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POLLINATION ECOLOGY OF *BRETSCHNEIDERA SINENSIS* (HEMSLEY), A RARE AND ENDANGERED TREE IN CHINA

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Abstract

Bretschneidera sinensis, a monotypic species mainly distributed in China, was listed in National Key Protected Wild Plants as a rare and tertiary relict tree. Pollination ecology of *B. sinensis* was studied to reveal its floral morphology, breeding system and possible bottlenecks for its weak natural regeneration. Results indicated that *B. sinensis* has an outcrossing-entomophily breeding system with protogyny and a high P/O ratio, and is pollinated by a variety of insects, with the main pollinators belonging to Hymenoptera. It was the integrative factor that contributed to its low fruit production. The fragmentation of the habitat with increased inbreeding might be the most important factor for the diminution of this rare species. Other factors in reproduction also cause the low fruit production, includes low numbers of flowering individuals, low pollen transfer efficacy, weak fruit retention and the short flowering season etc. Meanwhile, bottlenecks in subsequent life history stages, such as low survivorship of seeds and lack of seedlings were also observed which indicated reduced fitness for its population development.

Introduction

Faced with the problem of preserving rare species endangered by habitat destruction and fragmentation, the pollination ecology of endemic species with restricted geographic distributions has become a central concern of biologists (Anderson *et al.*, 2001). *Bretschneidera sinensis* Hemsl., mainly distributed in China, was listed in the China National Key Protected Wild Plants (First Group) and was regarded as a rare species in China Plant Red Data Book (Fu, 1992). As a tertiary relict, it is of great value for studies of phylogeny, ancient geography and ancient climate, and also a desirable subject for drawing attention for conservation because it is a representative of flora in the southern sub-tropical monsoon climate, a region that favored many rare and endemic species (Jin *et al.*, 2003). Previous studies of *B. sinensis* were mostly focused on phylogenetic relationships (Yang & Hu, 1985; Chaw & Peng, 1987; Tobe & Peng, 1990; Lu & Hu. 1994), seedling cultivation (Qiao *et al.*, 2009) and chemical constituents (Ma & He, 1992). No detailed information on pollination ecology is available, although this information is essential for the promotion of its natural regeneration. To this end, the objectives of our study are the following: 1) to describe the floral morphology and breeding system of *B. sinensis*; 2) to identify the important pollinators of *B. sinensis* and their pollen transfer efficacy; 3) to examine the production, germination, and viability of pollen; 4) to determine pollen type and stigma receptivity by histochemistry; and 5) to reveal the possible bottlenecks for its weak natural regeneration and to provide effective measures for its conservation.

Materials and Methods

Study site: Studies were carried out in Nankun Mountain (114°38′ E, 23°38′ N, Alt. 420-560 m; 96.6% forest coverage), a nature reserve located in Huizhou city, Guangdong Province, China. This reserve belongs to the southern sub-tropical monsoon climate, and the annual mean temperature and precipitation are 18.2°C and 2163 mm, respectively. With the permission and help of the local government, observation sites were chosen nearby 3 wild flowering individuals, and a tall observation stand was set up nearby the biggest one.

Floral morphology, nectar volume production and sugar content: Flowers were dissected to determine floral morphology, including floral architecture, design and display. Fifty flowers at different stages were collected at random and measured with a digital caliper, including the diameter, length of petals, stamens, filaments, anthers, styles and distance between stamens and pistil. Mean nectar volume production was measured with a 10 µl pipette from 30 flowers, and the sugar content was tested by hand refractometer (Atago N-8α).

Floral duration and mean flower lifespan: From April 2 to April 20, 2008, flower buds were monitored to determine the sequence of anthesis, average duration and opening time. A total of 121 flowers in 3 racemes from 3 individuals were marked before opening and then were observed hourly from bud stage to the shedding of tepals. The movement of the stamens and style during the flower lifespan also were recorded. According to development process, 5 stages in a flowers lifespan were identified, including: bud (1~4 d), early florescence (5 d), full bloom (6~8 d), late florescence (9 d), and wilt (10~12 d). Only buds with pink corollas could be calculated in the process, and the newly formed buds with green corollas were excluded because of the immaturity.

