**RESEARCH ARTICLE**

# Upper petal lip colour polymorphism in *Collinsia heterophylla* (Plantaginaceae): genetic basis within a population and its use as a genetic marker

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## Abstract

Understanding the genetics of a polymorphic trait is important to predict its likely evolution. In *Collinsia heterophylla*, the upper petal lip colour can be either be white or white with a purple band, while the lower petal lip colour is invariably purple. Because the corolla is only partly polymorphic, the polymorphism can not have evolved due to a mutation where a pigment was lost in the entire plant, which is common in other polymorphic species. In a previous study, high frequency of the purple band was found in populations with darker flowers, indicating possible selection for this trait. In this study, I determined inheritance of the colour polymorphism using two populations (one with only white morph and other with both morphs). I conducted experimental crosses within and between floral morphs to determine whether patterns of segregation in offspring conform to single-gene predictions. Data from $F_1$, $F_2$, $F_3$ and backcross progeny are consistent with a genetic model of one major locus with presence of the band being completely dominant, as indicated in earlier studies using distantly related populations. A novel finding in this study was that the two morphs did not show a difference in seed germination frequency or seedling survival. This trait can thus be valuable as a genetic marker. Even though more thorough ecological data are needed to understand the potential selection pressures on upper petal lip colour in *C. heterophylla*, its simple inheritance may indicate the possibility of fast evolutionary response to selective forces acting on this trait.

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## Introduction

A major goal in ecological and evolutionary genetics is to understand the direction and strength of natural selection, and to find the genetic basis of variation underlying ecologically important traits i.e., traits that influence fitness within a natural population (Orr and Coyne 1992; Feder and Mitchell-Olds 2003; Connor and Hartl 2004; Mitchell-Olds and Schmitt 2006). Despite recent development of genomic approaches in such research (Stinchcombe and Hoekstra 2008), it can still be of interest to study simple polymorphisms (co-occurrence of two or more discrete phenotypes within a population), as polymorphisms often show allelic differences at one or few gene loci and are relatively unaffected by the environment (Ford 1975). Recent studies involving polymorphic species have proved useful when investigating maintenance and evolutionary dynamics of genetic variation, and also in relation to a possible link between polymorphism and speciation (Sivero *et al*. 2000; Bell *et al*. 2004; Svensson and Abbott 2005; see also Connor 2006; Jorgensen *et al*. 2006; Schemske and Bierzychudek 2007; Gray and McKinnon 2007; Svensson *et al*. 2009).

In plants, corolla colour often varies within species and colour polymorphism is common, especially in species whose flowers normally contain pink, purple or blue anthocyanin pigments (Warren and Mackenzie 2001; Rausher 2008). Divergent selection on corolla colour can result from attraction of pollinators of varying preference, often related to frequency dependent behaviour of pollinators (Levin and

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Kerster 1967; Epperson and Clegg 1987; Stanton 1987; Irwin and Strauss 2005; Eckhart *et al*. 2006). It is, however, also well known that floral pigments can have pleiotropic effects on other aspects of plant performance, such as avoidance of herbivory and abiotic stress, which could maintain polymorphism in a variable environment (Schemske and Bierzychudek 2001; Warren and Mackenzie 2001; Coberly and Rausher 2003, Frey 2004; see also review by Strauss and Whittall 2006). Because it is still not known if selection by pollinators or by non-pollinating factors is more important for evolutionary transitions in flower colour (Rausher 2008), there is a clear need for further studies to understand maintenance of flower-colour polymorphism.

*Collinsia heterophylla* Buist (Plantaginaceae) has zygomorphic flowers with five-lobed corollas comprising one upper and one lower lip (Newsom 1929; Neese 1993). Corolla colour is generally white to pale purple on the upper lip and dark purple (or sometimes pale purple) on the lower lip. Some populations of *C. heterophylla* contain a variable proportion of plants with a coloured upper lip, often (but not always) expressed as a distinct dark band on the upper part of the upper lip (Weil and Allard 1964; Å Lankinen and J. A. Madjidian, unpublished data). The band was present in 10 out of 24 populations from all over California, and frequency of the white morph was bimodal (either high, 88%–100% or low, 0%–44%; Å Lankinen and J. A. Madjidian, unpublished data). We could not find a difference in pollination rate of the morphs, but we detected that the band was more frequent in populations with very dark flower colour, in the Sierra Nevada of northern California.

It is known that other species can be polymorphic only on parts of the corolla, such as when petal spots or marks are present versus absent (e.g., Lewis and Lewis 1955; Abbott 1981; Shore and Barrett 1987). This kind of polymorphism differs from when the whole corolla is involved, because it cannot have evolved due to a mutation where a pigment or a biochemically related substance was lost in the entire plant (cf. Warren and Mackenzie 2001; Armbruster 2002). In most other polymorphic species with petal spot or marks, however, the marks are of a different colour compared to that of the petals, indicating a modification of the floral pigment (Dorn and Bloom 1984), or that more than one pigment is involved (cf. Mol *et al*. 1998). Because of the pleiotropic effects of floral pigments (e.g. Warren and Mackenzie 2001, see above), it should be well worth investigating a polymorphism which does not seem to involve an additional colour, as presumably is in the case of *C. heterophylla*.

