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Molecularmarker diversity and hybrid sterility in indica-japonica rice crosses

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Abstract The partial sterility of hybrids between the indica and japonica rice subspecies of Asian cultivated rice is a serious constraint for utilizing inter-subspecific heterosis in hybrid rice breeding. In this study, we have investigated the relationship between molecular-marker polymorphism and indica-japonica hybrid fertility using a diallel set involving 20 rice accessions including 9 indica and 11 japonica varieties. Spikelet fertility of the resulting 190 F_1s and their parents was examined in a replicated field trial. Intra-subspecific hybrids showed much higher spikelet fertility than inter-subspecific hybrids except in crosses involving wide-compatibility varieties. The parents were surveyed for DNA polymorphism using 96 RFLP and ten SSR markers, which revealed extensive genetic differentiation between indica and japonica varieties. A large number of markers detected highly significant effects on hybrid fertility. The chromosomal locations for many of the positive markers coincided well with previously identified loci for hybrid sterility. The correlation between hybrid fertility and parental distance was low in both intra- and inter-subspecific crosses. The results suggest that the genetic basis of indica-japonica hybrid sterility is complex. It is the qualitative, rather than the quantitative, difference between the parents that determines the fertility of hybrids.

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Introduction

The strong hybrid vigor in the F_1s between the (*Oryza sativa* ssp. *indica*) and japonica (*O. sativa* ssp. *japonica*) subspecies of the Asian cultivated rice (*Oryza sativa* L.) has attracted a large amount of research interest with the hope of developing hybrid rice by making use of such heterosis. A major difficulty encountered in the development of such inter-subspecific hybrids is the partial sterility that occurs frequently in indica-japonica crosses (Kato et al. 1928). It has been shown that the fertility of indica-japonica hybrids varies widely from fully fertile to almost completely sterile, with the majority of the inter-subspecific hybrids showing significantly reduced fertility (Oka 1988; Liu et al. 1996).

Another common finding in the rice genetics literature is a significant genetic differentiation between indica and japonica varieties, which appears to be a major source of genetic diversity in the cultivated rice gene pool. Such indica-japonica differentiation has been detected in various rice samples using several classes of markers including isozymes (Glaszmann 1987; Morishima and Oka 1981), DNA restriction fragment length polymorphism (RFLP) (Zhang et al. 1992), simple sequence repeats (SSRs) (Yang et al. 1994), and randomly amplified polymorphic DNA (RAPD) (Mackill 1995).

Despite extensive studies of hybrid sterility and genetic differentiation between indica and japonica rice groups, little is known concerning the relationship between the hybrid sterility, on one hand, and indica-japonica differentiation, on the other hand. Detailed characterization of such a relationship will certainly enhance the understanding of the genetic basis of

hybrid sterility in indica-japonica crosses, and hence facilitate the utilization of the inter-subspecific heterosis.

The study reported in this paper was undertaken: (1) to assess the relationship between the level of genetic differentiation of the parents and the fertility of the indica-japonica hybrids using a 20-variety diallel set and (2) to infer the potential number and putative locations of the hybrid sterility genes involved in this diallel set.

Materials and methods

Rice materials and field experiments

The 20 rice accessions of this study, including 9 indica and 11 japonica varieties, were carefully selected with respect to the indica and japonica characteristics in order to have a wide range of representation of both rice groups (Table 1). Three accessions ("02428", "Dular" and "Ketan Nangka") have been reported to be wide-compatibility varieties which are able to overcome the partial sterility problem in indica-japonica crosses (Ikehashi and Araki 1984; Liu et al. 1992). Another three ("C57", "Minghui 63" and "Teqing") have been used as fertility restorers in hybrid rice-breeding programs (Lin and Min 1991). These 20 varieties were intermated in all possible non-reciprocal pairs to form a total of 190 crosses, consisting of 36 crosses of indica by indica, 55 crosses of japonica by japonica, and 99 crosses of indica by japonica. The F_1s and their parents (210 entries in total) were replicated three times in the 1994 rice growing season in the field at Jinzhou Agricultural Research Institute, Hubei Province, China. Spikelet fertility, scored as a percentage of filled and half-filled seeds in total florets, was measured on the basis of 5–10 true hybrid plants per plot. The details of the field experiment and data collection were described by Liu et al. (1996) except that one variety which was included in that report was not used in the present analysis.

