

Mapping AFLP markers associated with subspecific differentiation of *Oryza sativa* (rice) and an investigation of segregation distortion

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AFLP marker data were generated for a doubled haploid rice mapping population developed from a cross between IR64 (indica) and Azucena (japonica) varieties, as well as for a diverse set of rice germplasm. A total of 151 AFLP markers were incorporated into an existing restriction fragment length polymorphism framework map and compared with a published map. Markers associated with the indica–japonica subspecific differentiation in Asian rice were also mapped and were found to be distributed throughout the genome. Sources of mapping errors were examined and the possibility of such errors being caused by co-migration of bands and segregation distortion was explored. In the case of markers that show distorted segregation, it was concluded that one-third of these may be affected by gametophytic/sterility genes and a further one-third may arise because of association with the indica–japonica differentiation.

Keywords: AFLP, genetic map, germplasm, rice, segregation distortion.

Introduction

Efforts to enhance the use of crop genetic resources and the exploitation of conserved biodiversity more effectively by using molecular markers have led us to address some of the practical issues related to the maintenance of plant germplasm in a gene bank. This has included the designation of duplicate accessions (Virk *et al.*, 1995a) and taxonomic identification of rice species (Martin *et al.*, 1997). Additionally, we have demonstrated associations between molecular markers (RAPDs) and quantitative variation of interest to breeders that can lead to prediction of the agronomic potential of diverse cultivated rice stored in the gene bank at IRRI (Virk *et al.*, 1996a,b). Subsequently, such associations have been reported in diverse barley germplasm (Pakniyat *et al.*, 1997).

Asian rice has undergone ecogeographical differentiation, and the fundamental division is between indica and japonica types (Chang, 1976). Crosses between indica and japonica types result in heterosis, which is exploited by breeders in the development of new varieties (Zhang *et al.*, 1994).

Discrimination between indica and japonica types using morphological characters is not always easy, and material is frequently classified using isozyme data (Glaszmann, 1987). It is also possible to distinguish between the two types using RFLP (Zhang *et al.*, 1992), RAPD (Virk *et al.*, 1995b), AFLP (Mackill *et al.*, 1996) and ISSR (Parsons *et al.*, 1997) data. In this paper, the generation of AFLP marker data both in a widely studied doubled haploid rice-mapping population obtained from a cross between an indica variety ‘IR64’ and a japonica variety ‘Azucena’ (Huang *et al.*, 1994; Maheswaran *et al.*, 1997) and in a set of diverse rice material previously used by Virk *et al.* (1996a) is reported. This has allowed us to present the first example of mapped markers associated with subspecific differentiation in a plant species, namely indica and japonica types of Asian rice.

Possible causes of segregation distortion in the doubled haploid population were also investigated. Segregation distortion is the deviation of segregation ratios from the expected Mendelian fractions (Zamir & Tadmor, 1986; Lyttle, 1991) and has been reported in a wide range of plant species (see Xu *et al.*, 1997). There have been several previous reports

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of segregation distortion in rice (e.g. Lin *et al.*, 1992; Harushima *et al.*, 1996; Yamagishi *et al.*, 1996). We have identified sets of markers that appear to show segregation distortion, either because of association with gametophytic/sterility genes or because of the influence of the indica/japonica differentiation.

Materials and methods

A subset of 60 doubled haploid lines developed from a cross between IR64 and Azucena varieties of *Oryza sativa* was obtained from Dr Ning Huang along with RFLP data for the entire mapping population of 135 doubled haploid lines. Thirty-two diverse *O. sativa* accessions used by Virk *et al.* (1996a,b) were also used to generate AFLP data. Sixteen of these accessions belonged to the indica (isozyme group I; Glaszmann, 1987) and 16 to japonica (isozyme group VI) types (Virk *et al.*, 1996b).

