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台灣產玉葉金花生殖生物學與族群遺傳之研究

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台灣產玉葉金花生殖生物學與族群遺傳之研究

Reproductive biology and population genetics of *Mussaenda pubescens* (Rubiaceae) in Taiwan

計畫編號：NSC93-2621- B-002-024

執行期限：93 年 08 月 01 日至 94 年 07 月 31 日

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一、中文摘要

本研究在研究一個台灣獨特的例子-玉葉金花（*Mussaenda pubescens*），其在台灣北部和東北部的族群中，缺乏明顯的萼片來吸引傳粉者，但是結果率仍然很高。我們希望瞭解它是如何克服這個先天的劣勢。玉葉金花是台灣低海拔中常見的植物之一，它是一個雌雄異株的植物。玉葉金花常可以見到花萼其中的一個裂片尖端膨大成一個外形類似葉子、但顏色為白色或紅色的構造，稱為葉狀萼片（calycophylls, 或 floral semaphylls）。而玉葉金花名稱的由來，即源於它的花序中黃色的花瓣和白色的葉狀萼片而來。這些葉狀萼片不管在細胞形狀或細胞排列上，都與正常的花瓣類似，加上有著顯著的顏色，一般皆認為這些葉狀萼片在功能上與花瓣無異，而與吸引有效傳粉者有關。在前人與我們的觀察中發現，在台灣北部至新竹，及東北部以至南澳的玉葉金花族群中，都沒有膨大的花萼構造。我們選擇了台北縣樹梅坪，苗栗三義，和屏東南仁山的三個族群進行研究。其中只有樹梅坪的族群是沒有膨大的花萼構造。我們針對這些族群進行傳粉者，蜜腺分泌，花粉萌發率，結果率的觀察，以及利用 SEM 進行花器細部觀察。另外我們也同時利用分子工具進行譜系分析。我們所選用的是核內 ITS 的序列。藉由對玉葉金花的生殖生物學的研究，我們想探討東北部的族群中，是否有新的傳粉者，或者有其他的因素能幫它們克服失去膨大花萼的劣勢。本計畫原本為期兩年，但因經費僅支持一年，故本報告實為不完整的先期報告。

關鍵詞：玉葉金花，生殖生物學，分類

Abstract

Floral display is critical to attract pollinators for animal-pollinated plants. Loss of visual signal of flowers may well decrease the reproductive success, and eventually lead to extinction of such plant. We would like to investigate a unique case in Taiwan's *Mussaenda pubescens*, in which some populations may have survived the loss of showy floral signal. *Mussaenda pubescens* Ait., a member of Rubiaceae, has a distinct enlarged calyx-lobe which is generally thought to attract pollinators like butterflies. The high-contrast visual signals have been shown to be essential for high fruit sets in another *Mussaenda* species, *M. parviflora*. Based on our survey, we found many of the *M. pubescens* populations in northeastern Taiwan lack the showy semaphyll. The preliminary observation showed that these individuals are able to bear fruits, suggesting that cross-pollination still occurs and they somehow overcame the deficiency in pollinator attractants. We performed a survey for floral morphology, nectar secretion pattern, and SEM observation among the populations with and without enlarged semaphylls. We also performed a phylogenetic analysis using nrITS

sequences among three study sites of *M. pubescens*. This will help to understand the systematic identities with different semaphyll types in *M. pubescens*,. The original plan is to have a two-year experiment for long-term observation. Since this grant is funded only for one year. We have preliminary data shown here.

Keywords: *Mussaenda pubescens*, reproductive biology, systematics

二、計畫緣由與目的

Floral display is critical to attract pollinators for animal-pollinated plants. Loss of visual signal of flowers may well decrease the reproductive success, and eventually lead to extinction of such plant. We would like to investigate a unique case in Taiwan's *Mussaenda pubescens*, in which some populations may have survived the loss of showy floral signal.

