Arch. Biol. Sci., Belgrade, 63 (3), 799-809, 2011 DOI:10.2298/ABS1103799X

THE REPRODUCTIVE BIOLOGY OF *CALLIGONUM* L. IN RELATION TO *EX SITU* CONSERVATION IN A BOTANICAL GARDEN

KANG XIAOSHAN[1,2], PAN BORONG[3*], DUAN SHIMIN[3], SHI WEI[1,3] and ZHANG YONGZHI[1,3]

[1]*Key Laboratory of Biogeography and Bioresources in Arid Land, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences,* Urumqi 830011, PR China
[2]*Graduate University of Chinese Academy of Science,* Beijing 100049, PR China
[3]*Turpan Eremophytes Botanic Garden, Chinese Academy of Sciences,* Turpan 838008, PR China

Abstract - In this study, we observed the flowering phenology, breeding system, pollination and seed germination of four species of *Calligonum* (*C. calliphysa*, *C. rubicundum*, *C. densum* and *C. ebinuricum*) in the Turpan Eremophytes Botanic Garden, China. Our results showed that the species had overlapping flowering phenologies and were pollinated by similar pollination agents. Their breeding systems were self-compatible, and with signs of outbreeding, but not of hybridization with each other; the main isolation mechanism was post-zygotic isolation and they also had high seed germination rates. Therefore, they are suited to *ex situ* conservation in the Turpan Eremophytes Botanic Garden, and can supply sufficient seeds for renewal populations and the conservation of germplasm resources. Furthermore, these results provide theoretical support for the construction of a national germplasm resource garden of *Calligonum*, and for the introduction to the garden of other eremophyteplants and their conservation.

*Key words***:** *Calligonum, ex situ* conservation, flowering phenology, breeding system, hybridization, pollination, seed germination, botanic gardens.

UDC 582.665:581[58:069.029(510)

INTRODUCTION

Calligonum (Polygonaceae) species are shrubs or subshrubs that inhabit sand or desert areas. The genus is classified into four sections according to fruit morphological characters: (i) Sect. *Calliphysa*; (ii) Sect. *Pterococcus*; (iii) Sect. *Calligonum*; and (iv) Sect. *Medusae* (Bao and Grabovskaya-Borodina, 2003). There are ~35 *Calligonum* species that are native to North Africa, Asia and Southern Europe, and 23 species that are native to China, of which 22 are located in the Xinjiang Autonomous Region (Bao and Grabovskaya-Borodina, 2003; Zhang and Mao, 1989). Vegetation communities that include *Calligonum* species either as constructive or accompanying species are a distinct vegetation type in the deserts of Africa and Asia. *Calligonum* species are useful for sand control and are used extensively for windbreaks and sand fixing in China (Zhang and Mao, 1989).

We have investigated the distribution of *Calligonum* many times (between 2005 and 2010 during the growing season). Regrettably, *Calligonum* species are decreasing, with some no longer found in certain areas because of the effects of intensive agriculture, desertification, increased habitat fragmentation and use as a fuel. Many previous studies on *Calligonum* species have focused mainly on their ecology, taxonomy and evolution (Mao et al., 1983; Mao et al., 1986; Qiu, 1988; Kang et al., 2007, 2008, 2009; Zhuang et al., 2008; Li et al., 2009). For example, Tao and Ren (2004) studied the relationship of

19 *Calligonum* species by using isozyme data, and found that some species were closely related, such as *Calligonum rubicundum* and *Calligonum densum*. There have also been some reports on the flowering phenological characteristics of the genus (Yin et al., 1987; Wang et al., 1991).

However, little attention has been paid to the conservation of this genus. *Ex situ* conservation in botanic gardens might be a suitable means of protecting germplasm resources, such as *Calligonum* species, and the topic in general has attracted recent attention (e.g. Bossdorf et al., 2005; Schlaepfer et al., 2005; Oldfield, 2009; Swarts et al., 2009). Preliminary research is very important to ensure the long-term survival of species conserved *ex situ* and to protect their genetic diversity; for example, are plants bred *ex situ* able to adapt to new habitats? Is their growth affected by *ex situ* breeding and, if so, how? Are they able to set seed successfully, and are those seeds viable? Are the resulting plants purebred, or do they show signs of hybridization?

This paper reports on observations of the flowering phenology, breeding system, pollination and seed germination of four *Calligonum* species growing in the Turpan Eremophytes Botanic Garden (hereafter TEBG). The study investigated: (i) whether, and how, the phenology has changed under *ex situ* conservation compared with that in the field; (ii) whether hybridization occurs among the four species; (iii) whether the four species can grow normally *ex situ* and yield enough seed for reproduction and germplasm conservation; and finally, (iv) whether *ex situ* conservation is suitable for the long-term survival of these species.

