

## THE ECOLOGY, EVOLUTION, AND BIOGEOGRAPHY OF DIOECY IN THE GENUS *SOLANUM*: WITH PARADIGMS FROM THE STRONG DIOECY IN *SOLANUM POLYGAMUM*, TO THE UNSUSPECTED AND CRYPTIC DIOECY IN *SOLANUM CONOCARPUM*<sup>1</sup>

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- *Premise of the study:* Island plants are over-exploited and “under-explored.” Understanding the reproductive biology of plants, especially rare species, is fundamental to clarifying their evolution, estimating potential for change, and for creating effective conservation plans. Clarification of sexual systems like dioecy, and unusual manifestations of it in specific studies within *Solanum*, helps elucidate evolutionary patterns and genetic and ecological control of sex expression.
- *Methods:* Studies of reproductive systems of two Caribbean endemics, *S. polygamum* and *S. conocarpum*, combined multifaceted analyses of field populations and of multiple generations of greenhouse plants.
- *Key results:* The dioecy in both species is, like that in other solanums, largely cryptic, although the gender of *S. polygamum* flowers is obvious. The rare *S. conocarpum* is recognized as dioecious; floral gender is not obvious. Variation in sex expression facilitated experiments and promoted hypotheses on control and significance of morphological features and sex expression.
- *Conclusions:* Confirmed dioecy in at least 15 solanums is distributed across the genus, with perhaps 6 independent origins, and with crypticity in the form of morphologically hermaphroditic, but functionally unisexual, flowers characterizing all species. Dioecy is not more strongly associated with islands. Inaperturate pollen in pistillate flowers characterizes almost all, but not the two dioecious species studied herein. Dioecy in both species indicates leakiness (rare hermaphroditic flowers on male plants) that helps explain island colonization and radiation. Leakiness allowed confirmation—usually impossible for dioecious species—of self-compatibility for *S. polygamum*, and thus support for the hypothesis that dioecy evolved to promote outcrossing.

**Key words:** Baker’s Law; compatibility; conservation and endemics; cryptic/functional dioecy; leaky dioecy; island colonization; pollinator deception; sexual system evolution; *Solanum*; underlying synapomorphy.

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Thanks go to colleagues who called attention to a handsome and rare *Solanum* curiosity on St. John, *S. conocarpum*. In particular, that includes Gary Ray, who provided a great deal of help in the field in locating populations and plants, and providing a context for St. John ecology overall. D. Sapio and R. Balon (Resource Management, Virgin Islands National Park on St. Thomas) were helpful with permits, and Sapio, in particular with ideas and suggestions derived from monitoring the *S. conocarpum* plants in cultivation outside their offices/laboratories at 1300 Cruz Bay Creek, St. John. A. Stanford provided useful comments and suggestions on the work, and in her and her colleague’s paper on *S. conocarpum* ecology and genetics. C. Morse and M. Opel provided care in growing and keeping healthy finicky rare plants in the greenhouses. G. Breckon and F. Axelrod were valuable field partners in the vain search for *S. polygamum* on Vieques, and on Puerto Rico as well. Virge Kask and Jessica Machnicki produced the drawings of the flowers/flower parts, and E. Barbeau helped with figure preparation. L. Bohs generously provided unpublished phylogenetic information and background. It is worth noting too, the valuable resource that the “Solanaceae Source” provides for the research community, developed with a grant to S. Knapp, L. Bohs, M. Nee and D. Spooner from the National Science Foundation Planetary Biodiversity Inventory. Financial support was provided directly by The

Island plants are over-exploited and “under-explored,” the latter term used by Bill Baker in a Kew Science Blog recognizing the theme of island biodiversity as part of the 2014 International Day for Biological Diversity (<http://www.kew.org/discover/blogs/island-plant-diversity-endangered-and-under-explored>). Many island species are endemic and at least uncommon. Even those that are not strictly exploited, are, by virtue of their island habitat, of limited distribution and thus at minimum,

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more subject to the exploitation and abiotic vagaries that island biotas suffer worldwide. Understanding the reproductive biology of plants is fundamental to clarifying their evolution, estimating potential for change, and for establishing conservation programs (e.g., Ornduff, 1969; Anderson et al., 2002; Kaiser-Bunbury et al., 2010; Kueffer and Kaiser-Bunbury, 2014). Furthermore, if the plants of interest are also rare, or from restricted habitats (like island endemics), the importance of a full understanding of the impact of reproductive constraints is of even more significance for establishing effective conservation programs (Ellstrand and Elam, 1993; Crawford et al., 2011). Finally, elucidation of unusual sexual systems, like dioecy, helps clarify the evolution of sexual systems per se, and evolutionary patterns and options in angiosperms in general. We present a detailed analysis of the reproductive system of two West Indian island species that expands and deepens our understanding of the evolution of dioecy on islands in general, and of the unique dioecious system in *Solanum*, in particular. We consider, more broadly, the biogeography, ecological importance, and evolutionary history of *Solanum* dioecy *in toto*, and the implications of these particular studies for a general understanding of the evolution of dioecy in angiosperms.

Although unisexual and bisexual flowers were found in equal portions even up to the late Cretaceous (Friis et al., 2011), dioecy today is represented in only about 6% of angiosperms (Renner and Ricklefs, 1995; Renner, 2014). The dioecious system in *Solanum* is even more uncommon, it is usually cryptic and thus is often undetected. Nonetheless, the unusual manifestation of *Solanum* dioecy makes it worthy of study. The description of *S. polygamum* in the late 1700s (S. Knapp, 2014) constitutes the first suggestion of dioecy in the genus. The eminent Australian solanologist, David Symon, called much more attention to the topic with a little paper in *Taxon* almost 200 years later listing six additional dioecious species (Symon, 1970), all of them Australian. Symon followed that work with two papers in 1979 (Symon, 1979a, 1979b) where he recast these species as androdioecious, a very rare sex form in angiosperms, let alone in *Solanum* (androdioecy is a sexual system characterized by a species with two types of plants: some with only staminate flowers, and others with only hermaphroditic flowers). Anderson published the first of several papers based on intensive field, greenhouse, and laboratory studies on the biology of individual species, on the deceptively cryptic dioecy in a Mesoamerican *Solanum* (*S. appendiculatum*) in the same year (1979). Anderson and Symon (1989) followed this 10 years later with the results of a year-long study of the 9 Australian dioecious and 11 andromonoecious species (single plants bear both staminate and hermaphroditic flowers). And, approximately 10 years after that, Knapp et al. (1998) followed up with further analyses of pollen morphology of the dioecious solanums, including one other certain dioecious species (*S. confertiseriatum* from Ecuador), and one likely, but uncertain dioecious species (*S. crotonoides* from Hispaniola). Subsequently, Knapp has recognized a new species, *S. luculentum* (2010), that is cited as heterostylous and “probably dioecious” (cloud forests of northern South America). In addition, Martine et al. (2006), Brennan et al. (2006), Barrett (2013) and Martine et al. (2013) recognized at least three other northern Australian dioecious species (*S. cowiei*, *S. sejunctum*, *S. zoeae*) and Martine (personal communication) speculates that there are more to be discovered. The list of dioecious species of *Solanum* to date is given in Table 1.

As noted, dioecy in *Solanum* is not obvious, and has been described as “functional” (Anderson, 1979) or “cryptic” (Mayer and Charlesworth, 1991). These adjectives apply because all

dioecious solanums are morphologically hermaphroditic: staminate (or male, the term used interchangeably throughout) flowers bear pistils (though sometimes very highly reduced) and all pistillate (female) flowers bear nearly full-sized anthers. Thus, the expression of the dioecious condition is not obvious from floral morphology in some cases (e.g., *S. crotonoides*, Knapp et al., 1998), or not even apparent in others (e.g., *S. appendiculatum*, although oddly, it is this species where the male and female plants were previously treated as separate species—species based on trivial morphological characters; Correll, 1962; Anderson, 1979; Anderson and Levine, 1982). Furthermore, beyond the cryptic nature of the dioecy, unusual features like inaperturate pollen (pollen with no apertures [pores] or colpi [furrows]) in the anthers of the pistillate flowers characterize most of the known species (more below). The inaperturate pollen is highly unusual in the genus and family (Anderson and Gensel, 1976; Knapp et al., 1998). This inaperturate pollen is ‘non-functional’ in terms of affecting seed set. It cannot be induced to germinate (Levine and Anderson, 1986). However, it is functional in terms of impact on the overall reproductive biology in that such pollen does serve as the reward to the bees visiting (and vibrating) the flowers (Anderson and Symon, 1989). The retention of anthers in female flowers is not odd, but to be expected; the staminal column provides the ‘landing platform’ for the bees that visit the flowers (Buchmann, 1986)—without it, bees do not alight (Anderson and Anderson, unpubl. data). The retention of pollen in pistillate flowers may be of some significance in that all *Solanum* flowers lack floral nectararies (Anderson and Symon, 1985), and the only reward to pollinators is the pollen itself. Buchmann (1986) showed that American bumblebees are able to distinguish flowers that have been previously visited/buzzed, and spend less time on visited flowers than on virgin flowers. The documentation of dioecy in many clades across the increasingly clear phylogenetic tree of *Solanum* (Bohs, 2005; Weese and Bohs, 2007; Särkinen et al., 2013), and the explication particularly of the functional features of the reproductive biology/sexual system of these species, facilitates broader evolutionary considerations in *Solanum*, and, more importantly, for angiosperms in general.

The island homes of the solanums on which we focus herein (Caribbean Sea, West Indies, greater Antilles, and in particular, the small island of St. John of the Virgin Islands) have relatively small floras (Acevedo-Rodríguez and Strong, 2012), and, given the huge human impact via former agricultural practices on some islands (e.g., Picó, 2011), and more recently tourism on all of them, the remaining elements of the native flora are threatened (Acevedo-Rodríguez and Strong, 2012). The Solanaceae manifests considerable diversity in the American tropics, but less so in the West Indies (Knapp, 2014): Acevedo-Rodríguez and Strong (2012) rank the nightshades as the 14th largest family of native plant taxa in the West Indies. However, only about 2% of the total Virgin Islands flora is made up of nightshades, and then roughly half of that number is of cultigens, so the family, and the genus *Solanum*, though important agriculturally (e.g., Heiser, 1969), does not play a large role in the native flora. Nevertheless, what is particularly interesting, and what constituted the initial attraction for this study, is the presence of an island-endemic, little-studied, but first-suggested dioecious species of *Solanum*, *S. polygamum*.