Pollen grain numbers, ovule numbers and pollen-ovule ratio: A total number of 10 flowers just prior to opening from different racemes were examined, and the pollen grains were counted by using closed anthers to avoid contamination. The grains remained in suspension and were uniformly distributed in a Petri dish on a shaking machine for 10~12 minutes, and a compound microscope with counting plate was used to count the number of pollen grains in a field of view (working area on the counting Fig. 3 mm^2). The contents of multiple fields of view could be used to extrapolate the total number (Kannely, 2005), and the mean value was calculated and recorded. Number of ovules was just determined with a magnifying glass.

Pollen viability, pollen type and timing of stigma receptivity: Histological study of pollen was examined with fresh material stained with iodine/potassium iodide (I_2-KI) for starch and Sudan IV (0.09 g/ml in 95% ethanol) for lipids, respectively (Johansen, 1940).

In vitro pollen germination test was carried out by sitting drop culture at five sucrose concentrations (7%, 10%, 15%, 20% and 25%) with 1ppm boric acid as a preliminary test. Results showed that 20% with 1ppm boric acid sucrose was the best concentration according to the germination rate, broken rate, crimple rate and elongation of pollen tube 8h later. Subsequently, pollen viability in different stages was examined in 20% sucrose by sitting drop culture at 08:00 (Beijing time, the same as below) and hourly examined. Results were observed under a Leica DMLB photomicroscope.

Stigmas of different stages were stained with auramine O and observed by stereo-microscope to test the receptivity according to the normal implementation of bands (Dafni, 1992).

Insect activity: Pollinator observations were carried both day (06:00~18:00h) and night (19:00~6:00). In each case we recorded: 1) the behavior of the visitor, forage for nectar or pollen; 2) the frequency of visits to each plant; 3) the duration of each visit. Two racemes with 61 flowers and their visitors were investigated. Visitors were observed as they entered the observation area (6 m^2). Identities of the visitors and efficacy of foraging behavior were evaluated preliminarily in conjunction with descriptions of floral morphology. Visitors which met the criteria of pollinators were collected in the insect bottles and identified exactly by systematic experts. Finally the same specimen was examined under scanning electron microscope (SEM) for the presence and placement of pollen.

Breeding system: To determine the breeding system of *B. sinensis*, flowers were subjected to one of the following bagging treatments on Mar. 25, 2009. 1) Natural pollination without any treatment (control group); 2) Buds were paper-bagged without further manipulation to test for spontaneous self-crossing; 3) Buds were emasculated without bag; 4) Buds were set with mesh bag after emasculated; 5) Emasculated, hand-pollinated with pollen from different flowers in the same tree; 6) Emasculated, hand-pollinated with pollen from a different tree 150 m away. Bags were removed after the wilting stage so as not to hinder fruit development. Developing fruits were observed 2 wk later and counted in Apr 21, Jun 15, Jul 17 and Sep 26, 2009.

Results

Floral morphology with typical entomophilies characteristics: *Bretschneidera sinensis* is a perennial tree species, 8~20 m high, with many-flowered racemes in the branches and showy flowers clustered together in spiral type. The mean number of flowers in racemes is 20~45. As a deciduous plant, the terminal buds of *B. sinensis* form new leaves and flowers successively from March to April. The floral architecture was as follows: pedicels 2~4 cm, bilateral symmetry, campanulate corolla; calyx: 1.5~2.2 × 1.2~2.1 cm, hairy and 5-lobed; petals: pink, becoming darker under the sunshine, red striate, broadly spatulate to obovate-cuneate, 1.8~2 × 1~1.5 cm, base rounded, apex notched to form long corolla- tube, upper petal hoodlike, covering stamens and style (Fig. 1A). The mean diameter of the corolla from bud stage to full bloom stage was as follows: bud 0.5~2.0 cm, early florescence 2.0~2.5 cm, full bloom 2.5~3.5 cm. Some styles projected from the corolla just at the bud stage particularly, which could avoid self-pollination (Fig. 1B).

