QTL analysis of heterostyly in *Primula sieboldii* and its application for morph identification in wild populations

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**Background and Aims** *Primula sieboldii* is a perennial clonal herb that is distributed around the Sea of Japan and is endangered in Japan. Its breeding system is characterized by heteromorphic self-incompatibility, and the morph ratio within a population is very important for reproductive success. The aims of this study were to construct a linkage map, map the *S* locus as a qualitative trait and quantitative trait loci (QTLs) for floral morphological traits related to heterostyly, and predict the morph type in wild populations by using molecular markers for devising a conservation strategy.

**Methods** A linkage map was constructed with 126 markers. The QTLs for four floral traits and the *S* locus were mapped. Using the genotypes of loci that were located near both the *S* locus and the QTLs with large effects, morphs of 59 wild genets were predicted.

**Key Results** The linkage map consisted of 14 linkage groups (LGs). The *S* locus was mapped to LG 7. Major QTLs for stigma and anther heights were detected in the same region as the *S* locus. These QTLs exhibited high logarithm of the odds scores and explained a high percentage of the phenotypic variance (>85 %). By analysing these two traits within each morph, additional QTLs for each trait were detected. Using the four loci linked to the *S* locus, the morphs of 43 genets in three wild populations could be predicted.

**Conclusions** This is the first report of a linkage map and QTL analysis for floral morphology related to heterostyly in *P. sieboldii*. Floral morphologies related to heterostyly are controlled by the *S* locus in LG 7 and by several QTLs in other LGs. Additionally, this study showed that molecular markers are effective tools for investigating morph ratios in a population containing the non-flowering individuals or during the non-flowering seasons.

**Key words:** Heterostyly, floral morphology, QTL analysis, *Primula sieboldii*, morph identification, monitoring of morph ratio.

**INTRODUCTION**

Charles Darwin (1877) first suggested that heterostyly would promote cross-pollination between floral morphs by insect pollinators and that natural selection could result in reciprocal positions of the stigma and anther between morphs; this reciprocal positioning is an adaptation beneficial for promoting cross-pollination. Since then, the inheritance and molecular mechanisms of heterostyly have been studied in many heterostylyous plant species, such as those belonging to the genera *Primula*, *Turnera* and *Fagopyrum* (Barrett and Shore, 2008). Heterostyly and self-incompatibility are controlled by the *S* locus, which is a supergene complex consisting of at least 3–9 genes with diallelic polymorphisms in each one (Dowrick, 1956; Richards, 1997). It is presumed that these linked genes control not only morphological traits such as stigma or anther length, but also physiological traits related to self-incompatibility. Recently, with the development of new molecular techniques, molecular markers closely linked to the *S* locus have been identified in *Primula vulgaris* (Manfield et al., 2005; Li et al., 2007).

Many studies have been performed on heterostyly in the genus *Primula*. The sexual reproduction system of *Primula sieboldii* is characterized by typical heteromorphic self-incompatibility. Populations of *P. sieboldii* contain mainly two different forms (morphs) of flowers, long-styled and short-styled, and produce seeds only by cross-pollination between different morphs (usually called legitimate pollination). Washitani et al. (1994) and Nishihiro et al. (2000) indicated that both the stigma and anther exhibited continuous height variations within a wild population of *P. sieboldii*, and the relative heights of the stigma and anthers within a single plant (here termed the ‘relative position’) also varied rather continuously. In addition, Nishihiro et al. (2000) suggested that these floral morphological traits were targets of natural selection. Moreover, continuous variation of floral morphology or...
relative position was also reported in some distylose species, such as *Menyanthes trifoliata* (Thompson *et al.*, 1998), *Amsinckia spectabilis* (Bartowska and Johnston, 2009), and *Bouvardia ternifolia*, *Psychotria poeppigiana* and *Psychotria chiapensis* (Fairev and McDade, 2001).