AFLP analysis

Genomic DNA of the parental and doubled haploid lines was isolated from a small quantity of fresh leaf tissue (60 mg) according to the method of Virk *et al.* (1995b). The AFLP protocol developed by Vos *et al.* (1995) was essentially followed, with minor modifications. Genomic DNA (500 ng) was digested with 5 U each of *EcoRI* and *MseI* restriction enzymes. The *EcoRI* and *MseI* adapters were ligated to the ends of restricted fragments. The digested and ligated template DNA was preamplified using *EcoRI*-(EP;5'-GACTGCGTACCAATTCA-3') and *MseI*-(MP;5'-GATGAGTCC-TGAGTAAC-3')-directed primers. These primers contained a core sequence, restriction site and an additional selective nucleotide at the 3' end (A and C for *EcoRI* and *MseI* primers respectively). The preamplification was performed in a total volume of 25 μ L containing approximately 5 ng of restricted ligated template DNA, 30 ng each of primers (EP and MP), 0.1 mM of all four dNTPs, 1 U of *Taq* polymerase and 1 \times PCR buffer containing 1.5 mM MgCl₂. The mixture was overlaid with light mineral oil, and the amplification was performed in a Hybaid-Omnigene thermocycler. The preamplification profile was as follows: one cycle of 95°C for 1 min and 20 cycles of 94°C for 30 s, 56°C for 1 min and 72°C for 1 min. The preamplification products were then diluted 50-fold with 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0

Selective amplification was carried out using 20 primer pair combinations. Four *EcoRI* (E1 to E4 with EP+C, EP+A, EP+G and EP+T sequences respectively) and eight *MseI* end-directed (M1 to M8 with MP+AA, MP+AC, MP+AG, MP+AT, MP+TA, MP+TC, MP+TG and MP+TT sequences respectively) primers (Operon) were used. *EcoRI* adapter-directed primers were end labelled using [³³P]ATP and T4 polynucleotide kinase (Pharmacia). The selective amplification reaction was essentially the same as that for preamplification except that 5 μ L of 50-fold diluted preamplification products was used as template, and 5 ng of labelled *EcoRI* primer and 30 ng of unlabelled *MseI* primer were used. The amplification profile was as follows: one cycle of 95°C; one cycle of 94°C for 1 min, 65°C for 1 min, 72°C for 1 min followed by eight cycles with a stepwise reduction of 1°C in the annealing temperature to 57°C; 27 cycles of 94°C for 30 s, 56°C for 1 min, 72°C for 1 min. The reaction was stopped by adding 25 μ L of formamide loading buffer. The samples were denatured at 90°C for 5 min and then quickly cooled on ice. Three microlitres of the mix was loaded onto a 5% denaturing polyacrylamide gel [1 \times TBE (Sambrook *et al.*, 1989), 7.5 M urea]. Electrophoresis was performed at a constant 50°C for 2 h. The gel was cast in a Sequigen 38 \times 50 cm gel apparatus (Bio-Rad) and, after drying, exposed to Kodak Biomax film for 3–4 days.

RAPD assay

In addition to the AFLP markers, 12 RAPD bands were mapped. Five decamer Operon primers (OPF13, OPK11, OPC08, OPC10 and OPC14) were used following Virk *et al.* (1996b).

Data analysis

The AFLP bands were scored as present or absent. Each polymorphic marker was identified by the primer pair combination with the band number as a suffix. Data were coded such that band presence could be associated with one of the mapping population parents. The AFLP and RAPD marker data were combined with the RFLP data set of Huang *et al.* (1994), and linkage analysis was performed using MAPMAKER 3.0b (Lander *et al.*, 1987; Lincoln *et al.*, 1992) and JOINMAP 2.0 (Stam & Ooijen, 1996). The centromeres were placed at approximate positions following Singh *et al.* (1996). Map units (cM) were derived using the Kosambi (1944) function of MAPMAKER. This function was used to maintain

uniformity and for comparison of our map with that of Maheswaran *et al.* (1997).

Results and discussion

The construction of a genetic map is a complex and stepwise multipoint linkage analysis (Cloutier *et al.*, 1997). In our study, a LOD of 6.0 was used to map 295 markers, which means that, of 43365 pairwise comparisons, less than one pair would be expected to be declared erroneously linked. However, a source of mapping error is encountered with those markers that show segregation distortion. To minimize such errors, both JOINMAP and MAPMAKER were used to assign markers to linkage groups. The former uses modified LOD scores based on a chi-squared test for independence of segregation, therefore limiting the mapping errors introduced by segregation distortion (Stam & Ooijen, 1996).

