

Reproductive isolation of two sympatric louseworts, *Pedicularis rhinanthoides* and *Pedicularis longiflora* (Orobanchaceae): how does the same pollinator type avoid interspecific pollen transfer?

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To study the isolation mechanism of two commonly intermingled louseworts, *Pedicularis rhinanthoides* and *Pedicularis longiflora*, pollination biology in three mixed populations with the two species was investigated during a 3-year project. The results indicated that higher flowering density could help to enhance pollinator activity, and thus increase reproductive output. Bumblebees are the exclusive pollinator for the two louseworts and are essential for their reproductive success. Reproductive isolation between the two species is achieved by a combination of pre- and postzygotic isolation mechanisms. Although both species are pollinated by bumblebees, the present study indicates they successfully avoid interspecific pollen transfer due to floral isolation. Mechanical isolation is achieved by the stigma in the two species picking up pollen from different parts of the pollinator's body, whereas ethological isolation occurs due to flower constancy. Additionally, strong postzygotic isolation was demonstrated by non seed set after artificial cross-pollination even with successful pollen tube growth. We describe the hitherto unreported role of variation in the tightness and direction of the twist of the corolla beak in maintaining mechanical isolation between *Pedicularis* species. Although floral isolation in *Pedicularis* is incomplete, it is considered to be an important mechanism in maintaining species boundaries in the genus. © 2007 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2007, 90, 37–48.

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INTRODUCTION

Sympatric distribution between two or more closely-related species may serve to increase or decrease reproductive success. On one hand, the presence of simultaneously-flowering species can help to attract sufficient pollinators (pollination facilitation) and thus enhance the reproductive output (Rathcke, 1983; Kwak, 1988; Laverty, 1992; Kunin, 1993, 1997; Petanidou *et al.*, 1995; Ohashi & Yahara, 1998). On the other hand, it could lead to pollinator competition (Levin & Anderson, 1970). Moreover, unless assortative mating

is almost complete, interspecific pollen transfer will occur between the sympatric species (Gardner & Macnair, 2000). The potential adverse effects of such interspecific pollen transfer on reproductive success is displayed in phenomena such as stigma clogging or production of inviable seeds and unfit hybrids (Charlesworth, 1989; Gardner & Macnair, 2000), as well as in reduced seed set (Waser, 1978; Galen & Newport, 1988; Kwak & Jennersten, 1991; but see also Weller, 1979; Petanidou *et al.*, 1995).

Most species are separated by a number of 'isolation mechanisms' that form barriers to gene flow (Charlesworth & Charlesworth, 2000). Such isolation mechanisms can be subdivided into two main categories: prezygotic isolation and postzygotic isolation (external isolation and internal isolation) (Grant, 1981). Prezygotic isolation mechanisms consist of factors that can

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prevent interspecific pollen transfer among sympatric flowering plants (Grant, 1981, 1992, 1994a; Hodges & Arnold, 1994; Gardner & Macnair, 2000; Hodges *et al.*, 2004; Husband & Sabara, 2004). As an important external isolation mechanism, floral isolation between species is widespread in angiosperms with specialized animal-pollinated flowers (Grant, 1994b; Schemske & Bradshaw, 1999; Charlesworth & Charlesworth, 2000). Floral isolation, including mechanical isolation and ethological isolation (sometimes undistinguished), is based on an interspecific differentiation in floral characters, the floral mechanism of the species being specialized for pollination by different pollinators with different body forms or by pollinators with different behaviour traits (Grant, 1993, 1994a, b). The most common type of floral isolation exists when species are adapted to different groups of pollinators with different body sizes and shapes (Grant, 1994b). Cases where two sympatric species share the same type of pollinator are less studied. However, these cases could provide new insights into our understanding of evolution of floral isolation mechanisms (Grant, 1994a).

Even if interspecific pollen transfer occurs in coexisting species, internal reproductive barriers, including postzygotic isolation, can also interdict the gene flow (Petanidou *et al.*, 1995; Gardner & Macnair, 2000; Ando *et al.*, 2001). Internal reproductive barriers have the effect of preventing pollen germination and growth; preventing seed set or causing the formation of unviable hybrids. Internal reproductive barriers can additionally help to maintain the distinguishability of parental species (Sprague, 1962; Charlesworth, 1989; Gardner & Macnair, 2000). In nature, prezygotic isolation acting in combination with postzygotic isolation for reproductive isolation of sympatric species is a frequent occurrence (Sprague, 1962; Grant, 1994a, b; Gardner & Macnair, 2000; Ando *et al.*, 2001).

Pedicularis is a large hermaphrodite genus whose species display substantial variation in floral design from a narrow genetic basis (Macior, 1995a). More than 350 *Pedicularis* species have been recorded from China, with most being restricted to the south-west of the country (Hong, 1983; Yang, Holmgren & Mill, 1998). The Chinese Himalaya is regarded as the centre of origin and evolution of *Pedicularis*, and species with all the corolla types in the genus occur in the area (Li, 1951; Yang, Wang & Hong, 2003). Although *Pedicularis* species display substantial variation in floral design, they are almost exclusively pollinated by bumblebees, albeit without any species-specific pollinator (Wang & Li, 1998; Macior, Tang & Zhang, 2001). Quite commonly the blooming periods of two or more *Pedicularis* species in the alpine meadow of the Chinese Himalayas overlap from June to August (Macior & Tang, 1997; Wang & Li, 1998; Macior *et al.*, 2001; see also Macior, 1973, 1975; Adams, 1983 for North

America). Because louseworts are exclusively pollinated by bumblebees and exhibit overlapping blooming period, the prerequisites for interspecific pollen transfer between sympatric species are present. However, hybridization in genus *Pedicularis* is considered to be rare or absent (Macior, 1995b; Macior & Tang, 1997). It is clearly apparent that effective isolation mechanisms must exist between sympatric *Pedicularis* species.

