The transition to gender dimorphism on an evolutionary background of self-incompatibility: an example from *Lycium* (Solanaceae)[1]

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Populations of three North American species of *Lycium* (Solanaceae) are morphologically gynodioecious and consist of male-sterile (i.e., female) and hermaphroditic plants. Marked individuals were consistent in sexual expression across years and male sterility was present throughout much of the species' ranges. Controlled pollinations reveal that *L. californicum*, *L. exsertum*, and *L. fremontii* are functionally dioecious. Fruit production in females ranged from 36 to 63%, whereas hermaphrodites functioned essentially as males. Though hermaphrodites were mostly male, investigation of pollen tube growth reveals that hermaphrodites of all dimorphic species were self-compatible. Self-fertilization and consequent inbreeding depression are commonly invoked as important selective forces promoting the invasion of male-sterile mutants into cosexual populations. A corollary prediction of these models is that gender dimorphism evolves from self-compatible ancestors. However, fruit production, seed production, and pollen tube number following outcross pollination were significantly higher than following self-pollination for three diploid, cosexual species that are closely related to the dimorphic species. The data presented here on incompatibility systems are consistent with the hypothesis that polyploidy disrupted the self-incompatibility system in the gynodioecious species leading to the evolution of gender dimorphism.

Key words: breeding systems; dioecy; gynodioecy; *Lycium*; polyploidy; self-compatibility; self-incompatibility; Solanaceae.

Gender dimorphism is the presence of two sexual mating types (e.g., females and males in dioecy or females and hermaphrodites in gynodioecy) that coexist in populations. Many population genetic models for the evolution of gynodioecy from cosexuality propose that selection to promote outcrossing allows for the spread of male-sterile mutants (i.e., females) in cosexual populations (Lloyd, 1975; Charlesworth and Charlesworth, 1978a, b). This is because females, which do not produce pollen and are thus free from selfing, can invade selfing, hermaphroditic populations that are suffering inbreeding depression. Both theoretical (Lloyd, 1975; Charlesworth and Charlesworth, 1978a, b) and empirical studies (reviewed in Webb, 1999) provide evidence for the importance of selection for outcrossing in the evolution of gynodioecy. The models make explicit predictions concerning the sequence of evolutionary events and hypothesize that sexually dimorphic species evolve from self-compatible taxa that suffer from self-fertilization and inbreeding depression (Charlesworth and Charlesworth, 1979).

It is well established that physiological self-incompatibility is a mechanism to avoid self-fertilization and prevent inbreeding depression in plants (de Nettancourt, 1977; Jarne and Charlesworth, 1993; Matton et al., 1994). Baker (1959) was the first to point out, that because both dioecy and self-incompatibility are mechanisms to avoid inbreeding, they are unlikely to exist together in the same lineage. The obvious corollary, that gender dimorphism is more likely to evolve in groups that are self-compatible has been stated repeatedly (Baker, 1959; Thomson and Barrett, 1981; Baker, 1984; Charlesworth, 1985). However, controversy exists as to whether or not there is an association between self-compatibility and gender dimorphism (Givnish, 1982; Willson, 1982; Thomson and Brunet, 1990; Freeman et al., 1997). Thomson and Barrett (1981) found support for Baker's observations and argued that at both the family and generic levels, a negative correlation exists between self-incompatibility and dioecy. Givnish (1982), in an early attempt to statistically analyze the association, found no relationship between self-incompatibility and gender dimorphism and argued that ecological factors were at least as important as selection for outcrossing in the evolution of dioecy (see also Willson, 1982; Freeman et al., 1997). Charlesworth (1985), in the most thorough analysis to date, suggested that the available data are too fragmentary for an adequate test of the association. She found no statistical support for a negative association, though she supported Baker's (1959) original conclusion due to the low frequency of dioecy in lineages where self-incompatibility is known to occur. More recently, Freeman et al. (1997) argued that many dioecious species have close relatives with other outbreeding mechanisms (e.g., herkogamy, dichogamy, self-incompatibility, monoecy) and suggested that gender dimorphism, in these cases, is not likely due to selection for outcrossing (see also Bawa, 1980). However, they point out that these alternative outbreeding strategies appear to be present in species that evolve dioecy via monoecy and concede that, in gynodioecious taxa, females

may invade due to selection for outcrossing. Another possibility is that cytoplasmic male sterility may be responsible for the evolution of sexual dimorphism and in this case selection for outbreeding need not be involved either (Frank, 1989). Detailed studies of compatibility systems in the close relatives of dimorphic species and in the hermaphrodites of gynodioecious species are needed to resolve the controversy over the association between self-incompatibility and dioecy.

Here, we document morphological gynodioecy for the polyploid species *Lycium californicum* Nutt. Ex Gray, *L. exsertum* A. Gray, and *L. fremontii* A. Gray, including the presence and geographic distribution of male sterility. We assess the consistency of sexual expression for plants in natural populations across several years and report sex ratios in these populations. We also present results from controlled pollinations describing the breeding systems in populations of the dimorphic taxa. In particular, we investigated pollen limitation in females and hermaphrodites, agamospermy in females, and autogamy in hermaphrodites.

Recently, we suggested for *Lycium* that a polyploid event in the cosexual ancestor of the dimorphic lineage disrupted self-incompatibility, leading to the evolution of gender dimorphism as a result of selection for outcrossing (Miller and Venable, 2000). Here, we provide additional details of the crossing experiments described in Miller and Venable (2000), including data for females and comparison to the hermaphrodites in the dimorphic species. In addition, we compare pollen tube growth among dimorphic females, conspecific hermaphrodites, and three related diploid cosexual *Lycium* species (*Lycium berlandieri* Dunal, *L. pallidum* Miers, and *L. parishii* A. Gray). We show that more pollen tubes are present in females of dimorphic species compared to both conspecific hermaphrodites or cosexual species and that while cosexual species are self-incompatible, hermaphrodites of the dimorphic species are self-compatible. Further, we address some objections to our proposed scenario (Brunet et al., 2001), including those associated with determining the compatibility status of the dimorphic hermaphrodites.

