

Outcrossing rates and inferred levels of inbreeding depression in gynodioecious *Cucurbita foetidissima* (Cucurbitaceae)

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Sex-specific outcrossing rates and the inbreeding coefficient of adults in two populations of gynodioecious *Cucurbita foetidissima* were estimated using progeny array data from four allozyme loci to compare the frequencies of self-fertilization and the estimated levels of inbreeding depression to predictions from sex ratio theory. The frequencies of self-fertilization by hermaphrodites in both populations were similar and averaged 73 per cent. The outcrossing rate for females in one population was not different from unity whereas the estimated rate of outcrossing by females in the second population ($t = 0.593$, SE 0.178) indicated the occurrence of biparental inbreeding. Despite considerable self-fertilization by hermaphrodites, inbreeding coefficients of adult plants in both populations were not different from zero and thus inferred values of inbreeding depression were not different from one. Electrophoretically inferred levels of inbreeding depression are somewhat in excess of the value of 0.71 (SE 0.07) obtained in a previous field experiment which tested first-year survival of selfed and outcrossed seeds in this long-lived perennial. The high frequency of self-fertilization by hermaphrodites combined with severe inbreeding depression provides a strong selective force which, along with increased seed-set by females, is sufficient to maintain observed frequencies of females in natural populations of *C. foetidissima*.

Keywords: *Cucurbita foetidissima*, gynodioecy, inbreeding depression, outcrossing rate, self-fertilization, sex ratio.

Introduction

Genetic analysis of progeny arrays can provide useful tools for deducing mating patterns as well as selective processes such as inbreeding depression in natural populations (Clegg, 1980; Ritland & Jain, 1981; Ritland, 1990a). Such analyses can be used to test predictions of mating system theory as well as to corroborate evidence from field experiments. Here we use allozyme analysis of progeny arrays from female and hermaphrodite plants of gynodioecious *Cucurbita foetidissima* to estimate outcrossing rates and levels of inbreeding depression in two natural populations. Outcrossing rate and inbreeding depression estimates are used for testing predictions of sex ratio theory for gynodioecy, a sexual polymorphism in which populations consist of female and hermaphroditic individuals. Levels of inbreeding depression inferred from genetic marker data are compared with experimental estimates

for this long-lived perennial plant based on first-year survival in the field of hand-selfed and hand-outcrossed seeds.

Information on morph-specific outcrossing rates and on levels of inbreeding depression in gynodioecious species is of interest for a number of reasons. Models of gynodioecy in which male sterility is controlled by nuclear genes predict that females must gain at least a twofold advantage in female fitness relative to hermaphrodites to persist in natural populations (Lewis, 1941; Lloyd, 1974, 1975; Charlesworth & Charlesworth, 1978). As it is unlikely that male sterility will result in a twofold increase in seed production, large fitness increases in the seeds produced by females relative to hermaphrodites are expected. Such an advantage could result from the fact that the seeds of females must be outcrossed whereas inbreeding depression could reduce the fitness of seeds from self-compatible hermaphrodites. For this mechanism to play a major role in the maintenance of females in natural populations, however, hermaphrodites must self-fertilize at high frequency and their seeds must suffer large fitness losses as a result. Thus substantial

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genetic load must be maintained in these populations despite frequent selfing by hermaphrodites. Quantitative predictions concerning the frequency of self-fertilization by hermaphrodites necessary to explain the frequency of females in gynodioecious populations with nuclear sex determination are straightforward if levels of seed production and inbreeding depression are known (Lloyd, 1976; Charlesworth & Charlesworth, 1978).

Measurement of the selfing rate, together with estimates of the inbreeding coefficient of adults, can provide a noninvasive estimate of the level of inbreeding depression in natural populations (Ritland, 1990a; Dole & Ritland, 1993; Eckert & Barrett, 1994). Inbreeding depression is thought to be a principal selective force in mating system evolution and marker-based estimates may be useful where field estimates are difficult to obtain or unreliable, as is the case for long-lived perennial species where measurement of lifetime fitness is not feasible. However, the accuracy of inbreeding depression estimates based on marker data has been challenged because linkage between marker loci and deleterious fitness alleles can bias the estimate upwards (Charlesworth, 1991). The magnitude of this bias is difficult to assess making comparisons of values obtained from experimental and marker-based approaches useful (see e.g. Eckert & Barrett, 1994). In this paper we use allozyme data to estimate the outcrossing rates and levels of inbreeding depression in two populations of gynodioecious *Cucurbita foetidissima*, a perennial species for which field estimates of the sex ratio, seed production and inbreeding depression have been previously obtained (Kohn, 1988, 1989).

