A Genome-Wide Survey of Reproductive Barriers in an Intraspecific Hybrid

Yoshiaki Harushima,* Masahiro Nakagahra,† Masahiro Yano,‡ Takuji Sasaki‡ and Nori Kurata*

**Plant Genetics Laboratory, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan, †Institute of the Society for Techno-Innovation of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki 305-0854, Japan and ‡Rice Genome Research Program, National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305-8602, Japan*

Manuscript received December 21, 2000
Accepted for publication July 17, 2001

ABSTRACT

Genetic study of the reproductive barriers between related species plays an essential role in understanding the process of speciation. We developed a new method for mapping all possible factors causing deviations from expected Mendelian segregation ratios in F_2 progeny, which substantially contribute to reproductive isolation. A multiresponse nonlinear regression analysis of the allele frequencies of the markers covering an entire genome in the F_2 population was performed to estimate the map position and intensity of the reproductive barriers on each chromosome. In F_2 plants from a cross between a Japonica variety of rice, Nipponbare, and an Indica variety, Kasalath, the deviations of allele frequencies were well explained by 33 reproductive barriers. Of these, 15 reproductive barriers affected the allele transmission rate through the gametophyte and in 9 of these 15 cases, an Indica allele was transmitted at a higher frequency than a Japonica allele. The other 18 reproductive barriers altered the viability of the zygote via its genotype. Two zygotic reproductive barriers showed overdominance and 5 showed underdominance. The most pronounced reproductive barrier, mapped at 62.3 ± 0.4 cM on chromosome *3*, transmitted the Indica allele by 94% through the male gametophyte. The accuracy of the barrier position in the regression analysis was confirmed by progeny analysis. The regression analysis proved to be a powerful tool for detecting and characterizing every reproductive barrier, irrespective of whether it acted on the male or female gametophyte or the zygote.

IT is an accepted concept that biological species are groups of interbreeding populations that are reproductively isolated (Mayr 1942). One of the major challenges in biology is to understand the origin of species. In pursuit of this knowledge, the genetics of the reproductive barriers between closely related species have been studied extensively (reviewed by Dobzhansky 1951; Stebbins 1958; Coyne 1992; Wu and Palopoli 1994; Coyne and Orr 1998). Reproductive isolation may be achieved by a variety of mechanisms acting at various stages in the life history of an organism, for example, through the differential fitness of the gametophyte or zygote via different genes (Dobzhansky 1951; Stebbins 1958). Historically, the number and location of reproductive barriers have been estimated by observing their association with mapped morphological or biochemical trait loci and such studies have necessarily been limited to genetically well-characterized species, such as Drosophila. However, the recent availability of DNA markers covering the whole genome has allowed the genetics of reproductive barriers to be elucidated for many species.

One application of DNA markers is the analysis of quantitative trait loci (QTL) that seem to be responsible for reproductive isolation. For example, floral traits that cause pollinator discrimination (Schemske and Bradshaw 1999) have been analyzed as QTL of reproductive barriers (Bradshaw *et al.* 1995, 1998). There are four major limitations of QTL analysis in the study of reproductive barriers. First, sterility, an important reproductive barrier, is a phenotype of an individual plant or animal; however, sterility is not determined only by its own genotypes but also by genotypes of its progeny. A QTL approach without knowledge of the genotypes of the aborted gametes and zygotes would be difficult to use to analyze hybrid sterility. Second, the choice of traits investigated is restricted to those believed, *a priori*, to be involved in the isolation mechanisms. Third, the comparison of the isolation efficiency among different traits identified as reproductive barriers by QTL is difficult. Finally, the statistical sensitivity for the detection of QTL that affect sterility or inviability is weakened at the reproductive barrier by deviations of allele frequencies. If an allele at a QTL is an effective barrier in preventing progeny from having the allele, the allele frequency at the QTL will become too low to show statistical significance in the quantitative trait difference caused by the allelic difference.

The other application of DNA markers to the study of reproductive barriers is to analyze deviations from expected Mendelian segregation ratios. Hybrid sterility genes, hybrid breakdown genes, and gametophytic com-

Corresponding author: Yoshiaki Harushima, Plant Genetics Laboratory, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan. E-mail: yharushi@lab.nig.ac.jp

petition genes cause deviations from Mendelian expectation at such loci and also at the linked marker loci. There are several reports for studying reproductive barriers by the analysis of deviations from Mendelian segregation ratios of DNA markers covering a whole genome (Harushima *et al.* 1996; Xu *et al.* 1997; Gadau *et al.* 1999; Rieseberg *et al.* 1999; Jiang *et al.* 2000). Except for the study by Harushima *et al.* (1996), most studies reported deviations of allele frequencies of markers and/or segments and did not try to map factors causing these deviations. Fu and Ritland (1994), Mitchell-Olds (1995), and Cheng *et al.* (1996) developed maximum-likelihood methods for mapping a barrier, using flanking markers assuming a single barrier on a chromosome. Recently, Vogl and Xu (2000) developed a Bayesian method to map more than one barrier per chromosome in a backcross population. Although analysis of segregation ratios has not been performed for reproductive barriers, there was only one example of a whole-genome quantitative analysis. Locus positions and effects of inbreeding depression in a population from loblolly pine selfed seeds were estimated using a maximum-likelihood interval mapping procedure (Remington and O'Malley 2000). One of the reasons why there were few trials to map segregation-distorting loci in a whole genome is that there were few quantitative methods available for estimating the location and intensity of multiple reproductive barriers.

Here, we present a new method for mapping all possible factors acting as reproductive barriers causing deviation from Mendelian segregation ratios in F_2 progeny. A multiresponse nonlinear regression analysis was developed to estimate the map position and intensity of the reproductive barriers on each chromosome. As an application of the new method, the reproductive barriers of an intraspecific hybrid between a Japonica rice cultivar, Nipponbare, and an Indica rice cultivar, Kasalath, were analyzed, using a high-density linkage map (Harushima *et al.* 1998). A best-fit mathematical model was selected to describe the experimental observations of the allele frequencies of the DNA markers.