Molecular marker and laboratory assay

The 20 parental varieties were surveyed for DNA polymorphisms with two classes of markers: restriction fragment length polymorphisms (RFLPs) using a total of 96 probes, and simple sequence repeats (SSRs) using ten pairs of primers. These markers were selected at regular intervals from a published molecular-marker linkage map (Causse et al 1994). For the RFLP assay, the DNA sample of each parent was digested singly with three restriction endonucleases *Dra*I, *Eco*RI and *Hin*dIII. The procedures for DNA extraction and Southern-blot hybridization essentially followed those described previously by Saghai Maroof et al. (1984) and Zhang et al. (1992). The methods for SSR analysis were those described by Wu and Tanksley (1993) and Yang et al. (1994).

Data processing and statistical analysis

The molecular-marker data were processed using the scheme described by Zhang et al. (1994). Two kinds of banding patterns were resolved by various RFLP probes and SSRs. Banding patterns displayed by the majority of the RFLP probes and SSR primers were in accord with single-locus variation (single variable bands or the simultaneous occurrence of 2–3 major bands), and thus scored as genotypes. Multiple variable bands were detected by some of the RFLP probes and SSRs; they were scored by the presence or absence of individual bands. Also, restriction patterns resolved by different enzymes within probes were often perfectly correlated, only data from one of the enzymes for each probe were used in the analysis to avoid redundancy within probes. The same data-processing schemes were also applied to SSRs.

Table 1 A list of the varieties used in this study and their average spikelet fertility in inter-subspecific hybrids

Variety	Type	Source	Spiklet fertility (%)	
			Average	SD
Japonica				
Taichung 65	Cultivar	China (Taiwan)	31.5	24.7
Mudanjiang 8	Cultivar	China	30.8	25.6
Taihu Wanjing	Landrace	China	45.8	21.7
Zaoshajing	Cultivar	China	44.1	17.9
Balilla	Cultivar	Italy	37.8	19.9
Yaso	Cultivar	Japan	34.4	24.2
Nongken 58	Cultivar	China	43.9	20.3
Akihikari	Cultivar	Japan	39.8	19.2
C57	Cultivar[a]	China	52.4	12.0
02428	Cultivar[b]	China	62.6	14.1
Ketan Nangka	Landrace[b]	Indonesia	50.6	11.0
Indica				
Nanjing 1	Cultivar	China	32.0	15.6
Teqing	Cultivar[a]	China	47.2	14.7
Zhaiyeqing 8	Cultivar	China	35.5	14.8
Aijiao Nante	Cultivar	China	27.8	15.9
Shengli Xian	Cultivar	China	50.4	8.4
Minghui 63	Cultivar[a]	China	52.5	13.7
Indonesia Paddy Rice	Landrace	Indonesia	27.7	15.9
Yuchi 231-8	Cultivar	China	33.7	15.4
Dular	Landrace[b]	India	81.0	4.6

[a] Restorer lines in hybrid-rice production in China
[b] Reported to be a wide-compatibility variety

The genetic distance between parents was calculated as the percentage difference in marker genotypes over loci. The similarity of these 20 varieties revealed by the molecular markers was depicted with a cluster analysis using several algorithms, including single linkage, complete linkage (Sokal and Sneath 1963), and the Ward method (Ward 1963) provided by the statistical package Statistica (StatSoft 1991). The level of genetic diversity of each marker locus was calculated using a genetic diversity index ($1-\Sigma p_i^2$, where p_i is the frequency of the ith allele or banding pattern), which was subsequently partitioned into two components, one corresponding to genetic heterogeneity within indica and japonica groups and the other representing differentiation between the two groups. The marker genotypes of F_1 hybrids were deduced from the parental genotypes. A one-way analysis of variance (ANOVA) was conducted to detect the existence of a possible locus causing hybrid sterility in the vicinity of a marker, using marker types as the main effect and the entries within marker types as the error term.