The study of *S. polygamum* Vahl is interesting for three reasons. First, there are only 15–18 documented dioecious species in the large genus *Solanum* (see Table 1). Insights into the evolution of dioecy in angiosperms in general, its occurrence in

TABLE 1. Taxonomy, distribution, strength of dimorphism, and pollen type of verified Dioecious *Solanum* species.

Species	Taxonomy <sup>1</sup>	Distribution	Strength of floral dimorphy	Inaperturate pollen (IP), 3 porate (3Cp) or no pollen (NP) in pistillate flowers
<i>S. appendiculatum</i>	Potato group	Mexico, Central America	Very weak	IP
<i>S. leuculentum</i> <sup>2</sup>	Dulcamaroid	South America	?	?
<i>S. confertiseriatum</i> <sup>8</sup>	Geminata	South America	?	IP
<i>S. conocarpum</i> <sup>3</sup>	Geminata	Virgin Islands	Very weak	NP
<i>S. polygamum</i> <sup>3</sup>	Leptostemonum	Virgin Islands	Very Strong	NP
<i>S. crotonoides</i> <sup>8</sup>	Leptostemonum	Hispaniola	?	IP?3Cp?
<i>S. asymmetriphyllum</i>	Leptostemonum	Australia	Strong	IP
<i>S. carduiforme</i> <sup>4</sup>	Leptostemonum	Australia	Strong	IP
<i>S. cataphractum</i> <sup>4</sup>	Leptostemonum	Australia	Strong	?
<i>S. cunninghamii</i> <sup>4</sup>	Leptostemonum	Australia	Strong	IP
<i>S. cowiei</i> <sup>6</sup>	Leptostemonum	Australia	Strong	IP
<i>S. dioicum</i>	Leptostemonum	Australia	Strong	IP
<i>S. leopoldense</i>	Leptostemonum	Australia	Strong	IP
<i>S. petraeum</i>	Leptostemonum	Australia	Strong	IP
<i>S. sejunctum</i> <sup>5</sup>	Leptostemonum	Australia	Strong	IP
<i>S. tudunggae</i>	Leptostemonum	Australia	Strong	IP
<i>S. vansittartense</i> <sup>4</sup>	Leptostemonum	Australia	Strong	?
<i>S. zoeae</i> <sup>7</sup>	Leptostemonum	Australia	?	?

<sup>1</sup> Groups as recognized at: S. Knapp, 2014 Solanaceae Source Web Site, Natural History Museum.

<sup>2</sup> Knapp, 2010.

<sup>3</sup> Treated in this study.

<sup>4</sup> Symon, 1981; rare species, scanty material.

<sup>5</sup> Martine et al., 2006; Brennan et al., 2006.

<sup>6</sup> Martine et al., 2013.

<sup>7</sup> Barrett, 2013.

<sup>8</sup> Knapp, 2014 Solanaceae Source, and Knapp et al., 1998.

insular systems, and in particular in *Solanum* where nectariferous, pollen-only, hermaphroditic flowers predominate, are advanced by these studies: how does dioecy “work” with these characteristics, where did it come from, and what are the likely ecological and evolutionary corollaries for this manifestation of dicliny? Second, the reproductive biology of *S. polygamum* is of some interest because it is an island endemic, that is, at least, uncommon, and becoming less abundant (Aceveo-Rodríguez and Strong, 2012; personal communication Gary Breckon and Frank Axelrod). This formerly more-widespread species is today easily found only in the mostly protected areas of the U.S. National Park on St. John. Even with expert field help (G. Breckon and F. Axelrod), we could find no plants on Vieques, the small island off Puerto Rico, where it has been reported previously. In general, there are a very few dioecious solanums restricted to islands, and the nature of the dioecy of those reported is not known. The nonhermaphroditic solanums studied so far are mostly andromonoecious, also uncommon on islands (e.g., see Knapp et al., 1998; Anderson et al., 2006a; Anderson et al., 2014). A third reason making this a compelling case for study is that the dioecy in *S. polygamum* is more strongly expressed than that in any other *Solanum*. This species is a ‘local hero’, well known perhaps only among a subset of those who study the Solanaceae, or Antillean plants, because it is the most obvious dioecious species in *Solanum*. In spite of its recognized floral dimorphism, the nature of the dioecy has not been carefully studied.

The distribution of the second species of interest, *S. conocarpum* Dunal, is even more restricted (native distribution only on the island of St. John), with cultivated specimens on St. Thomas (A. Stanford, personal communication). Consequently, it is very little known outside of the thorough taxonomic work of Knapp on the “Geminata clade” to which it belongs (Bohs,

2005; Knapp, 2002, 2008), and its inclusion in comprehensive phylogenetic work by Weese and Bohs (2007). Stanford et al. (2002) first discussed its biology and conservation in an oral report at a Botanical Society of America meeting in 2002, where its known status at the time—four plants on St. John—was described. The genetic diversity estimates for those four plants, not surprisingly, were low. Gary Ray and others (Stanford et al. (2013)) have since found additional plants. Specifically, they discovered a population consisting of about 180 plants on one peninsula of St. John. That population, and a few other individuals on St. John (G. Ray, personal communication), constitutes all the native plants known for this species. Subsequently, there has been a keen interest in changing the listing status of *S. conocarpum*, and considerable interest by the National Park Service and the Fish and Wildlife Service in this very rare St. John endemic (D. Sapio, National Park Service, personal communication). According to a 12-mo petition finding, the Fish and Wildlife Service posted this species (also known by its common name, Marrón bacora) in February 2011 as a “US ESA: Priority 2 candidate (high threat magnitude, imminent threat immediacy, and species taxonomic distinctiveness)” (Federal Register Volume 76, Number 35, Pages 9722–9733, February 22, 2011). The rarity makes this species more important for study.

Rarity, however, does not constitute a reason to include *S. conocarpum* in the continuing study (by G. Anderson) of solanum dioecy. While engaged in field work in pursuit of *S. polygamum* in Puerto Rico (including Vieques) and the Virgin Islands, Anderson was introduced by Gary Ray and David Sapio to this “handsome” (that in itself is a little unusual for a *Solanum*) St. John endemic. As you will read, the reproductive biology of this unusual species turned out to be sufficiently interesting to merit intensive study of its reproductive biology as

well. The known, but little-studied strongly dioecious *S. polygamum* was the initial focus of these Caribbean studies. However, careful morphological study, combined with a range of field and greenhouse experiments of the sexual system of both *S. polygamum* and the (morphologically) weakly dioecious *S. conocarpum*, proved to be very interesting, and provided unexpected outcomes and insights into the function, evolution, ecology, and genetics of dioecy.

## MATERIALS AND METHODS

Representative vouchers, derived from greenhouse-grown plants from field-collected seeds for both species are in CONN (*S. conocarpum*: CONN00121092, CONN00121093, CONN00121094, CONN00121095; *S. polygamum*: CONN00121088, CONN00121089, CONN00121090, CONN00121091).

**Sex Ratio—Field**—Please note that “staminate” or “male”, and “pistillate” or “female”, will be used interchangeably throughout the rest of the manuscript.

***Solanum conocarpum***—We studied plants in the field and in the greenhouses. On St. John, we surveyed for flowering and fruiting status the single-remaining, large, natural population known for this species in October 2008. Most of the plants had been previously marked by G. Ray as part of an ongoing study of the ecology and population biology of this rare species. Our census consisted of scoring the reproductive status of every plant encountered. If plants were in flower, we could score gender based on style length. Multiyear studies of greenhouse plants have confirmed that styles of female plants are equal to or longer than the staminal column, usually by 0.5 mm or more. Some plants not in flower did bear fruits; for them, the gender is obvious. The more than 50 greenhouse-cultivated plants grown subsequently fully support this determination: no *S. conocarpum* plant scored unequivocally as a male has ever borne fruit (also see the crossing/hand pollination data below). Generally, plants were categorized as male if their flowers had styles shorter than the staminal column. Plants without flowers or fruit were recorded as vegetative.

***Solanum polygamum***—Field studies were carried out on four different occasions. Field searches were made in Puerto Rico (2007, 2009) and Vieques (in 2007) with no success: in particular, no extant *S. polygamum* were found in spite of a former distribution on these two islands (Acevedo-Rodríguez and Strong, 2012). Of course there may be some plants left, yet undiscovered, but this seems less likely given the help in the field of two of the most experienced Puerto Rican field biologists (Gary Breckon and Frank Axelrod). Extant plants are scattered, but common in the remaining natural scrub forests on St. John. Accordingly, notes on field morphology and distribution were made, and seeds and six cuttings were made on St. John in two field expeditions, in March and October of 2008. Gender is easily scored by obvious inflorescence and flower morphological differences between males and females (or fruit bearing in flower-less plants). Gender was scored in several populations, and also pooled for the St. John populations studied.

**Sex Ratio—Greenhouse**—About 100 seeds were planted, and germination was quite high for both species. About 50 seedlings of *S. conocarpum*, and nearly 40 of *S. polygamum* were grown to sexual maturity. After 36 mo post-germination, virtually all plants had flowered and were scored for gender.

From years of growing plants in greenhouses, we have determined that the style length alone is not the best feature to use in assigning sex for *S. conocarpum* (more discussion below). The most certain assignment comes from assessing anther shape. The difference in anther shape is obvious, as illustrated in the drawings of the flowers (Fig. 1): larger (both in terms of length and diameter) and straighter anthers characterize males, and smaller, more inflexed anthers are diagnostic of females. This anther character never fails to diagnose gender, and was used for accurate gender determination in the greenhouse. As noted, gender determination for *S. polygamum* is obvious, though the anther shape character is manifest there as well.

**Morphological Characters—Inflorescence Size**—The number of open flowers, buds, and floral scars per inflorescence was easily counted for both

species. Counts were made mostly from greenhouse-grown plants, but with samples from the field as well. The morphological characters following were assessed by hand measurements, with the aid of microscopy when required.

**Corolla Size and Number of Lobes**—The corolla diameter was measured of both alcohol-preserved field-collected flowers, and fresh flowers from greenhouse-grown plants with the usually very slightly campanulate corolla spread out flat.

**Staminal Column, Anthers, Anther Shape**—The length of stamens, width of the staminal column (in *S. conocarpum*), and the anthers were measured in both fresh and alcohol-preserved flowers. The shape of the anthers was scored as described above for male and female *S. conocarpum*.

