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Cryptic dioecy in *Mussaenda pubescens* (Rubiaceae): a species with stigma-height dimorphism

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• *Background and Aims* Evolutionary transitions from heterostyly to dioecy have been proposed in several angiosperm families, particularly in Rubiaceae. These transitions involve the spread of male and female sterility mutations resulting in modifications to the gender of ancestral hermaphrodites. Despite sustained interest in the gender strategies of plants, the structural and developmental bases for transitions in sexual systems are poorly understood.
• *Methods* Here, floral morphology, patterns of fertility, pollen-tube growth and floral development are investigated in two populations of the scandent shrub *Mussaenda pubescens* (Rubiaceae), native to southern China, by means of experimental and open-pollinations, light microscopy, fluorescence microscopy and scanning electron microscopy combined with paraffin sectioning.
• *Key Results Mussaenda pubescens* has perfect (hermaphroditic) flowers and populations with two style-length morphs but only weak differentiation in anther position (stigma-height dimorphism). Experimental pollinations demonstrated that despite morphological hermaphroditism, the species is functionally dioecious. The long-styled (L) morph possesses sterile pollen and functions as a female, whereas the short-styled (S) morph is female sterile and functions as a male. Self- and intra-morph pollinations of the S-morph were consistent with those expected from dimorphic incompatibility. The two populations investigated were both S-morph (male) biased. Investigations of early stages of floral development indicated patterns typical of hermaphroditic flowers, with no significant differences in organ growth between the floral morphs. Meiosis of microspore mother cells was of the simultaneous type with tetrads isobilateral in shape. The tapetal cells in anther walls of the L-morph became vacuolized during meiosis I, ahead of the uninucleate microspore stage in the S-morph. In the L-morph, the microspore nucleus degenerated at the tetrad stage resulting in male sterility. Microsporogenesis and male gametophyte development was normal in the S-morph. Failure in the formation of megaspore mother cells and/or the development of megagametophytes resulted in female sterility in the S-morph, compared with normal megasporogenesis in the L-morph.
• *Conclusions* In *M. pubescens*, cryptic dioecy has evolved from stigma-height dimorphism as a result of morph-specific sterility mutations.

Key words: Dioecy, distyly, female and male sterility, floral development, *Mussaenda pubescens*, stigma-height polymorphism, self-incompatibility.

INTRODUCTION

The flowers of most angiosperms are hermaphroditic with plants transmitting genes from generation to generation through female and male function. However, approx. 6–7 % of flowering plant species are dioecious with populations containing female and male plants (Renner and Ricklefs, 1995). The two main routes by which dioecy evolves from hermaphroditism are referred to as the 'gynodioecy pathway', in which populations with females and hermaphrodites (gynodioecy) are an intermediate condition, and the 'monoecy pathway', in which selection in ancestral monoecious populations on quantitative variation in sex allocation leads to dioecy (reviewed in Bawa, 1980; Geber *et al.*, 1999; Barrett, 2002). A less-common pathway by which dioecy evolves is from the floral polymorphism known as heterostyly or reciprocal herkogamy.

Heterostyly is a genetic polymorphism reported from approx. 28 angiosperm families and is characterized by populations composed of two or three floral morphs that differ reciprocally in the positions of their anthers and stigmas (reviewed in Ganders, 1979; Barrett, 1992; Barrett and Shore, 2008). Darwin (1877) proposed that sexual dimorphism could evolve from heterostyly as a result of gender specialization, with the long- and short-styled morphs (hereafter L- and S-morphs) no longer making equal contributions to offspring as paternal and maternal parents. Since then there has been considerable theoretical interest in this evolutionary transition, in part, because of the possibility that the selective mechanisms responsible for the transition may be independent of inbreeding avoidance (Lloyd, 1979; Beach and Bawa, 1980; Charnov, 1982; Muenchow and Grebus, 1989). However, relatively little is know about the early stages of this transition

including the genetic and developmental basis for the evolution of unisexuality and the selective mechanisms responsible for gender specialization.

Several families of flowering plants contain genera with both distylous and dioecious species [e.g. *Cordia*, Boraginaceae (Opler *et al.*, 1975), *Nymphoides*, Menyanthaceae (Ornduff, 1966), *Mussaenda*, Rubiaceae (Baker, 1958)]. In these groups there is some comparative evidence that dioecy has evolved from a distylous ancestral condition, through the spread of sterility mutations (reviewed in Ganders, 1979; Barrett and Richards, 1990). Detecting early stages in this transition involves recognizing the occurrence of 'cryptic dioecy', in which one or both of the functionally unisexual morphs are morphologically hermaphroditic. Cryptic dioecy is widely reported in flowering plants. However, in most instances it is not associated with transitions from heterostyly to dioecy (Bawa, 1980; Haber and Bawa, 1984; Kevan and Lack, 1985; Kawakubo, 1990; Mayer and Charlesworth, 1991; Kato and Nagamasu, 1995; Renner and Ricklefs, 1995; Aranha *et al.*, 2009). There is considerable functional variation in the expression of cryptic dioecy, depending on the degree of gender specialization exhibited by morphologically hermaphroditic plants (Lloyd, 1979; Mayer and Charlesworth, 1991; Humeau *et al.*, 1999; Naki and Kato, 1999; Dunthorn, 2004; Kawagoe and Suzuki, 2004). As far as is known, no study has examined the structural and developmental basis of gender dimorphism in taxa with stylar dimorphism.

Here, the occurrence of cryptic dioecy is reported in *Mussaenda pubescens* (Rubiaceae), a scandent shrub native to southern China. *Mussaenda* is a paleotropical genus of approx. 132 species (Alejandro *et al.*, 2005) and exhibits considerable diversity in floral biology and sexual systems, including monomorphic species, with either approach or reverse herkogamy, stigma-height dimorphism, distyly and dioecy (Baker, 1958; Richards and Koptur, 1993; Pailler and Thompson, 1997; Naiki and Kato, 1999; Faivre, 2000; Deng, 2007; Deng and Zhang, 2008). Hence, *Mussaenda* provides outstanding opportunities for investigating floral evolution and the origin of dioecy from stylar dimorphism.

Preliminary observations suggested that *M. pubescens* exhibited stigma-height dimorphism but that the style morphs differed in gender expression. The purpose of this study was therefore to confirm these observations experimentally and to address the following specific questions: (*a*) Which floral traits distinguish the style morphs and does the species possess a true stigma-height dimorphism? (*b*) What are the compatibility relationships of the style morphs, and is there evidence of male and female sterility in the L- and S-morphs, respectively? (*c*) Do the style morphs differ in floral ontogeny, microsporogenesis, megasporogenesis, and male and female gametophyte development? Following the presentation of the results, the ecological and genetic mechanisms that might be responsible for the evolution of gender specialization are considered.