MATERIALS AND METHODS

Study populations—*Lycium* is a genus of shrubby perennials found worldwide in arid to semi-arid environments. According to phylogenetic reconstructions, gender dimorphism has evolved a single time in North America (Miller, 2002), and previous studies have suggested that three species in North America have gender dimorphism: *L. exsertum* and *L. fremontii* (Chiang-Cabrera, 1981; Gilmartin, 1983) and *L. californicum* (J. S. Miller, personal observation). All other *Lycium* in North America (~18 spp.) are cosexual with all individuals producing perfect flowers (Chiang-Cabrera, 1981).

The six species of North American *Lycium* studied here were located in Pima and Pinal counties in south-central Arizona. Populations of the dimorphic species *L. californicum* and *L. fremontii* were located in lowland halophytic communities where they occur as the dominant or codominant species (32°46′48″ N, 111°37′48″ W; 32°47′06″ N, 111°42′22″ W; 33°15′40″ N, 111°56′38″ W). Dimorphic *L. exsertum* was located along sandy washes at Desert Peak (32°36′00″ N, 111°15′00″ W; Pinal Co., Arizona) and in Alamo Canyon at Organ Pipe National Monument (OPNM; 32°03′58″ N, 112°42′58″ W; Pima Co., Arizona). Cosexual *Lycium berlandieri* was studied along washes in the Casa Grande Mountains (32°47′31″ N, 111°41′56″ W; Pinal Co., Arizona). The study population of cosexual *L. parishii* was located along the U.S.-Mexican border inside OPNM along Puerto Blanco Drive (31°54′00″ N, 112°51′00″ W; Pima Co., Arizona). Study plants of cosexual *L. pallidum* were located southeast of Oracle in the foothills of the Santa Catalina Mountains

(32°32′28″ N, 110°42′44″ W; Pinal Co., Arizona). Voucher specimens are housed at the University of Arizona herbarium (ARIZ).

Male sterility, stability of gender expression, and sex ratios—Individual plants of *L. californicum*, *L. exsertum*, and *L. fremontii* have flowers that are either morphologically hermaphroditic or have reduced filaments and underdeveloped (apparently sterile) anthers. To confirm that male function was absent from plants with underdeveloped anthers, we collected recently opened flowers and mature floral buds from ten such plants and also from ten hermaphrodites. Anthers were removed from flowers and buds, stained with basic fuschin gel, and observed under a light microscope (10×) for the presence of pollen. Whenever populations of these species were encountered they were checked for the presence of female and hermaphroditic plants. In addition, herbarium specimens at the University of Arizona were examined for the presence of female flowers. Sex expression was easily assessed from the presence of functional stamens in hermaphrodites or reduced, nonfertile stamens in females.

We permanently tagged females and hermaphrodites and surveyed them repeatedly during the season and over several years to determine whether plants were consistent in gender expression. We monitored 60 females and 70 hermaphrodites of *L. fremontii* in two populations from 1995 to 2000. For *L. exsertum*, we surveyed 37 females and 57 hermaphrodites in one population from 1995 to 2000. We monitored 23 females and 29 hermaphrodites in a single population of *L. californicum* from 1997 to 2000. The ratio of female to hermaphroditic plants was determined in two or three populations for each of the dimorphic species (sample sizes varied from 45 to 251 plants per population for a total of 1000 plants).

Fruit and seed production—To determine whether the cosexual species and the hermaphroditic morphs of dimorphic species were self-compatible, we compared fruit and seed production of flowers pollinated with outcross pollen to those pollinated with self-pollen during the peak flowering of each species. Pollinations took place during fall (August–September) and spring (March–May) over four flowering seasons. The number of treatment plants per population ranged from 14 to 24 (mean = 17) in cosexual species and from 19 to 32 (mean = 25) in hermaphrodites of dimorphic species. On each plant, fine mesh bags were placed over unopened flower buds to prevent insect visitation. During the next mornings several recently opened flowers on which anthers had not yet dehisced were chosen haphazardly from within the bags (mean = 7.7 flowers per plant). These were emasculated and half were pollinated with outcross pollen (collected from at least two donors 10 m away) and half with self-pollen. The amount of pollen received by flowers exceeded the number of ovules present and was sufficient for full seed production. All treated flowers were re-covered to prevent external pollination by insects. Additional newly opened, unmanipulated, and uncovered control flowers were marked to assess natural levels of fruit and seed production on plants in cosexual species and hermaphrodites of dimorphic species. To determine whether the cosexual species and the hermaphroditic morphs of dimorphic species were autogamous, flower buds were marked and covered with fine mesh bags to preclude external pollination.

To investigate pollen limitation in females of the dimorphic species, we augmented pollen on flowers (mean = 6.1 flowers per plant) and compared fruit and seed production to unmanipulated control flowers (mean = 7.6 flowers per plant) on the same plant. We used 17 females of *L. californicum* and 25 females each of *L. exsertum* and *L. fremontii* in this experiment. For the augmentation treatment, both the pollen source and the application procedure were identical to those for the outcross treatment described above. Both augmented and control flowers were left open to experience natural pollination. Because polyploidy is often associated with agamospermy (Stebbins, 1971; Richards, 1997), we marked and covered floral buds on females of dimorphic species to determine if they were agamospermous. All treatments were monitored through fruit production (approximately 3–4 wk).

Data for fruit production were analyzed using a generalized linear model assuming a binomial response variable and a logit link function; chi-square values were adjusted to account for over-dispersion when necessary (PROC GENMOD, SAS Institute, 1989). The dependent variable was the total number

of flowers that produced fruit following a particular pollination treatment divided by the total number of flowers in that pollination treatment on a given plant. Effects included in the model were season, plant nested within season, pollination treatment, and the season by pollination treatment interaction. Season was tested for significance using the chi-square value corresponding to plant nested within season. When the season by pollination treatment interaction was not significant it was removed from the model. Pollinations of *L. berlandieri* and *L. parishii* were completed in a single season, so the season and season by treatment interaction were not included as effects for these analyses. Due to low fruit production for the hermaphrodites of dimorphic species, the analyses for the model described above did not converge and thus PROC GENMOD could not be used to analyze these data. In these cases, we used a general linear model (PROC GLM, SAS Institute, 1989) to analyze proportion fruit production (arcsine-square root transformed). Effects included in the model were as described above for conspecific females. Results of analyses for the hermaphrodites of the dimorphic species should be viewed as approximate because the residuals of these analyses were not normally distributed, although ANOVA is fairly robust to violation of this assumption (Zar, 1996).

