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# Heterostyly and pollinators in *Plumbago auriculata* (Plumbaginaceae)

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**Abstract**

Plants with hermaphrodite flowers risk conflict between male and female sexual function due to close proximity of sexual organs. Heterostyly, a genetic floral polymorphism characterized mainly by reciprocal herkogamy, may reduce this sexual conflict by increasing the precision of pollen transfer between morphs. This sexual organ reciprocity is often associated with various ancillary characters and a heteromorphic incompatibility system. Here we describe the morphometrics associated with heterostyly and ancillary characters in *Plumbago auriculata*. Using controlled pollination experiments, we show that this species has a heteromorphic incompatibility system. We also document the fauna of long-proboscid fly and butterfly pollinators in a *P. auriculata* population in KwaZulu-Natal, South Africa.


*Keywords:* Ancillary characters; Breeding system; Heterostyly; *Plumbago auriculata*; Pollination; South Africa

## 1. Introduction

Conflict between the male and female sex functions due to lack of precision in pollen transfer or through stigma interference in hermaphrodite flowers can be alleviated by various adaptations that promote cross-pollination (Barrett, 2002). One of these adaptive 'strategies' is heterostyly, a floral polymorphism primarily characterized by reciprocity in sex-organ levels (reciprocal herkogamy) between a "Pin" (Long-style) morph, with stigma above anthers, and a "Thrum" (Short-style) morph, with the reverse positioning (hereinafter termed L and S-morphs). Reciprocal herkogamy is usually accompanied by a sporophytic heteromorphic incompatibility system that prevents self- and intra-morph fertilizations, and a set of ancillary characters that differ between morphs, mainly related to pollen or stigma features (Ganders, 1979; Barrett, 1992; Dulberger, 1992).

Two different mechanisms are involved in the reduction of interference between reproductive functions of female and male organs in heterostylous plants. One is reciprocal sex-organ positions in the style morphs, whose function is apparently to increase male fertility by promoting more precise pollen dispersal among plants and thus reduce male gamete wastage through self-pollination (promotes male fitness). The second is self-incompatibility, which safeguards against self-fertilization and inbreeding depression (promotes female fitness) (Barrett, 2002).

Discrete sexual polymorphisms (i.e. dioecy and heterostyly) have been used as models for the evolution of reproductive strategies and in particular, sexual systems ever since Darwin (1877) first drew attention to their adaptive significance. To date, heterostyly is known to occur in at least 28 families of flowering plants (Barrett et al., 2000), including the family Plumbaginaceae, which provides one of the best examples of the complexity that heterostyly can attain.

Phylogenetic studies of the Plumbaginaceae have confirmed the monophyly of the two subfamilies Plumbaginoideae and Staticoideae, which are well differentiated by morphological, chemical, and molecular characters (Lledó et al., 1998, 2001). Plumbaginoideae comprise four genera, of which *Plumbago*,

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with approximately 20 species, is the largest. Heterostyly is known to occur in several different genera of both subfamilies (*Acantholimon*, *Armeria*, *Goniolimon*, *Limoniastrum* and *Limonium* in subfamily Staticoideae; and *Ceratostigma*, *Dyerophytum* and *Plumbago* in Plumbaginoideae) (Ganders, 1979 for heterostyly description). Three genera are found in southern Africa, *Dyerophytum* Kuntze, *Limonium* Mill. and *Plumbago* L. Dimorphisms in pollen size, pollen sculpturing and stigmatic surface occur throughout the family (Baker, 1948, 1953, 1966; Vuilleumier, 1967; Dulberger, 1975; Nowicke and Skvarla, 1977), accompanied in some cases by reciprocal herkogamy (e.g., some species of *Limonium*) but not in others (*Armeria*, *Limoniastrum*). Due to such a range of variation, Plumbaginaceae provides an ideal system in which to test hypotheses on the evolution of heterostyly. One of the most notable propositions to emerge from such studies in the family is that of Charlesworth and Charlesworth (1979), who hypothesized that heterostyly would have originated in an ancestor with strong inbreeding depression. The proposed evolutionary sequence starts with a change in pollen and a consecutive change in stigma types bringing about a heteromorphic incompatibility system and finally, only after incompatibility is established, does reciprocal herkogamy evolve. On the other hand, Lloyd and Webb (1992), following Darwin (1877) ideas, emphasized the initial evolution of reverse herkogamy as a mechanism promoting pollen transfer.