MATERIALS AND METHODS

Sampling of plant materials

This study was conducted from March to July 2006–2007 in two natural populations of *Mussaenda pubescens* located in the South China Botanical Garden and Nankunshan Nature Reserve of Guangdong Province, China. Voucher specimens (A. Li 001, 002) for the two populations were deposited in the herbarium of South China Botanical Garden, the Chinese Academy of Sciences (official acronym: IBSC). In both populations, the frequencies of floral morphs were counted to determine if a 1 : 1 ratio was evident, as occurs in many other species with stylar dimorphism. Chi-square tests were used to evaluate equality of morph representation. Flowers and buds from the two populations were also sampled and controlled pollinations, as described in detail below, were conducted.

Flower size measurements

Floral buds representing different developmental stages from individuals of the two floral morphs were collected. A total of 26 buds from ten individuals from each floral morph in both populations was sampled. Buds $<0{\cdot}5$ cm were measured under a stereomicroscope, whereas for larger-sized buds a digital micrometer was used. Buds were dissected to allow measurements of flower parts. Means and standard errors of floral organs in mature flowers were calculated using Microsoft Excel 2003 for Windows XP and *t*-tests were used to compare means of the floral traits, including floral tube length, anther height, stigma height, stigma lobe length, stigma–anther separation and length of hairs within the corolla tube.

Light microscopy

Flower buds were fixed in FAA solution (37 % formalin : glacial acetic acid : 50 % ethanol = 5 : 5 : 90). The samples were initially infiltrated under a vacuum at room temperature for 2 h and then preserved in FAA at room temperature. Samples were pre-stained with Ehrlichs haematoxylin, dehydrated in a graded ethanol series, embedded in paraffin, and then sectioned (8 μm in thickness) using a rotary microtome (Leica RM2016; Leica Microsystems, Germany). All sections were photographed using an Olympus BH41 microscope equipped with charge-coupled device.

Scanning electron microscopy

For observation using scanning electron microscopy, flower buds were fixed in 4 % glutaraldehyde, 0·1 M phosphate buffer at pH 7·0 and post-fixed in 1 % osmic acid, 0·1 M phosphate buffer at pH 7·0. After three washes in 0·1 M phosphate buffer, the samples were dehydrated through a graded ethanol series and subjected to freeze-drying (JFD-310; JEOL, Japan). Afterwards, samples were mounted on stubs and coated with gold in a sputter coater (JFC-1600; JEOL), and then examined and photographed under a scanning electron microscope (JSM-6360LV; JEOL). The length of hairs in corolla tubes was measured using SmileView (JEOL).

Controlled pollination studies

To evaluate the female fertility of morphs and to determine if there was evidence of an ancestral dimorphic self-incompatibility system, controlled hand-pollinations were conducted. For artificial pollinations, newly opened flowers from inflorescences

bagged with nylon mesh were used. Because flowers of both L- and S-morphs last for approx. 2 d, time intervals for stigma harvesting after pollination were designed as 1, 12, 24 and 48 h. Twenty flowers per treatment with five flowers per time interval were used. The following treatments were conducted: (*a*) self-pollination of the S-morph; (*b*) intra-morph pollination of the S-morph; (*c*) inter-morph pollination of the L-morph using pollen from the S-morph. Because anthers of the L-morph contained only a few misshaped pollen grains, it was not possible to conduct self-, intra-morph or inter-morph pollinations using this morph as a pollen donor. Prior to hand pollination, all flowers on inflorescences that were already in anthesis were removed and then they were covered with nylon mesh to exclude flower visitors. Once flowers had senesced the nylon mesh was removed and the fruits were allowed to mature before they were harvested and the seeds counted. Pistils were fixed after hand pollinations by clearing in 1 M NaOH until the tissues became transparent, washed with distilled water, and then stained with aniline blue according to Teng *et al.* (2005).

RESULTS

Morph ratios and floral morphology

Both populations of *M. pubescens* comprised two floral morphs that differed in style length. The L-morph has a long style with the stigma positioned above the anthers, whereas the S-morph has short styles with the anthers positioned above the stigma (Fig. 1). The stigma of the L-morph is visible above the throat of the corolla, while the anthers of the S-morph are above the stigma but hidden within the corolla throat (Fig. 1A, B). In the S-morph, anthers dehisce when flowers are fully expanded, whereas in the L-morph anthers are shrunken and contain very few misshaped pollen grains (Fig. 1C, D). Counts of floral morph in each population revealed a significant bias in favour of the S-morph [South China Botanical Garden: L-morph = 40, S-morph = 94 (χ^2= 11·34, $P <$ 0·001, $n = 134$); Nankunshan Natural Reserve: L-morph = 36, S-morph = 74 ($\chi^2 = 6{\cdot}77$, $P < 0{\cdot}01$, $n = 110$)].

Measurements of floral parts in the two floral morphs revealed significant differences in several traits with potential functional importance (Table 1). Most noteworthy is the fact that, although the two floral morphs exhibited clear difference in stigma height typical of distylous populations, the positions of anthers were much less differentiated. As a result, there was a lack of reciprocity between the lower organ levels. Short-level stigmas were positioned well below the anther heights of the L-morph ($t = 45{\cdot}6$, $P < 0{\cdot}01$, Fig. 1E, F). This arrangement resulted in a much smaller stigma–anther separation in flowers of the L-morph compared with the S-morph ($t = 22{\cdot}2$, $P < 0{\cdot}01$). Weak stamen level differentiation and

