EXPLORATION OF MOLECULAR METHODS FOR DETECTING INDIVIDUAL INBREEDING LEVELS IN *MIMULUS*

by

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Abstract

Inbreeding directly affects fitness and population dynamics by way of inbreeding depression and is particularly prevalent in plants and in small populations. While the importance of measuring fitness and inbreeding levels in natural populations is clear, it is difficult to do in practice. As an alternative, molecular methods have been developed for estimating individual inbreeding levels. These methods may also offer insight into fitness because in the presence of inbreeding depression, inbreeding level will negatively correlate with fitness. While some molecular methods for estimating inbreeding levels apply to any codominant marker, others have been specifically constructed for microsatellites, a type of genetic marker that roughly follows the stepwise mutation model (Ohta and Kimura, 1973). This model has provided the foundation for microsatellite-specific measures. Unlike conventional measures of inbreeding levels (e.g individual heterozygosity), microsatellite-specific measures possibly detect historical levels of inbreeding. The purpose of this thesis is to explore the correlation between various inbreeding measures and fitness.

A literature review of empirical and theoretical treatments of the first microsatellite-specific measure, d^2 (Coulson *et al.*, 1998), revealed that d^2 was sometimes, but not always, more correlated with fitness than was individual heterozygosity. The pattern in the literature suggests that populations may differ in their extent of historical inbreeding. The literature review also indicated that exploration of d^2 in closely related taxa of different selfing rates was still warranted. Furthermore, the analytical modeling of Tsitrone *et al.* (2001) provided the inspiration for the development of new microsatellite-specific measures that distinguish between small repeat differences but pool larger repeat differences together.

An empirical study of microsatellite-specific measures for detecting individual inbreeding levels was carried out in four taxa with known differences in selfing rates in the *Mimulus guttatus* species complex. Genotype-fitness correlations are driven by inbreeding levels, extent of inbreeding depression, and linkage disequilibrium, all of which are influenced by selfing rate. It was hypothesized that different measures might best correlate with fitness in inbreeding taxa than in outbreeders. However no genotype-fitness correlations were observed for any measure or in any of the species. This study shows that even in the presence of within-population variation in inbreeding levels, genotype-fitness correlations may not be detectable. Although inbreeding issues are most pressing in small populations, molecular methods for detecting inbreeding level do not perform well under these circumstances (Ritland, 1996; Tsitrone *et al.*, 2001).

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Chapter 1

Measuring inbreeding and fitness in natural populations: molecular methods

Introduction to inbreeding, inbreeding depression and fitness

Inbreeding affects population dynamics and persistence and is particularly prevalent in hermaphroditic plants and in small populations. In the plant kingdom, inbreeding, by way of self-fertilization is a common mating system component (Schemske and Lande, 1985). In small populations or populations with limited dispersal, mating between relatives is inevitable. As far back as Darwin (1876), the reduced fitness of inbred versus outbred offspring (termed inbreeding depression) has been thought to be a major player in the evolution of mating systems (Lande and Schemske, 1985). Inbreeding and inbreeding depression are of marked importance because they commonly occur in natural populations (Keller and Waller, 2002) and have direct impacts on fitness. Although inbreeding depression may lead to the evolution of inbreeding avoidance or self-incompatibility, the ability to self-fertilize persists in nature, presumably because it offers reproductive assurance for colonizers and annual plants (Stebbins, 1950).

Although fitness plays a major role in driving population dynamics, it is difficult to measure. Direct measurement of fitness is a laborious process that requires obtaining individual survivorship and fecundity values. As an alternative, indirect detection of fitness may be possible in populations with variation in inbreeding level; in the presence of inbreeding depression, inbreeding level (as measured by homozygosities for genetic markers) will negatively correlate with fitness. Rapid molecular methods for estimating inbreeding level involve genotyping tissue samples, and are far less labour intensive than is measuring fitness directly. Such rapid molecular assays for detecting inbreeding

level are highly desirable for studies of fitness, inbreeding and inbreeding depression in natural populations, particularly when pedigrees are unknown and fitness data is difficult to obtain directly. The main drawbacks to these methods are that they are usually associated with large error variance (Ritland, 1996) or low maximum correlations (Tsitrone *et al.*, 2001), particularly in small populations or with small sample sizes.

Focus of thesis

This thesis evaluates molecular measures designed to estimate inbreeding levels of individuals. Molecular measures that take the mutational process at microsatellite loci into account are the specific focus. These microsatellite-specific measures are presented as alternatives to individual heterozygosity (*HET*, number of heterozygous loci /total number of loci scored, also known as observed heterozygosity). Individual heterozygosity, originally applied to isozyme data, is limited to detecting inbreeding events in the parental generation and cannot detect historical events such as population bottlenecks, subpopulation mergers, or consanguineous matings several generations back. Heterozygosity-fitness correlations have been reported for a wide range of taxa including marine bivalves (Zouros *et al.*, 1988), great reed warblers (Hansson *et al.*, 2001) and pine trees (Ledig, 1986), reviewed in Mitton and Grant (1984), David (1998) and Keller and Waller (2002).

While *HET* scores alleles as being the same or different, microsatellite-specific measures incorporate the difference in repeat score at microsatellite loci, which may allow for the detection of historical levels of inbreeding. This led to the proposal of the microsatellite-specific measure d^2 (Coulson *et al.*, 1998), which is defined as:

$$(1)\ d^2 = \sum_{i=1}^{n} \frac{(l_{1i} - l_{2i})^2}{n},$$

where n is the number of loci, and l_{1i} and l_{2i} are the number of repeats for the first and second alleles, respectively, at locus i. The focus of this thesis is d^2 and related microsatellite-specific measures because of their unique potential for detecting historical levels of inbreeding.

As background, several topics relating to molecular methods for detecting inbreeding level and estimating fitness are reviewed. First, the genetic basis of inbreeding depression and its effects on genotype-fitness correlations are discussed. Second, an introduction is given to the relevant genetic markers. Third, mutational processes at these markers are described. Fourth, hypotheses explaining associations between marker genotype and fitness are given. Fifth, a review of literature related to the microsatellite-specific measure d^2 is presented, in which the current empirical and theoretical evidence is used to comment on the validity of d^2 and of microsatellite-specific measures in general. Finally, new microsatellite-specific measures for detecting genotype-fitness correlations are developed and presented.

The genetic basis of inbreeding depression

The genetic basis of inbreeding depression provides the foundation for genotype-fitness correlations. The genetic basis of inbreeding depression has been studied with both modeling (Charlesworth and Charlesworth, 1987; Charlesworth and Charlesworth, 1999; Lande and Schemske, 1985) and empirical approaches (e.g. Dudash and Carr, 1998; Johnston and Schoen, 1996). There are two main competing hypotheses for the genetic basis of inbreeding depression (Wright, 1977). First, inbreeding depression can be caused by deleterious recessive mutations whose effects are unmasked with inbreeding. Second, inbreeding depression can be due to overdominance because favorable genotypes at overdominant loci are less common in inbred offspring.

Empirical evidence points towards deleterious recessive mutations as the main cause of inbreeding depression (Charlesworth and Charlesworth, 1999; Dudash and Carr, 1998) although overdominance may play a role in some cases (Fu and Ritland, 1994).

Since the expression of inbreeding depression will increase with increasing homozygosity, a negative linear correlation between fitness and inbreeding level should exist (Charlesworth and Charlesworth, 1987). This relationship is predicted for a given level of inbreeding depression. Inbreeding depression itself, however, may evolve in response to inbreeding level. In persistently inbred populations (e.g. partial selfing), purging of deleterious mutations and changes in deleterious mutation frequency occur. Therefore, this relationship will not necessarily hold across populations with varying levels of inbreeding.

Codominant neutral genetic markers: isozymes and microsatellites

Neutral genetic markers are conventionally used to investigate selectively neutral evolutionary processes such as drift, mutation, migration, and gene flow. With measures such as *HET* and d^2, neutral genetic markers can potentially be used to study selective processes in populations that show variation in individual inbreeding levels; that is, inbreeding depression should be stronger in individuals with greater homozygosity at genetic markers.

The two main codominant genetic markers used in population genetic studies are isozymes and microsatellites. Isozymes were the first widely used codominant genetic marker in population genetics. They are alternate forms of functionally equivalent proteins that separate based on charge and size during electrophoresis. Although considered to be selectively neutral, they are involved in metabolic processes and must

be under some selection. Isozymes are moderately polymorphic but do not offer the variability of microsatellites.

The advent of microsatellite DNA technology in the early 1990's provided a type of genetic marker with greater statistical power than isozymes. Microsatellites consist of tandem repeats (one to six bases per repeat unit), and are an excellent tool for population genetic studies for several reasons. First, they are abundant in eukaryotic genomes (e.g. Valle, 1993). Second, they are predominantly found in noncoding DNA (Hancock, 1995) and thus are selectively neutral. Third, they are codominant. Fourth, they are hypervariable owing to their high mutation rates (e.g. Levinson and Gutman, 1987; Primmer *et al.*, 1996; Weber and Wong, 1993). Microsatellites separate according to size during electrophoresis, and the majority of allele size differences are due to differences in the number of repeat units.

Mutational processes at neutral loci

Mutational processes at neutral loci are an important consideration when developing measures for detecting inbreeding level, particularly at microsatellite loci where alleles can be linearly ordered according to allele size. If alleles of similar sizes are not independent, this may provide useful information for determining inbreeding level.

Mutational processes at neutral loci were first modeled using the infinite alleles model (IAM) (Kimura and Crow, 1964), where each mutation produces a novel allele. In 1973, an alternative model, termed the stepwise mutation model (SMM) (Ohta and Kimura, 1973), was introduced as a potential description of evolution at the electrophoretic level for loci with charge-based alleles such as isozymes (Ohta and Kimura, 1973). Allelic states are independent in the IAM and dependent in the SMM.

Although it is generally accepted that the IAM is better suited than the SMM for describing the mutational process at isozyme loci, the SMM is proving to be useful at modeling microsatellite mutations.

Stepwise mutation at microsatellite loci is attributable to two mechanisms (reviewed in Li *et al.*, 2002). First, slip-strand mispairing errors can occur during DNA replication. DNA mismatch repair systems cannot detect these errors, resulting in the gain or loss of repeat units. Second, unequal crossover during recombination in meiosis can result in changes in repeat number. Both mutational mechanisms play a role in microsatellite mutation, and account for the observed high mutation rates (Li *et al.*, 2002). Empirical evidence shows that most microsatellite mutations (~85%) indeed involve the gain or loss of exactly one repeat unit (Ellegren, 2000; Primmer *et al.*, 1996; Xu *et al.*, 2000). In violation of the SMM, mutation rate is not constant with microsatellite length (Ellegren, 2000; Carvalho-Silva *et al.*, 1999; Zhu *et al.*, 2000) nor are all mutations of a single step (e.g. Di Renzo *et al.*, 1994; Xu *et al.*, 2000; Zhu *et al.*, 2000). Although the SMM is not a perfect description of microsatellite mutation, its description of the mutation process is more accurate than the IAM; microsatellite loci are subject to a high degree of homoplasy and do follow a roughly stepwise mutation process.

There are alternative models such as the K alleles model (KAM), which is similar to the IAM but with a limited number of allelic states (K) and the two-phase model (TPM) (Di Renzo *et al.*, 1994), which allows for multistep mutations. These mutation models, however, are usually more difficult to incorporate into genetic measures than either the IAM or the SMM. Thus, when alternative genetic measures are available, the choice is most often between the extremes of the IAM and the SMM. This is the case for measures for estimating individual inbreeding levels.

Association between marker genotype and fitness: mechanisms

Correlations between marker genotype and fitness have been reported in several studies for both d^2 and *HET* (Keller and Waller, 2002). There are three proposed mechanisms for correlations between neutral genotype and fitness. First, the markers themselves may influence fitness. This mechanism may play a role for isozymes but is less important to consider for many DNA markers such as noncoding microsatellites. Second, allelic states at marker loci may be correlated with allelic states at fitness loci elsewhere in the genome (Ohta and Kimura, 1970). This is referred to as linkage disequilibrium and is often attributable to physical linkage. Third, homozygosity at marker loci may be correlated with genome-wide homozygosity (Ohta and Cockerham, 1974). This is called identity disequilibrium and is due to variation in inbreeding among individuals.

Associations between d^2 and fitness: empirical and theoretical evidence

In 1998 Coulson *et al.* introduced the microsatellite-specific measure d^2 and proposed that it could detect both recent and historical inbreeding events. A key feature of this measure is that it does not weight all heterozygosities equally. Specifically, greater weight is given to individual loci with alleles that differ greatly in size.

The inspiration for d^2 came from Goldstein *et al.* (1995) who developed a SMM-based genetic distance, which takes the difference in repeat score at microsatellite loci into account. Using computer simulation, Goldstein *et al.* (1995) showed that their measure was more reliable than Nei's distance (1972) (based on the IAM) provided mutation was mostly stepwise. The work of Goldstein *et al.* (1995) showed that the difference in repeat score at microsatellite loci is useful in calculating time since

coalescence at the population level. Using similar reasoning but with no mathematical or simulation-based proof, Coulson *et al.* (1998) applied the same logic at the individual level. They proposed the statistic, d^2, which they say "potentially captures variation in the relationship between the parents of an individual ranging from recent inbreeding through to population mixing," "is a genetic measure of the genetic distance between the gametes that formed the individual," and "focuses on events deeper in the pedigree than individual heterozygosity."