With a multilocus, large-throughput marker system such as AFLP, it is possible to encounter

problems caused by the chance co-migration, and hence superimposition, of nonallelic amplification products derived from a single genotype (Cloutier *et al.*, 1997). In a doubled haploid population, 3:1 or 15:1 segregation ratios for any band could be caused by co-migrating bands representing two or three loci respectively. Consequently, some putative segregation distortion may be accounted for by the superimposition of loci. One possible example is shown in Fig. 1, in which a band (E1M201*) with a strong signal was amplified from 'Azucena'. When scored as presence/absence in the 60 doubled haploid lines, a ratio of 45:15 was observed. It could be concluded that this locus shows segregation distortion. On the other hand, if intensity of bands was additionally scored as strong, faint or absent, a ratio of 28:17:15, was observed, fitting the expected ratio of 2:1:1 ($\chi^2 = 0.4$; $P > 0.05$) for faint and strong bands being superimposed. In the absence of proof of either one or the other situation, it would be advisable to omit such ambiguous data.

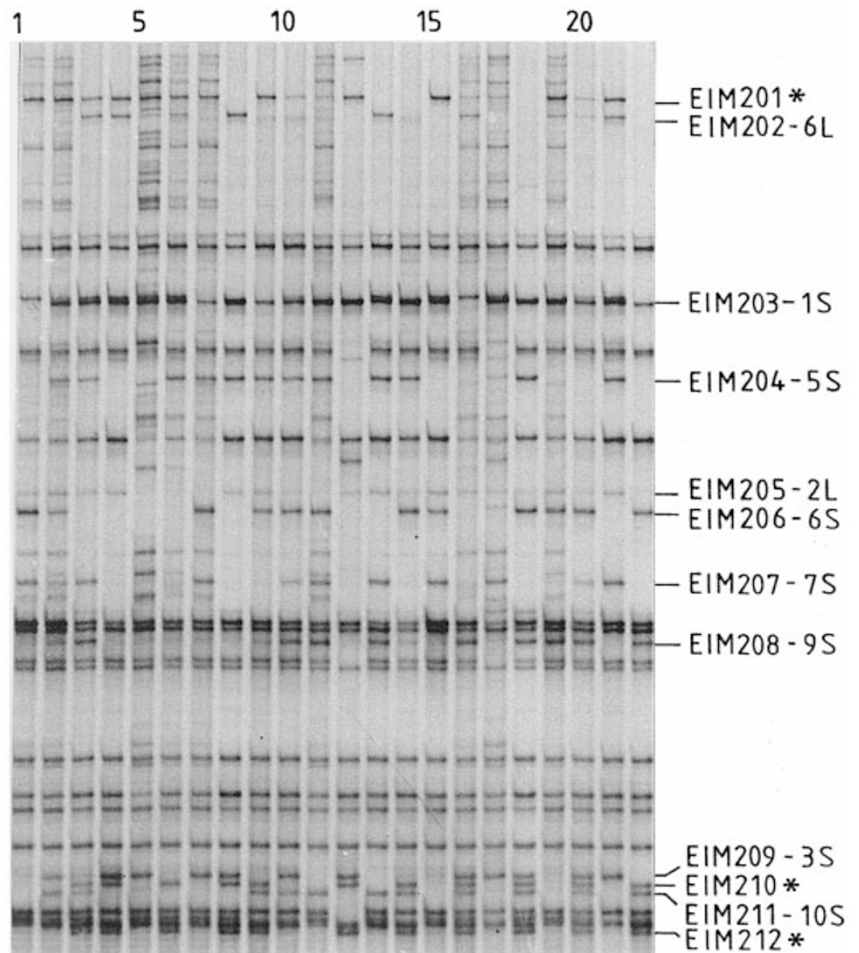


Fig. 1 AFLP marker profile of Azucena (lane 21), IR64 (lane 22) and some of the doubled haploid lines (lanes 1–20) of rice obtained from the primer combination E1M2. Markers are identified with band number and chromosomal location (Fig. 2) as a suffix (*markers not mapped).