Older studies suggest that presence of colour (independent of presence of a band) on the upper lip of *C. heterophylla* is determined by a gene for carmine, *Kn* (Hiorth 1930) and by two other genes of which one is closely linked with *Kn* (Goršič 1957). A common feature in these older studies is that crosses were made between distant populations, because the aim was rather to determine inheritance of traits deviating from the 'normal' type than investigating the genetic basis of variation between closely occurring populations or within a polymorphic population. Thus, the results of these studies are not informative in terms of the genetic basis of variability within populations. One important reason for establishing the genetic basis of corolla colour variation within a population is that, if determined by a single gene, it can be easily used as a genetic marker in experimental studies (e.g., for assessing paternity in mixed crosses). To be able to use a genetic marker it is further not only important to determine its inheritance, but also to explore the existence of indirect selection on the different morphs, especially at life stages prior to anthesis.

In this study, my aim was to investigate the genetic basis of the dark band on the upper lip of *C. heterophylla* using two isolated populations occurring at a fairly short distance from each other. The populations were similar in appearance apart from the fact that one consisted only of flowers without the dark band on the upper lip (white morph) and other consisted of both morphs. I made experimental crosses within and between upper-lip colour morphs originating from the same or different populations to determine whether the pattern of segregation was consistent with one dominant gene controlling presence of the dark band. Data were collected from $F_1$, $F_2$, $F_3$ and backcross progenies. I further recorded germination frequency and early survival in the progeny to evaluate if early selection on either morph distorted segregation pattern. In addition, in the polymorphic population I estimated the within-population morph and gene frequency of the two morphs.

## Materials and methods

### *Plant material*

*Collinsia heterophylla* is a self-compatible diploid ($2n = 14$) annual native to California Floristic Province (Newsom 1929; Neese 1993). Plants are widely distributed and grow in shady places and dry slopes (<1000 m). Depending on latitude and elevation, plants flower between March and June. Flowers are pollinated by a variety of native bees (Armbruster *et al*. 2002). Flowers are arranged in terminal whorls in spike-like inflorescences. They have four epipetalous stamens and one pistil. Newly opened flowers have undehisced anthers and stigmas that are not yet receptive. During the course of development of flower, the anthers dehisce usually one at a time over the course of 3–4 days. During this period the stigma becomes receptive and the style elongates. This eventually places the stigma in contact with the dehisced anthers, and selfpollination can occur (see Armbruster *et al*. 2002, for a more detailed description; see also Kalisz *et al*. 1999). Estimates of mean population outcrossing rates range from 0.32 to 0.64, based on allozyme markers (Charlesworth and Mayer 1995), and up to 0.94 + 0.27 based on morphological markers (Weil and Allard 1964). Flowers

develop into dry, dehiscent capsules, each containing up to 16 seeds (Armbruster *et al*. 2002).

The plants used in this study were dark purple on the lower lip and originated from two populations outside of Mariposa, California (about 30 km apart). Flowers appeared similar in both populations apart from presence or absence of the dark-purple band on the upper petal (figure 1); population 1 (Hornitos road, GPS coordinates: lat = 37.5020 N, long = 120.1236 W) had only the white morph, population 2 (Ferguson ridge, GPS coordinates: lat = 37.6536 N, long = 119.8866 W) had both morphs, but the white morph was uncommon (I did not attempt to quantify proportion of morphs in the field). Plants were raised from seeds collected approximately from 50 maternal plants (1–20 plants per maternal family) in each population and grown in an insect-free greenhouse between 2004 and 2006. Generally 1–3 plants per maternal family were used in experimental crosses.

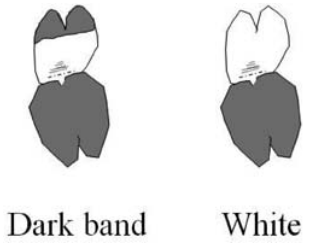


**Figure 1.** Outline of two distinct floral morphs in *Collinsia heterophylla* that either has a dark-purple band on the upper lip or is white on the entire upper lip. Population 1 of this study had only the white morph, while population 2 was polymorphic with a low proportion of the white morph. Both populations were dark purple on the lower lip.

### *Experimental crosses and analyses of segregation*

To evaluate the hypothesis of single-gene inheritance of the dark band, I performed a series of experimental crosses between and within populations (figure 2). Regarding the between-population crosses, I produced hybrids ($F_1$) and their selfed $F_2$ and $F_3$ progenies. In total, 21 parental crosses were made to produce the hybrids, involving seven maternal families without the band (population 1) and nine maternal families with the band (population 2) (see table 1). I assumed that plants with the band would be either 'homozygous' or 'heterozygous', under the expectation that presence of the band was dominant over its absence. The $F_2$ represent offspring from 17 of the crosses, while $F_3$ only represent offspring from a subset of five of the crosses. Backcrosses (BC) between $F_1$ hybrids (or occasionally $F_3$ of the white morph) and plants of either populations 1 and 2 respectively, were also made. The BC progeny were derived from 39 crosses involving eight maternal families from population 1 (of which five were used also in the previous crosses) and 10 maternal

