

Floral traits, pollination ecology and breeding system of three *Clematis* species (Ranunculaceae) in Yunnan province, southwestern China

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Abstract. Flowers of *Clematis* display showy corollas and diversified shapes. This diversity motivates study of adaptive evolution of flower–pollinator interactions and the functional association between floral traits and plant mating strategies. An integrative study was undertaken, which focused on pollination and reproductive biology of three species representing all three floral types of *Clematis*. Floral traits were measured, and pollinator assemblages were observed in the field. Bagging, hand-pollination and removal treatments were used to examine breeding systems. The inbreeding depression and pollen limitation were estimated by fruit-set and seed production obtained from pollination treatments. Their floral traits are distinctly different, but are highly associated with pollination syndrome and breeding system. Among them, *Clematis akebioides* and *C. rehderiana* may be facultative autogamy (the former was delayed selfing, and the later competing selfing), and *C. chrysocoma* may be nearly obligate outcrossing. These conclusions are reflected in their stamen–pistil ratios. The levels of inbreeding depression are negatively associated with autonomous self-pollination. Evolution of self-pollination in *C. akebioides* and *C. rehderiana*, and pollen limitation in reproduction of the three *Clematis* species are discussed. This present study, integrating with previous results, will help us to comprehensively recognise and understand the pollination system and reproductive characteristics of *Clematis*.

Introduction

The diversity of flowers and fruits largely reflects the diversity of species of flowering plants. Species classification depends heavily on the characteristics of flowers and fruits (e.g. Takhtajan 1997; Wu *et al.* 2003). Flower diversity has evolved as a consequence of adaptation to various biotic and abiotic environmental factors, particularly to pollinating agents (Grant and Grant 1965; Richards 1997; Gorelick 2001; Barrett 2002b; Strauss and Whittall 2006). One of the many dimensions of floral diversity is the evolution and variation of corollas, especially in animal-pollinated plants (Glover 2007). The diversification in floral form and function provides a compelling example of adaptive evolution and offers opportunities for investigating the functional association between floral traits and plant mating strategies (Barrett and Harder 1996; Barrett 1998, 2002a).

The genus *Clematis*, with ~350 species, is one of the largest genera in the Ranunculaceae. It is distributed in all continents except Antarctica (Wang and Bartholomew 2001; Wang and Li 2005), and exhibits significant diversification in south-western China, especially in Yunnan province (Wang and Bartholomew 2001; Jiang *et al.* 2007). *Clematis* spp. have been commonly used for ornamental purposes because their flowers have extraordinarily showy corollas and diverse shapes (Grey-Wilson 2000; Toomey *et al.* 2001). Based on the shape of sepals, *Clematis*

spp. can be classified into three types: (i) dish-like with spreading sepals; (ii) bell-like with ascending sepals; and (iii) tubular with erect sepals. Pollination observations of some *Clematis* species have been reported from Europe, North American and Japan (Knuth 1908; Dohzono and Suzuki 2002; Borkent and Harder 2007). However, pollination studies of this genus are rare in Asia, especially in south-western China. Furthermore, the diversity of *Clematis* flowers has attracted only a little attention in the study of adaptive evolution of flower–pollinator interactions and the functional association between floral traits and plant mating strategies (only in Dohzono *et al.* 2004).

Previously, pollination and reproductive studies of *Clematis* have been rare (Knuth 1908; Timmerman-Erskine and Boyd 1999; Dohzono and Suzuki 2002; Dohzono *et al.* 2004; Borkent and Harder 2007). Most of these studies focused on single species, with the exception of Knuth (1908) who described the visitor assemblages of seven species of *Clematis*. Timmerman-Erskine and Boyd (1999) explored which factors limited reproduction in the rare *C. scocialis*; Dohzono and his colleagues (Dohzono and Suzuki 2002; Dohzono *et al.* 2004) resolved the association of temporal change of calyx-tube length of *C. stans* with foraging behaviour of bumblebee; Borkent and Harder (2007) examined the correlation between dioecy in *C. ligusticifolia* and a generalised pollinator. Therefore,

it is valuable to make an integrated and comparative study in several species to understand pollination and reproductive characteristics of *Clematis*. This study compares three *Clematis* species that represent the three floral types and our aim is to: (i) characterise their floral traits, especially the stamen-pistil ratio; (ii) document floral visitor assemblages and behaviour of pollinating and non-pollinating floral visitors; (iii) characterise the degree of dependence on pollinators for seed production; and (iv) analyse the level of inbreeding depression and the index of pollen limitation. The pollination and reproductive biology of Chinese *Clematis* is integrated with previous studies from other regions to help us comprehensively recognise and understand the pollination system and reproductive characteristics of this genus around the world.

