REPRODUCTIVE BIOLOGY OF THE DIOECIOUS CANARY ISLANDS ENDEMIC *WITHANIA ARISTATA* (SOLANACEAE)[1]

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We confirmed functional dioecy of *Withania aristata* via field and greenhouse studies. Male flowers are significantly larger. Female flowers bear stamens with no pollen; males bear 220 000 grains. Stigmata of male flowers senesce in buds. Anatomical observations confirm more ovules in females and an ovarian nectary in both sexes. We detected nectar in female flowers in the greenhouse but found no nectar in males. Thus, males offer pollen and females nectar. Females bear large numbers of fruits and, infrequently, male plants bear few significantly smaller fruits with few seeds. Outcrosses of females (self crosses impossible without pollen) yielded fruits in young buds, older buds, and open flowers. Self crosses of male flowers succeeded only with very young buds. Although functionally dioecious, this species manifests self-compatibility; however, no fruits are produced autonomously. Bee species (*Lassioglossum*, *Amegilla*, *Apis*) visit flowers and mature buds. Bud visits in which bees force petal tips apart, coupled with self-compatibility, may explain infrequent fruit on males. Thus, dioecy in *W. aristata* seems to have evolved from self-compatible ancestors, that leaky dioecy may have been favored during colonization, and, that despite autogamy and a low floral visition rate, this endemic enjoys a high rate of reproductive success.

Key words: bees; Canary Islands; dioecy; flower morphology; leaky dioecy; reproductive biology; self compatibility.

Studies of reproductive biology inform the nature of species, adaptation, speciation, hybridization, and systematics (Ornduff, 1969; Anderson et al., 2002; Neal and Anderson, 2005). The Solanaceae shows a wide adaptive radiation that includes all forms of zoophily, i.e., its species can be pollinated by birds (e.g., Cocucci, 1999; Kaczorowski et al., 2005), moths (e.g., Arroyo and Squeo, 1990; Vesprini and Galetto, 2000; Raguso et al., 2003), butterflies (e.g., Cocucci, 1995, 1999), bats (e.g., Voss et al., 1980; Helversen, 1993; Sazima et al., 2003), bees (e.g., Anderson and Symon, 1988; Sazima et al., 1993; Bohs, 2000), and flies (e.g., Galetto et al., 1998; Cocucci, 1999). Similarly diverse are the floral rewards, which include nectar (e.g., Galetto and Bernardello, 1993, 2003), pollen (e.g., Symon, 1979; Lester et al., 1999; Connolly and Anderson, 2003), scents (e.g., Sazima et al., 1993; Passarelli and Bruzzone, 2004), and oil (e.g., Simpson and Neff, 1981; Cocucci, 1991). Within Solaneae, the solanaceous tribe with the most genera and species (Hunziker, 2001), about 75% of the genera are bee-pollinated (Cocucci, 1999), and, as in the Solanaceae generally, most flowers are hermaphroditic. However, the majority of the reproductively unusual dioecious taxa occur in this tribe (with the exception of *Symonanthus*

from another subfamily: Anthocercidoideae; Haegi, 1981; Hunziker, 2001). The few reported cases of dioecious species are in the genera *Solanum* (e.g., Levine and Anderson, 1986; Anderson and Symon, 1989; Knapp et al., 1998), *Deprea* (e.g., Sawyer and Anderson, 2000), *Dunalia* (e.g., Hunziker, 2001), *Lycium* (e.g., Minne et al., 1994; Miller and Venable, 2002), and *Withania* (Hepper, 1991; Hunziker, 2001).

The Solanaceae are not common on islands (Wagner et al., 1990; Marticorena et al., 1998; McMullen, 1999); thus the biology, systematics, and natural history of the insular species are of particular interest. Herein, we address the reproductive biology for the single endemic species of *Withania* on the Canary Islands, *Withania aristata* (Aiton) Pauq., for which the reproductive biology turns out to be much more interesting than it initially appears (e.g., Bramwell and Bramwell, 2001).

Withania, a small genus of 10–18 species—depending on species and generic boundaries—ranges from the Canary Islands, the Mediterranean region and northern Africa to India, China, and Japan (Hepper, 1991; Hunziker, 2001). Morphological and molecular data have generally indicated a systematic position among the physaloid genera in subfamily Solanoideae (Axelius, 1996; Olmstead et al., 1999).

The flora of the Macaronesian Canary Islands includes three *Withania* species (Bramwell and Bramwell, 2001). Two of these—*W. frutescens* Pauquy and *W. somnifera* (L.) Dunal—are introduced continental species. In fact, *W. somnifera* is extremely wide-ranging, from the Canary Islands and Europe to India and Australia (Hepper, 1991). The lone native species (locally known as "orobal") is the endemic *W. aristata* (Fig. 1), a frequent soft-wooded shrub found at low elevations on all the Canary Islands (Bramwell and Bramwell, 2001). *Withania* species, especially *W. somnifera* used in ethnobotanical practices (e.g., Chevallier, 1996), has been studied intensively because of its medicinal properties. Thus, although the reproductive biology of *W. aristata* has not previously attracted attention, the fruits, leaves,
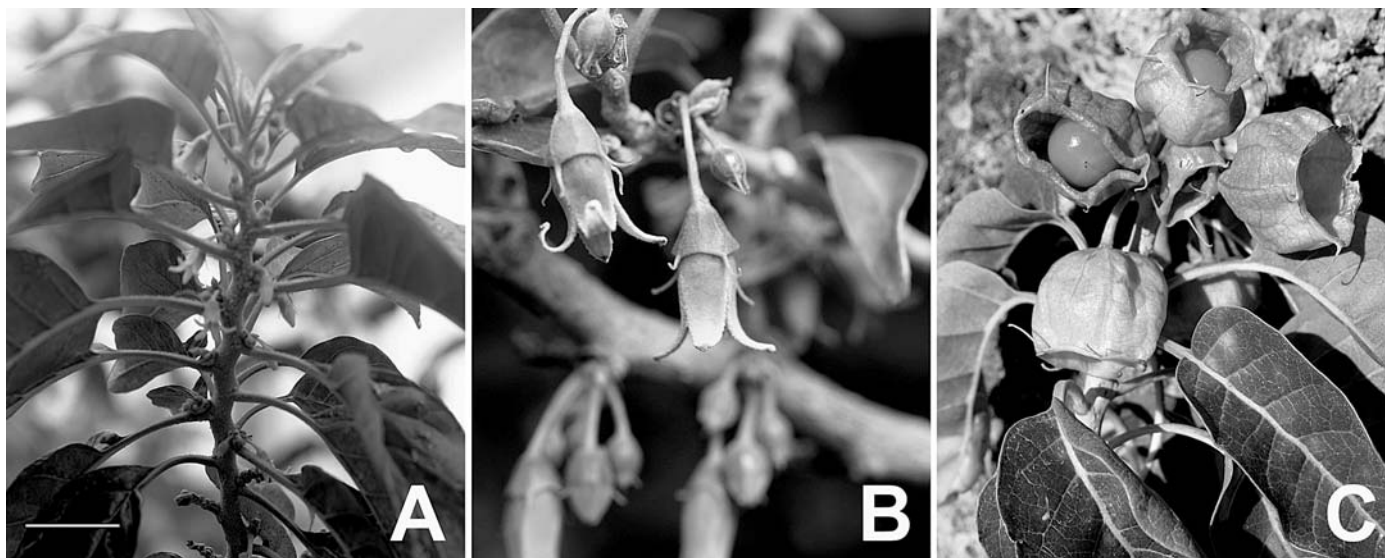
Fig. 1. Photographs of a *Withania aristata* female plant (population 5032). (A) Terminal branch. (B) Segment of branch with flowers. (C) Segment of branch with four fruits. Scale bars = 5 cm for A, 0.65 cm for B, 1.5 cm for C.

