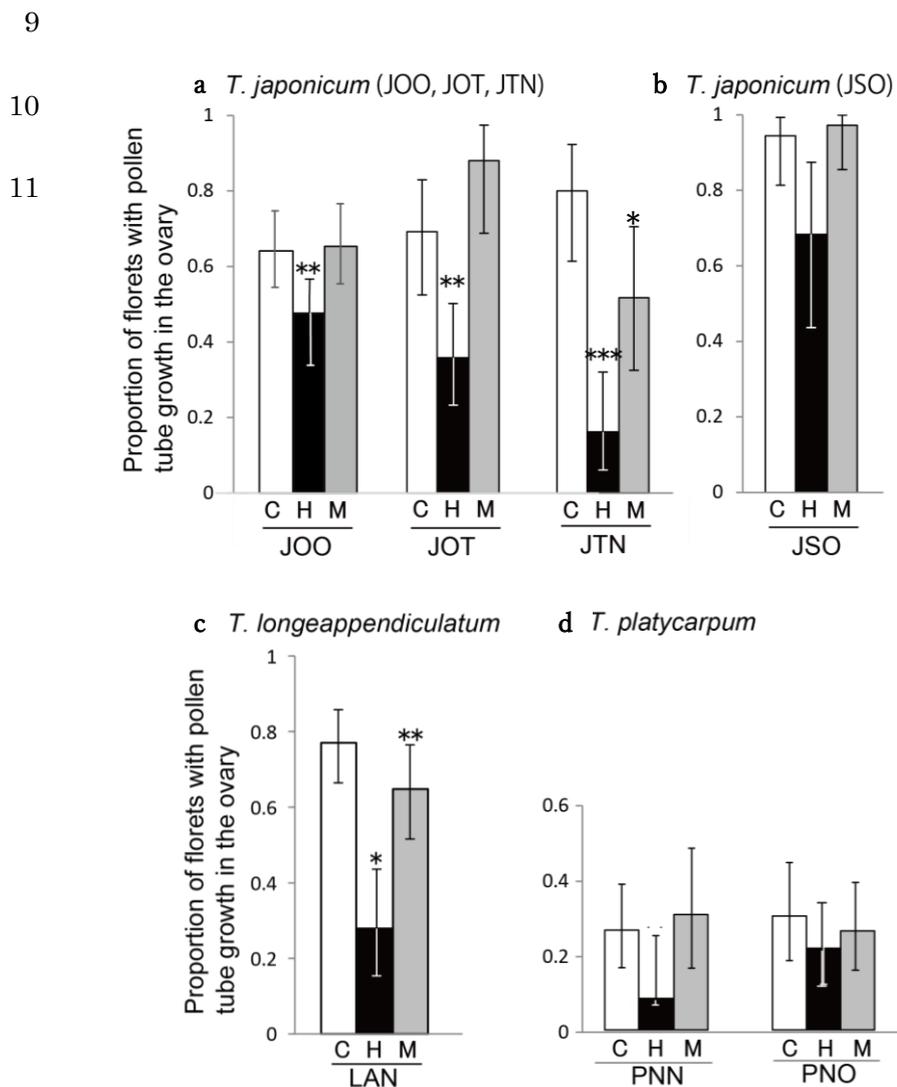
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16

1 **Fig. 1.** Map showing the approximate distributional ranges of the native *Taraxacum*
 2 species studied, and the locations of the sites included in this study and of site JSO,
 3 data from which (reported by Nishida et al. 2014) were used in our comparisons.
 4

