Theor Appl Genet (1995) 90: 182–188

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Molecular analysis of the inheritance of the *S-5* locus, conferring wide compatibility in Indica/Japonica hybrids of rice (*O. sativa* L.)

Received: 2 May 1994 / Accepted: 17 May 1994

Abstract RFLP analysis was conducted on a population derived from a three-way cross to determine the location of the hybrid sterility locus, *S-5*, in relation to mapped molecular markers and to identify markers that would be useful for selection in breeding. *S-5* is of interest to rice breeders because it is associated with spikelet sterility of F_1 hybrids in Indica/Japonica crosses. Identification of an S-5 allele which confers fertility in Indica/Japonica hybrids when introgressed into either the Indica or the Japonica parent has been reported. Varieties carrying this $S\text{-}5^n$ allele are known as "wide compatibility varieties (WCV)". Our data suggests that RFLP marker RG213 on chromosome 6 is closely linked to the *S-5* locus and can be efficiently used to identify wide compatibility (WC) lines. RG213 is a single-copy genomic clone that detects three bands of different molecular weights in DNA from Japonica ('Akihikari') and Indica ('IR36') varieties and WC line ('Nekken 2'). We demonstrate that the three alleles detected by this marker could be used to trace the inheritance of the "wide compatible" phenotype in breeders' material.

Key words Wide compatibility · *Oryza sativa* · RFLP markers · Hybrid sterility

Communicated by F. Salamini

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Introduction

There are two major sub-species of cultivated rice, Indica and Japonica, and F_1 sterility is common in hybrids resulting from Indica/Japonica crosses (Kato 1930).In 1939, Terao and Mizushima observed that crosses between Indica varieties and certain Japonica varieties known to originate in Indonesia (the Bulu's or varieties named 'Javanica' by Morinaga 1954) produced a higher frequency of fertile offspring than was typically observed in inter-subspecific crosses. They described the Indonesian 'Javanica' varieties as intermediate between Indica and Japonica because of their reproductive compatibility with both Indica and Japonica varieties. Several genetic mechanisms have been proposed to explain Indica/Japonica hybrid sterility, such as duplicate gametophytic lethal genes, a single hybrid sterility locus, and genes that retard gametic development (Oka 1953, 1974; Kitamura 1962). In the study described here, we focus on the inheritance of sterility due to female gamete abortion.In 1962, Kitamura proposed a single-locus model of inheritance of embryo sac sterility based on his observations of fertility in sets of near-isogenic lines constructed from Indica/Japonica crosses (Kitamura 1962). The chromosomal location of a hybrid sterility locus was reported in 1986, and the locus was named *S-5* (Ikehashi and Araki 1986). Ikehashi and Araki (1986) further suggested that a three-allele system at the *S-5* locus was capable of explaining both the partial hybrid sterility associated with Indica/Japonica hybrids and a phenomenon that they termed "wide compatibility (WC)", in which fertile hybrids resulted from crosses between the intermediate Javanica varieties and either Indica or Japonica varieties. Ikehashi and Araki 's work was based on populations derived from three-way crosses (Indica/Javanica//Japonica and Japonica/Javanica//Indica), and individuals were evaluated for fertility and for morphological markers. Among the several morphological loci showing clear differences between the Indica and Japonica subspecies, linkage was observed between percent spikelet fertility and three markers known to reside on chromosome 6 [Chro-

mogen (*C*), waxy endosperm (*wx*), and alkaline degeneration (*alk*)] (for a summary of the classical linkage map of rice, see Kinoshita 1993).

In the test of segregation distortion at *S-5* in a backcross population of Indica/Japonica//Indica hybrids, female gametes carrying the Japonica allele at *S-5* (referred to hereafter as $S\text{-}5^j$) were observed at a significantly lower frequency in $S\text{-}5^i/S\text{-}5^j$ heterozygotes than female gametes carrying the Indica allele at this locus (referred to as $S\text{-}5^i$) (Ikehashi and Araki 1986). This suggested that a larger proportion of $S\text{-}5^j$ gametes were aborted. When the segment of chromosome containing the *S-5* locus was derived from a Javanica cultivar, the allele was referred to as $S\text{-}5^n$, and no gamete abortion was observed in $S\text{-}5^i/S\text{-}5^n$ or $S\text{-}5^j/S\text{-}5^n$ heterozygotes (Ikehashi and Araki 1986). The Javanica donor was termed a "wide-compatibility variety (WCV)". In 1992, Yanagihara et al. (1992b) presented evidence for a second, independent hybrid sterility locus on chromosome 7 that confers semisterility and wide compatibility in different inter-subspecific cross combinations, suggesting that there are likely to be several similar loci in the rice genome. Evidence for the validity of the hypothesis has accumulated as several WC lines have been successfully utilized in hybrid rice breeding to produce fertile Indica/Japonica offspring (Ikehashi 1991).

