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Original Article

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

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Running title: QTL analysis of heterostyly in *P. sieboldii*

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1 **Abstract**

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

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

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

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

23

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

1 **INTRODUCTION**

2 Charles Darwin (1877) first suggested that heterostyly would promote cross-pollination
3 between floral morphs by insect pollinators and that natural selection could result in
4 reciprocal positions of stigma and anther between morphs; this reciprocal positioning is an
5 adaptation beneficial for promoting cross-pollination. Since then, the inheritance and
6 molecular mechanisms of heterostyly have been studied in many heterostylous plant species,
7 such as those belonging to the genera *Primula*, *Turnera*, and *Fagopyrum* (Barrett and Shore,
8 2008). Heterostyly and self-incompatibility are controlled by the *S* locus, which is a supergene
9 complex consisting of at least three to nine genes with diallelic polymorphisms in each one
10 (Dowrick, 1956; Richards, 1997). It is presumed that these linked genes control not only
11 morphological traits such as stigma or anther length, but also physiological traits related to
12 self-incompatibility. Recently, with the development of new molecular techniques, molecular
13 markers closely linked to the *S* locus have been identified in *Primula vulgaris* (Manfield *et al.*,
14 2005, Li *et al.,* 2007).

15 Many studies have been performed on heterostyly in the genus *Primula*. The sexual
16 reproduction system of *Primula sieboldii* is characterised by typical heteromorphic
17 self-incompatibility. Populations of *P. sieboldii* contain mainly two different forms (morphs)
18 of flowers, long-styled and short-styled, and produce seeds only by cross-pollination between
19 different morphs (usually called *legitimate pollination*). Washitani *et al.* (1994) and Nishihiro
20 *et al.* (2000) indicated that both the stigma and anther exhibited continuous height variations
21 within a wild population of *P. sieboldii*, and the relative heights of the stigma and anthers
22 within a single plant (here termed 'relative position') also varied rather continuously. In
23 addition, Nishihiro *et al.* (2000) suggested that these floral morphological traits were targets
24 of natural selection. Moreover, continuous variation of floral morphology or relative position
25 was also reported in some distylous species, such as *Menyanthes trifoliata* (Thompson *et al.*,

1 1998), *Amsinckia spectabilis* (Bartkowska and Johnston, 2009), and *Bouvardia ternifolia*,
2 *Psychotria poeppigiana*, and *Psychotria chiapensis* (Faivre and McDade, 2001).
3 These previous studies indicate that variation of the relative position within each morph
4 could not be explained by an *S* locus alone; thus, we hypothesised that floral morphological
5 traits related to heterostyly are controlled not only by an *S* locus, but also by several
6 quantitative trait loci (QTLs). Mather (1950) suggested the presence of two genes controlling
7 stigma and anther height other than the *S* locus, and this result supports our hypothesis. Thus
8 far, DeWinton and Haldane (1935) performed linkage analysis of various traits including
9 heterostyly in *P. sinensis*. Although they constructed the first linkage map for distylous
10 species, these traits were not analysed quantitatively, and this map was a partial map and not a
11 genome-wide map. For these reasons, the first aims of the present study were to perform QTL
12 analysis on four floral morphological traits and identify the location of the *S* locus to
13 understand the genetic foundation of floral morphological traits related to heterostyly.
14 *Primula sieboldii* E. Morren, a perennial clonal herb, is distributed in Japan, Korea,
15 northern China, and eastern Siberia (Yamazaki, 1993). It occupies a range of moist habitats,
16 from the understory of deciduous forests to well-managed grasslands. Overexploitation and
17 destruction of its habitats and horticultural collection are threatening wild populations with
18 extinction, and the species has become endangered in Japan; originally classified in the lower
19 rank of ‘vulnerable’, the species was reclassified to ‘near threatened’ in 2007 (Iwatsuki, 2008).
20 Seed production is limited in small populations in which the ratio of the two morphs is
21 imbalanced; this ratio is very important for reproductive success (Matsumura and Washitani,
22 2000). Based on a computer simulation, Washitani (1996) suggested that a decrease in the
23 frequency of short-styled morphs would caused inbreeding depression and genetic bottleneck
24 within a population, and proposed that the ratio of morphs in a population be monitored as a
25 means of active management. The second aim in the present study was to develop molecular

1 markers associated with the two morphs in a distylous wild population. These markers are
2 likely to be useful tools for monitoring the ratio of morphs in a population containing the
3 non-flowering ramets or during seasons when the ramets are not flowering.
4
5 **MATERIALS AND METHODS**
6 *Mapping population*
7 To construct a linkage map and perform QTL analysis, a mapping population (192
8 genets) was obtained using a four-way cross of four wild genets: HP05 and HT02, derived
9 from a population in Hidaka (42° 29′N, 142° 07′E), and YT112 and YP108, derived from a
10 population in Yatsugatake (35° 57′N, 138° 27′E). H and Y indicate the populations in Hidaka
11 and Yatsugatake, respectively. We selected these two populations, which differed between
12 each other by several microsatellite (SSR) markers (Honjo *et al*., 2009), and expected many
13 polymorphisms between these populations in the whole genome. T and P indicate short-styled
14 and long-styled morphs, respectively. Two F1 individuals, '5G14' and '2B1', were selected
15 from the progeny of crosses 'HP05 × YT112' and 'HT02 × YP108', respectively. 5G14 and
16 2B1 were then crossed to produce 192 genets as the second generation.
17
18 *Samples for morph prediction with molecular markers*
19 To examine the possibility of morph prediction with molecular markers, we used 59
20 genets of *P. sieboldii* from three wild populations in three regions of Japan: 26 from Hokkaido
21 (a lowland area in northern Japan, 42° 31′N, 142° 01′E,), 16 from Saitama (a lowland area in
22 central Japan), and 17 from Nagano (a highland area in central Japan, 36° 19′N, 138° 38′E).
23 The Hokkaido and Nagano populations were located near the populations from which the
24 mapping parents were sampled. For the Saitama population (35° 50′N, 139° 36′E), we used
25 genets that had been grown from seeds obtained from a wild population in the Saitama region

1 in a previous experiment (Washitani and Kabaya, 1988).