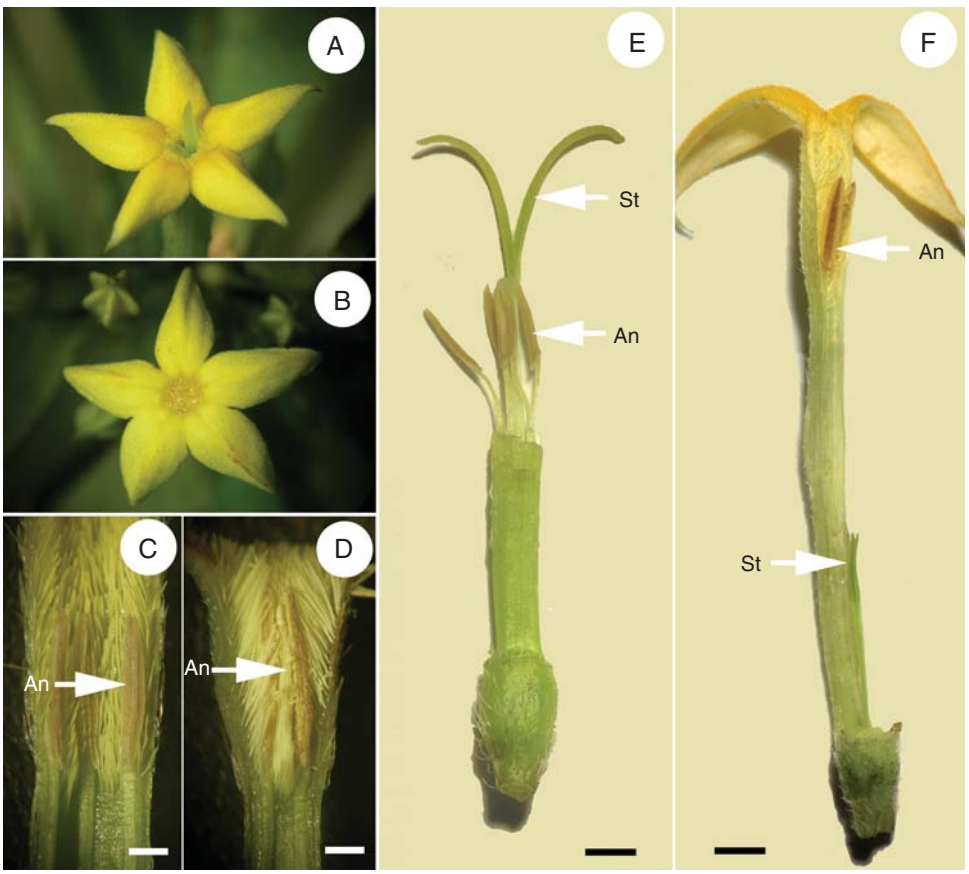

FIG. 1. Flowers of *Mussaenda pubescens* illustrating sex organ placement and floral tube hairs: (A) stigma protruding from the throat of the corolla in the L-morph; (B) anthers concealed in the corolla of the S-morph; (C) sterile anthers in the dense hairs of the L-morph; (D) anthers dehisced in the S-morph with the presentation of pollen grains in the dense upward-facing hairs of the corolla; (E and F) positions of the sex organs in the L-morph (E) and S-morph (F). An, Anther; St, stigma. Scale bars = 1 mm.

TABLE 1. *A comparison of floral traits in the long- (L) and short-styled (S) morphs of* Mussaenda pubescens

Floral traits	L-morph	S-morph	*t*-value
Floral tube length (mm)	20·18 ± 1·96**	22·34 ± 1·88	4·05
Anther height (mm)	10·70 ± 0·52**	14·64 ± 0·39	31·02
Stigma height (mm)	13·71 ± 1·03**	4·54 ± 0·44	41·7
Stigma lobe length (mm)	3·69 ± 0·46**	1·74 ± 0·40	16·25
Stigma–anther separation (mm)	3·95 ± 0·22**	9·17 ± 1·18	22·2
Length of hairs (μm)	546·80 ± 164·00**	808·15 ± 207·47	5·09

Values presented are means ± standard errors; sample size: $n = 26$ flowers per morph.

** $P < 0{\cdot}01$ based on *t*-tests.

the lack of reciprocity between dysfunctional anthers and stigmas of the S-morph confirm that populations of *M. pubescens* are best described as possessing stigma-height dimorphism rather than distyly. Difference between the floral morphs also involved the length of the upward-facing hairs in the corolla throats. Hairs in flowers of the S-morph were significantly longer than those of the L-morph ($t = 5{\cdot}09$, $P < 0{\cdot}01$).

Controlled pollinations

The results of hand pollinations were consistent with those expected if *M. pubescens* possessed an ancestral dimorphic incompatibility system (Fig. 2). Inter-morph pollinations of