In view of these conflicting hypotheses and in order to provide the baseline data necessary to study the evolutionary steps leading to heterostyly occurring in the family, (evident by the wide variation in stylar polymorphism), it is useful to further describe heterostyly in the members of Plumbaginaceae. To this end, we investigated various aspects of the floral biology of *Plumbago auriculata* in a natural population in KwaZulu-Natal, South Africa. In particular, we characterized the morphometrics of the stylar polymorphism and compared pollen and nectar features between morphs. We also investigated the incompatibility system and the pollinator spectrum in this population.

## 2. Materials and methods

### 2.1. Study species

*P. auriculata* is a shrub, 0.3–1.39 m high, with leaves thin in texture and with minute glandular dots; the petiole is winged at the base and auriculate. The leaves are weakly discolorous, greyish green beneath and sometimes with whitish scales apparently for light reflection. The species is a common constituent of lowland scrub in eastern South Africa up to 1623 m altitude, and blooms mostly between November and May, although there are often flowers at other times of the year. The salver-shaped (hypocrateriform) flowers are pale blue, actinomorphic, and grouped in terminal inflorescences (Pooley, 1998; Aubrey, 2001).

Our study was carried in a natural population on the eastern edge of the Umkomaas Valley between Richmond and Ixopo in South Africa (S29° 58.946 E030° 14.918).

### 2.2. Stylar polymorphism and proportion of morphs

One flower per plant from a total of 52 shrubs (24 L-morph and 26 S-morph) was collected and kept in 70% ethanol until measurement. The number of flowers and inflorescences in 24 random plants of both morphs was counted. Flowers were slit longitudinally and various morphological parameters measured with a caliper to the nearest 1 mm. The following measurements were recorded: (1) corolla tube length; (2) style length, up to the stigmatic surface; (3) stamen height, up to the midpoint of every anther (one stamen measured per plant) (Fig. 1). Differences between the two morphs were tested by applying a *t*-test for independent samples. Additionally, with the flowers collected we estimated the morph ratio in the population and calculated the reciprocity between sexual whorls following Sánchez et al. (2008).

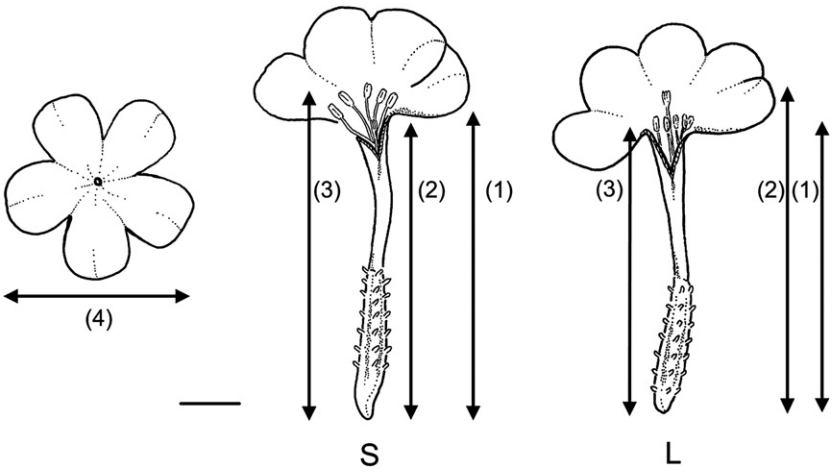


Fig. 1. Short-morph (S) and Long-morph (L) flowers of *Plumbago auriculata*. Numbers correspond to the morphometric measurements recorded for each flower: side view: (1) corolla length; (2) style length; (3) stamen height; front view: (4) corolla width. The top of the corolla tube was slit longitudinally to facilitate measurements. Scale bar = 10 mm.