MATERIALS AND METHODS

Study site and species

The study was conducted between 2007 and 2009 in the TEBG, which is located in eastern Xinjiang, China (40°51′N, 89°11′E; 76–95 m below sea level). It is the lowest-elevation botanical garden in the world. The climate in Turpan is characterized by low rainfall, high evapotranspiration, high temperature and desiccating winds. The annual mean temperature is 13.9°C (with a range of -28.0°C to 47.6°C), The average annual rainfall is 16.4 mm, but the annual mean evaporation is 2387.8 mm and the average annual relative humidity is 41%; there are 26.8 gale days annually, and maximum wind speed is 40 m/s (Yin 2004). Meteorological data were supplied by the TEBG (Fig. 1). The TEBG focuses on collecting and conserving the plant germplasm resources of the arid and semiarid areas of China and Central Asia.

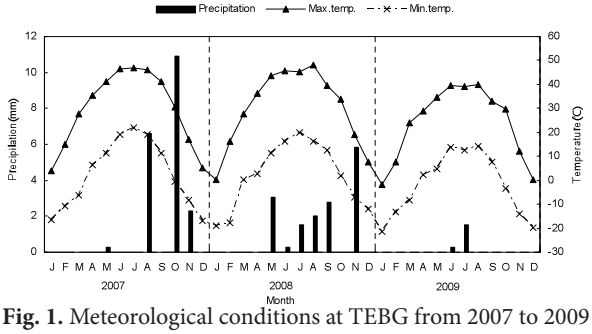

Fig. 1. Meteorological conditions at TEBG from 2007 to 2009

We selected a total of four species, one from each section: (i) *Calligonum calliphysa* (the only species in Sect. *Calliphysa*); (ii) *C. rubicundum* (Sect. *Pterococcus*; only found along the bank of the Irtysh River, Xinjiang, China); (iii) *C. densum* (Sect. *Calligonum*; only found in Huocheng, Xinjiang); and (iv) *Calligonum ebinuricum* (Sect. *Medusae*; a rare specieto China and that receives 3 protection in Xinjiang). These species were introduced to the TEBG from their endemic regions between 1973 and 1977 (*C. densum* was introduced from Huocheng, whereas the other three were from Jinghe; both areas are located north of Xinjiang). All species have been planted in the TEBG since 1977 (Yin, 2004), and currently show normal healthy growth.

Flowering phenology

Collection of flowering phenological information in the field

Phenological information relating to the *Calligonum* species was collected from both herbarium and field

investigations. We collected data from 2435 herbaria from 14 research institutions and colleges (e.g. Institute of Botany, Chinese Academy of Science (CAS); Xinjiang Institute of Ecology and Geography, CAS; Kunming Institute of Botany, CAS; Xinjiang University; Inner Mongol University). Field trips were conducted between 2005 and 2010 between March and September and examined plants over most of the current distribution of *Calligonum* in Xinjiang.

Flowering phenology at TEBG

To document the flowering phenology of the four species in the TEBG, we randomly selected 15 plants from each species; the distance between each plant was at least 15m. Selected plants were censused daily during the flowering periods between 2007 and 2009, with new flowers marked in each census. From these flowering data, four phenology parameters were derived, each of which had two levels (individual and population): (i) onset (date the first flower opened); (ii) peak flowering date (>50% of flower buds open); (iii) end date (date the last flower opened); and (iv) duration (difference between date of first and last flower opening).

The pairwise overlap in flowering phenology among all four species was determined and the percentage overlap was then calculated (Krebs, 1989) according to Equation 1:

$$P_{ij} = \left[\sum^{n}\left(\text{minimum } P_{di}, P_{dj}\right)\right]100 \quad (i = c, r, d, e; j = r, d, e; i \neq j) \quad [1],$$

where c=*C. calliphysa*, r=*C. rubicundum*, d=*C. densum* and e=*C. ebinuricum*; P_{ij}=percentage overlap between two species; P_{di}, P_{dj}=(number of plants of a species observed with flowers at each sampling date, d)/(total number of plants of a species observed with flowers in 2007, 2008 and 2009, combined for all sampling dates); and *n*=total number of sampling dates.

The flowering onset date was determined as described by Pickering (1995). Here, April 1 was defined as the first day (recorded as 1), April 2 as the second day (recorded as 2), and so on.

Mating system

The breeding systems of *C. calliphysa*, *C. rubicundum*, *C. densum* and *C. ebinuricum* were studied in a hand-pollination experiment in which more than 1973 flower buds were marked and bagged before they opened. Each flower of an individual plant was randomly assigned to one of the following treatments:

(i) bagging and no treatment, to test for spontaneous self-pollination;

(ii) bagging and self-pollination with pollen from the same flower, to test for self-incompatibility;

(iii) emasculation, bagging and cross-pollination with pollen from the same plant, to test for self-incompatibility;

(iv) emasculation, bagging and cross-pollination with pollen from another plant that was located ~10 m from the pollen recipient, to test for cross-compatibility;

(v) emasculation, bagging and no pollen, to test for apomixis;

(vi) emasculation, natural pollination and no bagging, to test whether pollinators are required;

(vii) bagging of the whole branch (~30cm in length), to test whether pollinators are required;

(viii) flower bagging with a net (mesh size ~0.8–1mm) to test whether anemophily occurs;

(ix) no bagging, to investigate natural pollination (this was the control treatment); and

(x) emasculation, with each species hybridized with the three other species (flowering of the four species was desynchronized in that *C. calliphysa*

and *C. rubicundum* finished flowering at the peak flowering point for *C. densum* and *C. ebinuricum*. Therefore, hybridization experiments between *C. calliphysa* and *C. densum*; *C. calliphysa* and *C. ebinuricum*; and *C. rubicundum* and *C. densum* were not completed).