MATERIALS AND METHODS

Mathematical models: The influence of a reproductive barrier on the genotype frequency of linked markers depends on whether it acts in the gametophyte or zygote. The simplest model is a reproductive barrier affecting the gametophyte. A single reproductive barrier alters the allele transmission rate (t_1) of the parent "A" at its locus (x_1) in the male (or female) gametophyte. t_1 can vary from 0 to 1, although Mendelian segregation is 0.5. The probability of transmitting genotype *A* at *x* in the gametophyte can be expressed as

$$(1 - \theta_1)t_1 + \theta_1(1 - t_1),$$

where θ_1 is the probability of recombination between *x* and x_1, and θ_1 can be expressed by the Kosambi map function, $\theta_1 = \frac{1}{2}\,\mathrm{Tanh}(2|x - x_1|)$ (Kosambi 1944). The probability of transmitting genotype *B* at *x* in the gametophyte can be expressed as

$$\theta_1 t_1 + (1 - \theta_1)(1 - t_1).$$

The expected frequencies of the homozygous genotype, *A*, the homozygous genotype, *B*, and the heterozygote, *H*, are

$$A = \frac{1}{2}(t_1 + \theta_1 - 2t_1\theta_1), \quad B = \frac{1}{2}(1 - t_1 - \theta_1 + 2t_1\theta_1), \quad H = \frac{1}{2}. \tag{1}$$

Suppose two reproductive barriers, (x_1, t_1) and (x_2, t_2), affect different gametophytes. The expected frequencies of the homozygous genotype, *A*, the homozygous genotype, *B*, and the heterozygote, *H*, are

$$\begin{aligned} A &= (t_1 + \theta_1 - 2t_1\theta_1)(t_2 + \theta_2 - 2t_2\theta_2),\\ B &= (1 - t_1 - \theta_1 + 2t_1\theta_1)(1 - t_2 - \theta_2 + 2t_2\theta_2),\\ H &= (t_1 + \theta_1 - 2t_1\theta_1)(1 - t_2 - \theta_2 + 2t_2\theta_2)\\ &\quad + (1 - t_1 - \theta_1 + 2t_1\theta_1)(t_2 + \theta_2 - 2t_2\theta_2), \end{aligned} \tag{2}$$

where $\theta_1 = \frac{1}{2}\,\mathrm{Tanh}(2|x - x_1|)$, $\theta_2 = \frac{1}{2}\,\mathrm{Tanh}, (2|x - x_2|)$.

Two reproductive barriers, (x_1, t_1) and (x_2, t_2), on the same chromosome affect the same gametophyte and $x_1 < x_2$. The expected frequencies of the homozygous genotype, *A*, the homozygous genotype, *B*, and the heterozygote, *H*, are as follows: When $x \le x_1$,

$$A = \frac{1}{2}(t_1 + \theta_1 - 2t_1\theta_1), \quad B = \frac{1}{2}(1 - t_1 - \theta_1 + 2t_1\theta_1), \quad H = \frac{1}{2}.$$

When $x_1 < x \le x_2$,

$$A = \frac{t_2\theta_2(1 - \theta_1 - 2\theta_2 + 2\theta_1\theta_2) + \theta_2\{t_1(-1 + 2\theta_1) + \theta_1(\theta_1 + \theta_2 - 2\theta_1\theta_2)\}}{2(\theta_1 + \theta_2 - 2\theta_1\theta_2)(1 - \theta_1 - \theta_2 + 2\theta_1\theta_2)},$$

$$B = \frac{\theta_1^2(1 - t_2 - \theta_2)(-1 + 2\theta_2) + \theta_2(1 - t_1 - \theta_2 + t_1\theta_2) - \theta_1\{t_2 - 2t_2\theta_2 + (-1 + \theta_2)(1 - 3\theta_2 + 2t_1\theta_2)\}}{2(1 - \theta_1 - \theta_2 + 2\theta_1\theta_2)(\theta_1 + \theta_2 - 2\theta_1\theta_2)},$$

$$H = \frac{1}{2}.$$

When $x_2 < x$,

$$A = \frac{1}{2}(t_2 + \theta_2 - 2t_2\theta_2), \quad B = \frac{1}{2}(1 - t_2 - \theta_2 + 2t_2\theta_2), \quad H = \frac{1}{2} \tag{3}$$

(see supplemental material at http://www.genetics.org/supplemental/). A single zygotic viability barrier alters the zygotic viabilities at x_v on a chromosome for each genotype. The viabilities of *B* and the heterozygote relative to the other genotype, *A*, are V_b and V_h, respectively. The viabilities of *A*, *B*, and *H* at *x* can be expressed as

$$\begin{aligned} V_{Ax} &= (1 - \theta_v)^2 + 2\theta_v(1 - \theta_v)V_h + \theta_v^2 V_b,\\ V_{Bx} &= \theta_v^2 + 2\theta_v(1 - \theta_v)V_h + (1 - \theta_v)^2 V_b,\\ V_{Hx} &= \theta_v(1 - \theta_v) + \{(1 - \theta_v)^2 + \theta_v^2\}V_h + \theta_v(1 - \theta_v)V_b, \end{aligned}$$

where θ_v is the probability of recombination between *x* and x_v and θ_v can be expressed by the Kosambi map function, $\theta_v = \frac{1}{2}\,\mathrm{Tanh}(2|x - x_v|)$. The expected frequencies of the homozygous genotype, *A*, the homozygous genotype, *B*, and the heterozygote, *H*, are

$$\begin{aligned} A &= \frac{V_{Ax} \cdot A_{gx}}{V_{Ax} \cdot A_{gx} + V_{Bx} \cdot B_{gx} + V_{Hx} \cdot H_{gx}},\\ B &= \frac{V_{Bx} \cdot B_{gx}}{V_{Ax} \cdot A_{gx} + V_{Bx} \cdot B_{gx} + V_{Hx} \cdot H_{gx}},\\ H &= \frac{V_{Hx} \cdot H_{gx}}{V_{Ax} \cdot A_{gx} + V_{Bx} \cdot B_{gx} + V_{Hx} \cdot H_{gx}}, \end{aligned} \tag{4}$$

where A_{gx}, B_{gx}, and H_{gx} are the expected frequency of each

genotype with gametophyte barriers, and 0.25, 0.25, and 0.5 are those expected without gametophyte barriers, respectively. When the viability of the heterozygote is an average of the viability of *A* and *B*, $V_h = (1 + V_b)/2$, the formula for the expected frequency of each genotype at a zygotic barrier on a chromosome is the same as that for a gametophytic barrier. Therefore, if there is a single gametophytic barrier on a chromosome, we cannot distinguish it from the zygotic barrier in the above case in the regression analysis of the F_2 population. We adopted a gametophytic model when there was little difference between the gametophytic and zygotic models assessed by the variance of the regression analysis. The regression analyses were performed on a Macintosh computer using original programs developed from Mathematica packages. The Mathematica packages are available from the National Institute of Genetics (http://www.shigen.nig.ac.jp/rice/seganalysis).

