

1 **Kinship between Mates Reduces Offspring Fitness in a Natural Population of**

2 *Rhododendron brachycarpum*

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10 Running title: Inbreeding depression in *Rhododendron*

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ABSTRACT

1
2 *Background and Aims* Reduction in offspring fitness resulting from mating between
3 neighbors is interpreted as biparental inbreeding depression. However, little is known
4 about the relationship between the genetic relatedness of parents and the biparental
5 inbreeding depression in their progeny in natural populations. Here, the effect of kinship
6 between mates on the fitness of their progeny and the extent of spatial genetic structure
7 in a natural population of *Rhododendron brachycarpum* were assessed.

8 *Methods* The kinship coefficients between 11782 pairs among 154 plants were
9 estimated *a priori* using six microsatellite markers in a natural population of *R.*
10 *brachycarpum*. Pairs of individuals were selected from the genotyped plants to vary the
11 level of the kinship coefficient from self-pollination to related plants and then to
12 unrelated plants. After a hand-pollination experiment among the 60 selected plants, the
13 offspring's fitness was measured at the following stages: seed maturation under natural
14 condition, and seed germination and seedling survival under greenhouse. In addition,
15 spatial autocorrelation analysis was performed to assess the genetic structure of the
16 population.

17 *Key Results* The offspring's fitness decreased significantly with increasing kinship
18 between mates. However, the magnitude and timing of this effect was not uniform
19 among the life-cycle stages. The standard measurements of inbreeding depression were
20 0.891 for seed maturation, 0.122 but not significant for seed germination, and 0.506 for
21 seedling survival. Local population structure was significant, and the physical distance
22 between parents mediated the level of inbreeding between mates.

23 *Conclusions* The level of inbreeding between mating individuals is a key determinant in
24 offspring fitness in *R. brachycarpum*, especially during seed maturation. Genetic

1 relatedness between parents caused inbreeding depression in their progeny. Therefore,
2 biparental inbreeding makes a limited contribution to reproduction and would be a
3 selective force to promote the effective movement of pollen since crosses from further,
4 unrelated, pollen have better survival.

5 **Key words:** biparental inbreeding depression, genetic structure, germination rate,
6 *Rhododendron brachycarpum*, seed maturation, selfing rate, survival rate.

7

INTRODUCTION

1
2
3 In angiosperms with self-compatible mating systems, inbreeding can arise as a result of
4 selfing (uniparental inbreeding) and/or mating between relatives (biparental inbreeding).
5 Although both modes of inbreeding can lead to reductions of fitness in the resulting
6 offspring (inbreeding depression; Darwin, 1876; Charlesworth and Charlesworth, 1987),
7 most empirical evidence of inbreeding depression in plants comes from uniparental
8 inbreeding in hermaphroditic species (reviewed by Husband and Schemske, 1996).
9 However, biparental inbreeding and its contribution to inbreeding depression are likely
10 to be ubiquitous in natural plant populations. In many plant species, seeds are dispersed
11 over short distances from the maternal plants, and related individuals are thus likely to
12 be neighbors and to cross among each other due to restricted flight distances by
13 pollinators (Fenster, 1991a; 1991b). Fine-scale genetic structure actually enhances
14 biparental inbreeding between plants surrounded by genetically similar neighbors
15 (Williams, 2007; Zhao, 2009). In addition, many experimental studies have confirmed
16 that mating between neighboring individuals results in reduced fitness compared to
17 mating between individuals growing at further distance from each other (e.g. Coles and
18 Fowler, 1976; Price and Waser, 1979; Fenster, 1991; Waser and Price, 1991; 1994;
19 Fisher and Matthies, 1997; Byers, 1998; Stacy, 2001; Nuortila et al., 2002; Robertson
20 and Ulappa, 2004). These empirical studies suggest that biparental inbreeding
21 depression occurred frequently in flowering plants. However, little is known about the
22 relationship between the genetic relatedness of parents and the biparental inbreeding
23 depression in their progeny in natural populations.

1 Measuring biparental inbreeding depression is a difficult task if prior information on the
2 relationships among individuals is lacking. For example, evidence of biparental
3 inbreeding depression was revealed by breeding experiments conducted over 3 years in
4 *Raphanus sativus* (Nason and Ellstrand, 1995) or over 10 years in *Cryptomeria japonica*
5 (Kurinobu et al., 1991). As an alternative to breeding over several generations under
6 artificial conditions, molecular markers allow the estimation of natural levels of
7 inbreeding in wild populations, which is especially beneficial in animals and self-
8 incompatible plants where crossing experiments are difficult (e.g. Slate et al., 2000;
9 Glémin et al., 2006; Jensen et al., 2007). For examples, “mixed mating model” has been
10 commonly used to assess occurrence of biparental inbreeding since the widespread
11 application of allozymes (Shaw et al., 1981; Ritland and Jain, 1981). In the mixed
12 mating model, selfing and biparental inbreeding are estimated together as the effective
13 selfing rate but the negative effect of biparental inbreeding cannot be quantified.
14 However, the development of highly polymorphic markers such as microsatellites
15 allows the estimation of genetic relatedness between mates, i.e. the magnitude of
16 biparental inbreeding in their progeny, with reference to their fitness at individual level
17 (e.g. Glémin et al., 2006; Teixeira et al., 2009). In addition, progress in statistical
18 methodologies has allowed the detection of relationships between genetic relatedness
19 and fitness using more robust methods such as General Linear Models (GLM) (Glémin
20 et al., 2006; Armstrong and Cassey, 2007). Therefore, molecular-marker based
21 methodology for quantifying biparental inbreeding depression under natural condition
22 has already been prepared.
23