These previous studies indicate that variation of the relative position within each morph could not be explained by an S locus alone; thus, we hypothesized that floral morphological traits related to heterostyly are controlled not only by an S locus, but also by several quantitative trait loci (QTLs). Mather (1950) suggested the presence of two genes controlling stigma and anther height other than the S locus, and this result supports our hypothesis. Thus far, DeWinton and Haldane (1935) performed linkage analysis of various traits including heterostyly in *Primula sinensis*. Although they constructed the first linkage map for distylose species, these traits were not analysed quantitatively, and this map was a partial one and not a genome-wide map. For these reasons, the first aims of the present study were to perform QTL analysis on four floral morphological traits and identify the location of the S locus to understand the genetic foundation of floral morphological traits related to heterostyly.

*Primula sieboldii*, a perennial clonal herb, is distributed in Japan, Korea, northern China and eastern Siberia (Yamazaki, 1993). It occupies a range of moist habitats, from the understorey of deciduous forests to well-managed grasslands. Overexploitation and destruction of its habitats and horticultural collection are threatening wild populations with extinction, and the species has become endangered in Japan; originally classified in the lower rank of ‘vulnerable’, the species was reclassified to ‘near threatened’ in 2007 (Iwatsuki, 2008). Seed production is limited in small populations in which the ratio of the two morphs is imbalanced; this ratio is very important for reproductive success (Matsumura and Washitani, 2000). Based on a computer simulation, Washitani (1996) suggested that a decrease in the frequency of short-styled morphs would cause inbreeding depression and a genetic bottleneck within a population, and that the ratio of morphs in a population be monitored as a means of active management. The second aim in the present study was to perform QTL analysis on these traits related to heterostyly in *P. sieboldii*, and expected many polymorphisms between these populations in the whole genome. T and P indicate short-styled and long-styled morphs, respectively. Two F1 individuals, ‘SG14’ and ‘2B1’, were selected from the progeny of crosses ‘HP05 × YT112’ and ‘HT02 × YP108’, respectively. SG14 and 2B1 were then crossed to produce 192 genets as the second generation.

**Samples for morph prediction with molecular markers**

To examine the possibility of morph prediction with molecular markers, we used 59 genets of *P. sieboldii* from three wild populations in three regions of Japan: 26 from Hokkaido (a lowland area in northern Japan, 42°31′N, 142°01′E), 16 from Saitama (a lowland area in central Japan) and 17 from Nagano (a highland area in central Japan, 36°19′N, 138°38′E). The Hokkaido and Nagano populations were located near the populations from which the mapping parents were sampled. For the Saitama population (35°50′N, 139°36′E), we used genets that had been grown from seeds obtained from a wild population in the Saitama region in a previous experiment (Washitani and Kabaya, 1988).

**DNA extraction and genotyping**

Genomic DNA was extracted from fresh leaves of each plant by using a modified cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). The genotypes of the 192 genets from the mapping population were determined with 140 pairs of SSR primers (Isagi *et al.*, 2001; Ueno *et al.*, 2003, 2005, 2009; Kitamoto *et al.*, 2005), 23 pairs of expressed sequence tag (EST)-SSR primers (Ueno *et al.*, 2011) and 40 single nucleotide polymorphism (SNP) primers (Ueno *et al.*, 2011). The PCR conditions for SSR primers followed the protocol described by Ueno *et al.* (2003, 2009). The PCR products were analysed using an ABI PRISM® 3100 Genetic Analyzer and GeneMapper software ver. 3.7 (Applied Biosystems). To map the S locus region, we also attempted to use the primers that were closely linked to the S locus reported by Li *et al.* (2007), but these primers did not amplify any DNA fragment from *P. sieboldii*.

**Construction of the linkage map**

Segregation data for the 157 markers indicated polymorphism between the parents and no segregation distortion (χ² goodness of fit; *P* < 0.01). JoinMap ver. 4 (van Ooijen, 2006) with the population type ‘CP’ was used for constructing the linkage map. The linkage map was constructed using regression mapping with Haldane’s function and the default setting in JoinMap. The grouping of markers was performed at a logarithm of odds (LOD) threshold ≥3.