In our study, 20 primer pair combinations yielded more than 200 AFLP bands polymorphic between Azucena and IR64. Some bands were not scorable in the doubled haploids, and others were difficult to associate with the RFLP framework map (Huang *et al.*, 1994). Consequently, only 151 AFLP along with 12 RAPD markers (Fig. 2) could be mapped satisfactorily. AFLP markers mapped to all 12 chromosomes (Fig. 2) and, overall, there was an equal distribution of Azucena and IR64 alleles in the doubled haploid lines.

The combined AFLP/RFLP/RAPD map shows a 24% increase in map length (427 cM) relative to the RFLP map (Table 1), which is less than that shown by Maheswaran *et al.* (1997), who observed a nearly 70% increase in map length. However, there was an average decrease of around 6 cM in distance between markers. The increase in map length can be attributed to gap filling between RFLP markers and addition of markers in the telomeric regions. The most pronounced increase in map length was for chromosomes 8, 9 and 10. This was caused primarily

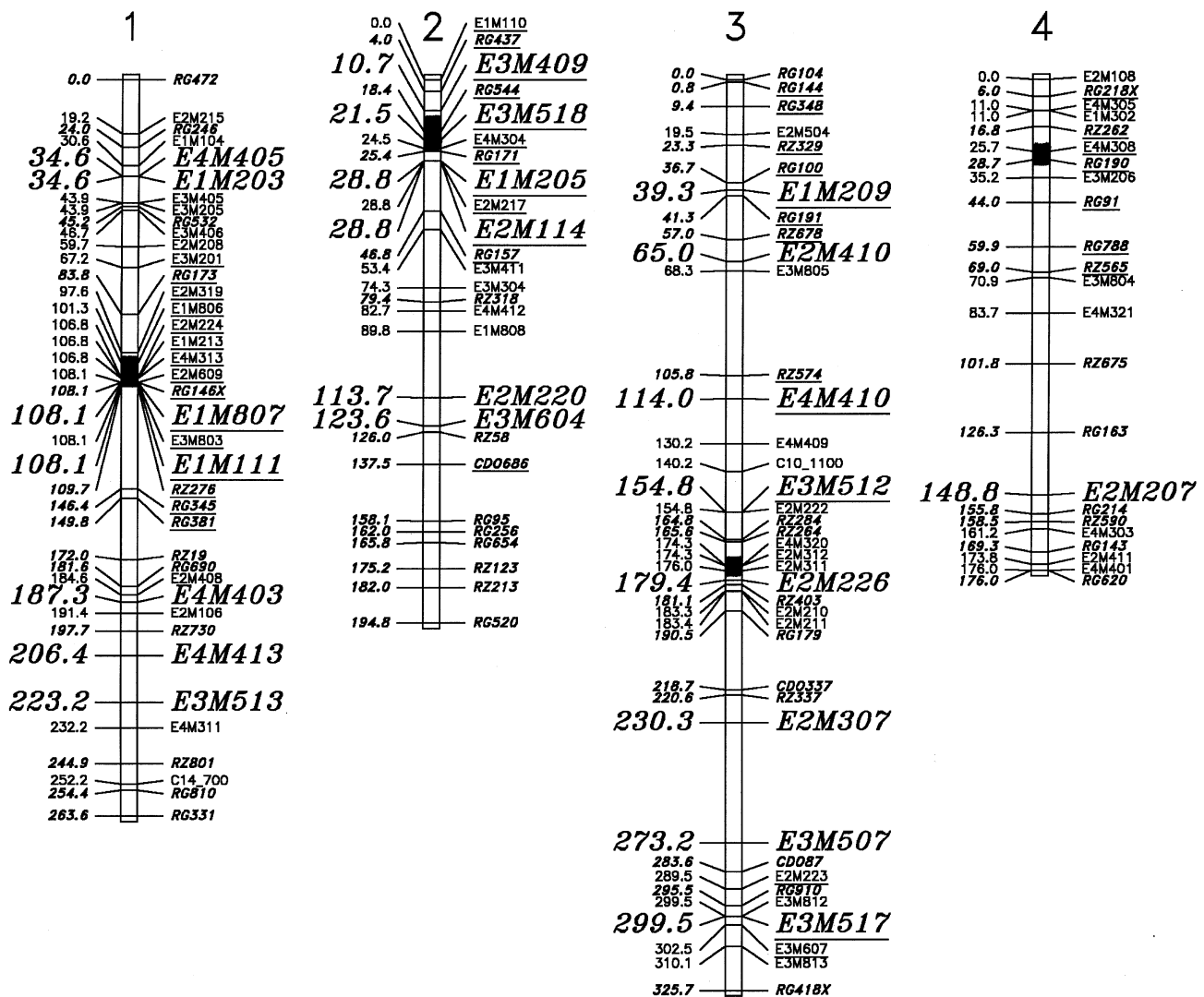