and bark are reported to have multiple uses in traditional medicine on the Canary Islands (Darias et al., 2001).

The reproductive systems in *Withania* are varied and at best incompletely understood, particularly in terms of functional field reproductive biology. This genus, like the family, is characterized by hermaphroditic flowers. However, as pointed out by Hunziker (2001), the Asian *W. coagulans* was the first member of the Solanaceae documented to be dioecious. Although the dioecy was not described in detail, it seems clear from the precise drawings of this species by Wight and Arnott (1850) and Hooker's (1852, p. 801) indication that it has "flores abortu dioeci," that some flowers bear functional stamens, and other plants have flowers with only shriveled, presumably nonfunctional stamens. Based on examination of herbarium collections, Hepper (1991) proposed *W. coagulans* as dioecious as well, with plants bearing either male flowers with short styles or female flowers with short, sterile anthers and long styles. *Withania adpressa*, from northwestern Africa, shows a similar dichotomy between male and female flowers, but Hepper (1991) was uncertain whether this species should be treated as monoecious or dioecious. The only article focusing on the reproductive biology of a *Withania* species (*W. somnifera*, Kaul et al., 2005) indicates that it has hermaphroditic flowers and is autogamous.

The reproductive biology of *W. aristata* presents a number of puzzling features. Flowers have been described as being either unisexual or hermaphrodite, with unclear differences in the fruit set of different individuals (Webb and Berthelot, 1845; Hepper, 1991; Bramwell and Bramwell, 2001; Hunziker, 2001). As part of a broader survey of the reproductive biology of many Canarian species (Anderson et al., 2005), our first studies of this endemic were confusing. Anthers seemed to dehisce and shrivel in many flowers, fruit set varied dramatically among plants, and the infrequent insect visits were by bees that only visited buds.

Thus, we were intrigued to turn our full attention to elements of the natural history of this notable and unusual island endemic. Here, via field and glasshouse analyses, we report detailed studies of the reproductive biology and mating system of this interesting taxon. We show that this solanaceous genus too, manifests some of the notable reproductive variation that characterizes an increasing number of carefully studied species in this family. Not only are the Solanaceae important economically (Heiser, 1987), but, surprisingly in many ways, they turn out to be remarkable in terms of reproductive biology as well, providing a number of reproductive variations that serve as examples of the benefit of detailed studies.

MATERIALS AND METHODS

Seven natural populations of *W. aristata* (Table 1) were studied in detail on Tenerife (Canary Islands, Spain) in January 2003, January and June 2004, and May 2005; vouchers have been deposited in the University of Connecticut G. S. Torrey Herbarium (CONN). For anatomical studies (accessions 5031, 5039, and 5061, Table 1), flowers were fixed in 70% ethanol, dehydrated in an ethanol-xylol series, and embedded in Paraplast (Oxford Labware, St. Louis, Missouri, USA). Serial cross and longitudinal sections were cut at 10 μm, mounted serially, and stained with safranin-fast green-hematoxylin and observed with a compound microscope.

Morphological observations and floral measurements were made using plants grown in pollinator-free greenhouses at the University of Connecticut, Ecology and Evolutionary Biology Conservatory (Storrs). This cultivated population consisted of six individuals (two male and four female) grown from seed (accession 4486, Table 1). Development of male and female flowers was tracked by measuring flowers at three stages (Fig. 2B, C): stage 1: buds ca. 3–4 days from opening, petals tightly closed, anthers undehisced, exposed portion of corolla about equal in length to the connate portion of the calyx; stage 2: buds ca. one day from opening, petals easily teased apart, anthers dehisced, exposed portion of corolla much longer than connate part of calyx; corolla tips are slightly parted, allowing particularly motivated floral visitors access (with mouth parts at least) to essentially closed buds; stage 3: fresh, fully opened flowers.

The length of anthers, length of styles from ovary to tip of stigma, length and width of ovaries, length of fused portion of calyces, and length of petals for 20 flowers per plant were measured. The free, filamentous, distal portion of the calyx lobes were also measured, but proved to be exceedingly variable, even from lobe to lobe within a flower, and thus, are not included here. The measurements from each flower were treated as independent for the statistical tests in Table 2. However, the same statistical results or similar trends were found when the comparisons were made among the six plants with data pooled from the various flowers of the same plant.

TABLE 1. *Withania aristata* populations studied (organized by latitude) from Tenerife, Canary Islands (Spain). Collection numbers are from Gregory J. Anderson.

Locality	Elevation (m a.s.l.)	Latitude	Dates of study: collection nos.
Punta de Teno, Casa Antigua	40	28°21′ N 16°54′ W	14–21 Jan 2003: 4500/01
			17–29 May 2005: 5031
Watertank at Buenavista	120	28°21′ N 16°52′ W	17–28 May 2005: 5034
Punta de Juan Centellas, abandoned vineyard	100	28°23′ N 16°41′ W	09–16 Jan 2003: 4483/86
			10–20 Jan 2004: 4673
			14–21 Jan 2003: 4487
			10–17 Jan 2004: 4664/4670
			17–29 May 2005: 5032/33, 5035/39
			22–30 May 2005: 5084/85
Road above Punta de Juan Centellas	120	28°23′ N 16°41′ W	19–29 May 2005: 5084/85
Bollullo Beach, parking area	65	28°25′ N 16°31′ W	18–29 May 2005: 5059
Road to Bollullo Beach	110	28°25′ N 16°31′ W	18–29 May 2005: 5060/61
Taganana, km 5	125	28°33′ N 16°11′ W	18–29 May 2005: 5055

The sex ratio of natural populations (voucher accessions for populations 5031, 5034, 5036, 5084, 5059, 5060, 5055; Table 1) was calculated counting all female and male plants available (a total of 241 plants in seven populations). In addition, for selected populations (accessions 4483, 4486, 5036, 5037, 5039, 5060, 5061, 5085; Table 1), we estimated fruit production per 50 cm of branch

length (three randomly chosen branches per plant, 10 plants per population), measured fruit diameter, and counted seed set per fruit.