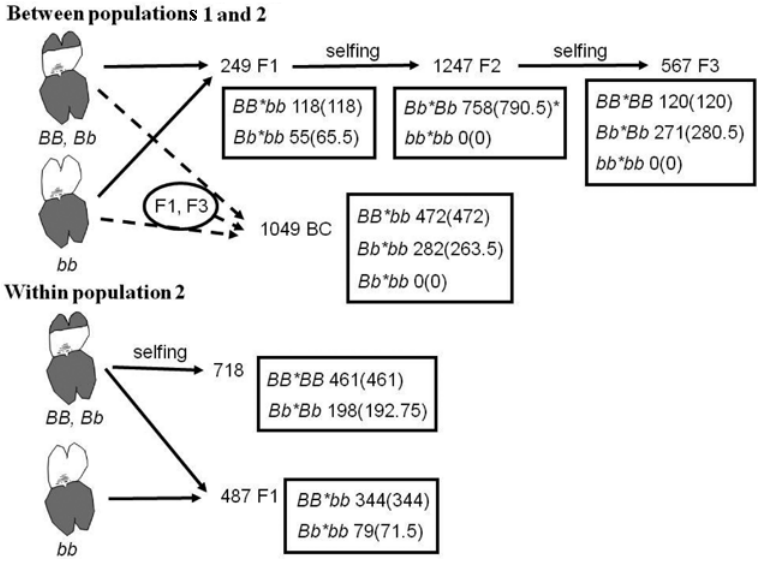


**Figure 2.** Experimental crosses and sample sizes of the resulting progeny used to evaluate if presence of a dark band on the upper petal lip was determined by a single gene *(B)* that was dominant over its absence *(b)* in *Collinsia heterophylla*. Crosses were conducted between and within two populations. The outcome of the crosses for each of the inferred genotypes is indicated in the boxes as observed (expected) number of progeny with the dark band (see tables 1–3 for further details). *Denote that the outcome was significantly different from expected ($P < 0.05$).

**Table 1.** Patterns of segregation in $F_1$, $F_2$ and $F_3$ and BC progenies from inter-population and intra-population crosses, as revealed by frequencies of floral morphs with or without a dark band on the upper petal (dark versus white).

| Cross type | Segregating progenies[a] | Inferred genotype(s)[b] | *n* Dark obs. (exp[b].) | *n* White obs. (exp[b].) | *P*[c] | *n* Crosses (families) |
|---|---|---|---|---|---|---|
| Between | $F_1$ | $BB \times bb$ | 118 (118) | 0 (0) | | 15 (14) |
| populations | $F_1$ | $Bb \times bb$ | 55 (65.5) | 76 (65.5) | ns | 6 (5) |
| 1 and 2 | $F_2$ | Selfing *Bb* | 758 (790.5) | 296 (263.5) | * | 14 (14) |
| | $F_2$ | Selfing *bb* | 0 (0) | 193 (193) | | 6 (5) |
| | $F_3$ | Selfing *BB* | 120 (120) | 0 (0) | | 3 (4) |
| | $F_3$ | Selfing *Bb* | 271 (280.5) | 102 (93.25) | ns | 4 (5) |
| | $F_3$ | Selfing *bb* | 0 (0) | 74 (74) | | 4 (5) |
| | BC | $BB \times bb$ | 472 (472) | 0 (0) | | 17 ($F_1$+10)[d] |
| | BC | $Bb \times bb$ | 282 (263.5) | 245 (263.5) | ns | 21 ($F_1$+7)[d] |
| | BC | $bb \times bb$ | 0 (0) | 50 (50) | | 1 ($F_1$+5)[d] |
| Within | $F_1$ | $BB \times bb$ | 344 (344) | 0 (0) | | 28 (30) |
| population | $F_1$ | $Bb \times bb$ | 79 (71.5) | 64 (71.5) | ns | 10 (15) |
| 2 | Selfing | Selfing *BB* | 461 (461) | 0 (0) | | 32 (28) |
| | Selfing | Selfing *Bb* | 198 (192.75) | 59 (64.25) | ns | 12 (12) |

[a]In $F_1$ and $F_3$ (unknown parental genotype), progeny with only the band present were assigned as originating from a homozygous parent (*BB*), while progenies from the other crosses were assigned to have had a heterozygous parent (*Bb*).

[b]Data was compared to a genetic model of one gene and two alleles, where presence of the dark band (*B*) is completely dominant over its absence (*b*).

[c]Heterogeneity *G*-test, see tables 2 and 3 for more detailed test statistics of expected frequencies; ns, $P > 0.5$; *, $P < 0.05$.

[d]Back-crosses (BC) were performed using seven $F_1$ plants, originating from crosses between plants of nine maternal families, see table 2.

families from population 2 (of which two were previously used). Some of the experimental crosses (both $F_1$ and BC) were made reciprocally with the same two individuals.

Within the polymorphic population (population 2), $F_1$ progeny were derived from 41 crosses using 32 maternal families with the band and seven maternal families without the band (figure 2; table 1). Further, a total of 40 maternal families used in crosses between or within populations (all possessing the band) were allowed to self in order to produce segregating offspring that would be expected to segregate according to the pattern found in the crosses (i.e. as 'homozygous' or 'heterozygous'). Selfed progeny were collected from 1–3 plants per maternal family. Most of these plants were used in the crosses.

Crosses were made on fully receptive (day four after flower opening), emasculated stigmas by adding pollen from a microscopic slide until the stigma was completely covered with pollen. Seed capsules were collected at ripening. Collected seeds were sown in a mixture of ordinary pot compost and sand (4:1) and kept in a refrigerator until they started germinating (approximately after 1.5 to 4 weeks). Plants were then kept in the greenhouse until they started flowering and it was possible to record presence or absence of the band.

A total of 4317 offspring from all crosses ($F_1$–$F_3$ and BC) were scored for presence or absence of the band (figure 2; see also table 1). Of these 2485 were produced from crosses where a mixture of both offspring morphs was expected. I used G-test to investigate whether observed morph frequency differed from that expected from a single-locus model with presence of the band showing complete dominance over absence of the band. When one observed value was 0 in these crosses, this cross was combined with another cross (reciprocal crosses when available or crosses involving one similar parent) to be able to perform the *G*-test. The overall fit of the genetic model was examined for all crosses of a particular type (i.e., six separate analyses, see tables 2&3) using a heterogeneity *G*-test, i.e. a replicated *G*-test combining goodness-of-fit pooled for all data and homogeneity of separate crosses (Sokal and Rohlf 1995).