**Pollen Presence, Stainability, Morphology (via Scanning Electron Microscopy—SEM)**—Fresh and field-collected alcohol-preserved flowers were both used. The number of grains was counted with a haemocytometer following the methods detailed in Anderson and Symon (1989). Pollen viability was assayed via staining (Aniline Blue in Lactophenol; also detailed in Anderson and Symon, 1989). SEM studies of pollen from fresh flowers (following standard techniques (as detailed in Anderson and Gensel, 1976) verified conclusions from light microscopy regarding pollen shape and morphology.

**Ovary and Style**—All of these were taken from either fresh or alcohol-preserved, field-collected flowers. Ovary diameter, and for *S. conocarpum*, ovary length (*S. polygamum* ovaries are largely spherical), were measured. Style length was measured from the top of the ovary to the tip of the stigma. Given the importance of relative style length (*vis a vis* the staminal column) as a key to identification of sex expression in dioecious and other non-hermaphroditic solanums (Anderson, 1979; Anderson and Symon, 1989), we also scored style length relative to the staminal column, but only for *S. conocarpum*. The scores consisted of the style length relative to the surrounding staminal column in intact living (not preserved) flowers. Thus, a style equal in length to the staminal column was scored as 1.0 (or 100%). A style only three-quarters as long as the staminal column (i.e., shorter than the staminal column) was scored as 0.75 (e.g., 75%), and a style that was half again longer than the staminal column was given a score of 1.5 (e.g., 150%). We used this method for two reasons. First, it gives a measure of what is likely much more important biologically, i.e., the position of the stigma relative to the staminal column. A style longer than the staminal column can much more easily and effectively receive pollen off the body of a visiting bee. Furthermore, and in a practical sense, using this nondestructive scoring method with living flowers allowed the relative style length (as an estimate of gender) to be used to preliminarily assign gender to flowers, and at the same time, to use the flowers in crossing experiments.

**Stigmata**—The overall shape (linear, globular, or strongly bilobed) was scored, the width measured, and stigmatic papillae scored as short, medium, or long. Estimates were also made of the amount of exudate on stigmata, from living flowers with a dissecting microscope, and in SEM studies.

**Ovule Quantity and Size**—Ovules can be very difficult to count. There is no known ovule-specific stain, so one must simply carefully dissect ovaries and count ovules under fairly high magnification. This was done primarily with the aid of a dissecting microscope, although sometimes counts were verified under (very) low power (60 $\times$ ) with a compound microscope. It is possible to count ovules because they stand out from ovary tissue in shape and texture; the drawback is that they are fairly delicate, and undoubtedly, in the process of dissection some are destroyed. Ovaries were dissected either without any stain, or with a light stain of Toluidine Blue dissolved in glycerin. The stain is useful because the ovary tissues take it up more quickly than the ovules do, so the ovules stand out. The glycerin is critical in that it prevents the delicate tissues from drying up under the long dissection required to separate and count the ovules. Ovules are elliptic; thus, both length and width were assessed at 100 $\times$  with a compound microscope. “Length” measures the longer of the two dimensions, and not necessarily the funiculus-micropyle dimension.

**Pollen Ovule (p/o ratio)**—The pollen ovule number cannot be calculated in any meaningful way for dioecious species without knowing population characteristics including the number of inflorescences, the number of flowers open/inflorescence, and the number of each sex form in a population at a given time. Thus, pollen ovule ratios per se are not considered.

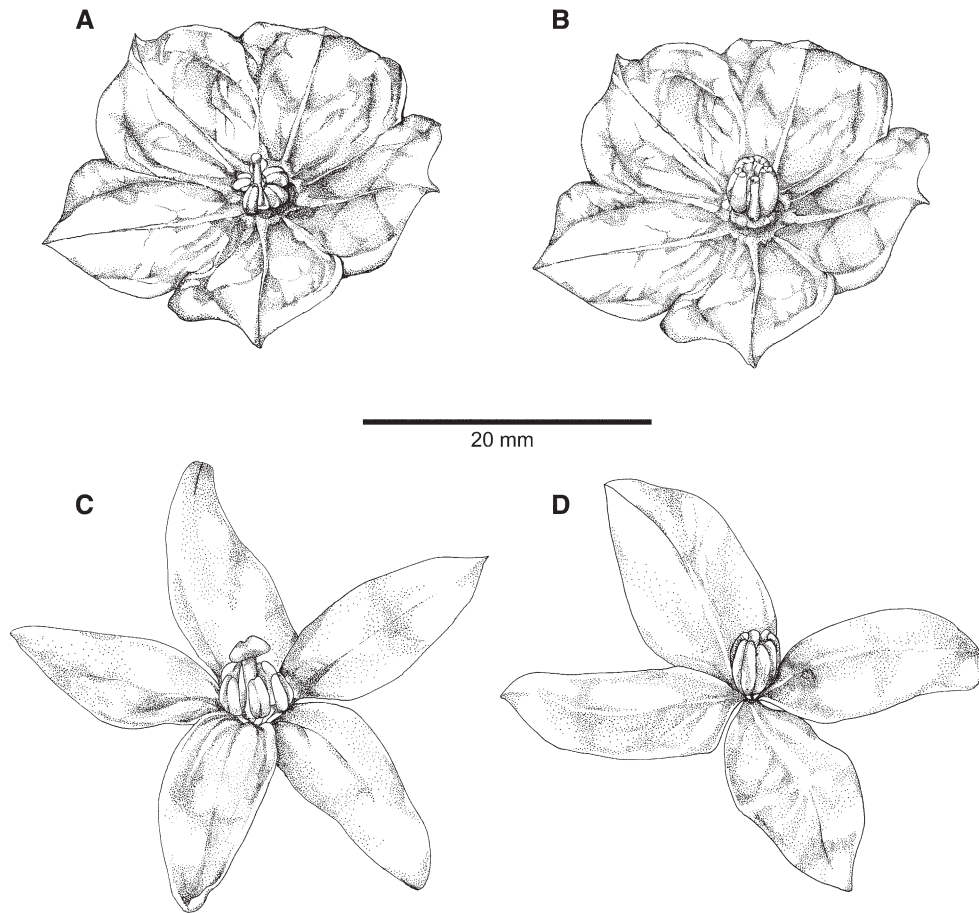


Fig. 1. Drawings of flowers, shown at same scale: (A) *Solanum conocarpum*—pistillate flower. (B) *Solanum conocarpum*—staminate flower (one stamen removed to show pistil). (C) *Solanum polygamum*—pistillate flower. (D) *Solanum polygamum*—staminate flower (pistil not shown, but highly reduced within flower).

**Pollination Observations**—No pollinators were seen on flowers of either species on St. John at any time over dozens of hours in the field. It is very likely the flowers are bee pollinated, like most solanums (e.g., Buchmann, 1986; Anderson and Symon, 1989; Connolly and Anderson, 2003), but the pollinator guild(s) and behavior remain to be studied.

**Crosses**—Crosses with *Solanum* flowers are relatively easy to make (Anderson and Levine, 1982), simply by holding anthers in a forceps, terminal pores facing a glass slide, and gently tapping the forceps on the edge of a microscope slide; the pollen is shaken out of the terminal pores onto the glass slide. Then, the actual application of pollen to the receptor flower is easily done by gently sliding the slide over the stigmatic surface through the mass of semi-sticky pollen visible on the slide. This method also allows one to judge successful transmission of pollen by observing the lines in the pollen mass on the slide that are created when the stigmata are passed through (and, of course, by verifying the pollen on the stigmata themselves). A slide (= a male parent) can be used to hold the pollen for several crosses.

Crosses were not made in all the combinations. Crosses, all with greenhouse plants, were made between short-styled flowers (= males) of *S. conocarpum*. Crosses among the staminate flowers of *S. polygamum* generally cannot be made because either the pistils are completely absent in male flowers, or they are so highly reduced that pollen cannot be transferred (however, exceptional flowers are produced rarely—see Discussion). Furthermore, crosses cannot be made between long-styled flowers (= females) of either species, because the anthers are devoid of pollen. Thus, the crosses done were all made in the greenhouses between nominal ‘males’ (short-styled flowers; staminate) as pollen donors and ‘females’ (long-styled flowers; pistillate) as pollen receptors.

## RESULTS

**Sex Ratios**—We scored 29 *S. conocarpum* plants for gender from the single large population on St John. Eight plants had no flowers or fruit and could not be scored. The remaining 21 were flowering or fruiting, and were scored as follows: 8 male: 13 female ( $\chi^2 = 1.19$ ,  $P = 0.275$ ). The greenhouse sex ratio was also not significantly different from 1:1 (29 male: 21 female;  $\chi^2 = 1.28$ ,  $P = 0.258$ ).

For *S. polygamum*, we scored gender at three field sites. The combined observations gave 23 no flowers: 19 males and 25 females (for male vs. female, the  $\chi^2 = 0.818$ ,  $P = 0.3657$ ). All of the greenhouse *S. polygamum* plants flowered. The ratio of male to female was 18:18, obviously a 1:1 ratio.

**Sex Ratio and Flowering Time**—The time (from seed germination) to first flowering was notable as well. For *S. conocarpum*, the females came to reproductive maturity much slower than the males. We tracked plants from seed germination to first flowering, and recorded the month the first plants flowered as ‘#1’, and the months following in succession out to 36 mo after first flowering. Most *S. conocarpum* plants (42) had flowered after 16 mo. The eight plants that had not flowered after 16 mo came into flower between 24 and 36 mo. Half of those very late flowering plants were male and half female—not surprising

given the sex ratio data reported above. However, that 1:1 ratio contrasts sharply with the pattern of the majority of plants tracked in the first 16 mo of flowering. As shown in Fig. 2, male plants came into flower much sooner than females. The difference between the pattern for male and female first flowering was analyzed with a log-rank test on survival curves using the Kaplan-Meier method on uncensored data (Proc LIFETEST SAS 9.1). The trajectories for time to first flowering were significantly different for males and females ( $\chi^2 = 7.84$ ,  $P = 0.005$ ).

The time to first flowering of males vs. females for *S. polygamum* was exactly the same, so no statistical test was performed.

**Morphological Characters (Table 2)—Inflorescence Size—**Over 100 inflorescences of *S. conocarpum* were counted on 36 different plants. Male inflorescences bore significantly more flowers than the female. The differences for *S. polygamum* were not tested for significance, because female inflorescences consist of only a single flower, as compared with nearly 15 flowers per male inflorescence.

**Corolla Diameter—**In both the field and greenhouse samples of *S. conocarpum*, the corollas are five-parted, and the male flowers are larger than females, but not significantly. Thus, flower gender cannot be predicted by humans, or presumably pollinators, based on flower morphology or size (see Fig. 1). Interestingly, the flowers of both sexes were on average about 13% larger in the greenhouse than the field, perhaps attributable to the better growing conditions.

