

A MANIPULATIVE EXPERIMENT TO ESTIMATE BIPARENTAL INBREEDING IN MONKEYFLOWERS

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Biparental inbreeding occurs when plants receive pollen from genetically related neighbors. The frequency of biparental inbreeding in natural populations is unknown but directly relevant to the evolution of plant mating systems. We suggest a simple manipulative experiment to distinguish the effects of biparental inbreeding from those of self-fertilization. The basis of the method is to compare the levels of inbreeding in plants with and without the potential to outcross with genetic relatives. We eliminate the potential for biparental inbreeding by transplanting seedlings to different locations within a population. The level of inbreeding is then estimated from homozygosity at molecular markers. This method is applied in a study of two natural populations of *Mimulus guttatus* (the yellow monkeyflower) using microsatellites as marker loci. In contrast to previous studies of this species, our study finds no evidence of biparental inbreeding in either population.

Keywords: inbreeding, mating systems, *Mimulus guttatus*, population structure.

Introduction

Since Darwin (1877a, 1877b, 1877c), evolutionary biologists have taken a great interest in the reproductive strategies of flowering plants. Many plants have elaborate adaptations to avoid self-fertilization and to successfully exchange pollen with other plants. However, outcrossing does not ensure outbred progeny. Natural populations frequently exhibit a distinct spatial structure owing to limited seed dispersal (Bradshaw 1972; Levin 1988). As a consequence, neighboring plants may often be genetic relatives. Since pollinators frequently move among neighboring plants (Pyke 1978; Waser 1986), outcrossed seed may result from matings between genetically related individuals. This process has been termed “biparental inbreeding” to distinguish it from self-fertilization (Uyenoyama 1986).

How frequent is biparental inbreeding in nature? The question is relevant to a number of evolutionary problems, including the prevalence and maintenance of mixed-mating reproductive systems. Mixed-mating plants reproduce by a mixture of outcrossing and self-fertilization. The selfing rate, s , is the proportion of seed derived from self-fertilization (many studies actually estimate the outcrossing rate, t , where $t = 1 - s$). Either s or t can be estimated from progeny arrays, i.e., sets of plants derived from a single mother that have been genotyped at molecular marker loci (Fyfe and Bailey 1951; Brown and Allard 1970; Clegg et al. 1978; Jain 1979; Ritland and Jain 1981). Intermediate estimates of s ($0 > s > 1$), which indicate mixed mating, have been obtained from many species (Lloyd 1979; Barrett and Eckert 1990; Vogler and Kalisz 2001).

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The classical mixed-mating model equates outcrossing with random mating. However, if outcrossing involves biparental inbreeding, estimates of the selfing rate will be upwardly biased (Ennos and Clegg 1982; Ritland 1984). Genetic studies of *Plantago lanceolata* illustrate the potential magnitude of this bias. Applying the mixed-mating model to molecular marker data yields estimates for the selfing rate of up to 16% (van Dijk et al. 1988; Tonsor et al. 1993). However, as this species is known to be self-incompatible, the elevated homozygosity must be attributable to biparental inbreeding and not selfing. Ellstrand et al. (1978) obtained even higher estimates for the apparent selfing rate in the self-incompatible *Helianthus annuus*. These examples prompt the question of whether mixed-mating systems are as common as widely believed.

The apparent abundance of mixed-mating species is surprising in light of results from evolutionary theory (Lloyd 1979; Lande and Schemske 1985). Simple models predict that mixed-mating systems are evolutionarily unstable. Populations should evolve to either complete outcrossing or complete selfing. However, subsequent theoretical work has identified a number of factors that can favor mixed-mating systems (Uyenoyama et al. 1993; Cheptou and Mathias 2001). One of these factors is biparental inbreeding (Uyenoyama 1986). The direct relevance of biparental inbreeding to both the estimation of mating system parameters and the evolution of these systems provides a compelling justification to estimate the frequency of this process in nature.