Seed number was analyzed for *Lycium berlandieri* and *L. parishii* using a general linear model (PROC GLM, SAS Institute, 1989) and included plant (random effect), pollination treatment (fixed effect), and the two-way interaction of plant by pollination treatment. *Lycium pallidum*, *L. exsertum*, and *L. fremontii* were analyzed using a general linear model that included pollination treatment as a fixed effect, season and plant nested within season as random effects, and the season by pollination treatment interaction.

Pollen tube growth—Controlled pollinations to evaluate pollen tube growth were conducted during spring 1998 and 1999 for cosexual *L. pallidum* and *L. parishii* and dimorphic *L. californicum*, *L. exsertum*, and *L. fremontii*. Fine mesh bags covered flowers for the duration of the experiment to prevent contamination of the pollen treatment. Sets of flowers on plants were emasculated prior to anther dehiscence and pollinated with either outcross or self-pollen as described above. Flowers were harvested after sufficient time to allow pollen tubes to grow through the style (as determined by pilot experiments). Flowers were fixed in formalin-acetic acid-alcohol for 24 h and transferred to 70% ethanol. The gynoecia were dissected from flowers and rinsed in tap water before softening in 8 mol/L NaOH for 5–8 h. Samples were rinsed for ~1 h to remove excess NaOH and stained in aniline blue for 8–12 h. Gynoecia were mounted on slides and pollen tubes were viewed with fluorescence microscopy. Since there is considerable variation in the site of action of the incompatibility reaction among species possessing gametophytic incompatibility, and since *Lycium cestroides* has been shown to have late-acting incompatibility (Aguilar and Bernardello, 2001), we deemed it wise to measure pollen tubes at three different levels of the style. The number of pollen tubes present at the top, halfway down, and at the base of the style were counted, and for each treatment on each plant, the number of pollen tubes present at each style position was averaged over replicate flowers.

In 1998 flowers were collected 24 h after pollination from 11 hermaphrodites of dimorphic *L. californicum* and 48 h after pollination from 14 hermaphrodites of dimorphic *L. fremontii*. Flowers from ten plants of cosexual *L. pallidum* and three plants of cosexual *L. parishii* were collected 48 h following pollination. In 1999, flowers were sampled at several time intervals following pollination and female plants (outcross pollen only) of dimorphic species were also included. For dimorphic *Lycium californicum*, one or two flowers from five females and five hermaphrodites were collected following 2, 4, 6, 8, and 24 h post-pollination. In the remaining species, one to three flowers on all plants were sampled at 6, 12, 24, and 72 h post-pollination. Eight females and nine hermaphrodites of dimorphic *L. exsertum*, four hermaphrodites and four females for dimorphic *L. fremontii*, and six plants of cosexual *L. parishii* were included in 1999. Sampling for *L. californicum* in both years was at shorter intervals than the other species since *L. californicum* has small flowers with short styles.

To test whether self-pollen was as successful as outcross pollen at growing through the style, we compared the relative success of pollen tube growth using data from paired pollinations (i.e., outcross and self) performed on the same plant and collected at the same time interval following pollination. The dependent variable in this analysis was the log ratio of outcross to self-pollen tubes calculated as the difference $[\log_{10}(\text{outcross} + 1) - \log_{10}(\text{self} + 1)]$. We used later collection times for each species (24 h for *L. californicum* and >24 h for all other species) to maximize the number of tubes present at the base of the style. Different plants were used in the two years so we combined the data from both 1998 and 1999. We analyzed the dependent variable using a split-plot ANOVA design including species and style position as fixed effects, plant nested within species as a random effect, and the two-way interaction of species by style position (JMP IN v3.2.1, SAS Institute, 1989–1999). We tested whether pollen tube growth was equal for outcross and self-pollen in the hermaphrodites of dimorphic species by setting the log ratio of outcross to self-pollen tubes for the cosexual species equal to zero by subtracting the mean value from each data point. We then reanalyzed the model and contrasted the dimorphic hermaphrodites to the cosexual species (i.e., to the null expectation that outcross equals self). Conversely, to test whether pollen tube growth was equal for outcross and self in the cosexual species, we set the dimorphic hermaphrodites to zero and contrasted the cosexual species to the dimorphic hermaphrodites.

We investigated differences in pollen tube number between females and hermaphrodites of dimorphic *Lycium exsertum* and *L. fremontii* and cosexual *L. parishii* by comparing the number of pollen tubes present 6, 12, 24, and 72 h following outcross pollination. Pollen tube number $[\log_{10}(x + 1)\text{-trans-}$ formed] was analyzed as a split-plot design and included species-morph combination, collection time, and style position as fixed effects, plant nested within species-morph as a random effect, and the two-way interactions of species-morph by collection time, species-morph by style position, and collection time by style position (JMPIN v3.2.1, SAS Institute, 1989–1999). Because sampling intervals were different than the other species, we tested for differences between females and hermaphrodites of *L. californicum* in a separate analysis.

RESULTS

Male sterility, stability of gender expression, and sex ratios—No pollen grains were present in the reduced anthers collected from flowers and buds on female plants, whereas abundant pollen was present in anthers from flowers on hermaphroditic plants. In addition, for all three dimorphic species, plants produced flowers that were either hermaphroditic or female, and field observations in populations confirmed the presence of a single floral type within plants with few exceptions. Females and hermaphrodites were present in all populations of dimorphic *L. californicum*, *L. exsertum*, and *L. fremontii* encountered in the field, and inspection of herbarium material indicated the presence of females throughout much of the species' ranges (Fig. 1).