1 Biparental inbreeding depression could be of interest to explain observed patterns of
2 pollen flow. During the past decade, paternity analysis using microsatellite markers in
3 various tree species has shown that a surprising amount of pollen originated from
4 distant sources (e.g. Dow and Ashley, 1998; Streiff et al., 1999, Lian et al., 2001;
5 Latouche-Hallé , 2004; Bacles et al. 2005; Cresswell, 2005; Goto et al., 2006). While
6 the density of pollen grains has generally been shown to exhibit a leptokurtic
7 distribution with a rapid decline as the distance from the donor increases (e.g. Handle,
8 1983; Campbell, 1991; Hardy et al., 2004; Robledo-Arnuncio and Gi, 2005), the
9 observation of relatively long distance movement may arise because progenies fertilized
10 by pollen from neighboring individuals may have decreased survival due to the effects
11 of biparental inbreeding depression. As supporting evidence, Isagi et al. (2007) have
12 clearly shown that effective pollen dispersal was enhanced by local kinship structure in
13 *Aesculus turbinata*. In the tree population, survival probability of progenies sired
14 through neighboring matings was lower than that through more outbred matings at
15 successive life stages. Therefore, biparental inbreeding depression can affect not only
16 the fitness of the resulting offspring but also the effective distance of pollen-mediated
17 gene flow. The tendency for pollen flow to be leptokurtic should lead to the following
18 expectations: (i) existence of local kinship structure in plant populations (reviewed by
19 Vekemans & Hardy, 2004), (ii) high likelihood of mating between neighboring
20 individuals (e.g. Handle, 1983; Hardy et al., 2004; Robledo-Arnuncio and Gi, 2005),
21 and (iii) selection against the offspring sired through inbred mating (Isagi et al., 2007).
22 Although the two former points have already been demonstrated in a number of studies,
23 little is known about the selective pressure for related mating through biparental
24 inbreeding depression under natural conditions. The purpose of the present study was to

1 estimate what extent biparental inbreeding affects the fitness of the resulting offspring
2 in a natural population.

3

4 In this study, microsatellites makers were applied to estimate kinship between mates
5 and to infer the magnitude of the inbreeding depression in a natural population of
6 *Rhododendron brachycarpum*. This species has shown mixed mating system like other
7 *Rhododendron* species (e.g. Kudo, 1993; Escaravage et al., 1997; Kameyama et al.,
8 2000; Ng and Corlett, 2000; Hirao et al., 2006; Ono et al., 2008), in which reproduction
9 occurs by both self-fertilization (selfing) and mating with other individuals
10 (outcrossing). Thus the genetic relatedness between mating individuals vary from self-
11 pollination to related plants and then to unrelated plants. Both uniparental and biparental
12 modes of inbreeding depression can be assessed together using a hand-pollination
13 treatment that provides a priori information on the genetic relatedness between mating
14 individuals in this species.

15

16 The primary objective of the study was to determine the effect of relatedness between
17 mating pairs on the probability of seed maturation in the field, and the extent of
18 inbreeding depression in seed germination and seedling survival as measured in
19 greenhouse. This was accomplished by performing a series of crosses, in which *a priori*
20 information about molecular markers was used to control crosses to be performed
21 among individuals that varied in their kinship coefficient from 0.5 (self-pollination) to 0
22 (outbred mating). Second, to illustrate the natural level of biparental inbreeding under
23 open-pollinated environment, mixed mating model (Ritland, 2002) was applied for
24 assessment of mating system in *R. brachycarpum*. In addition, the natural levels of

1 inbreeding were estimated at the successive stages from matured seeds to reproductive
2 adults to verify whether inbred mating is beneficial in this species. Finally, a spatial
3 genetic structure in the population was assessed to predict the extent of biparental
4 inbreeding depression.

5

6

MATERIALS AND METHODS

7

8 *Study site and species*

9 This research was conducted in a natural botanical garden at the Mt. Hakkoda Botanical
10 Laboratory of Tohoku University in Aomori Prefecture, northern Honshu, Japan
11 (40°38'N, 140°51'E). The altitude of the botanical garden (900 m asl) corresponds to the
12 transition zone from deciduous broad-leaved forest dominated by *Fagus crenata* to
13 subalpine coniferous forest dominated by *Abies mariesii* and *Betula ermanii*. The mean
14 monthly temperatures ranged from -9.3°C (January) to 20.5°C (August), and the mean
15 annual precipitation was 1696 mm between 1997 and 2006.

16 *Rhododendron brachycarpum* is an evergreen shrub that reaches heights of up to 3 m.
17 Flowering lasts for 2 to 4 weeks between late June and late July in the botanical garden
18 (Kikuchi, 1976). Adult plants produce axillary inflorescences of 7 to 20 cream-white
19 flowers. The protandrous flowers have five petals and ten stamens, and attract various
20 types of pollinators, including bumblebees, honeybees, small solitary bees, syrphid and
21 other flies, rove beetles, longicorn beetles, and lepidopterans. Fruits mature in October,
22 and include 200 to 400 tiny seeds that are dispersed by gravity or wind.

23

24 *Sampling, DNA extraction and genotyping*

1 In early June 2006, all of the adult plants in flower stage were mapped using a
2 Pathfinder Pro XR GPS receiver with differential correction module (Nikon-Trimble,
3 Tokyo, Japan) in a part of the botanical garden surrounded by footpaths (the “core area”
4 in Fig. 1). In addition, about 100 plants from outside the core area were chosen at
5 random and mapped (Fig. 1). From these mapped plants ($n = 154$), leaf samples were
6 collected for genotyping. DNA was isolated using the cetyltrimethyl ammonium
7 bromide extraction procedure (Stewart and Via, 1993). Six loci corresponding to
8 microsatellite markers were used in this analysis: RM2D2, RM3D1, RM3D2, RM9D6
9 (Naito et al., 1998), RM2D6 (Kameyama et al., 2001), and RM9D9 (Hirao et al., 2006).
10 The reaction mixture (6 μ L) included 10 ng of the template DNA, 1.2 pmol of primers
11 (the forward primers were fluorescent-dyed labeled), and 3 μ L of Multiplex PCR
12 Master Mix (Qiagen, Hilden, Germany). PCR amplification used a GeneAmp PCR
13 System 9700 (Applied Biosystems, Foster City, CA, USA) under the following
14 conditions: initial denaturation at 94°C for 15 min; 35 cycles of denaturation at 94°C for
15 30 s, annealing at 53°C for 90 s, and extension at 72°C for 1 min; and a final incubation
16 at 60°C for 30 min. The size of PCR products was determined using an ABI PRISM
17 3100 automated sequencer and GENESCAN analysis software (Applied Biosystems).

