

Population Structure and Its Effect on Haplotype Diversity and Linkage Disequilibrium Surrounding the *xa5* Locus of Rice (*Oryza sativa* L.)

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ABSTRACT

To assess the usefulness of linkage disequilibrium mapping in an autogamous, domesticated species, we have characterized linkage disequilibrium in the candidate region for *xa5*, a recessive gene conferring race-specific resistance to bacterial blight in rice. This trait and locus have good mapping information, a tractable phenotype, and available sequence data, but no cloned gene. We sampled 13 short segments from the 70-kb candidate region in 114 accessions of *Oryza sativa*. Five additional segments were sequenced from the adjacent 45-kb region in resistant accessions to estimate the distance at which linkage disequilibrium decays. The data show significant linkage disequilibrium between sites 100 kb apart. The presence of the *xa5* resistant reaction in two ecotypes and in accessions with different haplotypes in the candidate region may indicate multiple origins or genetic heterogeneity for resistance. In addition, genetic differentiation between ecotypes emphasizes the need for controlling for population structure in the design of linkage disequilibrium studies in rice.

THE ability to interpret patterns of molecular genetic diversity and to relate them to phenotypic variation will enhance the use of diverse genetic resource collections in crop improvement. Recently, a primary goal in genetic resource management has been to characterize the structure of diversity within a crop species (BROWN 1989). Increasingly, the focus is shifting to dissecting and understanding diversity in relation to genes underlying agronomic traits (BUCKLER and THORNSBERRY 2002; RAFALSKI 2002).

One possible approach to building the connection from genetic diversity to phenotype is linkage disequilibrium (LD) mapping, recently proposed as an alternative to traditional methods for mapping traits in plants (BUCKLER and THORNSBERRY 2002; NORDBORG and TAVARÉ 2002). Linkage disequilibrium is defined as the nonrandom association of alleles, and it can result from population structure, selection, drift, or physical linkage. The physical extent of linkage disequilibrium around a gene determines the effectiveness of this approach, and it is the result of many factors, including the rate of outcrossing, the degree of artificial or natural selection on the gene or region of the genome, the recombination rate, chromosomal location, population size and structure, and the age of the allele under study. In cultivated species, the extent of linkage disequilibrium will also be shaped by human selection and the bottlenecks associated with crop dispersal beyond the center of origin.

Recent studies in maize and Arabidopsis have provided contrasting results for the utility of linkage disequilibrium for fine mapping genes in plants on the basis of divergent estimates for the extent of linkage disequilibrium in these two plant genomes. In maize, an outcrossing species, significant linkage disequilibrium was detected only within a range from 100 bp to 7 kb on the basis of analysis of several genic regions (REMYN-TON *et al.* 2001; TENAILLION *et al.* 2001; THORNSBERRY *et al.* 2001); in the autogamous species *Arabidopsis thaliana*, significant linkage disequilibrium persisted for 250 kb in a single region (HAGENBLAD and NORDBORG 2002). Unlike maize, rice is predominantly autogamous, which is predicted to result in more extensive linkage disequilibrium, perhaps even genome-wide linkage disequilibrium. But in contrast to Arabidopsis, the domestication history of rice has presumably introduced numerous bottlenecks as well as diverse hybridization events followed by generations of selection for performance in diverse agricultural environments. Because many of the world's major crop species are autogamous, including many cereals, legumes, and Solanaceous species, the understanding of linkage disequilibrium in rice may assist in evaluating the utility of linkage disequilibrium mapping in other autogamous species.

In this article we provide an analysis of linkage disequilibrium in the genomic region containing *xa5*, a bacterial blight resistance allele whose identity is still unknown. The gene was first reported by PETPISIT *et al.* (1977) and is a recessive gene conferring race-specific resistance to *Xanthomonas oryzae* pv. *oryzae*. After its identification, subsequent screening of the genetic resources collection by the International Rice Research Institute

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(IRRI) resulted in a group of accessions with the *xa5* reaction profile to a panel of isolates, which was designated the "DZ192 group," named for the original donor of *xa5* (DZ192) to breeding lines such as IR1545 and isoline IRBB5 developed at IRRI. The gene has been mapped to the short arm of chromosome 5 (YOSHIMURA *et al.* 1984) and was subsequently localized to a bacterial artificial chromosome of ~136 kb (YANG *et al.* 1998) and to a region of ~70 kb (Blair *et al.* 2003).

The *xa5* resistance allele may be associated with only certain ecotypes of rice. Rice ecotypes are the result of intraspecific differentiation of *Oryza sativa* L. for diverse environmental conditions during the past 10,000 years since domestication (KHUSH 1997). Broad classification of rice into the subspecies *indica* and *japonica* fails to capture these evolutionarily distinct subgroups. For example, *indica* rices have traditionally included the *aus*, *aman*, and *boro* rices of Bangladesh as well as the *tjereh* rices of Indonesia. Within the *japonica* subspecies are the Japanese ecotype *nuda* and the Indonesian ecotype *bulu* (TAKAHASHI 1997). Allelism tests that showed *xa5* to be in higher frequency in rice accessions from Bangladesh and Nepal than from other Asian countries also suggested that *xa5* might be associated specifically with the *aus* and *boro* ecotypes (BUSTO *et al.* 1990). The divergence among the *aus*, *boro*, and *aman* ecotypes of Bangladesh has been shaped by the wet and dry cycles of the growing season (KHUSH 1997). Although the *xa5* resistance allele was found primarily in *aus* and *boro* ecotypes, the presence of the resistance allele was not assessed in light of a molecular genetic definition of ecotype that would provide the more precise evolutionary characterization required for statistical analysis. As noted previously, population structure resulting from ecotypic differentiation is critical because it could result in spurious associations in linkage disequilibrium analysis.