Coulson *et al.* (1998) calculated d^2 and measured fitness traits in a wild red deer (*Cervus elaphus*) population introduced to the Isle of Rum in 1845. Fitness traits were measured on all calves born during a 14-year period. Calves were typed at nine dinucleotide microsatellites, and inbreeding coefficient, individual heterozygosity and d^2 were calculated for each individual. Using regression models, the relationships between fitness and the three genetic variables were explored. While d^2 explained a significant amount of the variation in the fitness trait birth weight, *HET* did not. Consequently the authors concluded that the population was experiencing heterosis as a result of population mixing.

Following the initial d^2 study, the idea of d^2 was explored in habour seals (*Phoca vitulina*) (Coltman *et al.*, 1998, Table 1.1). A significant relationship between fitness and d^2 was found. These first two d^2 studies provided sufficient support for the new measure for other researchers to explore d^2 in several other taxa (Table 1.1).

One of the most powerful empirical studies is that of Slate and Pemberton (2002). They genotyped the same red deer population (but with different individuals) as Coulson *et al.* (1998) at 71 microsatellites, and indicated that, contrary to the previous report, *HET* was more correlated with fitness traits than d^2. Furthermore, there was no

evidence that d^2 scores were correlated across loci within an individual, as would be expected for an indicator of inbreeding level.

Overall, in the 11 species in which d^2 has been calculated and compared with *HET*, there is no consistent trend; in some studies d^2 was a better correlate of fitness than *HET*, while other studies showed the opposite trend. In five studies d^2 was more correlated with fitness than was *HET,* while in three studies *HET* showed the stronger correlation. In the remaining three studies neither measure was correlated with fitness. If natural populations differ in their extent of historical versus recent inbreeding, and if d^2 has the ability to detect historical inbreeding events, the pattern in the literature is expected. Populations with recent inbreeding should show a correlation between *HET* and fitness, while populations with historical inbreeding would only show a correlation between d^2 and fitness. Those with both historical and recent inbreeding may show both correlations.

Tsitrone *et al.* (2001) conducted analytical modeling of genotype-fitness correlations under the IAM and the SMM. The correlation coefficient between *HET* or d^2 at a single marker locus and fitness (as determined by true inbreeding level) was calculated for two inbreeding scenarios and for several population sizes and mutation rates. The first inbreeding scenario, admixture of two isolated populations, was invoked to represent historical long-term inbreeding. The second inbreeding scenario, partial selfing over a few generations (outcrossing rate, t=0.6), was invoked to represent short-term inbreeding. Both inbreeding scenarios result in variation in individual inbreeding levels and thus provide the potential to detect genotype-fitness correlations. The impacts of mutation model, population size, and mutation rates on the maximum correlation between fitness and genotypic index were determined. Under both inbreeding scenarios, *HET* was consistently more correlated with fitness than was d^2.

Furthermore, it was shown that d^2 does not detect fitness differences for large repeat differences (Figure 1.1). This is a notable problem because d^2 weighs large repeat differences more than small repeat differences. The analytical analysis of Tsitrone *et al.* (2001) does not support the use of d^2 in almost every circumstance evaluated. One situation, however, that remains to be explored is the behavior of d^2 (or other microsatellite-specific inbreeding measures) under different selfing rates. This could be done theoretically or could be done in an empirical study comparing inbreeders and outbreeders within a single plant genus.

The overall picture provided by the current body of d^2 literature shows that while empirical evidence sometimes favours the use of d^2, the two most definitive studies (one empirical, one theoretical) do not. The value of d^2 under a variety of mating systems (inbreeders and outbreeders) has not been adequately examined. Since inbreeding coefficient and inbreeding depression are related to mating system, an investigation of d^2 (and genotype-fitness correlations in general) under a variety of selfing rates is warranted. Additionally, other microsatellite-specific measures could be developed that incorporate allele size differently or that consider only small repeat differences between alleles.

Table 1.1. Summary of studies that examine d^2, *HET* and fitness. *N* = sample size. Note that when more than one study is available for the same population only the more extensive study is included.

Species	Scientific name	N	Population	# Microsatellites	Fitness trait(s)	Comment	Reference
Pacific Oyster	*Crassostrea gigas*	294	Breeding program	3	Growth rate	d^2 more correlated with fitness than *HET* but uses too few microsatellites.	Garnier-Gere *et al.* (2002)
Chinook Salmon	*Oncorhynchus tshawytscha*	200	Breeding Program	7	Reproductive traits	d^2 does not outperform *HET*.	Heath *et al.* (2002)
Red Deer	*Cervus elaphus*	346	Isle of Rum	71	Juvenile traits	d^2 does not outperform *HET*.	Slate and Pemberton (2002)
Gray Wolves	*Canis lupus*	30	Descendents from 4 pairs of wolves	27	Inbreeding coefficients calculated from pedigrees	Rejects use of d^2.	Hedrick *et al.* (2001)
Great Reed Warblers	*Acrocephalus arundinaceus*		Sibling dyads	5	Recruitment success	d^2 more correlated with fitness than *HET*.	Hansson *et al.* (2001)
Horseshoe Bat	*Rhinolophus ferrumequinum*	138	Bottleneck population	7	Survival	d^2 more correlated with fitness than *HET*.	Rossiter *et al.* (2001)
Rainbow Trout	*Oncorhynchus mykiss*	108, 109	2 cohorts	10	Weight/Length^3	Neither *HET* nor d^2 correlate with fitness.	Thelen and Allendorf (2001)
Ornate Dragon Lizard	*Ctenophorus ornatus*	29 adults, 44 offspring	Entire population	6	Adult fitness, offspring survival	Fitness is negatively correlated with d^2 and not correlated with *HET*. Neither correlates with offspring survival. Supports, to some degree.	LeBas (2002)
Natterjack Toad	*Bufo calamita*	5 spawn strings	Large natural population	5	Larval fitness traits	No correlation with fitness for either *HET* or d^2.	Rowe and Beebee (2001)
Common Frog	*Rana temporaria*	5 spawn strings	Large natural population	7	Larval fitness traits	No correlation with fitness for either *HET* or d^2.	Rowe and Beebee (2001)
Habour Seal	*Phoca vitulina*	258	700-1200 individuals	6	Birth weight, survival	d^2 more correlated with fitness than *HET*.	Coltman *et al.* (1998)

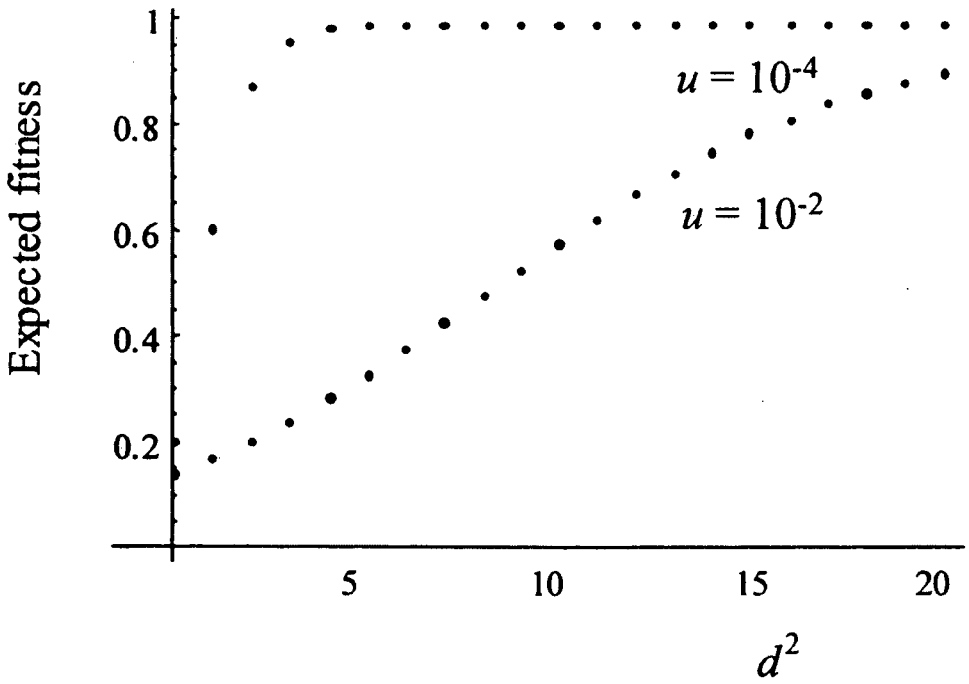

Figure 1.1. The expected value of the fitness trait as a function of d^2 at one locus under an admixture model assuming the SMM. The subpopulation size is 10^3 , the ancestor population size is 10^4, the divergence time between subpopulations in generations is 10^4, and μ is the mutation rate (Figure and figure text adapted from Figure 4 in Tsitrone *et al.* (2001)).

Alternative measures for detecting individual inbreeding levels

One major flaw of d^2 is that it puts more weight on large within-locus repeat differences between alleles. The modeling of Tsitrone *et al.* (2001) provided clear evidence that in the presence of inbreeding depression, fitness differences are only detectable for small repeat differences (Figure 1.1). Therefore, d^2 contains a great deal of useless information when large repeat differences are considered, and measures of individual inbreeding levels that pool together large repeat differences are justified. This has provided inspiration for the development of new microsatellite-specific measures where repeat differences greater than three are pooled together. Furthermore, measures that pool together large repeat differences will be less sensitive to departures from the SMM.

Another feature of d^2 is that within-locus repeat differences are squared. There is no hard evidence that this is necessary. Therefore, measures that incorporate the absolute allele span are presented.

The following measures are proposed:

(2) d-absolute (*dabs*):

$$d = \sum_{i=1}^{n} \frac{|l_{1i} - l_{2i}|}{n},$$

(3) *d - mini*:

$$d - mini = \sum_{i=1}^{n} \frac{a_i}{n},$$

$$\text{where } a_i = \begin{cases} 0 & if \quad |l_{1i} - l_{2i}| = 0 \\ 1 & if \quad |l_{1i} - l_{2i}| = 1 \\ 2 & if \quad |l_{1i} - l_{2i}| = 2 \\ 3 & if \quad |l_{1i} - l_{2i}| = 3 \\ 4 & if \quad |l_{1i} - l_{2i}| \geq 4 \end{cases},$$

(4) and d^2-mini:

$$d^2 - mini = \sum_{i=1}^{n} \frac{a_i^2}{n}.$$

Furthermore, a new multiple linear regression (*MLR*) approach is proposed, where independent variables measure the fraction of loci that have 0, 1, 2, 3 or more than 3 repeat differences. Such an approach may be more suitable than an average allele span over loci because the weight of a given allele span is determined by regression coefficients, not by the value of the allele span itself.

The following multiple linear regression model is proposed:

$$Fitness = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \varepsilon,$$

where X_1 is the fraction of homozygous loci, X_2 is the fraction of loci with a repeat difference of one, X_3 is the fraction of loci with a repeat difference of two, X_4 is the fraction of loci with a repeat difference of three, the βs are regression coefficients and ε is the error term.

A final, previously described measure for estimating individual inbreeding levels is considered in this thesis. Ritland's method-of-moments estimator (MME) (1996) weights the importance of homozygosities based on allele frequency. Homozygosities of low frequency alleles are more likely to indicate identity by decent than homozygosities involving common alleles. The MME does not take mutation into account, and could be described as a weighted variation of *HET*. Ritland's MME individual inbreeding coefficient is defined as:

$$(5)\ \hat{\rho} = \frac{\sum_{j,i} \frac{S_{ji} - P_{ji}^2}{P_{ji}}}{\sum_{i} (m_i - 1)}$$

where, *i* denotes the locus, *j* denotes allele *j* at locus *i*, *m* is the number of alleles at locus *i*, *S* is 1 if the two alleles at locus *i* are allele *j* and is 0 otherwise, and *P* is the estimated frequency of an allele. Ritland's MME assumes that allele frequencies are estimated from a large, reference population. If this assumption is violated, the measure is subject to large error variance but is still unbiased (Ritland, 1996).

Purpose of research

This thesis evaluates d^2 and genotype-fitness relationships with empirical data. In Chapter 2, four taxa in the *Mimulus guttatus* species complex (Scrophulariaceae) with different rates of self-fertilization are used to explore the relationship between fitness and several genetic measures of individual inbreeding levels. In Chapter 3, the implications of this research are discussed, and future research questions are presented.

Chapter 2

An empirical test of microsatellite-specific measures for detecting individual inbreeding levels in four taxa in the *Mimulus guttatus* species complex with different rates of self-fertilization

Introduction

In this chapter, genotype-fitness correlations are explored in partially selfing taxa in the *Mimulus guttatus* species complex. Partially selfing populations present the opportunity to empirically examine the effects of historical inbreeding on genotype-fitness correlations. It has been proposed that genetic measures incorporating mutational processes may be able to detect historical levels of inbreeding (Coulson *et al.*, 1998). While the particular measure of Coulson *et al.* (1998), d^2, probably does not detect historical inbreeding (Tsitrone *et al.*, 2001), other microsatellite-specific measures may be useful. New microsatellite-specific measures were proposed in Chapter 1, and these may be an improvement over d^2 because they only distinguish between small repeat differences. Prior inbreeding will not be the only factor affecting genotype-fitness correlations because partially selfing populations are also likely to vary in their amount of inbreeding depression and linkage disequilibrium.

Genotype-fitness correlations will behave differently under different selfing rates. In mating systems that generate no variation in individual inbreeding levels (e.g. large randomly mating populations; completely selfing populations), genotype-fitness correlations will not exist. In populations with mixed mating systems, genotype-fitness correlations should change with selfing rate. As the historical selfing rate increases, inbreeding depression is expected to decrease as recessive deleterious alleles are purged from a population (Charlesworth and Charlesworth, 1987). Additionally, as the

immediate selfing rate increases, genome-wide homozygosity increases, as genetic variation is converted from within to between individuals. Finally, the effective recombination rate is reduced with selfing, resulting in correlations between allelic states within a genome. As a consequence of these relationships, outbreeders should have high inbreeding depression, low linkage disequilibrium and very little homozygosity at highly polymorphic loci such as microsatellites. Conversely inbreeders should have low inbreeding depression, high linkage disequilibrium and high genome-wide homozygosity.

