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# Microsatellite and sequence-tagged site markers diagnostic for the rice bacterial leaf blight resistance gene *xa-5*

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**Abstract** Microsatellite and sequence-tagged site (STS) markers tightly linked to the bacterial leaf blight (BLB) resistance gene *xa-5* were identified in this study. A survey was conducted to find molecular markers that detected polymorphisms between the resistant (IRBB5) and susceptible ('IR24') nearly isogenic lines for *xa-5*, and between Chinsurah Boro II (CBII), an alternative source of *xa-5*, and a widely planted variety ('IR64') that lacks *xa-5*. Two $F_2$ populations, from the crosses 'IR24' × IRBB5 and CBII × 'IR64', were used to estimate linkage based on marker genotype and reaction to disease inoculation with *Xanthomonas oryzae* pv. *oryzae*. Two RFLP clones, RZ390 and RG556, were found to co-segregate with *xa-5* and were converted into STS markers. A microsatellite marker, RM390, was developed based on a simple sequence repeat in the 5′ untranslated region of the cDNA probe, RZ390, and found to co-segregate with resistance. Two other microsatellites, RM122 and RM13, were located 0.4 cM and 14.1 cM away from *xa-5*. A germplasm survey of diverse lines containing BLB resistance genes using automated fluorescent detection indicated the range of allelic diversity for each of the microsatellite loci linked to *xa-5* and confirmed their usefulness in following genes through the narrow crosses typical of a breeding program. The limited number of alleles observed at the microsatellite loci linked to the resistance gene in 35 *xa-5*-containing accessions suggested either a single ancestral origin or a few independent origins of the *xa-5* gene. PCR-based markers, like the ones developed in this study, are economical and easy to use, and have applicability in efforts to pyramid the recessive *xa-5* gene with other BLB resistance genes.

**Key words** Microsatellite · Sequence tagged site · Bacterial leaf blight resistance gene *xa-5* · Rice (*Oryza sativa*)

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## Introduction

The development of molecular markers diagnostic for the selection of resistance genes is a goal of many rice breeding programs. Several of the major resistance genes to the bacterial leaf blight (BLB) pathogen, *Xanthomonas oryzae* pv. *oryzae* , have been tagged with restriction fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD) markers (McCouch et al. 1992; Ronald et al. 1992; Yoshimura et al. 1992; Yoshimura et al. 1995a, b). However, both of these types of markers have limited application in a breeding program. Although RFLP markers are widely available for rice (Causse et al. 1994; Kurata et al. 1994), they have proven too technically cumbersome to be used for selection with large numbers of plants. Since RAPD markers are normally dominant, detect multiple loci and can have technical problems, they are less useful for selection. Therefore, new types of co-dominant, single-copy, polymerase chain reaction (PCR)-based markers are desirable. Recently, many RFLP markers have been converted into PCR-amplifiable sequence-tagged sites (STS) for specific locations in the rice genome (Inoue et al. 1994; Williams et al. 1991), and a group of microsatellite markers spread randomly throughout the rice genome has been developed (Wu and Tanksley 1993; Panaud et al. 1996; Chen et al. 1997). PCR-based markers closely linked to BLB resistance genes would be very useful for efficient marker-assisted selection.

Nineteen genes for BLB resistance have been identified (Kinoshita 1991), the majority of which are dominant and provide vertical, race-specific resistance (Ogawa and Khush 1989). While these genes can be phenotypically selected in backcross breeding programs, combining several genes at once requires screening with the appropriate bacterial strains to differentiate the overlapping phenotypic effects of these genes (Mew et al. 1993). Some of the BLB resistance genes, including *xa-5*, *xa-8*, *xa-9* and *xa-13*, are recessive, and progeny testing is required to detect them in the heterozygous state. Of the recessive resistance genes, *xa-5* was the first to be placed into a linkage group through trisomic analysis and with genetic studies using morphological markers (Yoshimura et al. 1984). Since then, nearly isogenic lines (NILs) with single BLB resistance genes in 'IR24' (*indica*) and 'Toyonishiki' (*japonica*) backgrounds have been developed by repeated backcrossing (Ogawa et al. 1988). These NILs have been used to characterize the resistance spectrum of the *xa-5* gene, both by itself (Ogawa et al. 1991) and in combination with other BLB resistance genes (Yoshimura et al. 1995b), and also to determine the approximate location of the *xa-5* gene in relationship to RFLP markers at the end of chromosome 5 (McCouch et al. 1992; Yoshimura et al. 1995a). The *xa-5* resistance gene is not near any previously described resistance genes, while several other BLB resistance genes show clear evidence of clustering. The genes *Xa-3*, *Xa-4*, *Xa-10* and *Xa-21* are all located on the short arm of chromosome 11, along with several genes for resistance to rice blast, while *Xa-1* and *Xa-2* are located together on chromosome 4 (Causse et al. 1994).

Here we report new PCR-based markers (microsatellite and STS) for the diagnosis of the *xa-5* resistance gene, map them in relation to previously described molecular markers on chromosome 5 and investigate their utility to evaluate BLB resistant and *xa-5* containing germplasm useful to disease resistance breeding programs in rice.

## Materials and methods

### Plant material

The resistant isoline containing *xa-5* (IRBB5), its susceptible recurrent parent ('IR24') and the donor parent which provided this resistance (IR1545-339) were used to survey marker polymorphisms. The IRBB5 isoline was the product of four backcrosses to 'IR24' (Ogawa et al. 1988), a variety that is susceptible to all Philippine races of the pathogen (Ogawa and Khush 1989).

A segregating $F_2$ population of 122 plants, derived from a cross between the resistant isoline, IRBB5, and the susceptible recurrent parent, 'IR24', was used to determine linkage between the microsatellites on chromosome 5 and the *xa-5* locus. A second population, consisting of 85 $F_2$ plants from the cross Chinsurah Boro II × IR64, was used to confirm the mapping of the *xa-5* linked markers. Chinsurah Boro II (CBII) is an *Aus* landrace from India which contains *xa-5* as well as a second recessive gene, *xa-13* (Singh et al. 1983; Ogawa and Khush 1989). 'IR64' is an *indica* variety that is widely-planted throughout tropical Asia and is known to contain the '*Xa-4*' gene and several quantitative trait loci (QTLs) for BLB resistance (R. Nelson, personal communication, IRRI).

