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Method for the mapping of a female partial-sterile locus on a molecular marker linkage map

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Abstract The female gametophyte is an absolutely essential structure for angiosperm reproduction, and female sterility has been reported in a number of crops. In this paper, a maximum-likelihood method is presented for estimating the position and effect of a female partial-sterile locus in a backcross population using the observed data of dominant or codominant markers. The ML solutions are obtained via Bailey's method. The process for the estimating of the recombination fractions and the viabilities of female gametes are described, and the variances of the estimates of the parameters are also presented. Application of the method is demonstrated using a set of simulated data. This method circumvents the problems of the traditional mapping methods for female sterile genes which were based on data from seed set or embryo-sac morphology and anatomy.

Introduction

Female sterility has been reported in a number of crops such as cotton, sorghum, tomato, tobacco, millet, alfalfa, Arabidopsis, soybean, and rice (Arthur et al. 1993; Bingham and Hawkins-pfeiffer 1984; Casady et al. 1960; Dhesi

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1966; Goldman et al. 1994; Hanna and Powell 1974; Honma and Phatak 1964; Kubo and Yoshimura 2005; Li et al. 2006; Pereira et al. 1997; Robinson-Beers et al. 1992; Stroman 1941; Wan and Mann 1967). This phenomenon is mainly caused by the abnormal development of female organs, including the development of the ovule, the formation of the embryo-sac, and the growth of the embryo.

According to the angiosperm's embryo genesiology, female sterility can be classified into three types. The first type is one in which there are no female organs or only incomplete female organs. The second type of female sterility is one in which the female organs lack a normal embryo-sac owing to blocked development of the ovule or the megasporocyte. The third type of female sterility is one in which there is a normal mature embryo-sac but abnormal development of the embryo after pollination (Li et al. 2006).

Research on female sterility in plants can have several applications. The selection against sterility for seed production improvement could be made easier (Rosellini et al. 1998). Female-sterile plants could be used as pollen parents for hybrid seed production (Bingham and Hawkins-pfeiffer 1984; Daskalov and Mihailov 1988). Female sterility could also help to prolong flower life in ornamental plants, or provide new ways to obtain seedless fruits. Non-seed crops by means of female sterility can be used to prevent the transgene containment in transgenic crops (Ficcadenti et al. 1999).

Genetic studies have been initiated to identify genes underlying female sterility. Casady et al. found that female sterility was conditioned by the complementary effect of two dominant genes in sorghum (Casady et al. 1960). Single recessive genes control female sterility in tomato, alfalfa, pearl millet, *Arabidopsis thaliana*, soybean, and cotton (Bingham and Hawkins-pfeiffer 1984; Hanna and

Powell 1974; Honma and Phatak 1964; Moore et al. 1997; Pereira et al. 1997; Stroman 1941). Wan and Mann reported that two duplicate recessive loci were responsible for female sterility in tobacco (Wan and Mann 1967). Liu et al. found that a polygenic system is involved in both megagametogenesis and postzygotic isolation in inter-subspecific hybrid rice (Liu et al. 2001). Kubo et al. found that novel epistatic genes underlying female sterility causing hybrid breakdown in an intraspecific cross of cultivated rice (Kubo and Yoshimura 2005). Such studies have been largely based on data collections obtained from the seed set or embryo-sac morphology. Seed set is often a trait with continuous distribution in the case of partial sterility, and individuals will be classified into different types according to a somewhat arbitrary criterion for linkage analysis (e.g., Kubo and Yoshimura 2005). Furthermore seed set tend to be influenced by other genetic and environmental factors. When the frequency of embryo-sac abortion is used as a quantitative trait, a great many panicles at the flowering stage must be used for the microscope observation, this work is very huge and time-consuming.

In the present paper, we present a method for mapping a female partial-sterile locus by using co-segregation data of molecular markers in a BC population. Bailey's method 1951 is employed to obtain the maximum-likelihood estimates. This method can account for the environmental effects on seed set and the differences in artificial criteria of sterility levels.