Since Ikehashi and Araki's publication in 1986, several studies have been undertaken to identify isozyme and restriction fragment length polymorphism (RFLP) markers linked to the *S-5* locus (Ikehashi and Araki 1988; Yanagihara et al. 1992a; Zheng et al. 1992; Liu et al. 1992), but different markers are reported by different researchers. In our study, we used RFLP, isozyme, and morphological markers to map the chromosomal region containing the *S-5* locus, thereby providing a unified version of markers in this area. We identify a molecular marker that can be used for selection of WCVs in rice improvement, describe a three-allele system observable at the RFLP level that coincides with the predicted three-allele system hypothesized by Ikehashi and Araki, and present evidence of the reliability of our markers for predicting the "wide-compatible" phenotype in breeding material.

Materials and methods

Population structure

A three-way cross, Indica/WCV//Japonica, was used to identify fertile and semi-sterile individuals, where the two types of heterozygotes, $S\text{-}5^i/S\text{-}5^j$ and $S\text{-}5^n/S\text{-}5^j$, were produced. The $S\text{-}5^i/S\text{-}5^j$ heterozygote is semisterile, caused by female gamete abortion as described above, while the $S\text{-}5^n/S\text{-}5^j$ counterpart is fertile.

Mapping population

A population of 254 plants from the three way cross, 'IR36'/'Nekken 2'//'Akihikari', were planted in an experimental field of the National Agricultural Research Center, Japan, in 1990. The fertility level of each plant was determined by counting fertile and sterile spikelets on the upper half of the panicles, as described in Yanagihara et al. (1992b). One hundred and sixty-one plants were randomly selected for RFLP analysis of markers known to reside in the *S-5* region of chromosome 6. Of those used in mapping, 39 plants with a fertility of more than 85% and 16 plants with a fertility of less than 50% were selected for linkage analysis of the *S-5* locus. The isozyme markers, *Est-2* and *Pgi-2*, were evaluated on the same plants following the procedure of Ishikawa et al. (1989).

Wide-compatibility-bred lines and their pedigrees

The analysis of *S-5* and RFLP markers in breeding material included the following WCVs (or WC lines) and their pedigree parents: WC lines in a Japonica background, 'Nekken 1', 'H90-123', 'Nekken 2', 'H90-125', and 'H90-126'; WC line in an Indica background, 'Shinkei 8953'; pedigree parents, 'Akihikari' (Japonica), 'Nihonmasari' (Japonica), 'GA3' (Japonica), 'Ketan Nangka' (WCV), 'Milyang 23' (Indica), and 'IR36' (Indica).

Clones

RFLP markers used in this study were a subset of those previously mapped to the *S-5* region of chromosome 6 (Tanksley et al. 1992). The clones in this region are designated with either a two- or three-letter suffix to denote the library from which they were derived and include rice genomic clones (RG), cDNA clones from rice (RZ), cDNA clones from oat (CDO), and a *waxy* clone from maize (kindly provided by Susan Wessler, University of Georgia).