Proportion germinated seeds and survival of seedlings to flowering were recorded to investigate if early selection on either morph distorted segregation pattern. Arcsine-transformed germination proportion was correlated to proportional deviation from expected frequency of the white morph. A positive relationship would be expected if the dark morph is better at germinating, and a negative one for the opposite pattern. An ANCOVA with germination proportion as the dependent variable and deviation from expectation as the covariate was used. This analysis allowed controlling for the factors: (i) selfing versus outcrossing, as selfed seeds are known to show lower germination percentage (Lankinen and Armbruster 2007), (ii) inter-population versus

**Table 2.** Patterns of segregation in inter-population $F_1$, $F_2$ and $F_3$, and BC progenies as revealed by frequencies of floral morphs with or without a dark band on the upper petal (dark versus white).

| Cross id[a] | Recipient[b] | Donor[b] | Prop. germ[c] | *n* Dark | *n* White | Exp. *n* white[d] | Total | *P*[e] |
|---|---|---|---|---|---|---|---|---|
| $F_1$, inferred genotype(s) *Bb* × *bb*[d] | | | | | | | | |
| A3 | 2.5 | 1.1 | 0.61 | 11 | 11 | 11 | 22 | |
| B4 | 1.1 | 2.5 | 0.75 | 13 | 14 | 13.5 | 27 | |
| B2 | 1.1.2 | 2.5 | 0.54 | 4 | 10 | 7 | 14 | |
| A4 | 2.5 | 1.7 | 0.42 | 8 | 5 | 6.5 | 13 | |
| B5 | 1.7 | 2.5 | 0.61 | 5 | 17 | 11 | 22 | ** |
| B8 | 1.50 | 2.104 | 0.41 | 14 | 19 | 16.5 | 33 | |
| | | | | 55 | 76 | 65.5 | 131 | |
| | | | | Pooled $G_1$ = 3.38 | | | | ns |
| | | | | Heterogeneity $G_5$ = 7.69 | | | | ns |
| | | | | Total $G_6$ = 11.1 | | | | ns |
| $F_2$ (selfing), inferred genotype(s) *Bb* × *Bb*[d] | | | | | | | | |
| A6 | 2.39 | 1.50 | 0.15 | 10 | 4 | 3.5 | 14 | |
| B9 | 1.50 | 2.39 | 0.02 | 1 | 0 | 0.25 | 1 | |
| B8 | 1.50 | 1.1.2 | 0.33 | 1 | 0 | 0.25 | 1 | |
| *A6+B9+B8* | | $\sum^f$ | *0.11* | *12* | *4* | *4* | *16* | |
| A9 | 2.126 | 1.56 | 0.56 | 36 | 15 | 12.75 | 51 | |
| A10 | 2.3 | 1.56 | 1.0 | 7 | 0 | 1.75 | 7 | |
| *A9+A10* | | $\sum^f$ | *0.59* | *43* | *15* | *14.5* | *58* | |
| A1 | 2.2 | 1.1 | 0.53 | 67 | 26 | 23.25 | 93 | |
| B3 | 1.1 | 2.2 | 0.44 | 41 | 23 | 16 | 64 | |
| A2 | 2.2 | 1.7 | 0.49 | 185 | 77 | 65.5 | 262 | |
| B6 | 1.19 | 2.39 | 0.23 | 24 | 13 | 9.25 | 37 | |
| A7 | 2.21 | 1.5 | 0.50 | 15 | 2 | 4.25 | 17 | |
| A8 | 2.21 | 1.56 | 0.54 | 34 | 10 | 11 | 44 | |
| A3 | 2.5 | 1.1 | 0.46 | 49 | 17 | 16.5 | 66 | |
| B4 | 1.1 | 2.5 | 0.54 | 48 | 16 | 16 | 64 | |
| B2 | 1.1 | 2.5 | 0.63 | 25 | 12 | 9.25 | 37 | |
| A4 | 2.5 | 1.7 | 0.70 | 101 | 41 | 35.5 | 142 | |
| B5 | 1.7 | 2.5 | 0.62 | 68 | 23 | 22.75 | 91 | |
| A12 | 2.50 | 1.56 | 0.42 | 46 | 17 | 15.75 | 63 | |
| | | | | 758 | 296 | 263.5 | 1054 | |
| | | | | Pooled $G_1$ = 5.21 | | | | * |
| | | | | Heterogeneity $G_{13}$ = 7.75 | | | | ns |
| | | | | Total $G_{14}$ = 12.9 | | | | ns |
| $F_3$ (selfing), inferred genotype(s) *Bb* × *Bb*[d] | | | | | | | | |
| A7 | 2.21 | 1.5 | 0.47 | 20 | 7 | 6.75 | 27 | |
| A8 | 2.21 | 1.56 | 0.60 | 76 | 18 | 23.5 | 94 | |
| A9 | 2.126 | 1.56 | 0.60 | 102 | 58 | 40 | 160 | ** |
| A12 | 2.50 | 1.56 | 0.56 | 73 | 19 | 23 | 92 | |
| | | | | 271 | 102 | 93.25 | 373 | |
| | | | | Pooled $G_1$ = 1.07 | | | | ns |
| | | | | Heterogeneity $G_3$ = 11.8 | | | | ** |
| | | | | Total $G_4$ = 12.7 | | | | * |
| BC, inferred genotype(s) *Bb* × *bb*[d] | | | | | | | | |
| | 2.4 | A4$F_1$(*bb*) | 0.81 | 18 | 21 | 19.5 | 39 | |
| | A4$F_1$(*bb*) | 2.4 | 1.0 | 7 | 12 | 9.5 | 19 | |
| | 2.4 | B8$F_1$(*bb*) | 0.83 | 16 | 9 | 12.5 | 25 | |
| | B8$F_1$(*bb*) | 2.4 | 0.91 | 31 | 12 | 21.5 | 43 | ** |
| | 2.4 | A9$F_3$(*bb*) | 0.94 | 19 | 26 | 22.5 | 45 | |