A total of 1973 flowers from 68 individual plants were used in the experiment (each treatment was repeated with at least 30 flowers, taken from at least five individual plants); the treatments were performed between 15 and 25 April 2008.

To ensure fruit and seed set among hand-pollinated flowers, the approximate timing of stigma receptivity and pollen viability were determined (the stigma receptivity of the four species was ~12 h; the pollen viability was ~12–24 h; data not shown). During late April to early May, the number of young fruit was counted and the ratio between the number of flowers treated/fruit produced was determined for each hand pollination treatment.

Floral visitors

Flower visitors were observed on ~0.5m^3 of a plant (for a total of four plants of each species) selected at random and the number and species of each visitor were recorded for a 30-min period each hour. A total of ~144 h of observations of floral visitors were made during the entire anthesis across the flowering periods. During the observation period, the behavior of each visitor in terms of the type of floral resource gathered (nectar only, pollen only, or both nectar and pollen), was recorded. Any contact with the stigma was also recorded and, where possible, the part(s) of the body, such as the ventral abdomen, tarsi or mandible that had made contact was noted. The behavior of the visiting species was analyzed and the animals classified as: (i) effective pollinators [when they collected the primary floral resource (nectar) and always came into contact with the stigma]; (ii) occasional pollinators [when the animals only collected nectar or pollen during rare visits (from one to five total sightings during the entire observation period) or when the visitors did not consistently come into contact with the stigma]; or (iii) nectar or pollen thieves (Inouye, 1980) (when the insects gathered reward without pollinating).

Specimens of the visiting insects were collected for identification and were then stored as voucher specimens in the entomological collection of the TEBG. Photographic records of visits were made to register and to better describe the visiting behavior.

The rate of seed germination

Calligonum species have an achene fruit type. Achenes are trigonous, ellipsoid or oblong-ovoid woody fruits; they have ribs with wings (Sect. Pterococcus) or bristles (Sect. Medusae), or ribs with wings and bristles at the wing margins (Sect. Calligonum), or overgrowing thin bladder-like membrane on the surface of reduced wings or bristles (Sect. Calliphysa) (Bao and Grabovskaya-Borodina, 2003).

Owing to their thick pericarp, seeds were subjected to one of two pre-treatments before the rate of germination was determined. The seeds were either soaked in either (i) concentrated sulfuric acid (SA) for 0.5 h, or (ii) distilled water for 48 h. For each pre-treatment, 1200 seeds were used (n=300 for each species), distributed equally in six dishes (60cm×40cm) with fine sand, and then deposited in germination chambers maintained at 35°C. The dishes were kept at a dampness level standardized during earlier tests to determine the time taken for germination. The dishes were observed for 60 days until no further germination occurred.

RESULTS

Flowering phenology in the field

The phenological information collected from herbarium and field-based investigations (2005–2010) showed that the flowering time of the four *Calligonum* species was from mid-May to early June in the field; some plants were found to flower continuously until early July.

Table 1. Summary of flowering phenological traits of the four Calligonum species at the individual and population levels[a,b]

	Calligonum calliphysa			*Calligonum rubicundum*			*Calligonum densum*			*Calligonum ebinuricum*		
Year	**2007**	**2008**	**2009**	**2007**	**2008**	**2009**	**2007**	**2008**	**2009**	**2007**	**2008**	**2009**
Individual-level observations												
Flowering onset	April 14 ±2.70	April 16 ±1.38	April 16 ±1.93	April 15 ±0.95	April 15 ±0.82	April 13 ±0.67	April 20 ±1.46	April 22 ±2.17	April 20 ±1.31	April 19 ±1.38	April 22 ±2.27	April 18 ±1.37
Onset range	10–16	15–18	14–20	14–17	15–17	13–15	18–23	18–24	17–21	17–22	19–26	16–20
Duration (d)	17±2.70	21±1.94	17±2.07	16±0.95	21±2.90	13±1.73	22±1.46	33±2.05	36±13.06	44±1.38	51±3.37	46±28.04
Duration range	13–25	14–25	13–19	15–18	10–28	11–16	17–25	21–44	25–58	36–50	39–57	19–80
Population-level observations												
Flowering onset	April 16	April 16	April 16	April 15	April 15	April 13	April 20	April 22	April 20	April 18	April 22	April 18
Peak flowering date	April 18	April 21	April 19	April 17	April 18	April 16	April 23	April 26	April 25	April 24	April 27	April 27
End date	May 10	May 10	May4	May2	May 12	April28	May 14	June 4	June 16	June 6	June 17	July 6
Duration (d)	25	25	19	18	28	16	25	44	58	50	57	80

[a] n=15 for each record.
[b]Where applicable, data shown are mean±standard deviation.