How might these factors affect correlations between genotypes at microsatellite loci and fitness? For a predominantly outbreeding species, a measure such as *HET* may not be informative since almost all loci will be heterozygous. However, owing to the high level of inbreeding depression, a measure that considers within locus allele span potentially could correlate with fitness, if the population had experienced historical inbreeding. In an inbreeding species with low inbreeding depression, high linkage disequilibrium and extensive homozygosity, *HET* may be an informative measure, provided there is still some level of heterozygosity (as with partial selfing).

Mating system and inbreeding depression in the Mimulus guttatus species complex

Overview of *Mimulus guttatus* species complex:

The plant genus *Mimulus* had been intensely studied with respect to the evolution of mating system. The *Mimulus guttatus* (yellow monkey flower) species complex (Simiolus section) consists of four major taxa usually referred to as species (*M. guttatus* Fisher, *M. nudatus* Greene, *M. laciniatus* Gray, *M. Tilingii* Regel). Several taxa, including *M. nasutus* Greene and *M. micranthus* Heller, are generally referred to as varieties of the bee-pollinated, mixed-mating *M. guttatus* Fisher. California is the

North American centre of diversity for this species complex, but populations of *M. guttatus* Fisher and *M. nasutus* can be found in Oregon, Washington and southern British Columbia (Vickery, 1978). Most species are annual, but, perennial varieties of *M. guttatus* Fisher do exist. *Mimulus* species are good model organisms because they are easy to rear, have short lifespans and produce abundant seed.

Four annual *Mimulus* taxa were used in this study (the mixed mating *M. nasutus*, *M. nudatus*, *M. laciniatus* and the predominant selfer, *M. micranthus*; illustrated in Figure 2.1). These taxa are listed in approximate order of increasing selfing (Ritland and Ritland, 1989). *M. nasutus* is a common *Mimulus* taxon found in wet habitats. *M. nasutus* exhibits wide variation in mating system (Ritland, 1990). While some populations of *M. nasutus* are cleistogamous, other large-flowered populations are indistinguishable from *M. guttatus* Fisher in terms of mating system (Ritland, 1990). A large-flowered population was chosen for this study. *M. nudatus* is a mixed-mating taxon occurring in serpentine substrate in seep habitats. Its range is restricted to north-western California, and is classified as rare (California Native Plant Society). *M. nudatus* is easily distinguishable from other taxa in the *Mimulus guttatus* species complex because of its narrow leaves, extensive branching, and small size. *M. laciniatus* occurs in grainy soils under moist conditions. Restricted to the high Sierra Nevada, *M. laciniatus* is classified as rare (California Native Plant Society). *M. laciniatus* is distinguishable by its highly dissected leaves, extensive branching, and small size. *M. laciniatus* undergoes more selfing than do *M. nasutus* and *M. nudatus*. Finally, *M. micranthus* is restricted to coastal northern California, and is a small-flowered, cleistogamous variety of *M. guttatus* Fisher. *M. micranthus* was chosen for this study to represent a primary selfing taxa.

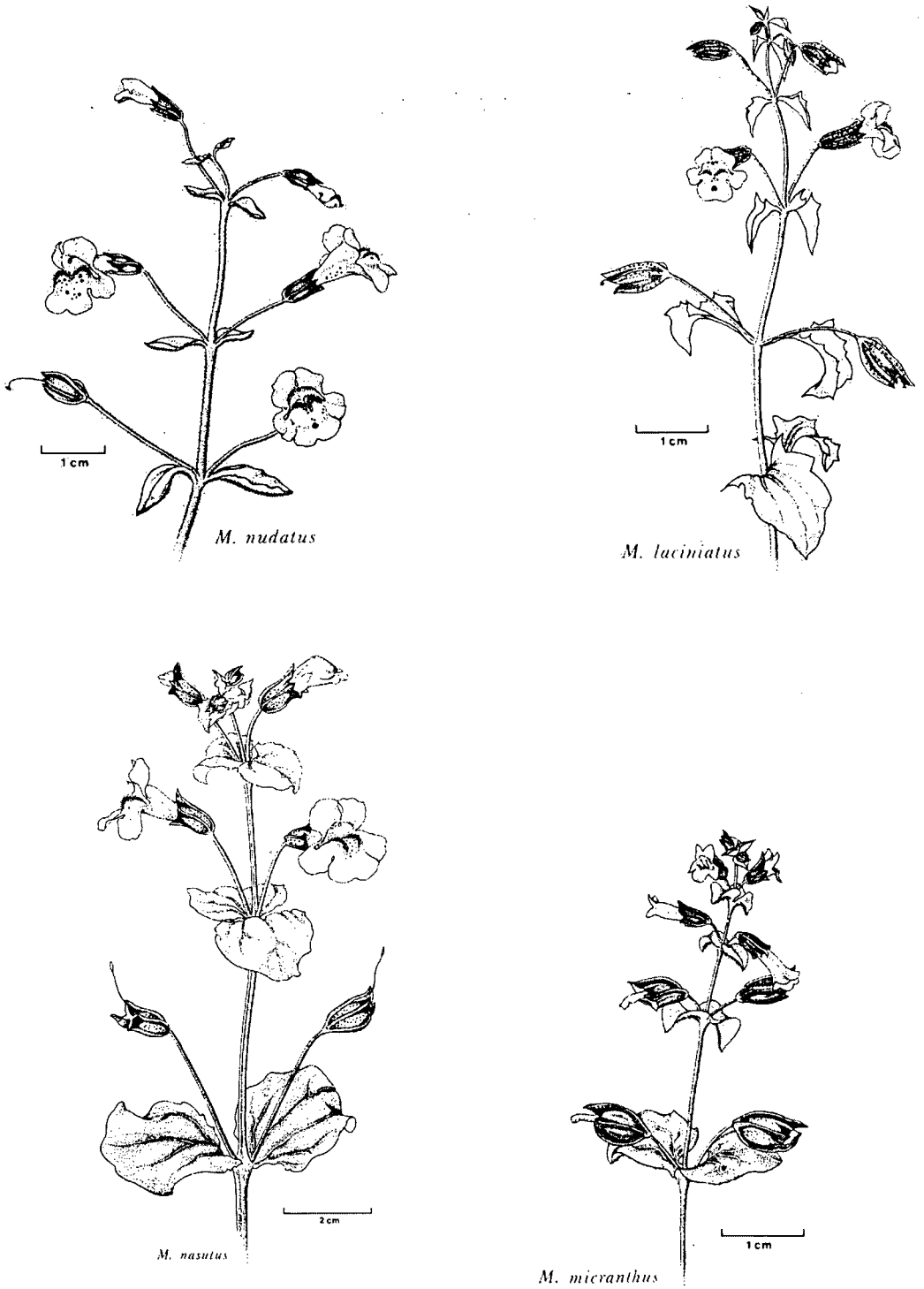

Figure 2.1. *Mimulus* taxa used in this study (adapted from Figure 1 in Ritland and Ritland (1989)).

Inbreeding depression in *Mimulus*:

It has been shown that closely-related and intercrossable *Mimulus* taxa exhibit a wide range of outcrossing rates (*t*) and inbreeding coefficients (Latta and Ritland, 1994; Dudash and Carr, 1998). Deleterious recessive alleles are mainly responsible for inbreeding depression in both *M. micranthus* (*t*=0.16) and *M. guttatus* Fisher (*t*=0.68-0.80) (Dudash and Carr, 1998). When inbreeding depression is caused by recessive deleterious mutations, inbreeding depression is expected to linearly decrease with increasing selfing rate (Charlesworth and Charlesworth, 1987).

The relationship between prior inbreeding level and inbreeding depression was explored in four *Mimulus* taxa (*M. guttatus* Fisher, *M. nasutus*, *M. glaucenscens*, *M. platycalyx*) by measuring the fitness of selfed versus outcrossed progeny in a controlled growth chamber experiment (Latta and Ritland, 1994). Plants grown from field-collected seed from 15 populations (15-30 seed parents per population) were crossed within a population to generate outbred plants. These plants were hand-pollinated to generate selfed and outcrossed progeny, from which inbreeding depression estimates were obtained. A negative relationship between prior inbreeding and inbreeding depression was observed but was only significant for the fitness trait height. Furthermore, for a given population-level inbreeding coefficient, a wide range of inbreeding depression was observed. Their study weakly suggests that deleterious mutations are purged with increased selfing in *Mimulus*.

Microsatellite markers for *Mimulus*:

The development of a large number of microsatellite primers for *M. guttatus* and *M. nasutus* (Awadalla and Ritland, 1997; Kelly and Willis, 1998; Sweigart *et al.*, 1999) provides the opportunity to explore genotype-fitness correlations in closely related taxa

with varying outcrossing rates. In this chapter, these primers are tested for cross-taxon amplification within the *Mimulus guttatus* species complex.

Hypotheses

In this study, four *Mimulus* species were genotyped at 11 microsatellite loci and assayed for three fitness traits in order to evaluate the relationship between genotype and fitness under a variety of outcrossing rates.

Based on the analytical modeling of Tsitrone *et al.* (2001), I hypothesize that d^2 will not explain a significant amount of variation in fitness is any of the species. However, I predict there is some information to be gained in distinguishing between repeat differences less than four, particularly in mixed mating populations with historical inbreeding. This is based on the modeling of Tsitrone *et al.* (2001) who provided evidence of fitness differences for individuals with alleles of small repeat differences. Thus I predict d^2*mini* or *dmini*, which pool together repeat differences greater than or equal to four, will explain the highest amount of variation in fitness. For predominant selfers, I predict that *HET* will be the most informative genetic measure because it has the power to detect recent outcrossing events. Detecting historical inbreeding in predominant selfers would be difficult because most individuals are homozygous unless they have undergone recent outbreeding.

Method

Field Collection

Seed capsules from 20 randomly selected plants were collected in June 2001 from one California population each for *M. nasutus*, *M. nudatus*, *M. laciniatus*, and *M.*

micranthus. The populations were all small in size, ranging from just over 20 to approximately 100 individuals. Population locations are given in Table 2.1.

Table 2.1. Taxa in the *Mimulus guttatus* species complex and their specific northern California population used in this study. All seed collections were made in June 2001. Population codes were assigned according to the Ritland lab system. Population 238 is previously described in Ritland (1990), and population 716 is previously described in Vickerey (1964). Populations 404 and 312 were located by K. Ritland.

Species	Population Code	Population Location	Elevation
M. nasutus	238	Barkerville, Hough Springs, Lake Co.	600m
M. nudatus	404	Knoxville, Napa Co.	600m
M. laciniatus	716	Hetch Hetchy, Yosemite National Park, Tuolumne Co.	1700m
M. micranthus	312	Bartlett Springs, Lake Co.	500m

Measurement of Fitness Traits

For each population, seeds were planted in six 40-cell trays in a 50:50 mixture of Promix and Perlite. Seed parents were randomly assigned to 12 adjacent cells within a tray. *Mimulus* seeds are minute and would have been difficult to randomize between trays without contamination of adjacent cells. Seedlings were germinated and grown in a growth chamber (model E15 with control panel 3244 or 4030 (Conviron)), one chamber per taxa, with 16°C days (16 hours) and 12°C nights (8 hours). Taxa were not randomized among chambers because they are morphologically distinct (Figure 2.1) and exhibit wide variation in average height, number of flowers and lateral growth patterns. The shorter taxa would have been shaded by the taller taxa, and taxa exhibiting extensive lateral branching would have crowded those without. Furthermore, germination was synchronized within a population but not among populations. These factors would have resulted in a heterogeneous environment for plants within a taxon, and would have made within-taxon comparisons unreliable. Trays were watered liberally and kept moist at all times. Following germination, seedlings were thinned to one per cell, and the remaining individuals were grown to senescence (~3 months). Within each growth chamber the trays were randomly relocated every seven days.

Plants within a taxon were harvested simultaneously and assessed for three fitness traits (number of flowers (including capsules and buds), above-ground height, above-ground dry mass). Due to time constraints, two of the twelve offspring per seed parent were randomly selected for genetic analysis. A 50mg leaf tissue sample was taken from each individual and was stored at –80°C for future genetic analysis. For most of the *M. micranthus* plants it was not possible to obtain a quality tissue sample because both the leaves and corollas are very small. The early, basal leaves are larger but these were dried up by the onset of senescence. As an alternative to a tissue

sample, selfed seeds from one seed capsule per individual were germinated on filter paper in a petri dish and were bulked to recover the parental genotype. The seed capsules almost certainly contained pure selfed seeds because *M. micranthus* is a cleistogamous species and was reared in a wind-free and pollinator-free environment.

Testing of Microsatellite Primers for Cross-Taxon Amplification

Twenty-nine microsatellite primer pairs designed for *M. guttatus* or *M. nasutus,* as described in Awadalla and Ritland (1997), Kelly and Willis (1998), and Sweigart *et al.* (1999), were tested for cross-taxon amplification in a panel of individuals from seven species within the *Mimulus guttatus* species complex. *M. guttatus* Fisher, *M. nasutus*, *M. micranthus*, *M. nudatus*, *M. glaucesens*, *M. platycalyx* and *M. laciniatus* were chosen because DNA for these taxa was available in the Ritland lab. Polymerase chain reaction (PCR) conditions were optimized for 11 loci that were both polymorphic and successful at cross-taxon amplification. Appendix A provides a summary of the primer testing. PCR conditions (using M13-tailed primers) for the selected loci are given in Table 2.2. Null allele detection was not possible at this stage because of low sample size.