Floral design and display: expressed in a long-styled form with short stamens at the onset of anthesis, and the mean distance between stamens and pistil was 0.19~0.29 cm. Filaments: 2.5~3 cm, hairy at base, recurving apically. Ovary and style hairy; style 3~4 cm, syncarpous gynoecium in axile placentation with 3 rooms, and 2 ovules per room, broad nectaries were at base of the staminal filament (Fig. 1C). The mean nectar volume production was 0.72±0.33 μl and the sugar content was 19.6%±1.97. All the characters above suggested a typical entomophily type.

Floral duration and convergent florescence of population: The convergent florescence of the population (20~25d) appears to be adaptation to lure more pollinators. Anthesis took place from below to above within a raceme except that the top flower opened quite early which was similar to the anthesis time of middle flowers, and it took about 11~13 days from bud to perish. The flowers often opened in the dusk and at night, and the anther split outwards longitudinally simultaneously (Fig. 1D). When anthesis was completed, the perianth and androecium would dry up and contract, then a developed abscission line in the receptacle allowed falling of wilted floral structures, including perianth, androecium and style.

Pollen grain numbers, ovule numbers and pollen-ovule ratio: The mean pollen grain number per flower was 75630~98568, and the ovule number was 6 in all, so the P/O is 12605~16428. According to criterion of Cruden (1977), this ratio indicates that *B. sinensis* belongs to an outcrossing and entomophily type.

Pollen type, morphology: Histochemistry results showed that the pollen grain was starchless and lipidic (Fig. 1E), which also indicated an entomophily type. Mature pollen grains were characterized by a thick and spiny exine and only 3 germinal furrows positioned isometrically on the round pollen grain (Fig. 4, A, B, C) which suggested a primitive type (Walker, 1974; Perveen *et al.*, 1994). The grains were quite easy to be separated in all manipulations which indicated its low conglutination trait.