In addition to the parents of the populations described above, 35 landraces and breeding lines containing the *xa-5* resistance gene (Sidhu et al. 1978; Singh et al. 1983) were screened to determine the allelic diversity for each of the microsatellite markers most closely linked to *xa-5*. These accessions were obtained from the International Rice Research Institute (IRRI). In some of these genotypes, the resistance genes *Xa-7* and *xa-13* were present in addition to *xa-5*. A group of eight differentials (Ogawa and Khush 1989) and 11 nearly isogenic lines (Ogawa et al. 1988), containing the BLB resistance genes *Xa-1*, *Xa-2*, *Xa-3*, *xa-5*, *Xa-4*, *Xa-7*, *Xa-10* , *Xa-11*, *xa-13*, *Xa-14* and *Xa-21*, and their recurrent parent 'IR24' were also included in the analysis of allelic diversity.

### Bacterial inoculation

Philippine strains of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) were used in inoculation experiments to determine resistant and susceptible phenotypes. A single inoculation of the race-1 strain, PXO 61, was used to study the segregation of *xa-5* in the 'IR24' × IRBB5 population. Double inoculation was used to distinguish *xa-5* and *Xa*-4 segregation for the CBII × 'IR64' population. The *xa-5* gene, but not the *Xa-4* gene, provides resistance to the race-2 strain, PXO 86, while both genes provide resistance to PXO 61. All plants were grown in the greenhouse and inoculated at 55 days after planting. Six of the youngest leaves of four tillers were inoculated by scissors-clipping at 5 cm below the leaf tips with a bacterial suspension having an $OD_{600} = 0.6$. The inoculum was prepared from bacteria revived from glycerol stocks maintained at Cornell University and grown for 48 h in nutrient yeast sucrose broth at 30°C. After inoculation the plants were maintained in a growth chamber with 11 h of light, relative humidity above 70%, night temperatures of 28°C and day temperatures of 32°C. At 14 days after inoculation the plants were scored as resistant or susceptible, and lesion length was measured for the six inoculated leaves. The controls used during inoculations were the four parents of the populations, the $F_1$s of each cross and the variety 'IR8', which is susceptible to both bacterial strains. The 35 *xa-5*-containing varieties were also inoculated with race 1 to confirm their disease reaction.

### RFLP analysis

DNA was extracted according to the method of McCouch et al. (1988) from a portion of the un-inoculated tissue, which was harvested at the time of BLB inoculation. RFLP survey filters were made from DNAs of the recurrent parent ('IR24'), isoline (IRBB5) and donor parent (IR1545-339) digested with 9 restriction enzymes (*Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Sca*I and *Xba*I). Separate parental survey filters were made for DNAs of CBII and 'IR64'. Genomic and cDNA probes from the high-density Cornell map (Causse et al. 1994) and the landmarker sets (NIAR Staff 1993) of the Japanese Rice Genome Program (JRGP) were selected, and Southern analysis was conducted according to Causse et al. (1994). Where the RFLP markers were polymorphic in the NIL parental survey, an introgression was interpreted to be present where the isoline had inherited a donor parent allele and absent where the isoline had inherited a recurrent parent allele (McCouch et al. 1991). The segregation of the polymorphic RFLP clones in each population was determined with mapping filters prepared with DNA from all the $F_2$ individuals digested with the appropriate restriction enzyme.

## Sequencing, homology search and PCR primer design

Two RFLP clones (Causse et al. 1994) were sequenced by the Cornell Biotechnology facility using M13 forward and reverse primers and an Applied Biosystem 373A automated sequencer: RZ390 was a 0.8-kb rice cDNA in a Lambda ZapII vector (Stratagene, La Jolla, Calif.), and RG556 was a 1.4-kb *Pst*I genomic clone in a pGEM4z vector (Promega, Madison, Wis.). The BLAST (basic local alignment search tool) algorithm (Altschul et al. 1990) was used to conduct a similarity search of standard, non-redundant DNA sequence databases maintained by the National Center for Biotechnology Information. The sequence information was used to design two sets of primers, from 19 to 24 bases long, for PCR amplification of RZ390 (Forward-5′ CCC TTG TTT CAG TGG CTC AG 3′/ Reverse-5′ TCG ATC TTT ACC GAA GTG G 3′) and RG556 (Forward-5′ ATA CTG TCA CAC ACT TCA CGG 3′/Reverse-5′ GAA TAT TTC AGT GTG TGC ATC 3′). These markers are referred to as STS390 and STS556, respectively. A simple sequence repeat near one end of the RZ390 clone was targeted with a second reverse primer (5′ CCA AGA TCA AGA ACA GCA GGA ATC 3′) to develop a microsatellite marker called RM390.

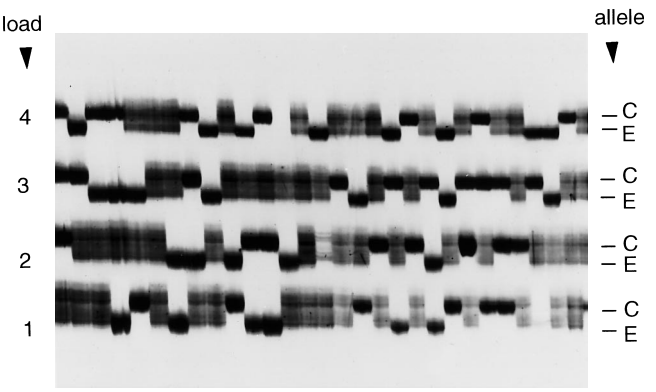


**Fig. 1** Multiplex mapping of a single microsatellite marker, RM122, for 122 $F_2$ individuals of the population 'IR24' × IRBB5, on a 4% polyacrylamide gel. Parental alleles for 'IR24' (*E*) and IRBB5 (*C*) are indicated for each of four (numbered) consecutive loadings of PCR amplification product samples

## STS analysis

The STS primers were used to amplify PCR products from genomic template DNA of the four parents of the segregating populations. The PCR conditions which optimized STS amplification were a hot start at 94°C for 2 min; 40 cycles of 94°C denaturing for 1 min; 55°C annealing for 1.5 min and 72°C extension for 2 min; and a 4 min extension at 72°C. For the parental survey, the PCR products were digested at the appropriate temperature with a panel of 31 restriction enzymes, with either four base-pair (*Alu*I, *Cfo*I, *Dpn*I, *Hae*III, *Hha*I, *Hpa*II, *Mbo*I, *Msp*I, *Mvn*I, *Rsa*I, *Sau*3A, *Spe*I, *Taq*I, and *Tru*9I), five base-pair (*Bst*NI, *Eco*RII and *Hin*fI) or six base-pair (*Apa*I, *Apo*I, *Acy*I, *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*cII, *Hin*dIII, *Pst*I, *Sca*I, *Xba*I and *Xho*I) recognition sequences. The digestion products were analyzed in 1 × TAE, 2% agarose gels. Both STS390 and STS556 were mapped by amplification of PCR products for the $F_2$ individuals of the two segregating populations, followed by digestion with the restriction enzyme, *Alu*I, and analysis on 1 × TBE, 2.5% Metaphor agarose (FMC, Rockland, Me.) gels.

