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CONTENTS

Proceedings of oral presentations
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PROCEEDINGS OF INTERNATIONAL GENETIC RESOURCES WORKSHOP ON THE GENUS *ORYZA*

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Distribution and diversification of *Oryza* species in Latin America

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Keywords *O. glumaepatula*, *O. alta*, *O. grandiglumis*, *O. latifolia*, Latin America

Four wild rice species, *Oryza glumaepatula*, *O. alta*, *O. grandiglumis* and *O. latifolia*, are distributed in the marshy area of tropical and sub-tropical Latin America (Fig. 1) *O. glumaepatula* is diploid having the same AA genome with cultivated rice, while, the rest three species are tetraploid with CCDD genome. They are considered as important genetic resources, however, their life-history traits and systematic relationships have not been well understood since the number of accessions so far examined was quite limited and seemed to be insufficient for elucidating entire variation of respective species In this study, we dealt with genetic diversity for wild rice species in Latin America using accessions collected from wide range of distribution areas of respective species Special reference was paid for whether *O. glumaepatula* is differentiated within species or not and how it is genetically related to

other AA genome wild species in the other continents.

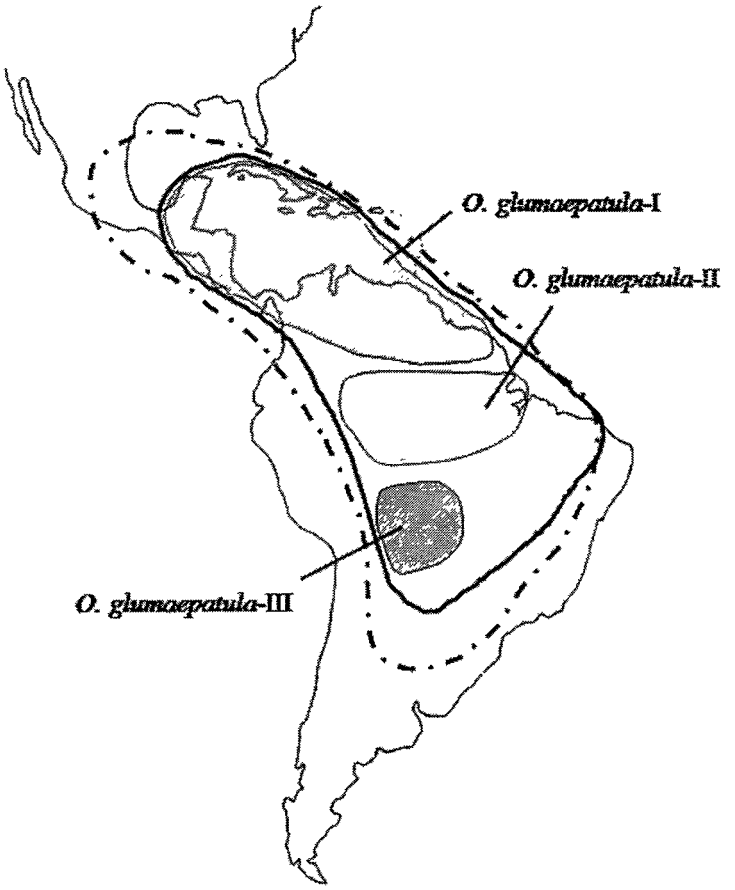

Fig. 1 Distribution of wild rice in Latin America. Solid line indicates distribution area of *O. glumaepatula*. Dotted line indicates distribution area of CCDD genome species, but *O. grandiglumis* has not been identified in Central America.

Diversification and bio-systematics of *O. glumaepatula*

We grew 57 accessions of *O. glumaepatula* together with related AA genome wild species, *O. rufipogon* from Asia and Oceania, *O. meridionalis* from Oceania, and *O. longistaminata* and *O. barthii* from Africa to examine the variability of 22 quantitatively measurable traits, 48 nuclear DNA markers, and 10 chloroplast DNA markers

Polymorphisms at quantitative traits, nuclear DNA markers, and chloroplast DNA markers were examined respectively by principal component analysis (PCA) Subsequently,

Euclidean distance for each pair of accessions was estimated to construct proximity matrices by using the principal component scores of the accessions obtained in respective PCA analyses A dendrogram using the UPGMA method was generated by each proximity matrix.

The dendrogram for quantitative traits analysis showed that *O. glumaepatula* accessions were divided into three groups (*O. glumaepatula*-I to III, respectively) *O. glumaepatula*-I was clustered with perennial accessions of *O. rufipogon* and can be regarded as perennial rice. *O. glumaepatula*-II and III were placed into independent clusters, respectively, and differed from any other taxa. Subdivision of *O. glumaepatula* accessions can be well explained by their geographical distributions Distribution area of three groups did not overlap with each other *O. glumaepatula*-I consisted of the accessions collected from Central America and north region of South America, and *O. glumaepatula*-II accessions were collected from the Amazon basin, and *O. glumaepatula*-III from the Pantanal swamp in south west Brazil (Fig. 1)

In the dendrogram constructed on the polymorphisms at nuclear DNA, all species tended to be separated from each other forming respective clusters, indicating that each species has its unique nuclear DNA type. Within the cluster of *O. glumaepatula*, arrangement of the accessions seemed to represent their geographical distributions

According to the dendrogram for chloroplast DNA analysis, accessions of *O. glumaepatula* were divided into two groups (*O. glumaepatula*-C1 and C2) C1 were closely

related to *O. barthii* C2 formed an independent cluster, though it contained mutations which were not found among the accessions of C1 but the accessions of *O. longistaminata*. Two chloroplast types of *O. glumaepatula* seem to have been derived from different lineages associated with two African species. Accessions belonging to *O. glumaepatula*-I and II in quantitative traits analysis exclusively carried C1 and C2 type chloroplast DNA, respectively. While, both C1 and C2 were found among the accessions of *O. glumaepatula*-III

O. glumaepatula proved to be a variable species and intra-specific differentiation was well recognized. The heterogeneity in chloroplast DNA found in this species inferred that the origin of *O. glumaepatula* is not monopyletic and there may have been several migrations of wild rice between the New world and other continents Nuclear DNA of *O. glumaepatula* was characterized by its own marker genotype and differentiated from that of other AA genome species, indicating that plants with different chloroplast DNA might introgress with each other in the process of their geographic expansions in Latin America. It is certain that *O. glumaepatula*-I and perennial accessions of *O. rufipogon* showed a similarity in quantitative traits However, no molecular evidence was obtained in the present study to support this view and most probably, such similarity is the result of convergence but not of common ancestry.

Diversification and bio-systematics of CCDD genome species

O. grandiglumis has well developed sterile lemma, which is the same length as lemma and

palea or sometimes longer than them, and by this character we can easily distinguish this species from two others Key characters dividing *O. latifolia* from *O. alta* are leaf width and spikelet length; leaves are narrower and spikelets are longer in *O. latifolia* than *O. alta*. These characters show an amount of variation within each species and sometimes species distinction is obscure.

Three CCDD genome species are considered to be genetically close with each other (Kiefer-Meyer *et al.* 1995) We examined variability at four regions of chloroplast DNA by SSCP method using accessions of three species Polymorphisms seemed to represent their geographical distributions rather than their species differentiation. It remains vagueness on taxonomy and systematic relationships among CCDD genome species

In Latin America, the destruction of the habitats of wild rice is not so serious as in Asia. Introgression of cultivars into wild rice, which is frequently occurring in Asia (Akimoto *et al* 1999) is also rare, since cultivation of rice is limited in this area. It is expected that wild rice in Latin America growing under diverse natural condition conserves primitive feature of wild rice, that are vanishing in Asian wild rice (Morishima 1994). In this sense, wild rice in Latin America is one of the valuable materials in studying ecology, population genetics and phylogenetics of wild rice.

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QTL analysis for several agronomic characters using four BC_2 populations between wild and cultivated rice species.

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Keywords: QTL analysis, the genus *Oryza*, wild species, backcross population, microsatellite markers

The genus *Oryza* consists of two cultivated species and about 20 wild species (Tateoka 1963) Genome analysis reveals that two cultivated species, *O. sativa* and *O. glaberrima*, share the same genome (AA) with five wild species, *O. rufipogon*, *O. glumaepatula*, *O. longistaminata*, *O. meridionalis*, and *O. barthii* Of these, *O. rufipogon* and *O. barthii* were thought to be ancestral species of *O. sativa* and *O. glaberrima*, respectively (Morishima *et al.* 1963). This was also confirmed by the phylogenetic studies using molecular markers, such as nuclear and chloroplast RFLP (Ishii *et al* 1988; Wang *et al* 1992) and microsatellite markers (Ishii *et al* 2001) In addition, Bautista *et al* (2001) analyzed phylogenetic relationships among A-genome species using four kinds of molecular markers. Their results indicate that *O. glumaepatula* was relatively close to the groups of *O. sativa* (including *O. rufipogon*) and *O. glaberrima* (including *O. barthii*) whereas *O. longistaminata* and *O. meridionalis* were highly differentiated from other A-genome species.