Materials and methods

Estimates of parameters

Consider a mating of *aarrbb* (P_1) × *AARRBB* (P_2), where *A–a* and *B–b* are two flanking dominant or codominant molecular markers, and *R–r* is a female sterile locus that is assumed to be located between markers *A* and *B* on the same chromosome. The order of the three loci is *A–R–B*. The recombination fraction between *A* and *R* is r_1, and that between *R* and *B* is r_2. No chiasma interference is assumed. The differential viability of female gametes with the genotype *r* is expressed as *s* ($0 < s < 1$) relative to that of normal gametes, *R*, that is, the proportion of viable female gametes among the female gametes with the genotype *r*. The relative viability of female gametes with the genotype *r* is expressed as $t = s/(s + 1)$, that is, the proportion of viable female gametes with the genotype *r* among all the female gametes.

The backcross (BC) *ARB/arb* × *arb/arb* will produce eight kinds of offspring. The *RR*, *Rr* and *rr* genotypes cannot be observed directly, and only the segregation data

of the four phenotypic classes of molecular markers *A* and *B* can be obtained. The expected gametic frequencies of eight genotypes and the expected relative frequencies of the four classes of BC phenotypes are shown in Tables 1 and 2, respectively.

Let n_i ($i = 1, 2, 3, 4$) be the observed counts of phenotype with respect to *A* and *B* and *n* be the total count of individuals in a BC population, then the likelihood is:

$$L = \frac{n!}{\prod_i n_i!} \prod_i p_i^{n_i}$$

where p_i ($i = 1, 2, 3, 4$) is the expected relative frequency of the *i*th phenotypic class of molecular markers *A* and *B*.

The log likelihood is:

$$\ln L = C + \sum_i n_i \ln p_i,$$

then the three equations for score are

$$S_{r_1} = \frac{\partial \ln L}{\partial r_1} = \sum_{i=1}^{4} \left(\frac{n_i}{p_i} \times \frac{\partial p_i}{\partial r_1} \right) = 0,$$

$$S_{r_2} = \frac{\partial \ln L}{\partial r_2} = \sum_{i=1}^{4} \left(\frac{n_i}{p_i} \times \frac{\partial p_i}{\partial r_2} \right) = 0,$$

$$S_t = \frac{\partial \ln L}{\partial t} = \sum_{i=1}^{4} \left(\frac{n_i}{p_i} \times \frac{\partial p_i}{\partial t} \right) = 0.$$

Table 1 Expected female gametic frequencies of eight genotypes

Genotype of gamete	Relative frequency	Viability of female gamete
ARB	$(1 - r_1)(1 - r_2)/2$	1
arb	$(1 - r_1)(1 - r_2)/2$	*s*
Arb	$r_1(1 - r_2)/2$	*s*
aRB	$r_1(1 - r_2)/2$	1
ARb	$(1 - r_1)r_2/2$	1
arB	$(1 - r_1)r_2/2$	*s*
ArB	$r_1 r_2/2$	*s*
aRb	$r_1 r_2/2$	1

Table 2 Expected phenotype frequencies in a backcross population

Genotype of markers	Expected relative frequency (p_i)	Observed counts (n_i)
AB/ab	$p_1 = (1 - r_1)(1 - r_2)(1 - t) + r_1 r_2 t$[a]	n_1
Ab/ab	$p_2 = r_1(1 - r_2)t + (1 - r_1)r_2(1 - t)$	n_2
aB/ab	$p_3 = r_1(1 - r_2)(1 - t) + (1 - r_1)r_2 t$	n_3
ab/ab	$p_4 = (1 - r_1)(1 - r_2)t + r_1 r_2(1 - t)$	n_4
Σ	1	*n*

[a] $t = \frac{s}{s+1}$

The maximum likelihood estimates can be obtained by solving these equations. As these equations cannot be solved by conventional algebraic methods, iterative calculation is needed to obtain the estimates.

