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Abnormalities in sexual development and pollinator limitation in *Michelia coriacea* (Magnoliaceae), a critically endangered endemic to Southeast Yunnan, China

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Abstract

Michelia coriacea (Magnoliacae) is a critically endangered tree, endemic to Southeast Yunnan province, China. Most of the individuals in the extant populations normally bear flowers, but fruit set and fertile seed production rates were only 6.7% and 0.2%, respectively. To identify possible causes of reproductive barriers, the following studies were carried out: examination of stamen and pistil development; experiments using controlled pollination; observations of behavior and frequency of floral visitors in both cultivated and natural populations. The results revealed that about 60% of pollen was abnormal and approx. 70% of ovules had delayed development. Hand pollination could effectively enhance the fruit set ($F_{4,25} = 35.139$, $P < 0.0001$) and seed set per fruit ($F_{4,25} = 85.022$, $P < 0.0001$). Both cultivated and wild *M. coriacea* had an extremely low frequency of floral visitors. Some beetles, a few species of Andrenidae and some *Bombus sp*. are likely to be the effective pollinators. The fruit set and seed set per fruit from controlled self-pollination and cross-pollination were significantly different ($P < 0.05$), and thus it is inferred, that inbreeding depression may be a contributing factor in the very low seed production. It appears that low seed set in *M. coriacea* is due to a combination of factors: abnormalities in pollen and ovules, low number of effective pollinators, and inbreeding depression.
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Keywords: *Michelia coriacea*; Critically endangered tree species; Yunnan of China; Rarity of species; Pollen limitation

Introduction

Michelia coriacea Chang and B. L. Chen (Magnoliaceae) is a newly described species distributed in southeast Yunnan Province of China (Chen, 1988; Liu and Wu, 1988). This species is a diploid ($2n = 38$; Zhang and Xia, 2005) usually small tree up to 10 m high and 20–45 cm diameter (Chen, 1988; Chen and Nooteboom, 1993). Recent detailed surveys confirmed that the species can grow to an excellent timber tree, up to 30 m high and 60 cm in diameter. It bears scented whitish or creamy yellow flowers with 6–7 tepals (originally described nine tepals; Chen, 1988). Anthesis lasts from February to April (Cicuzza et al., 2007; Sun and Yan, 2006).

Michelia coriacea is considered to be a critically endangered endemic tree (Cicuzza et al., 2007). Since 2003, we have carried out a comprehensive investigation of this species. The existing population of the species consists of about 300–500 individuals scattered on limestone mountain slopes or secondary shrubby woods

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and on roadsides at altitudes of 1400–1700 m (Cicuzza et al., 2007). The habitats have been badly degraded and fragmented due to heavy logging and vegetation destruction in the past decade, resulting in sub-populations that are isolated from each other. Although this species flowered well, its fruit and seed set were very low and seedlings/saplings were rarely found in the past three years that we have studied it. Thus, conservation measures based on scientific studies are urgently needed for this taxon.

Knowledge of reproductive biology is necessary for effective protection of endangered plants, especially for species with low seed set and small populations (Evans et al., 2004; Kaye, 1999; Kruckeberg and Rabinowitz, 1985; Spira, 2001; Xiao and Xu, 2006), and this is especially true when regeneration occurs exclusively via seeds (Bond, 1994; Schemske et al., 1994). In such species, population viability might be closely linked to seed dynamics, and conservation might depend on understanding the factors that limit seed production (Pavlik et al., 1993). When seed production is mediated by pollinators, seeds can be limited by pollen availability, scarcity and/or inefficiency of pollination vectors, and poor pollen quality (Bierzychudek, 1981; Burd, 1994; Larson and Barrett, 2000; Wilcock and Neiland, 2002). Pollen quantity and quality as well as abnormal gametic development can often result in limited seed production and subsequent population decline and eventual extinction. The study of plant species with low reproductive capabilities has been the subject of much recent research and has become an important aspect in plant conservation (Byers, 2004; Colling et al., 2004; Evans et al., 2004; Hedrick and Kalinowski, 2000; Pan et al., 2003; Rocha and Aguilar, 2001). In addition, self pollen can limit seed production through inbreeding depression (Bosch and Waser, 1999; Brown and Kephart, 1999). Fragmentation of habitat, human activities, and especially low sexual reproductive capability will limit regeneration and population maintenance (Evans et al., 2004; Pan et al., 2003).