Washitani *et al.* (1994) reported that self- or non-self incompatibility in *P. sieboldii* with self-incompatibility is assumed also to be controlled by an S locus. Therefore, the genotypes of short-styled and long-styled morphs were found...
to be heterozygotes (Ss) and homozygotes (ss), respectively, and the genotypes found within each morph type were also used for linkage analysis to map the S locus. In this study, based on the ratio of stigma height to anther height, the morphs with a ratio of relative positions of stigma and anther heights >1 were regarded as long-styled morphs and those with a ratio of relative positions <1 were regarded as short-styled morphs.

QTL analysis

In 2008, 599 ramets from the 192 genets were grown in a greenhouse at the University of Tsukuba (36°11’N, 140°10’E) to investigate their floral morphological traits. Clonally propagated ramets were planted into 12 cm pots in Metro-Mix 350 (Hyponex Japan, Corp.) in January. Four ramets from 74 genets, three ramets from 67 genets and two ramets from 51 genets were used as replications.

Four floral morphological traits were measured: corolla tube length, stigma height and anther height (maximum heights from the base of ovary), and anther length (Fig. 1). After 1 week of flowering, the first flower of each ramet was photographed alongside a scale marker (9 × 9 mm) with a digital camera (COOLPIX-850, Nikon, Tokyo). The floral traits were then measured using an image measurement program, Makijaku ver. 1.1 (Iwata, 2005).

The values for each genet used for the analysis were calculated as the average values of the corresponding ramets. Variance components were calculated by using restricted maximum likelihood (REML) tests to estimate the contribution of variance among genets and among ramets within a genet to the total phenotypic variance for each trait. The variation among ramets was assumed to represent environmental variation because each ramet of a given genet has an identical genotype, whereas the variation among genets was considered to represent genetic variation. Broad-sense heritability ($H^2$) was calculated by dividing the genotypic variance by the total variance (Falconer, 1981). Phenotypic correlation matrices were calculated to explore the relationships among all traits within each morph category or across both morphs. REML tests and phenotypic correlations were performed using JMP 6.0 statistical software (SAS Institute Inc.). The interval mapping method (Lander and Botstein, 1989) was used to detect QTLs for four traits by using MapQTL ver. 5 (van Ooijen, 2004). In addition, QTLs for each trait within each morph were also analysed, because minor QTLs might not be detected in a population segregating for a qualitative locus controlling heterostyly.

Genome-wide LOD thresholds with a P-value ≤0.05 were calculated for each trait and each morph by using a permutation test with 1000 iterations. Locations with a LOD score having a P-value ≤0.20 were considered suggestive of QTLs.

Prediction of morphs with molecular markers

Using the markers that were mapped near both the S locus and the QTLs with large effects, the relationships between genotypes and morphs in the mapping population were examined. Morphs of 59 genets derived from three wild populations were predicted on the basis of the genotype of each genet. In this study, the descriptors assigned by JoinMap ver. 4 software rather than fragment length were used as the genotype of each locus.

RESULTS

Linkage map

The linkage map consisted of 14 linkage groups (LGs) covering 602.9 cM from the results of linkage analysis of 157 markers (Fig. 2). Of the 157 markers, 31 were not linked with any other markers and could not be assigned to an LG. The number of LGs (n = 14) did not correspond to the basic chromosome number of P. sieboldii (n = 12; Yamaguchi, 1973), and the length of each LG varied from 3.3 to 108.4 cM. There were several densely mapped regions. Based on the assumption that the genotypes of the short-styled and long-styled morph are Ss and ss, respectively, the S locus was mapped to LG 7. Four markers were found within 1 cM of the S locus.