Materials and methods

Study species and sites

Three *Clematis* species, *C. chrysocoma* Franch., *C. akebioides* (Maxim.) Veitch and *C. rehderiana* Craib, were investigated from 25 April to 18 September in 2007 at two sites, Kuming Changchong Mountains (KCM) in the Yun-Gui Plateau, and Zhongdian Wengshui Village (Z WV) in the Hengduan Mountains. Both are in Yunnan Province, south-western China (see Appendix 1). *Clematis chrysocoma* belongs to sect. *Cheiroopsis*, and is a perennial ligneous herb; and *C. akebioides* belongs to sect. *Meclatis* and *C. rehderiana* belongs to sect. *Viorna*, and both are perennial woody lianas (Wang and Li 2005). *Clematis akebioides* and *C. rehderiana* occur along the roadside and frequently mix with each other or *Rosa* spp. or other shrubs, and *C. chrysocoma* often appears in open areas.

Floral biology

In order to observe variation in floral traits, 20 flowers from different individuals of the three *Clematis* species were randomly selected. The following data were recorded: the opening pattern of sepals, changes in sepal characters, dehiscence gradation of anthers, nectar production, floral odor, and floral lifespan. Nectar was simply identified by the naked eye and measured by 5- μ L capillaries (10–15 flowers each species). Floral scent was directly smelled by the nose. Floral lifespan represents flower state from opening to withering. Benzidine and hydrogen peroxide solution (1% $C_{12}H_{12}N_2$: 6% H_2O_2 : H_2O = 4: 11: 22) were used to test stigma receptivity and estimate dichogamy within flowers. Fifty flowers per species were collected to count numbers of stamens and pistils, and 40 flowers per species were used to measure length and width of sepals by vernier caliper. The length and width of sepals per flower were calculated by mean value of four (rarely five) sepals.

Correlation between stamen and pistil number within species was analysed by Pearson correlation (two-tailed). Datasets of floral lifespan, sepal size, stamen and pistil number, and stamen-pistil ratio had a normal distribution tested by a one-sample Kolmogorov–Smirnov or nonparametric test (NPar) ($P < 0.05$). Tukey's multi-comparison of one-way analysis of variance (ANOVA) were used to test the differences ($P < 0.05$) in floral lifespan for three species. Due to floral traits including sepal size, stamen number, pistil number, and stamen–pistil ratio violate the assumption of homogeneity of variance. A Kruskal–Wallis H test

of NPar was used to analyse the differences among the species. The differences between pairs of species were analysed by Mann–Whitney *U*-test of NPar ($P < 0.05$).

Pollinator observations

Observations of flower visitors to *C. chrysocoma* were made on 7, 12, 19 May 2007 from 10:00 h to 15:00 h, of *C. akebioides* on 12, 14, 15 August 2007 from 10:00 h to 16:00 h, and of *C. rehderiana* on 26, 28, 30, July 2007 from 10:00 h to 16:00 h. Because *C. chrysocoma* was scattered, one observer systematically walked through the population 2×100 m² and recorded visitor type and frequency. For *C. akebioides* and *C. rehderiana*, ~20 adjacent flowering plants for each species were chosen to record visitor type and frequency. Each record of all species was at intervals of 30 min. After being tracked and recorded, at least three individuals of each pollinator species were captured for identification and checking for the presence of pollen grains on their bodies. Voucher specimens of insect pollinators were deposited at the Kunming Institute of Botany (KIB), Chinese Academy of Sciences, Kunming.