It is well known that the production of well-developed seeds of a species plays an important role in population dynamics and persistence. Undoubtedly, the effective and long-term conservation of *M. coriacea* will depend on an understanding of causes of its poor seed production. Thus, this paper focuses on observations/examinations of sexual development and mating system of *M. coriacea*, while trying to identify the factors that lead to its low fruit/seed set. The following questions are particularly emphasized: (1) Do pollen and ovule of the sampled individuals have normal development? (2) If not, which process and what percent of abnormalities occurred? (3) Which pollinators were efficient in fruit and seed production? (4) Were pollinators sufficient or did they limit seed production?

Materials and methods

Study sites

This study was carried out at the Seedling Station of Wenshan Forest Bureau (SSWFB) (lat. 23°24′23.4″N, long. 104°15′5.9″E) and in a wild population in Xichou County (lat. 23°23′–23°25′N, long. 104°25′–105°57′E) in southeast Yunnan Province, China. The sampled *M. coriacea* cultivated at SSWFB were 15 years old.

Methods

Stamen and pistil development

Flower buds at successive developmental stages were collected every two weeks during the period from November 2006 to the following April at SSWFB. Each time, 15 flowers buds were collected and fixed in FAA (formalin:acetic acid:50% ethyl alcohol = 5:5:90 v/v). The customary methods of dehydration, infiltration, and embedding in paraffin were used. Samples were sectioned at a thickness of 5–7 μm for microspores and male gametophytes and 9–12 μm for macrospores and female gametophytes. The sections were stained with Heidenhain's iron-alum hematoxylin, and then observed with Olympus BX-51.

Breeding system

Pollination treatments were performed on six randomly chosen individual trees at SSWFB in March of 2007. Five pollination treatments were used on each tree to determine the mating system. Each pollination treatment was conducted on 10–15 flowers per tree. These five treatments were: (1) Floral buds were labeled and left untouched under open pollination conditions ("Open pollination"); (2) Floral buds were labeled and bagged to test for autogamy ("Autogamic pollination"); (3) The flowers were emasculated, labeled and bagged to test for agamospermy (asexual reproduction in flower, e.g. apomixis) ("Agamospermic test"); (4) Hand self-pollinations (pollen from the same tree) was performed on emasculated flowers; (5) Hand cross-pollinations (pollen come from different trees) were done on emasculated flowers. The treated flowering organs were bagged with water-proof paper bags. These bags were removed after anthesis of the target flowers.

Labeled fruits were harvested in late September of 2007. Seeds from the fruits were collected and viable seeds were distinguished by the color of the aril. Arils of fertile seeds were red, whereas those of aborted seeds were dark brown. The effect of pollination treatments was analyzed for two properties: (1) fruit set percentage [(number of fruits/number of flowers) $\times$ 100]; (2) percentage of seed set per fruit [(number of viable seeds/number of initial ovules) $\times$ 100].

Floral visitors and Pollinators

Four *M. coriacea* individuals were sampled, two from the cultivated trees at SSWFB and two from the wild population in Xichou County. The behavior of floral visitors was observed and recorded for ten flowers on each tree. Observations were conducted once an hour, for 8 h each day from 9:00 to 17:00 and persisted for four days at each site (from March 14 to March 17 at SSWFB; from March 21 and March 24 to March 26 at Xichou population). The frequency of floral visitors was counted. The climatic conditions were recorded during the flowering period. A sample of the floral visitors was collected and later identified. The frequency of floral visitors was computed by counting the number of times that the pollinator visited a flower, the length of the visit and the number of flowers on which they were observed.