Plant material and map construction: An F_2 population was produced by self-pollination of F_1 plants obtained by crossing an Indica variety, Kasalath, onto a Japonica variety, Nipponbare, as described previously (Kurata *et al.* 1994; Harushima *et al.* 1996, 1998). The seed fertility was averaged over 15 randomly selected panicles from randomly selected plants. The seed fertilities of Nipponbare, Kasalath, and the F_1 plants were 95.0, 96.3, and 95.6%, respectively. The *in vitro* germination rates of pollen of Nipponbare, Kasalath, and F_1 plants in a solution of 0.01% H_3BO_3, 0.03% $Ca(NO_3)_2$, and 17% sucrose were 80, 82, and 50%, respectively.

Construction of a high-density genetic linkage map with 2275 markers using 186 F_2 plants was described previously (Harushima *et al.* 1998).

Execution of the regression analysis: A multiresponse nonlinear regression analysis was performed to explain the observed allele frequencies of the markers on each chromosome. To avoid redundancy in the allele frequencies of cosegregated markers, markers mapped at different locations were used. To eliminate noise caused by a lack of plants with a scoring genotype, markers that scored in >176 of 186 F_2 plants were used. Ultimately, the allele frequencies of 1055 DNA markers were used for the regression analysis. The average number of scored plants for the 1055 markers was 183.9.

Progeny analysis: To determine the genotypes at a barrier locus of F_2 plants, genomic DNA was extracted from the bulked young leaves of ~100 F_3 seedlings for each F_2 plant. DNA extraction, electrophoresis, blotting, and hybridization with labeled marker probes, followed by examination with ECL detection systems (Amersham Pharmacia Biotech, Buckinghamshire, England), were performed as described previously (Kurata *et al.* 1994).

RESULTS

Regression analysis: The genotype segregation of adjacent linked markers tends to be similar, and, hence, when the frequencies of each allele in F_2 plants are plotted along a high-density linkage map, three continuous series of allele frequencies are obtained for each chromosome, corresponding to the genotypes of the heterozygote and the two homozygotes. A multiresponse nonlinear regression method was developed and used to analyze deviations from Mendelian segregation ratios of markers. In the regression analysis, mathematical models were fitted to the observed frequencies of alleles on an entire chromosome. In constructing the mathematical models for segregation frequency, the important issue was whether it affected the zygote or the gametophyte, and not whether it was involved in competition or abortion. In the models, a gametophytic reproductive barrier was described by two variables: the barrier position and the transmission rate of one parent allele to the progeny. In contrast, a zygotic reproductive barrier was described by three variables: the barrier position and the relative viabilities of one homozygote and the heterozygote to the other homozygote. Thus, the regression analysis could identify positions of reproductive barriers, distinguish between gametophytic and zygotic barriers, and determine their intensity by characterizing the deviations from Mendelian segregation ratios in an F_2 population. The importance of genetic interactions has been recognized in hybrid sterility, hybrid inviability, and gametophyte recognition (for example, Dobzhansky 1951; Oka 1988; Rieseberg *et al.* 1996, 1999; Gadau *et al.* 1999; Jiang *et al.* 2000). The mathematical models were made for mapping apparent factors that induce deviations in an F_2 population including any kinds of interactions, and the mapped locations of the factors by the regression analysis necessarily accounted for the results of interactions.

The expected allele frequency curves using simple mathematical models are shown in Figure 1. The allele frequency curves in Figure 1, A and B, were calculated by Equation 1 for a single gametophytic barrier and Equation 4 for a single zygotic barrier, respectively. The curves in Figure 1, C and D, were calculated using Equation 2 for one gametophytic barrier acting in each of the two different gametophytes. The curves in Figure 1, E and F, were calculated using Equation 3 for two gametophytic barriers acting in the same gametophyte. When gametophytic barriers on the same chromosome are gender specific and affect only male (or female) gametophytes, the segregation frequency of the heterozygote genotype is unaffected (Figure 1, A, E, and F). Deviation from the expected Mendelian heterozygous frequency occurs when either one zygotic barrier or two gametophytic barriers are involved, one affecting the male gametophyte and the other affecting the female gametophyte (Figure 1, B–D). When both male and female gametophytic barriers tend to transmit the same genotype, the heterozygous frequency is <50% (Figure 1C). On the other hand, the heterozygous frequency is >50% when male and female gametophytic barriers tend to transmit the opposite genotype (Figure 1D). Apparent overdominance is explained in two ways: when there are opposite gametophytic barriers in each gender and when there is a zygotic barrier. However, underdominance decreasing the frequency of the heterozygote by increasing both homozygotes is explained only by the presence of a zygotic barrier (Figure 1B). These characteristic features of allele frequencies of an F_2 population resulting from reproductive barriers give guidelines for applying mathematical models to the regression analysis; a flow chart is shown in Figure 2. Both allele frequency of heterozygote and coincidence of