Table 2.2. PCR conditions for loci selected from the primer testing. All PCR reactions were 10 µl reactions. * Indicates a locus has notable null allele problems observed in one or more of the surveyed populations. See text for details of PCR profiles 1 and 2. F primer, forward primer; R primer, reverse primer; T_a, annealing temperature. One µl Erica Haddelberg buffer (100mM Tris-HCl pH=8.0, 500mM KCl, 25mM $MgCl_2$, 0.1% gelatin, 1.6mg.ml BSA) and 1µl dNTP were used for all reactions.

Locus	DNA (ng)	Label (µl)	F Primer (µl)	R Primer (µl)	Taq (µl)	T_a (°C)	Cycles	PCR profile	Tail
003	10	0.3	0.5	0.5	0.2	55	35	2	F
019	60	1	1	1	0.2	53	35	2	F
217	40	1	1	1	0.2	54	35	2	F
230	20	0.5	1	1	0.2	54	35	2	R
267	20	1	1	1	0.2	51	35	1	R
356	30	1	1	1	0.2	57	35	2	R
367	30	0.5	0.5	0.5	0.2	54	35	1	R
372	20	0.5	1	1	0.2	55	35	2	F
374	30	1	1	1	0.2	53	35	1	R
261*	30	1	1	1	0.2	49	35	2	F
281*	50	0.5	1	1	0.2	50	35	2	R

Microsatellite Analysis

DNA Extraction:

DNA was extracted from leaf or seedling tissue using a modified CTAB extraction protocol (Doyle and Doyle, 1987). Tissue (~50mg) was placed in a 1.5ml tube and homogenized in liquid nitrogen. To each sample 700µl CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 20mM EDTA, 100mM TRIS-HCl pH = 8.0, 1% v/v 2-beta-mercaptoethanol) was added and incubated at 65°C for one hour with frequent vortexing. Subsequently a chloroform:isoamyl (24:1) extraction was carried out. The supernatant from each tube was transferred to a fresh 1.5ml tube and incubated with 1µl RNAse A (10mg/ml) at 37°C for 20 min. One µl Proteinase K (10µl/ml) was added and the tubes were incubated for an additional 20min at 37°C. A second chloroform:isoamyl extraction was then performed. DNA was precipitated from the supernatant with 100% cold isopropanol. The pellet was washed in 70% ethanol, air-dried, and resuspended in 100µl distilled sterilized water.

Polymerase Chain Reaction (PCR):

Approximately 40 individuals (two randomly chosen offspring per seed parent) per taxon were genotyped at 11 microsatellite loci using PCR. PCR conditions used were locus-specific and are summarized in Table 2.2. Depending on the locus, one of two PCR profiles was used for amplification in a PCT-100 thermocycler (MJ Research). Profile 1 involved denaturing at 95°C for 3min, followed by 30 to 35 cycles at 94°C for 45sec, annealing at the locus-specific annealing temperature (Table 2.2) for 45sec, extension at 72°C for 45sec, followed by one cycle at 72°C for 5min. Profile 2 involved denaturing at 94°C for 3min, annealing at 50°C for 1min, extension at 70°C for 1min, followed by 35 cycles at 94°C for 1min, annealing at the locus-specific temperature for

1min, extension at 72°C for 1min, followed by one cycle at 72°C for 10min. PCR reactions were run on 7% polyacrylamide gels on a LI-COR 4200 sequencer using denaturing electrophoresis. Gels were visualized using Base ImagIR (version 4.0a (LI-COR)), and loci were scored using RFLP scan (version 3.0 (Scanalytics, CSP Inc.) and by eye.

Null allele detection:

The loci were not formally tested for null allele because no method exists for doing so in species with partial selfing. However, null alleles were obvious at two loci (261, 281). These loci only amplified for some individuals (<85%). Adjustment of PCR conditions (e.g. DNA quantity, additional cycles, lower annealing temperature) failed to uncover the drop-out genotypes. Within a locus, drop-outs consistently occurred for the same individuals and were not related to drop-outs at other loci (data not shown), suggesting that it was not a DNA quality problem. The other loci do not appear to have null allele problems. At these loci, drop-out (<10%) genotypes are positively correlated across loci (data not shown). This indicates that drop-outs at these loci are due to poor DNA quality in a few individuals rather than true null alleles.

Statistical Analysis

Descriptive Statistics of Loci:

Loci were characterized using Genepop (web version, htttp://wbiomed.curtin.edu.au/genepop/index.html, Feb., 2003) which is an updated version of 1.2 (Raymond and Rousset, 1995). Specifically, the expected heterozygosity under Hardy-Weinberg equilibrium (H_e) and the observed heterozygosity (H_o) were calculated for each locus using Lorene's correction for H_e. The method of Weir and Cockerham (1984) was used for calculating the inbreeding coefficient (F). Genepop

was also used to test for heterozygote deficiency using the Markov Chain method with 1000 iterations (Guo and Thompson, 1992). Averages were calculated over all loci. Using the average F, the outcrossing rate, t, was estimated (Jain, 1979) as:

$$t = 1 - \frac{2F}{1+F}.$$

Calculation of individual inbreeding levels:

HET, d^2, *dabs*, d^2*mini*, *dmini* and Ritland's MME were calculated for each individual. A randomization test was carried out to distinguish between error variance in the estimation procedures and true variance in individual inbreeding levels within each population. Specifically, a C++ program was written in which genotypes were randomized without replacement at each locus among individuals. This generates data sets with no true variation in inbreeding levels among individuals but holds genotype frequencies constant. For each population, one thousand randomizations were carried out, and the variance of each measure of individual inbreeding levels was calculated for each randomization. The randomization procedure was used to test the hypothesis that the observed variance in individual inbreeding levels is greater than that expected due to sampling error.

Regression analysis and calculation of genetic measures:

Using SAS (version 8.02 (The SAS Institute)) PROC REC, linear regression analysis was carried out. Separate regression models were constructed using each of the three fitness traits as the response variable and one of several genetic measures as the predictor variable (Table 2.3). Each regression model was assessed for normality, homoskedasticity and lack of fit. For *M. nasutus* there was a strong tray effect so fitness traits were standardized by subtracting the tray mean from each observation and

then dividing by the tray mean. In all calculations missing genotypes were treated as failed PCR reactions rather than as null alleles.

Table 2.3. Variables used in regression analysis of fitness traits versus genetic measures for three *Mimulus* species (*M. nasutus*, *M. nudatus*, *M. laciniatus*). In total fifteen simple linear regressions and one multiple linear regression were preformed for each species.

Genetic measure	Fitness trait
HET	Above-ground height
d^2	Dry mass
d^2*-mini*	Number of flowers
d-abs	
d-mini	
MLR	

Results

Description of microsatellite loci

All four *Mimulus* taxa were genotyped at 11 microsatellite loci. However two loci (261,281; Table 2.2) were dropped because of obvious null allele problems. For *M. nasutus*, *M. micranthus* and *M. laciniatus* all nine loci successfully amplified (Table 2.4). For *M. nudatus* only seven loci successfully amplified; loci 003 and 367 did not yield scorable products. *M. nasutus* and M. *nudatus* had similar numbers of alleles per locus (10.7 and 9.9 respectively) whereas *M. laciniatus* had 4.8 alleles per locus and *M. micranthus* had only 1.4 alleles per locus. Polymorphisms were only detected at three loci in *M. micranthus*, and these loci were dominated by one allele. Due to the lack of allelic diversity found in *M. micranthus*, this taxon is excluded from any further discussion of genetic parameters. No linkage analysis was performed because of the high degree of inbreeding observed in all species. Conventional linkage analysis assumes Hardy-Weinberg equilibrium; this assumption is clearly violated in this study.

Allele size distributions are plotted for *M. nasutus* in Figure 2.2, *M. nudatus* in Figure 2.3, and *M. laciniatus* in Figure 2.4. At most loci, the allele size distributions are unimodel, with most alleles clustered together. However less continuous allele size distributions are found in *M. laciniatus,* and *M. nasutus* often has two or three clusters of alleles.

Table 2.4. Descriptive statistics at 11 microsatellite loci . *n*, number of individuals that amplified; n_a, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity under Hardy-Weinberg equilibrium; *F*, inbreeding coefficient; *SE*, standard error; no amp., no amplification.

Taxa (#individuals surveyed)	Locus	*n*	n_a	Size Range	H_o	H_e	*F*	*SE*(*F*)	P *F*>0
M. nasutus	003	36	19	134-190	0.67	0.94	0.2935	0.0000	0.0000
(38)	019	35	14	146-190	0.63	0.85	0.2656	0.0027	0.0033
	217	33	5	176-197	0.61	0.74	0.1784	0.0023	0.0280
	230	38	10	141-228	0.47	0.75	0.3681	0.0003	0.0003
	267	35	3	119-128	0.26	0.36	0.2884	0.0009	0.0137
	356	38	19	139-226	0.79	0.91	0.1385	0.0053	0.0097
	367	38	10	148-196	0.71	0.84	0.1559	0.0009	0.0019
	372	36	11	270-312	0.69	0.83	0.1542	0.0017	0.0022
	374	38	6	150-165	0.68	0.70	0.0258	0.0143	0.7361
	Average	36.3	10.7		0.61 (0.16)	0.77 (0.17)	0.2076		
M. micranthus	003	30	2	158, 168	0	0.13	*	*	*
(30)	019	30	1	147	*	*	*	*	*
	217	30	1	195	*	*	*	*	*
	230	30	2	189, 192	0.13	0.13	*	*	*
	267	30	1	125	*	*	*	*	*
	356	30	1	178	*	*	*	*	*
	367	30	1	190	*	*	*	*	*
	372	30	3	278, 284, 290	0.10	0.10	*	*	*
	374	30	1	153	*	*	*	*	*
	Average	30	1.44						

Taxa (#individuals surveyed)	Locus	*n*	n_a	Size Range	H_o	H_e	*F*	*SE*(*F*)	P *F*>0
M. nudatus	003	no amp.	*	*	*	*	*	*	*
(38)	019	37	19	156-204	0.54	0.93	0.4238	0.0000	0.0000
	217	34	4	189-201	0.59	0.66	0.1117	0.0012	0.0102
	230	32	12	169-208	0.31	0.91	0.6588	0.0000	0.0000
	267	38	5	107-131	0.61	0.75	0.1975	0.0002	0.0003
	356	38	23	136-289	0.79	0.93	0.1527	0.0119	0.0207
	367	no amp.	*	*	*	*	*	*	*
	372	38	5	273-285	0.53	0.62	0.1548	0.0013	0.0043
	374	38	1	141	*	*	*	*	*
	Average	36.4	9.85		0.56	0.80	0.283		
M. laciniatus	003	36	9	148-178	0.58	0.72	0.1919	0.02937	0.2937
(36)	019	33	3	158-208	0.03	0.14	0.792	0.0001	0.0002
	217	35	4	182-191	0.20	0.21	0.0593	0.0125	0.3714
	230	36	1	189	*	*	*	*	*
	267	36	3	125-134	0.14	0.27	0.488	0.0015	0.0180
	356	33	7	156-219	0.36	0.58	0.377	0.0033	0.0089
	367	33	10	148-223	0.13	0.76	0.828	0.0000	0.0000
	372	34	3	276-291	0.06	0.11	0.486	0.0023	0.0453
	374	35	3	150-180	0.09	0.21	0.592	0.0007	0.0025
	Average	34.2	4.78		0.20	0.38	0.477		

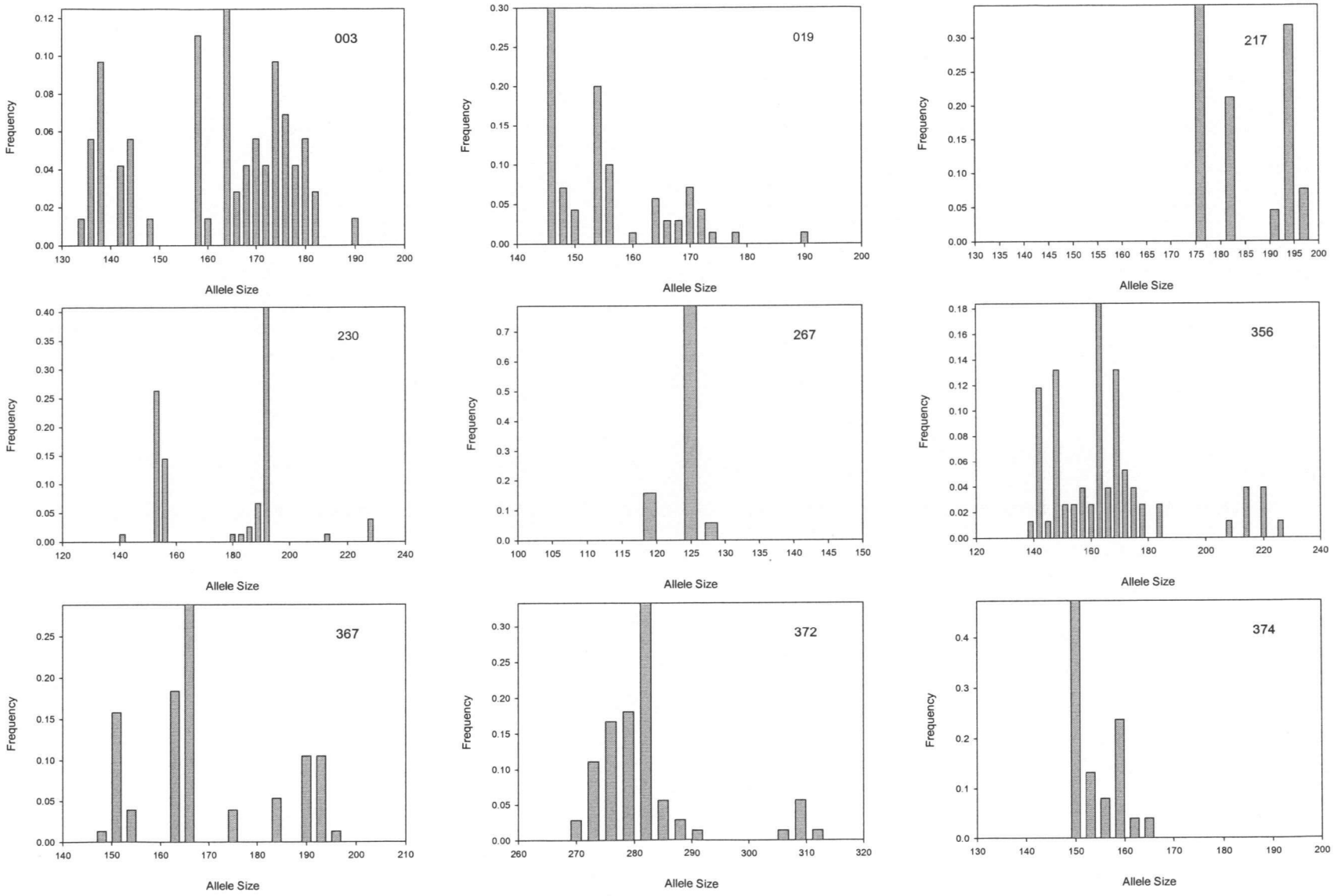

Figure 2.2. Allele size distribution for *M. nasutus* at nine microsatellite loci. Locus number is indicated on each graph.

