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Breeding systems of hermaphroditic and gynodioecious populations of the colonizing species *Trifolium hirtum* All. in California

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Summary. A multilocus procedure was used to estimate outcrossing rates in ten roadside populations of *Trifolium hirtum* in California. Three groups of populations were studied: cultivars, hermaphroditic, and gynodioecious (sexually dimorphic) populations. The multilocus outcrossing rate (t_m) varied from 0.05 to 0.43 among populations. Population level t_m estimates were significantly correlated with the observed heterozygosity in gynodioecious populations but not in hermaphroditic populations. The outcrossing rate of hermaphrodites and females was estimated in three gynodioecious populations; the estimates of t_m varied from 0.09 to 0.23 for hermaphrodites and from 0.73 to 0.80 for females. The distribution of outcrossing rates in gynodioecious populations is bimodal. Our results indicate that for the levels of selfing observed among hermaphrodites, inbreeding depression is likely to be a major factor in the maintenance of females in gynodioecious populations.

Key words: Gynodioecy – Colonizing species – Mating system – Isozymes – Population structure

Introduction

Gynodioecy is a breeding system where hermaphrodite and male-sterile (female) individuals coexist in an interbreeding population. Since male-steriles are selected against for the lack of their pollen-donor role, several theoretical models have identified the critical parameters and regions of the parameter space that allow the maintenance of females, either under nuclear or nucleocytoplasmic inheritance (Charlesworth and Charlesworth 1978; Charlesworth 1981). The mating system of the gynodioecious population is often of critical importance. In particular, the outcrossing rate of hermaphrodites affects the conditions for the maintenance of females. In fact, for one case of nuclear inheritance, differences in selfing rate among hermaphrodites may replace differences in seed fertility as a mechanism for maintaining females (Gregorius et al. 1982). Overdominance at a sex-controlling locus with nuclear inheritance is one possible mechanism for the maintenance of females. However, high selfing rates make the conditions for the maintenance of females more restrictive, requiring stronger heterozygote advantage (Ross and Weir 1975). In contrast, females are most likely to be maintained by overdominance at the sex-controlling locus if the outcrossing rates are high (Ross and Weir 1975). Under models of general heterosis, Charlesworth and Charlesworth (1978) found that the condition for the spread of a nuclear gene for male sterility is given by:

$$1+k>2(1-s\delta),$$

where k represents the mean ovule production of females relative to that of hermaphrodites, s is the selfing rate, and δ represents the inbreeding depression. Under such a scenario, the most favorable conditions for the establishment of gynodioecy occur when there are fairly high levels of both selfing and inbreeding depression and a higher ovule production by females relative to hermaphrodites (Charlesworth and Charlesworth 1978). Similarly, some of the conditions for the "protectedness" of gynodioecy critically depend on the values of the selfing rate of hermaphrodites when male sterility has a nucleocytoplasmic basis (Ross and Gregorius 1985). In a simulation study of a nucleocytoplasmic model, Frank (1989) found that as the selfing rate and inbreeding depression increase, the median percentage of females tends

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to increase. Therefore, under both modes of inheritance of male sterility, a detailed knowledge of the mating system is of critical importance.

Since the mating system determines the generational transition of genotypic frequencies in a population (Ritland 1988), it is no surprise to find empirical evidence that breeding systems have predictable and large effects on the population structure of plant populations (Jain 1975; Brown 1979). Several studies have documented significant correlations between outcrossing rates and population genetic structure in hermaphroditic (Holtsford and Ellstrand 1989) and heterostylous populations (Glover and Barrett 1987). However, very few studies have examined the relationship between mating system and population genetic structure in gynodioecious populations.

We have recently observed that male sterility occurs within Californian populations of *Trifolium hirtum*. In this paper we present some data on the variation in the mating system of hermaphroditic and gynodioecious populations of *Trifolium hirtum*, as a part of a broader study dealing with the maintenance of male sterility and its role in the colonizing ability in rose clover. Rose clover is an annual legume native to the Mediterranean region that was introduced into California during the 1940s as a desirable forage species for range lands (Love 1985). Apparently, the introduction of rose clover in California was not accompanied by a reduction in its genetic diversity. In fact, the number of multilocus genotypes has increased, and the polymorphic loci that were introduced have been maintained (F. Molina-Freaner and S. K. Jain, in preparation). During the late 1960s this species was observed actively colonizing roadside areas in several counties (Jain and Martins 1979). Jain and Martins (1979) measured the outcrossing rates of a group of hermaphroditic populations from pasture and roadside sites in California. Although not significantly different, the outcrossing rates were slightly higher in roadside colonies (0.051) than in pasture populations (0.038). In order to survey the mating system variation in roadside habitats of California we used isozyme polymorphisms to estimate the multilocus outcrossing rates of ten roadside populations. The inheritance and geographic distribution of male sterility will be reported in another paper.

