Is *ex situ* Conservation Suitable for *Calligonum* L.? A Research Program in Turpan Eremophyte Botanical Garden

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Abstract

In this study, we observed the flowering phenology, breeding system, pollination and seed germination of four species of *Calligonum* (Polygonaceae) (*C. calliphysa*, *C. rubicundum*, *C. densum* and *C. ebinuricum*) in Turpan Eremophyte Botanical 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. They also had high seed germination rates. Therefore, they are suitable to *ex situ* conservation in the Turpan Eremophyte Botanical Garden, and can supply sufficient seeds for renewal population and conservation of germplasm resources. These results provide theoretical support to the construction of a national germplasm resource garden of *Calligonum*.

Key words: *Calligonum*, *ex situ* Conservation, Flowering Phenology, Breeding System, Hybridization, Pollination, Seed Germination, Botanical Gardens.

Introduction

Calligonum (Polygonaceae) species are shrubs or subshrubs that inhabit sand or desert areas. There are ~35 *Calligonum* species that are native to North Africa, Asia and South Europe, classified into four sections according to fruit morphological characters. Twenty three species are native to China, out of which 22 are located in the Xinjiang Autonomous Region (Bao & Grabovskaya-Borodina 2003; Zhang & Mao 1989). Vegetation communities that include *Calligonum* species are distinct 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 & Mao 1989).

Many previous studies on *Calligonum* species have focused mainly on their ecology, taxonomy and evolution (Mao *et al.* 1983; Mao & Pan 1986; Qiu 1988; Kang *et al.* 2007; Li *et al.* 2009). There are some reports on the flowering phenology for some species of the genus (Yin 1987; Wang & Yin 1991). Our sampling in the last five years suggest that geographical distribution of *Calligonum* is decreasing, probably due to effects of intensive agriculture desertification, increased habitat fragmentation and their use as fuel.

Despite these problems, little attention has been paid to the conservation of this genus. *Ex situ* conservation in botanical gardens might be a suitable way to protect 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 & Dixon 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, and are those seeds viable? Are the resulting plants pure bred, or is there any possibility of hybridization with each? Hybridization is a risk in mixed-species collections (Snogerup 1979), with novel hybrids being generated from artificial sympatry. Therefore, tests of hybridization test can be used to assess the effectiveness of *ex situ* conservation.

This paper reports the observations of the flowering phenology, breeding system, pollination and seed germination of four *Calligonum* species growing in Turpan Eremophyte Botanical 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

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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). The climate in Turpan is characterized by low rainfall, high evapotranspiration, high temperature and dry 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 2,387.8 mm and the average annual relative humidity is 41%. There are 26.8 annual gale days, and maximum wind speed is 40 m/s (Yin 2004). Meteorological data were supplied by the TEBG (Figure 1). The TEBG focuses on collecting and conserving the plant germplasm resources of arid and semiarid areas of China and Central Asia.

We selected a total of four species, one from each botanical section: i) *Calligonum calliphysa* (the only species in Sect. *Calliphysa*); ii) *C. rubicundum* (Sect. *Pterococcus*; only found along the bank of the Irtys River, Xinjiang, China); iii) *C. densum* (Sect. *Calligonum*; only found in Huocheng, Xinjiang); and iv) *Calligonum ebinuricum* (Sect. *Medusae*; a rare species to China and that receives three protection in Xinjiang). These species were introduced to 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 in the north of Xinjiang). All species have been planted in TEBG since 1977 (Yin 2004), and currently show normal and health growth.

Calligonum have bisexual flowers that occur in groups of two to four in 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 & Grabovskaya-Borodina 2003). The pollen presentation pattern is “Gradual pollen presentation”, and when pollen has viability, the stigmas also have receptivity (no dichogamy) (unpublished data). The nectary belong to the torus type (Lin 1989; Wang *et al.* 2010).

Collection of flowering phenological information in the field

Phenological information relating to *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 of CAS (Chinese Academy of Sciences); 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 during March to 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 TEBG, we randomly selected 15 plants from each species, with a distance between each plant being of at least 15 m. Selected plants were censused daily during the flowering periods between 2007 and 2009, with new flowers marked

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

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) (Equation 1) by

$$P_{ij} = \left[\sum_{d_i}^n (\text{minimum } P_{d_i}, P_{d_j}) \right] 100 \quad (1)$$

where P_{ij} is the percentage overlap between two species, i and j , P_{d_i} and P_{d_j} are the proportions of number of plants of a species observed with flowers at each sampling date, d , is respect to the total number of plants of a species observed with flowers in 2007, 2008 and 2009 (combined for all sampling dates), and n equals the total number of sampling dates.