As the number of independent parameters to be estimated equals to the number of independent pieces of information, or degrees of freedom, Bailey's method (Bailey 1951) can be used to find the MLE's for the parameters by equating observations to their expected values. Doing so gives equations that are easier to solve than those from setting the scores equal to zero. The equations are:

$$\begin{cases} (1 - r_1)(1 - r_2)(1 - t) + r_1 r_2 t = n_1/n \\ r_1(1 - r_2)t + (1 - r_1)r_2(1 - t) = n_2/n \\ r_1(1 - r_2)(1 - t) + (1 - r_1)r_2 t = n_3/n \end{cases}$$

These equations can be algebraically solved, giving the MLE's:

$$\begin{cases} t = \dfrac{1}{2} + \dfrac{u}{2n(2n_2 + 2n_3 - n)} \\ r_1 = \dfrac{1}{2} - \dfrac{u}{2n(2n_1 + 2n_3 - n)} \\ r_2 = \dfrac{1}{2} - \dfrac{u}{2n(2n_1 + 2n_2 - n)} \end{cases}$$

where

$$u = \sqrt{n(n - 2n_1 - 2n_2)(2n_2 + 2n_3 - n)(2n_1 + 2n_3 - n)}.$$

Significance test of the estimates

Let $\theta_1 = r_1$, $\theta_2 = r_2$, $\theta_3 = t$, the expected Fisher information matrix $\boldsymbol{I}$ is given by

$$I(\theta) = (I_{ij}).$$

The elements of the matrix will be obtained as follows (Weir 1990):

$$I_{ij} = -\frac{\partial^2 \ln L(\theta)}{\partial \theta_i \partial \theta_j}, \ (i, j = 1, 2, 3)$$

For large samples, the inverse of the expected information matrix provides the variances and covariances of the MLE's (Brandt 1983). For the three parameters θ_i the expected information matrix is

$$\varepsilon[I(\theta)] = \begin{bmatrix} -\varepsilon\left(\frac{\partial^2 \ln L}{\partial r_1^2}\right) & -\varepsilon\left(\frac{\partial^2 \ln L}{\partial r_1 r_2}\right) & -\varepsilon\left(\frac{\partial^2 \ln L}{\partial r_1 t}\right) \\ -\varepsilon\left(\frac{\partial^2 \ln L}{\partial r_2 r_1}\right) & -\varepsilon\left(\frac{\partial^2 \ln L}{\partial r_2^2}\right) & -\varepsilon\left(\frac{\partial^2 \ln L}{\partial r_2 t}\right) \\ -\varepsilon\left(\frac{\partial^2 \ln L}{\partial t r_1}\right) & -\varepsilon\left(\frac{\partial^2 \ln L}{\partial t r_2}\right) & -\varepsilon\left(\frac{\partial^2 \ln L}{\partial t^2}\right) \end{bmatrix}$$

The expected value is found by replacing each count n_i, wherever it occurs, by its expected value. Inverting this matrix gives the large-sample variances and covariances:

$$\{\varepsilon[I(\theta)]\}^{-1} = \begin{bmatrix} \mathrm{Var}(\hat{r}_1) & \mathrm{Cov}(\hat{r}_1, \hat{r}_2) & \mathrm{Cov}(\hat{r}_1, \hat{t}) \\ \mathrm{Cov}(\hat{r}_2, \hat{r}_1) & \mathrm{Var}(\hat{r}_2) & \mathrm{Cov}(\hat{r}_2, \hat{t}) \\ \mathrm{Cov}(\hat{t}, \hat{r}_1) & \mathrm{Cov}(\hat{t}, \hat{r}_2) & \mathrm{Var}(\hat{t}) \end{bmatrix}$$

For large samples, the MLE's $\hat{\theta} = (\hat{\theta}_1, \hat{\theta}_2, \hat{\theta}_3)$ is unbiased, and has the multivariate normal distribution with mean θ and variance $\{\varepsilon(I(\theta))\}^{-1}$

$$\hat{\theta} \sim N\left(\theta, \{\varepsilon(I(\theta))\}^{-1}\right)$$

This allows tests of hypotheses to be set up very easily. For the test of parameter t, the null hypothesis is $H_0: t = 0$; for the test of parameters r_1 and r_2, the null hypotheses are $H_0: r_1 = 0.5$ and $H_0: r_2 = 0.5$, respectively. The test statistics used are the standard normal distribution deviations (u-test statistics) defined as the differences between the estimates of the parameters and the hypothesized values divided by the standard errors of the estimates, respectively.