For *S. polygamum*, there are significant differences; the gender of the flowers is clearly different. First, in addition to the number of flowers/inflorescence cited above, the number of corolla lobes is clearly different. Generally, staminate flowers are 4-lobed and pistillate flowers 5-lobed. Pistillate flowers are larger, but not significantly. However, they appear larger because of number of corolla lobes and thus attractive tissue (see Fig. 1). It would seem likely that pollinators could easily distinguish the staminate and pistillate flowers.

**Androecium—Staminal Column—**Table 2 shows that there are a number of significant differences in the staminal column for both species, including stamen and anther lengths.

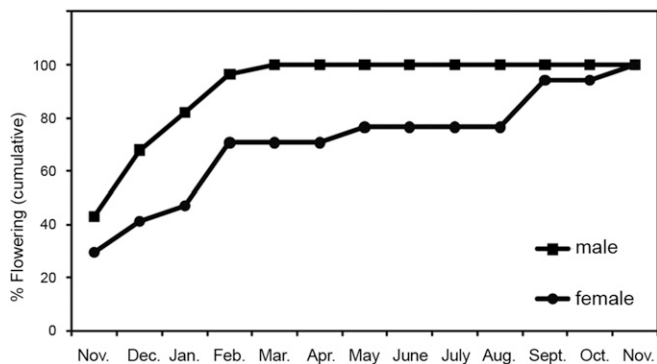


Fig. 2. Time of first flowering of *S. conocarpum* plants grown from seeds. The y-axis is the cumulative percentage of plants that had flowered by the month indicated (see Methods for further explanation). ●, —female or pistillate plants. ■, —male or staminate plants.

Perhaps most important for macromorphological gender characterization is that the anthers of male and female *S. conocarpum* have different shapes. The smaller anthers on the female show a slight inward arc (vs. straight-sided in male flowers), and a smaller girth (see Fig. 1). The easiest, and most certain way to assess gender is by using this feature of stamen morphology.

**Pollen Quantity—**Pollen is present and abundant in the flowers of male flowers of both species. Generally, there is no pollen found in any of the female flowers. That generalization applies to virtually all pistillate flowers of both species, however, it is not absolutely true. In a few female flowers of each species, we found a few good pollen grains. Some of the grains were collapsed, but most were ‘good’ (=stainable, like the pollen from the male flowers, which translates to viable) and tricolporate. The few female flowers that did have grains contained from 20–500 grains/flower, with a median number/flower of about 100 in *S. conocarpum*. Attempts to remove the grains and use them in crosses proved fruitless.

The number of pollen grains in the staminate flowers of *S. conocarpum* is very high (~300 000 grains on average). Staminate flowers of *S. polygamum* include much less pollen (~110 000). These numbers are comparable to those reported for the American (Anderson, 1979) and Australian (Anderson and Symon, 1989) dioecious males.

**Pollen Morphology—**SEM analysis of the pollen from male flowers of both *S. conocarpum* and *S. polygamum* indicates that it is typical of the genus in being tricolporate (Anderson and Gensel, 1976) (Fig. 3). In contrast with all of the other dioecious species of *Solanum* studied in detail except possibly *S. crotonoides* (Knapp et al., 1998), the female flowers do not bear inaperturate pollen (Anderson and Symon, 1989; Knapp et al., 1998). Instead, as noted above, we found the flowers are largely devoid of pollen, and Knapp et al. (1998) reported no pollen in anatomical sections of female flowers of *S. polygamum*. We found the few pollen grains in the few pistillate flowers that bore them in most instances to be similar to the tricolporate grains of the male flowers, although sometimes the grains are collapsed and abnormally shaped.

**Pollen size—**For both *S. conocarpum* and *S. polygamum*, several greenhouse plants were sampled for pollen grain size (from male flowers only). On average, for both species, the grains were about 25  $\mu\text{m}$  in diameter, a size also typical for *Solanum* (Anderson and Gensel, 1976).

**Pollen Fertility—**Pollen stainability of both *S. conocarpum* and *S. polygamum* varied somewhat with the age of the plants. The first round of flowering on plants grown from seed (i.e., the first time the plants reached sexual reproduction) showed somewhat lower stainability (median around 75%: 40 flowers from 20 male plants) in comparison with subsequent bouts of flowering where almost all grains were stained ( $\bar{x} = 97\%$ , s.d. = 0.02; from 49 flowers on 10 male plants). The few tricolporate grains in the few female individuals (sample of 11 flowers) with pollen, had a stainability similar to the males.

**Gynoecium—Style length and Stigmata—*Solanum conocarpum*—**The styles of pistillate plants are nearly twice the

TABLE 2. Macro- and micromorphological characteristics of staminate and pistillate flowers of *Solanum conocarpum* and *S. polygamum*.

Features	<i>Solanum conocarpum</i> Male/staminate	<i>Solanum conocarpum</i> Female/pistillate	<i>Solanum polygamum</i> Male/staminate	<i>Solanum polygamum</i> Female/pistillate
# flowers / inflorescence	7.8 ( <i>n</i> = 56/20) 3.93 <sup>***a</sup> GH	3.7 ( <i>n</i> = 47/16) GH	14.8 ( <i>n</i> = 14/14) GH	1.0 GH+ F
# corolla lobes—GH	5	5	4.3 ( <i>n</i> = 42/15) -4.5 <sup>***</sup>	5.2 ( <i>n</i> = 26/12)
Corolla diameter (mm) —F	25.3 ( <i>n</i> = 29/15) -0.83 <sup>NS</sup>	24.1 ( <i>n</i> = 12/6)	20 ( <i>n</i> = 19/6) -1.06 <sup>NS</sup>	20.9 ( <i>n</i> = 9/5)
Corolla diameter (mm) —GH	28 ( <i>n</i> = 125/27) 0.82 <sup>NS</sup>	27 ( <i>n</i> = 105/22)	33 ( <i>n</i> = 14/14) -0.84 <sup>NS</sup>	35 ( <i>n</i> = 10/10)
Staminal column—diameter—F	3.3 ( <i>n</i> = 29/15) 0.81 <sup>NS</sup>	3.5 ( <i>n</i> = 12/6)	3.8 ( <i>n</i> = 19/6) 2.09 <sup>NS</sup>	3.4 ( <i>n</i> = 9/5)
Stamen length—F	4.4 ( <i>n</i> = 29/15) -7.24 <sup>***</sup>	3.3 ( <i>n</i> = 12/6)	3.8 ( <i>n</i> = 19/6) 12.52 <sup>***</sup>	2.3 ( <i>n</i> = 9/5)
# anthers	5 GH + F	5 GH + F	4.4 ( <i>n</i> = 47/15) -4.08 <sup>***</sup> GH	5.2 ( <i>n</i> = 26/12) GH
Anther Length—F	3.5 ( <i>n</i> = 29/15) -8.09 <sup>***</sup>	2.5 ( <i>n</i> = 12/6)	2.9 ( <i>n</i> = 19/6) 14.2 <sup>***</sup>	1.9 ( <i>n</i> = 9/5)
Pollen stainability—GH	97% ( <i>n</i> = 49/10)	No pollen; but see text	88% ( <i>N</i> = 12)	No pollen; but see text
# Pollen grains/flower—GH	295 000 ( <i>n</i> = 18/7)	0 (but see text)	109 300 ( <i>n</i> = 10/10)	0 (but see text)
Style length—F	2.9 ( <i>n</i> = 29/15) 7.21 <sup>***</sup>	4.7 ( <i>n</i> = 12/6)	Strongly reduced pistil	3.6 ( <i>n</i> = 9/5)
Style length proportional <sup>b</sup> —GH	53% ( <i>n</i> = 155) 27.26 <sup>***</sup>	170% ( <i>n</i> = 155)	Strongly reduced pistil	No data
Style past or (less than) staminal column—F	(1.0) ( <i>n</i> = 29/15)	2.5 ( <i>n</i> = 12/6)	Strongly reduced pistil	2.1 ( <i>n</i> = 9/5)
Stigma width—F	0.5 ( <i>n</i> = 30/15) 1.66 <sup>NS</sup>	0.6 ( <i>n</i> = 12/6)	Strongly reduced pistil	2.0 ( <i>n</i> = 9/5)
Ovary diameter—F	0.95 ( <i>n</i> = 29/15) 4.37 <sup>***</sup>	1.3 ( <i>n</i> = 12/6)	Strongly reduced pistil	2.4 ( <i>n</i> = 9/5)
Ovary length—F	1.3 ( <i>n</i> = 29/15) 3.31 <sup>***</sup>	1.8 ( <i>n</i> = 12/6)	Strongly reduced pistil	1.3 ( <i>n</i> = 9/5)
Ovules/flower—GH <sup>c</sup>	Range = 0 - 135 $\bar{x}$ = 78 ( <i>n</i> = 17/15) -2.96 <sup>**</sup>	116 ( <i>n</i> = 11/8)	Strongly reduced pistil	74 ( <i>n</i> = 7/4)
Ovule size $\mu\text{m}$ (L x W) —GH	144 × 113 ( <i>n</i> = 39/12) -4.51 × -3.07 <sup>***</sup>	185 × 140 ( <i>n</i> = 33/8)	Strongly reduced pistil	230 × 155 ( <i>n</i> = 24/4)
# Seeds/fruit—F	—	50 ( <i>n</i> = 9)	—	47 ( <i>n</i> = 19)
# Seeds/fruit—GH	—	38 ( <i>n</i> = 13)	—	82 ( <i>n</i> = 16)

All measurements are means and in millimeters unless otherwise noted. *n* = Sample size with the number of measurements as a numerator, and number of individual plants as the denominator (where available). F = Fluid-stored field-collected material or counts. GH = Greenhouse-grown fresh material.

<sup>a</sup>All statistical tests were *t* tests (two-sample assuming unequal variances) comparing staminate and pistillate flowers within the same species. No tests were performed if the values were essentially identical, or if the two floral forms were obviously different. The numbers given are the *t*-values, with superscript symbols also indicating significance levels: \*\*\**p* ≤ 0.005; \*\**p* ≤ 0.01; \**p* < 0.05; NS = no significant difference.

<sup>b</sup>Style length as a percentage of the height of the staminal column in an intact flower. Thus, percentages ≤99% are for styles shorter than the staminal column, and those ≥100% are for styles that exceed the staminal column; see Methods for detailed explanation. The two *t*-values are for L × W comparisons, respectively.