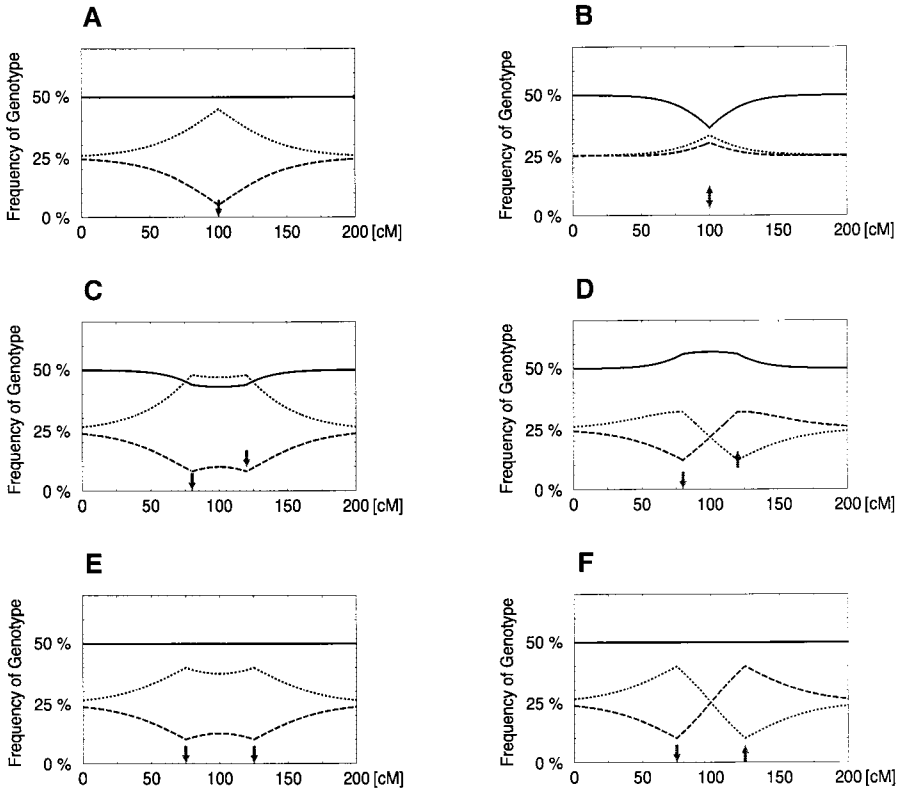

FIGURE 1.—The allele frequency curves calculated using simple reproductive barrier models. The allele frequencies of codominant markers in the F_2 population were calculated along a 200-cM length chromosome using equations for simple reproductive barrier models (see *Mathematical models*). (---) *A* homozygote; (···) *B* homozygote; (—) heterozygote. The locations of reproductive barriers are indicated by arrows. Up and down arrows indicate the positions of gametophytic barriers that increase the *A* and *B* alleles, respectively. Gametophytic barriers affecting different genders are noted by different vertical arrows (C and D). The double-headed arrows indicate the positions of barriers that affect zygotic viability. (A) A gametophyte barrier at 100 cM transmits the *A* allele by 10% of the total (x_1 = 1.0, t_1 = 0.1 in Equation 1). (B) A zygotic barrier at 100 cM alters the relative viability of the *B* homozygote and heterozygote to the *A* homozygote by 1.1 and 0.6, respectively (x_v = 1.0, V_b = 1.1, V_h = 0.6 in Equation 4). (C) Gametophytic barriers at 80 and 120 cM affect different gametophytes, and both tend to transmit the *A* allele by 20% (x_1 = 0.8, t_1 = 0.2, x_2 = 1.2, t_2 = 0.2 in Equation 2). (D) Two gametophytic barriers affect different gametophytes and these tend to transmit the opposite allele (x_1 = 0.8, t_1 = 0.2, x_2 = 1.2, t_2 = 0.8 in Equation 2). (E) Gametophytic barriers at 75 and 125 cM affect only male gametophytes, and both tend to transmit the *A* allele by 20% (x_1 = 0.75, t_1 = 0.2, x_2 = 1.25, t_2 = 0.2 in Equation 3). (F) Gametophytic barriers affect only male gametophytes, and these tend to transmit the opposite allele (x_1 = 0.75, t_1 = 0.2, x_2 = 1.25, t_2 = 0.8 in Equation 3).

peak locations of allele frequencies are important factors to apply to mathematical models. If a reproductive barrier at a locus affects both male and female gametophytes, we cannot distinguish it from a zygotic reproductive barrier.

Execution of the regression analysis: To study reproductive barriers in an intraspecific hybrid between Nipponbare and Kasalath, a regression analysis of allele frequencies in the high-density linkage map (HARUSHIMA *et al.* 1998) was performed. The allele frequencies of 1055 DNA markers in the 186 F_2 plants were measured and plotted along the genetic linkage map for each chromosome (Figure 3). Deviations from the expected Mendelian segregation ratio (25% for each homozygote

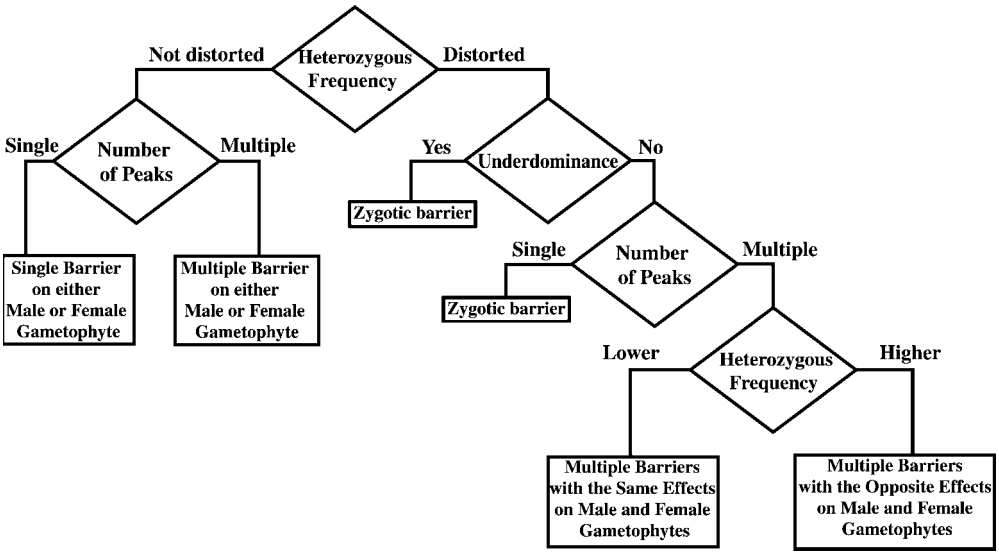

FIGURE 2.—A flowchart for applying mathematical models to the regression analysis. "Number of peaks": When each allele frequency has a single or no peak and the peaks are located at the same location in the map, number of peaks is a "single." The other cases are "multiple."