Fig. 2 Molecular map (Kosambi units) from the rice cross IR64 × Azucena showing the location of 151 AFLP, 12 RAPD and previously mapped RFLP (Huang *et al.*, 1994, in bold type). The markers are coded as follows: for example, E2M215 = 15th polymorphic marker (in declining size) obtained using primers E2 and M2 (see Materials and methods). Details of the sizes of markers can be obtained by contacting the authors. Markers showing segregation distortion are underlined. Markers that are indica or japonica specific are in large font. Solid lines along the chromosomes represent centromeric regions (Singh *et al.*, 1996).

by an addition of markers either on the short arm of chromosomes 8 and 9 or on a large telomeric segment on the short arm of chromosome 10.

A perusal of the current map (Fig. 2) reveals that there are 14 large (>25 cM) segments of chromosome devoid of markers; these occur on all chromosomes except numbers 2 and 12. In this population, Maheswaran *et al.* (1997) used *Pst*I and *Mse*I restric-

tion enzymes rather than *Eco*RI and *Mse*I as used here. Although we do not have access to their data, it is possible to make some general comparisons. For instance, four of the 14 regions devoid of markers on our map were filled on the map of Maheswaran *et al.* (1997). Conversely, eight of 19 gaps in the map of Maheswaran *et al.* (1997) were filled on our map. A further examination of these two maps, in