The multiple testing procedure (MTP) proposed by Dudoit and van der Laan (2008) is used to obtain rejection regions (i.e., cut-offs) for the test statistics. The null distribution Q_0 is estimated by the distribution of the null shift and scale-transformed bootstrap test statistics using Procedure 2.3 (Dudoit and van der Laan 2008),

$$Z_n^B(m, b) \equiv \sqrt{\min\left\{1, \frac{\tau_0(m)}{\mathrm{Var}\left[T_n^B(m, \cdot)\right]}\right\}} \\ \times \left(T_n^B(m, b) - E\left[T_n^B(m, \cdot)\right]\right) + \lambda_0(m).$$

where B is the number of bootstrap samples, n is the sample size, $T_n^B(m, b)$ is an M-vector of test statistics for the bth bootstrap sample, $m = 1,\ldots, M$, corresponding to the M null hypotheses, $\lambda_0(m)$ and $\tau_0(m)$ are the user-supplied null values, here we take $\lambda_0(m) = 0$ and $\tau_0(m) = 1$, for the test statistics used are the standard normal distribution deviations. The bootstrap estimator of the common cut-off $\gamma_0(\alpha)$ is obtained using Procedure 4.21 for family-wise error rate (FWER) control (Dudoit and van der Laan 2008).

Monte Carlo simulation

Simulations were carried out based on one chromosome of length 130 cM with 11 markers. The map distance between markers 3 and 4 was 30 cM, the map distances between other adjacent pairs of markers were 10 cM. A single female sterility locus was simulated at position 40 cM, that is, between markers 3 and 4. The following factors were considered in the simulations: the intensity of viability and sample size of the mapping population. The intensity of viability ranges from 0.1 to 0.9 with fixed sample size 200. The sample size ranges from 50 to 500 with fixed intensity of viability 0.2. The simulation was replicated 1,000 times

under each setting. The means and standard deviations of the 1,000 replicates were used to evaluate the performance of each parameter combination. The purpose of the simulation was to demonstrate that the method works well and the test statistic behaves as expected, and to investigate the range of parameter values where the method works best.

Results

An example of the result obtained by analyzing a data set of 200 individuals with the proposed procedure is shown in Table 3. In this example, the estimates of the three parameters are all positive only in the interval flanking by marker M3 and M4, in which the putative sterile gene is located, and the estimated values are very close to the real values of these parameters. The estimates in all other intervals are not all positive. To obtain rejection region for the test statistics, we resampled this simulated dataset, using the above bootstrap strategy. One thousand bootstrap samples were generated. The bootstrap estimators of the common cut-off at $\alpha = 0.05$ and $\alpha = 0.01$ were $\gamma_0(0.05) = 2.82$ and $\gamma_0(0.01) = 4.46$, respectively. The u-test statistics for the three estimates are listed in Table 4. Table 4 shows that only in interval M3-M4, the u-test statistics of the three parameters are all positive and significant at the level of $\alpha = 0.01$. The u-test statistics of the three parameters in other intervals are either not all positive or not all significant. This example indicates that the method works well and the test statistic behaves as expected.

The results of analyzing simulated 1,000 data sets with the procedure are listed in Figs. 1, 2, 3 and 4 for the mean bias and the mean standard deviations of the estimates. As expected from the example shown in Table 1, the sterile locus was detected in all the data sets. When differential

Table 3 Estimates of parameters when $s = 0.1$ and sample size $= 200$

Intervals	r_1	r_2	t
M1–M2	0.090605	−0.000080	0.225347
M2–M3	0.090486	−0.000046	0.164747
M3–M4	0.090398	0.165329	0.090666
M4–M5	−0.000443	0.090780	0.226192
M5–M6	−0.000431	0.090528	0.275904
M6–M7	−0.000715	0.090852	0.316502
M7–M8	−0.001161	0.091188	0.349865
M8–M9	−0.000673	0.090137	0.377007
M9–M10	−0.002969	0.091893	0.399266
M10–M11	−0.004339	0.092029	0.417764
Real value	0.090635	0.164840	0.090909