Counts of pollen grains and ovules per flower, as well as estimates of pollen viability, were obtained from flower buds preserved in 70% ethanol for three flowers per plant. The accessions examined (see Table 1) were 5031 (10 male
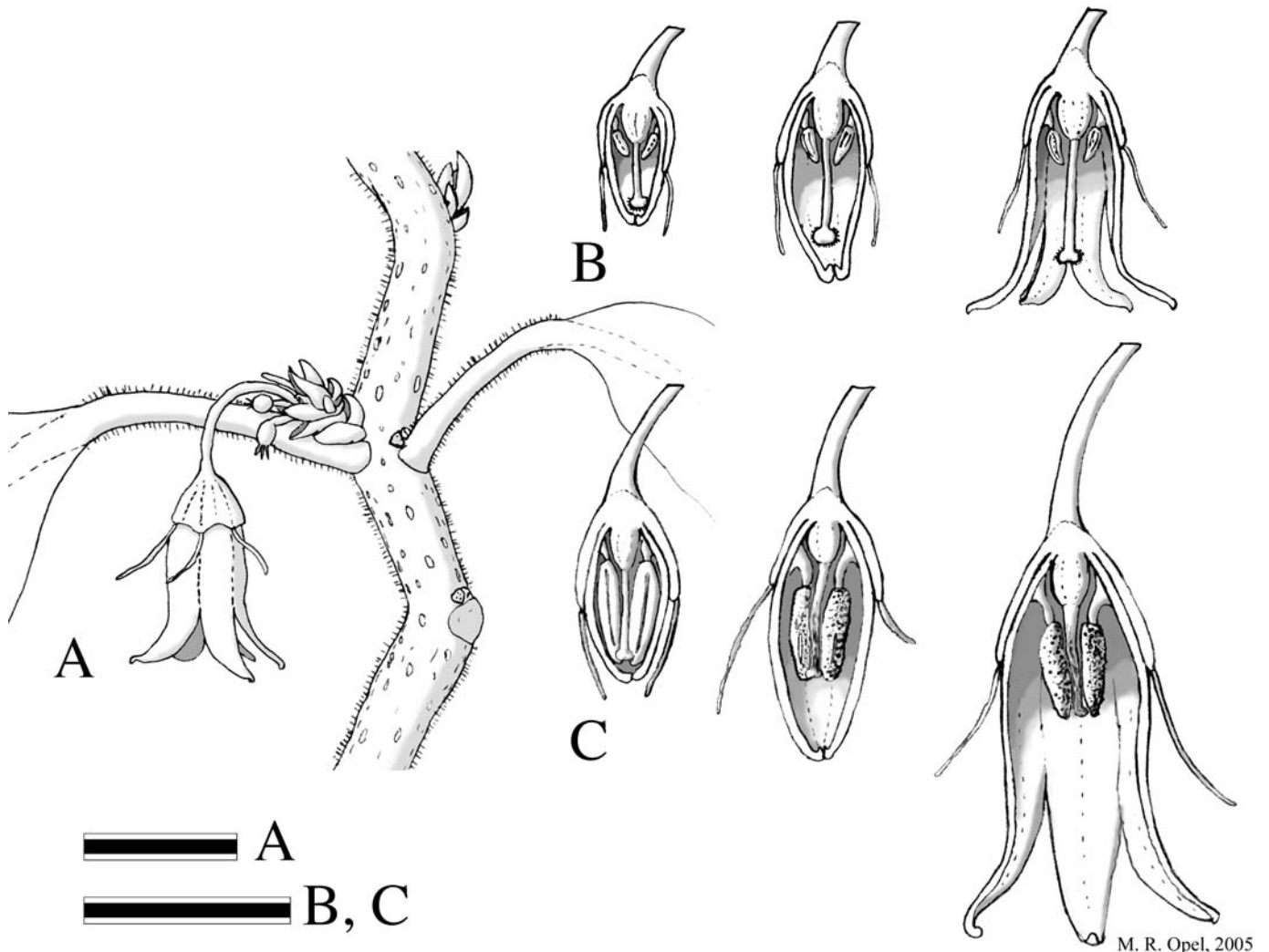

Fig. 2. Illustrations of *Withania aristata* shoot and flowers (population 4486). (A) Segment of a long shoot of a male plant, with a short shoot bearing flowers developing in the axil of one leaf. (B) Three developmental stages of female flowers (stage 1, 2, and 3, respectively). (C) Three developmental stages of male flowers (stage 1, 2, and 3, respectively). All scales = 1 cm.

TABLE 2.   Lengths of floral parts from male and female early buds (stage 1), late buds (stage 2), and open flowers (stage 3; see Fig. 2 and text for details) of *Withania aristata* from fresh material of cultivated plants. Data are means (mm $\pm$ SD). Significant differences (*t*-test, $P < 0.05$) between different stages and sexes are indicated by different superscript letters.

Flower type and stage	Anther length	Style length	Calyx length	Corolla length	Ovary length	Ovary width
Female, stage 1	1.9 [a] $\pm$ 0.2	2.9 [a] $\pm$ 0.4	2.9 [a] $\pm$ 0.3	6.3 [a] $\pm$ 0.7	2.0 [a] $\pm$ 0.3	1.5 [a] $\pm$ 0.1
Female, stage 2	1.8 [a] $\pm$ 0.2	5.2 [b] $\pm$ 0.7	3.2 [b] $\pm$ 0.3	10.7 [b] $\pm$ 0.9	2.0 [a] $\pm$ 0.2	1.7 [b,c] $\pm$ 0.2
Female, stage 3	1.7 [b] $\pm$ 0.2	6.7 [c] $\pm$ 0.5	3.2 [b] $\pm$ 0.3	13.1 [c] $\pm$ 1.2	2.2 [b] $\pm$ 0.2	1.8 [b] $\pm$ 0.2
Male, stage 1	4.9 [c] $\pm$ 0.2	4.7 [d] $\pm$ 0.3	3.7 [c] $\pm$ 0.3	7.6 [d] $\pm$ 0.4	2.3 [a] $\pm$ 0.2	1.4 [a] $\pm$ 0.2
Male, stage 2	4.2 [d] $\pm$ 0.4	5.6 [b] $\pm$ 1.0	4.0 [d] $\pm$ 0.5	14.0 [e] $\pm$ 1.1	2.1 [c] $\pm$ 0.3	1.6 [c] $\pm$ 0.3
Male, stage 3	3.7 [e] $\pm$ 0.4	5.5 [b] $\pm$ 0.8	4.5 [e] $\pm$ 0.5	18.7 [f] $\pm$ 1.4	2.4 [b] $\pm$ 0.2	1.8 [b] $\pm$ 0.1

plants), 5036 (one male plant), 5037 (10 female plants), 5038 (one male plant), 5039 (one male plant), and 5061 (one male plant). Pollen grains and ovules were counted using buds prior to anther dehiscence. Ovaries were gently squashed on microscope slides and their ovules counted. Pollen grain numbers per flower were estimated with a haemocytometer, using the methods of Lloyd (1965) as modified by Anderson and Symon (1989). Viability of ca. 400 pollen grains per flower was tested by staining with 1% aniline blue in lactophenol. Nectar sugar concentration was measured with a Bausch and Lomb hand refractometer (Rochester, New York, USA).