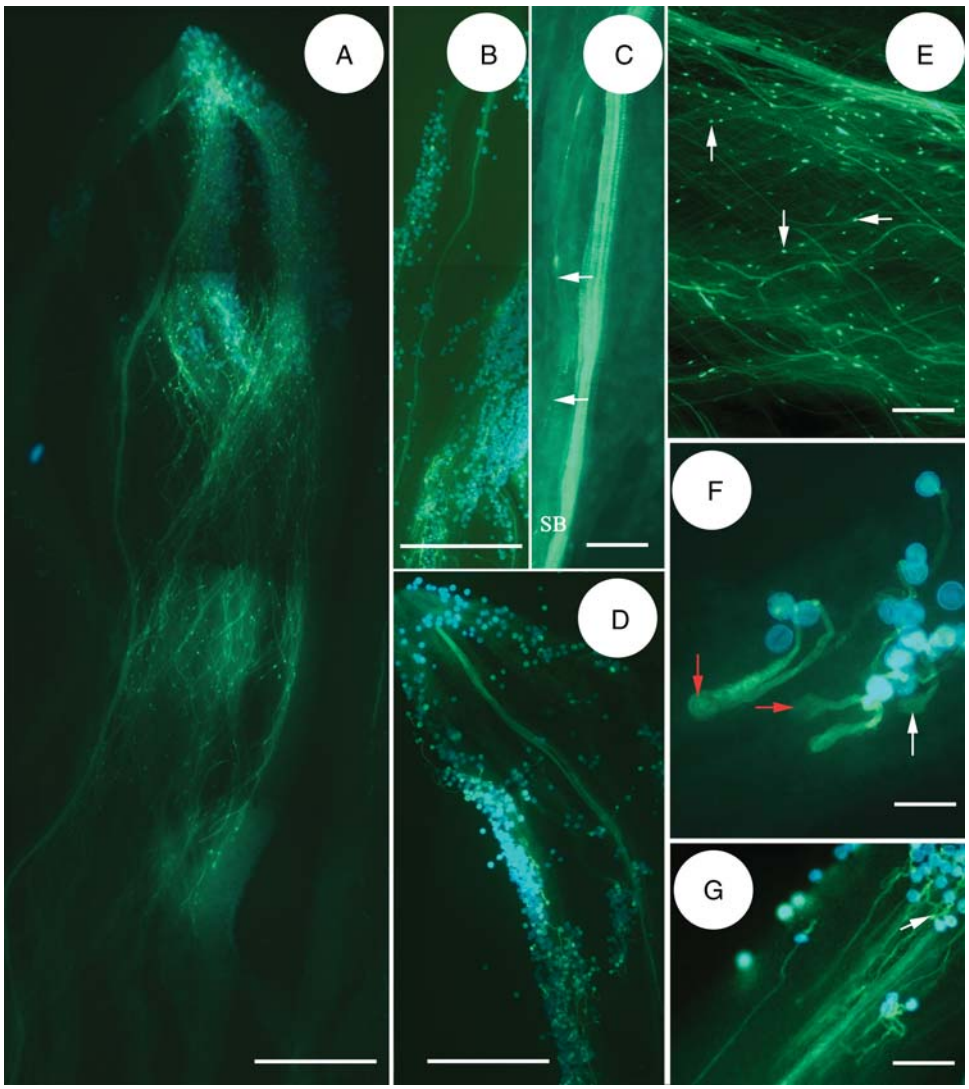

FIG. 2. Pollen-tube growth in the stigma lobes and style of *Mussaenda pubescens* following self-, intra-morph and inter-morph pollination: (A) L-morph stigma lobes and style 24 h after inter-morph pollination, showing normal pollen germination and pollen-tube growth; (B) S-morph stigma lobes and style 24 h after intra-morph pollination, showing pollen-tube growth arrested on the stigma; (C) pollen tubes arrived at the base of S-morph style (SB) after intra-morph pollination; (D) S-morph stigma lobes and style 24 h after self-pollination, showing pollen-tube growth arrested on the stigma; (E) callose plugs (white arrowheads) present at intervals in the pollen tubes in the L-morph style after inter-morph pollination; (F) pollen tubes with slightly swollen tip (red arrowheads) in the S-morph style after intra-morph pollination; (G) pollen tubes with rare callose plugs (white arrowhead) in the S-morph style after self-pollination. Scale bars: (A–C) = 500 μm; (D, E, G) = 100 μm; (F) = 50 μm.

the L-morph resulted in pollen tubes that extended from the stigmatic surface through the stylar canal to the ovary, with callose plugs formed at regular intervals (Fig. 2A, E). In contrast, in the S-morph, pollen tubes were arrested within the stigma 48 h after self- and intra-morph pollination (Fig. 2B, D). However, a few pollen tubes were observed at the base of the style in intra-morph pollination of the S-morph (Fig. 2C), and a significant percentage of pollen tubes showed swelling at their tips (Fig. 2F) and callose plugs were seldom observed (Fig. 2F, G). As expected from these observations, no seed was produced from self, intra-morph or open pollination of the S-morph. In contrast, inter-morph pollination of the L-morph resulted in 92·4 % ± 6·1 (standard error) ($n = 48$) fruit set and a similarly high level of fruit set was observed from open pollination (91·4 % ± 5·5, $n = 97$).

Floral ontogeny and morph differentiation

Floral ontogeny in *M. pubescens* was divided into 14 developmental stages according to a series of landmark events from initiation until the opening of the flower (Table S1 and Fig. S1 in Supplementary data, available online). Up until stage 10 it was not possible to distinguish organ growth between the floral morphs. Early floral development proceeded from a bulging apical meristem, which subsequently extended in a tangential direction (stage 2–3; Figs S1A and S2A in Supplementary data). Five sepal primordia were initiated sequentially at the edge of the floral apex and gradually enlarged to form a calyx (stage 4; Fig. S1B–F). The inner five petals (tepals) arose almost simultaneously alternate with the sepals, and then increased in size (Fig. S1F–I), and finally the petals closed together (stage 5; Figs S1J and S2D). Ahead of the folding of petals, the five stamen primordia emerged synchronously and were alternately positioned between the petal primordia (stage 6; Figs S1K and S2E).

With the depression at the centre of the receptacle, the carpel primordia initiated separately (Figs S1L and S2F), then differentiated into style and ovary lumen by intercalary growth (Figs S1M, N and S2F). The middle of the style fused, forming two separate stigma lobes. Simultaneously, the placentae with ovules emerged forming two newly formed locules (Figs S1O–P and S2G). Thus, a morphologically hermaphroditic flower of *M. pubescens* was evident at this stage (stage 8; Fig. S2G).

At stage 8, stigmas of the L-morph were located below the anthers. Since the filaments were fused to the corolla, the stamens elongated concomitantly during corolla-tube elongation. However, when anthers were about 3·8 mm in length, they stopped elongating, even though flower buds were still expanding, and at stages 12, 13 and 14 stigmas were now positioned above anthers. At anthesis, stigmas protruded from the corolla, with the base of the stigmas and the top of the anthers at about the same height (Fig. 1A, E). The floral ontogeny of short-styled flowers was similar to that of long-styled flowers before stage 9. However, at stage 10, anthers of S-morph raised above the stigma. At anthesis, both anthers and stigma were hidden in the corolla tube in flowers of the S-morph (Fig. 1B, F).

Pollen development in the L-morph

At stage 9, four large-size archesporial cells with large nuclei, positioned at the corners below the single-layered epidermis of young anthers (Fig. 3A), were divided into primary parietal cells and primary sporogenous cells by periclinal division (Fig. 3B). The primary parietal cells were closely arranged and divided to form secondary parietal cells, which gave rise to endothecium, middle layer and tapetum by continuous periclinal and anticlinal division (Fig. 3C–E). The middle layer cells were ephemeral and only existed for a short time. In meiosis I of microspore mother cells, the middle layer began to disintegrate, and then disappeared completely at the microspore stage (Fig. 3H–K).

The tapetal cells were small and nearly square shaped, with obvious nuclei and nucleoli. At the stage of microspore mother cells, the volume of tapetal cells increased, and some vesicles were present in the cytoplasm (Fig. 3E). During meiosis I of microspore mother cells, the increasing cytoplasmic vesicles accumulated and fused with each other, then vacuolized significantly at the tetrad stage (Fig. 3I). At the free microspore stage, the tapetal cytoplasm and nucleus disintegrated entirely (Fig. 3J, K).

Secondary sporogenous cells were of large size and were converted into microspore mother cells with increased size (Fig. 3D, E). An isobilateral tetrad with four microspores was formed through simultaneous cytokinesis (Fig. 3F, G). Subsequently, the free uninucleate microspores were produced by separation of the tetrad (Fig. 3H, J). The microspore nuclei degraded gradually, and ruptured into debris (Fig. 3J, K), leading to anther atrophy and male sterility (Fig. 3L).

Pollen development in the S-morph

Microsporogenesis in flowers of the S-morph resembled that observed in flowers of the L-morph before degradation of the nucleus at the microspore stage (Supplementary data Table S1 and Fig. 4). However, in the S-morph the tapetum disintegrated at the free microspore stage, lagging behind the L-morph in which disintegration of the tapetum began during meiosis I of the microspore mother cells. As the isolated microspores increased in volume, the expanded vacuole pushed the central nucleus to the cell edge, i.e. mononuclear side phase (Fig. 4G, H). Later, by mitosis the nucleus divided into two unequal nuclei (Fig. 4I), a smaller nucleus near the cell wall and the other in the middle. Subsequently, the deposition of callose between the two nuclei contributed to the production of male gametes with a vegetative cell and a germ cell, i.e. two-celled pollen grain. During this period, the pollen tube cytoplasmic bulge could be seen in the germination aperture (Fig. 4I). After that, the pollen tube cytoplasmic bulge gradually reduced and at anthesis it disappeared completely.

