

# Pollinator-syndrome driven changes in the mating systems of two Cape Legume Species

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## ABSTRACT

The challenge in answering the question of whether or not plants evolve different mating systems to accommodate their respective pollinators lies in finding a pair of closely related species differing only in pollination syndromes. Furthermore, it has been hypothesized that Non-Flying Mammal Pollination (NFMP) arises from a bird pollinated ancestor as a result of their limited distributions. *Liparia splendens* subsp. *splendens* and *L. parva* are two genetically indistinguishable species that are thought to differ in pollination syndromes and co-occur with similar distributions, densities and have sympatric pollinators. Speculations that closely related sister species *L. splendens* subsp. *splendens* and *L. parva* are bird and nonflying mammal pollination (NFMP) respectively have been confirmed here. Furthermore, mating system divergence in terms of nectar volume and sugar concentration, pollen ovule ratios and self-compatibility has been investigated. There was no difference in nectar volume between the two species investigated, however, nectar concentrations have been found to be significantly higher in *L. parva*, which is thought to have evolved through selective pressures of pollinator preference. Similarly, pollen ovule ratios in *L. parva* (22663) are statistically higher than that of *L. splendens* subsp. *splendens* (17360), which is predicted to facilitate gene-flow between populations. Both species have been shown to have early-acting self-incompatible (ESI). Similar genetic variation and gene-flow of the two species in question suggest that NFMP is similar to that of bird pollination in its ability to maintain high levels of genetic diversity. The case of *Liparia* provides a basis to reject the hypothesis of NFMP evolution from a matrix of bird pollinated ancestors due to similar pollinator efficiencies.

## INTRODUCTION

The high diversity of plant species in the Cape region of South Africa has resulted in a wide range of pollination strategies; typically insect, bird and Non Flying Mammal Pollinated (NFMP) (Wiens, *et al* 1983; Rebello, 1987; Johnson *et al* 1997). Recent studies on pollination in the Cape have focused on the ability of pollinators to drive evolution; a concept known as the Geographic Pollinator Mosaic Hypothesis (GPMH). Because not all pollinators are everywhere, a plant expanding its range may be forced to adapt to local pollinators in a novel environment, ultimately leading to speciation. For example, Johnson *et al.* (1997) showed how floral tube length of the *Disa draconis* in the Cape region varies geographically and was correlated with the proboscis length of local long-tongued fly pollinators. Similar speciation events have occurred across pollination syndromes; Wiens *et al.* (1983) suggested that NFMP in *Proteaceae* evolved from a bird pollinated ancestor through a mechanism which is a subset of GPMH known as the Restricted Population Hypothesis (RPH). This describes how NFMP plant species exist in small metapopulations in a matrix of bird pollinated relatives; a bird flying through this matrix may be more likely to visit the more abundant species while ignoring the small populations. In contrast, given that

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rodents are generally residential, they may be expected to visit small populations within their foraging range (Wiens, *et al* 1983).

Regardless of the evolutionary processes involved, NFMP species generally have low-growing geoflorus flowers, such as in the *Proteaceae*, therefore accommodating for their non-aerial pollinators (Wiens *et al*, 1983). In contrast to NFMP species, plants relying on birds as a means of pollination are often equipped with brightly coloured flowers as an attractant (Shrestha, *et al* 2013). In addition to morphological changes, pollinators have also been known to drive changes in phenology; particularly in the Cape, NFMP species generally flower during the winter season (July-August), when rich nectar becomes a highly attractive resource when insects are inactive in the stressful conditions of Cape winters (Wiens *et al*, 1983). Alternatively, bird pollinated species flower during summer (December-March), when warm temperatures are more accommodating for bird activity (Wiens, *et al* 1983). Therefore, evolutionary factors contributing to the reduction in plant size (such as resprouting) and changes in phenology may indirectly be responsible for the evolution of NFMP by placing flowers close to the ground and providing nectar during times when it's most needed by small mammals.

While it is clear that pollinators can be responsible for changes in morphology and temporal variations in flowering, it has also been hypothesized that pollinator-syndromes may include changes in mating systems (Mitchell *et al*, 2009). However, studying pollinator driven selection of mating systems in plants has proved to be a difficult challenge due to confounding factors that may be responsible for these changes. For example, the question of whether or not the differences in pollen production between bird and NFMP species are entirely attributable to the behavior of their respective pollinators is dubious if other factors contributing to these changes are not controlled for. One major confounding factor here is the case of genetics. Phylogenetically unrelated plants may vary in pollen production simply due to their differences in genetics, which makes identifying the cause of varying pollen production difficult, especially with increasing phylogenetic divergence. For example, Wiens *et al* (1983) noted differences in mating systems between *Proteaceae* differing in pollination syndromes. Specifically, nectar concentrations are thought to have co-evolutionary relationships with the pollinators they intend to attract (Baker & Baker, 1975; Freeman & Worthington, 1985; Spira 1981). Furthermore, Cruden (1997) suggested that pollen: ovule ratios are an indication of the mating systems of a particular species. However there lies no confidence in attributing these changes to the effects of their respective pollinators as the differences may have arisen through other selective pressures acting on mating system divergence. The challenge in answering the question of whether or not plants evolve different mating systems to accommodate their respective pollinators therefore lies in finding a pair of closely related species that differ only in pollination syndromes. While studies on floral divergence with pollinator shifts is fairly common (Waterman *et al*, 2009), sister species differing in pollination syndromes is rare, and as a result so are appropriate studies addressing such questions.

Boatwright, *et al* (2008) has shown that sister species *Liparia splendens* subsp. *splendens* and *L. parva* of the Cape region are genetically indistinguishable and are thought to differ in pollination syndromes. The implication of the RPH/GPMH model is that bird pollination is superior to that of NFMP (Wiens, *et al* 1983). If this assumption is true, we might expect to see limited gene flow and increased levels of inbreeding within NFMP populations, such as in the case of *L. parva*. However, compared to bird pollinated species, a plant subject to limited gene flow due to poor pollinator efficiency might evolve more dependable mating systems to compensate for this loss. The case of *L. splendens* subsp. *splendens*

and *L. parva* therefore provides an excellent opportunity to investigate pollinator-syndrome driven changes in plant mating systems.

