



Reproductive biology of *Magnolia sinica* (Magnoliaceae), a threatened species with extremely small populations in Yunnan, China



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ABSTRACT

Magnolia sinica is one of the most threatened trees endemic to Southeast Yunnan. Based on our investigations, only 52 individuals and eight populations are found in the wild. *M. sinica* has been categorized as Critically Endangered on the IUCN Red List and identified as a “Plant Species with Extremely Small Populations (PSESP)”. Its fruit/seed set is very low and seedlings are rarely found in the wild. It is hypothesized that it may encounter obstacles to reproductive success. This study, therefore, focuses on its reproductive biology, knowledge of which is essential for effective conservation. Flowers of this species are protogynous and nocturnal, and possess a two-day rhythm of sexual presentation. For the first night of anthesis, the flowers are in the pistillate stage during which tepals open at dusk and close approx. 1 h later (except for the open outer ones). They remain closed until the next afternoon, when flowers, now in the staminate stage, re-open and remain so until the tepals drop. Nocturnal beetles enter into the flowers and remain trapped throughout the night as the flower closes, during which time they feed on tepals. Pollen-gathering bees are found to visit the re-opened flowers and the beetles are released during this stage. Two species of Pleocomidae and Curculionidae beetles appear to be effective pollinators.

M. sinica is a self-compatible, pollinator-dependent species, and its fruit/seed set can be significantly increased by hand-pollination. No functional seed dispersers have been found in its extant natural habitats. These findings suggest that it may face both pollination and seed disperser insufficiencies in its current fragmented habitats, which may account for its low regeneration. Here we propose conservation strategies based on our findings.

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Magnolia sinica (Law) Noot. (Magnoliaceae), a rare tree species endemic to Southeast Yunnan, China, was first described as *Manglietiastrum sinicum* Law in 1979 (Law, 1979). Most Chinese botanists often call this species *M. sinicum* and also use its common name *huagaimu* (Sun et al., 2012; Wang et al., 2016). Its other synonyms are *Manglietia sinica* (Chen and Nootboom, 1993) and *Pachylarnax sinica* (Xia et al., 2008). Because the species has been referred to as *M. sinica* internationally, *M. sinica* is also used in the present study.

M. sinica distributes in south subtropical monsoon broadleaved evergreen forests and scatters at altitudes between 1339 and 1707 m (Wang et al., 2016). Recent anthropogenic activities, including deforestation for commercial cultivation (e.g. *Amomum tsao-ko* Crevost et Lem. and *Cunninghamia lanceolata*) and habitat destruction, have lead both to the reduction of its population size and to serious habitat fragmentation. This species is categorized as Critically Endangered on the IUCN Red List (Cicuzza et al., 2007; Rivers et al., 2016) and has been identified as a “Plant Species with Extremely Small Populations (PSESP)” (State Forestry Administration of China, 2012; Ren et al., 2012; Ma et al., 2013; Volis, 2016) for priority conservation in China. It has also been targeted as one of the 20 species approved by the Yunnan government for urgent rescue action before 2015 (Wang et al., 2016). In the field, a total of 52 individuals of *M. sinica* were isolated in eight isolated populations. Of these 52 individuals, nine are young trees

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with a DBH (diameter at breast height) of less than 22.5 cm (Wang et al., 2016).

Although *M. sinica* flowers well, its fruit/seed set is low and seedlings are rarely found in the wild. Conservation via *ex situ* cultivation in gardens and reinforcement/reintroduction in the wild have been conducted by Kunming Botanical Garden (Sun, 2013). However, the rarity of seedling/saplings in the wild limits the potential for the natural regeneration of this species. Therefore, despite *ex situ* and *in situ* conservation trials, effective conservation measures based on scientific studies are imperative.

Knowledge of reproductive biology is essential for the effective protection of endangered plants, especially for species with small populations (Spira, 2001; Evans et al., 2004; Xiao and Xu, 2006). Successful reproduction is crucial in maintaining a viable population size, which is of critical concern to highly endangered taxa facing extinction (Pandit and Babu, 2003; Gong et al., 2014). In species relying on seeds to recruit new individuals into populations, population viability may be closely related to seed dynamics and conservation measures may depend on understanding the factors that limit seed production (Pavlik et al., 1993; Zhao and Sun, 2009) and dispersal. When seed production is mediated by pollinators, it can be influenced by pollinator abundance or behavior (Bierzychudek, 1981; Larson and Barrett, 2000). It may be limited by pollen, because self-pollen may cause reduction in seed production through inbreeding depression (Bosch and Waser, 1999; Brown and Kephart, 1999; Zhao and Sun, 2009). Seed production in particular, as a seed source for offspring, may directly affect seed dispersal. Seed dispersal may also be influenced by disperser abundance or behavior, if seed dispersal is mediated by dispersers.