The objective of this research is to describe the diversity and the decay of linkage disequilibrium in one region of the rice genome. This region consists of a small telomeric area on the short arm of chromosome 5 that harbors *xa5*. Our goals were (1) to characterize the extent of linkage disequilibrium in the region containing *xa5* in resistant accessions and to determine if it is possible to reduce the number of candidate genes, (2) to analyze haplotype diversity in the context of population structure to determine the distribution of the resistance allele among ecotypes, and (3) to make predictions about the allelic diversity underlying the *xa5* phenotype.

MATERIALS AND METHODS

Plant material: The 114 rice accessions used in this study (listed in Table 1) were obtained from the IRRI in the Philippines and from the National Plant Germplasm System Small Grains Collection in Aberdeen, Idaho. A subset of the sample had previously been allele tested for *xa5* (OLUFOWOTE *et al.*

1977; SIDHU *et al.* 1978; SINGH *et al.* 1983); see Table 1. Plants were grown in a greenhouse and genomic DNA was extracted using a cetyltrimethylammonium bromide protocol (COLOSI and SCHAAL 1993).

Bacterial blight inoculation and evaluation: Accessions were evaluated for their disease response at 60 days after sowing following inoculation with *X. oryzae* pv. *oryzae* (*Xoo*) isolates representing Philippine races one (PXO61), two (PXO86), and four (PXO71; MEW 1987). The inoculum was prepared as in BLAIR *et al.* (2003), except that the bacteria were cultured for 4 days on modified Wakimoto's medium. Inoculation was performed using the leaf-clipping method (KAUFFMANN *et al.* 1973). Three leaves were inoculated for each race, and different bacterial isolates were inoculated on separate tillers. IR24 and IRBB5 were included as susceptible and resistant controls, respectively. Plants were maintained in growth chambers with 11 hr of daylight, night temperatures of 28°, and day temperatures of 32°. Lesions were measured 14 days after inoculation. Lesion lengths generally showed a bimodal distribution, and this distribution was used as the basis for defining resistant and susceptible reactions. For typing of disease response, each plant was classified as resistant if the mean lesion length was between 0 and 3 cm. Plants with mean lesion lengths >6 cm were classified qualitatively as susceptible. Plants with lesion lengths intermediate to these two classes were classified as moderately resistant.

Markers: Twenty-one simple sequence repeats (SSRs) distributed on the 12 chromosomes of rice were employed to analyze population structure (RM11, RM14, RM105, RM109, RM152, RM174, RM202, RM206, RM215, RM225, RM228, RM230, RM232, RM235, RM259, RM317, RM335, RM400, RM481, RM467, and RM415; as summarized in CHEN *et al.* 1997; TEMNYKH *et al.* 2000, 2001; <http://www.gramene.org>). This number of markers seemed reasonable because it has been shown that 15–20 unlinked SSRs are sufficient to detect population stratification in humans (PRITCHARD and ROSENBERG 1999). In addition, 13 amplicons were analyzed for single nucleotide polymorphisms (SNPs) and used for haplotype and linkage disequilibrium analysis using primer pairs described by BLAIR *et al.* (2003) and summarized in Table 2.

PCR amplification: The SSRs and SNP amplicons were generated using the following PCR conditions: 95° for 4 min; 30 cycles of 94° for 1 min, 55° for 2 min, 72° for 2 min; and a 1-hr extension at 72° to promote nontemplate addition of adenine by Taq.

Genotyping: PCR products were size separated on 4% polyacrylamide gels using an ABI Prism 377 DNA analyzer (Applied Biosystems, Foster City, CA). SSRs were analyzed with GenScan 3.1.2 software (Applied Biosystems) and scored with Genotyper 2.5 software (Applied Biosystems).

DNA sequencing: A total of 10 µl of quantified PCR product was treated with 10 units exonuclease I and 2 units shrimp alkaline phosphatase and incubated at 37° for 15 min followed by 80° for 15 min. Single-pass sequencing was performed by automated sequencing using an ABI Prism 3700 DNA analyzer (Applied Biosystems) at the Cornell BioResource Center (Ithaca, NY). Because rice is a diploid, predominantly selfing species and therefore predominantly homozygous, direct sequencing of PCR products resulted in a monomorphic sequence. Sequences were aligned using Sequencher 4.0.5 (Gene Codes, Ann Arbor, MI) for base calling and CLUSTAL W (THOMPSON *et al.* 1994) with manual quality control for insertion/deletions. The ends of fragments were trimmed to remove low-quality sequence. The resulting sequences are listed in Table 2, along with the putative gene content of each fragment on the basis of TIGR annotation (<http://www.tigr.org>). Singletons, SSRs, and polymorphisms resulting from the expansion/contraction of polyA and polyTs were eliminated