## Microsatellite analysis

Microsatellite primer pairs (Research Genetics, Huntsville, Ariz.) for three loci on chromosome 5, RM13 (Panaud et al. 1996), RM122 and RM164 (Wu and Tanksley 1993), and one locus on chromosome 11, RM21 (Panaud et al. 1996), were used in the parental survey. Total plant DNA was used as the template for PCR amplification, with a hot start of 92°C for 5 min; 35 cycles of 92°C denaturing for 1 min; 55°C annealing for 1 min and 72°C extension for 2 min and a 4-min final extension at 72°C. Polymorphisms between the parents were analyzed by running the PCR products from the genotypes in adjacent lanes on 40-cm-long 4.5% denaturing polyacrylamide gels (PAGE) run for 1.5 h at 75 constant watts and silver stained according to the manufacturer's instructions (Promega). Multiplexing, which is the sequential loading of independent PCR amplification products on a single PAGE gel at intervals of about 20 min, was used for genotyping all the individuals of a population for a single locus (Fig. 1) and for the simultaneous mapping of several different microsatellite loci (Fig. 2). For the diversity analysis, the microsatellites were loaded individually and compared to two size standards (markers V and VIII from Boehringer-Mannheim, Indianapolis, Ind.) that were used at regular intervals throughout the lanes of the PAGE gel.

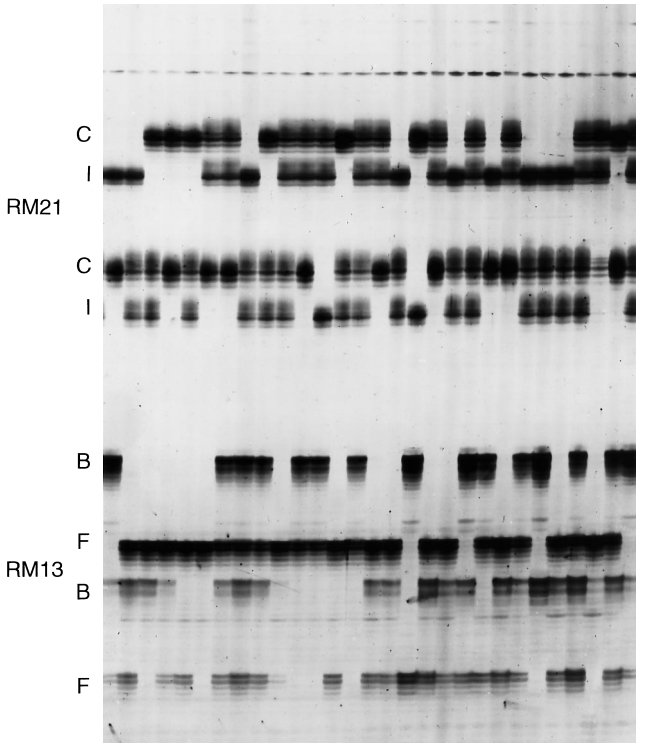


**Fig. 2** Simultaneous mapping of two unlinked microsatellite markers, RM13 on chromosome 5 and RM21 on chromosome 11, for the population 'IR24' × IRBB5 on a single 4% polyacrylamide gel by multiplexing. Parental alleles for 'IR24' (*C*, *B*) and IRBB5 (*I*, *F*) are indicated and correspond to the designation in Table 2

## Identification of fluorescently labeled microsatellite alleles

To obtain more accurate size estimates, we genotyped the varieties with different alleles observed in silver-stained PAGE gels using automated fluorescent detection. The forward primers of the RM13, RM21 and RM122 markers were labeled with the fluorescien dyes HEX, TET and FAM (Research Genetics), respectively. PCR amplification of the microsatellites was carried out as described above.

The three microsatellite PCR products for each variety were mixed together, diluted 1:1 with water and combined with loading dye, formamide and a size standard (TAMRA-350) in preparation for co-electrophoresis. The samples were run in a 6% polyacrylamide gel for 10 h with a 373A automated sequencer. The data were collected and analyzed by Genescan software (Applied Biosystems). The size of the bands for each allele, in base-pairs, was estimated by the local Southern method, which compares the migration of the sample fragment to that of adjacent bands of the internal-lane size standards.The polymorphism information content (PIC) for the microsatellite markers RM13, RM122, RM390 and RM21 was calculated according to Anderson et al. (1993), based on the allele patterns of all the varieties used.

### Linkage analysis

Full multipoint linkage analysis for the segregating polymorphic markers and the *xa-5* resistance gene was conducted with MAPMAKER V. 2.0 (Lander et al. 1987) for each of the individual populations. For the 'IR24' × IRBB5 population, the maximum-likelihood map order for markers was determined with a LOD score threshold of 3.0, and was used as a fixed sequence framework for integrating linkage data from the CBII × 'IR64' population. JOINMAP V. 1.4 (Stam 1993) was used to calculate the combined recombination frequencies for the two populations together and to align the markers into a consensus map. All map distances (centiMorgans, CM) are reported in Kosambi units, and critical LOD score thresholds of 3.0 and 0.05 were used for determining linkage groups (linklod) and for the calculation of map distances (maplod) in JOINMAP V. 1.4. The segregation ratios of individual markers were calculated with the software program QGENE V. 2.0 (Nelson 1994), and skewing was indicated when the ratio deviated significantly from the expected.

## Results

### Homology of RFLP clones tightly linked to *xa-5* and development of PCR-based markers

Previous studies demonstrated that the RFLP clones RG556 and RZ390 were tightly linked to *xa-5* (McCouch et al. 1992; Yoshimura et al. 1995a). Using 450 bp of sequence data from each end of the RG556 genomic clone we found no homology with any of the sequences in the GenBank, EMBL or DDBJ databases. Full-length sequence data (823 bp) were obtained for the RZ390 cDNA clone, which was found to have 95% overall sequence similarity to a rice cDNA (EMBL accession number X75670) previously reported by Smith et al. (1994) that encoded cytochrome b5. The 5′ untranslated region (5′ UTR) of the RZ390 clone was almost twice as long (240 bp) as that of the cytochrome b5 cDNA (132 bp) reported by Smith et al. (1994).