<sup>c</sup>The variance in ovule number is high for *S. conocarpum* male flowers; the smallest ovaries seem to show arrested development, with ovules uncountable and unmeasurable in 5 of 17 samples.

length of the staminate (Table 2). This conclusion is confirmed by the two different, and independent measurements of length—the absolute measurements of field-collected samples, and then length estimates based on comparisons with staminal length (these expressed as percentages—as explained in Methods above). The sizes of the stigmata of male and female plants of *S. conocarpum* from preserved field samples were not significantly different from each other (Table 2). However, results from an unpublished study by Patel (2012), using light microscopy and SEM, showed that it is possible to identify stigmatic differences. These are shown in Fig. 4. The most extreme male flowers bear stigmata that are more or less linear with the style, punctiform, while the most extreme female flowers have styles topped by stigmata that are somewhat capitate and nascently bilobed.

*Solanum polygamum*—We only made field comparisons, which were easy in that virtually all staminate flowers possessed

pistils that were, at best, vestigial. The stigmata on pistillate flowers are huge, averaging 2 mm in the greatest dimension (Table 2), and are generally bilobed.

*Ovary Size*—Both the diameter and length of *S. conocarpum* ovaries were significantly different between males and females (Table 2).

*Ovule quantity and Size*—A number of staminate and pistillate plants of *S. conocarpum* from the greenhouse populations were assayed for ovule quantity. Overall, male flowers bear significantly fewer and smaller ovules than females (Table 2), but, in this case, the average values do not portray the complete story. The ovary in male flowers is always present, though in many it is highly reduced. In the smallest ovaries (5 of the 17 dissected), the ovules are ‘nascent’, smaller, and cubical, and not the usual ovulate shape that mature ovules assume. These

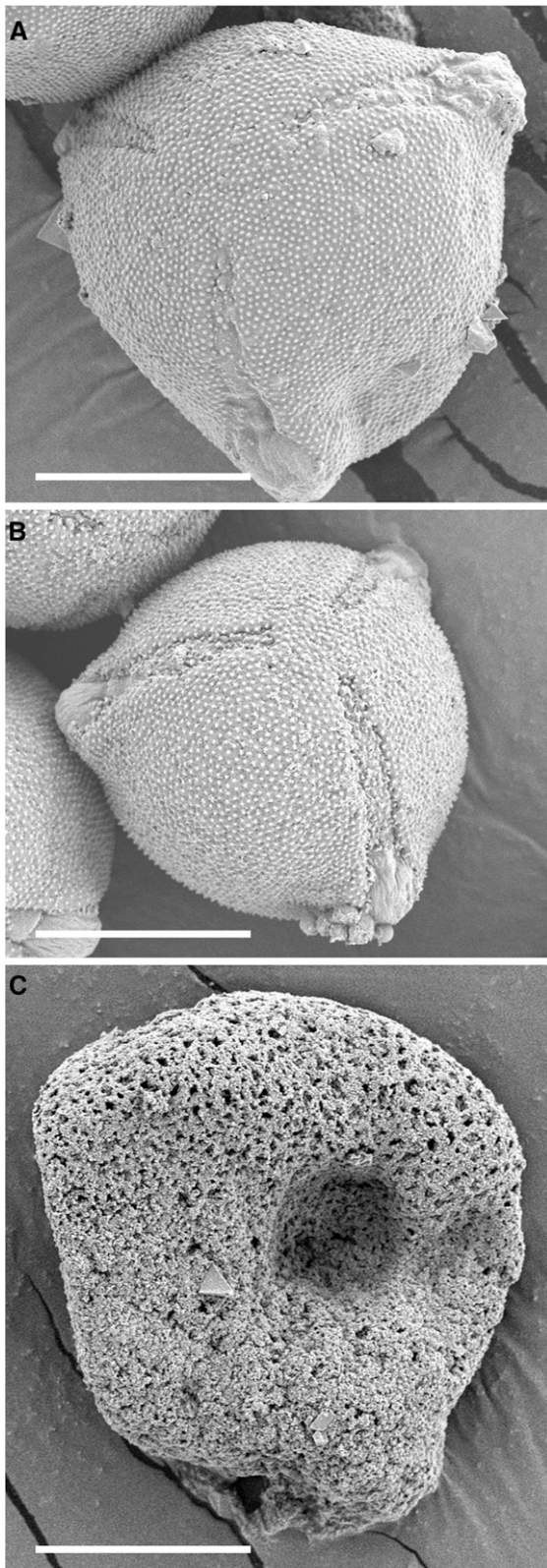


Fig. 3. Scanning electron micrographs of pollen, shown at same scale (scale bar = 10  $\mu$ m). (A) *Solanum polygamum*—staminate flower. (B) *Solanum conocarpum*—staminate flower. (C) *Solanum conocarpum*—pistillate flower.

nascent ovules are also much more delicate on dissection, seemingly not fully formed.

There is also an interesting, and strong positive correlation between the size of the ovary (length or height in this case) and length of the style (an  $r^2$  value of 0.87 for 14 pistils sampled from greenhouse-grown plants). Thus, the pistils that demonstrate the strongest male response in terms of style length (i.e., shortest) also have the smallest (shortest) ovaries. And, it is only in these flowers that the diminutive ovules described above are found.

A few ovule counts and measurements were made for the pistillate flowers of *S. polygamum* as well. The males were not assayed because the ovaries in staminate flowers are virtually always reduced to the point to make such an analysis untenable, and for our purposes here, uninformative.

**Seed Quantity**—The number of seeds for both *S. conocarpum* and *S. polygamum* was counted from field-collected plants and from greenhouse fruits (the result of hand pollinations). There were differences between the numbers for each species, but for both species in each setting the range for the number of seeds was wide. Thus, the meaning of these numbers is not clear, except to indicate that greenhouse cultured plants are healthy and that hand crosses approximate natural seed set.

**Intercompatibility—*Solanum conocarpum***—Almost 60% (57%) of the 155 crosses made were successful (= produced fruits with good seeds). The raw data from the crosses were sorted in various ways to analyze patterns. The style length (here we used the proportional measure of style length, e.g., the style length as a percentage of the staminal height) of flowers used as pollen receptors (females) was more than three times as long as those used as pollen donors (males) (Table 3). It is perhaps not surprising that the styles of plants used as males in successful crosses were shorter ( $\bar{x}$  male style length = 41 units; and significantly so;  $t = -4.773$ ;  $P = 0.000$ ) than those involved in crosses that failed ( $\bar{x} = 69$  units). The style lengths of the females in successful crosses were longer ( $\bar{x} = 172$  vs. 167 units in the failures), but not significantly ( $t = 0.887$ ;  $P = 0.376$ ).

All 31 crosses involving pollen from male flowers applied to stigmata of male flowers from other plants (i.e., the equivalent of an 'outcross') were unsuccessful. Self-crosses (pollen from anther to stigma within the same flower) in greenhouses were also unsuccessful. This conclusion is based as well on examination of stigmata of a number of male flowers that had some pollen deposited autonomously (presumably via normal greenhouse operations agitating the flowers during watering, handling in other experiments, etc., from the anthers to the sometimes adjacent stigma). In addition, in spite of the autonomous self-pollination taking place, there were no fruits set on any male plants. This may mean that this species is self-incompatible (SI), but it is equally or more likely to mean that the pistils of male flowers are nonfunctional. This lack of functionality may be due to stigmatic differences (e.g., a lack of sufficient stigmatic exudate perhaps) rather than genetic-based self-incompatibility. The latter was proposed as a possible explanation for the similar failure of male  $\times$  male crosses in the dioecious *S. appendiculatum* (Anderson, 1979).

***Solanum polygamum***—Only a few crosses (i.e., the legitimate crosses involving pollen from staminate flowers applied to pistils of pistillate flowers) were done because the success rate of such crosses was so high (80–90% of the more than



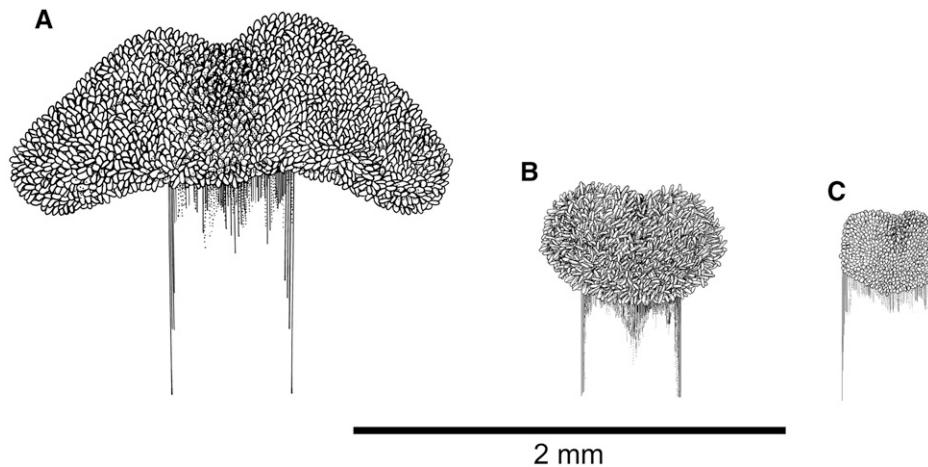


Fig. 4. Drawings of stigmata. (A) *Solanum polygamum*—pistillate flower. (B) *Solanum conocarpum*—pistillate flower. (C) *Solanum conocarpum*—staminate flower.

30 crosses done yielded fruits with viable seeds), and because there is no question related to gender expression indicated by morphology in this species.

As noted in the Methods, crosses involving pollen from male flowers applied to stigmata of other male flowers are mostly impossible to do because pistils in the vast majority of male flowers are tiny or essentially absent. However, in the few crosses we were able to do, all were unsuccessful. Presumably, self-crosses of male flowers are also unsuccessful because stigmata are generally not normally functional, perhaps for the same reasons as suggested above for *S. conocarpum*. That is, odd males bear pistils that have styles that raise the stigmata close to the anther pores at the top of the staminal column. In such instances, the stigmata of male flowers regularly had pollen on them (as a result of movement of the plants in greenhouse ventilation, and/or watering, etc.). No spontaneous fruits were set on such flowers—with one exception. One fruit set spontaneously on one plant once, a plant that otherwise bore clearly male flowers, i.e., flowers with no or diminutive pistils. This fruit had a few seeds, which have been germinated and are part of ongoing study (Anderson, unpubl. data) of the control of sexual expression and selection for hermaphroditism.

