

珍稀濒危物种格木传粉方式和交配系统的初步研究

朱鹏^{1,2,3}, 王峥峰^{1,2*}, 叶万辉^{1,2}, 曹洪麟^{1,2}, Saravanan THAVAMANIKUMAR⁴

(1. 中国科学院华南植物园, 中国科学院退化生态系统植被恢复与管理重点实验室, 广州 510650; 2. 中国科学院华南植物园, 数字化植物园广东重点实验室, 广州 510650; 3. 中国科学院大学, 北京 100049; 4. *Department of Forest and Ecosystem Science, University of Melbourne, Creswick, VIC 3363, Australia*)

摘要: 为研究格木(*Erythrophleum fordii* Oliv.)的传粉方式和交配系统,在鼎湖山国家级自然保护区对珍稀濒危树种格木进行野外观察,花的微器官(花粉和柱头)用扫描电镜观察,并进行控制授粉实验和遗传分析。结果表明,格木具有颜色亮丽和散发香味的总状花序,格木花粉包被厚重的粘性物质,柱头没有易于捕获花粉的结构,风媒传粉没有坐果,推断格木应该是通过虫媒传播花粉,或者主要是通过虫媒传播。遗传分析和在不同繁殖环境的两棵目标树的坐果情况表明,格木偶尔可以自交产生后代,但异交占据着绝对的优势,鼎湖山格木种群的异交率达到 90.6%。格木传粉方式和交配系统的研究有利于对格木进行保护和保育。

关键词: 格木; 传粉方式; 交配系统; 遗传分析

doi: 10.3969/j.issn.1005-3395.2013.01.005

Preliminary Studies on Pollination and Mating System of Rare and Endangered Plant *Erythrophleum fordii* Oliv.

ZHU Peng^{1,2,3}, WANG Zheng-feng^{1,2*}, YE Wan-hui^{1,2}, CAO Hong-lin^{1,2}, Saravanan THAVAMANIKUMAR⁴

(1. *Key Laboratory of Vegetation Restoration and Management of Degraded Ecosystems, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China*; 2. *Guangdong Key Laboratory of Digital Botanical Garden, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China*; 3. *University of Chinese Academy of Sciences, Beijing 100049, China*; 4. *Department of Forest and Ecosystem Science, University of Melbourne, Creswick, VIC 3363, Australia*)

Abstract: Although a few efforts have been taken to conserve rare and endangered species *Erythrophleum fordii* Olive., information about its pollination and mating system still remains unknown. The pollination and mating system of *E. fordii* through performing the field observation, SEM observation of pollen and stigma, controlled pollination experiment and genetic analyses in Dinghushan Nature Reserve, China were studied. *Erythrophleum fordii* has brightly colorful inflorescences and sends forth delicate odor, its pollen is coated by strong mucous substance and its stigmas have not specific structure for pollen capturing, and meanwhile the inflorescences pollinated by wind did not fruit at all, therefore it was inferred that *E. fordii* might be entomophilous. According to genetic analyses and fruiting of two object trees under contrasting reproductive situations, *E. fordii* can be selfing occasionally but out-crossing is dominant with out-crossing rate in the Dinghushan *E. fordii* community for 90.6%. This preliminary elucidation of pollination and mating system would benefit the conservation of *E. fordii*.

Key words: *Erythrophleum fordii*; Pollination; Mating system; Genetic analysis

Received: 2012-04-11

Accepted: 2012-04-26

Supported by National Basic Research Program of China (973 Program) (2007CB411600), the Knowledge Innovation Project of The National Key Technology Rand D Program (2008BAC39B02), the Chinese Forest Biodiversity Monitoring Network and the Foreign Exchange Program National Founder (31011120470)

Author: ZHU Peng, PhD student, major in molecular ecology. E-mail: rocerzhu2008@126.com