Plants of all three species were consistent in their sexual expression across years, and the presence of switching was essentially absent. None of the *L. californicum* or *L. exsertum* plants changed sex expression across the 4 or 6 yr, respectively. One individual plant of *L. fremontii*, originally identified as a hermaphrodite in 1995, was recorded as female in the following year and then as hermaphrodite in subsequent years. As this plant was growing interspersed with at least two other plants, this switch may have been a mistaken observation. The frequency of females and hermaphrodites in natural populations was not significantly different from a 1 : 1 ratio (pooled $G = 10.29$, df $= 7$).

Fruit and seed production—Fruit production for hermaphrodites in all three dimorphic species was near zero (<4%) for all treatments, and there were no significant differences among self, outcross, and controls with the exception of *L. fremontii* (Table 1A). In this species, the controls had significantly higher fruit production than either pollination treatment (for both
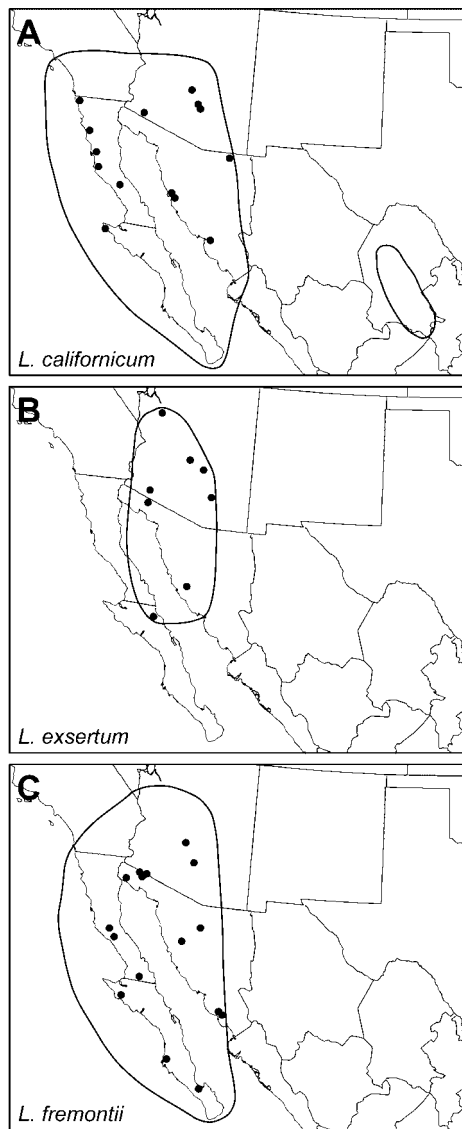
Fig. 1. Location of male-sterile plants in populations of (A) *Lycium californicum*, (B) *Lycium exsertum*, and (C) *Lycium fremontii*, as determined from field observations and herbarium specimens housed at the University of Arizona herbarium (ARIZ). Species distributions are outlined and are taken from Chiang-Cabrera (1981).

not suggest higher seed production for outcrossed flowers in hermaphrodites of dimorphic species.

In contrast to hermaphrodites, females in the dimorphic species had high fruit production in both pollination treatments (Table 1A). Although, on average, pollen augmentation slightly increased fruit production over controls in females of *L. californicum* and *L. exsertum*, these differences were not statistically significant (*L. californicum*, $\chi^2 = 2.76$, df = 1, $P > 0.10$; *L. exsertum*, $F_{1,24} = 2.82$, $P > 0.20$). In contrast, fruit production was significantly lower with augmented pollen than for controls in females of *L. fremontii* ($\chi^2 = 6.02$, df = 1, $P < 0.02$). Fruit production did not vary among seasons for any species, but maternal plant was significant for females of all three dimorphic species (*L. californicum*, $\chi^2 = 26.56$, df = 14, $P < 0.03$; *L. exsertum*, $F_{23,24} = 37.96$, $P < 0.001$; *L. fremontii*, $\chi^2 = 85.64$, df = 21, $P < 0.0001$). However, for *L. californicum*, the interaction between pollen treatment and season was significant ($\chi^2 = 10.48$, df = 2, $P < 0.006$), indicating differences in pollen limitation across seasons. Specifically, in this species, augmented fruit production was higher than control fruit production in two seasons (spring 1997, 1998), but control fruit production was higher during the third season (fall 1997). In the absence of pollen application and pollinators, no flowers on females of *L. californicum* (98 flowers on ten plants), and only three of 178 flowers on 13 plants of *L. fremontii* and two of 94 flowers on 13 plants of *L. exsertum* produced fruit.

Pollen augmentation increased seed number per fruit by more than 1.5 times compared to control fruits for females of dimorphic *L. exsertum* and *L. fremontii* (Table 1B; *L. exsertum*, $F_{1,49} = 26.70$, $P < 0.0001$; *L. fremontii*, $F_{1,4.15} = 10.61$, $P < 0.03$). Because *L. californicum* has only two ovules and consistently produces a two-seeded fruit, pollen limitation for seed number does not seem to occur and was not evaluated in this species. Seed number did not vary among seasons for either *L. exsertum* or *L. fremontii*, but maternal parent explained a significant portion of the variation in seed number for both species (*L. exsertum*, $F_{15,49} = 3.62$, $P < 0.0004$; *L. fremontii*, $F_{19,210} = 7.10$, $P < 0.0001$). In addition, the season by pollination treatment interaction was significant for *L. fremontii* ($F_{3,210} = 3.35$, $P < 0.02$), indicating that the magnitude of pollen limitation varied across seasons. Specifically, seed number for augmented fruits of *L. fremontii* ranged from 1.5 times that of control fruits in spring 1999 to 2.5 times that of control fruits in fall 1997.