The aim of my paper is therefore to verify speculations of bird pollination of *L. splendens* subsp. *splendens* (Rebelo, 1987) and NFMP of *L. parva* (Letten & Midgely, 2009), as well as investigate mating system divergence between the two sister species in terms of pollen ovule ratios, nectar sugar concentration and volume as well as self-compatibility. Although *L. parva* has already been suggested as NFMP (Letten & Midgely, 2009), the methods used in this study are speculative as visitation and pollination was noted in an unnatural environment. However, it is hypothesized that *L. splendens* subsp. *splendens* and *L. parva* are bird and NFMP pollinated respectively. Furthermore, *L. parva* is predicted to compensate for poor NFMP pollinator efficiency by producing higher pollen ovule ratios, as well as increasing nectar sugar concentrations to accommodate for the preferences of NFMP (Wiens, *et al* 1987). Given the prediction that *L. parva* has evolved more dependable mating systems to cope with reduced NFMP efficiency, I hypothesize that self-compatibility is unlikely for both species.

## METHODS AND MATERIALS

### *Study sites*

Both study sites are situated in the southern horn of the Table Mountain National Park. The Cape region experience Mediterranean-type climate, receiving winter rainfall and summer droughts. Red Hill (34°11'30''S, 18°23'40''E) is approximately 250m above sea-level. 6800m north-west as the crow flies is Kommetjie – the second site used in this study. The Kommetjie site (34°09'06''S, 18°20'10''E) is approximately 130m above sea level, situated above a small town. Both sites have been burnt at least two years prior to the study.

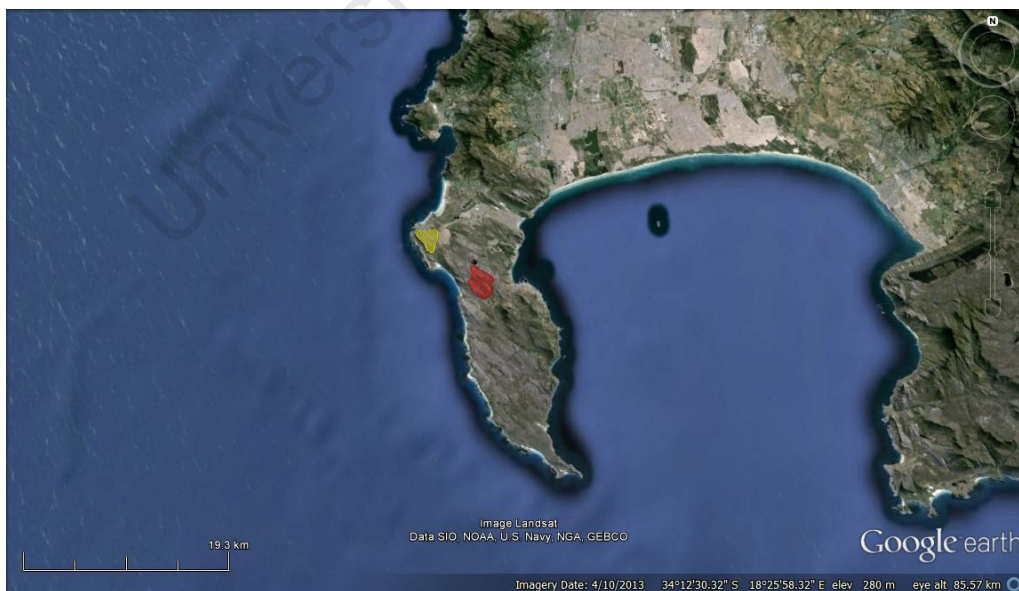


Figure 1: Google Earth image showing Red Hill (red polygon) and Kommetjie (yellow polygon) sites.

### *Self-compatibility*

To test for differences in seed-set between allogamy and xenogamy, 69 *L. splendens* subsp. *splendens* inflorescences were bagged in the budding stage of floral development from 36 separate individuals. Inflorescence condition and individual flower development was monitored prior to flower treatment. Throughout the duration of the bagging period, 32 inflorescences were discarded prior to the experiments due to fatality from unknown natural causes. Seven flowers were self-fertilized (pollen from own anther admitted to stigma) from seven different individuals to simulate xenogamous pollination. Similarly, an additional 14 flowers were treated on seven inflorescences; one flower selfed and one flower cross fertilized (pollen from a different individual applied admitted to stigma to simulate allogamous pollination). Furthermore, 12 bags were left alone to test for autonomous autogamy. After two months, seed set was recorded on all treated flowers.

Eight *L. splendens* subsp. *splendens* and 10 *L. parva* inflorescences, each from separate individuals, were collected and placed in water in a phytotron for two weeks. Flower development was monitored every second day in order to determine the capacity for autonomous autogamy. Development was categorized into four stages: (1) flower still in bud, (2) keel petal separates from corolla, (3) stigma covered in pollen penetrates corolla tube and (4) stigma devoid of pollen penetrates corolla tube. The proportion of stigmas penetrating the corolla with pollen was then calculated to get an estimate of autonomous autogamy. Furthermore, flowers were dissected at stages 1, 2 and 3 in order to determine to the position of the anthers in relation to the stigma at the various stages of floral development. This will allow for inferences to be made on the protandrous/protogynous nature of floral development.

In order to determine whether or not self-incompatibility exists and whether *L. splendens* subsp. *splendens* and *L. parva* are early or late acting, pollen tubes of experimentally pollinated flowers were examined under a fluorescent microscope; ten flowers of *L. splendens* subsp. *splendens* and *L. parva* were collected and placed in water in a phytotron. Ten and eight flowers from each species were self and cross fertilized, respectively. Following the methods of Zapata & Arroyo (1978), forty-eight hours after the fertilization, gynoecia were removed from the flower and stored in Carnoy's solution (1 glacial acetic: 3 95% ethanol) to prevent any further metabolic activity. Stigmas were then rinsed in distilled water twice for an hour to remove any excess alcohol. This was followed by an 8M NaOH (sodium hydroxide) treatment for three hours to soften the tissue. After two additional hour long distilled water rinses, the stigma were submerged in 20% H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide) for four hours in order to amplify the effects of the stain. Stigmata were rinsed again prior to the addition of the stain. An aniline blue stain was used and prepared using the addition of 21ml 1% aniline blue (0.2g Gurr aniline blue), 7ml K<sub>3</sub>PO<sub>4</sub> (potassium phosphate) (1.4g of grains/pellets in 7ml distilled water) and 182ml distilled H<sub>2</sub>O to make a 200ml 1% aniline blue stain solution. The stain solution was left in a 4°C fridge overnight to decolour. Stigmata were then mounted in glycerol and a drop of stain, after which were examined using the Zeiss axiocam camera, attached to the microscope. Pollen tubes were then noted and photographed at the stigma and approximately 0.5cm and 1cm into the style.