Wild species have wider genetic variation than cultivated species. Using wild rice species, many useful traits have been identified and transferred to elite breeding lines. They

are usually controlled by single genes and can be easily identified by phenotypic screening. On the other hand, quantitative traits, such as plant size and yield, are associated with many genes, and the manner of inheritance to succeeding generations appears to be very complicated. Therefore, limited studies on quantitative trait loci (QTLs) using wild rice species has so far been conducted. However, recently several kinds of molecular markers have been developed for various crop species, and their molecular linkage maps which covered almost the whole genome have been constructed. These molecular markers have made it possible to analyze QTL effect and identify the chromosomal locations of QTLs (Tanksley and McCouch 1997). In rice, Xiao *et al.* (1996) gave the first report on wild QTL analysis. They found that the low-yielding wild rice ancestor, *O. rufipogon*, had alleles that can enhance yield of highly productive breeding variety. The similar results were also reported using different populations, i.e., *O. sativa* (upland rice) X *O. rufipogon* (Moncada *et al.* 2001) and *O. sativa* X *O. glumaepatula* (Brondani *et al.* 2002). These all indicate the usefulness of wild germplasms improving rice varieties.

In this study, a single accession of closely-related wild species, *O. rufipogon* from Myanmar (NIG acc. no. W630) and a single accession of distantly-related, *O. meridionalis* from Australia (NIG acc. no. W1627) were respectively backcrossed with two typical rice cultivars, *O. sativa* Japonica Nipponbare and Indica IR36, and four BC_2 populations consisting of approximately 200 plants each were produced. In order to identify the useful QTL alleles from closely-related and distantly-related wild species, QTL analysis was carried out in the BC_2 generation. A total of 11 morphological characters (days to heading, photosynthesis activity, culm length, panicle length, number of tillers, yield, 100-seed weight, seed length, seed width, grain length, grain width) were evaluated for four BC_2 populations. As for yield, a small scale of yield testing was also performed in the next generation of BC_2F_2 at two locations. Besides the morphological evaluation, DNA was extracted from BC_2

plants and their 12 chromosomal regions were surveyed with about 80 microsatellite markers Single-point QTL analysis was carried out using qGene software (Nelson 1997) in order to identify the useful QTL alleles from wild species

Wild QTL analysis using *O. rufipogon*

A total of 55 and 51 QTLs were identified at a significance of $P<0.01$ with the backcrossed populations of Nipponbare and IR36, respectively Of these, 29 (52.7 %) and 28 (54.9 %) had wild alleles increasing trait values. Since a single wild plant was used as a donor parent for two backcross populations, effect of the wild QTL alleles in the background of two typical rice cultivars was examined, and six QTL locations were found to be common between two populations. Regarding the wild QTL alleles increasing yield, one QTL location on chromosome 1 was detected in both Indica and Japonica background populations, whereas two (on chromosomes 1 and 2) and three (on chromosomes 5, 7 and 11) were found to be specific to Japonica and Indica background, respectively.

Wild QTL analysis using *O. meridionalis*

A total of 108 and 80 QTLs were identified in the backcrossed populations of Nipponbare and IR36, respectively Of these, 47 (43.5 %) and 36 (45.0 %) had wild alleles increasing trait values. In the background of two typical rice cultivars, 22 QTL locations were found to be common between two populations. Regarding the wild QTL alleles increasing yield, one QTL location on chromosome 8 was detected in both Indica and Japonica background populations, whereas two (on chromosomes 1 and 12) and three (on chromosomes 1 and 3) were found to be specific to Japonica and Indica background, respectively.

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Comparative Cytogenomics and Physical Mapping in the Oryzeae.

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Keywords

comparative genomics, physical mapping, FISH, Oryza, chromosomes

Text

Plant genomes, including those within the tribe Oryzeae, differ in DNA content, chromosome number and morphology, ploidy and the fraction of repetitive sequences in the genome. However, even with these varying characteristics, there is still considerable conservation of gene content among plant species (Hulbert et al 1990) Even more remarkable is the conservation of gene order (colinearity), especially between more closely related species such as rice, sorghum, maize, wheat and millet (Devos and Gale 2000) These observations beg the question: what evolutionary forces have acted on related genomes such that the gene content and order is remarkably similar but chromosome number, ploidy, and DNA content vary drastically among species? Furthermore, how or in what manner have these genomes been remodeled such that gene colinearity has been maintained?

Physical mapping of related plant genomes gives us the opportunity to directly observe the physical relationship of genetic synteny or colinearity between related species Comparing the molecular and physical maps of rice and maize will permit geneticists to make inferences about the mechanisms by which gene content and colinearity were maintained in the 50 million years since the last common ancestor (Doebley et al 1990) However, the tribe Oryzeae, which represents 10-12 million years of independent evolution, allows us to look in more detail at early chromosomal events Within the genus *Oryza,* the genome sizes vary from 343 to 1,691 Mb (megabases) per haploid genome, and there are both diploid and autopolyploid species (Table 1) Outside the genus *Oryza* but within the tribe Oryzeae, the genus *Zizania* has three putative duplicated chromosomes (Kennard et al 2000) and a genome approximately twice the size of *O. sativa.*

To exploit this genomic diversity and begin to model plant genome evolution, especially in the cereals, a toolbox is being made for comparative and evolutionary genomics (http //www.omap.org) This toolbox will consist of BAC (bacterial artificial chromosome) libraries for several *Oryza* genomes (Table 1) These BAC libraries will be fingerprinted, end sequenced and aligned to the sequenced *O. sativa* genome. To gain further insights into chromosome evolution by comparative physical mapping of *Oryza* genomes, we are using FISH (fluorescence *in situ* hybridization) of individual BACs or sets of overlapping BACs (contigs) to *O. sativa* and related *Oryza* genomes

Table 1 Oryza species being used for comparative genomics

Species	**Ploidy**	**Genome Size (Mb)**	**Genome Designation**
Oryza sativa	2x	430	AA
O. rufipogon	2x	760	AA
O. glaberrima	2x	809	AA
O. punctata	2x	539	BB
O. officinalis	2x	1,201	CC
O. minuta	4x	1,691	BBCC
O. australiensis	2x	1,054	DD
O. latifolia	4x	1,127	CCDD
O. schlechteri	4x	1,568	HHKK
O. ridleyi	4x	1,568	HHJJ
O. brachyantha	2x	343	FF
O. granulata	2x	907	GG

FISH can be done to either mitotic/meiotic chromosomes (Cheng et al 2001) or extended DNA fibers (fiber-FISH) (Jackson et al 2000) Each target, chromosomes or fibers, has its own advantages and limitations for genome analysis (Table 2) FISH analysis of centromere repeats cloned from *O. sativa* (Dong et al 1998) showed that some are present at the centromeres of several Oryzeae species, including *Z. palustris,* while others that evolve more quickly were found only in a subset of the most closely related *Oryza* species (Hass et al in press) For instance, RCS2, the major centromeric satellite repeat in *O. sativa,* was present primarily in other AA-

genome species, while RCS1, derived from a centromere specific retrotransposon (Dong et al 1998), was found in all the Oryzeae species tested and in several cereal species (Jiang et al 1996; Hass et al 2003)

Table 2. Comparison of FISH techniques for plant genome analysis

Technique	Resolution	Advantages	Disadvantage
FISH	1-2 Mb	Visualize duplications and chromosomal location	Adjacent clones must be separated by several Mb
fiber-FISH	<5 kb	High resolution, measure overlaps between adjacent clones	Lose chromosomal reference and cannot easily determine duplications

We are also using FISH to analyze the structure of specific regions of the rice genome across related *Oryza* species This work was begun with a BAC contig from the short arm of chromosome 10, however, it soon became apparent that this region is mostly heterochromatic (Rice Chromosome 10 Sequencing Consortium 2003) and that many of the sequences present in the BACs were degenerating to fast to use heterologous probes to related genomes Thus, two regions on chromosome 1 were chosen for 1) high gene density and 2) availability of a sequenced BAC/PAC contigs Using a gene-rich BAC contig the comparative FISH analysis has been more successful, however, the utility of *O. sativa*-derived BACs as heterologous probes on related Oryza genomes is limited. In fact, the best results are obtained on the related AA and BB genome species This is in stark contrast to our observations in the Brassicaceae (Jackson et al 2000) where we have used *Arabidopsis thaliana* BACs as heterologous probes on related species spanning 10-35 million years of evolution (Lagercrantz 1998)

In the Oryzeae, we are in the beginning stages of developing a molecular toolbox (BAC libraries, physical maps and cytological tools) that can be used to more fully understand the evolutionary forces effecting the remodeling of *Oryza* genomes

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Genetic potential of wild species of the genus *Oryza* and their use in rice improvement

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Key words: *Oryza*, Wild species, Genes, Rice improvement

ABSTRACT

The genus *Oryza* has 22 wild species and two cultivated species The cultivated as well as the wild species grow in a wide range of habitats world wide ranging from below sea level to high altitudes The two cultivated species, *O. sativa* and *O. glaberrima* have been domesticated for several thousand years and have pantropical distribution (Vaughan et al 2003) Rice production and productivity in tropical and temperate countries are severely affected by several biotic and abiotic stresses due to lack of stress resistance genes to protect rice plant against new biotypes or pathotypes of pests as well as adverse soil and environmental conditions Some of the major biotic stresses of rice are diseases such as bacterial blight (BB), blast (Bl), sheath blight (ShB), Tungro virus (RTV), dwarf virus (DV)

and insects such as brown planthopper (BPH), whitebacked planthopper (Wph) and stem borer. The major abiotic stresses are drought, cold, submergence, aluminum toxicity and salinity. It is essential to widen the genepool of cultivated rice by incorporating genes from diverse genetic sources. The wild species of *Oryza* are a rich source of beneficial genes for rice improvement (Brar and Khush 1997)

Transfer of useful genes from wild species into cultivated rice genotypes has been extremely difficult because of crossability and recombination barriers Nevertheless, several beneficial genes from wild *Oryza* species have been transferred into rice cultivars across crossability and recombination barriers (Table 1) The genes from wild species could be transferred using advanced techniques of tissue culture and chromosome manipulation through production of monosomic alien addition lines (Jena and Khush 1989; 1990; Brar and Khush 1997)

Table 1 Some wild *Oryza* species, useful genes, associated DNA markers, chromosome location and gene transfer mechanisms in rice.