A 3-year study project was conducted to ascertain the mechanisms of reproductive isolation in sympatric populations of two louseworts, *Pedicularis rhinanthoides* Schrenk ex Fisch. et Mey. and *Pedicularis longiflora* Rudolph. These populations are common in the alpine meadows of the Sichuan Himalayas; however, no hybrid between the species has been found within the populations. The present study aimed to determine the nature and mode of action of isolation mechanisms responsible for reproductive isolation between the two species with similar pollinators. To achieve this objective, detailed comparative observations on the pollination ecology of the two species, as well as artificial treatments to investigate potential hybridization between the species, were conducted.

MATERIAL AND METHODS

STUDY SPECIES AND POPULATION

Intermingled populations of *P. rhinanthoides* and *P. longiflora* commonly occur in the alpine wet meadows of the Sichuan Himalayas. The corolla structure of the two species is shown in Figure 1. Flowers of the two species are nectarless; and have a corolla beak and a relatively long corolla tube. The flower of *P. rhinanthoides* is red, whereas that of *P. longiflora* is yellow. According to Li (1951), flowers with a corolla beak and a long tube represent the derived type of corolla structure in the genus. Species with this corolla type are restricted to the Himalaya. The stigmas in the two species have an expansive surface and 'gully like' structure. This is the form of stigma with the highest capability of pollen retention among species in the genus *Pedicularis* (Yang *et al.*, 2002).

Three subpopulations (A, B, and C) in the Sichuan Himalayas with intermingled *P. rhinanthoides* and *P. longiflora* were used in this study. The characters of each subpopulation are summarized in Table 1. In all the subpopulations, *Pedicularis* species were found to coexist with plants of other families, such as species of the cuckoo-bud (Ranunculaceae) and gentians (Gentianaceae). Flowering density was evaluated by the number of flowers of any species that were blooming in a 1 m × 1 m quadrat. Five such quadrats were randomly selected during the period of high simultaneous blooming and used to obtain the average flowering density of each subpopulation. All the artificial polli-

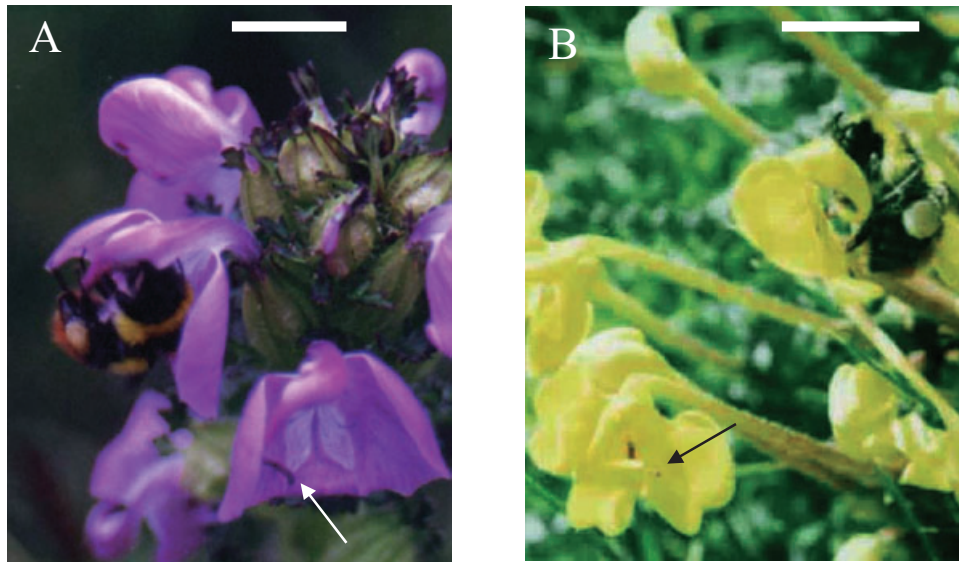


Figure 1. Corolla structure, stigma position, and bumblebee pollination of the two species. Arrows indicate the position and direction of stigma due to twist of corolla beak. A, *Pedicularis rhinanthoides*; B, *Pedicularis longiflora*. Scale bar = 1 cm.

Table 1. Characters of the three mixed subpopulations

Mixed subpopulation	A	B	C
Location	30°01'61" , 101°51'20"E	30°02'40"N, 101°50'35"E	30°08'98"N, 101°52'28"E
Habitat	Wet meadow	Shrubby hillside	Wet meadow
Elevation (m)	3520	3700	3680
Time of pollination observation (h)			
2001	60	30	10
2002	100	60	6
2003	40	40	4

nation treatments and detailed pollination observations were conducted in subpopulation A. From the other two subpopulations comparative data was obtained on the parameters: measurements of blooming period; identity of pollinators and their corbicula loads; pollinator frequency; flower constancy for each pollinator species; analysis of stigmatic pollen load; and fruit set.