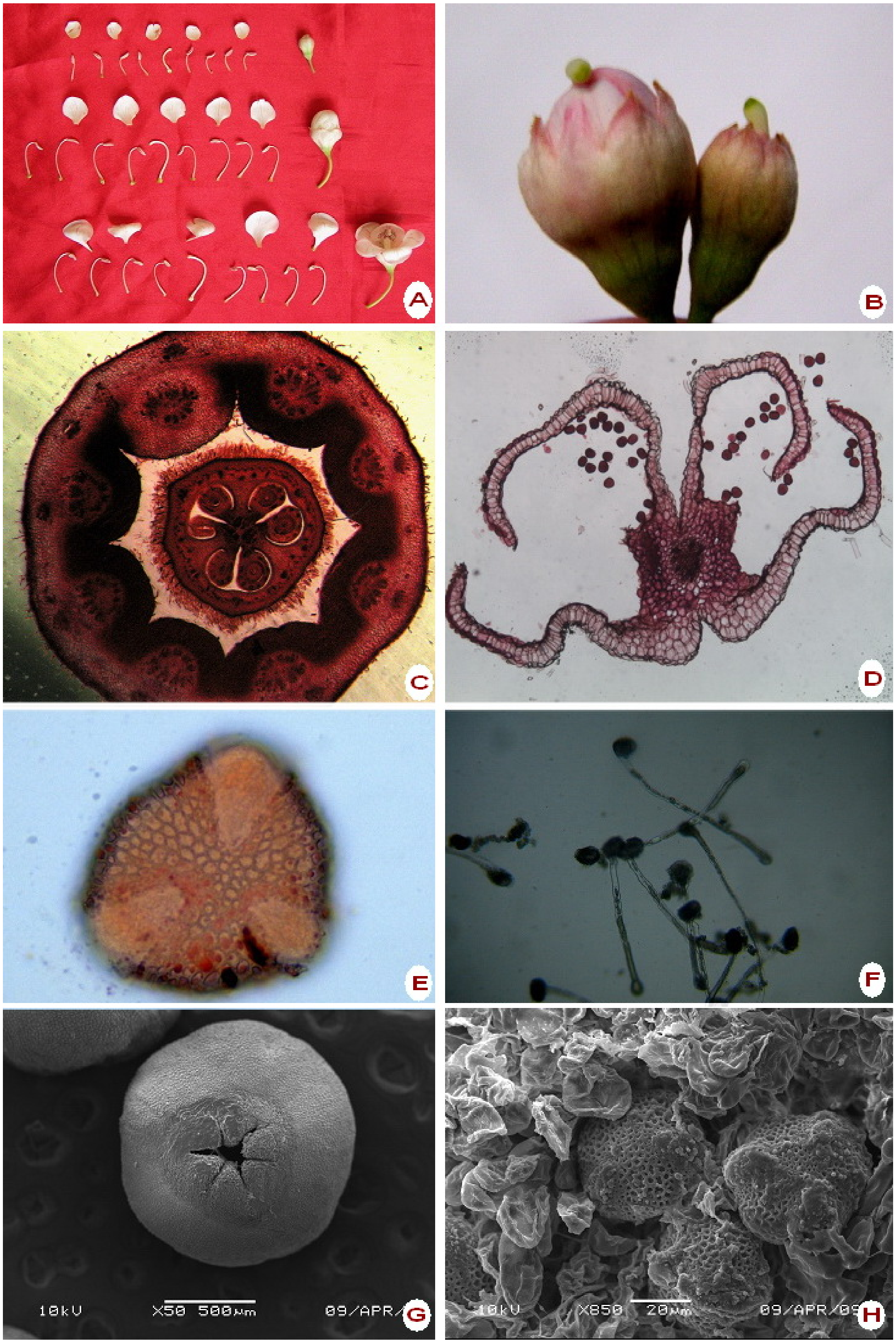

Fig. 1. Morphological characteristics of the flower of *B. sinensis*.
A. Floral structures from bud stage to full-bloom stage, B. Styles projecting from the corolla at bud stage, C. Nectaries at base of the staminal filament, ovule, placenta and hairy germen were also shown, D. Release of pollen grain, E. Pollen was stained by Sudan IV, F. Germination of pollen, G. Receptivity face of the stigma, H. Pollen on the stigma

Viability of pollen and stigma: Pollen viability tests indicated that the germination process was quite long that pollen tubes could reach 3~5 multiple of the grain 16 h later (Fig. 1F), and the germination rate reached a high level only at full-bloom stage (Fig. 2). It was also noticed that pollen viability would reduced sharply (21%-38%) if the pollen grains were watered by rain, which indicated that continual rain would bring side effects not only to the visiting frequency of pollinators but also the pollen viability.

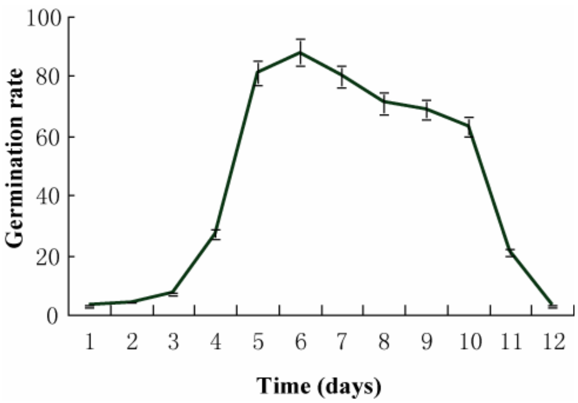

Fig. 2. Change of Pollen viability in different stages
Note: small buds (1~2 d), big buds (3~4 d), early florescence (5 d), full bloom (6~9 d), and wilt (10~12 d)

Histochemistry results showed that stigma receptivity occurred from the bud stage to the full-bloom stage, which indicate a protogyny system in conjunction with pollen viability in *B. sinensis* (Table 1). Also the low fluid secretion of the surface, big size of pollen (48~53 µm) in contrast to the relatively small receptive surface of the stigma (767-813 µm) all showed the low receptive ability of the stigma (Fig. 1G, H).

Table 1. Change of stigma receptivity in different stages.