**Table 2** (*contd.*)

| Cross id[a] | Recipient[b] | Donor[b] | Prop. germ[c] | *n* Dark | *n* White | Exp. *n* white[d] | Total | *P*[e] |
|---|---|---|---|---|---|---|---|---|
| | $A9F_3$(*bb*) | 2.4 | 0.59 | 8 | 8 | 8 | 16 | |
| | $A3F_1$ | 2.104(*bb*) | 0.82 | 10 | 3 | 6.5 | 13 | |
| | $A4F_1$ | 2.104(*bb*) | 0.77 | 26 | 24 | 25 | 50 | |
| | $A3F_1$ | 1.1(*bb*) | 1.0 | 22 | 13 | 17.5 | 35 | |
| | $A3F_1$ | 1.7(*bb*) | 0.92 | 6 | 6 | 6 | 12 | |
| | $A3F_1$ | 1.8(*bb*) | 1.0 | 11 | 11 | 11 | 22 | |
| | $A3F_1$ | 1.28(*bb*) | 1.0 | 13 | 18 | 15.5 | 31 | |
| | $B4F_1$ | 1.5(*bb*) | 0.91 | 16 | 16 | 16 | 32 | |
| | $B4F_1$ | 1.7(*bb*) | 1.0 | 8 | 8 | 8 | 16 | |
| | $B4F_1$ | 1.54(*bb*) | 1.0 | 13 | 12 | 12.5 | 25 | |
| | $B2F_1$ | 1.7(*bb*) | 0.88 | 4 | 3 | 3.5 | 7 | |
| | $B2F_1$ | 1.50(*bb*) | 0.92 | 13 | 11 | 12 | 24 | |
| | $B5F_1$ | 1.2(*bb*) | 0.92 | 12 | 10 | 11 | 22 | |
| | $B8F_1$(*bb*) | 2.104 | 0.74 | 7 | 10 | 8.5 | 17 | |
| | $B2F_1$(*bb*) | 2.104 | 1.0 | 22 | 12 | 17 | 34 | |
| | 1.5(*bb*) | $A1F_1$ | 0.77 | 7 | 10 | 8.5 | 17 | |
| | | | | 282 | 245 | 263.5 | 527 | |
| | | | | Pooled $G_1 = 2.60$ | | | | ns |
| | | | | Heterogeneity $G_{20} = 22.5$ | | | | ns |
| | | | | Total $G_{21} = 25.1$ | | | | ns |

[a]Crossing identity starting with A denotes crosses where population 2 was used as recipient and population 1 as donor, while B denotes the opposite pattern.
[b]Origin of recipients and donors were denoted by a number starting with 1 (population 1) or starting with 2 (population 2). The second number denotes maternal family.
[c]Proportion of seeds germinated.
[d]Data was compared to a genetic model of one gene and two alleles, where presence of the dark band (*B*) is completely dominant over its absence (*b*).
[e]Expected frequencies were tested with a heterogeneity *G*-test, examining both pooled goodness-of-fit and heterogeneity; ns, $P > 0.5$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
[f]When the observed frequency was 0, the result of this cross was combined with another cross (shown in italics).

**Table 3.** Patterns of segregation in intra-population $F_1$ and selfing progeny, as revealed by frequencies of floral morphs with or without a dark band on the upper petal (dark versus white).

| Recipient[a] | Donor[a] | Prop. germ[b] | *n* Dark | *n* White | Exp. *n* white[c] | Total | *p*[d] |
|---|---|---|---|---|---|---|---|
| $F_1$, inferred genotype(s) $Bb \times bb$[c] | | | | | | | |
| 2.127 | 2.20 (*bb*) | 1.0 | 1 | 0 | 0.5 | 1 | |
| 2.127 | 2.55 (*bb*) | 0.56 | 7 | 16 | 11.5 | 23 | |
| | $\sum$[e] | 0.57 | 8 | 16 | 12 | 24 | |
| 2.9 | 2.17 (*bb*) | 0.59 | 7 | 10 | 8.5 | 17 | |
| 2.10 (*bb*) | 2.9 | 0.12 | 4 | 0 | 2 | 4 | |
| | $\sum$[e] | 0.34 | 11 | 10 | 10.5 | 21 | |
| 2.49 | 2.13 (*bb*) | 0.20 | 0 | 1 | 0.5 | 1 | |
| 2.13 | 2.49 | 0.22 | 2 | 2 | 2 | 4 | |
| | $\sum$[e] | 0.22 | 2 | 3 | 2.5 | 5 | |
| 2.51 | 2.13 (*bb*) | 0.31 | 6 | 5 | 5.5 | 11 | |
| 2.17 (*bb*) | 2.51 | 0.19 | 4 | 3 | 3.5 | 7 | |
| 2.118 | 2.20 (*bb*) | 0.39 | 10 | 4 | 7 | 14 | |
| 2.5 | 2.55 (*bb*) | 0.31 | 8 | 3 | 5.5 | 11 | |
| 2.7 | 2.13 (*bb*) | 0.28 | 3 | 5 | 4 | 8 | |
| 2.53 | 2.55 (*bb*) | 0.50 | 13 | 5 | 9 | 18 | |
| 2.54 | 2.17 (*bb*) | 0.86 | 14 | 10 | 12 | 24 | |