FLORAL BIOLOGY

Blooming period and peak blooming for each species in the three mixed subpopulations were recorded from 2001–3. Blooming period was taken to be the date from the blooming of the first individual to the fading of the flowers on the last individual. The peak blooming period approximately equals the period during which no less than 60% of the individuals in a population are simultaneously in bloom. To examine flower function during anthesis, 15 randomly-selected

flowers from 15 individuals in subpopulation A were labelled and the onset of stigma sensitivity and pollen dehiscence were recorded. At least 20 flowers randomly selected from ten individual plants in subpopulation A were harvested before pollen dehiscence and immediately fixed in formalin alcohol acetic acid (FAA) solution for measurement of pollen and ovule production in the laboratory. Another 20 blooming flowers were picked randomly, fixed in FAA solution and used to study pollen morphology and pollen grain size by scanning electron microscopy.

POLLINATION AND REPRODUCTIVE SUCCESS

The periods during which pollination observations were made in each subpopulation during each year of the study are shown in Table 1. Pollinators were captured immediately after visiting the flower for identification in the laboratory. The pollinators' preferences with regard to ecological factors, including the

weather, air, temperature, and relative humidity, were also noted. Flower constancy and pollination frequency of each pollinator were measured in all of the three mixed subpopulations. To detect the flower constancy of each pollinator, at least 15 individual insects of each species in the pollinator fauna were tracked during a whole flying trip in the population. Pollination frequency was estimated as the cumulative period of time spent by pollinators on the flowers of *Pedicularis* species in a 1 m × 1 m quadrat during a 1-h observation period. Five randomly-selected quadrats were used during the peak blooming period to calculate the average pollination frequency of each of the subpopulations. To assess pollinator constancy and potential interspecific pollen transfer for each of the two study species in the three subpopulations, we examined the corbicular pollen load of each pollinator ($N > 10$) and the stigmatic pollen load ($N > 30$). Analysis of insect corbicular pollen load and stigmatic pollen load followed Macior (1995b) and Yang *et al.* (2002), respectively.

More detailed pollination observations and analysis were conducted in subpopulation A. Individuals of each of the bumblebee species visiting the two louseworts were carefully observed during flower visitation to ascertain their pollinating behaviour. Particular attention was paid to how the insect makes a landing on the flower; the specific parts of the insect's body to which pollen gets attached; and how the insect transfers the pollen grains to stigma. Flowers ($N > 20$) of the two species subsequent to undergoing this kind of pollination were harvested and fixed in FAA solution for analysis of stigmatic pollen load.

Pollination rate and fruit set were used in this study to evaluate pollination success and reproductive success. Pollination rate was calculated as the proportion of flowers whose stigmas had pollen deposit within a batch of 100 flowers harvested in the afternoon at peak blooming period from 30 randomly-selected individuals. To measure pollination rate, pistils were dissected carefully from the flowers and observed under a fluorescence microscope (Nikon E-600) after treatment with 8 mol L⁻¹ NaOH for 24 h, followed by dyeing in 0.1% aniline blue. At least 25 individuals of both *Pedicularis* species in each subpopulation were harvested for measurement of fruit set. Fruit set was calculated as the number of fruits with seeds divided by the total number of flowers on the plant.

ARTIFICIAL TREATMENTS

To detect reproductive success without pollinator service, ten individual plants of each species in subpopulation A were selected and enclosed with fine bridal veil netting before opening of the flowers. All the indi-

viduals were later harvested and checked for fruit and seed set.

Although a hybrid between the two species has not been found in nature, interspecific hand-pollination [*P. rhinanthoides* × *P. longiflora* (♀)] was conducted in subpopulation A. With the aim of determining the factors preventing formation of hybrids, ten individual plants of *P. longiflora* that had previously been enclosed in fine bridal veil netting were used for interspecific hand-pollination experiments during the period of highest simultaneous blooming. Following hand-pollination, 25 flowers were harvested 24 h later. Another 25 flowers were harvested 48 h after hand-pollination. The flowers were fixed immediately in FAA solution for examination under fluorescence microscope in the laboratory. The remainder of the treated flowers were retained on the plant still enclosed to provide information on fruit and seed set.

RESULTS

FLORAL TRAITS, POLLINATION, AND REPRODUCTIVE SUCCESS OF THE TWO SPECIES

Anthesis of individual flower in *Pedicularis rhinanthoides* is 5–7 days, whereas that of *P. longiflora* is 3–4 days. In both species, the blooming period of enclosed flowers is 2–3 days longer than that of exposed flowers. Stigmas of both species are receptive at the beginning of flower opening and, at this time, the anthers are also ready for dehiscence. Reproductive data for the two study species are summarized in Table 2. Both species received a high level of pollinator service, and had a relative high fruit-set under open pollination. However, flowers separated from pollinators recorded fruit and seed set values of close to zero (Table 2). Although stigmatic pollen load under open pollination is higher than ovule number per ovary for both species ($F = 7.34$, $P = 0.01$ for *P. rhinanthoides*; $F = 9.14$, $P = 0.005$ for *P. longiflora*, respectively), most of the ovules in the two species do not form mature seeds as indicated by the very low seed per capsule (Table 2).