* Corresponding author. E-mail: wzf@scib.ac.cn

Erythrophleum fordii Oliv. is a precious hardwood tree species belonging to the informal group *Dimorphandra* of the tribe Caesalpinieae (Fabaceae: Caesalpinioideae) and naturally occurring in tropical and subtropical areas of South China and Vietnam^[1-2]. In addition to *E. fordii*, 15 other congener species can also be found in tropical areas of Africa, tropical and subtropical areas of Eastern Asia and North Australia^[2]. The *E. fordii* trees produce quite hard, heavy, durable and erosion-resistant wood, popularly called as ‘iron wood’, which is generally used for furniture, bridge, shipbuilding, etc.^[3]. In addition, *E. fordii* is also a species with medicinal properties^[4] used by native Chinese as invigoration and blood circulation promoting agents^[5]. Because of its superior wood and medicinal uses, it has been historically over-exploited leading to the serious population reduction^[6]. To protect it from further population reduction, it has been listed in both China and Vietnam plant red data book^[7-8] and also placed into the ‘endangered’ category by IUCN^[1].

Erythrophleum fordii normally occurs as a canopy tree species in the south subtropical evergreen broad-leaved monsoon forest, thus it plays an important role in maintaining some forest communities in the south subtropical areas. Few studies have been conducted on the conservation biology of *E. fordii*, such as endangered mechanism^[6], seed germination^[9], *in situ* and *ex situ* conservation^[10], population dynamics^[11-12], phenotypic variation of fruit and seed related to geographic distribution^[13], and so on. However, we still know little about the pollination and mating system of *E. fordii*, which are limited factors for endangered species’ conservation. Therefore, we expected to learn about the pollination and mating system of *E. fordii* to promote our conservation for this endangered precious species and in turn for its communities.

1 Material and Methods

1.1 Species and study site

Erythrophleum fordii has small single flowers

with ten stamens and one pistil, which form racemes and in turn panicle^[14]. Single small flower does not have colorful corolla but only the calyx-like green corolla^[14]. The flowering of *E. fordii* is prosperous yearly after maturing but fruiting is different among years^[6].

Our study site is located in the first national nature reserve of China, Dinghushan Nature Reserve (23°10' N / 112°34' E). This reserve is designated into the MAB (Man and the Biosphere) programme in 1979. The main vegetation is lower subtropical evergreen broadleaf forest^[11]. In contrast to the disturbed surrounding forests, the Dinghushan Nature Reserve comprises rare primary forests of at least 400 years of age (www.unesco.org). The annual average rainfall is about 1956 mm with distinct dry and wet season, of which the dry season occurs between September and February with an annual average temperature of 20.9°C^[15].

In the core area of the reserve, there is a small *E. fordii* dominated community (Circa 2 hm²), the unique *E. fordii* community. Additionally, one mature single *E. fordii* individual was found near the office area (referred to MST later) in the reserve with strong human disturbance, 1.5 km away from the *E. fordii* community. Apart from the mature *E. fordii* individuals in the community and the MST, no other mature *E. fordii* individuals could be found in the reserve. Comparing mature *E. fordii* individuals in the community which could exchange their pollens easily, MST has a quite different reproductive environment with receiving pollens from other *E. fordii* individuals hardly because of its isolated status.

1.2 Flower visitor observation

Since the tree height of a mature *E. fordii* is normally between 20 m and 30 m, we set up bamboo shelves with a platform on the top of shelves surrounding the trunk of trees to facilitate flower visitor observation, flower specimen collection and controlled pollination experiment later. One mature tree in the community (referred to PT later) and the MST were selected as the object trees. During

fluorescence (May) flower visitors were observed on the top of the object trees in the morning (9:00 – 11:30 am) and afternoon (2:00 – 5:30 pm) in 10 consecutive days. We could not capture visitor specimens to conduct further research in the laboratory because the insect visitors observed normally were far from our platform.