### 2.3. Pollen characterization

To evaluate the number of pollen grains produced by flowers of each morph, two previously bagged flowers from each of 10 plants per morph were randomly collected before anther dehiscence and kept in 70% ethanol. A single anther was removed from each flower using a dissecting needle and placed on a microscope slide. Under a binocular microscope the anther was split, squashed on a drop of aqueous acid fuchsin (pollen stained dark red) and all pollen grains were counted under a microscope at 400×. The number of pollen grains produced per flower was estimated by multiplying the total pollen produced per anther by 5 (five anthers per flower). The pollen to ovule ratio (P/O) was calculated (Cruden, 1977). Differences in the pollen production by morph and pollen size were tested using a *t*-test for independent samples.

Scanning electron microscopy was used for the characterization of pollen size and shape. Pollen grains were placed on double-sided tape on metallic stubs and viewed at 15 kV in a Philips XL30 Environmental Scanning Electron Microscope (ESEM).

### 2.4. Nectar characterization

Twenty-four hour floral nectar production was assessed in ten plants of each of the two morphs. Three flower buds per plant were randomly chosen and enclosed using a mesh net to exclude pollinators. Twenty four hours after anthesis, nectar production was measured by inserting 1 µl microcapillary tubes. Sugar concentration was determined with a low-volume hand refractometer (Eclipse Handheld Refractometer, Bellingham & Stanley Ltd, Tunbridge Wells, UK). Corolla tube length (from the bottom of the corolla tube to the union of the petal tube and limb) and width (in the union of the petal tube and limb) were measured for every flower from which nectar was extracted. A *t*-test was used to analyze differences between morphs. The relationships between nectar features and morphological characteristics were analyzed through Spearman correlations.

### 2.5. Hand-pollination experiments

Controlled hand-pollination experiments were carried out in the field in order to determine if *P. auriculata* shows the typical sporophytic self-incompatibility system exhibited by many heterostylous species of Plumbaginaceae. Seven plants of each morph were randomly chosen and inflorescences covered using nylon gauze bags to exclude visitors. On each plant 4–8 mature buds on the point of opening were tagged and emasculated, and early the next morning the newly opened flowers were (a) self pollinated using pollen from flowers on the same plant, (b) cross-pollinated with pollen from another plant of the same morph (intra-morph crosses: L × L and S × S), (c) cross-pollinated with pollen from another plant of the opposing morph (inter-morph crosses: L × S and S × L) or (d) left unmanipulated to test spontaneous autogamy. For inter- and intra-morph crosses we used a mixture of pollen from different flowers that were situated 100–200 m away from the experimental plants. Two days after pollen supplementation one style per treatment was collected from each plant and placed in 70% ethanol for subsequent examination of pollen tube development. Pistils were cleared and softened with sodium hydroxide (8 N for 2 h), rinsed in distilled water and stained overnight with aniline blue (0.05% prepared in potassium phosphate 0.1 M) (Dafni et al., 2005).

Fruit development was checked after 2 weeks. All fruits produced were analyzed and seed production was determined by opening surgically the ripening fruits and counting the developing seeds. Fruit set after open pollination was assessed in the same tagged plants from three randomly selected inflorescences per plant. The number of pedicels (assuming that each pedicel represents a flower produced) plus actual fruits produced were counted.

### 2.6. Pollinators

The pollinator spectrum was investigated by monitoring floral visits for 20 different 15-minute periods over four days in November 2009 (total 5 h, 39 L-morph and 66 S-morph plants visited). Insect flower visitors were collected and taken to the laboratory for identification. Voucher specimens of insect pollinators are deposited in the Natal Museum, Pietermaritzburg.

The pollen loads transported by the proboscis of different visitors were determined by using cubes of stained glycerine jelly (2 × 2 × 2 mm), which were placed onto a pin and pressed onto the insect proboscis. The jelly was then transferred to a slide, melted, and all pollen grains counted. We expected that possible pollen losses due to visitors being shaken during capture would be similar across species and the comparisons among them would thus be meaningful.

All analyses were carried in SPSS 16 software. Statistical significance was assumed if the null hypothesis could be rejected at the $P = 0.05$ level in all cases.