Table 4 u-test statistics for the estimates of parameters when $s = 0.1$ and sample size $= 200$

| Intervals	r_1	r_2	t
M1–M2	7.919858	−0.018197	16.088622
M2–M3	8.711110	−0.054103	13.247007
M3–M4	7.533738	12.357016	7.552628
M4–M5	−0.057140	7.918253	16.127858
M5–M6	−0.020254	7.033731	18.417759
M6–M7	0.002158	6.147920	20.296449
M7–M8	0.015215	5.280514	21.872325
M8–M9	0.081509	4.415252	23.191852
M9–M10	0.042901	3.741640	24.273694
M10–M11	0.075820	3.070720	25.195821
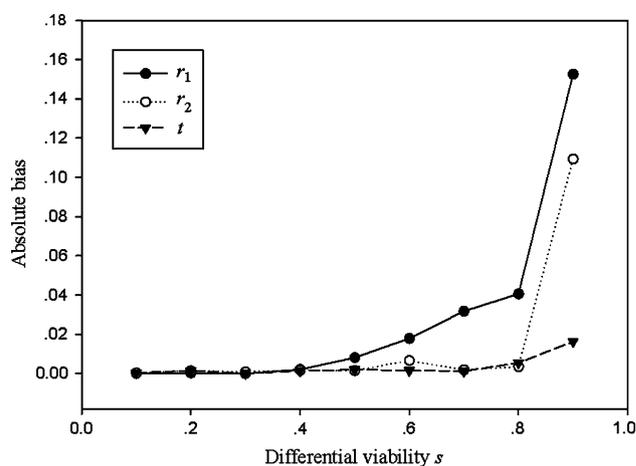
Fig. 1 The influence of differential viability s on the bias of the estimates

viability s was less then 0.5, the estimates of the position and effects of the sterile locus were almost unbiased (Fig. 1) and the standard deviations of the estimates were very small (Fig. 2). When differential viability s is larger then 0.5, the bias and standard deviations of the estimates increased very sharply. So this method may be more effective in the case where the effect of the sterile gene is strong.

Figures 3 and 4 show that with the increase of sample size, the bias and the standard deviations of the estimates decrease. When the sample size was larger than 150, the decrease of biases were not obvious.