Wild species	Genome	Genes*	DNA markers	Chromosome	Transfer mechanism
O.nivara	AA	*GS*	-	-	Homologous recombination
O.longistaminata	AA	*Xa*-21	RG103	11	Homologous recombination
O.rufipogon	AA	*RTV*	-	-	Homologous recombination

O.rufipogon	AA	*QAlRr*1 1 *QAlRr*3 1 *QAlRr*9 1	RM252 RG391 RM201	1, 3 9	Homologous recombination
O.officinalis	CC	*Bph*-6(t)	$OPA16_{938}$	11	Rare recombination
O.minuta	BBCC	*Pi*-9(t)	pB8, RG64	6	Rare recombination
O.australiensis	EE	*Bph*-10(t)	RG457	12	Rare recombination

* QTL = Quantitative Trait Loci

Recent advances in molecular biology and subsequent development of highly saturated molecular map of rice have made it possible to identify the useful genes of wild species on the chromosomes of *O. sativa* (Jena et al 1992; McCouch et al 2002; Jena et al 2003; Nguen et al 2003) The newly developed molecular breeding methods have made use of beneficial genes of wild species for the genetic improvement of rice cultivars The BB resistance gene *Xa*-21 from the wild *Oryza* species, *O. longistaminata* and BPH resistance gene from *O. officinalis* have been successfully introgressed into rice cultivars and have expressed broad spectrum of resistance to BB pathotypes and BPH biotypes respectively in Asian countries (Song et al 1995, Jena et al 2003) These findings have paved the way for rice breeders and geneticists to make use of valuable genetic resources of wild species of *Oryza* in rice improvement With the completion of a highly reliable genome sequence of *Oryza sativa* by International Rice Genome Sequencing Project (IRGSP), it would be possible now to transfer numerous beneficial genes from wild *Oryza* species into rice cultivars to improve the yield potential, grain quality and stress resistance.

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Wild Rice Core Collection and Its Use for The Study of Gene Diversity

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Key words: wild species, core collection, gene diversity, reproductive barrier

Near 2,000 wild rice accessions including 9 genomes and more than 21 species have been collected in our National Institute of Genetics (NIG) from all over the world spent more than fifty years. We have selected representative accessions from each species and prepared a core collection consisting of 44 strains for rank 1, 64 strains for rank 2 and 171 supplemented accessions in rank 3, as a resource for the study of genetic diversity and evolution in genus Oryza. Phenotype characters, habitat, place of origin, photographic images and related information are also being gathered. These information is accessible to our database Oryzabase at http://www.shigen.nig.ac.jp/rice/oryzabase/top/top.jsp. Data examples and future activities in the Oryzabase will be introduced.

Though studies of evolutionary relationship among these Oryza species have been performed by several workers using PCR technique, specific sequences or isozyme analysis (Aggarwal *et al.* 1999; Ge *et al.* 1999; Chen *et al.* 2002; Cheng *et al.* 2003), diversity in gene members and sequences including regulatory elements have not been elucidated yet. Our new attempt to search genome and species specific genes in a few genome species is now going to start. Difference of gene expression and sequence diversity, as well as presence or absence of certain kind of gene members would be major attribute of the genetic diversification of

species. Identification of genome or species specific genes and highly variable genes in the sequence or at the expression level will be carried out by the use of EST analysis and microarray analysis. Plans and a few examples will be shown in my talk.

As for the gene/genetic diversity among species and sub-species, there are several other approaches to find them. One of the direct approach to detect gene/genetic barriers between sub-species, those are in a step of evolutionary processs for speciation, is finding of barriers which cause segregation distortion in the crossed progenies. Genome-wide survey was carried out to detect almost all reproductive barriers between Nipponbare and Kasalath and in other two cross combinations (Harushima *et al.* 2001 and 2002) More than 30 barriers were detected in each three combination and were quite different among three crosses. We are interested in their function and one of the prominent barriers are now in the process of gene identification by map-based cloning.

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Transgene escape and its biosafety concerns in rice (*Oryza*)

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Key words: Gene flow, introgression, ecological biosafety, wild rice, GM crop

Rice (*Oryza sativa* L.) is one of the most important world's cereal crops, providing staple food for nearly one half of the global population. More than 90% of rice is grown and consumed in Asia where about 55% of the world's population lives (Lu 1996), reflecting the importance of rice in Asian people's daily life, in addition to its significances in cultural aspects in this area. Rice is also one of the earliest world's crops to which transgenic biotechnology has been effectively applied for its genetic improvement (Ajisaka *et al* 1993; Yahiro *et al* 1993). Therefore, genetically modified (GM) rice is not any more a novel term to publics of many societies. To date, no GM rice varieties have yet been officially approved for their extensive commercial cultivation anywhere in the world, but genes conferring traits such as high protein content, special nutritional compounds, disease and insect resistance, virus resistance, herbicide resistance, and salt tolerance, have been successfully transferred into different rice varieties through transgenic technology (Ajisaka *et al*, 1993; Yahiro *et al*, 1993; Matsuda 1998; Messeguer *et al* 2001; Brooks and Barfoot 2003). It is predicted that, as an important world cereal crop, GM rice varieties, like many other GM crops that enhance yields, improve human health, and make agriculture more sustainable (Snow 2003), will be released into environment for commercial production in the near future, after their necessary food and environmental biosafety assessments are accomplished by the authorized agencies (Brooks and Barfoot 2003).

Like many other new developments, transgenic biotechnology and the GM products have evoked most intense debates on their biosafety concerns worldwide (Bergelson *et al* 1998; Schiermeier 1998; Crawley *et al* 2001; Ellstrand 2001; Prakash 2001). Biosafety issue has become a crucial constraint to the further development of transgenic biotechnology and wider application of GM products, including GM rice. Transgene escape from the GM rice varieties and its potential environmental consequences is among the most controversial biosafety concerns across the world (Lu *et al*, 2003). Terminologically, **transgene escape** refers in general to a gene or a group of genes introduced to a crop variety by a genetic engineering method moving to its non-GM counterparts or wild relative species (including weedy biotypes) through **gene flow** (including pollen flow and seed dissemination). Cross-pollination between GM rice and non-GM rice varieties or its wild relatives is the major pathway for transgene escape. Accordingly, there are two types of transgene escape, i.e., crop-to-crop and crop-to-wild transgene escape that are usually discussed by publics.

It is argued that the **crop-to-crop** transgene escape might contaminate non-GM rice varieties, affecting the purity of these rice varieties, as well as the strategic deployment of GM- and non-GM rice in a given agricultural system. When normal rice varieties are mixed

with individuals of GM rice, the exporting trade of rice, particularly to these countries with a rigid biosafety control, would be considerably influenced, and even cause some legal difficulties. The **crop-to-wild** transgene escape may lead to the persistence and dissemination of transgenes in wild or weedy rice populations through sexual reproduction and/or vegetative propagation. If the transgenes are responsible for resistance to biotic and abiotic stresses (such as drought and salt tolerance, and herbicide resistance), these genes could significantly enhance ecological fitness of wild and weedy rice species, and make the host wild plants more invasive, which could probably cause unpredictable environmental consequences in certain ecosystems. On the other hand, when transgenes escape to wild rice populations through outcrossing, the rapid spread of the resulted hybrids and their transgene-carrying progeny would result in contamination of the original wild rice populations, and even lead to the extinction of endangered wild rice populations in local ecosystems by the so-called swarm effect (Kiang *et al* 1979; Ellstrand and Elam 1993). This will jeopardize *in situ* conservation of wild rice germplasm. In addition, the perennial hybrids between GM rice and their wild relatives carrying transgenes may serve as a "bridge" to spread their transgenes to other wild related species through outcrossing, causing even more significant ecological consequences.

Transgene escape and its ecological consequences have been extensively discussed worldwide. Will transgene escape occur in rice through outcrossing? Will transgene escape pose an environmental safety problem in rice ecosystems? These questions relating to the biosafety of GM rice need to be adequately addressed for scientific and public understanding. Normally, the occurrence of transgene escape from GM rice needs to meet the following three prerequisites. Spatially, GM rice and its non-GM counterparts/wild relatives should have a sympatric distribution, i.e. growing in the same vicinity and also in a close contact. In terms of crop-to-wild transgene escape, temporally, the flowering time (including flowering duration within a year and flowering time within a day) of GM rice and its wild relatives should overlap considerably; and biologically, GM rice and its wild relative species should have a sufficiently close relationship, also the resulted interspecific hybrids should be able to reproduce naturally. Consequently, the knowledge of geographic distribution patterns, flowering habits, and genetic relationships of cultivated rice and its wild relative species will be essential for predicting transgene escape in rice. Similarly, data on the actual gene flow frequencies between different rice varieties, and between cultivated and wild species are also important for predicting transgene escape and its potential ecological consequences, based on which strategies to minimize transgene escape can be developed.