Breeding system

To examine the breeding system, and the contribution of insect visitors and floral organs to pollen transport, seven experimental treatments were performed (Table 1): (1) open pollination: flowers were marked before anthesis and then permitted natural visitation by insects; (2) bagged treatment: flowers about to open were enclosed in 1 mm \times 1 mm nylon nets until withered; (3) hand self-pollination: flowers were bagged before anthesis, and freshly opened flowers were hand-pollinated with self-pollen and then bagged again; (4) hand cross-pollination: treatments followed hand self-pollination, but pollen sources were collected from other individuals at least 5–15 m away from pollen recipients; (5) removal of sepals only; (6) removal of anthers only; and (7) removal of stamens only (i.e. anthers and filaments) (except *C. chrysocoma* because it is without a nectary at the base of filaments). For *C. akebioides* and *C. rehderiana*, the seven treatments were manipulated on seven flowers on the same plant, and 20–60 plants were randomly chosen. For *C. chrysocoma*, the first four treatments were manipulated on four flowers on the same plant, and the latter two treatments were manipulated on two flowers on the same plant, because only a few plants had more than four plants blooming synchronously. They were marked and collected for fruit and seed count when fruits were mature. The autonomous selfing index was defined as the fruit-set or seed production of bagged flowers divided by that of hand-crossed flowers (Lloyd and Schoen 1992).

The Crosstabs test was used to compare the differences ($P < 0.05$) of fruit-set among (six or) seven treatments within species. The seed production dataset had a normal distribution, tested with a one-sample Kolmogorov–Smirnov test of NPar ($P < 0.05$). Tukey's multi-comparison test of one-way ANOVA were used to test the differences ($P < 0.05$) in seed production among (six or) seven treatments within species.

Inbreeding depression and pollen limitation

The experimental data of fruit-set and seed production obtained from breeding systems were used to calculate an index of

Table 1. Floral traits of the three *Clematis* species (mean \pm s.e.)

One-way ANOVA with Tukey's multi-comparison test was used to analyse the differences of floral lifespan among three species. Kruskal–Wallis H test of nonparametric tests (NPar) was used to analyse the differences of sepal size, stamen and pistil number, and stamen–pistil ratio among three species, and Mann–Whitney *U*-test of NPar was used to compare the differences between them. The different lowercase letters indicating the means were significantly different ($P < 0.05$)

Floral characters	<i>C. chrysocoma</i>	<i>C. akebioides</i>	<i>C. rehderiana</i>	Statistical analysis
Dehisced gradation of anthers	From outer to inner	From outer to inner	From inner to outer	–
Nectar production	Nectarless	Nectar	Nectar	–
Floral odour	Odourless	Odour	Odourless	–
Floral colour	White	Orange	Yellow	–
Floral lifespan (days)	7.55 \pm 0.17 ^a <i>n</i> = 20	5.00 \pm 0.16 ^b <i>n</i> = 20	4.85 \pm 0.17 ^b <i>n</i> = 20	<i>F</i> = 50.80***
Sepal size				
Length (cm)	2.31 \pm 0.05 ^b <i>n</i> = 40	3.03 \pm 0.08 ^a <i>n</i> = 40	2.01 \pm 0.03 ^c <i>n</i> = 40	χ^2 = 67.65***
Width (cm)	1.53 \pm 0.04 ^a <i>n</i> = 40	1.01 \pm 0.02 ^b <i>n</i> = 40	0.60 \pm 0.01 ^c <i>n</i> = 40	χ^2 = 104.27***
No. of stamens per flower	100.02 \pm 3.14 ^a <i>n</i> = 50	45.34 \pm 0.7 ^b <i>n</i> = 50	50.82 \pm 0.37 ^b <i>n</i> = 50	χ^2 = 113.51***
No. of pistils per flower	50.12 \pm 2.17 ^b <i>n</i> = 50	141.98 \pm 2.39 ^a <i>n</i> = 50	36.14 \pm 0.38 ^c <i>n</i> = 50	χ^2 = 108.74***
Stamen–pistil ratio	2.13 \pm 0.08 ^a <i>n</i> = 50	0.32 \pm 0.05 ^c <i>n</i> = 50	1.41 \pm 0.02 ^b <i>n</i> = 50	χ^2 = 120.70***

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

inbreeding depression and pollen limitation. Inbreeding depression was calculated as $\delta = 1 - (w_s/w_o)$, where w_s is the fitness of progeny from within-flower selfing and w_o the fitness of progeny from outcrossing (Lande and Schemske 1985; Charlesworth and Charlesworth 1987). Cumulative inbreeding depression was calculated as the fitness ratio for fruit-set (*f*) and seed production (*s*), that is, $\delta = 1 - [(w_{sf}/w_{of}) \times (w_{ss}/w_{os})]$ (Husband and Schemske 1996; Carrió *et al.* 2008). The level of inbreeding depression is always less than one, where zero indicates no inbreeding depression. Positive values indicate that outcrossed progenies are more advantageous than selfed ones, whereas negative values mean the opposite.

