BUZZING BEES AND THE EVOLUTION OF SEXUAL FLORAL DIMORPHISM IN AUSTRALIAN SPINY *SOLANUM*

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ABSTRACT

The flower morphology and reward availability of animal pollinated plants are intrinsically related to the foraging behaviour and preferences of their pollinators. However, it is often difficult to test how pollinator preferences may have helped to shape floral morphology because the morphology of many animal pollinated flowers is an adaptive compromise to optimise both male and female function. This may be overcome by studying the foraging decisions of pollinators in relation to flower morphology of species with unisexual flowers. The inherent difficulty of studying diclinous species is that in nearly all of these species the flowers of different sexes do not only differ in morphology, but also in reward type: male flowers offer pollen and possibly nectar, while female flowers offer nectar only.

Solanum is an ideal genus to investigate evolutionary links between pollinators and flower morphology for two reasons. First, it demonstrates a large variation in sex system with hermaphrodite, andromonoecious and dioecious species. The diclinous species of *Solanum* have evolved sexual dimorphisms involving floral size and the number of flowers per inflorescence. This variation allows the evaluation of floral morphology in a phylogenetically informed way. Second, pollen is the only reward, and is present in apparently equal amounts in both male and hermaphrodite/female flowers. This allows the investigation of sexual floral morphology in the absence of differences in reward type and amount. The genus *Solanum* is further suitable for such investigations because it relies for pollination on a relatively small number of buzz-pollinating bee species.

The main objective of this study was to examine how sexual dimorphisms in floral display and reward availability influence bee foraging behaviour, as this could lead to an understanding of the evolution of floral traits in association with changes in sex systems in the Australian members of *Solanum* subgenus *Leptostemonum*. To investigate this, buzz pollinating bees were tested for their responses to dimorphisms in three floral traits: corolla size, flower number and style length. Although *Amegilla murrayensis* had an initial preference for larger flower size, this preference quickly disappeared in the absence of differences in rewards among flowers. Clusters of flowers were more attractive than solitary flowers, even when the clustered flowers were smaller in size. In another experiment, *Amegilla chlorocyanea* showed no differences in the number of buzzes and time spent on each buzz between two floral morphs of andromonoecious *Solanum elaeagnifolium* that differed in their style length. Furthermore, foraging decisions by individual bees were analysed in relation to variation in pollen availability. *Amegilla chlorocyanea* showed no difference in the total number of times they buzzed pollen-full and pollen-empty flowers before they left the patch. However significant differences observed between first and second visits to flowers indicated that bees could perceive recent visitation by a bee and adjust their visitation behaviour.

Since an overall lack of support was found for the evolution of floral sexual dimorphism as a direct response to bee foraging preferences, a phylogenetic analysis was performed to investigate other possible explanatory models for the evolution of floral dimorphism in the diclinous species of *Solanum*. First, molecular phylogeny was inferred based on three gene region sequences of

71 Australian members of *Solanum* subgenus *Leptostemonum.* The analysis showed that the evolution of andromonoecy from hermaphroditism is most likely preceded by the evolution of large fruit, and thus the selection for large fruit size is the main driving force for the evolution and maintenance of andromonoecy in this group.

DECLARATION

I, Arthur Selwyn Mark, certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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ARTHUR SELWYN MARK Date .03.2014

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CHAPTER ONE - INTRODUCTION

Floral structures for animal pollination

Almost three-quarters of flowering plants in the world rely on pollinators to transfer pollen among flowers (Berenbaum et al. 2007). Plants and their pollinators have a mutualistic relationship: pollinators transfer pollen to another flower and plants supply dietary requirements to the pollinators. This mutualism has led to coevolution of plants and their pollinators. The diversification in floral features such as shape, colour, odour and rewards has evolved in conjunction with the morphology and behaviour of pollinators and vice-versa. The plant-pollinator interaction has been fascinating ecologists ever since coevolution theory, wherein both the pollinators and plants undergo reciprocal evolutionary change, was proposed (Darwin 1876; Thompson 1994).

Plants have evolved diverse floral forms. When they reflect some form of specialisation involving particular groups of pollinators, the convergent phenotypic traits are called 'pollination syndromes' (Faegri and van der Pijl 1979 ; Stebbins 1970). Fenster et al. (2004) defined a pollination syndrome as 'a suite of floral traits, including rewards, associated with the attraction and utilisation of a specific group of animals as pollinators'. These specialised floral traits reflect adaptive responses to selection pressures by functional groups of pollinators. Since the direction of selection on floral traits largely depends on pollinator morphology and behaviour, different pollinators belonging to a functional group are expected to have similar behaviour on a flower and exert similar selection pressures on floral traits (Armbruster et al. 2000; Fenster et al. 2004).

Specialisation in pollination

The concept of 'pollination syndrome' in plants demands some sort of specificity in pollination, which means pollination is achieved only by a small group of potential pollinators. Floral traits that have evolved as a result of specialisation in pollination act as mechanisms through which plants attract potential pollinators and filter the visits from inefficient pollinators. Such specialised pollination syndromes involve various combinations of floral features including shape, size, colour, symmetry, scent, floral display, rewards and flowering time. For instance, flowers with shorter corolla tubes are frequently visited by short-tongued bees (Schemske and Horvitz 1989). Some species of *Dalechampia* are pollinated exclusively fragrance-collecting male *Euglossine* bees (Armbruster 1993). And odourless bilabiate flowers coloured white, purple, yellow and orange with large floral tube offering rich nectar corresponded to the preference of long-tongued solitary bees (Momose et al. 1998).

Although specialisation in floral visitation and pollination has been largely studied in bisexual plants, some diclinous plants have also been analysed for the existence of strong correlations between floral traits and pollinator groups. Bawa (1980) proposed that dioecious species have evolved more generalised flowers in the presence of unspecialised multiple pollinators. However, this hypothesis refuted by clear evidence for presence of specialised flowers adapted to specific pollinators in some dioecious species (Renner and Feil 1993).

Specialisation in unisexual species

Specialisation in floral traits, including floral rewards, is closely associated with the mating success of plants. Floral traits become fixed throughout a

population in the long-term when they convey higher fitness. Many unisexual species have acquired inter-sexual variation in primary and secondary floral traits, i.e., traits that are associated respectively with pollinator reward and attraction (Delph 1996; Delph et al. 1996). It is often assumed that these variations are a response to the foraging behaviour of the pollinators. Vamosi and Otto (2002) modelled inter-sexual variation in floral display and its role in pollen delivery events in dioecious plants and suggested that the benefits of male floral attractiveness could wholly depend on the abundance of pollinators and their foraging behaviour. Ashman (2000) demonstrated that pollinator selectivity can exert selection pressure to increase petal length and the maintenance of stamens in females of gynodioecious wild strawberry (*Fragaria virginiana*). In some species, female flowers without nectar reward produce pollen grains, but they are either sterile or inaperturate (Mayer and Charlesworth 1991). These flowers offer rewards to pollinators that play no role in the transfer of gametes. But reliable pollination by specialised pollinators has enabled some diclinous species to evolve a female floral morph without reward (Renner and Feil 1993). Furthermore, some dioecious species have temporal shifts in the production of floral rewards that may improve pollen transfer from male to female flowers. For example, males flower first followed by female flowers that open later in some species (Bullock and Bawa 1981; Clark and Clark 1987; Forero-Montaña and Zimmerman 2010; Pickering and Hill 2002; Symon 1979b).

Thus, it is likely that some sexual floral dimorphisms evolve in response to the foraging behaviour and preferences of effective pollinators. Therefore, studying the foraging decisions of pollinators in relation to flower morphology of

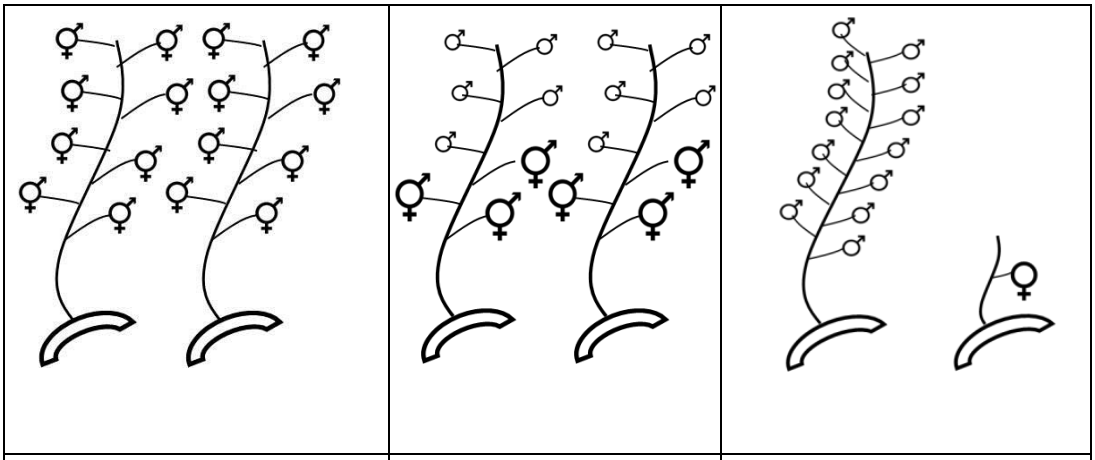
dioecious species should provide greater understanding of whether pollinators impose any selection on plants to become dimorphic. However, this is often problematic, because the sexes do not only differ in their morphology. They also differ in their reward type and structure, as female flowers generally do not produce pollen, and are likely to offer more nectar (Mayer and Charlesworth 1991; Willson and Ågren 1989).

Differences in rewards are not encountered in the dioecious and andromonoecious species in the genus *Solanum*, where female and male flowers produce equal quantities of pollen (Anderson and Symon 1989). Furthermore, the genus has andromonoecious, dioecious and hermaphrodite species. Therefore, this genus provides a model system that allows investigation of the evolution and maintenance of sexual floral dimorphism in relation to pollinator foraging behaviour, in the absence of differences in reward structure. In this thesis I investigate the possible influence of bee foraging behaviour on the evolution and maintenance of floral dimorphisms in the Australian *Solanum* species.

Australian *Solanum* species and their floral structures

The genus *Solanum* (Solanaceae) constitutes ~1400 species that are widely distributed throughout the world (Olmstead et al. 2008). In Australia, there are approximately 185 *Solanum* species of which 146 species belong to subgenus *Leptostemonum*, the spiny *Solanum* (Barker 2010). The flowers of *Solanum* are generally categorized as bisexual, however, unisexual forms such as andromonoecy and dioecy have been recognized in the subgenus *Leptostemonum* (Symon 1979b, Table 1). The andromonoecious species have one or a few hermaphrodite flowers below a cyme of few to many male flowers, while

dioecious species bear erect cymes of numerous male flowers and solitary hermaphrodite flowers on separate individuals. In subgenus *Leptostemonum*, the andromonoecious sex system occurs multiple times in 13 of 22 described sections with each representing presumed independent origins among distantly related species groups (Whalen 1984; Whalen and Costich 1986). There are about 15 dioecious species in four out of 13 clades within genus *Solanum* (Knapp et al. 1998; Martine et al. 2009; Knapp 2010). Thirteen of these species are in the subgenus *Leptostemonum,* and are present only in Australia (Martine et al. 2009; Barrett 2013; Brennan et al. 2006; Martine et al. 2011). In this rare sexual form, female flowers produce anthers that are filled with inaperturate, sterile pollen grains (Anderson and Symon 1989; Knapp et al. 1998).



Hermaphroditism	**Andromonoecy**	**Dioecy**
⚥	⚥ + ♂	♀, ♂
Hermaphrodite flowers only	Hermaphrodite flowers and male flowers in a single inflorescence	Functionally female flowers and male flowers on separate plants

Figure 1.1. Description of sexual forms of *Solanum* flowers

Floral dimorphisms

In *Solanum*, dimorphism in the floral traits is found between the sexes and it is likely that these differences are associated with the sexual functions of the flowers. Anderson and Symon (1989) described and analysed the differences in floral morphologies between male and female flowers of the 19 andromonoecious and dioecious Australian *Solanum* species. They considered flower number, corolla size, style length and diameter, ovary diameter, ovule length, anther length, pollen quantity and flowering period. They found that number of male flowers in an inflorescence ranges between 12 and 60, while female flowers are mostly solitary. Furthermore, in dioecious and andromonoecious species, the average corolla size of female flowers is respectively 1.43 and 1.26 times larger than the corolla size of male flowers. Male and female flowers further differ from each other in gynoecial characters, such as style length, style diameter, ovule length and ovary diameter. These floral traits are rudimentary in male flowers. Male plants started flowering 2-4 days prior to female plants, with up to three flowers simultaneously in anthesis in an inflorescence. Male flowers were open for a few days. Thus, these diclinous species of *Solanum* have developed clear patterns in flower size differences between the sexes, in the proportion of male to female flowers that are flowering stage at one time, and in the flowering period (Anderson and Symon 1989; Symon 1979b). Interestingly, there was no apparent dimorphism in anther size and number of pollen grains, but there were strong differences in pollen morphology between the sexes.

Reward structure

In the genus *Solanum*, flowers bear poricidal anthers that are firmly erect, which form a cone shape around the style and dehisce pollen through small terminal pores (Symon 1979b). The poricidal anthers may be present in two arrangements within flowers of *Solanum* species (Glover et al. 2004). Firstly, the commonly found 'salt cellar' type in which free anthers are presented in a loose cone without contacting each other, from which pollen can be collected from individual anthers. Secondly, the robust 'pepper pot' type presents the anthers in a tightly bound cone, in which they are fused by interlocking trichomes and release pollen from apical pores or slits. *Solanum* flowers don't produce nectar and both sexes offer pollen in equal amounts (Anderson and Symon 1989; Levine and Anderson 1986). Pollen grains from male flowers are tricolporate, while female flowers bear inaperturate pollen (Anderson and Gensel 1976; Anderson and Symon 1989; Knapp 1991). These inaperturate pollen grains cannot develop pollen tubes or fertilise ovules (Knapp et al. 1998; Levine and Anderson 1986). *Solanum* flowers offering only pollen have evolved morphological adaptations to conceal this reward (Vogel 1978). Pollen can only be accessed by female bees that can vibrate the flowers to expel it (Buchmann 1983). They are called 'buzz-pollinated' flowers. These flowers rely only on a small number of relatively large bees that are able to buzz the flowers, but they are also visited by a number of other bees that may collect pollen by 'milking' the anthers (Buchmann 1983). As nectar is not produced by *Solanum* flowers, the existence of a single pollinator reward in these species, i.e. pollen, offers a unique opportunity to explore how floral dimorphisms are associated with the foraging behaviour of bees.

Pollinators and their associations with floral traits of *Solanum*

Pollinators of Australian Solanum

In Australia, the main visitors of buzz-pollinated *Solanum* flowers include species in the genera *Amegilla*, *Trichocolletes*, *Lipotriches*, *Xylocopa* and *Trigona* (Anderson and Symon 1988). The social bee *Trigona* was observed to dig the pollen out of anthers, making more intra-plant movements and favouring self-pollination. In contrast, bees that collect pollen from anthers by buzzing are likely to cause outcrossing through pollen transfer between plants (Anderson and Symon 1988). Buzzing bee species were observed to forage along traplines of *Solanum* flowers by visiting a single flower in an area and then moving to another, presumably in a distant *Solanum* patch (Anderson and Symon 1988).

Buzz pollination

The vibrations produced by bees during buzz pollination loosen the pollen in the locules, which results in rapid expulsion of pollen grains through the pores of the anther (Buchmann 1983). Bees that use buzzing sonication for pollen collection occur in three out of five families within Apoidea and in more than 50 genera including *Bombus*, *Xylocopa*, *Colletes*, *Anthophora*, *Amegilla*, and *Nomia* (Buchmann 1983; De Luca and Vallejo-Marín 2013; Thorp 2000). When buzzing a flower, the bee holds the anther cone or the style with its legs or jaws, curls its body under the anthers to collect the pollen, and then vibrates the anther by contracting its indirect flight muscles (Buchmann 1983). The biophysical properties of the buzzing mechanism have been measured in some of these bees. The fundamental frequencies generated when buzzing flowers ranges between 100 and 400 Hz, which is largely determined by the magnitude of vibration of the

thoracic flight muscles (De Luca and Vallejo-Marín 2013; King et al. 1996). In contrast, honey bees generate slower thoracic acceleration and are not capable of collecting pollen from anthers using sonication (King and Buchmann 2003).

Pollination syndromes of buzz pollinated Solanum *flowers*

There is convergent floral morphology among buzz-pollinated plant species, which is often referred to as the *Solanum*-type flower or solanoid flower (Faegri 1986; Vogel 1978). These solanoid flowers offer no nectar reward, but produce a relatively large amount of pollen for pollinator attraction (Buchmann 1983; Vogel 1978). These flowers have evolved a common strategy in regards to pollen presentation that balances the need for pollinator attraction and the chance of pollen being inaccessible for pollination. The strategy involves the concealment of pollen within poricidal anthers and partitioned pollen release through staggered anther dehiscence (Buchmann 1983; Buchmann et al. 1977; Harder and Thomson 1989). Poricidal anthers contain small amounts of tapetal fluid that clump the pollen grains together. Centrifugal force generated through stamen vibration modes is required to remove pollen from the apical pores (King and Buchmann 1996). The gradual dehydration of the tapetal fluid has enabled a timed pollen release mechanism (King and Buchmann 1996).

Behavioural responses of bees to sexual floral dimorphisms

As mentioned above, flower morphology influences foraging behaviour of bees. Therefore, it is possible that the evolution of sexual floral dimorphisms can be understood from the perspective of the pollinators' responses to dimorphism in individual floral traits. In this thesis, I investigate whether the bees respond differentially to the different traits of male and female *Solanum* flowers.

Bee foraging responses to floral display

Most diclinous species of *Solanum* have evolved floral dimorphism in which female flowers are solitary and larger than male flowers (Symon 1979b; Symon 1981). The larger corollas could represent not only a sex-role specialisation, but also an adaptation to promote outcrossing, i.e, they may attract more pollinators for pollen dispersion and/or attract pollen-carrying insects to stigmas. For solitary female flowers, larger size may be considered important, as they are more easily seen from a distance by bees. A negative correlation between searching time and flower size indicates that foraging efficiency of pollen-collecting bees is lower on smaller flowers as bees find them more difficult to detect (Spaethe et al. 2001). Ohashi and Yahara (2001) recognized two types of pollinator responses to increased floral display size: larger floral displays attract more pollinators per unit of time, and the number of flowers that individual pollinators probe per plant also increases with flower display size. Hence variation in floral display size is expected to influence the foraging behaviour of bees, make a substantial difference in the distribution of pollination events, and ultimately affect plant fitness. Whether sexual dimorphisms displayed in flower size and flower numbers among diclinous species of *Solanum* influence bee foraging behaviour has not been previously studied. Since there are no known differences in the number of pollen grains between the sexes, bee foraging responses to sexual dimorphism in floral display are not known to be influenced by reward. This presents a unique opportunity to study the role of pollinators in the evolution of floral sexual dimorphism in *Solanum*.

Bee foraging responses to style length differences

The transition from hermaphroditism to andromonoecy in *Solanum* spp. has involved the loss of female function in some of the distal hermaphrodite flowers of an inflorescence, which have a rudimentary pistil with a short style and a non-functional ovary. It has been assumed that style length differences between male and hermaphrodite flowers increase their respective reproductive functions (Bertin 1982). Short styles in male flowers facilitate efficient pollen removal without interference, while hermaphrodite flowers with styles that protrude beyond the anthers may receive more pollen from bees (Diggle and Miller 2004; Elle and Meagher 2000; Solomon 1986). Although this pistil interference hypothesis has been theoretically discussed in earlier studies (Bertin 1982; Diggle and Miller 2004; Elle and Meagher 2000; Solomon 1986). Recently, Quesada-Aguilar et al (2008) found that the two floral morphs of *Solanum carolinense* differing in their style length had differential benefits to reproductive success. Bees removed more pollen from short-styled floral morphs that served as better pollen donors, while more pollen was received on long-styled morphs that served as better pollen recipients (Quesada-Aguilar et al. 2008). Therefore, differential bee responses to pistil lengths could influence pollen removal from male and hermaphrodite flowers and pollen deposition on hermaphrodite flowers. However, there has been no evidence that style length differences influence pollinator behavior. This result prompted Quesada-Aguilar et al. (2008) to hypothesise an important role for pollinator foraging interactions with style length differences between floral morphs in the evolution of andromonoecy in *Solanum*. However, so far, the influence of style length on pollen collecting behaviour has been shown for some, but not for other species of *Solanum* (Quesada-Aguilar et al. 2008;

Vallejo-Marín and Rausher 2007). In this thesis, I experimentally examine whether bees respond to style length differences in their visitation frequency.

Bee foraging responses to reward availability

Symon (1979b) observed that the production of male flowers initiates pollinator visitation through supplying a large amount of pollen before female plants of dioecious *Solanum* spp. come into flower. This strategy of having few open flowers each day and flowering over a long period is expected to maximize outcrossing (Harder and Barrett 1995). Female bees foraging on *Solanum* flowers could establish traplining behaviour as a response to the temporal distribution of pollen rewards, which can be either through timed-pollen release of single flowers or through opening new flowers sequentially. In American *Solanum douglasii* and *Solanum xanthi*, pollen grains are released gradually over the course of a day (Buchmann et al. 1977). The pollen grains are released from anther cones in response to frequent buzzes by bees. Further studies on *Solanum laciniatum* have shown that the apportioning of pollen availability can stimulate bees to revisit and collect newly available pollen grains from an anther cone (King and Buchmann 1996).

Bees' flower handling times and the numbers of buzzes per flower are influenced by the availability of pollen. Buchmann and Cane (1989) have demonstrated that sonicating bees are capable of assessing the pollen returns on a flower-by-flower basis and tailor their harvesting efforts to match pollen availability in *Solanum elaeagnifolium*. Thus, the ability of bees to assess pollen returns can influence their foraging decisions, especially distances travelled and the frequency of revisits. If bees could assess the pollen returns before landing or

immediately after landing, they make short visits to flowers that are empty. In another study, the foraging patterns of buzzing bees at individual flowers of *Solanum wendlandii* were found to be more resource-dependent than time-dependent (Shelly et al. 2000). The ability of bees to assess the difference in pollen availability should produce differences in flower handling times and numbers of buzzes between male and female flowers, as male flowers are likely to be emptied earlier than female flowers. It is still unknown whether the ability to assess pollen availability is universal among buzzing bees, and how individual bees respond to variations in pollen availability in nearby flowers.

Associations between floral dimorphisms and sex systems in *Solanum* - an evolutionary perspective

There is diverse interspecific variation in the production of male flowers are observed in andromonoecious *Solanum*. Factors contributing to this diverse sexual expression are phenotypic plasticity, inter and intra inflorescence architectural effects and total flower production (Miller and Venable 2003). Besides its evolutionary sexual lability, subgenus *Leptostemonum* also shows considerable interspecific variation in floral and fruit characters (Anderson and Symon 1989; Diggle 1994; Miller and Diggle 2007; Whalen and Costich 1986). These make this subgenus a particularly interesting model system to study the evolution of sex systems and their association with floral and fruit characters. The variation in reproductive traits may reflect constrained phylogenic origins in distant ancestors or they may reflect localised selection pressures that involved multiple speciation events, e.g. through the influence of pollinators and seed dispersal vectors. Phylogenetic comparative approaches are commonly used to

determine whether variation in character traits present among extant species has evolved in conjunction with speciation events (Blomberg et al. 2003; Felsenstein 1985; Pagel 1997, 1999). This approach is used in this thesis to study the floral traits found in subgenus *Leptostemonum*.

Previous studies in subgenus *Leptostemonum* have examined the associations between characters present among species in an attempt to explain causes and mechanisms involved in the evolution of sex systems (Anderson and Symon 1989; Bertin 1982; Diggle and Miller 2004; Martine and Anderson 2007; Symon 1979b; Whalen and Costich 1986). When phylogenetic independent contrast analyses were employed, the corolla sizes of fruiting flowers showed a positive correlation with the strength of andromonoecy, which is defined as the proportion of male flowers in an inflorescence, among the species of sections *Acanthophora* and *Lasiocarpa* (Miller and Diggle 2007). Similarly, Symon (1979a) recognized a trend of an increase in fruit size and a reduction in the number of fruits in an inflorescence among andromonoecious species. Subsequently, Whalen and Costich (1986) demonstrated a correlation between the number of male flowers and fruit size among the species of *Solanum* sections *Acanthophora* and *Lasiocarpa*, indicating an association between strong andromonoecy and large fruits. Such correlations between the expression of sex system and morphological traits have not been tested among the Australian species of subgenus *Leptostemonum* in a phylogenetic context. The presence of phylogenetically correlated changes could suggest causal mechanisms and the adaptive significance of character transitions in the evolutionary history of *Leptostemonum*.

The observation that andromonoecy is associated with increased fruit size and reduced fruit number (Miller and Diggle 2007; Symon 1979a; Whalen and Costich 1986) requires an explanation. Among hypotheses evaluated by Whalen and Costich (1986), the most likely explanation was that resource limitation of fruit set would cause selection for large fruits and therefore that the underlying mechanism was a trade-off between fruit size and number (Diggle 1995; Miller and Diggle 2007; Stephenson 1981; Whalen and Costich 1986). An increase in the costs of producing fruits and seeds would select for female-biased sex allocation at the plant level (Bertin 1982; Spalik 1991) and a reduction of the number of functional pistils (Bertin 1982; de Jong et al. 2008). It was thus proposed that species having large fruits and many or large seeds are more likely to evolve andromonoecy than small-fruited species (Bertin 1982; Whalen and Costich 1986).

If this explanation for the evolution of the association between flower size and sex system is correct, then the evolution of large fruit would precede the evolution of andromonoecy, and may be correlate with a large female flower (Miller and Diggle 2007). Furthermore, a large female flower may preclude a reversal to hermaphroditism. Support for these associations has been found in correlations between the proportion of male flowers and fruit size among the andromonoecious species of subgenus *Leptostemonum* section *Acanthophora* and *Lasiocarpa* (Whalen and Costich 1986, Miller and Diggle 2007). However, these hypotheses have so far not been tested in a phylogenetically informed way and analyses to date were hampered by the lack of a phylogeny of the Australian *Solanum* subgenus *Leptostemonum*.

Objectives of this study

The two main objectives of this thesis are to understand how floral traits have evolved in association with changes in sex systems in the Australian members of *Solanum* subgenus *Leptostemonum*, and to examine how sexual dimorphisms in floral display and reward availability influence bee foraging behaviour, using blue-banded *bees*, specifically *Amegilla murrayensis* and *A. chlorocyanea* as model species. The study aims are to:

1. Evaluate whether sexual dimorphisms in floral display (corolla size and number of flowers in the display) influence bee foraging behaviour
2. Examine whether bees have different foraging responses to style length dimorphisms
3. Examine the bees' ability to assess pollen availability in flowers, and test whether bees respond to sequential release of pollen from anthers
4. Investigate floral trait evolution and explore the extent of correlated evolution between sex systems and reproductive characters in subgenus *Leptostemonum* within a phylogenetic context

CHAPTER TWO - DOES FLORAL SEXUAL DIMORPHISM INFLUENCE BEE FORAGING BEHAVIOUR?

Abstract

Elucidating the role of sexual differences in floral display in attracting pollinators should contribute to the understanding of the evolution of floral dimorphism in species with unisexual flowers. To test whether such dimorphism in floral display is selected by pollinator preferences, one needs to disentangle the influences of reward and floral display in pollinator attraction. I experimentally investigated how floral sexual dimorphism influences flower visitation by female blue-banded bees, *Amegilla murrayensis*, in a glasshouse. The bees initially visited large flowers more frequently than small flowers, but this difference was not maintained in the absence of a difference in reward. The visitation of bees to clustered flowers was proportional to flower number but not influenced by flower size. While a cluster may present a more attractive display, sequential opening of clustered flowers also offers increased opportunities for pollen dispersal through pollen release over a long period of time. This can be particularly valuable when pollinators are rare. Dioecious *Solanum* species show sexual dimorphism in flower size and number with no apparent difference in reward. While floral sexual dimorphism may serve to improve pollen uptake and deposition in these species, it is unlikely to be a direct evolutionary response to selection through pollinator preferences.

Introduction

The evolution of separate sexes from hermaphroditism is one of the major and least well understood transitions in evolutionary history (Barrett 2002; Charlesworth 1999; Charlesworth and Charlesworth 2010; Charlesworth 2006). The evolutionary transitions between sexual systems of plants, especially the evolution of unisexual flowers from bisexual flowers, have been explored by investigating the microevolutionary processes governing reproductive trait transitions that have occurred over the course of evolutionary history (Barrett 2008; Spigler and Ashman 2012; Torices et al. 2011). These studies showed that separation of sexes is often accompanied by the evolution of morphological differences between sexes in floral traits that are associated with pollinator attraction (Delph 1996; Delph et al. 1996). Elucidating the role of sexual differences in floral traits in attracting pollinators should contribute to the understanding of selective pressures that lead to floral dimorphism during reproductive transitions in flowering plants.

A range of intraspecific sexual differences can be found in floral traits of plants with unisexual flowers (Delph 1996). For example, analysis of a qualitative dataset of 314 animal-pollinated species with dimorphic unisexual flowers showed that the corolla size of male flowers is larger than females in 55% of the cases (Delph et al. 1996). In addition, male inflorescences often have more flowers (Eckhart 1999; Lloyd and Webb 1977; Delph 1996) and emit relatively more volatile substances than female flowers (Ashman 2009). Flower size and number seem to function in pollinator attraction, as indicated by observations of

higher visitation rates to displays of larger or more numerous flowers (Goulson 1999).

The reason male flowers are larger in size and number than female flowers is often explained by two assumptions. Firstly, a strong intrasexual selection is assumed through male fertility to attract more pollinator visits to male flowers (Bell 1985). When pollinators are abundant, pollinator attraction largely acts as a selection agent on pollen dispersal from males to females, thus favouring higher resource allocation for attractive floral display in males (Wilson et al. 1994; Bell 1985). Investment in showy male flowers is thought to increase pollinator attraction and male reproductive success through increased transfer of pollen to female flowers (Vamosi and Otto 2002).

