Ability of the endangered species *Papaver fauriei* to produce hybrids with a cultivated poppy (*Papaver* sp.)

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Abstract

*Papaver fauriei* is an endemic and endangered species that grows only on the gravelly alpine slopes of Mt. Rishiri, Japan. Cultivated poppy (*Papaver* sp.), the species name of which is unknown, has been introduced to the natural habitats of *P. fauriei* through human activities. Because the appearance and internal transcribed spacer (ITS) sequences of these two poppies are highly similar, it is of concern that they could produce hybrids in their natural habitats. Thus, first, the ability of these two poppies to produce hybrids was analyzed by artificial fertilization in this study. A large number of seeds were produced by reciprocal crosses between *P. fauriei* and the cultivated poppy, comparable with the number of seeds obtained by self- or cross-fertilization of *P. fauriei* or the cultivated poppy. In addition, high germination was observed for seeds obtained from crosses between the two poppies, and deleterious phenotypes, such as albinism and dwarfism, were not detected in the F1 generation. These results indicate that after pollination, there is no reproductive isolation between the two poppies. Second, we sequenced the internal transcribed spacer (ITS) region of 240 poppy individuals collected from the gravelly alpine slopes of Mt. Rishiri, and 66 showed the sequence of *P. fauriei*, whereas 174 showed the sequence of the cultivated poppy. However, the ITS sequence that confirms hybridism between the two poppies was not detected in these individuals, indicating that hybridization of *P. fauriei* and the cultivated poppy rarely occurs under natural conditions. Unknown mechanism(s) appear to prevent cross-pollination between the two poppies.

Keywords: artificial hybridization, conservation of endangered plants, hybrid incompatibility, internal transcribed spacer, special protection zone of Rishiri National Park.

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Introduction

Mt. Rishiri (1721 m above sea level) lies at the center of Rishiri Island, Hokkaido, Japan (Fig. 1a). As some plant and animal species are endemic to Mt. Rishiri, the high-altitude mountainous region of Mt. Rishiri is designated a National Park Special Protection Zone, where cutting and collecting plants, capturing animals, sowing and transplanting plants, and stocking of animals are prohibited (http://www.env.go.jp/nature/park/rel_ctrl/index.html). *Papaver fauriei* (Fedde) Tatew. & Miyabe, a small yellow poppy, is an endemic plant on Mt. Rishiri (Miyabe & Tatewaki 1936; Gardner 1999). *Papaver fauriei* is listed as a threatened vascular plant (EN, endangered species) in the Japanese Red List (http://www.env.go.jp/houdou/gazou/8886/10251/2777.pdf); therefore, projects to conserve this species are expected.

*Papaver fauriei* is a dwarf poppy with hairy, serrated leaves, yellow petals and spherical capsules (Fig. 1b,c). The pollination pattern of *P. fauriei* is not known; however, as *P. fauriei* has large, colored flowers, it is thought
to be an entomophilous plant. We observed small bees (families Andrenidae and/or Halictidae) and syrphid flies (family Syrphidae) visiting *P. fauriei* flowers in the mountain area (unpublished results), although it is uncertain whether these insects aid pollination.

*Papaver fauriei* is found only on gravelly alpine slopes of Mt. Rishiri (Fig. 1b), whereas many small yellow poppies (hereafter, cultivated poppies, Fig. 1d) are found in the gardens of houses and public spaces in seaside towns on Rishiri Island. The size and appearance of cultivated poppies are highly similar to those of *P. fauriei* (Kondo et al. 2012). In the previous study, we evaluated sequence polymorphisms between the cultivated poppy and several *Papaver* species native to the Far East, including *P. fauriei*, to elucidate the origin of the cultivated poppy. Internal transcribed spacer (ITS) sequences were used, which are often conserved in each species yet show several polymorphisms between closely related species; they are known to exhibit polymorphisms among *Papaver* species (Carolan et al. 2006). The ITS sequences of the cultivated poppies resembled those of *P. fauriei* and *P. miyabeanum* and were most similar to the sequences of *P. miyabeanum*, which is endemic to the Kuril Islands (Yamagishi et al. 2010). However, because the ITS sequences found in the cultivated poppy were not found in the wild species, we were unable to specify whether the cultivated poppy was *P. miyabeanum*, *P. fauriei* or any other *Papaver* species.