18

19 *Estimation of kinship coefficient*

20 The following measures of genetic variation were calculated as mean values across the
21 six loci: the number of alleles per locus (A), observed heterozygosity (H_o), expected
22 heterozygosity or gene diversity (H_e), and Wright’s inbreeding coefficient (f , following
23 the method of Weir and Cockerham, 1984). The significance of f across the loci was
24 obtained using randomized procedures provided in version 2.9 of the FSTAT software

1 (Goudet, 1995). The multilocus kinship coefficients between individuals i and j (F_{ij})
 2 were estimated according to the method of Loiselle et al. (1995), which is defined as
 3 average across multiallelic (multilocus) estimate of the kinship coefficient for each
 4 allele k ;

$$5 \quad F_{ij} = (P_i - \overline{P_k})(P_j - \overline{P_k}) / \overline{P_k}(1 - \overline{P_k}) + 1/2(n-1)$$

6 where p_i and p_j are the frequencies of allele k in individuals i and j , respectively, and $\overline{P_k}$
 7 is the average frequencies of allele k in the population with sample size n . The estimated
 8 values of kinship coefficient were approximate half of pedigree relatedness, that is, 0.25
 9 full sibs and 0.125 half sibs (Loiselle et al., 1995). These calculations were implemented
 10 for all pairs of the 154 samples using version 1.1 of SPAGeDi software (Hardy and
 11 Vekemans, 2002).

12

13 *Pollination experiment*

14 Previously, pairs of the genotyped plants for the pollination treatment were listed to
 15 vary the kinship coefficient from self-pollination (assuming $F_{ij} = 0.5$), to related plants
 16 and then to unrelated ones (assuming $F_{ij} = 0.0$). For the hand-outcrossed pollinations,
 17 mating pairs were selected to generate high estimated kinship ($F_{ij} > 0.1$) to facilitate the
 18 detection of biparental inbreeding depression. In late June 2006, 60 plants were selected
 19 and one to two inflorescences per plant were bagged using fine-mesh nets before flower
 20 opening. Both the hand-self and hand-outcross pollinations were conducted at least once
 21 for each flower in the bagged inflorescences. Self and outcross pollen was collected
 22 from freshly dehisced anthers of selected plants. Particularly, outcrossed biparentally
 23 inbred pollen donors were chosen to have high estimated kinship ($F_{ij} > 0.1$), but were
 24 chosen from spatially distant individuals to minimize the correlation between spatial

1 genetic structure and inbreeding depression. Pollinated inflorescences were bagged
2 again until the flowers wilted. In addition, more than 80 flowers of intact plants were
3 marked to estimate mating system parameters (described below) and survival
4 probabilities under open-pollinated treatments. Ripe fruits were collected and counted in
5 October 2006. To assess the success of sexual reproduction, the numbers of matured
6 seeds and aborted seeds were counted for every fruit using a microscope in the
7 laboratory. Matured seeds were relatively large and plump, whereas aborted seeds were
8 small and shrunken (Fig. 2). Seeds were considered to be mature if they were 2.8 ± 0.3
9 mm long and full. Seed set ratio was calculated as the number of matured seeds per total
10 number of matured seeds plus aborted ones.

11

12 *Seed germination and seedling survival*

13 Offspring fitness in seed germination and seedling survival was measured under
14 uniform conditions. In April 2007, 9 to 60 seeds per fruit were sown on 0.8% agar
15 medium in 9-cm-diameter Petri dishes and stored at 4°C for 2 months as a moist chilling
16 treatment. The total numbers of seeds sowed was 544 from 34 hand-selfed fruits, 2056
17 from 32 hand-outcrossed fruits, and 3045 from 56 open-pollinated fruits. After the
18 moist chilling treatment, the dishes were transferred to germination chambers to
19 incubate under a temperature regime of 30/25°C at 12-h intervals. The numbers of
20 germinated seedlings were counted after 30 days of incubation to calculate germination
21 rate as the number of germinated seedling per sowed seed. At the beginning of July
22 2007, more than five germinants per cross were transferred into peat pots filled with
23 acid garden compost when true leafs emerged. The total numbers of the transplanted
24 seedlings were 144 for hand-self, 272 for hand-outcross, and 472 for open-pollination.

1 The pots were placed in a greenhouse under natural sunlight at Hokkaido University,
 2 Sapporo (elevation 17 m asl.). Survival rates of the seedlings were observed at 1 month
 3 intervals until November 2007. The seedling survival rate was determined as the
 4 numbers of survival seedlings per transplanted germinant at the final observation.

5

6 *Mating system*

7 Using 61 seedlings from 26 open-pollinated plants for the measurement of fitness until
 8 seed germination, the selfing rate (estimated from multilocus genotypes, s_m , and from
 9 the average single-locus estimate, s_s) at fertilization was estimated based on Ritland's
 10 mixed-mating model (Ritland & Jain 1981; Ritland 2002). The primary selfing rate (s_p),
 11 which represents the selfing rate at the time of pollination, was also estimated by
 12 adjusting the observed multilocus estimate of selfing rate (s_m) to account for any
 13 inbreeding depression using the following formula (Maki, 1993);

$$14 \quad s_p = \frac{s_m}{1 - \delta + s_m \delta}$$

15 where δ was the degree of inbreeding depression during seed maturation (as described
 16 below).