Mussaenda pubescens Ait. (*sensu* Yang 1998), a member of Rubiaceae, has a distinct enlarged calyx-lobe which is generally thought to attract pollinators like butterflies (Broges et al. 2003). Showy and modified inflorescences are quite common in the Rubiaceae. They can be classified as 'extrafloral semaphyll', which referring to showy bracts and leaves, and 'floral semaphyll', which are enlarged and showy calyx-lobes (Leppik, 1977; Claβen-Blockhoff, 1996). Together they are found in nearly 40 genera in Rubiaceae. The enlarged calyx-lobes are extraordinary in that it looked like a leaf in shape and clearly petiolate, but has many petal features (Leppik, 1977; Claβen-Blockhoff, 1996), such as vivid color and having loose mesophylls. The genus *Mussaenda*, with about 100 species in Africa and Asia, is the best-known genus with such enlarged showy calyx-lobes. Although the semaphylls of *Mussaenda* are mostly leaf-shaped, they are very colorful (white or red) and show a different venation pattern and are three times thicker than the foliage leaves. Furthermore, the semaphylls contain mesophyll with large spongy cells only, and the upper epidermis is papilliate, which increases the brilliant effect of the blades (Weber, 1955; Claβen-Blockhoff, 1996). Such high-contrast visual signals are crucial in successful advertising to the pollinators (Bell 1985; Giurfa et al. 1996; Ne'eman & Kevan 2001).

There are at least two *Mussaenda* species in Taiwan (Yang, 1998), *Mussaenda macrophylla* Wall. and *M. pubescens*. The first one is only found in Lanyu. The latter species is very common in forests at low to medium elevations of Taiwan, and is the target for our study. Yang (1998) placed *Mussaenda parviflora* Matsumune and *Mussaenda taiwaniana* Kanehira as synonyms of *M. pubescens*, although variations among examined specimens suggest a more careful re-evaluation is necessary. Despite the rather confusing taxonomic status, some population level divergence has been observed by several early studies. In particular, Yamazaki (1964) and Chao (1964) described that in some individuals of *M. taihokuensis* (=*M. parviflora sensu* Chao 1970, =*M. pubescens sensu* Yang 1998), they have smaller or completely absent petaloid semaphylls, and only possess normal sepals in their flowers. However, there is no further study to show if they are still able to reproduce successfully since they lost the distinct structure to attract pollinators. Moreover, the whole genus of

Mussaenda has distylous flowers and many species, including *M. parviflora*, have been thought to be functional dioecious (Jayaweera 1963; Chao 1964; Naiki & Kato 1999). Therefore, self-pollination is impossible in this taxon.

Based on co-PI (T. Y. Yang) and our own survey, we found many of the *M. pubescens* populations in northeastern Taiwan do lack the showy semaphyll. The preliminary observation showed that these individuals are able to bear fruits, suggests that cross-pollination still occurs and they somehow overcame the deficiency in pollinator attractants. This is intriguing because in another species *Mussaenda frondosa* from India, it has been showed that removing the showy sepals will significantly reduce butterfly visits as well as fruit sets in these plants (Borges 2003). For the case in Taiwan, the loss of showy semaphylls affiliated with unreduced fruit set suggests that there might be a pollinator shift in populations of *M. pubescens* to assure the reproductive success. The new pollinator might not need the showy semaphyll as the guidance, but still can effectively transfer pollens among individuals. In order to understand the reproductive ecology of *M. pubescens*, we performed a survey for floral morphology, nectar secretion pattern, and pollen germination experiment, among the populations with and without enlarged semaphylls. We also performed a survey for genetic divergence and phylogenetic analysis using molecular markers among three study sites of *M. pubescens*. This will help to understand the degree of divergence among individuals with different semaphyll types in *M. pubescens*, and provide information for taxonomic status of this potential species complex. We used nuclear ribosomal DNA internal transcribed spacer (nrITS) sequences to examine the genetic distance among individuals.

The plant is usually found at the edge of lowland forests, and flowers from April to June. Since the project starting time will be after the flowering peak, we had proposed on a two-year span of work. Unfortunately, this grant was only funded for one year, therefore, many of the experiment was not able to conduct. We decided to perform some basic observation on floral biology and conduct molecular phylogeny for *Mussaenda pubescens.*

三、研究方法，結果與討論

(1) Sample collection and germination test

Samples collection and voucher information were shown in Table 1. Mature fruits from Shu-Mei-Pin (Taipei Co.) and Nanjenshan (Pingtung Co.), were collected and seeds were taken out for counting and for germination tests.