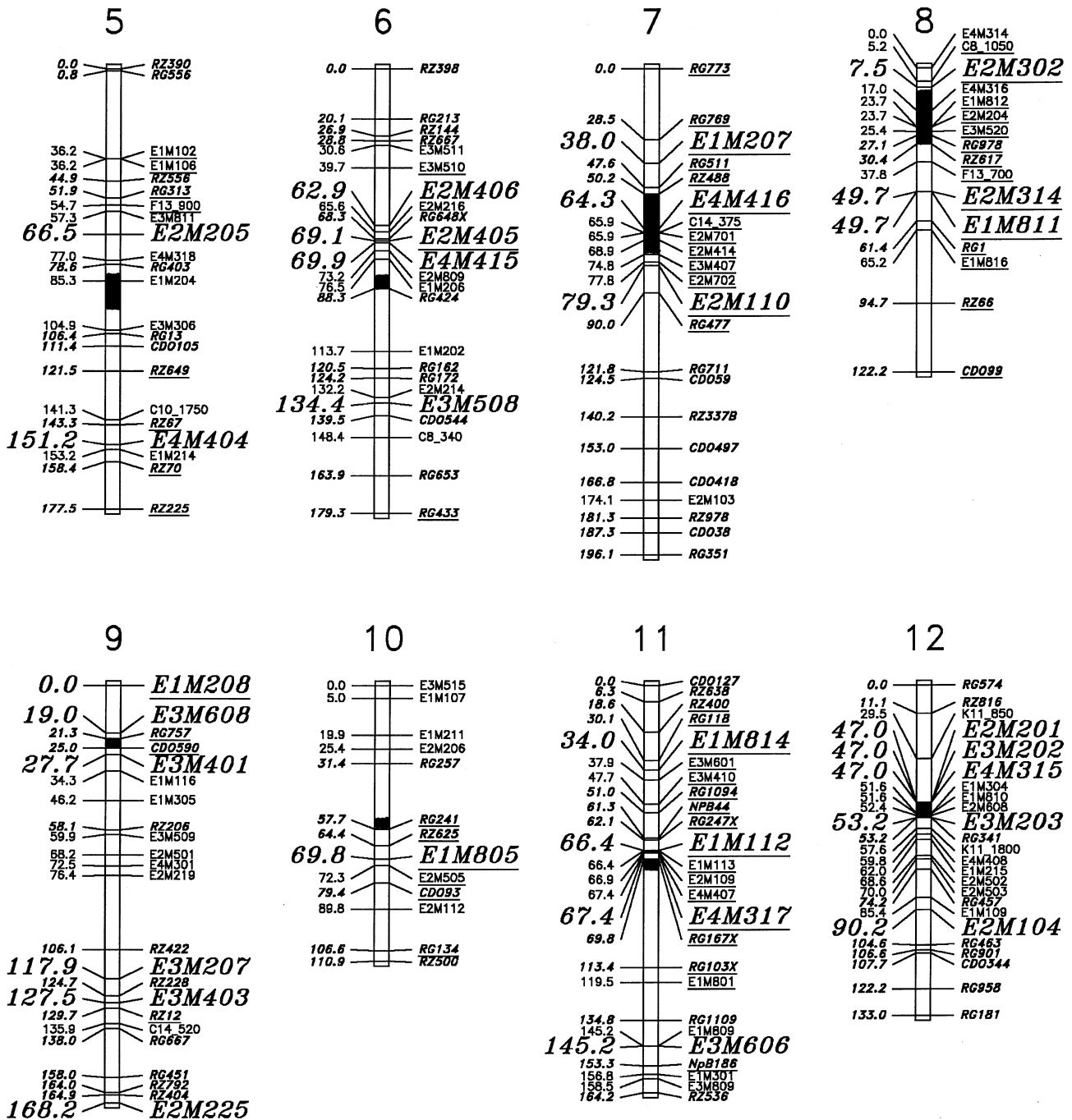


Fig. 2 Continued

comparison with the RFLP framework map (Huang *et al.*, 1994), indicates an overall better coverage of the short arms of chromosomes 9 and 11 and the long arms of chromosomes 2, 6, 9 and 10 in the AFLP maps. It would appear that, even after combining AFLP markers obtained from two different sets of restriction enzymes (*EcoRI/MseI* and *PstI/MseI*), markers are still needed on 10 regions of seven chromosomes, particularly chromosomes 3 and 8. This suggests that using further pairs of enzymes for restricting genomic DNA would be fruitful.

In all, 54 mapped AFLP markers in this cross showed segregation distortion. Of these, 10 and nine markers mapped to the vicinity of loci previously designated as gametophytic (*ga-9*; chromosome 1)

Table 1 A comparison of the combined AFLP-RFLP map with the RFLP map of rice

Chromosome	Increase caused by AFLP markers (cM)	% increase
1	40.4	18
2	25.4	15
3	64.7	25
4	30.0	21
5	30.4	21
6	22.0	13
7	24.6	14
8	43.1	54
9	50.2	42
10	47.4	75
11	22.0	15
12	26.7	25
Average	35.6	24

or sterility (*S3*; chromosome 11) genes, respectively (Xu *et al.*, 1997), pointing to an obvious cause of segregation distortion. To study the molecular genetic basis of segregation distortion exhibited by the remaining 35 AFLP markers, we have scored these mapped, distorted AFLP markers in a diverse set of 32 *O. sativa* accessions. These 32 accessions comprised equal numbers of indica and japonica types. It was possible to score 31 of the 35 AFLP markers that showed segregation distortion in this diverse set. Using chi-squared analysis, it was shown that 17 of these markers were either indica or japonica specific ($P < 0.05$). Of these, nine and eight loci showed over-representation of indica or japonica alleles respectively (Table 2). Such results show that the predominance of indica or japonica alleles is equally distributed among those loci that show segregation distortion ($\chi^2 = 0.06$, $P > 0.05$). This is in contrast to the widely held belief that japonica alleles are a predominant cause of distortion.