17 To estimate the amount of biparental inbreeding, two measurements were used: (i) the
 18 difference between the single locus and multilocus estimates of the selfing rate ($s_s - s_m$),
 19 and (ii) the correlation of selfing among loci (r_s). The former measurement, $s_s - s_m$, has
 20 been widely used to demonstrate biparental inbreeding but tends to greatly
 21 underestimate, especially when estimates are based on few loci (reviewed by Griffin
 22 and Eckert, 2004). While the estimate of r_s corresponds to the proportion of the
 23 effective selfing rate due to true uniparental selfing, with the remainder of the effective

1 selfing rate due to biparental inbreeding (Ritland, 2002). These mating system
 2 parameters were estimated using the software MLTR (version 3.3, revised May 2008;
 3 Ritland, 2002). Standard errors of these mating system parameters were estimated from
 4 1000 bootstrap replications.

5

6 In addition, the selfing rate under the open pollinated conditions was estimated based on
 7 the performance of progeny at three different life stages (seed maturation, seed
 8 germination, and seedling survival) using the following formula (Charlesworth, 1988);

$$9 \quad s = (p_x - p_w) / (p_x - p_s)$$

10 where p_x is cumulative fitness of progenies under open pollination, p_w is that of
 11 controlled outcross-progenies (assuming $F_{ij} = 0$), p_s is that of selfed progenies
 12 (assuming $F_{ij} = 0.5$) to infer the predicted values by the logistic analyses as described
 13 below.

14

15 *Estimation of inbreeding depression*

16 Inbreeding depression was analyzed using generalized linear mixed models (GLMMs),
 17 in which the number of surviving offspring at each life-history stage (i.e. matured seeds,
 18 germinated seeds, and survival seedlings) in n trials (i.e. prepared ovule, sowed seeds,
 19 potted seedling) follows a binomial distribution with a logit-link function. In the
 20 GLMM, the effect of kinship coefficient between mates (the fixed effect) on offspring
 21 survival was assessed using a logistic regression (for details, see Glémin et al., 2006).

22 The random-effects error structure for survival probability was defined as non-
 23 independent of the maternal plants. For selfed offspring, the kinship coefficient was
 24 adjusted as $(1 + F_{ii}) / 2$, where F_{ii} is the inbreeding coefficient of their maternal plants

1 (Glémin et al., 2006). The GLMM analysis was conducted using glmmML function
2 implemented in version 2.7 of R software (R Development Core Team, 2008). To
3 examine whether self-pollinated samples were over-weighted in the results, the GLMM
4 analysis was performed for only hand-outcross. In addition, the relationships were also
5 examined using a GLM to assess the influence of ignoring the dependence of the
6 maternal individuals; for this analysis, glm function provided by the R software was
7 used. To compare the goodness-of-fit of the above models, Akaike's information
8 criterion (AIC) was calculated. AICs are interpreted as the probability that a model is
9 the best model, given the observed data and the set of candidate models. The standard
10 measurement of inbreeding depression, $\hat{\delta}$, was calculated by comparing the fitness of
11 selfed individuals (assuming $F_{ij} = 0.5$) with the fitness of controlled outcrosses
12 (assuming $F_{ij} = 0$) inferring the predicted values by logistic analyses. For each life
13 history stage (seed maturation, seed germination, and seedling establishment), the
14 magnitude of the inbreeding depression was assessed.

15

16 *Spatial genetic structure*

17 To assess the spatial genetic structure, spatial autocorrelation analysis was performed
18 based on the kinship coefficient. To graphically represent the spatial genetic structure,
19 average kinship coefficients were calculated for nine distance classes (with the upper
20 limit of the classes equal to 5, 10, 20, 30, 40, 50, 70, 100, and >100 m). The degree of
21 spatial genetic structure was quantified using the statistic $Sp = -b / (1 - F_1)$, where b is
22 the slope of the linear regression of the kinship coefficient against the natural logarithm
23 of the physical distance, and F_1 is the kinship coefficient for the first distance class
24 (Vekemans and Hardy, 2004). Standard errors for the Sp statistics were estimated using

1 the jackknife procedure across all six loci. The significance of the genetic structure was
 2 tested by comparing b with the values obtained after 9999 permutations of the spatial
 3 coordinates for the individuals. In the same way, the 95% confidence interval was
 4 obtained for the average kinship coefficient at a particular distance using 9999
 5 permutations. These analyses were conducted using version 1.1 of SPAGeDi software
 6 (Hardy and Vekemans, 2002). In addition, the biparental inbreeding depression
 7 corresponding to spatial genetic structure inferred from Ishihama et al. (2005) was
 8 estimated but using the following formula;

$$9 \quad \delta_{Bi} = 1 - \frac{1 + \exp(-(\beta_1 + \beta_2 \overline{F_{ij}}))}{1 + \exp(-\beta_1)}$$

10 where δ_{Bi} is the degree of biparental inbreeding, $\overline{F_{ij}}$ is the average kinship coefficient
 11 for each distance class to infer the observed value of the spatial genetic structure, and β
 12 ₁ and β ₂ are the intercept and the coefficient for the kinship, respectively, in the logistic
 13 regression for estimation of inbreeding depression as described above. The cumulative
 14 amount of biparental inbreeding depression was calculated as the product from seed
 15 maturation to seedling survival.