### *Nectar*

Nectar was extracted from 26 and 10 mature flowers from *L. splendens* subsp. *splendens* and *L. parva* respectively using a micro-syringe. After the total volume (µL) of nectar was recorded, a refractometer

was used to determine sugar concentration in % sugar. A paired t-test was performed at 95% confidence on the nectar and volume between species in order to determine statistical differences.

#### *Pollen & Ovule*

Pollen ovule ratios were calculated using modified methods from Zapata & Arroyo (1978). Seventeen *L. splendens* subsp. *splendens* and 16 *L. parva* flowers were picked prior to dropping their keel petals in order to ensure maximum development as well as to prevent any loss of pollen. Flowers were submerged in 3ml 95% glacial acetic acid in 1ml epindorphs and centrifuged at 1850 rpm (rotations per minute) for 15 minutes as to soften the tissue. After previous chemicals were decanted, 3ml of a dissolving solution (9 acetic anhydride: 1 sulphuric acid) was added to each of the epindorphs and placed in a 100°C water bath for 25 minutes. Epindorphs were removed from the water bath and centrifuged at 1850rpm for 10 minutes in the dissolving solution to isolate the pollen from the dissolved plant material. Dissolved plant material was decanted and isolated pollen was rinsed with distilled H<sub>2</sub>O. Pollen was then stored in 1ml 70% ethanol. A vortex was then used to distribute the pollen evenly throughout the ethanol solution, after which total pollen per flower was estimated using a hemocytometer. Ovules were counted under a dissection microscope and averaged. A pollen ovule ratio was then calculated by dividing the average pollen count by the average number of ovules. A 95% confidence paired t-test was performed on the two pollen ovules ratios in order to determine statistical differences.

#### *Pollinator observations*

A total of 11 hours was spent observing 47 *L. splendens* subsp. *splendens* inflorescences at Red Hill. Little Acorn camera traps were used to observe *L. parva* visitation. Forty-eight hours was spent observing 16 *L. parva* inflorescences at Red Hill, and an additional 48 hours spent observing 20 inflorescences at Kommetjie. Visitation events and visiting species were recorded for each species.

A visitation event can be defined here as a pollinator visiting on one or many flowers in the same inflorescence. For example, if a pollinator spent time foraging on many flowers within an inflorescence, one visitation event was noted. However, if a pollinator spent time on two inflorescences from the same plant, two visitation events were recorded. *L. parva* was visited in certain cases in which no role of pollination was evident; these visitation events have been termed as “robbing”. “Inflorescence hours” was calculated by multiplying the amount of inflorescences observed by the time spent observing them; this allows for a standardized time effort for both species. Inflorescence hours was then divided by 24 and then further by the amount of visitations in order to get a rate represented as average visitations per day for a single inflorescence.

## RESULTS

#### *Self-Compatibility*

Four of the cross-fertilization breeding experiments on *L. splendens* subsp. *splendens* produced seed pods which averaged 2.66 seeds per pod. One self-fertilization experiment produced one seed; however the

seed was poorly developed. Due to the low success of breeding experiments in *L. splendens* subsp. *splendens* flowers, this method of determining self-compatibility was discontinued for the remaining species, *L. parva*.

The floral development of *L. splendens* subsp. *splendens* was not consistent throughout all flowers monitored. Flowers develop at varying rates; certain flowers went through stages 1-4 overnight (see Fig. 1), whereas others remained in stage 1 throughout the 14 days of monitoring. 62.5% of *L. splendens* subsp. *splendens* flowers had emerged stigma covered in selfed pollen (Fig. 1; C), whereas the remainder of flowers (37.5%) had stigma void of any pollen (Fig. 1; D). Interestingly, one particular flower went through stages 1-4 and returned to stage 3, re-covering the stigma in its own pollen after it had been removed. Furthermore, two other flowers went from stage 3 to stage 2 and back to stage 3, which suggests that stigmata occasionally emerge and disappear in the space of at most 48 hours.