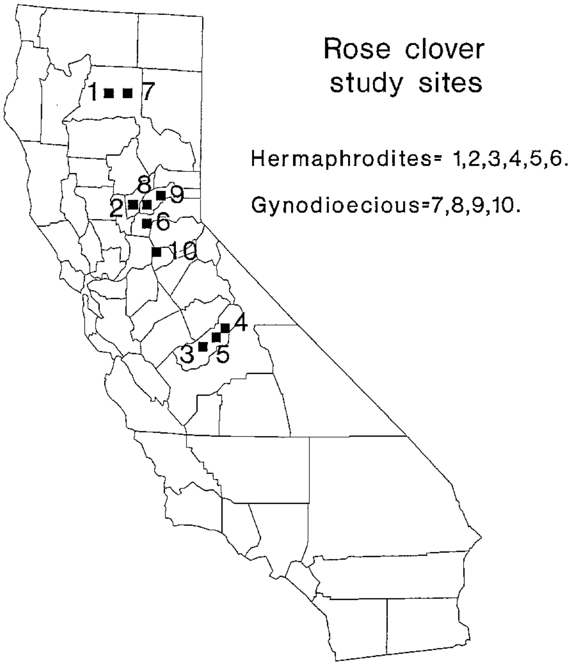

Fig. 1. Location of populations. The numbers refer to the list of populations of Table 1

Table 1. Populations used for outcrossing rate estimation

Group	Population	Year	Family	Individuals/ family
Cultivars				
	1. Deschutes (Hykon)	1988	49	5
		1989	80	5
	2. Grass Valley (Hykon)	1988	60	5
Hermaphroditic populations				
	3. Madera (A)	1989	80	5
	4. Madera (B)	1989	80	5
	5. Madera (C)	1990	40	5
	6. Auburn	1988	50	5
		1989	80	5
		1990	30	5
Gynodioecious populations				
	7. Bear Creek	1988	50	5
		1989	80	5
		1990	54 (27/27)[a]	5/10[a]
	8. Nevada City	1988	50	5
		1989	80	10
		1990	75 (29/46)[a]	5/10[a]
	9. Tahoe National Forest	1989	59	5
	10. Sacramento	1990	60 (30/30)[a]	5/10[a]

[a] Hermaphrodites and females, respectivley

Material and methods

Seed families were collected over a 3-year period (1988–1990) during June-July from a total of ten populations of rose clover in California. The number of families, number of individuals per family, and the years in which populations were analyzed are shown in Table 1. The locations of populations used in this study are shown in Fig. 1. The selected sites include populations from the south, middle, and north part of the Central Valley and Sierra Foothills. Three groups of populations were included (Table 1): (a) two populations (1 and 2) that represent the Australian cultivars that have been introduced by the California Highway Division (F. Molina-Freaner and S. K. Jain, in preparation); (b) hermaphroditic populations; and (c) gynodioecious populations that include the range of variation in male sterility found in California (Molina-Freaner and Jain, in preparation). During 1988 five populations were studied, and in each population infrutescences (heads) were collected from randomly chosen plants. During 1989 seven populations were analyzed, and 80 seed families were collected from six of the seven populations (Table 1). Within each population, 20 families were collected at each of four patches separated at least 8 m from each other along a linear transect. At each patch, plant density was qualitatively scored using an arbitrary scale: less than 4 individuals (low), between 4 and 12 individuals (medium), and more than 12 individuals (high) in a quadrat of 25 by 25 cm. In three populations the average plant size per patch was estimated by harvesting and taking the mean above-ground dry weight of a sample of 50 individuals per patch. In two sites with gynodioecious populations (Bear Creek and Nevada City) the percentage of male sterility was estimated by sampling flowers from each of 50 individuals around each of the four patches that were sampled. The anthers were observed under a microscope and the sex phenotype was determined. Pearson's and/or Spearman's rank correlation coefficients were calculated among the multilocus outcrossing rate, plant density, plant size, and percentage of male sterility of different patches. During 1990 five populations were studied, and 200 individuals were tagged and their sex phenotype determined by taking one flower from each of them at the three gynodioecious populations. At the end of the season seed families were collected from individuals whose sex phenotype was known.