Stage		Color change	Air bubble	Number of pollen grains on stigma
Bud		Pale blue	+	0
Early florescence		blue	++	21
	1d	blue	++	112
full bloom	2d	blue	++	116
	3d	Dark blue	+	121
Late florescence	4d	Dark blue	+	Surface destroyed
Wilt		Dark brown	-	Surface destroyed

Note: + small amount of air bubbles, ++ large amount of air bubbles, - means no air bubbles

Pollinators: The main pollinators for *B. sinensis* were Hymenopteran and Lepidopteran insects, and different insects contacted the flower in different ways to get pollen or honey from nectar (Table 2). Furthermore, no typical night activity insects were found to help the pollination. Available information about the pollination was that the size of pollinators corresponds with the carrying ability. For hymenopterans, large-sized insects were *Xylocopa* spp. (including *Xylocopa collaris*), which were assumed to be the most active pollinators and were always the first one to enjoy the honey in the early morning (Beijing time 6:00-7:00). *Bombus* and *Apis cerana* would enjoy the honey and pollen later just after *Xylocopa spp*. Finally *Vespa*, *Talbotia naganum* and *Syrphidae* enjoyed the remaining honey. Additional evidence to test the carrying effectiveness of different hymenopteran insects was the body hairs for the adhesion and load-carrying ability, and *Xylocopa*were was also the most efficient one just because of its flinty hairs which like many strong ropes to carry more large grains easily (Fig. 4, E, F, G, H).

Table 2. Pollinators and visiting frequency, mean duration and reward type
(Apral 13~15, 2008, 06:00~ 18:00 Beijing time).

Time (March 2008)	Total visiting frequency			Mean duration of each visit (second)	Nectar (N) Pollen (P)	Touch style abdomen (A) Back (B)
Visitor	rainy (13)	rainy (14)	sunny (15)			
Bombus sp.	6	1	2	8~10	N, P	A,B
Apis cerana	28	15	4	10~12	N, P	A,B
Xylocopa sp.	3	3	11	10~15	N	B
Xylocopa collaris	4	5	3	6~8	N	B
Vespa sp.	2	1	2	4~6	N	B
Talbotia naganum	2	1	6	4~12	N	A
Syrphidae sp.	18	11	6	6~15	N	A,B

Figure 3 indicated that 6:00, 9:00, 12:00 and 15:00 were the high-frequency visiting period. With the reduction of pollen and nectar, pollinators would reduce the visiting frequency and total time on the flowers:

Other co-flowering species (*Rhododendron* sp., *Mucuna birdwoodiana*, *Paulownia tomentosa*, *Rhodoieia Championi* and *Ehretia thyrsiflora*) were visited by the same insects. A few pollen grains of *B. sinensis* could be transferred because of their large size and low conglutination character, which suggested low competitive ability with the other co-flowering species (Fig. 4, D).

Breeding system: According to our field investigations in Nankun Nature Reserve, the distribution area of *B. sinensis* was narrow and concentrated, and only 7 mother trees were found (Qiao *et al*., 2010). Results of mating system experiments (Table 3) indicated that fruit set rate of flowers with hand-pollination from a different tree was the highest (52.94%) whereas flowers that were hand-pollinated with pollen from a different flower in the same tree was quite low (18.92%), which suggested that the breeding system belonged to a outcrossing type. All the immature fruits fell down finally in September which showed that the fruit retention force of this rare plant was very weak.

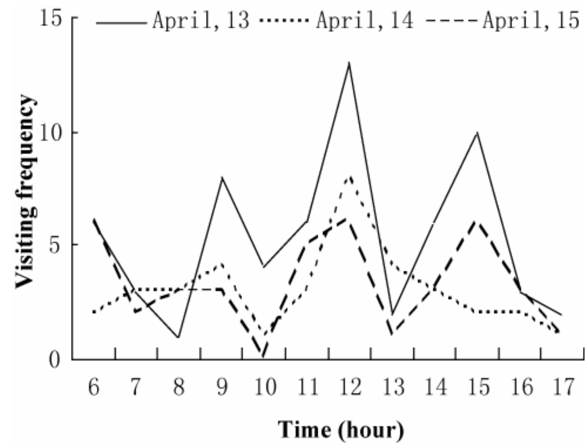

Fig. 3. Total visiting frequency of pollinators at different times.

Table 3. Breeding system experiments.