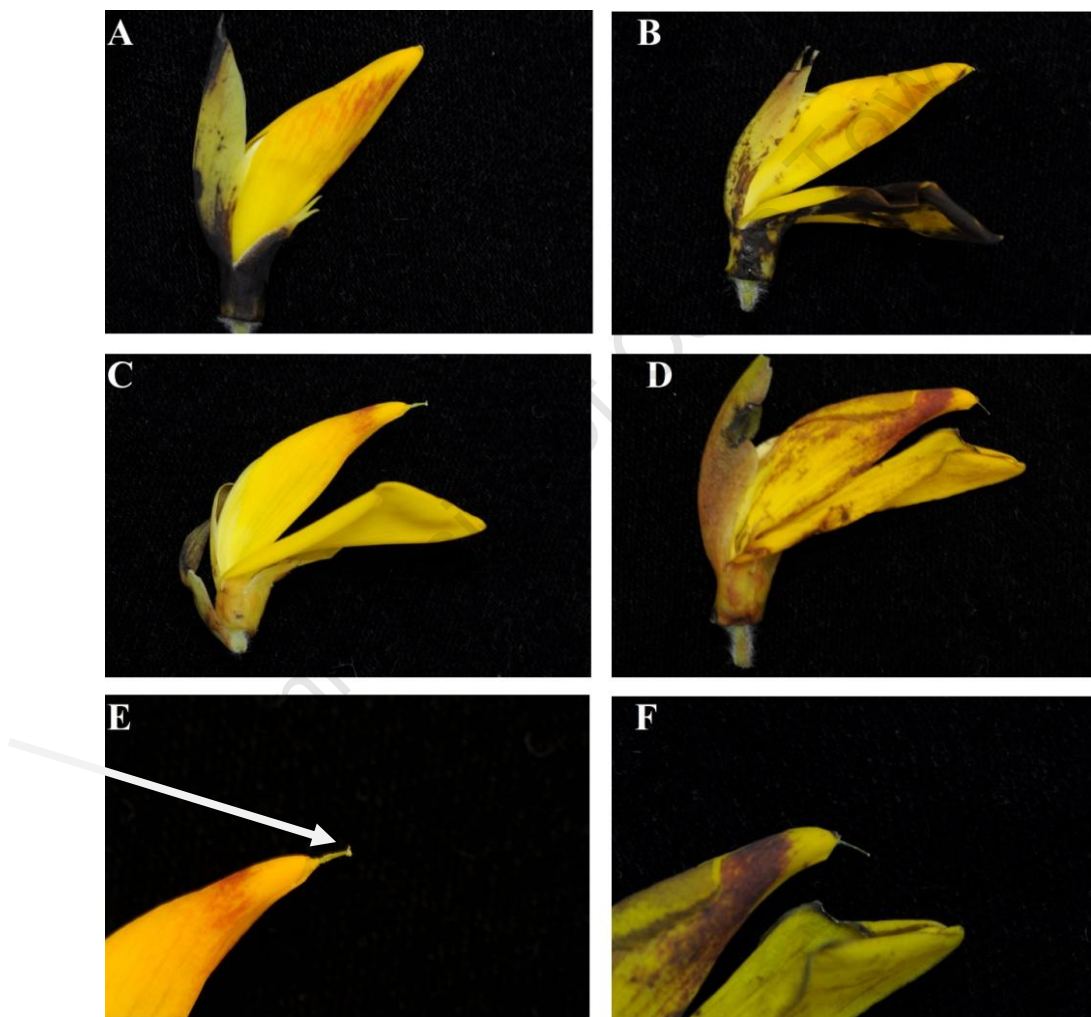


Figure 1: Development of *L. splendens* subsp. *splendens* in the laboratory. Photograph (A) represents stage 1 indicative of the flower still in the bud stage. Photograph (B) represents stage 2 as the keel separates from the main corolla tube. Stage 3 is represented by photograph (C) and photograph (D) represents stage 4. Photographs (E) and (F) represent a close-up images of the styles of photograph (C) and (D) respectively; note how stage 3 has pollen present on the stigma (photograph E) whereas stage 4 does not (photograph F). Arrow indicates self-pollen on the stigma.

*L. parva* had floral development dissimilar to that of *L. splendens* subsp. *splendens* in that none of the florets monitored had emerging stigmas. However, when florets were manually triggered by simulating a pollinator (photographs D & E, figure 2), selfed pollen was present on emerging stigmata. *L. parva* was similar to *L. splendens* subsp. *splendens* in that floral development was not consistent throughout the 10 flowers monitored; some flowers remained in stage 1, while others may have reached their final development stage (stage 3, figure 2, photograph C) over night.

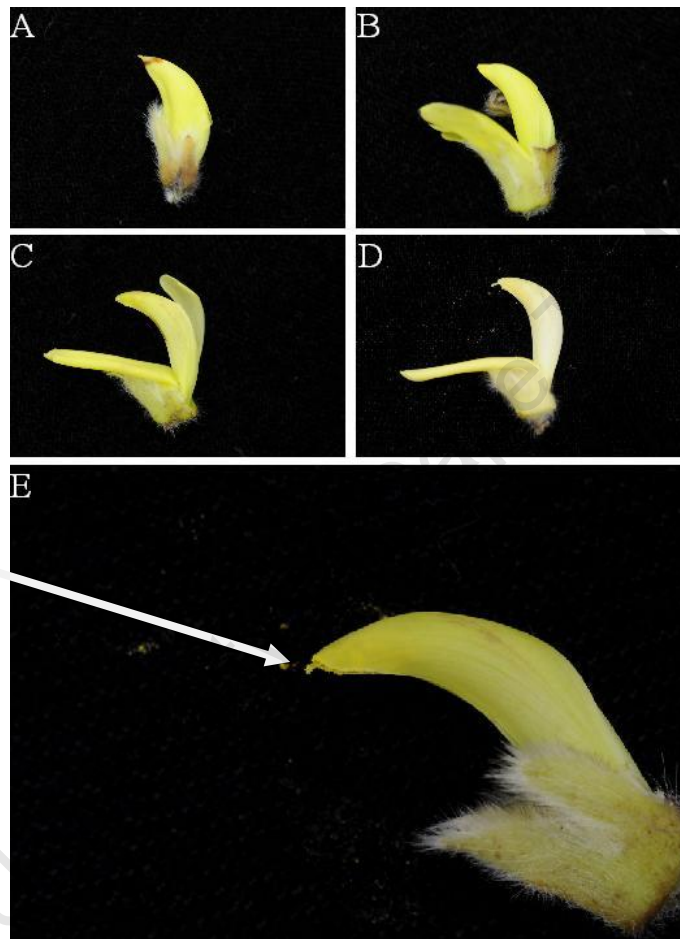


Figure 2: Development photographs of *L. parva*. Photograph (A) represents stage 1 indicative of the flower still in the budding phase. Photograph (B) stage shows the keel separating from the main corolla axis, following the description of floral development stage 2. A fully mature flower is shown in photograph (C), or stage 3 of floral development. Photographs (D) and (E) show stigma (arrow) covered in self-pollen after florets were triggered simulating a pollinator. Arrow indicates self-pollen on the stigma.

Dissection photographs (Fig. 3) show that the anthers and stigma of both *L. splendens* subsp. *splendens* and *L. parva* are spatially separated throughout all stages of development in that the stigma are consistently longer than the anthers. However, dissections of stages 2 & 3 for both species revealed compact anthers and stigma at the end of their corolla tubes; although stigma are always longer than anthers, if they are unable to penetrate the corolla tube it is likely they will receive pollen from their own anthers regardless of the differences in pollen/stigma lengths. The restrictions of the corolla tube on

stigma growth further emphasize the mechanical capabilities of *L. splendens* subsp. *splendens* and *L. parva* for autonomous autogamy.