Results

Polymorphism of the markers

Ninety two of the 96 RFLP probes used in this study detected polymorphism with at least one restriction enzyme, and all ten pairs of SSR primers detected polymorphism (Fig. 1). These 92 polymorphic RFLP probes and ten SSRs, referred to as 102 "markers", generated a total of 241 pieces of non-redundant information including 155 scored as genotypes and 86 scored by bands. Of the 241 pieces of non-redundant information, 17 were obtained from the ten SSR markers and the remaining 224 were from the 92 RFLP markers.

For ease of description, we henceforth refer to each of the 241 non-redundant pieces of information as a "locus". Thus, there were two alleles at each of the 86 "loci" scored by the presence or absence of individual bands. Of the 155 loci that were scored as genotypes, nine were SSRs that resolved an average of 4.8 alleles per locus, and the remaining 146 were RFLP which detected 2.6 alleles per locus. Thus SSRs are much more polymorphic than RFLPs.

Genetic similarity of the rice varieties

Exactly the same grouping of rice varieties was obtained by the cluster analysis using several different algorithms (simple linkage, complete linkage and the Ward method). The dendrogram obtained using complete linkage is given in Fig. 2 for illustration purposes. The 20 varieties were clearly separated into two groups, all the 11 japonica varieties were tightly clustered in one group and all the nine indica varieties were in the other group. There also seemed to be sub-groupings within each group. However, the dissimilarity between the sub-groups was small compared to the level of difference between the two main groups.

Indica-japonica differentiation

The amounts of genetic differentiation between indica and japonica varieties obtained by partitioning the diversity of the individual loci are illustrated in Fig. 3, which range from zero ($<2.5\%$) differentiation at six loci to complete differentiation ($>97.5\%$) at 19 loci, with an average of 40.8% over the 241 loci. The average differentiation (33.4%) over the 17 SSR loci was not significantly lower than the bulk of the RFLP loci (41.3%). It should be noted that, based on the formula that we used in partitioning the diversity, 100% differentiation was obtained only when the indica and japonica groups were each fixed for a different allele. Differentiation would be less than complete if there was polymorphism within groups, even though the allelic compositions were completely different between the groups. In fact, there was a number of loci at which indica and japonica varieties had completely different alleles while the amount of differentiation was much less than 100% (data not shown).

Fertility of various crosses

Spikelet fertility differed widely among the 190 F_1 hybrids (Fig. 4). All F_1s of intra-subspecific crosses (indica × indica and japonica × japonica) showed normal fertility ($\geq 60\%$). In contrast, the majority of the F_1s from inter-subspecific crosses (indica × japonica or japonica × indica) showed partial to complet sterility; exceptions were found only in crosses involving wide-compatibility varieties as the parents and in a few other crosses.

The spikelet fertility averaged over the inter-subspecific hybrids from each parent showed that the parents differed significantly in their influence on the fertility of the hybrids in indica-japonica crosses (Table 1). Among the inter-subspecific hybrids derived from wide-compatibility varieties, those involving "Dular" as one of the parents were highly fertile; most hybrids involving "02428" were also highly fertile, whereas the fertility of hybrids derived from "Ketan Nanka" was lower. There were also significant differences in fertility among the inter-subspecific hybrids derived from non-wide compatibility varieties. The japonica variety C57 and indica varieties Minghui 63, Shengli Xian and Teqing produced more fertile hybrids than other varieties; three of these four varieties are restorer lines commonly used in hybrid-rice production (Table 1).