During the 3 years of study, no other effective pollinators except bumblebees and no species-specific pollinators were observed for the two species. All the bumblebees observed visiting flowers of the two species collected pollen. Bumblebees land on the base of the floral beak and cling to the beak, making special wing vibrations that cause pollen to fall out of the hood and become dispersed over those parts of the bee's body that are exposed by the cleft in the corolla beak. The pollen grains are soon collected into the pollen baskets during grooming. However, the bees have an area on their bodies that is not accessible for grooming. These special 'pollination areas' correspond exactly to the position of stigma, which protrudes from

Table 2. Floral traits and reproductive success for the two species (mean \pm standard deviation)

Species	<i>Pedicularis rhinanthoides</i>	<i>Pedicularis longiflora</i>
Pollen size*	(21.83 \pm 0.63) \times (19.13 \pm 0.81)	(32.54 \pm 0.90) \times (31.96 \pm 0.91)
Pollen production	124736 \pm 21359	22688 \pm 3074
Ovule production	28.54 \pm 2.63	30.64 \pm 2.76
Pollen-ovule ratio	4436	746
Pollination rate	0.933	0.905
Fruit set		
Open pollination	85.6%	85.7%
Enclosed	4.8%	3.0%
Stigmatic pollen load	33.35 \pm 5.96	36.40 \pm 6.72
Seeds per capsule		
Open pollination	5.05 \pm 1.17	4.57 \pm 2.01
Enclosed	0.31	0.24

Data from subpopulation A in 2001–02.

*Polar axis diameter (μm) \times equatorial axis diameter (μm).

the tip of the corolla beak in both study species. The highest pollination frequencies for bumblebees in the study populations were recorded from 10.00 h to 15.00 h. The air temperature and humidity values were 20–25 °C and 45–55%, respectively.

For both species the queen bee is only an occasional pollinator, and has a very low level of flower constancy (Table 3). The worker bee is a frequent and more constant pollinator. Table 3 shows the different pollinator types and their flower constancy for *P. rhinanthoides* and *P. longiflora*. For *P. rhinanthoides*, the most important pollinator is *Bombus richardsi* (worker), followed by *Bombus mirus* (worker) and *Bombus yunnanicola* (worker). For *P. longiflora*, *B. yunnanicola* (worker) is the most frequent pollinator. On a single flying trip, most bumblebees tend to go from plant to plant of the same flower type, whereas they skip over plants with different flower traits. During our field observation of the three subpopulations over 3 years, only *B. richardsi* (worker) was observed pollinating flowers of both *P. rhinanthoides* and *P. longiflora* during a single flying trip in subpopulation A in 2002 (Table 3) and approximately 26 instances of this occurrence were recorded. Examination of the corbicular pollen loads of these bumblebees indicated that they consisted of pollen from *P. longiflora* (60%), *P. rhinanthoides* (26.5%), and from plants of other families (13.5%).

TEMPORAL VARIATION IN BLOOMING PERIOD FOR THE TWO SPECIES

Flowers in *P. rhinanthoides* blossom earlier than in *P. longiflora*, however, the blooming periods of the two species overlap (Fig. 2). The pattern was almost the same for all the three subpopulations in all 3 years. In

the same year, blooming periods of the subpopulations A and B were almost the same, whereas subpopulation C is in peak blooming 3–4 days later than the other two subpopulations.

VARIATION IN FLORAL TRAITS BETWEEN THE TWO SPECIES

Flowers of *P. rhinanthoides* have a much higher pollen production than those of *P. longiflora*. However, ovule production is similar for flowers of both species. Consequently, pollen-ovule ratio is much higher in *P. rhinanthoides* than in *P. longiflora*. Pollen size in *P. longiflora* is bigger than that of *P. rhinanthoides* (Table 2). In addition, the pollen grain in *P. longiflora* is spherical, whereas it is ellipsoidal in *P. rhinanthoides* (see polar and equatorial axis diameters in Table 2), a character that made it easy to distinguish pollen grains of the two species in insect corbicular and stigmatic pollen loads.

The differences between *P. rhinanthoides* and *P. longiflora* in the structure of their corolla beaks have an important effect on the pollination process in the two species (Fig. 1). First, the direction of the twist of the corolla beak is different in the two species. For *P. rhinanthoides*, it twists to the left, whereas in *P. longiflora* it twists to the right. In addition, the beak that encloses the anthers has a left and downward facing cleft in the flower of *P. rhinanthoides*, whereas it has a right-facing cleft in that of *P. longiflora*. A potential consequence of this difference is that pollen grains of the two species may tend to be deposited on different parts of the bumblebee's body. Only those parts of a bumblebee's body that are aligned to the cleft in the corolla beak can receive pollen grains whereas those facing the direction opposite

Table 3. Pollinator type for each species in the three mixed subpopulations and its flower constancy

Pollinator type	<i>Pedicularis rhinanthoides</i>			<i>Pedicularis longiflora</i>		
	Pollination observation	Flower constancy*	Pollination observation	Pollination observation	Flower constancy	Inter-pollination
<i>Bombus atrocinctus</i> (Queen)	None	–	2002A	2002A	7%	None
<i>Bombus lucorum</i> (Worker)	2002A	36%	None	None	–	None
<i>Bombus mirus</i> (Worker)	2001A, C; 2002A, B, C; 2003A	79%	None	None	–	None
<i>Bombus richardsi</i> (Queen)	2002A	19%	2002A	2002A	13%	None
<i>Bombus richardsi</i> (Worker)	2001A, B, C; 2002A, B, C; 2003A, B, C	86%	2001A, B, C; 2002A, B, C; 2003A, B, C	2001A, B, C; 2002A, B, C; 2003A, B, C	66%	2002A
<i>Bombus yunnanicola</i> (Worker)	2001A; 2002A, B	61%	2001A, B, C; 2002A, B, C; 2003A, B, C	2001A, B, C; 2002A, B, C; 2003A, B, C	84%	None