Results

Development of stamen and pistil

Abnormalities were detected both in the development of anthers and ovules. The anther was tetrasporangiate and the mature anther wall consisted of epidermis, endothecium, 2–3 middle layers and glandular tapetum of 1–2 cells thick. The secondary sporogenous cells which were derived by mitosis of primary sporogenous cell functioned directly as microsporocytes enlarging further. At this stage, $40.1\pm6.1\%$ from 500 sections presented sunken and clumped microsporocytes (Fig. 1). Modified simultaneous cytokinesis took place by furrowing, followed by meiosis, yielding in the developmental process $57.6\pm9.7\%$ degenerated microspores (Fig. 2). Of the microspores studied, $40.1\pm6.1\%$ were with callus accumulation, $50.7\pm5.8\%$ crumpled, and $52.18\pm7.3\%$ formed a bone shaped appearance by shrinking. Microspores released from the tetrads developed into two-celled pollen. The mature pollen grains were shed at this stage. At the time of pollen release $60.2\pm6.6\%$ of them were noted to be aborted (Fig. 3; Table 1).

The ovules of *M. coriacea* are anatropous, bitegmic and crassinucellar. The meiotic division of the megaspore mother cell resulted in a linear tetrad. The chalazal megaspore of the tetrad was functional. The functional megaspore underwent three successive nuclear divisions to give rise to an eight-nucleated embryo sac which corresponds to the *Polygonum* type.

Among 501 observed ovules, approx. 70% had delayed development, detected at stage of the uni-nucleate or bi-nucleate embryo sac. Of all, $24.9\pm9.4\%$ of them remained at the stage of the megaspore mother cell (Fig. 4). A $30.7\pm9.8\%$ of the total observed ovules remained at the uni-nucleate embryo sac stage (Fig. 5), and $14.5\pm9.9\%$ of them remained at the bi-nucleate embryo sac stage when the flower withered (Fig. 6; Table 2).