Prior to electrophoresis, seeds from each family were scarified and germinated on petri dishes. The seed coat was removed 24 h after scarification, and crude extracts from the cotyledons and emerging radicle were electrophoresed in 12% starch gels. Three enzymes systems (PGI, ME, and EST) determined by a previous study to be polymorphic (Molina-Freaner and Jain, in preparation) were used for the estimation of mating system parameters. The exceptions were 'Deschutes' and 'Grass Valley' (cultivars, Table 1), where only two loci were used. Two buffer systems were used to assay the three enzyme systems (F. Molina-Freaner and S. K. Jain, in preparation). A histidine gel was used to assay PGI-2 and ME, and a TRIS-borate gel was used to score EST. The genetic control of the PGI-2, ME, and EST zones was worked out from segregation data obtained from progenies of heterozygous plants. The three zones showed simple Mendelian control.

Estimates of multilocus outcrossing rate (t_m) were calculated using MLT (Ritland 1990), a program that is based on the multilocus model of Ritland and Jain (1981). The most likely maternal genotype of each family was inferred by the method of Brown and Allard (1970), and the outcrossing rate was estimated via the Newton-Raphson method. Standard errors of the t_m estimates were calculated by the bootstrap method. Each estimate of the standard error is based on 100 bootstraps. The goodness-of-fit of the data to the assumptions of the mixed mating model was evaluated by a Chi-square test. For the gynodioecious populations (in 1990) the multilocus outcrossing rate estimates were calculated for hermaphrodites and females using ML2T (Ritland 1990). In this program it is assumed that all parameters are identical for hermaphrodites and females, with the exception of the outcrossing rates of the two groups. The outcrossing rate of the population was estimated in this case as a mean, weighted by the frequencies of females and hermaphrodites.

Mean observed heterozygosities (H) for the three loci were calculated for the set of maternal genotypes from each population. When the heterozygosity of hermaphrodite and female mothers was statistically different, the calculated mean heterozygosity of the population was weighted by the frequencies of females and hermaphrodites. Wright's fixation index (F) was estimated for each population as a minimum variance average over loci for the set of maternal genotypes. The expected value of the equilibrium coefficient of inbreeding due to selfing alone was calculated from the multilocus outcrossing rate t_m as $F_{eq} = (1 - t_m)/(1 + t_m)$. The quantity ΔF represents deviation of the fixation index among mothers (F) from the expected value that assumes selfing to be the only source of departure from the Hardy-Weinberg equilibrium.

Results

The estimates of t_m for each of the four patches analyzed during 1989 are shown in Table 2. The average range of variation across patches for the set of six populations was 0.24. However, this variation was not correlated with either plant density or plant size (Table 3). The only exception was Madera B, where t_m was negatively correlated with plant density. In contrast, in both gynodioecious populations the percentage of male sterility was positively correlated with t_m (Table 3).

The estimates of t_m for hermaphrodites of gynodioecious populations varied from 0.099 to 0.235 (Table 4), while t_m ranged from 0.737 to 0.807 in females (Table 4). The difference between t_m and t_s (mean of single-locus

Table 2. Spatial variation within populations for the multilocus estimate of outcrossing rates (t_m) in rose clover during 1989. Standard error in parentheses

Population	Patch 1	Patch 2	Patch 3	Patch 4
Deschutes	0.32 (0.09)	0.27 (0.13)	0.08 (0.04)	0.42 (0.14)
Madera A	0.30 (0.14)	0.27 (0.08)	0.09 (0.03)	0.11 (0.03)
Madera B	0.29 (0.11)	0.23 (0.10)	0.10 (0.09)	0.14 (0.07)
Auburn	0.17 (0.04)	0.21 (0.05)	0.10 (0.03)	0.10 (0.04)
Bear Creek	0.22 (0.05)	0.00 (0.04)	0.20 (0.06)	0.13 (0.03)
Nevada City	0.47 (0.11)	0.49 (0.08)	0.12 (0.06)	0.13 (0.02)