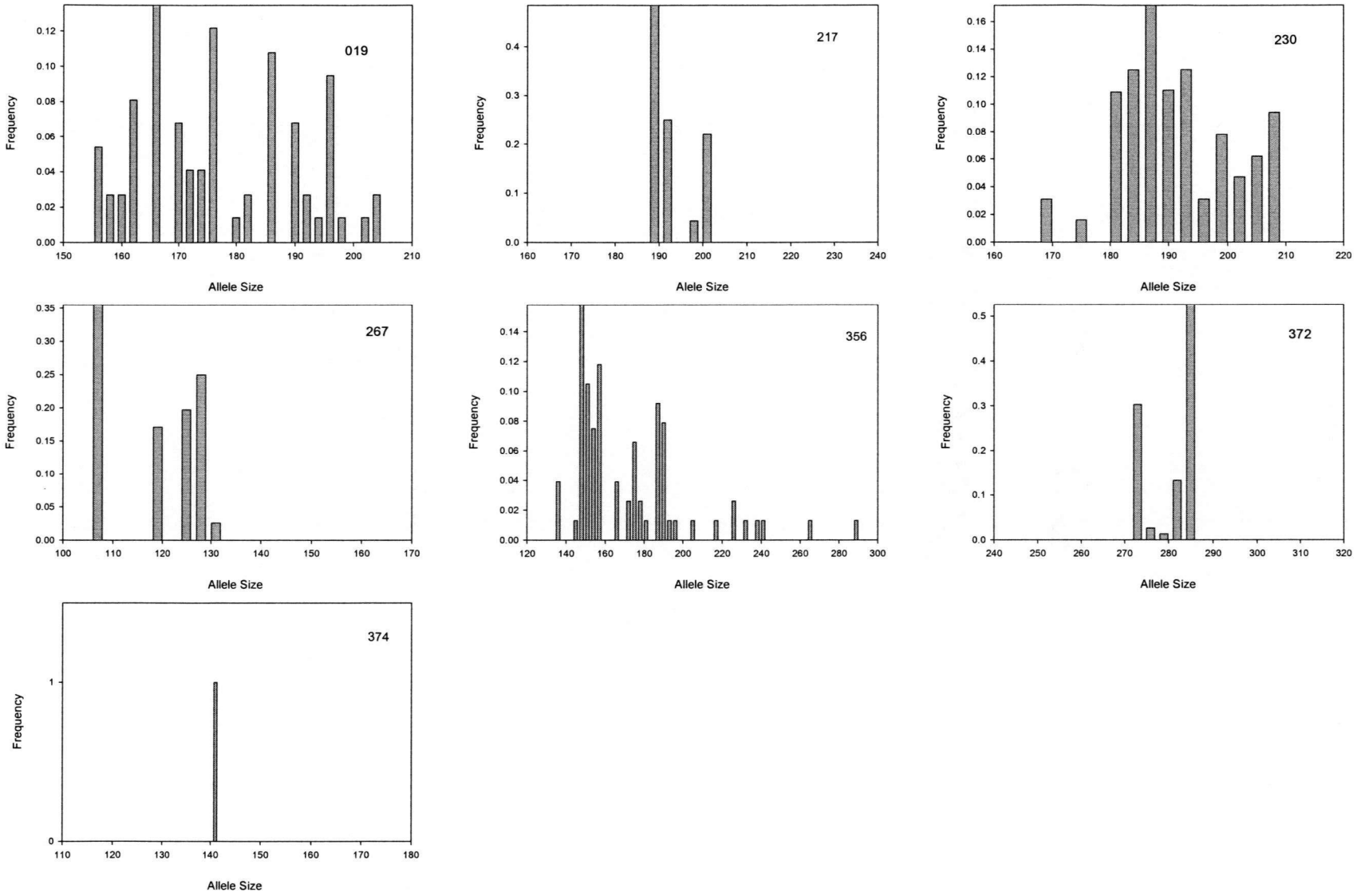

Figure 2.3. Allele size distribution for *M. nudatus* at seven microsatellite loci. Locus number is indicated on each graph.

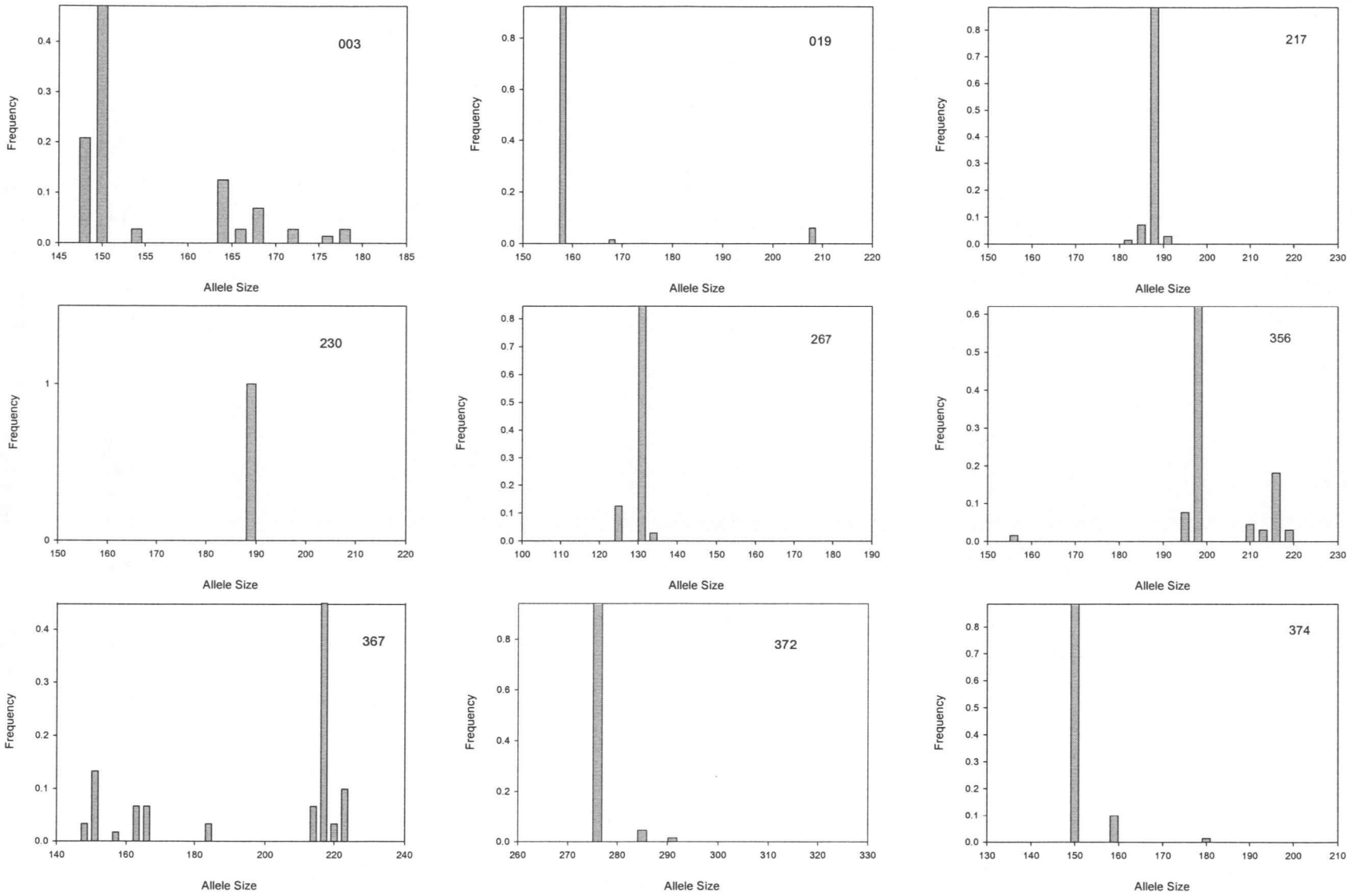

Figure 2.4. Allele size distribution for *M. laciniatus* at nine microsatellite loci. Locus number is indicated on each graph.

Inbreeding coefficients

The inbreeding coefficients estimated apply to seedlings from natural populations and may be different from the inbreeding coefficients of reproductive individuals in the same population. However they still should provide a relative ranking of inbreeding in the study populations. *M. nasutus* had the lowest inbreeding coefficient (f=0.21), and *M. laciniatus* had the highest (f=0.48) and *M. nudatus* had an inbreeding coefficient of f=0.28. Using the method of Jain (1979), this converts to 35% selfing for *M. nasutus*, 43% for *M. nudatus* and 65% for *M. laciniatus*.

Inbreeding coefficients were also calculated at the individual level using Ritland's Method-of-Moments Estimator (MME) (1996). The distribution of individual inbreeding coefficients for each species is presented in Figure 2.5. Although the mean inbreeding levels differ slightly from those estimated using the method of Weir and Cockerham (1984), their ranking remains the same across taxa.

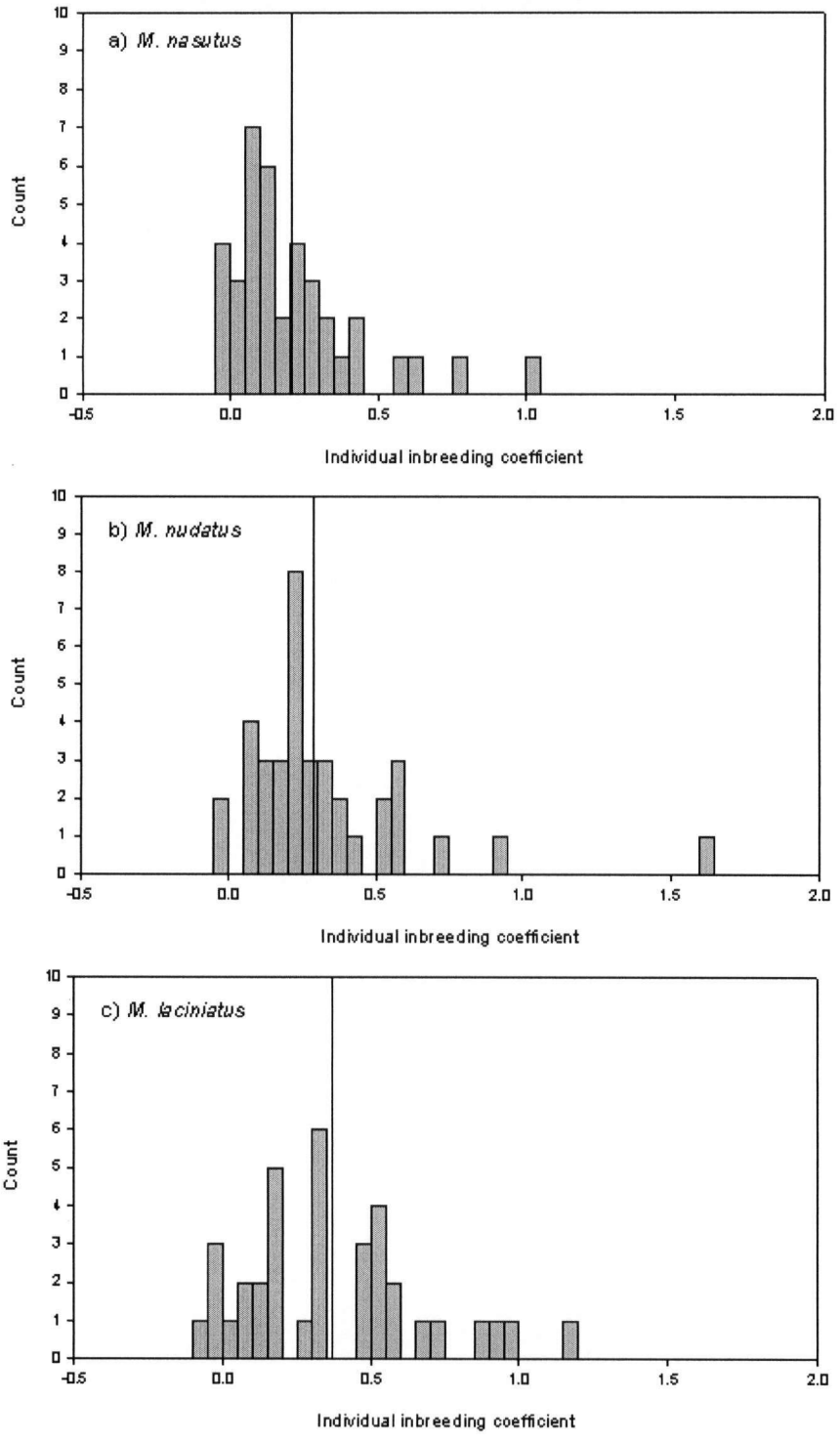

Figure 2.5. Frequency distribution of individual inbreeding coefficients at the seedling stage calculated using Ritland's Method-of-Moments Estimator (1996) for a) *M. nasutus* (*n* = 38, *mean* = 0.21, *SD* = 0.23, *SE* = 0.04), b) *M. nudatus* (*n* = 38, *mean* = 0.34, *SD* = 0.33, *SE* = 0.05) and c) *M. laciniatus* (*n* = 36, *mean* = 0.36, *SD* = 0.31, *SE* = 0.05). Vertical line indicates mean.