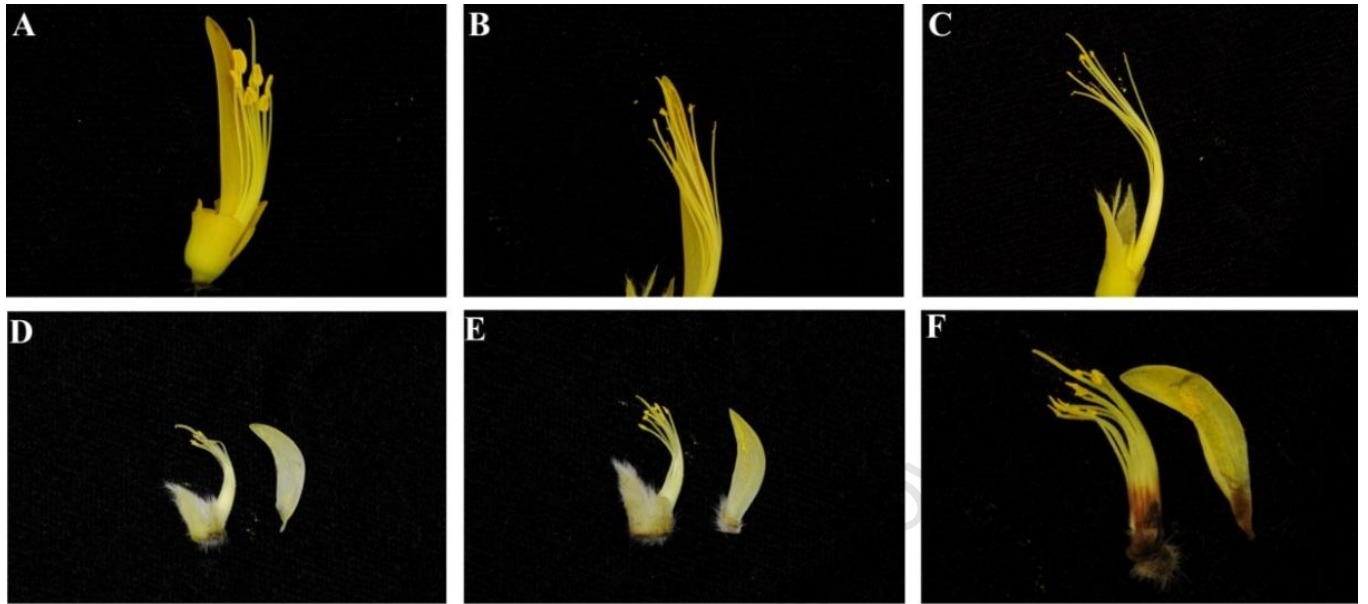


Figure 3: Dissection photographs of *L. splendens* subsp. *splendens* (A-C) and *L. parva* (D-F). Photographs A, B & C represent development stages 1, 2 & 3 of *L. splendens* subsp. *splendens*, respectively. Similarly, photographs D, E & F represent development stages 1, 2 & 3 of *L. parva*.

Pollen-tube analysis of *L. splendens* subsp. *splendens* and *L. parva* as a result of self-fertilization reveal that both species have early acting self-incompatible (ESI) (Fig.4); self-pollen is capable of germinating, however, pollen tubes are rejected approximately half way down the style for both species. Pollen-tubes as a result of cross-fertilization were observed growing into the ovary.

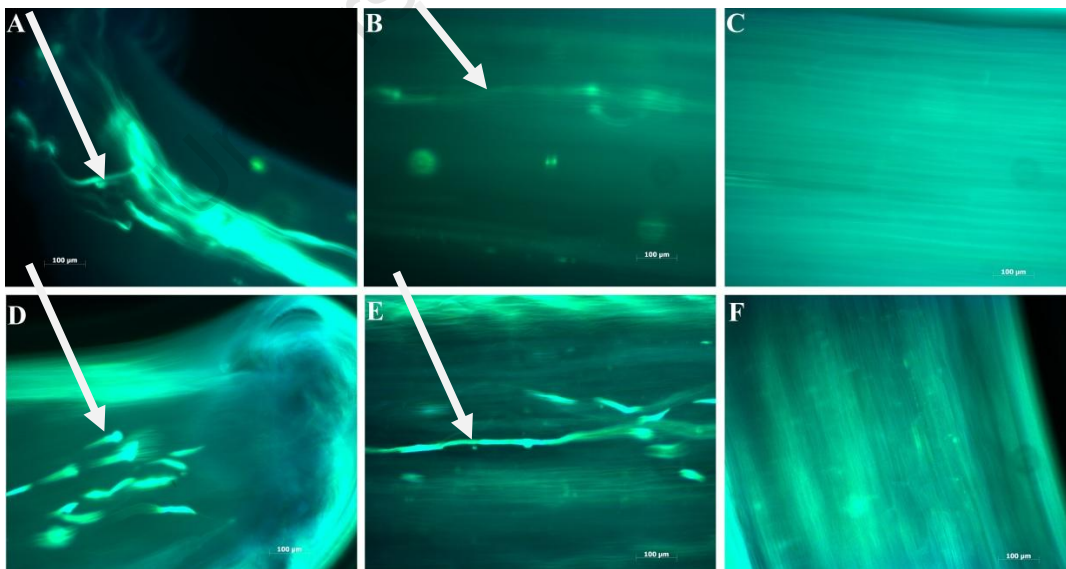


Figure 4: Self-pollen tube germination of *L. splendens* subsp. *splendens* (A-C) and *L. parva* (D-F). Photographs (A) & (D) are taken at the stigma, photographs (B) & (E) at 0.5cm from the stigma and (C) & (F) taken 1cm from the style. Arrows indicate pollen tubes.

## Nectar

Despite *L. splendens* subsp. *splendens* flowers being almost twice the size of *L. parva*, nectar volume production between the two species is not significantly different from one another ( $p=0.34$ ,  $t=0.97$ ,  $df=34$ ). However, *L. parva* had approximately twice the sugar concentration of *L. splendens* subsp. *splendens* ( $\bar{x}=32.00\mu\text{L}$  &  $\bar{x}=16.32\mu\text{L}$  respectively) and is statistically highly significant ( $p < 0.01$ ,  $t=-9.43$ ,  $df = 34$ ).

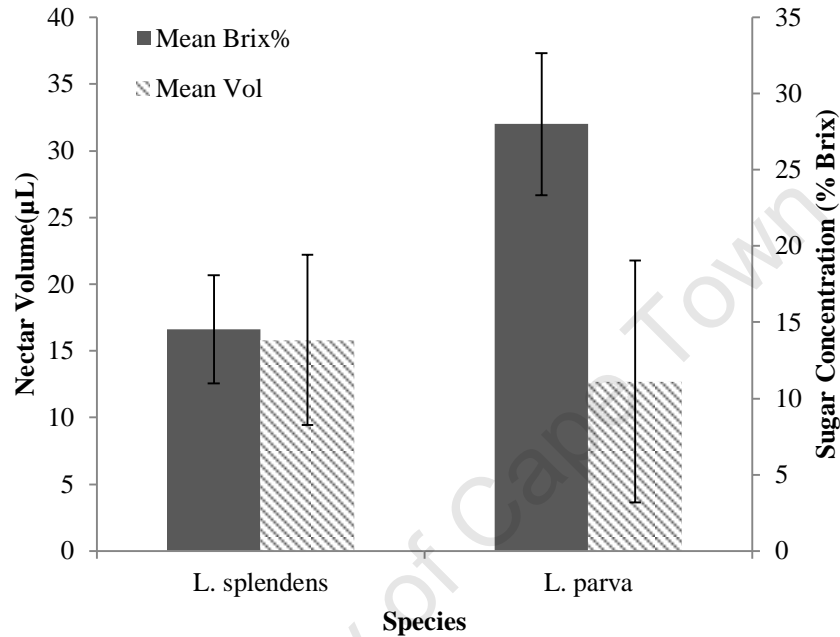


Figure 5: Nectar Volume ( $\mu\text{L}$ ) and Sugar Concentration of *L. splendens* subsp. *splendens* and *L. parva*.