(2) Floral morphology

Flowers were collected from selected sites. Pollen grains were examined for the germination rate in various percentage of sucrose solution. Detailed epidermal observations of floral parts were conducted with Scanning Electronic Microscopy (SEM). Nectar volume is measured by using 2μl

microcapillary tubes (Drummond, PA, USA). Sugar concentration of the nectar was measured by a pocket refractometer (Bellingham & Stanley, Kent, England). All the procedure will be according to Naiki & Kato (1999).

(3) DNA isolation and phylogenetic analysis

Total genomic DNAs were isolated from fresh collected or frozen leaves using the 2X CTAB procedure described by Doyle and Doyle (1987), the method has been modified and routinely used in our lab to isolate plant genomic DNAs. The obtained DNAs from plants with known floral type were subjected to PCR amplification of nrITS region, and compared with the published *Mussaenda* data matrix (Alejandro et al. 2005), and subjected to phylogenetic analysis using neighbor joining criteria.

四、研究結果與討論

(1) Sample collection

All the samples collected are shown in Table 1, for their collection localities and voucher information. The vouchers are in either dried specimens or in -80°C freezer.

Table 1. Sample collection and voucher information. Taxa with asterisks are used for phylogenetic analysis.

Voucher	Date	Locality		Voucher	Date	Locality
CYC 086	7/13/2004	屏東南仁山		CYC174*	9/8/2003	蘭嶼天池
CYC 087*	7/13/2004	屏東南仁山		CYC175*	2/24/2005	台北樹梅坪
CYC 148*	3/16/2005	台北樹梅坪		CYC176*	4/21/2005	新竹打牛崎
CYC 150	4/2/2005	蘭嶼青蛇山		Hu 1461	1/14/2004	台北菁桐古道
CYC 152	4/2/2005	蘭嶼青蛇山		Hu 1508	4/29/2004	台北灣潭
CYC 154*	4/2/2005	蘭嶼天池		Hu 1520	5/12/2004	台北泰平
CYC 161	5/26/2005	台北北豹子廚山		Hu 1527	7/20/2004	苗栗勝興
CYC 162	5/26/2005	台北南豹子廚山		Hu 1528	7/20/2004	苗栗勝興
CYC 163*	6/8/2005	宜蘭雙連埤		Hu 1535	4/22/2005	新竹內灣
CYC 164	6/8/2005	宜蘭武荖坑		Hu 1536	5/4/2005	台北湖桶古道
CYC 165	6/8/2005	宜蘭武荖坑		Hu 1539	5/4/2005	宜蘭五峰奇
CYC 166*	6/21/2005	新竹尖石		Hu 1542	5/18/2005	台北泰平-三分二
CYC 167	6/21/2005	新竹尖石		Hu 1552	5/25/2005	台北柑腳山
CYC 168	6/21/2005	新竹水田林道		Hu 1565	7/12/2005	台北烏山古道
CYC 171	7/10/2005	屏東大漢林道		Hu 1575*	9/29/2005	台北大桶山
CYC 172*	10/6/2005	台中科博館		Hu 1578	10/7/2005	台中科博館
CYC 173	10/6/2005	台中科博館		Hu 1584	11/8/2005	台北西坑林道

(2) Seed collection and germination test

The seeds of *Musaenda pubescens* are black when mature. Over two hundred seeds per fruit were observed. All matured seeds collected from Pingtung (with normal enlarged sepals) germinated in 2 weeks. However, in two different seed collections from CYC 148 and Hu 1575, both of which do not have enlarged sepals, fail to germinate.

(3) Floral morphology

Pollen grains germinate 12 out of 20 (60%) in 30% sucrose solution, and all germinate (15/15) in 40% sucrose solution. The pollens did not germinate or only shown swollen in apertures in 20% or lower, as well as 50% and 60%, sucrose solution.

Nectar volume was not measured since they are too few. We have collected nectar from several flowers for unopened and opened flowers of individuals of Shu-Mei-Ping at about 6 am. The Brix reading is 34%.

The SEM of epidermal cells from petals, normal sepals, and enlarged sepals are shown in Fig. 1. The epidermal cells of petals show typical papillate morphology found in many flowers (Fig. 1A). The normal sepals shown flat epidermal cells (Fig. 1B, C), whereas the adaxial side of enlarged sepals also show papillate cell type (Fig. 1D, E), but is different from the shape seen in the petals (Fig. 1A). The abaxial side of enlarged sepals retains the flat epidermal cell type (Fig. 1F).