TABLE 1
Rice accessions studied

Accession name	Ecotype from passport data	Country of origin	IRGC no. or USDA no. (PI)
ARC5756 ^{a,b}	Unknown	India	IRGC 20220
Ashmber	Aman	Bangladesh	IRGC 27522
Aus 449 ^{a,b}	Unknown	Bangladesh	IRGC 29230
Aus251 ^{a,b}	Unknown	Bangladesh	IRGC 29043
Aus32 ^{a,b}	Unknown	Bangladesh	IRGC 28895
Ausha Boro	Boro	Bangladesh	IRGC 27508
Badal 1 ^b	Aus	Bangladesh	IRGC 37362
Bageri ^{a,b}	Unknown	Nepal	IRGC 16193
Bangaluwa ^{a,b}	Unknown	Nepal	IRGC 16268
Bashiraj	Aman	Bangladesh	IRGC 26298
Battiboro	Boro	Bangladesh	IRGC 37005
Beri	Aus	Bangladesh	IRGC 25841
Bhim Dhan	Unknown	Nepal	PI 596818
Biolam	Aman	Bangladesh	IRGC 49070
BJ1 ^{b,c}	Unknown	India	IRGC 27526
Boro	Boro	Bangladesh	IRGC 27511
Boro	Aus and Boro	Bangladesh	IRGC 49159
Burimural	Aus	Bangladesh	IRGC 49164
Chandra Har	Aman	Bangladesh	IRGC 31754
Chikon Shoni ^b	Aus	Bangladesh	IRGC 64771
Chinsurah Boro II ^d	Unknown		
Cut Keureusek	Unknown	Indonesia	IRGC 66540
DB3 ^{a,b}	Unknown	Bangladesh	IRGC 8631
Devarasi ^{a,b}	Unknown	Nepal	IRGC 16173
DF1 ^{a,b}	Unknown	Bangladesh	IRGC 8365
Dhan	Unknown	Nepal	PI 549215
Dharia ^b	Aus	Bangladesh	IRGC 64773
Dholai Moni	Boro	Bangladesh	IRGC 53492
Dholi Boro	Boro	Bangladesh	IRGC 27513
DL5 ^{a,b}	Unknown	Bangladesh	IRGC 8593
DNJ142 ^{a,b}	Unknown	Bangladesh	IRGC 8426
Dudhi ^{a,b}	Unknown	Nepal	IRGC 16256
Dumpja Kaap	Unknown	Bhutan	IRGC 64890
DV139 ^{a,b}	Unknown	Bangladesh	IRGC 8870
DV29 ^{a,b}	Unknown	Bangladesh	IRGC 8816
DV32 ^{a,b}	Unknown	Bangladesh	IRGC 8818
DV85 ^{a,b}	Unknown	Bangladesh	IRGC 8839
DV86 ^{a,b}	Unknown	Bangladesh	IRGC 8840
DZ192 ^b	Unknown	Bangladesh	PI 403366
DZ78 ^{a,b}	Unknown	Bangladesh	IRGC 8555
Garia ^b	Aus	Bangladesh	IRGC 25854
Ghuni Boro	Boro	Bangladesh	IRGC 27515
Girresa	Unknown	Sri Lanka	IRGC 66512
Gokhue Saier	Unknown	Nepal	IRGC 16195
Janeri	Unknown	Nepal	PI 400662
Jumli Marshi	Unknown	Nepal	PI 596816
Jumula 2 ^b	Unknown	Nepal	PI 400021
Kal Shoni ^b	Aus	Bangladesh	IRGC 64780
Kaliboro 600 ^{a,b}	Unknown	Bangladesh	IRGC 29367
Kalo	Unknown	Nepal	PI 596824
Karar Vadui	Aus	Bangladesh	IRGC 53527
Katar Vadui	Aus	Bangladesh	IRGC 53527
Kathmandu Valley no. 1 selection	Unknown	Nepal	PI 400718
Kaun Kaek	Unknown	Cambodia	IRGC 22894
Khagrai Digha	Aman	Bangladesh	IRGC 53530
Koimurali ^b	Aus	Bangladesh	IRGC 49222
Kumri Boro	Boro	Bangladesh	IRGC 27518

(continued)

TABLE 1
(Continued)

Accession name	Ecotype from passport data	Country of origin	IRGC no. or USDA no. (PI)
Kuning	Unknown	Indonesia	IRGC 24999
Laita Parsum	Aman	Bangladesh	IRGC 26388
Lakhi Jhota	Aus	Bangladesh	IRGC 34711
Lakhi Jhota	Aus	Bangladesh	IRGC 34711
Laksmi Bilash	Aman	Bangladesh	IRGC 26705
Laksmilota ^b	Aus	Bangladesh	IRGC 27564
Lal Ahu ^{a,b}	Unknown	Nepal	IRGC 16121
Lal Chamara ^b	Aman	Bangladesh	IRGC 26499
Lal Sar ^{a,b}	Unknown	Nepal	IRGC 16185
Lalaka Gadur ^{a,b}	Unknown	Nepal	IRGC 16255
Latu	Unknown	Indonesia	IRGC 18093
Lekat Lembayung	Unknown	Indonesia	IRGC 66585
Lua Lon	Unknown	Vietnam	IRGC 16719
Machhapuchhre	Unknown	Nepal	PI 602664
Mansat-3	Unknown	Myanmar	IRGC 33341
Matury ^{a,b}	Unknown	Nepal	IRGC 16190
Meritam	Unknown	Indonesia	IRGC 18267
Mery	Aus	Bangladesh	IRGC 34722
Mery	Aus	Bangladesh	IRGC 34722
Mimidam	Unknown	Bangladesh	IRGC 25897
Mimidim	Unknown	Bangladesh	IRGC 25798
Molladigha	Aus	Bangladesh	IRGC 25906
Mudjaer	Unknown	Indonesia	IRGC 18296
Myawutyi	Unknown	Myanmar	IRGC 33416
Nakhi ^{a,b}	Unknown	Nepal	IRGC 16254
Narikel Jhupi ^b	Unknown	Bangladesh	IRGC 64792
Ngaja	Unknown	Bhutan	IRGC 64917
Ngasein Kalagyi	Unknown	Myanmar	IRGC 33498
Pakhi	Unknown	Nepal	PI 400664
Pankiraj ^{a,b}	Unknown	Bangladesh	IRGC 24139
Phcar Tien P65	Unknown	Cambodia	IRGC 23174
PI180060-1 ^{a,b}	Unknown	India	IRGC 3687
Pidi Niyam Wee	Unknown	Sri Lanka	IRGC 66529
Putuje	Unknown	Nepal	PI 391237
Rabija	Unknown	Nepal	PI 596831
Rakhoil ^b	Aus	Bangladesh	IRGC 64793
Ratomansara	Unknown	Nepal	PI 549235
Rerm Bilash ^{a,b}	Unknown	Nepal	IRGC 16273
RP291-20 ^b	Unknown	India	IRGC 40033
Sada Niniya	Aman	Bangladesh	IRGC 53569
Sajani ^{a,b}	Unknown	Nepal	IRGC 16177
Sampatti	Aus	Bangladesh	IRGC 25920
Shaitan Dumra	Aus	Bangladesh	IRGC 25921
Shoru	Aman	Bangladesh	IRGC 26523
Sokan Dhan ^{a,b}	Unknown	Nepal	IRGC 16250
Tally ^{a,b}	Unknown	Nepal	IRGC 16146
Tepi Boro	Boro	Bangladesh	IRGC 27519
Thapachini	Unknown	Nepal	PI 420983
Tolil14 ^{a,b}	Unknown	Malaysia	IRGC 13836
Trang Chum	Unknown	Vietnam	IRGC 16778
Zaneli	Unknown	Nepal	PI 403534