2

3 *DNA extraction and genotyping*

4 We extracted genomic DNA from fresh leaves of each plant by using a modified CTAB
5 method (Murray and Thompson, 1980). The genotypes of the 192 genets from the mapping
6 population were determined with 140 pairs of SSR primers (Isagi *et al.*, 2001; Ueno *et al.*,
7 2003; Kitamoto *et al.*, 2005; Ueno *et al.*, 2005; Ueno *et al.*, 2009), 23 pairs of expressed
8 sequence tag-simple sequence repeat (EST-SSR) primers (Ueno *et al.*, unpubl. res.), and 40
9 single nucleotide polymorphism (SNP) primers (Ueno *et al.*, unpubl. res.). The PCR
10 conditions for SSR primers followed the protocol described by Ueno *et al.* (2003, 2009). The
11 PCR products were analysed using an ABI PRISM® 3100 Genetic Analyzer and GeneMapper
12 software ver. 3.7 (Applied Biosystems). To map the *S* locus region, we also attempted to use
13 the primers that were closely linked to the *S* locus reported by Li *et al*. (2007), but these
14 primers did not amplify any DNA fragment from *P. sieboldii*.

15

16 *Construction of the linkage map*

17 Segregation data for the 157 markers indicated polymorphism between the parents and
18 no segregation distortion ($\chi 2$ goodness of fit; $P < 0.01$). We used JoinMap ver. 4 (van Ooijen,
19 2006) with the population type 'CP' for constructing the linkage map. The linkage map was
20 constructed using regression mapping with Haldane's function and the default setting in
21 JoinMap. The grouping of markers was performed at logarithm of odds (LOD) threshold ≥ 3.

22 Washitani *et al*. (1994) reported that self- or non-self intramorph pollination (illegitimate
23 pollination) resulted in no or little seed set in the same genet and indicated high
24 self-incompatibility in *P. sieboldii*. From this result, we considered that heterostyly in *P.*
25 *sieboldii* with self-incompatibility is assumed to also 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; 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.

We measured four floral morphological traits (Figure 1): corolla tube length, stigma height and anther height (maximum heights from the base of ovary), and anther length. After 1 week of flowering, the first flower of each ramet was photographed alongside a scale marker (9 mm × 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. We calculated variance components 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

1 genetic variation. We calculated broad-sense heritability (H^2) by dividing the genotypic
2 variance by the total variance (Falconer, 1981). Phenotypic correlation matrices were
3 calculated to explore the relationships among all traits within each morph category or across
4 both morphs. REML tests and phenotypic correlations were performed using JMP 6.0
5 statistical software (SAS Institute Inc.). The interval mapping method (Lander and Botstein,
6 1989) was used to detect QTLs for four traits by using MapQTL ver. 5 (van Ooijen, 2004). In
7 addition, we also analyzed QTLs for each trait within each morph, because minor QTLs might
8 not be detected in a population segregating for a qualitative locus controlling heterostyly.
9 Genome-wide LOD thresholds for with a P-value $\leq$ 0.05 were calculated for each trait and
10 each morph by using a permutation test with 1000 iterations. Locations with a LOD score
11 having a P-value $\leq$ 0.20 were considered suggestive QTLs.
12
13 *Prediction of morphs with molecular markers*
14 Using the markers that were mapped near both the *S* locus and the QTLs with large
15 effects, we examined the relationships between genotypes and morphs in the mapping
16 population. Morphs of 59 genets derived from three wild populations were predicted on the
17 basis of the genotype of each genet. In this study, we used the descriptors assigned by
18 JoinMap ver. 4 software rather than fragment length as the genotype of each locus.
19
20 **RESULTS**
21 *Linkage map*
22 The linkage map consisted of 14 linkage groups (LGs) covering 602.9 cM from the
23 results of linkage analysis of 157 markers (Figure 2). Of the 157 markers, 31 were not linked
24 with any other markers and could not be assigned to a linkage group. The number of linkage
25 groups (n = 14) did not correspond to the basic chromosome number of *P. sieboldii* (n = 12;