QTL analysis

The mapping population exhibited bimodal distributions for stigma height and anther height (Fig. 3). These distributions were divided into two distinct groups by morphs, but there was quantitative variation in the average values of the genets for the two traits within each morph. For corolla tube length and anther length, the average values and variation within the short-styled morph were larger than those within the long-styled morph, but the distributions of the morphs overlapped with each other (Fig. 3).
FIG. 2. Linkage map of *P. sieboldii*. Abbreviations: A, mapped using all individuals from both morph classes; L, mapped within a long-styled morph; S, mapped within a short-styled morph. Thick vertical lines indicate QTLs with $P < 0.05$; double vertical lines indicate suggestive QTLs ($0.05 < P < 0.20$).
Stigma height and anther height exhibited very high heritability (0.96), indicating that these traits were not affected greatly by the surrounding environment (Table 1). Heritabilities of these two traits were larger than those of the other two traits (corolla tube length and anther length). When each morph was analysed separately, heritabilities within the long-styled morph were lower than those in the short-styled morph, with anther lengths of both long- and short-styled morphs exhibiting the lowest heritability among all the traits (0.48 and 0.57, respectively).

The phenotypic correlation between the two non-bimodal traits (corolla tube length and anther length) was examined across both morphs and was found to be weak but statistically significant (r = 0.413, P < 0.05, d.f. = 190). Because the other two traits (stigma and anther heights) exhibited a bimodal distribution, their correlations across the two morphs were not examined. Within each morph, corolla tube length was strongly correlated with anther height (r = 0.861 in the long-styled morph and r = 0.951 in the short-styled morph; Table 2). These strong correlations were thought to be due to the dependent relationship between corolla tube length and anther height, because the anther arises from inside the corolla tube. The other correlations were low (|r| < 0.7), although most of them were statistically significant (Table 2).

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From the results of the QTL analysis for both morphs together, 12 QTLs for all traits were detected in seven LGs (Table 3). QTLs for all traits were detected at the same position in LG 7. In particular, QTLs for the two traits with bimodal distribution (stigma height and anther height) exhibited extremely high LOD scores relative to the scores of other QTLs. The phenotypic variance explained (PVE) by the QTLs in LG 7 was 91.2 and 85.6% for stigma height and anther height, respectively. These QTLs were detected between the em2ca038 and ga1277 marker loci, where S was also mapped. Because no other QTLs for these two traits were detected in any LG, these QTLs are likely to control stigma height and anther height qualitatively.

<table>
<thead>
<tr>
<th>Trait</th>
<th>All</th>
<th>Long</th>
<th>Short</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corolla tube length</td>
<td>0.77</td>
<td>0.73</td>
<td>0.76</td>
</tr>
<tr>
<td>Stigma height</td>
<td>0.97</td>
<td>0.63</td>
<td>0.86</td>
</tr>
<tr>
<td>Anther height</td>
<td>0.96</td>
<td>0.69</td>
<td>0.77</td>
</tr>
<tr>
<td>Anther length</td>
<td>0.57</td>
<td>0.48</td>
<td>0.57</td>
</tr>
</tbody>
</table>

AH, anther height; AL, anther length; CTL, corolla tube length; SH, stigma height.

Significant correlations are indicated in bold (P < 0.05). Upper right side of matrix, long-styled morph; lower left side of matrix, short-styled morph.

When each morph was analysed separately, heritabilities within the long-styled morph were lower than those in the short-styled morph, with anther lengths of both long- and short-styled morphs exhibiting the lowest heritability among all the traits (0.48 and 0.57, respectively).

The phenotypic correlation between the two non-bimodal traits (corolla tube length and anther length) was examined across both morphs and was found to be weak but statistically significant (r = 0.413, P < 0.05, d.f. = 190). Because the other two traits (stigma and anther heights) exhibited a bimodal distribution, their correlations across the two morphs were not examined. Within each morph, corolla tube length was strongly correlated with anther height (r = 0.861 in the long-styled morph and r = 0.951 in the short-styled morph; Table 2). These strong correlations were thought to be due to the dependent relationship between corolla tube length and anther height, because the anther arises from inside the corolla tube. The other correlations were low (|r| < 0.7), although most of them were statistically significant (Table 2).