To aid the recovery of *P. fauriei* populations in their natural habitats on Mt. Rishiri, seeds of the cultivated poppy have been collected from seaside towns and sown at the National Park Special Protection Zone of Mt. Rishiri on several occasions. Therefore, ITS sequences of poppy plants grown in the mountain area were analyzed in the previous study (Yamagishi et al. 2010). Some poppy plants in the mountain area were found to carry the ITS sequences of cultivated poppies, indicating that the cultivated poppy is established in the National Park Special Protection Zone. Thus, this non-indigenous species was introduced to natural habitats through human actions (Yamagishi et al. 2010). The cultivated poppy will be removed from the Special Protection Zone to conserve the endangered species.

Closely related species or subspecies sometimes hybridize naturally in wild habitats; for example, hybrids between *Hemerocallis fulva* and *H. citrina* (Hasegawa et al. 2006), between *Rhododendron kiusianum* and *R. kaempferi* (Kobayashi et al. 2007), and between *Lilium japonicum* and *L. auratum* (Yamamoto et al. 2018), are observed in natural habitats where both parental species grow. Because *P. fauriei* resembles the cultivated poppy in terms of phenotype and ITS sequence, it is possible that these are closely related species or subspecies. Since the cultivated poppy was established in the National Park Special Protection Zone, *P. fauriei* and the cultivated

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**Fig. 1** (a) The location of Rishiri Island (45°10′N, 141°14′E, shown by a red arrow). (b) *Papaver fauriei* growing on gravelly alpine slopes of Mt. Rishiri. Scale bar = 5 cm. (c) Flower of *P. fauriei*. (d) Flower of the cultivated poppy (*Papaver* sp.). Scale bars in c and d = 5 mm.
poppy have grown in the same habitats on Mt. Rishiri for a few decades (Yamagishi et al. 2010). Thus, there is concern that P. fauriei and the cultivated poppy can hybridize in Mt. Rishiri habitats. Such hybridization would not be desirable for the conservation of P. fauriei, because genes from the congeners can undergo introgression or invade endemic species (Parsons & Hermanutz 2006; Kothera et al. 2007).

Two examinations were conducted in the present study. First, the ability of P. fauriei to produce hybrids with a cultivated poppy was evaluated. Papaver fauriei and the cultivated poppy were artificially hybridized and the fertility of their progeny plants was analyzed. Second, as the cultivated poppy plants were found in the mountain area (Yamagishi et al. 2010), ITS regions of all poppy plants grown in the habitats of P. fauriei in the mountain area have been sequenced to remove poppy plants exhibiting the ITS sequences of the cultivated poppy from the Special Protection Zone; the results during the past 8 years are summarized in this report. The poppy plants grown in the mountain area were also screened for hybrid plants between P. fauriei and the cultivated poppy using these ITS sequences to ascertain whether hybrid plants are growing in the Special Protection Zone.

Materials and methods

Plant materials

Seeds of pure P. fauriei plants, which were identified by their ITS sequence, were sampled in the summer of 2010 from the mountainous region of Mt. Rishiri, Rishiri Island, Japan, with permission from the Ministry of the Environment. Seeds of the cultivated poppy (Papaver sp.) were collected in the summer of 2010 from a residential area of Rishiri-fuji town, Rishiri Island, Japan. The seeds of both poppies were sown in early spring in 36-cm-diameter plastic pots, which were filled with a mixture (2:1:1) of a commercial nursery soil containing total N 0.4 g/kg, total P 1 g/kg and water-soluble K 0.6 g/kg (Kureha-engei-baiyodo, Kureha Corporation, Tokyo, Japan), a clay loam soil (Akadama soil) and a weathered pumice soil (Kanuma soil). They were grown in a greenhouse (unheated and natural photoperiod) at the experimental farm of Hokkaido University, Sapporo, Japan. Under these conditions, these poppy plants set flowers within 4 months from sowing. Once the flower buds were visible, these plants were moved to a growth incubator set at 20 °C with a 16-h photoperiod, in 2011, to hybridize artificially. In 2012, P. fauriei, the cultivated poppy and their progeny plants were grown in the glasshouse, where artificial hybridization was carried out.

Artificial hybridization

Hand-pollination was carried out in July using 2–11 flowers on the plants grown under the artificial conditions described in the previous sub-section. At 1 or 2 days before anthesis, anthers were removed and female organs were bagged. Then, fresh pollen collected from P. fauriei or cultivated poppies was placed on stigmas a few days after anthesis. Hybridization combinations are shown in Table 1. When green fruits turned to brown and dried, they were harvested; then, mature seeds were counted using a stereomicroscope. Large and swollen seeds were regarded as mature. Immature seeds were not evaluated in this experiment, because such seeds appeared like dust and were too small to count. Thus, the percentage of mature seeds could not be calculated from the total (mature plus immature) number of seeds.