1 Yamaguchi, 1973), and the length of each linkage group varied from 3.3 cM to 108.4 cM.
2 There were several densely mapped regions. Based on the assumption that the genotypes of
3 the short-styled and long-styled morph are *Ss* and *ss*, respectively, the *S* locus was mapped to
4 LG 7. Four markers were found within 1 cM of the *S* locus.
5
6 *QTL analysis*
7 The mapping population exhibited bimodal distributions for stigma height and anther
8 height (Figure 3). These distributions were divided into two distinct groups by morphs, but
9 there was quantitative variation in the average values of the genets for the two traits within
10 each morph. For corolla tube length and anther length, the average values and variation within
11 the short-styled morph were larger than those within the long-styled morph, but the
12 distributions of the morphs overlapped with each other (Figure 3).
13 Stigma height and anther height exhibited very high heritability (>0.96), indicating that
14 these traits were not affected greatly by the surrounding environment (Table 1). Heritabilities
15 of these two traits were larger than those of the other two traits (corolla tube length and anther
16 length). When each morph was analysed separately, heritabilities within the long-styled
17 morph were lower than those in the short-styled morph, with anther lengths of both long- and
18 short-styled morphs exhibiting the lowest heritability among all the traits (0.48 and 0.57,
19 respectively).
20 The phenotypic correlation between the two non-bimodal traits (corolla tube length and
21 anther length) was examined across both morphs and was found to be weak but statistically
22 significant (r = 0.413, $P < 0.05$, df = 190). Because the other two traits (stigma and anther
23 heights) exhibited a bimodal distribution, we did not examine their correlations across the two
24 morphs. Within each morph, corolla tube length was strongly correlated with anther height (r
25 = 0.861 in the long-styled morph, and 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).

From the results of the QTL analysis for both morphs together, 12 QTLs for all traits were detected in seven linkage groups (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 linkage group, these QTLs are likely to qualitatively control stigma height and anther height.

From the results of the QTL analysis within each morph, we detected one QTL for stigma height (36.3% PVE) and two QTLs for anther height (20.8% and 15.8% PVE) 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 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

1 were examined across both morphs combined, five and three QTLs, respectively, were
2 detected in addition to the QTL in LG 7. The QTLs (including that in LG 7) cumulatively
3 accounted for 73.0% and 51.2% of the phenotypic variation for corolla tube length and anther
4 length, respectively. This result indicated that these two traits were controlled by more QTLs
5 than were the other traits (stigma height and anther height). When corolla tube length was
6 analysed separately within each morph, there were two QTLs in the long-styled morph that
7 together accounted for 32.0% of the phenotypic variation and three QTLs in the short-styled
8 morph that together accounted for 60.3% of the phenotypic variation. For anther length, three
9 QTLs detected in the long-styled morph together accounted for 75.1% of the phenotypic
10 variation and one QTL in short-styled morph accounted for 30.5% of the phenotypic variation.
11 One QTL each for corolla tube length and anther length was detected in LG 9 and LG 11,
12 respectively, at the same position in both morphs, suggesting that these QTLs control each
13 trait regardless of the morph.
14
15 *Association between morphs and molecular markers linked to the S locus*
16 Table 4 presents the relationships between the two morphs in the mapping population
17 and the genotypes at each of the four marker loci mapped within 1 cM of the *S* locus region.
18 There was a strong association between the genotypes at each locus and the morph type,
19 suggesting that these loci would be effective for identifying the morphs of wild genets. By
20 examining the linkage relationships between each allele of ga1277 and the allele at the *S* locus,
21 we concluded that allele ‘c’ was linked to the *S* allele and that alleles ‘a’, ‘b’, and ‘d’ were
22 linked to the *s* allele, because nearly all genets with genotypes ‘ad’ and ‘bd’ possessed the
23 long-styled morph and nearly all genets with allele ‘c’ possessed the short-styled morph. For
24 ga495, ga0821, and PS-4, we concluded that allele ‘g’ was linked to the *S* allele and alleles ‘e’
25 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 2 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, we constructed a *P. sieboldii* map containing 14 linkage groups (602.9 cM). 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

1 heritabilities (H^2 > 0.95) (Figure 3 and Table 1), suggesting the existence of a major gene
2 controlling these traits. Thus, for each of these two traits, we detected a QTL with a large
3 LOD score and high PVE (85.6 and 96.1%) in the same region of LG 7 in which the *S* locus
4 was mapped. These results revealed that the *S* locus of *P. sieboldii* is located in LG 7. Our
5 results support many previous studies (Barret and Shore, 2008) that demonstrated that
6 heterostyly of *Primula* is controlled by a single locus. This locus has been called the *S* locus,
7 which is actually a supergene in which genes controlling stigma height and anther height are
8 tightly linked. Although QTLs for corolla tube length and anther length were also detected in
9 LG 7, the PVE by these QTLs was much smaller than that by the QTLs for the other three
10 traits related to heterostyly. The low PVE values revealed that these QTLs were different from
11 the *S* locus and that there were other loci controlling corolla tube length and anther length in
12 LG 7.
13 For the two traits controlled by a single QTL with large effects (stigma height and anther
14 height), one or more additional QTLs were detected in each morph (Table 3a, 3b). Among the
15 nine QTLs for these two traits, only the QTLs for anther height in LG 9 were detected in both
16 morphs, suggesting that the QTL with PVE of approximately 20–25% controlled anther
17 height regardless of the morph, and the other seven QTLs were specific to a morph. We
18 suggest, therefore, that these specific QTLs are involved in the variation observed for each
19 trait within a particular morph. Of particular interest is the fact that these three traits are
20 controlled by the *S* locus in LG 7 as well as by several QTLs in other LGs. These additional
21 QTLs are likely to generate a wide range of variation within each morph. The finding that
22 suggested the existence of morph-specific QTLs is also interesting. If these QTLs are actually
23 specific to a morph, we could detect a significant effect of interaction between the *S* locus and
24 the QTLs. However, we could not detect the interaction effect exactly for the following two
25 reasons. The first reason is because we use "Interval-mapping method", in which QTL is

assumed to be existed between two linked marker loci, and is estimated using likelihood ratio tests. Therefore, the genotypes of the marker loci are not correspond with the genotype of morph-specific QTL itself. The second reason is because the recombination occurs between QTL and a locus depending on the marker distance, we consider that this linkage map with some large gaps is insufficient for examining the relationships with *S* locus. In the future, we would like to try to examine the interaction between the genotypes of *S* locus and QTLs by using statistical test (i.e. two-way ANOVA, when we are able to construct a finer linkage and narrow the QTL region).

