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Hiroshi Takahashi[1] and Masayuki Maki[2*]: **Seed abortion due to inbreeding depression in the threatened saprophytic *Petrosavia sakuraii* (Petrosaviaceae)**

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Inbreeding and loss of genetic diversity are unavoidable in small populations of threatened species (Frankham et al. 2002). Inbreeding increases homozygotes with deleterious alleles and leads to expression of harmful effects, i.e., inbreeding depression. Outcrossing populations normally accumulate deleterious mutations as heterozygotes and their effects are hidden unless such alleles are homozygous. However, as a population size decreases, the homozygotes will increase and the effects of inbreeding depression will grow. This is the case for many threatened populations of both plants and animals. In hermaphroditic plants, the dominant sexual type of angiosperms, autogamy is a more important factor causing inbreeding depression because after only one time self-fertilization of heterozygotes in a locus, half of the sibs will be homozygotes at a locus. Habitat fragmentation of a plant species often causes pollinator shortages, leading to self-pollination and subsequent inbreeding depression (Eckert et al. 2010).

Petrosavia sakuraii (Makino) J. J. Sm. ex. Steenis is a very small perennial plant, which rarely grows in the dark understory of mixed forests containing both evergreen and deciduous trees (Mizuno et al. 1974) or in coniferous forests (Ohba 1984). This species lacks chlorophyll and has degenerate leaves because it is mycoheterotrophic. Takahashi et al. (1993) showed that the species is self-compatible; all flowers set fruits in bagging experiments, indicating that the plants can produce fruits without pollination. This species is listed as "endangered" (EN), based on IUCN category in the Red List of vascular plants of Japan (Environment Agency of Japan 2007). To afford insights into the conservation of *P. sakuraii*, in this study, we estimate the magnitude of inbreeding depression in the seed production by crossing experiments in a natural population of *P. sakuraii* and compared seed set between selfed and outcrossed flowers by counting viable seeds and aborted seeds in the laboratory.

Materials and methods

Plant material

Petrosavia sakuraii is an approximately 10–20 cm tall, achlorophyllous perennial, distributed in the central part of mainland Honshu and Amami-Oshima Islands in Japan, Taiwan, and China (Satake 1982; Chen and Tamura 2000; Ohashi 2000). This plant has degenerate leaves, and the flowers are approximately 2–3 mm in diameter. It flowers from early July to mid-August, and sets fruits from late September to late October. Each individual normally produces one or two shoots with an inflorescence generally containing three to 20 flowers. The main pollinators of the species were halictid bees, although a few dipteran species visited the flowers (Takahashi et al. 1993). The flowers do not open at a definite time of day and their stigmas are receptive from flower opening, while the inner and outer anthers dehisce approximately after 24 h and 48 h, respectively, after flower opening.

Hand-pollination experiments

Hand-pollinations were conducted in a population of *P. sakuraii* located in Takayama City, Gifu Prefecture, Japan, in early August 2009. This population consisted of over 100 individuals of *P. sakuraii*. The habitat was the understory of a plantation of *Chamaecyparis obtusa* and most individuals occurred in humid soil on the upper slope.

In the previous study (Takahashi et al. 1993), the cross-pollinated flowers of the first day of flowering produced the fruits with slightly smaller rate (97%) than those of the bagging, in which the fruit sets were almost 100 % at second and third days of flowering. They also showed that the number of seeds in a fruit was smaller in the cross-pollinated flowers than in the bagged and the untreated flowers. This may be because the emasculation treatment in the cross-pollinated flowers influenced the seed production as the bagged flowers were not emasculated and were intact. In addition, since they showed only the number of seeds in a fruit, seed sets were not examined in the pollination experiments. Therefore, to examine the effects of selfing on seed production, it is necessary to use emasculated ones in both outcrossed and selfed flowers and to examine the difference of seed abortion rate between those crossing experiments. Accordingly, in this study, we conduct selfing and outcrossing experiments using flowers that were emasculated before they open.

Two or three days before flowering, a total of 22 whole plants were chosen haphazardly in at least a few meter intervals and covered with nylon-mesh domes to prevent pollinator access. Just after opening, the flowers whose anthers had never dehisced, were emasculated using forceps. Among these flowers, the outcrossed pollinating treatments were performed by using mixed pollen grains obtained from the flowers of 3–5 other randomly selected individuals. The selfed treatments were conducted by pollinating with pollen grains from another non-emasculated flower of the same individual. Pollen grains were gathered from dehisced anthers of non-emasculated flowers of non-treated individuals using forceps and were placed in a Petri dish until the pollinations. The stigmas of the second or three day flowers were adequately covered by the pollen grains. After pollinations, the plants were covered again with the nylon-mesh domes until the flowers withered. In principle, the selfed and outcrossed treatments were conducted for one or two flower(s) per individual, respectively.

Approximately two months later (mid-October 2009), each fruit from the outcrossed or selfed treatments was collected and dried in envelopes at room temperature. To examine the effects of the hand-pollination on seed production, we also collected fruits that were not subjected to any treatment as open-pollinated treatment.

Seed set counts

The fruits were dissected with a forceps, and mature seeds and aborted ovules were discriminated under a dissecting microscope: it was rather easy to distinguish mature and aborted ones, possibly including those failing in fertilization, in this manner due to their size difference. In total, 30 and 32 fruits were examined for the outcrossed and selfed treatment, respectively. For open-pollinated treatment mature seeds and aborted ovules of 28 fruits were also counted.

The significance values of differences in the number of viable seeds (Ns), aborted ovules (Na), and total number of ovules (Nt) among the treatments were tested by logistic regression using the program R (R Development Core Team 2010). These significance values were corrected for multiple tests with the sequential Bonferroni correction (Rice 1989).