In all three cosexual *Lycium* species, outcross pollination resulted in significantly greater fruit production than self-pollination (Fig. 2A). In addition, some plants have higher fruit production than others, regardless of pollination treatment, as indicated by a significant plant main effect for all three species (*L. berlandieri*, $F_{13,23} = 19.97$, $P < 0.001$; *L. pallidum*, $F_{12,26} = 34.96$, $P < 0.001$; *L. parishii*, $\chi^2 = 37.45$, df = 23, $P < 0.03$). Fruit production did not differ among seasons in *L. pallidum* ($F_{1,12} = 0.31$, $P > 0.50$), the only cosexual species for which data were collected in multiple seasons. Outcrossed flowers also produced more fruit than control flowers in all three cosexual species, significantly so in *L. pallidum* and *L. parishii* (Fig. 2A). Autogamous fruit production was rare and occurred in only one of 65 flowers on 13 plants of *L. parishii* and was never observed in either *L. berlandieri* or *L. pallidum*.

Outcrossed fruits produced 2–8 times more seed than selfed fruits in the three cosexual species (Fig. 2B). This difference was significant for *L. berlandieri* and *L. parishii*, but not for

treatments, $F_{1,60} \geq 4.39$, $P < 0.05$). Maternal plant was significant for both *L. exsertum* ($F_{22,45} = 2.10$, $P < 0.02$) and *L. fremontii* ($F_{29,60} = 5.29$, $P < 0.0001$), suggesting that particular hermaphrodites have a greater propensity for female function. There were no significant main effects of season or interactions involving season. Autogamous fruit production was absent from hermaphrodites in all three dimorphic species. Due to low fruit production in hermaphrodites of dimorphic species, it was not possible to statistically analyze the data for seed number per fruit. However, the single hermaphroditic plant of *L. fremontii* that produced fruit for both outcross and self treatments had 20 and 44 seeds, respectively. Average seed number for the outcross treatment in *L. exsertum* hermaphrodites was 9.6 (five fruits from two plants), while the single self-fruit contained seven seeds. These limited data do

TABLE 1.   Fruit and seed production among pollination treatments in females (F) and hermaphrodites (H) of dimorphic *Lycium* species. Values are (A) average fruit production among plants (number of plants in parentheses) and (B) average number of seeds per fruit among plants (number of plants, number of fruit in parentheses) for a particular pollination treatment. For within-species comparisons, means sharing the same superscript do not differ significantly.

	L. californicum		*L. exsertum*		*L. fremontii*	
	F	H	F	H	F	H
A) Fruit production						
Outcross	—	0.000 (17)[a]	—	0.035 (24)[a]	—	0.008 (30)[a]
Self	—	0.000 (19)[a]	—	0.007 (24)[a]	—	0.004 (32)[a]
Control	0.36 (17)[a]	0.012 (19)[a]	0.41 (25)[a]	0.011 (23)[a]	0.63 (25)[a]	0.015 (32)[b]
Augment	0.41 (17)[a]	—	0.54 (25)[a]	—	0.49 (25)[b]	—
B) Seed production						
Outcross	—	—	—	—	—	—
Self	—	—	—	—	—	—
Control	—	—	10.4 (12, 29)[b]	—	16.0 (23, 138)[b]	—
Augment	—	—	17.1 (14, 38)[a]	—	25.6 (23, 99)[a]	—

L. pallidum, which, despite a large difference (25 vs. 11 seeds per fruit), had a low sample size due to the low fruit production of self crosses. Maternal plant explained a significant portion of the variation in seed number in *L. parishii* ($F_{23,33.6} = 2.43$, $P < 0.01$) and *L. berlandieri* ($F_{11,10.9} = 3.19$, $P < 0.04$) but not in *L. pallidum* ($F_{10,29} = 1.50$, $P > 0.19$). Seed number did not differ among seasons in *L. pallidum* ($F_{1,14.9} = 0.01$, $P > 0.93$).

Pollen tube growth—Outcross pollen germinates and produces more pollen tubes than self-pollen in both cosexual *L. pallidum* and *L. parishii* (Table 2, a priori contrast of cosexual species to zero; Fig. 3). In contrast, outcross and self-pollen-tube growth was equivalent in hermaphrodites of dimorphic *L. californicum*, *L. exsertum*, and *L. fremontii* (Table 2, a priori
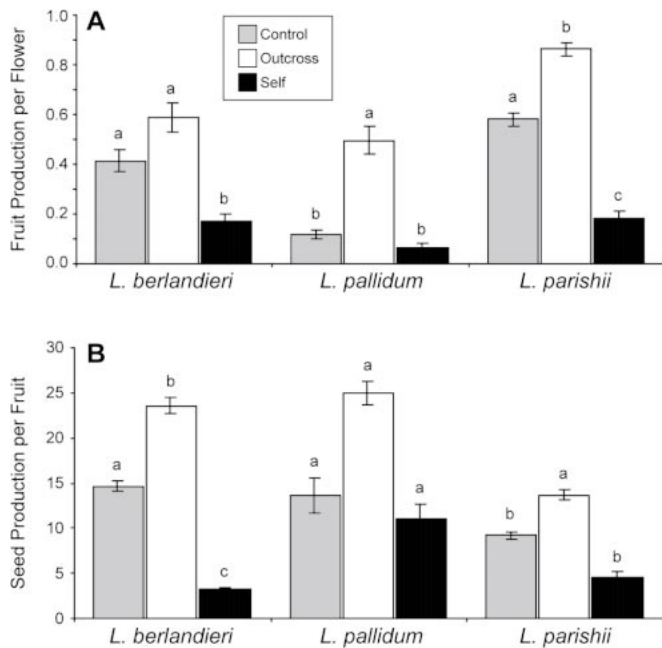

Fig. 2.   Means ± 1 SE for (A) fruit and (B) seed production for three species of cosexual *Lycium*. Flowers were pollinated with either outcross (open bars) or self- (closed bars) pollen and compared to control (shaded bars) fruit and seed production. Significance is indicated by lowercase letters; pollination treatments sharing the same letter are not statistically significant.

contrast of dimorphic hermaphrodites to zero; Fig. 3). Consequently, the log ratio of outcross to self-pollen-tube number was significantly greater in the cosexual species compared to the hermaphrodites of dimorphic species (Table 2).