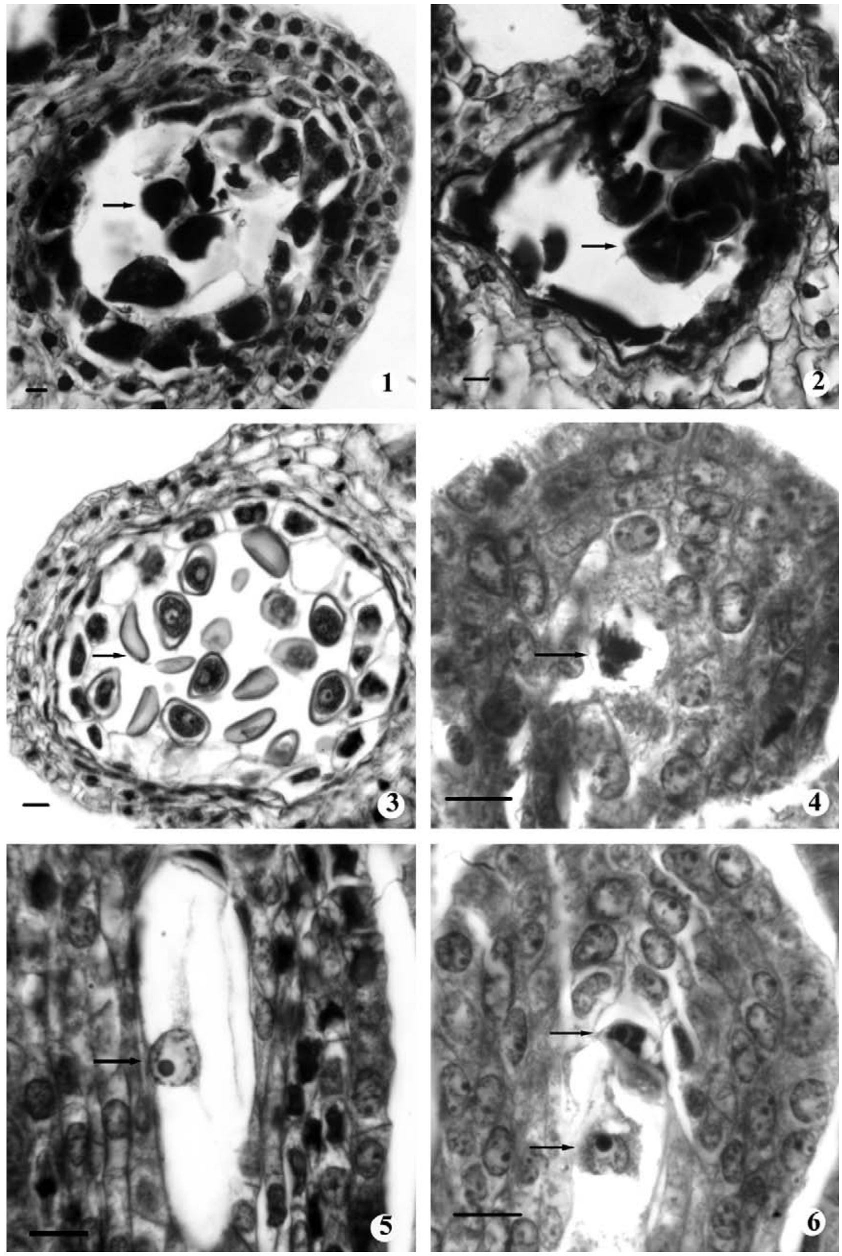
Flowers, fruits and seed production

Flowers of *M. coriacea* were hermaphroditic and protogynous, with 6–7 ivory-white or yellowish white tepals, with 81.6 ± 8 stamens, and 20.8 ± 5.1 carpels per flower. Each carpel bore 6.9 ± 1.1 ovules. A single flower persisted for approx. six days. Upon opening, the flowers were functionally female. At the very beginning of anthesis tepals were initially closed and started to partly open about 2–3 days after anthesis. At this time stigmas became brown, and then the anthers began to dehisce. By days 4–5 tepals began to unfold, and the following day they had completely unfolded. Gradually, the anthers were abscising, rendering the flower inviable and withered.

Pollination treatments had a highly significant effect on fruit set ($F_{4,25}=35.139$, $P<0.0001$) and seed set per fruit ($F_{4,25}=85.022$, $P<0.0001$). Only two aggregate fruits from a total of 60 flowers were formed in the autogamic pollination test and no fruit from the agamospermic test was observed. The average fruit set from hand self-pollination, hand cross-pollination, and open pollination were 23.3%, 40%, and 6.7%, respectively. The seed set per fruit from those pollination treatments were 0.6%, 1.6%, and 0.2%, respectively (Fig. 7). Fruit set and seed set per fruit from both hand pollination treatments were significantly larger than those from open pollination ($P<0.01$: Fig. 7). Fruit set and seed set per fruit from hand self-pollination and hand cross-pollination showed a significant difference ($P<0.05$: Fig. 7).

Floral visitors

We observed approx. 432 visitors during three sunny days and 64 visitors on one overcast day in the sampled 20 flowers at SSWFB (Table 3). These visitors belonged to three orders of insects: Coleoptera, Diptera, and Hymenoptera. Visitors were less frequent on overcast days; the mean frequency of floral visitors was 1.8 per hour on sunny days and 0.8 per hour on overcast days. The highest frequency of floral visitors was $3.63\,h^{-1}$ from 13:00 to 14:00 on sunny days and $1.9\,h^{-1}$ from 14:00 to 15:00 on overcast days (Fig. 8). Less than 90 individual insects (36 Chrysomelid beetles, 30 Andrenid flies and 21 *Bombus*/Apidae) appeared to be effective pollinators. Their mean frequency of visitation was only $0.27\,h^{-1}$ (Table 3). The beetles were observed to forage for pollen and to crawl on stigmas and stamens in



Figs. 1–6. Abnormalities in pollen and ovule development in *M. coriacea*. Black arrows point to abnormalities. (1) Abnormal development of microspore mother cell; (2) Microspore agglutinated and forming tetrads that are bone-shape; (3) Aborted microspores; (4) Delayed development of megaspore mother cells; (5) Uni-nucleate embryo sac with delayed development; (6) Bi-nucleate embryo sac with delayed development. Micropylar end is on the upper side. Scale bars = 10 μm.