## 3. Results

### 3.1. Flower morphometrics and pollen features

Two well-differentiated morphs were discriminated after the morphometric analysis. One morph presents the stigmas above the anthers (L-morph) and the other, stigmas below them (S-morph). Significant differences were found in corolla measurements (mean ± SE) between the two morphs, with a larger corolla in the S-morph (20.0 ± 0.00 mm in L-morph; 22.1 ± 0.08 mm in S-morph). As expected, the L-morph presented significantly larger styles than the S-morph (27.9 ± 0.07 mm in L-morph; 20.0 ± 0.00 mm in S-morph). There were also significant differences for the stamen height between morphs (Table 1), with anthers in the S-morph placed higher (23.3 ± 0.10 mm) than the L ones (20.0 ± 0.00 mm). See Table 1 for the results of the comparisons of all characters. The value for the reciprocity index was 0.013. Style length and stamen height were standardized by corolla length for graphical representation (Fig. 2).

The proportion of morphs (L:S) in the population was 1.17:1 ($N = 52$). The mean number of inflorescences per plant was 40.4

Table 1
Differences in floral display, floral morphology and nectar features in the two morphs of *Plumbago auriculata*.

| | *N* (L, S) | Mean±SD | | *df* | *t* |
|---|---|---|---|---|---|
| | | L | S | | |
| Number of inflorescences | 10, 14 | 23.80±19.90 | 40.43±36.58 | 20.8 | 1.301 |
| Corolla length (mm) | 28, 24 | 20.0±0.00 | 22.1±4.10 | 23 | 2.460* |
| Style length (mm) | 28, 24 | 27.9±4.20 | 20.0±0.00 | 27 | 9.950** |
| Stamen height (mm) | 28, 24 | 20.0±0.00 | 23.3±4.80 | 23 | 3.391** |
| Pollen production | 20, 19 | 1329±456.7 | 1261±338.5 | 37 | 0.525 |
| Pollen width (μm) | 16, 13 | 52.55±2.61 | 56.12±1.98 | 27 | 4.076** |
| Pollen length (μm) | 9, 6 | 57.17±2.86 | 66.13±3.17 | 13 | 5.701** |
| Nectar production (μl) | 10, 10 | 0.87±0.17 | 1.20±0.42 | 18 | −2.278* |
| Nectar concentration (%) | 10, 10 | 23.48±4.82 | 22.47±1.85 | 18 | 0.542 |

Values are means±1SD and differ significantly (between morphs) at *$P<0.05$, **$P<0.01$.

in S-morph and 23.8 in L-morph, but no significant differences were found between morphs (Table 1).

There were no significant differences between morphs for pollen production (Table 1). This means that there were also no differences found for the P/O ratio values, since there is only one ovule per flower. Pollen in the S-morph was significantly more elongated and wider than pollen of the L-morph (Table 1). Although some differences were found in the exine sculpturing in pollen grains, with the S-morph pollen tending to be more verrucate, these were not consistent across morphs (Fig. 3).

### 3.2. Nectar characterization

Significant differences in nectar volume, but not for nectar concentration, were found between morphs (Table 1). The S-morph produced more nectar (1.20±0.42 μl) than the L-morph (0.87±0.17 μl). Concentration ranged from 19.5 to 36.5% sucrose equivalents. Regarding relationships between nectar characteristics and floral morphology, there was a significant positive correlation only between corolla width and nectar volume (Spearman's $R=0.299$, $P=0.0213$).

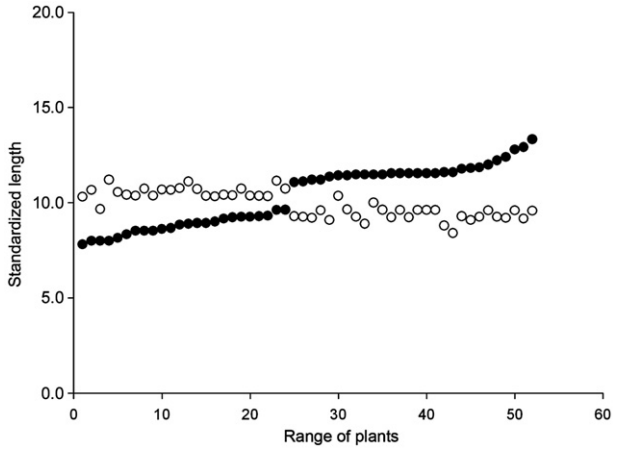


Fig. 2. Variation in the standardized height of style (●) and stamen length (○) in *Plumbago auriculata*. Individuals in each plot are ordered by increasing style length.