In this article, we describe a simple field manipulation to estimate the effects of biparental inbreeding in a population. The basic idea is to compare the levels of inbreeding in plants with and without the potential to outcross with genetic relatives. While a population will exhibit spatial structure (relatedness among neighboring plants) if seed dispersal is limited, biparental inbreeding requires that both seed and pollen dispersal are limited (table 1). We can thus eliminate the potential

Table 1
Predicted Population Structure under Different Models of Pollen and Seed Dispersal

Model	Seed dispersal	Pollen dispersal	Genetic relatedness among neighbors	Biparental inbreeding	Homozygosity among progeny of natives and transplants
1	Not limited	Not limited	No	No	Equal
2	Not limited	Limited	No	No	Equal
3	Limited	Not limited	Yes	No	Equal
4	Limited	Limited	Yes	Yes	Higher for natives

for biparental inbreeding by transplanting a seedling to a different location within the population. This ensures that a transplant's neighbors will not be genetic relatives, or that they will at least be no more closely related than two random individuals from that population. Any outcrossed progeny of transplanted individuals will be fully outbred with inbreeding coefficient $f = 0$. In contrast, a native (untransplanted) individual may have related neighbors, and if pollen dispersal is limited, their outcrossed progeny will have an average $f > 0$ (as a result of biparental inbreeding).

Both the spatial genetic structure of a population and the level of inbreeding can be estimated with molecular markers. We use highly polymorphic microsatellite loci to investigate both spatial structure and inbreeding in two populations of *Mimulus guttatus* ($2n=28$; Scrophulariaceae). Commonly known as the yellow monkeyflower, *M. guttatus* is a self-compatible wild flower that occurs throughout western North America (Vickery 1978). Because *M. guttatus* self-fertilizes to some extent in the field, we can partition offspring into three different categories: those derived from selfing (a fraction s of the total), those derived from outcrossing between unrelated plants (a fraction t of the total), and those derived from matings between relatives (a fraction r of the total). Transplanting eliminates matings between relatives and should reduce the average f of progeny (table 1). The predicted difference in offspring homozygosity between transplants and natives depends on both the frequency of biparental inbreeding, as measured by r , and the average relatedness of mates, as measured by the coefficient of coancestry (see Lynch and Walsh 1998, p. 135). While the transplant method can estimate the overall contribution of biparental inbreeding, additional information about the spatial distribution of genotypes and the movement of pollinators is necessary to decompose this contribution into its components (see Ritland 2002).

Material and Methods

The two populations considered in this study, Iron Mountain and Dexter, are annual or winter annual. Iron Mountain is a well-studied site in the Cascade Mountains of Oregon (Willis 1993, 1996). Dexter is a low-elevation site near the town of Dexter in the Willamette Valley (also in Oregon). In June 1997, we initiated two experiments at Iron Mountain. The first investigates the spatial distribution of genotypes within this population. Plants were collected along two parallel transects and genotyped at five microsatellite loci. Along each transect, two plants were collected at each sample point, and

sample points were separated by 0.5 m. This design allows estimation of genetic structure at multiple scales. The relatedness of nearest neighbors can be estimated from the genetic similarity of plants derived from the same sample point. Comparisons among pairs at varying distances provide a picture of the larger "neighborhood structure" of the population (e.g., Loiselle et al. 1995). While we give a brief account of the results in "Discussion," a complete description of the methods and results of this spatial genetic analysis is given in Sweigart et al. (1999).

The second experiment, i.e., the transplant experiment, was performed at both Iron Mountain and Dexter. Four parallel transects were established at each site. Each transect was 25 m long and at least 1 m distant from other transects within the site. Fifty seedlings were tagged along each transect (they were uniformly distributed at 0.5-m intervals) and numbered sequentially. Plants with even identification numbers (2, 4, 6, ..., 200) were randomly transplanted to another even-numbered position within the array. Odd-numbered plants were dug out of the ground and then placed back into the same position to control for the effects of removal. Plants were periodically monitored through maturation and flowering. Fruits were collected from all plants that survived and flowered. We also attempted to preserve and collect tissue from the maternal plants, although many died when removed from the field.

Seeds from these fruits were subsequently planted in the greenhouse, and five plants per family were grown to flower. Corolla tissue was collected from these plants and stored for DNA extraction. Because of germination failure or failure to flower, the number of plants successfully genotyped was fewer than five for many of the families. We used the general PCR reaction conditions and thermocycle programs described in Kelly and Willis (1998) for genotyping, except that the 5' primers were end labeled with infrared (IRD) dyes for visualization with a Li-Cor automated sequencing system. A detailed description of the procedures is given in Fishman et al. (2001). Briefly, the PCR products were resolved on 18 cm of denaturing polyacrylamide gels run on a Li-Cor 4000L automated sequencer according to the gel preparation and loading protocols of Remington et al. (1998). Before gel loading, 5 μ L of formamide loading dye was added to each reaction. The samples were denatured by heating to 75°–85°C for 2 min and then immediately chilled to 4°C. We loaded 1–2 μ L of dye/product mixture into each sample lane (48-well comb) and also ran parental genotypes and/or IRD-labeled size standards in the outermost lanes. We used electrophoretic run parameters of 1000 V, 35 mA, 25 W, 50°C plate temperature, scan speed 3,

signal filter 3, and 16-bit pixel depth for the collection of TIFF image files. We scored fragments by eye in the TIFF image files using the program RFLPSCAN 3.0 (1998; Scanalytics, Fairfax, Va.). The segregating fragments were assigned molecular weights by the program according to the molecular weight standards and the previously determined parental allele sizes.