IRGC, International Rice Germplasm Center; PI, plant introduction.

^a SIDHU *et al.* (1978).

^b xa5 phenotype.

^c OLUFOWOTE *et al.* (1977).

^d SINGH *et al.* (1983).

from linkage disequilibrium and diversity analyses to exclude variation potentially introduced by sequencing error.

Analysis: Population structure was evaluated on the basis of three different analyses of genotypic data from the 21 SSRs:

genetic distance, the model-based program "Structure" (<http://pritch.bsd.uchicago.edu/>), and F_{ST} (WRIGHT 1969) implemented in Genepop software (<http://wbimed.curtin.edu.au/genepop/>). Genetic distance was calculated using D_C

TABLE 2
Amplicon names, lengths, and matches to genes in the TIGR gene index

Marker name	Length	Gene	Score	Function
RS1	148	None		
RS2	345	None		
RS4	283	None		
RS5	139	None		
RS6	254	None		
RS7	334	None		
RS8	250	None		
RS9	180	None		
RS11	321	BE039874	245 8.5e06	Unknown
RS12	243	None		
RS13	363	None		
RS14	225	None		
RS15	367	TC84724	261 2.1e06	Putative tRNA synthase
RS16	233	TC92643	438 1.3e14	Unknown
RS17	217	None		
RS18	237	TC93981	491 1.1e17	Unknown
RS19	239	None		
RS21	347	BI807280	239 5.6e06	Unknown

(CAVALLI-SFORZA and EDWARDS 1967). Phylogenetic reconstruction was based on the neighbor-joining method (SAITOU and NEI 1987) implemented in PowerMarker, a free genetic analysis software package distributed by Kejun Liu (kliu2@unity.ncsu.edu). Linkage disequilibrium, diversity, and recombination analyses of sequence data were performed using SITES (http://lifesci.rutgers.edu/heylab/ProgramsandData/Programs/SITES/SITES_Documentation.htm) and dipdat software (<http://home.uchicago.edu/rhudson1/source/misc/dipld/>). Linkage disequilibrium was plotted as the squared correlation coefficient r^2 . The minimum set of recombination intervals was calculated as in HUDSON and KAPLAN (1985). Association tests were performed using Strat software (<http://pritch.bsd.uchicago.edu/>).

RESULTS

Significant divergence among ecotypes was found by using all measures for population structure. Distance-based analysis of 84 accessions detected two major clusters, as illustrated in Figure 1. Although ecotypic designation was not available for all accessions, enough samples of *aus*, *boro*, and *aman* ecotypes were available to anchor the ecotype identities of the clusters (Table 1). The first group consisted of the Bangladeshi *indica* rice ecotype called *aman*, breeding lines and landraces from Nepal of unknown ecotype, and a single accession from Malaysia, hereafter referred to as *indica*. The second group was populated by *aus* and *boro* ecotypes, mainly from Bangladesh and Nepal, as well as accessions of unknown ecotype.

Results from model-based analysis generally concur with the relationships determined by genetic distance analysis. In this study, the model-based analysis gave high significance levels for several theoretical population sizes, but the highest posterior probability was for

a model with three populations. These results provided evidence for substructure within the *indica* group, formalizing the subclusters into two populations: one consisting mainly of *aman* ecotypes and another consisting of Nepali breeding lines. The third model-derived population corresponded to the *aus-boro* group of the distance-based analysis. Only four accessions differed in their population assignment by the two approaches; these were individuals that clustered with the *indica*'s in the genetic distance analysis but were assigned to the *aus-boro* group in the model-based analysis.

When F_{ST} values were computed using the model-based population subdivisions for two and three populations (Table 3), they showed a high degree of population structure (overall F_{ST} for two populations = 0.89; overall F_{ST} for three populations = 0.85). There was a higher F_{ST} for the pairwise comparison of the *aus-boro* group with the *indica* group than between the two *indica* groups, indicating that the *aus-boro* subgroup was more differentiated from the *indica* groups than either was from the other. Because the sample size was small for the *indica* group and because several samples were breeding lines that were closely related, these two groups were treated as one for the remaining analyses. The population structure data support a hypothesis of hierarchical levels of divergence within rice, with greater divergence between the *indica* and *aus-boro* groups and no detectable divergence between the *aus* and *boro* ecotypes at this level of genomic resolution. This suggests that the divergence between the *indica* and *aus-boro* ecotypes is more ancient than that between the *aus* and *boro* ecotypes.