Treatments	Number of flowers	April 21 Number and set rate of fruit		June 15 Number of fruit	July 17 Number of fruit	September 26 Number of fruit
		Number	Set rate (%)			
Natural pollination (CR)	98	32	32.65	10	7	0
Paper-bagged	12	0	0.00	0	0	0
Emasculated without bag	3	1	33.33	1	1	0
Emasculated, mesh-bagged	17	0	0.00	0	0	0
Emasculated, hand-pollinated with pollen from different flower in the same tree	37	7	18.92	2	2	0
Emasculated, hand-pollinated with pollen from different tree 150 m away	34	18	52.94	6	3	0

Discussion

Possible bottlenecks for its low fruit production: It was the integrative factors that contributed to the low fruit production, including: habitat fragmentation and destruction, low numbers of flowering individuals, low pollen transfer efficacy and weather disaster as well.

Pollination systems of many rare plants in China are under increasing threat from anthropogenic sources, including fragmentation of habitat, changes in land use and modern agricultural practices. The number of flowering individuals of *B. sinensis* has been reduced dramatically because of habitat limitation and human disturbance in recent years (Qiao *et al.*, 2010), and as a result, increased inbreeding depression could be caused which might be the most important factor in the diminution of this rare species.

Belonging to an outcrossing-entomophilous breeding system, *B. sinensis* had many typical characteristics to lure pollinators, including a short flowering season, many-flowered racemes, bright pink corolla color, long corolla-tube, bilateral symmetry, and broad nectar at base of the stamen filament etc. However, the low conglutination of pollen and 3 germinal furrows of pollen, relatively small receptive surface, and low fluid secretion of the stigma all resulted in its low fecundity as primitive characteristics. Furthermore, co-flowering species might be highly significant to the reproductive success of rare plants, whether by attracting pollinators to the area, or by providing additional recourses, or competing for pollinator visits (Tepedino & Sipes, 1997); (Abid *et al*., 2010). SEM results suggested that the pollen grains of *B. sinensis* were bigger than other co-flowering species, whereas the eusocial insect (*Bombus sp*., *Apis cerana*) bodies always carried smaller pollen grains that were easier to store in reserve. Furthermore, some proportion of the pollen they deposit on stigmas may be heterospecific, while female function might be reduced through physical blocking of limited stigmatic surface with heterospecific pollen (Mosquin, 1971; Horvitz & Schemske, 1988; Waser & Fugate, 1986). Accordingly, *B. sinensis* had a typical primitive reproduction strategy which appeared high input (pollen grains) in contrast low output (seeds) and was thought to represent a pattern of “compensation” described by Bond (1994).