**Table 3** (*contd.*)

| Recipient[a] | Donor[a] | Prop. germ[b] | *n* Dark | *n* White | Exp. *n* white[c] | Total | $p^d$ |
|---|---|---|---|---|---|---|---|
| | | | 79 | 64 | 71.5 | 143 | |
| | | | Pooled $G_1 = 0.50$ | | | | ns |
| | | | Heterogeneity $G_9 = 10.6$ | | | | ns |
| | | | Total $G_{10} = 11.1$ | | | | ns |
| Selfing, inferred genotype(s) $Bb \times Bb$[c] | | | | | | | |
| 2.104 | 2.104 | 0.38 | 5 | 1 | 1.5 | 6 | |
| 2.118 | 2.118 | 0.53 | 27 | 7 | 8.5 | 34 | |
| 2.127 | 2.127 | 0.48 | 17 | 6 | 5.75 | 23 | |
| 2.2 | 2.2 | 0.07 | 2 | 1 | 0.75 | 3 | |
| 2.4 | 2.4 | 0.14 | 12 | 6 | 4.5 | 18 | |
| 2.5 | 2.5 | 0.29 | 0.21 | 3 | 6 | 24 | |
| 2.7 | 2.7 | 0.22 | 13 | 1 | 3.5 | 14 | |
| 2.9 | 2.9 | 0.52 | 24 | 9 | 8.25 | 33 | |
| 2.49 | 2.49 | 0.15 | 7 | 3 | 2.5 | 10 | |
| 2.51 | 2.51 | 0.44 | 20 | 8 | 7 | 28 | |
| 2.53 | 2.53 | 0.14 | 9 | 3 | 3 | 12 | |
| 2.54 | 2.54 | 0.81 | 41 | 11 | 13 | 52 | |
| | | | 198 | 59 | 64.25 | 257 | |
| | | | Pooled $G_1 = 0.58$ | | | | ns |
| | | | Heterogeneity $G_{11} = 6.96$ | | | | ns |
| | | | Total $G_{12} = 7.55$ | | | | ns |

[a]Identity of recipients and donors were denoted by a number indicating 'population × maternal family'.

[b]Proportion of seeds germinated.

[c]Data was compared to a genetic model of one gene and two alleles, where presence of the dark band (*B*) is completely dominant over its absence (*b*).

[d]Expected frequencies were tested with a heterogeneity *G*-test, examining both pooled goodness-of-fit and heterogeneity; ns, $P > 0.5$.

[e]When the observed frequency was 0, the result of this cross was combined with another cross.

intra-population crosses, and their interaction. Because all germinated seedlings survived to flowering, seedling survival was not included in further analysis.

For plants grown from seeds collected directly from the wild, frequency of floral morphs was determined (number of maternal families, 43 (population 1); 45 (population 2), only collected from plants of the dark morph). In the polymorphic population (population 2), frequency of progeny with the white morph originating from 'homozygous' or 'heterozygous' maternal plants ($f_{white}$) was used to determine gene frequency of the white allele $(1 - p)$ under the single-gene model (assuming Mendelian inheritance and deviation from Hardy–Weinberg equilibrium caused by partial selfing with the selfing rate S):

$$f_{white} = 1 - \left( \frac{p^2 + F(p(1-p))}{p^2 + F(p(1-p)) + 2p(1-p)(1-F)} + \frac{2p(1-p)(1-F)}{p^2 + F(p(1-p)) + 2p(1-p)(1-F)} \left(1 - \frac{1}{2}(1-p)\right)\right), \quad (1)$$

where $p$ is gene frequency of the dark allele and $F$ the equilibrium inbreeding coefficient, $F = S/(2 - S)$ (see Wright 1965).

## Results

### *Appearance of the dark band on the upper lip*

In general, it was easy to determine whether the dark band was present or not, even though the band varied in colour, intensity, evenness and size. Colour of the band mostly consisted of two colours, purple and carmine, but some individuals only had one of the two colours. In plants lacking a distinct band, it was quite common to see some colour on the upper lip (e.g., a streaky or a more irregular appearance). There was little variation in appearance of the dark band within individual plants. Appearance of the band was similar in 'homozygous' and 'heterozygous' plants.

### *Segregation following experimental crosses between populations 1 and 2*

In the $F_1$ generation produced by crosses between the two populations, either all offspring resulting from a cross had a dark band or the offspring segregated 1 : 1 for the two morphs

(table 1; figure 2). This is consistent with a genetic model of one gene and two alleles where presence (*B*) is dominant over absence (*b*), and individuals possessing the band (population 2) are either homozygous (*BB*) or heterozygous (*Bb*). Among crosses involving one heterozygous parent there was some heterogeneity; one cross produced significantly more offspring without the band than the expected frequency of 1:1 (table 2).

Offspring produced by selfing of hybrid plants of the dark morph ($F_2$ and $F_3$ generations) segregated close to the expected frequencies of 3:1 (tables 1 and 2; figure 2). In the $F_2$ generation there was, however, a significant but small deviation in favour of plants with the white morph when testing the goodness-of-fit of all data (table 2). This deviation was not significant when all crosses were pooled together (table 2). In the $F_3$ generation, there was no significant deviation from the expected value, but some heterogeneity was caused by one cross showing more white offspring than expected (tables 1 and 2; figure 2).

Six of the $F_2$ plants did only produce $F_3$ progeny with the band, indicating that they were homozygous for presence of the band. The proportion of homozygous plants in the $F_2$ generation was a little lower that expected, but the proportion did not significantly differ from expectation ($6/23 = 0.26$ *BB* compared to expected, $0.25/0.75 = 0.33$, binomial test, $P > 0.1$).