16

17

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RESULTS

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20 *Genetic variation*

21 In adult plants, 6 to 25 alleles were detected per locus with a mean of 16.7 (Table 1).

22 Expected heterozygosity (H_e) ranged from 0.676 to 0.913, and averaged 0.815 across

23 the loci. The mean inbreeding coefficient (f) across the loci did not deviate significantly

1 from the Hardy–Weinberg expectation ($f = -0.013$; $P > 0.05$ randomization test),
2 indicating a randomly mating population.

3

4 *Mating*

5 Under the open-pollinated condition, the single locus and multilocus selfing rate was
6 0.212 (± 0.062 SE) and 0.161 (± 0.059), respectively. Both $s_s - s_m$ and the correlation of
7 selfing among loci (r_s) indicated that significant biparental inbreeding occurred ($s_s - s_m$
8 = 0.051 ± 0.040 ; $r_s = 0.675 \pm 0.185$). The difference $s_s - s_m$ indicated that about 25%
9 ($0.051/0.212$) of the effective selfing rate was due to biparental inbreeding, while the
10 correlation of selfing among loci indicated that biparental inbreeding contributed to
11 33% ($1 - r_s = 0.325$) of the effective selfing. The primary selfing rate (s_p) at the time of
12 pollination, which based on multilocus selfing rate after adjusting for inbreeding
13 depression, was 0.638 ± 0.119 .

14

15 *Magnitude and timing of inbreeding depression*

16 Owing to heavy damage by insects, fruit were collected from only 34 of the 60
17 pollinated plants. Almost all of the hand-pollination flowers including selfed ones
18 developed into fruits if not insect damaged them. The total numbers of fruits collected
19 was 34 for hand-self and 52 for hand-outcross plants.

20

21 Seed maturation of the hand-pollinated fruit depended significantly on the kinship
22 coefficient (Table 2, Fig. 3A). The GLMM predicted that seed maturation was very
23 lower in self-fertilized flowers than in randomly outcrossed flowers ($\hat{\delta} = 0.891$). On the
24 other hand, at the germination stage the effect of the kinship coefficient on fitness was

1 not significant ($\hat{\delta} = 0.122$; $P > 0.1$, Table 2, Fig. 3B), while seedling survival depended
2 significantly on the level of inbreeding ($\hat{\delta} = 0.506$, $P < 0.01$, Table 2; Fig. 3C). The
3 magnitude of inbreeding depression during seedling survival was lower than that during
4 seed maturation. Cumulative fitness from seed development to seedling survival
5 decreased by 94.6% in selfed offspring compared with outcrossed offspring. Under the
6 open-pollination environment, the survival probabilities of the offspring were $0.436 \pm$
7 0.200 (mean \pm SD) for seed maturation, 0.400 ± 0.239 for seed germination, and 0.268
8 ± 0.196 for seedling survival. The selfing rate of open-pollinated offspring, which was
9 estimated from Charlesworth's formula (1988), decreased progressively from 0.404 at
10 seed maturation to 0.151 at seed germination, and then to 0.023 at seedling survival.
11 GLMMs provided better fits to the data than GLMs (which ignored random effects),
12 indicating the importance of considering the maternal factor (AIC values in GLMM vs
13 GLM: 5391 vs 10923 for seed maturation, 239.7 vs 849.7 for seed germination, and
14 90.3 vs 164.9 for seedling survival). Standard deviations of the random effect were
15 1.56, 1.42, and 0.384, for seed maturation, seed germination, and seedling survival,
16 respectively. Even if GLMM analysis was conducted for only hand-outcross fruit to
17 examine whether self-pollinated samples biased the results, the estimated effect of the
18 kinship coefficient on seed maturation was significant (Table 2, Fig 3A), supporting
19 occurrence of biparental inbreeding depression. However, for seedling survival, the
20 effect of the kinship coefficient was not detected when self-pollination was excluded
21 (Table 2, Fig. 3C).

22

23 *Predicted biparental inbreeding depression based on genetic structure*

1 The correlogram for the kinship coefficients across pairs of individuals is shown in
2 Figure 4A. Significantly positive kinship coefficients were detected within a distance of
3 20 m, and a significantly negative kinship coefficient was detected beyond a distance of
4 100 m (randomization test; $P < 0.05$). The kinship coefficients clearly decreased with
5 increasing physical distance, suggesting a significant spatial genetic structure ($b =$
6 -0.009 , $Sp = 0.0096$; $P < 0.001$). The estimated inbreeding depression as a function of
7 the spatial genetic structure show that the cumulative fitness components progressively
8 decreased as a function of distance class such in the distance classes of 0-5, 5-10, 10-20
9 and 20-30m fitness decrease by 20.1%, 11.8%, 4.5%, and 1.4%, respectively (Fig. 4B).

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11

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DISCUSSION

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Occurrence of biparental inbreeding depression

14
15 The level of inbreeding between mating plants is a key determinant of offspring fitness
16 in *R. brachycarpum*, especially for seed maturation. Several observations indicate that
17 early-acting inbreeding depression plays the dominant role in reducing self-fertility,
18 rather than pre- or post-zygotic incompatibility. (1) Almost all of selfed-pollinated
19 flowers produced fruits even if no matured seeds. (2) A higher proportion of flattened
20 and shriveled seed were present in selfed than outbred fruit (Fig. 2). These observations
21 indicate that self-fertilized ovules were aborted at post-zygotic stage. Studies of other
22 *Rhododendron* species have shown that self pollen tubes can grow into the style and
23 fertilize ovules equally well as pollen tubes produced from cross-pollination (Palsa et
24 al., 1989). (3) The aborted seeds ranged in sizes (Fig. 2), suggesting that seeds were

1 aborted at different stages of development after fertilization. If late-acting self-
2 incompatibility caused, embryos are aborted at a uniform stage in development (Seavy
3 and Bawa, 1986). (4) Seed set of self-pollinated fruits showed largely variation across
4 individuals from 0.000 to 0.478. Variable expression across individuals is considered by
5 some to be a hallmark of inbreeding depression due to mutational genetic load (Seavey
6 and Carter 1994). Thus, early-acting inbreeding depression but not late-acting self-
7 incompatibility was responsible for early selection at seed maturing stage in this
8 species. Therefore, biparental inbreeding depression can take account into the decrease
9 in seed-set ratio with increasing kinship between mates