The pollen limitation index was expressed as $L = 1 - (P_o/P_s)$, where P_o is the reproductive output of open-pollinated controls, while P_s is that of hand cross-pollination controls (Larson and Barrett 2000). The level of pollen limitation generally ranges from zero to one, where zero indicates no pollen limitation. Sometimes, the values are negative (i.e., greater fertility from natural than supplemental pollination), likely resulting from experimental or Type I statistical errors (Young and Young 1992; Burd 1994; Larson and Barrett 2000).

Results

Floral biology

The corolla type of *C. chrysocoma* is dish-like, that of *C. akebioides* is bell-like, and that of *C. rehderiana* is tubular (Fig. 1). *Clematis chrysocoma* bloomed earlier than the other two species. All three species are protogynous. Floral traits of the studied species are listed in Table 1.

Anthers of *C. chrysocoma* and *C. akebioides* dehisced from outer to inner, and those of *C. rehderiana* did the opposite. *Clematis chrysocoma* produced no nectar or nectar in small amounts. The other two evidently produced nectar (0.6–3.0 μ L) at the base of filaments. *Clematis akebioides* smells sweet, and the other two have no odor detectable to humans. The floral lifespan of *C. chrysocoma* was ~6–9 days, which is remarkably longer than the other two species (Table 1). Tukey's multi-comparison test of one-way ANOVA showed that

the mean value of lifespan of *C. chrysocoma* was significantly longer than the other species (Table 1). The sepals of *C. akebioides* were longer than in the others species, and the sepals of *C. chrysocoma* were wider than in the other species (Table 1).

The mean value of stamen–pistil ratio for *C. chrysocoma* was higher than for *C. akebioides* and *C. rehderiana* (Mann–Whitney *U*-test, $Z = -8.62$, $P < 0.001$, and $Z = -7.08$, $P < 0.001$, respectively), and the latter invested significantly more in stamens relative to pistils compared with the former ($Z = -8.62$, $P < 0.001$). Additionally, correlations between stamens and pistils for *C. chrysocoma* and *C. akebioides* were distinctly positive ($r = 0.566$, $P < 0.001$, and $r = 0.523$, $P < 0.001$, respectively), but for *C. rehderiana* were not ($r = 0.104$, $P > 0.1$).

Pollination systems

Insects that consistently contacted both anthers and stigmas within flowers of one species and had pollen grains deposited on their bodies were regarded as pollinators (Dafni 1992). Based on field observation and checking for pollen on specimens using a microscope, two species of Syrphidae (*Syrphus* sp. and *Episyrphus* sp.) and one species of Megachilidae (*Lithurgus* sp.) were considered effective pollinators of *C. chrysocoma* (Fig. 1A–C, Table 2). When the flies *Syrphus* sp. and *Episyrphus* sp. were visiting, they licked anthers of *C. chrysocoma*, and pollen grains adhered to their feet and abdomen and were deposited within the flower or were transferred to other flowers (Fig. 1A, B). The pollinating behaviour of *Lithurgus* sp. was similar to the two flies but they collected pollen grains on their scopa (Fig. 1C). Among the three pollinators, *Syrphus* sp. was the most frequent pollinator (Table 2). Numerous beetles stayed on the floral sepals, but they only dallied with each other or gnawed sepals and stamens (Fig. 1D).

Clematis akebioides and *C. rehderiana* were primarily pollinated by three bumblebee species (Fig. 1E–K). When the bees entered the blooming flowers (Fig. 1E, F, H, J) or broke into unopened flowers (Fig. 1G, I, K) to collect nectar from the base of