Offspring produced in backcrosses between $F_1$ (or $F_3$) plants and populations 1 and 2, respectively, segregated as expected from the one-gene predictions (table 1; figure 2). There was no significant heterogeneity, but in one cross the band was significantly more common than predicted, i.e. the opposite to the deviations observed in the $F_2$ and $F_3$ generations (table 2). In total, three out of the 45 inter-population crosses deviated significantly from expectation (table 2), which was not significantly more than that expected by chance (2.25 crosses with a significance level of $P = 0.05$; binomial test $P > 0.1$). Reciprocal crosses made in two out of the three cases showing deviation did not differ significantly from expectation (table 2).

When the expected outcome instead was that all offspring in a cross should be of only one of the two morphs, the result was always exactly as the one predicted, e.g., the band never appeared in offspring predicted to be without (table 1; figure 2). Segregation in reciprocal crosses did not differ depending on if a particular floral morph was used as recipient or pollen donor (paired *t*-test; proportion of offspring without band following *Bb* × *bb* crosses or *Bb* selfing: df = 8, $P > 0.1$; table 2).

#### *Segregation following experimental crosses within population 2*

Crosses performed within population 2 showed the same general result as crosses between populations; segregation pattern of offspring was in line with those expected if a single gene controls presence of the band (tables 1 and 3; figure 2). A difference compared to the outcome between populations was that no significant deviations from expectation were found.

Progeny from plants that had been assigned as either heterozygotes or homozygotes depending on the outcome in inter-population or intra-population crosses, always showed the predicted segregation pattern also in their selfed progeny (cf. tables 2 and 3 for heterozygotes).

#### *Germination proportion and deviation from expected morph frequency*

Germination proportion differed substantially among crosses, ranging between 0.02 and 1.0 in inter-population crosses (table 2), and between 0.07 and 0.86 in intra-population crosses (table 3). There was no detectable relationship between germination frequency and deviation from expected frequency of the white morph (ANCOVA controlling for selfing versus outcrossing and inter-population versus intra-population crosses), $F_{1,61} = 2.01$, $P = 0.16$). This suggests that the two morphs did not differ significantly in germination ability.

#### *Floral morph and gene frequency within wild populations*

As expected, plants of population 1 originating directly from the wild showed only the white morph. In population 2, seeds collected from maternal families of only the dark morph resulted in 4% plants of the white morph (eight plants out of 200 originating from 45 maternal families). Gene frequency of the white allele in population 2 was calculated to range between 0.22 and 0.31 for the known estimates of the outcrossing rate (0.94–0.32, Weil and Allard 1964; Charlesworth and Mayer 1995), assuming that inheritance of the band is determined by a single gene in a simple Mendelian fashion.

## Discussion

In this study on *C. heterophylla*, presence of a dark band on the upper lip segregated in accordance with dominant single-gene predictions in progeny ($F_1$–$F_3$, BC) produced both between two similar populations and within a polymorphic population. Low germination percentage did not significantly distort segregation pattern and all seedlings survived to flowering, indicating that the floral morphs are not differently affected by indirect selection during the juvenile stages.

By crossing populations of *C. heterophylla* originating from several counties in California, Goršič (1957) identified three genes responsible for upper petal lip colour, of which the *Kn* gene was dominant and the other genes (colour, *O*, and intensity, *In*) modified its appearance. In the present study involving two populations from the Sierra Nevada that differed in upper petal lip colouration, but were otherwise similar, segregation pattern of progeny was in line with a single dominant gene determining the presence or absence

of a dark, distinct band on the upper lip. Appearance of the band varied considerably among plants, indicating influence of modifying genes. The previously described *Kn* gene (Hiorth 1930; Goršič 1957) is probably equivalent to the gene coding for the band (see Weil and Allard 1964). On the other hand, the connection between the *Kn* gene and the other gene for colour, *O*, as found by Goršič (1957) is not in accordance with my results. In his study the *O* allele was never expressed unless the basic *Kn* allele was present. In the present study, however, some colour (similar to presence of the *O* allele of the *O* gene in figures 29 and 30 in Goršič 1957) was found on the upper lip also when the distinct band was absent. This rather suggests a gene controlling colour independent of whether the band is present or not. Presumably, colour on the upper lip may have a different genetic basis in different populations. For example, in the field, we found one polymorphic population (in Santa Barbara county) where plants varied both in colour on the upper lip and in presence versus absence of a distinct band (Å. Lankinen and J. Madjidian unpublished data).

In general, a faster evolutionary response to selection in polymorphic traits should be expected when only one or few genes influence the traits, when the novel morphs are controlled by dominant alleles and when different genetic mechanisms can give rise to the same morph(s) (Haldane 1924; Lande 1983; Hoekstra 2006). In species with polymorphic petal colouration, it is common that the polymorphism is determined by simple inheritance of one or few genes either with complete or incomplete dominance (Ennos and Clegg 1983; Gottlieb and Ford 1988; Levin and Brack 1995; Sangwan and Lodhi 1998). In the present study, it was not possible to detect a difference between homozygote and heterozygote plants possessing the band. This result indicates that if the dark morph arises in a population only consisting of the white morph, selection on the dark morph should lead to a rapid evolutionary response (Haldane 1924; Lande 1983). This is in line with the suggestion that adaptation may often involve major genes rather than many genes with small effects (Orr and Coyne 1992; Orr 1998; Bradshaw and Schemske 2003; Hoekstra *et al*. 2004).