10

11 Marker-based methods were used to create biparentally inbred progeny and the effect of
12 this on progeny fitness was then assessed under natural conditions. Some empirical
13 evidence for biparental inbreeding depression was provided by breeding plants under
14 experimental fields (e.g. Kurinobu et al., 1991; Nason and Ellstrand, 1995; Teixeira et
15 al., 2009), but in the present study biparental inbreeding depression during seed
16 maturation was assessed in the field. It was found that higher values of genetic
17 relatedness between parents caused the higher cost for their offspring fitness. Namely,
18 the magnitude of biparental inbreeding depression depended on genetic relatedness
19 between mating partners in a natural population.

20

21 *Reproductive success through uniparental and biparental inbreeding*

22 In the open-pollinated environment, self-pollination dominated at the time of pollination
23 ($s_p = 0.638$). Much of the self-pollen on stigmas should result from geitonogamy
24 because of the large display of protoandrous flowers without autogamy in this species

1 (Hirao, unpublished). Thus, frequent geitonogamous pollination should lead to a high
2 cost for reproduction due to severe inbreeding depression on selfed progenies. At the
3 successive stages, the selfing rate decreased progressively from seed maturation to seed
4 germination and then to seedling survival. In addition, the inbreeding coefficient of the
5 adult trees ($f = -0.013$; $P > 0.05$) indicated that inbred progenies were removed from the
6 population over time. Although self-pollination appears to be assurance of reproduction,
7 this benefit could often outweighed by severe inbreeding depression and seed
8 discounting (e.g., Herlihy & Eckert 2004). Therefore, self-pollinations including
9 geitonogamous pollinations were not beneficial for reproduction in *R. brachycarpum*.
10 The uniparental mode of inbreeding depression is a selective force to promote outcross
11 in this species.

12

13 As the other mode of inbreeding depression, biparental inbreeding occurred in a natural
14 population of *R. brachycarpum*. The amount of biparental inbreeding contributed to
15 about 25-33% of the effective selfing under the open-pollinated environment. Although
16 the pollen-dispersal distances of *R. brachycarpum* are unknown, the investigations in
17 another species *Rhododendron* has showed the high likelihood of neighboring mates
18 (Kameyama et al., 2000; 2001; Escaravage and Wagner, 2004). The physical distance
19 between parents mediated the level of inbreeding between mates, resulting from a
20 significant spatial genetic structure (Fig. 4). Because spatial genetic structure was found
21 to exist within 20 m of a focal plant, this suggested that, on average, pollen arriving
22 from within 20 m will suffer from some degree of biparental inbreeding. For example,
23 neighboring mates within 5 m, in which average kinship corresponded with the level of
24 half-sib ($F_{ij} = 0.125$), would decrease the offspring's fitness by about 80% (Fig. 4).

1 Biparental inbreeding depression was a substantial selective pressure against the
2 progeny resulting from neighboring mates in natural populations that exhibit distance-
3 dependent genetic structure. Therefore, biparental inbreeding depression would be a
4 selective force to promote effective pollen movement between unrelated individuals in
5 this species.

6

7 *Magnitude and timing of biparental inbreeding depression*

8 The magnitude of inbreeding depression in *R. brachycarpum* was not uniform among
9 the life-cycle stages. It was high for seed maturation, but low for seed germination and
10 seedling survival. Inbreeding depression is generally expressed more strongly in the
11 harsh environment of natural habitats compared with the regulated environment in a
12 greenhouse (Dudash, 1990). Thus, these estimates of inbreeding depression for the
13 germination ability and seedling survival may be underestimated because of artificial
14 conditions. However, these results were consistent with the general pattern of the
15 magnitude and timing of inbreeding depression in angiosperms, in which stronger
16 inbreeding depression occurred during seed maturation rather than during the latter
17 stages of seed germination to seedling survival (Husband and Schemske, 1995; Hufford
18 and Hamrick, 2003; Goodwillie and Knight, 2006; reviewed in Husband and Schemske,
19 1996). That is, pre-seed-dispersal processes should be directly affected by intensive
20 selection caused by inbreeding depression including biparental inbreeding depression.
21 Accordingly, the selective force responsible for long-distance pollen movement could
22 be more intensive at a pre-seed-dispersal stage rather than at post-seed-dispersal stages.
23 Although Isagi et al. (2007) showed that biparental inbreeding depression promotes
24 effective gene flow over longer distances during seedling establishment as post-seed-

1 dispersal stages, this driving forces promoting long-distance pollen movement should
2 also be verified at pre-seed-dispersal stage. Application of paternity analysis of single
3 pollen grains on stigmas (Matsuki et al., 2007; Hasegawa et al., 2009) by comparison
4 with that of matured seeds will identify the intensive selection for the long-distance
5 pollen movement caused by biparental inbreeding depression at pre-seed-dispersal stage.

6

7 *Accuracy of the marker-based approach*

8 Empirical studies of inbreeding depression have suggested that offspring fitness varied
9 greatly among maternal families (Sorensen, 1969; Fowler and Park, 1983; Goodwillie
10 and Knight, 2006). In the present study, the logistic models considering the random
11 effect of maternal families were strongly supported as a means of evaluating inbreeding
12 depression. The wide variance in progeny fitness among families has often been
13 explained by differences in genetic load (e.g. Sorensen, 1969) but the design examined
14 in this study included non-genetic (environmental) factors such as soil conditions and
15 the light environment, especially in terms of the seed maturation. More accurate
16 estimate of the variance in genetic load could come from carefully designed
17 experiments such as mutual pollination exchanging between pollen-donors and pollen-
18 recipients, where genetic load should be explained by genetic components descended
19 from “two parental plants” not but by one side of parents as “a maternal plant”.