The progeny from Iron Mountain were scored at five loci: AG 19, AAT 9, AAT 211, AAT 300, and AAT 356. The progeny from Dexter were scored at four loci, which included all of the above except AAT 9. These loci were known to be highly polymorphic. The data from the spatial genetic study yielded estimates for gene diversity (Weir 1996, p. 151) at these loci of 0.93, 0.79, 0.83, 0.81, and 0.89, respectively (Sweigart et al. 1999). These numbers represent the expected frequency of heterozygotes at a locus for a population with the same allele frequencies but no inbreeding (in Hardy-Weinberg equilibrium).

Results

At Iron Mountain, 34 transplants and 30 natives produced sufficient seed to genotype progeny (a total of 118 progeny from transplants and 143 from natives). Not all progeny were successfully genotyped for all five markers. At Dexter, we obtained seed from 25 transplants and 27 natives (a total of 95 progeny from each type of parent). The results are displayed in table 2. The number of distinct alleles that were identified in the sample (natives and transplants combined) is given for each marker locus.

A simple test for biparental inbreeding is to compare levels of heterozygosity among transplanted and native families. As each type of family is derived from parents uniformly distributed over each population, there should be no difference in allele frequencies between classes. Thus, under the null hypothesis of no biparental inbreeding, we expect the frequency of heterozygotes at each locus to be the same within transplanted and native families. For each family, we determined the fraction of individuals that were heterozygotes at each locus. The average values of these proportions (across families) for each locus are given in the last two columns of table 2. There are no significant differences between transplanted and native families at any locus in either population.

Averaging within families was used because a preliminary inspection revealed that individuals within a family are not statistically independent with regard to their determination as outcrossed or selfed. There is a positive correlation of selfing among siblings in the terminology of Ritland (1989). Averaging is thus required because each offspring does not provide independent information about the underlying probabilities of selfing or biparental inbreeding. The negative results in table 2 also prompt a comment on statistical power. Power is a concern because there are substantial quantitative differences in average heterozygosity at some markers. In principle, greater power could be achieved by using a statistic that integrates over loci. However, as most of the differences in table 2 are in the wrong direction (with lower heterozygosity in progeny of transplants), it seems unlikely that such an analysis would change our conclusions. As discussed in greater detail below, a more complete analysis is possible when both maternal and offspring genotypes are known.

Table 2

Number of Alleles and Observed Heterozygosities (with SE) at Iron Mountain and Dexter			
Locus	Number of alleles	Average H transplant	Average H native
Iron Mountain:			
1. AG 19	25	0.65 (0.06)	0.79 (0.05)
2. AAT 9	26	0.49 (0.06)	0.54 (0.07)
3. AAT 211	13	0.54 (0.06)	0.49 (0.06)
4. AAT 300	20	0.37 (0.06)	0.51 (0.07)
5. AAT 356	29	0.55 (0.07)	0.68 (0.05)
Dexter:			
1. AG 19	28	0.77 (0.07)	0.66 (0.07)
2. AAT 211	11	0.64 (0.06)	0.57 (0.08)
3. AAT 300	27	0.40 (0.08)	0.47 (0.08)
4. AAT 356	22	0.58 (0.06)	0.58 (0.08)

Discussion

We find no evidence of biparental inbreeding in two populations of *Mimulus guttatus*. This result is surprising given that bumblebees (*Bombus* sp.) are an important pollinator of *M. guttatus* (Robertson et al. 1999; MacNair and Gardner 1998) and that these animals routinely move among neighboring plants when foraging (Pyke 1978). However, biparental inbreeding requires not only localized mating but also genetic relatedness among neighboring plants (table 1). The spatial genetic study of Iron Mountain reveals that the genetic relatedness of neighboring plants is near zero (Sweigart et al. 1999). While we do not have comparable spatial genetic data for Dexter, the results in table 2 indicate that the inbreeding that does occur in this population must be due to self-fertilization. Thus, both Iron Mountain and Dexter can be safely classified as genuine mixed-mating populations.