In inter-population crosses, but not in intra-population crosses, there were some deviation from the expected ratio in segregating offspring. Even though the number of deviating crosses was not more commonly occurring than expected by chance, deviation was found in both directions and reciprocal crosses did not show the same pattern of deviation, and there was a slight tendency for the white morph to be overrepresented. Because of the long pathway involved in production of anthocyanin pigments (Harborne 1967), it can be hypothesized that some plants of the white morph inherited the major gene for presence of the band but in that it was inactivated by a mutation of a gene influencing the pathway of anthocyanin production (cf. Gerats *et al*. 1982; Bonas *et al*. 1984). If the white morph indeed can arise through multiple genetic mechanisms (cf. Hoekstra 2006; Rausher 2008), this may enhance the evolutionary rate of invasion of the white morph.

Floral polymorphism often involves a coloured morph and a white morph, where the white morph in most cases is the secondary one (Rausher 2008). In *C. heterophylla*, on the other hand, most populations do not have a band on the upper lip, possibly indicating that the dark band is a derived trait. Other species within the genus *Collinsia* (ca. 20) can be either light or dark on the upper lip. To my knowledge, however, only one other species (*C. tinctoria*) has been detected sometimes to have a distinct dark band on the upper lip. *C. tinctoria* is usually white or pale purple on both lips and have one or more very thin bands of a different colour on the middle part of the upper lip (as well as thin marks and spots on the lower lip). Dark varieties of this species show colour as a band on the upper lip just above the thin band. This uncommon dark variety has in fact been found close to some polymorphic populations of *C. heterophylla* in the Sierra Nevada (Å. Lankinen and J. A. Madjidian, personal observation). Interestingly, at least one of these populations of *C. tincoria* consisted of a mixture of plants with white, pale purple and dark purple flowers. A difference compared to the polymorphism in *C. heterophylla*, is that the floral polymorphism of *C. tinctoria* involved the entire corolla and not only the upper lip. *C. tinctoria* and *C. heterophylla* differ in flowering time and do not seem to hybridize in the field. According to recent molecular evidence; however, *C. tinctoria* has a hybrid ancestry involving the *C. bartsiifolia* / *C. corymbosa* lineage and the *C. heterophylla* lineage (B. G. Baldwin, S. Kalisz and W. S. Ambruster unpublished observations and personal communication). It is conceivable that the common history of the two species accounts for the similar band pattern. It is also possible that the occurrence of polymorphic populations of both species in the same area suggest parallel selection pressures on floral traits.

In our recent field study of 24 populations of *C. heterophylla* across California, we found a positive connection between darkness of flowers and a high frequency of the band (A. Lankinen and J. Madjidian, unpublished data). Interestingly, we also detected that populations with a high frequency of the band had higher ground coverage of vegetation; possibly indicating that the dark band (or maybe being as dark as possible) has some kind of selective advantage in certain environments. Populations may then be polymorphic because the dark band has only fairly recently been introduced and the gene is currently increasing towards fixation. Gene frequency of the white allele within the polymorphic population of this study was calculated to 22–31% using the known range of the outcrossing rate of this species (94–32%; Weil and Allard 1964; Charlesworth and Mayer 1995). According to this range the expected frequency of the white morph should be between 4.8% and 9.6%, depending on the outcrossing rate of our study population. However, we generally found a bimodal distribution of morph frequencies in the field, so that in some populations the fre-

quency of the white morph can be very high (> 88%) (Å. Lankinen and J. A. Madjidian, unpublished data). Other possibilities for the occurrence of the colour polymorphism in *C. heterophylla* are that it is selectively neutral or that it is maintained by conflicting selection pressures related to pollinators or to pleiotropic effects on other aspects of plant performance (e.g., Schemske and Bierzychudek 2001; Warren and Mackenzie 2001; Irwin and Strauss 2005; Eckhart *et al*. 2006). So far, we do not have any evidence for fitness differences of the two alleles. Neither Weil and Allard (1964) nor our preliminary results (Å. Lankinen and J. A. Madjidian, unpublished data) could show a difference in pollination behaviour regarding the two morphs in *C. heterophylla*. In the present study, germination ability did not significantly distort segregation pattern and both floral morphs seem to perform equally well under greenhouse conditions (though we need more detailed studies to confirm plant performance later in the life cycle). These results suggest that the polymorphism is selectively neutral. Even so, it can be hypothesized that the two morphs perform differently in the wild. In polymorphic *Lotus corniculatus*, for example, lighter or darker keel colour influenced internal flower temperature differently depending on the weather (Jewell *et al*. 1994), which in turn may affect pollen performance and male reproductive success (cf. McKee and Richards 1998). In future, it would be interesting to investigate further how the dark band is related to fitness both in the greenhouse and in the field.

In conclusion, progeny analysed from crosses between and within two similar populations of *C. heterophylla* indicate that a single gene determines presence or absence of a dark band on the upper petal, where presence is a completely dominant trait. Appearance of the band varied considerably suggesting that several modifying genes influence the major locus. Fast evolutionary response of this trait might be predicted in natural populations. These results were mainly in agreement with older studies conducting crosses between distantly related populations. A novel finding was that the two morphs did not show a difference in seed germination frequency or seedling survival. The lack of early selection on either morph in connection with the simple inheritance of this polymorphism indicate its usefulness as a genetic marker e.g. to determine paternity following mixed crosses. Further, in future studies, it would be of interest to investigate how morph frequencies vary over time and how the dark band is related to selection by pleiotropic fitness effects, especially to understand how the colour polymorphisms could be maintained in present-day populations of *C. heterophylla* (cf. Rausher 2008).

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