Studies have clearly shown geographical distribution patterns and genetic relationships of cultivated rice and its wild relative species in the genus *Oryza*. It is known that the genus comprises two cultivated species and over 20 wild species with ten different genome types, i.e. the AA, BB, CC, BBCC, CCDD, EE, FF, GG, JJHH, and JJKK genomes (Vaughan 1994; Ge *et al* 1999), widely distributed in the pan-tropics and subtropics (Vaughan 1994). Species that contain different genomes have significant reproductive barriers. Therefore, genetically speaking, such species are distantly related and spontaneous hybridization between these species with different genomes is extremely difficult. Cultivated rice contains the AA genome and is relatively easy to cross with its close relative species (including weedy rice) that also contain the AA genome. Theoretically, transgene escape from GM rice varieties will merely occur to the wild rice species with the AA genome.

Research data have demonstrated that apart from the two cultivated rice species (*O. sativa* and *O. glaberrima*), these wild relatives, *O. rufipogon* and *O. nivara* from Asia, *O. longistaminata* and *O. barthii* from Africa, *O. glumaepatula* from Latin America, and *O. meridionalis* from northern Australia and New Guinea, also contain the AA genome. The AA-genome wild *Oryza* species is distributed across a significantly wide geographic region in different continents, and Asian cultivated rice (*O. sativa*) shares sympatric distribution with these wild species in many areas, particularly in Southeast and South Asia, Central Africa, and Latin America. The weedy rice is usually found in rice fields alongside cultivated rice. Data on geographic distribution evidently indicate that spatially transgenes from cultivated rice have a great potential to escape to its wild relative species.

Flowering habits of cultivated rice grown in different parts of the world vary considerably depending on differences in local cultivation time and seasons, and in varietal types. The flowering and pollinating time of different wild rice species or different populations of the same species also varies significantly across different geographic regions. Our studies of a selected *O. rufipogon* population found in Hunan Province of China and two cultivated rice varieties showed that both of the flowering period in a year and flowering time in a day had considerable overlap between *O. rufipogon* and the two rice varieties. Our additional experimental data also showed that pollen grains of *O. rufipogon* and a cultivated rice variety could be viable in air for more than 60 minutes (Song *et al.* 2001). These results suggest that outcrossing between *O. rufipogon* and cultivated rice will take place, if the two species are grown near to each other.

Data on interspecific crossability, meiotic chromosome pairing, and fertility in the F_1 hybrids can be used to estimate genetic relationships of the cultivated rice and its AA-genome wild relatives. If cultivated rice has relatively high crossability with its wild relatives, normal meiosis, and comparatively high fertility in the F_1 hybrids, the transgenes will easily escape to wild relative species through outcrossing and persist in environment. Transgenes would also disseminate through reproduction or vegetative propagation if the hybrids and their progeny were perennial. Results from our interspecific hybridization showed that most of the AA-genome wild rice species have relatively high compatibility with the cultivated rice, extremely high chromosome pairing formed in meioses of the F_1 hybrids with the wild rice, and spikelet fertility of the F_1 hybrids was relatively high under bagged self-pollination conditions. These data indicate a high opportunity of transgene escape from GM rice to its wild relatives, in terms of the close genetic relationships of cultivated rice with its AA-genome wild relatives.

In order to obtain data on the actual gene flow frequencies between different cultivated rice varieties, and between cultivated rice and its wild species under natural conditions, we conducted a series of experiments involving GM-, non-GM rice varieties, weedy rice, and *O. rufipogon*. The experimental data showed that gene flow frequencies between different rice varieties were very low (less than 0.5%), although with a certain variation, comparable gene flow frequencies were observed between cultivated rice and weedy rice. However, gene flow from a cultivated rice variety Minghui-63 to the Hunan *O. rufipogon* population under four different experimental designs were significantly variable, with the maximum frequency of ca. 3% under the special cultivation conditions, indicating clearly that gene flow from cultivated rice to the widely distributed *O. rufipogon* would occur considerably in nature.

Our studies demonstrated that transgene escape from GM rice to non-GM rice varieties, and to its wild relatives is possible to occur, although its extent might vary considerably between different varieties or different species (populations). This result is accordant with many other conclusions based on gene flow or introgression studies of rice species, although the gene flow frequencies between GM- and non-GM rice varieties were comparatively low (e.g. Messeguer *et al.* 2001). It is therefore a very important biosafety strategy to establish an effective buffering isolation zone between GM rice and non-GM rice varieties, particularly between GM rice and its closely related wild species, to avoid or significantly minimize transgene escape, given that the spatial, temporal, and biological conditions for rice transgene escape are satisfied in many rice producing countries or regions. In addition, our further research activates should be focused more on ecological consequences of rice transgene escape, which is still a controversial issue that have received an extensive attention by publics, scientists and government agencies. Although it is difficult to assess and monitor potential ecological consequences caused by transgene escape within a limited period, long-term and continued accumulation of basic knowledge on ecological impacts of transgene escape is essential to increase our understanding of GM rice biosafety, and reduce our biosafety concerns caused by GM rice. Moreover, there should be more scientific inputs allocated for investigating scientific questions on ecological consequences of rice transgene escape. This will allow us to effectively assess and manage the potential ecological risks resulting from rice transgene escape, which will in return promote the development and safe use of transgenic rice varieties.

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Cytological diversity of FISH loci of a tandem repeat DNA sequence in *Oryza sativa* and its related species

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Keywords: *Oryza sativa*, wild rice, cytological diversity, fluorescence *in situ* hybridization (FISH), *Os48*, tandem repetitive DNA sequence

Genetic diversity in genus *Oryza* and domestication of rice has been investigated based on their morphological and molecular characteristics From the viewpoint of cytology, diversity in the genus *Oryza* has been revealed by various studies from genome organization analyses based chromosome numbers (diploid and tetraploid) and genome groups (A-J) to the molecular biological analyses based on physical mapping by the fluorescence *in situ* hybridization (FISH) of ribosomal RNA genes (rDNA) and the repetitive DNA sequences (Uozu *et al* 1997, Shishido *et al* 2000) These cytological studies revealed inter-species diversity in the genus *Oryza*. There are few cytological studies on the intra-species diversity, however, there are many molecular studies on intra-species diversity in cultivated rice, *O. sativa*, based on, for example, the organellar DNA (Ishii *et al* 1988), transposable elements (Mochizuki *et al* 1993, Cheng *et al* 2003), RFLP and RAPD polymorphism (Suh *et al* 1997)

Os48 (*Rc48*) is a representative tandem repeat sequence in cultivated rice, *Oryza sativa* (Wu & Wu 1987) This 355-bp sequence is found in only *Oryza* A genome species (Wu *et al* 1991) In previous FISH studies this sequence was mapped on rice chromosomes Eight loci were detected in *indica* variety Zhongxian 3037 and two were found in *japonica* variety Wuyujing 8 (Cheng *et al* 2001) A tandem repeat sequence in the A genome, TrsA, which shows high similarity to *Os48*, was visualized at six loci in the *indica* varieties IR8 and IR36 and two in *japonica* variety Nipponbare (Ohmido & Fukui 1997, Ohmido *et al* 2000) These FISH results showed that there are difference in the number of *Os48* between rice varieties and suggested intra-species cytological diversity in *O. sativa*.

To reveal cytological intra-specific diversity among varieties of *O. sativa*, FISH of *Os48*

with tyramide signal amplification (TSA, Okada *et al* 2000) was employed. Cytological diversity was shown in the differences in the number of FISH signals The number of FISH loci was almost the same among all *japonica* varieties, including the temperate (paddy and upland habitats) and tropical (*javanica*) varieties But the *indica* varieties showed significant differences in the number of the FISH loci These FISH results reveal two points about cytological diversity of rice chromosomes, one is intra-species cytological diverse has accumulated in rice chromosomes and the other is narrow cytological diversity is observed in *japonica* while wide diversity is observed in *indica*. Additional FISH studies of *O. rufipogon* will shed light on the domestication of cultivated rice from a cytological viewpoint

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Phylogenetic analysis of *Oryza* species based on simple sequence repeats and its flanking nucleotide sequences from the genomes of cytoplasmic organelle

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Keywords: *Oryza*, rice, simple sequence repeat, chloroplast, mitochondria

Simple sequence repeats (SSRs) have widely been used to detect allelic variation in the nuclear genome among species in many living organisms. Earlier studies using SSRs polymorphism were based on size variation of amplified PCR products that includes SSR plus flanking sequence. However, it was difficult to distinguish whether the size differences are due to the length of the SSR itself or that of its flanking sequence. Rice is the only crop where entire genome information is available both in chloroplast (Hiratsuka *et al.* 1989) and mitochondrion (Notsu *et al.* 2002). The complete SSR information is available for the chloroplast genome of rice (Ishii and McCouch 2000) whereas mitochondrial SSRs have not been studied yet from the completely sequenced genome. Chloroplast and mitochondrial genomes are maternally inherited and their characteristics can effectively be used to trace back the genetic relationships among species, particularly to study the evolution of polyploid species. Hence, the present study was undertaken to assess the level of genetic variation in the

genomes of cytoplasmic organelle based on SSRs, and to characterize the phylogenetic relationships of genus *Oryza* based on these information.

A total of 44 accessions of genus *Oryza* were analyzed. Two accessions of *Leersia tisserantii* were used as an outgroup. The SSR information of the rice mitochondrial genome was obtained from the entire sequence (Notsu *et al.* 2002). A total of seven mitochondrial and five chloroplast SSR regions were analyzed. Polymorphism was identified by nucleotide sequence determination and subsequent phylogenetic analysis was performed using the most parsimony method.