1.3 Stigma and pollen observation

Fresh single flowers and anther specimens were collected and fixed in 4% glutaraldehyde in 0.1 mol L⁻¹ phosphate buffer at pH 7.4 and then brought back for stigma, anther and pollen examination using light microscopy (LM) and scanning electron microscopy (SEM) in the laboratory. When doing the observation with SEM, the fixed samples were rinsed three times in the same buffer and fixed again with 1% osmium tetroxide. Samples were then mounted on metal stubs using double-sided adhesive tape and sputter-coated with platinum using JFC-1600 auto fine coater (JEOL Ltd., Tokyo, Japan). The stigmas were observed with a ZEISS-510Meta LSM (German) and a JSM-6360LV SEM (JEOL Ltd., Tokyo, Japan), anthers with a LM and a JSM-6360LV SEM and pollen grains with a JSM-6360LV SEM.

1.4 Controlled pollination experiment

Controlled pollination experiment was designed with five treatments for each object tree: (1) Net bagging (NB): four panicles without any single flower blooming were bagged using four nylon net bags (1.5 mm × 1.5 mm) to protect from animal pollination but allow wind pollination and tagged; (2) Paper bagging (PB): four panicles without any single flower blooming were bagged using four closed paper bags to test self-pollination and tagged; (3) Human-assisted pollination with pollens from the same tree (HPS); (4) Human-assisted pollination with pollens from the other object tree (HPO); (5) We also tagged four panicles without any treatment as control. However, during doing HPS and HPO, we found that the followers were strongly impaired after androecium wipe-off because small single flowers (approximately width × height = 2 mm × 10 mm and length of stamen =

4 mm) are too squashing together at the raceme, so we doubted the impairment actually would affect the ability of fruiting, thus finally we did not complete HPS and HPO.

1.5 Examination of mating system using genetic analyses

The exhaustive survey for *E. fordii* individuals in the *E. fordii* community by setting up 10 m × 10 m small survey boxes over the whole community. Overall, 528 *E. fordii* individuals were found in the whole community, but only 498 individuals were sampled for further genetic analyses. We did not sample those small individuals of $H < 0.3$ m and less than 10 leaves to avoid anthropogenic mortality leading to disturbing population's natural regeneration. Tree height (H) and diameter at breast height (DBH) were measured for individuals of $H \geq 2$ m and only H was measured for individuals of $H < 2$ m. According to Huang^[6] and our field observation, based on DBH and/or H all sampled individuals were classified into four cohorts: (i) adult (DBH ≥ 15 cm, $N = 78$), (ii) juvenile (DBH < 15 cm and $H \geq 2$ m, $N = 29$), (iii) sapling ($2 \text{ m} > H \geq 0.5$ m, $N = 124$) and (iv) seedling ($H < 0.5$ m, $N = 267$). And under MST we found three small individuals (0.3 – 0.5 m at tree height, Table 1), which plus MST were sampled for further genetic analyses either.

Total DNA was extracted from tissue preserved in dry condition with silica gel using the cetyltrimethyl ammonium bromide (CTAB) method^[16]. Nine microsatellites isolated previously^[17] were used to genotype all the samples. PCR amplified products were separated by 6% denaturing polyacrylamide gel electrophoresis (PAGE) and visualized with silver staining. Two genetic analyses were carried out to study the mating system including genotype comparison and mating rate calculation based on parentage analysis.

Genotype comparison Due to MST is an isolated adult in office area, the small individuals found under it were unlikely to be grown up from seeds delivered from other *E. fordii* trees given its

seed properties of gravity dispersal and not attracting birds, but rather produced by MST. Therefore we did direct genotype comparison between MST and the three small individuals under it to check whether the small individuals had come from the out-crossing of MST with pollens from other trees or from selfing of MST. If small individuals are from selfing, we would not expect to find alleles in them that are not identical to those of MST and we could also expect to find the inclination of the heterozygous loci in MST becoming homozygous loci in small individuals.

Parentage analysis Parentage analysis can statistically assign individuals with their most likely parents and it has been used as an effective method to investigate the mating system. We performed the parentage analysis by using Cervus^[18–20] with using all adults as candidate parents ($n = 78$) for saplings ($n = 124$) and seedlings ($n = 267$). The type of parentage analysis used here was the model of parent pair (sex unknown) considering self fertilization. Once individuals were assigned with their most likely parents, we then calculated both mating rates of out-crossing and selfing of *E. fordii* in the *E. fordii* community. In the assignments, only the individuals assigned with one pair of parents with the mother and the father being the same tree were viewed as selfing, all other cases including one parent, one pair of parents with the mother and the father being different trees and multiple pairs of parents were viewed as out-crossing.