Analysis of the *xa5* phenotype in relation to population structure analysis of the accessions confirmed the presence of *xa5* in the Bangladeshi *aus* and *boro* eco-

Model-based population assignments

- Indica 1
- Indica2
- Aus-boro

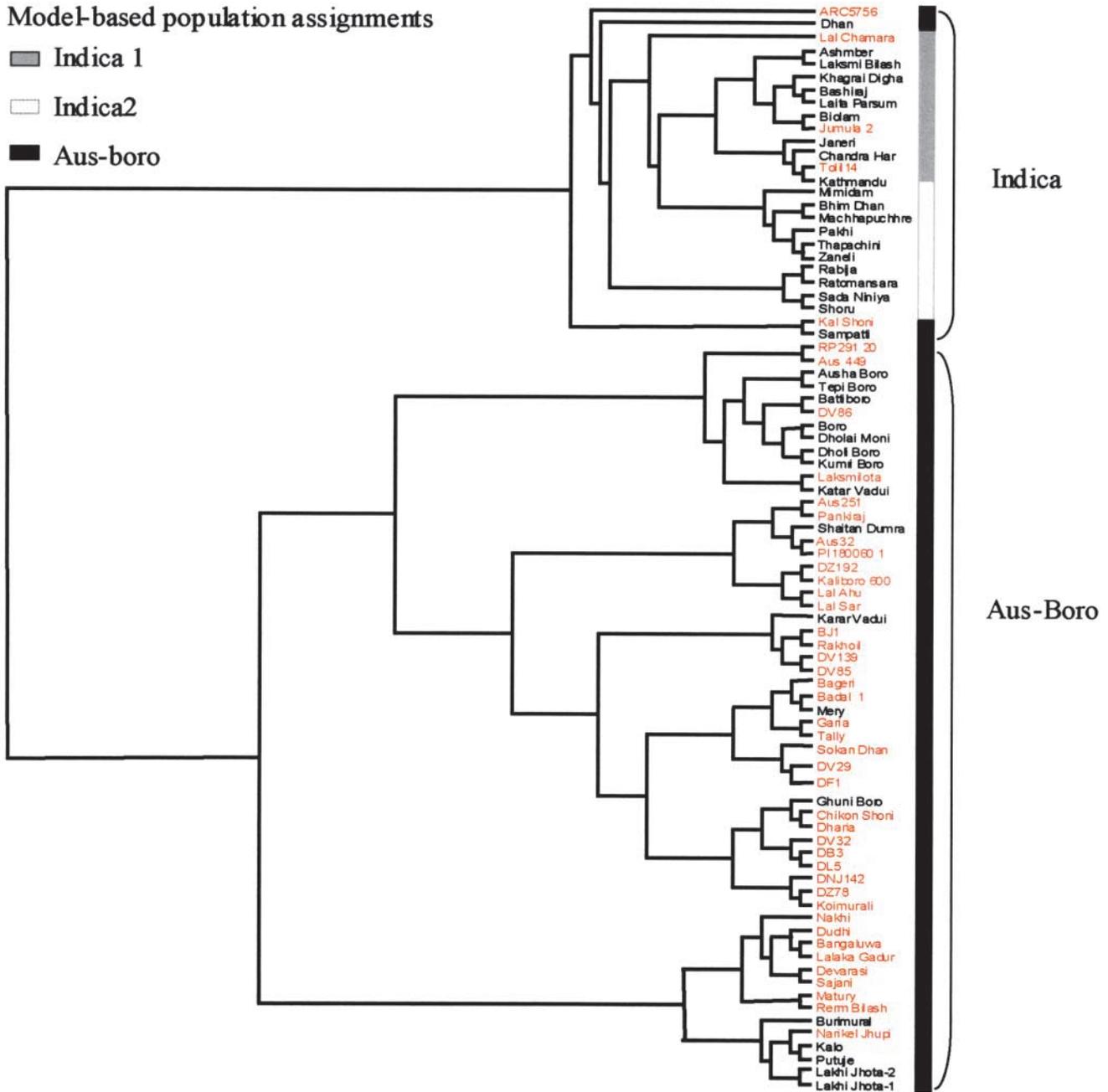


FIGURE 1.—Neighbor-joining tree of 84 rice accessions based on D_c (CAVALLI-SFORZA and EDWARDS 1967) using 21 unlinked SSR markers. Resistant accessions are indicated in red.

types. Of the 45 resistant rice accessions for which genotypic information was available, all were assigned to the *aus-boro* subgroup except three accessions originating from Malaysia, Bangladesh, and Nepal (accessions Tolil 14, Lal Chamara, and Jumula 2). The presence of the *xa5* phenotype outside of the *aus-boro* group could indicate gene flow or multiple origins.

Linkage disequilibrium in the 70-kb *xa5* region was extensive but potentially informative in reducing the candidate region for *xa5* described in BLAIR *et al.* (2003). Because the accessions showed significant population structure, *indica*'s and *aus-boro*'s were analyzed separately. Linkage disequilibrium, measured as r^2 , showed

significant linkage disequilibrium for the distal 45 kb of the candidate region for resistant accessions from both *indica* and *aus-boro* accessions (Figure 2), a pattern that was not observed in the susceptible groups. Association tests showed all sites to be equally significant due to the low frequency of recombination events in the haplotypes (data not shown).