Seed germination

Mature seeds were used within 2 weeks of collection. Four or five replicates of 30 seeds for each treatment were sown in two layers of moistened filter paper using plastic Petri dishes (55 mm in diameter). The Petri dishes were placed in an incubator at 30 °C for 12 h in light (30 μmol/m’s) and at 20 °C for 12 h in darkness. Seeds with a radicle were considered to have germinated and were counted and removed from the dishes every 1 to 3 days, for 30 days after sowing.

ITS sequencing

The ITSs in P. fauriei, the cultivated poppy and the plants derived from artificial fertilization between these poppies were sequenced to determine whether the artificially crossed plants were hybrids. Methods for total DNA isolation, PCR amplification of ITS regions and direct sequencing of the PCR products have been described previously (Yamagishi et al. 2010).

Statistical analysis

Analysis of variance (ANOVA) was used to assess the statistical significance of differences in the number of mature seeds per fruit and seed germination (%) between fertilization combinations. Then, as the statistical significance was detected in the number of mature seeds per fruit, a post hoc analysis using Tukey’s honestly significant difference test was further performed using R version 3.2.1 (http://cran.r-project.org/doc/manuals/r-release/R-intro.pdf).

Screening for P. fauriei, the cultivated poppy and their hybrids

As a part of the project to conserve P. fauriei, which is mainly governed by the Informative Center for Natural
In 2012, all poppy plants grown in the habitats of *P. fauriei* on Mt. Rishiri were labeled individually, and ITS sequences were determined to identify whether these were *P. fauriei* or the cultivated poppy; the latter were removed from the Special Protection Zone. During the past 8 years (2008–2015), 240 poppy plants, in total, were found, their leaf segments (approximately 5 mm in length) were collected with permission from the Ministry of the Environment, and ITS regions were sequenced using the same methods as those described previously (Yamagishi et al. 2010). In addition, the 240 individuals were screened for hybrid plants between *P. fauriei* and the cultivated poppy using the ITS sequences of the 240 poppy plants.

### Results

**The ability of *P. fauriei* to produce hybrids with a cultivated poppy**

Similarities between the phenotypes and ITS sequences of *P. fauriei* and the cultivated poppy suggest that these poppies are closely related species or subspecies. Thus, artificial hybridization was carried out in 2011 to test the ability of *P. fauriei* to produce viable seeds with the cultivated poppy. In the self- and cross-fertilization of *P. fauriei* or the cultivated poppy, and the reciprocal crosses between *P. fauriei* and the cultivated poppy, all the flowers set seeds (Table 1). The number of mature seeds per fruit varied, ranging from 444 to 1410 (Table 1). Similar numbers of mature seeds set in the self- or cross-fertilization of *P. fauriei* (764 or 810 seeds, respectively) and in the *P. fauriei* × cultivated poppy crosses (928 seeds). The number of mature seeds obtained in cultivated poppy × *P. fauriei* crosses (444 seeds) was similar to that obtained in the cross-fertilization of cultivated poppy (493 seeds), although this was smaller than that obtained from the self-fertilization of cultivated poppy (1410 seeds). These results indicate that reciprocal crosses between *P. fauriei* and the cultivated poppy produce many mature seeds.

Germination of these seeds was analyzed in 2011 under artificial conditions (30/20 °C, day/night) to confirm their viability. More than 98% of seeds germinated (Table 1), and no significant differences were observed between the cross-combinations by ANOVA.

The hybrid plants derived from the crosses between the two poppies showed a similar phenotype to *P. fauriei* and were not distinguishable from the parents by their morphology. To determine hybridism, the ITS regions in *P. fauriei*, the cultivated poppy and the hybrid plants were sequenced. The clearest nucleotide polymorphism was observed at nucleotide position 498, where all *P. fauriei* plants showed ‘T’, whereas all the cultivated poppy plants exhibited ‘C’ (Yamagishi et al. 2010). In the plants derived from the crosses between these two poppies, a mixed nucleotide of ‘Y (T plus C)’ appeared at this position (Fig. 2), indicating that these plants were hybrids.