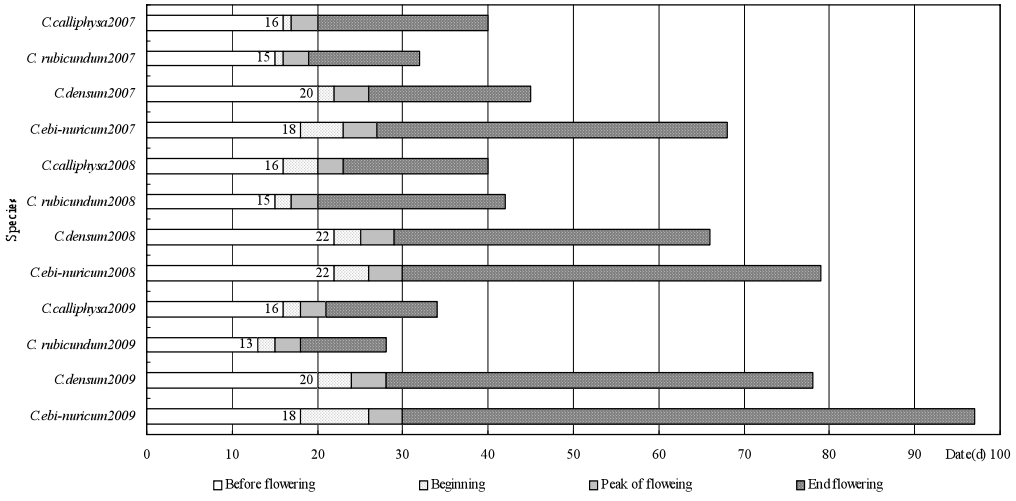

Fig. 2. Duration and overlapping of flowering in the four *Calligonum* species at the population level in 2007, 2008 and 2009 (n=15 for each species). Note that the x-axis shows the flowering date; April 1was the first day from which flowering was counted

Flowering phenology at the TEBG

In the TEBG, the four species flowered in succession. The flowering duration of *C. calliphysa* and *C. rubicundum* was from mid-April to early May, whereas that of *C. densum* and *C. ebinuricum* was from late April to mid-June; some individual plants of *C. densum* and *C. ebinuricum* continued to flower sporadically until early July (Table 1). Therefore, the timing of flowering differed for plants in the field and those in the TEBG.

Most of the flowering phenology characteristics of *C. calliphysa* and *C. rubicundum* were similar, as was the case for *C. densum* and *C. ebinuricum*. The duration of flowering was longer for *C. densum* and *C. ebinuricum* than for *C. calliphysa* and *C. rubicundum*, probably because the first two species produce buds continuously during the flowering period.

The flowering periods of *C. calliphysa* and *C. rubicundum* overlapped significantly (Fig. 2); the percentage overlap calculated for these two species

Table 2. Percentage flowering overlap for the four *Calligonum* species in 2007, 2008 and 2009

		Calligonum rubicundum	*Calligonum densum*	*Calligonum ebinuricum*
Calligonum calliphysa	2007	100%	13.23%	17.12%
	2008	79.99%	23.90%	19.96%
	2009	93.69%	24.38%	23.96%
Calligonum rubicundum	2007	/	1.80%	3.37%
	2008	/	13.66%	9.73%
	2009	/	10.49%	7.33%
Calligonum densum	2007	/	/	79.82%
	2008	/	/	60.22%
	2009	/	/	73.73%

Table 3. Comparison of fruit set of the four *Calligonum* species under each pollination treatment[a,b]

Treatment	Species			
	Calligonum calliphysa	Calligonum rubicundum	Calligonum densum	Calligonum ebinuricum
	Percentage fruit set (*n*)			
No emasculation, bagged, self-pollination	0 (31)	0 (34)	0 (36)	0 (31)
Bagged, hand self-pollination	3.33±0.07 (30)	2.86±0.06 (33)	0 (30)	2.86±0.06 (35)
Emasculation, bagged, hand geitonogamy	79.52±0.07 (34)	88.57±0.06 (34)	86.67±0.07 (30)	92.14±0.11 (36)
Emasculation, bagged, hand cross-pollination	91.43±0.08 (35)	70.00±0.07 (30)	94.29±0.08 (34)	100 (35)
Emasculation, bagged, no pollination	0 (32)	0 (30)	0 (31)	0 (30)
Emasculation, unbagged, natural pollination	39.05±0.05 (33)	84.28±0.01 (32)	45.00±0.07 (40)	15.00±0.06 (40)
Branch bagged	0 (30)	0 (30)	0 (30)	0 (30)
Flower bagged with net	12.50±0.09 (40)	7.50±0.11 (40)	17.5±0.07 (40)	22.50±0.10 (40)
Unemasculation, unbagged, natural pollination	40.49±0.01 (205)	27.83±0.03 (79)	30.45±0.01 (289)	47.14±0.02 (140)