Controlled-pollination experiments

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) autonomous pollination: bagging and no treatment, to test for spontaneous self-pollination; ii) selfing: bagging and pollination with pollen from the same flower, to test for self-compatibility; iii) geitonogamous selfing: emasculation, bagging and pollination with pollen from the same plant, to test for self-compatibility; iv) crossing: emasculation, bagging and pollination with pollen from another plant that was located ~10 m from the pollen recipient, to test for cross-compatibility; v) apomixis: emasculation, bagging and no pollen, to test for apomixis; vi) natural pollination: emasculation, and no bagging, to test whether pollinators are required; vii) autonomous pollination via geitonogamy: bagging of the whole branch (~30 cm in length), to test whether pollinators are required; viii) anemophilous pollination: flower bagging with a net (mesh size ~ 0.8-1 mm) to test whether anemophily occurs; ix) control: no bagging; and x) hybridization: emasculation and cross-pollinations with three other species.

To ensure fruit and seed set among hand-pollinated flowers, the approximate timing of stigma receptivity and pollen viability was determined (the stigma receptivity of the four species was ~12 hours; the pollen viability was ~12-24 hours; unpublished data).

Floral visitors

Flower visitors were observed on ~ 0.5 m³ 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 minutes period each hour for three days (from 7:00 AM to 9:00 PM). The behavior of the visiting species was analyzed and the animals classified as: i) effective pollinators; ii) occasional pollinators; or iii) nectar or pollen thieves (Inouye 1980).

Specimens of the visiting insects were collected for identification and were then stored as voucher specimens in the entomological collection of the TEBG.

The rate of seed germination

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 hours; or ii) distilled water for 48 hours. For each pre-treatment, 1200 seeds were used (total seed = 2400, $n = 600$ for each species from 10 individuals). Distributed equally in six dishes (60 × 40 cm) with fine sand ($n = 50$ /per dish), 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 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 (e.g. *C. rubicundum* and *C. ebinuricum*).

The flowering phenology characters at Turpan Botanical Garden

In the TEBG, 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 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 (Table 2); the percentage overlap calculated for these two species was 80-100%, whereas the percentage overlap for *C. densum* and *C. ebinuricum* was

Table 1. Summary of flowering phenological traits of the four *Calligonum* species at the individual and population levels (n = 15 for each record, data shown are mean ± SE).

Year	Calligonum calliphysa			Calligonum rubicundum			Calligonum demum			Calligonum ebinuricum		
	2007	2008	2009	2007	2008	2009	2007	2008	2009	2007	2008	2009
Individual-level observations												
Flowering onset	April 14 ± 0.01	April 16 ± 0.01	April 16 ± 0.01	April 5 ± 0.001	April 5 ± 0.001	April 13 ± 0.001	April 20 ± 0.01	April 22 ± 0.01	April 20 ± 0.001	April 19 ± 0.001	April 22 ± 0.01	April 8 ± 0.001
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 ± 2.53	21 ± 3.75	17 ± 1.69	16 ± 0.33	21 ± 1.71	13 ± 0.52	22 ± 3.24	33 ± 4.31	36 ± 4.62	44 ± 0.79	51 ± 2.74	38 ± 6.30
Duration range	13-25	14-25	13-19	15-18	10-28	11-16	17-25	21-44	25-58	36-50	39-57	19-62
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	May 4	May 2	May 12	April 28	May 14	June 4	June 16	June 6	June 17	June 18
Duration (d)	25	25	19	18	28	16	25	44	58	50	57	62

60.2-79.8%. 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. 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 ending 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 (Table 1, 2).

The breeding 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 apomixis does not occur in these species. However, fruit set did occur when flower were bagged with meshed net suggesting that anemophily does occur. Hybridization among the four species did result in no fruit set, shriveled set or empty fruit (setting fruit but no seed), and the results (%) were showed in Table 4. (The flowering of the four species was desynchronized in

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

		<i>Calligonum rubicundum</i>	<i>Calligonum densum</i>	<i>Calligonum ebinuricum</i>
<i>Calligonum calliphysa</i>	2007	100	13.2	17.1
	2008	80.0	23.9	20.0
	2009	93.7	24.4	24.0
<i>Calligonum rubicundum</i>	2007	-	1.8	3.4
	2008	-	13.7	9.7
	2009	-	10.5	7.3
<i>Calligonum densum</i>	2007	-	-	79.8
	2008	-	-	60.2
	2009	-	-	73.7

Table 3. Comparison of fruit set of the four *Calligonum* species under each pollination treatment (*n* = is the total number of flowers manipulated in each treatment, data shown are mean ± SE).