Table 1. Abnormal development of stamina of *Michelia coriacea*.

Stage of stamen development	Percent of abnormalities of stamen development (each stage among 500 sections) (%)
Microsporocyte	40.14 ± 7.2
Microsporocyte meiosis	57.61 ± 9.7
Microspore release	60.22 ± 6.6

Table 2. Delayed pistil development of *Michelia coriacea*.

Stage of pistil development	Percent of delayed development of pistil (among 501 ovules) (%)
Megaspore mother cell	24.89 ± 9.4
Uni-nucleate embryo sac	30.70 ± 9.8
Bi-nucleate embryo sac	14.46 ± 9.9

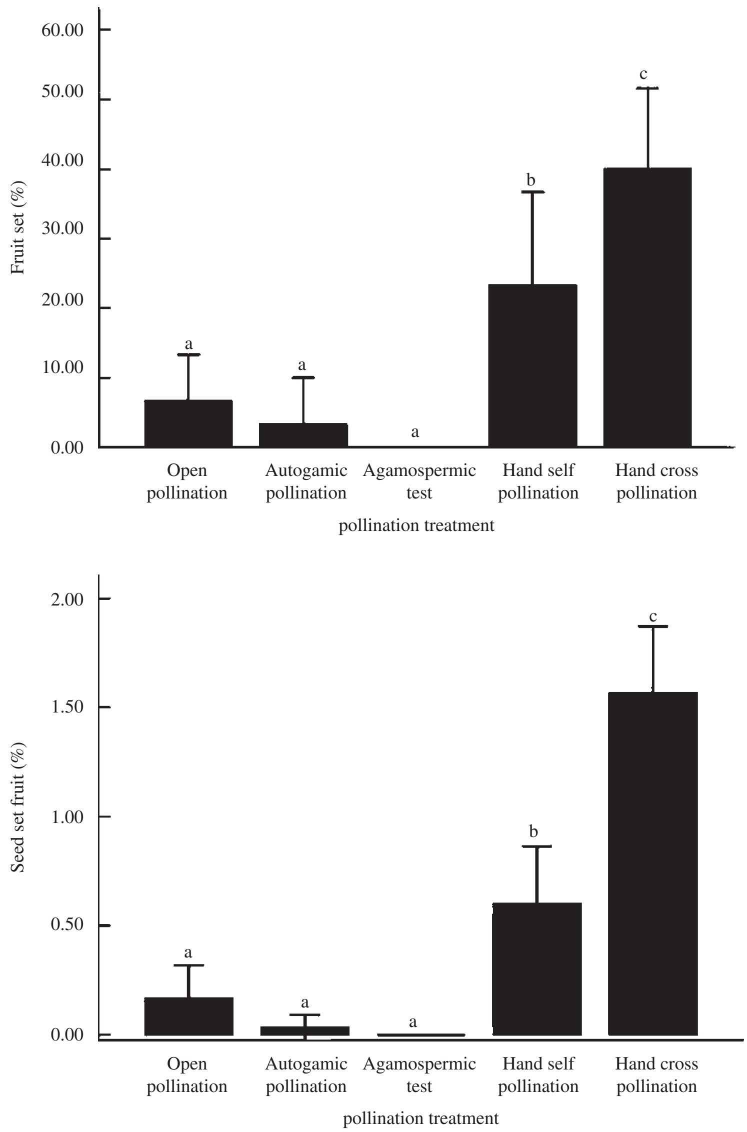


Fig. 7. Mean values of fruit set and seed set per fruit for six sampled trees with respect to the five types of pollination protocols. Vertical bars show the standard deviation. Different letters above the columns indicate significant differences between the types of pollination for each variable ($P < 0.05$).

different flowers. *Bombus* and the species of Andrenidae were also seen to transfer pollen from one flower to another. The most frequent floral visitor, a fly, *Fannia sp.*, which appeared to sun-bask on the corollas, might not be effective pollinator.