20

21 Estimates of the kinship coefficient with using six microsatellite markers showed
22 considerable sampling variance, because precise estimates of the kinship coefficient
23 require more than dozens of loci (for details, see Glémin et al., 2006). Although the
24 kinship coefficients are expected to range from 0 (assuming outbred mating) to 0.5

1 (assuming self-pollination), some estimates of the kinship coefficient showed negative
2 or more than 0.5. The wider range of estimations of the kinship coefficient will decrease
3 the slope of regression for assessment of inbreeding depression, thereby causing
4 underestimation. However, it seems unlikely that this sampling error biased the
5 interpretation of the hypothesis that the magnitude of inbreeding depression depends on
6 genetic relatedness and its expression differs among the life-cycle stages.

7

8 *Conclusion*

9 Genetic relatedness between parental plants caused biparental inbreeding depression in
10 their progeny. Both uniparental and biparental modes of inbreeding depression make a
11 limited contribution to reproductive fitness and then to genetic diversity in this species.
12 Under distance-dependent genetic structure, the presence of biparental inbreeding
13 increases the effective movement of pollen since crosses from further, unrelated, pollen
14 have better survival. I again find it “adaptionist” thinking to say that the leptokurtic
15 dispersal curve of pollen is adaptive –perhaps its existence has enabled plants to
16 “choose” more unrelated pollen from the pool of pollen that arrives to stigma, but the
17 curve itself is no adaptive –since clearly the plant would “choose” to have it not to
18 leptokurtic.

19

20

21

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5

6

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- 16
- 17

1 Figures captions

2

3 Fig. 1. Distribution of sampled individuals of *Rhododendron brachycarpum* in the
4 study plot. The location of the plants sampled for hand-pollination treatments are shown
5 as closed circles. The area surrounded by the bold line is the “core plot” in which all
6 flowering adults of the species were mapped. The open squares represent lodges for
7 researchers.

8

9 Fig. 2. Fully mature seeds from outcross-pollinated fruits (*left*) and presumed aborted
10 seeds from self-pollinated fruits (*right*) of *Rhododendron brachycarpum*. Scale bars,
11 5mm.

12

13 Fig. 3. Relationships between the kinship coefficient and offspring fitness at three life-
14 cycle stages (A, seed maturation; B, germination; and C, seedling survival). Open and
15 closed circles represent hand-self and hand-outcross treatments, respectively. Fitted
16 curves predicted by the generalized linear mixed model are presented when the effect of
17 the kinship coefficient was significant (see Table 2). Solid and dashed curves represent
18 the overall samples analyzed and the samples after selfing was excluded, respectively.
19 Dashed horizontal lines indicate the level of offspring fitness under open-pollination
20 conditions.

21

22 Fig. 4. Spatial genetic structure and predicted inbreeding depression. (A) Correlogram
23 for the kinship coefficient among pairs of *Rhododendron brachycarpum* individuals,
24 expressed for nine distance classes (values on the *x*-axis represent the upper limit of

- 1 each distance classes). Dashed lines represent the 95% confidence interval for the null
- 2 hypothesis that assumes no genetic structure based on 9999 randomizations. Asterisks
- 3 indicate significance ($P < 0.05$) for each distance class. (B) Predicted inbreeding
- 4 depression was calculated as a function of the spatial genetic structure for each stage.

1 Table 1. Characteristics of the six microsatellite markers used for the genetic analysis
 2 based on 154 individual genotypes of *Rhododendron brachycarpum*.

Locus	<i>A</i>	<i>H_o</i>	<i>H_e</i>	<i>F</i>
RM2D2	16	0.770	0.825	0.067
RM2D6	25	0.914	0.913	-0.001
RM3D1	6	0.733	0.676	-0.085
RM3D2	22	0.892	0.897	0.006
RM9D6	16	0.813	0.783	-0.038
RM9D9	15	0.828	0.794	-0.043
Mean	16.7	0.825	0.815	-0.013

3 *A*, number of alleles; *H_o*, observed heterozygosity; *H_e*, expected heterozygosity or gene
 4 diversity; *f*, Wright's inbreeding coefficient. The mean *f* across loci was not
 5 significantly positive according to a randomization test ($P < 0.05$).

6

1 Table 2. Analysis of the generalized linear mixed model for the relationship between
 2 offspring fitness, kinship coefficient (F_{ij}), at the three life-cycle stages (seed maturation,
 3 seed germination, and seedling survival). Numbers in parentheses are in the case of
 4 excluding had-self pollinations.

	Estimate	SE	df	<i>P</i>
Seed maturation				
Intercept	0.660 (0.781)	0.282 (0.302)	83 (49)	0.0190 (0.0098)
F_{ij}	-6.430 (-8.144)	0.085 (0.291)	83 (49)	<0.0001 (<0.0001)
Seed germination				
Intercept	-0.8175 (-0.847)	0.354 (0.401)	38 (22)	0.0209 (0.0345)
F_{ij}	-0.3665 (-0.097)	0.265 1.228	38 (22)	0.1666 (0.9371)
Seedling survival				
Intercept	-1.171 (-1.224)	0.198 (0.233)	53 (30)	< 0.0001 (<0.001)
F_{ij}	-1.702 (-1.406)	0.655 (1.623)	053 (30)	0.0094 (0.3860)

5 Effect of the kinship coefficient was tested using Wald Z-statistic test. The data used in
 6 this analysis are plotted in Figure 2.