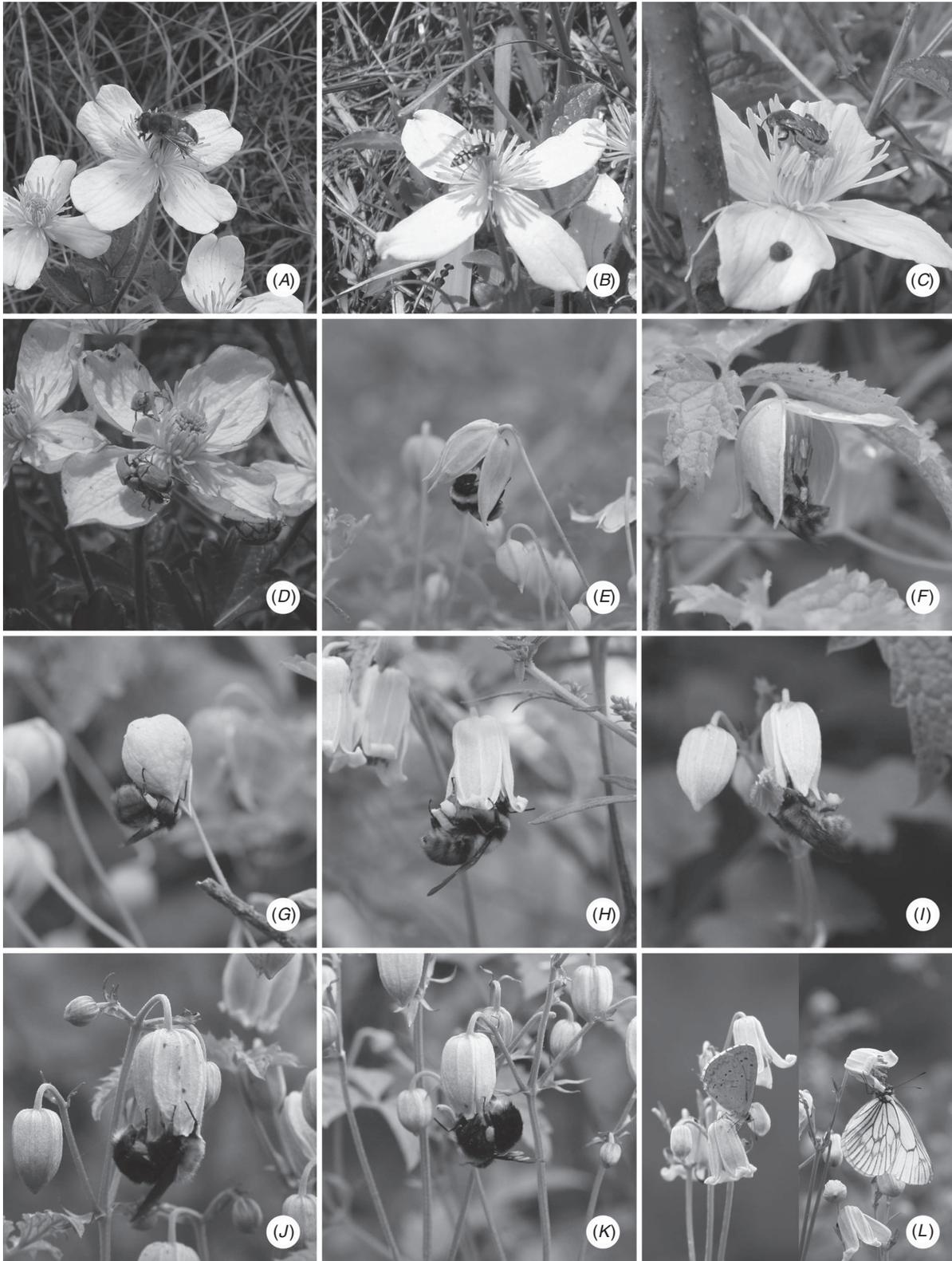


Fig. 1. Flower visitors of the three *Clematis* species. (A–D) *C. chrysocoma*: (A) *Syrphus* sp., and (B) *Episyoohus* sp. were licking anthers pollen with their tongues; (C) *Lithurgus* sp. was collecting pollen by its legs; (D) beetles were playing out of the flower. (E–G) *C. akeioides*: (E) *Bombus richardsi* and (F) *B. lucorum* were visiting opened flowers; (G) *B. richardsi* managed to enter an unopened new flower. (H–L) *C. rehderiana*: (H) *B. richardsi* and (J) *B. atrocinctus* were visiting opened flowers; (I) and (K) managed to enter an unopened new flower; (L) butterflies were nectar-robbers.

Table 2. The frequency of different insect visitors to three *Clematis* species

Species	No. of hours	Insect visitors	No. of visitors	Pollinator ratios (%)
<i>C. chrysocoma</i>	15	<i>Syrphus</i> sp.	158	79.0
		<i>Episyrphus</i> sp.	16	8.0
		<i>Lithurgus</i> sp.	26	13.0
<i>C. akebioides</i>	18	<i>Bombus richardsi</i>	111	80.4
		<i>B. lucorum</i>	8	17.4
		<i>B. atrocinctus</i>	19	2.9
<i>C. rehderiana</i>	18	<i>B. richardsi</i>	171	82.2
		<i>B. lucorum</i>	8	3.8
		<i>B. atrocinctus</i>	19	9.1

a calyx, pollen grains were stuck on their thoraxes and venters, and deposited on to stigmas or transferred to other flowers. Among the three bumblebees, *Bombus richardsi* was the most frequent pollinator of *C. akebioides* and *C. rehderiana* (Table 2). In addition, two butterflies were frequently observed staying on the flowers of *C. akebioides* and *C. rehderiana* and robbing nectar through the gap between sepals (Fig. 1L).