## Pollen & Ovule

Pollen ovule ratios between *L. splendens* subsp. *splendens* and *L. parva* are statistically different from one another ( $p = 0.02$ ,  $t=-2.55$ ,  $df = 31$ ) (Fig. 5). The difference in pollen ovule ratios described arises from differences in pollen production and not rather through differences in the number of ovules produced. Ovule counts for *L. splendens* subsp. *splendens* ( $\bar{x}=6.4$ ) and *L. parva* ( $\bar{x}=6.9$ ) are not statistically different from one another ( $p < 0.01$ ,  $t=-1.28$ ,  $df=31$ ), however; *L. parva* produces more pollen ( $\bar{x}=157222$ ) per flower than *L. splendens* subsp. *splendens* ( $\bar{x}=111307$ ) with high significance ( $p < 0.01$ ,  $t=-8.14$ ,  $df=31$ ).

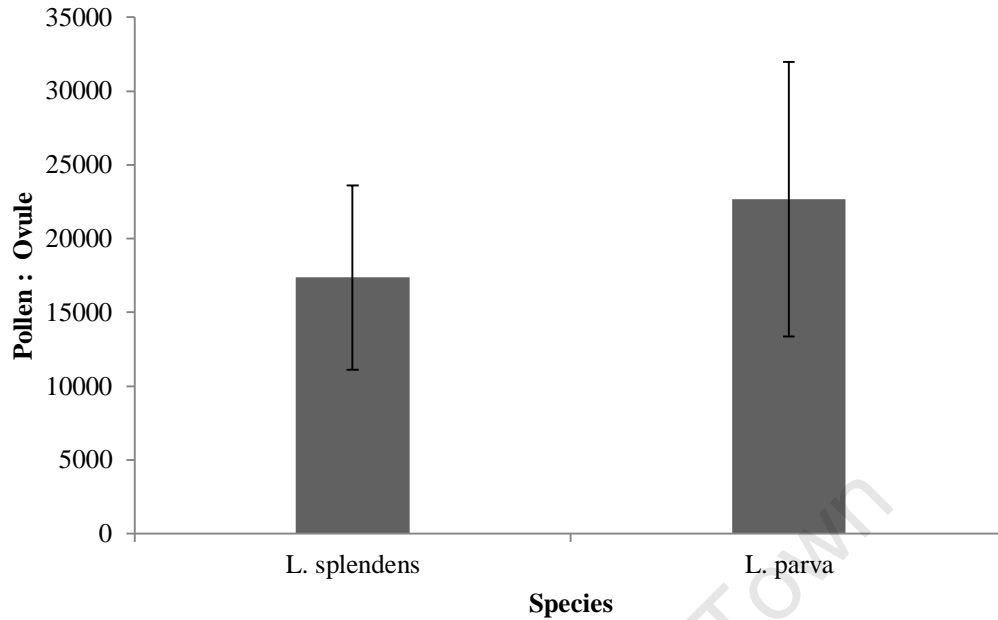


Figure 6: Pollen ovule ratios of *L. splendens* subsp. *splendens* and *L. parva*

### Pollination

The data obtained in this study suggests that *L. splendens* subsp. *splendens* and *L. parva* are largely pollinated by Orange Breasted Sunbirds (OBS) *Anthobaphes violacea* (Fig. 6) and the Cape Spiny Mouse *Acomys subspinosus* (Fig. 7) respectively. Visitation rate is far higher in *L. splendens* subsp. *splendens* than in *L. parva*. In addition, 960 inflorescence hours at the Kommetjie site produced no evidence of *A. subspinosus*, let alone pollination events; however, *L. parva* had a visitation rate of 0.31 visitations per day at Red Hill. Furthermore, in addition to having no pollination, *L. parva* experienced robbing by OBS at the Kommetjie site, whereas no OBS visitation was found at Red Hill.

Table 1: Visitation data of *L. splendens* subsp. *splendens* and *L. parva*.

Species	Site	Flower hours	Pollination events	Robbing events	Visiting Species	Visitation rate(visitations/day)
<i>L. splendens</i> subsp. <i>splendens</i>	Red Hill	520	55	0	<i>Anthobaphes violacea</i>	2.54
<i>L. parva</i>	Red Hill	768	10	0	<i>Acomys subspinosus</i>	0.31
<i>L. parva</i>	Kommetjie	960	0	8	<i>Anthobaphes violacea</i>	0

## DISCUSSION

### *Pollination*

Factors contributing to the differences in visitation rate between *L. splendens* subsp. *splendens* and *L. parva* might be; (1) that rodents are generally under more threat of predation than birds and might expect to see lower visitation of NFMP species in the area, and (2) that rodents are generalist foragers, only partially relying on the nectar of *L. parva* as food source. On the other hand, birds, in particular OBS, are nectarivores and might be expected to visit *L. splendens* subsp. *splendens* more frequently as they depend on the resources provided by these flowers as a major food source. Interestingly, at the Kommetjie site, where no visitation on *L. parva* by *A. subspinosus* was found, video footage of a rare Cape feline *Genetta tigrina* was found. Furthermore, it is suspected that rodents at the Kommetjie site may be under predation by domestic cats from the surrounding town. Kommetjie therefore assumes unnatural conditions for a pollination study, however; visitation rates given for Red Hill are thought to be appropriate for natural conditions.

Visitation of *L. parva* by OBS is assumed to play no role in pollination; the bill length of OBS is longer than that of the anther-nectary length (Fig. 8), suggesting appropriate pollen deposition is unlikely to occur. In one particular robbing event, pollen was deposited on the bill of an OBS, which was subsequently removed by shaking the head from side to side. Furthermore, all but one OBS robbed *L. parva* from the side of the corolla tube, which is unconventional for natural *Liparia* pollination; *L. splendens* subsp. *splendens* visitation by OBS occurs by approaching a flower directly, resulting in pollen deposition on the back of the head (Fig. 7).