Controlled pollinations, as well as additional counts of pollen grains, ovules, and estimates of pollen viability were carried out using plants grown in the greenhouse. Crosses were attempted utilizing all six plants in all possible combinations (given that males have pistils, but females produce no pollen): male × self, male × nonself male, and female × male. Each crossing combination was treated as a separate event, rather than pooling the data by plant. This approach is supported by the fact that the combinations generally succeed or fail; i.e., there are not gradations of crossing success (except for the female × male comparison among the three floral stages).

The fate of unmanipulated flowers in the pollinator-free greenhouse environment was also tracked. Because insects were observed forcing their way into unopened flower buds in nature, we considered the possibility of bud pollination in this species, and crosses were attempted at all three stages of floral development outlined above.

The diameter of fruits was measured at maturity, and seeds were extracted, counted, and then sown in soil-less potting mix. Seed pots were kept evenly moist in warm greenhouse conditions, and germination was assessed after 2 months. Additional germination trials were carried out with open-pollinated, field-collected seeds from the following accessions: 5036 (♀), 5037 (♀), 5038 (♂), 5039 (♂), 5060 (♀), and 5061 (♂).

Insects visiting *W. aristata* flowers or buds were observed, photographed, or collected. Observations were made in all wild populations in all field expeditions for a total of c. 100 plants for more than 100 h, at the sites given in Table 1. Periods of observation ranged from 10 min to 1 h during daylight hours (from 0900 to 1600 hours). In addition, from July to September 2005, two female and two male plants from the greenhouse-cultivated plants were placed in the garden of the Torrey Life Sciences Building (Storrs) and exposed to non-native North American pollinators for observations of bee behavior and fruit set. Insects were deposited in the Biological Collections at the Ecology and Evolutionary Department (University of Connecticut) and were identified by Francisco La Roche-Brier.

RESULTS

Floral features—Wild plants flower abundantly and simultaneously (both sexes) year round, with a peak in the northern hemisphere spring. Flowers are pentamerous, actino-morphic, and pendant (Figs. 1, 2) producing a weak odor that is similar to lilacs (genus *Siringa*, Oleaceae). Both buds and open flowers are green to chartreuse. The calyx is gamosepalous and campanulate with five long linear lobes (Figs. 1B, 2). The corolla is campanulate with five lobes as long as or slightly longer than the tube (Figs. 1B, 2).

A superficial examination of the flowers suggests that there are no sexual differences, thus perhaps explaining the treatment in floras as regular hermaphrodite flowers. However, both male

and female plants can be recognized with striking differences in fruit production among individuals. Effectively, female plants bear many fruits at a time, whereas male plants have a large number of flowers, but bear no fruits (with unusual exceptions, see *Fruit features*). Although the corolla of male flowers is significantly larger, it is not an obvious feature, and the variation of corolla lengths masks any sexual difference. Thus, we conclude that no secondary sexual differences in architectural or other vegetative characters can be documented.

The sex ratio in wild populations is 1 : 1 (total of 126 male and 115 female plants, i.e., 52.3% vs. 47.7%; χ^2-tests, $P > 0.05$, no significant differences from the expected ratio). We observed no signs of monoecy, e.g., female plants bearing some flowers with functional anthers, in either wild or greenhouse plants.

Even though both flower types have all floral whorls, male and female flowers can be differentiated. The outer whorls of male flowers are significantly larger than those of female flowers at the same developmental stage, with the calyx and corolla can be up to 40% longer in males (Table 2). But, as noted, the variation among flowers and plants is great enough that this is not a reliable character. In both flower types, stamens are included, equal in size, and inserted at the base of the corolla tube, forming a staminal column surrounding the ovary (Fig. 2B, C). In addition, anthers are tetrasporangiate and bithecal (Fig. 3A, B) having longitudinal dehiscence that occurs in stage 2 buds. The differences are related to the presence of pollen—anthers in female flowers are sterile and have no pollen (Fig. 3A, C), whereas stamens in male flowers have pollen (Figs. 3B, D)—and to statistical differences in anther length (longer in males in all developmental stages [Table 2]; supported as well by the analyses, not shown, with the data pooled by plant). In both sexes, anthers reach their maximum size early, notably in stage 1 buds, shrinking by the time the flowers open; this phenomenon is more marked in male flowers. In female flowers, anthers have no pollen (Fig. 3A, C) and, as soon as flowers open, their thecae become dry and brown. On the other hand, male flowers yield large numbers (more than 200 000) of pollen grains that are highly stainable (= viable) (Fig. 3B, D; Table 3). Some pollen is still present in open flowers with shriveled anthers.