Table 3. Pearson's (r) and Spearman's (r_s) correlation coefficients between the multilocus outcrossing rate (t_m) and plant density (d), plant size (s), and percentage of male sterility (% ms) for several populations of rose clover during 1989

Variables/ parameter	NC	BC	MA (B)	MA (A)	AUB	DE
t_m and d						
r_s	0.10	−0.50	−0.85*	−0.05	0.40	−0.05
t_m and s						
r	−0.79	0.04	−0.04			
r_s	−0.70	−0.20	−0.15			
r_m and % ms						
r	0.96*	0.90				
r_s	0.94*	0.93*				

* $P < 0.05$

NC, Nevada City; BC, Bear Creek; MA (B), Madera B; MA (A), Madera A; AUB, Auburn; DE, Deschutes

Table 4. Multilocus outcrossing rate (t_m) and average single-locus outcrossing rate (t_s) for hermaphrodites and females in three gynodioecious populations of rose clover during 1990. Standard errors in parentheses

Population	% females	Hermaphrodites			Females		
		t_m	t_s	t_m-t_s	t_m	t_s	t_m-t_s
Nevada City	39	0.235 (0.05)	0.218 (0.05)	0.017	0.737 (0.06)	0.681 (0.06)	0.056
Bear Creek	12	0.099 (0.05)	0.094 (0.04)	0.005	0.807 (0.05)	0.752 (0.05)	0.055
Sacramento	08	0.188 (0.07)	0.168 (0.06)	0.020	0.742 (0.05)	0.708 (0.05)	0.034

Table 5. Multilocus outcrossing rates (t_m), equilibrium inbreeding coefficient (F_{eq}), minimum variance fixation index among mothers (F), $\Delta F(=F-F_{eq})$, and observed heterozygosity among mothers (H) for several populations of rose clover

Group/Population	Year	t_m (SE)	F_{eq}	F (SE	ΔF	H (SD)
Cultivars						
Deschutes	1988	0.08 (0.03)	0.83	0.73 (0.12)	−0.10	0.04 (0.02)
	1989	0.25 (0.03)	0.59	0.50 (0.11)	−0.09	0.07 (0.03)
Grass Valley	1988	0.26 (0.05)	0.57	0.34 (0.18)	−0.23	0.12 (0.04)
Hermaphroditic populations						
Madera (A)	1989	0.12 (0.03)	0.77	0.50 (0.07)	−0.27	0.16 (0.04)
Madera (B)	1989	0.16 (0.02)	0.71	0.71 (0.05)	0.00	0.14 (0.04)
Madera (C)	1990	0.29 (0.05)	0.54	0.57 (0.11)	0.03	0.04 (0.03)
Auburn	1988	0.10 (0.02)	0.80	0.37 (0.08)	−0.43*	0.27 (0.06)
	1989	0.14 (0.02)	0.75	0.45 (0.05)	−0.30*	0.21 (0.05)
	1990	0.25 (0.05)	0.59	0.44 (0.12)	−0.15	0.17 (0.07)
Gynodioecious populations						
Bear Creek	1988	0.24 (0.05)	0.61	0.54 (0.06)	−0.07	0.24 (0.06)
	1989	0.11 (0.02)	0.80	0.72 (0.09)	−0.08	0.14 (0.03)
	1990	0.18 (0.06)	0.30	0.25 (0.08)	−0.05	0.37 (0.07)
Nevada City	1988	0.32 (0.06)	0.50	0.29 (0.11)	−0.21	0.33 (0.07)
	1989	0.21 (0.01)	0.64	0.53 (0.05)	−0.11	0.24 (0.05)
	1990	0.43 (0.07)	0.34	0.16 (0.10)	−0.18	0.37 (0.06)
TNF	1989	0.05 (0.01)	0.89	0.42 (0.13)	−0.47*	0.17 (0.05)
Sacramento	1990	0.23 (0.07)	0.43	0.42 (0.10)	−0.01	0.27 (0.06)

Significant differences of ΔF are denoted by $P<0.05$

estimates) was small and always positive, and a little larger for females. The levels of heterozygosity were the same for female and hermaphrodite mothers in Nevada City and Bear Creek populations (0.38 vesus 0.37 and 0.37 versus 0.37, respectively). In contrast, females showed a higher level of heterozygosity in Sacramento (0.35 versus 0.23). As expected, the progeny of females showed higher levels of heterozygosity in all populations (data not shown).