Discussion

Female sterility has been reported in several species and sterility genes can act gametophytically (Pereira et al. 1997, and references therein) or sporophytically (Klucher et al. 1996; Rosellini et al. 1998, and references therein).
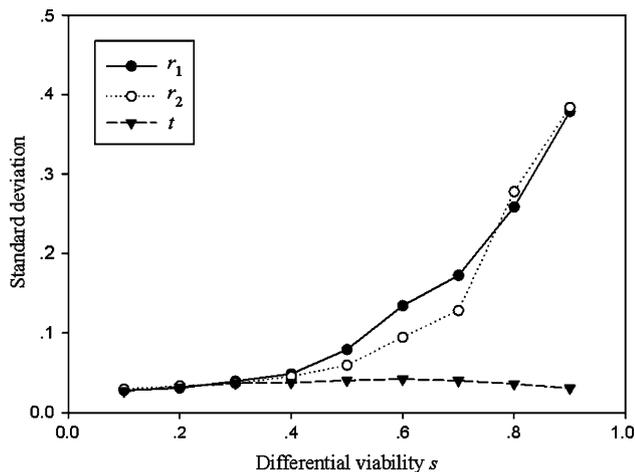
Fig. 2 The influence of differential viability s on the standard deviation of the estimates
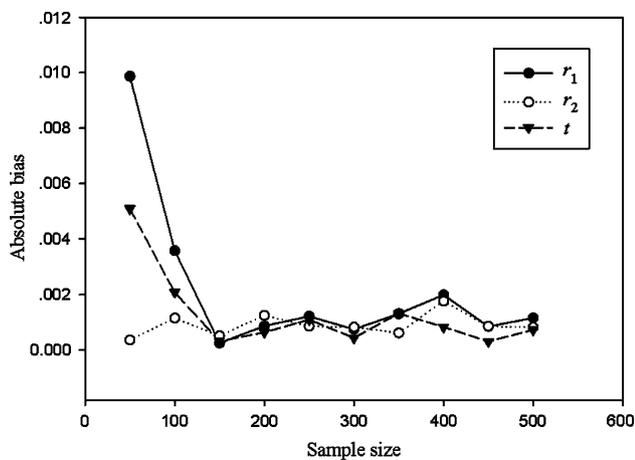
Fig. 3 The influence of sample size on the bias of the estimates
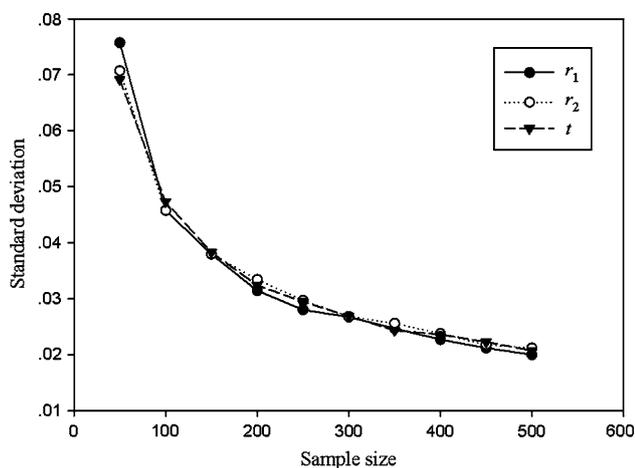
Fig. 4 The influence of sample size on the standard deviation of the estimates

Mapping of this kind of sterility genes is very important theoretically and practically. The traditional mapping methods for female sterile genes were based on data from seed set or embryo-sac morphology. Seed set tends to be influenced by environments and the criteria for sterility are arbitrary to some extent. And test crosses of progeny from fertile F2 plants must be conducted to determine the genotype of F2 plants (Kato and Palmer 2003a, b). Unless an easily scorable marker is available, collecting data on embryo-sac morphology is time-consuming and it is impractical to examine many plants. The approach presented in this paper circumvents these problems, which take advantage of the segregation distortion exhibited by molecular markers linked to female sterile genes.

It has become clear that plants contain two broad classes of mutations that exhibit fundamentally different segregation patterns. Sporophytic mutations affect sporophytically expressed genes and generally exhibit Mendelian 3:1 segregation patterns. Gametophytic mutations, by contrast, affect gametophytically expressed genes and exhibit apparent non-Mendelian segregation patterns (Drews et al. 1998). The extent of segregation distortion depends both on the distance between the markers and the sterile genes liked with them and on the effects of the sterile genes. Hence the segregation data of molecular markers can be used to estimate the locations and effects of the sterile genes. Chromosomal regions that cause distorted segregation ratios have been referred to as segregation-distorting loci (SDL) (Vogl and Xu 2000). And methods have been developed to map SDLs (Vogl and Xu 2000; Luo and Xu 2003; Luo et al. 2005; Wang et al. 2005). These methods involved the effects of both sporophytically expressed genes and gametophytically expressed genes, gametic selection and zygotic selection can be only separated form each other using statistical technology. In the practice of plant breeding, many female sterile mutations are found to be controlled by single recessive genes (Bingham and Hawkins-pfeiffer 1984; Hanna and Powell 1974; Honma and Phatak 1964; Moore et al. 1997; Pereira et al. 1997; Stroman 1941). In this case, much more simple method can be developed for the mapping of gametophytically expressed female sterile genes. The method suggested in this paper seems to be more practical for plant breeders. A saturated molecular map is not needed for the experimental population. Polymorphic markers can be screened using bulked segregant analysis and pairs of markers showing significant distorted segregations should be used as flanking markers to locate the putative female sterile gene. The computation is also very simple, no special packages is needed. The genotype data of the markers can be analyzed using the Microsoft Excel or even with a calculator.