The gynoecium in both flower types is bicarpellate and bilocular. Each carpel bears many ovules on an axilar placenta (Fig. 3E, F). Interestingly, there are no statistical differences between the ovary size of either flower type (Table 2). On the other hand, there are important sex-related differences in the course of development of the stigma and style (Fig. 2B, C). In females, the stigma remains pale green and covered with turgid papillae throughout bud development, and for the c. 4-day life of the open flower. In addition, the style lengthens significantly
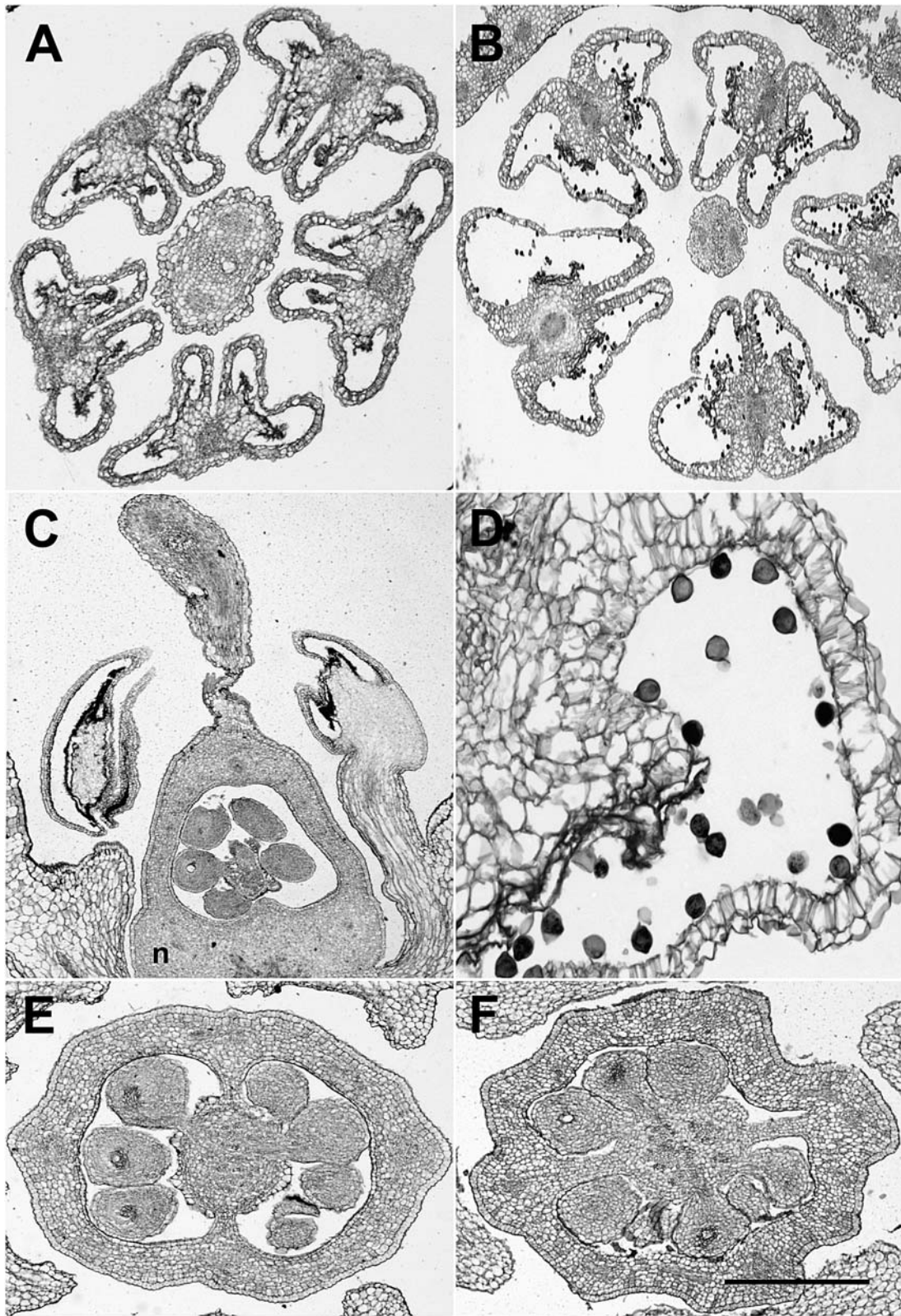
Fig. 3.   Anatomical features in *Withania aristata* (population 5035). (A) Detail of female flower cross-section showing style and five sterile anthers with no pollen. (B) Detail of male flower cross-section showing style and five anthers with pollen. (C) Detail of female flower longi-section showing gynoecium, nectary (n) and two staminodes. (D) Thecae cross-section showing two microsporangia united with pollen grains. (E) Detail of female flower cross-section showing ovary. (F) Detail of male flower cross-section showing ovary. Scale bars = 300 μm for A, 0.5 mm for B, E, F, 0.8 mm for C, and 130 μm for D.

TABLE 3. Pollen and ovule counts, pollen/ovule (P/O) ratio, and pollen viability for *Withania aristata* flowers from wild plants ($N = 50$ flowers for each measurement, 10 from each population; accession nos. 5036, 5038, 5039, 5061, 5037, see Table 1). Data are means $\pm$ SD. Significant differences (*t*-test, $P < 0.05$) between sexes are indicated by different superscript letters.

Flower type	Pollen/flower	Ovules/flower	P/O ratio	Pollen viability (%)
Female	0 [a]	17.7 [a] $\pm$ 1.3	0 [a]	—
Male	220 000 [b] $\pm$ 42 000	13.3 [b] $\pm$ 2.5	17 200 [b] $\pm$ 4600	81 $\pm$ 14.6

TABLE 4. Number of fruits per 50 cm of branch (populations 5036, 5038, and 5039), fruit diameter, and seed set (both from populations 5031, 5036/39, 5085, 5061 and 5060) according to flower type of *Withania aristata*. See Table 1 for population data. Significant differences (*t*-test, $P < 0.05$) between sexes are indicated by different superscript letters.

Flower type	No. fruits/50 cm of branch			Fruit diameter (mm)			Seeds/fruit		
	Mean $\pm$ SD	Range	*N*	Mean $\pm$ SD	Range	*N*	Mean $\pm$ SD	Range	*N*
Female	70 [a] $\pm$ 19.9	46–92	30	9.7 [a] $\pm$ 1.3	8–12.3	100	11.9 [a] $\pm$ 4.6	3–24	100
Male	0.2 [b] $\pm$ 4.5	0–10	30	6.3 [b] $\pm$ 0.5	1.5–9	39	4.3 [b] $\pm$ 1.4	0–8	39

at each stage of floral development in females (Fig. 2B, Table 2). In contrast, the stigma and style of male flowers shows signs of senescence, such as wilting and a brownish color in stage 2 buds, i.e., buds that will open in about 24 h. By the time male flowers open (stage 3), the stigma and distal parts of the style are usually shriveled, brown-black, and completely nonfunctional. Thus, the style of male flowers reaches its maximum length by stage 2, growing no further in the final phases of floral development (Fig. 2C, Table 2). In recently opened flowers (stage 3), the style is generally longer in females (Table 2); in these flowers, the stigma is presented well above the tips of the vestigial anthers (Fig. 2B). In contrast, in male flowers the brown/wilted stigma generally extends to about the same level as the anther tips (Fig. 2C); however, there is considerable variation in this trait and the stigma may be either entirely hidden or held just above the staminal column. The condition of the style is more reliable: the wilted, brown style and stigma in male flowers is obvious, and is also nonreceptive at anthesis.

All plants bear flowers with normal-looking ovules (Fig. 3E, F). Male and female flowers have a significantly different number of ovules, with females bearing more ovules (Table 3). The pollen/ovule ratio in males is about 17 200 (Table 3).