Figure 7: Orange Breasted Sunbird visitation on *L. splendens* subsp. *splendens*. Note the deposition of pollen on the forehead.

## Nectar

In this study we hypothesized the *L. parva*, being NFMP, would have higher nectar sugar concentrations as to accommodate for the tendency of small mammals to prefer sweeter nectar (Wiens, *et al* 1983). Given the phylogenetic proximity of the two species in question, the higher concentrations of nectar found in *L. parva* can be assumed to have evolved through selective pressures of pollinator preference. Wiens *et al* (1983) reported on similar nectar concentrations for bird and NFMP in the *Proteaceae* and described these differences as “adaptations” to the respective pollinator-syndromes. Although variations in nectar concentrations in plant species may be relatively distinct between NFMP and bird pollinated species, they should be used conservatively when determining unknown pollination-syndromes. Through experimental tests, humming birds were found to prefer nectar with higher sugar concentrations, regardless of the concentration of their naturally visited plant species (Hainsworth & Wolf, 1976; Stiles, 1976; Tamm & Gass, 1986). If nectar concentrations really are an adaptation to pollinator preference, this would support the hypothesis that NFMP species evolve from a bird pollinated ancestor as there would appear to be very little selective pressure for a bird pollinated species to reduce their sugar concentrations. To my knowledge, this is the first definitive evidence suggesting co-evolutionary adaptations between nectar and the pollinators they intend to attract.

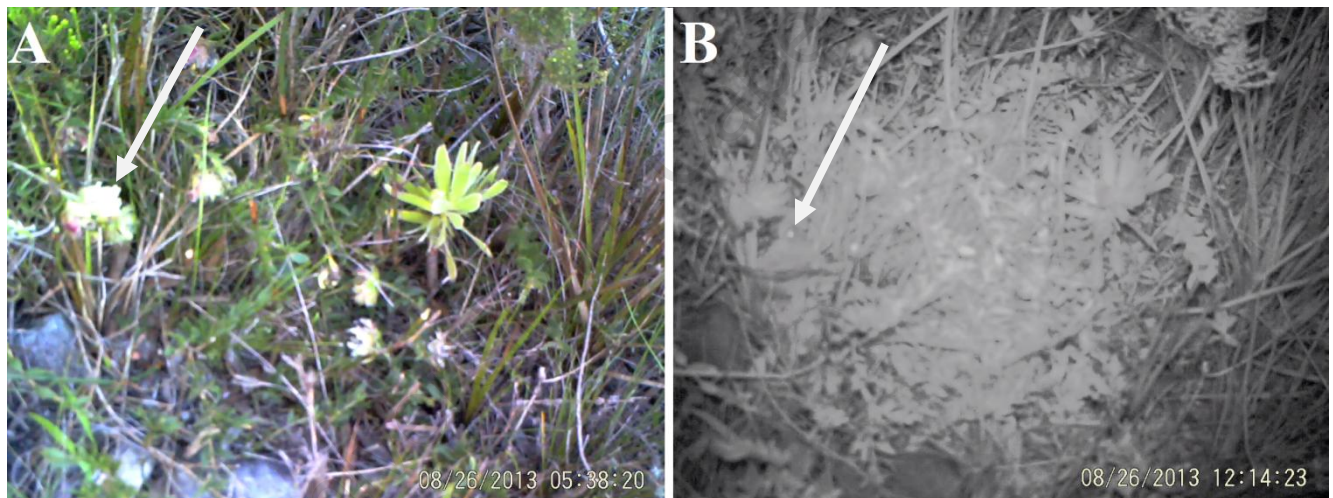


Figure 8: *Achomys* visitation of *L. parva*. Note position of visited flower in photograph (A), and *Achomys* visitation on marked flower in photograph (B). Arrow (A) indicates the flower which was visited by *Achomys* represented by arrow in (B).

## Pollen Ovule

As suggested by Cruden (1977), the pollen ovule values given in this paper indicate that both *L. splendens* subsp. *splendens* and *L. parva* are obligate xenogamous. Identifying mating systems by using these pollen ovule criteria may not be accurate for all species, particularly as in the case of *Liparia* as both species investigated here have pollen ovule ratios far superior to that of the maximum reported by Cruden (1977). However, a study investigating 32 *Fabaceae* species report on a pollen ovule range of 911-23000 (Galloni, *et al* 2007). Both species of *Liparia* investigated here lie within the upper quartile of this range and whether or not the numbers produced by Cruden (1977) are applicable for all families, high pollen production in general is likely to be indicative of xenogamy. *L. splendens* subsp. *splendens* and *L. parva*

both seem to produce relatively high pollen ovule ratios, therefore supporting the classification of obligate xenogamy.

Flower size in *Fabaceae* has been shown to be proportional to pollen production in that larger flowers generally produce larger amounts of pollen (Galloni, 2007); the exception to this rule found in the case of *Liparia* suggests a strong evolutionary selection for high pollen production in the smaller flowers of *L. parva*. Given that both species are incapable of autogamy and that seed set is limited by pollen production (Ehrlen, 1992), it would appear that *L. parva* produces more pollen to compensate for a lower NFMP efficiency.

#### *Self-compatibility*

Breeding experiments in *Fabaceae* in general are rarely successful (Soboda, et al 2012; Galloni, 2007), which has been attributed to hand-pollination experiments failing to mimic the actions of their natural pollinators (Lord & Kohorn, 1986). Often in *Fabaceae*, the presence of a stigmatic cuticle prevents pollen from germinating if it is not ruptured by the specific actions of their respective pollinators (Sahai, 2008). This mechanism has evolved to prevent autonomous autogamy in that if pollen lands on the stigma of the same flower as a result of their close proximity, because there is a lack of pollinators the stigmatic cuticle remains intact and pollen is therefore unable to germinate (Aronne, 2012; Valtuena, 2008). In addition to this, hand-pollination experiments in *Fabaceae* are difficult purely because of the naturally low seed set; Amorim (2013) reported on a 5% seed set in the *Inga sessilis*. There seems to be a similar case for that of *Liparia*, in particular *L. splendens subsp. splendens*, as of the 69 flowers xenogamously hand-pollinated, a seed set of 5.8% was recorded in this study. Whether or not this value indicates a true reflection of the natural seed set, or whether the hand-pollination experiments performed simply did not sufficiently rupture the stigmatic cuticle may be something for further investigation.