2 Results

2.1 Flower visitors

The beetles (Cerambycidae), butterflies

(Papilionidae), bees and wasps were observed to be the main visitors of *E. fordii* (Fig. 1). We did not find any birds visit the inflorescences.

2.2 Stigma and pollen observation

Stigma has an obvious ornament-absent stigmatic cavity (Fig. 2: A). Pollen grains released as isopolar, tricolporate monads with a size of a mean P (polar length) $\times$ mean E (equatorial width) = $19.4 \mu\text{m} \times 36.9 \mu\text{m}$ and the surface of pollen grains were psilate-finely perforate or perforate (Fig. 2: D,E). Strong sticky coats were observed on the surface of pollen grains making them tightly stick to each other (Fig. 2: C – E).

2.3 Controlled pollination

In the controlled pollination experiment, the treatments of both PB and NB of two object trees did not bear any fruits. For the Control, MST did not bear fruits either while PT did. For the whole fruiting of two object trees, MST was found only having sparse fruits while PT having a mass of fruits.

2.4 Examination of mating system using genetic analyses

Genotype comparison between the three small individuals under MST and the MST (Table 1) showed that four loci (Gm 1048, Gm 2024, Gm 4040 and Gm 4058) which were heterozygous in MST had two, three and four loci becoming homozygous in O-1, O-2 and O-3 respectively with all alleles identical to the alleles in MST. All the other loci which were homozygous in MST were still homozygous in all the three small individuals with identical alleles between tree small individuals and MST.

Table 1 Genotype comparison between mature single tree found in office area (MST) and three small individuals under MST

Individual name	DBH or Height	Microsatellite locus								
		Gm 1048	Gm 1052	Gm 2024	Gm 2062	Gm 2065	Gm 4040	Gm 4058	Gm 5b3	Gm 65
MST	42.8 cm	2/3	2/2	2/4	1/1	5/5	6/9	3/5	4/4	2/2
O-1	0.33 m	2/2	2/2	4/4	1/1	5/5	6/9	3/5	4/4	2/2
O-2	0.42 m	2/2	2/2	2/2	1/1	5/5	9/9	3/5	4/4	2/2
O-3	0.36 m	2/2	2/2	2/2	1/1	5/5	6/6	3/3	4/4	2/2

MST: Mature single *E. fordii* isolated in office area; O-1, O-2, O-3: No. 1, No. 2, and No. 3 offspring under MST, respectively.