Breeding system

For all three species, both fruit-set and seed production of (six or seven treatments were remarkably different (Table 3). The two hand-pollination treatments indicated that all three *Clematis* species were self-compatible. The autonomous selfing indices of *C. chrysocoma*, *C. akebioides* and *C. rehderiana* were 0.29, 0.97 and 0.80 for fruit-set, respectively, and 0.23, 0.76 and 0.84 for seed production, respectively. For *C. akebioides* and *C. rehderiana*, these indices were larger than 0.76, indicating that they had high potential autonomous selfing rates in the absence of pollinators. In contrast, *C. chrysocoma* primarily depended on pollinators to achieve pollination. Self-fertilisation including autonomous selfing and hand selfing remarkably reduce seed production in all three species (Table 3).

Removal of anthers and stamens (except *C. chrysocoma*) showed that insect-mediated cross-pollination played a very important role in sexual reproduction of all three *Clematis*,

although trimmed flowers produced less fruits and seeds than intact ones (Table 3). Removal of anthers had less effect on fruit-set than removal of stamens in *C. akebioides* and *C. rehderiana*, and had less effect on seed production in the former, but had no effect in the latter (Table 3). Additionally, removal of sepals reduced fruit-set and seed production in all three *Clematis*, especially of *C. chrysocoma* (Table 3).

Inbreeding depression and pollen limitation

The levels of inbreeding depression of *C. chrysocoma*, *C. akebioides* and *C. rehderiana* were 0.13, -0.03 and 0.05 for fruit-set, respectively, and 0.65, 0.12 and 0.20 for seed production, respectively. The cumulative values of inbreeding depression of *C. chrysocoma*, *C. akebioides* and *C. rehderiana* were 0.71, 0.10 and 0.25, respectively. In *C. akebioides*, these values were near zero, indicating the absence of high inbreeding depression, at least in these phases of fruiting and seed development. *Clematis chrysocoma* experienced high inbreeding depression, especially in the phases of seed development. Although inbreeding depression of *C. rehderiana* was lower in the phase of fruiting, it was significantly increased in the phase of seed development.

Pollen limitation indices of *C. chrysocoma*, *C. akebioides* and *C. rehderiana* were 0.10, 0.06 and 0.13 for fruit-set, respectively, and 0.02, -0.02 and -0.04 for seed production, respectively. In all three *Clematis* species, these indices were near zero, indicating that they did not experience serious pollen limitation under natural pollination in our study populations.

Discussion

Traditional classifications of *Clematis* always emphasise floral forms as infra-genera criteria (Wang and Li 2005 for a review). According to our surveys, floral traits of our three studied species representing three sections of *Clematis* are functionally distinct. The dish-like flowers of *C. chrysocoma* are obviously different from the other two species (Table 1). Variation in floral forms would directly influence divergence of floral characters, pollination system and breeding system (Darwin 1877; Grant and Grant 1965; Fenster *et al.* 2004). Recent molecular phylogenetic studies demonstrate that similar floral forms of *Clematis* are scattered in different clades (Miikeda *et al.* 2006;

Table 3. Fruit-set and seed production of the three *Clematis* species under different pollination treatments

The different lowercase letters show the significant differences ($P < 0.05$) by crosstabs test (2×2) for fruit set, and by Tukey's multi-comparison test of one-way ANOVA analysis for seed production