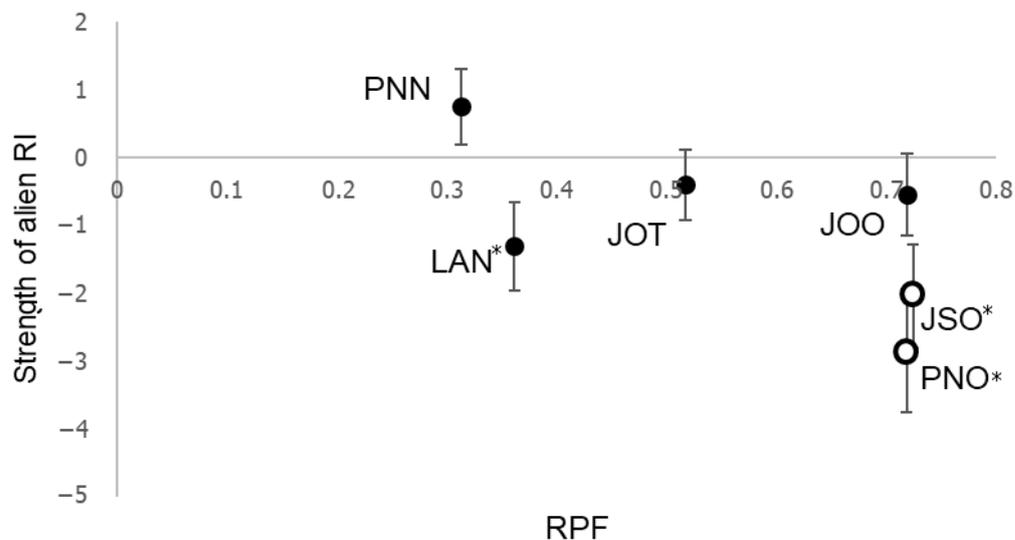


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1 **Fig. 2.** Proportion of florets in a population in which a pollen tube penetrated the ovary
 2 following conspecific (C), heterospecific (H), or mixed (M) pollination: **a)** *Taraxacum*
 3 *japonicum* (JOO, JOT, JTN); **b)** *T. japonicum* (JSO); **c)** *T. longeappendiculatum*
 4 (LAN); and **d)** *T. platycarpum* (PNN, PNO). The bar graph in **b)** is modified from
 5 Nishida et al. (2014). Asterisks indicate significant differences between the results
 6 following the heterospecific or mixed pollination and the results following conspecific
 7 pollination (Wald test with Holm's adjustment; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).
 8 Error bars show 95% confidence intervals.



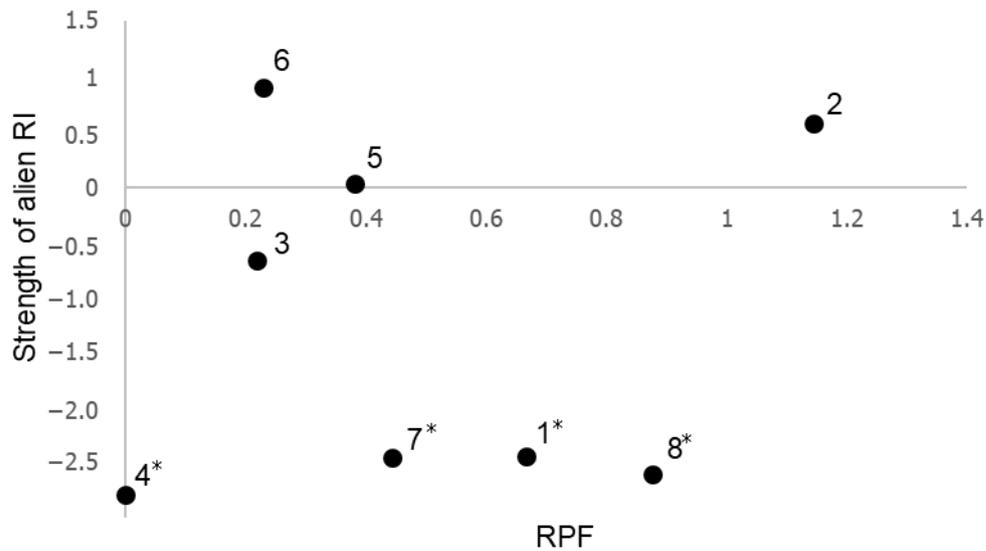
1 **Fig. 3.** Relationship between the relative pollen tube penetration frequency (RPF;
2 frequency of penetration following heterospecific pollination / frequency following
3 conspecific pollination) in ovaries and the strength of alien RI (as indicated by the
4 GLMM coefficient, which is a measure of the effect of relative abundance of the alien
5 on seed set) suffered by native *Taraxacum* populations, namely, *T. japonicum* (JOO,
6 JOT, JSO), *T. longeappendiculatum* (LAN), and *T. platycarpum* (PNN, PNO). Note
7 that the strength of alien RI is larger when the value of the vertical axis is smaller. Data
8 of population JSO are based on previously reported results (Nishida et al. 2014, 2017).
9 Populations for which a significant and negative effect of the alien's relative
10 abundance on seed set was reported by Nishida et al. (2014, 2017) are marked by an
11 asterisk. Closed (open) circles indicate populations that had (did not have) significantly
12 more florets with the ovary penetrated by pollen tubes following heterospecific
13 pollination than following conspecific pollination.



15

16

1 **Fig. 4.** Relationship between the relative pollen tube penetration frequency (RPF;
 2 frequency of penetration following heterospecific pollination / frequency following
 3 conspecific pollination) in ovaries and the strength of alien RI (as indicated by the
 4 GLM coefficient, which is a measure of the effect of mixed pollination on seed set) in
 5 eight native *Taraxacum japonicum* individuals. The numbers by the circles correspond
 6 to the individual plant numbers in Table 3. Individuals for which a significant and
 7 negative effect of relative abundance of the alien on seed set are marked by an asterisk.
 8 Note that the strength of alien RI is larger when the value of the vertical axis is smaller.
 9



10

Table 1. Strength of RI from the alien to the native in *Taraxacum*, as reported in the literature, as indicated by GLMM analysis results for the effect of the relative abundance of alien individuals on seed set of natives individuals, based on data obtained by field surveys.