In the wild population where 20 flowers of *M. coriacea* were monitored, the following observations were made: 140 visitors during three sunny days and 23 visitors during one overcast day (Table 4). Most of visitors were the same as at SSWFB, except that some species of Sphecidae were found and *Fannia sp.* did not appear. The effective pollinators were the same as at SSWFB but appearing at much lower frequency, namely 11 Chrysomelid beetles, 13 members of Andrenidae, and 7 bumblebees. The mean frequency of the floral visitors was less than $0.1\,h^{-1}$ (Table 4). More than 53% of all the floral visitors were a species of Sphecidae. As these wasps flew around the tree, and then shortly visited the flowers, it is not clear if they are effective pollinators. The mean frequency of floral visitors was 0.58 and $0.28\,h^{-1}$ on sunny days vs. overcast days. The greatest frequency of floral visitors was $1.3\,h^{-1}$ from 13:00 to 14:00 on sunny days and $0.7\,h^{-1}$ from 14:00 to 15:00 on overcast days (Fig. 8).

Discussion

Abnormal gametic development appears to be widespread in endangered species of Asiatic Magnoliaceae. Abortion of pollen and ovule is one of the important

Table 3. List of floral visitors to *Michelia coriacea* observed at SSWFB.

Floral visitor	*N*	Percentage (%)	Mean floral visitor frequency (times h^{-1})
Coleoptera:			
Chrysomelideae[a]	36	7.26	0.11
Meloidae	85	17.14	0.27
Diptera:			
Fanniidae (*Fannia sp.*)	144	29.03	0.45
Hymenoptera:			
Apidae[a]	21	4.23	0.07
Andrenidae[a]	30	6.05	0.09
Trigonalidae	72	14.52	0.23
Vespidae	108	21.77	0.34
Total	496		

[a]Effective pollinators to *M. coriacea*.

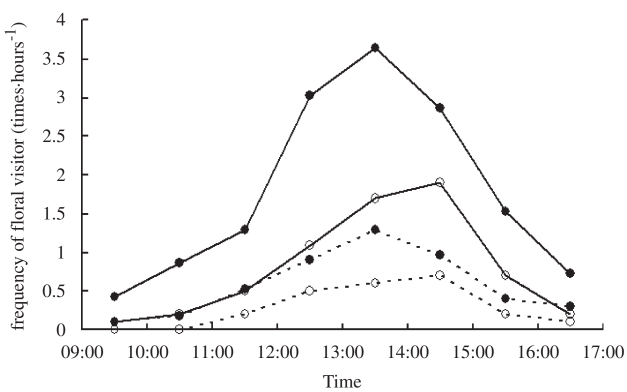


Fig. 8. Mean frequency of floral visitors on a sunny day (closed circle) and an overcast day (open circle) from 9:00 to 17:00 in SSWFB (solid line) and the Xichou Population (broken line).

Table 4. List of floral visitors to *Michelia coriacea* observed in the Xichou Population.

Floral visitor	*N*	Percentage (%)	Mean frequency of floral visitor (times h^{-1})
Coleoptera			
Chrysomelideae[a]	11	6.75	0.03
Meloidae	21	12.88	0.07
Hymenoptera			
Apidae[a]	7	4.29	0.02
Andrenidae[a]	13	7.98	0.04
Sphecidae	87	53.37	0.27
Trigonalidae	11	6.75	0.03
Vespidae	13	7.98	0.04
Total	163		

[a]Effective pollinators to *M. coriacea*.