Megasporogensis and megagametophyte development of the L-morph

At stage 10 in the L-morph, some cells in the placenta began division and projected inwards, forming spherical ovule primordia in the locule. An archesporial cell, distinguishable by large size and dense cytoplasmic contents, originated from a

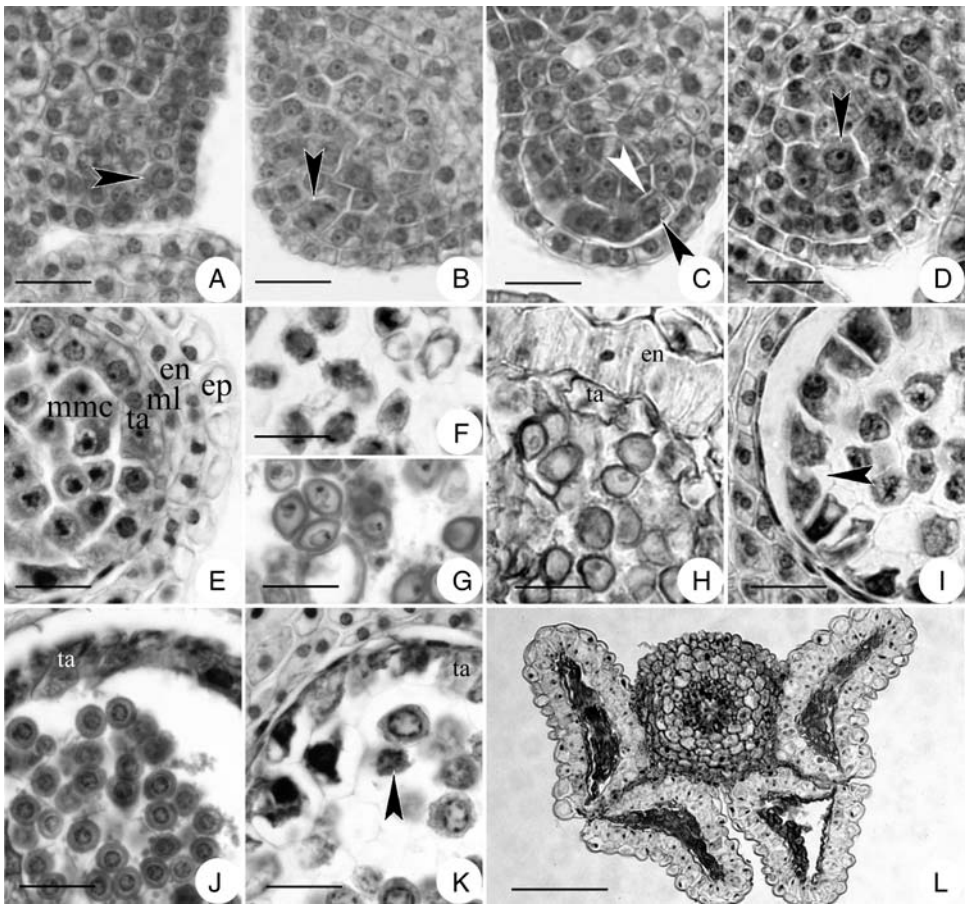

FIG. 3. Microsporogenesis and male gametophyte development in the L-morph of *Mussaenda pubescens*: (A) archesporial cell (arrow); (B) archesporial cell periclinal division (arrow); (C) a primary parietal cell (black arrow) and a primary sporogenous cell (white arrow) produced by archesporial cell division; (D) secondary sporogenous cells (arrow); (E) microspore mother cells (mmc) surrounded by complete anther wall consisting of epidermis (ep), endothecium (en), middle layer (ml) and tapetum (ta); (F) meiosis I of microspore mother cells; (G) microspore tetrad with atrophic nucleus; (H) free microspore stage showing abnormal microspore and degraded tapetum (ta); (I) tapetum cells cytoplasm contracted (arrow) when the microspore mother cells are in meiosis; (J) tapetum cells (ta) degradation in uni-nucleate microspore; (K) the nuclei of microspores are degraded (arrow); (L) shrivelled anther. Scale bars: (A–K) = 20 μm; (L) = 100 μm.

cell below the nucellar epidermis (Fig. 5A). Increasing in size, the archesporial cell developed directly into the megaspore mother cell, surrounded by a single nucellar epidermis cell layer (Fig. 5B). Soon, the megaspore mother cell started meiotic division, producing two dyad cells, and then a linear tetrad (Fig. 5D, E). Due to asynchronous growth, the funicle was bent towards one side, which resulted in anatropus ovules. Of the four linearly arranged megaspores, the three near the micropyle degraded, and the remaining one developed into a functional megaspore. Subsequently, the nucleus in the megaspore moved to the middle, and this was followed by three mitoses. Finally, an eight-nucleate embryo sac was produced of the *Polygonum* type (Fig. 5F, G, L).

Megasporogensis and megagametophyte development in the S-morph

In the S-morph, four megaspores resulted from meiosis and were arranged in a linear shape, similar to that observed in the L-morph (Fig. 6A–C, E). However, these four megaspores became degraded in a short time (Fig. 6D). Very few functional megaspores formed and differentiated into a two-nucleate embryo sac, but they often suffered from atrophic deformation at this stage (Fig. 6H). Extremely few two-nucleate embryo sacs developed further. In a sample of 233 degenerated embryo sacs counted, 8·6 %, 11·2 % and 18·5 % degenerated at megaspore mother cell stage, megaspore dyad stage and the megaspore tetrad stage, respectively, and a further 9·4 % degenerated during megagametogenesis, while the degeneration stage of the remaining 52·3 % could not be identified.