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From the results of the QTL analysis within each morph, one QTL for stigma height (36.3 % PVE) and two QTLs for anther height (20.8 and 15.8 % PVE) were detected in the long-styled morph (Table 3). Meanwhile, in the short-styled morph, three QTLs each for stigma height (22.4, 43.1 and 14.9 % PVE) and anther height (15.6, 24.9 and 24.8 % PVE) were detected. The QTL for stigma height was detected in a region near the \( S \) locus in LG 7. The number of QTLs affecting these two traits in the short-styled morph was greater than that in the long-styled morph. The QTLs for these two traits within the long-styled and short-styled morph explained 36.3 and 80.4 % (stigma height), respectively, and 36.6 and 65.3% (anther height), respectively, of the phenotypic variation in the mapping population. QTLs for anther height in LG 9 were found in both morphs; all of the other QTLs were morph specific.

When the traits with a continuous distribution, i.e. corolla tube length and anther length, were examined across both morphs combined, five and three QTLs, respectively, were detected in addition to the QTL in LG 7. The QTLs (including that in LG 7) cumulatively accounted for 73.0 and 51.2 % of the phenotypic variation for corolla tube length and anther length, respectively. This result indicated that these two traits were controlled by more QTLs than were the other traits (stigma height and anther height). When corolla tube length was analysed separately within each morph, there were two QTLs in the long-styled morph that together accounted for 32.0 % of the phenotypic variation and three QTLs in the short-styled morph that together accounted for 60.3 % of the phenotypic variation. For anther length, three QTLs detected in the long-styled morph together accounted for 75.1% of the phenotypic variation and one QTL in the short-styled morph accounted for 30.5 % of the phenotypic variation. One QTL each for corolla tube length and anther length was detected in LG 9 and LG 11, respectively, at the same position in both morphs, suggesting that these QTLs control each trait regardless of the morph.

**Association between morphs and molecular markers linked to the \( S \) locus**

Table 4 presents the relationships between the two morphs in the mapping population and the genotypes at each of the four marker loci mapped within 1 cM of the \( S \) locus region. There was a strong association between the genotypes at each locus and the morph type, suggesting that these loci would be effective for identifying the morphs of wild genets. By examining the linkage relationships between each allele

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**Table 3. Summary of map locations, LOD scores and contributions of QTLs obtained by interval mapping**

<table>
<thead>
<tr>
<th>Morph</th>
<th>Linkage group</th>
<th>Position (cM)</th>
<th>Nearest marker</th>
<th>Maximum LOD score*</th>
<th>( P )-value</th>
<th>PVE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stigma height</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Both morphs</td>
<td>7</td>
<td>83-13</td>
<td>ga1277</td>
<td>100-11 (37.2)</td>
<td>0.000</td>
<td>91.2</td>
</tr>
<tr>
<td>Long-styled morph</td>
<td>3</td>
<td>58-58</td>
<td>106I23</td>
<td>3.27 (3-8)</td>
<td>0.142</td>
<td>36-3</td>
</tr>
<tr>
<td>Short-styled morph</td>
<td>4</td>
<td>65-14</td>
<td>ga9008</td>
<td>4.23 (3-8)</td>
<td>0.026</td>
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<tr>
<td></td>
<td>7</td>
<td>75-50</td>
<td>ga1000</td>
<td>5.97 (3-8)</td>
<td>0.003</td>
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</tr>
<tr>
<td></td>
<td>11</td>
<td>19-35</td>
<td>ga1508</td>
<td>3.19 (3-8)</td>
<td>0.162</td>
<td>14-9</td>
</tr>
<tr>
<td><strong>Anther height</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Both morphs</td>
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<td>83-13</td>
<td>ga1277</td>
<td>79.92 (19-1)</td>
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<td>85.6</td>
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<td>2-47</td>
<td>207B22</td>
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</tr>
<tr>
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<td>11</td>
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<td>205O19</td>
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<tr>
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<td>9</td>
<td>2-47</td>
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<tr>
<td></td>
<td>12</td>
<td>6-00</td>
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<td><strong>Corolla tube length</strong></td>
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<td>5</td>
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<tr>
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<td>15-41</td>
<td>202B22</td>
<td>3.94 (3-9)</td>
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<td>Short-styled morph</td>
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LOD, logarithm of odds; PVE, phenotypic variance explained.