factors restricting reproductive success of *Liriodendron chinense* (Qin and Li, 1996; Yin and Fan, 1997). Liao et al. (2000) stated that abnormal development was very common in the development of embryo and endosperm, leading to abortion of seeds of *Manglietia glauca*. Pan and Gong (2002) found that about 79.1% ovules of *Manglietia insignis* were degenerated, resulting in low rate of seed formation, and Tang et al. (2003) reported that abnormal phenomena in the process of reproduction of *Tsoongiodendron odorum* caused this species to be endangered. Wang et al. (2005a) concluded that development of ovule and pollen were partially abnormal in *Magnolia biloba*. Similar key factors in restricting seed production of *M. coriacea* were the fact that we found 60% abnormal pollen development and approx. 70% delayed development in ovules. We speculate that such a high percentage of abortion and degeneration of pollen and ovules may be the main cause leading to the extremely poor seed production of the species. Although the vegetation where *M. coriacea* occurs has been badly destroyed and its habitat fragmented, our current genetic analysis on the species indicates that *M. coriacea* maintains an unexpectedly high genetic diversity (PPB = 93.00%, $H = 0.264$, $I = 0.410$) and low genetic differentiation (Gst = 0.1197), which might indicate that there was a major destruction in the past few decades (unpublished data). It can also be inferred that the vegetation destruction and habitat fragmentation might be one of the factors accelerating abortion of pollen and ovules of the species, as it has been reported also from *Leymus chinensis* (Poaceae) (Teng et al., 2006).

It is well known that plants rely on vectors transferring pollen to their stigmas for fertilization. It could be that in fragmented populations plant–pollinator interactions may become disrupted and reproduction may be reduced because of insufficient pollination (Ægisdóttir et al., 2007; Chacoff et al., 2008; Colling et al., 2004; Kearns et al., 1998; Moody-Weis and Heywood, 2001). Plants in small populations are often less attractive to pollinators and may be visited less frequently (Rathcke and Jules, 1993). It is certain that both adequate fertile pollen and pollinators are essential to produce seeds. Our observations indicated that very few seeds were produced when *M. coriacea* flowers were caged, demonstrating that selfing could take place without insect vectors, but did so very infrequently (similar findings: Chacoff et al., 2008). Hand pollination could significantly improve seed set over that from open-pollinated flowers ($P<0.01$). SSWFB is located in the same climatic region as the natural habitat of *M. coriacea*, but in the latter location, individuals of the species are widely scattered across the greatly disturbed habitat. The natural population of *M. coriacea* experiences a distinctly lower frequency of floral visitors ($0.1\,h^{-1}$) than the cultivated plants at SSWFB ($0.27\,h^{-1}$), and these figures in general are far lower than those found for other species of the same family, such as *Magnolia sieboldii* (Wang et al., 2005b) and *Liriodendron chinense* (Huang et al., 1999). The low frequency of effective pollinators visiting *M. coriacea* might be related directly or indirectly with its scarcity and with habitat destruction.

Inbreeding depression is of major concern in the management and conservation of endangered species (Hedrick and Kalinowski, 2000). Inbreeding can strongly reduce both individual and population viability (Colling et al., 2004; Keller and Waller, 2002), and negative effects of small population size on seed production have been found in many species (Ke′ry et al., 2001; Morgan, 1999). In small populations, the local density of plants is often reduced and few insects will effect cross-pollination (Kunin, 1997; Roll et al., 1997). The total number of *M. coriacea* known is less than 500, and these plants are individually distributed in Xichou and Malipo Counties, which extend over 4190 km^2 (Cicuzza et al., 2007). The fruit and seed set of *M. coriacea* from hand-effected self-pollination were significantly lower than those from hand cross-pollination ($P<0.05$). It appears therefore likely that *M. coriacea* is self-compatible in principle, but exhibits inbreeding depression in its natural habitat.

In conclusion, the breeding system of *M. coriacea* is intermediate between autogamy and xenogamy. The fact that hand cross-pollinated flowers yielded significantly more fruits and seeds than open-pollinated flowers indicates that under natural conditions low levels of pollination and fertilization occur in this plant. Its rarity results most probably from a combination of factors: high levels of abnormal development of pollen and ovules, relatively few potential pollinators, especially in natural scattered populations with few plants. In addition, destruction of habitat has isolated populations, leading probably to more inbreeding. Based on this, further genetic studies of *M. coriacea* should render a better understanding of the amount of variability that is still remaining in this taxon.

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