### 3.3. Hand-pollination experiments

Only inter-morph treatments produced fruits after hand pollination. There were no significant differences between morphs neither in seed production nor in natural fruit production (Table 2). Pollen tubes were only produced from inter-morph fertilizations, and their number decreases basipetally down the style (Table 3).

### 3.4. Pollinators

During the period of this study (November and December, 2008), long-proboscid flies (*Philoliche aethiopica*, Tabanidae) were the primary visitors to flowers of *P. auriculata* at the

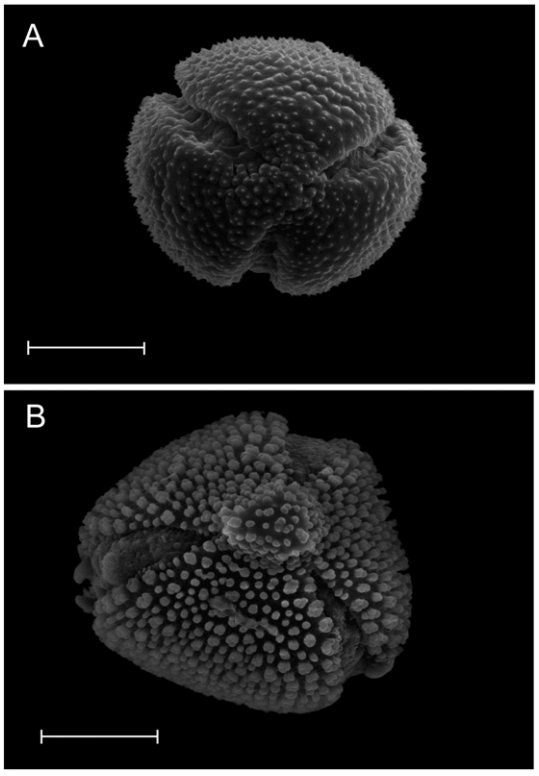


Fig. 3. Pollen grains of *Plumbago auriculata*. A) L-morph; B) S-morph. Scale bar=20 μm.

Table 2
Mean fruit set (%) of flowers of *Plumbago auriculata* after hand-pollination experiments.

| Treatment | *N* (L, S) | Fruit set (%) mean±SD | | *df* | *t* |
|---|---|---|---|---|---|
| | | L | S | | |
| Intra-morph | 11, 12 | 0 | 0 | | |
| Inter-morph | 12, 13 | 66.67±49.24 | 53.85±51.89 | 23 | −0.632 |
| Autogamy | 12, 14 | 0 | 0 | | |
| Insect exclusion | 14, 14 | 0 | 0 | | |
| Open pollination | 12, 19 | 26.72±25.58 | 23.11±22.86 | 29 | −0.409 |

*N*, total number of plants (L-morph and S-morph). Values differ significantly (between morphs) at $*P<0.05$, $**P<0.01$.

Richmond study site with occasional visits by the large swallow-tail butterflies, *Papilio demodocus* and *P. nireus*, smaller *Pieris* butterflies (Lepidoptera) and small, pollen-collecting bees in the family Halictidae (Table 4 and Fig. 4). Individuals of *P. aethiopica* carried the most pollen: up to 144 pollen grains per proboscis of ±20 mm long (Table 4). Unfortunately no information on the pollen loads or proboscis length was obtained for *Papilio* butterflies, but our observations suggest that they are potentially important pollinators of *P. auriculata*. Long tongue pollinators should be able to reach nectar produced by the plant (proboscis length longer or close to 20 mm).

## 4. Discussion

Flowers of *P. auriculata* show reciprocal herkogamy, with anthers and style differing in lengths between morphs and positioned reciprocally in flowers of both morphs. Reciprocity in the population of *P. auriculata* that we investigated was very high (i.e., low index value, 0.013) compared to published results for other species-values of 0.020–0.040 in distylous species of *Lithodora diffusa*, *L. moroccana* and *L. oleifolia* (Sánchez et al., 2008). Differences in corolla size between morphs have been associated with increases in reciprocity (Ganders, 1979). The longer corolla in the S-morph promotes reciprocal positioning of anthers to stigmas in the L-morphs where the style protrudes out of the corolla (Ganders, 1979). Marked differences in exine sculpturing on pin (L-morph) and thrum (S-morph) pollen are known in members of Plumbaginaceae (Baker, 1953, 1966), however, in *P. auriculata* the differences did not appear to be consistent in our surveyed samples. Differences in pollen size are usually associated with differences in pollen production (Ganders, 1979). In the *P. auriculata* population studied here, the L-morph produced greater numbers of smaller grains compared to the S-morph. The functional significance of these differences is unclear (see Dulberger, 1992, for a review).