Using data collected in 1999, we compared outcross pollen tube number between conspecific females and hermaphrodites and cosexual *L. parishii*. In addition to the expected differences due to collection time and style position, there were differences among individual plants in the number of pollen tubes present in styles (Table 3). The species-morph by collection time interaction was also significant (Table 3), but nonetheless, females generally had higher numbers of pollen tubes than conspecific hermaphrodites and cosexual species at all collection times (Fig. 4). More pollen tubes are present in the styles of dimorphic females compared to both conspecific hermaphrodites and cosexual *L. parishii* (Table 3; Fig. 4), whereas pollen tube number is equal for dimorphic hermaphrodites and cosexual *L. parishii* (Table 3; Fig. 4). The significant collection time by style position interaction reflects that at the early collection times pollen tubes had not reached the base of the style. As in the other dimorphic species, the female morph of dimorphic *L. californicum* had more pollen tubes than the hermaphroditic morph ($F_{1,9.4} = 7.62$, $P = 0.0213$) and some plants had more pollen tubes regardless of other treatments ($F_{8,54} = 3.29$, $P = 0.0039$). Collection time was also significant indicating the continued growth of pollen tubes over the course of the experiment ($F_{4,54} = 42.83$, $P < 0.0001$).

DISCUSSION

Male sterility, stability of gender expression, and sex ratios—Plants of *Lycium californicum*, *L. exsertum*, and *L. fremontii* produce flowers that have either reduced stamens and abortive anthers (females) or are morphologically perfect and have both male and female sex organs (hermaphrodites). Pollen was absent from flowers on female plants examined in the field or laboratory and male-sterile plants were found throughout the ranges of all three dimorphic species. Recently, Freeman et al. (1997) predicted that for species that evolve dioecy via the gynodioecious pathway, plants are not apt to show lability in sex expression. Consistent with this idea, lability of gender expression both within and among seasons was absent for the three dimorphic species. Plants are apparently canalized for gender (Lloyd and Bawa, 1984), and the ratio of female to hermaphrodite plants does not differ from 1 : 1.

TABLE 2.   General linear model analyzing the log ratio of the number of outcross to self-pollen tubes for hermaphrodites of dimorphic *Lycium californicum, L. exsertum,* and *L. fremontii* and cosexual *L. pallidum* and *L. parishii.*

Source†	df	Type III MS	F
Species	4	1.935	7.88****
Plant (species)	44	0.268	7.49****
Style position	1	0.037	1.05
Species × style position	4	0.082	2.28
Dimorphic hermaphrodites vs. cosexual species	1	6.645	27.07***
Dimorphic hermaphrodites vs. zero	1	0.016	0.06
Cosexual species vs. zero	1	6.603	26.91***

*** $P < 0.001$ and **** $P < 0.0001$.

† F test denominator df = 40 for all effects, except for species and the contrasts, where df = 45.3.

Gender specialization in Lycium—Our crosses demonstrate that the three morphologically gynodioecious species of North American *Lycium* are, in fact, functionally dioecious. Fruit production in females of *L. californicum, L. exsertum,* and *L. fremontii* greatly exceeds that of hermaphrodites, which in consequence function primarily as males (Table 1). Gender dimorphism is uncommon in Solanaceae, known only from three genera: *Solanum* (Anderson, 1979), *Deprea* (Sawyer and Anderson, 2000), and *Lycium* (Minne et al., 1994; Miller, 2000). In *Lycium*, there have been at least two separate origins of gender dimorphism, one occurring in North America and (at least) one other in Africa (Miller, 2002). Interestingly, all cases of gender dimorphism reported in Solanaceae to date are cryptic in nature, often going undetected (Mayer and Charles-
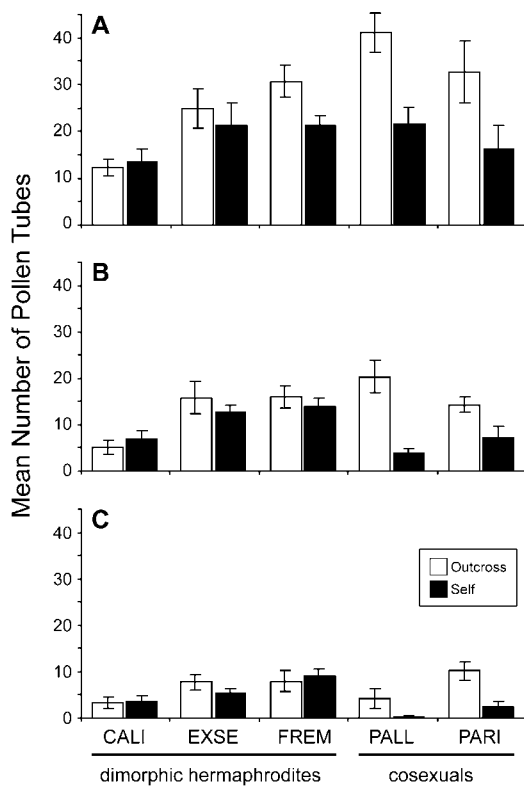

Fig. 3.   Means (± 2 SE) of pollen tube number following pollination with either outcross (open bars) or self- (closed bars) pollen for the hermaphrodites of three dimorphic species and two cosexual species of *Lycium*. Pollen tube number was counted at the (A) top, (B) middle, and (C) base of the style. Species abbreviations are CALI, *L. californicum*; EXSE, *L. exsertum*; FREM, *L. fremontii*; PALL, *L. pallidum*; and PARI, *L. parishii*.

worth, 1991; Sawyer and Anderson, 2000). In North American *Lycium*, male-sterile plants appear perfect but anthers lack viable pollen, and though hermaphroditic plants are morphologically perfect, they produced little fruit or seed in this study.