Treatments	Flowers	<i>C. chrysocoma</i>		<i>C. akebioides</i>		<i>C. rehderiana</i>			
		Fruit set (%)	Seed production (mean \pm s.e.)	Fruit set (%)	Seed production (mean \pm s.e.)	Fruit set (%)	Seed production (mean \pm s.e.)		
Open pollination	40	87.5 ^a	48.49 \pm 1.82 ^a	60	86.7 ^{a,b}	111.31 \pm 3.55 ^a	60	85.0 ^b	32.65 \pm 0.87 ^a
Bagged	40	25.0 ^d	11.38 \pm 2.09 ^c	40	90.0 ^{a,b}	83.19 \pm 3.86 ^b	40	77.5 ^{b,c}	26.23 \pm 1.12 ^{b,c}
Hand self-pollination	40	85.0 ^{a,b}	17.26 \pm 1.66 ^{b,c}	40	95.0 ^a	96.08 \pm 3.18 ^{a,b}	40	92.5 ^{a,b}	24.89 \pm 1.06 ^c
Hand cross-pollination	40	97.5 ^a	49.59 \pm 1.69 ^a	40	92.5 ^a	109.14 \pm 3.12 ^a	40	97.5 ^a	31.28 \pm 0.83 ^{a,b}
Sepals removed	20	50.0 ^c	27.30 \pm 2.56 ^b	20	60.0 ^c	91.67 \pm 5.75 ^{a,b}	20	80.0 ^{b,c}	30.94 \pm 1.65 ^{a,b}
Anthers removed	20	60.0 ^{b,c}	24.50 \pm 2.23 ^b	25	72.0 ^{b,c}	92.11 \pm 5.58 ^{a,b}	30	86.7 ^b	26.16 \pm 1.29 ^{b,c}
Stamens removed	–	–	–	20	55.0 ^c	86.91 \pm 4.64 ^b	20	60.0 ^c	26.83 \pm 1.90 ^{b,c}
Statistical analysis		$\chi^2 = 69.97^{***}$ $F = 64.85^{***}$		$\chi^2 = 29.71^{***}$ $F = 8.31^{***}$		$\chi^2 = 18.03^{**}$ $F = 9.07^{***}$			

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Jiang 2010). Homoplasy of floral forms may be associated with pollination agents (Kevan and Baker 1983; Leppik 1988; Richards 1997).

The nectarless *C. chrysocoma* as well as *C. vitalba*, *C. recta* and *C. ligusticifolia* (Knuth 1908; Borkent and Harder 2007) with dish-like shapes and white coloured flowers may be highly associated with generalised pollinators (e.g., flies) as a traditional pollination syndrome (Stebbins 1970; Faegri and van der Pijl 1979; Kevan and Baker 1983; McCall and Primack 1992). Nevertheless, the nectar of *C. akebioides* as well as *C. balearica* (= *C. cirrhosa* var. *halearica*) and *C. alpine* (= *Astragene alpina*) (Knuth 1908) with bell-like flowers, and *C. rehderiana* as well as *C. integrifolia* (Knuth 1908) and *C. stans* (Dohzono and Suzuki 2002) with tubular flowers, may be highly associated with a specialised pollinator, that is, bumblebees. Based on this present study and integrated with previous results, the pollination system of *Clematis* can be classified by two functional groups of pollinators, that is, fly pollination and bumblebee pollination (Knuth 1908; Fenster *et al.* 2004). Therefore, we suggest that the bell-like and tubular flowers may correlate with specialised pollination, and the dish-like flowers with generalised pollination, which can be explained by the pollination syndrome hypothesis (Faegri and van der Pijl 1979; Kevan and Baker 1983; Richards 1997; Fenster *et al.* 2004), although this hypothesis has been questioned (Waser *et al.* 1996; Ollerton *et al.* 2009).

The large sepal, showy colour and abundant reward of these three *Clematis* species indicates that they are outcrossing species (Cruden 1977; Dafni 1992). However, their autonomous selfing indices indicate that *C. rehderiana* and *C. akebioides* may be facultative autogamous species, while on the other hand *C. chrysocoma* may be a nearly obligate outcrossing species. This conclusion is reflected by their stamen-pistil ratios. The two bumblebee-pollinated *Clematis* show higher potential selfing than the fly-pollinated one, because the pollination failure of *C. akebioides* and *C. rehderiana* might be more severe than that of *C. chrysocoma*. First, the specialised pollination systems might be easily collapsed (Bond 1994; Waser *et al.* 1996; Johnson and Steiner 2000). In addition, *C. chrysocoma* prefers open and less-disturbed habitats, and its flowering time is in the dry season. However, *C. akebioides* and *C. rehderiana* were primarily distributed along the disturbed roadside or riverside, and their flowering time is in the rainy season. *Clematis akebioides* and *C. rehderiana* did not obviously experience pollinator limitation in our study populations, because abundant plants exhibit synchronous blooming. When they are invading new disturbed areas, selfing could provide reproductive assurance (Kalisz *et al.* 2004; Fenster and Marten-Rodriguez 2007). Apparently, *C. akebioides* and *C. rehderiana* favour cross-fertilisations as well as other species with mixed-mating strategies (Barrett and Harder 1996; Herlihy and Eckert 2002). Recently, our molecular phylogenetic result shows that *C. akebioides* and *C. rehderiana* belong to the *Meclatis* group and the *Connatae* group, respectively (Jiang 2010), which imply that self-pollination may be lost or gained independently in different lineages of *Clematis* (Barrett *et al.* 1996; Barrett 2002a).