The most widely used test for biparental inbreeding employs a comparison between single and multilocus statistics (Ritland and Jain 1981; Shaw et al. 1981; Ritland 1990). Biparental inbreeding will reduce t_s , the average single locus estimate for the outcrossing rate, relative to t_m , the multilocus estimate. Ritland (2002) has recently generalized his estimation programs for t_s and t_m (now titled MLTR) to accommodate microsatellite loci. We applied MLTR to our data and found that natives and transplants yield indistinguishable estimates for t_s and t_m at both Iron Mountain and Dexter (results not shown). Because biparental inbreeding should reduce t_s relative to t_m in natives but not transplants, this analysis is fully consistent with the heterozygosity analysis in table 2.

The agreement of results from the spatial genetic and transplant experiments at Iron Mountain is reassuring. The transplant method indicates the absence of biparental inbreeding where it should not be occurring. Will the transplant method correctly identify biparental inbreeding in populations in which it does occur? C. Muis and C. Eckert (unpublished manuscript) have recently compared direct (transplant) and indirect (t_s vs. t_m) methods to estimate biparental inbreeding in *Aquilegia canadensis*. Using genetic data from two allozyme loci, they document significant spatial genetic structure in three of four populations of this species. There is thus substantial opportunity for biparental inbreeding. However, estimates of the

difference between t_s and t_m suggest that biparental inbreeding is infrequent. Because the indirect method is known to underestimate the incidence of biparental inbreeding (Brown 1990), Muis and Eckert (2002) performed a transplant experiment in two of their populations. Higher homozygosity in the progeny of natives indicates that almost 20% of outcross matings are between relatives. Thus, the indirect method underestimates the actual frequency of biparental inbreeding by more than tenfold. These results provide strong support for the transplant method.

In contrast to this study, Ritland and Ganders (1987) did find evidence for biparental inbreeding in populations of *M. guttatus* from Washington State and British Columbia (see also Leclerc-Potvin and Ritland 1994). They used a different estimation method that relies on information about the spatial distribution of genotypes. In contrast to Iron Mountain, each of the Ritland and Ganders (1987) populations exhibit significant spatial structure. These differences are not surprising given that *M. guttatus* is really a species complex composed of many populations with varying reproductive biologies (Grant 1924; Vickery 1978). Previous studies have demonstrated interpopulation differences in floral morphology (Waser et al. 1982), amount of genetic variation for floral traits (Carr and Fenster 1994; Robertson et al. 1994), selfing rate (Ritland and Ganders 1987; Willis 1993; Awadalla and Ritland 1997), and timing of selfing over the life span of the flower (Dole 1990, 1992; Dudash and Ritland 1991; Leclerc-Potvin and Ritland 1994; Arathi et al. 2002). We can now add the incidence of biparental inbreeding to this list.

While the diversity of reproductive attributes among *M. guttatus* populations certainly merits detailed consideration, the main purpose of this article is to introduce the transplant method for estimating biparental inbreeding. In this application of the method, we used variation at highly polymorphic microsatellite loci. These loci provide excellent materials to estimate mating system parameters. Because each locus has a large number of relatively infrequent alleles, an outbred individual will be heterozygous at most microsatellite loci. For the loci considered in this study, the probability of heterozy-

gosity ranges from 0.79 to 0.93 per locus (Sweigert et al. 1999). Multilocus homozygosity is thus a compelling indicator of inbreeding. With highly polymorphic loci, homozygosity is strongly linked to identity by descent. Inferring the latter from the former is an essential feature of mating system estimation.

In this study, we were unable to determine the genotypes for most of the maternal parents. Future applications of the transplant method should endeavor to obtain these data because they allow several important refinements of the analysis. First, since the maternal parents are sampled from known positions within the population, these data are directly informative about the spatial genetic structure of the population (Heywood 1991). Second, knowledge of the maternal genotype greatly facilitates analysis of the progeny. For the families in this study for which we were able to determine maternal genotype, outcrossed progeny can easily be distinguished from self-fertilized progeny by simple inspection of their genotypes. Selfed offspring only have alleles that are present in the mother, whereas outcrossed progeny are very likely to have a foreign allele at one or more loci. When it is possible to subdivide progeny in this way, biparental inbreeding can be measured as increased homozygosity within outcrossed progeny. This greatly increases statistical power and should allow transplant experiments to detect even low levels of biparental inbreeding.

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