For *P. sieboldii*, the stigma in the long-styled morph can more easily receive legitimate pollen as compared to 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 3a), 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 best of our knowledge, our study is the first QTL analysis of floral morphology in a heterostylous 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 *S* genes from these species, evaluating heterostyly as mainly a qualitative trait. Here, to quantitatively evaluate floral morphology related to heterostyly, we performed QTL analysis in a different perspective from previous studies. Our results indicated that corolla tube length

1 and anther length were controlled by a number of QTLs with small effects (Table 3d, 3e). We
2 revealed that stigma height and anther height, both of which are most closely related to
3 heterostyly, are controlled qualitatively by a single major QTL in LG 7, but also modified
4 quantitatively by several QTLs with small effects. In addition, the existence of some QTLs
5 other than the *S* locus in *P. sieboldii* could be proved as Mather (1950) suggested the presence
6 of two genes controlling stigma and anther height other than the *S* locus in *P. sinensis*. Our
7 genome-wide QTL mapping will be a useful tool for interpreting floral evolution, especially
8 the evolution of heterostyly.
9
10 *Effectiveness of morph identification using molecular markers*
11 From the genotypes of four loci (Table 5), we could predict the morphs of 43 genets from
12 three wild populations. Of these 43 genets, only the genet 'H-7' from Hokkaido had a
13 genotype that did not predict the actual morph. The most likely explanation for this apparent
14 discrepancy is recombination between the 'PS-4' locus and *S* locus, which are separated by
15 0.66 cM. Considering that recombination occurred in only 2 out of 190 genets in the original
16 mapping population (Table 4), we believe that morph prediction using these four markers is
17 an effective method to investigate the ratio of morphs in wild populations. Although the
18 short-styled morph is expected to be heterozygous (genotype '*Ss*') at the *S* locus, there were
19 seven short-styled genets from the Hokkaido population (27%) that scored as having a
20 homozygous *SS* genotype (genotype 'gg') at the ga0821 locus. Based on the map distance
21 between the *S* locus and the ga0821 locus (0.658 cM), recombination between the two loci
22 does not occur frequently. Instead, the mostly likely explanation is that these genets have null
23 alleles (i.e. alleles with no DNA fragment amplification because of sequence polymorphism in
24 the primer sites) at the ga0821 locus, causing some plants heterozygous for the 'g' allele to
25 appear homozygous. When inbreeding coefficients within each population (1-H_o/H_e; H_o:

1 observed heterozygosity, H_e: expected heterozygosity) were calculated using the investigated
2 wild genets, we found values of -0.045, 0.053, 0.474, and -0.050 for ga1277, ga0495, ga0821,
3 and PS-4, respectively. The value for ga0821 was high compared to those of the other marker
4 loci. Because inbreeding affects the whole genome and results in increased inbreeding
5 coefficients for all marker loci (not just one, as observed here), the notably higher inbreeding
6 coefficient for ga0821 might not be the result of inbreeding. This result provides further
7 evidence that null alleles exist in ga0821 in wild populations and that genotypes scored as
8 'gg', 'ee', and 'ff' may in fact be 'g/null', 'e/null', and 'f/null', respectively. Although allele
9 'g' is linked to the *S* allele and genotype 'g/null' does not directly affect the morph prediction
10 because it would be short-styled in any case, genets with genotypes 'e/null' and 'f/null' would
11 not necessarily be long-styled (i.e. because the null allele could be an *S* allele). For this reason,
12 it would be better not to use genotypes 'ee' and 'ff' in ga0821 for morph prediction. The
13 morphs of some genets, for example H-12, could be predicted on the basis of the genotypes at
14 PS-4. Because the use of loci at which null alleles are detected reduces the accuracy of morph
15 prediction, it is better to predict the morphs by using other markers.
16 In this study, morphs were predicted in the wild genets using only alleles that were
17 previously detected in the mapping population and clearly related to either the *S* or *s* alleles of
18 the *S* locus (Table 4). Because SSR markers are highly polymorphic, a given locus may have
19 many alleles, some of which are unsuitable for morph prediction. To solve this problem, EST
20 or SNP markers that have alleles common across wild populations might be more effective for
21 morph identification than are SSRs.
22 The proportion of genets for which the morph could be predicted differed greatly among
23 the three populations (Table 5). The proportion of Saitama genets that could be predicted was
24 noticeably lower than that of the other two populations, and only five genotypes could be
25 predicted. The difference in prediction accuracy between Saitama and the other two

1 populations may be due to the materials we used for mapping. The parents of the mapping
2 population originated from populations near where the genets of the Hokkaido and Nagano
3 wild populations were sampled. Because the wild Saitama population was not located near the
4 Hokkaido and Nagano populations, there were many undetected alleles (i.e. alleles that were
5 not detected in the mapping population) in the Saitama population; thus, the estimation
6 accuracy was low. However, we could estimate the relationship between a particular
7 previously undetected allele and the alleles at the *S* locus, as all genets with allele 'u_1' at locus
8 ga0495 were observed to be short-styled, and thus, this allele is thought to be linked to allele
9 *S*. Therefore, previously undetected alleles can be useful for morph estimation in wild
10 populations when we can directly observe the morph for a sufficient number of individuals
11 and correlate it to the genotype.
12 In this study, four markers linked to the *S* locus were proven to be effective tools for
13 predicting the morph of *P. sieboldii*. For population management, we can monitor the ratio of
14 morph types of non-flowering individuals and during the non-flowering period with these
15 useful markers.
16

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5

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5
6

1 **Figure captions**

2 **FIG. 1.** Schematic diagram of the four floral traits investigated in this study. (1), corolla tube
3 length; (2), stigma height; (3), anther height; and (4), anther length.