[a]Data are means±SE.
[b]*n* = is the total number of flowers manipulated in each treatment.

was 79.99–100% (Table 2), whereas the percentage overlap for *C. densum* and *C. ebinuricum* was 60.22–79.82%. However, the percentage overlap for *C. calliphysa* and *C. densum* for *C. calliphysa* and *C. ebinuricum* for *C. rubicundum* and *C. densum* and for *C. rubicundum* and *C. ebinuricum* was < 25% in all cases.

In addition, overlap in the flowering period between any two species was greater than that among all four species (Fig. 2). The flowering periods of the four species overlapped by 13, 19, and 7 d in 2007, 2008 and 2009, respectively. The flowering periods of *C. calliphysa* and *C. rubicundum* overlapped by 17, 25 and 13 d, and those of *C. densum* and *C. ebinuri-*

Table 4. Fruit set for the four *Calligonum* species under different hybridization treatments[a,b]

Species cross	*Calligonum calliphysa* ♀	*Calligonum rubicundum* ♀	*Calligonum demum* ♀	*Calligonum ebinuricum* ♀
	Percentage fruit set (*n*)			
C. calliphysa ♂	N/A	0 (39)	/	/
C. rubicundum ♂	3.33±0.07 (28)	N/A	/	5.00±0.07 (39)
C. demum ♂	/	/	N/A	0 (39)
C. ebinuricum ♂	/	/	2.50±0.06 (39)	N/A

[a]Data are mean±SE.
[b]Not applicable as the treatments were not completed.

Table 5. Details of the main visitors to *Calligonum* flowers, their reward and visit outcome

Flower visitor	Reward	Visit	Pollination
	Bees		
Apis mellifera	Nectar and pollen	Yes	Yes
Halictus sp.	Nectar and pollen	Yes	Yes
	Flies		
Lasiopticus sp.	Nectar	Yes	Occasionally
Musca domestica	Nectar	Yes	No
Muscidae	Nectar	Yes	No
Calliphora vicina	Nectar	Yes	No
	Butterflies		
Plebejus argus	Nectar	Yes	No
	Others		
Formicidae	Nectar	Yes	No

cum overlapped by 25, 44, and 58 d in 2007, 2008 and 2009, respectively. The peak flowering periods of *C. calliphysa* and *C. rubicundum* occurred at the same time as the start of the flowering period in *C. densum* and *C. ebinuricum*. When flowering was coming to an end in the first two species, flowering in the second two species was at its peak. Consequently, flowering phenology was divergent among the four species, but the divergence did not result in separate periods of flowering for each species.

Mating systems of the four Calligonum species

The results of the pollination experiment suggest that the four *Calligonum* species have similar mating systems (Table 3), as both geitonogamy and cross-pollination conducted by hand yielded better fruit sets compared with natural pollination. When pollinators were excluded by bagging the flowers, no fruits were produced, which indicates that spontaneous self-pollination does not occur. The self-pollination treatment resulted in very low (if any) fruit set, indicating that the plants are not self-fertile. Geitonogamy pollination yielded fruit which suggests that there is a degree of self-compatibility within each species. Both exclusion of pollinators and emasculation resulted in no fruit set, which indicates that apomixia does not occur in these species. However, fruit set did occur when flowers were bagged with a meshed net, suggesting that anemophily does occur. Hybridization among the four species did result in fruit set (Table 4), whereas interspecific hand pollination did not yield any viable seeds. Moreover, in this treatment, some pollen tubes were able to germinate on the style successfully (Fig. 3).

Floral visitors

Calligonum have bisexual flowers that occur in groups of two to four in the leaf axils. The perianth is persistent and comprises five parts. The tepals are green or red with a broad white margin abaxially, ovate, unequal, and not accrescent in fruit. There are 12–18 stamens and the filaments are connate at the base. The four styles are short and stigmas are capitate (Bao and Grabovskaya-Borodina, 2003); The nectary belongs to the torus type (Lin, 1989; Wang et al., 2010).

Table 6. Rate of seed germination under different pre-treatment conditions[a,b,c]

Treatment	Germination rate (%)			
	Calligonum calliphysa	*Calligonum rubicundum*	*Calligonum densum*	*Calligonum ebinuricum*
Seed soaked in concentrated SA for 0.5 h	83.33±0.05A	77.33±0.14AB	60.33±0.17B	91.33±0.07A
Seed soaked in distilled water for 48 h	67.00±0.12c	72.00±0.11bc	88.67±0.07a	84.00±0.08ab

[a]n=2400.
[b]Data shown are the mean±standard deviation.
[c]Different lower- and upper-case letters denote significant differences at the P<0.05 level.