## DISCUSSION

In concordance with expectations from studies of other solanums (e.g., Anderson and Symon, 1989), both sexes bear

morphologically hermaphroditic flowers that are functionally, and thus cryptically, dioecious. For both species studied, as for all other dioecious solanums, the short-styled flowers functioned as males, and long-styled flowers as females. Fruits were set with the latter, and not with the former, and in contrast with all the other dioecious solanums studied in detail to date, pollen was available for pollinators only from the male flowers. Other dioecious solanums studied in detail (but not all recognized dioecious solanums—see Table 1) include inaperturate pollen in the stamens of female flowers. Thus, the sexual role of flowers characterized by the length of the style is further confirmed for *Solanum* as a universally effective means of distinguishing functional gender. Within the strongly dioecious *S. polygamum*, style length differences are obvious. The pistils in male flowers are tiny, often so reduced that it is very hard to see them with the naked eye or hand lens, vs. those in female flowers where the pistils are very large, with not only robust styles, but also notable ovaries and bilobed stigmata (see Fig. 4). In the (morphologically) weakly dioecious *S. conocarpum*, the situation is different: staminate flowers generally produce visible styles that are much shorter than the staminal column, and pistillate flowers that bear styles that are longer (often much longer) than the staminal column. But there can be considerable variation in style length among the flowers on an individual of either gender (see more below). In terms of male primary sexual characteristics, both genders of both species bear stamens, but the anthers are larger in the male flowers, and, as pointed out in the Methods section (and shown in Fig. 1), the anther shape (arched inward,

TABLE 3. Style length and success of hand pollinations with pollen from male flowers applied to stigmata of female flowers for *Solanum conocarpum*.

	Gender of flower	
	Male	Female
Style length all flowers ( $n = 155$ )	53 <sup>a</sup> (s.d.=37.9)	170 <sup>a</sup> (s.d.=37.7)
Style length of flowers in successful crosses ( $n = 88$ )	41 (s.d.=33.8)	172 (s.d.=35.8)
Style length of flowers in crosses that failed ( $n = 67$ )	69 (s.d.=37.4)	167 (s.d.=40.1)
$t$ -test value (two-sample assuming unequal variances) comparing successful and failed crosses within each sex	0.00***	0.38 <sup>NS</sup>

155 crosses attempted; 57% successful.

\*\*\*  $p = 0.005$ .

<sup>a</sup>Style length is a percentage of the staminal column, values are means; see Methods for further explanation.

nearly connivent in female flowers) in the morphologically-gender-ambiguous *S. conocarpum* serves as an unequivocal macromorphological character to signal functional gender of flowers—even when the style length character is dubious. The presence of abundant pollen in the anthers of male flowers, and not in female flowers, in both species is also a certain guide to sexual function—but the presence of pollen, of course, is not an easily observed field character.

Regardless of the style length variation, the crossing data offer indisputable evidence that plants with short styles, and pollen-filled anthers, are males; i.e., staminate-flowered plants, and those with long styles, and empty, inwardly arched, anthers, are females—i.e., pistillate-flowered plants. And, regardless of the notable variation in style length among flowers on individual plants, all those on “male” plants are male, and all those on “female” plants are female. This is what generally is expected for dioecious species, although it is much tempered by the ambiguity of functional or cryptic dioecy in solanums where female flowers bear anthers, and male flowers pistils (Anderson and Symon, 1989).

The variation in style length of flowers of both sexes, in particular in *S. conocarpum*, suggests interesting scenarios. For instance, the styles on individual male plants vary from 0–100% the length of the staminal column. Of particular interest is that this style length variation is also associated with crossing success. That is, there is a significant difference in siring success: male flowers with shorter styles are much more likely to sire fruits than males with longer styles. The crossing data (Table 3), including detailed analysis of the 10 individuals used as sires in multiple (i.e., more than five) crosses, shows that this phenomenon is at the level of the individual flower; there is no clear pattern supporting the conclusion that some plants are more effective males than others. That is, male flowers with shorter styles are significantly ‘better’ males than those flowers—still from the same staminate flowered plant—with longer styles. ‘Better’ means greater success siring fruit. A regression analysis of male flowers comparing the percentage of successful fruit set with style length supports this conclusion as well ( $r^2=0.54$ ;  $F=12.69$ ;  $P=0.004$ ). Interestingly, there seems to be a threshold: male flowers with styles  $>80\%$  the length of the staminal column were more or less associated with failure to yield fruits in crosses (Fig. 5). These unusual results lead to a fundamental question: what is it about male flowers with shorter styles that often yields more successful crosses? In a small sample, we examined the pollen quantity and quality (as assayed by Aniline Blue stainability—a measure of viability). In 49 counts of pollen stainability from flowers of 10 plants, we could find no pattern of association: shorter-styled flowers did not bear a higher percentage of stained pollen. A smaller sample (16 flowers/5 plants) of pollen quantity also failed to show any association: shorter-styled flowers do not bear more pollen. Thus, we are left with no obvious explanation for the differential performance of pollen from shorter vs. longer-styled male flowers from the same plant. Perhaps this variation in sexual performance among flowers—even from the same inflorescence—is not so surprising in that an analogous phenomenon characterizes gynoecea in andromonoecious plants (including other solanums, e.g., Anderson and Symon, 1989; Diggle, 1991; Anderson et al., 2014). In andromonoecious solanums, the base of an inflorescence bears hermaphroditic flower(s), and the distal end of an inflorescence, staminate flowers. The floral specializations in an andromonoecious inflorescence constitute differences in pistil function, not anther function, in adjacent flowers.

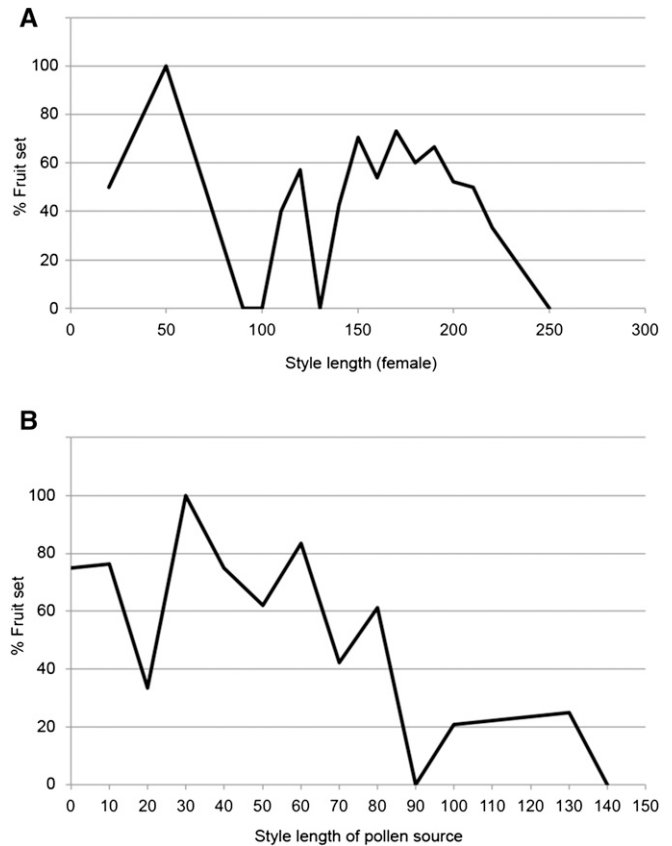


Fig. 5. Association of fruit set and style length, style length values on the y-axis are given as a percentage of the staminal column (see Methods for more explanation). (A) The proportion of pistillate flowers setting fruit at different style lengths. (B) The proportion of fruit set induced by pollen from staminate flowers with the style length shown.

But, this kind of variation establishes a precedent for differential performance from adjacent flowers that, in the case of *S. conocarpum*, applies to pollen function. These differences in sex expression of adjacent flowers are presumably based on developmental genetics and gene expression at the level of an individual flower. Perhaps a similar mechanism exists in *S. conocarpum* where the gene products that promote longer styles in male flowers have a pleiotropic affect on components of pollen performance.

Performance in male flowers seems to be correlated with morphology. Style length is strongly positively correlated with ovary size (shorter styles and smaller ovaries), and, furthermore, the only male flowers that had underdeveloped (‘nascent’) ovules were those with the very smallest ovaries and shortest styles—a case of arrested development perhaps. Thus, although the shorter-styled flowers were not ‘stronger’ males in terms of pollen quantity or viability, they were ‘weaker’ females—at least in terms of morphology, and, if they are like some other dioecious solanums, perhaps in terms of development as well (C. Martine, personal communication). These intriguing and unusual observations are part of some on-going studies (Anderson, unpubl. data).

On the female side, where there is significant variation in style length, there is not in flower performance. The difference between the styles of female flowers that produced fruit and those that did not was not significant. Furthermore, there are no

significant differences in the regression analysis of female style length on the percentage of fruiting success ( $F = 0.234$ ;  $P = 0.635$ ), and, as shown in Fig. 5, there is no obvious inflection point where styles are too long or too short to allow fruit set. Thus, styles on female flowers are equally functional whether they just exceed the staminal column, or are very long.

**Distribution, phylogeny and evolution of dioecy in *Solanum*—Biogeography**—There do not seem to be any obvious biogeographic patterns manifest in the distribution of *Solanum* dioecy. The majority of dioecious species occur in northern Australia, but are all part of one major clade. There are (at least) 10 dioecious species in this general region. The other 5–6 dioecious species are scattered in the New World, where the genus is most diverse (Raven and Axelrod, 1974). However, there is no geographic consistency beyond that. Three species occur in the plexus of diversity for *Solanum*, in Latin America (*S. appendiculatum*—Mexico/Guatemala, *S. confertiseriatum*—Ecuador, *S. leuculentum*—northern Andes), and 2–3 grow on islands in the Caribbean (*S. conocarpum*, *S. polygamum*, and the possibly dioecious *S. crotonoides*). In general, it may be that there is a higher proportion of dioecy on islands than continents (e.g., Anderson, 1984; Baker and Cox, 1984), but the dioecious members of *Solanum* do not add anything to strengthen that argument: there is no special association of the insular habitat and dioecy in the genus *Solanum*.