DISCUSSION

The major finding of the present study is that *M. pubescens* is functionally dioecious, despite possessing morphologically hermaphroditic flowers. This condition is an example of cryptic dioecy. Populations of *M. pubescens* contain two style-length morphs with complementary gender roles. The L-morph possesses sterile pollen and functions as a female and the S-morph exhibits female sterility and serves as a pollen parent. Sex organ placement in the two morphs

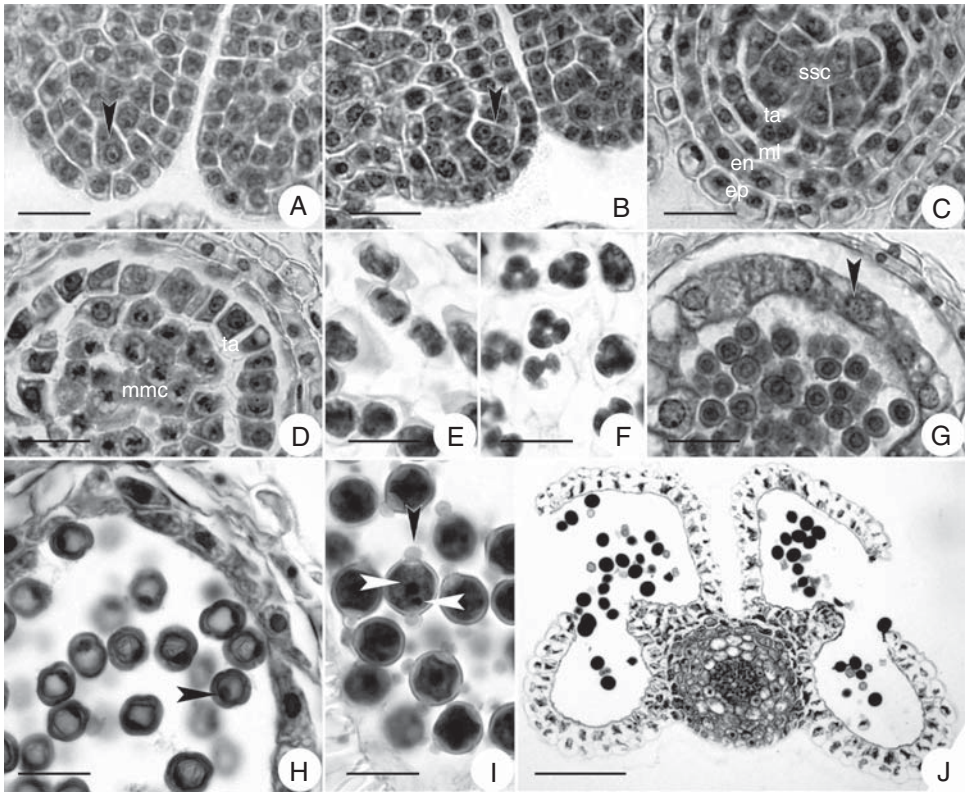

FIG. 4. Microsporogenesis and male gametophyte development in the S-morph of *Mussaenda pubescens*: (A) archesporial cell (arrow); (B) archesporial cell with periclinal division into primary parietal cell and primary sporogenous cell (arrow); (C) secondary sporogenous cells (ssc) surrounded by anther wall consisting of epidermis (ep), endothecium (en), middle layer (ml) and tapetum (ta); (D) microspore mother cells (mmc) produced by secondary sporogenous cell mitosis; (E) meiosis II of microspore mother cells; (F) meiosis II of microspore mother cells; (G) free microspore (arrow); (H) tapetum cells (ta) degraded in uni-nucleate microspore with nucleus near lateral (arrow); (I) two-nucleate pollen grains (white arrows) with cytoplasmic bulge (black arrow); (J) mature fertile anther. Scale bars: (A–I) = 20 μm; (J) = 100 μm.

indicates that the ancestral sexual system was likely to have been stigma-height dimorphism, a relatively uncommon floral polymorphism often associated with distyly. This discussion considers the relationships between stigma-height dimorphism and distyly and the ecological and genetic mechanisms responsible for the evolution of gender specialization and origins of cryptic dioecy.

Stigma-height dimorphism and distyly

Morphological measurements of anther heights in the L- and S-morphs of *M. pubescens* revealed a significant difference in their relative placements to stigmas (Table 1). Anthers in the L-morph were positioned well above short-level stigmas, resulting in a lack of reciprocity between lower-level sex organs and a difference in the degree of herkogamy between the floral morphs. Stigma–anther separation in the L-morph was weakly developed in comparison with the S-morph. These features are not typical of most distylous species, which possess reciprocal herkogamy and balanced reciprocity between both sets of anthers and stigmas.

The arrangement of sex organs in the two floral morphs of *M. pubescens* is characteristic of species possessing stigma-height dimorphism, a floral polymorphism involving style length not anther height (reviewed in Barrett *et al.*, 2000). However, distyly and stigma-height dimorphism sometimes merge into one another, and distinguishing between them can be somewhat arbitrary (Barrett and Richards, 1990; Dulberger, 1992). Stigma-height dimorphism occurs in genera with distyly (e.g. *Anchusa*, *Linum*, *Lithodora*, *Narcissus* and *Primula*; reviewed in Barrett *et al.*, 2000; Ferrero *et al.*, 2009), and in these cases the polymorphism may represent the transitional stage between stylar monomorphism and distyly envisioned by Darwin (1877), and predicted by theoretical models of the evolution of distyly (Charlesworth and Charlesworth, 1979; Lloyd and Webb, 1992). However, stigma-height dimorphism also occurs sporadically in families with no heterostylous taxa [e.g. *Chlorogalum angustifolium*, Liliaceae (Jernstedt, 1982); *Epacris impressa*, Epacridaceae (O'Brien and Calder, 1989); *Kalmiopsis leachiana*, Ericaceae (Barrett *et al.*, 2000)], indicating that the polymorphism is not always associated with the evolutionary build-up of distyly. The occurrence of both distyly and stigma-height dimorphism in Rubiaceae probably indicates that the two polymorphisms are functionally associated and evolutionarily related. If this is the case, stigma-height dimorphism is most likely the antecedent condition to cryptic dioecy in *M. pubescens.*

Rubiaceae has more distylous species than any other family (Darwin, 1877; Bir Bahadur, 1968; Anderson, 1973; Barrett and Richards, 1990). Although previous investigations of dimorphic *Mussaenda* species indicate that this genus may