DNA extraction, digestion, and blotting

DNA was extracted from mature rice leaves that were harvested from F_1 plants (derived from a three way cross) and air-dried overnight at 55°C in a wind-oven (Constant Temperature Oven, DN63, Yamato) as described in Tai and Tanksley (1991). Where F_1 plants provided insufficient amounts or poor quality DNA for RFLP analysis, bulked leaves from 3 to 5 plants of the F_2 generation were used to reconstruct the original genotype. In a two-allele marker system (two alleles were observed for most of the RFLP markers evaluated in this study), when 5 F_2 plants were used to reconstruct the F_1 backcross genotype, the probability that an F_1 heterozygote would be misconstrued as a japonica homozygote was $P< 0.016$. (This can be calculated by the equation, $P=(1/4)^n$, where n=number of plants in an F_2 line.) Dried leaves were ground with a coffee grinder or with a mortar and pestle in liquid nitrogen. DNA was extracted using sodium dodecyl sulfate-potassium acetate, essentially as described in Dellaporta et al. (1983). The DNA was digested with the restriction enzymes *BamH*I, *Dra*I, *EcoR*I, *EcoR*V, *Hin*dIII, and *Xba*I and separated on 0.9% agarose gels by electrophoresis using a 1 × NEB buffer (0.1 *M* Tris-aceticacid, 1 m*M* EDTA, 12.5 m*M* sodium acetate, pH 8.1). The size-separated genomic DNA was transferred to either Hybond N or Hybond N^+ nylon membranes (Amersham) using the transfer methods described in the manufacturer's instructions.

Nonradioactive labelling

Clones were labelled using the Dig DNA labelling kit (Boehringer Mannheim) according to the manufacturer's instructions, or by polymerase chain reaction (PCR) labelling according to Panaud et al. (1993), but with a Dig-dUTP: dATP ratio of 8:2. Prehybridization and Southern hybridization were carried out essentially according to Panaud et al. (1993) with a clone concentration of 100–150 ng/50 ml.

Washing and band detection

After a 10-h incubation, filter washing and band detection for Hybond N was conducted according to the instructions accompanying

the Dig chemiluminescent detection kit (Boehringer Mannheim). Band detection for Hybond N^+ was conducted using a blocking buffer containing 5% nonfat dry milk and 0.5–1% polyvinyl pyrrolidone to reduce background and applying the instructions accompanying the AMPPD detection reagent, Western Light (Tropix). Luminograms were developed after exposing to filters for 15 min to 12 h.

Map construction

Linkage analysis of the morphological, isozyme, and molecular markers on rice chromosome 6 was performed by Map Manager (Manly 1993) and by Mapmaker version 2.0 (Lander et al. 1987). Quantitative trait locus (QTL) mapping was performed by Mapmaker/QTL (Lincoln et al. 1992) to evaluate the proportion of phenotypic variance explained by markers associated with the *S-5* locus.

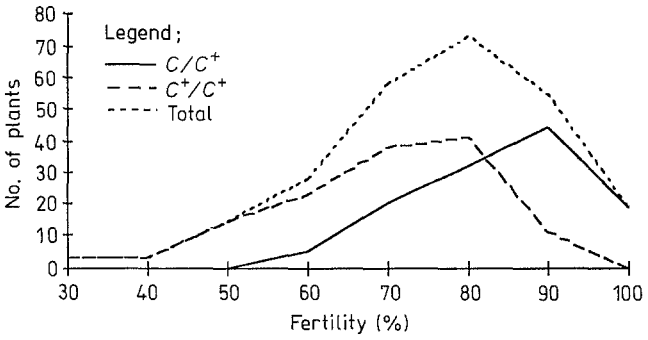

Fig. 1 Frequency distribution of percentage fertility in 254 plants derived from 'IR36'/'Nekken 2'//'Akihikari'

Results and discussion

Distribution of fertile and semisterile plants

An almost continuous frequency distribution was observed when fertility and semisterility were evaluated in the population of 254 plants derived from the three-way cross 'IR36'/'Nekken 2'//'Akihikari' (Yanagihara et al. 1992a) (Fig. 1). If the wide-compatibility character were highly heritable and controlled by a single gene, a bi-modal distribution with a 1:1 allele frequency would be expected. On the basis of Ikehashi and Araki 's model (1986), this would result from the two possible allele combinations, $S\text{-}5^i/S\text{-}5^j$ (semisterile) and $S\text{-}5^n/S\text{-}5^j$ (fertile). The fact that a continuous distribution was observed suggests that environmental and/or genetic factors affected our ability to accurately evaluate the phenotype. The distribution of fertility has been investigated in a large number of similar three-way crosses (Ikehashi and Araki 1986, 1988; Ikehashi et. al. 1991). In some cases, fertile versus semisterile classes were clearly separable, while in other cases continuous variation was observed.