Although reciprocal herkogamy in Plumbaginaceae is normally accompanied by differences in ancillary characters (i.e., pollen size and production; and papillae features) and a heteromorphic incompatibility system, as in *Dyerophytum* (Weber-El Ghobary, 1986), *Limonium* (Baker, 1966), in some cases, e.g., *Armeria* (Baker, 1966), there is a lack of reciprocal herkogamy, despite both the ancillary characters and the incompatibility being present. In *P. auriculata* the incompatibility system prevents selfing and intra-morph fertilization. As documented in other Plumbaginaceae, pollen rejection occurs at the stigmatic level, which is typical of a sporophytic incompatibility system (Baker, 1966). It seems that there is no gender specialization since no differences in fruit production were found between morphs.

The observed differences in pollen size may be linked to the incompatibility mechanism since it has been proposed that a unique gene controls the pollen morphology together with the incompatibility reaction of the pollen tube while another gene controls stigma morphology as well as its incompatibility reaction (Baker, 1966). However, lack of information on stigma morphology prevents us from drawing conclusions about the presence of morphological differentiation related to the incompatibility system. This aspect certainly warrants further investigation.

Although the data is somewhat limited, the main pollinators of *P. auriculata* in the Richmond population appear to be long-proboscid flies, butterflies and, less frequently, small bees (Fig. 4). Most insects, with the exception of bees, carry pollen on their proboscis and there was trend for insects with longer proboscides to have the largest pollen loads (Table 4). Long-proboscid flies and butterflies are nectar feeders, although some Bombyliidae can move pollen from their proboscis to a position where they can suck it up and ingest it (Szucsich and Krenn, 2000). Halictidae observed visiting the flowers of *P. auriculata*

Table 3
Number of pollen tubes growing in the styles of *Plumbago auriculata* after treatments.

| Treatment | *N* (L, S) | Distal mean±SD | | Medial mean±SD | | Proximal mean±SD | |
|---|---|---|---|---|---|---|---|
| | | L | S | L | S | L | S |
| Intra-morph | 7, 6 | 0 | 0 | 0 | 0 | 0 | 0 |
| Inter-morph | 4, 7 | 12±1.63 | 9.86±6.23 | 4.25±2.21 | 4±2.89 | 2.25±1.70 | 2.28±1.70 |
| Autogamy | 7, 8 | 0 | 0 | 0 | 0 | 0 | 0 |
| Insect exclusion | 5, 8 | 0 | 0 | 0 | 0 | 0 | 0 |

Positions: Distal, at the stigma level; medial, in the middle of the style; Proximal, at the level of the ovary.

Table 4
Insect visitors to flowers of *Plumbago auriculata* and their pollen loads.

| Orden | Insect | Total number of foraging bouts observed (%) | | No. captured insects | Number of pollen grains $x \pm SD$ | Proboscis length (mm) |
|---|---|---|---|---|---|---|
| | | L | S | | | |
| Diptera | *Philoliche aethiopica* | 19 (18.8) | 16 (15.8) | 7 | 61±17.78 | 21.6±0.4 |
| Hymenoptera | *Nomia* sp | 1 (0.9) | 2 (1.9) | 1 | 0 | 3.5 |
| Lepidoptera | *Colotis auxo* | | 5 (4.9) | 1 | 1 | 15.0 |
| | *Pieris* sp | 7 (6.9) | 26 (27.7) | 2 | 7±4.00 | 21.5±3.5 |
| | *Papilio* sp | 12 (10.8) | 16 (13.8) | | – | – |

are probably pollen collectors as they are unable to reach the nectar (Fig. 4D).