In addition to length polymorphism in SSR sequence itself, our results revealed the occurrence of many deletions/insertions in the flanking region of SSRs. Base substitutions were also identified in SSRs. In conclusion, the length polymorphism in PCR products of the SSR region was recognized from the deletions/insertions in their flanking region as well as among SSR itself by sequence determination. Previously, the deletions/insertions always misled polymorphic information because the internal substitutions of SSRs were not taken into account. Results obtained both by chloroplast and mitochondrial SSR analyses showed a similar relationship in principle among species although analysis of chloroplast SSR showed more detailed taxonomic tree. The most parsimonious tree for the genus *Oryza* showed clearly the separation of the complexes in the genus *Oryza* (Fig. 1). However, the species relationships written the *O. officinalis* complex and *O. sativa* complex could not be well

discerned due to insufficient polymorphism among the complex. The species of CC genome showed clear differences with BB, whereas, it was more closely related to BBCC and CCDD genome. Our results strongly suggests that CC genome is the maternal parent for the BBCC and CCDD species analyzed. This is the first report on phylogenetic relationships among different rice species based on mitochondrial and chloroplast SSRs and their flanking sequences.

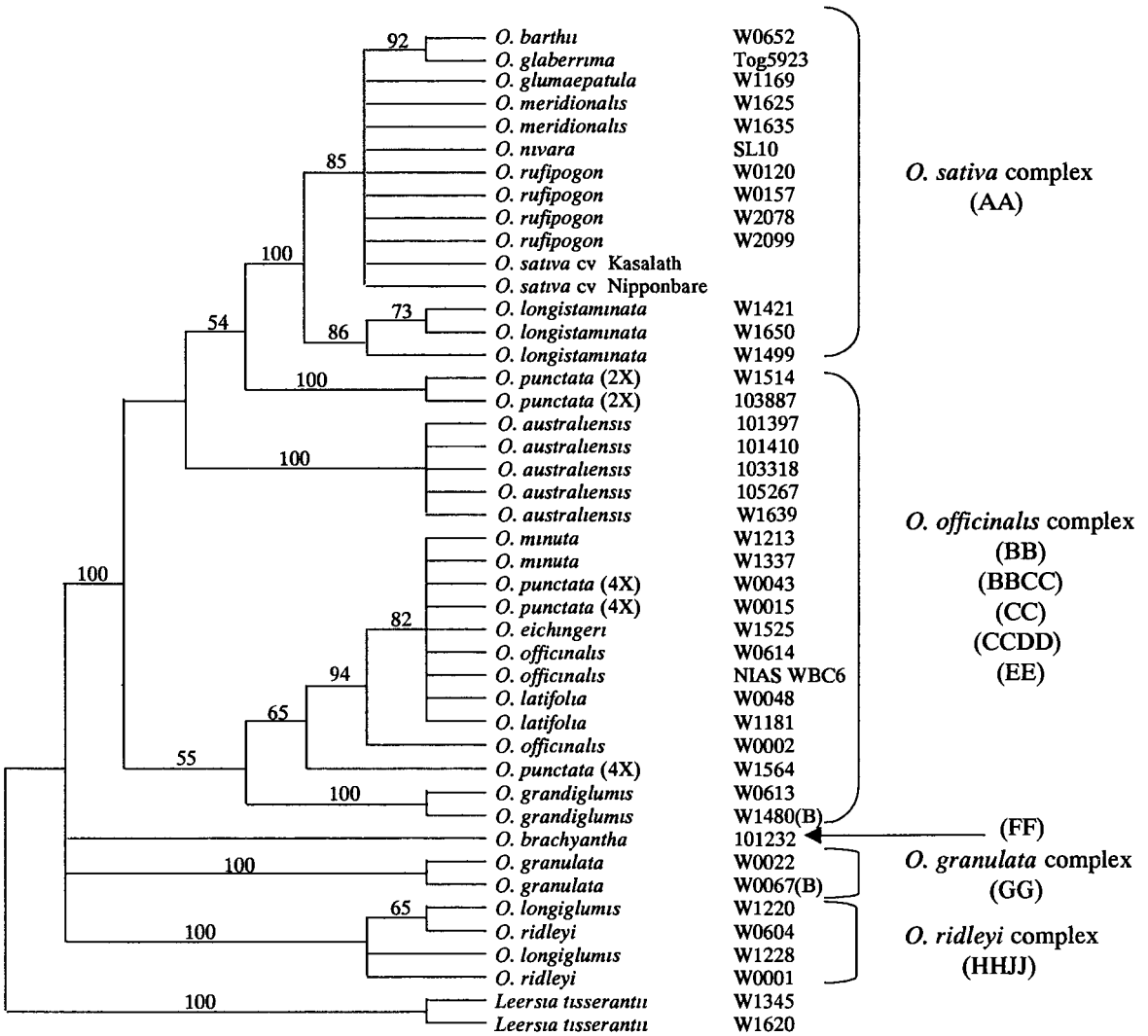

Fig. 1 Phylogenetic analysis of genus *Oryza* based on variation of SSR loci and their flanking sequences in organelle genome. The numbers above the nodes represent bootstrap values expressed as percentage of 10,000 bootstrap replications

Acknowledgments

DNAs from four *O. australiensis* accessions and one *O. brachyantha* accession were kindly provided by Dr. Ishii of Kobe University, Japan. We thank him for his valuable comments on our results. We thank Ms. Nohara for her technical assistance. Leaf materials were collected from plants grown at doomed greenhouse, National Institute of Agrobiological Sciences, Tsukuba.

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Origin of Cultivated and Wild Rice with AA Genome: Based on the Interspersion Patterns of SINEs

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Keywords; *p-SINE1*, *Oryza* genus, AA Genome, insertion polymorphysm, phylogeny

Species of *Oryza* genus

Oryza genus comprises approximately 22 species distributed in Asia, Africa, Australia, and Central and South America. Based on interspecific crossing, subsequent cytogenetic analysis and genomic DNA hybridization, all species in the *Oryza* genus are classified into six diploid genome types (AA, BB, CC, EE, FF and GG) and four tetraploid genome types (BBCC, CCDD, HHJJ and HHKK). Among AA genome species, there are two cultivated (*O. sativa* and *O. glaberrima*) and five wild species (*O. rufipogon, O. Barthii, O. glumaepatula, O. longistaminata, O. meridionali*). The cultivated species, *O. sativa* and *O. glaberrima,* are thought to be derived from *O. rufipogon* and *O. Barthii,* respectively. *O. sativa* strains have been classified morphologically into indica and japonica (Kato et al., 1928). The *O. rufipogon* strains have been classified into two ecotypes, annual and perennial (Morishima et al.,1992). Although *O. sativa* is thought to be a domesticated species of *O. rufipogon,* both species show remarkably high intraspecific variation leading to various speculation for the origin of *O. sativa.*

Discovery of *p-SINE1*

p-SINE1 is the first plant SINE identified in the *Waxy* gene in *O. sativa* (Umeda,

et al., 1991). It is 122 bp in average size and contains a promoter for RNA polymerase III, and an A-T rich region with a variable length of T-rich pyrimidine tract at the 3' end (Fig. 1). *p-SINE1* was estimated to be present in the *Oryza* genome in high copies, with 6500 and 3000 copies per haploid genome of *O. sativa* and *O. glaberrima*, respectively (Motohashi et al., 1997). Many *p-SINE1* members have been identified in the genome of *O. sativa* by inverse PCR (IPCR) or genomic library screening. Some members showed interspecific insertion polymorphism among species with AA genome. These polymorphic members might have retrotransposed into the respective loci during the divergence of the rice species with AA genome (Mochizuki et al., 1992; Hirano et al., 1994). Recently, many *p-SINE1* transcripts have been isolated by 5' RACE-PCR from various kinds of organs of *O. sativa* strains in our laboratory (Tsuchimoto et al., unpublished). Some of these transcripts had a sequence starting at the precise 5' end of *p-SINE1*, suggesting that they are the products from the internal pol III promoter of *p-SINE1*, and are derived from some transcriptionally active members. These suggest that the *p-SINE1* members were active in transposition during the divergence of AA genome species, and may be still presently active in transposition.

RA subfamily, a recentry amplified *p-SINE1*

We collected forty-seven members of *p-SINE1* at different loci in the genome of *O.sativa* ,japonica type, as well as indica type. The presence or absence of these members at particular loci was determined by PCR in strains with the AA genome, *O. sativa, O. rufipogon, O. glumaepatula, O. meridionalis, O. longistaminata,* and *O. barthii.* Seventeen *p-SINE1* members were found polymorphic, indicating that they may have been amplified during the divergence of the rice species. Among those polymorphic members, some showed insertion polymorphism in between *O. sativa* and *O. rufipogon.* This means that these *p-SINE1* members were amplified only recently after the divergence of *O. sativa* and *O. rufipogon* and therefore have not yet become fixed in the *O. sativa* and *O. rufipogon* populations. Alignment of the nucleotide sequences of the polymorphic

members showed that they share three common mutations; two in the A- and B-box sequences in the pol III promoter and one in the distal end region (Fig. 1). Some of the members are transcribed, and thus, indicate that *p-SINE1* members with three diagnostic mutations have been amplified in recent evolutionary time. We therefore named them RA (Recently Amplified) subfamily members (Cheng et al., 2003).

p-SINE1 - insertion polymorphism and phylogenetic analysis of *O. sativa* and *O. rufipogon*.