Following interspecific hybridization, hybrid plants sometimes show sterility at the first filial generation (F1). In 2012, all flowers from cross- or self-pollinated F1 plants produced fruits; these fruits included 337–634

### Table 1  
Number of fruits that set seeds, number of mature seeds per fruit, and the germination ratio of these seeds obtained by artificial fertilization among *Papaver fauriei*, the cultivated poppy (*Papaver* sp.) and their hybrids

<table>
<thead>
<tr>
<th>Fertilization combination</th>
<th>Number of fruits</th>
<th>Number of mature seeds per fruit, average ± SD†</th>
<th>Seed germination (%), average ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pollinated</td>
<td>Set seeds</td>
<td></td>
</tr>
<tr>
<td>In 2011</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Self-fertilization of <em>P. fauriei</em></td>
<td>8</td>
<td>8</td>
<td>764 ± 222 ab</td>
</tr>
<tr>
<td>Cross-fertilization of <em>P. fauriei</em></td>
<td>7</td>
<td>7</td>
<td>810 ± 210 abc</td>
</tr>
<tr>
<td><em>P. fauriei</em> × cultivated poppy</td>
<td>9</td>
<td>9</td>
<td>928 ± 92 bc</td>
</tr>
<tr>
<td>Self-fertilization of cultivated poppy</td>
<td>2</td>
<td>2</td>
<td>1410 ± 172 c</td>
</tr>
<tr>
<td>Cross-fertilization of cultivated poppy</td>
<td>11</td>
<td>11</td>
<td>493 ± 376 a</td>
</tr>
<tr>
<td>Cultivated poppy × <em>P. fauriei</em></td>
<td>4</td>
<td>4</td>
<td>444 ± 299 a</td>
</tr>
<tr>
<td>In 2012</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Self-fertilization of (<em>P. fauriei</em> × cultivated poppy) F1</td>
<td>6</td>
<td>6</td>
<td>599 ± 71 z</td>
</tr>
<tr>
<td>Cross-fertilization of (<em>P. fauriei</em> × cultivated poppy) F1</td>
<td>7</td>
<td>7</td>
<td>485 ± 170 yz</td>
</tr>
<tr>
<td>Self-fertilization of (cultivated poppy × <em>P. fauriei</em>) F1</td>
<td>6</td>
<td>6</td>
<td>634 ± 153 z</td>
</tr>
<tr>
<td>Cross-fertilization of (cultivated poppy × <em>P. fauriei</em>) F1</td>
<td>7</td>
<td>7</td>
<td>337 ± 171 xy</td>
</tr>
<tr>
<td>Self-fertilization of <em>P. fauriei</em></td>
<td>6</td>
<td>6</td>
<td>192 ± 68 x</td>
</tr>
<tr>
<td>Self-fertilization of cultivated poppy</td>
<td>9</td>
<td>9</td>
<td>613 ± 138 z</td>
</tr>
</tbody>
</table>

nt, not tested; SD, standard deviation. † The same letters indicate that the values are not statistically significant (*P* < 0.05) by Tukey’s honestly significant difference test.
mature seeds per fruit, whereas 192 and 613 seeds were set by the self-fertilization of *P. fauriei* and the cultivated poppy, respectively (Table 1). Thus, F1 plants produced an equal or higher number of seeds compared with *P. fauriei* and the cultivated poppy. Seeds from cross- and self-fertilization of the F1 plants exhibited more than 96% germination (not significant between fertilization combinations by ANOVA, Table 1). These results indicate that the F1 hybrids obtained from *P. fauriei* and the cultivated poppy are fertile.

Screening for the hybrid plants in the National Park Special Protection Zone

The results shown in the previous sub-section indicate that *P. fauriei* has an ability to produce fertile hybrid plants with the cultivated poppy, suggesting the possibility that such hybrid plants might exist in their natural habitats; thus, we screened for hybrid plants in the mountain area. Since 2008, the project to conserve *P. fauriei* has sequenced the ITS regions of poppy individuals grown in the National Park Special Protection Zone. During the past 8 years, 66 and 174 of 240 poppy plants exhibited ‘T’ (*P. fauriei*) and ‘C’ (cultivated poppy), respectively, at nucleotide position 498 (Table 2); the latter 174 plants had been removed from the Special Protection Zone. At the nucleotide position 498, however, the mixed nucleotide ‘Y’ was not found (Table 2), indicating that hybrid plants between these poppies were not present in the mountain area.

Discussion

First, the ability of *P. fauriei* and the cultivated poppy to produce hybrids was analyzed by artificial fertilization in this study. Reciprocal crosses between the two poppies produced a large number of seeds, and these hybrid seeds showed a high level of germination. Hybrid weakness, including albinism, dwarfism and seed sterility, was not detected in the F1 generation.

The numbers of mature seeds per fruit, following hand-pollination, varied among cross combinations in 2011 and 2012 (Table 1), which may be attributable to the considerable fruit size variation within the *P. fauriei* plants and within the cultivated poppy plants. Because *P. fauriei* and the cultivated poppy are wild plants, individual poppies and their F1 hybrids have different genotypes, which may lead to variation in plant and fruit sizes.