A putative recombination event was detected on the distal side of the candidate region only, raising the question of how far linkage disequilibrium extended on the proximal side of the candidate region. To observe a decay of linkage disequilibrium, five additional amplicons spanning an additional 45 kb were analyzed in resistant

TABLE 3

Overall and pairwise estimates of F_{ST} for 21 SSR loci, using model-based population subdivisions

Subdivision	Indica	Overall
Aus-Boro	0.2103	—
Combined	—	0.8914

Subdivision	Indica 1	Indica 2	Overall
Indica 2	0.1762	—	—
Aus-Boro	0.2654	0.2462	—
Combined	—	—	0.8483

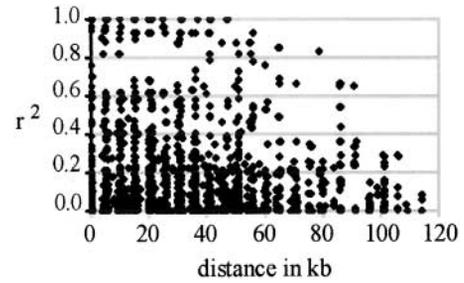


FIGURE 3.—The decay of linkage disequilibrium between all pairs of SNP loci in the region, shown as a function of the distance between the loci. Linkage disequilibrium was measured as r^2 .

accessions and added to the previous data set. Results confirmed that extensive linkage disequilibrium was present; r^2 approaches 0.1 only after 100 kb (Figure 3).

Analysis of haplotype diversity for *xa5* indicates that the *xa5* resistance phenotype either derives from multi-

ple origins or is genetically heterogeneous. Sequence diversity and haplotype structure were assessed in a larger sample of 114 accessions at 13 amplicons in the *xa5* candidate region. Additional accessions not analyzed previously were included for two purposes: to serve

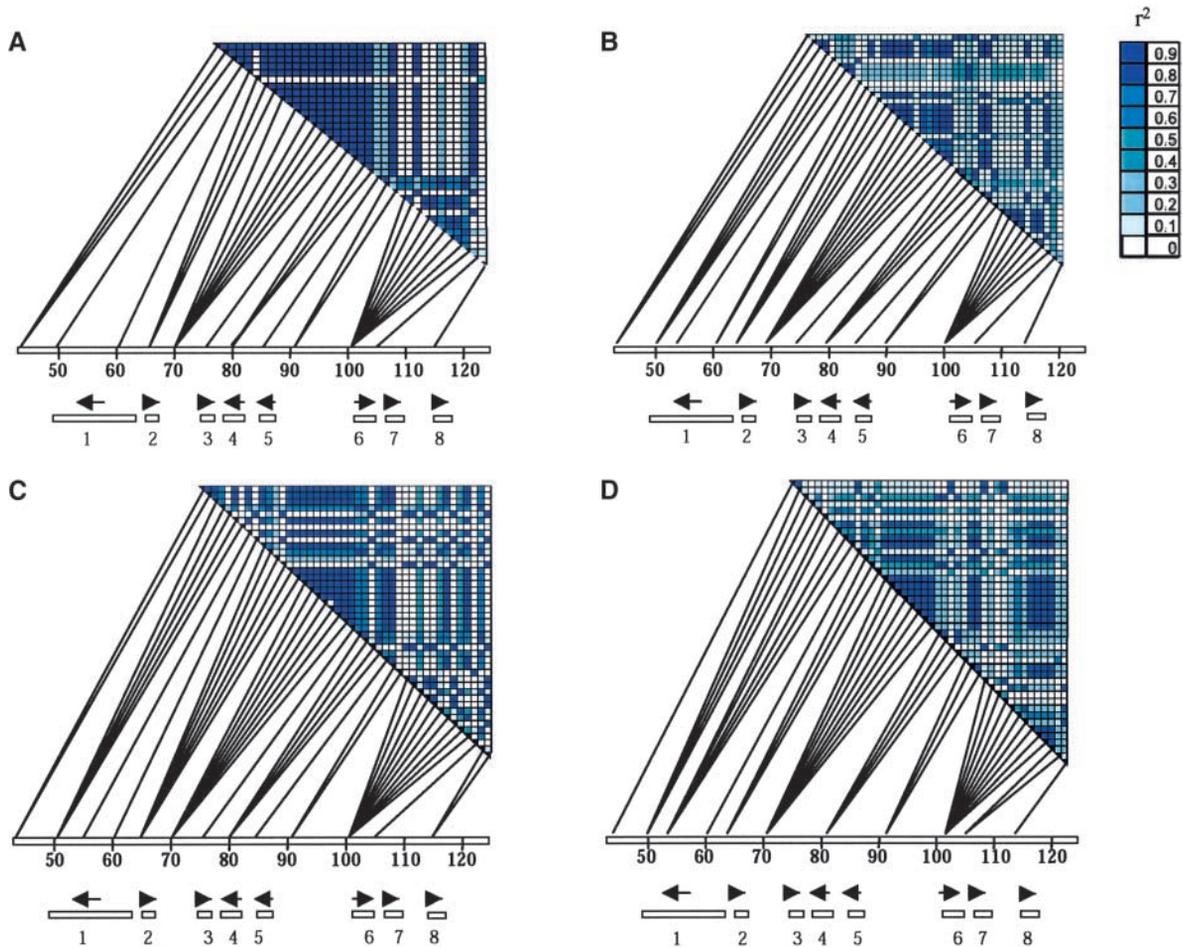


FIGURE 2.—Pairwise value for r^2 between all pairs of SNP loci, analyzed by population and phenotype: (A) *aus-boro* resistant. (B) *aus-boro* susceptible. (C) Non-*aus-boro* resistant. (D) Non-*aus-boro* susceptible. The shade of blue indicates the value for r^2 . The position of each site in the candidate region is indicated by lines that connect the loci with a chromosomal segment, labeled with physical distance measured in kilobase pairs. Candidate genes, represented by numbered boxes below the chromosome segment, are 1, putative ABC transporter; 2, putative TFIIa small subunit; 3, putative 23.6-kD protein; 4, putative tRNA synthase; 5, putative 46.2-kD protein; 6, putative 61.5-kD kinase; 7, hypothetical 33.3-kD protein; 8, putative cysteine protease. Arrows indicate the direction of transcription.