Detecting loci contributing to hybrid sterility

Highly significant effects ($P<0.01$) on spikelet fertility were detected for 182 of the 241 loci in the entire data set of 210 entries (190 F_1s and 20 parents) by the

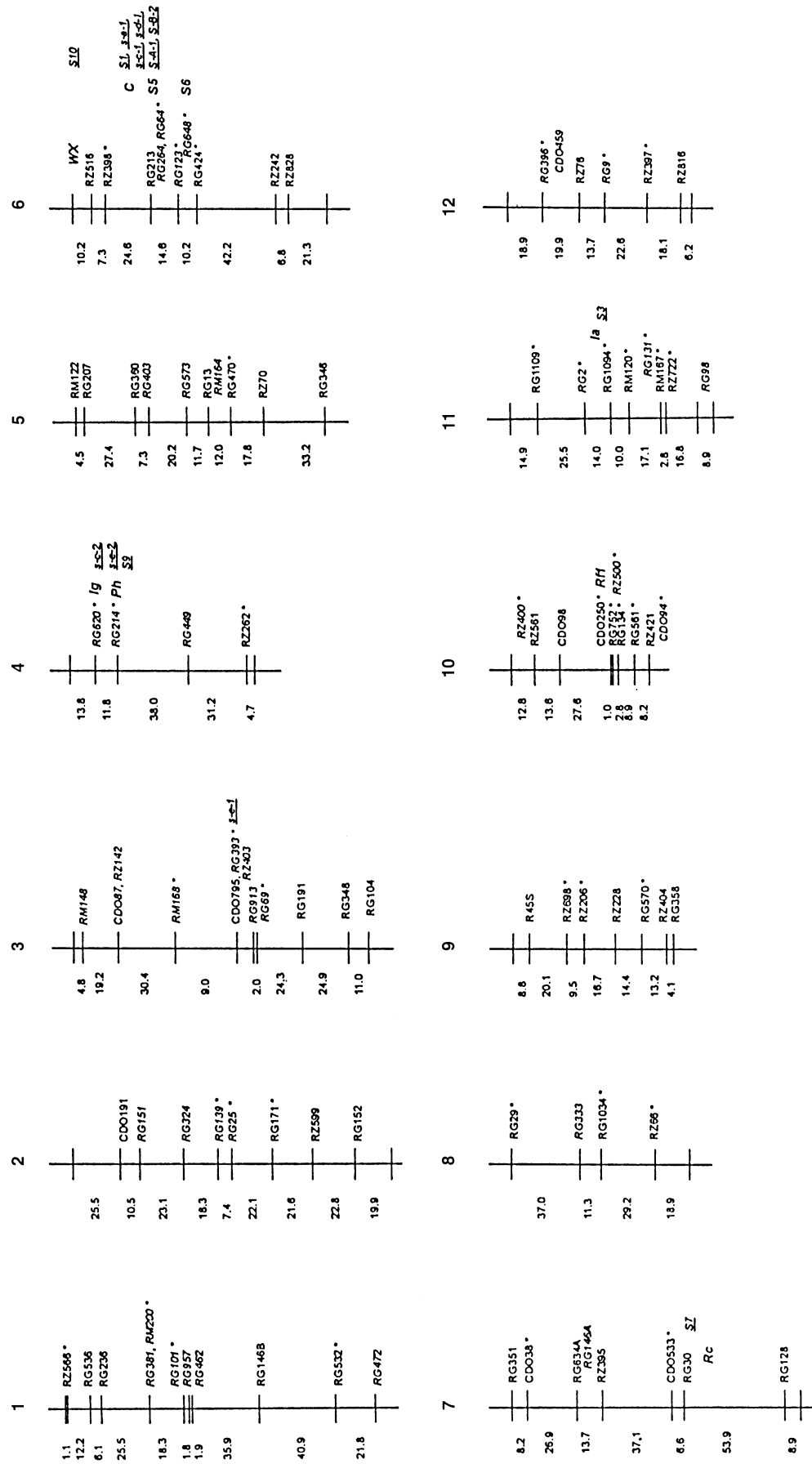

Fig. 1 Distribution of the molecular markers used that are polymorphic among the 20 varieties. There are five markers (*RG163*, *RG447*, *RM1*, *RM2* and *RM163*) whose chromosomal locations are not known. The marker distances (cM, on the left of the chromosomes) are essentially according to Causse et al. (1994). Chromosomal locations for markers set in *italicized* face are approximate. Those marked with an *asterisk* are positive markers determined by a one-way analysis of variance using the partial diallel set excluding the data from hybrids involving "Dular" and "02428". Those in *bold face* are known gene loci taken from Causse et al. (1994), and the ones *underlined* are genes for hybrid sterility whose approximate map locations are deduced based on the data from previous studies (see Discussion section). A *horizontal line* on the end of a chromosome indicates the position of the very last marker on the end in the map of Causse et al. (994) that is not used in this study