We collected 101 strains of *O. sativa* and *O. rufipogon*. By using PCR, we investigated the presence or absence of 24 *p-SINE1* members, including 22 RA polymorphic members at particular loci in each rice strain. RA members of *p-SINE1* showed insertion polymorphism among these rice strains. To show the relationships among those, each strain was "bar-coded" based on the presence or absence of *p-SINE1* members at the respective loci, then a phylogenetic tree was constructed by the neighbor-joining method based on the bar codes given to all the strains (Fig. 2).

In the phylogenetic tree, the rice strains are divided into four major groups, I - IV (Cheng et al., 2003). *O. sativa* strains are clearly separated into two groups, I and II. The two ecotype strains of *O. sativa*, Japonica and Indica, can be distinguished almost exclusively by the presence or absence of *p-SINE1* members. *O. rufipogon* strains are divided among all four groups on the tree (Fig. 2), indicating that *O. rufipogon* shows remarkably high intraspecific variation. Interestingly, all the annual *O. rufipogon* strains, except for one strain, are present in group II, whereas the perennial strains are in each of the other three groups, I, III and IV (Fig. 2). supporting the idea that the annual strains are derived from the primitive perennial strain (Morishima et al.,1992). Because annual type strains appear to form a distinct group in the *O. rufipogon* population (Fig. 2), differentiation into the annual type should be considered an intraspecific variation, as reported previously (Oka 1988).

Polyphyletic origin of *O. sativa*

The phylogenetic tree shows the japonica strains of *O. sativa* and six perennial strains of *O. rufipogon* are in group I (Fig. 2), indicative that the japonica and perennial strains of this group originated from a common source, probably *O. rufipogon* of the perennial type. The indica strains of *O. sativa* and annual strains of *O. rufipogon* are both in group II (Fig. 2). Most indica strains are clearly distinct from the annual strains, indicating that both types of strains are originated from another common source. When we consider the fact that the *O. sativa* cultivars are essentially perennial plants (Morishima et al.,1992), the source is most probably *O. rufipogon* of the perennial type. However, a few indica strains, including those from Assam (India) and Nepal, are clustered with the annual strains (Fig.2). This suggests that these indica strains may have originated in the annual *O. rufipogon* population by recent domestication. These findings show that the *O. sativa* strains originated polyphyletically.

Evolutionary relationships among rice species with AA genome based on the SINE insertion polymorphysm

We further identified new *p-SINE1* members showing interspecific insertion polymorphism from representative strains of four wild rice species with AA genomes. The *p-SINE1* insertion patterns of the strains of cultivated rice species, *O. sativa* and *O. glaberrima*, were very similar to those of wild rice species, *O. rufipogon* and *O. barthii*, respectively. Phylogenetic analysis based on the *p-SINE1* insertion patterns showed that the strains of each of the five wild rice species formed a cluster (Fig. 3). The strains of *O. longistaminata* appear to be distantly related to those of *O. meridionalis*. They appear to be distantly related to those of the other three species, *O. rufipogon*, *O. barthii* and *O. glumaepatula*. A phylogenetic tree including a hypothetical ancestor showed that the strains of *O. longistaminata* are related most closely to the hypothetical ancestor. This indicates that *O. longistaminata* and *O. meridionalis* are diverged early, whereas the other

species are diverged relatively recently, and suggests that the *Oryza* genus with AA genome might have originated in Africa, rather than in Asia (Cheng et al.,2002).

Acknowledgement We thank Drs. N. Kurata and Q. Xue for providing the rice strains. We are grateful to Drs. H. Morishima and Y. Sano for the information on the ecotypes of the *O. rufipogon* strains and for their critical reading of the manuscript. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and from the Ministry of Agriculture, Forestry and Fisheries of Japa

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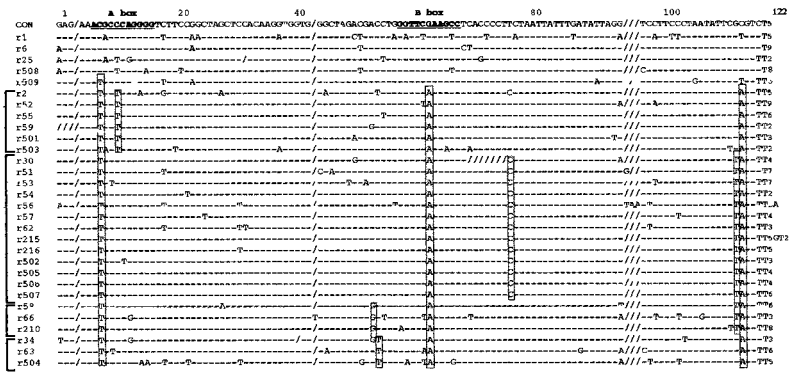

Fig 1 Nucleotide sequences of the *p-SINE1* members, most, except r1, showing insertion polymorphism among the rice species A concensus sequence(CON) of *p-SINE1* (Motohashi et al 1997) is shown at the top A-box and B-box of the pol III promotor are shown in baldface letters RA subfamily diagnostic mutations are boxed. RA subfamily members may be further divided into four groups as indicated by the square bracket

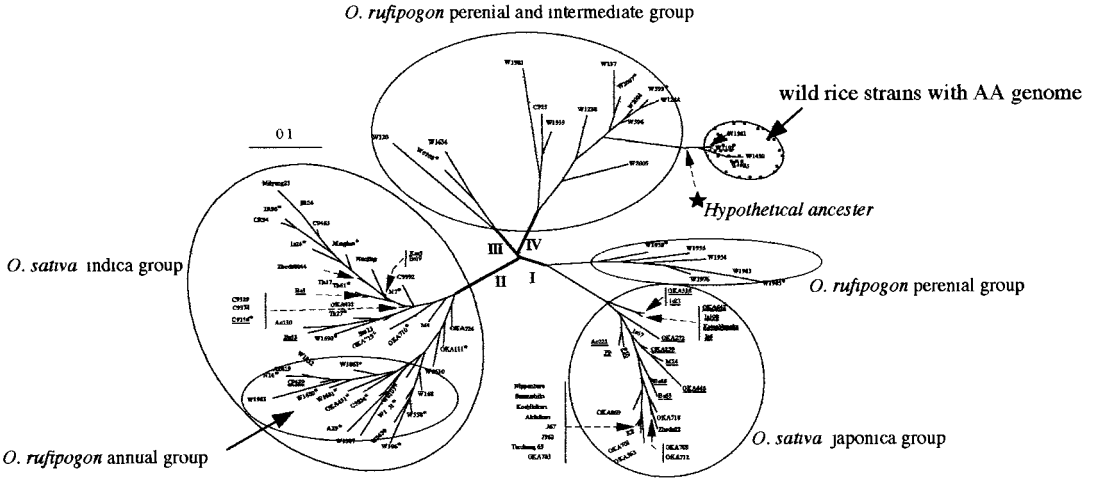

Fig 2 A phylogenetic tree showing relationships among the *O. sativa* and *O. rufipogon* strains The tree was constructed by the NJ method based on the pattern of the presence or absence of *p-SINE1* members The four groups (I-IV) identified are shown by four deep internal branches indicated by thick lines Each major groups are circled. The strains with hatched box belong to *O. sativa* indica group The large star indicate a hypothetical ancestor with no *p-SINE1* insertion

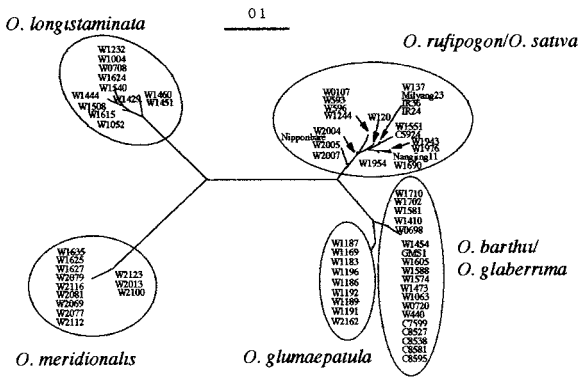

Fig 3 A phylogenetic tree showing relationships among the 72 strains with AA genome The tree was constructed by the NJ method based on the pattern of the presence or absence of *p-SINE1* members All strains were clustered into five groups, corresponding to *O. longistaminata, O. meridionalis, O. glumaepatula, O. barthii/O. glaberrima* and *O. rufipogon/O. sativa* Note that the strains of *O. barthii* and *O. glaberrima* are in the same cluter so as those of *O. rufipogon/O. sativa*

The Complete Nipponbare genome sequence as a standard for *Oryza* genome research

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Keywords genome sequence, *in silico* mapping, Nipponbare, Kasalath, *Oryza sativa*

The genome sequence of Nipponbare, a *japonica* type variety of *Oryza sativa* has been almost completed by the international sequencing consortium, IRGSP. All sequence data for BAC/PAC clones correctly aligned along the 12 rice chromosomes using DNA markers and fingerprint data has been read with at least 99.99% accuracy. As of August 2003, IRGSP has constructed a physical map covering 96% of the Nipponbare genome which has a total size of 400 Mb The genome sequence provides the most basic information necessary to understand phenotypic variations and to clarify how these differences are genetically controlled. Rice is one of the major cereal crops and an important food source for more than half of the word's population. Improvement of rice production has always been a major concern to guarantee a stable food supply particularly in rice consuming regions in Asia, Africa and Latin America where a drastic population increase is predicted in the next 20 years The accurate genome sequence of Nipponbare is expected to now widely used for gene identification corresponding to phenotype by genetic and reverse-genetic methods For example, precise tagging of target phenotype using DNA markers with sequence information facilitates efficient application of map-based cloning method in rice. So far, several important disease resistance and morphological trait-related genes have been identified. The sequence information is also indispensable for rapid identification of corresponding candidate gene within the specific genomic region tagged with DNA markers Reverse-genetic method is now commonly used to identify the function of a gene with known sequence by phenotypic analysis of plants in which the function of the gene is impaired. This approach is greatly facilitated with the utilization of the rice retrotransposon, *Tos17* which moderately transposes during tissue culture and serves as an efficient insertional mutagen. The accurate genome sequence of Nipponbare can be useful for corresponding gene identification and analysis of the functions of more than 40-60,000 predicted genes

in the rice genome.