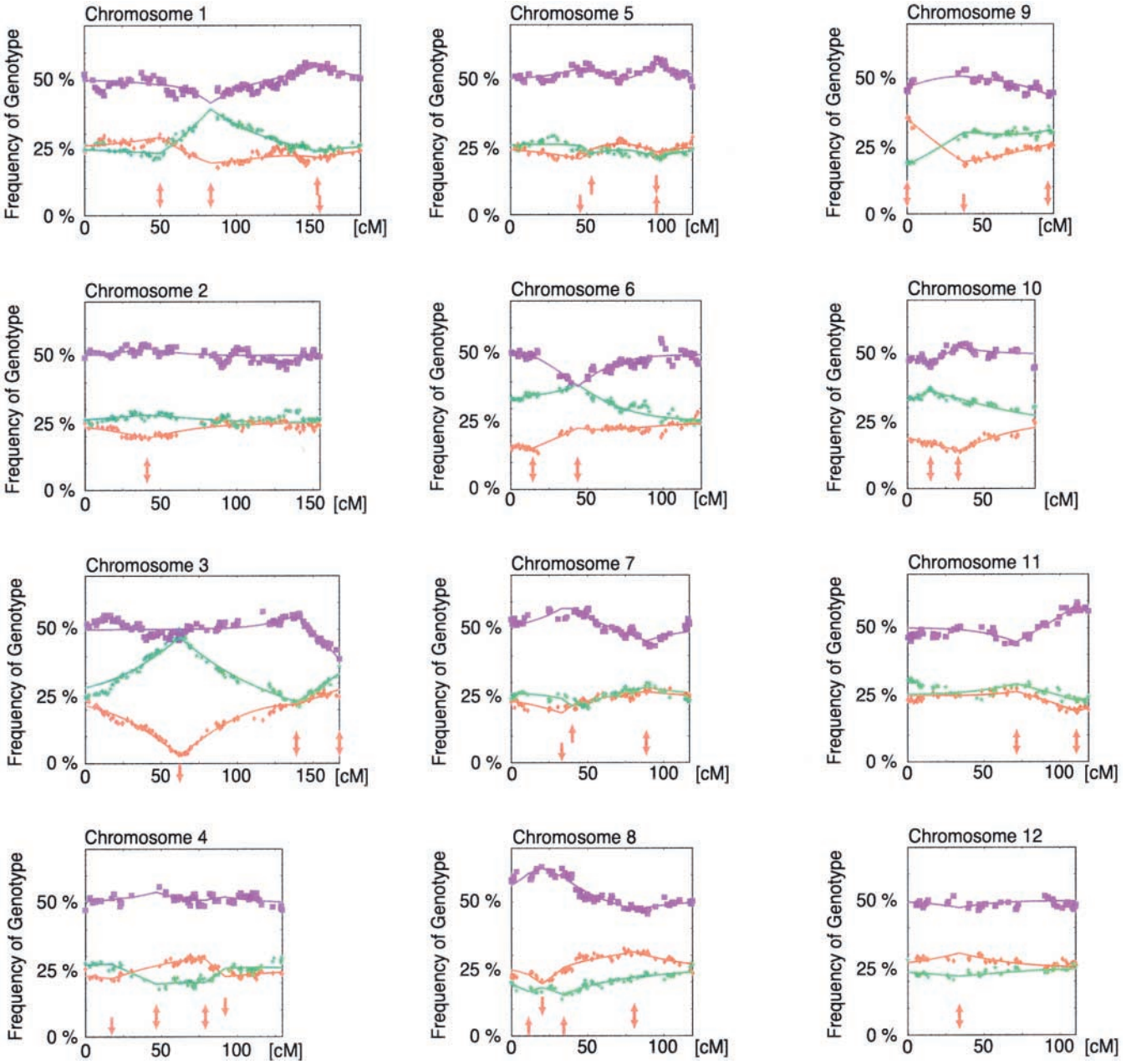

FIGURE 3.—Frequencies of each allele of DNA markers and the best-fitted regression curves are plotted along the genetic linkage map. For each chromosome, the left and right ends correspond to the short and long arm ends of the genetic linkage map that covers 1521.6 cM in the Kosambi function on 12 chromosomes (HARUSHIMA *et al.* 1998). The frequency of Nipponbare homozygous genotypes (orange diamonds), Kasalath homozygous genotypes (green stars), and heterozygous genotypes (purple squares) of the individual markers that scored more than 176 genotypes and mapped at different locations are plotted at the marker positions. This analysis used 1055 markers. The best-fitted regression curves for each allele frequency on the chromosome are also presented in the respective colors. The arrows represent reproductive barriers as in Figure 1. The up and down arrows indicate gametophytic barriers that prefer to transmit Japonica and Indica alleles, respectively.

and 50% for the heterozygote) were observed for all chromosomes. For each chromosome, several models with different initial guesses were applied following the guidelines in Figure 2. In some cases, it was hard to distinguish gametophytic barriers from zygotic barriers. For example, nine models with 14 different initial guesses were applied to explain genotype frequencies on chromosome *1*. The model with a gametophytic reproductive barrier in each gender and two zygotic barriers gave the smallest variance in the 14 analyses (Tables 1 and 2 and Figure 3). The deviations from Mendelian segregation ratios at ~50 cM and at ~80 cM were explained by two zygotic barriers, and the deviations at ~150 cM were explained by two gametophytic barriers on different genders. However, the deviations at ~50 cM were also explained with a slight increment of the

TABLE 1

Gametophytic reproductive barriers detected in the F_2 population of a cross between Nipponbare and Kasalath

Chr	Position (cM)	Marker	Gender	Alternative	T_J (%)	SS	*J* (%)	*I* (%)	*H* (%)	χ^2
1	153.2 ± 1.2	L819	G1	Z_1	66.2 ± 0.4		33.1	16.9	50.0	9.8[b]
1	154.7 ± 1.2	L819	G2	Z_1	33.3 ± 0.4		16.7	33.3	50.0	10.4[b]
3	62.3 ± 0.4	C582			5.8 ± 0.5	D	2.9[a]	47.1[a]	50.0	72.7[b]
4	17.4 ± 2.1	C820	G1	Z	38.5 ± 1.0		19.3	30.8	50.0	4.9
4	92.0 ± 1.4	R738	G2		30.0 ± 3.0		15.0[a]	35.0[a]	50.0	14.9[b]
5	45.7 ± 0.8	C282A	G1		33.2 ± 0.5		16.6	33.4	50.0	10.5[b]
5	53.2 ± 0.9	R413	G2		63.9 ± 0.6		32.0	18.0	50.0	7.2
5	95.7 ± 0.8	C1402	G2		34.2 ± 0.4		17.1	32.9	50.0	9.3[b]
5	95.8 ± 0.8	C1402	G1		67.6 ± 0.4		33.8	16.2	50.0	11.6[b]
7	33.2 ± 1.0	V177	G1	Z_2	27.6 ± 0.6		13.8[a]	36.2[a]	50.0	18.7[b]
7	40.1 ± 0.7	R1488	G2	Z_2	70.1 ± 0.5		35.1[a]	15.0[a]	50.0	15.0[b]
8	11.3 ± 1.7	G278	G1		76.1 ± 0.7		38.1[a]	11.9[a]	50.0	25.4[b]
8	20.3 ± 0.7	R2367	G2		25.1 ± 0.4	D	12.6[a]	37.4[a]	50.0	23.0[b]
8	34.4 ± 1.3	V115	G1		76.8 ± 0.7	D	38.4[a]	11.6[a]	50.0	26.7[b]
9	37.2 ± 2.2	S2660			31.4 ± 1.3		15.7	34.3	50.0	12.9[b]

Chr, chromosome number; position, genetic distance from the short arm end of the genetic map; marker, closest marker name; gender, which gender the gametophytic barrier affects; G1 and G2, different gender gametophytic barriers on the same chromosome, *e.g.*, when G1 is a male gametophytic barrier. G2 is a female gametophytic barrier on the same chromosome. However, the G1 barriers on the different chromosomes do not always affect the same gametophyte. Alternative, alternative types of barriers that could explain the allele frequencies with slight increment of variance. A single zygotic barrier could replace two pairs of barriers on chromosomes *1* and *7*. T_J, the Japonica transmission rate through the gametophyte. SS corresponds to detection of segregation distorting reproductive barriers by simple scoring that counts major segregation distortion peaks beyond a certain range, 15.5–34.5% for homozygotes and 36.5–63.5% for heterozygotes (Harushima *et al.* 1996). D, barriers detected by simple scoring in a previous study (Harushima *et al.* 1996). *J, I,* and *H* are the respective expected frequencies of the Japonica homozygote, Indica homozygote, and heterozygote at each reproductive barrier, assuming that a single barrier is acting on the chromosome. χ^2 for the segregation was calculated from *J, I,* and *H* for 186 plants.