There is a circular nectary at the base of the ovary (Fig. 3C) in both sexes. However, nectar is very hard to observe in flowers from wild populations. In fact, in most flowers examined, we found no evidence of nectar. In contrast, nectar is easily detected in female flowers from greenhouse grown plants, but rarely in male flowers. Greenhouse-grown female flowers produce small amounts of nectar (<3 μL) with a mean sugar concentration of 36% ($N = 25$ flowers, four individuals).

Fruit features—The fruit is a globose berry, dark green when immature, turning orange-red when ripe, and surrounded throughout development by a conspicuous accrescent calyx (Fig. 1C). In greenhouse conditions, berries mature about 9 weeks after hand pollination. Fruits are often shed with the dried calyx and the pedicel still attached. Sporadically, the calyx expands and persists for weeks, or even months, around a presumably unpollinated ovary. Such false fruit set seems to be especially likely on vigorously-growing shoots on female plants.

By late spring (i.e., May) on Tenerife, females bear large numbers of fruits (Table 4). Infrequently, some males produce fruits (Table 4). When male and female fruit sizes and seed numbers are statistically compared, female fruits are larger and bear more seeds (Table 4).

Experimental crosses—Controlled pollinations of females with pollen from males have the potential to be successful in all stages of flower development (Table 5); the percentage of fruit set varied significantly based on the stage of the ovule parent. Buds in stage 2 (i.e., just prior to flowers opening), are the most receptive, yielding fruit in almost 100% of the crosses (Table 5). On the other hand, stage 1 buds yielded the lowest percentage of fruit (60%) in hand pollinations, as well as producing fruits that were significantly smaller and had fewer seeds than those from flowers in the other two stages (2, 3). Stage 3 flowers (fully open) produced intermediate fruit set, with fruits of the same size and seed number as stage 2 fruits (Table 5).

TABLE 5. Results of controlled greenhouse pollinations of *Withania aristata*, with ovule parent in early bud (stage 1), late bud (stage 2), or open flower (stage 3; see Fig. 2 and text for details). Data are percentage of fruit set (number of crosses attempted), mean fruit diameter in mm $\pm$ standard deviation (number of fruits measured), and mean seed number per fruit $\pm$ standard deviation (number of fruits examined). Significant differences in fruit diameter and seed number between cross types and stage (*t*-test, $P < 0.05$) are indicated by different letters in superscript. Significant differences of fruit set percentages (χ^2-test; $P < 0.05$) are indicated by different letters in superscript.

Ovule parent $\times$ pollen parent	Fruit set	Fruit diameter	Seed set
Female $\times$ male			
Stage 1	60% [a] (73)	8.6 [a] $\pm$ 1.4 (34)	8.7 [a] $\pm$ 4.7 (43)
Stage 2	96% [b] (73)	9.5 [b] $\pm$ 0.9 (49)	11.8 [b] $\pm$ 3.9 (69)
Stage 3	71% [c] (111)	9.5 [b] $\pm$ 0.9 (53)	11.9 [b] $\pm$ 3.8 (78)
Male $\times$ self			
Stage 1	41% [d] (46)	8.2 [a] $\pm$ 0.8 (12)	9.1 [a] $\pm$ 4.0 (18)
Stage 2	0% [e] (23)	—	—
Stage 3	0% [e] (24)	—	—
Male $\times$ nonself male			
Stage 1	50% [d] (50)	8.4 [a] $\pm$ 0.8 (20)	9.2 [a] $\pm$ 3.8 (25)
Stage 2	0% [e] (21)	—	—
Stage 3	0% [e] (26)	—	—

TABLE 6. Germination percentages of *Withania aristata* seeds from fruits of controlled greenhouse pollinations (ovule parent listed first) and from open-pollinated fruits from wild plants (5036 ♀, 5037 ♀, 5038 ♂, 5039 ♂, 5060 ♀, 5061 ♂; see Table 1 for population data). Seeds resulting from female × male crosses were considered together, disregarding the flower stage of the ovule parent. Significant differences (χ^2-test; $P < 0.05$) are indicated by different superscript letters.

Seeds from crosses	Female × male (%)	Male × self (%)	Male × nonself male (%)	Female (wild, %)	Male (wild, %)
Germination (%)	49 [a]	61 [b, c]	70 [c]	61 [a, b]	27 [d]
No. seeds	1453	140	131	110	86

Unexpectedly, self pollinations of male flowers did succeed (Table 5), but only when the flowers were pollinated at stage 1, i.e., very young buds with still turgid stigmata/styles. This indicates that, although dioecious, this species is self-compatible (SC). Crosses between different male plants were also successful (Table 5) and showed no significant differences from the fruit set obtained with male selfed buds. Comparatively, success rates were lower for males, either selfed or outcrossed, than for females pollinated at the same developmental stage (Table 5), but still, the final outcome yielded a respectable 41–50% fruit set. The fruits produced by manipulated male flowers were similar in size and seed number to the ones obtained with female flowers as ovule donors (Table 5).

Unmanipulated flowers in pollinator-free greenhouse conditions never yielded fruits, in either females ($N = 750$ flowers) or males ($N = 600$ flowers) in the two successive years of cultivation. Cultivated plants exposed to pollinators in a Connecticut garden for three summer months (July to August) were visited by wasps and small bees common in the area. In this environment, the exposed female plants set copious fruit, but the male plants set none.

Seeds produced by all plant types on Tenerife are viable and germinate, as do seeds from all greenhouse crosses (Table 6). In general, germination rates are moderate, reaching a maximum of 70%. However, seeds field-collected from male plants showed significantly lower germination rates (27%; Table 6). There are no statistical differences among the rates of seed germination from field-collected female plants and artificial crosses of female or selfed males (Table 6). Preliminary observations of young plants derived from these experimental sowings reveal no discernable effects of cross type on plant size or apparent health.

Visitors—Four bee species from three genera visited *W. aristata* flowers on Tenerife (Table 7). Two *Lassioglossum*

TABLE 7. Insects collected visiting flowers of *Withania aristata* on Punta de Juan Centellas (Tenerife). Collection numbers are from Gregory J. Anderson.

Species	Date: collection no.	Hour
Amegilla quadrifasciata (♀)	10 Jan 2004: 4673	1100
Amegilla quadrifasciata (♂)	14 Jan 2004: 4685	1000
Apis mellifera (worker)	13 Jan 2004: 4681	1215
Apis mellifera (worker)	13 Jan 2004: 4682	1230
Apis mellifera (worker)	14 Jan 2004: 4686/88	1000
Lassioglossum loetum (♀)	10 Jan 2004: 4672	1300
Lassioglossum loetum (♀)	12 Jan 2004: 4680	1200
Lassioglossum loetum (♀)	14 Jan 2004: 4699, 4701	1000
Lassioglossum viride subsp. *viride* (♀)	10 Jan 2004: 4670, 4671	1200
Lassioglossum viride subsp. *viride* (♀)	13 Jan 2004: 4683	1230
Lassioglossum viride subsp. *viride* (♀)	14 Jan 2004: 4689, 4690/98, 4700	1000

species (Halictidae) endemic to all Canary Islands, the native *Amegilla quadrifasciata* (Anthophoridae), and, much less frequently, the introduced European bee (*Apis mellifera*, Apidae) were detected. Bees were observed in open flowers, but more frequently probed mature flower buds of both sexes, especially stage 2 buds. Bees were observed chewing at the tips of the petals of closed buds in their attempts to gain entry.