Species	Site	GLMM results	References
<i>T. japonicum</i>	JOO	-0.546 n.s.	Nishida et al. 2017
<i>T. japonicum</i>	JOT	-0.402 n.s.	Nishida et al. 2017
<i>T. japonicum</i>	JTN	-0.357* [†]	Nishida et al. 2017
<i>T. japonicum</i>	JSO	-2.040**	Takakura et al. 2009
<i>T. longeappendiculatum</i>	LAN	-1.310*	Nishida et al. 2012
<i>T. platycarpum</i>	PNN	0.752 n.s.	Nishida et al. 2017
<i>T. platycarpum</i>	PNO	-2.867**	Nishida et al. 2017

* $P < 0.05$, ** $P < 0.001$, Wald test.

[†]The effect of mixed pollination on seed set compared to conspecific pollination was reanalyzed by assuming that the relative abundance of the alien species was 0.5 and 0, respectively (see text for details).

Table 2. Percentage of florets with pollen tube penetration to the ovary and the GLMM analysis results for the effect of heterospecific pollination on pollen tube penetration, compared with conspecific pollination in *Taraxacum japonicum* (JOO, JOT, JSO, JTN), *T. longeappendiculatum* (LAN), and *T. platycarpum* subsp. *hondoense* (PNN, PNO).

Recipient (female)	Site	No. of florets (individuals) observed			Percentage of florets with pollen tube penetration to the ovary		
		Conspecific	Heterospecific	Mixed	Conspecific	Heterospecific	Mixed
<i>T. japonicum</i>	JOO	85 (11)	116 (11)	77 (11)	61.2%	44.0%	63.6%
<i>T. japonicum</i>	JOT	40 (3)	53 (3)	25 (3)	69.2%	35.8%	88.0%
<i>T. japonicum</i>	JTN	30 (3)	37 (4)	29 (2)	80.0%	16.2%	51.7%
<i>T. japonicum</i>	JSO [†]	36 (5)	19 (5)	36 (5)	94.4%	68.4%	97.2%
<i>T. longeappendiculatum</i>	LAN	79 (10)	43 (6)	63 (8)	77.2%	27.9%	65.1%
<i>T. platycarpum</i>	PNN	68 (7)	48 (5)	36 (4)	26.5%	8.3%	30.6%
<i>T. platycarpum</i>	PNO	53 (5)	60 (6)	61 (7)	30.2%	21.7%	26.2%

[†]Data from Nishida et al. (2014).

*Wald test.

Table 2 (continued)

GLMM results		
Coefficient \pm SE	Z value	<i>P</i> *
-0.96 \pm 0.33	-2.95	0.003
-1.62 \pm 0.51	-3.19	0.001
-4.31 \pm 1.12	-3.85	0.000
-2.17 \pm 1.30	-1.67	0.095
-9.44 \pm 4.40	-2.15	0.032
-1.67 \pm 0.83	-2.02	0.043
-0.45 \pm 0.43	-1.03	0.302

Table 3. Percentages of florets with pollen tube penetration to the ovary following conspecific (CF) and heterospecific (HF) pollination; ratio of HF to CF (RPF); and the results of GLM analyses of the effect of relative abundance of the alien species on seed set of the native species in eight *Taraxacum japonicum* individuals of population JOO.

Individual number	Number of florets tested (conspecific, heterospecific pollination)	Pollen tube penetration			RI (GLM) [†]		
		CF, Conspecific	HF, Heterospecific	RPF = HF/CF	Coefficient ± SE	Z value	P*
1	6, 9	100.0%	66.7%	0.667	-4.87 ± 1.01	-4.83	0.000
2	6, 7	50.0%	57.1%	1.143	1.16 ± 1.22	0.95	0.342
3	7, 8	57.1%	12.5%	0.219	-1.33 ± 1.34	-1.00	0.319
4	8, 3	25.0%	0.0%	0.000	-5.58 ± 1.06	-5.29	0.000
5	8, 7	37.5%	14.3%	0.381	0.06 ± 0.93	0.07	0.944
6	8, 7	62.5%	14.3%	0.229	1.80 ± 0.84	2.14	0.032
7	4, 6	75.0%	33.3%	0.444	-4.91 ± 0.88	-5.58	0.000
8	7, 10	57.1%	50.0%	0.875	-5.21 ± 0.87	-5.99	0.000

[†]Analyses of data obtained by Takemori et al. (2019). *Wald test.