RZ390 and RG556 were converted into sequence tagged sites (see Materials and methods) and a single DNA fragment was produced by each PCR reaction. The STS556 product was the same size as the genomic clone, RG556 (1.4 kb). The STS390 product (1.2 kb) was larger than the RZ390 cDNA clone (0.8 kb), indicating the presence of at least one intron in the cytochrome b5 gene. Both STS markers were monomorphic between the parents of the two mapping populations; however, restriction digestions produced useful polymorphisms. For STS556, 4 enzymes (*Alu*I, *Bst*NI, *Hin*fIII and *Tru*9I) of the 31 used produced polymorphisms between both sets of parents. For STS390, none of the enzymes produced a polymorphism between CBII and 'IR64', and only the *Alu*I digestion produced a polymorphism between 'IR24' and IRBB5.

The presence of a simple sequence repeat at the end of the 5′ untranslated region (5′ UTR) of the RZ390 clone allowed us to design primers defining a new microsatellite marker. A block of five tandem (CT) repeats located 71 nucleotides from the end of the clone was developed as a microsatellite marker, which was named RM390. The primers consistently amplified a single, strong PCR product from rice genomic DNA template that included both the CT repeats and an adjacent AT-rich region (sequence data not shown). RM390 was monomorphic for the parents of the 'IR24' × IRBB5 population and polymorphic for the parents of the CBII × 'IR64' population.

### Bacterial leaf blight resistance

Leaf inoculation of the $F_2$ individuals and controls produced the expected phenotypes. Infection by race 1 of *Xoo* clearly differentiated the susceptible genotypes 'IR8' and 'IR24', which had lesions averaging over 15 cm in length from the resistant genotypes IR1545 and IRBB5, which had typical brown necrotic lesions averaging less than 1 cm long. The $F_1$ of the cross 'IR24' × IRBB5 was susceptible, as predicted for the recessive gene *xa-5*. The observed phenotypic classes for the $\chi^2$ individuals fit the expected 3:1 ratio of susceptible: resistant in a $\chi^2$ test ($P > 0.75$). The average lesion length was 17.6 cm (SE = 0.82) for the susceptible individuals and 0.8 cm (SE = 0.06) for the resistant individuals in this population. 'IR64' was susceptible to race 2 and resistant to race 1, while CBII was resistant to both races. The reaction to race 2 was used to determine *xa-5* resistance in the CBII × 'IR64' population for which the $F_2$ segregation fit the expected 3:1 ratio ($P > 0.85$).

### High-resolution linkage map for the *xa-5* region on chromosome 5

The IRBB5 isoline was shown to contain a large introgression from the donor source, that included *xa-5* and spanned the entire short arm of chromosome 5 (Fig. 3). The two microsatellite loci, RM13 and RM122, that had been located to this arm of chromosome 5 by Panaud et al. (1996) and Wu and Tanksley (1993), respectively, were polymorphic in the NIL survey and diagnostic for the introgression. In previous studies of the same parents (McCouch 1992; Yoshimura et al.

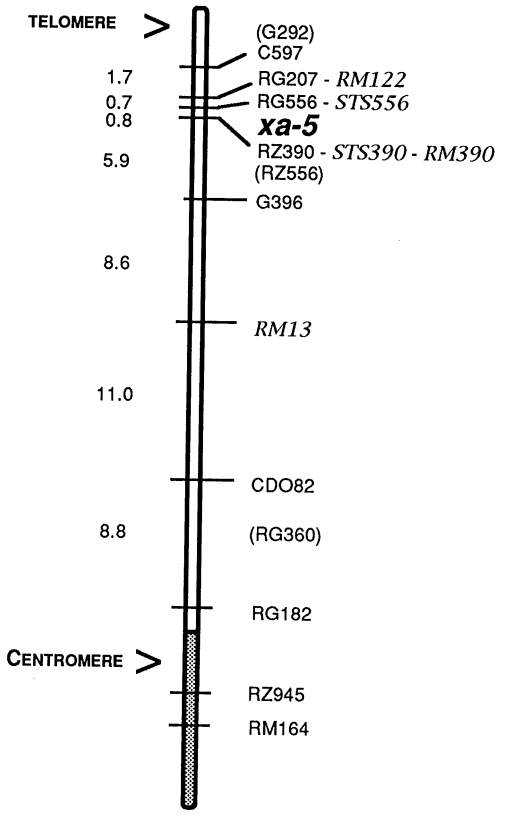


**Fig. 3** Genetic linkage map for the IRBB5 introgression (*non-stippled*) on the short arm of chromosome 5 based on the segregation data for 207 $F_2$ individuals of two populations, 'IR24' × IRBB5 and Chinsurah Boro II × 'IR64', showing the placement of *xa-5* in relation to 5 PCR-based markers (*italicized*) and 10 RFLP markers (*non-italicized*). The map distances (cM) were determined with JOINMAP V.1.4 (Stam 1993) and are reported in Kosambi units. The locations of the centromere (Singh et al. 1995) and the telomere (Kurata et al. 1994) as well as the orientation of the long and short arms of chromosome 5 are indicated. The non-introgressed region of this chromosome in the IRBB5 isoline (*darkly stippled bar*) and the markers which define the end of the introgression are shown.

1995a), 6 RFLPs were identified in the region: RG207, RG556, RZ390, CDO82, RG360 and RG182. In our survey, we detected polymorphisms between the isolines for four additional RFLP clones, G292, C597, G396 (NIAR Staff 1993) and RZ556 (Causse et al. 1994), that were located in this region. It is interesting to note that while most clones were single-copy, a cluster of linked clones, i.e. RZ390, RG556 and RZ556, detected multiple bands in Southern analysis. In these cases one band was polymorphic for one or more enzymes, allowing confirmation of the chromosome map position of at least one member of each sequence family.

The present study used the combined data for 207 $F_2$ plants from the two segregating populations to determine the location of *xa-5* on chromosome 5 in relation to the new microsatellite and STS markers as well as the RFLP markers (Causse et al. 1994; NIAR Staff 1993). The integrated genetic linkage map for chromosome 5 (Fig. 3) which is based on the JOINMAP analysis of the combined data for the two populations, shows the placement of three microsatellites, RM13, RM122 and RM390, and 2 STS markers, STS556 and STS390, in relation to *xa-5* and to 8 RFLP markers. The *xa-5* resistance gene (scored as a dominant/recessive phenotype in the $F_2$ generation) showed perfect co-segregation with 2 co-dominant RFLP markers, RZ390 and RG556, and their respective STS markers. However, RG556 and STS556 were located 0.4 cM away from RZ390, STS390 and RM390 on the integrated map. Therefore, the most likely location of the *xa-5* gene is the short genetic interval between these two sets of markers.