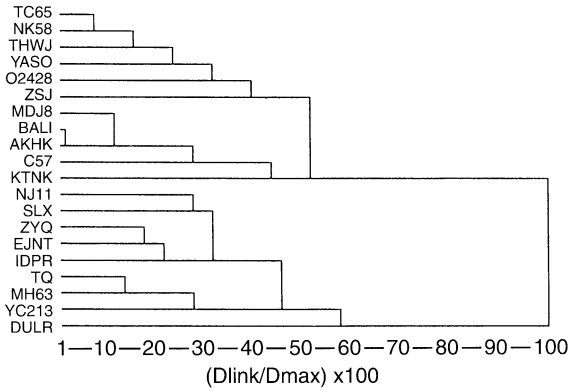

Fig. 2 Cluster dendrogram of 20 indica and japonica varieties used in this study as resolved by complete linkage using molecular-marker difference as the distance measure

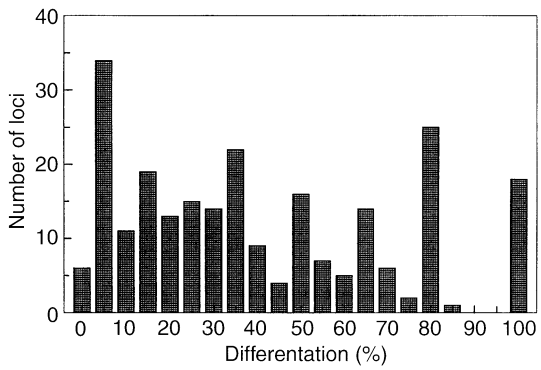

Fig. 3 Distribution of the amount of indica-japonica differentiation as detected by the 241 marker loci. See text for the definition of a locus

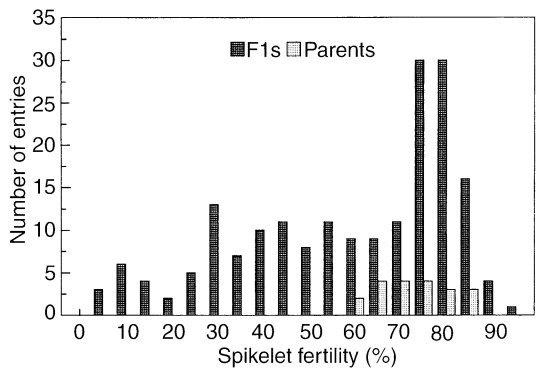

Fig. 4 Distribution of fertility in the 190 hybrids and their parents of the diallel set involving 20 indica and japonica varieties

ANOVA (data not shown). This is expected because of the strong indica-japonica differentiation. The indica × indica hybrids were homozygous or heterozygous for the indica alleles at most of the loci, and highly fertile. Similarly, japonica hybrids were homozygous or heterozygous for the japonica alleles at most of the loci, and also highly fertile, whereas the inter-subspecific hybrids were heterozygous for the indica and japonica alleles at most of the loci and showed various degrees of sterility. Such large differences of fertility and marker genotypes between the intra- and inter-subspecific crosses over-dominated the detection by the ANOVA.

To detect the loci that may have effects on the fertility of inter-subspecific hybrids, the ANOVA was performed again using only the 99 indica-japonica hybrids. One-hundred loci involving 59 markers (data not shown) detected highly significant effects ($P < 0.01$) on fertility. However, close inspection of the results from the analyses of variance showed that the effects detected by most of the loci was due to a "rescuing" effect by the wide-compatibility variety Dular (and in a few cases also by "02428"). A highly significant effect was detected whenever "Dular" had a different marker allele from the rest of the indica parents, while the marker genotypes of "Dular" were frequently different from other varieties in this data set (Fig. 2).