*Percentage of pollen grains of a certain species out of the pollen load of bumblebee captured from the species. *N* > 10, except for *B. atrocinctus* (Queen), *N* = 3, and *B. richardsi* (Queen), *N* = 5 for *P. rhinanthoides*, *N* = 4 for *P. longiflora*.

to the cleft have no chance for pollen deposition. Second, the further structural difference in the flower of the two species is in the degree of twist of the corolla beak. The twist is tighter in *P. longiflora* than in *P. rhinanthoides*. This difference results in a different position of the stigma, which protrudes from the tip of the corolla beak in both species. In the flower of *P. rhinanthoides*, the stigma is at a lower, inflexed position, whereas it is at an upper extrorse position in *P. longiflora*. In addition, the distance from the stigma to the anther is longer in *P. rhinanthoides* than in *P. longiflora*. We observed that, owing to these subtle structural differences, the stigma in the flowers of the two species contacts a different area of the insect's body during bumblebee pollination.

VARIATION OF BUMBLEBEE BEHAVIOUR IN POLLINATING FLOWERS OF THE TWO SPECIES

The flowers of the two species are pollinated sternotribically by bumblebees during foraging for pollen (Fig. 1). The behaviour of different bumblebees when visiting flowers of the same species is similar. However, careful observation revealed a subtle difference in the pattern in which pollen is deposited on the pollinator's body in the flowers of *P. longiflora* and those of *P. rhinanthoides*. During pollination, the anthers of *P. rhinanthoides* disperse pollen grains onto the caudal abdomen of the bumblebee, which is precisely the area that comes into contact with the stigma of the next flower to be visited. In *P. longiflora*, the pollen grains are deposited onto the prothorax of the bee's body. Correspondingly, although the stigma in the flower of *P. longiflora* has little chance of coming into contact with the caudal abdomen, it readily brushes against the prothorax of the insect during flower visitation.

NATURAL INTERSPECIFIC POLLEN TRANSFER

Although corbicular pollen load of all bumblebees is mixed, among 30 flowers of each species in the three subpopulations, we found a very low level of pollen interference on the stigmas of each species in all the subpopulations (Fig. 3A). Even for flowers of the two species picked after being visited by the interpollinating bumblebee (*B. richardsi*) in a single flying trip, a surprisingly high percentage of stigmas with only conspecific pollen were found (Fig. 3B).

FLOWERING DENSITY, POLLINATOR FREQUENCY, AND FRUIT SET

Figure 4 shows the flowering density, pollinator frequency and fruit set of each subpopulation and the trends of these parameters among the three subpopu-

lations. The data are means calculated from the values obtained in the 3 years of the study. Flowering density in subpopulations A and C is significantly higher than that in subpopulation B ($F = 22.41$, $P = 0.0001$ for A and B, $F = 21.43$, $P = 0.0001$ for C and B, respectively) whereas that in subpopulations A and C does not show any significant difference ($F = 0.05$, $P = 0.83$). A similar pattern was observed in pollinator frequency and

fruit set among the three subpopulations (pollinator frequency: $F = 18.94$, $P = 0.002$ for A and B, $F = 20.03$, $P = 0.002$ for C and B, and $F = 0.66$, $P = 0.44$ for A and C; fruit set: $F = 53.20$, $P < 0.0001$ for A and B, $F = 31.09$, $P < 0.0001$ for C and B, and $F = 4.75$, $P = 0.04$ for A and C; Fig. 4). Fruit set was taken as the ratio of the total number of fruits to the total number of flowers per plant for both *P. rhinanthoides* and *P. longiflora*.

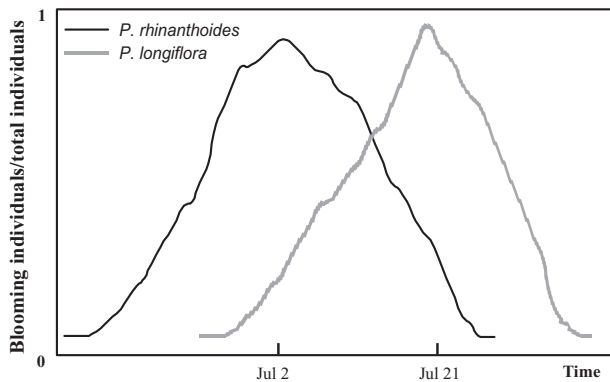


Figure 2. Blooming period of the two species in subpopulation A, 2002. The pattern was almost the same for all three subpopulations in all 3 years of the study. Additionally, in the same year, the blooming period of mixed subpopulations A and B was almost the same, whereas subpopulation C was 3–4 days later than A and B. The blooming period in 2001 was 5–7 days later than 2002, whereas that in 2003 was 12–15 days later than 2002.