Materials and methods

Study species

Cucurbita foetidissima (Cucurbitaceae) is a xerophytic clonal perennial native to grasslands and deserts of the southwestern US and northern Mexico. Two sex morphs occur in all populations examined. Monoecious plants (hereafter called hermaphrodites) are self-compatible and produce staminate and pistillate flowers similar to those of cultivated squashes. Gynodioecious plants (females) produce only pistillate flowers and comprise on average 32 per cent of individuals in natural populations. Plants have never been observed to change sex and females produce on average 1.5 (SE=0.2) times as many seeds as hermaphrodites (Kohn, 1989).

Cucurbita foetidissima usually inhabits disturbed sites, chiefly roadsides in the area of this study where populations consist of a series of patches each

separated from the next by from tens of metres to a few kilometres. Within patches, from one to a few hundred ramets may occur but usually less than 20. Allozyme analysis indicates that multiple ramets occurring within a patch frequently represent clonal copies of the same genotype (Kohn & Casper, 1992).

Seed collection and allozyme assays

Fruits were collected in early September of 1992 from two populations in southern Arizona. The first location was along USA highway 80 from Granite Pass to 25 km south of the Arizona–New Mexico state line. The second was along Arizona highways 82 and 83 both north and southwest of Sonoita, AZ, but within 20 km of the intersection of the two highways. These populations correspond to the US80 and AZ82-83 populations of Kohn (1989).

One or two ripe fruits were taken from a single individual of each sex that occurred in each patch and were transported to the laboratory. Only a single individual of a given sex was sampled in each patch to avoid sampling clones of the same genotype. Whereas this sampling constraint ensured that seed families represented different maternal genotypes, it made the collection of large numbers of families difficult given the overall rarity of patches and the fact that 74 per cent of patches contain plants of only one sex (Kohn, 1989). After fruit collection, seeds were removed, dried overnight at 40°C and then stored at room temperature until assayed electrophoretically. Ten seeds from a single fruit from each individual were prepared for electrophoretic analysis by soaking overnight in water, removing the seed coats and homogenizing in phosphate grinding buffer (Soltis *et al.*, 1983). The homogenate was absorbed onto Whatman no. 3 filter paper wicks and inserted into 11.5 per cent starch gels. Allozyme variation was assayed at four loci, *ADH-2*, *PGM-2*, *PGI-1* and *PGI-2*, which were known to be polymorphic in the US80 population based on previous work (Kohn & Casper, 1992). Two buffer systems were used: Tris–EDTA–borate pH 8.6 for *ADH*, and histidine–citrate pH 6.5 for *PGM* and *PGI*. Running buffers and staining recipes were as in Soltis *et al.* (1983). All loci were diallelic, except for the triallelic *ADH-2* locus.

Outcrossing rates of females and hermaphrodites and population-level inbreeding coefficients were estimated for each population using a Macintosh Power PC computer and the *MLT* and *ML2T* programs of Ritland (1990b). The *MLT* program implements the multilocus mating system estimation model of Ritland & Jain (1981). *ML2T* estimates the outcrossing rates of two morphs within a population given the assumption that the outcross pollen pools are the same for both

morphs. As the ML2T model uses the entire population data set to calculate pollen allele frequencies, it is expected to give more reliable morph-specific outcrossing rate estimates than those produced by MLT using each morph as a separate population. Standard errors of outcrossing rates and inbreeding coefficients were estimated from 250 bootstrap analyses using families as the unit of observation. Inbreeding depression and its bootstrapped standard error were estimated using the population-level values of s and F produced by the MLT program and the equation:

$$\delta = 1 - \frac{2F(1-s)}{s(1-F)}, \quad (1)$$

where δ is the inbreeding depression defined as one minus the fitness of a selfed seed relative to an outcrossed seed, s is the frequency of self-fertilization and F is the inbreeding coefficient of adult plants (Ritland, 1990a). Population-wide estimates of s and F from the MLT program were used for inbreeding depression estimation because no model exists to estimate morph-specific levels of inbreeding depression in populations with two morphs that differ in outcrossing rate. In addition, the population estimate of F produced by MLT may be more reliable as fewer parameters are estimated than by the ML2T. Uncertainty in the estimate of F is the chief source of large standard errors when inferring levels of inbreeding depression from allozyme data (Ritland, 1990a). The sex ratios of the samples did not differ from previous sex ratio censuses in these same populations (Kohn, 1989).