Neutral genotype and fitness

No significant relationships between variation at microsatellite loci and fitness traits were detected in linear regression analysis (Table 2.5; data for dry mass and number of flowers not shown). One statistically significant regression between genotype and fitness was detected for *M. nasutus*, but this taxon showed a strong tray effect, with tray 3 being significantly different from the rest (Figure 2.6). Besides producing plants that scored high for all fitness traits, tray 3 had higher *HET* than average. These two factors combined make direct interpretation of the *M. nasutus* data difficult. Therefore, additional analyses were performed with fitness traits standardized by tray for *M. nasutus*. A summary of the regression analysis for height is given in Table 2.5, and plots of height versus *HET*, d^2, d^2*mini*, *dabs*, *dmini* and individual inbreeding coefficient are given for *M. nasutus* (Figure 2.7 and Figure 2.8), *M. nudatus* (Figure 2.9) and *M. laciniatus* (Figure 2.10). Height was chosen for these plots because it showed a stronger correlation with inbreeding coefficient than the other fitness traits in Latta and Ritland (1994). Furthermore, height, dry mass and number of flowers were highly correlated in each of the three species. No relationship between height, dry mass, and number of flowers, and any of the genetic variables were found for *M. nudatus* or *M. laciniatus* (data for dry mass and number of flowers not shown). The analysis was omitted for *M. micranthus* due to insufficient microsatellite diversity (mostly monomorphic).

The results of the multiple linear regression using frequency of loci exhibiting 0 to 3 repeat differences as predictor variables was not statistically significant for any of the fitness traits in any of the populations (Table 2.6).

Table 2.5. Summary of linear regression using Height or Standardized height as the dependent variable. Standardized height is Height standardized by the tray average. *indicates regression slope is significantly different from 0 at α=0.05.

Population	Dependent variable	Predictor variable	r^2	F value	p-value
238	Height	*dabs*	0.04	1.54	0.22
404	Height	*dabs*	0.01	0.23	0.64
716	Height	*dabs*	0.01	0.25	0.62
238	Height	d^2	0.01	0.35	0.56
404	Height	d^2	0.00	0.01	0.94
716	Height	d^2	0.02	0.68	0.41
238	Height	*dmini*	0.09	3.60	0.07
404	Height	*dmini*	0.01	0.43	0.52
716	Height	*dmini*	0.00	0.09	0.77
238	Height	d^2*mini*	0.08	3.20	0.08
404	Height	d^2*mini*	0.02	0.56	0.46
716	Height	d^2*mini*	0.00	0.03	0.87
238	Height	*HET*	0.11	4.35	0.04*
404	Height	*HET*	0.00	0.00	0.99
716	Height	*HET*	0.02	0.52	0.48
238	Standardized height	*dabs*	0.01	0.37	0.55
238	Standardized height	d^2	0.03	1.10	0.30
238	Standardized height	*dmini*	0.00	0.02	0.88
238	Standardized height	d^2*mini*	0.00	0.01	0.90
238	Standardized height	*HET*	0.02	0.62	0.44

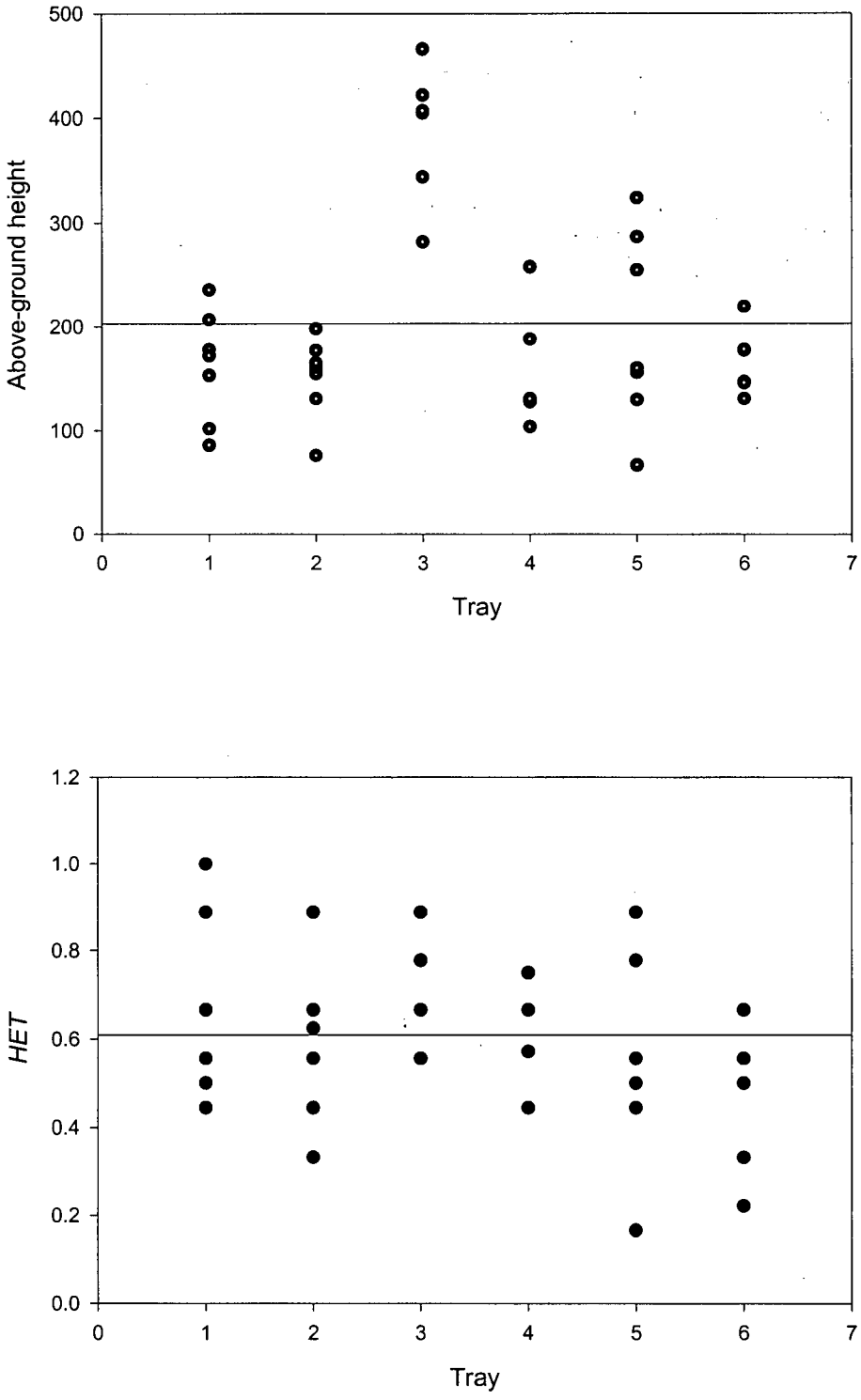

Figure 2.6. Height and *HET* of each individual used in this study plotted by tray for *M. nasutus*. The solid line represents the overall average height on the top graph and the overall average *HET* on the bottom graph.

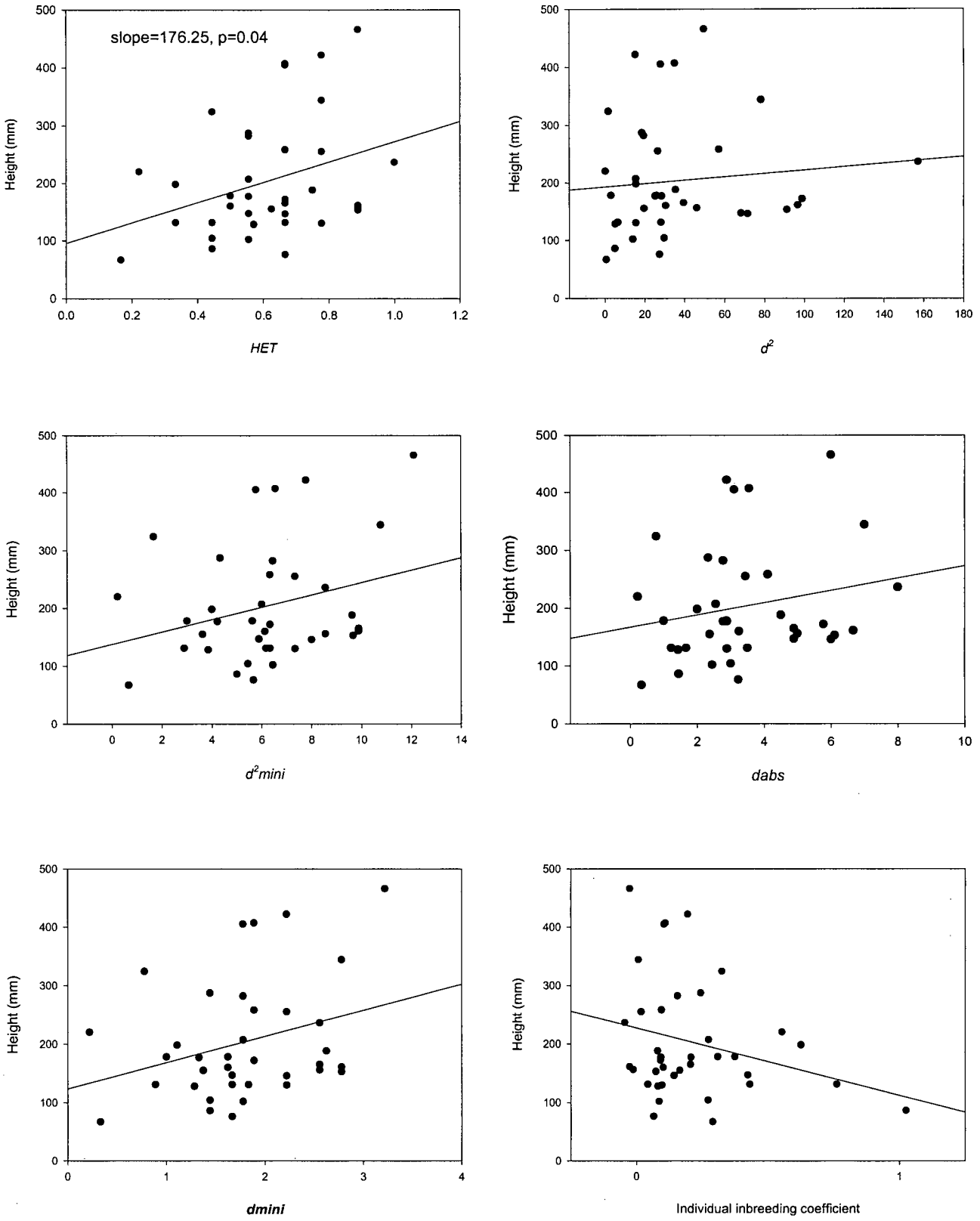

Figure 2.7. Height versus six genotypic measures in *M. nasutus*. Each point represents one individual.