EFFECTS OF INTERSPECIFIC HAND POLLINATION

Interspecific hand pollination (*P. rhinanthoides* × *P. longiflora* (♀)) did not yield any viable seeds. In some limited instances, it yielded shrunken or empty seeds. However, pollen grains from *P. rhinanthoides* germinated successfully on the stigma and penetrated the style of *P. longiflora*. Our experiments demonstrated that more than 90% (35 of 38) stigmas of *P. longiflora* hand-pollinated with pollen of *P. rhinanthoides* had successful pollen germination. Moreover, pollen tubes of the pollen grains of *P. rhinanthoides* successfully penetrated through the long style of *P. longiflora* and entered into the ovary within 24 h.

DISCUSSION

Because exclusion of insects from the *Pedicularis* plants completely prevents fruiting, whereas open-pollinated plants have a relatively high fruit set, pol-

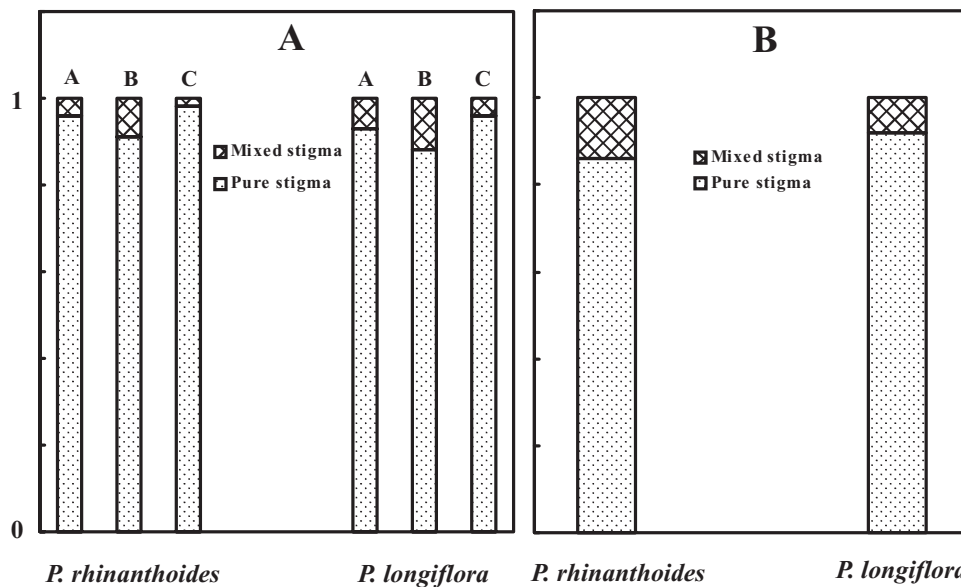


Figure 3. Percentage of pure stigma to mixed stigma among stigmas examined. A, randomly selected stigmas, data from average of three study years ($N > 90$ for each subpopulation); B, stigmas pollinated by intervisited of *Bombus richardsi* (worker) in a single flying trip, data from subpopulation A in 2002 ($N > 20$).

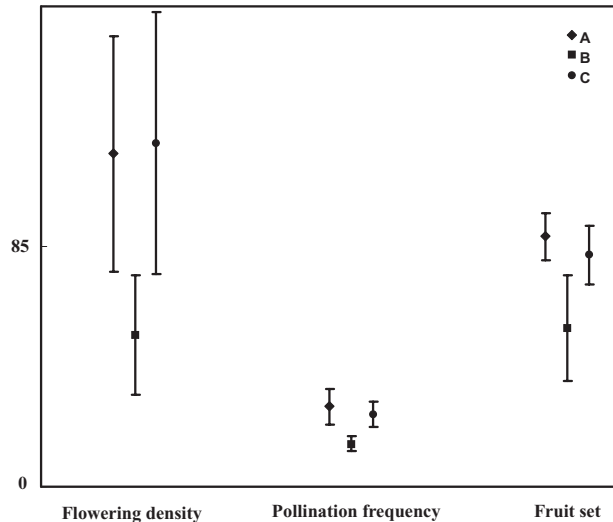


Figure 4. Flowering density, pollination frequency, and fruit set in the three mixed subpopulations. Data are from three study years. The units for flowering density, pollination frequency, and fruit set are the number of blooming flowers m⁻², the number of individual bumblebees h⁻¹ m⁻², and fruits per 100 flowers, respectively. Fruit set here is averaged for *Pedicularis rhinanthoides* and *Pedicularis longiflora*.

len transfer by *Bombus* species is therefore essential for reproductive success in *P. rhinanthoides* and *P. longiflora* (Wang & Li, 1998; Macior *et al.*, 2001). In addition, the differences in fruit set of the three mixed subpopulations display a similar trend to that of pollinator service. However, the observed low seed production per capsule cannot be ascribed to inadequacy of pollinators because high stigmatic pollen loads have been recorded in the two species (Yang, Sun & Guo, 2005).

POLLINATION FACILITATION AND POTENTIAL FOR INTERSPECIFIC POLLEN TRANSFER

The occurrence of simultaneously-flowering species can help to attract sufficient pollinators (pollination facilitation), which then increases reproductive output (Rathcke, 1983; Kwak, 1988; Laverty, 1992; Kunin, 1993, 1997; Petanidou *et al.*, 1995; Ohashi & Yahara, 1998). In the three mixed subpopulations used in the present study, preliminary examination showed that pollinator frequency follows the same trend as the density of blooming flowers per unit area. The same trends were observed in fruit set. The pattern of two or more species with overlapping blooming periods is common in genus *Pedicularis*. We suggest that the significance of this sympatric pattern of occurrence may be to increase pollinator abundance.