Results

Outcrossing rates for hermaphrodites were similar in the two populations with an average of 73 per cent of seeds produced through self-fertilization (Table 1). For females, the outcrossing rate estimate for the AZ82-83

population was not significantly different from unity whereas it was substantially lower in the US80 population, although the standard error associated with this estimate is large because of the small sample of families. Despite considerable selfing by hermaphrodites, estimates of the inbreeding coefficient of adults were not different from zero in either population. The lack of excess homozygosity in the adult population indicates that selfed seeds are not contributing to adult generation plants. Thus inferred values of inbreeding depression in these populations are not different from one (Table 1).

Discussion

The electrophoretic evidence indicates that self-fertilization by hermaphrodites is frequent, accounting for more than 70 per cent of their naturally pollinated seeds. However, few if any inbred offspring contribute to the adult plants currently found in populations as the inbreeding coefficients of adults from US80 and AZ82-83 populations were not significantly different from zero. If, as assumed by the model, selfing rates measured today are indicative of those that occurred when the current cohort of adult plants were themselves seeds, then inbreeding depression has apparently eliminated all or nearly all selfed seeds before adulthood. There is little reason to suspect that selfing rates have changed drastically during recent times. Native pollinators, squash bees of the genera *Peponapis* and *Xenoglossa*, continue to be the predominant pollinators of *C. foetidissima* (Hurd & Linsley, 1964; J. R. Kohn, personal observation). However, patch sizes and patch frequencies may have been altered by increased human disturbance over the last 200 years and such demographic changes could affect the mating system.

Are levels of self-fertilization and inbreeding depression sufficient to explain the current frequency

Table 1 Morph-specific multi- and single-locus outcrossing rates (t_m , t_s) and population-level estimates of the inbreeding coefficient (F) and level of inbreeding depression (δ) in two populations of gynodioecious *Cucurbita foetidissima*

Population	t_m	t_s	F	δ	N_{seeds}	N_{families}
US80 (total)	0.451 (0.114)	0.378 (0.108)	-0.114 (0.106)	1.28 (0.3)	334	35
Females	0.593 (0.178)	0.542 (0.185)			124	13
Hermaphrodites	0.272 (0.50)	0.208 (0.042)			220	22
AZ82-83 (total)	0.546 (0.072)	0.512 (0.068)	-0.015 (0.001)	0.96 (0.07)	346	38
Females	0.881 (0.125)	0.794 (0.129)			162	17
Hermaphrodites	0.264 (0.140)	0.219 (0.118)			184	21

Standard errors are in parentheses.

Morph-specific and population-level estimates were produced by Ritland's (1990b) ML2T and MLT programs, respectively.

of females in natural populations? The sex ratio under nuclear sex determination in gynodioecious populations in which inbreeding depression is the sole cause of fitness differences in the seeds produced by females vs. hermaphrodites can be given by:

$$\frac{P}{1-p} = 1 - 2(1-s\delta) \frac{m_h}{m_f} \quad (\text{Lloyd, 1976; Charlesworth \& Charlesworth, 1978})$$

where p is the proportion of females in the population, m_h and m_f refer to the average seed production of hermaphrodites and females, respectively, s is the selfing rate of hermaphrodites and δ is the value for inbreeding depression. Previous investigation has shown that the ratio m_h/m_f is approximately 0.67 and that natural populations contain 32 per cent females on average (Kohn, 1989). In this study, the average frequency of self-fertilization by hermaphrodites was 0.73 and the value of inbreeding depression was 1.0. Thus the predicted proportion of females in the population is 38 per cent, a value slightly in excess of the observed frequency of females in natural populations. Given the imprecision with which individual parameters are measured, the most important conclusion to be drawn is that high levels of selfing by hermaphrodites combined with severe inbreeding depression and increased seed production by females all appear to contribute strongly to the maintenance of high frequencies of females in natural populations. In combination, these selective forces are strong enough to maintain high frequencies of females even under the stringent conditions imposed by nuclear male sterility. Two studies have suggested that sex determination in *C. foetidissima* is nuclear with females heterogametic (Yousef, 1976; Dossey *et al.*, 1981). However, much work remains to be done to confirm the mode of sex determination in *C. foetidissima*. If sex determination involves cytoplasmic-nuclear interactions, as is known for many plant species, conditions for the maintenance of females are less stringent than under nuclear sex determination (Charlesworth, 1981; Delaney *et al.*, 1981; Gregorius & Ross, 1984; Ross & Gregorius, 1985; Couvet *et al.*, 1986; Frank, 1989; Guyon *et al.*, 1991).