*Significant LOD thresholds (\( P < 0.05 \)) calculated by permutation test are in parentheses.

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of ga1277 and the allele at the S locus, we concluded that allele ‘c’ was linked to the S allele and that alleles ‘a’, ‘b’ and ‘d’ were linked to the s allele, because nearly all genets with genotypes ‘ad’ and ‘bd’ possessed the long-styled morph and nearly all genets with allele ‘c’ possessed the short-styled morph. For ga495, ga0821 and PS-4, we concluded that allele ‘g’ was linked to the S allele and alleles ‘e’ and ‘f’ were linked to the s allele. For each marker, the two genets for which the morph did not match the predicted phenotype were assumed to contain recombination between the S locus and the marker.

In 43 of the 59 wild genets tested, the morph could be predicted from the genotypes of one or more of the four loci (Table 5). Of these genets for which a prediction was made, only one genet (H-7) in the Hokkaido population had a morph different from that expected on the basis of genotype. Meanwhile, we could not predict the morphs of the other 16 genets because these genets had alleles that were not detected in the mapping population and exhibited an unclear linkage relationship with the S or s alleles. The number of previously undetected alleles (designated ‘u’) ranged from two for the PS-4 locus to 13 for the ga0821 locus. Many alleles not found in the mapping population were detected in the Saitama populations, and the prediction accuracy (31%) was considerably lower than those of the Hokkaido (88%) and Nagano (82%) populations.

**DISCUSSION**

**Linkage map and QTLs for floral morphology**

In this study, a *P. sieboldii* map containing 14 linkage groups (602.9 cM) was constructed. This is the first linkage map of wild *P. sieboldii*. Although the map contained more linkage groups than the actual number of chromosomes of *P. sieboldii* (n = 12), it should prove to be a very effective map for identification of the S locus region. This map enabled us to detect QTLs for floral morphological traits, and it will play an important role in future studies of floral morphology and self-incompatibility in *Primula*.

Corolla tube length and anther length, which exhibited a continuous distribution in the mapping population, were controlled by a number of QTLs with small effects (PVE <20% for each one; Table 3), suggesting that these traits are polygenic characters. In contrast, stigma height and anther height all had bimodal distributions and high broad-sense heritabilities (H² > 0.95; Fig. 3 and Table 1), suggesting the existence of a major gene controlling these traits. Thus, for each of these two traits, we detected a QTL with a large LOD score and high PVE (85.6 and 96.1%) in the same region of LG 7 in which the S locus was mapped. These results revealed that the S locus of *P. sieboldii* is located in LG 7. Our results support many previous studies (Barret and Shore, 2008) that demonstrated that heterostyly of *Primula* is controlled by a single locus. This locus has been called the S locus, which is actually a supergene in which genes controlling stigma height and anther height are tightly linked. Although QTLs for corolla tube length and anther length were also detected in LG 7, the PVE by these QTLs was much smaller than that by the QTLs for the other three traits related to heterostyly. The
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<td>f₁u₁₂</td>
<td>f₁u₁₂</td>
<td></td>
</tr>
<tr>
<td>Nagano</td>
<td>N-15</td>
<td>Long</td>
<td>u₁₂u₁₂</td>
<td>u₁₂u₁₂</td>
<td>u₁₂u₁₂</td>
<td>f₁u₁₂</td>
<td>f₁u₁₂</td>
<td></td>
</tr>
<tr>
<td>Nagano</td>
<td>N-16</td>
<td>Short</td>
<td>bu₁₂</td>
<td>u₁₂u₁₂</td>
<td>u₁₂u₁₂</td>
<td>f₁u₁₂</td>
<td>f₁u₁₂</td>
<td></td>
</tr>
<tr>
<td>Nagano</td>
<td>N-17</td>
<td>Short</td>
<td>bu₁₂</td>
<td>u₁₂u₁₂</td>
<td>u₁₂u₁₂</td>
<td>f₁u₁₂</td>
<td>f₁u₁₂</td>
<td></td>
</tr>
</tbody>
</table>