The second assumption is that female fertility is resource constrained, and not limited by pollen receipt, which is seemingly achieved in few pollinator visits. Therefore selection on attractive traits would be less strong through female function (Wilson et al. 1994). However, this assumption may not be valid as it has been observed that the number of pollinator visits to female flowers affects pollen receipt. Pollen limitation often occurs and can be the main determinant of female fertility (Vamosi and Otto 2002; Ashman et al. 2004). This should exert selection pressure on attractive female traits to ensure sufficient pollen receipt (Knight et al. 2005; Delph and Ashman 2006). Furthermore, a comparison across several species indicates that the strength of sexual selection through male or female fertility for attraction may be pollinator-context dependent and may vary among plant species (Ashman and Morgan 2004). While there is evidence that strongly supports the existence of selection through female function on attractive traits in

response to pollen limitation (Ashman et al. 2004; Burd 1994), it is still unclear how selection on attractive male flower display increases pollinator attraction and the realization of male fertility, i.e. pollen transfer (Ashman and Morgan 2004). I experimentally assess the effects of pollinator visitation in shaping the selection for sexual differences in floral display. The results particularly pertain to the evolution of floral dimorphism in dioecious species, because the separation of male and female plants maximizes opportunities for selection of sexual dimorphism.

A comparative evaluation of the role of floral sexual dimorphism in attracting pollinators is generally hampered by confounding factors. Pollinators usually visit flowers to obtain a reward, which is advertised by means of showy floral display. In dioecious species, the type and amount of reward on offer to pollinators often differ between male and female flowers. Female flowers may offer only nectar, only sterile pollen or no reward, while male flowers reward pollinators with pollen either with or without nectar (Renner and Feil 1993; Mayer and Charlesworth 1991). These differences between male and female flowers in the type and quantity of rewards make it difficult to disentangle the influences of reward and flower morphology on pollinator visitation.

Furthermore, apart from resulting in differential attraction of pollinators, the different visual and olfactory signals produced by the sexes may provide information about the reward present in the flowers (Cohen and Shmida 1993). This implies that there is a coevolutionary nexus between the pollinators' choice of foraging on the most rewarding floral display and the increased investment in display in relation to the reward. Support for this has been found in the strong

positive correlation between the corolla size and reward in several hermaphrodite species, indicating that large flower display often signals a larger reward (Plowright 1981; Stanton and Preston 1988; Galen and Plowright 1985; Cohen and Shmida 1993; Robertson et al. 1994; Gómez et al. 2008; Mione and Anderson 1992). Furthermore, bees foraging on dimorphic flowers prefer larger male flowers over small female flowers and this preference has been observed to increase with flower display dimorphism (Glaettli and Barrett 2008; Huang et al. 2006; Vaughton and Ramsey 1998). However, it is as yet unclear whether the bees' foraging preference for showy male floral displays is the result of a learnt association between large floral displays and high rewards, or whether it is simply the result of increased detectability.

Thus to identify the role of pollinators in the evolution of sexual floral dimorphism, one needs to disconnect the differences in morphology from the differences in reward. Australian dioecious *Solanum* species are an ideal genus to illustrate this, as they display sexual floral dimorphism without apparent differences in reward structure. Flowers do not produce nectar and both sexes offer pollen in equal amounts (Anderson and Symon 1989; Levine and Anderson 1986), but the pollen from female flowers is inaperturate and sterile (Knapp et al. 1998). The sexual floral dimorphism in dioecious *Solanum* pertains to distinct differences in corolla size, and number of flowers per inflorescence (Anderson and Symon 1989). The average corolla size of female flowers is 1.43 times larger than male flowers. Female plants bear inflorescences with a single flower, while males have 12-60 flowers per inflorescence, and display one to three open flowers at any one time. Thus, Australian dioecious *Solanum* species present an example

of sexually dimorphic flowers with no apparent quantitative differences in the reward between male and female flowers.

The pollinators of dioecious *Solanum* are large buzz pollinating bees (Anderson and Symon 1988). These bees collect pollen from both male and female flowers. Solitary and primitively social bees are central place foragers with a foraging range established around their nests, which remains stable during their lifetime (Cresswell et al. 2000; Schoener 1979). They make repeated sequential visits to flowering patches within their foraging range in a more-or less fixed order (Thomson et al. 1997). While foraging in a flowering patch, bees learn to associate the reward just experienced with signals present in the flowers. This associative learning forms short-term reward expectations which enable bees to optimize their foraging by matching their flower choices with reward expectations (Gil et al. 2007; Blarer et al. 2002). However, it is not clear whether differences in floral display *per se* suffice to influence bee visitation.

In this paper, I investigate whether the differences in floral display, such as those seen in dioecious *Solanum* spp., result in differences in visitation by bees, and how associative learning, as happens in central place foraging bees, influences visitation of sexually dimorphic flowers. In the experiment, sets of artificial flowers were used that differed in size and or display, but not in reward, i.e. sugar feeders donning artificial corollas that could continuously supply the reward during bee visits. My experiments tested whether dimorphism in floral display influences initial visitation and whether these preferences are maintained during successive visits to dimorphic floral displays that differ in flower size and number of flowers displayed, but not in reward. I use female *A. murrayensis* Rayment, a

buzz pollinating blue-banded bee. I use my results to evaluate the possible factors involved in the evolution of sexual dimorphism in floral display of dioecious species in general, and of dioecious *Solanum* spp. in particular.

Materials and methods

Materials

Experimental arena

I conducted experiments at the Waite Campus, the University of Adelaide, Australia, in a glasshouse (7 x 4 x 3.5 m) between October and December in 2011 and 2012. Supplemental lighting with high-frequency flicker (350 Hz) fluorescent lamps was used to keep 14h:10h alternating light: dark schedule in the glasshouse. All experiments were conducted between 11 AM and 1 PM.

Bees

Amegilla murrayensis has a wide distribution in Australia (Leijs et al. in preparation) and is known to collect pollen on *Solanum* spp. (Hogendoorn et al. 2007; Anderson and Symon 1988). I sourced female *A. murrayensis* from the ongoing breeding culture (Hogendoorn et al. 2007). There were always 3-4 bees foraging in the glasshouse and new females were added regularly to replace the dead ones. These bees were not marked individually. Mud-brick blocks were placed in the glasshouse as substrate for bee nesting (Fig. 2.1). The experimental glasshouse contained flowering tomato (Improved Apollo variety) and silver-leaf nightshade (*Solanum elaeagnifolium* Cavanilles) in pots that supplied bees with pollen for provisioning their brood cells (Fig. 2.1).

Artificial flowers

Feeders with sugar water were the only source of carbohydrates in the glasshouse. The feeders consisted of a glass tube (5 mm diameter) donned with a five-lobed fake flower corollas made of purple polyester fabric (Fig. 2.2). These artificial flowers were uniform in size and colour. A hollow nylon yellow tube (10 mm length, 1.5 mm diameter) was inserted through the flower and formed the central nectar guide. This tube was inserted into a 0.2 mm hole near the bottom of the glass tube. Glass tubes were filled with 30% sugar water by weight and closed using rubber stoppers. Feeders were renewed every third day to prevent any fungal growth. They were supported at a height of 1.5 m from the floor and access by ants to feeders was prevented applying a barrier of Tanglefoot™. Since there was *ad libitum* sugar water in the feeders, there was no depletion of reward during the observation. In experiments, bees visited artificial flowers either from the nest or after visiting one of the many pollen producing *Solanum* spp. plants.

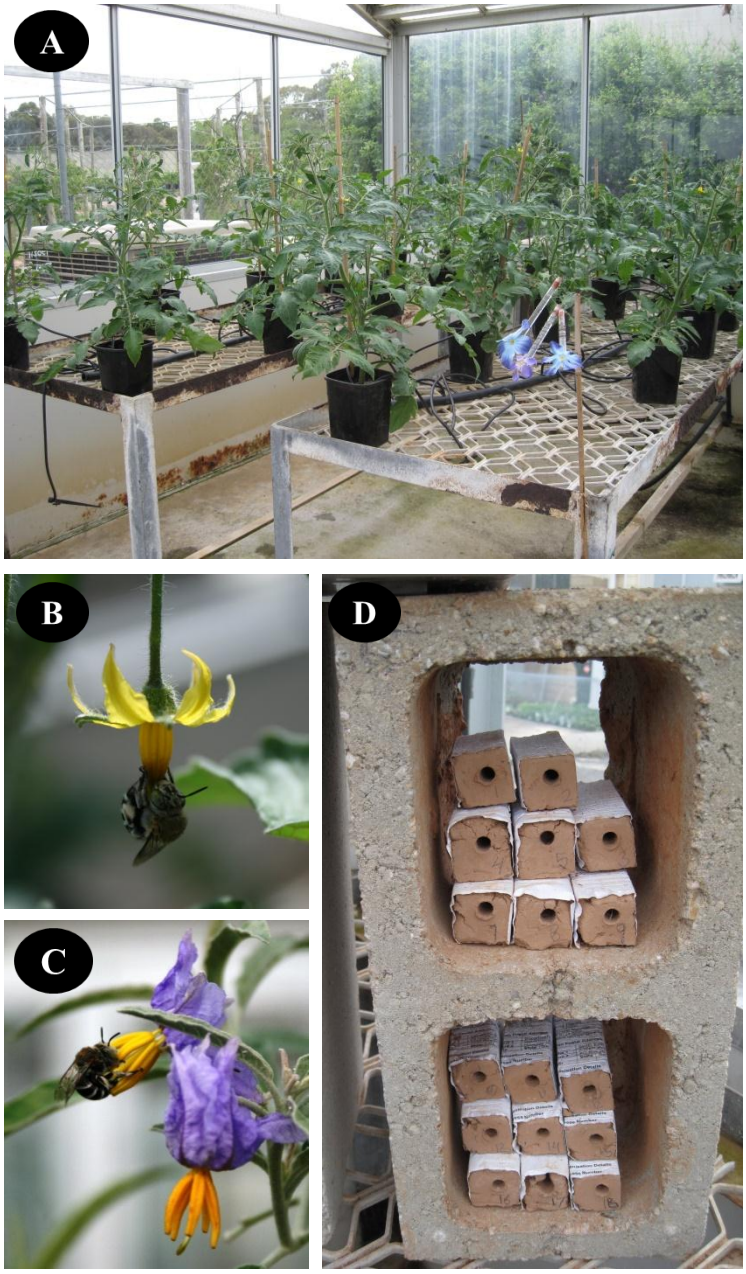


Figure 2.1. Experiments were conducted in a glass house (A), where nesting female blue-banded bee, *Amegilla murrayensis*, pollen-forages on the flowers of tomato (B) and nightshades (C). Mud brick blocks (D) were placed for bee nesting in the glasshouse.

Figure 2.2. Feeders consisted of glass tubes filled with sugar water, and donned artificial flower corollas. These feeders served as carbohydrate source for bees in the glasshouse.

Experimental design

Training

Before each experiment, I trained the bees to visit a single location ('Training Site') near the centre of the glasshouse with two identical feeders (70 mm diameter) for at least 24 hours (Fig. 2.3). This time period was based on imperfect memory retention reported for bees (Keasar et al. 1996). Feeders were removed 30 minutes prior to each experiment, and placed back at the same spot after the experiment.

Experiment 1: do bees have a preference for large flowers?

To investigate whether bees have any preference for large flowers, I offered choices between two differently sized flowers using artificial feeders. I also investigated whether this preference was maintained during successive foraging visits. I presented the bees with flowers of two diameters: 20 mm and 35 mm on either side of the 'Training Site', placed 2 m away from each other (Fig. 2.3). The 35 mm flowers had a surface area three times larger than the 20 mm flowers. Since I had 3 to 4 female bees foraging in the glasshouse, four feeders in each diameter size were used to avoid competition between foraging bees for flowers. Four feeders of each of these two sizes were arranged in the shape of half a circle with their centers 5 cm apart, all at the same height. Observations on bee visitations to individual flowers of two different sizes were recorded for 60 minutes. The placement of experimental flowers was alternated between the replicates. The proportion of visits to large and small flowers was recorded over 20 replicates of 60 minute observation. A visit was defined from when a bee landed on a flower until it left the flower.

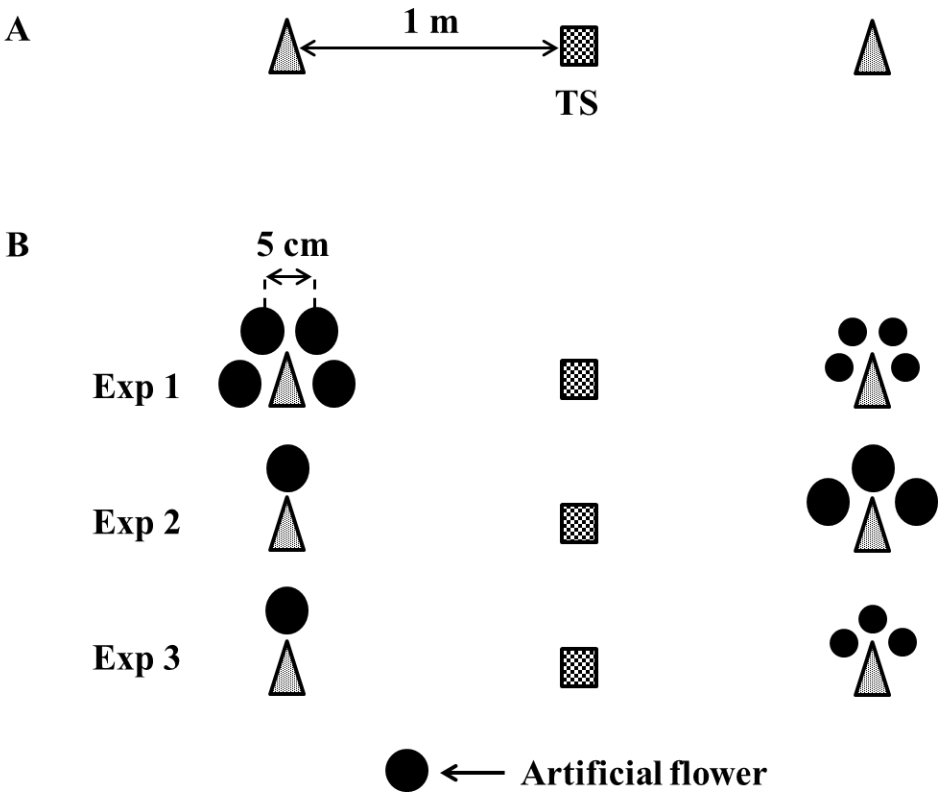


Figure 2.3. Schematic diagram of experimental setup. A. Arrangement of artificial flowers. B. Arrangement of artificial flowers in each experiment. 'TS' denotes 'training site'.

Experiment 2: are clustered flowers more attractive than solitary flowers?

To investigate whether the number of flowers displayed at a time influences bee foraging behaviour I presented the same 20 mm diameter flowers in clusters and solitarily. The bees were given a choice between clustered and solitary flowers that were placed at 1 m distance from the 'Training Site' on either side (Fig. 2.3). The clustered flowers contain three feeders placed next to each other, spaced 5 cm apart. Bee visitations to clustered and solitary flowers were recorded for 60 minutes. The proportion of visits to individual flowers and clusters was recorded over 10 replicates.

Experiment 3: visitation to large solitary and small clustered flowers

In this experiment, I combined the flower size with number of flowers displayed at a time and studied whether flower size has any influence on bee foraging preferences for solitary and clustered flowers. I offered bees a choice between a cluster of three small flowers (20 mm diameter) and one large solitary flower (35 mm diameter) each at 1 m distance from the 'Training Site' (Fig. 2.3). Observations of bee visits to flowers over 60 minutes were recorded and repeated 10 times.

Data analysis

In flower choice experiments, I investigated whether bees had preferences between large and small flower displays by examining the size of the first flower visited and the number of visits to flowers of each display size during 10 min intervals. To test whether the observed proportions of first flowers visited did not differ significantly from the expected 50%, two-tailed binomial tests were performed. One-sample *t* tests were used to test the null hypothesis that the proportions of bee visits to flowers with different morphology did not deviate from 50% of overall visits during 10-min foraging intervals. Similarly, bee visits to flowers presented in clusters and solitarily using same sized flowers and different sized flowers were tested for significant difference from 50% of overall visits using *t* tests. To test whether bees visit flowers in equal frequency, I examined the hypothesis that the proportions of bee visitations to cluster of three flowers and solitary flower follow 3:1 ratio. As a post hoc analysis to examine the effect of flower size on bee visitations to flower clusters, I conducted independent sample *t* tests on the proportions of bee visits to clustered flowers and small

clustered flowers recorded in two different experiments. In all *t* tests involving proportions of visits, proportions were arc-sine transformed to achieve an underlying normal distribution.

Results

Experiment 1: do bees have preference for large flowers?

In 85% of the replicates (n=20), the large flower was the first flower visited. Thus the bees initially showed a preference for the larger flowers (Binomial test, $P = 0.003$). This preference was maintained during the first 30 minutes (Fig. 2.4) but gradually decreased and no significant difference in the number of bee visits to large and small flower size was observed after 30 minutes [$t = 3.87$, $P = 0.001$ (0-10 min); $t = 3.62$, $P = 0.002$ (10-20 min); $t = 3.2$, $P = 0.005$ (20-30 min); $t = 1.4$, $P = 0.18$ (30-40 min); $t = -0.12$, $P = 0.9$ (40-50 min); $t = -0.83$, $P = 0.42$ (50-60 min), $df = 19$].

Experiment 2: are clustered flowers more attractive than solitary flowers?

In 90% of replications (n=10), clustered flowers were visited first (Binomial test, $P = 0.02$) and this proportion did not change during the course of an hour [$t = 3.85$, $P = 0.004$ (0-10 min); $t = 2.70$, $P = 0.02$ (10-20 min); $t = 2.56$, $P = 0.03$ (20-30 min); $t = 3.12$, $P = 0.01$ (30-40 min); $t = 2.43$, $P = 0.038$ (40-50 min); $t = 3$, $P = 0.01$ (50-60 min), $df = 9$] (Fig. 2.5A). Thus the bees preferred clusters over solitary flowers. The proportions of bee visitation to cluster of three flowers did not deviate from the expected 75% of total visitations recorded during 10 min intervals indicating that bees visited flowers in equal frequency.

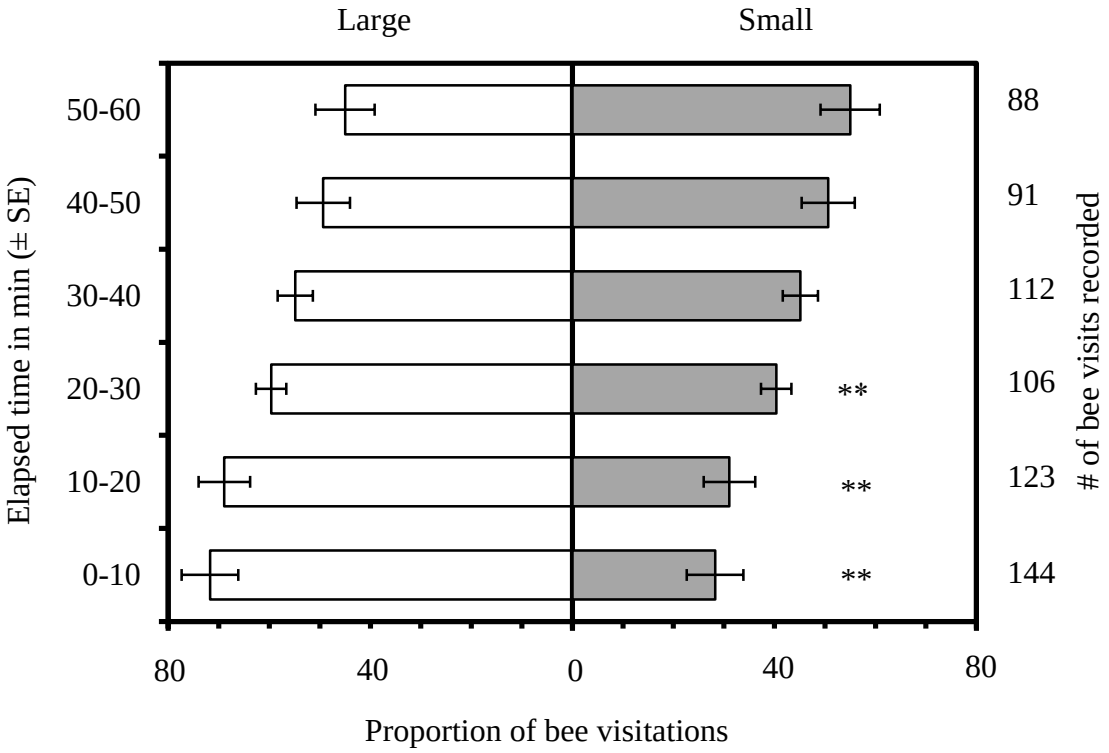
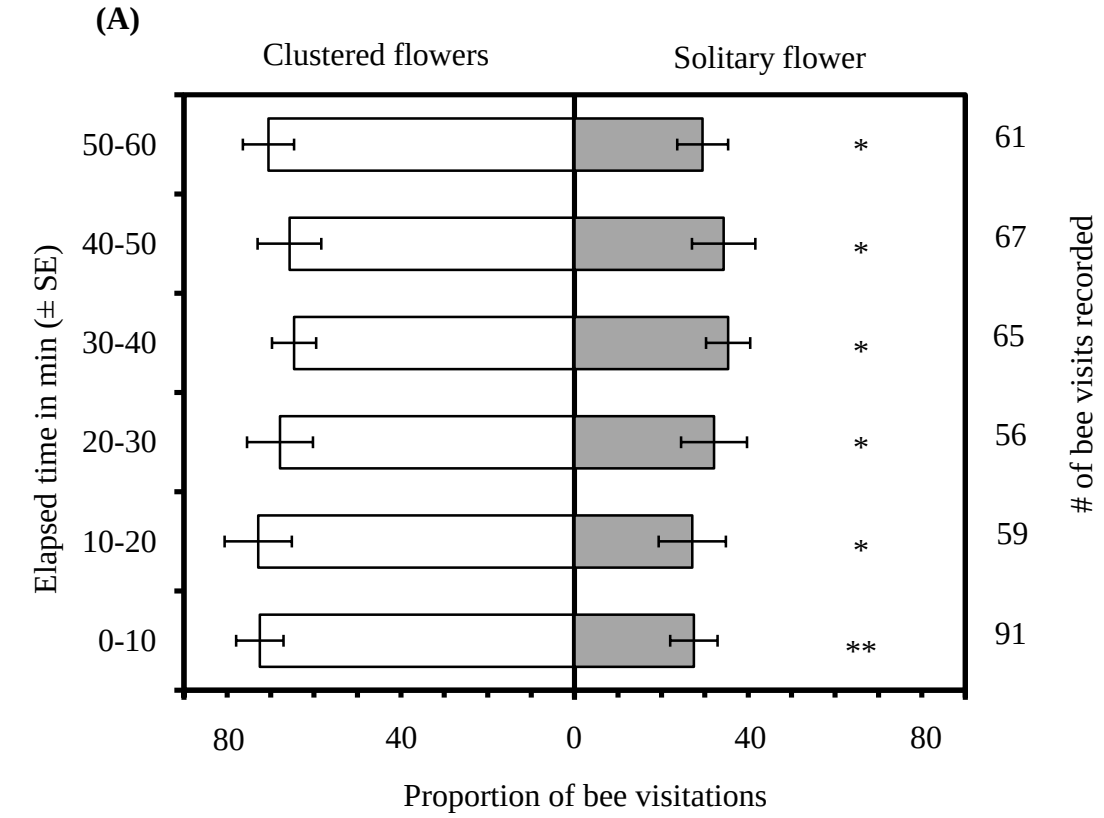


Figure 2.4. Average proportions (± SE) of bee visitations to large (35 mm diameter) and small (20 mm diameter) flowers over one hour after first presentation (n=20 replicates). Proportions of bee visits that were significantly different from the expected 50% are indicated (** = $P < 0.01$).

Experiment 3: visitation to large solitary and small clustered flowers

In 90% of replications (n=10), the cluster of small flowers was visited first (Binomial test, P=0.02). Throughout the observation time, the clusters of small flowers were visited more frequently than the large solitary flowers [t = 2.753, P = 0.02 (0-10 min); t = 5.716, P = 0.00 (10-20 min); t = 5.07, P = 0.001 (20-30 min); t = 4.2, P = 0.002 (30-40 min); t = 5.38, P = 0.001 (40-50 min); t = 3.12, P = 0.017 (50-60 min), df = 9] (Fig. 2.5B). The proportions of bee visitations to the


(A)
Clustered flowers
Solitary flower
50-60
40-50
30-40
20-30
10-20
0-10
*
*
*
*
*
**
61
67
65
56
59
91
Elapsed time in min (± SE)
# of bee visits recorded
80
40
0
40
80
Proportion of bee visitations

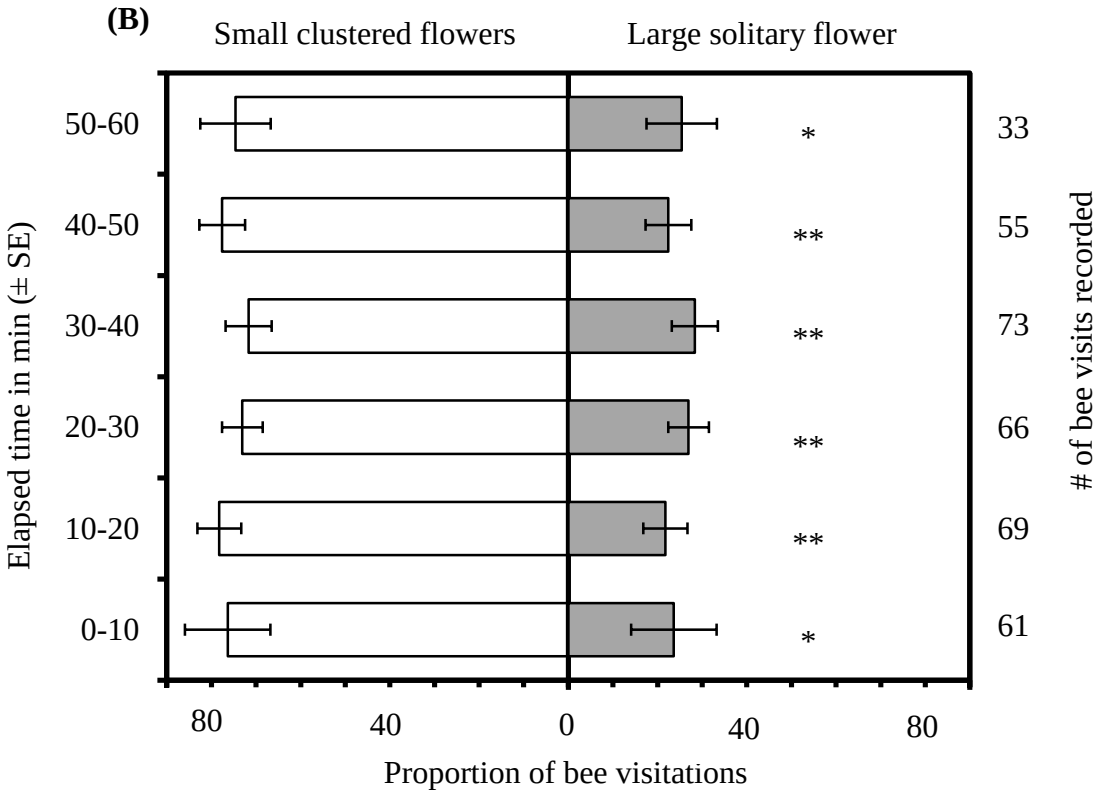

(B)
Small clustered flowers
Large solitary flower
50-60
40-50
30-40
20-30
10-20
0-10
*
**
**
**
**
*
33
55
73
66
69
61
Elapsed time in min (± SE)
# of bee visits recorded
80
40
0
40
80
Proportion of bee visitations

Figure 2.5. Average proportions (± SE) of bee visitations to flowers presented in clusters and solitarily using same sized flowers (20 mm) (A) and different sized flowers (20 vs. 35 mm) (B) over one hour after first presentation (n=10 replicates). Proportions of bee visits that were significantly different from the expected 50% are indicated (* = $P < 0.05$; ** = $P < 0.01$).

cluster of three small flowers and solitary flower are consistent with expected 3:1 ratio showing that bee visitation is directly proportional to the number of flowers presented. Moreover, flower size had no effect on bee visitations to clustered flowers as there were no significant differences in the proportions of bee visits between clustered flowers and small clustered flowers [t = -0.224, P = 0.82 (0-10 min); t = -0.966, P = 0.35 (10-20 min); t = -1.05, P = 0.31 (20-30 min); t = -0.777, P = 0.45 (30-40 min); t = -1.36, P = 0.19 (40-50 min); t = -0.066, P = 0.95 (50-60 min), df = 18].

Discussion

Bees' response to flower display dimorphism

While the bees preferred large over small flowers during their initial foraging bouts, this preference gradually decreased over time and disappeared within 30 minutes, indicating that an association between reward expectation and flower size can rapidly disappear in the absence of actual reward differences. The initial preference for larger flowers corroborates earlier findings (Galen and Newport 1987; Stanton and Preston 1988; Conner and Rush 1996; Eckhart 1991). However, these studies only investigated the bees' initial preference, thus not taking into account the possibility that bees adjust their expectations over time on the basis of experience. My results indicate that in the absence of a difference in

reward, any preference for large flowers would quickly disappear with experience. Therefore, a size-based preference would only be found in relatively inexperienced bees (Makino and Sakai 2007; Makino et al. 2007).

My data support the notion that the association of flower size and reward might be an innate expectation of high reward. In many studies, reward size has been shown to increase with flower size (Plowright 1981; Stanton and Preston 1988; Galen and Plowright 1985; Cohen and Shmida 1993; Robertson et al. 1994; Gómez et al. 2008; Mione and Anderson 1992). Furthermore, bees that often encounter highly-rewarding large flowers during their foraging visits over a period of time develop preference for large flowers with an initial expectation of more rewards (Cohen and Shmida 1993; Blarer et al. 2002). Goulson (1999) suggests that bees tend to keep a high expectation of the reward available in a flower to be visited next while foraging in a patch, and use visual and olfactory cues from floral traits that are associated with reward production in choosing the rewarding flowers. The associative learning of reward availability with floral size enables experienced bees to choose a rewarding floral size when initially preferred flowers become unrewarding (Blarer et al. 2002). Furthermore, my data show that bees' initial size preferences quickly disappear when reward size is not different among flower sizes, which corroborates the findings of Abraham (2005). With reward-based foraging behaviour, experienced bees show no preferences for flower sizes that have the same amount of reward. Thus, a larger corolla size can only be effective in attracting more pollinators when it provides an honest signal of the quality or quantity of the reward.