Treatment	Species			
	<i>Calligonum calliphysa</i>	<i>Calligonum rubicundum</i>	<i>Calligonum demum</i>	<i>Calligonum ebinuricum</i>
Percentage fruit set (<i>n</i>)				
No emasculation, bagged, self-pollination	0 (31)	0 (34)	0 (36)	0 (31)
Bagged, hand self-pollination	3.3 ± 0.1 (30)	2.9 ± 0.1 (33)	0 (30)	2.9 ± 0.1 (35)
Emasculation, bagged, hand geitonogamy	79.5 ± 0.1 (34)	88.6 ± 0.1 (34)	86.7 ± 0.1 (30)	92.1 ± 0.1 (36)
Emasculation, bagged, hand cross-pollination	91.4 ± 0.1 (35)	70.0 ± 0.1 (30)	94.3 ± 0.1 (34)	100 (35)
Emasculation, bagged, no pollination	0 (32)	0 (30)	0 (31)	0 (30)
Emasculation, unbagged, natural pollination	39.1 ± 0.1 (33)	84.3 ± 0.01 (32)	45.0 ± 0.1 (40)	15.0 ± 0.01 (40)
Branch bagged	0 (30)	0 (30)	0 (30)	0 (30)
Flower bagged with net	12.5 ± 0.1 (40)	7.5 ± 0.1 (40)	17.5 ± 0.1 (40)	22.5 ± 0.1 (40)
Unemasculation, unbagged, natural pollination	40.5 ± 0.01 (205)	27.8 ± 0.03 (79)	30.5 ± 0.01 (289)	47.1 ± 0.02 (140)

Table 4. Fruit set for the four *Calligonum* species under different hybridization treatments (n = is the total number of flowers manipulated in each treatment, mean ± SE).

Species cross	<i>Calligonum calliphysa</i> ♀	<i>Calligonum rubicundum</i> ♀	<i>Calligonum demum</i> ♀	<i>Calligonum ebinuricum</i> ♀
	Percentage fruit set (n)			
<i>C. calliphysa</i> ♂	N/A	0 (39)	-	-
<i>C. rubicundum</i> ♂	3.3 ± 0.1(28)	N/A	-	5.0 ± 0.1(39)
<i>C. demum</i> ♂	-	-	N/A	0 (39)
<i>C. ebinuricum</i> ♂	-	-	2.5 ± 0.1(39)	N/A

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), so interspecific hand pollination did not yield any viable seeds.

Floral visitors

Same types of pollinating and visiting species were found for each of the four species. The major pollinators were *Apis mellifera* L. and *Halictus* sp., which collected pollen and nectar. 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. The other species recorded were nectar thief, they were Flies (*Lasioticus* sp., *Musca domestica*, Muscidae, and *Calliphora vicina*), Butterflies (*Plebejus argus*) and others (Formicidae).

Seed germination

The seed germination of the four species under the two different pre-treatments were relatively high, and the seed germination rates were as follows: *C. calliphysa* were 83.3 ± 0.1 (i) and 67.0 ± 0.1 (ii); *C. rubicundum* were 77.3 ± 0.1 (i) and 72.0 ± 0.1 (ii); *C. demum* were 60.3 ± 0.2 (i) and 88.7 ± 0.1 (ii); *C. ebinuricum* 91.3 ± 0.1 (i) and 84.0 ± 0.1 (ii).The difference between the two pre-treatments was not significant (*C. calliphysa*, *F* = 9.401, *P* > 0.05; *C. rubicundum*, *F* = 0.537, *P* > 0.05; *C. densum*, *F* = 14.751, *P* > 0.05 and *C. ebinuricum*, *F* = 2.854, *P* > 0.05). This suggests that most seeds were able to germinate and develop normally.

Discussion

Even in the occurrence of flowering overlap, the absence of hybridization among the four species of *Calligonum*, as well their high seed viability allow the conservation of these species in botanical gardens.

Temporal heterogeneity in flowering periods among sympatric species often contributes significantly to their

isolation (Sprague 1962; Levin 1971; Adams 1983; Grant 1992, 1994). 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 time was earlier of the former two 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 three years of the study, and even *C. calliphysa* and *C. rubicundum* were found to overlap completely in one instance. 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 1994). 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, 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 botanical garden. We can conclude that they are suitable for *ex situ* conservation in TEBG.

Genetic diversity is one of the important aims of *ex situ* conservation. The genetic diversity conservation dose not

only do good to the long-term survival of species but also provides information to improve the understanding and appropriate management of plant populations (Kramer & Havens 2009; Swarts & Dixon 2009). Therefore, there will be a continued need for botanical gardens to conserve and maintain the genetic diversity of *Calligonum*, as there will be for other such threatened species.

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