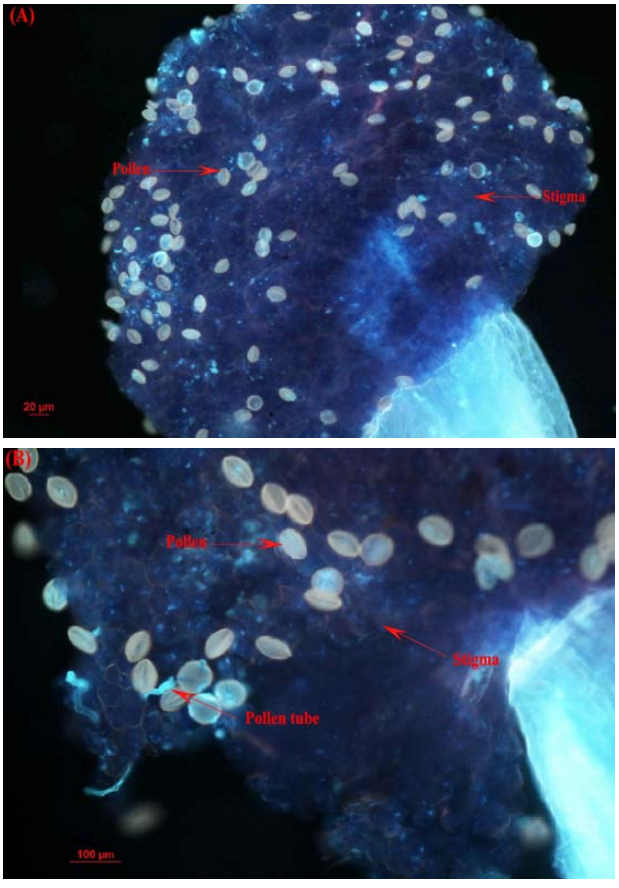

Fig. 3. Interspecific pollen on stigma (A) and growth of the pollen tube (B) (as viewed using a fluorescence microscope).

Similar types of pollinating and visiting species were found for each of the four species (Table 5). The major pollinators were *Apis mellifera* L. and *Halictus* sp., which collected both pollen and nectar and pollinated the flowers. Pollen was collected in pollen baskets located on the third legs of the insects, although pollen occasionally adhered to their chests and could then come into contact with the stigmas when the insect was feeding. Bee pollination was achieved only while bees were collecting pollen; these insects usually visited nearby flowers on the same plant and always paid repeat visits to the same flowers. Other species recorded were observed as visitors, but extracted nectar from the flowers rather than being involved in pollination (Table 5).

Seed germination

The seed germination of the four species under the two different pre-treatments was relatively high (Table 6). The difference between the two pre-treatments was not significant among the four species (F=9.401; F=0.537; F=14.751 and F=2.854, P>0.05); moreover under the same pre-treatment, the interspecies differentiation was also not very significant. This suggests that most seeds were able to germinate and develop normally.

DISCUSSION

Our study of the reproductive biology of four species of *Calligonum* will provide practical knowledge to support the introduction and conservation of these and other plants to the TEBG and perhaps other botanic gardens worldwide.

Hybridization is a risk in mixed-species collections (Snogerup, 1979), with novel hybrids being generated from artificial sympatry. Therefore, tests of hybridization can be used to assess the effectiveness of *ex situ* conservation. Temporal heterogeneity in flowering periods among sympatric species often contributes significantly to their isolation (Sprague, 1962; Levin, 1971; Adams, 1983; Grant, 1992, 1994a). On the one hand, the flowering periods of *C. calliphysa*, *C. rubicundum*, *C. densum*, and *C. ebinuricum* did have a degree of overlap; the peak flowering date of the former two was earlier than for the latter two species. The biological significance of the difference in peak blooming period is especially important, because this is the period in which flowers are most likely to be fertilized (Willson, 1983; Burd, 1995). On the other hand, the overlap of flowering period among the four species differed over the 3 years of the study, and even *C. calliphysa* and *C. rubicundum* were found to overlap completely in one instance. Therefore, although flowering among the four species was divergent, the resulting temporal isolation is not sufficient and reliable enough to prevent gene flow entirely. Therefore, the difference in peak flowering period could influence the establishment of reproductive isolation, even though temporal separation is not complete (Grant, 1994a). Therefore, hybridization is theoretically possible among these four species.

The results of the hand-pollination experiments suggest that the four species of *Calligonum* are self-compatible (geitonogamous, but not autophilous) and require pollinators for successful seed set. In addition, there is no apomixis. Furthermore, because exclusion of pollinators resulted in the absence of fruit set, pollinators would seem to be necessary for the sexual reproduction of these *Calligonum* species.

The crosses produced from these four species either did not yield seeds or the seeds were shriveled or empty. This suggests the existence of a strong internal isolation mechanism within each of these species. In addition, interspecific pollen can germinate and grow a pollen tube successfully, which indicates that the internal reproductive barrier must operate after fertilization in these four sympatric species.