4

5 **FIG. 2.** Linkage map of *P. sieboldii*. Abbreviations: A, mapped using all individuals from both
6 morph classes; L, mapped within long-styled morph; S, mapped within short-styled morph.
7 Thick vertical lines indicate QTLs with $P < 0.05$; double vertical lines indicate suggestive
8 QTLs ($0.05 < P < 0.20$).

9

10 **FIG. 3.** Frequency distributions of the four traits assessed in the mapping population. Black
11 bars, short-styled morph (Short); grey bars, long-styled morph (Long). Values next to the keys
12 for each graph show average values and variances (in parentheses).

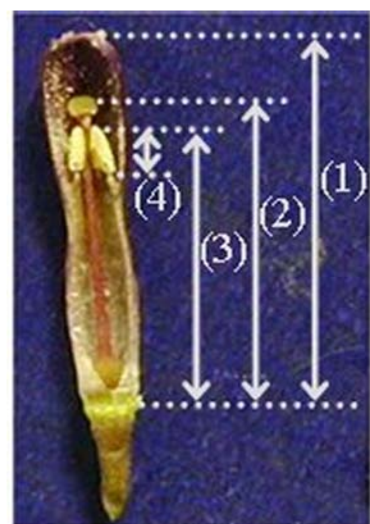

FIG. 1