Although RZ390 and RG556 hybridized to multiple restriction fragments, the PCR-based markers RM390, STS390 and STS556 represented single loci that mapped to the region of interest on chromosome 5. The new microsatellite marker RM390 co-segregated with RZ390, the RFLP clone from which it was derived, in the CBII × 'IR64' population; and as expected the CBII allele of RM390 co-segregated with *xa-5* resistance. Polymorphism was not detected for STS390 in the CBII × 'IR64' population, so this marker was mapped only in the 'IR24' × IRBB5 population, where it co-segregated with *xa-5* and RZ390. The STS556 marker was co-dominant and mapped to the same location in both populations as the RFLP marker RG556.

Other PCR-based markers flanked the interval that contained *xa-5*. The microsatellite marker RM122 was 0.4 cM distal to the interval containing *xa-5*. In previous studies by Yoshimura et al. (1995a) and McCouch et al. (1992), no recombinants were observed between *xa-5* and the markers RG207, RG556 or RZ390, namely the interval shown here to contain RM122, probably because smaller numbers of $F_2$ individuals were used. The third microsatellite identified in the region, RM13, was 14.1 cM proximal to the interval containing *xa-5*.

The positions of 3 JRGP markers, G292, C597 and G396, were integrated with the 7 markers from the Cornell map. Two JRGP markers, G292 and C597, were distal to the interval containing *xa-5*, while G396 was 5.5 cM proximal to this interval. C597 and G396 have been converted into STS markers by our lab (data ot shown) and by Inoue et al. (1994), respectively. RZ556 and RG360 were placed onto the maximum-likelihood map order for the 'IR24' × IRBB5 population with a LOD score below 3.0 in multipoint analysis and were not included in the integrated map. CDO580, a previously untested chromosome 5 marker in the region, was monomorphic with the panel of 9 enzymes.

The end of the chromosome 5 introgression in the IRBB5 isoline was defined by the RFLP marker RG182. The IRBB5 isoline showed the recurrent parent allele for both the microsatellite marker RM164 and the RFLP marker RZ945; therefore both these

markers were outside the introgression. A spurious introgression on chromosome 11, detected with RFLPs by McCouch (1990), could be diagnosed in the present study by the microsatellite marker RM21.

### Germplasm analysis

The diversity of alleles in BLB resistant genotypes was measured for 4 microsatellite markers (Table 1). Microsatellite alleles could easily be differentiated in silver-stained gels (Fig. 4), and automated fluorescent detection was very reliable for obtaining accurate size estimates for those alleles (Table 2). Bulk DNA representing more than 5 plants per accession was used to uncover any seed mixtures. None of the samples showed more than one allele per locus for the four microsatellites tested (Table 1), indicating that the landraces were not heterogeneous for these loci. For the 35 varieties tested, the microsatellite RM21 showed the highest degree of polymorphism (PIC = 0.72) with ten alleles ranging in size from 130 to 164 bp (Table 2). Of the microsatellites on chromosome 5, RM122 was more informative (PIC = 0.64) than RM13 (PIC = 0.56). However, RM13 had 7 alleles ranging in size from 129 to 145 bp, while RM122 had 5 alleles ranging in size from 231 to 250 bp (Table 2). The RM390 locus showed the least polymorphism (PIC = 0.46) of any microsatellite tested, with only 2 alleles detected. For this locus, the two alleles were estimated to be approximately 175 and 181 bp based on the evaluation of silver stained gels (Fig. 4).

An analysis of the frequency of the alleles found in the *xa-5* containing germplasm showed that specific alleles (indicated in parenthesis) predominated for the *xa-5*-linked microsatellites RM13 (f) and RM122 (c and e) on chromosome 5 (Table 2). In contrast, the unlinked microsatellite RM21 on chromosome 11 had no single predominant allele but rather five alleles (d, e, g, h, i), each with a frequency of 8.5% or more, among the *xa-5* source varieties. The distribution of microsatellite alleles was not correlated with the country of origin of the *xa-5*-containing germplasm (Table 1). However, the single southeast Asian variety among the genotypes, 'Tolil 14' from Malaysia, had rare microsatellite alleles for all three loci discussed above. 'Tolil 14' could be a *javanica* variety, while all of the other *xa-5*-containing varieties are believed to be *indica* rices (Sidhu et al. 1978).

The germplasm analysis showed correlation between the subspecific origin of the varieties analyzed and the microsatellite alleles detected for RM13, RM122 and RM21. The eight BLB differentials contributed new alleles to those observed in the 35 *xa-5*- containing genotypes. The two *japonica* BLB differentials, Kogyoku and Chugoku 45, had different alleles than the *indica* BLB differentials, Tetep and Cas209 (Table 1). Meanwhile, the IRRI varieties 'IR8', 'IR20', 'IR24' and 'IR64' had a less predictable set of alleles, undoubtedly due to their varied and complex pedigrees. As expected from their pedigree relationship, IRBB5 had the same allele as the donor parent IR1545-339 and the original source of *xa-5* resistance gene, DZ192, for all of the *xa-5*-linked microsatellites. Except for IRBB2 and the RM21 locus, the NILs for other BLB resistance genes contained the same allele at all four microsatellite loci as the recurrent parent, 'IR24' (Table 1).

## Discussion

Microsatellites were the most practical markers for tagging and for marker-assisted selection of the targeted BLB resistance gene. Microsatellite markers have advantages for applied plant breeding because they are co-dominant, are based on PCR amplification, represent single loci and detect high levels of polymorphism (Morgante and Olivieri 1993). Microsatellite markers provide the high levels of polymorphism needed to follow genomic segments through the narrow crosses and closely related pedigrees of a rice breeding program (Panaud et al. 1996). In our parental and NIL surveys, the microsatellite markers detected a high level of polymorphism, despite the fact that the *xa-5* donor and the recurrent parents were both *indica* rices. Microsatellites were especially effective for whole genome analysis of NILs, because many loci could be screened simultaneously on a single polyacrylamide gel at minimal cost and effort. Multiple loading (multiplexing) of a single polyacrylamide gel at regular intervals with the same microsatellite marker facilitated the evaluation of large populations (Fig. 1). A typical wide sequencing gel, with 64 well combs, loaded five times could regularly analyze the genotypes of 320 individuals. Such high throughput genotyping would be useful for map-based cloning efforts and for QTL or gene-tagging studies with large mapping populations. The mixing of two or three microsatellite markers of different sizes followed by co-electrophoresis also permitted the efficient use of resources (Fig. 2) and enabled the simultaneous positive and negative selection of several regions of the genome.