Long, long-styled morph; short, short-styled morph.

Bold letters indicates the genotypes useful for morph identification.

*Morph identified by visual confirmation.

† 'u' indicates alleles that were not detected in the mapping population. ‘–’ indicates a possible null genotype.
‡ '×' indicates morphs that could not be predicted from the genotypes of the four markers. # indicates morph for which the predicted type was different from the observed type.

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low PVE values revealed that these QTLs were different from the S locus and that there were other loci controlling corolla tube length and anther length in LG 7.

For the two traits controlled by a single QTL with large effects (stigma height and anther height), one or more additional QTLs were detected in each morph (Table 3). Among the nine QTLs for these two traits, only the QTLs for anther height in LG 9 were detected in both morphs, suggesting that the QTL with PVE of approx. 20–25% controlled anther height regardless of the morph, and the other seven QTLs were specific to a morph. We suggest, therefore, that these specific QTLs are involved in the variation observed for each trait within a particular morph. Of particular interest is the fact that these three traits are controlled by the S locus in LG 7 as well as by several QTLs in other LGs. These additional QTLs are likely to generate a wide range of variation within each morph.

The finding of possible morph-specific QTLs is also interesting. If these QTLs are actually specific to a morph, we will have confirmed a significant effect of interaction between the S locus and the QTLs. However, at this stage, our mapping approach (interval-mapping method) does not allow us to resolve such an interaction. Once a finer linkage map is available to resolve these narrow QTL regions, we will be able to confirm or refute these putative morph-specific QTLs.

For *P. sieboldii*, the stigma in the long-styled morph can more easily receive legitimate pollen as compared with the short-styled morph because a taller stigma makes better contact with the bodies of pollinators (Matsumura and Washitani, 2002). In addition, Nishihiro et al. (2000) suggested the possibility of natural selection for higher stigma height of short-styled morphs. Thus, it is thought that stigma height might be associated with female fitness and could become a target of natural selection. In this study, we detected three QTLs for stigma height in the short-styled morph, all of which differed from the single QTL found in the long-styled morph (Table 3), indicating that continuous variation in stigma height within the short-styled morph was caused by several QTLs specific to that morph. These loci might be a target of natural selection.

Although, to the best of our knowledge, our study is the first QTL analysis of floral morphology in a heterostylos species, the inheritance of floral morphology and heterostyly has been studied in many species, including the genera *Primula*, *Lythrum* and *Turnera* (Barrett and Shore, 2008). Recently, researchers have also attempted to identify and clone genes that control heterostyly quantitatively, we performed QTL analysis from a different perspective from that in previous studies. Our results indicated that corolla tube length and anther length were controlled by a number of QTLs with small effects (Table 3). We revealed that stigma height and anther height, both of which are most closely related to heterostyly, are controlled qualitatively by a single major QTL in LG 7, but also modified quantitatively by several QTLs with small effects. In addition, the existence of some QTLs other than the S locus in *P. sieboldii* could be proved, as Mather (1950) suggested the presence of two genes controlling stigma and anther height other than the S locus in *P. sinensis*. Our genome-wide QTL mapping will be a useful tool for interpreting floral evolution, especially the evolution of heterostyly.