Gametophytic self-incompatibility has been reported in *Papaver rhoas* (Franklin et al. 1995; Wilkins et al. 2015), whereas *P. somniferum* is self-compatible (Miller et al. 2005). In 2011, self- and cross-fertilization in *P. fauriei* produced a similar number of mature seeds, and self-

<table>
<thead>
<tr>
<th>Year</th>
<th><em>P. fauriei</em></th>
<th>Cultivated poppy</th>
<th>Hybrid</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>28</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>2009</td>
<td>5</td>
<td>69</td>
<td>0</td>
</tr>
<tr>
<td>2010</td>
<td>6</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>2011</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2012</td>
<td>6</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>2013</td>
<td>5</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>2014</td>
<td>5</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>2015</td>
<td>6</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>66</td>
<td>174</td>
<td>0</td>
</tr>
</tbody>
</table>

Internal transcribed spacer sequences were used to determine whether poppy plants were *P. fauriei*, the cultivated poppy or their hybrid.
fertilization produced more mature seeds than cross-fertilization in the cultivated poppy (Table 1). Thus, both *P. fauriei* and the cultivated poppy should be self-compatible.

Artificial hybridization between *P. fauriei* and the cultivated poppy can produce a large number of mature seeds (Table 1). Pollen germination, pollen tube growth, double fertilization, and embryo and endosperm development are often restricted during hybridization between distinct species, which leads to low seed fertility; however, fertilization and seed development can occur successfully during hybridization between *P. fauriei* and the cultivated poppy.

Postzygotic hybrid incompatibility has been reported in hybrids between closely related species or subspecies. It results in deleterious phenotypes, such as seed death, necrosis and hybrid sterility, and generally acts as a mechanism that isolates species (Sweigart et al. 2007; Burkart-Waco et al. 2012; Blevins et al. 2017). More than 98% of the seeds germinated from the F1 generation of the crosses between *P. fauriei* and the cultivated poppy (Table 1), indicating that most of the hybrid seeds were viable. No necrosis or dwarfism was found in the hybrid plants (data not shown), and F1 plants set large numbers of seeds by self- and cross-fertilization (Table 1). At the second filial generation, seeds derived from self- and cross-fertilization of the F1 plants exhibited more than 96% germination (Table 1). Thus, postzygotic hybrid incompatibility was not recognized in the hybrids between *P. fauriei* and the cultivated poppy. These results indicate that reproductive isolation does not act between *P. fauriei* and the cultivated poppy after pollination. However, we cannot rule out the possibility that hybrid incompatibility will occur under natural conditions. In this study, plants were grown in a nutrient-rich soil in a relatively warm climate but, in nature, *P. fauriei* plants grow on nutrient-poor volcanic soils, at high latitude in a harsh, high-altitude environment. Further research will be needed to test this hypothesis.

Second, we screened for hybrid plants between *P. fauriei* and the cultivated poppy in the mountain area; no hybrids (i.e. with mixed nucleotides, ‘Y’, at position 498) were recorded. Similar cases have been reported, indicating that hybrids of *Polemonium kiushianum* and non-indigenous horticultural congeners are not detected in the semi-natural grasslands of Mt. Aso, Japan (Matoba et al. 2011). However, interspecific hybrids are often found in the mixed zones where both parental species grow (Hasegawa et al. 2006; Parsons & Hermanutz 2006; Kobayashi et al. 2007; Kothera et al. 2007; Yamamoto et al. 2018).

Because post-pollination reproductive barriers do not appear to isolate *P. fauriei* and the cultivated poppy, this indicates that unidentified mechanisms prevent cross-pollination between these poppies under natural conditions. The shape, color and fragrance of flowers, and flowering time, are important factors that prevent cross-pollination among different species (Galliot et al. 2006; Hasegawa et al. 2006; Cooley et al. 2011; Klahre et al. 2011; Shang et al. 2011). However, flower morphology, including color and shape, was highly similar between *P. fauriei* and the cultivated poppy. In our observation of *P. fauriei* and cultivated poppies in the mountain area, several flowers opened in succession in one individual during the summer season; therefore, their flowering time was long and overlapped. Thus, flower characteristics and flowering time are unlikely to prevent cross-pollination. *Papaver fauriei* was shown to be self-compatible. Given that pollinators should be active only on sunny days in a cold and windy climate in Mt. Rishiri, one of the most probable mechanisms to prevent hybrid production is that self-fertilization is dominant in *P. fauriei* under natural conditions. Future research should investigate the mechanisms that prevent cross-pollination between these poppies.

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