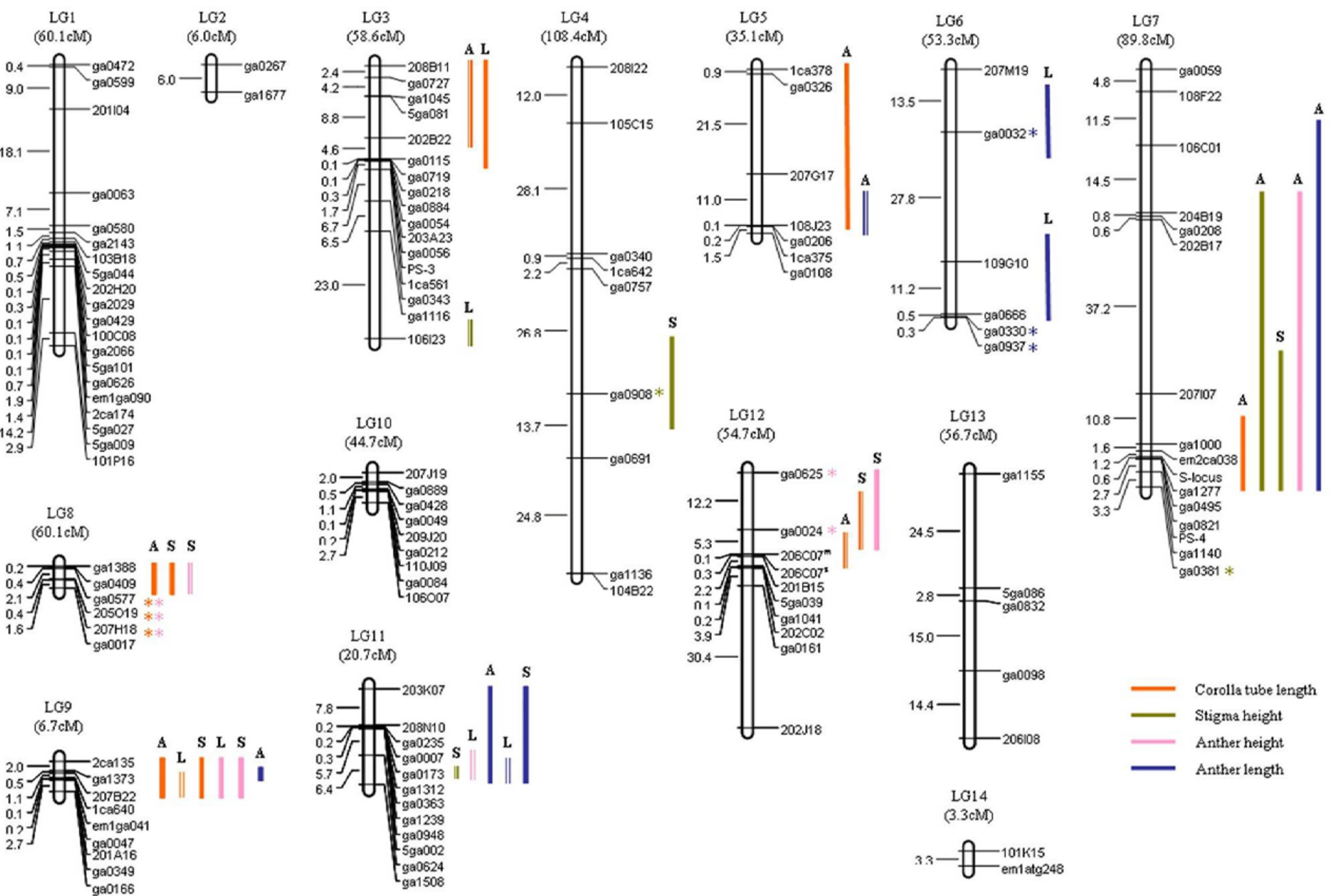

FIG. 2

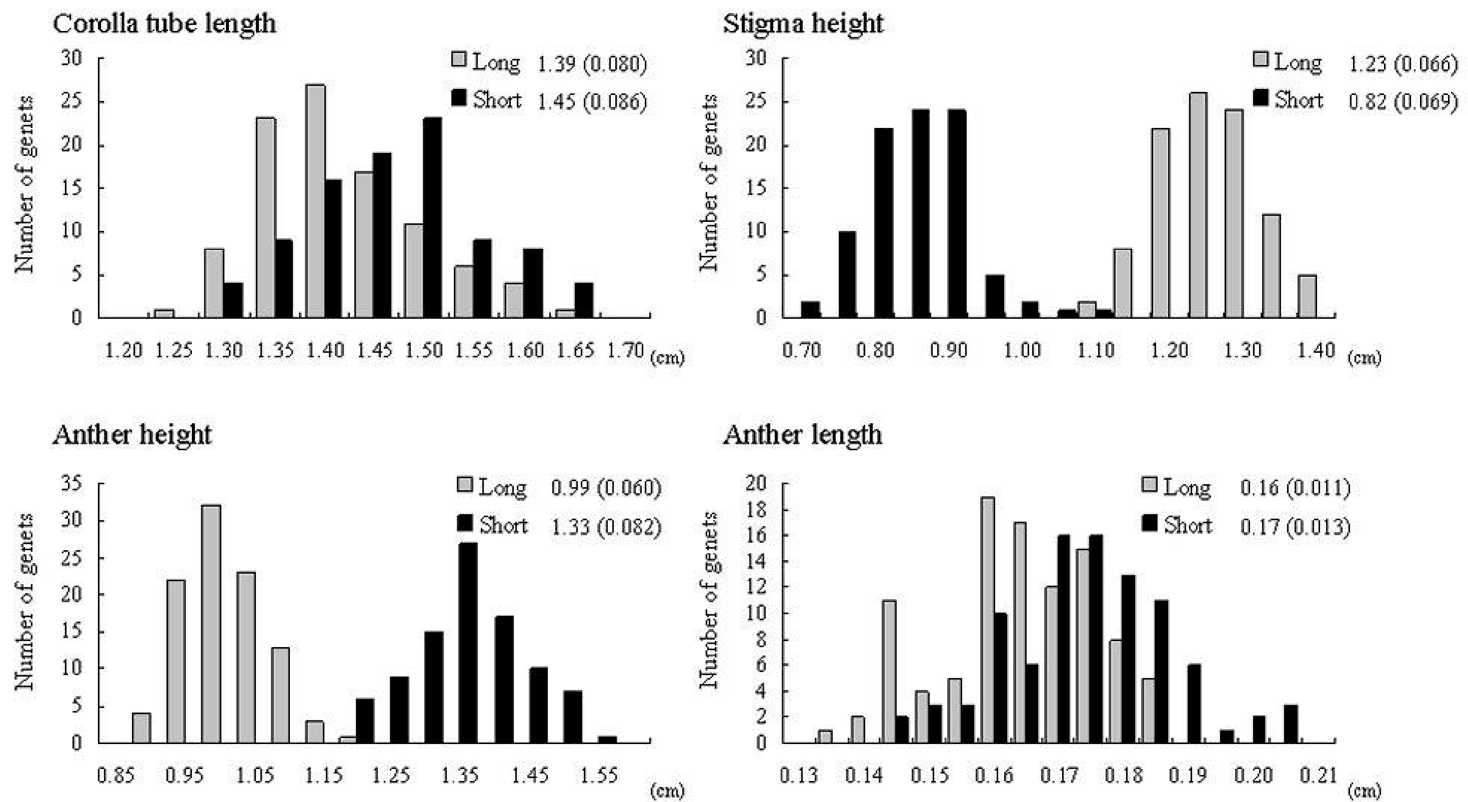

FIG. 3

TABLE 1. *Broad-sense heritability (H^2) for traits measured in this study*

Trait	All	Long	Short
Corolla tube length	0.77	0.73	0.76
Stigma height	0.97	0.63	0.86
Anther height	0.96	0.69	0.77
Anther length	0.57	0.48	0.57

Abbreviations: All, all individuals; Long, long-styled morph; Short, short-styled morph

TABLE 2. *Phenotypic correlation matrix for the four traits within each morph*

	CTL	SH	AH	AL
CTL	-	**0.358**	**0.861**	0.209
SH	**0.572**	-	**0.357**	**0.278**
AH	**0.951**	**0.606**	-	**0.440**
AL	**0.461**	**0.303**	**0.549**	-