However, I cannot exclude the possibility that the large flower size may simply present a better visual stimulus that can be easily detected by bees from a long distance. Bees use long-wavelength photoreceptor (L-receptor) signals to detect flowers from a distance, and receive stronger signals on the L-receptor from brightly-centered flowers that are larger in size (Hempel de Ibarra and Vorobyev 2009). My artificial flowers fit that model and therefore may have been easier to detect from a longer distance.

The sustained preference for clusters of flowers over solitary flowers of the same size corresponds with findings from earlier studies that bees preferentially visit larger flower displays (Mitchell et al. 2004; Ohara and Higashi 1994; Ohashi and Yahara 1998; Thomson 1988; Robertson and Macnair 1995; Goulson et al. 1998; Grindeland et al. 2005; Miyake and Sakai 2005; Goulson 1999). It seems possible that this is also due to easier detection of conspicuous display of flower clusters from a longer distance. Interestingly, in my experiments, the clusters consisting of same sized flowers and clusters of small flowers received equal proportions of bee visitations. Thus, given the distances used in these experiments, bees preferred clusters of flowers, regardless of flower size differences. Hence, it is likely that bees were attracted by the number of flowers in the display, corroborating the findings of Wertlen *et al*. (2008) for the detectability of groups of discs by bees.

Is floral sexual dimorphism in dioecious **Solanum** *a response to bee foraging?*

Dioecious male and female *Solanum* flowers have more or less the same reward, but have differences in corolla sizes and the number of flowers per inflorescence (Anderson and Symon 1989). Since male and female flowers are on

separate individuals, bee movement from male to female flowers is essential for effective pollen transfer. My experiments indicate that flower size alone is not enough to cause any differences in visitation rates by experienced central place foraging bees, because after a time period, the bees have obtained complete information about the expected reward size in the foraging patch by learning (Thomson et al. 1997; Williams and Thomson 1998). Therefore, it is unlikely that flower size dimorphism in dioecious *Solanum* has evolved in response to initial bee preferences for large flowers. However, a large display with numerous flowers signals higher rewards and attracts more bee visitations. Thus, higher visitation rates to clustered flowers may lead to increased pollen dispersal from male to female flowers.

Based on these considerations, I put forward a number of non-exclusive explanations for the evolution of sexual dimorphism in flower number per inflorescence and flower size in dioecious *Solanum*.

For most dioecious species that lack nectaries and produce pollen as the only reward, a larger number of flowers in male inflorescences is reported (Anderson and Symon 1989; Kawagoe and Suzuki 2004, 2002; Sawyer and Anderson 2000). My findings suggest that clusters of flowers, irrespective of flower size differences, attract more visitors than solitary flowers. This would be advantageous if pollen uptake is the main limiting factor for pollination. However, this should be verified. Another benefit of the production of a series of flowers in male inflorescences is that it enables the male plants to release the pollen over a long period of time (Harder and Thomson 1989; Lloyd and Yates 1982) and allows a protracted flowering period in male inflorescences. This could

substantially improve effective pollen dispersal, particularly when bee visitation is rare (Glaettli and Barrett 2008; Thomson 2006; Yakimowski et al. 2011). Furthermore, there may be a trade-off between flower size and number of flowers per inflorescence, a life history constraint that is commonly found in angiosperms (Sargent et al. 2007). Such a flower size-number trade-off in the male inflorescences of dioecious *Solanum* spp. may be the result of greater opportunities for effective pollen dispersal events through increasing the number of male flowers.

Apart from the potential for prolonged pollen release and the trade-off between flower size and number, flower size differences may also be related to the difference in the timing of flower opening between male and female flowers. In dioecious species of Australian *Solanum,* male plants initiate anthesis before females (Anderson and Symon 1989; Martine 2006). I assume that the pollen collecting bees that use a trapline to forage on *Solanum* flowers initially visit male flowers and later include female flowers in their trapline. The larger flower size of the solitary female flowers may either enhance detectability, or indicate a fresh source of rewards at a time when pollen in the male flowers has been somewhat depleted.

In conclusion, in the absence of reward differences, it is unlikely that flower size dimorphism in dioecious *Solanum* is a strategy to directly manipulate bee foraging activity. The larger number of flowers in male inflorescences may reflect such a strategy, but this is only valid if pollen dispersal from male flowers, and not pollen deposition on female flowers is the limiting factor in reproduction. This could be verified. Furthermore, the dimorphism in floral display could be the

result of a combined need to release pollen over a long period of time, and thereby sustain rare pollinator visits, and also signal true differences in reward that may exist due to sexual differences in flowering times. Further establishment of how these life-history strategies of dioecious plants influence bee visitation, and improve the chances of pollination require more extensive experimentation in the field.

CHAPTER THREE - ON THE EVOLUTION OF ANDROMONOECY IN *SOLANUM* AND POLLINATOR VISITATION RESPONSES TO STYLE LENGTH DIFFERENCES

Abstract

The transition from hermaphroditism to andromonoecy in *Solanum* has involved suppression of gynoecial development, i.e., the production of a rudimentary pistil with a short style in the distal flowers. The difference in style length between hermaphrodite and male flowers seems to be a functional strategy to provide either sexual morph an advantage to increase its reproductive function. In order to understand the functional significance of the reduction in pistil size in male flowers, pollinator foraging responses to floral morphs differing in their style length must be determined. Do style length differences in floral morphs influence bee visitation patterns? In two choice experiments, I presented blue-banded bees (*Amegilla chlorocyanea*) with natural or experimentally manipulated floral morphs of andromonoecious *Solanum elaeagnifolium* that differed in their style length. I observed no differences in the number of buzzes during a single visit or the average duration per buzz between the two floral morphs. Thus, I find no support for the hypothesis that the short style in male flowers of andromonoecious *Solanum* species is the result of selection to avoid interference with foraging bees.

Introduction

The species-rich genus *Solanum* (Solanaceae) displays a range of sexual forms, which include hermaphroditism, andromonoecy and dioecy (Symon 1979). The andromonoecious sex system has evolved multiple times independently in distantly related sections within this genus (Whalen and Costich 1986). Andromonoecy in *Solanum* is often present in the form of developmental phenotypic plasticity, with one or a few bisexual flowers below a cyme of few to many male flowers (Diggle 1991, 1994; Miller and Diggle 2003). Transitions from hermaphroditism to andromonoecy involve the loss of female function in some of the distal hermaphrodite flowers of an inflorescence through producing a rudimentary pistil with a short style and non-functional ovary (Diggle 1994; Miller and Diggle 2003). To date, four compelling hypotheses exist to explain the evolution and maintenance of andromonoecy *Solanum*: the resource allocation hypothesis (Bertin 1982; Solomon 1986; Elle 1999; Vallejo-Marín and Rausher 2007a), the pollen donation hypothesis (Anderson and Symon 1989; Elle and Meagher 2000; Vallejo-Marín and Rausher 2007a; Quesada-Aguilar et al. 2008), the pollen receipt hypothesis (Vallejo-Marín and Rausher 2007b; Quesada-Aguilar et al. 2008) and the pistil interference hypothesis (Solomon 1986; Elle and Meagher 2000; Diggle and Miller 2004; Quesada-Aguilar et al. 2008).

The primary focus of three of these hypotheses, i.e. pollen donation, pollen receipt and pistil interference, is the selective advantage of producing male flowers over fruiting and non-fruiting hermaphrodite flowers in terms of pollen removal and pollen deposition. The reduced pistil with the short style in male flowers makes them morphologically different from hermaphrodite flowers. These

hypotheses assume that style length differences between hermaphrodite and male flowers provide either sexual morph an advantage to increase their respective reproductive function. The short style length in male flowers should facilitate more efficient pollen removal without interference, while the long style length in hermaphrodite flowers should ensure improved pollen reception from visiting pollinators (Diggle and Miller 2004; Elle and Meagher 2000; Solomon 1985). To date, only two studies have investigated pollinator interactions with style length differences and their relevance to sex specific fitness in order to explain the evolution and maintenance of andromonoecy in *Solanum*.

Firstly, Vallejo-Marín & Rausher (2007a) experimentally manipulated the style length of the flowers of *Solanum carolinense* to investigate the whether this affects visitation rates of bumblebees (*Bombus pennsylvanicus* and *B. impatiens*). The bees did not display any preference for either type of flower, and the authors concluded that no differences in the bee visitation rates between two floral morphs could have resulted in equal pollen siring success from hermaphrodite and male flowers (Vallejo-Marín and Rausher 2007a). However, this conclusion largely rests on the assumption that increased pollinator visitation rates lead to increased chances of siring success through increased pollen removal. This raises the question whether floral morphs differing in their style length influence the amount of pollen removed from and deposited on a flower.

In the second study of the same species (*S. carolinense*), Quesada-Aguilar et al. (2008) examined the effect of style length differences on the amount of pollen removed from anthers and deposited on the stigma. They showed that short-styled floral morphs serve as better pollen donors, while long-styled morphs are more

efficient pollen recipients. Behavioral observations showed that the frequency of contact events or buzzes on short and long-styled flowers was equal for halictid bees (*Augochloropsis metallica* and *Lassioglossum* spp.), whereas bumblebees (*B. impatiens*) performed more buzzes on long-styled flowers. The authors concluded that variations in the foraging responses of pollinator bees resulted in differential benefits to reproductive success of the floral morphs of *S. carolinense*. Furthermore, the existence of variations in foraging patterns among pollinator species implied that different pollinators would impose different selection pressures on floral morphs. Based on this, Quesada-Aguilar et al. (2008) suggested that andromonoecy in *S. carolinense* has evolved in response to the selection from the most plausible pollinators, bumblebees, and favoured the hypothesis that short and long style floral morphs are a strategy to increase both male and female fitness, respectively.

Both studies recorded the visitation patterns of bumblebees, which are considered to be the main pollinators of *S. carolinense*. The bumblebees in the study by Quesada-Aguilar et al. (2008) performed more buzzes on long-styled than short-styled morphs in a single visit, while they removed more pollen from short-styled than from long-styled morphs. This suggests that fewer buzzes are required to remove pollen from short-styled morphs than from long-styled morphs. Thus, more pollinator visits would be expected on short-styled flowers. However, Vallejo-Marin & Rausher (2007a) did not find a preference for the experimentally manipulated short-styled floral morph of *S. carolinense* by bumblebees. Taken together, these studies suggest that floral morphs can influence the amount of pollen removed and deposited, but without affecting

pollinator visitation patterns. Thus, while pistil size has been shown to influence sex specific reproductive fitness through pollen donation and pollen reception, it is still unclear whether style length differences influence pollinator visitation patterns in andromonoecious *Solanum*.

In this study I examined whether bees have different visitation responses in terms of the numbers of buzzes and buzzing times to differences in style length between hermaphrodite and male flowers. I observed the visitation patterns of blue-banded bees (*Amegilla chlorocyanea*) on the flowers of andromonoecious *Solanum elaeagnifolium* (silver-leaf nightshade). I compared buzzing behaviour on manipulated and unmanipulated hermaphrodite flowers and on hermaphrodite and male flowers. I hypothesised that if pistil interference occurs, then there should be differences in foraging behaviour between manipulated and unmanipulated hermaphrodite flowers. I also discuss the implications of my results for the role of pollinators in the evolution and maintenance of andromonoecy.

Methods

S. elaeagnifolium is a perennial herbaceous species that is native to South & Central America (Boyd et al. 1984). It is a weed in many parts of the world, including Australia (Cuthbertson et al. 1976). Individual plants produce inflorescences bearing both hermaphrodite and male flowers, so this species can be described as andromonoecious (Fig. 3.1A). Each inflorescence generally has 3-5 hermaphrodite flowers at the base and 1-3 male flowers at the distal end. Male flowers of *S. elaeagnifolium* have a much reduced pistil with a short style and a

Figure 3.1. (A) Silver-leaf nightshade (*Solanum elaeagnifolium*) plants produce both long-style hermaphrodite and short-style male flowers in an inflorescence. (B) Pollen-foraging female blue-banded bees (*Amegilla chlorocyanea*) regularly visited these nightshade flowers.

non-functional ovary that does not mature into fruit, even when flowers are hand-pollinated (A.S. Mark-unpublished data). There is no sexual dimorphism in flower size, apart from a positional effect on corolla size variation between hermaphrodite and male flowers, which is also found in other andromonoecious *Solanum* species (Diggle and Miller 2004). Flowers do not produce nectar and offer only pollen as reward. As in other *Solanum* species, flowers bear poricidal anthers that are firmly erect, and dehisce pollen through small apical pores (Symon 1979). Out of 4-8 flowers present in an inflorescence, two flowers are usually displayed at any time.

The plants used in my experiments were grown from seeds harvested from an open-pollinated plant in a reserve near Adelaide, South Australia. Seedlings were grown in 2.5 l pots containing cocopeat in a greenhouse. To measure the pollen availability during a first bee visit, I used an electric toothbrush to vibrate the anthers of 20 hermaphrodite and 20 male flowers for 10-15 seconds. To collect the pollen, anther pores were positioned inside a 2 ml centrifuge tube. I added 300 µl of distilled water to the pollen and then loaded 40 µl of pollen suspension onto a haemocytometer to count the pollen grains under a compound microscope. The number of pollen grains was counted in each of the corner 1 mm^2 regions on both sides of the haemocytometer (eight fields per sample). Pollen counting was repeated three times and average pollen number per unit volume was calculated.

Experiments were conducted outdoors in the sensory garden at the Waite Campus of the University of Adelaide between January and March 2012. Native blue-banded bees, *Amegilla chlorocyanea*, were regularly observed foraging on

flowers in the garden at the time. Two weeks before the experiments, I placed around 25-30 potted flowering nightshade plants in the garden to train pollen collecting bees to visit them. Female blue-banded bees are capable of buzzing the anther to release pollen from apical pores, and are known to collect pollen from *S. elaeagnifolium* and other *Solanum* species (Anderson and Symon 1988; Hogendoorn et al. 2007; Thorp 2000). Female *A. chlorocyanea* included the nightshade flowers in their foraging routes and regularly visited them for pollen (Fig. 3.1B). All experiments were conducted on sunny days between 8:30 and 11:00 AM.

I conducted two experiments to examine whether style length in *S. elaeagnifolium* flowers influences bee visitation pattern. In the first experiment, I studied bee visits to pairs of similar-sized hermaphrodite and male flowers to determine if there is a natural preference for either flower type. In the second experiment, I examined whether reduced style length influenced visitation patterns by observing bee foraging behaviour on pairs of similar-sized hermaphrodite flowers in two floral morph treatments: normal and with manually reduced style length. Style length was reduced using fine scissors. This excluded the possibility that differences other than style length between male and hermaphrodite flowers influence bee behavior.

On the day prior to the experiment, I covered opening flower buds using fine netting bags with pull strings to avoid any bee visits and allocated them to either treatment. In each experiment, I uncovered one pair of treatment flowers and placed the pots in such a way that the experimental flowers were 10 cm from each other and at the same height above the ground (Fig. 3.2). Visitation

Figure 3.2. Experimental set up displaying short- and long-styled floral morphs placed 10 cm from each other was allowed for two bee visits.

responses on the first two bee visits to the pairs of experimental flowers were observed in 22 and 20 replicates in the first and second experiments, respectively. A visit began when a bee buzzed the first flower of the pair, and ended when the bee left to forage elsewhere, e.g. to adjacent flower patches. The bees always visited both of the treatment flowers, and they regularly switched between the flowers during a visit. The second bee visit followed within 10 minutes from the first visit, and was either from a different bee or from a same bee that visited first, but after visiting adjacent non *Solanum* flower patches. In some cases, the treatment flowers did not receive a second bee visit within 10 minutes after the first bee visit. To characterize the bee visitation patterns on treatment flowers, I

recorded the number of buzzes per flower and average time spent on each buzz, i.e., buzzing time using *The Observer* event-recording software (Noldus 1991).

A two-way ANOVA was performed to examine the interaction effect of observation date and floral morph treatments on the numbers of buzzes and buzzing times. As there was no effect of observation date and no interaction, I subsequently compared the visitation of the flowers using independent sample *t* tests during first and second bee visits. For statistical analysis, all datasets were log-transformed to achieve equal variances. Means are presented with their standard errors.

Results

There was no difference in the number of pollen grains released after manual vibration of hermaphrodite (245,353 ± 19,163 SE) and male (214,703 ± 39,022 SE) flowers ($t_{38} = 0.79$, $P = 0.21$).

The bees did not respond differently to the two floral morphs across three observation dates in the number of buzzes ($F_{2,38,1^{st}\ visit} = 0.86$, $P = 0.43$; $F_{2,22,2^{nd}\ visit} = 1.95$, $P = 0.18$) and buzzing time ($F_{2,38,1^{st}\ visit} = 0.76$, $P = 0.47$; $F_{2,22,2^{nd}\ visit} = 0.06$, $P = 0.81$). There was no significant difference in the number of buzzes performed on hermaphrodite and male flowers during the first visit ($t_{42} = 0.59$, $P = 0.55$) or the second visit ($t_{26} = 0.004$, $P = 0.99$; Fig. 3.3A). There was no difference in the average time spent per buzz on hermaphrodite and male flowers during first ($t_{42} = 0.07$, $P = 0.95$) and second bee visits ($t_{26} = 1.1$, $P = 0.28$; Fig. 3.3B).

In the second experiment, there was no effect of observation date on either the number of buzzes on normal hermaphrodite flowers and hermaphrodite

flowers with an experimentally reduced style ($F_{3,32,1}^{st}{}_{visit} = 0.19$, $P = 0.89$; $F_{3,30,2}^{nd}{}_{visit} = 1.67$, $P = 0.19$) or the average duration of buzzes ($F_{3,32,1}^{st}{}_{visit} = 0.03$, $P = 0.99$; $F_{3,30,2}^{nd}{}_{visit} = 0.46$, $P = 0.71$). There was no significant difference in the number of buzzes performed on hermaphrodite flowers with manually reduced style length and flowers with normal style length during the first ($t_{38} = 0.64$, $P = 0.52$) and second bee visits ($t_{36} = 0.49$, $P = 0.62$; Fig. 3.4A). The mean time per buzz did not differ significantly between the two floral morphs during the first ($t_{38} = 0.07$, $P = 0.94$) and second bee visits ($t_{36} = 1.38$, $P = 0.18$; Fig. 3.4B).

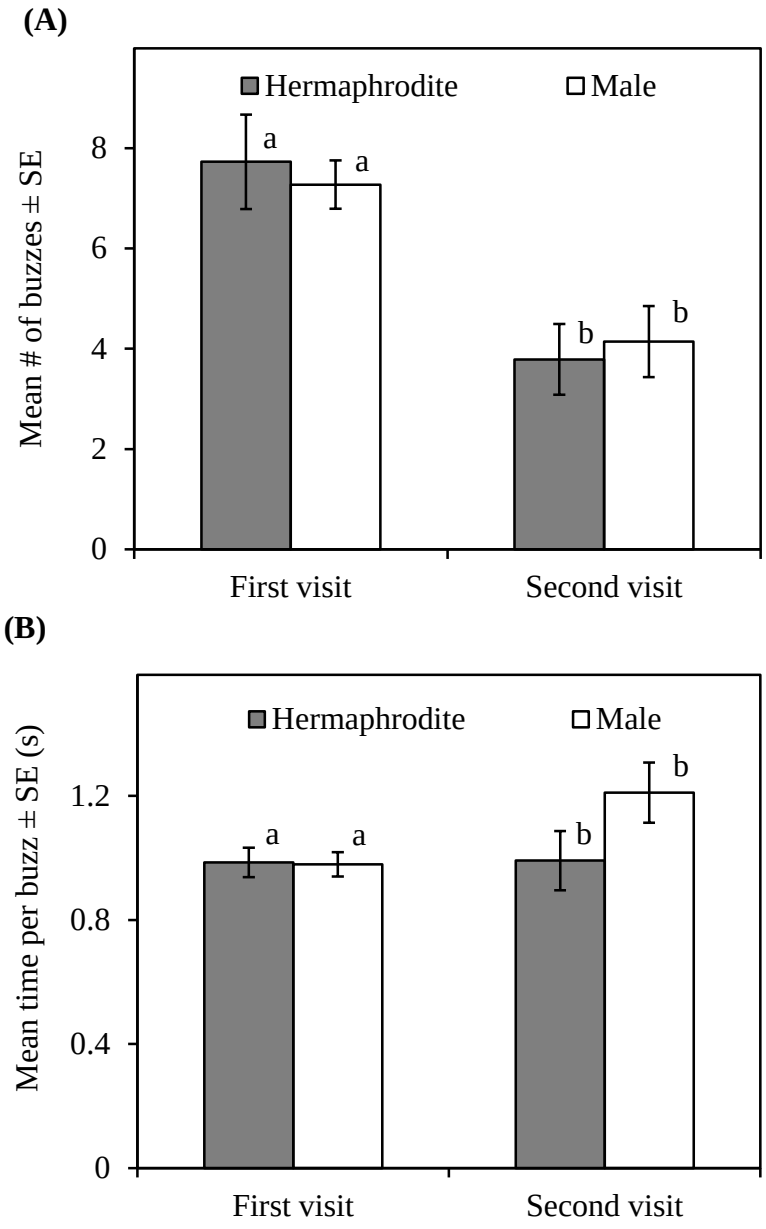


Figure 3.3. A comparison of the mean number of buzzes (A) and mean buzzing time (B) recorded in hermaphrodite and male flowers of *Solanum elaeagnifolium* during two visits by *Amegilla chlorocyanea*. The same letters above the bars indicate no significant differences between the two floral morphs in each bee visit. Number of samples of each floral morph is 22 flowers.

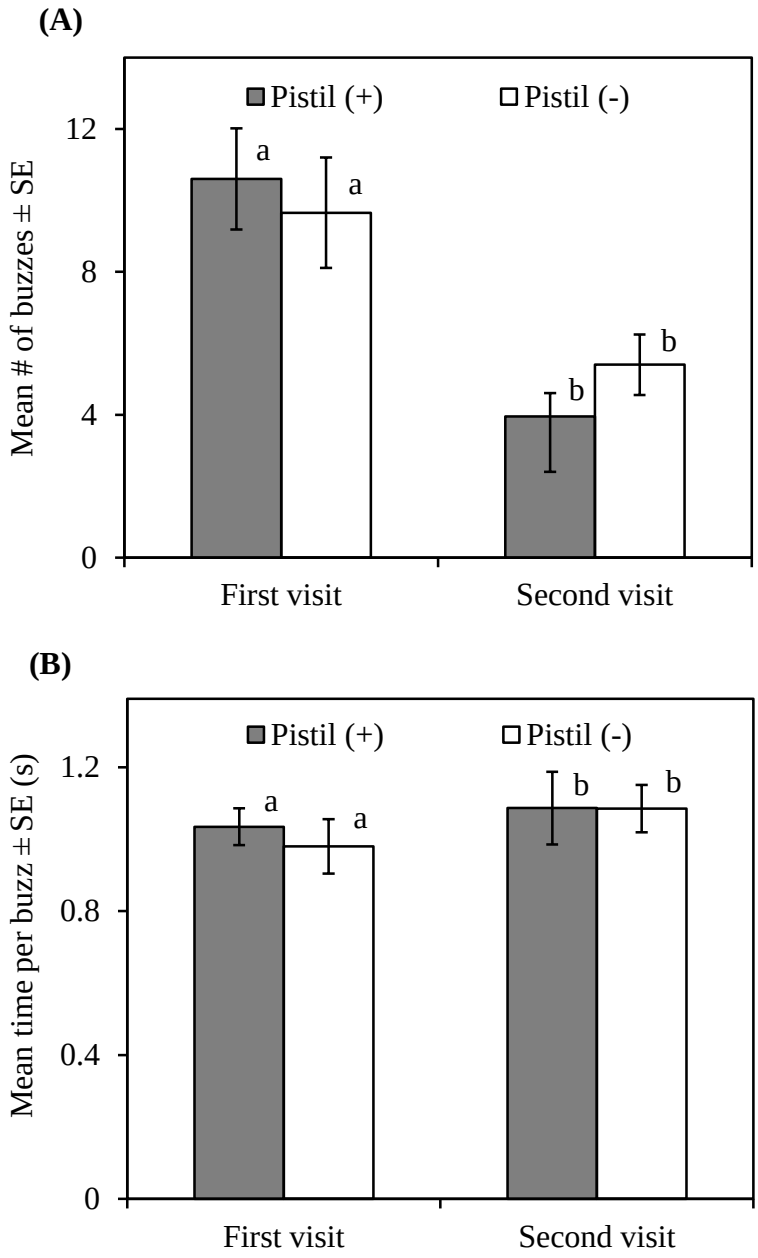


Figure 3.4. A comparison of the mean number of buzzes (A) and mean time per buzz (B) recorded during the first two visits by *Amegilla chlorocyanea* to hermaphrodite flowers of *Solanum elaeagnifolium* with pistils (+) and without pistils (-).The same letters above the bars indicate no significant differences between the two floral morphs in each bee visit. The number of samples of each floral morph is 20 flowers.

Discussion

In andromonoecious *S. elaeagnifolium*, floral morphs that differed in their style length did not elicit any difference in the visitation patterns of blue-banded bees in my outdoor experiments. During the first and second bee visits, there were no differences in either the number of buzzes or time spent on each buzz between the floral morphs. So my results suggest that pollinator bees don't respond in their visitation patterns to style length differences and thus short styles in male flowers of andromonoecious *Solanum* species are unlikely to be driven by bees' avoidance of a long style.

Quesada-Aguilar et al. (2008) showed that the number of buzzes in a single visit by bumblebees positively influenced the positive relationship found between style length and the amount of pollen removed from the anther and likewise, the negative relationship of style length with the amount of pollen deposited on the stigma. These findings indicate that any selection on style length to increase either pollen removal or pollen deposition should not affect the number of buzzes to floral morphs. In contrast, the number of buzzes by bumblebees significantly differed between long-styled and short-styled flowers of *S. carolinense* in the same study (Quesada-Aguilar et al. 2008). However, as predicted, small halictid bees that are able to buzz the anthers displayed equal numbers of buzzes to short- and long-styled flowers in the same study (Quesada-Aguilar et al. 2008). Interestingly, Vallejo-Marín & Rausher (2007a) reported that bumblebee visitation to the two floral morphs of *S. carolinense* differing in their style length was the same in frequency and duration. Consistent with previous studies, the present study also showed that visitation patterns of *Amegilla* bees to short- and

long-styled floral morphs were not different in the number of buzzes and buzzing time during a single visit. Therefore, I suggest that differences in style length between hermaphrodite and male flowers of andromonoecious *Solanum* species do not change bee visitation patterns. This raises the question of whether bee visitation patterns influence the amount of pollen removed from the anther and deposited on the stigma.

In a number of *Solanum* species, the protruding stigma was found to interfere with the ability of bees to grasp the anther cone and buzz it, and thus to interfere with efficient pollen removal (Diggle and Miller 2004; Elle and Meagher 2000; Solomon 1985). However, bees, especially those of medium size, do not always attempt to hold all five anthers together when the anthers are free and form a loose anther cone, as in *S. elaeagnifolium*. They often grasp and buzz two or three anthers together (ASM, personal observations). Even though the advantage of transferring more pollen to bees on a single visit was found for the short-styled floral morphs of *S. carolinense* (Quesada-Aguilar et al. 2008), this did not make them preferable over long-styled morphs to bumblebees (Vallejo-Marín and Rausher 2007a). Furthermore, when a bee visited the flowers of *S. carolinense*, the stigma protruding above the anthers of the long-styled floral morph was more likely to touch the bee's corbiculum, and thus received more pollen (Quesada-Aguilar et al. 2008). In addition, the short style morph would likely fail to remove a large amount of pollen from bees that collected it from long-styled morphs (Quesada-Aguilar et al. 2008). Irrespective of pistil interference, bees would be likely to re-visit the long-styled morph repeatedly to collect pollen rather than forego the reward. Therefore, I suggest that pistil interference in the form of style

length differences may be functionally significant to the flowers, as short-styled flowers are more advantageous for pollen collection, whereas long-styled flowers are advantageous for pollen deposition in andromonoecious *Solanum*.

While having no responses to style length differences, pollen collecting bees usually display a preference for flowers based on the amount of reward offered (Harder 1990; Robertson et al. 1999). Bees also have the ability to assess pollen availability using visual and olfactory signals from floral traits that are associated with rewards, and adjust their visitation frequency to flowers that are depleted of pollen (Buchmann and Cane 1989; Goulson 1999; Harder 1990). In *Solanum*, pollen is concealed in poricidal anthers that could limit bees' ability to detect the differences in pollen reward available in flowers without any floral cues (Buchmann and Cane 1989). While style length is unlikely to be an indicator of pollen availability, bruises left on the anthers after several buzzing events may perhaps provide visual signals to bees (Larson and Barrett 1999). Consistent with this, I found significant differences in the number of buzzes between first and second bee visits, regardless of floral morphs visited in the present study. However, with reward-based foraging, the visitation patterns of *A. chlorocyanea* did not differ between hermaphrodite and male flowers that had the same amount of pollen reward. My results therefore indicate that the style length difference between hermaphrodite and male flowers of andromonoecious *Solanum* species is unlikely to influence bee visitation patterns that are largely determined by the reward present in the flowers.

I also found no difference between male and hermaphrodite flowers in the number of buzzes they received during the first visit. This was not unexpected and

indicates equal pollen availability. The effectiveness of pollination often relies on more than a single visit by a bee and any difference in the reward would disappear after the first visit, as the pollen in buzz pollinated flowers reverts to a standing reserve.

In conclusion, my study suggests that the style length differences between male and hermaphrodite andromonoecious *Solanum elaeagnifolium* do not influence the visitation patterns of *A. chlorocyanea*. Nevertheless, it is possible that style length influences pollen removal and deposition, more pollen is removed from short-styled flowers, and there is an increased pollen deposition on long-styled flowers, as showed by Quesada-Aguilar et al. (2008). Thus, while the pollen removal and deposition hypotheses are upheld, my results do not support the pollen interference hypothesis.