(4) Phylogenetic analysis

A total of ten nrITS sequences was obtained from *Mussaenda pubescens* in Taiwan, including three individuals with non-enlarged sepals and seven with enlarged sepals (Table 1). Phylogenetic analysis based on neighbor joining criteria is shown in Fig. 2. All ten nrITS we obtained shown exactly identical sequences, and to the sequence published in the name '*Mussaenda parvifolia*' (AJ848674), which was collected by Naiki from unknown locality in Taiwan (Alejandro et al. 2005). There is a sequence AJ846856 under the name '*Mussaenda pubescens*' by the same study, and was collected by Naiki in Hong Kong. There are two nucleotide differences between this and all of our sequences. At this moment we still uncertain about the identity of *Mussaenda* species in Taiwan, as the sequences are too conservative. Further analysis using trnL intron is ongoing, which hopefully can provide more information. Morphological examination is carried out by Aleck Yang, and no conclusive result is available at the moment.

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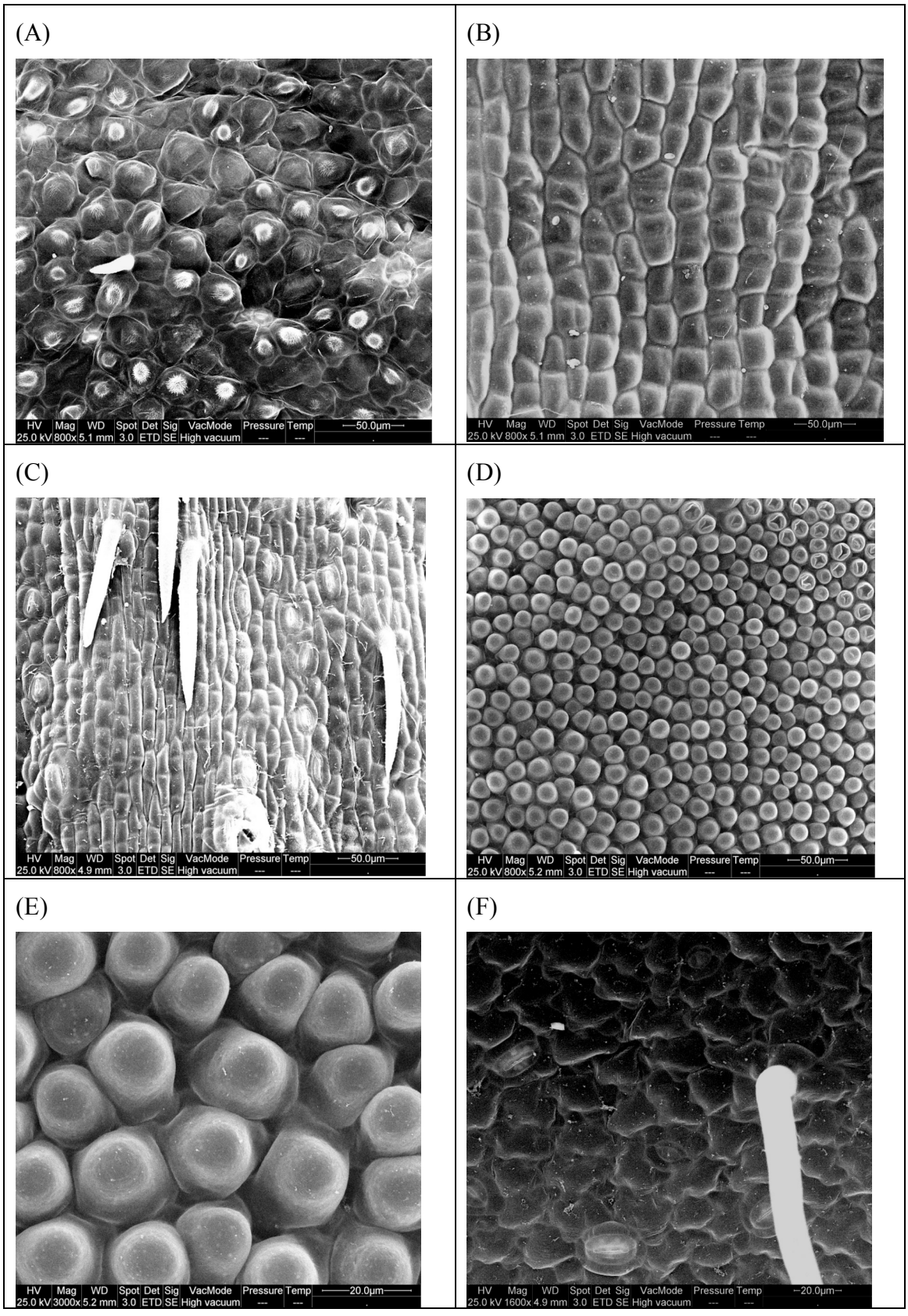