Compared with external isolation, internal isolating mechanisms are relatively reliable. External isolation mechanisms are easily affected by environmental conditions and break down when environmental changes upset an existing equilibrium (Grant, 1992). Pre-zygotic isolation acting in combination with post-zygotic isolation is very frequent in nature (Sprague, 1962; Grant, 1994a, b; Gardner and Macnair, 2000; Ando et al., 2001; Yang, 2007). Macior (1973, 1977, 1982, 1983) predicted that external isolation associated with internal isolation is the most important factor maintaining species boundaries in the genus *Pedicularis*. Our research also revealed external isolation associated with internal isolation in *Calligonum* species. In addition, the fact that most of the seeds collected were able to germinate following either pre-treatment suggests that the four *Calligonum* species would be able to yield enough viable seeds to help renew the population and conserve this important germplasm resource.

Consequently, under *ex situ* conservation, the flowering phenological time differed from that recorded in the field, but the four species showed normal healthy growth, yielded viable seeds, and were therefore able to complete their life cycle in the environment of a botanic garden. Therefore, we can conclude that they are suitable for *ex situ* conservation in the TEBG.

As many populations of *Calligonum* species occur in isolated and fragmented habitats, it is likely that at least some of them suffer from a limiting pollinator service, which, when combined with an unpredictable climate and little rainfall, could result in little if any seed set. Therefore, there will be a continued need for botanic gardens to conserve *Calligonum*, as there will be for other such threatened species.

Acknowledgments - This work was supported by an International Cooperation Project from the Ministry of Science and Technology (MOST), People's Republic of China, in a grant

for studies on Calligonum L. in middle Asia and platforms for its germplasm resource (Grant No. 2008DFA31360). We are grateful to Professor Renxin Huang, Department of Life Science and Technology, Xinjiang University, China for insect identification; and Professor Amots Dafni, Laboratory of Pollination Ecology, Institute of Evolution, University of Haifa, Israel, and Marina Olonowa, Tomsk State University, Novosibirsk, Russia, for their comments on the article. We also thank International Science Editing for their language editing.

REFERENCES

Adams, V.D (1983). Temporal patterning of blooming phenology in *Pedicularis* on Mount Rainier. *Can. J. Bot*. **61**: 786-791.

Ando, T., Nomura, M., Tsukahara, J., Watanabe, H., Kokubun, H., Tsukamoto, T., Hashimoto, G., Marchesi, E., and *I.J. Kitching* (2001). Reproductive isolation in a native population of *Petunia sensu* Jussieu (Solanaceae). *Ann. Bot-London*. **88**, 403-413

Bao, B.J., and *A.E. Grabovskaya-Borodina* (2003). Flora of China. **5**, 325-329. Beijing Science Press, Beijing.

Bossdorf, O., Auge, H., Lafuma, L., Rogers, W.E., Siemann, E., and *D. Prati* (2005). Phenotypic and genetic differentiation between native and introduced plant populations. *Oecologia*. **144**, 1-11.

Burd, M (1995). Ovule packaging in stochastic pollination and fertilization environments. Evolution. 49, 100-109.

Inouye, D.W. (1980). The terminology of floral larceny. *Ecology*. **61**, 1251-1253.

Gardner, M., and *M. MacNair* (2000). Factors affecting the coexistence of the serpentine endemic *Mimulus nudatus* Curran and its presumed progenitor, *Mimulus guttatus* Fischer ex DC. *Biol. J. Linn. Soc*. **69**, 443-459.

Grant, V. (1992). Floral isolation between ornithophilous and sphingophilous species of *Ipomopsis* and *Aquilegia*. *Proceedings of the National Academy of Sciences of the United States of America.* **89**, 11828-11831.

Grant, V (1994a). Mechanical and ethological isolation between *Pedicularis groenlandica and P. attllens* (Scrophulariaceae). *Biologisches Zentralblatt.* **113**, 43-51.

Grant, V (1994b). Modes and origins of mechanical and ethologicalisolation in angiosperms. *Proceedings of the National Academy of Sciences of the United States of America*. **91**, 3-10

Kang, X.S., Zhang, Y.Z., and *B.R. Pan* (2007). Review on the Research of Classification of *Calligonum* L. *Journal of Xinjiang University (Natural Science Edition)*. **24**(4), 454-459.

Kang, X.S., Zhang, Y.Z., and *B.R. Pan.* (2008). Research on Fruits of Morphological Characteristics of *Calligonum ebinuricum* population. *Acta Bot. Boreal. Occident. Sin*. **28**(6), 1213-1221.

Kang, X.S., Pan, B.R., Zhang, Y.Z., Duan, S.M., and *W. Shi* (2009). Study on variation of fruit characters of the Chinese endemic species *Calligonum ebinuricum* in population. *Bulletin of botanical research*. **6**(12), 747-752.

Krebs, C.J. (1989). Ecological Methodology. Harper and Row Publishers. 654. New York, USA.