CHAPTER FOUR - BEES' ABILITY TO ASSESS POLLEN COLLECTION FROM PORICIDAL ANTHERS OF INDIVIDUAL FLOWERS

Abstract

Flowers of buzz-pollinated plants produce no nectar and conceal the amount of pollen present. Thus, bees foraging on such flowers need to assess pollen availability to prevent foraging in non-rewarding patches and maximize foraging efficiency. I presented buzz-pollinating blue-banded bees with pollen-full and pollen-emptied flowers of silver leaf nightshade (*Solanum elaeagnifolium*) to examine how bees modify their foraging behaviour in response to pollen availability. I recorded the number of buzzes and the duration of each buzz during bee visits. The foraging responses of individual female *Amegilla chlorocyanea* were the same on rewarding pollen-full flowers and pollen-emptied flowers during the first visit, but there were significant differences between first and second visits to treatment flowers. Bee foraging responses during visits to individual flowers at time intervals were found to be significantly different, however the decline in the number of buzzes was gradual. The present study provides further evidence that bees can assess pollen availability, but not necessarily at the individual flower level, and can also modify their foraging behaviour in response to pollen availability.

Introduction

Bees forage in such a way that would improve their efficiency, and in turn accomplish pollination success in flowers. Bee-pollinated plants often produce both nectar and pollen as reward for pollination service, but some species offer only pollen. Floral rewards are often variable in availability within individual flowers (Simpson and Neff 1983). Thus, bees have various foraging strategies including detection and rejection of less rewarding flowers by associating rewards with subtle visual and olfactory signals to increase the chance of more reward in the flowers they visit (Goulson 1999). The behavioural responses of bees to interfloral variation in reward availability have been extensively studied, but largely in relation to nectar reward. For instance, the nectar foraging bees are known to assess the nectar available within a flower, and respond by avoiding non-rewarding flowers and spending longer times on more-rewarding flowers (e.g. Cnaani et al. 2006; Cresswell 1999; Goulson 1999; Rathcke 1992). In contrast, few studies have been conducted to establishing the relationship between bee foraging behaviour and pollen availability in flowers.

Experiments investigating whether pollen-collecting bees discriminate between flowers on the basis of pollen variability among individual flowers have produced equivocal results. Hodges and Miller (1981) reported that pollen-collecting bumble bees did not distinguish between flowers, and had a relatively constant probability of leaving a flower after any pollen collection time. Similarly, Haynes and Mesler (1984) concluded that bumble bees did not respond to pollen availability while moving among flowers. However, other authors concluded that bumble bees foraging for pollen differentiated recently visited flowers from

unvisited flowers and avoided revisiting them (Dobson et al. 1999; Gori 1989; Pellmyr 1988; Zimmerman 1982). Similar discrimination between flowers on the basis of visitation rate was also found in bumblebees foraging on *Campanula rotundifolia* (Cresswell and Robertson 1994) and *Mimulus guttatus* (Robertson et al. 1999), which implies that visitation can serve as a proxy for assessment of pollen availability. Likewise, a halictid bee, *Agapostemon nasutus*, accepted only fresh flowers and avoided flowers that were just visited while collecting pollen from nectarless *Sida glabra* flowers (Goulson et al. 2001).

In the experiments described above, the bees' ability to recognize and avoid rewardless flowers without landing is not surprising when flowers have freely exposed anthers, because bees might directly assess the visible pollen (Cresswell and Robertson 1994). However, bees do not have the same visual cue when pollen is concealed within the poricidal anthers of buzz-pollinated plants, and it is unknown whether they have similar foraging responses to flowers with concealed pollen.

In flowers with poricidal anthers, the potential pollen reward is hidden from the bee before it sonicates the flower. A pollen-collecting bee makes a series of short buzzes during each flower visit with short pauses when they transfer pollen from their body into their scopae (Buchmann 1983). If bees monitor the quantity of pollen as it is being collected, then this should be reflected in their foraging behaviour, including flower handling time, number of buzzes and the frequency of groomings to transfer pollen, could differ between full and pollen-depleted flowers. Hence, such bees' foraging responses to pollen returns from flowers have been considered as a measure of bees' ability to assess the pollen concealed

within poricidal anthers of flowers. The number of floral visits in a given observation period may not be a good indicator of bees' response to pollen availability, as visits made to treatment flowers were not significantly different (Burkart et al. 2014; Connolly and Anderson 2003; Luo et al. 2008; Shelly et al. 2000; Vallejo-Marín et al. 2009).

Several studies have shown that buzz-pollinating bees modify their foraging behaviour in response to pollen returns while collecting pollen from poricidal flowers. Buchman and Cane (1989) removed the pollen rewards of flowers of *Solanum elaeagnifolium* by sealing the anther pores with glue or manually emptying them. Two species of bees that visited these unrewarding flowers elicited fewer buzzes and shorter handling times compared to their visits to flowers that offered pollen (Buchmann and Cane 1989). However, in a similar study with *Solanum carolinense*, Connolly and Anderson (2003) reported that both pollen rewarding flowers and flowers that had their anther pores sealed were equally visited during observations, but the duration of first bee visits to flowers with sealed anthers was shorter. In another study, carpenter bees buzzed the flowers of *Solanum stramoniifolium* with glued anthers less often than control flowers, nevertheless both flowers had nearly an equal number of bee visits (Burkart et al. 2014). Furthermore, Shelly et al. (2000) also found that flower handling times of three buzzing species of bees on flowers of *Solanum wendlandii* were longer on recently introduced fresh flowers during initial visits compared to concurrent visits to flowers that were introduced two hours earlier, even though both types of flowers had equal numbers of bee visits (Shelly et al. 2000). In a similar study with pollen-hidden *Dodecatheon*, Harder (1990) measured the

duration of buzzes elicited in the pairs of experimental flowers, and found that bumble bees buzzed rewarding flowers longer than empty flowers. These studies have provided evidence that bees are capable of assessing pollen returns from individual flowers that do not visually display the pollen availability and adjusting their foraging behaviour in response to actual pollen returns from flowers.

Here, I investigated whether individual bees recognize pollen depletion by examining bee foraging responses to flowers that have been emptied by recent bee visit and manual pollen extraction. I studied the foraging behaviour of buzz-pollinating female blue-banded bees (*Amegilla chlorocyanea*) by recording the number of buzzes and the duration of each buzz on the flowers of silver leaf nightshade (*Solanum elaeagnifolium*), that offer only pollen as reward. In addition, I also examined how the number of buzzes and the duration of each buzz are modified over time as novel pollen becomes available through the gradual release of pollen from anthers. This was done by investigating the foraging behaviour of bees on individual flowers over three time periods.

Methods

Silver leaf nightshade (*S. elaeagnifolium*), is a perennial herb species, native to South & Central America (Boyd et al. 1984). It is a weed in many parts of the world, including Australia (Cuthbertson et al. 1976). Flowers do not produce nectar and offer pollen as the only reward to bees. As in other *Solanum* species, pollen is concealed within poricidal anthers, which dehisce pollen through small apical pores. These buzz-pollinated flowers are visited by relatively large bees that can curl their body to hold anthers together and vibrate them to release the pollen from the apical pores (Buchmann 1983).

Experiments were conducted with plants that originated from the seeds from fruits of a single plant that were collected near Adelaide, South Australia. Seeds were germinated and grown in 2.5L pots using cocopeat soil in a greenhouse. Flowers to be used in experiments were tagged and covered using fine netting bags with pull strings on the day prior to the experiment to prevent any bee visits.

To investigate the total amount of pollen available on the first visit, pollen was manually collected in a 2 ml centrifuge tube by vibrating the flower with an electric tooth brush. 300 µl of distilled water was added to the pollen and then 40 µl of pollen suspension was loaded into a haemocytometer to count the pollen grains under a compound microscope. The number of pollen grains was counted in each of the corner 1 mm^2 regions on both sides of the haemocytometer (eight fields per sample). Pollen counts were repeated three times to estimate the mean pollen load per flower.

I conducted two experiments to examine whether bees respond to the amount of pollen available within the flower. The outdoor experiments were conducted in the sensory garden at the Waite Campus of the University of Adelaide between January and March 2012 on sunny days between 8:30 AM and 12:30 PM. To attract the local blue-banded bees to forage in this spot, I placed 25-30 potted flowering nightshade plants in the garden from two weeks prior to the start of my experiments until all my observations were finalized. I regularly observed native blue-banded bees, *Amegilla chlorocyanea*, foraging in the garden for pollen and nectar. Female blue-banded bees are capable of buzzing to release the pollen grains from poricidal anthers, and are also reported to collect pollen from *Solanum* spp. (Anderson and Symon 1988; Hogendoorn et al. 2007; Thorp

2000). The females of *A. chlorocyanea* included the nightshade flowers in their regular foraging routes. They usually started foraging on nightshade flowers around 8 AM in the morning.

In the first experiment, I investigated whether bees adjusted their foraging behaviour to the reward received. I did this by studying bee visits to pairs of equal-sized fresh flowers, each assigned to one of two treatments: pollen-full and pollen-emptied. Plants were positioned so that the treatment flowers were at the same height from the ground and 10 cm from each other. Pollen-emptied flowers were prepared by vibrating the flowers for 10-15 seconds continuously using an electric tooth brush. I observed bee foraging responses to 20 pairs of flowers during the first bee visit to the flowers on three different dates. I also observed the effect of the treatment on the second bee visit in seven instances. The second visit occurred within 10 minutes after the first visit, and was either by a different bee or by the same bee that visited first, but after it visited other flowers.

In the second experiment, I studied whether bees modify their foraging behaviour during revisitation to the same flowers at different intervals. To investigate this, I allowed bees to visit equal-sized flowers during 1-hr time intervals. On the day of an experiment, individual flowers were introduced in pairs to allow single bee visits, and were covered in between time intervals. The foraging responses of the bees were recorded on 40 individual flowers of each type on four different dates. Each flower was allowed to receive a single visit during the interval of 1 hr. As not all flowers that were presented after 3 and 4 hours were visited by bees, I analyzed the data collected from 32 flowers that received bee visits during all of the three times.

In both experiments, I recorded how many times the bees buzzed an individual flower (a) before switching to the other flower (i.e. during one 'buzzing bout'), (b) before leaving the patch ('number of buzzes per visit') using the *Observer* software (Noldus 1991). I also recorded the time spent per buzz and calculated the mean for each flower visit ('buzzing time').

I used a two-way ANOVA to analyse the number of buzzes and buzzing times in pollen-full and pollen-emptied flowers during two bee visits. I also conducted independent sample *t* tests to examine the differences in the number of buzzes during first five buzzing bouts between situations when first encountered flowers were pollen-full or pollen-emptied. To test how bees' responses changed over hourly revisitation to the same flowers, I conducted ANOVA for repeated measures followed by post-hoc tests with Bonferroni correction. The data were transformed with the square root before statistical analysis to meet the assumption of equal variances.

Results

The amount of pollen present in a single flower was in the range of 84,000 to 350,000 pollen grains per flower (mean = 225,274 ± 18,189 SD; N = 40).

In the first experiment, the pollen-collecting blue-banded bees always visited both flowers during a visit to the patch. They often switched between the flowers and there were between one and four buzzing bouts per flower. Bees buzzed pollen-full and pollen-emptied silver leaf nightshade flowers with equal frequency during two visits (Fig. 4.1A; $F_{1,50} = 0.01$, $P = 0.9$). The number of buzzes during the

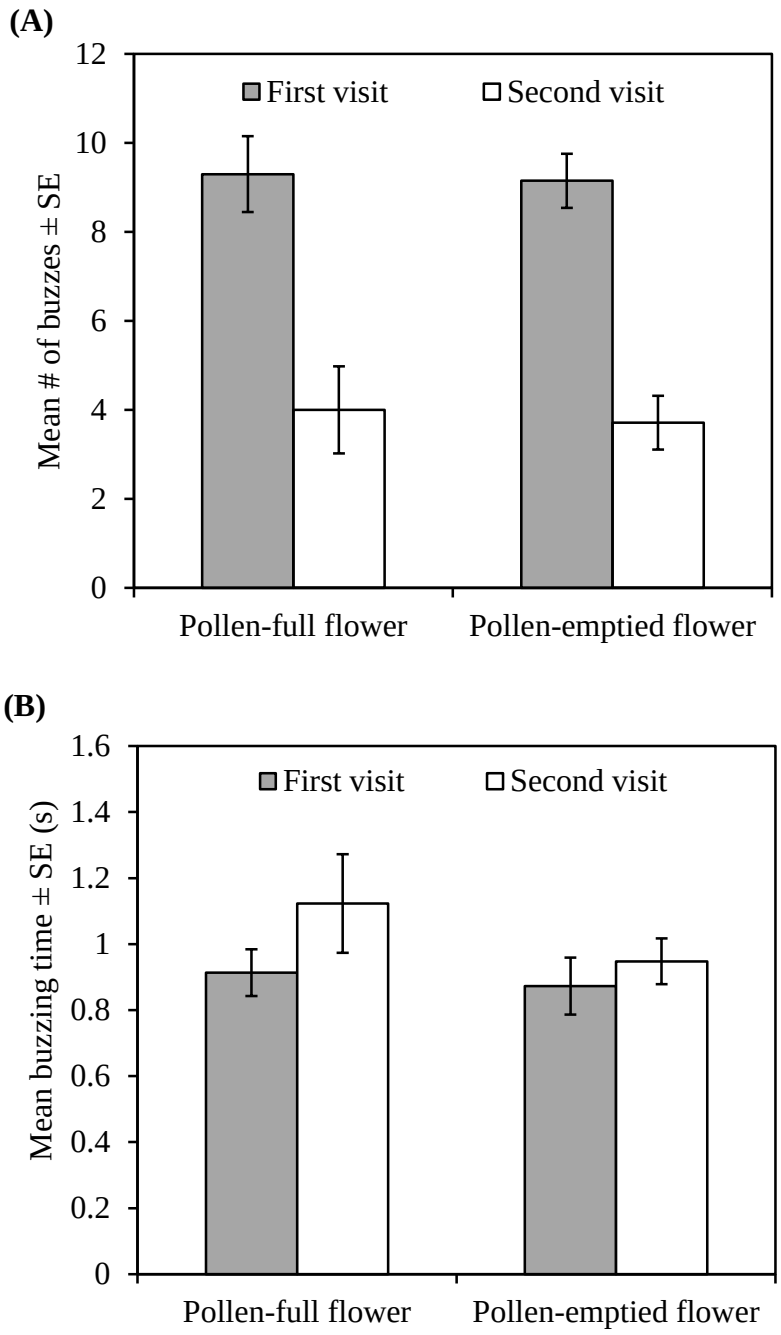


Figure 4.1. Number of buzzes (A) and buzzing time (B) were recorded at pollen-full flowers and pollen-emptied fresh flowers during two bee visits. The numbers of flowers in each treatment was 20 and 7 during first and second visits respectively.

first two buzzing bouts of the first bee visit was not significantly different in both scenarios when first encountered flower was full of pollen or pollen-emptied (Fig. 4.2; t = 1.11, P = 0.28). After the first two buzzing bouts, the number of buzzes

per bout became significantly lower (Fig. 4.2; t = 7.17, p = 0.001).Bees adjusted the number of buzzes on the second visit to flowers that were just visited. The bees buzzed significantly more frequently during the first than the second visit to the same flower, and this was found for both the pollen-full and the pollen-emptied flowers. Compared to the first visit, second visits elicited only half the number of buzzes (Fig. 4.1A, $F_{1,50}$ = 38.7, P = 1.0E-8). There were no interactions between first and second bee visits and treatment on the number of buzzes ($F_{1,50}$ = 0.02, P = 0.89), or between treatment and observation dates in either the number of buzzes ($F_{2,51}$ = 0.88, P = 0.41) or buzzing time ($F_{2,51}$ = 0.58, P = 0. 56). Mean buzzing time on pollen-emptied flowers was not different from that on pollen-full flowers (Fig. 4.1B, $F_{1,50}$ = 2.15, P = 0.15). However bees spent a longer time buzzing the flowers during their second visit than in the first visit ($F_{1,50}$ = 4.4, P = 0.04).

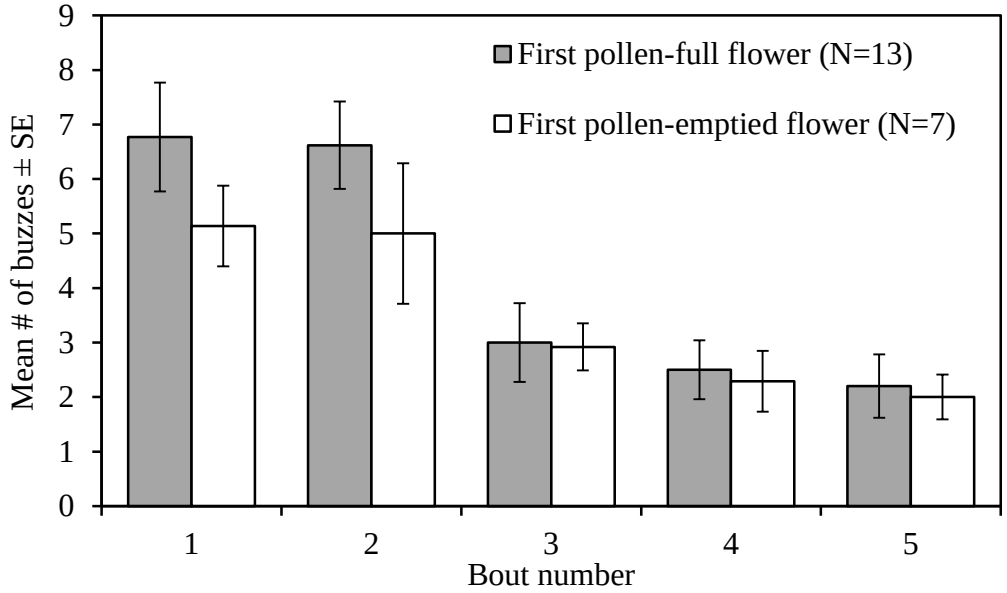


Figure 4.2. Comparison of the number of buzzes recorded during the first five buzzing bouts of the first bee visit between scenarios when first encountered flower was pollen-full or pollen-emptied.

There was a gradual decline in the number of buzzes during visits to individual flowers at time intervals. Over the single visits to the same flower at a one hour interval, the mean number of buzzes decreased from 11.65 during first visit to 8.5 and 6.6 in the second and third visits respectively (Fig. 4.3A). The mean number of buzzes differed significantly across all three visits in time intervals (repeated measures ANOVA $F_{2,62}$ = 17.39, P = 1.0E-6), and both the differences between first and the second and the second and the third buzz were significantly different (pairwise t-tests with Bonferroni correction: first and second buzz: t = 3.91, P = 0.0002; second and third buzz: t = 2.47, P = 0.01). The duration of the individual buzzes was the same across three visits at 1-hr time intervals (Fig. 4.3B, $F_{2,62}$ = 0.44, P = 0.65).

Discussion

Female *A. chlorocyanea* did not seem to assess the pollen reward during buzzing, as pollen-emptied and pollen-full flowers received equal numbers of buzzes of equal duration during their first visit. The non-significant difference in the number of buzzes performed during the first buzzing bout further supports this conclusion. However, the number of buzzes decreased with the buzzing bout number, and flowers that were visited a second time received fewer than half the number of buzzes than flowers that were visited for the first time. This demonstrates that the bees adjust the number of buzzes over time.

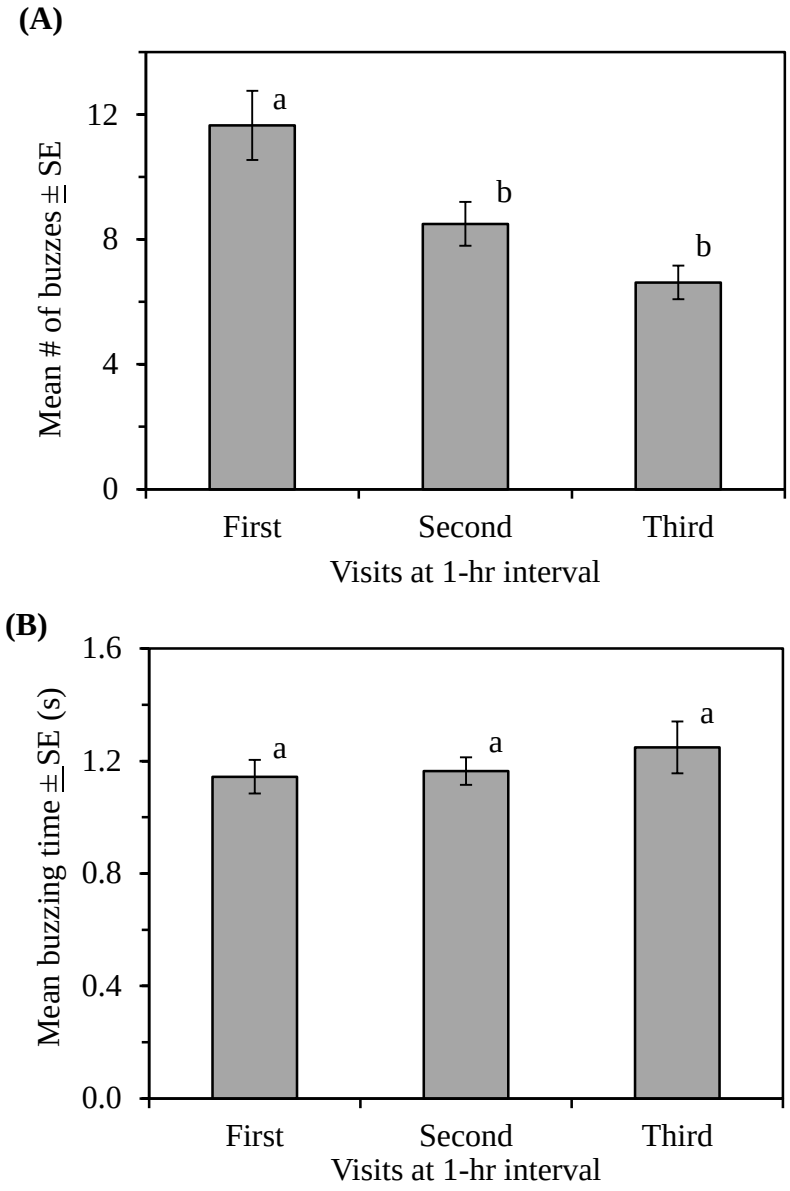


Figure 4.3. Number of buzzes (A) and buzzing time (B) were recorded during three bee visits to flowers at intervals of 1-hr. Sample size was 32 flowers. Different letters above the bar indicate significant differences in mean values between time intervals.

The finding that the number of buzzes and buzzing time per visit were the same for both pollen-full and pollen-emptied flowers appears to be in contrast with most previous studies (Buchmann and Cane 1989; Burkart et al. 2014;

Connolly and Anderson 2003; Harder 1990; Shelly et al. 2000). My results are in conformity with those of Luo et al. (2008), who found that the duration of first visits made by carpenter bees to buzz-pollinated flowers of *Melastoma malabathricum* were not different between flowers with sealed anthers and control flowers. The lack of difference in the foraging behaviour between experimental flowers may otherwise indicate that pollen availability in one flower provides little information about pollen availability in an adjacent flower as suggested in Harder (1990). I therefore suggest that, although there seems to be some raised expectation of increased reward when an empty flower is buzzed directly after a full flower was encountered, it seems that, on the whole of the visit to a patch, the individual bees do not assess pollen availability at the flower level. The lack of differences in foraging responses between treatment flowers during second bee visit was not unexpected, because even in pollen-full flowers, pollen grains left in the anthers are not released immediately after the first visit.

The observation that there are significant differences in the number of buzzes and buzzing duration between the first and the second visit was also reported for carpenter bees foraging on *M. malabathricum* flowers (Luo et al. 2008). It is interesting to note that the individual buzzes lasted longer during the second visit. Such longer and more intense buzzes may serve to collect the remaining pollen from nearly depleted flowers. It is to be expected that, on encountering a patch with a full flower, the bees may be motivated to move quickly to other flowers nearby to remove any large amounts of pollen before the competitors have discovered them. The bees could then return to already visited flowers to collect the remnant pollen that requires relatively more effort to extract.

Furthermore, the differential foraging responses by female *Amegilla* to pollen availability found between first and second visits indicate that bees somehow recognize recently visited flowers. Because my earlier results did not indicate an assessment of pollen availability during buzzing, this recognition must be based on other cues than the amount of available pollen. In general, bees may use visual and olfactory stimuli as signals of pollen availability to make indirect pollen assessment in flowers (e.g. Dobson et al. 1999). While floral odour, including from pollen grains, has been reported in many *Solanum* species (Buchmann et al. 1977), Morse et al. (2012) did not find any evidence for flower size and floral scent being used by bumble bees to assess the pollen availability in flowers of commercial tomato (*Solanum lycopersicum*). The differences in bee foraging responses between first and second visits to treatment flowers may be attributed to the bees' ability of recognizing bite marks that were left on the anthers after frequent buzzes from previous visitors. In addition it is possible that the same individuals remembered the visited patch upon their return. Since I did not monitor the identity of the bees, this cannot be verified using my data. A third possibility is that the bees recognized a recent visit because the flower was marked with a volatile substance. The foraging strategy of using scent marks on a flower is prominent among traplining bees (Gilbert et al. 2001; Goulson et al. 2000; Schmitt and Bertsch 1990). Gilbert et al. (2001) suggested that the marking behaviour allows traplining bees to avoid just-emptied flowers, but at the same time also encourages revisits when the scent mark is faded. In my study, the number of buzzes was higher when the bees revisited after one hour, than when they revisited within ten minutes (Fig 4.1A and 4.3A). This observation supports

the hypothesis that bees recognise flowers that were previously visited using either memory or flower marking, as bite marks on the anthers remain after the first visit.

The gradual decrease in the foraging patterns of female *Amegilla* across three floral visits at the time interval of 1-hr corresponds with the changes in pollen availability in flowers over the course of a day through the sequential release of pollen grains from poricidal anthers (King and Buchmann 1996). The apportionment of pollen availability, which has been reported in many species including *Solanum* (Buchmann et al. 1977; King and Buchmann 1996; Passarelli and Cocucci 2006), entices bees to revisit the same flowers. Bees might perhaps receive nearly an equal amount of pollen during their second and third visits to individual flowers in time intervals, as the number of buzzes was not significantly different between the visits. In my observations, female *Amegilla* made repeated sequential visits to flowering patches within their foraging range in a fixed order and this behaviour was described as traplining foraging (Thomson 1996). Foraging responses to replenishment of pollen availability over time in flowers may have arisen among traplining bees as a result of prior foraging experience in their foraging path (Thomson 1996).

In summary, my study suggests that *Amegilla chlorocyanea* has the capability to recognize visited from non-visited flowers, and will buzz visited flowers fewer times. In an another study (Chapter 3), I found that female *Amegilla* were not affected by style length differences in flowers of *S. elaeagnifolium*, as bee foraging patterns did not differ between hermaphrodite and male flowers that had the same amount of pollen reward. Experiments with artificial flowers

(Chapter 2) also revealed that the foraging decisions of female *Amegilla* were reward-dependent, and any preferences for large flower sizes would diminish when flowers of different sizes offer the same amount of reward. Thus, the ability of buzz-pollinating bees to assess pollen rewards from poricidal anthers may ensure that bees revisit the flowers to collect pollen grains retained and later released from the anthers of *Solanum* flowers, which cannot use nectar to ensure revisitation.

CHAPTER FIVE - CROSS DRESSING IN AUSTRALIAN SPINY *SOLANUM*: PHYLOGENY, ANCESTRAL STATE RECONSTRUCTION AND CORRELATED EVOLUTION OF SEX SYSTEM AND MORPHOLOGICAL CHARACTERS

Abstract

The evolution of andromonoecy through production of male flowers at the distal end of an inflorescence has been hypothesised to be a developmental response to an increased allocation of limited resources to female fitness involving large flowers and fruits. This implies an association between large fruit, large flowers and the sex system. In this study, I investigated the evolutionary origins of sex systems and the morphological characters corolla size and fruit based on three gene regions of 71 Australian members of the subgenus *Leptostemonum*, which show interspecific variations in sexual expressions and associated morphological characters. I then tested for correlated evolution to determine whether transitions from one state to another in sex systems are accompanied by shifts in corolla size and fruit diameter. Phylogenetic evidence indicates that the sex system is highly variable with multiple independent evolutionary transitions from andromonoecy to hermaphroditism. The frequent increases in corolla size from the ancestral states were mostly of recent origin, while both increases and decreases in fruit diameter occurred at constant evolutionary rate from common ancestor to extant species. The rates of transition in correlated evolution of sex systems and morphological characters indicate that (1) the forward shifts from small to large in

corolla size and fruit diameter were present in both sex systems, but reversals occurred only in hermaphrodites; (2) the evolution of andromonoecy from hermaphroditism is likely to be preceded by increases in fruit diameter and corolla size.

Introduction

The genus *Solanum* (Solanaceae) constitutes ~1400 species worldwide of which around 450 species belong to subgenus *Leptostemonum*, the 'spiny *Solanum*' (Whalen 1984; Levin et al. 2006; Olmstead et al. 2008). In Australia the subgenus is represented by about 146 species (Barker 2010). Species within the subgenus *Leptostemonum* display a range of sex systems including hermaphroditism, andromonoecy and dioecy (Whalen and Costich 1986). The evolution of andromonoecy in this subgenus involves a transition in which the female function in flowers at the distal end of an inflorescence is lost. Andromonoecy is often considered a form of phenotypic plasticity with one or a few bisexual flowers below a cyme of few to many male flowers, and a potential unisexual transitional stage between hermaphroditism and dioecy (Diggle 1994; Miller and Diggle 2003). It occurs within 13 of 22 described sections of this subgenus, with each potentially representing independent origins among distantly related species groups (Whalen 1984; Whalen and Costich 1986). The degree of andromonoecy is highly labile, showing interspecific variations in the production of male flowers within species of this subgenus (Whalen and Costich 1986). Andromonoecy appears to have further evolved into the dioecious sex system in which male and functional female flowers are found on separate individuals in the subgenus *Leptostemonum* section *Melongena* (Symon 1979b; Martine et al. 2009;

Martine et al. 2006). This subgenus of *Solanum* provides a unique opportunity to evaluate the diversification of sex systems and evolutionary origin of andromonoecy.