Twenty AFLP bands showed segregation distortion that could not be associated with known gametophytic/sterility genes or with the indica/japonica differentiation in this study. Other possible reasons for such distortion in a doubled haploid population could be selective regeneration *in vitro* during the production of the doubled haploid population.

In the present study, 60 of an original set of 135 doubled haploid lines were used (Maheswaran *et al.*, 1997). As expected, the selection of those lines showing the highest frequencies of normal segregation and normal fertility had an effect on the extent of segregation distortion. In fact, 54 RFLP markers showed segregation distortion in the original 135 lines, whereas this number was reduced to 28 in the 60 selected doubled haploid lines. In the light of this

Table 2 A summary of markers showing segregation distortion not attributable to *ga-9* or *S3* genes in rice

Chromosome number	Number of distorted AFLP markers scored in double haploid lines	Number of distorted markers scorable in diverse set of 32 accessions	Number of distorted markers showing over-representation in the 32 accessions	
			Indica alleles	Japonica alleles
2	7	7	0	4
3	6	6	1	3
4	1	1	0	0
5	2	2	0	0
6	2	1	1	0
7	7	5	3	0
8	7	7	3	0
9	1	1	1	0
10	2	1	0	1

observation, it is clear that our assessment of segregation distortion using this selected sample of the original doubled haploid population is actually an underestimate of the overall distortion that occurred.

A further potential problem with the use of AFLP markers is the co-migration of nonallelic amplification products derived from different genotypes and, with regard to the work presented here, this can be divided into three categories. First, if co-migrating, nonallelic fragments are produced from two homozygous parents, these bands do not segregate. Therefore, co-migration leads to the loss of potentially useful markers rather than an error in mapping. Problems could arise, however, in the second situation, in which such PCR markers are obtained from a range of accessions in order to calculate indices of diversity or to classify material into subtypes; there are rarely any inheritance data associated with such studies (and none in the present work). These factors are relevant to the present study because we have defined the indica and japonica subtypes using cluster analysis of AFLP data. However, we are confident about the accuracy of this classification of rice accessions, as we have shown that the same results are obtained using isozyme, AFLP and RAPD banding data (data not shown) and because classifications based on these techniques have been shown to agree with those obtained using RFLP data (Zhang *et al.*, 1992).

In the present study, there is a third example of the assumption that co-migrating bands are allelic. It is assumed that those bands that have been mapped using the doubled haploid population are homologous with co-migrating bands in the set of 32 indica and japonica accessions. We have provided no direct evidence that this is the case; this would be technically demanding, requiring either cloning and sequencing of large numbers of amplification products and/or following the segregation of markers after crossing pairs of rice accessions. However, one parent of the cross leading to the doubled haploids was a japonica type (Azucena) and the other an indica type (IR64); the 50 mapped AFLP bands used were all selected on the basis that chi-squared testing ($P < 0.05$) showed them to be strongly indica or japonica specific. Nonallelism between bands scored in the mapping population and bands scored in the landrace accessions would therefore require the unlikely combination of chance co-migration of amplified fragments and chance indica or japonica specificity.

The availability of the above data allowed us, for the first time, to study the genomic distribution of

taxonomically important markers at the subspecific level. Altogether, 50 AFLP markers were defined as indica or japonica specific ($P < 0.05$ using the chi-squared test). These 50 markers are distributed almost all over the genome (Fig. 2); there is some under-representation on two chromosomes (4 and 10) and some aggregation at the top and bottom of chromosome 9. This general distribution supports the hypothesis that indica and japonica types were domesticated from different populations of a common ancestor (probably *O. nivara*) and possess much overlapping variation (Khush, 1997).

Data for mapped AFLP markers scored on a set of diverse *O. sativa* germplasm are now available. These data will be presented in forthcoming papers to address several interesting questions beyond the scope of this study. The first of these will be a study of the effects of using mapped markers on the measurement of diversity. Next, our findings about the underlying genetic causes of the reported associations between molecular markers and quantitative variation of interest to breeders will be reported (Virk *et al.*, 1996a,b; Pakniyat *et al.*, 1997).

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