Fig. 1. SEM of epidermal cells. (A) Adaxial side of mature petal. (B) Adaxial side of normal sepal. (C) Abaxial side of normal sepal. (D) Adaxial side of enlarged sepal. (E) A higher resolution of (D). (F) Abaxial side of enlarged sepal.

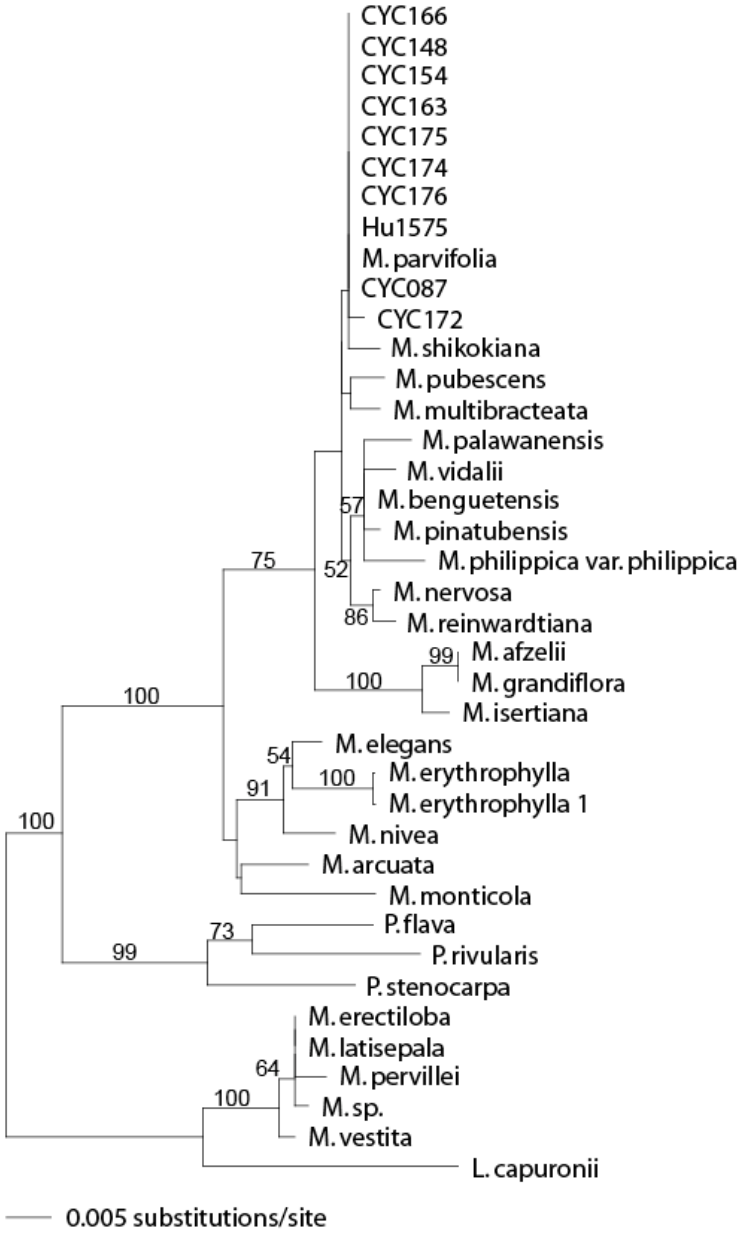

Fig. 2. Neighbor-joining tree of nrITS sequences of *Mussaenda* species. Our sequences are shown in CYCXXX and Hu1575. The other *Mussaenda* sequences are obtained as in Alejandro et al. (2005).