Levin, D.A. (1971). The origin of reproductive isolating mechanism in flowering plants. *Taxon.* **20**, 91-113.

Li, D.L., Wang, J.H., and *Q.L. Ma* (2009). Numerical Taxonomy of Fruit Characteristic of *Calligonum* L. *Journal of Desert Research*. **29**(4), 718-722.

Lin, S.Q. (1989). Nectar Plant. 208-209. China Forestry Publishing House. Beijing.

Macior, L.W. (1973). The pollination ecology of *Pedicularis* on Mount Rainier. *Am. J. Bot*. **60**, 863-871.

Macior, L.W. (1977). The pollination ecology of *Pedicularis* (Scrophulariaceae)in the Sierra Nevada of Califolia. *Bulletin of the Torrey Botanical Club*. **104**, 148-154.

Macior, L.W. (1982). Plant community and pollinator dynamics in the evolution of pollination mechanism in *Pedicularis*. In: Armstrong JA, Powell JM, Richards AJ, eds. Pollination and evolution. Royal Botanic Gardens, Sydney, Australia.

Macior, L.W. (1983). The pollination dynamics of sympatric species of *Pedicularis* (Scrophulariaceae). *Am. J. Bot.* **70**, 844-853.

Mao, Z.M., Yang, Y., and *C.G. Wang* (1983). Studies on chromosome numbers and anatomy of young branches of *Calligonum* of Xinjiang in relation to the evolution of some species of the genus. Acta. *Phytotax. Sin*. **21**(1), 44-49.

Mao, Z.M., and *B.R. Pan* (1986). The classification and distribution of the genus *Calligonum* L. in China. *Acta. Phytotax. Sin*. **24**(2), 98-107.

Oldfield, S.F. (2009). Botanic gardens and the conservation of tree species. *Trends Plant Sci*. **14** (11), 581-583.

Pickering, C.M. (1995). Variation in flowering parameters within and among five species of Australian alpine *Ranunculus. Aust. J. Bot*. **43**, 103-112.

Qiu, G.Y. (1988). The Pollen Characters and the Evolutional Relationships of *Calligonum*. *Journal of Inner Mongolia forestry college (Science)*. **2**, 73-81.

Schlaepfer, M.A., Sherman, P.W, Blossey, B., and *M.C. Runge* (2005). Introduced species as evolutionary traps. *Ecol. Lett*. **8**, 241-246.

Snogerup, S (1979). Cultivation and continued holding of Aegean endemics in an artificial environment. In: Synge H and Townsend H (eds) Survival or Extinction: The Practical Role of Botanic Gardens in the Conservation of Rare and Threatened Plants. 85-90.The Bentham-Moxon Trust, Royal Botanic Gardens, Kew, UK.

Sprague, E.F. (1962). Pollination and evolution in *Pedicularis* (Scrophulariaceae). *Aliso*. **5**: 181–209

Swarts, N.D., and *K.W. Dixon* (2009). Perspectives on orchid conservation in botanic gardens, *Trends Plant Sci*. **14** (11): 590-598.

Tao, L., and *J. Ren* (2004). Study on relationship and cluster of *Calligonum* by isozyme electrophoresis analysis. *Acta. Bot. Boreal. Occident. Sin*. **24**(9): 1708-1713.

Wang, Y., and *L.K. Yin* (1991). The phenology on 19 rare and endangered desert plant species. *Arid Zone Research*. **4**(4): 25-32.

Wang, H., Wang, X., Wang, Y.X., Yang, B.Y., and *Y.C. Jiang* (2010). Developmental anatomy of the floral nectaries in *Calligonum ebinuricum*. *Bulletin of Botanical Research*. **30** (3): 262-266.

Willson, M.F. (1983). Plant reproductive ecology. John Wiley & Sons, New York, USA.

Yang, C.F., Gituru, R.W., and *Y.H. Guo* (2007). Reproductive isolation of two sympatric louseworts, *Pedicularis rhinanthoides* and *Pedicularis longiflora* (Orobanchaceae): how does the same pollinator type avoid interspecific pollen transfer? *Biol. J. Linn. Soc*. **90**: 37-48.

Yin, L.K. (1987). Observations on the phenology of 68 species in Turpan Eremophytes Botanic Garden. *Arid Zone Research*. **4**(4): 25-32.

Yin, L.K. (2004) Turpan Eremophytes Botanic Garden of the Chinese Academy of Sciences-a developing conservation and research base of plan t resources diversity in desert area. *Arid Zone Research*. **21**(Suppl.): 1-4.

Zhang, D.M., and *Z.M. Mao* (1989). A study on the *Calligonum* desert in Xinjiang. *Arid Zone Research*. **2**: 13-18.

Zhuang, Y.L., Zhao, W.Z., Xie, G.X., and *G.L. Hu* (2008). The features of *Calligonum mongolicum* population and its expansion process in sandy habitat. *Acta Ecologica Sinica*. **28** (4): 1399 -1407