[a] Values beyond the range.

[b] Highly significant distortion ($\chi^2 > 9.2$).

variance by two gametophytic barriers on the different genders, and the deviations at ~150 cM were also explained by a zygotic barrier. The other models failed to explain the allele frequencies without large increment of the variance.

The reproductive barriers detected by the regression analysis with the smallest variance are listed in Tables 1 and 2 for all chromosomes. The regression curves for allele frequencies based on these models are overlaid on the observed allele frequencies of markers in Figure 3. The distortions in allele segregation frequencies on all chromosomes were well explained by 33 reproductive barriers: 15 gametophytic (Table 1) and 18 zygotic (Table 2). Some of the allele frequency deviations from Mendelian expectations could also be explained by either the zygotic barrier or gametophytic barriers on the different gender. These barriers are indicated in the "Alternative" column in Tables 1 and 2. The number of barriers to explain allele frequencies varied from 31 to 38 using alternative explanations.

Of 15 gametophytic reproductive barriers, 9 barriers preferentially transmit Indica alleles, $T_J < 50\%$, and 6 barriers preferentially transmit Japonica alleles, $T_J > 50\%$. The highest and lowest Japonica transmission rates of the gametophytic barriers were 76.8% at 34.4 cM on chromosome *8* and 5.8% at 62.3 cM on chromosome *3*, respectively.

We also detected both overdominance and underdominance loci in zygotic viability. Overdominance occurs when the viability of the heterozygote is greater than that of both homozygotes; in other words, V_H is greater than both 1 and V_I in Table 2. Two zygotic reproductive barriers showed overdominance: one at 138.9 cM on chromosome *3* and one at 110.6 cM on chromosome *11*. Underdominance occurs when V_H is less than both 1 and V_I. Six zygotic reproductive barriers, on chromosomes *3*, *6*, *7*, *8*, *9*, and *11*, demonstrated underdominance. Underdominance barriers that lowered only heterozygote viability, while the viabilities of both homozygotes were the same, were found on chromosomes *7*, *9*, and *11*. The zygotic barrier on chromosome *8* seemed to be a "Japonica vigor" barrier rather than an underdominance barrier, because the viabilities of both the Indica homozygote and the heterozygote were lowered to almost the same degree. The zygotic barrier on chromosome *12* is also a Japonica vigor barrier. Zygotic barriers that decreased only the Japonica homozygotes were at 40.8 cM on chromosome *2* and at 33.2 cM on chromosome *10*. A zygotic barrier that decreased only the Indica homozygote was at 46.6 cM

TABLE 2

Zygotic reproductive barriers detected in the F_2 population of a cross between Nipponbare and Kasalath

Chr	Position (cM)	Marker	Type	Alternative	V_I	V_H	SS	J (%)	I (%)	H (%)	χ^2
1	49.8 ± 1.0	R2151S		G1, G2	0.51 ± 0.03	0.70 ± 0.02		34.5[a]	17.5	48.0	11.0[b]
1	83.2 ± 0.8	S13849			2.69 ± 0.15	1.23 ± 0.04	D	16.3	43.6[a]	40.1	35.2[b]
2	40.8 ± 1.6	Y2724R			1.45 ± 0.03	1.37 ± 0.03		19.3	27.9	52.8	3.4
3	138.9 ± 1.1	S770	o	G1, G2	0.77 ± 0.05	1.22 ± 0.06		23.8	18.4	57.8	5.7
3	167.2 ± 16.3	L375	u		1.29 ± 0.08	0.67 ± 0.25	DD	27.6	35.5[a]	36.9	15.1[b]
4	46.6 ± 2.2	S10644		G2	0.70 ± 0.05	0.99 ± 0.04		27.1	19.0	53.9	3.6
4	78.9 ± 1.4	M132		G1	0.41 ± 0.05	0.63 ± 0.06		37.6[a]	15.2[a]	47.1	19.3[b]
6	14.4 ± 1.5	B2F7		G1, G2	2.26 ± 0.12	1.87 ± 0.08	D	14.3[a]	32.3	53.4	13.0[b]
6	43.7 ± 0.8	P126	u		1.20 ± 0.05	0.67 ± 0.03	D	28.3	33.9	37.8	12.3[b]
7	88.6 ± 1.4	S13453	u		1.09 ± 0.02	0.85 ± 0.02		26.4	28.7	44.9	2.1
8	80.6 ± 1.8	S1609	u		0.79 ± 0.02	0.78 ± 0.01		29.8	23.6	46.6	2.3
9	0.0 ± 1.6	C152			0.39 ± 0.02	0.57 ± 0.02	D	39.5[a]	15.4[a]	45.1	23.4[b]
9	92.3 ± 1.0	C506	u		1.09 ± 0.02	0.82 ± 0.01		26.9	29.2	43.9	2.9
10	15.1 ± 1.2	S11777			1.52 ± 0.09	1.06 ± 0.05	D	21.6	32.8	45.6	6.2
10	33.2 ± 1.2	Y1053R			1.80 ± 0.11	1.74 ± 0.09	D	15.9	28.7	55.4	8.2
11	71.4 ± 1.9	V117	u		1.06 ± 0.04	0.79 ± 0.03		27.4	29.0	43.6	3.1
11	110.6 ± 1.2	R2968	o	G1, G2	1.20 ± 0.05	1.60 ± 0.06		18.5	22.2	59.3	7.0
12	33.8 ± 1.7	R367		G1, G2	0.72 ± 0.01	0.78 ± 0.01		30.5	22.0	47.5	3.2

Type, the type of zygotic barrier; o and u, overdominance and underdominance, respectively; V_I and V_H, the relative viabilities of the Indica homozygote and the heterozygote to the Japonica homozygote, respectively. The relative viability of the Japonica homozygote is 1 by definition. The other columns are the same as in Table 1. Either zygotic barrier at 46.6 or 78.9 cM on chromosome *4* could be replaced with slight increment of variance by a zygotic barrier.

[a] Values beyond the range.