Beside separation of sexes, the Australian species in the subgenus *Leptostemonum* also presents interspecific variation in traits that are usually associated with sexual functions including corolla size and fruit diameter (Symon 1979a, 1987; Anderson and Symon 1989). The variations in reproductive traits may reflect constrained phylogenic origins in distant ancestors or they may reflect localised selection pressures that involved multiple speciation events. The estimation of phylogenetic effects can elucidate the patterns of variation in character expression. Hence, this study first examined the phylogenetic relationships within the subgenus to understand the relationships among species. Then, the patterns of sexual character state evolution were investigated by reconstructing the ancestral states to identify the sequence and frequency of character transitions in a phylogenetic context.

Andromonoecy is associated with increased fruit size and reduced fruit number (Symon 1979b; Whalen and Costich 1986; Miller and Diggle 2007). It has been suggested that resource limitation associated with fruit set and producing large fruits causes a trade-off between fruit size and number (Stephenson 1981; Whalen and Costich 1986; Diggle 1995; Miller and Diggle 2007). Under resource limitation the high costs of producing fruits and seeds leads to female-biased sex allocation at the flower level, and this can cause a reduction in the number of functional pistils (Bertin 1982; Spalik 1991). It was thus proposed that species that bear large fruits and many or large seeds are more likely to evolve

andromonoecy than small-fruited species (Bertin 1982; Whalen and Costich 1986; de Jong et al. 2008). Among andromonoecious species, Whalen and Costich (1986) found a correlation between the proportion of male flowers and fruit size among the species of subgenus *Leptostemonum* section *Acanthophora* and *Lasiocarpa*. The same was further established when accounting for shared evolutionary history (Miller and Diggle 2007). In addition, the corolla size of fruiting flowers was found to be positively correlated with the strength of andromonoecy, i.e., the proportion of male flowers in an inflorescence among species (Miller and Diggle 2007).

Correlations between sex system and morphological traits have not been investigated among the Australian species of subgenus *Leptostemonum* in a phylogenetic context. This study explores mating system evolution to evaluate whether transitions from one state to another in the mating system are accompanied by shifts in corolla and fruit diameter. In cases where associations were detected, the direction of transition was evaluated. Any phylogenetically correlated changes should provide insights into the causal mechanisms and the adaptive significance of character transitions.

In this paper, a phylogeny of the Australian species of *Solanum* subgenus *Leptostemonum* was reconstructed and then used to explore patterns of character evolution by (1) determining the patterns of directional evolutionary change in morphological traits, and (2) then examining the phylogenetic dependence, mode and tempo of character changes that were mapped onto speciation events. Subsequently, (3) the ancestral character states at the nodes in the phylogenetic tree were reconstructed to investigate the occurrence and frequency of forward

and backward shifts between character states that were defined as binary using critical measurement values. Furthermore, (4) testing for correlated evolution between sex system and each of morphological traits: corolla size and fruit diameter was performed to examine whether the evolution of andromonoecy are conditional upon changes in these traits or whether these traits are more likely to evolve in the presence of andromonoecy.

Materials and Methods

Taxon sampling

This study includes a large sample (71 species) of the Australian members of *Solanum* subgenus *Leptostemonum*. For outgroups, five species from clades that formed a sister to Old World clade within subgenus *Leptostemonum* (Levin et al. 2006) and one species from *Brevantherum* clade, which is closely related to *Leptostemonum* within *Solanum* genus were selected (Weese and Bohs 2007).

Sequence alignment

I obtained DNA sequence data for the ITS (Internal Transcribed Spacer region of nuclear ribosomal DNA, composed of ITS1, 5.8S gene and ITS2), waxy (nuclear gene GBSSI composed of 3' end of exon 1 through 3' end of exon 10) and *trnT-trnF* (entire chloroplast region between *trnT* and *trnF* genes) from GenBank. Information on authors and GenBank accession numbers for all sequences sampled in this study are given in the Supporting Information Table S1. Sequence alignment for each gene region was conducted using ClustalW with default parameter settings (Larkin et al. 2007) as included in MEGA v.5 (Tamura et al. 2011). The concatenated dataset comprises alignment of 4092 characters on 77 species. Alignment gaps were coded as missing characters.

Inferring phylogeny

A Bayesian Inference (BI, Box 5.1) analysis was conducted with a gene region-partitioned sequence matrix of 4092 characters using MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003) to infer the phylogeny of the Australian subgenus *Leptostemonum*. A general time reversible model with a proportion of invariable sites, coupled with gamma-distributed rates across sites (GTR+I+G) was found to be the best-fit model of nucleotide substitution in the jModelTest 2.1.4 (Darriba et al. 2012; Guindon and Gascuel 2003). Comparison of marginal likelihoods demonstrated that the relaxed clock model was strongly favoured over a strict clock model. Two independent runs were performed with four Metropolis-coupled Monte Carlo Markov chains (MCMC) per generation (1 cold and 3 heated chains) simultaneously for 8 million generations, with sampling every 100 generations. To ensure adequate sampling of posterior distribution and determine

Box 5.1 Bayesian inference

A number of methods including the parsimony method, distance methods, and the maximum likelihood method have been used to infer phylogenetic trees. The advantages of Bayesian inference of phylogeny over other methods include easy interpretation of results, the ability to incorporate prior information, and some computational advantages. In a Bayesian analysis, inferences of phylogeny are based upon the posterior probabilities of phylogenetic trees. The posterior probability of a phylogenetic tree is the probability that the tree is correct, assuming that the model is correct. The MrBayes program uses the Metropolis-coupled Markov chain Monte Carlo method $(MC)^3$ to approximate the posterior probabilities of trees.

Source: (Huelsenbeck and Rannala 2004; Huelsenbeck and Ronquist 2001)

the burn-in, stationarity was assessed graphically using Tracer v.1.5 by plotting log likelihood values of all the sampled parameters against generation number. Two other convergence diagnostic methods namely, Estimated Sample Size (ESS) and Potential Scale Reduction Factor (PSRF) were also used with Tracer v.1.5 to confirm whether analysis achieved adequate sampling of posterior distribution. Although stationary distribution was reached after first 10% generations, the first 25% of sampled trees were conservatively removed in the further analysis. All the estimated parameters had ESS above 200 with PSRF approaching at 1 as the runs converge (Supporting Information Table S2). The detailed information on the choice of substitution models, different priors used and testing methods for species convergence in the MCMC chains during phylogenetic analysis are given in Appendix S1. A 50% majority-rule consensus tree was constructed from post burnin trees with posterior probabilities (PP) for each clade. Trees were viewed in FigTree v.1.3.1. The internal nodes with >90% posterior probability support are indicated with numbers from 1-30 on the phylogenetic tree. In order to account for phylogenetic uncertainty, the last 1000 sampled trees of the Bayesian phylogenetic analyses were used in the Bayesian analyses of character trait evolution.

Patterns of character state evolution

For the analysis of character state evolution, two characters: corolla size of fruiting flowers and fruit diameter were examined using continuous datasets (Supporting Information Table S3). Specimens from the State Herbarium of South Australia (AD) were examined for the corolla size, otherwise morphological descriptions in Symon (1981) and Bean (2004) were used. I first investigated

whether the evolution of traits followed a directional change random-walk model (directional, Model B) or a standard constant-variance random walk model (drift, Model A) using the Bayesian MCMC framework in the BayesTraits program (Pagel and Meade 2006). The most supported model of trait evolution was used to estimate three branch length scaling parameters: lambda (λ), kappa (κ) and delta (δ) for each continuous trait with MCMC framework in the BayesTraits (Box 5.2). The lambda values correspond to the contribution of shared evolutionary history in the phylogeny to the observed covariance among species in trait values. The kappa parameter measures the contribution of different branch lengths to evolutionary changes in traits, while delta scales the contribution of path lengths (i.e. distance from root to tip on the phylogenetic tree) to the rates of trait evolution as time progresses. More details on the estimation of three scaling parameters are explained in Appendix S1. I tested whether these estimated scaling parameters, which take branch length into account, were consistent with Brownian motion model, which assumes that phenotypic difference between lineages increases in proportional to the time since they shared a common ancestor. The estimated parameter values were incorporated as 'ModelFile' in the estimation of ancestral state values.

Box 5.2. Three scaling parameters and their interpretation when applied to trait evolution on a phylogeny

Parameter	**Action**	**0**	**<1**	**1**	**>1**
lambda	Assess contribution of phylogeny	Star phylogeny (species independent)	phylogenetic history has minimal effect	default phylogeny	not defined
kappa	Scale branch lengths in tree	punctuational evolution	stasis in longer branches	default gradualism	longer branches more change
delta	Scale total path (root to tip) in tree	Scale total path (root to tip) in tree	not defined	temporally early change important (adaptive radiation)	default gradualism

Source: (Pagel and Meade 2013)

Ancestral states reconstructions

I reconstructed ancestral values of corolla size and fruit diameter at 30 well supported internal nodes using MCMC framework in BayesTraits program. The estimated posterior distribution of model parameter (α) along with three scaling parameters (λ, κ and δ) were incorporated as 'ModelFile' in the MCMC chain after adjusting the data deviation parameter to achieve 20-40% acceptance for estimated ancestral states. I used 'MRCA' or 'Most Recent Common Ancestor' command in BayesTraits to specify the internal nodes for ancestral state reconstruction. I also used weighted squared-change parsimony in Mesquite 2.74 (Maddison and Maddison 2011) to reconstruct ancestral states of corolla size and fruit diameter at each node using the consensus tree.

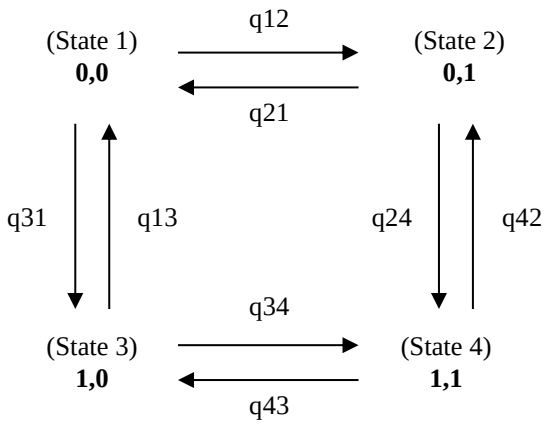
I used the MultiState program implemented in BayesTraits to reconstruct the ancestral states of three discrete characters: sex system, corolla size and fruit diameter. The continuous datasets of corolla size and fruit diameter did not have a bimodal distribution, and therefore I avoided using an arbitrary cut-off value to categorise them into small and large. I instead used the reconstructed ancestral state value at the base of the Australian species to create binary states for corolla size and fruit diameter. Corolla size was coded as small (≤2.32 cm) or large (>2.32 cm), while fruit diameter was coded as small (≤1.47 cm) or large (>1.47 cm). The sex system of species in this study is either hermaphroditism or andromonoecy. A reversible jump (RJ-) MCMC analysis was run for 20 million iterations sampling every 300th iteration and discarding the first 1 million iterations as burn-in. After exploring different prior combinations, rate deviation (rd) was fixed at 80 with exponential prior for rate coefficients seeded by hyperprior distribution on the interval 0 to 5 to achieve an acceptance rate of proposed change between 20 and 40%. This RJ-MCMC approach tests simultaneously the five possible transition rate parameter models of character state changes by visiting them in proportion to their posterior probabilities and reconstructs ancestral states (Appendix S1, Supporting Information Table S4). Ancestral state reconstructions were performed using 'MRCA' command that calculates the proportion of likelihood associated with each of the alternative character states on a given node (Pagel et al. 2004).

Box 5.3. Dependent or Correlated model of evolution

The dependent or correlated model assumes that the traits are correlated and the rate of change in one trait is dependent on the state of the other. The dependent model has 8 parameters, q12, q13, q21, q24, q31, q34, q42 and q43.

Parameter	Dependent on	Trait …	Transition
q12	Trait 1=0	2	$0 \rightarrow 1$
q13	Trait 2=0	1	$0 \rightarrow 1$
q21	Trait 1=0	2	$1 \rightarrow 0$
q24	Trait 2=1	1	$0 \rightarrow 1$
q31	Trait 2=0	1	$1 \rightarrow 0$
q34	Trait 1=1	2	$0 \rightarrow 1$
q42	Trait 2=1	1	$1 \rightarrow 0$
q43	Trait 1=1	2	$1 \rightarrow 0$

In other words, such a model describes possible transitions among the four paired states that each represents the combined states of two binary variables or traits. This is shown in the diagram below. Subscripts *i* and *j* for the transition parameters *q* identify the beginning and ending states, respectively, of each transition. The values 1, 2, 3, and 4 correspond to the state pairs {0,0}, {0,1}, {1,0}, and {1,1}. Thus, q_{12} describes the transition between state {0,0} and state {0,1} over a short time interval *dt*. Dual transitions in which the states of both variables instantaneously change are assumed not to occur.



Testing correlated evolution

To test for associations between sex system and (a) corolla size, (b) fruit diameter and (c) corolla size and fruit diameter, I used BayesDiscrete program in BayesTraits, which directly estimates the correlated evolutionary change in the binary characters taking branch length into account (Pagel and Meade 2006). A Bayesian MCMC framework in this program uses binary discrete characters to run two alternative models: (1) the independent model in which two binary characters evolve independently; (2) the dependent model which describes correlated evolution for the combination of two binary characters (Pagel and Meade 2006). The correlated or dependent model detects whether change in one character is more likely to evolve in the presence of a particular state in another character (Box 5.3). For the analysis, a uniform hyperprior with exponential distribution on the interval of 0 to 5 was used while rate deviation was set to achieve the recommended acceptance rates (20-40%). The RJ-MCMC chain was allowed to run for 20 million iterations with a sampling frequency of 300 and a burnin of first 1 million iterations. Bayes Factor (BF) values were calculated comparing the harmonic means from alternative models to determine the best fitting model of character evolution (see Box 5.4.and Appendix S1 for more information). The probable directions of evolutionary changes from the reconstructed ancestral state in two binary characters were determined by examining the posterior distributions of the rate coefficients.

Box 5.4. Bayes Factor values

In a Bayes factor (BF) test, the marginal likelihood values for the independent and dependent models are compared. The test statistic is

ln(BF) = 2(*ln*[harmonic mean(complex model)] – *ln*[harmonic mean(simple model)]

Log (Bayes Factor) *ln*(BF)	Interpretation
< 2	Weak evidence
> 2	Positive evidence
5-10	Strong evidence
> 10	Very strong evidence

Source: (Pagel and Meade 2013)

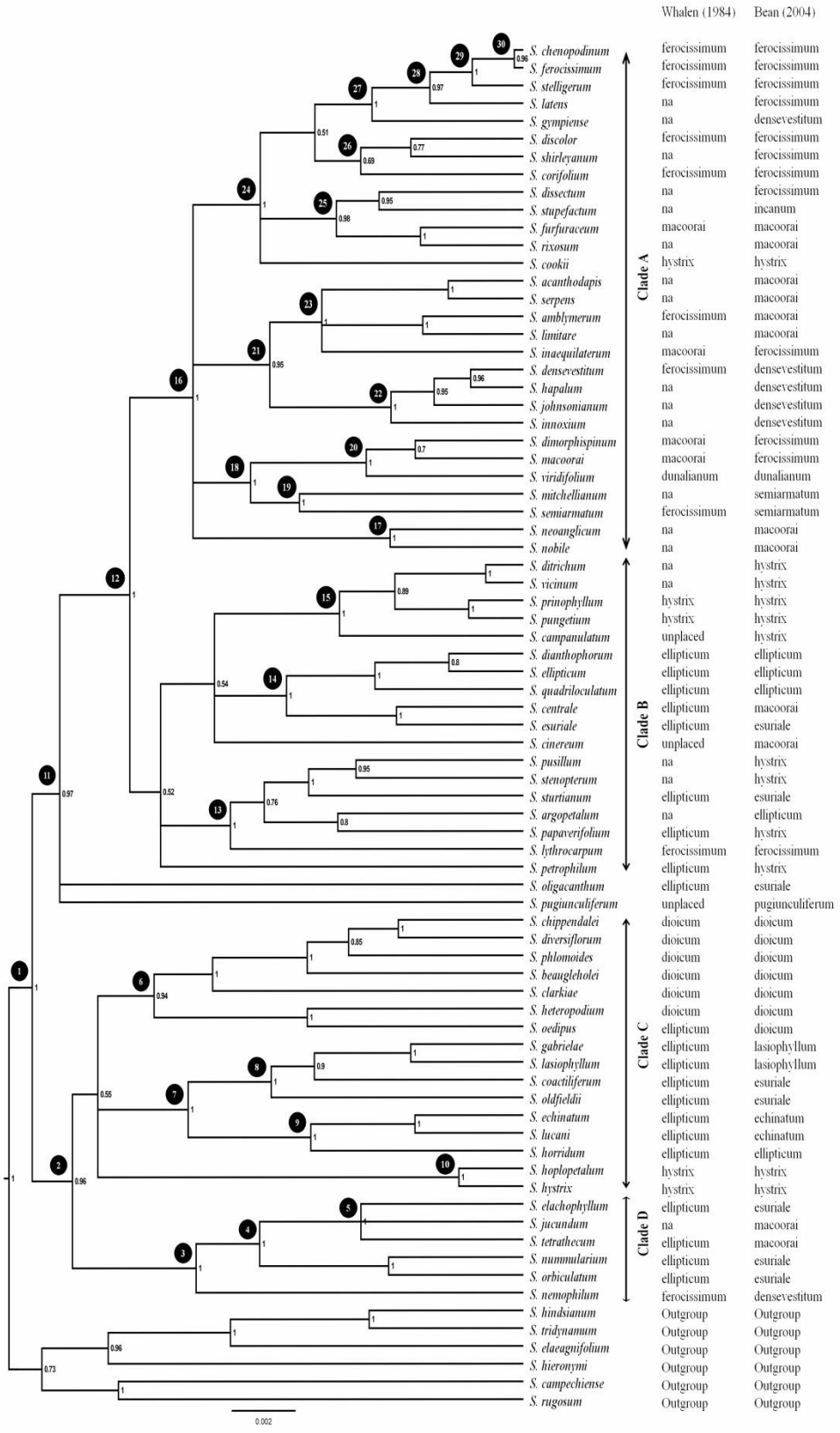
Results

Phylogenetic inference

Phylogenetic inferences were based on DNA sequence data from two nuclear gene regions (662 bp of ITS and 1706 bp of GBSSI) and one chloroplast spacer region (1725 bp of *trnT-trnF*) for a total of 4092 aligned nucleotide sites from 77 *Solanum* species. Consensus phylogenies from three replicate Bayesian analyses using the combined dataset had identical topologies. A 50% majority rule consensus tree inferred from the first Bayesian run resolved two major clades with strongly supported posterior probability values within subgenus *Leptostemonum* (Fig. 5.1). Species relationships recognised from the consensus tree offered no support to the classification of species groups presented in the earlier taxonomic treatments (Whalen 1984; Bean 2004). Two partially resolved clades were

detected within first major clade, the Clade A consisting of species largely from *ferocissimum-macoorai* species complex, whilst species belonging to *ellipticum-hystrix* species complex formed the Clade B. The second major clade consisted of one partially resolved Clade C and one fully resolved Clade D. The Clade C comprised of three subclades recognising *ellipticum-dioicum-hystrix*. Species from *ellipticum* species complex formed the clade D. None of the large species groups recognised by Whalen (1984) and Bean (2004) namely *ferocissimum, ellipticum, macoorai, hystrix* and *esuriale* were recovered as monophyletic on the phylogeny.

Figure 5.1. A Bayesian 50% majority rule consensus tree of *Solanum* subgenus *Leptostemonum* inferred from the combined ITS, GBSSI and *trn*T-*trn*F dataset of 77 species. Values at branches refer to posterior probabilities in support of each node. The well-supported internal nodes (>90 PP) used for ancestral state reconstruction analyses are given numbers from 1-30. The species group complex recognised in this study and species group classifications from previous taxonomic treatments (Whalen 1984; Bean 2004) are given next to species in the phylogeny.


Whalen (1984)
Bean (2004)
S. chenopodinum ferocissimum ferocissimum
S. ferocissimum ferocissimum ferocissimum
S. stelligerum ferocissimum ferocissimum
S. latens na ferocissimum
S. gympiense na densevestitum
S. discolor ferocissimum ferocissimum
S. shirleyanum na ferocissimum
S. corifolium ferocissimum ferocissimum
S. dissectum na ferocissimum
S. stupefactum na incanum
S. furfuraceum macoorai macoorai
S. rixosum na macoorai
S. cookii hystrix hystrix
S. acanthodapis na macoorai
S. serpens na macoorai
S. amblymerum ferocissimum macoorai
S. limitare na macoorai
S. inaequilaterum macoorai ferocissimum
S. densevestitum ferocissimum densevestitum
S. hapalum na densevestitum
S. johnsonianum na densevestitum
S. innoxium na densevestitum
S. dimorphispinum macoorai ferocissimum
S. macoorai macoorai ferocissimum
S. viridifolium dunalianum dunalianum
S. mitchellianum na semiarmatum
S. semiarmatum ferocissimum semiarmatum
S. neoanglicum na macoorai
S. nobile na macoorai
S. ditrichum na hystrix
S. vicinum na hystrix
S. prinophyllum hystrix hystrix
S. pungetium hystrix hystrix
S. campanulatum unplaced hystrix
S. dianthophorum ellipticum ellipticum
S. ellipticum ellipticum ellipticum
S. quadriloculatum ellipticum ellipticum
S. centrale ellipticum macoorai
S. esuriale ellipticum esuriale
S. cinereum unplaced macoorai
S. pusillum na hystrix
S. stenopterum na hystrix
S. sturtianum ellipticum esuriale
S. argopetalum na ellipticum
S. papaverifolium ellipticum hystrix
S. lythrocarpum ferocissimum ferocissimum
S. petrophilum ellipticum hystrix
S. oligacanthum ellipticum esuriale
S. pugiunculiferum unplaced pugiunculiferum
S. chippendalei dioicum dioicum
S. diversiflorum dioicum dioicum
S. phlomoides dioicum dioicum
S. beaugleholei dioicum dioicum
S. clarkiae dioicum dioicum
S. heteropodium dioicum dioicum
S. oedipus ellipticum dioicum
S. gabrielae ellipticum lasiophyllum
S. lasiophyllum ellipticum lasiophyllum
S. coactiliferum ellipticum esuriale
S. oldfieldii ellipticum esuriale
S. echinatum ellipticum echinatum
S. lucani ellipticum echinatum
S. horridum ellipticum ellipticum
S. hoplopetalum hystrix hystrix
S. hystrix hystrix hystrix
S. elachophyllum ellipticum esuriale
S. jucundum na macoorai
S. tetrathecum ellipticum macoorai
S. nummularium ellipticum esuriale
S. orbiculatum ellipticum esuriale
S. nemophilum ferocissimum densevestitum
S. hindsianum Outgroup Outgroup
S. tridynamum Outgroup Outgroup
S. elaeagnifolium Outgroup Outgroup
S. hieronymi Outgroup Outgroup
S. campechiense Outgroup Outgroup
S. rugosum Outgroup Outgroup
Clade A
Clade B
Clade C
Clade D
0.002

Patterns of character evolution

The constant-variance random walk model of trait evolution was the best fitting model for estimating scaling parameters and ancestral state reconstructions. In the MCMC analysis using continuous character values, this constant-variance model resulted in higher likelihood values than the directional change random walk model (Supporting Information Table S5). With an estimated λ value close to 1 (λ = 0.75), fruit diameter was consistent with a Brownian motion model of trait evolution, while corolla size had an intermediate lambda values ($0 < \lambda < 1$) (Table 5.1). These two characters were phylogenetically non-independent traits with lambda estimates significantly greater than 0. Scaling parameter kappa (κ) estimates for corolla size and fruit diameter were consistent with a Brownian motion model of evolution (κ = 1) supporting character evolution dependent on branch length (Table 5.1). Kappa estimates were different from zero rejecting the punctuated mode of evolution in corolla size and fruit diameter. The tempo of fruit diameter evolution had constant evolutionary rates from root to tips (δ = 1) following the Brownian motion model (Table 5.1). Corolla size with δ = 2.14 had more evolutionary changes arising in more recent species.

Overall, there is a general trend of equal rate of gains and reversals in three characters investigated for evolutionary patterns using the Bayesian rj-MCMC approach that visits different models in proportion to their posterior probabilities. For sex system, the most sampled parameter model was the one in which the transitions from hermaphroditism to andromonoecy is constrained to zero ($q01 = 0$) (Table 5.2). The one parameter model of equal forward and backward transition rates ($q01 = q10$) was the next supported model (41%), while other models were

rarely visited. For corolla size evolution, the MCMC chain sampled half of its time in the restricted model ($q10 = 0$) in which transitions from large to small is constrained to zero, followed by a model where backward and forward transitions are at equal rates ($q01 = q10$) (Table 5.2). Only two models were frequented in the chain for the evolution of fruit diameter and out of these equal transition rates had substantially more support than the model where reversal to small fruit size did not occur ($q10 = 0$) (Table 5.2). The frequent sampling of restricted models in the chain makes transitions such as hermaphroditism to andromonoecy and large to small corolla size and fruit diameter less likely.

Table 5.1. Scaling parameters: lambda (λ), kappa (κ), delta (δ) for two characters estimated using MCMC framework in the constant-variance random walk model in BayesTraits Beta v1.1. Bayes Factors (BF) are given for those instances where the mean likelihood value in the model in which parameters are fixed (v = 0 and 1) is significantly different from the model in which parameters are estimated (v = est).

Character	Parameter estimate	Log harmonic mean of likelihood value				
		v = est	v = 0	BF	v = 1	BF
$\lambda_{\text{Corolla size}}$	0.47	-85.83	-89.27	6.9	-90.04	8.4
$\lambda_{\text{Fruit diameter}}$	0.75	-70.62	-79.53	17.8	-71.31	1.37
$\kappa_{\text{Corolla size}}$	0.93	-91.05	-94.84	7.6	-90.67	-0.76
$\kappa_{\text{Fruit diameter}}$	0.70	-71.33	-72.15	1.6	-71.03	-0.60
$\delta_{\text{Corolla size}}$	2.14	-88.12			-90.31	4.4
$\delta_{\text{Fruit diameter}}$	1.77	-70.96			-70.19	1.77

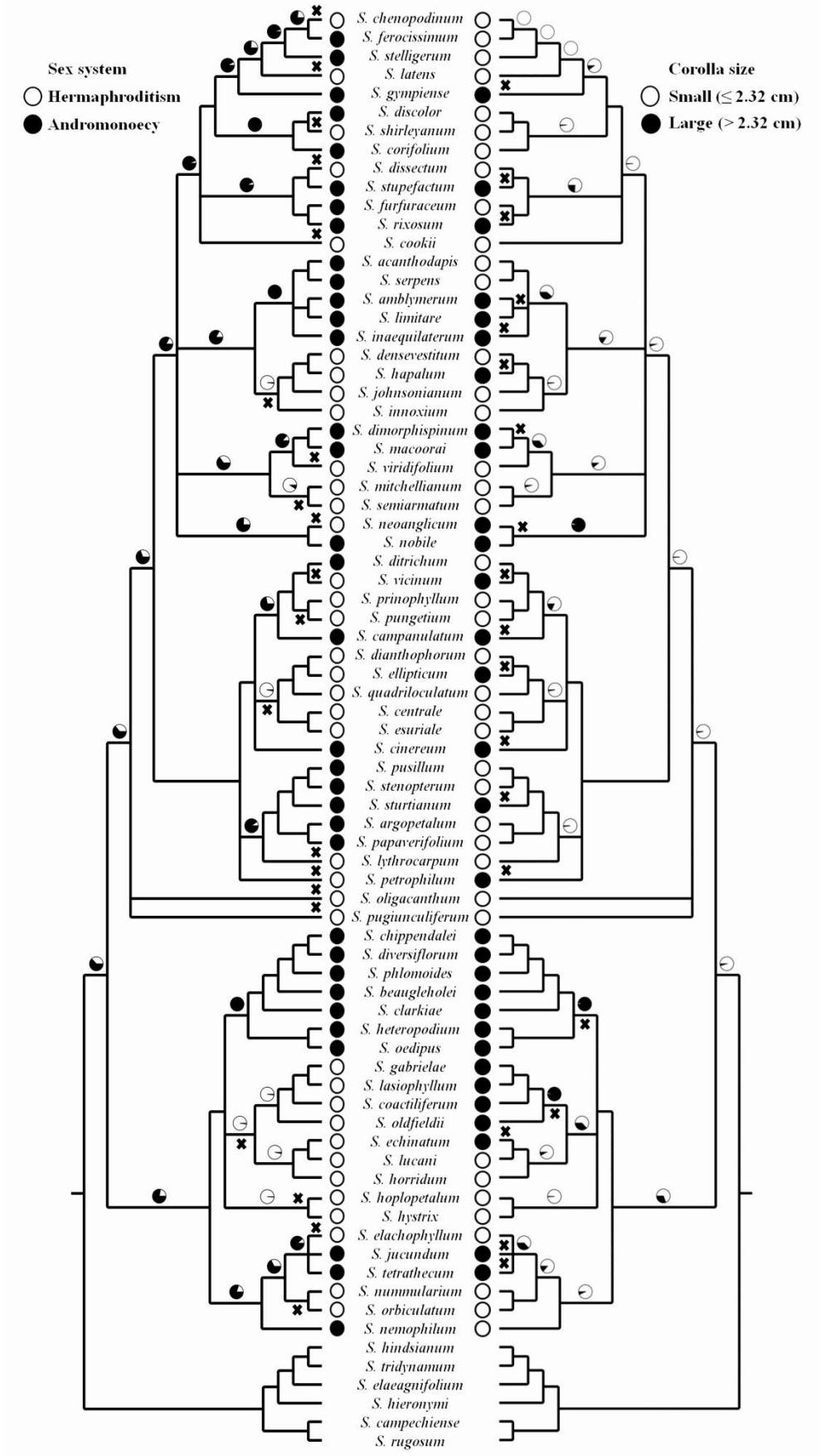
Table 5.2. Percentages of the most frequented parameter models sampled in posterior sample drawn from 63333 post-burnin iterations during the Bayesian MCMC framework for four discrete characters. For sex systems: 0 = hermaphroditism, 1 = andromonoecy; For corolla size and fruit diameter: 0 = small, 1 = large.