Rice is produced in a wide range of environmental and climatic conditions, and ca. 120,000 varieties are cultivated around the world. Each variety has unique characteristics adapted to each cultivating condition and differences in phenotype account for enormous genomic diversity This indicates that there are at least 120,000 differences in the genome sequences of cultivated rice, *O. sativa*. These differences range from subtle changes involving one or a few nucleotides among closely related varieties to more conspicuous changes such as insertions or deletions commonly found between *japonica* and *indica* ecotypes More defined differences can be expected between *O.sativa* and its wild relatives such as *O.glaberrima* and *O.rufipogon*, which carry favorable characteristics not found in cultivated species At present, map-based cloning following a detailed genetic analysis using chromosomal substitution lines has made it possible to identify genes involved in complex traits controlled by more than two loci Analysis of flowering time in rice clearly has shown that subtle differences in corresponding genes between Nipponbare and an indica ecotype Kasalath concertedly contribute to the difference in phenotype. This suggests that similar variation in nucleotide sequences in genes responsible for the same phenotype exists in other rice varieties The Nipponbare genome sequence with an accurate positional assignment as may serve as a standard for clarifying variation across a wide taxonomical range.

We tried to construct an accurate physical map of Kasalath by *in silico* mapping of BAC-end sequences using the Nipponbare genome as reference sequence. A BAC-based physical map of an indica ecotype is expected to facilitate genome-wide practical applications such as map-based cloning or position-directed detailed genome sequencing. Using ca. 78,000 Kasalath BAC-end sequences, BAC clones were assigned to chromosome 1 resulting in 22 contigs with a total length of 38 7 Mb corresponding to 89% of the chromosome. This strategy can be applied to other varieties of *O.sativa* and might be promising for other *Oryza* species by introducing less stringent condition for *in silico* mapping.

Our knowledge on the diversity of *Oryza* species at the genome level is very limited, but we must re-evaluate the gene resources to discover many unknown genes which may have been activated during the domestication of wild relatives to cultivated rice. It may also provide an accurate estimate of the evolutionary relationships which may elucidate the genetic mechanisms of species diversification. Also information on relation of variation of nucleotide sequence such as SNPs with variation of phenotype may provide an effective tool for improvement of rice plants based on breeding strategies

Semi-sterile perennial wild rice (*Oryza rufipogon*) as the progenitor of *japonica* cultivar

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We have been collecting wild rice (*Oryza rufipogon* and *O. nivara*) in Southeast Asia since 1983. After 1990s, we collected stem nodes in stead of seed-samples, in case that the population shows extremely low seed productivity. Here we report genetic traits among 85 samples. To classify those samplers into indicia- and japonica-homologues, coda types (deletion or non-deletion types at PRF 100 regions; Chen et al. 1993, and sequence at the PS-ID region; Nakamura et al. 1997) were determined. The results obtained are as follows.

1. Samples used were likely classified as *O. rufipogon* (or, perennial type of *O. rufipogon* in a broad sense), but scarcely be done as *O. nivara*.
2. Of the samples, 50 ones showed low seed productivity and were classified into two types. First type shows extremely strong seed sterility, while they bore a lot of panicles. Second type shows poor panicle bearing throughout a year (Fig. 1).

Fig. 1 A perennial wild rice population in Mekong delta (Photo taken in November, but no panicle was born)

We saw the second type only in Mekong delta with a few exceptions. On the other hand, first type was seen more widely.

3. We compared the proportion of plants having *japonica*-homologous cpDNA (eg. Non deletion at ORF 100 region and/or 6C7A or 7C6A PS-ID types) and *indica* -homologous cpDNAs (eg. Deletion at ORF 100 region and/or 6C8A or 7C7A PS-ID types, see Nakamura et al. 1997) in both of the stem-sampled and the seed-sampled populations. In the stem-sampled populations, *japonica*-homologous cpDNA was significantly frequent compared to homologue of *indica* (data will be shown at the conference site).

From these facts, and also Yamanaka et al. (2002), we consider that *japonica*-homologous *rufipogon* was originally vegetative propagator (Fig. 2).

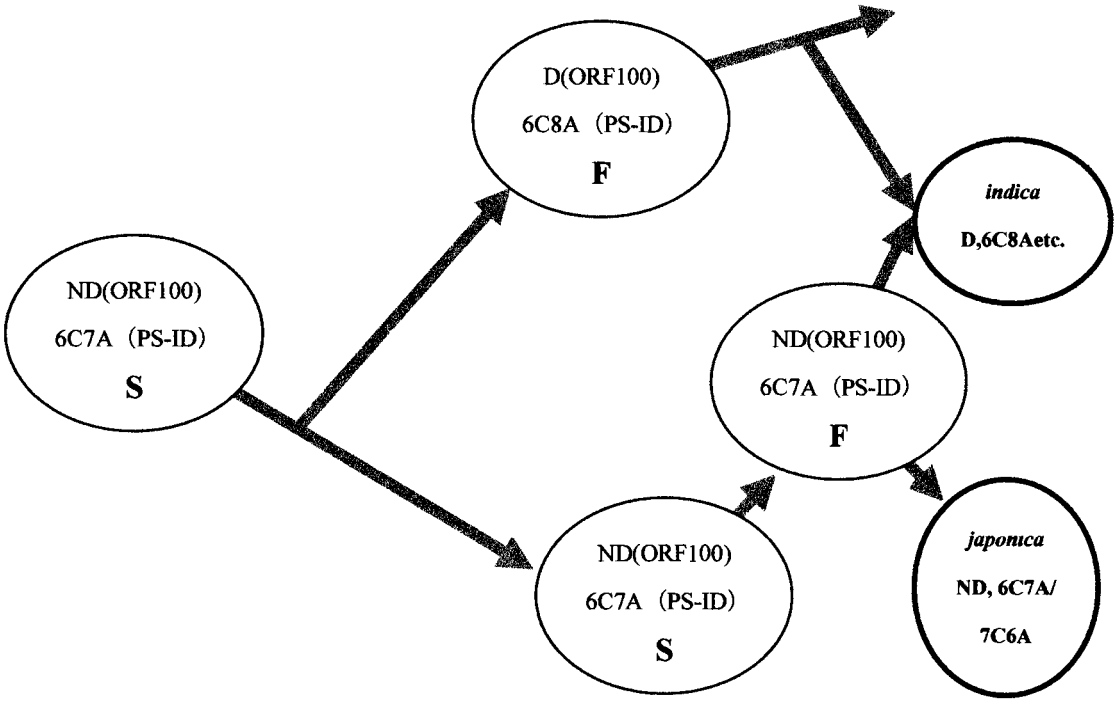

Fig.2 Schematic figure showing evolution of rice based on cpDNA types. ND: Non-deletion at ORF100 region, 6C7A etc. are PS-ID types (see Nakamura et al. 1997). S: Sterile, F: Fertile.

The experimental results obtained by using collected seed samples may cause mislead, because the seeds of such sterile populations are likely resultants of outcrossing by pollen grains derived from outside of the population.

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Genus, genomes and species: what do they mean in relation to *Oryza*?

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1. What is the genus *Oryza*?

Genera are delimited based on key morphological traits Traits that distinguish *Oryza* species include presence and shape of sterile lemma and morphology and anatomy of leaves The genus *Oryza* is most closely related to the widely distributed genus *Leersia* (Fig. 1) In 1965 three species that were previously considered *Oryza* species were transferred to the genus *Leersia*. Two species, *O. brachyantha* and *O. schlechteri*, remain in the genus *Oryza* but are on the boundary between *Oryza* and *Leersia*. *O. brachyantha* is closer to the genus *Oryza* than *Leersia* although it shares with *Leersia* anatomical features of the leaf midrib rather than *Oryza*. Distinct features of this species are that it has the smallest genome size of *Oryza* species and its narrow ecological adaptation to laterite (iron) rock pools where it can be sympatric with *Oryza barthii* *O. schlechteri* is also closely related to *Leersia* but scanning microscopy of the spikelets confirmed that it is an *Oryza* species due to the presence of coriaceous palea and lemma and sterile lemma. However, overall habit and morphology of *O. schlecteri* are more similar to *Leersia* species that are also polyploid. Therefore *O. brachyantha* and *O. schlechteri*, while both very different from each other, could be considered species that link to the genus *Oryza* to *Leersia*.

One much studied species, *Porteresia coarctata*, was previously in the genus *Oryza* but detailed taxonomic studies showed it is sufficiently different from other *Oryza* species to have been placed in its own genus The morphological traits on which the genus *Porteresia* was established are morphological and anatomical features of leaves and caryopsis

2. Where are species and genomes of *Oryza* distributed?

Genome designation in the genus *Oryza* is based on chromosome pairing at meiosis of F_1 hybrids between different taxa. This cytological approach provides a measure of the divergence of the whole genome. Recently other rapid approaches to determining genome designation have been employed such as total genomic DNA

hybridization (Aggarawal et al, 1997) and gene phylogenies (Ge et al, 1999) Total genomic DNA hybridization is a whole genome approach and results from this method seem reasonable. However, use of gene phylogenies requires confirmation at the whole genome level for genome designations to be accepted. Genome diversity is a better approach to understanding *Oryza* evolution than species diversity since species are rather difficult to distinguish from one another, intermediate accessions between species are known and different rice workers have different opinions regarding the number of species in the genus *Oryza*.