Species of Plumbaginaceae were included in Baker's (1966) comparative studies of breeding systems and the evolutionary buildup of the distylous syndrome, as a result of which he speculated that pollen dimorphism would have arisen at an early stage along with the genetic incompatibility system. This would be followed by modifications in style and stamen lengths leading to reciprocal herkogamy. Our observations therefore suggest that *Plumbago* is at the end of this particular evolutionary pathway.

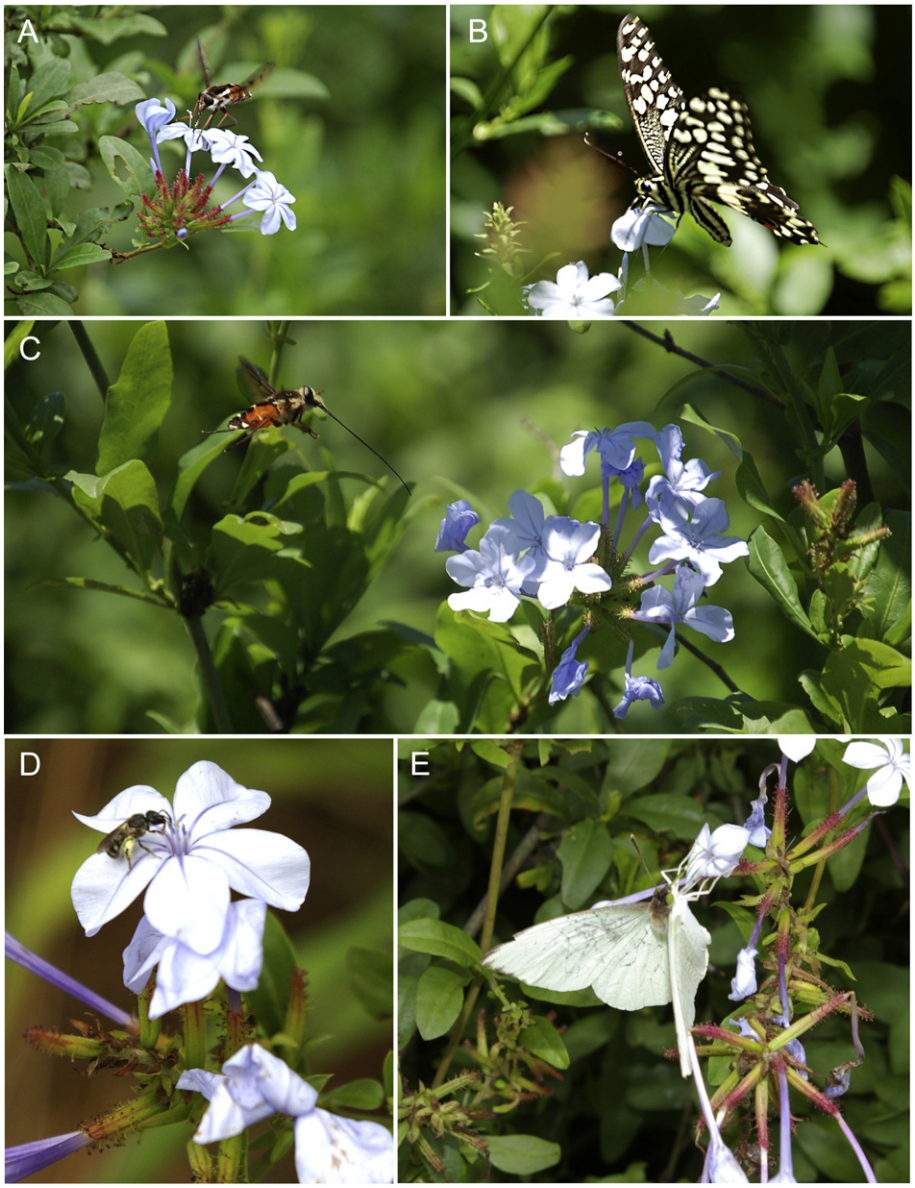


Fig. 4. Pollinators of *Plumbago auriculata* in the Richmond population. A, C) *Philoliche aethiopica*; B) *Papilio demodocus*; D) *Nomia* sp; E) *Pieris* sp.

However, a complete phylogenetic framework for Plumbaginaceae is required in order to reconstruct the patterns of character evolution in the family and thereby test Baker's hypothesis.

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