Inbreeding depression (δ) at the stage of seed production was given as $\delta = 1 - Ss/So$, where Ss and So are the averages of seed set by the selfed and outcrossed treatments, respectively (Charlesworth et al. 1990).

Results and discussion

No significant differences were observed in total ovule number within a flower (Nt) in the three treatments (Table 1). The number of viable seeds (Ns) was significantly higher in the outcrossed treatment (112.5) and the open-pollinated treatment (157.6) than in the selfed treatment (61.6). As a result, the number of aborted ovules including those that failed in fertilization (Na) was significantly higher in the selfing treatment (115.1) than in the outcrossed treatment (66.2) or the open-pollinated treatment (28.8) (Table 1). The numbers of seeds fertilized tend to be larger in the popu-

Table 1. Mean values for the number of viable seeds (*Ns*), aborted ovules including those that failed in fertilization (*Na*), total number of ovules (*Nt*), and seed set in the three pollination treatments (outcrossed, selfed, and open-pollinated). The standard deviations are given in parentheses. Significant differences in the seed set between pairwise comparisons of each treatment were tested with the logistic regression and corrected with sequential Bonferroni correction. The different superscript letters denote significance between treatments

	Ns	*Na*	*Nt*	Seed set
Outcrossed	112.5[a] (78.8)	66.2[a] (67.0)	178.8[a] (34.7)	0.610[a] (0.392)
Selfed	61.6[b] (57.2)	115.1[b] (118.5)	176.7[a] (26.7)	0.364[b] (0.350)
Open-pollinated	157.6[a] (48.5)	28.8[a] (36.1)	186.5[a] (40.8)	0.850[c] (0.188)

lation examined in this study than the corresponding values obtained in the other population in Takahashi et al. (1993) (112.5 vs 42.1–61.6 in the outcrossed treatments, and 157.6 vs 138.5 in the open-pollinated treatment), suggesting that there is difference in resources available for seed production between these two populations.

The seed set values were significantly higher in the outcrossed treatment (0.610) and the open-pollinated treatment (0.850) than in the selfed treatment (0.364), and also significant difference was observed between the outcrossed treatment and the open-pollinated treatment (Table 1). These results suggest that the emasculation treatment negatively affected the seed set even in this study as in Takahashi et al. (1993). However, rendering the emasculation conditions equivalent between the outcrossed and the selfed treatments, we could examine the effect of selfing on the seeds set, which differed from the previous study (Takahashi et al. 1993).

Both late-acting self-incompatibility and early-acting inbreeding depression are considered factors that can cause abortion of developing selfed seeds (de Nattencourt 2001). Although distinguishing these two factors is often difficult, one difference between them is that no or almost no seed production after selfing in all individuals will be found in late-acting self-incompatibility (Seavey and Bawa 1986). In the present study, however, since most of the selfed flowers did not produce high numbers, but still some seeds, the lower seed set in the selfed treatment would have been the result of early-acting inbreeding depression. Another difference expected between the two systems is size variation among aborted seeds, but we could not test it because of the very small sizes of them. Based on these results, inbreeding depression at the stage of seed production (δ) was calculated as 0.403 (= 1−0.364/0.610). Husband and Schemske (1986) reported that the inbreeding depression at seed production averaged among 31 outcrossing species was 0.32 (SE 0.053). Considering the averaged value, the inbreeding depression estimated by the present study suggested that severe inbreeding depression was manifested at the stage of seed formation.

As germinating the seeds of *P. sakuraii* is very difficult, probably because of the parasitic nature of its fungi (H. Takahashi unpublished data), we cannot estimate the outcrossing rate in natural populations of the species or the magnitude of inbreeding depression in the late stages of the life cycle. Husband and Schemske (1996) reviewed the magnitude and timing of inbreeding depression in plants. Selfing species express the majority of their inbreeding depression late in the life cycle, while outcrossing species express their inbreeding depression at both the early and late stages of their life cycle. Therefore, more severe inbreeding depression will manifest throughout all stages of the life cycle of *P. sakuraii*. Furthermore, inbreeding depression is more severe in outcrossing species than in selfing species, suggesting that *P. sakuraii* is potentially a highly outcrossed species, at least in the population examined in this study.

From the viewpoint of conservation practice in populations of *P. sakuraii*, the remnant populations should be preserved first. Also, preservation of the pollinator fauna is necessary to avoid an increase in selfing rates because higher selfing would cause more seed abortion and more developmental failures in later stages of the life cycle of *P. sakuraii* due to inbreeding depression. Because human disturbances negatively impact plant-pollinator interactions (Eckert et al. 2010), assessing anthropogenic habitat modifications and their effects on threatened plant species such as *P. sakuraii* is urgent.

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高橋　弘[1]・牧　雅之[2]：サクライソウ集団における近交弱勢による種子結実率の低下

サクライソウはサクライソウ科に属する小型の菌従属性植物である。この種は国内では数集団が現存するのみで，絶滅が危惧されている。本研究では，サクライソウの保全を考えるうえで，集団の存続に影響を及ぼすと考えられる近交弱勢を推定することを目的とし，岐阜県高山市のサクライソウの1集団において，人工交配実験を行い，近交弱勢による種子結実率の低下について調べた。自家交配を行った花では，他家交配を行った花よりも結実率が大きく低下した。これらのデータをもとに計算される，種子結実段階の近交弱勢はおおよそ0.4となった。近交弱勢は種子形成後にも現れると考えられるので，世代を通しての近交弱勢はさらに大きなものとなる可能性がある。

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