Character	Percentage of models sampled					$q01/q10$
	$q01 = q10$	$q01 = 0$	$q10 = 0$	$q01 > q10$	$q01 < q10$	
Sex system	41.20	55.35	2.95	0.26	0.24	0.40
Corolla size	47.58	0	51.88	0.27	0.28	2.09
Fruit diameter	85.39	0.04	14.37	0.11	0.09	1.16

Ancestral state reconstructions

The Bayesian framework yielded reliable ancestral reconstructions for all three discrete characters except in some nodes with ambiguous assignment of character states (Fig. 5.2 and 5.3; Supporting Information Table S6). Ancestral states were considered unambiguous if the mean posterior probability for a node adopting a state was ≥ 0.8. For sex system, the two third of nodes offered the unambiguous decision of plesiomorphic state with one or the other state across all trees in the reconstruction analysis. The equivocal support for andromonoecious

Figure 5.2. Ancestral state reconstructions for sex system mapped on the left cladogram and for corolla size on the right using pie charts at internal nodes numbered from 1 to 30. Mean posterior probability values for character states used in the pie charts are provided in Table S7. Filled cross refers to origin of forward transitions in character states. Circles at the tips of the tree indicate the character states of extant species.


Sex system
Hermaphroditism
Andromonoecy
Corolla size
Small (≤ 2.32 cm)
Large (> 2.32 cm)
S. chenopodinum
S. ferocissimum
S. stelligerum
S. latens
S. gympiense
S. discolor
S. shirleyanum
S. corifolium
S. dissectum
S. stupefactum
S. furfuraceum
S. rixosum
S. cookii
S. acanthodapis
S. serpens
S. amblymerum
S. limitare
S. inaequilaterum
S. densevestitum
S. hapalum
S. johnsonianum
S. innoxium
S. dimorphispinum
S. macoorai
S. viridifolium
S. mitchellianum
S. semiarmatum
S. neoanglicum
S. nobile
S. ditrichum
S. vicinum
S. prinophyllum
S. pungetium
S. campanulatum
S. dianthophorum
S. ellipticum
S. quadriloculatum
S. centrale
S. esuriale
S. cinereum
S. pusillum
S. stenopterum
S. sturtianum
S. argopetalum
S. papaverifolium
S. lythrocarpum
S. petrophilum
S. oligacanthum
S. pugiunculiferum
S. chippendalei
S. diversiflorum
S. phlomoides
S. beaugleholei
S. clarkiae
S. heteropodium
S. oedipus
S. gabrielae
S. lasiophyllum
S. coactiliferum
S. oldfieldii
S. echinatum
S. lucani
S. horridum
S. hoplopetalum
S. hystrix
S. elachophyllum
S. jucundum
S. tetrathecum
S. nummularium
S. orbiculatum
S. nemophilum
S. hindsianum
S. tridynamum
S. elaeagnifolium
S. hieronymi
S. campechiense
S. rugosum

ancestor at the basal node (63%) was lost subsequently and hermaphroditism has evolved independently six times in the internal nodes and 14 times in the extant species (Fig. 5.2). There was no event of andromonoecy evolving from hermaphroditism after it was lost during the speciation events. The rate of transition from andromonoecy to hermaphroditism was two times higher than the rate of transition from hermaphroditism to andromonoecy ($q01/q10 < 1$) (Table 5.2).

Ancestral reconstruction analyses for continuous character datasets conducted in the MRCA approach and the weighted squared change parsimony methods yielded almost identical values (Supporting Information Table S7). There have been trends of both increases and decreases in the corolla size during evolution along various lineages leading to extant species. The reconstructed ancestral corolla size of 2.32 cm at basal node was larger than the mean calculated from the observed values of each extant species. The Bayesian ancestral reconstruction analysis of corolla size strongly supported an ancestor with corolla size ≤2.32 cm in 21 out of 30 internal nodes with mean probability above 80% (Fig. 5.2; Supporting Information Table S6). The evolution of larger corolla size from the ancestral size was detected at least six times in the internal nodes and

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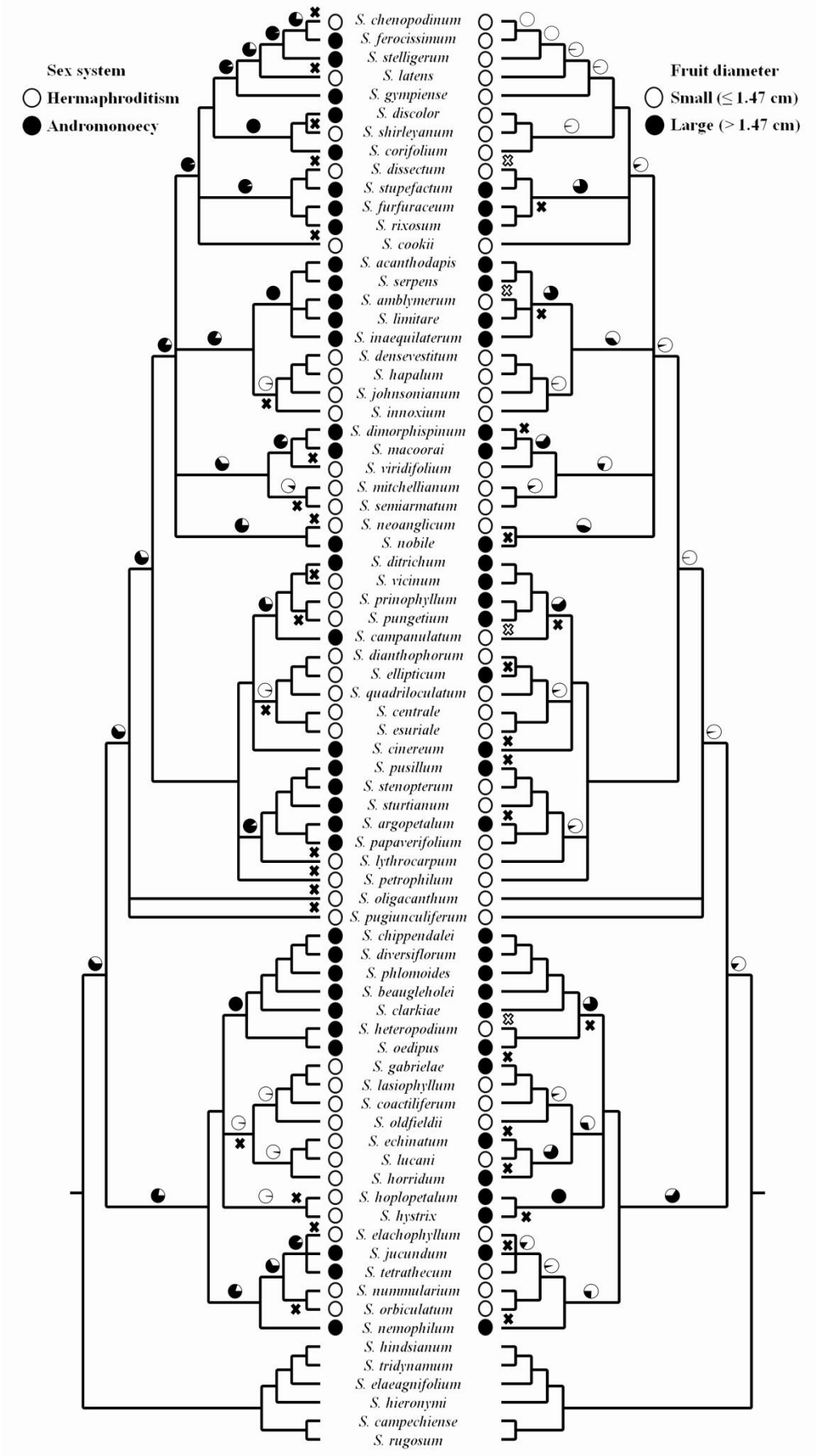
Figure 5.3. Ancestral state reconstructions for sex system mapped on the left cladogram and for fruit diameter on the right using pie charts at internal nodes numbered from 1 to 30. Mean posterior probability values for character states used in the pie charts are provided in Table S7. Filled cross refers to origin of forward transitions in character states; open cross denote reversals. Circles at the tips of the tree indicate the character states of extant species.


Sex system
Hermaphroditism
Andromonoecy
Fruit diameter
Small (≤ 1.47 cm)
Large (> 1.47 cm)
S. chenopodinum
S. ferocissimum
S. stelligerum
S. latens
S. gympiense
S. discolor
S. shirleyanum
S. corifolium
S. dissectum
S. stupefactum
S. furfuraceum
S. rixosum
S. cookii
S. acanthodapis
S. serpens
S. amblymerum
S. limitare
S. inaequilaterum
S. densevestitum
S. hapalum
S. johnsonianum
S. innoxium
S. dimorphispinum
S. macoorai
S. viridifolium
S. mitchellianum
S. semiarmatum
S. neoanglicum
S. nobile
S. ditrichum
S. vicinum
S. prinophyllum
S. pungetium
S. campanulatum
S. dianthophorum
S. ellipticum
S. quadriloculatum
S. centrale
S. esuriale
S. cinereum
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S. stenopterum
S. sturtianum
S. argopetalum
S. papaverifolium
S. lythrocarpum
S. petrophilum
S. oligacanthum
S. pugiunculiferum
S. chippendalei
S. diversiflorum
S. phlomoides
S. beaugleholei
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S. oedipus
S. gabrielae
S. lasiophyllum
S. coactiliferum
S. oldfieldii
S. echinatum
S. lucani
S. horridum
S. hoplopetalum
S. hystrix
S. elachophyllum
S. jucundum
S. tetrathecum
S. nummularium
S. orbiculatum
S. nemophilum
S. hindsianum
S. tridynamum
S. elaeagnifolium
S. hieronymi
S. campechiense
S. rugosum

12 times in the extant species independently (Fig. 5.2). Majority of species in the Clade C have evolved larger corolla size (>2.32 cm). There are 31 species (44%) having corolla size larger than the ancestral size (2.32 cm). This pattern of frequent transitions from small to large corolla size in this subgenus was supported by two times higher rate coefficients found in forward transitions ($q01>q10$) (Table 5.2).

The ancestral state reconstructions of fruit diameter inferred fruits of 1.47 cm diameter at the basal node (Supporting Information Table S7). The most recent common ancestor at 18 internal nodes on the tree had small fruits (≤1.47 cm diameter) in the ancestral reconstruction analysis of fruit diameter (mean probability = 0.80) (Fig. 5.3; Supporting Information Table S6). In my sample of Australian subgenus *Leptostemonum*, 40 species (56%) produce fruits with smaller than the reconstructed ancestral size. Species belonging to Clade C produce fruits in larger size than the ancestral size. Fruit diameter revealed 5 shifts from small to large fruit diameter in the internal nodes, while 12 extant species have evolved larger fruit size independently (Fig. 5.3). The reversals to small fruit diameter from the ancestor with large fruits occurred four times in the extant species. The analysis of transitions in the binary dataset of fruit diameter revealed multiple gains from plesiomorphic small size to large fruit size. However, model sampling results showed equal rate coefficients for transitions between small and large fruit size ($q01/q10 = 1.16$) (Table 5.2).

Testing correlated evolution

There was a 'strong' evidence for correlated evolution between sex system, corolla size and fruit diameter (Table 5.3). A comparison of harmonic means of likelihood values across trees revealed that the dependent model of evolution is a better fitting model for these two character combinations. The character combination between corolla size and fruit diameter was not supported for correlated evolution as independent model was best fitting the discrete dataset (Table 5.3). Therefore, character combinations lacking significance for correlated evolution were not included in further analysis.

Table 5.3. A comparison of the harmonic means of likelihood values estimated from independent and dependent model of evolution to test correlated evolution in character combinations using Bayes Factor tests. In the log-scale, Bayes Factor values greater than 2 suggest positive evidence and values greater than 5 are taken as strong evidence for correlated evolution.

Character combination	Harmonic mean of likelihood value		Bayes Factor
	Independent model	Dependent model	
Sex system Vs corolla size	-99.80	-96.60	6.38
Sex system Vs fruit diameter	-96.76	-92.46	8.59
Corolla size Vs fruit diameter	-96.82	-97.40	-1.16

Table 5.4. Description and mean posterior distributions of the transition rate coefficients of correlated evolution between sex system and morphological traits such as corolla size and fruit diameter estimated from all visited models in rj-MCMC approach using BayesTraits. The proportion of times each rate coefficient was assigned to zero bin ('Z') is given in parenthesis.

Evolutionary transition		Sex system vs corolla size	Sex system vs fruit diameter
Forward transitions			
*q*12	Size grown larger in hermaphrodite sex system	60.67 (0)	62.04 (3)
*q*13	Andromonoecious sex system evolved in presence of small size	32.89 (34)	10.02 (79)
*q*24	Andromonoecious sex system evolved in presence of large size	44.74 (16)	64.44 (1)
*q*34	Size grown larger in andromonoecious sex system	62.70 (0)	68.26 (0)
Reversal transitions			
*q*21	Size grown smaller in hermaphrodite sex system	54.07 (6)	65.28 (1)
*q*31	Hermaphrodite sex system evolved in presence of small size	62.79 (0)	68.53 (0)
*q*42	Hermaphrodite sex system evolved in presence of larger size	10.44 (70)	6.57 (83)
*q*43	Size grown smaller in andromonoecious sex system	12.57 (71)	45.30 (30)

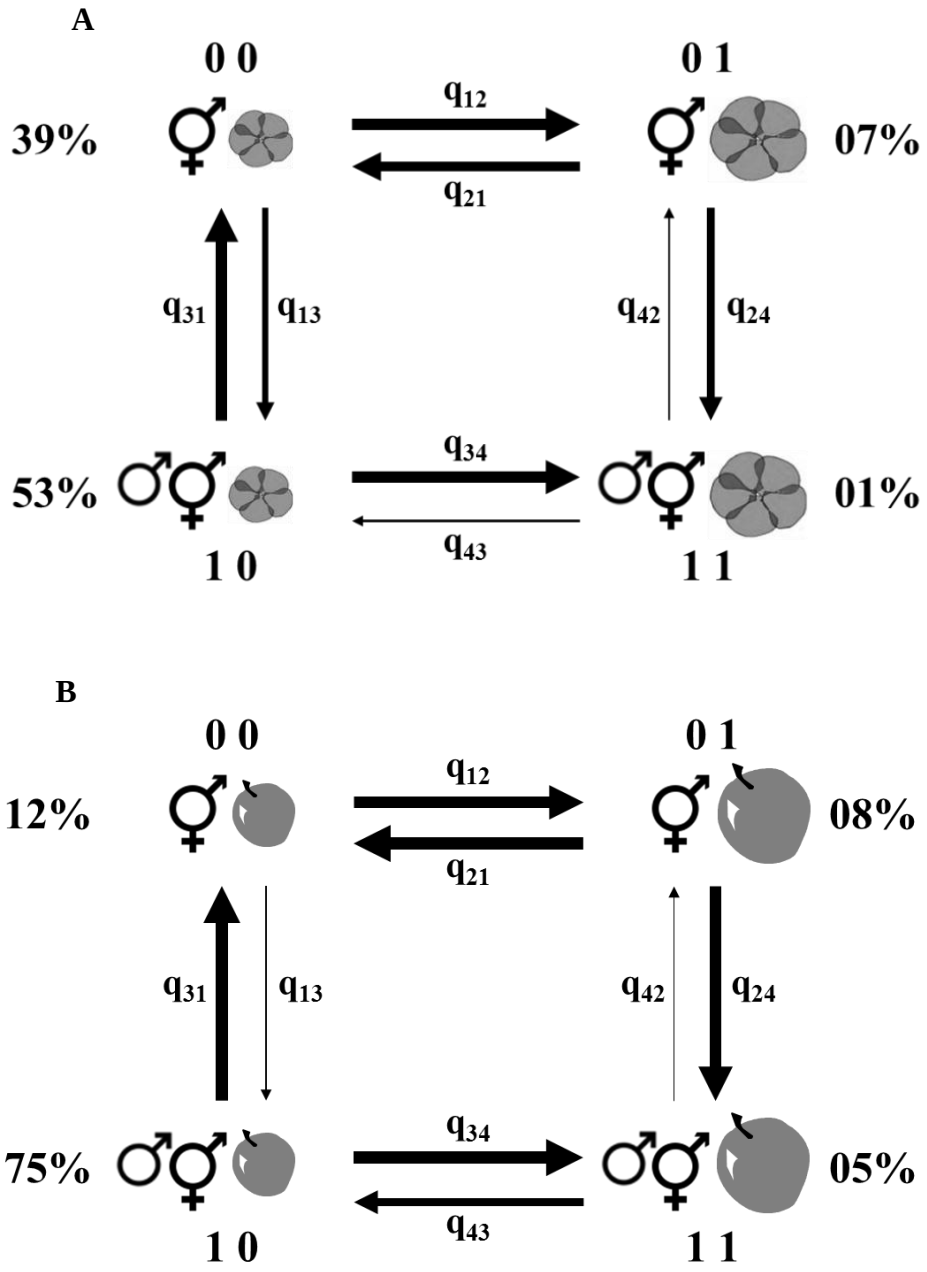

Figure 5.4. A dependent model showing most probable evolutionary paths for transitions between (A) sex system (0 = hermaphrodite; 1 = andromonoecious) and corolla size (0 = small; 1 = large), (B) sex system and fruit diameter (0 = small; 1 = large) with four combinations (00, 01, 10, 11) in the correlated evolution. The thickness of arrows corresponds to transition rate coefficients estimated between character states. Mean ancestral state probabilities in the combinations are given in percentage.

The rates of forward and reverse transitions in sexual system were correlated with changes in corolla size (Fig. 5.4). In subgenus *Leptostemonum*, the corolla size of bisexual flowers in nearly two third of andromonoecious species is large (62%), while hermaphroditic species have predominantly smaller corolla (73%). The dependent model of evolution reconstructed andromonoecy with small corolla size as the ancestral character state combination with 53% mean posterior probability (Fig. 5.4A). However, the next probable ancestral state, hermaphroditism with small corolla size, accounts for 39% of posterior probability, which makes the ancestral state ambiguous. Transition rate coefficients reveal that the andromonoecy with small corolla size as the plesiomorphic state is likely to evolve either large corolla size or hermaphroditism in equal rate ($q34=q31$) (Table 5.4). Once established, flowers with small corollas are less likely to reverse back to an andromonoecious sex system, which is reflected in smaller transition rate and frequent visits (34%) to the $q13 = 0$ model. When hermaphroditism with small corolla size is assumed as the plesiomorphic state, the high transition rate of $q12$ supports forward transition in which large corolla size is gained first in the hermaphroditism. Then being a rare character combination, hermaphroditism with large corolla size is more likely to evolve towards either small corolla size ($q21$) or andromonoecy ($q24$). Transition from andromonoecy to hermaphroditism occurred only when the corolla size was small ($q31>q42$), whereas increase in corolla size was not conditional upon sex system ($q34 = q12$) (Table 5.4). In both pathways, the high proportion of times the rj-MCMC chain set the rate parameters $q42$ and $q43$ to zero (Table 5.4) indicates

that once established, the derived character combination of an andromonoecy with large flowers seldom lost in the evolutionary history.

About 70% of species (28) with small fruit are hermaphrodites, while only 29% hermaphroditic species (9) have large fruits. As I found strong evidence for correlated evolution between sex system and fruit diameter (BF > 5), I investigated the evolutionary transition pathways leading to these correlations (Table 5.3). The ancestral state combination is inferred as the andromonoecious sex system having small fruit diameter with 75% mean posterior probability (Fig. 5.4B). When andromonoecy with small fruit is assumed as plesiomorphic state, the posterior distributions of rates suggest that the evolution of hermaphroditism in the presence of small fruit ($q31$) and the evolution of large fruit in andromonoecy ($q34$) occurred at the same transition rates (Table 5.4). These two derived character state combinations are less likely to revert back to the ancestral state, which is reflected in the sampling of models: $q13 = 0$ and $q43 = 0$ in higher frequency in the Markov chain. From the character state combination, hermaphroditism with small fruit, a transition to andromonoecy with large fruit is more likely through an intermediate pathway, first evolving large fruit in hermaphroditic sex system ($q12$) followed by evolving andromonoecy ($q24$) (Fig. 5.4B). The transition from the intermediate character combination, hermaphroditism with large fruit diameter, which is found only in 9 species, is expected either in the direction of reducing the fruit diameter ($q21$) or evolving andromonoecy in the presence of large fruit diameter ($q24$).

Discussion

Phylogenetic relationships

My phylogenetic analysis of 71 Australian species of subgenus *Leptostemonum* using the three gene regions clearly recognised two clades at a basal split. There was an ambiguity in the resolution of subsequent internal nodes. While inadequate phylogenetic resolution reflected in several polytomies may indicate evolution of species lineages in a short time-span and therefore with few molecular differences (Weller and Sakai 1999), a strong overall posterior probability support was found for the species relationships. A comparison of the placement of closely related species recognised in the phylogenetic analysis with earlier taxonomic treatments of this subgenus by Symon (1981), Whalen (1984) and Bean (2004) was made (Supporting Information Fig. S1). Symon (1981) in his authoritative treatment placed species included in this study in six sections, largely in *Oliganthes*, *Melongena* and *Graciliflorum* sections. According to Whalen (1984)'s classification of species group within subgenus *Leptostemonum*, study species were recognised in six species groups. Bean (2004)'s treatment that made some amendments and alterations to Whalen (1984)'s description with new groups established from *ellipticum* and *ferocissimum* species groups placed my study species in 13 species groups.

Phylogenetic relationships inferred from this study did not support the classification of several species groups proposed in the previous treatments. Whalen (1984)'s species groups: *ellipticum*, *macoorai*, *ferocissimum* and *hystrix* seemed to have species forming independent lineages on the phylogenetic tree. Species belonging to *ellipticum* group were present in Clade B, C and D, while

macoorai group species were nested within in Clade A. For many informal species groups presented in Bean (2004), the validation was not possible because of the inadequate species representation from those species groups in my phylogenetic analysis. Whalen (1984) recognized Symon (1981)'s treatment of Australian species in section *Melongena* in two species groups: *dioicum* and *hystrix*. Further, Whalen (1984) also proposed a close relationship between *dioicum*, *hystrix* and *ellipticum* groups referring to similarity between *Solanum oedipus* and *S. hystrix*, and *S. dioicum* and *S. echinatum*. Species relationships inferred in the Clade C on the phylogenetic tree are consistent with above mentioned Whalen (1984)'s species group treatment. Molecular studies testing the monophyly of section *Melongena sensu* Symon (1981) gained little support, but identified species of *dioicum*, *hystrix* and *ellipticum* groups *sensu* Whalen (1984)'s in five distinct clades (Martine et al. 2009; Martine et al. 2006). In support of this species relationship, the current phylogeny also recognized three separate subclades in clade C (Fig. 5.1).

The unplaced taxa in previous treatments (Symon 1981; Whalen 1984) such as *Solanum stupefactum*, *S. companulatum*, *S. ditrichum* and *S. cinereum* were examined for species affinity with species groups in the current phylogeny. The species, *S. companulatum*, *S. ditrichum* and *S. cinereum* seemed to show affinity with Clade B. *S. stupefactum*, placed in the *incanum* group by Bean (2004) appeared to have close relationship with *Solanum dissectum* of Clade A. Martine et al. (2009; 2006) also found similar species relationships among these species in their molecular analyses. Overall, the results of current phylogenetic analysis

provide reliable species affinities among species groups, but additional sampling of remaining species is needed to further resolve species relationships.

Patterns of character evolution

In the subgenus *Leptostemonum*, the shared evolutionary history had significant effect on the variation in corolla size and fruit diameter. Changes associated with cladogenic events were gradual for these two characters ($\kappa = 1$). The difference in phylogenetic signal (λ) between corolla size and fruit diameter indicates that the values of these characters across species represent different responses to evolutionary history. This also means that both these characters have distinct functions and therefore less integrated in the character evolution. The strong phylogenetic signal in fruit diameter relative to the corolla size suggests that shared common ancestry of a lineage constrains fruit diameter. Otherwise, variation in fruit diameter among species that are evolved largely in response to any strong selective pressure would obscure the phylogenetic affinity (Blomberg et al. 2003). This was the case in corolla size. The scaling parameter for tempo of evolution, delta (δ) value close to 1.0 in fruit diameter infers continuous evolution of these traits at constant rate from base to extant species. Here it is assumed that accelerated evolutionary changes in fruit diameter accompanied species diversification in the topology. The strong influence of phylogenetic history in the evolution of fruit diameter is in agreement with phylogenetic conservatism commonly found in the fruit traits of many species (Jordano 1995). However, other developmental and physiological constraints imposed by floral traits could possibly limit the rate of evolutionary change in fruit diameter as well (Wolfe and Denton 2001; Torices and Méndez 2010; Diggle 2003; Stephenson 1981). In

addition, evolutionary changes in fruit diameter also showed to be correlated with different disperser types (Jordano 1995).

Corolla size seems to exhibit a homoplastic pattern of evolution with similar sizes encountered in distantly related species, but not necessarily in closely related lineages. Furthermore, the intermediate lambda value for corolla size ($\lambda = 0.47$) suggests that present day changes in corolla size among species are seemingly an adaptive response to different selective pressures from external source. The tempo (δ) of corolla size evolution with significant departure from Brownian motion shows inter-specific variation developed more rapidly at the later phase of evolution. Therefore, the rate of changes in corolla size is predicted to be higher in late than in early diverging lineages. Such phenotypic plasticity in corolla size is mainly attributed to high demand for strong pollinator attraction, architectural effects, flower number and size trade-off, reinvesting the resources saved through reducing flower numbers and adaptation to the microenvironment in the habitat (Emms 1993; Diggle 2003; Solomon 1985; Fenster et al. 2004; Harder and Johnson 2009). However, the lack of phylogenetic resolution, and intraspecific variations might have also contributed the lower phylogenetic signal in corolla size.

Ancestral state reconstructions

In my phylogeny, a high degree of sexual variation was inferred in the subgenus *Leptostemonum* representing extreme lability of sex systems along speciation events. While andromonoecy was reconstructed as the plesiomorphic state of the basal node, this was only weakly supported (63%). A little less than the half of species (48%) are andromonoecious species in subgenus

Leptostemonum. Hermaphroditism is inferred as the apomorphic state of at least six internal nodes within subgenus *Leptostemonum*. The transitions between sex systems largely occurred in one direction and changes from andromonoecy to hermaphroditism were twice as likely as those from hermaphroditism to andromonoecy. The reverse transitions from derived sex system hermaphroditism to andromonoecy were not inferred during species diversification. It is still unclear whether initial andromonoecy evolved from hermaphroditic ancestry within Australia or just before dispersal into Australia, followed by repeated reversals to hermaphroditism, as indicated by the weakly supported posterior probability for plesiomorphic andromonoecy at the basal node. In subgenus *Leptostemonum*, Symon (1979b) and Whalen and Costich (1986) suggested that andromonoecy and dioecy are the two derived sex systems from hermaphroditism.

It would be interesting to know what features make the species in this subgenus so sexually labile. In the sections *Acanthophora* and *Lasiocarpa*, andromonoecy has evolved as a developmentally plastic response in terms of suppression of gynoecium development at individual flowers, but within the constraints set by inflorescence architecture (Diggle 1994; Miller and Diggle 2003). Factors including phenotypic plasticity, inter and intra inflorescence architectural effects and total flower production are attributed for diverse sexual expressions among andromonoecious species (Miller and Diggle 2003). Furthermore, variation in the lability of sex expression among andromonoecious *Solanum* species explains the production of male flowers at the distal end of an inflorescence as a fixed developmental trait in some species and labile in others (Miller and Diggle 2003). Lability in sex expression mediated through floral

developmental changes perhaps act as an evolutionary mechanism which affects the plasticity and stability of sex system, which was highlighted for *Wurmbea* (Case et al. 2008; Vaughton and Ramsey 2012).

Ancestral state reconstruction with discrete dataset strongly inferred small corolla size as the plesiomorphic state of the Australian species of this subgenus. The evolution of increase in corolla size from the ancestral size (2.32 cm) was largely noticed in Clade C and D (nodes from 5 to 8), while the ancestors of Clade A and B have evolved reduced corolla size (≤2.32 cm) (Supporting Information Table S6). Interestingly, there was no further reduction of corolla size in the subsequent internal nodes of Clade A and B. In support of this, the change from large to small corolla size was less frequent in the analysis of binary state transitions. The increase in corolla size seems to have evolved independently in many extant species within this subgenus (Fig. 5.2), demonstrating a labile nature of corolla size with potential to accumulate large phenotypic variation within species in response to selection pressure. There are notable differences in corolla size among sister species, indicating the evolutionarily lability of corolla size in this subgenus (Fig. 5.2). For example, *Solanum dianthophorum* and *S. ellipticum*; *S. densevestitum* and *S. hapalum*; *S. dissectum* and *S. stupifactum*; *S. furfuraceum* and *S. rixosum*. Lability in floral characters in general and corolla size in particular has been documented in other plant groups (Prather 1999; Miller and Diggle 2003; Kimball and Crawford 2004; Pérez et al. 2006; Boyd 2003), and lability may be critical in speciation and reproductive isolation. The possibilities for association between corolla size and sex system evolution are discussed below.

Small fruit diameter was inferred as the plesiomorphic state of Australian *Leptostemonum* with a high posterior probability at the basal node. The reconstructed ancestral fruit diameter value (1.47 cm) was congruent with observed mean values of extant species. The frequent and independent evolution of large fruit size occurred quite early among species in the node 2 and also more recently towards extant species. The increase in fruit size involves placental elaboration and an increase in locule numbers through forming a septum during fruit development (Symon 1987). Furthermore, the presence of large fruits in phylogenetically distinct lineages indicates multiple evolutionary origins. The reverse transitions from large to small fruit diameter are rare and inferred only four times. The reduction in fruit diameter implies simple non-elaborated placentas and a degenerated septum with few seeds per fruit (Symon 1987). Overall, the frequent sampling of model, $q01=q10$, suggests equal rates of transitions between small and large fruit diameter, however the slightly but not significantly higher rate of $q01$ over $q10$ indicates more transitions from small to large fruit than vice-versa. Gene regulation studies conducted in few *Solanum* species including commercial varieties of tomato and eggplant and closely related wild species have identified mutations in few loci that account for variations in fruit size (Frary et al. 2000; Tanksley 2004; Doganlar et al. 2002; Lippman and Tanksley 2001). Some of these genes might contribute to fruit size variations in many of *Solanum* species through causing mutations in the number of locules (Tanksley 2004).