As noted earlier, one drawback associated with the increased pollinator service in mixed populations of sympatric species is the potential for increased interspecific pollen transfer. Therefore, we would expect the existence of effective isolation mechanisms between sympatric *Pedicularis* species. Grant (1994a) cited the case of genus *Pedicularis* as an example for studying reproductive isolation, especially floral isolation (Macior, 1982). A series of detailed studies on pollination biology of *Pedicularis* have been published by Sprague (1962), Koeman-Kwak (1973), Kwak (1979), Philipp *et al.* (1996), Wang & Li (1998), Eriksen, Molau & Svensson (1993), and especially by Macior and his colleagues (Macior, 1968, 1969, 1970, 1973, 1975, 1977, 1983, 1986, 1988, 1990, 1995b, c, 1996; Macior & Sood, 1991; Macior & Tang, 1997; Macior *et al.*, 2001). Unfortunately, studies focusing on reproductive isolation in the genus are very few (but are discussed by Macior, 1973, 1977, 1982, 1983). Grant (1994a) surveys the floral isolation between *P. attollens* and *P. groenlandica* by analysing the results of the studies by Sprague (1962) and Macior (1977). Here, we discuss the reproductive isolation (especially floral isolation) between *P. rhinanthoides* and *P. longiflora* in the light of the more detailed data obtained from our present study.

TEMPORAL ISOLATION

Temporal isolation can eliminate the possibility of interspecific pollen transfer among sympatric species (Grant, 1992, 1994a). Sprague (1962) and Adams (1983) report the existence of temporal variation in flowering periods in sympatric *Pedicularis* species, as also found in the present study (Fig. 2). The biological significance of the difference in peak blooming period is especially important because, in general, flowers in peak blooming period tend to have greater possibilities of being fertilized (Willson, 1983; Burd, 1995). In *P. rhinanthoides* and *P. longiflora*, the difference in peak flowering periods potentially may influence the establishment of reproductive isolation even though the temporal separation is not complete (Grant, 1994a).

MECHANICAL ISOLATION

The most common type of mechanical isolation exists when flowers of two or more species are adapted to different groups of pollinators with different body sizes and shapes (Grant, 1994b). This classical case of mechanical isolation obviously does not occur in the two study species because both of them are pollinated by bumblebees (Grant, 1994a; see also Kephart & Theiss, 2004). Considering that different sympatric *Pedicularis* species are occasionally pollinated by one

bumblebee during a single flying trip (Sprague, 1962; in this study: *B. richardsi*), it would be interesting to find out how mechanical reproductive isolation operates in these plants. Grant (1994a) reported mechanical isolation in *Pedicularis*, which is effected by the stigma picking up pollen from different parts of the pollinator's body. Additionally, Macior (1982) pointed out that floral morphology in *Pedicularis* closely corresponds to insect form and behaviour, with residual pollen being transported in one of three specific positions on insects' bodies that are readily accessible to a stigma but not to insect grooming. The results of our study lend support to the findings of this earlier work. Due to differences in corolla structure between the two species, *P. rhinanthoides* disperses pollen grains onto the caudal abdomen of the pollinating bumblebee, which is precisely the region that will come into contact with the stigma during visitation to the next flower of the same species. For *P. longiflora*, pollen grains will be deposited on the prothorax of the bumblebee and, correspondingly, the stigma in the flower of this species will pick up pollen grains from the prothorax of the insect. Even though insect corbicular pollen load is mixed, examination of stigmas shows that stigmatic pollen load exhibits a high degree of purity. Moreover, examination of the stigmas of flowers subsequent to interspecific pollination by *B. richardsi* (workers) also indicates that pollen mixing due to interspecific pollination is minimal. This rather unexpected situation can be attributed to mechanical isolation.

The evolutionary significance of the substantial variation of corolla beak in *Pedicularis* remains unclear up to now, although such a 'beak' has been regarded as a derived floral trait that can enhance pollination efficiency (Robart, 2000). In the present study, we highlight the role of corolla beak in causing mechanical reproductive isolation in genus *Pedicularis*. The different shapes of corolla beak arising from the varying degrees and direction of twisting of the corolla beak in genus *Pedicularis* creates different positions and directions of the stigma, and different patterns of pollen dispersal. This allows different *Pedicularis* species to have different pollination syndromes, and thus increase the chances of mechanical reproductive isolation. We suggest that the evolution of the corolla beak in *Pedicularis* was in response to the need to avoid interspecific pollen transfer (by forming an isolating mechanism) in the presence of pollinator competition since *Pedicularis* species are far in excess of the species of bumblebees pollinating the genus.

ETHOLOGICAL ISOLATION

Grant (1994b) recognized four types of ethological isolation. He further pointed out that the type of etholog-

ical isolation in genus *Pedicularis* is due to flower constancy (Grant, 1994a), which is a very common type of ethological isolation and is found in many other taxa (Grant, 1994a,b). Our observation that bumblebees display a high level of constancy in visiting flowers of *Pedicularis* clearly supports the existence of ethological isolation between the two *Pedicularis* species (Macior, 1982, 1995b, c; Macior & Tang, 1997; Macior *et al.*, 2001). This position was supported by the results of analysis of insect corbicular pollen loads, which indicated a relatively high level of purity. However, the ethological isolation between the two *Pedicularis* species obviously cannot be complete as demonstrated by the mixed corbicular pollen loads and the occasional observations of bumblebees indiscriminately visiting flowers of the two species during a single foraging foray (Sprague, 1962; Macior, 1995b, c).