While the combined effects of selfing and inbreeding depression appear to contribute strongly to the maintenance of females in *C. foetidissima*, this is by no means a general result in studies of gynodioecious taxa. Inbreeding depression appears to play a leading role in the maintenance of females in gynodioecious *Bidens* (Schultz & Ganders, 1995) and *Shiidea* (Sakai *et al.*, 1989). However, in many other gynodioecious

species, females gain advantages from either maternal effects on seed survival or from direct benefits of male sterility genes on survival, growth and reproduction rather than through inbreeding avoidance (see for example van Damme & van Delden, 1984; Shykoff, 1988; Delph 1990a,b; Ashman, 1992; Eckhart, 1992). In some species, both inbreeding depression and developmental advantages contribute significantly to the advantages that accrue to females (Assoud *et al.*, 1978; Kesseli & Jain, 1984).

One goal of this study was to compare electrophoretically inferred levels of inbreeding depression with values determined from the field experiment reported in Kohn (1988). In that study the level of inbreeding depression from seed through the first year of growth was 0.71 (SE = 0.07), a value somewhat lower than the ones obtained in this study using the electrophoretic inference. The value of inbreeding depression obtained from the field experiment may be an underestimate because plants were not followed to maturity or because experimental planting conditions (1 cm below the soil surface at 26 cm spacing) might have been less severe than average for naturally dispersed seeds. Experiments may commonly underestimate the true value of inbreeding depression if experimental conditions are more benign than the natural situations to which seeds disperse (Dudash, 1990; Barrett & Kohn, 1991). In contrast, the value for inbreeding depression obtained using electrophoretic data may be an overestimate if the genes responsible for inbreeding depression are linked to marker alleles (Charlesworth, 1991). Despite the difference in estimates of inbreeding depression from electrophoretic and experimental data sets, both methods indicate that inbreeding depression is a strong selective force acting in natural populations of *C. foetidissima*.

To date, we are aware of only one other study that directly compared inbreeding depression values from experimental and electrophoretic approaches. Eckert & Barrett (1994) obtained estimates of inbreeding depression from greenhouse data for two populations of *Decodon verticillatus* and from 10 populations examined electrophoretically. In their study, as in this one, inbreeding depression values measured by the experimental approach were lower than those estimated by electrophoresis, although both approaches suggested that the fitness consequences of selfing were severe. In *Bidens sandvicensis*, the mean level of inbreeding depression measured experimentally was 0.91 whereas electrophoretic evidence indicated no excess homozygosity relative to Hardy-Weinberg expectations among adult plants despite population-level selfing rates averaging 0.61 (Schultz & Ganders, 1995). Thus inferred levels of inbreeding depression in

Bidens, if calculated, would not differ from unity and would slightly exceed values measured experimentally. If this pattern of lower experimental than electrophoretic estimates of inbreeding depression continues, it may be difficult to determine which estimation procedure is producing the majority of bias. Nevertheless, the ability to measure inbreeding depression rapidly and in a number of different populations, and the qualitative agreement between electrophoretic and experimental values, makes the electrophoretic approach a valuable tool.

It may at first seem surprising that high levels of inbreeding depression occur in a species that regularly self-fertilizes as selfing might be expected to purge the population of genetic load thus reducing the value of inbreeding depression. However, our data showing high rates of inbreeding depression despite frequent self-fertilization are consistent with estimates of inbreeding depression and outcrossing rates in at least one other gynodioecious perennial (*Bidens sandvicensis*; Schultz & Ganders, 1995) and in many other plants with more conventional breeding systems. Husband & Schemske (1995) compiled estimates from the literature on inbreeding depression and its correlation with outcrossing rate and life history (annual, shrubby perennial, woody perennial). They showed that long-lived species which self-fertilize at frequencies up to at least 50 per cent often suffer heavy inbreeding depression, usually expressed early in the life cycle. In a related paper, Lande *et al.* (1994) show that if genome-wide rates of deleterious mutation are sufficiently high and the selfing rate does not exceed some threshold value (near 0.7 for genome-wide mutation rates near 1), high levels of inbreeding depression will be maintained at equilibrium even if load is caused by highly deleterious recessive mutations. High genome-wide mutation rates may occur in plants, particularly those that are long-lived, because plants do not separate germ and soma and mutations that occur in somatic cell lines leading to reproductive meristems will be passed along in gametes (Klekowski, 1984, 1988). High levels of load can also be maintained at equilibrium in mixed-mating species if the load is highly polygenic (Lande & Schemske, 1985; Charlesworth & Charlesworth, 1987).