DISCUSSION

Floral morphology and anatomy and results from experimental crosses support the conclusion that *W. aristata* is dioecious. David Symon called Hepper's attention to the possibility that this species was dioecious, based on informal observation of cultivated plants in Australia (see Hepper, 1991). In spite of these hints at the unusual sexual system in *Withania*, detailed studies have not followed.

Both male and female flowers have all whorls, but there are striking differences between them. Male flowers bear a much larger calyx, corolla, and anthers, and female flowers bear anthers, but they are devoid of pollen. Although the ovaries show no differences in the first stage of bud growth, female flowers subsequently develop longer styles. Most important, female stigmata are functional during anthesis, including from the early bud stage we identified, but in male flowers stigmata become nonfunctional well before flowers open—hence, their treatment as males. Plants with male flowers do not produce fruits, but exceptionally, fruits are found on male plants in the field in very low quantities. These observations are in accordance with the results of experimentally selfed or outcrossed males, where hand pollinations of flowers at an early bud stage (before the stigma and style wither) yielded around 50% fruit set. Thus, *W. aristata* is self compatible, in accordance at least with most other dioecious and andromonoecious solanums known (Anderson and Symon, 1989). The high pollen/ovule ratio for male flowers supports Cruden's (1977) category for obligate xenogamy, and is analogous to the pollen/ovule ratio for male flowers of another dioecious species, *Solanum appendiculatum* (Mione and Anderson, 1992). Thus, the presumably residual self-compatibility reflects phylogenetic ancestry, but not current reproductive biology.

Although we did not calculate the flower/fruits ratio for plants in the field, our extensive observations support a high percentage of fruit set on female plants. Given the impossibility of selfing in female flowers (no pollen), the lack of support for autogamous (or apomictic) fruit set in experimental greenhouse settings of either sex, and, in spite of few floral visitors being observed at any given time, we conclude that these flowers enjoy a high rate of cross pollination. All visitors observed were bees, endemic, native, or introduced (Izquierdo et al., 2004). The behavior of native bees forcing their way into unopened (but mature) buds, coupled with the self-compatibility

of *W. aristata*, may be responsible for the infrequent fruit set in male plants which, otherwise, does not occur spontaneously. For instance, in the two male, cultivated specimens that we grew for 2 years in greenhouses and in a gardens, we never obtained fruits without manipulation.

Little information is available for pollinators of other *Withania* species. The only report is for *W. somnifera*, a species with smaller, nonpendant, generalist flowers, visited in India by bees, butterflies, and flies (Kaul et al., 2005).

Flowers of each gender may be considered as offering different rewards. Females offer nectar and males pollen. In greenhouse-grown plants, females always produced nectar and males did not. In other dioecious species, Eckhart (1999) found that the quantity of nectar may differ between male and female flowers, but Eckhart's survey did not include Solanaceae. Interestingly, anatomical observations in both flower types suggest that the nectary should be functional. This species produces tiny amounts of nectar in comparison to other solanaceous, nectariferous flowers of the same size (e.g., Galetto and Bernardello, 1993, 2003). And, as noted, in the field, we essentially could not detect nectar production. Presumably, this lack of nectar is due to visitor activity or environmental conditions (it is dry and virtually always windy along the coast where the plants grow). The floral nectary is similar to those observed in other Solanaceae (Hunziker, 2001).

Recent accounts suggest that about 10% of flowering plant species are dioecious, but these studies also show that dioecy has evolved repeatedly, being represented in nearly half of the angiosperm families (Renner and Ricklefs, 1995; Gerber et al., 1999; Webb, 1999). Within the Solanaceae, cosexuality is common and dioecy is comparatively rare (Sawyer and Anderson, 2000; Hunziker, 2001). Gender dimorphism has evolved independently in six lineages, primarily in the tribe Solaneae (Sawyer and Anderson, 2000) and has been reported for as few as six of the more than 90 genera that comprise the family and only about 20 species (i.e., less than 1% of the species). Interestingly, virtually all dioecious cases in the Solanaceae are referred to as functional dioecy (Anderson, 1979; Anderson and Symon, 1989) or cryptic dioecy (Mayer and Charlesworth, 1991), as we report here for *Withania*. Clearly, functional or cryptic dioecy is a regular phenomenon apparently not just in *Solanum*, but in other members of the Solanaceae. It is important to understand this syndrome, and its extent, because the reproductive system has a clear impact on the systematics, diversity, and evolution of a lineage (Anderson et al., 2002). The existence and nature of such cryptic systems stimulates the study of other taxa that might otherwise continue to be considered typically hermaphroditic. And, the anatomy, morphology, development, and distribution of characters may help us to understand the evolution of fundamental features of flowers such as pollen (e.g., Zavada and Anderson, 1997; Zavada et al., 2000) and pistils.

Our data suggest that dioecy in *W. aristata* has evolved from self compatible (SC) ancestors. In *Withania*, only the hermaphroditic *W. somnifera* was analyzed previously. It is also SC, but, in contrast, is highly selfing and has a low pollen/ovule ratio (817; Kaul et al., 2005). Self compatibility is likely ancestral in *Withania*, as is true for much of *Solanum* (Whalen and Anderson, 1981). In several plant groups, gender dimorphism is purported to have evolved from SC hermaphroditic ancestors, a fact that has been interpreted as a mechanism to promote outcrossing and to avoid self-fertilization and the resulting negative consequences of inbreeding depression (Lloyd, 1976; Charlesworth and Charlesworth, 1978a, b; Anderson and Symon, 1989; Sakai and Weller, 1999). However, in some other Solanaceae, such as *Lycium* (Miller and Venable, 2002) and possibly *Deprea* (Sawyer and Anderson, 2000), gender dimorphism has evolved on a phylogenetic background of self-incompatibility (Richman and Kohn, 2000). Unfortunately, no data are available on the mating systems of the other dioecious genera of the family (*Dunalia* and *Symonanthus*), so broader conclusions are hard to draw at this point. But, it is just such studies that are needed in order to provide the foundation for a generalized understanding of the evolution of mating and breeding systems.