Separation of fertile and semisterile groups

Fertility can be understood as the successful completion of the reproductive process in the presence of delicately balanced genetic and environmental conditions. Highly fertile plants, therefore, represent the expression of their genetic potential in terms of reproduction. Sterility, on the other hand, results when genetic, physiological, or environmental factors disrupt the normal development of the male and/or the female gametes, or the zygote (Liedl et al. 1993), or when fertilization itself is inhibited. In some cases, the genetic and environmental causes of sterility can be distinguished if enough is known about the mechanism to target evaluation at a specific stage in plant development, a specific organ, or tissue, or if the appearance of the symptoms can be correlated with a known environmental stress. However, both genetic and environmental sterility mechanisms generally produce similar effects when sterility is evaluated at the end of the reproductive process, i.e., as percent spikelet sterility in mature plants. Therefore, semisterile plants, as evaluated in this study, are less reliable as a reflection of genotype than are fertile plants.

The average spikelet fertility in Indica/Japonica hybrids grown under conditions similar to those in this study is 64–67% (Ikehashi and Araki 1984; Ikehashi 1991). We would expect to observe similar levels of fertility for $S\text{-}5^i/S\text{-}5^j$ genotypes in this study and considerably higher levels of fertility in $S\text{-}5^n/S\text{-}5^j$ genotypes if the basic model proposed by Ikehashi and Araki (1986) is applicable.

In an attempt to test the usefulness of the single-locus hypothesis to explain our results, we used multipoint analysis to determine the map location of *S-5*. To do this, we defined our groups of fertile and semisterile plants using very conservative cut-off points. We defined the high fertility group as plants with 85% or more fertile spikelets (Table 1). This group consisted of 39 plants, and the phenotype could be estimated with a high degree of certainty. The opposite tail of the distribution contained plants with less than 49.9% fertility. This represented a more stringent cut-off point to minimize the environmental variance associated with the low-fertility group. There were 16 plants in this group. The plants with a spikelet fertility between 85% and 50% were not used in estimating the map location of *S-5* because genetic and environmental factors were most likely to be confounded in the intermediate fertility range.

Map location of the *S-5* locus

A map of the region of chromosome 6 containing the *S-5* locus is illustrated in Fig. 2. It includes 7 RFLP, 2 isozyme, and 2 morphological markers, and was constructed on the basis of 161 plants from the population of 'IR36'/'Nekken 2'//'Akihikari'. When linkage relationships were estimated by multipoint analysis using Mapmaker, Version 2.0, the *S-5* locus mapped 1.6 cM away from *Est-2* and was flanked on the other side by DNA marker, RG213 (4.4 cM away) (Fig. 2).

Because of the continuous distribution for percent spikelet fertility observed in the population analyzed in this study, we also used a quantitative model to analyze the lo-

Table 1 Recombination values of markers in various fertility level

Fertility level (%)		*Wx*	RZ516	CDO475	*C*	*Est-2*	RG213	*Alk*	*Pgi-2*	CDO96	RG424
	In fertile group										
Over 90% 1)	Normal	10	11	11	17	17	17	14	15	14	14
	Recombinant	7	6	4	0	0	0	1	1	2	3
	RCV. (%)	41.2%	35.3%	26.7%	0.0%	0.0%	0.0%	6.7%	6.3%	12.5%	17.6%
85.0%–89.9% 2)	Normal	16	18	16	20	21	21	16	14	17	16
	Recombinant	6	4	3	1	1	1	2	3	3	6
	RCV. (%)	27.3%	18.2%	15.8%	4.8%	4.5%	4.5%	11.1%	17.6%	15.0%	27.3%
80.0%–84.9% 3)	Normal	8	12	10	13	13	10	9	11	10	7
	Recombinant	10	9	7	7	7	8	10	7	11	13
	RCV. (%)	55.6%	42.9%	41.2%	35.0%	35.0%	44.4%	52.6%	38.9%	52.4%	65.0%
1) + 2)	RCV. (%)	34.2%	26.7%	21.2%	2.4%	2.3%	2.3%	8.9%	11.9%	13.8%	22.5%
1) + 2) + 3)	RCV. (%)	41.3%	32.1%	27.9%	13.3%	13.2%	16.3%	23.5%	20.9%	26.6%	36.6%
	In sterile group										
60.0%–64.9%	Normal	21	21	19	19	19	15	16	16	16	14
	Recombinant	2	3	3	4	5	5	7	5	7	7
	RCV. (%)	8.7%	12.5%	13.6%	17.4%	20.8%	25.0%	30.4%	23.8%	30.4%	33.3%
50.0–59.9%	Normal	21	22	20	23	19	19	21	19	21	18
	Recombinant	2	3	5	3	5	5	5	5	4	7
	RCV. (%)	8.7%	12.0%	20.0%	11.5%	20.8%	20.8%	19.2%	20.8%	16.0%	28.0%
49.9% and less	Normal	11	13	11	15	16	12	15	12	12	10
	Recombinant	5	3	4	1	0	2	0	1	4	6
	RCV. (%)	31.3%	18.8%	26.7%	6.3%	0.0%	14.3%	0.0%	7.7%	25.0%	37.5%
Total	Normal	87	97	87	107	105	94	91	87	90	79
	Recombinant	32	28	26	16	18	21	25	22	31	42
	RCV. (%)	26.9%	22.4%	23.0%	13.0%	14.6%	18.3%	21.6%	20.2%	25.6%	34.7%