Pollen limitation for seed number but not fruit production was observed in females of dimorphic *L. fremontii* and *L. exsertum* (Table 1), suggesting that current levels of pollinator visitation are below that needed for full seed set (but see discussion in Bawa and Webb, 1984). If pollen limitation is consistently strong, selection favoring attractive traits (e.g., nectar production, corolla size) might be expected to evolve for females. However, as is common in other gynodioecious species (e.g., Puterbaugh, Wied, and Galen, 1997, reviewed in Eckhart, 1999), there are several morphological correlates associated with male sterility in *Lycium*, such as shorter corolla tubes and more narrow corolla diameters (Miller and Venable, in press). Thus, it is possible that the basis for pollen limitation in females of *L. exsertum* and *L. fremontii* is that pollinators discriminate against the smaller, less conspicuous flowers of females (e.g., Ashman, Swetz, and Shivittz, 2000). We found no evidence for autogamous self-pollination in hermaphrodites and little evidence for agamospermy in females for any of the polyploid dimorphic species. For females of *L. exsertum* and *L. fremontii* it is likely that the few flowers that set fruit were due to pollinator visitation to unopened buds prior to bagging (J. S. Miller, personal observation). Polyploid, dimorphic species of *Lycium* in southern Africa also show no evidence for agamospermy (Minne et al., 1994).

The basis for such low fruit set in hermaphrodites of the dimorphic species is puzzling. For all three dimorphic species, hermaphrodites have larger ovaries than those on conspecific females and ovule number does not differ between females and hermaphrodites for any of the dimorphic species (J. S. Miller, unpublished data). In addition, fruit is produced on hermaphrodites in natural populations of the dimorphic species, and seed collected from hermaphrodites of *L. fremontii* germinates comparably to conspecific females in the greenhouse (J. S. Miller, unpublished data). It is possible that the disproportionately shorter style and smaller stigma of hermaphrodites (compared to females; Miller and Venable, in press) is an early manifestation of specialization of hermaphrodites towards male function. For example, in African dimorphic *Lycium*, hermaphrodite plants of the functionally dimorphic species sometimes have completely vestigial styles and stigmas, but otherwise intact ovaries (Minne et al., 1994). The retention of ovaries in hermaphrodites of dimorphic *Lycium* (in both North America and Africa) may be due to the role of the ovary in nectar production, as *Lycium* species produce nectaries near

TABLE 3.   General linear model analyzing the number of pollen tubes following the outcross pollination treatment for females and hermaphrodites of dimorphic *Lycium exsertum* and *L. fremontii* and cosexual *L. parishii*.

Source†	df	Type III MS	F
Species-morph	4	0.751	3.13*
Plant (species-morph)	24	0.271	3.86****
Collection time	3	3.461	49.22****
Style position	1	3.770	53.62****
Species-morph × collection time	12	0.158	2.24*
Species-morph × style position	4	0.047	0.67
Collection time × style position	3	0.273	3.89*
Dimorphic females vs. dimorphic hermaphrodites	1	1.672	6.96*
Dimorphic females vs. cosexual *L. parishii*	1	1.608	6.70*
Dimorphic hermaphrodites vs. cosexual *L. parishii*	1	0.035	0.15

* $P < 0.05$ and **** $P < 0.0001$.

† F test denominator df = 128 for all effects, except for species-morph and the contrasts, where df = 26.3.

the base of the ovary (Hitchcock, 1932; cf. Sawyer and Anderson, 2000).

Breeding systems in cosexual Lycium—The three cosexual species of *Lycium* studied here were self-incompatible based on fruit and seed production (Fig. 2). Experimental pollinations reveal that outcrossing results in a 19-fold increase, on average, in seed production per flower compared to selfing, and it is unlikely that self-pollen would be successful at fertilizing many ovules in the presence of mixed pollen loads.

Fruit production appeared to be pollen limited in the cosexual species *L. parishii* and *L. pallidum* (Fig. 2A), and seed production was pollen limited in cosexual *L. berlandieri* and *L. parishii* (Fig. 2B). Our results for pollen limitation are consistent with a recent comparative analysis demonstrating that self-incompatible species are often pollen limited (Larson and Barrett, 2000). Interestingly, for all species, neither fruit nor seed production differed among seasons perhaps suggesting that these perennial species effectively buffer environmental variability in these parameters. In addition, we found little evidence for autogamy in the cosexual species.

Our data show reduced fertility following self- compared to outcross pollination, which we interpret as evidence for self-incompatibility. However, self-incompatibility in cosexual *Lycium* is apparently leaky, as observed in many other species

(Ascher, 1984), and some selfed seed is produced raising the possibility that our results may also be due, at least in part, to early-acting inbreeding depression. Indeed, in such leaky self-incompatible species, any seed that is produced from self matings is likely to experience inbreeding depression.

Pollen tube growth—In *Lycium pallidum* and *L. parishii*, pollen tube growth following outcrossing was more successful than following selfing (Table 2; Fig. 3), and evidence of the self-incompatibility reaction (i.e., thick, irregular callose deposition and haphazard pollen tube growth; de Nettancourt, 1977) was observed in self-pollinations. Similarly, pollen tube studies suggest self-incompatibility in *Grabowskia duplicata* (Bianchi et al., 2000), a taxon that phylogenetic analyses place within *Lycium* (Miller, 2002), and Aguilar and Bernardello (2001) have documented late-acting self-incompatibility in *Lycium cestroides*. Gametophytic self-incompatibility is well documented in Solanaceae (de Nettencourt, 1977; Matton et al., 1994; Richman, Uyenoyama, and Kohn, 1996), and molecular work confirms self-incompatibility in *Lycium andersonii* (Richman and Kohn, 2000), which is the sister species of cosexual *L. berlandieri* and a close relative of the dimorphic taxa studied here (Miller, 2002). In addition, phylogenetic analyses of *S*-alleles demonstrate that allelic divergence predates the speciation of several Solanaceous genera including *Lycium* (Richman and Kohn, 2000). The important implication of this work is that self-incompatibility is the ancestral condition for *Lycium* (cf. Brunet et al., 2001).