Testing correlated evolution

Changes in two morphological traits, corolla size and fruit diameter, had significant association with changes in sex system. However, there were no associations between changes in corolla size and in fruit diameter, which was surprising, for the sections *Lasiocarpa* and *Acanthophora* in the subgenus *Leptostemonum*, a positive and significant interspecific correlation between fruit and flower size was found (Miller and Diggle 2007). The genetic constraints and co-regulation in the development of floral parts, as reviewed in Ashman and Majetic (2006), have been invoked for an increase in fruit size in relation to flower size to accommodate the enlarged placenta of an ovary. My results indicate that these traits evolve independently to certain extent, perhaps due to differential selection pressures.

The reconstructed ancestral state combination was andromonoecious sex system with small corolla size, which coincides with the ancestral states of individual characters, but was found only in 13 contemporary species. My results supported correlated evolution between sex system and corolla size, and suggested that forward transitions appears first in the corolla size ($q34$), as found in the Clade C that has evolved large corolla without changing the sex system. After evolving large corolla size, andromonoecious species undergo neither reduction in corolla size nor reversal to hermaphroditism in the evolutionary history. Instead, the derived state: andromonoecy with large corolla size further evolved towards dioecy, where functional female flowers that produce sterile pollen and male flowers occur on separate plants (Anderson and Symon 1989; Martine et al. 2009; Martine et al. 2006). Interestingly, transitions from andromonoecy to

hermaphroditism, as recognized largely in the extant species, occurred only in the lineages with small corolla size.

Analyses testing the association between evolutionary changes in fruit diameter and sex system provided strong support for correlated evolution of these traits in the subgenus *Leptostemonum*. The andromonoecious sex system with small fruit diameter was reconstructed as the plesiomorphic state combination. This was in line with the plesiomorphic character states of the individual characters. In the evolutionary history of subgenus *Leptostemonum*, the evolution of andromonoecious from hermaphrodite sex system has initiated early in the species divergence, while the increase in fruit diameter followed changes in sex system. The evolutionary pathways analysis suggests that changes in sex system from hermaphroditism towards andromonoecy are likely to precede changes in fruit diameter. In addition, it is unlikely for hermaphroditic species bearing small fruits to evolve andromonoecy without an increase in fruit size. Therefore, I suspect that the plesiomorphic state of hermaphrodite sex system with small fruit diameter may not have been recognized as the plesiomorphic state combination.

From the ancestral state combination, the andromonoecious sex system with small fruits is likely to evolve either towards hermaphroditism without changing the fruit size or to large fruit diameter without a change in the sex system. The latter combination has a very small probability of reversal to a hermaphroditic sex system. The hermaphroditic species with small fruit is very unlikely to reverse back to andromonoecy, but much more develop into large-fruited hermaphroditic species. Based on the probabilities of these pathways, intermediate state combination, the transition to large fruit clearly preceded the evolution of

andromonoecy. Bertin (1982) also hypothesised the evolution of andromonoecy from large-fruited hermaphroditic species, because it is likely that plants having large fruits would tend to reduce investment in functional pistils without costing female fitness. This notion had found theoretical support through evolutionary modelling, as the production of large fruit predisposes towards the evolution of male flowers (de Jong et al. 2008). My data support a transition to large fruit diameter preceding a transition in sex system as large fruit diameter.

The maintenance of male flowers among andromonoecious species may further act as a mechanism that regulates the reallocation of resources to the female function of basal bisexual flowers (Bertin 1982; Solomon 1986; Whalen and Costich 1986; Miller and Diggle 2007). There was a positive significant correlation between fruit size and the proportion of male flowers in an inflorescence among andromonoecious *Solanum* sections *Acanthophora* and *Lasiocarpa* noticed (Whalen and Costich 1986; Miller and Diggle 2007). Experimental studies by Vallejo-Marin and Rausher (2007) also supported for selection acting through increased female fitness in favour of increasing the number of male flowers in *S. carolinsense*.

Thus, I find that the evolution of andromonoecy from hermaphroditism in the Australian members of subgenus *Leptostemonum* is most likely preceded by the evolution of a large fruit, and also by a large corolla size. Taking this further, the selective factors involved in the production of large fruit along with the advantage of having large corolla size may be of major importance in the evolution of andromonoecy. Fruit size, as a function of ovary size, is generally determined by growth parameters such as the number of ovules in the ovary and

the number of ovules successfully fertilized, and cell enlargement capacity (Gillaspy et al. 1993; Bohner and Bangerth 1988b; Bohner and Bangerth 1988a). The larger fruit may be the result of strong stochastic uncertainty in pollination success, as such stochastic variation in mating success is associated with packaging of large number of ovules per flower in a taxonomically broad sample of angiosperm species (Burd et al. 2009). The unpredictable windfall success of pollination could have occurred more often among the species of subgenus *Leptostemonum* in the evolutionary history. The distribution of Australian *Solanum* species is largely in the areas of stochastic environment with unpredictable rainfall, long dry season, and frequent bushfire. The large buzz pollinating bees that pollinate *Solanum* flowers may occur in low densities in these areas. Fruiting flowers with large corolla size could certainly increase the probability that bees will first transfer pollen collected from other plants on the stigma before visiting male flowers. Furthermore, as *Solanum* flowers do not produce nectar, pollen receipt by flowers through bee visits is influenced by the presence of nectar producing flowering plants in the vicinity, which may also introduce additional stochasticity.

CHAPTER SIX - DISCUSSION

Summary of findings

This study addresses the possible influence of buzz-pollinating bees on the evolution and maintenance of sexual floral dimorphisms in unisexual *Solanum* species. The flower morphology and reward availability of animal pollinated plants are intricately related with the foraging behaviour and preferences of their pollinators. The genus *Solanum* has three sexual forms in its lineage and is an ideal study system to conduct research into evolutionary links between pollinators and flower morphology as it relies on a relatively small number of buzz-pollinating bee species. *Solanum* flowers have evolved a number of sex-related strategies that have the potential to influence the behaviour of pollen foraging bees. They include sexual dimorphisms in flower size and flower numbers, style length differences and reward availability. In chapters 2-4, I investigated the effects of sexual differences in floral traits and reward availability on bee visitation and pollen collecting behaviour. In chapter 5, the existence of an association between the evolution of floral morphology and sex system was also evaluated within a phylogenetic context. In this chapter, I summarise the key findings of this study. I also discuss the combined implications for the understanding of the evolution of floral dimorphism in *Solanum* and the role of bees in shaping this dimorphism.

Bee foraging responses to floral display

In chapter 2, experiments on bee visitations to artificial flowers offering equal rewards were conducted in a glasshouse. The main question was how do

differences in flower display influence bee foraging behaviour? When female *Amegilla murrayensis* were given a choice between two differently sized artificial flowers, the bees' initial preference for large flower size disappeared quickly in the absence of differences in rewards among flowers. In the choice test between solitary and clustered flowers, bees preferred clusters of flowers regardless of flower size differences. Results demonstrated that bee visitation is directly proportional to the number of flowers displayed, but not influenced by flower size. Therefore, flower size is unlikely to influence the foraging behaviour of pollen collecting bees.

Importantly, if I had observed for a shorter period of time, I would have concluded that the bees have a preference for large flowers. The bees' initial preference could be the result of an expectation that large flowers are more rewarding than small flowers, which is supported by observations that reward size increases with flower size (Cohen and Shmida 1993; Galen and Plowright 1985; Gómez et al. 2008; Plowright 1981; Robertson et al. 1994; Stanton and Preston 1988). Furthermore, large flowers have a larger visual stimulus and bees that use long-wavelength photoreceptor (L-receptor) signals can detect them from a longer distance (Hempel de Ibarra and Vorobyev 2009). Therefore, it is not surprising that a number of previous studies recorded that bees prefer large over small flowers (Conner and Rush 1996; Eckhart 1991; Galen and Newport 1987; Stanton and Preston 1988), but several of these studies only tested the initial preference.

Bees optimise their foraging by adjusting their reward expectations over time on the basis of foraging experience (Blarer et al. 2002). Thus, when flowers of the initially preferred size become less rewarding or unrewarding, bees choose

the more rewarding flowers irrespective of size (Gil et al. 2007). Therefore, large flowers can only be effective in attracting more bee visits when they provide an honest signal of the reward availability. In support of this view, bees showed preferences for a large display with many flowers, as they signalled both higher attractiveness and reward.

The lack of an effect of flower size on foraging preferences demonstrates that it is unlikely that sexual dimorphism in flower size in the diclinous species of *Solanum* evolved in direct response to bee foraging preferences. This implies that, to understand the evolution of sexual dimorphisms in floral display in the diclinous species of *Solanum* pertaining to corolla size and flower numbers (Anderson and Symon 1989), other possible explanations need consideration for the production of numerous small male flowers. It is important to realise that there may be an association between the numbers and the sizes of the flowers. The production of a series of male flowers allows plants to release the pollen over a longer period of time. Such an extended flowering period was shown to benefit male flowers through increased pollen dispersal (Glaettli and Barrett 2008; Thomson 2006; Yakimowski et al. 2011). The evolution of smaller male flowers could then be the result of a trade-off between flower size and number on flowers per inflorescence, which is commonly found in many angiosperms (Sargent et al. 2007). Bees, as a vector of pollen, are then implicated in the evolution of increased numbers of male flowers, but are not necessarily the driving force behind this evolution.

Bee foraging responses to style length differences

Besides flower size dimorphism in *Solanum*, the sex system transition from hermaphroditism to andromonoecy has involved the suppression of gynoecial development, i.e., the production of rudimentary pistils with short styles in the distal flowers (Diggle 1991; Diggle 1994). In chapter 3, the results of an experiment to test whether style length differences in floral morphs of *Solanum elaeagnifolium* influence the visitation patterns of female *Amegilla chlorocyanea* were reported. Bees showed no differences in the number of buzzes and time spent on each buzz between floral morphs that differed in their style lengths during their first and second visits. Therefore, there is no evidence that pistil length interferes with bee visits.

This implies that the evolutionary loss of a long style in male flowers was not driven by the bees' avoidance of the long pistil. Therefore, other explanations for the differences in style length must be considered. Style length differences can be functionally significant. It has been shown that flowers of *Solanum* species with long styles protruding beyond the anthers receive more pollen from bees on their stigmas, while those with short styles facilitate efficient pollen removal without pistil interference (Diggle and Miller 2004; Elle and Meagher 2000; Quesada-Aguilar et al. 2008; Solomon 1985). Furthermore, it is also possible that pistil reduction is simply the result of the suppression of gynoecial development (Diggle 1991) rather than being an adaptive response to pollinator behaviour.

Bee foraging responses to reward availability

The experiments in chapter 2 and 3 revealed that the blue-banded bees did not change their visitation patterns in response to sexual dimorphisms displayed in

floral displays and style lengths in *Solanum*. Since the flowers of *Solanum* bear poricidal anthers that visually conceal pollen availability, the bees cannot use a visual assessment to determine the amount of pollen available. In chapter 4, I investigated how variation in pollen availability between flowers influences the foraging behaviour of bees by studying the foraging patterns of individual female *A. chlorocyanea* on reward-manipulated flowers of *S. elaeagnifolium*. During the first two buzzing bouts on the experimental flowers, the bees buzzed both flowers more when they had initially landed on a full than on an empty flower. There was no difference in the total number of times they buzzed full and empty flowers before they left the patch. However there were significant differences between first and second visits to emptied flowers. Thus, when they found only empty flowers, they buzzed both flowers only half the number of times. Such foraging responses of individual bees appear to be in contrast with previous studies (Buchmann and Cane 1989; Connolly and Anderson 2003; Harder 1990; Shelly et al. 2000). This is probably because previous studies measured the behavioural responses to pollen availability made by different bees and not by individual bees.

Results also showed that there was a gradual decline in the number of buzzes during visits to individual flowers at time intervals. The timed-pollen dispensing mechanism in *Solanum* species is expected to facilitate a release of nearly equal amounts of pollen from flowers in time intervals (Buchmann et al. 1977; King and Buchmann 1996; Passarelli and Cocucci 2006). Foraging responses to pollen replenishment over time in flowers are likely in traplining bees, including *Amegilla* spp., as they make repeated sequential visits to flowering patches in a fixed order (Thomson et al. 1997). The differential opening time of

female and male flowers will enhance overall visitation in patches of flowers and therefore the chances of successful pollen transfer. Overall, this study suggests that buzz-pollinating bees have the capability of recognising pollen availability. They respond to rewardless flowers by buzzing them fewer times, but this pollen assessment is unlikely to occur at the individual flower level. In addition, because the bees will increase the number of buzzes in a patch after encountering a full flower, the time differential opening of male and female flowers in *Solanum* could be a strategy to enhance cross pollination.

Evolutionary perspective on associations between floral traits and sex systems

As bees do not respond consistently to variation in corolla size (Chapter 2), it is unlikely that the evolution of floral dimorphism is due to selective pressures from pollinators. However, selection mediated through coevolution with pollinator foraging preferences is not necessarily the only possible explanation for the evolution of floral traits in *Solanum*. To examine the evolutionary history of floral and fruit size evolution, and the potentially correlated evolution between sex systems and flower and fruit sizes, I studied the interspecific variation in sex systems and reproductive traits in chapter 5. The phylogenetic inferences were based on the three gene region sequences of 71 Australian members of *Solanum* subgenus *Leptostemonum*. Phylogenetic analyses revealed a high degree of sex system lability, with multiple independent evolutionary transitions between sex systems. The ancestral state for *Leptostemonum* was inferred to be andromonoecy. The reversals towards hermaphroditism were conditional upon a reduction in corolla size. The analysis indicated that fruit size evolved at constant rate from

common ancestor to contemporary species. An increase in fruit diameter always preceded the evolution of andromonoecy from hermaphroditism, whereas the reversal transitions from andromonoecy to hermaphroditism were associated with a reduction in fruit diameter.

The findings lend support to a theoretical sex allocation model for the evolution of andromonoecy that predicts that the production of male flowers is favoured when the production costs of additional large fruits are prohibitive (de Jong et al. 2008). My study strongly supports the importance of fruit size in the evolution of andromonoecy from hermaphroditism. The evolution of andromonoecy is most likely preceded by the evolution of a large fruit, while reversals are accompanied by the loss of large fruit size. There was also some support for an increased corolla size in the fruiting flower.

Conclusion

This study has contributed to the understanding of the evolution of floral traits in general and the evolution of floral dimorphism in *Solanum* in particular. The research was sparked by questions regarding the role of pollinators in the evolution of floral sexual dimorphism. *Solanum* was taken as a model system, because there is no difference in the amount and type of the reward.

In Australian *Solanum*, the evolution of dicliny is associated with the evolution of smaller male flowers and a decreased pistil size. Experiments showed that the foraging decisions of the bees relate to the reward rather than to the size of the flower and that blue-banded bees, the main pollinators of Australian *Solanum*, are not bothered by the long style in functionally female *Solanum* flowers. Thus the sexual floral dimorphism in the diclinous species of *Solanum* is

unlikely to be a direct evolutionary response to selection through pollinator preferences, even though the dimorphisms probably contribute to plant reproductive success. However, two of the dimorphisms may have evolved to enhance bee visitation frequency and therefore chances of pollination. First, a reduction in the size of male flowers increases the possibilities of partitioning the pollen availability over time through the production of more flowers and this enhances visitation frequency by bees. Secondly, the temporal differences in reward availability increased the bee visitation frequency. Therefore, this dimorphism in timing of flower opening may have evolved in response to pollinator activity.

Phylogenetic analysis of the highly variable sex system and the associated morphological characters in the Australian species of *Solanum* subgenus *Leptostemonum* showed that the evolution of andromonoecy from hermaphroditism is more likely to be preceded by the increases in fruit diameter and corolla size. Thus, selective factors involved in the evolution of large fruit may be also responsible for the evolution of flowers that are functionally and morphologically male. This supports the notion that pollinators are not necessarily important in the evolution of andromonoecy. However, once this transition has occurred, the enhancement of visitation through extended pollen partitioning and timed differences in presentation between the sexes may be the result of selection through pollinators.

Difficulties and future directions

To fully understand whether bees impose any selection on plants to become sexually dimorphic in flower size and flower numbers, one needs to study the

bees' foraging responses in species with unisexual flowers that do not differ in their reward size or type. To investigate how these differences in floral display between male and female flowers influence bee foraging behaviour, I initially intended to conduct experiments using dioecious *Solanum* species that are endemic to the North Kimberley of northern Western Australia and the Kakadu-Alligator River region of the Northern Territory (Martine 2006). Since conducting field experiments in the remote places of Western Australia during the flowering time proved to be impossible, seeds were sourced through contacting local seed collectors and taxonomists in the state herbarium. For germination, the seeds were treated with different methods including smokewater, gibberellic acid (2.89 mM), scarification and soaking for 12 and 24 hrs, and were planted in different media such as water agar, sand, cocopeat, perlite/vermiculite and a sand-soil mix (75-25%). However, growing large number of seedlings of dioecious *Solanum* in the greenhouse was found to be difficult. Therefore, this study used artificial flowers and flowers of silver-leaf nightshade which offering similar characteristics as those found in dioecious *Solanum* species. It would be good to repeat the experiments with dioecious flowers. The use of such flowers would also allow investigations to assess the nutritious value of inaperturate pollen produced by female flowers.

My study suggested that later opening of female flowers increase the number of visits by bees to both male and female flowers. Female flowers open later in the season (Anderson and Symon 1989; Symon 1979) and the effect of this has not been as yet investigated. It is possible that the later opening of female flowers is a strategy that influences visitation by bees. For example, the presence

of male flowers could allow bees to set up traplines, which they revisit day after day (Thomson et al. 1997). When bees move along an established trapline, female flowers with full rewards may be spotted quicker as they open after the male flowers. Furthermore, the strategy may enhance cross pollination, as bees often start foraging at the bottom of an inflorescence (Best and Bierzychudek 1982; Harder 1990). These are hypotheses that require further study

The phylogenetic relationships inferred from this study did not support the monophyly of several species groups proposed in the previous treatments (Bean 2004; Symon 1981; Whalen 1984), which may have been caused by inadequate species sampling. The validity of the species groups recognised on the basis of morphological characters should be further explored by additional sampling of species to provide a more comprehensive molecular phylogeny of the Australian *Solanum* subgenus *Leptostemonum*. Furthermore, it would be interesting to explore the species diversification through evolutionary history within the subgenus in a biogeographical context of extant species, as some closely related species appear to have distributions in different biogeographical regions.

Significance of this study

Studying the bee foraging patterns on unisexual flowers in relation to sexual floral dimorphisms has provided some understanding about strategies evolved to enhance pollen transfer from male to female flowers in diclinous *Solanum* species. The findings of this study provide a new context for viewing patterns of character evolution and examining their ecological and developmental basis. This study also reveals evolutionary changes in sex systems occurred during the recent past in the different lineages of Australian *Solanum* species. This study offers an

evolutionary perspective to the growing body of evidence that transitions between sex systems have to be seen in a more holistic way by studying the functional correlation of reproductive characters.

The Australian *Solanum* species mainly occur in environments that are unpredictable with respect to rainfall, with long dry seasons and frequent bushfires. Most of these species are currently under threat or very rare in the wild (Smith 2010). The frequent evolutionary changes in fruit size indicate a strong stochasticity in dispersal success present among diclinous species. Overall, the scientific information derived from this study is critical in conserving threatened *Solanum* species. This study has developed a comprehensive molecular phylogeny of the Australian *Solanum* subgenus *Leptostemonum* species and the inferred phylogeny can be used to describe species relationships and biogeographical affinities.

This study complements previous studies in providing plausible explanations for the evolution of andromonoecy in the Australian *Solanum* species. Besides some implications on species conservation, this study also provided some insights on floral display strategies that can attract and encourage pollinators for inter-plant movements. A similar approach may be employed by increasing the floral display to attract more pollinator visits in commercial hybrid seed production.

SUPPORTING INFORMATION

Appendix S1. Detailed information of methods used in this study.

Phylogenetic inference

The *Solanum* subgenus *Leptostemonum* constitutes ~450 species worldwide of which around 146 species recorded in Australia (Barker 2010). A comprehensive taxonomic monograph of Australian *Solanum* species by Symon (1981) divided this subgenus into six sections. In a subsequent classification, Whalen (1984) recognized 33 species groups worldwide including six groups from Australia based on morphological and biogeographical affinities. More recently, Bean (2004) proposed 13 species groups for the Australian members of subgenus *Leptostemonum* by amending some of Whalen's (1984) species groups. Using DNA sequence data from two nuclear regions (ITS and GBSSI) and one chloroplast spacer region (*trnS-trnG*), Levin, Myers and Bohs (2006) identified 10 clades within subgenus *Leptostemonum*, and placed the Australian species covered in the study in an Old World clade. However, these studies did not include numerous species described by Symon (1981), Whalen (1984) and Bean (2004). Subsequently, the phylogenetic relationships of the Australian section *Melongena sensu* Symon (1981) were studied using ITS and *trnK-matK* gene regions and also examined for the monophyly of the section *Melongena* (Martine et al. 2006; Martine et al. 2009). However a comprehensive phylogeny of the Australian species of subgenus *Leptostemonum* to describe species relationships and affinities remains to be established. This study analyses phylogenetic relationships within the subgenus in order to get a better understanding of species diversification in the evolutionary history. I included 71 Australian species of

Subgenus *Leptostemonum* in this study. I had six outgroup species. Five outgroup species (*S. campechiense, S. elaeagnifolium, S. hindsianum, S. hieronymi* and *S. tridynamum*) belong to the clades that are closely related to the Old World clade, in which Australian species are placed within the subgenus (Levin et al. 2006). Another outgroup species, *S. rugosum,* was chosen from *Brevantherum* clade, which is a sister subgenus to *Leptostemonum* within *Solanum* genus (Weese and Bohs 2007).

To infer the phylogeny of the Australian members of subgenus Leptostemonum, the Bayesian Inference (BI) analysis was conducted with the gene region-partitioned sequence matrix of 4092 characters using MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003). Prior to this analysis, I identified the best-fitting substitution model for my sequence dataset according to the Akaike information criterion (AIC) using jModelTest 2.1.4 (Darriba et al. 2012; Guindon and Gascuel 2003). A general time reversible model with a proportion of invariable sites, coupled with gamma-distributed rates across sites (GTR+I+G) obtained the smallest AIC score and incorporated as a nucleotide substitution model in the Bayesian analysis. The priors for parameters such as substitution rates, state frequencies, gamma shape and the proportion of invariable sites were unlinked between partitions, while the prior probability of topology and distribution on branch lengths were linked. Partition-specific rate multiplier was allowed to vary with partition rates with a dirichlet (1,1,1). The comparison of the marginal likelihoods of three models (non-clock, strict clock and relaxed clock) using harmonic mean method demonstrated that the relaxed clock model was strongly favoured. The independent gamma rates (IGR) model was used for clock

rate variation across lineages with default prior rate at which the variance of branch length increases over time (Igrvarpr=exponential (10)). To speed up convergence, a 'hard' constraint was added forcing all species but outgroup to form a monophyletic group. Two independent runs were performed with four Metropolis-coupled Monte Carlo Markov chains per generation (1 cold and 3 heated chains) simultaneously for 8 million generations sampling every 100 generations. To improve chain-swapping, temperature for heating chains was set to 0.2 considering the acceptance rates for swaps between chains (20-80%). To ensure adequate sampling of posterior distribution and determine the burn-in, the graphical assessment of stationarity was performed using Tracer v.1.5. Average standard deviation of split frequencies between chains dropped below 0.01 which is a very good indication of convergence. I also used two other convergence diagnostic methods namely, Estimated Sample Size (ESS) and Potential Scale Reduction Factor (PSRF) to confirm whether analysis achieved adequate sampling of posterior distribution. All the estimated parameters had ESS above 200 with PSRF approaching at 1 as the runs converge (Supporting Information Table S2). The first 25% of sampled trees were conservatively removed in the further analysis. A 50% majority-rule consensus tree was constructed from post burnin trees with posterior probabilities (PP) for each clade.

Patterns of character state evolution

Phylogenetic comparative approaches are commonly used to determine whether variation in character traits present among extant species has evolved in the influence of speciation events along evolutionary history (Blomberg et al. 2003; Felsenstein 1985; Pagel 1997, 1999). When a character trait evolves in a

phylogenetically conservative mode showing higher phylogenetic signal, then closely related species are more similar than expected by their shared evolutionary history (Blomberg et al. 2003). The patterns of the evolution of two continuous characters: corolla size and fruit diameter were traced on the phylogenetic tree. I first investigated whether the evolution of traits followed a directional change random-walk model (directional, Model B) or a standard constant-variance random walk model (drift, Model A) using the Bayesian MCMC framework in the BayesTraits beta V1.1 program (Pagel and Meade 2006). In drift model A, a single parameter, the instantaneous variance of evolution (α) was measured while allowing traits to evolve according to the Brownian motion model. Model B, in addition to variance of evolution parameter (α), estimated a directional evolutionary change parameter (β) using a regression of trait values across species against total path length from the base to the tips of the phylogenetic tree. I used log-Bayes Factor, which is twice the difference of the harmonic mean of the log likelihood values of the two models of trait evolution, to determine the model that fits the data best. In the log-scale, Bayes factor values greater than 2 suggest a positive evidence, greater than 5 is strong evidence, and above 10 is taken as very strong evidence (Pagel and Meade 2006).

I then estimated three branch length scaling parameters: lambda (λ), kappa (κ) and delta (δ) for each continuous trait under the most supported model of trait evolution with MCMC framework in the BayesTraits beta V1.1 program (Pagel and Meade 2006; Pagel 1999). The Lambda (λ) parameter values correspond to the contribution of shared evolutionary history in the phylogeny to the observed covariance among species in trait values. A λ value of 0.0 suggests no

phylogenetic association of trait evolution i.e. patterns of variation in trait values among species are independent of shared ancestry. If the trait was evolved following the tree topology, λ takes the value of 1.0 indicating phylogenetic signal consistent with Brownian model. An intermediate λ value indicates the overestimation of covariance among species by the phylogeny. The parameter kappa (κ) measures the contribution of different branch lengths to evolutionary changes in traits. Kappa > 1.0 suggests longer branches accelerating trait evolution (i.e. gradual model), while the increased rate of trait evolution in short branches gives rise to the κ value of less than 1.0. Kappa value close to 0.0 corresponds to the punctuational mode of evolution in which changes in traits are independent of branch length. The delta (δ) parameter scales the contribution of path lengths (i.e. distance from the root to the tip in the phylogenetic tree) to the rates of trait evolution as time progresses. When longer paths accelerate the trait evolution over time, the δ takes the value of greater than 1.0, suggesting a species-specific adaptation. A $\delta < 1.0$ is consistent with shorter paths contributing more to the rates of trait evolution indicating adaptive radiation (i.e. the early evolution of traits in the evolutionary history). The delta value close to 1.0 indicates that the rates of trait evolution are constant from root to the tips of phylogeny. I tested whether estimated all three scaling parameters (λ, κ and δ) take the value of 1.0 indicating they are consistent with Brownian motion of evolution. The Brownian motion model of evolution assumes that phenotypic difference between lineages increases in proportional to the time since they shared a common ancestor. I compared the harmonic means of the log likelihood values from the models in which scaling parameters were fixed at the value 0.0, freely estimated or set at the

default value 1.0 for every iteration. The log Bayes Factor values were used to test whether the harmonic mean of log likelihood of the model in which scaling parameters are estimated is significantly different from the model in which they are fixed to 1.0 and 0.0. I then incorporated the estimated parameter values as 'ModelFile' in the estimation of ancestral state values for continuous traits.

Ancestral states reconstructions and correlated evolution

Interspecific variations in sexual systems and its morphological characters were observed among the Australian species of subgenus *Leptostemonum*. While considering phylogenetic dependence, Weller and Sakai (1999) cautioned that using labile characters that evolve frequently with equal transition rates for gains and losses might present considerable uncertainty in detecting the pattern of character evolution at ancestral nodes when parsimony based approaches were used. Therefore, the existence of evolutionary lability in sex system and associated reproductive traits among the species of subgenus *Leptostemonum* is expected to cause problems in evaluating the ecological correlates of character traits within a phylogenetic framework. Recent phylogenetic approaches using continuous-time Markov models in Bayesian framework offers a method for mapping characters on trees to study character evolution and for identifying character correlation while accommodating uncertainty in assigning ancestral states and tree itself (Huelsenbeck et al. 2003; Nielsen 2002; Pagel et al. 2004; Ronquist and Huelsenbeck 2003). Pagel and Meade (2006) further developed a Bayesian approach using reversible jump-MCMC method that estimates posterior descriptions of the rate coefficients for the evolutionary pathways correlating two character traits, by which evolutionary hypotheses on gains and losses of character

states in various trait combinations can be tested. This approach in the BayesTraits beta v1.1 program (Pagel and Meade 2006) was attempted in the resultant phylogeny of this study to explore the direction of character trait transitions, and to test the correlated evolution between sex systems and corolla size and fruit diameter.

I reconstructed ancestral states of three discrete characters such as sex system, corolla size and fruit diameter using MultiState program implemented in BayesTraits Beta v1.1 (Pagel and Meade 2006). A continuous-time Markov or Monte Carlo Markov Chain (MCMC) model of trait evolution that allows multiple changes between binary states of a discrete trait in each branch of the phylogenetic tree was performed to estimate transition rate parameters (Pagel et al. 2004). This model calculates the posterior probability distributions of transition rate parameters on a branch while simultaneously accounting for phylogenetic uncertainty, and estimates the rate of coefficients, forward rate (q_{01}) and backward rate (q_{10}), which correspond to the gains and losses between two states. This reversible-jump MCMC approach tests simultaneously the five possible transition rate parameter models of character state changes by visiting them in proportion to their posterior probabilities and reconstructs ancestral states (Supporting Information Table S4). Ancestral state reconstructions for 30 well supported nodes with > 90% Bayesian posterior probabilities and with extant species having more than one character state were performed using 'AddMRCA' command that calculates the proportion of likelihood associated with each of the alternative character states on a given node (Pagel et al. 2004).