resistant haplotypes in the *aus-boro* group, allele-tested accession *Aus* 449 had a haplotype that was distinct from the others, and it was very similar (1/66 sites differed) to a haplotype found in accessions showing complete susceptibility or moderate resistance to *Xoo* race 1. Within phenotypically resistant non-*aus-boro* accessions that had not been allele tested for *xa5*, there were 4 additional related haplotypes, but there they are not supported by accessions that were allele tested for *xa5*, so this may indicate genetic heterogeneity for resistance.

In general, each haplotype was found in a single subpopulation, and frequently several closely related haplotypes were found in the same subpopulation. Haplotypes 1–9, 11, 15, 16, and 20–22 were found in *indica* ecotypes. *Aus-boro* accessions contained haplotypes 10, 12, 13, 14, and 23–26. The apparent restriction of a haplotype to a specific, genetically defined subpopulation did not preclude a wide geographical distribution. The detection of the global distribution of haplotypes was hindered because of the strategy to sample accessions from Bangladesh and Nepal, where the resistance allele was expected to be in highest frequency. However, the example of haplotype 3, which was present in rice collected from Vietnam, Cambodia, Indonesia, Myanmar, and Bangladesh, attests to the global spread of certain haplotypes and suggests that geographic origin may be a poor indicator of genetic distance.

The data showed a high-enough level of diversity both within and between populations for effective mapping and indicated a higher role for mutation than for recombination in generating the observed haplotype diversity. The sequenced amplicons containing the 66 variable sites were predominantly noncoding, although parts of five amplicons had significant matches to genes in the TIGR gene index (Table 2). Of the variable sites, 4 were insertion-deletions ranging in length from 1 to 33 bp and 62 were SNPs.

Variation in the *xa5* region was similarly distributed between the *indica* and *aus-boro* ecotypes, despite the much larger sample size for the *aus-boro*'s. Specifically, 50 sites varied within the *aus-boro* subpopulation and 53 sites within the *indica*'s. The 13 additional variable sites were found in outgroups and nonallele-tested accessions of *xa5*. Many haplotypes ($n = 11$) differ from their most similar haplotype by a single site, indicating an important role for mutation in generating haplotype diversity. In contrast, the minimum set of recombination intervals is four, indicated in Figure 4. There is evidence for recombination only in haplotypes 7, 17, and 23.

DISCUSSION

The analysis of population structure underscores the need for genetic analysis of ecotypic differentiation if linkage disequilibrium and association mapping approaches are to be of value in rice improvement. The divergence between *indica* and *aus-boro* ecotypes de-

tected by SSRs in the present study had been observed in previous studies employing isozymes (15 loci), amplified fragment length polymorphisms (AFLPs; 179 bands), and randomly amplified polymorphic DNAs (RAPDs; 35 bands; GLASZMANN 1987; ZHU *et al.* 1998; PARSONS *et al.* 1999). The mixing of *aus* and *boro* genotypes was noted in the isozyme and RAPD studies, but was not addressed in the AFLP analysis. The fact that 21 SSR markers give similar results to the other marker systems attests to the utility of multi-allelic, highly variable SSR markers for detecting population differentiation as well the depth of population structure in rice. It contrasts with an outcrossing species such as maize, with a different evolutionary history, where a greater number of markers may be required to detect population structure. It is interesting to note that the differentiation between *aus* and *boro* rices, which is meaningful in the farming system, is invisible with this low genomic resolution. This could indicate a recent divergence between *aus* and *boro* rices or continuing gene flow between them.

The frequency of nucleotide polymorphisms in this sample was 1 SNP per 100 bp. This is lower than that of maize, where the frequency of SNP polymorphism in US elite inbred germplasm was 1 SNP per 48 bp in noncoding regions and 1 SNP per 131 bp in coding regions (BHATTRAMAKKI *et al.* 2002). The estimate for SNP frequency in rice in this study is more similar to preliminary data from sorghum, which, like rice, has low outcrossing rates (PEDERSEN *et al.* 1998), where the estimated frequency is 1 SNP per 102 bp (average $n = 25.45$; M. HAMBLIN, personal communication). However, because this study included only a few *indica*'s from outside of Bangladesh, and therefore represents a small sampling of their wide geographic range and no tropical or temperate japonicas or aromatic rices, the accessions included in this study do not represent the full diversity of rice. It is possible that different genomic regions and sampling will offer different views of both the frequency and the distribution of polymorphism and recombination.