The multilocus outcrossing rates (t_m) varied from 0.05 to 0.43 among populations (Table 5). For the cultivars, t_m ranged from 0.08 to 0.26 (range = 0.18); for hermaphroditic populations, from 0.10 to 0.29 (range = 0.19); and for gynodioecious populations, from 0.05 to 0.43 (range = 0.38). Within populations, t_m varied among years from 0.08 to 0.25 (range = 0.17) for Deschutes, and from 0.10 to 0.25 (range = 0.15) for Auburn. For the gynodioecious populations, t_m ranged from 0.11 to 0.24 (range = 0.13) for Bear Creek and from 0.21 to 0.43 (range = 0.22) for Nevada City. Chi-square tests of the goodness-of-fit of genotypic data were not significant for the cultivars and hermaphroditic populations, suggesting that the data fit the assumptions of the mixed mating model. In contrast, some loci from three gynodioecious populations violated the assumptions of the mixed mating model: Pgi and Est for Bear Creek in 1988 ($P<0.05$), Pgi and Est for Nevada City in 1989 ($P<0.01$), and Me for Tahoe National Forest (TNF) in 1989 ($P<0.05$).

In all populations the estimates of fixation indices were positive and the values usually greater than 0.3 (Table 5). Although statistical differences were detected in just three cases, the equilibrium inbreeding coefficients (given by $F_{eq}=1-t_m/1+t_m$) were in most cases greater than the fixation indices, and therefore the value of ΔF was consistently negative (Table 5).

The mean heterozygosity of the gynodioecious group was statistically higher than that of the other two groups (cultivars and hermaphrodites) showing, as expected, that male sterility tends to increase the heterozygosity of

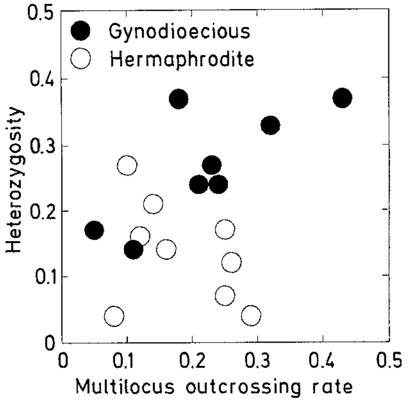

Fig. 2. The mean heterozygosity of maternal plants plotted as a function of the multilocus outcrossing rate (t_m) of gynodioecious and hermaphroditic populations of rose clover

Table 6. Mean heterozygosity (three loci) and single-locus heterozygosity among groups of populations of rose clover

Group	*n*	Pgi (SD)	Est (SD)	Me (SD)	Mean H (SD)
Cultivars	3	0.01 (0.01)	0.23 (0.11)	0.00 (0.00)	0.07a (0.04)
Hermaphrodites	6	0.15 (0.10)	0.22 (0.11)	0.12 (0.07)	0.16a (0.07)
Gynodioecious	8	0.23 (0.12)	0.27 (0.11)	0.30 (0.10)	0.27b (0.09)

Significant differences ($P < 0.05$) for the mean heterozygosity are denoted by different letters

the population (Table 6). The higher heterozygosity of the gynodioecious group is mainly due to the heterozygosity of Pgi and Me. This is particularly true for the hermaphroditic population because in the case of the cultivars, Pgi and Me tend to be fixed (Table 6). There seems to be a linear correlation between the multilocus outcrossing rate (t_m) and the mean observed heterozygosity (H) ($r = 0.75$, $P < 0.05$) for the gynodioecious group (Fig. 2). However, such a relationship is not detected ($r = -0.39$, $P = 0.28$) from the hermaphroditic group (Fig. 2).