[b] Highly significant distortion ($\chi^2 > 9.2$).

on chromosome *4*. A zygotic barrier that increased only the Indica homozygote was at 15.1 cM on chromosome *10*. The viabilities of the heterozygote of the other five zygotic reproductive barriers were between those of the two homozygotes. The highest and lowest relative viabilities for an Indica homozygote to a Japonica homozygote were 2.69 at 83.2 cM on chromosome *1* and 0.39 at the short arm end on chromosome *9*, respectively.

Direct comparison of the intensity of gametophytic and zygotic reproductive barriers is difficult, because the types of deviations from Mendelian segregation ratios caused by the reproductive barriers are different. To compare the intensity of reproductive barriers, we calculated χ^2 for Mendelian segregation as an index by the expected frequencies of genotypes at the barrier locus for 186 plants, assuming that a single barrier is acting on the chromosome (Tables 1 and 2). To classify barrier intensity as strong or weak, the critical value of χ^2 at probability level 0.01 for 2 d.f. was 9.2. Thirteen out of 15 gametophytic barriers were strong (Table 1); on the other hand, 11 of 18 zygotic reproductive barriers were weak (Table 2). The highest χ^2 was 72.7 of the gametophytic barrier at 62.3 cM on chromosome *3*.

Progeny analysis: The accuracy of the position of the reproductive barrier detected by the regression analysis can be confirmed by tests of the progeny of F_2 plants. If a barrier is strong enough and separated from others, the genotype at the barrier locus in the F_2 plant can easily be determined by Southern hybridization of the linked marker to genomic DNA using the bulked F_3 progeny. The progeny test for genotyping an F_2 plant at a barrier locus is performed by comparing the band intensities between the Japonica and Indica alleles of linked heterozygous restriction fragment length polymorphism (RFLP) markers. The F_3 Southern band intensity ratio of the Japonica band to the Indica band of a heterozygous marker is 1:1 when the barrier is not active; *e.g.*, the mother F_2 plant is homozygous at the barrier loci. However, the intensity ratio of the Southern band will be different from 1:1 when the barrier locus is also heterozygous; *e.g.*, deviation from Mendelian segregation ratios by the barrier also occurs in subsequent generations. The gametophytic reproductive barrier at 62.3 cM on chromosome *3* was separated enough from the other barriers, and the Japonica allele was rarely transmitted through males (or females). When the barrier is heterozygous in the F_2 plant, the bulked Southern band ratio of Japonica to Indica is expected to be 1:3.

The genotypes of F_2 plants with a crossover near the barrier and Southern blot analysis of their bulked F_3 progenies by a linked RFLP marker are shown in Figure 4. In the bulked F_3 progenies of F_2 plant nos. 91, 148, and 71, the heterozygous marker S11433 showed that the Southern band intensity ratios of the Nipponbare allele to the Kasalath allele were 1:3. This showed that the genotypes at the barrier locus should be heterozygous in these F_2 plants and the barrier caused deviation from Mendelian segregation ratios. On the other hand,

A

Locus Name		S11433	R2906S	C1135	C198	C582	C730	C603
Position		56.3	57.2	59.6	61.5	62.0	63.4	65.0
F_2 Plant No.	91	H	H	H	H	H	H X	K
	148	H	H	H	H	H	H X	K
	71	H	H	H	H	H X	N	N
	53	H	H	H	H X	K	K	K
	147	H	H	H	H X	N	N	N

B

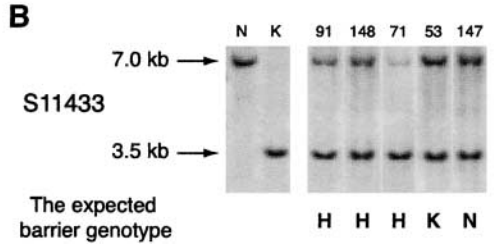

FIGURE 4.—Mapping of a gametophytic reproductive barrier on chromosome *3* by progeny analysis. (A) The genotypes of the F_2 plants with a crossover near the reproductive barrier are shown. N, K, and H are a Nipponbare homozygote, Kasalath homozygote, and heterozygote, respectively. X denotes a crossover position. (B) Southern blot analysis of *Bgl*II-digested genomic DNA of Nipponbare, Kasalath, and 100 bulked F_3 seedlings from respective F_2 plants using an RFLP marker of S11433. The F_2 plant numbers are indicated at the top of each lane. Considering the hybridization signal intensity of the allelic bands, the expected genotypes at the barrier loci in the F_2 plant are also indicated at the bottom.

the F_3 band ratios from F_2 plant nos. 53 and 147 were 1:1. This showed that the barrier locus was homozygous and deviation from Mendelian segregation ratios did not occur. These results showed that the barrier locus cosegregates with C582 at 62.0 cM and was present between C198 at 61.5 cM and C730 at 63.4 cM. The position of the barrier locus estimated by the progeny analysis agreed well with the estimate at 62.3 ± 0.4 cM by the regression analysis.

DISCUSSION

Deviation from Mendelian segregation ratios is an important feature caused by genetic reproductive barriers that prevent the free exchange of genes. Hence, analysis of allele frequency is very useful to survey the reproductive barriers in an entire genome. As a simple analysis of allele frequency, we previously proposed a method of mapping reproductive barriers by plotting genotype frequency along the linkage map and counting major distortion peaks beyond a certain range (HARUSHIMA *et al.* 1996). In this article, we propose a new method using quantitative analysis of allele frequency to characterize reproductive barriers. The results obtained by the new method are compared with those of the previous method.

We previously detected 11 reproductive barriers in the same F_2 population (HARUSHIMA *et al.* 1996). However, the regression analysis identified 33 reproductive barriers, including 10 of the 11 previously reported barriers (indicated by "D" in the column "SS" in Tables 1 and 2). There were three reasons for this difference in barrier detection: a different explanation of deviation from Mendelian segregation ratios, a difference in sensitivity, and the effects of neighboring barriers. One example of a different explanation is the distortion at the long arm end of chromosome *3* (Figure 3 and Table 2). We previously reported that there were two gametophytic barriers affecting both male and female gametophytes; however, we now consider that the assignment of a zygotic barrier is appropriate (only zygotic barriers can cause underdominance distortion). The regression analysis is more sensitive in detecting distortion caused by a barrier on a chromosome. We ignored many deviations in the previous report, since the distortion peaks did not exceed the set range. The decomposition of neighboring reproductive barriers by regression analysis improves the accuracy of the evaluation of the effects of each reproductive barrier on allele frequencies. The expected allele frequencies at a barrier locus were calculated using barrier parameters, while neglecting other barriers on the same chromosome (Tables 1 and 2). The expected allele frequencies of 13 out of 33 barriers exceeded the range that was used previously, 15.5–34.5% for homozygotes and 36.5–63.5% for heterozygotes, and are identified by footnote *a* in Tables 1 and 2. Seven of these 13 strong barriers appeared identical to those detected in our previous study. The remaining 3 previously reported barriers were zygotic barriers at 43.7 cM on chromosome *6* and at 15.1 and 33.2 cM on chromosome *10*. The apparent effect of these three reproductive barriers on genotype frequencies appeared to be heightened by neighboring zygotic reproductive barriers. On the other hand, 6 out of the 13 strong barriers were previously undetected, because apparent effects on allele frequencies were lowered by neighboring reproductive barriers. These were at 49.8 cM on chromosome *1*, at 78.9 and 92.0 cM on chromosome *4*, at 33.2 and 40.1 cM on chromosome *7*, and at 11.3 cM on chromosome *8*. Thus, the present regression analysis proved to be a powerful tool for detecting and characterizing all effective reproductive barriers, both gametophytic and zygotic.