STS markers are easily developed once sequence data are available for cloned genomic DNA (Inoue et al. 1994); however, they are usually less polymorphic than the RFLPs from which they were derived (Ghareyazie et al. 1995). The use of STS markers for the selection and incorporation of resistance genes for rice blast has been recommended (Hittalmani et al. 1995). In our study, we found that polymorphisms between the parents of the mapping populations for the 2 STS markers used in this study were only detectable after digestion with a panel of multiple restriction enzymes. Therefore mapping of the STS markers required the additional costly and time-consuming step of digesting

**Table 1** Allele variation for four microsatellite (RM) loci in 35 *xa-5*-containing varieties, eight BLB resistance differentials, 11 nearly isogenic lines for bacterial leaf blight resistance genes and their recurrent parent

| Variety | Origin | BLB gene | Microsatellite allele | | | |
|---|---|---|---|---|---|---|
| | | | RM122 | RM390 | RM13 | RM21 |
| Sources of xa-5 | | | | | | |
| Tolil14[a] | Malaysia | *xa-5* | a | a | b | j |
| Aus32[a] | Bangladesh | *xa-5* | c | a | f | h |
| Aus251 | Bangladesh | *xa-5* | e | a | f | i |
| Aus449 | Bangladesh | *xa-5* | e | b | f | i |
| DB3 | Bangladesh | *xa-5* | e | a | e | i |
| DF1 | Bangladesh | *xa-5* | e | a | f | d |
| DL5 | Bangladesh | *xa-5* | e | a | f | i |
| DNJ142 | Bangladesh | *xa-5* | e | b | e | i |
| DV29 | Bangladesh | *xa-5* | e | b | f | d |
| DV32 | Bangladesh | *xa-5* | e | b | e | e |
| DV139 | Bangladesh | *xa-5* | e | b | f | h |
| DZ192 | Bangladesh | *xa-5* | c | a | f | i |
| Kaliboro 600 | Bangladesh | *xa-5* | c | a | f | i |
| Pankiraj[a] | Bangladesh | *xa-5* | c | a | f | h |
| Bageri[a] | Nepal | *xa-5* | e | b | e | h |
| Bangaluwa[a] | Nepal | *xa-5* | c | a | f | e |
| Devarasi | Nepal | *xa-5* | b | a | f | e |
| Dudhi | Nepal | *xa-5* | b | a | f | e |
| Lal Ahu | Nepal | *xa-5* | b | a | f | i |
| Lal Sar | Nepal | *xa-5* | c | b | f | i |
| Lalaka Gadur | Nepal | *xa-5* | c | a | f | e |
| Matury | Nepal | *xa-5* | c | a | f | i |
| Nakhi[a] | Nepal | *xa-5* | c | a | f | e |
| Rerm Bilash | Nepal | *xa-5* | c | a | f | i |
| Sajani | Nepal | *xa-5* | b | a | f | e |
| Sokan Dhum[a] | Nepal | *xa-5* | e | a | c | g |
| Tally | Nepal | *xa-5* | e | b | f | g |
| ARC5756 | India | *xa-5* | b | a | a | d |
| PI180060-1[a] | India | *xa-5* | c | a | f | i |
| RP 291-20 | India | *xa-5* | e | b | f | i |
| DZ78 | Bangladesh | *xa-5*, *xa-7* | e | a | e | i |
| DV85[a] | Bangladesh | *xa-5*, *xa-7* | e | b | f | g |
| DV86 | Bangladesh | *xa-5*, *xa-7* | e | b | f | i |
| BJ1 | India | *xa-5*, *xa-13* | e | b | f | i |
| Chinsurah Boro II[a, b] | India | *xa-5*, *xa-13* | e | b | f | i |
| BLB differentials | | | | | | |
| Kogyoku[a] | Japan | *Xa-1* | d | a | g | f |
| Tetep[a] | Vietnam | *Xa-1*, *Xa-2* | e | b | e | i |
| Chugoku 45[a] | Japan | *Xa-3* | d | a | g | f |
| IR20[a] | IRRI | *Xa-4* | e | a | c | i |
| IR64[a, b] | IRRI | *Xa-4* | d | a | a | a |
| IR1545-339 | IRRI | *xa-5* | c | a | f | i |
| Cas 209[a] | IRRI | *Xa-10* | e | a | d | b |
| IR8[a] | IRRI | *Xa-11* | e | a | b | i |
| Nearly isogenic lines | | | | | | |
| IRBB1 | IRRI | *Xa-1* | e | a | b | c |
| IRBB2 | IRRI | *Xa-2* | e | a | b | i |
| IRBB3 | IRRI | *Xa-3* | e | a | b | c |
| IRBB4 | IRRI | *Xa-4* | e | a | b | c |
| IRBB5[a, b] | IRRI | *xa-5* | c | a | f | i |
| IRBB7 | IRRI | *xa-7* | e | a | b | c |
| IRBB10 | IRRI | *xa-10* | e | a | b | c |
| IRBB11 | IRRI | *xa-11* | e | a | b | c |
| IRBB13 | IRRI | *xa-13* | e | a | b | c |
| IRBB14 | IRRI | *xa-14* | e | a | b | c |
| IRBB21 | IRRI | *xa-21* | e | a | b | c |
| IR24[a, b] | IRRI | None | e | a | b | c |
| Total no. of alleles | | | 5 | 2 | 7 | 10 |

[a] Varieties used to confirm allele sizes through automated fluorescent detection
[b] Varieties that were used as parents in crosses to produce the two $F_2$ populations used in this study