Bumblebees exhibit a high level of flower constancy in their foraging behaviour. Individual bees tend to go from plant to plant of the same flower type, whereas they skip over plants with different flower traits (Heinrich, 1976). This is the foundation of ethological isolation. Grant (1994a) suggests that the occasional interspecific visits of bumblebees are for two reasons. First, young inexperienced workers are inconstant at first but soon become constant (Heinrich, 1979). Second, experienced bumblebees working on *Pedicularis* interrupt their activity at intervals and make episodic side trips. These behaviours are also probably the main contributors to the mixed corbicular pollen load. The occasional interspecific bumblebee visits observed in subpopulation A might merely be due to the longer observation time because analysis of corbicular pollen loads in *B. richardsi* indicated a similar percentage of pollen grains of *P. longiflora* on both bumblebees displaying flower constancy in their visitation behaviour and those exhibiting interspecific visitation.

Although the existence of pollinator-mediated plant speciation in species with generalized pollinators has been questioned recently (Waser, 1998; Johnson & Steiner, 2000), floral isolation in such sympatric species has been found to play a role in maintaining reproductive isolation, which is necessary for plant speciation (Grant, 1949; 1994a; Kephart & Theiss, 2004; Rieseberg & Wendel, 2004). *Pedicularis* species have no species-specific pollinator; however, ethological isolation in combination with mechanical isolation clearly plays an important role in reproductive isolation, as indicated by the results of the present study. This condition, where the two forms of isolation act in concert, has been reported in many other studies for other taxa (Kwak, 1978; Nilsson, 1983; Suzuki, 1984; Nilsson *et al.*, 1987). Even in cases where floral isolation is incomplete, Grant (1992) noted, 'It does not mean that it is unimportant as a species-separating mechanism. Floral isolation does not act alone; it oper-

ates in conjunction with other external isolating mechanisms, which are also incomplete. Two or three incomplete isolating mechanisms acting jointly can bring about a high degree of reproductive isolation'.

INTERNAL REPRODUCTIVE BARRIERS

A cross of *P. rhinanthoides* × *P. longiflora* (♀) does not yield seeds and only seldom does it yield shriveled or empty seeds. This suggests the existence of a strong internal isolation mechanism between the two sympatric species. Sprague (1962) and Macior (1983) also predict that internal barriers are operative between sympatric *Pedicularis* species. Additionally, the successful germination of interspecific pollen and growth of the pollen tube indicate that, for the two sympatric species, the internal reproductive barrier must operate after fertilization. Compared with external isolation, internal isolating mechanisms are relatively robust. External isolations are fragile because they may work effectively under stable environmental conditions but break down when environmental changes upset an existing equilibrium (Grant, 1992). Prezygotic isolation acting in combination with postzygotic isolation is frequent in nature (Sprague, 1962; Grant, 1994a, b; Gardner & Macnair, 2000; Ando *et al.*, 2001). In Macior's series of studies on *Pedicularis*, he also predicts that floral isolation in conjunction with internal isolation is the most important factors maintaining the species boundaries in the genus (Macior, 1973, 1977, 1982, 1983). The results of the present study fit very well into this prediction.

THE NECESSITY OF FLORAL ISOLATION IN PEDICULARIS

Because internal isolation strongly exists between the two sympatric *Pedicularis* species, their evolving of floral isolation would appear to be superfluous; however, Kephart & Theiss (2004) state that floral isolation can reduce pollen wastage, stigma clogging, or the production of inviable or unfit offspring, even in species with strong post-mating barriers to hybridization. This argument explains the observed successful germination of interspecific pollen and growth of the pollen tube for the two sympatric species in the present study. Unless floral isolation is effective, interspecific pollen (unfit pollen) will occupy the stigma, clog the style, and prevent the ovule from forming viable seed (yield useless seed). Furthermore, conspecific pollen grains deposited later on the stigma may be denied the opportunity to fertilize the ovule and yield functional offspring.

Recent studies have indicated that plants show flexibility in adjusting their strategies of reproductive isolation according to the changes of the environment. Wendt *et al.* (2002) found that reproduction can be

facilitated by selfing among three sympatric *Pitcairnia* species by the absence of temporal isolation, geographical isolation, and isolation via postpollination processes (Ando *et al.*, 2001). The current pattern of high diversity in flower structure in *Pedicularis* has been regarded as a result of relatively rapid evolution (Macior, 1995a; Yang *et al.*, 2003). The results of the present study suggest that the plants have evolved to utilize several partially effective external isolation mechanisms in combination with internal isolation to accomplish reproductive isolation. The evolution of a richer repertoire of reproductive isolation mechanisms rather than reliance on a single mechanism may be in response to the stochastic pollination environment imposed by the highly variable climate in mountainous habitats where sympatric *Pedicularis* species commonly bloom simultaneously. The sympatric isolation mechanisms described in the present study are likely more widespread and have played an important role in the floral evolution of *Pedicularis*.

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