When the female sterility is controlled by sporophytically expressed genes and the individuals can be classified

according to the fertility completely, the standard binary QTL mapping methods may be used to locate the female sterility genes. But in the case of female partial sterility controlled by one recessive gametophytically expressed gene, the phenotypes of that locus cannot be observed directly. What we can use is the genotype data of markers linking closely to the sterility gene. The seed set as a result of the female partial sterility and environmental effect will show a continuous distribution and individuals cannot easily be classified into different genotypes based on their

$$
\mathbf{X} = (x_{ij}) =
\begin{bmatrix}
\dfrac{\partial p_1}{\partial r_1} & \dfrac{\partial p_1}{\partial r_2} & \dfrac{\partial p_1}{\partial t} \\[2mm]
\dfrac{\partial p_2}{\partial r_1} & \dfrac{\partial p_2}{\partial r_2} & \dfrac{\partial p_2}{\partial t} \\[2mm]
\dfrac{\partial p_3}{\partial r_1} & \dfrac{\partial p_3}{\partial r_2} & \dfrac{\partial p_3}{\partial t} \\[2mm]
\dfrac{\partial p_4}{\partial r_1} & \dfrac{\partial p_4}{\partial r_2} & \dfrac{\partial p_4}{\partial t}
\end{bmatrix}
$$

$$
=
\begin{bmatrix}
-(1-r_2)(1-t)+r_2t & -(1-r_1)(1-t)+r_1t & -(1-r_1)(1-r_2)+r_1r_2 \\[1mm]
(1-r_2)t-r_2(1-t) & (1-r_1)(1-t)-r_1t & r_1(1-r_2)-(1-r_1)r_2 \\[1mm]
(1-r_2)(1-t)-r_2t & (1-r_1)t-r_1(1-t) & -r_1(1-r_2)+(1-r_1)r_2 \\[1mm]
-(1-r_2)t+r_2(1-t) & -(1-r_1)t+r_1(1-t) & (1-r_1)(1-r_2)-r_1r_2
\end{bmatrix}
$$

Appendix

Derivation of the formula:

$$
L(r_1, r_2, t) = \frac{n!}{n_1! n_2! n_3! n_4!} p_1^{n_1} p_2^{n_2} p_3^{n_3} p_4^{n_4}
$$

$$
\ln L = \ln \frac{n!}{n_1! n_2! n_3! n_4!} + n_1 \ln p_1 + n_2 \ln p_2 + n_3 \ln p_3 \\
+ n_4 \ln p_4
$$

seed settings. So this trait cannot be treated as a binary QTL mapping problem.

When the segregation distortion results from female sterility, the heterozygote must be used as female parent in a BC in order to make use of the information provided by the partially sterile female gametophytes. Single markers can be used only when the female gametophytes are completely sterile, in which case there will be two genotypes in a BC population and there will be only one parameter (map distance) to be estimated. When the female gametophytes are partially sterile, flanking markers should be used in a BC population, for there are three parameters to be estimated. When the female sterility is controlled by one single gametophyte-expressed gene, the method presented in this paper is expected to work well. If there are more than one such gene on the same chromosome which influence female sterility, epistasis between these genes should be taken into account. And new approaches should be developed to address this kind of problem.

$$
S_{r_1} = \frac{\partial \ln L}{\partial r_1} = \left(\frac{n_1}{p_1} - \frac{n_3}{p_3} \right) x_{11} + \left(\frac{n_2}{p_2} - \frac{n_4}{p_4} \right) x_{21}
$$

$$
S_{r_2} = \frac{\partial \ln L}{\partial r_2} = \left(\frac{n_1}{p_1} - \frac{n_2}{p_2} \right) x_{12} + \left(\frac{n_3}{p_3} - \frac{n_4}{p_4} \right) x_{32}
$$

$$
S_t = \frac{\partial \ln L}{\partial t} = \left(\frac{n_1}{p_1} - \frac{n_4}{p_4} \right) x_{13} + \left(\frac{n_2}{p_2} - \frac{n_3}{p_3} \right) x_{23}
$$