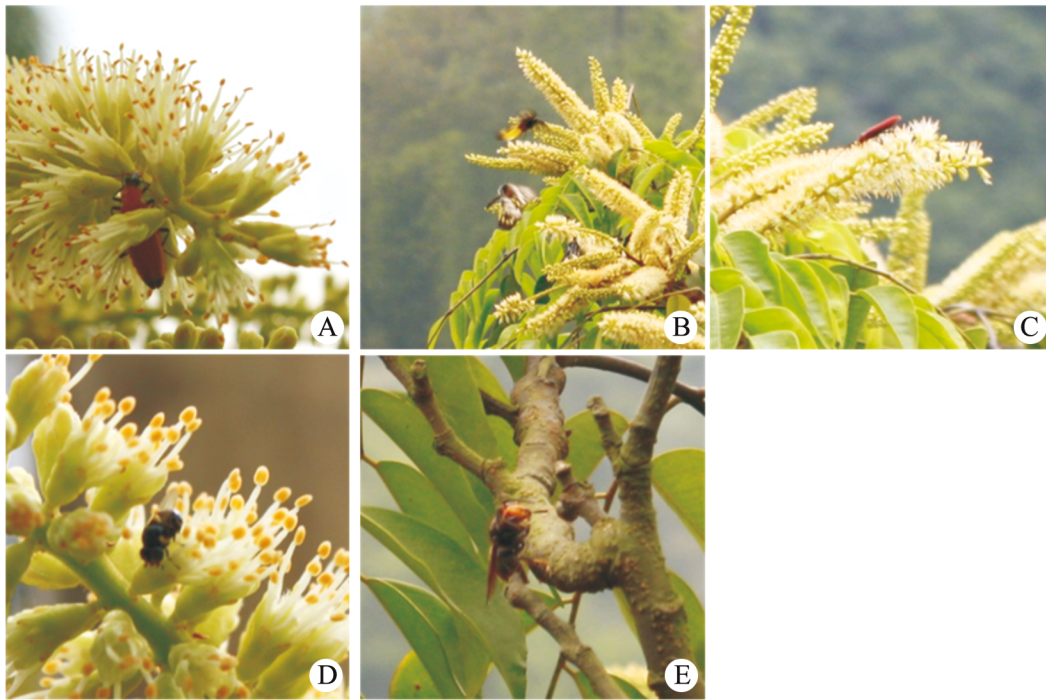


Fig. 1 Flower visitors during florescence. Beetle (A,C), butterflies (B), bee (D) and wasp (E).

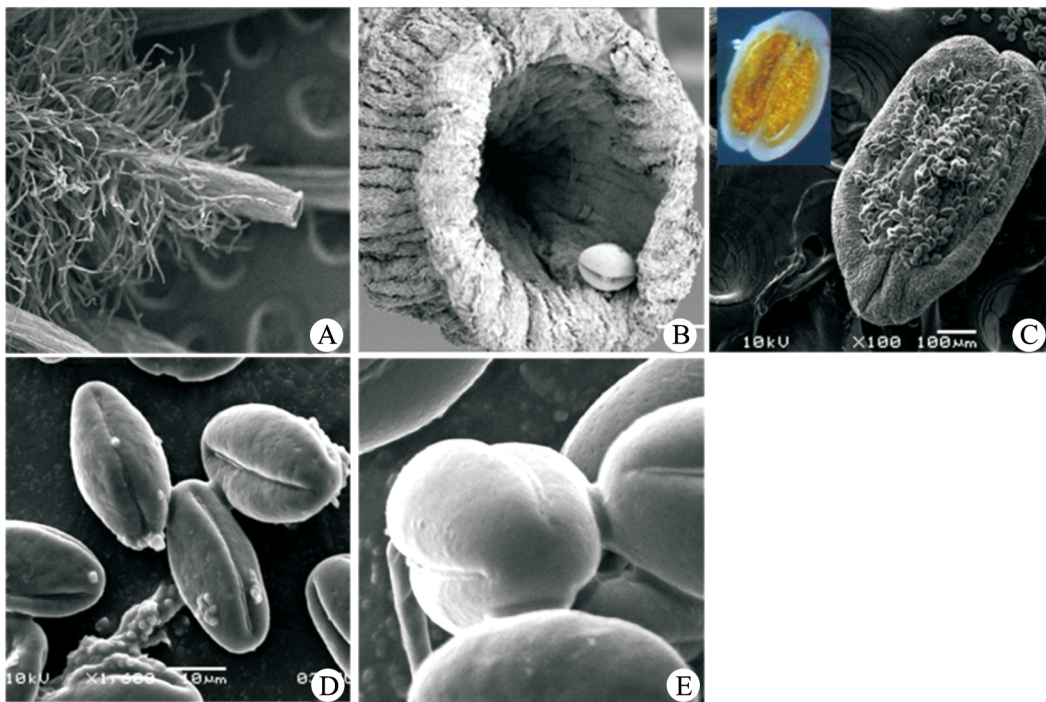


Fig. 2 Stigma and pollens under SEM. A. Style and stigma; B. Close-up stigma of *Erythrophleum africanum* showing one pollen grain within stigmatic cavity^[24]; C. Dehiscent anther (insert in C for the dehiscent anther examined under LM); D – E. Pollens.

In parentage assignment (Table 2), 385 individuals out of 391 seedlings and saplings were assigned with parents from adults in the *E. fordii* community, of which 36 individuals were assigned with one pair of parents having mother and father being the same tree, i.e. selfing rate of 0.094, and 349 individuals were

assigned with parents having mother and father being different trees, i.e. out-crossing rate of 0.906.