If self-fertilization greatly reduces seed fitness, why do hermaphrodites of *C. foetidissima* self-fertilize so frequently? Some self-fertilization in cosexual individuals may be unavoidable, especially in species that are prone to clonal spread (Handel, 1985). Even within a single ramet of a monoecious plant, the ability to coordinate within and among branches so that only pistillate or staminate flowers are open at a given time may be limited. Physiological self-incompatibility would be another way to limit self-fertilization but there may

be no genetic variation for this in *C. foetidissima* as self-incompatibility is apparently unknown in the family Cucurbitaceae (East, 1940; Nettancourt, 1977). Finally, some forms of self-fertilization are not selected against even when inbreeding depression is severe, for instance when self-fertilizations occur after all outcross pollen has arrived and fertilized ovules (Lloyd, 1979, 1992). We have no direct information on the mode of self-fertilization occurring in *C. foetidissima*. However, female and hermaphrodite individuals have similar pistillate flower to fruit ratios and females set 80 per cent as many seeds per fruit as hermaphrodites (Kohn, 1989). As females receive enough outcross pollen to ensure 80 per cent of the per-flower seed-set of hermaphrodites, lack of outcross pollen deposition may, at best, be a partial answer to the question of why high levels of self-fertilization occur in hermaphroditic individuals.

For population AZ82-83 rates of biparental inbreeding appear to be low. The estimated outcrossing frequency of females was 0.88 and was not statistically different from unity whereas the value of $t_m - t_s$ for the population, another estimator of biparental inbreeding (Ritland, 1984), was also negligible (0.034, SE = 0.02). In the US80 population, however, estimated levels of biparental inbreeding were considerable. The multilocus outcrossing frequency of females was estimated to be only 0.593 (SE = 0.178) and the $t_m - t_s$ value for hermaphrodites was 0.073 (SE = 0.02). The small sample size of females from this population makes interpretation of their low effective outcrossing rate problematic and the $t_m - t_s$ estimate is modest; however, it is possible that levels of biparental inbreeding differ from one population to another because of differences in population structure or pollinator behaviour. In seven estimates of the outcrossing rate of females in gynodioecious Hawaiian *Bidens* populations, values ranged between 0.75 and 1.0 with a mean of 0.85 (Sun & Ganders, 1988). Finally, it is notable that in a field experiment, survival of naturally-pollinated seeds of females from the US80 population was 21 per cent less frequent than survival of cross-pollinated seeds of hermaphrodites (Kohn, 1988). While this difference was not statistically significant, it could indicate that seeds of females suffer some deleterious effects of biparental inbreeding. In gynodioecious Hawaiian *Bidens*, Schultz & Ganders (1995) also found slightly lower fitness of naturally pollinated seeds from females compared with those from cross-fertilized hermaphrodites whereas both seed types had considerable fitness advantages over selfed seeds. Using hand-pollinations among siblings, Ashman (1992) demonstrated that biparental inbreeding could significantly reduce the fitness of seeds produced by females of gynodioecious *Sidalcea oregana* spp. *spicata*.

Gynodioecy provides outstanding opportunities to examine selection on reproductive traits. Compensation for male sterility in terms of increased female reproductive success can provide some of our best estimates of the true cost of male function, evaluated in the currencies of growth, seed production and survival (Assoud *et al.*, 1978; Kesseli & Jain, 1984; Kohn, 1989; Delph, 1990a,b; Eckhart, 1992; Ashman, 1992, 1994). In addition to increased seed production, the advantages of outcrossing may compensate females for their lack of male reproductive success and thus gynodioecious plants can provide model systems to study the power of inbreeding depression to select on mating modifiers. In this study, electrophoretic evidence confirmed that hermaphrodites frequently self-fertilize in gynodioecious *Cucurbita foetidissima* and provided very large inferential estimates of inbreeding depression in this species. Large estimated inbreeding depression is consistent with, although somewhat greater than, field experimental measures of this parameter for this species. This paper is one of the first to compare directly field and electrophoretic estimates of inbreeding depression. Similarity between the estimates provides confirmation that marker-based methods will provide useful estimates of inbreeding depression, a parameter of prime importance for mating system evolution, particularly for plants in which experimental approaches are not feasible. In addition, the occurrence of high inbreeding depression in this species underscores the fact that genetic load may not be purged by moderate rates of self-fertilization, a finding consistent with recent theoretical models that assume high genome-wide mutation rates in perennial plants.

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