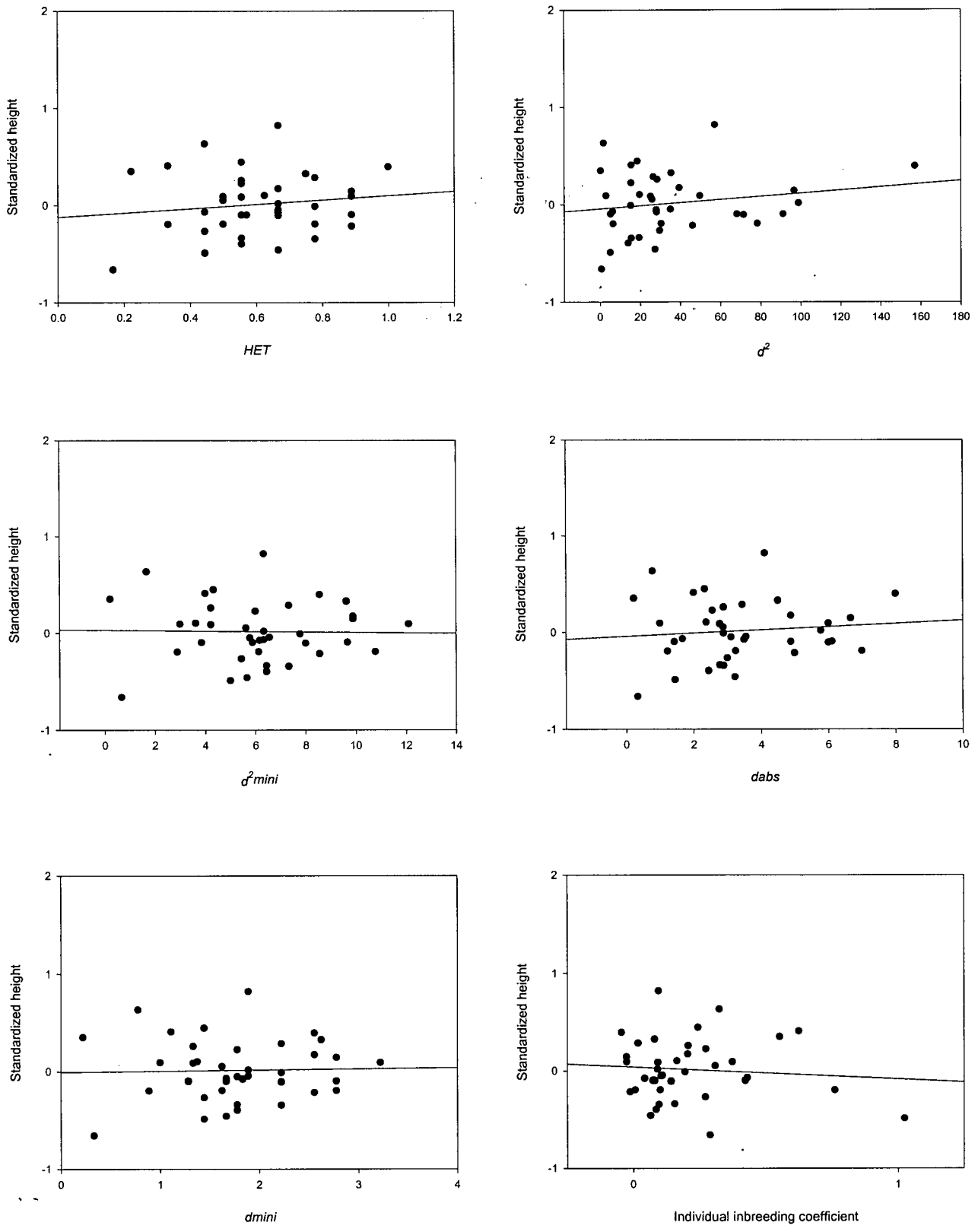

Figure 2.8. Standardized height versus six genotypic measures in *M. nasutus*. Each point represents one individual.

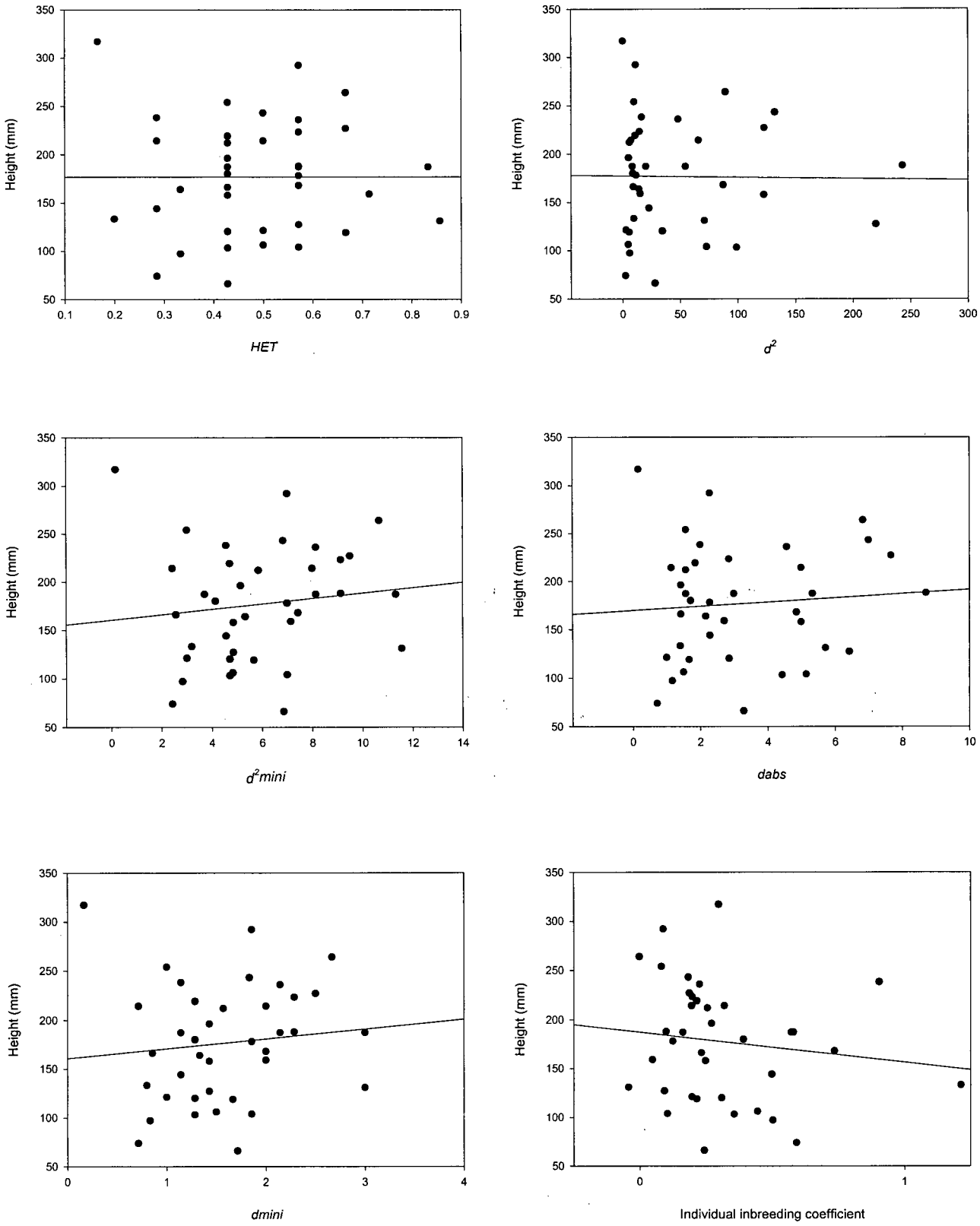

Figure 2.9. Height versus six genotypic measures in *M. nudatus*. Each point represents one individual.

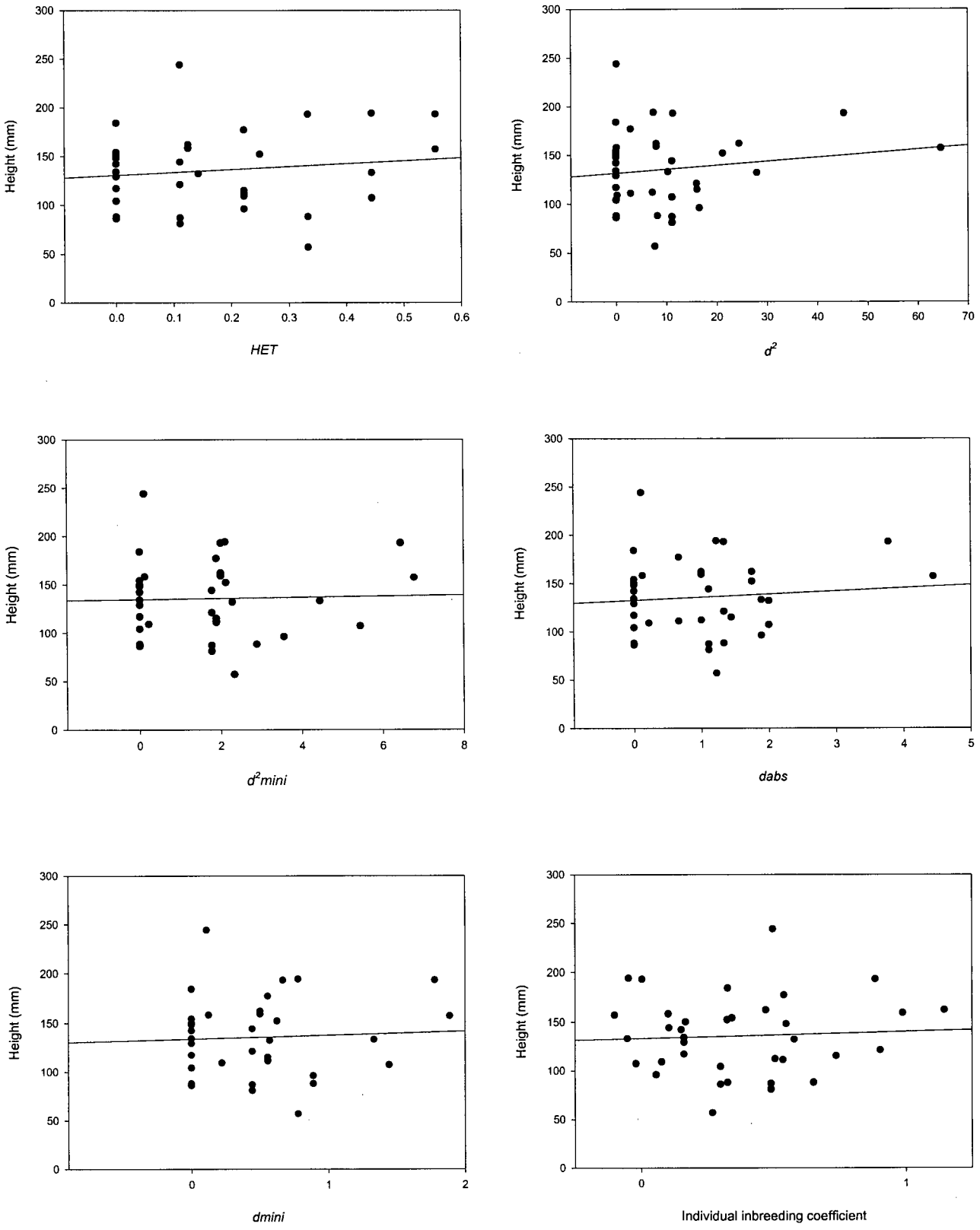

Figure 2.10. Height versus six genotypic measures in *M. laciniatus*. Each point represents one individual.

Table 2.6. Summary of *MLR* approach. No violations of the *MLR* assumptions were found. *Adj.*R^2 , adjusted R^2 .

Species	Response variable	*F*	*P-value*	*Adj.* R^2
M. nasutus	Height	1.81	0.15	0.08
M. nasutus	Standardized height	0.82	0.52	-0.02
M. nudatus	Height	0.57	0.68	-0.05
M. laciniatus	Height	0.69	0.61	-0.04

Variation in individual inbreeding levels: error variance versus true variation

Variation in individual inbreeding levels (as measure by *HET*, d^2, *dabs*, d^2*mini*, *dmini*, and Ritland's MME) was observed. However, it is important to tease apart variance due to the error in the estimation procedures from true variation in individual inbreeding levels. For *M. nasutus*, observed variance was significantly greater than that expected by error variance for all measures of individual inbreeding levels, with the exception of d^2 (Table 2.7). For *M. nudatus*, observed variance was not greater than that expected by error variance for all measures. For *M. laciniatus*, observed variance was significantly greater than that expected by error variance for most measures of individual inbreeding levels, with the exception of d^2 and Ritland's MME (Table 2.7).

Table 2.7. Summary statistics for genetic measures of individual inbreeding coefficients. Mean, *SD*, and variance of each population are given. P-value is reported for the probability that the observed variance is greater than the variance expected from the estimation procedure alone. *indicates significance at α=0.05. §indicates significance at α=0.10. *SD*, standard deviation.

Taxa	Measure	Mean	*SD*	Variance	p-value
M. nasutus	*HET*	0.6097	0.1893	0.03485	0.058§
	d^2	35.1909	33.8059	1142.844	0.119
	dabs	3.3928	1.9141	3.6638	0.045*
	d^2*mini*	6.1147	2.6792	7.1783	0.056§
	dmini	1.7787	0.6780	0.4597	0.030*
	Ritland's MME	0.2168	0.2262	0.051146	0.005*
M. nudatus	*HET*	0.4852	0.1561	0.02437	0.840
	d^2	45.1695	58.7988	3457.3	0.516
	dabs	3.2192	2.1842	4.7707	0.247
	d^2*mini*	5.8478	2.6687	7.1218	0.504
	dmini	1.5831	0.6462	0.41762	0.546
	Ritland's MME	0.3447	0.3026	0.0916	0.520
M. laciniatus	*HET*	0.1951	0.1872	0.03506	<0.001*
	d^2	11.0384	15.4482	238.647	0.370
	dabs	1.1114	1.1810	1.395	0.030*
	d^2*mini*	1.92626	2.0432	4.1750	0.001*
	dmini	0.5483	0.5640	0.3181	<0.001*
	Ritland's MME	0.3592	0.2890	0.0835	0.852

Discussion

In this study, correlations between selectively neutral marker genotype and fitness were examined in several *Mimulus* taxa with different selfing rates. *Mimulus* presented a good system for such an exploration because fitness traits are known to correlate with population inbreeding level, and selfing rate varies between taxa (Ritland and Ritland, 1989; Latta and Ritland, 1994). *A priori*, it was hypothesized that genotype-fitness correlations would behave differently in more inbreeding taxa versus more outbreeding taxa because they experience different levels of historical inbreeding, inbreeding depression and linkage disequilibrium. It was hypothesized that *HET* would be the genotypic measure most correlated with fitness in species with high selfing, and *d-mini* or d^2*mini* would be the most informative measure in predominantly outcrossing species. The results of this study are not in agreement with these predictions. In fact, there were no significant genotype-fitness correlations in any population for any genotypic measure.

This result may indicate that genotype-fitness correlations within populations are not detectable in *Mimulus*. It is difficult to determine if this null result is genuine, or if it is due to the power of this study. In order to address this issue, biological and experimental causes of the null result in this study must be explored. In the following section, the implications of the inbreeding coefficients of and the distribution of individual inbreeding coefficients in the populations is discussed. Subsequently, sources of experimental error are explored.