3 Discussion

For non-hydrophytic plants, anemophily and

Table 2 Results of parentage assignment and from which the rates of selfing and out-crossing calculated

Types of parent assignment for offspring	Number	Rate
SP (Selfing)	36	0.094 [†]
DP (Out-crossing)	349	0.906 [†]
One parent	21	---
One pair of parents	302	---
Multiple pairs of parents	26	---
No assigned parents	6	---
Total of offspring	391 [#]	---

SP: Offspring having mother and father being the same tree; DP: Offspring having mother and father being different trees; †: Calculated from excluding the offspring with no assigned parents; #: Sum of saplings ($n = 124$) and seedlings ($n = 267$).

entomophily are two main pollination types of the cross-pollination. Anemophilous plants are usually characterized by the reduction of the normal means of attracting and rewarding animal visitors like dull-colored corolla and faint scent, while entomophilous plants often develop the inverse means^[21–22]. For *E. fordii*, the inflorescences with mass of single small flowers having orange anthers tightly distributing on them produce a very bright color and the flowers also send forth a delicate odor, which would attract animal visiting. Meanwhile, in our field survey, beetles, butterflies, bees and wasps were observed visiting on the flowers during florescence. In addition, the pollen grains of *E. fordii* were found to be clothed by strong mucous substance, which would increase the dispersing difficulty for pollen by wind, although they were found to belong to pollens of relative small size according to Harder's^[23] review and have psilate-finely perforate or perforate ornamentation surface. The stigma of *E. fordii* does not have any specific structures promoting pollen capturing while only has an ornament-absent stigmatic cavity, similar to some other *Erythrophleum* species^[24], which would make the stigma have a low ability to capture the pollens delivered only by wind. Furthermore, in our controlled pollination experiment, net bagging of both object trees that was designed to test wind pollination did not bear fruits at all. Accordingly, *E. fordii* might deliver its pollen by insects rather than by wind, i.e. entomophilous plant. However, more evidences from direct observation of pollination behaviors of insects are needed for further confirming the inference here.

Petit & Hampe^[25] summarized that many trees can self but not one is prominently selfing^[26]. According to the direct genotype comparison between the MST and the three small individuals found under it which were likely produced by MST, we found that the four loci which were heterozygous in MST had two, three and four loci becoming homozygous in O-1, O-2 and O-3, respectively, with all alleles identical to the alleles in MST, and meanwhile all the other loci which were homozygous in MST were still homozygous in three small individuals. Give the isolated status of the MST, the long distance between MST and *E. fordii* community which might be the only heterogenous pollen source for MST and the selfing inclination exhibited from genotype comparison, the three small individuals might have been produced by the selfing of MST, especially the O-3 which had all loci being homozygous. Further, through parentage analysis between seedlings/saplings and adults in the *E. fordii* community, 36 seedlings/saplings were assigned with one pair of parents having mother and father being the same tree, which also gave the evidence that *E. fordii* can be selfing. However, the selfing rate was quite lower than out-crossing rate (90.6%), showing that *E. fordii* is dominantly out-crossing. Meanwhile, in our field survey, we found that the inflorescences without controlled pollination treatment of two object tree (PT and MST) had quite different amounts of fruits with PT having a mass of fruits but MST only having exiguous fruits. PT was growing in the *E. fordii* community while MST is isolated in the office

area, so PT could easily obtain heterogenous pollens from other trees accompanying with it but MST inversely. The contrasting reproductive situations and the contrasting productions of PT and MST also suggested that *E. fordii* should be a dominantly out-crossing species and might have some mechanism to constrain selfing although *E. fordii* can be selfing.

Acknowledgements We thank Prof. HUANG Zhong-liang for his help in setting up the bamboo shelves and WU Lin-fang for his help to collect the samples in the field. We also thank HU Xiao-ying for her help on the microscopy and XU Gou-liang and Prof. WU Hong-ji for their helps to identify insects.

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