To eliminate the confounding effect caused by wide-compatibility varieties in the detection of loci for hybrid sterility, data from hybrids involving "Dular" and "02428" were excluded from the analysis (hybrids from "Ketan Nanka" were not excluded because this variety showed very little wide-compatibility). In this case, 64 loci involving 44 markers detected significant effects ($P < 0.01$) on hybrid sterility (Fig. 1). Of the 44 markers, one was from chromosome 5, two from chromosome 7, three each from chromosomes 2, 3, 4, 8, 9 and 12, four from chromosome 1, five from chromosome 6, and seven from each of chromosomes 10 and 11.

Correlation between marker distance and hybrid fertility

As expected, there was a large negative correlation (-0.711, $P < 0.01$) between marker distance and hybrid fertility, indicating that fertility was inversely related to the genetic dissimilarity between the parents. However, the results need to be viewed with caution for two reasons.

First, it can be clearly seen from Fig. 5 that there is a discontinuity in the x-axis that separated the distance measures into two disjoint classes: as intra-subspecific class (indica × indica and japonica × japonica) with all the distance measures $< 43\%$, and an inter-subspecific class (indica × japonica or japonica × indica) with all the distance measures $> 49\%$. This discontinuity parallels the fertility distribution of the F_1s (Fig. 4). Thus, the highly negative correlation between fertility and marker distance was largely dominated by the difference between the two groups of crosses: small distance associated with high fertility featured by the intra-subspecific crosses and large distance associated with low fertility exhibited by the inter-subspecific crosses. To

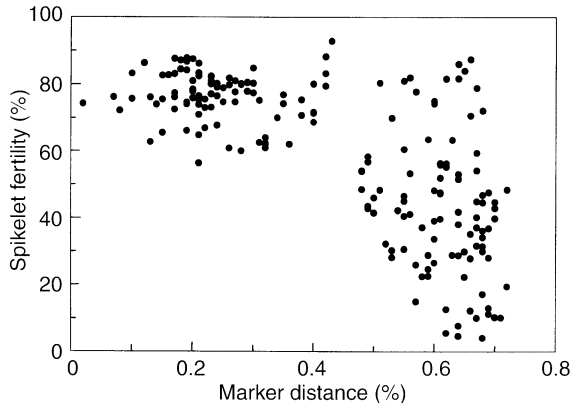

Fig. 5 The relationship of hybrid fertility and marker distance between the parents in the 190 F_1 hybrids of the diallel cross

remove such an overwhelming effect, these two types of F_1s were separated and the correlations were again calculated. The resulting correlation coefficients were -0.095 between marker distance and fertility ($P > 0.05$) in intra-subspecific crosses, and -0.235 ($P < 0.05$) in inter-subspecific crosses.

There is also a further complication caused by the wide-compatibility varieties. Those F_1s that showed both large distances and high fertility (located in the upper right of Fig. 5) were exclusively from crosses involving two parents, "Dular" and "02428". When these crosses were excluded from analysis, the correlation between parental distance and hybrid fertility in the intersubspecific crosses became -0.302 ($P < 0.01$). Thus, overall, the correlation between marker distance and hybrid fertility was very low in both intra- and intersubspecific crosses.

Discussion

The main objective of this study was to assess the relationship between molecular diversity and hybrid fertility in indica-japonica crosses. The analysis has clearly demonstrated that indica-japonica differentiation is the major determinant of the hybrid sterility, and hybrids of intra-subspecific crosses have much higher fertility on the average than do inter-subspecific crosses. Consequently, a majority of the markers that differentiate between the indica and japonica varieties detected significant effects on fertility. It should be noted, however, that such a high proportion of positive markers is not because all the markers themselves are located in the vicinity of genes controlling hybrid fertility. Rather, it is largely a result of the very close associations among the subspecies-specific alleles at various marker loci which differentiate the multilocus combinations into essentially two large arrays extending to almost the entire genome. As a result, any of the loci that are involved in the complex will show apparent effects on the fertility of hybrids between the two groups. It should also be noted that the low correlations between marker distance and hybrid fertility, observed in both intra- and inter-subspecific crosses, indicate that the total amount of difference between the parents makes very little contribution to the fertility of the hybrids.