Inbreeding depression values for fruit-set and seed production are negatively associated with autonomous selfing indices in the three *Clematis* species, which is in agreement with the generalisation of Husband and Schemske (1996). *Clematis*

chrysocoma, an obligate outcrossing species, exhibits substantial inbreeding depression in the phases of fruiting and seed development. *Clematis akebioides* with high selfing potential experiences low inbreeding depression in both analysed stages. Inbreeding depression value of *C. rehderiana* for fruit-set is equal to the average value of selfing species, but the value for seed production is up to the average value of outcrossing species, which indicate that inbreeding depression in selfers relative to outcrossers may increase from early to late life stages (Husband and Schemske 1996). Inbreeding depression values should be further calculated at seed germination, seedling and other later stages of development because most selfing species express inbreeding depression late in their life cycle (Husband and Schemske 1996).

Generally, fruits and/or seeds of animal-mediated crossing species are frequently limited by pollen source (Burd 1994; Larson and Barrett 2000; Knight *et al.* 2005). However, hand-supplemental pollination does not always enhance fruit-set and/or seed production, because plants had no pollen limitation or limitation by other factors (Young and Young 1992; Ashman *et al.* 2004; Wesselingh 2007). In the present study, we found that all three *Clematis* do not experience evident pollen limitation. First, three species are frequently pollinated by flies and/or bees. Additionally, efficient outcrossing by bumblebees integrated with high selfing potential increase pollination success for *C. akebioides* and *C. rehderiana*, and longer floral lifespan and pollen-dispensing mechanisms improve outcrossing pollination of *C. chrysocoma* by generalised flies (Harder and Thomson 1989). For the endangered species *C. socialis*, Timmerman-Erskine and Boyd (1999) find inadequate pollination limits reproduction. Because our treatments represent fractional flowers and only a single time of plant life-stage, this result might be inaccurate (Knight *et al.* 2006). Nevertheless, pollen limitation has probably been overestimated by previous reviews, because most publications are biased against nonsignificant results (Knight *et al.* 2006).

In summary, our study shows that variation in floral forms of *Clematis* would directly influence divergence of floral characters, pollination systems and breeding systems. The pollination system of *Clematis* can be classified by two functional groups of pollinators, that is, fly pollination and bumblebee pollination. The stamen-pistil ratios indicating their outcrossing levels are validated by field investigation of the breeding system. Self-pollination may be lost or gained independently in different lineages of *Clematis*. The levels of inbreeding depression are negatively associated with autonomous self-pollination. All three *Clematis* do not experience pollen limitation under natural pollination.

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Appendix 1. Corolla types, distribution and field data of the three studied *Clematis* species

	<i>Clematis chrysocoma</i>	<i>C. akebioides</i>	<i>C. rehderiana</i>
Corolla types	Dish-like	Bell-like	Tubular
Habits	Perennial woody herb	Perennial woody liana	Perennial woody liana
Study sites	Kunming Chongchong Mts. 25°07'05.98"N, 102°42'11.30" E	Zhongdian Wengshui 28°28'01.68"N, 99°48'26.22"E	Zhongdian Wengshui 28°28'01.68"N, 99°48'26.22"E
Habitats	Meadows	Along road or stream	Along road or stream
Altitude	2300 m	3240 m	3240 m
Flowering phenology	Late April–early June	Early July–early September	Late June–late August
Field study time	25 April to 20 June	25 June to 18 September	25 June to 18 September
Distribution*	Yunnan, W Guizhou, W and S Sichuan	NW Yunnan, W Sichuan, E and SE Xizang, Gansu, W Nei Mongol, Qinghai	NW Yunnan, W Sichuan, E Xizang, S Qinghai; Nepal

*Based on *Flora of China* (Wang and Bartholomew 2001); E, eastern; NW, north-western; S, southern; W, western.