Inbreeding coefficients, distribution of individual inbreeding levels, and implications

Population level :

The inbreeding coefficients at the seedling stage for the populations used in this study were 0.21 for *M. nasutus*, 0.28 for *M. nudatus* and 0.48 for *M. laciniatus*. Assuming no biparental inbreeding or self/cross pollen competition, this is equivalent to 35% selfing for *M. nasutus*, 43% selfing for *M. nudatus* and 65% for *M. laciniatus*. It is noteworthy that no highly outcrossing population is represented in this study. Since inbreeding coefficients are estimated from genetic data it was impossible to foresee that no primarily outcrossing population was sampled. It is possible that no significant inbreeding depression is present in any of the populations used in this study since inbreeding depression is expected to decrease with selfing rate. However empirical evidence suggests that inbreeding depression does not necessarily decrease with inbreeding level (reviewed in Husband and Schemske, 1996; data for *Mimulus* in Latta and Ritland, 1994). Even without high inbreeding depression, genotype-fitness correlations can occur because linkage disequilibrium is prevalent in species with high rates of self-fertilization.

M. micranthus was chosen for this study as a species that represents a predominantly selfing species. However this end of the inbreeding spectrum was also lost because *M. micranthus* showed virtually no allelic diversity at the assayed microsatellite loci, and consequently no inbreeding coefficient could obtained. *M. micranthus* is a mainly cleistogamous species and therefore extremely inbred. However, the lack of allelic diversity in *M. micranthus* was a surprising result because isozyme diversity has been observed in *M. micranthus* and was used to estimate an inbreeding coefficient (f=0.73) in Ritland and Ritland (1989). The lack of diversity at microsatellite loci in *M. micranthus* likely indicates that the rate of random genetic drift is

extremely high due to small effective population size. Random genetic drift seems to be a much stronger force in this population than mutation. Alternatively, selection may account for the maintenance of allelic diversity at isozyme loci, and the lack of microsatellite variation may be more indicative of the true extent of neutral genetic variation in *M. micranthus*.

Individual level:

The distribution of individual inbreeding coefficients within a population plays an important role in generating genotype-fitness correlations; variation in individual inbreeding levels is required for these correlations to exist. Figure 2.5 shows the variation in individual inbreeding coefficients (as estimated by Ritland's MME) in the studied populations. However, *M. nasutus* is the only species that shows significantly more variation in individual inbreeding coefficients than is expected from error in the estimation procedure. In *M. nasutus*, genotype-fitness correlations were generally not detectable. This indicates that even in the presence of variation in individual inbreeding coefficients, genotype-fitness correlations are not always detectable.

Variance in individual inbreeding coefficients is not significant for *M. nudatus* and *M. laciniatus*. Ritland's MME (1996) is unbiased, but subject to error high variance in small populations. This is because it assumes allele frequencies are estimated from a large, stable reference population. The allele frequencies in this study, however, were estimated from individuals sampled from a small population. In small populations, stochastic changes in allele frequency are significant, and this likely the case for *M. laciniatus*, were other measures of inbreeding levels that do not consider allele frequency showed significant variation. For *M. nudatus*, the data set appears to have low power. For all measure of individual inbreeding levels, the observed variation was indistinguishable from variance expected from the estimation procedures. For *M.*

nasutus and *M. laciniatus*, true variation in individual inbreeding levels was detectable for most measures.

Inbreeding coefficient and inbreeding depression:

Previous studies (Latta and Ritland, 1994; Dudash and Carr, 1998) provide evidence of inbreeding depression in *Mimulus*, ranging from 0% to 68% among populations, including in populations with intermediate inbreeding coefficients (Latta and Ritland, 1994). Thus a lack of inbreeding depression is likely not the cause for the results of this study. Conversely, while Latta and Ritland (1994) estimated inbreeding depression at 47% (*SE*=0.07) for *M. nasutus*, only four of six populations showed statistically significant inbreeding depression. No previous inbreeding depression estimate is available for the *M. nasutus* population included in this study. Although this population does show variation in individual inbreeding levels, it is possible that it does not have a sufficient level of inbreeding depression to result in any genotype-fitness correlations, irrespective of the genetic measure used. Inbreeding depression estimates are not available for *M. nudatus* and *M. laciniatus*, however, very low inbreeding depression is expected in both species because they experience high levels of selfing.

Any level of selfing will have some effect on inbreeding depression through purging of deleterious mutations. Studies such as that of Latta and Ritland (1994), however, do show that a significant amount of inbreeding depression can be observed in populations with high selfing and that variation in inbreeding depression for a given outcrossing rate is remarkable. It is impossible to determine if inbreeding depression is operating in the study populations, but based on previous *Mimulus* work, it is likely present at least in *M. nasutus*.

Experimental error may contribute to the null result

There are several criticisms that can be made of this experiment and its potential to detect genotype-fitness correlations. The first is that fitness traits were being measured on growth-chamber reared plants rather than in the field. However growth chambers allow for simultaneous sowing of seeds, uniform growth conditions and a chance to collect good tissue samples for DNA extraction and to measure fitness characteristics at senescence. Furthermore both Latta and Ritland (1994) and Dudash and Carr (1998) were able to detect inbreeding depression in growth chamber and greenhouse experiments. Thus fitness traits do vary between selfed and outcrossed progeny in *Mimulus* in artificial environments. The artificial environment was likely not behind the null result.

A second criticism of this study is that the variability in microenvironment in the growth chambers was too large to allow for the detection of genotype-fitness correlations. There is some validity to this argument. Although trays were rotated within a growth chamber every seven days, there were observable differences between trays in terms of soil fungi, rusts and germination success. This problem could have been averted if only two offspring per seed parent had been planted and thus all individuals from one population would have fit in one tray. However this experiment initially had a larger sample size and was only cut down to a more manageable sample size after the growth chamber phase of the experiment was completed.

A third problem is one of size. Both the relatively small sample size and the small population sizes used in this study can present problems for detecting genotype-fitness correlations. Theoretical treatment of genotype-fitness correlations make this abundantly clear (Ritland, 1996; Tsitrone *et al.*, 2001). Ritland's MME, *HET* and d^2 perform best when estimated from large, stable populations. In the case of Ritland's

MME, allele frequencies weigh the importance of homozygosity at a locus. In this study, allele frequencies were estimated from individuals sampled from small populations. In small populations, allele frequencies are subject to large stochastic changes due to random genetic drift. Therefore, the poor performance of Ritland's MME in two of the populations is likely due to inaccurate allele frequency estimates. The other measures of individual inbreeding levels do not incorporate allele frequency. Random genetic drift may obscure their ability to detect genotype-fitness correlations. The small population sizes are the main cause for concern in this experiment.

Conclusions

In this study, genotype-fitness correlations were not observed in three *Mimulus* species of varying selfing rates. This study does not lend support to the use of d^2 or any other microsatellite-specific genetic measure as a correlate of fitness. Selfing rates were sufficiently high to suggest the possibility of low inbreeding depression (a major driving force behind genotype-fitness correlations), but the high selfing rates also result in linkage disequlibrium between allelic states at marker loci and fitness loci. This study shows that even in the presence of within-population variation in inbreeding level (as observed in *M. nasutus* and *M. laciniatus*), genotype-fitness correlations may not exist. Genotypic measures that relate to inbreeding level cannot always be used to estimate relative fitness in natural populations. There is particular concern about applying these measures to small populations. Although genotype-fitness correlations do occur in nature (Keller and Waller, 2002), this study, as well as previous studies (Table 1.1) shows they are by no means a universal phenomenon.

While this study does not lend support to the use of genotypic measures for detecting fitness in natural populations, it by no means ends the debate on the subject.

Three new genotypic measures were developed and introduced. These measures, inspired by the analytical modeling of Tsitrone *et al.* (2001), should be explored in data sets where heterozygosity is a correlate of fitness. In this study, significant variation in individual inbreeding levels was detected, as indicated by several microsatellite-specific measures. However, d^2 was consistently the least useful measure of individual inbreeding levels. The microsatellite-specific measures proposed in this thesis were able to detect significant variation in individual inbreeding levels. This suggests that pooling together large repeat differences is indeed useful. These new microsatellite-specific measures do warrant further exploration, both theoretically and empirically.

Chapter 3

General conclusion

The purpose of this thesis was to evaluate molecular measures of individual inbreeding coefficients and to correlate these measures with fitness in populations with variation in individual inbreeding levels. Microsatellite-specific measures were the particular focus of this study. Correlations were not found between neutral genotypic measures and fitness in any of the *Mimulus* species examined. This study emphasizes that genotype-fitness correlations are by no means implied by variation of individual inbreeding levels within a population.

There is a general problem woth studying inbreeding in natural populations. The best-case scenario involves knowing pedigrees and calculating inbreeding coefficients directly. However this type of information is nearly impossible to obtain in many cases, especially in plants. Without pedigree information, individual inbreeding coefficients, relatedness, *HET*, d^2, and related measures can be estimated using marker genotypes. Theoretical treatment has shown that these methods (with the exception of d^2) can perform well in large populations but are increasingly problematic as population size decreases (Ritland, 1996; Tsitrone *et al.*, 2001). This presents a fundamental problem because inbreeding effects are most prominent and of greater impact in small populations. Researchers often ignore the fundamental problem of using molecular methods in small populations and still apply these measures to their data. I believe that this is a major problem that is rarely addressed in the empirical literature. In some larger populations, genotype-fitness correlations are likely to exist (Tsitrone *et al.*, 2001). Genotype-fitness correlations are most likely to be detectable in large populations of mixed mating plants, or in substructured populations (Tsitrone *et al.*, 2001). These scenarios have large populations with inherent variation in individual

inbreeding levels. Exploring genotype-fitness correlations with small populations is ill-advised.

There is a need for further theoretical studies genotype-fitness correlations in general. The idea of d^2 is receiving very little support from the population genetics community and was even discredited by the same research group that initially proposed it (Slate and Pemberton, 2002). The data in Chapter 2 further contributes to evidence against d^2. As was shown in the randomization procedure, d^2 scores were not correlated across loci within an individual, as would be expected for any measure of inbreeding. This was a significant result because other microsatellite-specific measures (those proposed in Chapter 1) were correlated across loci within an individual. These new microsatellite-specific measures were proposed based on modeling (Tsitrone *et al.*, 2001) that showed fitness differences for small d^2. These measures do detect differences in individual inbreeding levels and should be subjected to simulation-based or analytical modeling.

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Appendix A

Table A.1. Cross-species amplification of microsatellite primers designed for *M. guttatus* or *M. nasutus*. A "y" indicates successful amplification and a "n" indicates no amplification, based on a panel of individuals of each taxon.
The number of individuals tested per taxon is indicated in under each taxon's name. Intra-taxon polymorphism observed in this test panel is indicated by a "p." "m" indicates monomorphic across all taxa. "*" indicates multiple banding pattern not indicative of a microsatellite loci.

Locus	Repeat	*M. guttatus* (4-5)	*M. nasutus* (3)	*M. micranthus* (2-3)	*M nudatus* (3-5)	*M. glaucesens* (1-2)	*M. platycalyx* (2-3)	*M. laciniatus* (1)	*M. Tilingii* (2-3)
019	(AG)25	y,p	y,p	y,p	y,p	y,p	y,p	y	y,p
211	(AAT)9	y,p	n	n	y	y	n	y	y
217	(AAT)13	y,p	y,p	y,p	y	y,p	y	y	y,p
222	(AAT)10	y	y,p	y	y	y,p	y	n	y,p
225	(AAT)18	y,p	y,p	y	y	y	y	n	y
230	(AAT)20 C	y,p	y,p	y	y,p	y	y,p	y	y,p
233	(AAT)17	y	n	y	y	n	n	n	n
240	(AAT)10	y,p	y,p	y	n	y,p	y	y	y,p
261	(AAT)24	y,p	y,p	y	y,p	y	y	y	y,p
265	(AAT)28	n	n	n	n	n	n	n	n
267	(AAT)12	y,p	y,p	y,p	y	y,p	y	y	y
272	(AAT)21	y	y	y	n	y	n	y	y
278	(AAT)11	y,p	y,p	y	y	y,p	y	y	y
281	(AAT)26 C	y,p	y,p	y,p	n	y,p	y,p	y,p	y,p
300		y	n	y	y	y	y	y	y
308	(AAT)40	y	y	n	y	y,p	y,p	n	y,p
356	(AAT)20	y	y,p	y	y,p	y	y	y,p	y
364	(AAT)9	y	n	y	n	n	y	y	n

Locus	Repeat	*M. guttatus* (4-5)	*M. nasutus* (3)	*M. micranthus* (2-3)	*M nudatus* (3-5)	*M. glaucesens* (1-2)	*M. platycalyx* (2-3)	*M. laciniatus* (1)	*M. Tilingii* (2-3)
367	(AAT)8	y,p	y,p	y	n	y,p	y,p	y	y
372	(AAT)22 C	y,p	y,p	y	y	y,p	y,p	y	y,p
374	(AAT)10	y,p	y,p	y	y	y,p	y,p	y	y,p
Mimct-8	(AT)16(GA)36	*	*	*	*	*	*	*	*
Mimct-10	(AT)11(GA)>8	*	*	*	*	*	*	*	*
Mimct-12[1]	COMPLEX	y,p	y,p	y,p	y	y	n	y,p	y,p
Mimct-14	(CA)16(CT)16	*	*	*	*	*	*	*	*
Mimct-15	COMPLEX	*	*	*	*	*	*	*	*
Mimca-b	COMPLEX	m	m	m	m	m	m	m	m
Mimct-c	COMPLEX	*	*	*	*	*	*	*	*
Mimct-k	COMPLEX	*	*	*	*	*	*	*	*

[1] Referred to as locus 003 in thesis.