**Phylogeny**—Dioecy is found in 4 of the 13 major clades of *Solanum* recognized by Bohs (2005). The largest subgenus in *Solanum*, *Leptostemonum* (and the clade by the same name) includes all 10+ Australian species, as well as 1 of the 2 Caribbean species studied here (*S. polygamum*), although it is likely that the phylogenetically problematic *S. polygamum* had an independent origin within *Leptostemonum*. Two dioecious species occur in the Geminata clade (including *S. conocarpum* studied herein), and one each in the Dulcamaroid clade and the Potato clade; their phylogenetic relationship is not yet known. Clearly there is a phylogenetic concentration of dioecy in the *Leptostemonum* clade. This is also the clade with most of the other nonhermaphroditic forms reported for *Solanum*, most of which are andromonoecious (Symon, 1979b; Whalen, 1984; Anderson and Symon, 1989; Knapp et al., 1998; Levin et al., 2006). The overall syndrome of dioecy is similar in many respects among these species, except for the lack of pollen in female flowers in the two species studied herein and a few others, and in the strength of the expression of the dimorphism (see Table 1). The dioecious species that are not part of the *Leptostemonum* clade are not from any particular easily identified location or habitat. They also share little with each other (other than the standard features associated with dioecy in *Solanum*) in terms of morphology, habitat, or distribution. It seems this broad overall similarity in expression of dioecy covering nearly 20 species, and among a wide range of clades, might be taken as an example of underlying synapomorphies (e.g., Saether, 1979; see more discussion of this in the next section).

**Evolution of dioecy in *Solanum***—Detailed study of the two species considered herein brings us to the point where we know a good deal about the features of the dioecious system of most of the 15 certain dioecious solanums. There are three other species published that are very likely dioecious, but require a bit more study, and there are several likely dioecious species to be identified (e.g., Brennan et al., 2006; Knapp, 2010; Barrett,

2013; C. Martine personal communication), but what we have learned about these two additional species, one of the ‘most’ dioecious (*S. polygamum*) and one of the ‘least’ dioecious (*S. conocarpum*; although *S. appendiculatum* qualifies as the species with the least expression of obvious dioecious features in *Solanum*), does allow speculation on the nature of dioecy in this overwhelmingly hermaphroditic-flowered genus. Dioecy has arisen more than 100 times in the angiosperms (Charlesworth, 2002), and, as noted above, multiple times in a number of distantly related clades in the Solanaceae. The largest dioecious radiation in *Solanum* is in one clade, *Leptostemonum*, and in one place, northern Australia (Anderson and Symon, 1989; Martine et al., 2006; Martine, et al., 2009; Martine et al., 2011); that would seem to qualify as a radiation. Most of the other examples are of single examples of dioecy. In spite of this, there is an impressive singularity of many elements: all species are represented by sexes that bear morphologically hermaphroditic flowers with marked differences in style length belying actual, functional, and thus cryptic, gender. Although in most other dioecious angiosperms the female flowers are as might be expected, mostly anther-less (and thus also pollen-less), that is not the case in *Solanum*. For all dioecious solanums, the female flowers bear anthers, and the vast majority have anthers with pollen. The pollen in the female flowers is ‘functional’ as a pollinator reward (viable, filled with the same nutrients as the pollen in male flowers; Levine and Anderson, 1986), but it is possibly unique (in a dioecious species), and certainly unusual, in being inaperturate, and thus not capable of germination. Hence, the inaperturate pollen plays no role in sexual reproduction (other than possibly spatially displacing functional tricolporate pollen on stigmata). A minority of the species (20%), including both of those studied here, have no (or virtually no) pollen in the anthers of the female flowers. This poses a challenge for understanding the energetics of pollination. Solanums are nectarless (Anderson and Symon, 1985), the only reward to pollinators is the pollen that is ‘buzzed’ out of the anthers. And bees, the primary pollinators, recognize flowers that have been buzzed previously, and spend less time on them (Buchmann, 1986; Anderson and Anderson unpubl. data with greenhouse experiments including bumblebees and *S. vespertilio*). The argument made for the presence of the inaperturate pollen that cannot germinate in the females is that it provides a pollinator reward, while the lack of germinability facilitates the unimodal, i.e., female, sexual role for the pistillate flowers (Anderson and Symon, 1989). Pistillate flowers without the pollen reward still bear the pollinator-attracting anthers, but the bees might spend less time on such flowers that they identify as pollen-less, just as they do on flowers previously visited and buzzed (and thus, with smaller quantities of pollen). These attractive (anthers present) but reward-less (no pollen) flowers, would seem to constitute an example of pollinator deception. Field studies of how that pollen-absent condition impacts effective pollinator behavior remain to be done.

Thus, given the relatively uniform morphology of the dioeciousness in the species studied, and the independent phylogenetic lineages, it seems clear that this is a good example of an homoplasy, parallel evolution, a good case of, and for, underlying synapomorphies (e.g., Saether, 1979; Funk, 1982; Brooks, 1996; Wake, 1996)—where the same features of dioecy are expressed in independent, but related, lineages under the appropriate ecological/evolutionary forces. However, the cases of documented dioecious solanums (including the two reported herein) without pollen in pistillate flowers suggest that there are

variations in the expression of the genes associated with the manifestation of dioecy. Identifying these genes would be of considerable interest in the effort to understand the processes associated with underlying synapomorphies, and, more broadly, of the evolution of sexual systems in *Solanum* and in angiosperms in general. Hypotheses that combine evolutionary and ecological elements, not apparently associated with biogeography or phylogeny, seem to offer the best explanations for the occurrence of dioecy in the genus (e.g., as suggested in Anderson and Symon, 1989).

**Sex Ratios**—The pattern most often reported for dioecious species is a 1:1 sex ratio, although virtually all of the reports are for secondary sex ratios (i.e., from the field; Rottenberg, 1998). The primary (the sex ratio at first flowering, usually determined with cultivated plants) and secondary sex ratios (i.e., in natural settings, and plants of unknown ages) of *S. conocarpum* and *S. polygamum* are male biased, but statistically equivalent to 1:1. The only other study of dioecious *Solanum* with a large enough sample to make comparisons regarding sex ratio, is with *S. appendiculatum* (Anderson, 1979). In this Central American potato relative, the primary sex ratio (in greenhouse populations) is significantly female biased (2F:1M), but the secondary sex ratio (in the wild) is 1:1. There are not enough data to suggest any pattern for the genus, nor sufficient data from the studies of *S. conocarpum* or *S. polygamum* to suggest an unusual method of sex determination, as the primary sex ratio data for *S. appendiculatum* implies.

One ecologically interesting variation on sex ratio issues was manifest in the much longer time it took for female plants of *S. conocarpum* to flower for the first time (from the seedling stage) compared with the males. This pattern was not found for *S. polygamum*, and was not observed in *S. appendiculatum*, although it tends to be the pattern in many other species (Lloyd and Webb, 1977). Given that both species studied herein are relatively long-lived perennial plants, the time to initial flower production is of interest in terms of individual biology, if not for sex ratios per se. Long term monitoring of greenhouse plants has not indicated any difference in flowering rate, or much seasonality for these tropical species, once plants have first flowered. However, biologically, or perhaps physiologically, this extended presexual phase is of interest in that it may signal a higher threshold age (as a proxy for size?) that must be reached before female plants flower. This would presumably be related not so much to flowering itself, but to plants being of sufficient size to support subsequent fruit growth on female plants (i.e., fruit development is a plant cost, not a flower cost). Obviously, the cost of reproduction for male plants is simply the cost of the flowers, hence flowering, and the contribution of genes (via pollen flow) to the next generation can occur with a less energetic effect on plants.

**Compatibility and the evolution of dioecy**—The questions relating to the driving forces for the evolution of dioecy have been particularly interesting and vexing. The ever-increasing effectiveness of phylogenetic studies obviously facilitates assessing the distribution of compatibility in lineages and hypotheses of likely ancestral forms in dioecious lineages. With full dioecy, however, it is usually impossible to perform the direct experiments that test for compatibility. Thus, it is generally not possible to address directly whether the dioecy in a lineage might be best explained by genetic (for ensuring outcrossing) or

ecological factors (e.g., Bawa, 1980; Thomson and Barrett, 1981; Givnish, 1982; Anderson and Stebbins, 1984; Weller et al., 1990; Charlesworth, 1999; Barrett, 2010; Cheptou, 2012). In that context, the unusual cryptic/functional dioecy in *Solanum* manifests morphologically hermaphroditic flowers that in rare instances offer the chance to test the actual compatibility within a dioecious species. We carried out such studies with both *S. conocarpum* and *S. polygamum*. Given that neither species includes any extractable pollen in the anthers of female flowers, self crosses are performed only with male flowers. From the few female flowers of *S. conocarpum* we did attempt to dissect out the very few pollen grains they sometimes include, but we were unsuccessful. The pollen quantity is too small, the grains are too small to separate from the tapetal tissue, and as the SEM photos imply, perhaps those few pollen grains are also not functional. Thus, the experimental crosses necessarily must involve pollen from male flowers applied to stigmata of the usually very diminutive pistils within those flowers. For *S. conocarpum*, that is easier, because the pistils in male flowers are reduced, but often are still large enough to work with. Nonetheless, all the self-crosses we performed with males of *S. conocarpum* failed. Similarly, all the self-crosses with male flowers of *S. polygamum* failed. The crosses with male flowers of *S. polygamum* were even harder to perform (and likely failed) because the pistils are often so diminutive that they are probably nonfunctional. These results imply that perhaps both species are SI. This is discussed further in the section on “leakiness” below, but here we add a caveat. Given the reduction of the pistil in pistillate flowers of dioecious solanums, crosses testing for compatibility may fail for reasons other than presumed functioning of the incompatibility system (Gametophytic Self Incompatibility (GSI) in the Solanaceae; de Nettancourt, 1977). That is, with these two species, and with other dioecious and andromonoecious solanums (e.g., Anderson, 1979; Anderson and Symon, 1989; Anderson et al., 2014), self-pollinations may fail not because the plants are SI, but because the reduced pistils of staminate flowers have much smaller stigmata, and stigmata often lack exudate. Thus, even pollen carefully applied with the aid of a hand lens frequently does not stick to these small stigmata, and the few grains that might occasionally stay in place on stigmata will not be stimulated to germinate by virtue of the lack of exudate on the diminutive stigmata. Furthermore, as noted above, it is possible that the megagametophyte is not completely developed (Martine, personal communication).