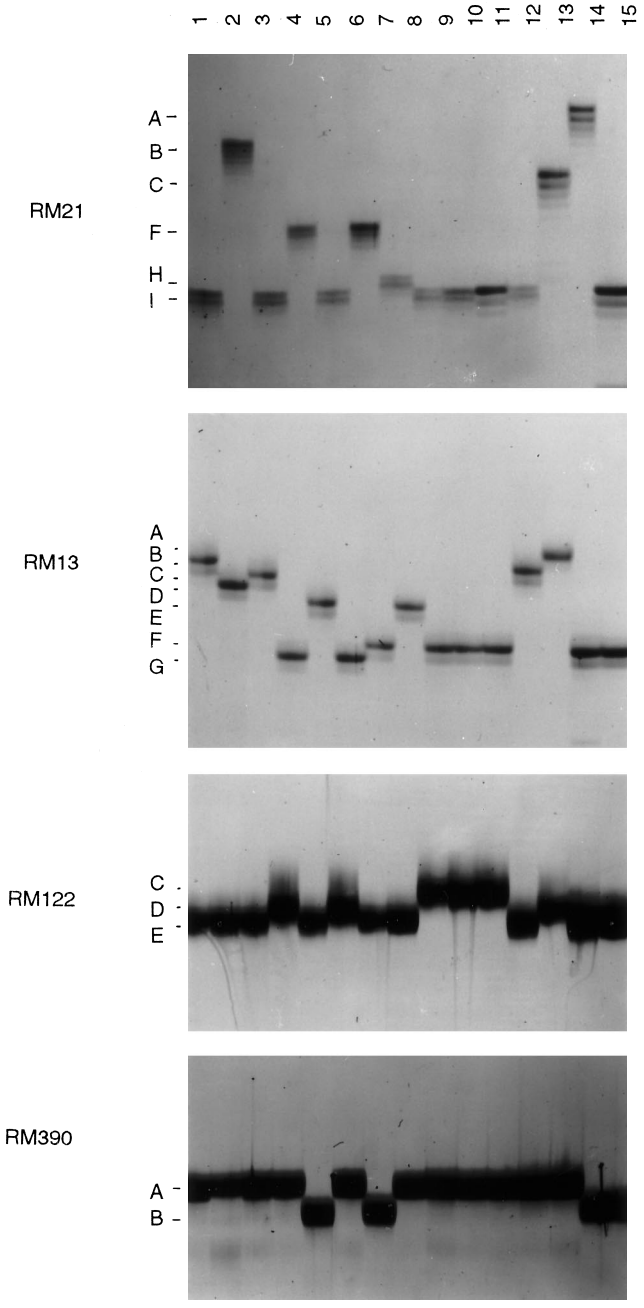


**Fig. 4** Allelic diversity of four microsatellite loci, RM21, RM13, RM122 and RM390, for the bacterial leaf blight resistance differentials or mapping parents, 'IR8' (*1*), Cas 209 (*2*), 'IR20' (*3*), Chugoku 45 (*4*), Tetep (*5*), Kogyoku (*6*), DV85 (*7*), DZ78 (*8*), DZ192 (*9*), IR1545-339 (*10*), IRBB5 (*11*), 'IR24' (*12*), IR64 (*13*), Chinsurah Boro II (*14*) and BJ1 (*15*). Allele designations (*A–I*) correspond to molecular-weight estimates from Table 2

the PCR product. An advantage of the 2 new STS markers was that they were single-copy and represented the specific loci of interest on chromosome 5 even though the RFLP clones RZ390 and RG556, from which they were derived, hybridized to multiple bands in Southern analysis. Homologous RFLP loci for the

**Table 2** Allele designation, allele length in base pairs (bp) with standard deviation (SD) and allele frequency among 35 varieties containing *xa*-5 (see text) for three microsatellite loci, RM13, RM21 and RM122, analyzed by automated fluorescent detection and Genescan software (ABI)

| Allele | length (bp) | SD[a] | Frequency (%) |
|---|---|---|---|
| | RM 13 | | |
| A | 145.5 | 0.06 | 2.9 |
| B | 142.5 | 0.07 | 2.9 |
| C | 140.5 | 0.25 | 2.9 |
| D | 138.5 | n.a. | 0 |
| E | 136.1 | 0.38 | 14.3 |
| F | 130.6 | 0.07 | 77.1 |
| G | 128.7 | 0.00 | 0 |
| | RM 21 | | |
| A | 164.3 | n.a. | 0 |
| B | 158.0 | n.a. | 0 |
| C | 151.3 | n.a. | 0 |
| D | 149.4 | n.a. | 8.5 |
| E | 146.8 | 0.00 | 20.0 |
| F | 142.4 | 0.00 | 0 |
| G | 139.2 | 0.00 | 8.5 |
| H | 133.9 | 0.18 | 11.4 |
| I | 132.2 | 0.80 | 48.6 |
| J | 130.1 | n.a. | 2.9 |
| | RM 122 | | |
| A | 249.8 | n.a. | 2.9 |
| B | 242.7 | n.a. | 14.3 |
| C | 241.0 | 0.37 | 31.4 |
| D | 235.2 | 0.19 | 0 |
| E | 231.0 | 0.55 | 51.4 |

[a] Standard deviation based on determination of allele length in varieties containing identical alleles in silver-stained gels. Unique alleles have no estimate of standard deviation

RZ390 and RG556 clones are known to exist on chromosome 1 (Li et al. 1995) and chromosome 7 (Causse et al. 1994), respectively. The homologue of the cDNA clone, RZ390, that encodes cytochrome b5 but does not contain the same 5′ UTR found in our clone (Smith et al. 1994) could represent the duplicate copy of this gene. To identify the chromosome 5 locus we developed a forward primer from sequence specific to the 5′ untranslated region of RZ390. In practical terms, both new STS markers were co-dominant, single-locus and easier to use than the RFLPs from which they were derived. An advantage of both microsatellites and STSs was that small-scale DNA extractions were sufficient for implementation of these PCR-based assays. In contrast, screening with RFLPs was tedious and costly because of the need for large amounts of DNA, multiple enzyme digestions, duplicate filters and numerous single-probe hybridizations.

The effective use of marker-assisted selection in backcross or pedigree breeding programs requires that the marker and the target gene be very tightly linked (Michelmore 1995). Two of the microsatellite markers described, RM390 and RM122, as well as both of the newly designed STS markers meet this requirement.

The use of 2 tightly linked markers flanking *xa-5* should preclude the misclassification of genotypes (Hittalmani et al. 1995). Marker-assisted selection could simplify the process of combining the recessive *xa*-5 resistance gene with other dominant BLB resistance genes that have overlapping effects or race specificities. Another useful marker for pyramiding disease resistance traits is the microsatellite RM21, which is known to be located in the region of chromosome 11 (Panaud et al. 1996), which contains the cloned *Xa-21* gene (Song et al. 1995) as well as other genes for blast and BLB resistance (Causse et al. 1994). The pyramiding of BLB resistance genes is of interest to study the complementary interactions of these genes (Yoshimura et al. 1995a) and to produce new varieties with a broader spectrum and potentially more durable resistance (Mew et al. 1993).