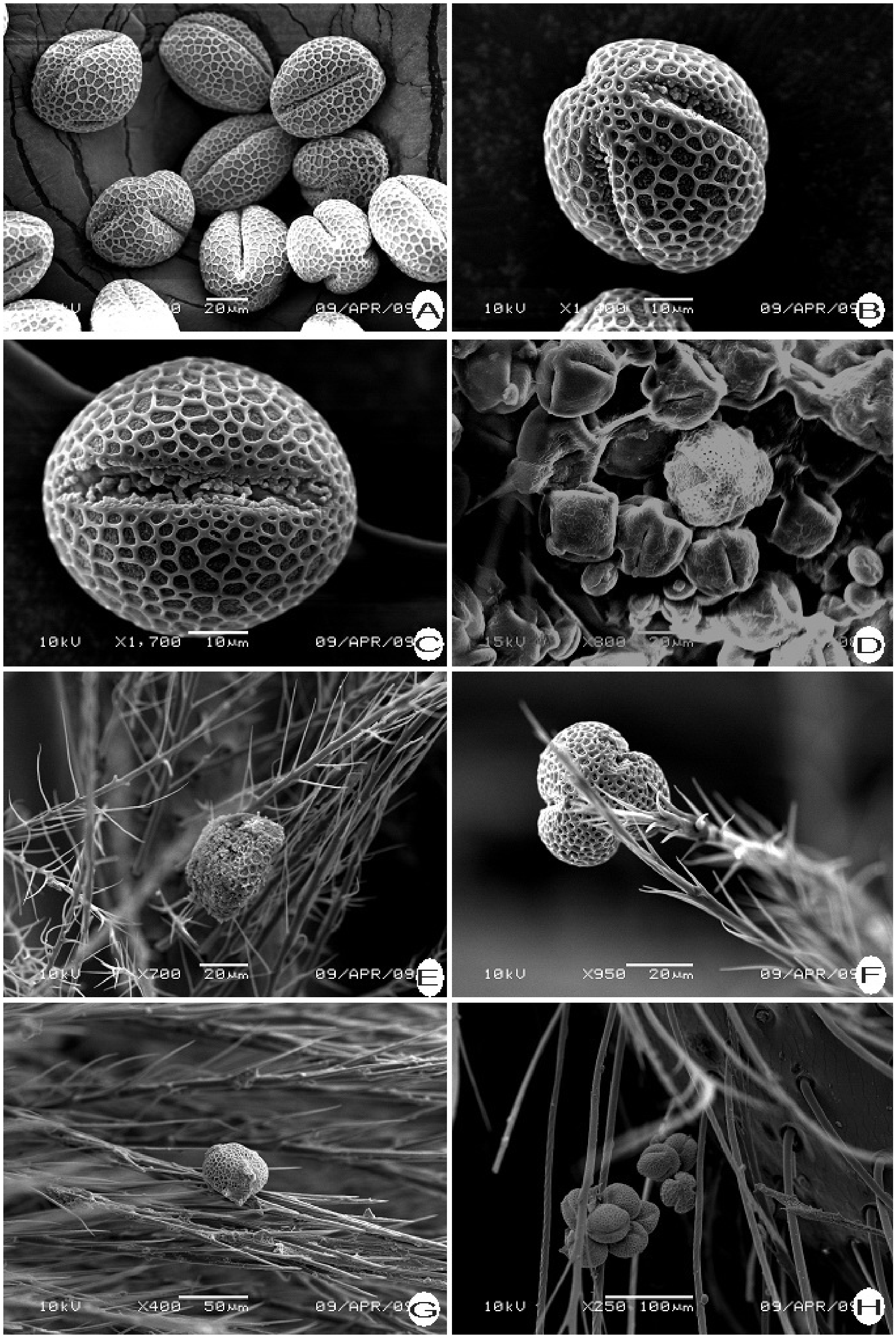

Fig. 4. Morphological characteristics of the pollen of *B. sinensis* and carrying status of different pollinators.
A. Different morphology of the pollen grains, B. Polar view of the pollen grain, showing 3 germinal furrows, C. Germinal furrow of the pollen, D. Few grains of *Br. sinensis* were observed in contrast to other grains of co-flowering species, under SEM, E. Pollen grain carried by *Apis cerana,* under SEM, F. Pollen grain carried by *Bombus* sp.*,* under SEM, G. Pollen grain carried by *Xylocopa collaris*, under SEM, H. Pollen grain carried by *Xylocopa* sp., under SEM

Weather also plays a key role in the reproduction process in *B. sinensis*. Long-term observation showed that unfavorable weather (precipitation, strong or gusty winds) probably have negative impacts on pollinators' activity and long development of fruit later; especially the familiar long precipitation in the April in many habitats of *B. sinensis* would reduce the pollen viability dramatically. Although the convergent flowering season of a population (20~25 days) could attract more pollinators, it also causes weak resistance to unfavorable weather which can reduced the visiting frequency of middle-sized insects that have low volitation ability.

Other bottlenecks in subsequent life history stages for the weak natural regeneration: It is recognized that limited population recruitment of rare species is probably correlated with seed germination, long-term survival of seedlings and fecundity in future generations (O'Malley & Bawa, 1987; Fischer & Matthies, 1998). In our further research, other bottlenecks such as low survivorship of seeds and lack of seedlings were also observed in *B. sinensis* which indicated the reduced fitness in subsequent life history stages. Large losses occur between seed dispersal and seedling emergence, and the survival and distribution of *B. sinensis* is associated mainly with the mechanisms ensuring germination and seedling development at favorable time and place (Qiao *et al.*, 2009; Qiao *et al.*, 2011).

Conservation strategies: According to the results above, conservation of habitats and primary pollinators are the main tasks for local government. Hand pollination and controlled release of native *Apis cerana*, *Xylocopa* sp., and *Bombus* sp., are recommended on sunny days to reduce the risk in the rainfall. In addition, fruit-protecting reagents could be utilized to improve fruit retention. And from a conservation standpoint, long-term surveys of population size (seasonally and annually) and *in situ* conservation should be undertaken together to facilitate its persistence.

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