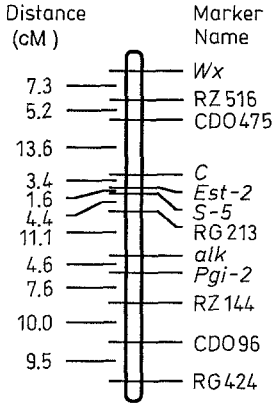

Fig. 2 Linkage map of RFLP, isozyme and morphological markers on chromosome 6, including the estimated position of *S-5*

cation of *S-5*. On the basis of interval analysis with Mapmaker/QTL, 44.8% of the phenotypic variance associated with spikelet fertility was explained by the *S-5* locus (LOD 19.9). This analysis suggested that *S-5* lies approximately 1.4 cM from *Est-2* and 2.0 cM from *C*, a location precisely on the opposite side of *Est-2* as when estimated by multipoint analysis using MapMaker, Version 2. Both approaches suggested that *S-5* is located less than 1.5 cM from the isozyme marker *Est-2* and that flanking markers *C* and RG213 are linked at distances of less than 5 cM.

Since the identification of *S-5*, five independent loci for hybrid sterility have been identified (including unpublished data, Wan and Ikehashi). 'Ketan Nangka', the donor of the $S\text{-}5^n$ allele in the improved variety, 'Nekken 2', showed hybrid sterility with some varieties at *S-7*, *S-8*, and *S-9* (Yanagihara et al. 1992b; Wan et al. 1993a, b). It is possible that genetic factors other than *S-5* contribute to the semisterility of hybrids in this population. Analysis of the population with markers covering the entire rice genome would allow us to investigate this possibility. On the other hand, a refinement of the phenotypic assay would allow us to distinguish among the factors causing panicle sterility, female gamete abortion (the mechanism associated with *S-5*), non-viability of pollen, zygote abortion, or environmental stress, and would contribute directly to our ability to map this locus with precision.

Three-allele marker system

While most molecular markers included in this study showed two alleles, corresponding to the Japonica and Indica genomes, a third allele was detected for *Est-2* (as previously reported by Nakagahra 1977) and RG213 (Fig. 3). In both cases, the third allele was detected in 'Nekken 2'. The allele of RG213 was uncommon in Indica and Japonica rice germ plasm, and its origin could be traced to the original Javanica donor of the WC character, 'Ketan Nangka'. The two markers detecting this Javanica-specific allele were both closely linked to the *S-5* locus, and pro-

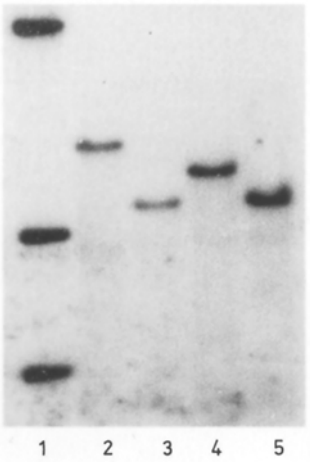

Fig. 3 Autoradiogram illustrating three alleles at RG213. *Lane 1* Lambda *Hin*dIII digest with bands of 23 kb, 9.4 kb and 6.6 kb, *lane 2* 'Akihikari' (Japonica), *lane 3* 'Nekken 2' (WC line), *lane 4* 'IR36' (Indica), *lane 5* 'Ketan Nangka' (WCV)