$$
\frac{\partial^2 \ln L}{\partial r_1 r_1} = -\left(\frac{n_1}{p_1^2} + \frac{n_3}{p_3^2} \right) x_{11}^2 - \left(\frac{n_2}{p_2^2} + \frac{n_4}{p_4^2} \right) x_{21}^2
$$

$$
\frac{\partial^2 \ln L}{\partial r_1 r_2} = \left(\frac{n_1}{p_1} - \frac{n_2}{p_2} - \frac{n_3}{p_3} + \frac{n_4}{p_4} \right) + \left(n_2 \frac{x_{21}}{p_2^2} - n_1 \frac{x_{11}}{p_1^2} \right) x_{12} \\
+ \left(n_3 \frac{x_{11}}{p_3^2} - n_4 \frac{x_{21}}{p_4^2} \right) x_{32}
$$

$$
\frac{\partial^2 \ln L}{\partial r_1 t} = \left(\frac{n_1}{p_1} + \frac{n_2}{p_2} - \frac{n_3}{p_3} - \frac{n_4}{p_4} \right) - \left(n_1 \frac{x_{11}}{p_1^2} + n_4 \frac{x_{21}}{p_4^2} \right) x_{13} \\
- \left(n_2 \frac{x_{21}}{p_2^2} + n_3 \frac{x_{11}}{p_3^2} \right) x_{23}
$$

$$\frac{\partial^2 \ln L}{\partial r_2 r_1} = \left(\frac{n_1}{p_1} - \frac{n_2}{p_2} - \frac{n_3}{p_3} + \frac{n_4}{p_4}\right) + \left(n_2\frac{x_{21}}{p_2^2} - n_1\frac{x_{11}}{p_1^2}\right)x_{12}$$
$$+ \left(n_3\frac{x_{11}}{p_3^2} - n_4\frac{x_{21}}{p_4^2}\right)x_{32}$$

$$\frac{\partial^2 \ln L}{\partial r_2 r_2} = -\left(\frac{n_1}{p_1^2} + \frac{n_2}{p_2^2}\right)x_{12}^2 - \left(\frac{n_3}{p_3^2} + \frac{n_4}{p_4^2}\right)x_{32}^2$$

$$\frac{\partial^2 \ln L}{\partial r_2 t} = \left(\frac{n_1}{p_1} - \frac{n_2}{p_2} + \frac{n_3}{p_3} - \frac{n_4}{p_4}\right) - \left(n_1\frac{x_{12}}{p_1^2} + n_4\frac{x_{32}}{p_4^2}\right)x_{13}$$
$$+ \left(n_2\frac{x_{12}}{p_2^2} + n_3\frac{x_{32}}{p_3^2}\right)x_{23}$$

$$\frac{\partial^2 \ln L}{\partial t r_1} = \left(\frac{n_1}{p_1} + \frac{n_2}{p_2} - \frac{n_3}{p_3} - \frac{n_4}{p_4}\right) - \left(n_1\frac{x_{11}}{p_1^2} + n_4\frac{x_{21}}{p_4^2}\right)x_{13}$$
$$- \left(n_2\frac{x_{21}}{p_2^2} + n_3\frac{x_{11}}{p_3^2}\right)x_{23}$$

$$\frac{\partial^2 \ln L}{\partial t r_2} = \left(\frac{n_1}{p_1} - \frac{n_2}{p_2} + \frac{n_3}{p_3} - \frac{n_4}{p_4}\right) - \left(n_1\frac{x_{12}}{p_1^2} + n_4\frac{x_{32}}{p_4^2}\right)x_{13}$$
$$+ \left(n_2\frac{x_{12}}{p_2^2} + n_3\frac{x_{32}}{p_3^2}\right)x_{23}$$

$$\frac{\partial^2 \ln L}{\partial t t} = -\left(\frac{n_1}{p_1^2} + \frac{n_4}{p_4^2}\right)x_{13}^2 - \left(\frac{n_2}{p_2^2} + \frac{n_3}{p_3^2}\right)x_{23}^2$$

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