Whereas the cosexual species are self-incompatible, analyses of the ratio of outcross to self pollen tube number indicates that the hermaphrodites of the three dimorphic species are self-compatible. Determining the compatibility status of the hermaphrodite mating type for the dimorphic species is complicated since hermaphrodites are functionally male (i.e., they have low fruit and seed set). However, while fruit production by hermaphrodites is low (especially compared to conspecific females), it is not uncommon to find fruit on hermaphrodite plants in the field, and in crossing experiments hermaphrodites retain the ability to produce fruit. When fruit are set, seed number in open-collected fruit is slightly, but not significantly, higher in hermaphrodites compared to females for both *L. exsertum* (12.4 seeds/fruit in hermaphrodites vs. 8.7 seeds/fruit in females; $F_{1,35} = 2.87$, $P = 0.10$) and *L. fremontii* (19.1 seeds/fruit in hermaphrodites vs. 16.5 seeds/fruit in females; $F_{1,126} = 2.33$, $P = 0.13$), suggesting that hermaphrodites also retain the ability to set abundant seed. Seed number also does
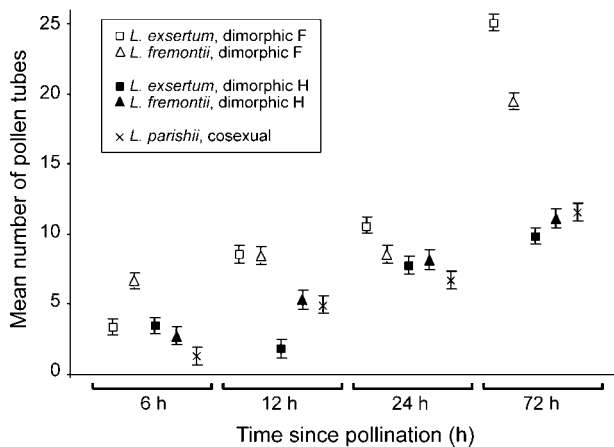

Fig. 4.   The mean (± 1 SE) number of pollen tubes following outcross pollination for females (F) and hermaphrodites (H) of dimorphic species and cosexual *Lycium parishii* as a function of the amount of time following pollination that styles were collected.

not differ between females and hermaphrodites in dimorphic *L. californicum* and is fixed at two seeds per fruit. On average in this study, pollinations on hermaphrodites resulted in ~1% fruit set, making experimental crossing to test for incompatibility intractable. For this reason, we evaluated female fertility prior to seed production (i.e., pollen tube growth). For dimorphic hermaphrodites, pollen tube number following both outcross and self-pollination was similar, whereas in cosexual species pollen tube number was higher following outcross compared to self-pollination (Fig. 3). More to the point, self- and outcross pollen tube number in the hermaphrodites of dimorphic *L. exsertum* and *L. fremontii* are similar to pollen tube number following outcross, but not self-, pollination in the cosexual species (Fig. 3). Thus, the greater number of self-pollen tubes in dimorphic hermaphrodites compared to cosexual species can be attributed to self-fertility in dimorphic hermaphrodites and is not confounded with reduced female function because pollen tube number is similar between dimorphic hermaphrodites and cosexuals (cf. Brunet et al., 2001).

The significantly greater number of pollen tubes present in females of the dimorphic species compared to both conspecific hermaphrodites and cosexual *L. parishii* (Table 3; Fig. 4) is intriguing. This result may be due to the larger stigmatic surfaces of females, which may act as reservoirs for pollen grains to continually germinate and thus accumulate in the style. Increased pollen tube number may have an important advantage for females if pollen tube competition in the style results in more fit offspring (Mulcahy and Mulcahy, 1975; Bertin, 1990; Shykoff, 1992).

Conclusions—Baker's hypothesis (1959) and population genetic models for the evolution of gender dimorphism (Lloyd, 1975; Charlesworth and Charlesworth, 1978a, b) predict that gender dimorphism should evolve in self-compatible groups. However, we have found that gender dimorphism in *Lycium* has evolved on a phylogenetic background of self-incompatibility (Richman and Kohn, 2000; Miller, 2002) and that only hermaphrodites of the polyploid, dimorphic species are self-compatible. Elsewhere we have proposed that polyploidy can be a trigger for the evolution of gender dimorphism in self-incompatible groups (Miller and Venable, 2000) by causing the breakdown of self-incompatibility. Indeed, there is much empirical work demonstrating the effect of polyploidy on self-incompatibility in Solanaceae and other gametophytically controlled groups (reviewed in Stone, 2002). A further test of this hypothesis could be conducted by experimentally inducing polyploidy in diploid species to determine the specific effects of polyploidy on incompatibility in *Lycium*. In addition, molecular characterization of *S*-allele diversity in the dimorphic species would help to clarify the number of times self-incompatibility has been lost in this group.

Webb (1999) cites other examples where the breakdown of outcrossing mechanisms initiates selection for separate gender function. For example, in *Aralia* (Araliaceae) increased inbreeding due to the breakdown of dichogamy (temporal segregation of sexual function) with an increase in clone size has resulted in the establishment of gender dimorphism (Barrett, 1984). Webb (1999) suggests that the breakdown of outcrossing mechanisms may frequently be the starting point for the evolution of gender dimorphism. However, the cited scenarios usually involve a background of self-compatibility. This is perhaps the best-documented case of the evolution of dimorphism

following the breakdown of self-incompatibility. Though it is well known that polyploidy is associated with the evolution of autogamy or apomixis (Stebbins, 1971; Richards, 1997), we found no evidence for either here. *Lycium* is exceptional in that polyploidy facilitates transition to an outbreeding strategy.

Detailed studies of particular evolutionary transitions can provide important insights into breeding system evolution. Inclusion of *Lycium* in a study of the distribution of self-compatibility and gender dimorphism would incorrectly conclude that the two traits were not related. However, closer inspection of the dimorphic species and the distribution of compatibility systems reveals that the pattern in *Lycium* is consistent with both Baker's original predictions and theoretical models for the evolution of gynodioecy.

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