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

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

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

a) Stigma height

Morph	Linkage group	Position (cM)	Nearest marker	Maximum LOD score[a]	P-value	PVE (%)
Both morphs	7	83.13	ga1277	100.11 (37.2)	0.000	91.2
Long-styled morph	3	58.58	106I23	3.27 (3.8)	0.142	36.3
Short-styled morph	4	65.14	ga0908	4.23 (3.8)	0.026	22.4
	7	75.50	ga1000	5.97 (3.8)	0.001	43.1
	11	19.35	ga1508	3.19 (3.8)	0.162	14.9

b) Anther height

Morph	Linkage group	Position (cM)	Nearest marker	Maximum LOD score[a]	P-value	PVE (%)
Both morphs	7	83.13	ga1277	79.92 (19.1)	0.000	85.6
Long-styled morph	9	2.47	207B22	4.96 (3.7)	0.003	20.8
	11	14.35	ga0624	3.25 (3.7)	0.115	15.8
Short-styled morph	8	2.77	205O19	3.38 (4.0)	0.125	15.6
	9	2.47	207B22	5.71 (4.0)	0.002	24.9
	12	6.00	ga0625	4.49 (4.0)	0.019	24.8

c) Corolla tube length

Morph	Linkage group	Position (cM)	Nearest marker	Maximum LOD score[a]	P-value	PVE (%)
Both morphs	3	0.00	208B11	3.70 (3.8)	0.063	14.4
	5	22.35	207G17	3.84 (3.8)	0.044	10.6
	7	83.13	ga1277	4.59 (3.8)	0.016	10.5
	8	0.00	ga1388	5.29 (3.8)	0.004	12.0
	9	2.47	207B22	7.79 (3.8)	0.000	17.2
	12	17.90	201B15	3.55 (3.8)	0.086	8.3
Long-styled morph	3	15.41	202B22	3.94 (3.9)	0.050	17.7
	9	2.47	207B22	3.29 (3.9)	0.181	14.3
Short-styled morph	8	2.77	205O19	4.93 (3.8)	0.008	21.9
	9	2.47	207B22	5.09 (3.8)	0.005	22.5
	12	12.22	ga0024	3.47 (3.8)	0.105	15.9

d) Anther length

Morph	Linkage group	Position (cM)	Nearest marker	Maximum LOD score[a]	P-value	PVE (%)
Both morphs	5	35.07	ga0108	3.39 (3.8)	0.104	7.9
	7	83.13	ga1277	7.86 (3.8)	0.000	17.4
	9	2.47	207B22	4.32 (3.8)	0.017	9.9
	11	12.64	ga0624	5.73 (3.8)	0.003	16.0
Long-styled morph	6	10.00	ga0032	4.93 (3.9)	0.006	34.4
	6	45.27	109G10	4.15 (3.9)	0.024	26.9
	11	20.75	ga1508	3.10 (3.9)	0.200	13.8
Short-styled morph	11	12.64	ga0624	5.48 (3.9)	0.004	30.5

Abbreviation: LOD, logarithm of odds; PVE, phenotypic variance explained.

[a] Significant LOD thresholds ($P<0.05$) calculated by permutation test are in parentheses.

TABLE 4. *Relationships between morph and genotype at four loci tightly linked to the S locus*

ga1277 [0.64cM]	Genotypes			
	ac	ad	bc	bd
Long-styled	1	41	0	56
Short-styled	49	0	41	1

†a = 269, b = 273, c = 250, d = 257

ga0495 [0.64cM]	Genotypes			
	ee	ef	eg	fg
Long-styled	56	41	0	1
Short-styled	1	0	41	49

†e = 240, f = 242, g = 230

ga0821 [0.658cM]	Genotypes			
	ee	ef	eg	fg
Long-styled	56	42	0	1
Short-styled	1	0	42	49

†e = 119, f = 114, g = 108

PS-4 [0.66cM]	Genotypes			
	ee	ef	eg	fg
Long-styled	55	42	0	1
Short-styled	1	0	42	49

†e = 203, f = 201, g = 207

Values in table indicate the number of genets of each morph type with each genotype. Values in brackets at upper left of each section indicate the map distance between the indicated marker and the *S* locus

† indicates the fragment length of each allele at each locus. Note that an allele is defined by a locus-letter combination; for example, the “e” locus at ga0821 is distinct from the “e” locus at PS-4.

TABLE 5. *Results of morph prediction for genets in wild populations based on the genotypes at four marker loci*