**Effectiveness of morph identification using molecular markers**

From the genotypes of four loci (Table 5), the morphs of 43 genets from three wild populations could be predicted. Of these 43 genets, only the genet ‘H-7’ from Hokkaido had a genotype that did not predict the actual morph. The most likely explanation for this apparent discrepancy is recombination between the ‘PS-4’ locus and the S locus, which are separated by 0.66 cM. Considering that recombination occurred in only two out of 190 genets in the original mapping population (Table 4), we believe that morph prediction using these four markers is an effective method to investigate the ratio of morphs in wild populations. Although the short-styled morph is expected to be heterozygous (genotype ‘S’s’) at the S locus, there were seven short-styled genets from the Hokkaido population (27%) that scored as having a homozygous SS genotype (genotype ‘gg’) at the ga0821 locus. Based on the map distance between the S locus and the ga0821 locus (0.658 cM), recombination between the two loci does not occur frequently. Instead, the most likely explanation is that these genets have null alleles (i.e. alleles with no DNA fragment amplification because of sequence polymorphism in the primer sites) at the ga0821 locus, causing some plants heterozygous for the ‘g’ allele to appear homozygous. When inbreeding coefficients within each population (1 – H/S; where Hs is the observed heterozygosity and Hs is the expected heterozygosity) were calculated using the investigated wild genets, values of –0.045, 0.053, 0.474 and –0.050 were found for ga1277, ga0495, ga0821 and PS-4, respectively. The value for ga0821 was high compared with those of the other marker loci. Because inbreeding affects the whole genome and results in increased inbreeding coefficients for all marker loci (not just one, as observed here), the notably higher inbreeding coefficient for ga0821 might not be the result of inbreeding. This result provides further evidence that null alleles exist in ga0821 in wild populations and that nulls scored as ‘gg’, ‘ee’ and ‘ff’ may in fact be ‘g/null’, ‘e/null’ and ‘f/null’, respectively. Although allele ‘g’ is linked to the S allele and genotype ‘g/null’ does not directly affect the morph prediction because it would be short-styled in any case, genets with genotypes ‘e/null’ and ‘f/null’ would not necessarily be long-styled (i.e. because the null allele could be an S allele). For this reason, it would be better not to use genotypes ‘ee’ and ‘ff’ in ga0821 for morph prediction. The morphs of some genets, for example H-12, could be predicted on the basis of the genotypes at PS-4. Because the use of loci at which null alleles are detected reduces the accuracy of morph prediction, it is better to predict the morphs by using other markers.

In this study, morphs were predicted in the wild genets using only alleles that were previously detected in the mapping population and clearly related to either the S or s alleles of the S locus (Table 4). Because SSR markers are highly polymorphic, a given locus may have many alleles, some of which are unsuitable for morph prediction. To solve this problem, EST or SNP markers that have alleles common
across wild populations might be more effective for morph identification than are SSRs.

The proportion of genets for which the morph could be predicted differed greatly among the three populations (Table 5). The proportion of Saitama genets that could be predicted was noticeably lower than that of the other two populations, and only five genotypes could be predicted. The difference in prediction accuracy between Saitama and the other two populations may be due to the materials we used for mapping. The parents of the mapping population originated from populations near where the genets of the Hokkaido and Nagano wild populations were sampled. Because the wild Saitama population was not located near the Hokkaido and Nagano populations, there were many undetected alleles (i.e. alleles that were not detected in the mapping population) in the Saitama population; thus, the estimation accuracy was low. However, we could estimate the relationship between a particular previously undetected allele and the alleles at the $S$ locus, as all genets with allele ‘u1’ at locus ga0495 were observed to be short-styled, and thus this allele is thought to be linked to allele $S$. Therefore, previously undetected alleles can be useful for morph estimation in wild populations when we can directly observe the morph for a sufficient number of individuals and correlate it to the genotype.

In this study, four markers linked to the $S$ locus were proven to be effective tools for predicting the morph of *P. sieboldii*. For population management, the ratio of morph types of non-flowering individuals and during the non-flowering period can be monitored with these useful markers.

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**LITERATURE CITED**