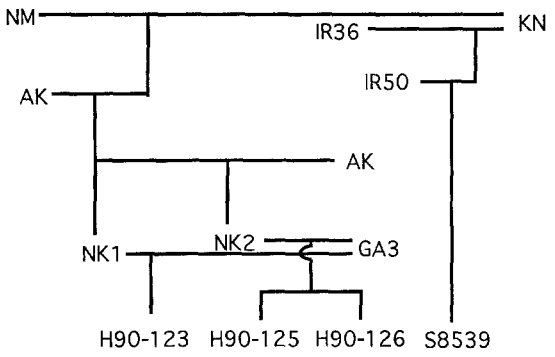

Fig. 4 Illustration of WC lines and their pedigrees(♀ on *left*, ♂ on *right*). Note; H90-125 and H90-126 are sib lines selected for short stature and apiculous color. (*NM* 'Nihonmasari', *NK1* 'Nekken 1', *AK* 'Akihikari', *NK2* 'Nekken 2')

vide molecular evidence of genetic divergence between the Indica, Javanica, and Japonica genomes in this region of chromosome 6. The fact that we observed a three-allele system at both a DNA and an isozyme marker closely linked to the *S-5* locus is of interest in terms of the hypothesis on allelic interaction proposed by Ikehashi and Araki in 1986.

We suggest that the molecular markers identified here will be useful when Indica/Javanica or Japonica/Javanica crosses are used in a breeding program, especially when the parents do not segregate for the visually scorable marker, *C*. The possibility of selecting for wide compatibility early in the life of a plant and without the laborious process of a second step of crossing to determine the allele at *S-5* is of great interest in hybrid rice breeding. Selection for wide compatibility based on *C* has been both efficient and effective, but the identification of a cloned DNA marker near *S-5* makes it possible to move toward a more in-depth characterization of this very interesting region of the rice genome.

It is noteworthy that three alleles have been detected by markers other than *Est-2* and RG213in this region of chromosome 6. RG64, which is not consistently present in WCV materials, has three alleles (Second and Maheswaran, IRRI, The Philippines, personal communication), one of which is a Javanica-specific allele. RG64 has previously been reported to be linked to the *S-5* locus (Zheng et al. 1992), as well as to the blast resistance gene, *Pi-2* (Yu et al. 1991), and to the photoperiod sensitivity gene, *Se-1* (Mackill et al. 1993). Further molecular characterization of this region will undoubtedly yield interesting insights into some of our agronomically important genes in rice.

Use of markers in selection

To investigate the reliability of using marker-assisted selection to identify material with wide compatibility, lines of known performance and pedigree were analyzed for their marker profiles (Fig. 2).The pedigrees of the lines analyzed in this study are summarized in Fig. 4. As can be seen in Fig. 5 (map and graphical genotype), the advanced wide-combatibility sib-lines, 'Nekken 2', 'H90-125', and 'H90-126', show identical molecular profiles in the *S-5* region of the genome. They have a small segment of Javanica-derived DNA in the region bordered by *C* and RG213 and that segment is surrounded by DNA from the 'Akihikari' (Japonica) parent. The two earlier WCVs, 'Nekken 1' and 'H90-123', share the recombination event between RZ450 and the morphological marker, *C*, but contain a larger segment of Javanica-derived DNA that extends down beyond RFLP marker RG264. In selecting for a return to the Japonica plant type in subsequent generations, breeders were able to narrow down the segment of Javanica-derived DNA.

Line S8539 was derived from an 'IR50'//'IR36'/'Ketan Nangka' cross with the objective of introgressing the wide compatibility character into an Indica background. This line contains a segment of Javanica-derived DNA similar to that found in 'Nekken 1' and 'H90-123', but it is heterozygous at RZ450. In the breeding program in which these lines were developed, 'Nekken 1' and '2' were being developed simultaneously, and selection for *C* was imposed in an effort to obtain wide-compatible lines, based on Ikehashi and Araki's results (1986). The outcome of this selection can be clearly seen in Fig. 5. This study indicates that the chromosomal segment containing the putative $S\text{-}5^n$ allele spans a region marked by *C*, *Est-2*, and RG213. It also suggests that combinations of these markers should provide a reliable way for breeders to select for wide-compatible lines containing *S-5*.