Differentiation of *japonica* and *indica* subspecies was observed in the germplasm we tested and has been reported in previous assessments of genetic diversity in rice using microsatellites (Xiao et al. 1996; Yang et al. 1994). Our interest in this study, however, was to apply the microsatellite markers to the practical purpose of dissecting finer levels of genetic diversity among BLB-resistant genotypes. The analysis of genetic variation at resistance gene loci using hypervariable PCR-based markers has been recommended for the screening of wild or cultivated germplasm for alternate resistance sources and for the selection of novel resistance haplotypes (Michelmore 1995). We obtained the high resolution needed to identify the hypervariable microsatellite alleles by automated fluorescent detection, which has proven benefits for efficient data acquisition (Kresovich et al. 1995; Reed et al. 1994). Less polymorphic types of markers, such as RFLPs and isozymes, generally do not allow the differentiation of closely-related genotypes (Becker and Huen 1995). In this regard, 3 of the microsatellite markers we used, RM13, RM21 and RM122, were very informative due to their high degree of polymorphism, while the microsatellite locus RM390 was not. The lack of allelic diversity for RM390 may be explained by its location in the transcribed region of the cytochrome b5 gene rather than in non-transcribed DNA. Simple sequence repeats occurring in transcribed DNA may cause disruptions through frameshift mutations and small-scale insertions/deletions that limit the number of alleles possible at these loci (Morgante and Olivieri 1993). The lack of polymorphism in transcribed sequences is not limited to microsatellites. Notably, fewer enzymes produced polymorphisms with the STS marker developed from the cDNA clone RZ390 than with the STS marker developed from the genomic clone RG556. The level of polymorphism for plant microsatellites identified mainly from expressed sequences found in public databases has been shown to be related to the length of the simple sequence repeat they contain (Akkaya et al. 1992; Becker and Huen 1995). Therefore, a microsatellite such as RM390, encompassing fewer than ten repeating units, may tend to be inherently less polymorphic.

None of the *xa-5*-linked microsatellites showed a specific unique allele present in all of the *xa-5* containing varieties that could be used to predict the presence of the gene in untested germplasm without disease screening. The allelic diversity for the linked microsatellites in the *xa-5*-containing varieties may indicate either that several alleles of the *xa-5* resistance gene were generated independently from multiple mutation events or, alternatively, that a single mutation event occurred hundreds or thousands of years ago and the gene has been widely dispersed since that time. The first hypothesis is supported by the fact that the germplasm represented a wide array of landraces from a large geographic area. The *xa-5* gene has been found in a total of 40 genotypes (of which we used 35 in our analysis) by allelism tests performed by Olufowote et al. (1977), Singh et al. (1983) and Sidhu et al. (1978, 1979). These genotypes are not likely to be closely related, except in cases where breeding lines were from the same national agricultural program. In support of the second hypothesis, it is noteworthy that the *xa-5* gene is most prevalent among landraces belonging to isozyme group II (*Aus* and *Boro* ecotypes), which is a distinctive subgroup derived from the *indica* subspecies (Busto et al. 1990). This suggests that the center of origin for the *xa-5* gene is likely to be the Indian subcontinent (Pakistan, Nepal, India or Bangladesh). Trade routes throughout southeast Asia could have long-ago distributed the *xa-5* resistance gene from a single source found in a limited region to multiple gene pools in new environments where it could have been useful against the prevalent, unadapted races of the pathogen. Several donor varieties for the *xa-5* gene have been identified in more distant countries of Southeast Asia (Malaysia and Indonesia) (Sidhu et al. 1978, 1979), and varietal grouping based on the reaction pattern to the Philippine races of the pathogen suggests that *xa-5* may also be found in a few select varieties from Burma, Cambodia, Vietnam, Sri Lanka (Ogawa et al. 1986), China, Laos and Thailand (Busto et al. 1990). Meanwhile, the *xa-5* pattern of race resistance has not been found for any varieties from Korea, Japan, Taiwan or the Philippines (Busto et al. 1990).

If a single *xa-5* mutation is the source of the resistance gene in all the donor sources, the evolution of several alleles at closely linked microsatellite loci may reflect the antiquity of this gene. The Bangladeshi breeding line DZ192 was the original source of *xa-5* used since the early 1970s to construct the genotype IR1545-339 ('IR24' × DZ192) and the isoline IRBB5 ('IR24'/5/IR1545-339) (Ogawa et al. 1991). Since these genotypes share the same allele for the *xa-5*-linked loci, the microsatellites can be assumed to be mutationally stable over at least the past 25 years of the pedigree of the IRBB5 isoline. Similarly, Panaud et al. (1996) reported long periods of stability for microsatellite alleles

as reflected in the pedigree of 'IR8', which encompasses more than 50 years of breeding and production. However, the precise mutation rate for rice microsatellite loci and the exact time-frame necessary for the evolution of new microsatellite alleles would be difficult to ascertain. The predominance of certain alleles among the *xa-5*-containing varieties for the chromosome 5 microsatellites that we found in this study indicates that linkage disequilibrium may exist between these alleles and *xa-5*, which can be taken as molecular evidence of a single, albeit ancestral, source of the resistance gene. In contrast, little indication of linkage disequilibrium was found between *xa-5* and the unlinked microsatellite RM21 on chromosome 11, which had a wider distribution of alleles among the germplasm tested. The evolutionary relationships among possible alleles of the *xa-5* gene and clarification of its probable origin awaits the cloning of the actual gene and in-depth structural and functional analysis.

Linkage analysis for the IRBB5 introgression on chromosome 5 integrated markers from two highly saturated maps of the region, provided an estimate of the size of the DZ192-derived introgression and showed that the *xa-5* resistance gene was located very close to the telomere. The parental origin of a critical section of chromosome 5, from *xa-5* to CDO82, was obscure in previous studies (McCouch 1990; Yoshimura et al. 1995a) due to monomorphism for the majority of the RFLP clones in the region. We were able to confirm that the IRBB5 introgression on this chromosome was continuous down the entire short arm because the alleles for the marker loci, RZ556, G396 and RM13, all proximal to *xa-5*, were inherited from the donor source. This introgression may include the centromere, which is located between RG182 and RZ945 (Singh et al. 1995). The mapping of 2 RFLP markers, G292 and C597, distal to *xa-5* allowed us to estimate the genetic distance from the resistance gene to the telomere of this chromosome, which has been mapped with a telomere-specific repeat clone, TEL1 (Kurata et al. 1994). Chromosomal regions adjacent to telomeres are believed to have higher rates of recombination than the rest of the genome and therefore a low ratio of genetic to physical distance, which makes genes in these regions good candidates for map-based cloning by chromosome landing (Tanksley et al. 1995). The molecular markers which co-segregated or were closely-linked to *xa-5* will be used for screening large-insert DNA libraries with the aim of physically mapping the region and eventually cloning the resistance gene.

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