Although the regression analysis of allele frequencies is a powerful way to map reproductive barriers causing deviation from Mendelian segregation ratios, the disadvantage of this method is that we cannot identify the isolation mechanism of each barrier detected. When a regression analysis is performed on the F_2 population, we can distinguish whether the barriers act at a zygotic or gametophytic level, but we cannot distinguish whether the gametophytic barriers are on the male or female side. Moreover, we cannot distinguish whether the barriers induce abortion or involve gametophyte competition. Seed and pollen fertility could be used to

consider the mechanism of barrier action. The high seed fertility of the Nipponbare × Kasalath F_1 plants suggests that significant abortion of the female gametophytes and zygotes does not occur. However, we detected 18 zygotic reproductive barriers and 6 pairs of gametophytic reproductive barriers affecting both male and female gametophytes. There are two possibilities to explain this inconsistency. One is that some deviations were due to chance. Because a genetic linkage map is constructed that has an equal probability of apparent recombination frequency on the map, deviations by chance in gametophyte or zygote would pose as those that were due to reproductive barriers. Elimination of barriers that showed weak effect by a χ^2 test after the regression analysis (Tables 1 and 2) would be one possible way to distinguish false barriers. One-half of zygotic reproductive barriers were weak, which partly explains the inconsistency between the high seed fertility of the F_1 plants and the large number of zygotic barriers. The other possibilities are that the zygotic reproductive barriers detected in this cross must affect seed germination and the viability of the F_2 plants and female gametophytic reproductive barriers must affect selection and competition in the female gametophyte.

Since Nipponbare, Kasalath, and F_1 are viable, sterility of F_1 and inviability of F_2 would be caused by unfavorable gene interactions. Gene interactions in reproductive isolation has been claimed to be important (for example, Dobzhansky 1951; Oka 1988; Wu and Palopoli 1994; Rieseberg *et al.* 1996, 1999; Gadau *et al.* 1999; Jiang *et al.* 2000). We performed analysis of all pairwise interactions between marker loci for segregation. These results suggested that some zygotic reproductive barriers detected here interact with other loci (these results will be published elsewhere).

Gene interactions between segregated loci affect the apparent barrier intensities. When interaction of barriers does not induce the deviations at the barrier loci, we cannot map the barrier loci by regression analysis. For example, if different genotype combinations of two genes in a gametophyte cause its abortion (neither a combination of genotype *A* at one locus and genotype *B* at another locus nor vice versa survive), one-half of male gametophytes cannot survive. However, the allele frequencies at these gene loci show no deviation and we cannot detect the barriers by regression analysis. Because a segregated interactive locus always contains favorable genotypes to survive, the apparent deviations caused by the barrier interacting with segregated loci become smaller than those caused by the barrier that interacts with nonsegregated loci (cytoplasmic factors or parental loci). If a gametophytic barrier interacts with a segregated locus of a gametophyte, one-half of the unfavorable genotype must survive and the transmittance rate cannot exceed the range from 0.33 to 0.66. As the number of interactive segregated loci increases, the permissible range of the transmission rate becomes narrow and the apparent deviations reduce. Since the transmission rates of the four gametophytic barriers at 62.3 cM on chromosome *3* and at 11.3, 20.3, and 34.4 cM on chromosome *8* exceeded the range, these barriers would not interact with the other gametophytic loci.

An interaction with a linked locus also affects the apparent recombination frequency between the barrier locus and the interactive locus. If two linked loci with different genotypes induced hybrid sterility, the recombinant gametophytes were aborted and the apparent recombination frequency between them was lower than the intrinsic one. The interaction affects the apparent genetic distances in the region between the two barriers; however, it does not affect the order of markers including barrier loci. Since we used the apparent recombination fractions in the regression analyses, the mathematical models can well explain the observed data. Although the total map length is influenced by the decrease of the apparent recombination frequencies, the relative barrier location in the linkage map is not influenced by the interaction. When interactive barriers in gametophyte or zygote are not on the same chromosome or a barrier interacts with cytoplasmic factors or parental loci, the genetic distances in the linkage map are not influenced at all.

Many genes for reproductive barriers in rice have been detected using different crosses and phenotypic markers. These are listed in the reports of the Committee on Gene Symbolization, Nomenclature, and Linkage Groups of the Rice Genetic Cooperative (Kinoshita 1995). It is difficult to discuss the correspondence between the barriers detected in this study and the genes reported previously because the positions of the previously reported genes are not precise in the molecular linkage map.

This is the first quantitative analysis of allele frequencies that surveys all reproductive barriers causing deviations from Mendelian segregation ratios in an entire genome. Our method is easily applicable to a backcross population, although we cannot distinguish whether the barriers act at the zygote or gametophyte in the analysis of a backcross population. This represents the beginning of studies that will lead to an understanding of the genetics of reproductive isolation. How does each barrier contribute to reproductive isolation? What gene or element is the barrier? If a real gene is involved in reproductive isolation, what is its biological significance in a self-pollinating plant like cultivated rice? Further studies are necessary to elucidate the nature of individual reproductive barriers. Reciprocal backcross experiments will verify whether the reproductive barrier acts in the zygote, in the male gametophyte, or in the female gametophyte. The development of a near-isogenic line for each reproductive barrier is necessary to identify its isolation mechanism and to clone the barrier as a gene or an element. We are planning to isolate the most prominent barrier on chromosome *3* by positional cloning.

We thank H. Morishima, anonymous reviewers, and the editor for valuable comments and suggestions. This work was partly supported by a grant from the Ministry of Agriculture, Forestry, and Fisheries of Japan (Rice Genome Project MP-1117).

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Communicating editor: C.-I Wu