Estimates of linkage disequilibrium are important as an indicator of how useful linkage-disequilibrium-based trait mapping approaches may be compared to other available methods on the basis of the tradeoff between population size and informativeness. If linkage disequilibrium declines rapidly, genome scans will require an excessive marker density, but the testing of candidate genes is feasible. If linkage disequilibrium is too large, resolution may be low, but genome scans are viable. The linkage disequilibrium decay at 100 kb observed in this study would require an average of one marker per centimorgan (1 cM = 200–300 kb; FENG *et al.* 2002; SASAKI *et al.* 2002), and these results suggest that linkage disequilibrium mapping strategies could provide greater resolution (because of the higher recombination rate) than primary quantitative trait locus (QTL) mapping, where populations with 200–300 individuals are typically surveyed with 150–200 markers and result

in QTL typically 10–20 cM in size. However, to generate a whole-genome scan that captures the resolution offered by LD mapping, such a study would require the use of ~1500 well-distributed markers. Thus, most applications of LD mapping are likely to be limited to regions previously delimited by QTL analysis or by candidate gene studies. In these cases, association mapping offers the advantage of exploring the relationship between phenotype and a broad array of genotypic variants at a favorable level of resolution in a specified target region. Because linkage disequilibrium is likely to extend beyond a single gene in rice, the application differs greatly from maize where genes already known to be associated with a trait can be tested to identify the functional nucleotide polymorphisms (THORNSBERRY *et al.* 2001). More studies will be required to determine if the extent of linkage disequilibrium reported here is typical of other subpopulations and loci in rice.

In this sample, significant linkage disequilibrium ($r^2 \geq 0.1$) persisted between sites up to 100 kb apart. This is the same order of magnitude as linkage disequilibrium observed at the *FRIGIDA* flowering time locus in *A. thaliana*, where significant linkage disequilibrium was detected between pairs of sites up to 250 kb apart (HAGENBLAD and NORDBORG 2002; NORDBORG *et al.* 2002). As expected, these estimates differ greatly from the limited linkage disequilibrium observed in outcrossing species like maize where linkage disequilibrium frequently decays at distances between 100 bp and 1.5 kb (REMINGTON *et al.* 2001; TENAILLON *et al.* 2001; THORNSBERRY *et al.* 2001). In addition, it is possible that the *xa5* locus is under selection and would therefore be predicted to have more extensive linkage disequilibrium than a locus evolving neutrally.

The resolution of the origin of *xa5* and the allelic diversity for resistance was not possible with this data set. The *xa5* phenotype was found predominantly within the genetically defined *aus-boro* subpopulation. However, the presence of the phenotype in a few accessions in the *indica* group raises the possibility of independent origins of this phenotype in different subpopulations, particularly when haplotypic data are considered. Within the *aus-boro* subpopulation one very common haplotype was associated with the *xa5* reaction profile; however, very different haplotypes were associated with resistance in *indica* ecotypes.

Several lines of evidence suggest genetic heterogeneity for the resistance phenotype. For instance, some resistant, allele-tested accessions had haplotypes highly similar to susceptible accessions (compare haplotype 18 to 19 and haplotype 6 to 7). It is possible that the relevant differences lie in unsequenced regions and that recombination has not broken the linkage. These pairs of haplotypes could be useful for examining candidate genes for evidence of mutations because they would be expected to be highly similar at most positions. Another possibility is that susceptibility is being caused by another

locus, because the Philippine *Xoo* races contain multiple avirulence (*avr*) proteins, which could interact with susceptibility alleles at other loci in the rice genome.

More evidence for genetic heterogeneity is that some non-allele-tested, resistant accessions from the presumed *indica* group originating in Southeast Asia have a haplotype that differs from the *aus-boro* resistant haplotype and is identical to some susceptible accessions. Because these accessions were not allele tested, it is possible that another locus confers the phenotype, a hypothesis that could be confirmed by genetic mapping. Alternately, it could be a different resistance allele at this locus; if the recessive nature of the gene is indicative of a knock-out mutation, the phenotype could be achieved by many possible nucleotide changes. Once again, it is also possible that the relevant mutation could be so recent that recombination has not occurred to sufficiently reduce linkage disequilibrium.

Genetic heterogeneity for a trait would require careful sampling if linkage disequilibrium and association mapping were to be employed. If alleles in rice have arisen after the diversification into subpopulations and their isolation has been enforced by limited gene flow, this situation would represent a violation of the common assumption for association mapping, the common disease common variant hypothesis, which proposes that common variants are responsible for the genetic risk for certain diseases (LANDER 1996). This would reduce the power to detect the association between genotype and phenotype and suggests that larger sample sizes could be necessary. At this time, little information is available on the distribution of alleles in subpopulations of rice. In a study of the haplotype at the *waxy* locus that confers glutinous texture to rice, the glutinous haplotype was found mainly in temperate and tropical *japonica*'s and in only a few *indica* accessions (OLSEN and PURUGGANAN 2002). This trait would be expected to be under strong selection due to cultural preference, so one might expect limited gene flow. It is not known how this would differ for traits affecting biotic or abiotic stress resistance.

A similar example of genetic heterogeneity was found for the early flowering *FRIGIDA* locus in Arabidopsis. The early flowering haplotype in Central Asia differs from that found in the rest of the early-flowering accessions (HAGENBLAD and NORDBORG 2002), and eight independent loss-of-function mutations at this locus conferring early flowering have been identified (LE CORRE *et al.* 2002). Both rice and Arabidopsis are predominantly autogamous, and therefore the expectation of a single origin of a phenotype that occurs across subpopulations may be less plausible than in outcrossing species. This has implications for sampling in future linkage disequilibrium or association studies. Isolated populations, employed in the study of human diseases, may find their plant counterpart in the subpopulations of autogamous crop species, which can have the advan-

tage of a greater likelihood of having a single origin for a phenotype (SHIFMAN and DARVASI 2001). However, because linkage disequilibrium may extend beyond a single gene, studies will require large sample sizes to capture rare recombination events.

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