Most dioecious *Solanum*, with the exception of *S. appendiculatum*, are derived from SC progenitors (Whalen and Anderson, 1981; Levine and Anderson, 1986; Anderson and Symon, 1989; Knapp et al., 1998). There are other similarities between *Solanum* and *Withania* sexual systems. The dioecy is cryptic in both, in the sense that the sexes are morphologically hermaphroditic, but functionally unisexual. The scent of both is weak or absent. Both are pollinated by bees, though *Solanum* flowers require "buzzing" to extract the pollen from poricidal anthers, whereas the pollen in *Withania* falls free of the longitudinally dehiscent anthers. Pollen is a significant reward in both, though it is the only reward in *Solanum*, as is the case for the male flowers of *W. aristata*. And the sex ratio is the same for both (1 : 1). There are a number of differences as well. The flowers in *Withania* are comparatively dull in coloration (green vs. white or yellow), *Solanum* flowers completely lack nectar in both sexes, and though most species of the dioecious solanums seem to have evolved on a platform of self-compatibility, males never bear seed-bearing fruits (G. J. A. observed apparent "fruits" on males of *S. appendiculatum* in the field in Mexico; however, these were all seedless, bearing only larvae of an ovary-parasitic insect).

There are associations—as documented by Renner and Ricklefs (1995) for other dioecious species in general—between dioecy and various ecological and morphological traits in *W. aristata*. *Withania* is biotically dispersed, and has a shrubby growth form and a tropical distribution. Effectively, seed dispersal is endozoic; *W. aristata* fruits are reddish, fleshy, and edible (often bird-associated dispersal features). Two endemic lizards in the genus *Galottia* are reported to be natural dispersal agents in the Canaries (Valido and Nogales, 1994, 2003; Valido et al., 2003). Valido and Nogales (1994) also reported that seeds passed through a lizard gut had a significantly increased germination rate. Birds may be dispersers as well, considering the rich bird fauna in the Canary Islands (Delgado, 2001) and the fruit features of *W. aristata* (e.g., orange fruits at maturity).

Some oceanic islands have a large proportion of endemic dioecious species (Bawa, 1980; Givnish, 1982; Sakai and Weller, 1999), among which New Zealand and Hawaii are notable (Carlquist, 1974; Sakai et al., 1995a, b; Webb, 1999). In contrast with these two archipelagoes, the Canary Islands bear only 3% (Helfgott et al., 2000), a percentage close to that of other islands such as the Azores, Galapagos, Reunion, Aldabra, and Bermuda (Baker and Cox, 1984). The general argument for dioecy on islands is that selection for outcrossing in small, colonizing, hermaphroditic populations favors separation of the sexual functions (Carlquist, 1974; Baker, 1967; Bawa, 1980; Thompson and Barrett, 1981). Alternatively, the incidence of dioecy on islands may simply reflect its

incidence in the source flora of the nearest continents (Baker and Cox, 1984).

Withania aristata shows weak gender plasticity, in that a few male plants sometimes produce a very few fruits. This kind of dioecy—perhaps akin to subdioecy (see Delph and Wolf, 2005) or leaky dioecy (Baker and Cox, 1984)—has been reported in different plant families and island systems for several species (e.g., Baker and Cox, 1984; Cox, 1990; Weller et al., 1990; Sakai and Weller, 1991; Ladley et al., 1997; Percy and Cronk, 1997; Humeau et al., 1999, 2000; Litrico et al., 2005). On one hand, "leaks" in the dioecious system, in an evolutionary sense, are probably not adaptive, but instead reflect the origins of dioecy, the strength (weakness) of the selection for full sexual separation, and/or the relative recency of the dioecious system. But on the other hand, leaky dioecy may facilitate the establishment after long-distance dispersal of colonizers with sexual variability (Baker and Cox, 1984). The ability of isolated males to undergo occasional sexual reproduction may be important and heavily selected for, preventing the extinction of subpopulations that have become unisexual through drift (Percy and Cronk, 1997). Apropos to *W. aristata*, we can only speculate, but we did observe one exemplar population where there was only one female plant (out of seven). Thus, the evolution of dioecy in *W. aristata* may be relatively recent, and gender plasticity may have been favored during colonization of the archipelago. Its founders may have expressed sexual variation when they first arrived on the islands. The existence of a related species from Morocco and Algeria (*W. adpressa*) with unisexual flowers suggests this possibility, although its reproductive system has not been studied (Hepper, 1991). There are other explanations possible for the leakiness. For instance, Percy and Cronk (1997) also suggest that when full dioecy is approached, the selection pressure for further loss of female function might be negligible.

As is well known, there are many more recorded vascular plant extinctions from islands than from continental areas (Reid and Miller, 1989; Frankham, 1997). Island species, generally with few populations and few individuals, are especially vulnerable to human-induced disturbance (e.g., direct predation, habitat degradation or loss, introduction of plant and animal exotic species, loss of pollinators; Groombridge, 1992; Vitousek et al., 1995; Whittaker, 1998; Vamosi et al., 2006). The Canary Islands have been inhabited by humans for ±3 millennia, but serious human disturbance began in the 15th century when the islands were annexed by Spain (Fernández-Palacios and Martín-Esquivel, 2001). Human impact has grown. The main threats today are the continuous loss of natural habitat to tourist and residential developments, agriculture, overgrazing, invasive plants, off-road vehicles, and fires (Bramwell, 1994), together with natural erosion. In contrast to the 100+ endemic species in the archipelago that are endangered (Bañares et al., 2003), *W. aristata* is fairly widespread (Bramwell and Bramwell, 2001). In addition, unlike dioecious species from other archipelagoes (e.g., Farwig et al., 2004), *W. aristata* naturally produces good fruit set. Thus, this distinctive island endemic may not be in immediate danger of extinction. Nonetheless, like other endemics care must be taken to conserve this species, whose persistence also means preserving the plant–pollinator relationship (Nabhan et al., 1998; Vamosi et al., 2006) and the natural areas where its populations grow. The unusual reproductive system we document herein increases the importance of such preservation. Protection of *W. aristata* presents some challenges because populations are restricted to lower elevations (from sea level to 600 m) where human disturbance is higher (from Canary banana and wine-grape production and seaside tourism), and inexorably, there are fewer undeveloped, "wild" areas.

Our studies suggest that the reproductive biology of other *Withania* species should be studied in detail, particularly those known to have unisexual flowers (the Asian *W. coagulans* is known to be dioecious), and some sort of dicliny is suggested (from morphological studies of specimens) for *W. adpressa* from North Africa and *W. qaraitica* from Oman. Phylogenetic analyses of the group would provide a foundation critical to tracing the evolution of the different mating systems. Finally, reproductive studies need to include living plants and experimental studies to accompany field and specimen study. Our results imply that it is imperative to perform careful reproductive studies even at the mature bud stages.

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