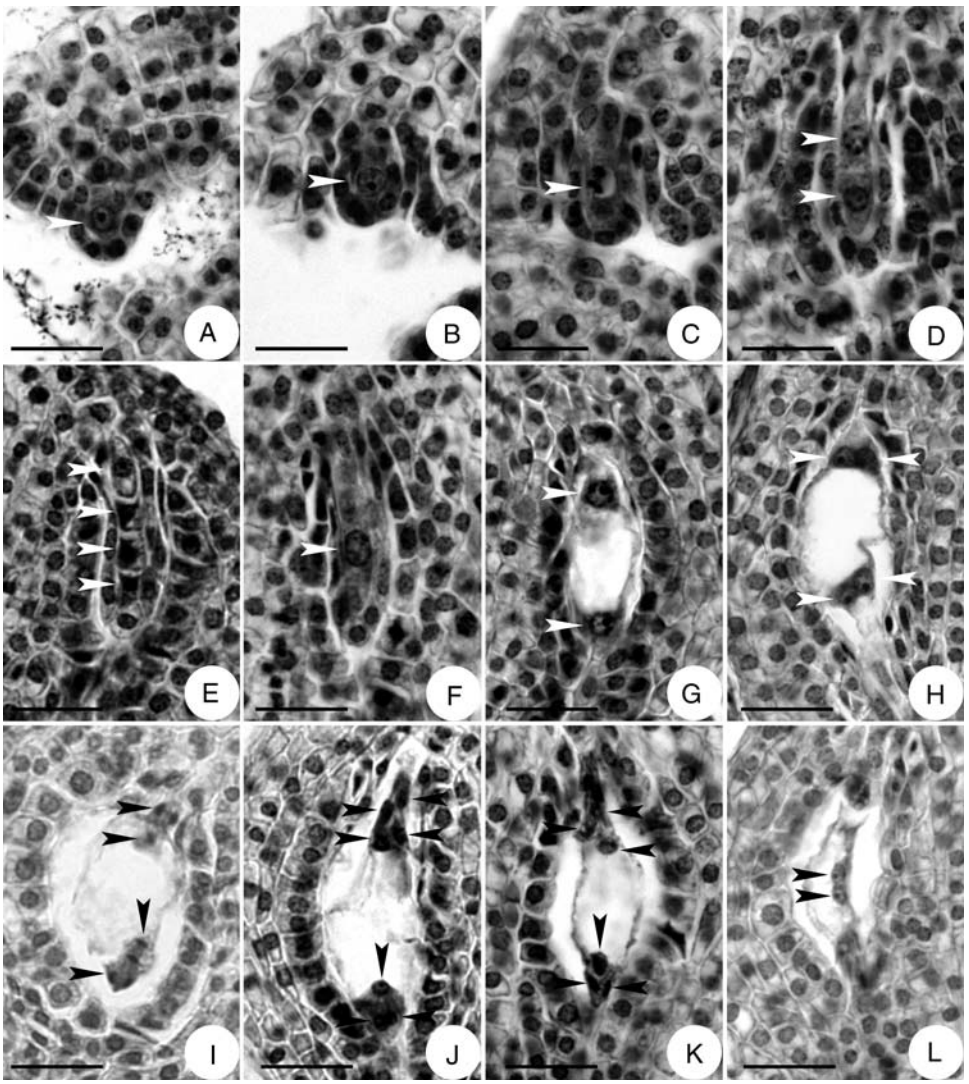

FIG. 5. Megasporogenesis and female gametophyte development in the L-morph of *Mussaenda pubescens*: (A) archesporial cell (arrow); (B) elongated archesporial cell developed into megaspore mother cell (arrow); (C) meiosis in megaspore mother cell (arrow); (D) megaspore dyed (arrows); (E) megaspore tetrad (arrows); (F) the chalazal megaspore developed into functional megaspore; (G) two-nucleate embryo sac (arrow); (H) four-nucleate embryo sac (arrows); (I) mitosis in four-nucleate embryo sac (arrow); (J–L) seven-celled eight-nucleate embryo sac. Scale bars = 20 μm.

be especially prone to deviations from reciprocal herkogamy and the evolution of gender specialization (Baker, 1958; Hamilton, 1990; Richards and Koptur, 1993; Pailler and Thompson, 1997; Naiki and Kato, 1999; Faivre and McDade, 2001; Deng, 2007). The present observations of pollen-tube growth are consistent with those reported for distylous species (Barrett and Cruzan, 1994), including members of the Rubiaceae (Bawa and Beach, 1983). Because of pollen sterility in the L-morph it was not possible to conduct illegitimate pollinations; however, when these were conducted on the S-morph they revealed that the vast majority of pollen tubes ceased growth in the stigma, with only occasional pollen tubes reaching the base of the style. These results are consistent with what would be predicted if the S-morph possesses a remnant dimorphic incompatibility system. However, the extent to which ovule sterility in the S-morph might influence pollen–pistil interactions is unclear, so this conclusion should be considered tentative.

Surveys of morph frequency in species with stigma-height dimorphism commonly report L-morph-biased morph ratios, probably as a result of asymmetrical mating (Barrett *et al.*, 1996; Baker *et al.*, 2000; Barrett and Hodgins, 2006). However, the two populations of *M. pubescens* investigated in this study both contained an excess of the S-morph (male) rather than the L-morph (female). Although it would be premature to generalize from such a limited sampling of populations, the occurrence of male-biased sex ratios is very common in dioecious species, occurring over twice as often as female-biased sex ratios (Barrett *et al.*, 2010). Several mechanisms associated with differences in the costs of reproduction between females and males account for this difference, and these may have contributed to the male-biased sex ratios observed. Interestingly, in the two population of *M. pubescens* investigated male individuals started flowering earlier and finished later than females, a common feature of dioecious species (Lloyd and Webb, 1977). More extensive