Population	Genet	Observed morph[1]	Genotype[2]				Predicted morph[3]
			ga1277	ga0495	ga0821	PS-4	
Hokkaido	H-1	Long	du_2	u_4u_8	-	**ee**	Long
Hokkaido	H-2	Long	au_1	fu_2	fu_1	fu_2	×
Hokkaido	H-3	Long	du_2	u_4u_8	u_1u_1	**ee**	Long
Hokkaido	H-4	Long	**ad**	fu_4	ff	**ef**	Long
Hokkaido	H-5	Long	**ad**	fu_4	ff	**ef**	Long
Hokkaido	H-6	Long	**dd**	u_4u_4	u_1u_1	**ee**	Long
Hokkaido	H-7	Short	au_6	fu_4	fu_9	ff	Long*
Hokkaido	H-8	Long	du_1	u_4u_4	u_1u_1	**ee**	Long
Hokkaido	H-9	Long	du_1	u_2u_4	u_1u_1	eu_2	×
Hokkaido	H-10	Long	**ad**	fu_4	ff	**ef**	Long
Hokkaido	H-11	Long	**ad**	fu_4	ff	**ef**	Long
Hokkaido	H-12	Long	au_2	fu_7	ff	**ef**	Long
Hokkaido	H-13	Long	du_2	u_4u_8	u_1u_1	**ee**	Long
Hokkaido	H-14	Long	du_2	u_4u_8	u_1u_1	**ee**	Long
Hokkaido	H-15	Short	$\mathbf{cu_6}$	$\mathbf{gu_4}$	$\mathbf{gu_9}$	**fg**	Short
Hokkaido	H-16	Short	$\mathbf{cu_2}$	$\mathbf{gu_7}$	**gg**	**eg**	Short
Hokkaido	H-17	Short	**cd**	$\mathbf{gu_4}$	**gg**	**eg**	Short
Hokkaido	H-18	Short	u_2u_6	$\mathbf{gu_4}$	$\mathbf{gu_9}$	**fg**	Short
Hokkaido	H-19	Short	du_2	$\mathbf{gu_4}$	**gg**	**eg**	Short
Hokkaido	H-20	Short	**cd**	$\mathbf{gu_4}$	**gg**	**eg**	Short
Hokkaido	H-21	Short	**cd**	$\mathbf{gu_4}$	u_3u_3	**eg**	Short
Hokkaido	H-22	Short	$\mathbf{cu_6}$	$\mathbf{gu_4}$	u_3u_9	**fg**	Short
Hokkaido	H-23	Short	$\mathbf{cu_1}$	$\mathbf{gu_2}$	u_3u_3	$\mathbf{gu_2}$	Short
Hokkaido	H-24	Short	du_2	$\mathbf{gu_4}$	**gg**	**eg**	Short
Hokkaido	H-25	Short	**cd**	$\mathbf{gu_4}$	**gg**	**eg**	Short
Hokkaido	H-26	Short	$\mathbf{cu_2}$	$\mathbf{gu_8}$	**gg**	**eg**	Short
Saitama	S-1	Long	u_2u_5	**ef**	eu_8	eu_1	Long
Saitama	S-2	Long	u_2u_2	u_2u_2	u_2u_2	u_2u_2	×
Saitama	S-3	Long	du_2	u_2u_9	u_3u_3	eu_2	×
Saitama	S-4	Long	du_2	u_2u_9	u_3u_3	eu_2	×
Saitama	S-5	Long	du_2	u_2u_9	u_3u_3	eu_2	×
Saitama	S-6	Long	bu_2	fu_2	u_2u_7	fu_2	×
Saitama	S-7	Short	u_2u_2	u_1u_2	u_1u_2	$\mathbf{gu_2}$	Short
Saitama	S-8	Long	u_2u_2	u_2u_2	u_2u_2	u_2u_2	×

Population	Genet	Observed morph[1]	Genotype[2]				Predicted morph[3]
			ga1277	ga0495	ga0821	PS-4	
Saitama	S-9	Long	du_2	u_2u_9	u_3u_3	eu_2	×
Saitama	S-10	Long	u_2u_2	u_2u_2	u_2u_2	u_2u_2	×
Saitama	S-11	Long	u_2u_2	u_2u_2	u_2u_2	u_2u_2	×
Saitama	S-12	Long	bu_2	fu2	u_2u_7	fu_2	×
Saitama	S-13	Short	u_2u_6	u_1u_4	u_1u_6	**eg**	Short
Saitama	S-14	Short	u_2u_5	eu_1	u_1u_{13}	**eg**	Short
Saitama	S-15	Long	u_2u_4	u_2u_6	u_2u_9	fu_2	×
Saitama	S-16	Long	u_5u_6	**ee**	u_6u_{13}	ee	Long
Nagano	N-1	Short	du_2	u_1u_5	u_1u_9	**fg**	Short
Nagano	N-2	Long	du_1	**ee**	u_6u_{10}	**ef**	Long
Nagano	N-3	Long	du_4	eu_4	u_6u_6	**ef**	Long
Nagano	N-4	Long	u_2u_5	u_3u_5	u_9u_{11}	**ef**	Long
Nagano	N-5	Long	u_1u_1	eu_3	$u_{14}u_{15}$	**ee**	Long
Nagano	N-6	Long	u_2u_4	u_4u_8	u_4u_6	**ef**	Long
Nagano	N-7	Long	u_2u_4	u_2u_4	u_1u_5	eu_2	×
Nagano	N-8	Long	u_1u_2	u_2u_3	u_1u_{11}	eu_2	×
Nagano	N-9	Long	u_2u_4	u_4u_8	u_4u_5	**ef**	Long
Nagano	N-10	Long	u_1u_1	u_2u_5	u_1u_9	fu_2	×
Nagano	N-11	Long	du_2	fu_5	u_9u_9	**ef**	Long
Nagano	N-12	Long	u_4u_4	u_4u_4	u_6u_6	**ee**	Long
Nagano	N-13	Long	du_1	**ee**	fu_{12}	**ef**	Long
Nagano	N-14	Long	du_1	**ee**	fu_{12}	**ef**	Long
Nagano	N-15	Long	u_2u_2	u_5u_8	u_4u_9	**ff**	Long
Nagano	N-16	Short	bu_2	u_1u_3	u_1u_{11}	**eg**	Short
Nagano	N-17	Short	bu_2	u_1u_5	u_1u_9	**fg**	Short

Abbreviations: Long, long-styled morph; Short, short-styled morph

Bold letters indicates the genotypes useful for morph identification.

[1] Morph identified by visual confirmation.

[2] "u" indicates alleles that were not detected in the mapping population. "-"indicates a possible null genotype.

[3] "×" indicates morphs that could not be predicted from the genotypes of the four markers. *

indicates morph for which predicted type was different from the observed type.