The fact that all of the advanced material ('Nekken 2', 'H90-125', and 'H90-126') carries *C* is not surprising, given the selection criteria that was imposed, but it is interesting that all of this material carries *Est-2* and RG213 as well. In marker-assisted backcross breeding experiments, we aim to break the linkage between *Est-2* and RG213, and that between *C* and *Est-2*, in order to determine more precisely where the locus or loci controlling the wide-compatibility character is located within this region of DNA.

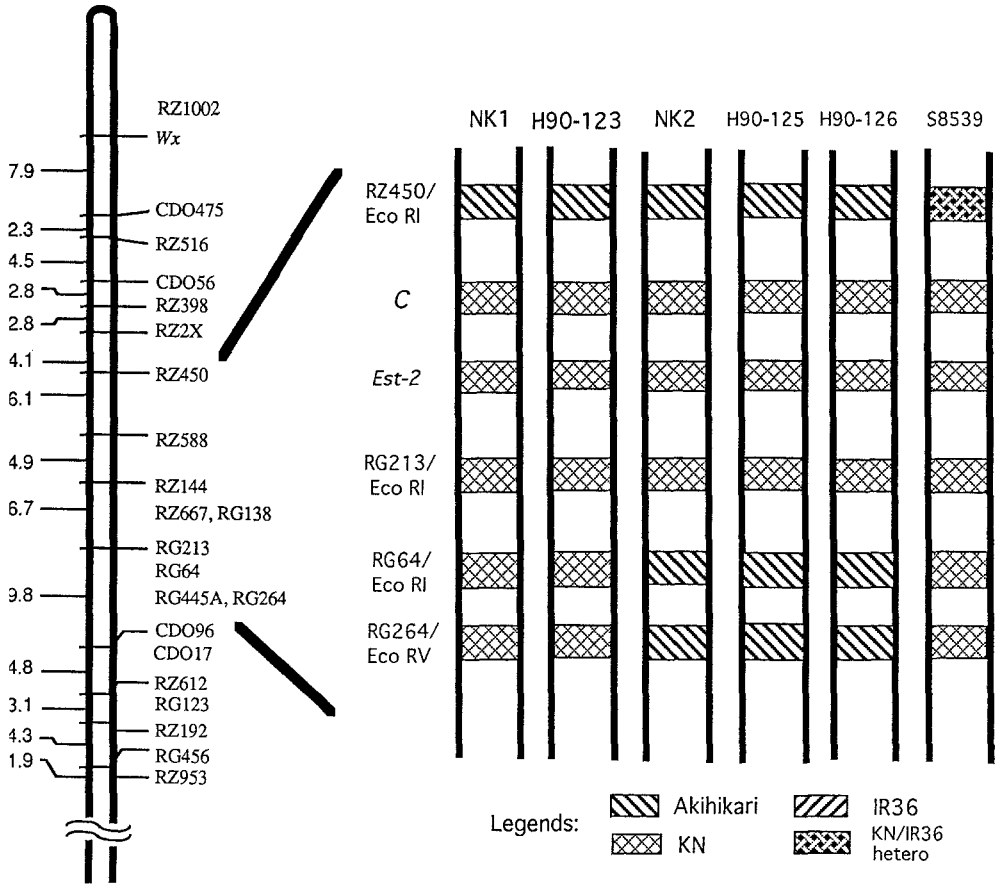

Fig. 5 Graphical genotype around *S-5* locus of WC lines

In addition to *S-5*, *S-7* and *S-8* have been reported to be hybrid sterility genes causing female gamete abortion and carrying wide compatibility alleles (Yanagihara et al. 1992; Wan et al. 1993). Accumulating such wide-compatibility genes in useful genetic backgrounds should give us valuable lines for use in hybrid rice breeding.

Acknowledgements We gratefully acknowledge the field assistance and helpful discussions provided by Drs. H. Kato and H. Araki, the technical assistance of Drs. T. Ishii and O. Panaud, and help in formatting by Ms C. Morehouse.

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