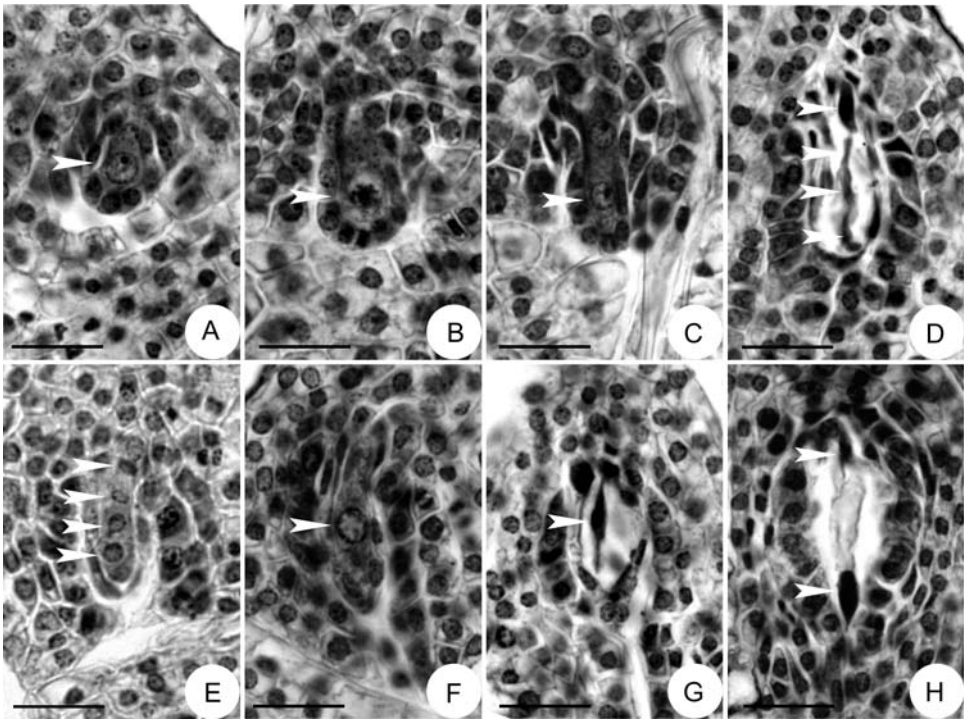

FIG. 6. Megasporogenesis and female gametophyte development in the S-morph of *Mussaenda pubescens*: (A) archesporial cell (arrow); (B) megaspore mother cell undergoing meiosis (arrow); (C) megaspore dyed (arrow); (D) megaspore tetrad degenerated (arrows); (E) normal megaspore tetrad (arrows); (F) a chalazal megaspore developed into functional megaspore (arrow); (G) functional megaspore degenerated (arrows); (H) two-nucleate embryo sac degenerated. Scale bars = 20 μm.

sampling of populations is required to confirm these observations.

Ecological mechanisms responsible for gender differentiation

Several pathways for the evolution to dioecy are recognized with occasional transitions to sexual dimorphism from hermaphroditic dimorphic ancestors including heterodichogamy and distyly (Lloyd, 1979; Bawa, 1980; Ross, 1982; Thomson and Brunet, 1990; Barrett, 2002; Pannell and Verdú, 2006). Baker (1958), following Robertson (1892), proposed an ecological mechanism that might explain the evolutionary transition from distyly to dioecy. He proposed that pollen transfer from short-level anthers of the L-morph to stigmas of the S-morph had become impeded as a result of the 'choking hairs' that characterize the floral tubes of *Mussaenda* species. With relaxed selection to main reciprocal cross-pollination, sterility mutations in the pollen and ovules of the L- and S-morph, respectively, would lead to unisexuality and gender dimorphism as a result of disuse of short-level organs. This hypothesis could provide a plausible mechanism for how cryptic dioecy has evolved in *M. pubescens*.

Beach and Bawa (1980) proposed that the shift to dioecy in distylous groups may be initiated by a change in the pollinators that visit distylous populations. They argued that a loss in long-tongued pollinators, capable of mediating pollen transfer between short-level organs, would result in unidirectional pollen transfer with the S-morph serving as a paternal parent and the L-morph as a maternal parent. However, there is no evidence in *Mussaenda* that such an effect results from the absence of long-tongued pollinators. Pollinator observations of *M. pubescens* (Lai *et al.*, 2009) and *M. parviflora* (Naiki and Kato, 1999) revealed that long-tongued butterflies and hawkmoths capable of mediating pollen transfer between short-level organs commonly visit populations. Unfortunately, since in both species short-level anthers of the L-morph contain no functional pollen, comparisons of pollen transport between the two morphs to investigate the effectiveness of pollen dispersal are not possible. Investigations of pollen transfer in other *Mussaenda* species would be valuable to reveal what role the floral tube hairs play in the pollination process. Floral tube hairs are widely distributed in the genus and occur in typical distylous species (e.g. *M. macrophylla*), dioecious species (e.g. *M. erosa*, *M. kwangtungensis* and *M. pinbianensis*) and monomorphic species (e.g. *M. lancipetala* and *M. simpliciloba*) (Deng, 2007; Deng and Zhang, 2008).

Floral ontogeny and developmental basis of sterility

Comparative analysis of floral ontogeny and development in *M. pubescens* has provided detailed histological and morphological landmarks of male and female reproductive organogenesis in both morphs. The present results demonstrated that differentiation of flowers of the L- and S-morph occurred at a comparatively late stage, similar to previous studies of distylous *Primula* (Webster and Gilmartin, 2003). Microsporogenesis in both morphs of *M. pubescens* was of the simultaneous type, consistent with previous studies on non-dimorphic Rubiaceae species in which the tetrad is formed in one step after the completion of the second

meiotic division (Vinckier and Smets, 2005). Although species of Rubiaceae have been reported to possess pollen grains with three nuclei at maturity (El-Ghazaly *et al.*, 2001), pollen in *M. pubescens* was bicellular when shed, in accordance with previous studies on *Psychotria* and a few other genera (Vuilleumier, 1967; Souza *et al.*, 2008).

Recent studies of the post-meiotic developmental programme of microsporogenesis indicate that degradation of the tapetum resulting from programmed cell death is necessary to produce fertile pollen grains (Li *et al.*, 2006). Any change in this programme, including premature or delayed degradation of the tapetum, can lead to male sterility (Kawanabe *et al.*, 2006; Shi *et al.*, 2009). In the present study, the occurrence of tapetum programmed cell death in the S-morph fits well with normal cases of tapetum development following meiosis, while degradation of tapetum in the L-morph was precocious, indicating that tapetum pre-degradation probably accounts for the male sterility in this morph. Pollen development in *M. pubescens* differs from that of distylous *Psychotria ipecacuanha* (Rubiaceae), in which pollen development of both morphs is similar to that observed in most dicotyledons (Souza *et al.*, 2008).

Investigation of megasporogenesis and female gametophyte development in Rubiaceae is somewhat fragmentary (Robbrecht, 1988; Igersheim and Robbrecht, 1993; De Toni and Mariath, 2008). As in most Rubiaceae, *M. pubescens* has inferior, bicarpellate and bilocular ovaries. Flowers of the L-morph exhibited numerous small ovules per locule, whereas only one gametophyte is formed per ovule, in contrast to some Rubiaceae in which more than one gametophyte is formed at different development stages to ensure successful fertilization (Mariath and Cocucci, 1997). Female gametophyte development in the L-morph was of the *Polygonum* type, as in most angiosperm species (Johri, 1984). However, in the S-morph, megasporogenesis and megagametogenesis appeared abnormal, ceasing at different stages, with most at the tetrad stage, resulting in the absence of functional ovules.

In summary, the present studies of floral organogenesis in the L- and S-morph of *M. pubescens* indicate how cryptic dioecy develops in this species. Male sterility in the L-morph results from earlier degradation of the tapetum, while female sterility of the S-morph appears to be mainly due to the failure of functional megaspore establishment. Further studies to identify the relevant genes responsible for sterility, and their relationship with the loci governing stylar dimorphism, would be useful for understanding the proximate genetic and developmental mechanisms governing transitions to functional dioecy.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: SEM micrographs of *Mussaenda pubescens* flower buds showing floral development. Figure S2: Longitudinal sections of *M. pubescens* flower buds showing floral development. Table S1: Developmental stages of long-styled and short-styled flowers of *M. pubescens*.

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