Figure 9: Orange Breasted Sunbird robbing *L. parva*. Note lack of deposition of pollen on head.

The floral development of both *L. splendens* subsp. *splendens* and *L. parva* indicates that autonomous autogamy is likely; the majority of the flowers in *L. splendens* subsp. *splendens* were subject to self-pollen deposition on the stigmas simply through structural changes in flower morphology leading up to anthesis. As the stigmas of *L. parva* rarely penetrated the corolla tube without pollinator facilitation to activate the pump mechanism, the ability for autonomous autonomy in this species is less obvious. However, dissections of the flowers through the three major stages of development (Fig. 3) reveal relatively close proximity of anther and stigma through all stages of development, particularly in the final stages. As the corolla tube acts as a barrier to the growth of the stigma, a reduction in anther-stigma distance would suggest increased likelihood of autonomous autogamy. The same seems to be the case for *L. splendens* subsp. *splendens*, so whether or not selfed-pollen is visible on the stigma, for both species, the likelihood of autonomous autogamy appears to be relatively high.

The ability of a plant to reproduce autogamously depends on mechanical and biological factors; the pollen of a flower may be physically capable of reaching its own stigma, however, whether pollen tube germination will be accepted to produce viable seeds depends on compatibility pathways (Arroyo, 1981). For example, Amorim (2013) found that *Inga sessilis* is incapable producing pollen tubes from selfed-pollen, whereas Borges, *et al* (2009) noted the autogamous pollen tube penetration of the ovules in *Caesalpinia echinata*, however no seeds were set. Both of these species are self-incompatible (SI), but rejection of self-pollen occurs in different ways; early acting (ESI) and late acting self-incompatible (LSI), respectively.

TABLE 2: Standard diversity indices for *L. parva* and *L. splendens*. Number of alleles (N<sub>A</sub>), observed heterozygosity (H<sub>O</sub>), expected heterozygosity (H<sub>E</sub>), and Exact test for significant departure from Hardy-Weinberg equilibrium for each microsatellite locus for *L. parva* and *L. splendens*. (Table extract from Illing *et al*, in prep.)

Locus	Allele size range (bp)	<i>L. parva</i> (rodent)					<i>L. splendens</i> (bird)				
		N <sub>A</sub>	H <sub>O</sub>	H <sub>E</sub>	p-value	F <sub>IS</sub>	N <sub>A</sub>	H <sub>O</sub>	H <sub>E</sub>	p-value	F <sub>IS</sub>
LspIAAT2	292-310	7	0.504	0.744	<0.001	0.326	4	0.343	0.513	<0.001	0.332
LspIXGT2	223-243	8	0.416	0.709	<0.001	0.414	4	0.500	0.500	0.961	0.0001
LparXAG2	394-426	10	0.624	0.770	0.002	0.189	7	0.615	0.643	0.786	0.043
LparXAG3	340--394	20	0.677	0.843	<0.001	0.197	16	0.663	0.695	0.001	0.046
Mean		11.25	0.555	0.766			7.8	0.530	0.587		
Std Error		5.17	0.102	0.049			4.9	0.123	0.083		

Illing *et al* (in prep.) found very low levels of inbreeding in the Cape Peninsula populations of *L. splendens* subsp. *splendens* and *L. parva* (Table. 2) which supports the breeding system classification of obligate xenogamous. Within *Liparia*, self-incompatibility and their relatively long-lived life histories (due to their ability to resprout) may be evolutionary adaptations to purge the otherwise likely effects of inbreeding depression. For example, Severns *et al* (2011) found reduced population viability in the threatened legume *Lupinus oreganus* as a result of high levels of geitonogamy and inbreeding; *Liparia* may well have suffered the same fate had it not been for the selection against self-fertilization.

Furthermore, inflorescence-type flowering in *Fabaceae* is relatively rare and as a result may increase the likelihood of geitonogamy; having many flowers in close proximity to one another may increase the likelihood of visitation within a plant rather than between plants. Therefore, having relatively large pollinators may have driven the evolution to have larger, more attractive inflorescences rather than a single, solitary floret. The behavior of both pollinators is such that visitation of various flowers on a single plant is common, and as a result, geitonogamous visitations may have been a frequent mode of pollination had it not been the evolution of their self-incompatibility. Soboda *et al* (2012) found that in the legume *Hedysarum boreale*, seeds produced xenogamously are far more likely to survive; the low survivability of autogamous seeds, presumably through inbreeding depression, may therefore be a further driver in the evolution of obligate xenogamy in the harsh conditions of the Cape Peninsula.

NFMP has been shown by Illing *et al* (in prep.) to result in significant levels of gene-flow in *L. parva* populations of the Cape Peninsula. Although increased pollen production here may marginally facilitate gene-flow, this seems to be a relatively insignificant adaptation to dealing poor pollinator efficiency. If *L. parva* populations were suffering genetic consequences as a result of limited gene-flow, we might expect to see the evolution of self-compatibility. Therefore, NFMP should not be seen as a “fail safe” option for small, fragmented populations under threat of reduced visitation by birds. On the contrary, NFMP is likely to be as efficient at moving pollen over large distances. In addition to this, genetic diversity (Table. 2) in *L. parva* is similar to that of its bird-pollinated sister species, *L. splendens* subsp. *splendens*. Given that both species are myrmecochorous, self-incompatible and that population size/density between species appears to be similar, the role of both pollinators investigated here can be assumed to be similar in their abilities to maintain high levels of genetic diversity.

*L. parva* seems to disprove the evolutionary origins of NFMP as proposed by the GPMH; both species of *Liparia* here seem to display equal distributions and densities and have relatively wide-spread sympatric pollinators. The implication of the GPMH/RPH model is that birds are superior to NFMP in terms of pollinator efficiency; the results provided by this study prove that this is not always the case. The evolution of rodent pollination in *L. parva* may therefore have been the consequence of changes previously mentioned by Wiens, *et al* (1983), rather than the result. These include (1) the low growing nature of *L. parva*, as a result of their ability to resprout, may have evolved in response to dealing with fire-prone ecosystems, rather than to accommodate for NFMP, and (2) that producing nectar during winter is simply less demanding on resources. Other possible factors leading to the reduction in plant stature, and as a result NFMP, may include an allometric reduction in leaf and inflorescence size which results in reduced water loss, which can be seen as an attractive adaptation to drought-prone summers of the Cape Peninsula (Midgley & Bond, 1989).

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