However, an unanticipated event occurred with the strongly dioecious *S. polygamum*. A fruit was formed in 2011 with a few viable seeds (bearing about 50% of standard seed number) on one staminate-flowered plant (from the nearly 20+ male plants producing thousands of flowers over several years in the greenhouses). As noted elsewhere above, almost all pistils in male flowers of *S. polygamum* are so small they are hard to discern without magnification. Presumably, the staminate flower producing the fruit must have been one of the rare variants with a larger pistil (more following). Given that the research greenhouses are pollinator free, the fruit had to have been formed via autogamy or apomixis. Apomixis is unlikely given this single spontaneous fruit (on a male-flowered plant), and that there have been no spontaneous fruits on the pollen-less pistillate-flowering plants (where fruits are easily produced in experimental out-crossing studies). Furthermore, the plants grown from these seeds produced a small percentage of flowers with larger pistils, some pistils approximating the size of a typical

distillate flower: i.e., they are essentially hermaphroditic flowers, because they also bear anthers, and anthers with viable and typical tricolporate pollen. A high percentage of these anomalous functionally hermaphroditic flowers on otherwise staminate-flowered plants set fruits after hand self-pollination (the detailed data are part of an on-going study by G. Anderson and collaborators on the genetics and evolution of the sexual system in *Solanum*). That is, the flowers on the subsequent generations of plants (which were all male) are fully self-compatible (SC). Thus, the “most dioecious” *Solanum* described—*S. polygamum*—is SC. The SC would, in fact, be expected given that *S. polygamum* is in the large *Leptostemonum* clade of *Solanum*. Most of the species tested therein, and in fact, most solanums are SC; SI has been shown to be mostly resident in the potato group and its close relatives (Whalen and Anderson, 1981). Overall, the anomalous hermaphroditic flower production and the SC demonstrated suggest that the evolution of dioecy in this species is perhaps associated with promotion of genetic diversity. This has been suggested for other dioecious solanums as well (Anderson and Symon, 1989), but these are the first direct data to provide support.

As noted above, this serendipitous fruit set also opens the door for speculation on the genetic control of sex expression. The fact that all ~25 of the first two generations of offspring (the original one, and one derived from hand selfing the male flowers) from the anomalous fruit have been male suggests that there are no sex chromosomes, or that, if there are, the males are the homomorphic sex. Charlesworth (2002) indicates that “...the majority of plants studied with sex chromosomes have heterozygous males...” Following that logic, one might expect (although the sample size here is small) that some female-flowered plants would result (an expected 2XY: 1XX ratio). Furthermore, sex might be determined by the expression of female suppressor genes and/or many more options (Charlesworth, 2002). However, sex determination in plants is notoriously complex, ranging from sex chromosomes in a few species, to hormonal, or even pheromonal control (Charlesworth, 2002, Tanurdzic and Banks, 2004). Perhaps, however, the original fortuitous fruit set provides an opportunity to study this complex phenomenon. And, as noted, this fortuitous event has also launched an on-going multigenerational study of the back-selection from dioecy to hermaphroditism.

**Islands, compatibility, dioecy and leaky dioecy**—Self-compatibility and dioecy are opposites in terms of their biological effect, but both are often associated with islands (e.g., Baker, 1955; Baker and Cox, 1984; Carlquist, 1966), the former with enhancement of successful colonization, and the latter with promotion of diversity subsequent to establishment. Conjecture about their importance is limited by the ability to identify their predominance in insular floras: dioecy is easy to score, but SC presents a real challenge. Sometimes dioecy can be scored by inspection of herbarium specimens, so that gives a better first approximation of its extent. Although the percentages vary, a notable portion of several insular floras are dioecious (e.g., Baker and Cox, 1984; Sakai et al., 1995; Bernardello et al., 2001). Such scoring would include species like *S. polygamum*, where the sexual system is obvious from Fig. 1, but, simply scoring specimens would not—and in fact, it did not—include species like *S. conocarpum*. In the latter, dioecy can only be identified by detailed study, and often detailed study requiring experimental manipulations with living plants.

Inspection of herbarium specimens, and/or descriptions extracted from floras are often not sufficient.

Like cryptic dioecy, SC cannot be reliably scored from herbarium specimens or floras. Although abundant fruit set is a hint, it is only that. The only certain way to score SC requires intensive fieldwork, or subsequent growing of plants from seed collections, and hand pollination experiments. As a result, there are fewer data on SC for whole island floras. The relative paucity of data means less information to test principles such as the generalization about the strong link between SC and island floras. In its most popular version, this generalization is touted as “Baker’s Rule” (Baker, 1967) or “Law” (Baker, 1955), although Baker objected to the latter as too strong a statement. However, in spite of the significant questions raised about the general applicability of Baker’s Rule (e.g., Carlquist, 1966), the few comprehensive studies of island floras support Baker’s general principle, i.e., a significant portion of the natives/endemics are SC—although not necessarily autogamous (e.g., Anderson et al., 2013; Crawford et al., 2013; McMullen, 2009; Anderson et al., 2001, Bernardello et al., 2001). There is an important caveat even with these careful contemporary studies of the breeding systems of island plants: the *current* compatibility status of species on older archipelagos does not necessarily indicate that those species were SC or SI at the point of initial colonization. Nonetheless, studies of current compatibility are a good starting point.

Given the constraints on successful colonization of dioecious species or SI species for that matter, i.e., there must be at least two diaspores, there are legitimate questions regarding the frequency of successful island colonization by fully dioecious species. The solution is what we too often do not recognize in science, i.e., a less-than-perfect system. About 30 years ago, Baker and Cox coined the term, “leaky dioecism” and recognized its importance for island colonization (Baker and Cox, 1984). Many studies have followed (e.g., Humeau et al., 1999; Anderson et al., 2006b; Venkatasamy et al., 2007; Martine et al., 2014), and others have made similar convincing arguments about leaky self-incompatibility, in particular for facilitating island colonization (e.g., Reinartz and Les, 1994; Levin, 1996; Humeau et al., 1999; Mena-Ali and Stephenson, 2007; Crawford et al., 2009). The leakiness, a less-than-perfect system of self-incompatibility or dioecy, allows the colonizers to have it both ways: the first to arrive are able to reproduce sexually at a level sufficient for establishing populations, and subsequent generations benefit from the genetic diversity that dioecy or self-incompatibility promote. The leaky dioecy in solanums seems to support the growing consensus on the general pattern (e.g., Lloyd and Webb, 1977; Humeau et al., 1999; of Crane, 2013) where it is the male flowers that show the leakiness.

The two dioecious solanums discussed herein offer different perspectives on these scenarios. The expression of dioecy in *S. conocarpum* is morphologically highly cryptic, and phylogenetic comparisons imply that the close relatives (Knapp, 2008, 2014) of *S. conocarpum* are not dioecious. Results with male × male crosses described above suggest that *S. conocarpum* is SI. As discussed in the section above, however, failures in crosses to test compatibility in those rare and lucky instances where one can conduct such experiments with dioecious species are a bit less informative than successes are. The failures may also indicate a loss of function of the pistils, or of the stigmata and perhaps the loss of stigmatic exudate, via reduction, rather than the actual genetic features associated

with SI. The hypothesis that SC characterizes ancestral forms of *S. conocarpum* instead relies on standard phylogenetic analyses: i.e., derived from comparisons with species within Geminata and the clades related to it. This approach suggests that the progenitors of *S. conocarpum* would have borne hermaphroditic flowers that were SC, and the cryptic dioecy of this species likely would constitute a post-dispersal development. The other dioecious species in Geminata, *S. confertiseriatum*, taxonomically is placed quite distantly from *S. conocarpum* (and thus is presumably phylogenetically distinct as well) (Knapp, 2008), and manifests a different brand of dioecy (there is inaperturate pollen in the pistillate flowers; Knapp et al., 1998). Thus, we might speculate that the island colonists were SC and not dioecious.

The in situ development of dioecy implicated for *S. conocarpum* is generally considered less common, i.e., most insular dioecy is projected to be from dioecious colonists (e.g., Baker and Cox, 1984; Sakai et al., 1995). In contrast, *S. polygamum* is in the clade (Leptostemonum) with most of the dioecious species (which is still a small number) in the genus, but issues with sequence alignment have made its specific phylogenetic position difficult to determine (L. Bohs, personal communication, Weese and Bohs, 2007). Thus, there are yet no identified closest relatives to provide phylogenetic clues about its likely reproductive status as a colonist. However, given the strength of expression of dioecy in this species, and the existence of dioecy in the clade, it is at least more likely that the island colonists of this species were dioecious. And, as described above, we have direct evidence of SC for this species. The errant, and fortuitous, fruit set on a single *S. polygamum* male plant indicates unequivocally that this dioecious species is SC. Baker and Cox (1984) cite unpublished data by Baker that indicate he felt that "...in those cases that have been examined experimentally, hermaphroditic flowers in dioecious species are self-compatible" (p. 249). Thus, it is quite reasonable to hypothesize that dioecious *S. polygamum* was successful as an island colonist perhaps facilitated by leaky dioecy and SC.

## CONCLUSIONS

Finally, this study is another illustration of how combining a wide range of approaches, including systematics, phylogeny, ecology, and reproductive biology, is often needed to understand the basic biology of plants—even for something as apparently obvious as dioecy. These detailed experimental studies of what appeared to be an obvious system revealed unexpected insights into the nature of, and control of, sex expression. Multifaceted approaches may be particularly important for endemics and rare plants—descriptors that characterize many island species (e.g., Bateman et al., 2013). Such approaches, including especially the characterization of breeding and sexual systems, are necessary to establish effective conservation programs. The documentation of dioecy in the rare island endemic *S. conocarpum* will certainly dictate a new conservation plan. This species is even more rare now, in the sense that there are likely only 100–150 seed-producing (pistillate) plants extant (roughly one-half of the number previously estimated), and protection or restoration must ensure that both sexes are present and in sufficient quantities to effect good seed set. Dioecy in *Solanum* is uncommon, but also uncommonly interesting, given that all species are morphologically hermaphroditic. Consequently, the dioecy is best recognized as cryptic and functional. Dioecy is focused

in the *Leptostemonum* clade, but is not restricted to a single radiation within that clade, and in fact is spread across many clades in this highly speciose genus. In spite of this phylogenetic diversity, the dioecious species share most of the fundamental features of this sexual system, pointing to selection on a common genome. The only obvious biogeographic radiation is in northwestern Australia, and there is no particular linkage to island species, unless we consider the radiation in isolation in Australia as an island phenomenon. Leakiness in the dioecious expression for the island species studied herein facilitated support for two important hypotheses: first, that such leakiness facilitates island colonization, and second, that many dioecious progenitors were self-compatible. The former adds support to a growing consensus in island biology, that leaky systems in sexual systems (e.g., dimorphic flowers, in monoecy, dioecy, etc.) and in breeding systems (SI vs. SC) resolve the conundrum of colonization and subsequent radiation. The likely ancestral SC offers a confirmation, generally only inferred via cladistic analyses, that supports a genetic argument for the evolution of dioecy.

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