Studies on the reproductive biology of threatened species with low reproductive capabilities have been the focus of recent research and have become an important aspect of conservation management. Despite extensive attention and critical conservation status, little information is available on the reproduction of *M. sinica*. The current study was undertaken to gain knowledge of the reproductive characteristics of *M. sinica* and aims to address the following issues: (1) its floral biology, especially the flowering process; (2) its pollination biology and the role of pollinators in fruit set; (3) its breeding system; (4) the characteristics of seed dispersal and seed germination of the species in the natural habitats.

1. Methods and materials

1.1. Study sites

The pollination biology of *M. sinica* was investigated from 2014 to 2015 in Jingping County of Honghe Hani-Yi Autonomous Prefecture. The population there comprises four individuals. One is located beside a mountain trail in Zhongliang village and the other three are located in the thick forest on Luoguoping Mountain. Reproductively mature individuals of *M. sinica* are usually tall trees. The tree in Zhongliang village was chosen as the experimental subject because of its convenience and easy access, and a 17 m high stand was built around it for experiments. All observations and experiments on the reproduction of the species were carried out on this tree, in the middle of March when *M. sinica* began flowering.

The seed dispersal experiment was conducted in November 2014 in a natural habit of *M. sinica* in Chinese fir seed orchards (label DLS-T), located in Dalishu Township of Maguan County in Wenshan Zhuang-Miao Autonomous Prefecture.

Further seed germination experiments were conducted in November 2014, at three natural localities: DLS-T, Zhongliang village of Jingping County in Honghe Hani-Yi Autonomous Prefecture (label ZL-V), and Miechang Township of Maguan County in

Wenshan Zhuang-Miao Autonomous Prefecture (two plots, label MC-T-1 and MC-T-2).

1.2. Pollen viability and stigma receptivity

The examination of pollen viability and stigma receptivity was conducted on three flowers per day over five consecutive days at full-bloom stage in both 2014 and 2015.

Pollen viability was examined using the MTT method (Dafni, 1992). After pollen has been mixed with MTT solution and left for 10 min, viable pollen will turn dark purple in color whereas inviable pollen will turn tawny-yellow or remain unchanged. Pollen was taken from anthers at different flowering stages and mixed evenly with 1% MTT on two slides. The numbers of viable and inviable pollen grains were counted over three separate views per slide, using a light microscope.

Stigma receptivity was examined by the benzidine-hydrogen peroxide method (Dafni, 1992). After soaking in a benzidine-hydrogen peroxide solution, a receptive stigma will show peroxidase activity and turn blue with a mass of bubbles surrounding it. The depth of the blue color indicates the intensity of receptivity. At different flowering stages, two stigmas per flower were picked and assessed for mucus secretion. They were then soaked in the benzidine-hydrogen peroxide solution. The changes of color and occurrences of bubbles were observed by eye.

1.3. Flowering dynamics

Based on primary observations, flower buds that are about to open can easily be recognized by the softness of their bud tips and their stronger fragrance, and some mature flower buds will open simultaneously at the same time every dusk over the flowering duration of the tree. In every flowering-season over two consecutive years, five flowers were labeled and their flowering stages were observed continuously until the tepals wilted.

1.4. Observation of floral visitors

Observations of floral visitors were carried out from 8:00 AM to 22:00 PM over four consecutive days, spreading across two flowering seasons: March 2014 and April 2015. During observations, five or six flowers were randomly labeled per day. Visitors to these flowers were caught using tweezers and kept in 70% ethyl alcohol for subsequent identification in the laboratory.

1.5. Breeding system

To evaluate the breeding system of *M. sinica*, flower buds that would definitely blossom at dusk were selected randomly, marked and caged with sulfate paper bags (30 × 25 cm) every day in the morning at the full-bloom stage in both 2014 and 2015. Five pollination treatments were assigned at the moment when the flowers started to open. Autonomous self-pollination (label SPON, n = 12) was tested by bagging flowers to exclude visitors. Self-compatibility was tested by bagging emasculated flowers and transferring their own pollen by hand (label SELF-S, n = 22) and bagging emasculated flowers and being hand-pollinated with pollen from other flowers at the same tree (label SELF-D, n = 62). To test for xenogamy (label CROSS, n = 47), flowers were treated similarly but were hand-pollinated with pollen from other trees. Control flowers were unmanipulated (label OPEN, n = 150).

The marked flowers were harvested in early November, when fruits were collected to assess fruit and seed set.

The self-compatibility index (SCI) was used to determine the breeding system of *M. sinica* and was obtained as mean percentage

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