A large number of genes for hybrid sterility have been identified previously. According to the list compiled by Kinoshita (1991, 1993), chromosomal locations for 16 of these genes have been determined. Ten of the 16 genes are located on chromosome 6, three on chromosome 4 and one on each of chromosomes 3, 7 and 11, respectively. Thus, it would be very interesting to relate the chromosomal locations between the genes for hybrid sterility and the positive markers identified in the analysis of the partial diallel data not involving "Dular" and "02428".

Among the ten genes for hybrid sterility on chromosome 6, the S_5 locus has been located on the RFLP linkage map (Liu et al. 1992; Zheng et al. 1992; Yanagihara et al. 1995). The approximate location of S_6 on the molecular-marker linkage map can also be determined (Causse et al. 1994). The S_{10} locus is tightly linked to the *wx* locus (Sano et al. 1992), while the location of S_8 on the molecular-marker linkage map cannot be unambiguously determined based on the available data. The remaining six loci are linked either to the *wx* locus at distances of 21–33 recombination units, or to the *C* locus at distances ≤ 10 recombination units (Kinoshita 1993). Thus, it can be inferred that the majority of these genes for hybrid sterility correspond to the map locations of the positive markers on this chromosome (Fig. 1). The three hybrid-sterility genes reported on chromosome 4 are located at distances of 15–31 recombination units from either the *lg* or *Ph* locus (Kinoshita 1993), the locations of the fertility genes on the molecular-marker linkage map have yet to be resolved. However, it is likely that the effects detected by markers RG602 and RG214 are caused by some or all of the three loci. One of the positive markers on chromosome 7, CDO533, corresponds to the chromosomal location of S_7, which is closely linked to the *Rc* locus (Yanagihara et al. 1992). Moreover, the six positive markers, clustered in the middle of chromosome 11, correspond to the location of S_3, which is very tightly linked to the *la* locus (Sano 1983). Furthermore, the hybrid sterility locus *s-e-1*, which is linked to the *bc-1* locus on chromosome 3 (Oka 1974), also seems to have a good correspondence with the positive markers from this chromosome (RG393 and RG69, Fig. 1). Thus, the coincidence between the locations of positive markers and the known hybrid-sterility genes indicates that marker-based analysis of such a partial diallel set is also very useful for detecting hybrid-sterility genes, as in the case of marker-based analysis of barley powdery mildew resistance in the diallel set of Saghai Maroof et al. (1994).

On the other hand, there are still a number of positive markers on several chromosomes where no known genes for hybrid sterility have been reported. Additionally, there are a number of genes for hybrid sterility whose chromosomal locations have not been determined (Kinoshita 1991). It is likely that some of the remaining positive markers, and at least those clustered on certain chromosome regions (Fig. 1), correspond to genes for hybrid sterility. Interestingly, the five positive markers clustered on the bottom half of chromosome 10 correspond to a locus for fertility restoration (*Rf1*) in male-sterile lines (Causse et al. 1994) The presence of fertility restoration gene(s) on chromosome 10 has also been observed in other studies (e.g. Bharaj et al. 1995; Q. Zhang et al. unpublished data). In connection with the present observation that the indica-japonica hybrids involving restorer lines as one of their parents showed higher spikelet fertility, this seems to imply that genes for fertility in indica-japonica hybrids and those for fertility restoration in the male-sterility system may be functionally related.

In summary, the analysis suggests that many loci exist in the rice genome that can exert significant effects on the fertility of indica-japonica hybrids. There is very little correlation between the genetic differentiation of the parents and hybrid fertility and it is therefore the qualitative, not the quantitative, difference between the parental genotypes that determines the fertility of the hybrids.

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