Discussion

In rose clover the overall range of variation in population outcrossing rates is restricted to values below 0.5 (Table 5). However, the range of variation exhibited by the hermaphroditic populations is clearly larger than the range observed in inbreeding species such as *Bromus mollis* (Brown et al. 1974) or *Hordeum spontaneum* (Brown et al. 1978). The range observed in rose clover is more similar to that observed in *Lupinus affinis* and *L. bicolor* (Harding et al. 1974). Compared to values reported in studies of intraspecific variation in the mating system of other gynodioecious species (Kesseli and Jain 1984; Wolff et al. 1988), rose clover exhibits higher selfing rates. The range reported here is larger than that reported earlier for pasture and roadside populations using morphological markers (Jain and Martins 1979), suggesting that the range of variation of t has probably increased in recent years. The values of t_m reported here may be partially responsible for the high number of multilocus genotypes and the widespread maintenance of the three polymorphic loci introduced to California (F. Molina-Freaner and S. K. Jain, in preparation).

In general, the estimated values of the inbreeding coefficient, F, correspond to their expectations based on the multilocus estimate of t (Table 5). The difference between F and F_{eq} (ΔF) is in most cases negative (Table 5), suggesting an excess of heterozygotes with respect to the expected value if inbreeding was due only to selfing. These values of ΔF exhibit a pattern similar to the one observed for some inbreeders, referred to as the "heterozygosity paradox" by Brown (1979). However, because there are many factors that may bias the estimation of t (Ennos and Clegg 1982; Hedrick and Ritland 1990), and hence the expected value of F_{eq}, the "paradox" may be more the result of the lack of precision of outcrossing estimates than of any biological process.

As expected, the outcrossing rate has an effect on the heterozygosity of the gynodioecious populations (Fig. 2). Because male sterility is correlated with t_m, this relationship is reflected in the levels of heterozygosity observed in gynodioecious populations as compared to hermaphroditic populations (Table 6). However, other factors may be involved within the hermaphroditic populations because there seems to be no correlation between t_m and H (Fig. 2). The relationship between t_m and H in gynodioecious populations is further supported by the correlation found between t_m and male sterility among patches in two gynodioecious populations (Table 3). In general, there was no detectable effect of plant density and size on t_m (Table 3). As has been found in other cases, the effects of these ecological factors are highly idiosyncratic, depending on particular attributes of the species in question (Barrett and Eckert 1990).

The estimates of t_m for hermaphrodites and females of rose clover are clearly non-overlapping (Table 4). The pattern observed in rose clover is quite different from patterns observed in other gynodioecious species where intraspecific variation in t_m has been reported (Table 7). In *Thymus vulgaris*, *Limnanthes douglasii*, and *Plantago coronopus* (Table 7) the estimates of t for hermaphrodites are greater than 0.4. Other species like *Silene vulgaris* (Charlesworth 1989) and *Bidens* spp (Sun and Ganders 1988) show high outcrossing rates for hermaphrodites. In that respect, rose clover is probably more similar to the cases of male sterility in cultivated inbreeders like barley (Jain and Suneson 1964). However, in order to show that

Table 7. Outcrossing rates (t) of hermaphrodites (her) and females (fem) in gynodioecious species

Species	t (her)	t (fem)	Reference
Thymus vulgaris	0.51–0.90	?	Valdeyron et al. (1977)
Limnanthes douglasii	0.75–0.95	?	Kesseli and Jain (1984)
Plantago coronopus	0.45–0.90	0.97–1.00	Wolff et al. (1988)
Trifolium hirtum	0.09–0.23	0.73–0.80	This study

the distribution of outcrossing rates in gynodioecious populations of rose clover is really bimodal, it is necessary to estimate t_m for individual families of hermaphrodites and females.

If nuclear control of male sterility is assumed, overdominance at the sex-controlling locus is an unlikely mechanism for the maintenance of females because under the levels of selfing observed for rose clover, the conditions for the maintenance of females are quite restrictive (Ross and Weir 1975). On the other hand, the estimated levels of selfing suggest that regardless of whether male sterility is under nuclear or nucleocytoplasmic control, inbreeding depression (or general heterosis) is a possible or indeed a more likely mechanism for the maintenance of females (Charlesworth and Charlesworth 1978; Frank 1989). The relative excess of heterozygotes indicated by the negative values of ΔF is at least consistent with such a mechanism. Experiments designed to measure the level of inbreeding depression in the field are in progress and will be reported in another paper.

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