For continuous traits: corolla size and fruit diameter, ancestral state reconstructions at 30 well supported internal nodes were performed in two steps using MCMC framework with 'AddMRCA' command in the BayesTraits beta v1.1 program (Pagel and Meade 2006). Firstly, I estimated the posterior distribution of model parameter (α) along with three scaling parameters (λ, κ and δ) using constant-variance random walk model (model A). These model parameters were incorporated in the next run for reconstruction of ancestral states at internal nodes after adjusting the data deviation parameter to achieve the 20-40% acceptance of estimated ancestral states. I also used the weighted squared-change parsimony method in the Mesquite 2.74 (Maddison and Maddison 2011). This method minimizes the sum of the squares of character state changes along the branches on the phylogeny (Maddison 1991) and, in taking branch length into account, and estimates ancestral states using weighted average of all the character trait values at neighbouring terminal and internal nodes (Hardy 2006).

Table S1. List of species included in this study, their GenBank accession numbers for GBSSI, ITS and *trnT-trnF* gene sequences and author information. [#](Stern et al. 2009); *(Martine et al. 2006); [$](Levin et al. 2006); ^(Bohs and Olmstead 2001); [¥](Stern and Bohs 2012); [€](Weese and Bohs 2007)

Sl.No	Species	GBSSI	ITS	*trnT-trnF*
1	*S. acanthodapis*	GQ163633[#]	GQ163571[#]	GQ163485[#]
2	*S. amblymerum*	GQ163634[#]	GQ163572[#]	GQ163486[#]
3	*S. argopetalum*	GQ163635[#]	GQ163573[#]	GQ163487[#]
4	*S. beaugleholei*	GQ163637[#]	GQ163489[#]	DQ364737*
5	*S. campanulatum*	AY996388[$]	DQ180395*	AY996488[$]
6	*S. campechiense*	AY996389[$]	AF244728^	DQ180475*
7	*S. centrale*	GQ163640[#]	GQ163575[#]	GQ163492[#]
8	*S. chenopodinum*	AY996393[$]	AY996492[$]	DQ180396*
9	*S. chippendalei*	GQ163641[#]	DQ364734*	GQ163493[#]
10	*S. cinereum*	AY996394[$]	AY996493[$]	DQ180397*
11	*S. clarkiae*	AY996396[$]	AY996495[$]	DQ855033*
12	*S. coactiliferum*	GQ163642[#]	GQ163576[#]	GQ163494[#]
13	*S. cookii*	GQ163643[#]	GQ163577[#]	GQ163495[#]
14	*S. corifolium*	GQ163644[#]	GQ163578[#]	GQ163496[#]
15	*S. densevestitum*	GQ163646[#]	GQ163579[#]	GQ163498[#]
16	*S. dianthophorum*	GQ163647[#]	GQ163499[#]	GQ163580[#]
17	*S. dimorphispinum*	GQ163648[#]	GQ163581[#]	GQ163500[#]
18	*S. discolor*	GQ163650[#]	GQ163582[#]	GQ163502[#]
19	*S. dissectum*	GQ163651[#]	GQ163583[#]	GQ163503[#]

Table S1. *Continued*

Sl.No	Species	GBSSI	ITS	*trnT-trnF*
20	*S. ditrichum*	GQ163652[#]	GQ163584[#]	GQ163504[#]
21	*S. diversiflorum*	AY996408[$]	AY996505[$]	DQ855023*
22	*S. echinatum*	AY996411[$]	AY996507[$]	DQ180398*
23	*S. elachophyllum*	GQ163653[#]	GQ163585[#]	GQ163506[#]
24	*S. elaeagnifolium*	AY996413[$]	AY996508[$]	DQ180399*
25	*S. ellipticum*	GQ163654[#]	GQ163586[#]	GQ163507[#]
26	*S. esuriale*	GQ163655[#]	GQ163587[#]	GQ163508[#]
27	*S. ferocissimum*	AY996415[$]	AY996510[$]	DQ180400*
28	*S. furfuraceum*	AY996417[$]	AY996512[$]	DQ180401*
29	*S. gabrielae*	GQ163656[#]	GQ163588[#]	GQ163509[#]
30	*S. gympiense*	GQ163660[#]	GQ163592[#]	GQ163513[#]
31	*S. hapalum*	GQ163661[#]	GQ163593[#]	GQ163514[#]
32	*S. heteropodium*	GQ163662[#]	GQ163516[#]	DQ364733*
33	*S. hieronymi*	AY996423[$]	AY996517[$]	GQ163517[#]
34	*S. hindsianum*	AY996424[$]	AY996518[$]	DQ180402*
35	*S. hoplopetalum*	GQ163663[#]	DQ364752*	GQ163518[#]
36	*S. horridum*	GQ163664[#]	GQ163594[#]	GQ163519[#]
37	*S. hystrix*	GQ163665[#]	DQ364753*	GQ163520[#]
38	*S. inaequilaterum*	GQ163666[#]	GQ163595[#]	GQ163521[#]
39	*S. innoxium*	GQ163667[#]	GQ163596[#]	GQ163524[#]
40	*S. johnsonianum*	GQ163668[#]	GQ163597[#]	GQ163525[#]
41	*S. jucundum*	GQ163669[#]	GQ163598[#]	GQ163526[#]

Table S1. *Continued*

Sl.No	Species	GBSSI	ITS	*trnT-trnF*
42	*S. lasiophyllum*	GQ163672[#]	GQ163601[#]	GQ163530[#]
43	*S. latens*	GQ163673[#]	GQ163602[#]	GQ163531[#]
44	*S. limitare*	GQ163675[#]	GQ163603[#]	GQ163533[#]
45	*S. lucani*	GQ163677[#]	GQ163604[#]	GQ163535[#]
46	*S. lythrocarpum*	GQ163678[#]	GQ163605[#]	GQ163536[#]
47	*S. macoorai*	GQ163679[#]	GQ163606[#]	GQ163537[#]
48	*S. mitchellianum*	GQ163680[#]	GQ163607[#]	GQ163539[#]
49	*S. nemophilum*	AY996446[$]	AY996535[$]	AY998447[$]
50	*S. neoanglicum*	GQ163682[#]	GQ163609[#]	GQ163541[#]
51	*S. nobile*	GQ163683[#]	GQ163610[#]	GQ163542[#]
52	*S. nummularium*	AY996448[$]	AY996537[$]	GQ163543[#]
53	*S. oedipus*	GQ163684[#]	GQ163611[#]	GQ163544[#]
54	*S. oldfieldii*	GQ163685[#]	GQ163612[#]	GQ163545[#]
55	*S. oligacanthum*	GQ163686[#]	GQ163613[#]	GQ163546[#]
56	*S. orbiculatum*	GQ163687[#]	GQ163614[#]	GQ163547[#]
57	*S. papaverifolium*	GQ163688[#]	GQ163615[#]	GQ163548[#]
58	*S. petrophilum*	AY996454[$]	AY996542[$]	GQ163550[#]
59	*S. phlomoides*	GQ163690[#]	GQ163616[#]	GQ163551[#]
60	*S. prinophyllum*	AY996456[$]	AY996544[$]	DQ180407*
61	*S. pugiunculiferum*	AY996458[$]	AY996545[$]	GQ163552[#]
62	*S. pungetium*	GQ163691[#]	GQ163617[#]	GQ163553[#]
63	*S. pusillum*	GQ163692[#]	GQ163618[#]	GQ163554[#]

Table S1. *Continued*

Sl.No	Species	GBSSI	ITS	*trnT-trnF*
64	*S. quadriloculatum*	GQ163693[#]	GQ163619[#]	GQ163555[#]
65	*S. rixosum*	GQ163695[#]	GQ163621[#]	GQ163557[#]
66	*S. rugosum*	DQ169046[€]	JN542598[¥]	DQ180490[€]
67	*S. semiarmatum*	GQ163696[#]	GQ163622[#]	GQ163559[#]
68	*S. serpens*	GQ163697[#]	GQ163623[#]	GQ163560[#]
69	*S. shirleyanum*	GQ163698[#]	GQ163624[#]	GQ163562[#]
70	*S. stelligerum*	AY996469[$]	AY996555[$]	DQ855018*
71	*S. stenopterum*	GQ163699[#]	GQ163626[#]	GQ163563[#]
72	*S. stupefactum*	GQ163700[#]	DQ364757*	GQ163564[#]
73	*S. sturtianum*	GQ163701[#]	GQ163627[#]	GQ163565[#]
74	*S. tetrathecum*	GQ163702[#]	GQ163628[#]	GQ163566[#]
75	*S. tridynamum*	AY996474[$]	AY996559[$]	DQ180412*
76	*S. vicinum*	GQ163705[#]	GQ163631[#]	GQ163569[#]
77	*S. viridifolium*	GQ163706[#]	GQ163632[#]	GQ163570[#]

Table S2. Model parameter summaries based on a total of 1200002 samples from 2 runs. Each run produced 800001 samples of which 600001 samples were included.

Parameter	Mean	Variance	95% HPD interval		Median	min ESS*	avg ESS*	PSRF+
			Lower	Upper				
TH{all}	0.016	0.000	0.013	0.019	0.016	206.360	213.190	1.013
TL{all}	0.556	0.001	0.489	0.624	0.554	325.740	328.770	1.011
r(A<->C){1}	0.110	0.000	0.083	0.137	0.110	3440.790	3750.080	1.000
r(A<->G){1}	0.282	0.000	0.240	0.322	0.281	2669.530	2835.290	1.000
r(A<->T){1}	0.077	0.000	0.059	0.095	0.077	4647.520	4879.970	1.000
r(C<->G){1}	0.121	0.000	0.090	0.155	0.121	3488.120	3490.410	1.000
r(C<->T){1}	0.295	0.000	0.256	0.338	0.294	2742.000	2906.630	1.000
r(G<->T){1}	0.116	0.000	0.090	0.142	0.115	4032.170	4065.910	1.000
r(A<->C){2}	0.062	0.000	0.044	0.081	0.061	3509.350	3598.540	1.000
r(A<->G){2}	0.243	0.001	0.197	0.290	0.242	1828.030	1848.050	1.000
r(A<->T){2}	0.056	0.000	0.030	0.083	0.055	2568.670	2621.620	1.000
r(C<->G){2}	0.090	0.000	0.071	0.110	0.090	2558.170	2671.830	1.000
r(C<->T){2}	0.472	0.001	0.419	0.528	0.472	1536.710	1617.370	1.000
r(G<->T){2}	0.077	0.000	0.054	0.102	0.076	2632.300	2868.060	1.001
r(A<->C){3}	0.198	0.000	0.159	0.238	0.197	3503.370	3528.700	1.000

Table S2. *Continued*

Parameter	Mean	Variance	95% HPD interval		Median	min ESS*	avg ESS*	PSRF+
			Lower	Upper				
r(A<->G){3}	0.205	0.000	0.164	0.245	0.204	3620.880	3676.620	1.000
r(A<->T){3}	0.040	0.000	0.027	0.054	0.040	5121.270	5559.330	1.000
r(C<->G){3}	0.086	0.000	0.049	0.124	0.085	3234.840	3332.330	1.000
r(C<->T){3}	0.243	0.001	0.198	0.286	0.242	3217.940	3375.410	1.000
r(G<->T){3}	0.228	0.001	0.184	0.273	0.227	2976.270	3090.490	1.000
pi(A){1}	0.284	0.000	0.264	0.303	0.284	4487.270	4856.730	1.000
pi(C){1}	0.189	0.000	0.174	0.207	0.189	4083.730	4522.130	1.000
pi(G){1}	0.202	0.000	0.185	0.219	0.202	5083.420	5167.190	1.000
pi(T){1}	0.325	0.000	0.304	0.345	0.325	4991.340	4991.770	1.000
pi(A){2}	0.158	0.000	0.134	0.181	0.158	3209.930	3210.580	1.000
pi(C){2}	0.387	0.000	0.356	0.422	0.387	1926.130	2151.250	1.000
pi(G){2}	0.328	0.000	0.296	0.359	0.328	2124.150	2453.880	1.000
pi(T){2}	0.127	0.000	0.108	0.148	0.126	2075.620	2103.600	1.000
pi(A){3}	0.369	0.000	0.347	0.390	0.368	4337.610	4759.330	1.000
pi(C){3}	0.155	0.000	0.139	0.170	0.155	5198.680	5640.780	1.000
alpha{1}	74.856	3959.866	0.690	185.407	61.965	2846.480	3378.820	1.000

Table S2. *Continued*

Parameter	Mean	Variance	95% HPD interval		Median	min ESS*	avg ESS*	PSRF+
			Lower	Upper				
alpha{2}	0.476	0.018	0.273	0.756	0.449	946.010	1068.300	1.001
alpha{3}	60.726	3996.667	0.401	181.646	35.059	1776.720	1966.340	1.002
pinvar{1}	0.394	0.007	0.201	0.518	0.413	795.320	919.850	1.000
pinvar{2}	0.341	0.006	0.187	0.490	0.343	918.340	997.240	1.001
pinvar{3}	0.467	0.014	0.182	0.616	0.506	510.990	575.420	1.004
m{1}	0.650	0.001	0.584	0.714	0.649	2169.900	2325.390	1.000
m{2}	3.288	0.014	3.051	3.525	3.288	1591.210	1643.690	1.000
m{3}	0.468	0.001	0.414	0.523	0.468	2485.830	2745.630	1.000
igrvar{all}	0.001	0.000	0.001	0.002	0.001	283.560	324.480	1.000

* Convergence diagnostic (ESS = Estimated Sample Size); min and avg values correspond to minimal and average ESS among runs. ESS value below 100 may indicate that the parameter is under sampled.

+ Convergence diagnostic (PSRF = Potential Scale Reduction Factor; Gelman and Rubin, 1992) should approach 1.0 as runs converge.

Table S3. Details of character traits: sex system, corolla size and fruit diameter for species included in this study. Range value is given next to mean corolla size and fruit diameter.

Sl.No	Species	Sex system	Corolla (mm)	Fruit (mm)
1	*S. acanthodapis*	Andromonoecy	10; 8-12	16.5;15-18
2	*S. amblymerum*	Andromonoecy	24; 20-28	13.5;11-16
3	*S. argopetalum*	Andromonoecy	8; 7-9	15
4	*S. beaugleholei*	Andromonoecy	30; 25-40	30; 25-35
5	*S. campanulatum*	Andromonoecy	28.2; 20-30	11; 8-14
6	*S. centrale*	Hermaphroditism	20; 15-25	12.5; 10-15
7	*S. chenopodinum*	Hermaphroditism	17.5; 10-20	7.5; 5-9
8	*S. chippendalei*	Andromonoecy	30; 25-35	26.5; 20-30
9	*S. cinereum*	Andromonoecy	30; 28-35	20.5; 15-24
10	*S. clarkiae*	Andromonoecy	25.3; 22-30	37.5; 20-40
11	*S. coactiliferum*	Hermaphroditism	25; 20-30	11.5; 8-15
12	*S. cookii*	Hermaphroditism	20; 15-22	12.5; 10-15
13	*S. corifolium*	Andromonoecy	20	9.5; 10-15
14	*S. densevestitum*	Hermaphroditism	11.5; 10-13	7; 6-8
15	*S. dianthophorum*	Hermaphroditism	7; 5-10	9.5; 9-10
16	*S. dimorphispinum*	Andromonoecy	28; 15-30	32.5; 25-35
17	*S. discolor*	Andromonoecy	5; 3-7	12; 10-14
18	*S. dissectum*	Hermaphroditism	12.5; 10-15	8; 7-9
19	*S. ditrichum*	Andromonoecy	22.5; 20-25	23.5; 21-26
20	*S. diversiflorum*	Andromonoecy	25.4; 22-30	25; 20-30
21	*S. echinatum*	Hermaphroditism	25; 20-30	15; 10-20

Table S3. *Continued*

Sl.No	Species	Sex system	Corolla (mm)	Fruit (mm)
22	*S. elachophyllum*	Hermaphroditism	17.5; 10-25	13; 12-14
23	*S. ellipticum*	Hermaphroditism	25; 20-30	17.5; 1.5-20
24	*S. esuriale*	Hermaphroditism	15; 10-20	13.5; 12-15
25	*S. ferocissimum*	Andromonoecy	20	7.5; 6-9
26	*S. furfuraceum*	Andromonoecy	13; 10-25	18; 15-20
27	*S. gabrielae*	Hermaphroditism	25; 20-30	15; 10-20
28	*S. gympiense*	Andromonoecy	25; 20-30	5.5; 4.5-7
29	*S. hapalum*	Hermaphroditism	27.5; 25-30	5.25; 4-6.5
30	*S. heteropodium*	Andromonoecy	35	12.5; 10-15
31	*S. hoplopetalum*	Hermaphroditism	22.5; 15-30	17.5; 15-20
32	*S. horridum*	Hermaphroditism	17.5; 15-20	17.5; 15-20
33	*S. hystrix*	Hermaphroditism	20; 15-25	20; 15-25
34	*S. inaequilaterum*	Andromonoecy	27.5; 20-35	15; 11-18
35	*S. innoxium*	Hermaphroditism	8; 7-9	3.75; 3-4.5
36	*S. johnsonianum*	Hermaphroditism	8.5; 6-11	5.4; 5-5.8
37	*S. jucundum*	Andromonoecy	25; 20-30	15; 14-16
38	*S. lasiophyllum*	Hermaphroditism	27.5; 25-30	12.5; 10-13
39	*S. latens*	Hermaphroditism	17.5; 15-20	5.25; 4-6.5
40	*S. limitare*	Andromonoecy	25; 20-30	15.5; 14-17
41	*S. lucani*	Hermaphroditism	22.5; 20-25	12.5; 10-15
42	*S. lythrocarpum*	Hermaphroditism	10	10; 8-11
43	*S. macoorai*	Andromonoecy	25; 20-30	20; 15-25

Table S3. *Continued*

Sl.No	Species	Sex system	Corolla (mm)	Fruit (mm)
44	*S. mitchellianum*	Hermaphroditism	8.5; 7-10	8.5; 8-9
45	*S. nemophilum*	Hermaphroditism	25; 20-30	7; 4.5-9
46	*S. neoanglicum*	Andromonoecy	22; 18-25	16; 14-17
47	*S. nobile*	Andromonoecy	27; 21-33	21; 18-24
48	*S. nummularium*	Hermaphroditism	22.5; 20-25	12.5; 10-15
49	*S. oedipus*	Andromonoecy	50; 45-55	17.5; 15-20
50	*S. oldfieldii*	Hermaphroditism	32.5; 25-40	7.5; 5-10
51	*S. oligacanthum*	Hermaphroditism	22.5; 20-25	7.5; 4-11
52	*S. orbiculatum*	Hermaphroditism	22.5; 20-25	12.5; 10-15
53	*S. papaverifolium*	Andromonoecy	22.5; 20-25	11; 10-12
54	*S. petrophilum*	Hermaphroditism	27.5; 25-30	9; 8-10
55	*S. phlomoides*	Andromonoecy	45; 40-50	32.5; 30-35
56	*S. prinophyllum*	Hermaphroditism	12.5; 10-15	17.5; 15-20
57	*S. pugiunculiferum*	Hermaphroditism	10	9.5; 9-10
58	*S. pungetium*	Hermaphroditism	17.5; 15-20	27.5; 25-30
59	*S. pusillum*	Andromonoecy	8.5; 7-10	15
60	*S. quadriloculatum*	Hermaphroditism	22.5; 20-25	13.5; 10-17
61	*S. rixosum*	Andromonoecy	25; 20-30	20; 15-24
62	*S. semiarmatum*	Hermaphroditism	17.5; 15-20	10; 8-12
63	*S. serpens*	Andromonoecy	22.5; 20-25	15; 14-16
64	*S. shirleyanum*	Hermaphroditism	15	4.75; 3.5-6
65	*S. stelligerum*	Andromonoecy	20; 15-25	8.5; 6.5-10

Table S3. *Continued*

Sl.No	Species	Sex system	Corolla (mm)	Fruit (mm)
66	*S. stenopterum*	Andromonoecy	22.5; 20-25	10
67	*S. stupefactum*	Andromonoecy	27.5; 25-30	32; 30-34
68	*S. sturtianum*	Andromonoecy	35; 30-40	12; 9-15
69	*S. tetrathecum*	Andromonoecy	30; 20-40	14.5; 12-17
70	*S. vicinum*	Hermaphroditism	25; 20-30	20; 10-30
71	*S. viridifolium*	Hermaphroditism	15; 12-18	8; 6-10

Table S4. Description of the transition rate parameter models of trait evolution sampled in the RJ-MCMC framework of BayesTraits program using discrete character dataset.

Rate parameter	Model	Interpretation
q01 = q10	One parameter model (00)	Equal rates of gaining new state and reversal of ancestral state from new state
q01 = 0	Restricted model (Z0)	Rate of gaining new state is constrained to zero
q10 = 0	Restricted model (0Z)	Rate of reversal of ancestral state is constrained to zero
q01 > q10	Two parameter model (10)	Rate of gaining new state is higher than the rate of reversal of ancestral state
q01 < q10	Two parameter model (01)	Rate of reversal of ancestral state is higher than the rate of gaining new state

Table S5. Constant-variance random-walk (Model A) and directional change random-walk (Model B) models of trait evolution were tested to determine the best fitting model for two characters by comparing the harmonic means of likelihood values from two models using Bayes Factors (BF) in BayesTraits.

Character	Harmonic mean of likelihood value		BF
	Model A	Model B	
Corolla size	-85.56	-86.53	1.9
Fruit diameter	-71.16	-71.42	0.53

Table S6. Ancestral states reconstructed in three discrete characters using the Bayesian MCMC framework with MRCA approach in the BayesTraits program at 30 well supported internal nodes. Mean posterior probabilities of ancestral state being '0' is given. The subtraction of the probability of state '0' from one is the mean posterior probability of having state '1' as ancestral state. Significant posterior probabilities, P(0) < 0.2 and P(0) > 0.8, are indicated in bold.

Node	Sex system 0 = hermaphroditism 1 = andromonoecy	Corolla size 0 = small (≤ 2.32 cm) 1 = large (> 2.32 cm)	Fruit diameter 0 = small (≤ 1.47 cm) 1 = large (> 1.47 cm)
1	0.37	**0.95**	**0.86**
2	0.22	0.69	0.34
3	**0.20**	**0.93**	0.76
4	0.32	**0.86**	**0.95**
5	**0.11**	0.63	**0.84**
6	**0.01**	**0.03**	0.24
7	**0.98**	0.63	0.72
8	**0.98**	**0.03**	**0.93**
9	**0.97**	**0.92**	0.30
10	**0.99**	**0.99**	**0.01**
11	0.39	**0.97**	**0.96**
12	0.31	**0.99**	**0.99**
13	**0.10**	**0.98**	**0.92**
14	**0.97**	**0.97**	**0.94**
15	0.28	**0.82**	0.38

Table S6. *Continued*

Node	Sex system 0 = hermaphroditism 1 = andromonoecy	Corolla size 0 = small (≤ 2.32 cm) 1 = large (> 2.32 cm)	Fruit diameter 0 = small (≤ 1.47 cm) 1 = large (> 1.47 cm)
16	**0.17**	**0.95**	**0.95**
17	0.24	**0.04**	0.57
18	0.35	**0.89**	**0.80**
19	**0.91**	**0.95**	**0.91**
20	**0.13**	0.64	0.35
21	**0.17**	**0.84**	0.64
22	**0.98**	**0.98**	**0.98**
23	**0.00**	0.61	0.21
24	**0.06**	**0.99**	**0.91**
25	**0.07**	0.76	0.23
26	**0.08**	**0.99**	**0.99**
27	**0.08**	**0.90**	**0.99**
28	0.24	**1.00**	**0.99**
29	**0.05**	**1.00**	**1.00**
30	0.23	**1.00**	**1.00**

Table S7. A comparison between observed mean (OM) and reconstructed ancestral values of corolla size and fruit diameter using weighted squared-change parsimony method (SCP) in Mesquite 2.74 and MRCA approach in BayesTraits program at 30 well supported internal nodes.

Node	Corolla size (cm)			Fruit diameter (cm)		
	OM	Ancestral value		OM	Ancestral value	
		SCP	MRCA		SCP	MRCA
1	2.17	2.30	2.32	1.46	1.47	1.47
2	2.72	2.45	2.58	1.79	1.59	1.59
3	2.33	2.37	2.46	1.39	1.50	1.52
4	2.35	2.36	2.44	1.35	1.43	1.44
5	2.42	2.39	2.46	1.42	1.42	1.42
6	3.44	2.90	2.80	2.59	1.99	1.93
7	2.50	2.53	2.55	1.31	1.49	1.47
8	2.75	2.66	2.58	1.16	1.30	1.35
9	2.17	2.29	2.48	1.50	1.52	1.48
10	2.13	2.16	2.39	1.88	1.86	1.76
11	1.92	2.19	2.15	1.31	1.39	1.42
12	1.93	2.09	1.97	1.33	1.34	1.36

Table S7. *Continued*

Node	Corolla size (cm)			Fruit diameter (cm)		
	OM	Ancestral value		OM	Ancestral value	
		SCP	MRCA		SCP	MRCA
13	1.78	1.88	1.93	1.22	1.26	1.28
14	1.79	2.00	1.96	1.33	1.42	1.41
15	2.11	2.22	2.04	1.99	1.71	1.69
16	1.89	1.99	1.93	1.22	1.31	1.34
17	2.60	2.44	2.05	1.40	1.38	1.34
18	1.88	1.88	1.94	1.58	1.34	1.36
19	1.30	1.72	1.94	0.93	1.22	1.28
20	2.27	2.09	2.05	2.02	1.71	1.65
21	1.83	1.92	1.93	1.08	1.20	1.20
22	1.39	1.43	1.86	0.54	0.71	0.92
23	2.18	2.08	1.95	1.51	1.35	1.31
24	1.83	1.92	1.92	1.16	1.29	1.27
25	1.95	1.94	1.91	1.95	1.66	1.47
26	1.33	1.65	1.90	0.88	0.97	1.07

Table S7. *Continued*

Node	Corolla size (cm)			Fruit diameter (cm)		
	OM	Ancestral value		OM	Ancestral value	
		SCP	MRCA		SCP	MRCA
27	2.00	1.97	1.92	0.69	0.83	0.88
28	1.88	1.90	1.90	0.72	0.75	0.85
29	1.92	1.92	1.92	0.78	0.78	0.81
30	1.88	1.88	1.89	0.75	0.75	0.81

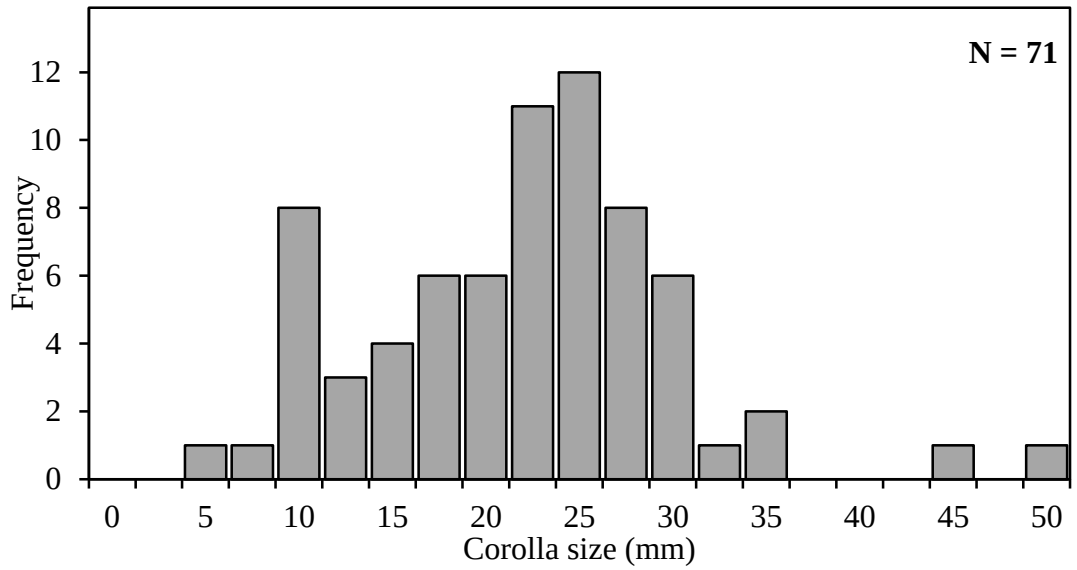

Figure S1. Frequency distribution of corolla size (mm) among the Australian *Solanum* species included in this study

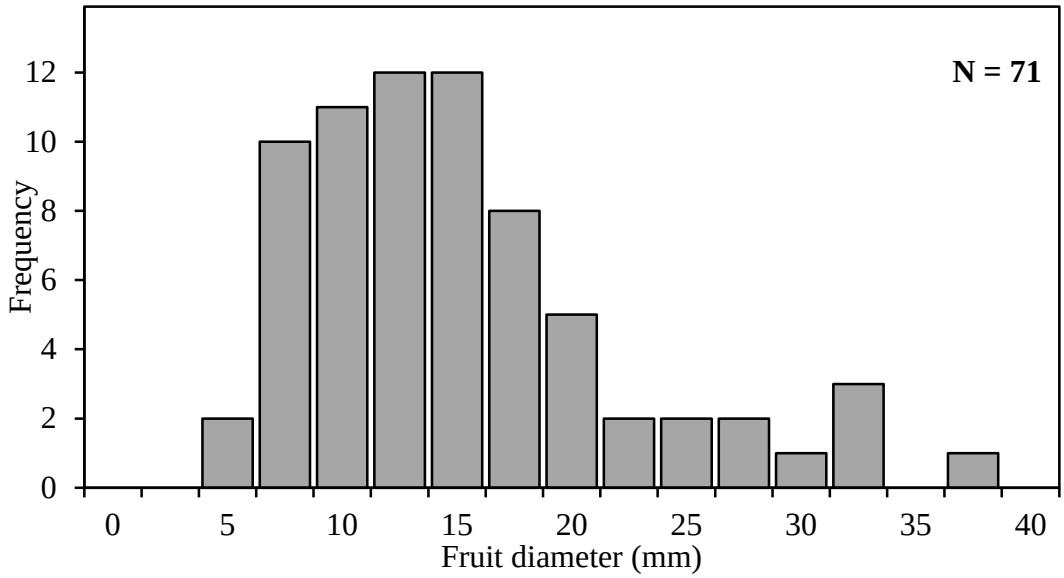

Figure S2. Frequency distribution of fruit diameter (mm) among the Australian *Solanum* species included in this study

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