Fig. 1 (Hirao)

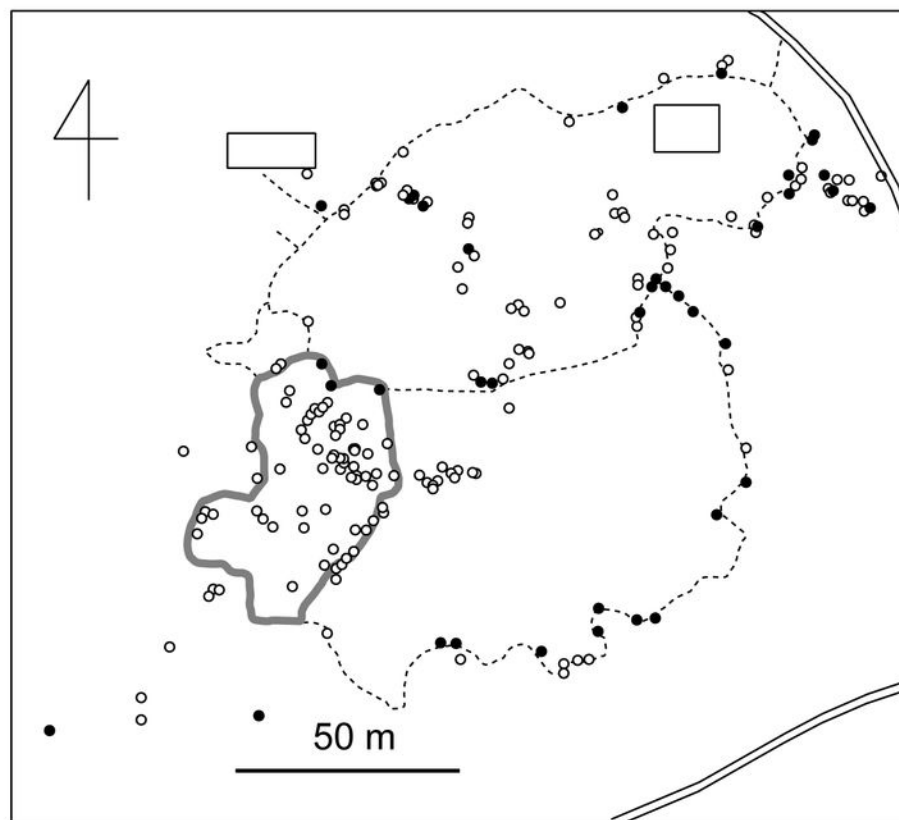


Fig. 2 (Hirao)



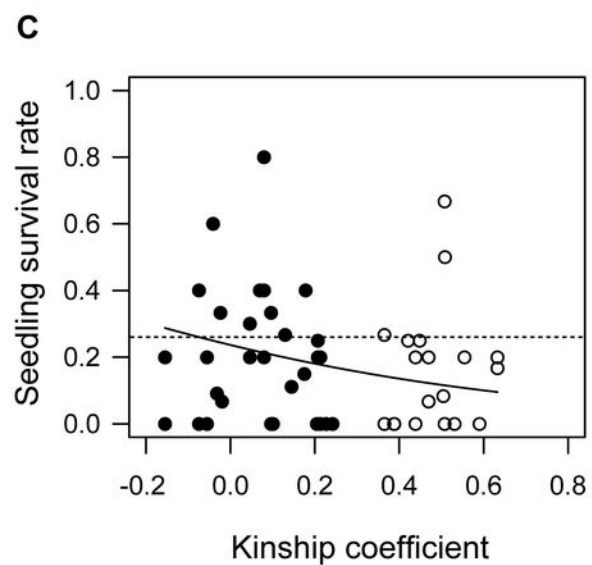
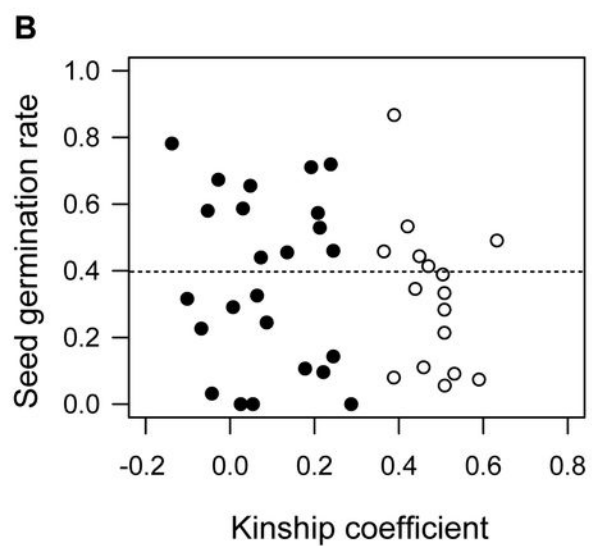
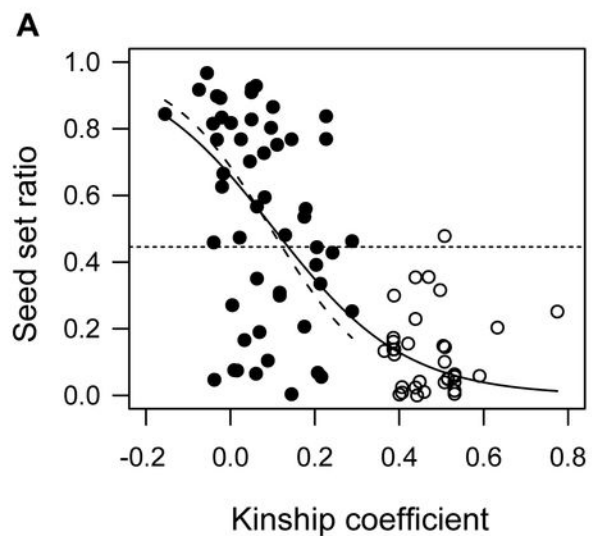


Fig. 4 (Hirao)