The distribution of genomes of the genus *Oryza* reveals that more genomes are to be found in Australia-New Guinea than anywhere else. Latin America has *Oryza* species with 3 genomes, Africa with 4, Asia with 7 and Australia-New Guinea with 8. Comparison of the main islands of the Malay Archipelago also reveals highest genome diversity on New Guinea (Table 1)

Fig. 1. Relationships among genera within the tribe *Oryzeae*. Size of box approximates to the number of species (from Vaughan, 2003)

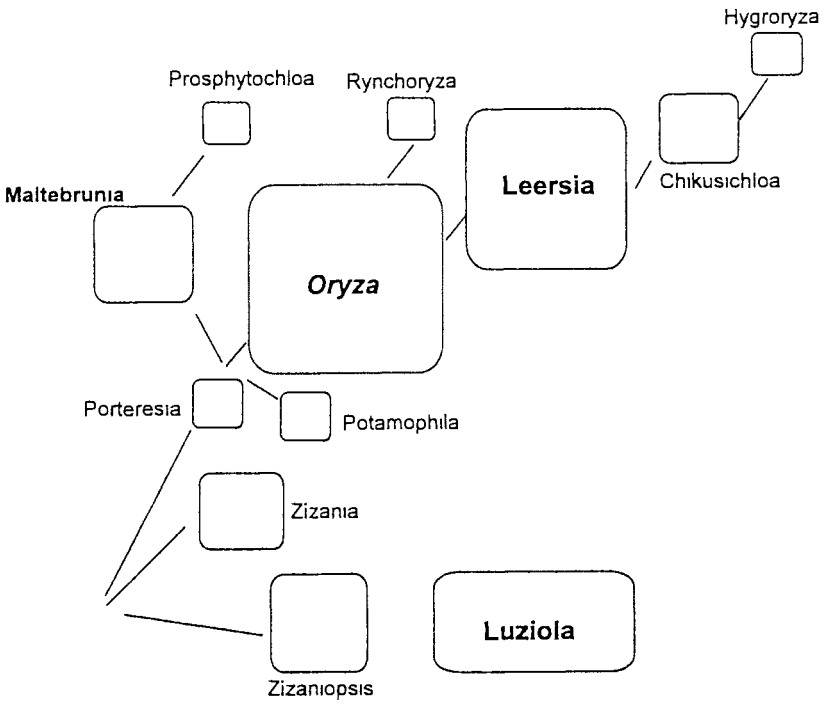


Table 1. *Oryza* species and their genomes on the largest of the islands in the Malay Archipelago.

Island	Number of species[1]	Number of genomes[2]
Borneo	4	5
Mindanao	4	4
New Guinea	7	7
Sumatra	5	5

[1] Borneo (*O. meyeriana, O. officinalis, O. ridleyi, O. rufipogon*), Mindanao (*O. meyeriana, O. minuta, O. officinalis, O. rufipogon*), New Guinea (*O. longiglumis, O. ridleyi, O. minuta, O. officinalis, O. rufipogon, O. meridionalis, O. schlechteri*), Sumatra (*O. granulata, O. meyeriana, O. officinalis, O. ridleyi, O. rufipogon*) It might be expected that a member of the *O. granulata* complex is present in New Guinea since species from this complex are present to the west and southeast of New Guinea. However, to date no reports of species from this complex in New Guinea have been reported.

[2] In this table it is assumed based on distribution and morphology that the two genomes of *O. schlecteri* are different from others in the genus *Oryza*.

3. Ecological factors, speciation and *Oryza* species complexes

Understanding the species complexes in the genus *Oryza* requires consideration of the their characteristic habitats

Only species in the *O. granulata* complex do not occur in seasonal or permanently wet habitats *O. granulata* complex species maybe in lowland or upland habitats Species in both the *O. granulata* and *O. ridleyi* complexes occur in forests, thus, shaded habitats The *O. ridleyi* complex species are always in lowlands particularly along rivers banks of Southeast Asia and New Guinea.

O. sativa complex and *O. officinalis* complex species have been put in the same taxonomic section, section *Oryza*. However, these two complexes are genetically well diverged and ecologically have evolved in response to different primary selection pressures The *O. sativa* complex has evolved in aquatic habitats and the main (but not only) selection pressure resulting in diversity of species in this complex is response to the hydrological regime - thus annual and perennial life cycle reflect lack or abundance of water on an annual basis Regional variation within some species of the *O. sativa* complex is sometimes very clear. However, in some locations a single population may consist of

plants exhibiting annual, perennial and intermediate life cycles due to local variation in the hydrological regime, for example, a lakeside habitat Human selection during domestication of rice must have targeted high seed producing wild rice ecotypes and this does not strictly correspond to life history.

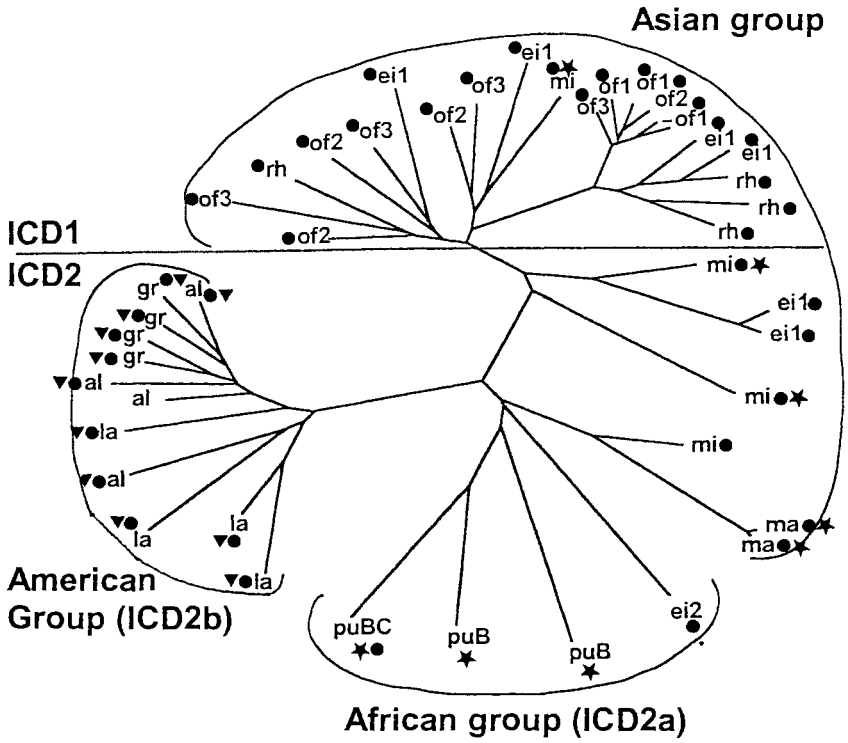
The *Oryza officinalis* complex in contrast to the *O. sativa* complex exhibits diversity primarily in response to light A measure of habitat disturbance, a characteristic associated with weedy plants appears common to most species in the complex and thus populations of species in this complex today tend to be small and fragmented. Population size maybe one reason, in contrast to species in the *O. sativa* complex, species in this complex have not been domesticated. A full understanding of why species in this complex were not domesticated needs investigation and advances in *Oryza* genome studies may provide this

The *O. officinalis* complex in contrast to the *O. sativa* complex includes several allotetraploid species The three CCBB species appear from results presented at this workshop (see abstract by Nishikawa et al), to have their maternal genome from CC genome species These three allotetraploid species are of shaded or partially shaded habitats Among diploid BB and CC genome *Oryza* species only one species, *O. eichingeri* (CC), is found in shaded habitats (see Vaughan 2003b; Vaughan et al 2003c)

We have undertaken several studies of the *O. officinalis* complex and species radiation within this complex (Fig. 2) The results show the high level of diversity in the diploid CC genome species *O. eichingeri* that may be closest to the ancestral type for the *O. officinalis* complex.

There are scientific questions of relevance to cultivated rice for which answers may be sought in the *O. officinalis* complex. Among these, reflecting comments above are; Do species in the *O. officinalis* complex have the capacity for domestication (seed size and production of some of the *O. officinalis* complex species are not much different from presumed progenitors of rice)? Why has autopolyploidy not successfully occurred in *Oryza* species? Why is genome size markedly larger in species of the *O. officinalis* complex? Why is genome radiation a marked characteristic of the *O. officinalis* complex but not *O. sativa* complex even though distribution is similar and genetic studies suggest that these two complexes may have evolved over a similar time span (cf. Second, 1984)

Fig. 2. Diversity in *O. officinalis* complex based on sequence diversity of cloned integrase coding domain of a gypsy like retrotransposon (modified from Shcherban et al, 2001) ★ BB genome; ● CC genome; ▼ DD genome; species indicated by first two letters of their specific name.



4. Future directions

i Despite intensive collecting of *Oryza* genetic resources there are important locations yet to be explored. Two vast river systems not yet explored are (a) the Orinoco of South America where wild *Oryza* species are known to exist and (b) the Mamberamo-Pegunungan Foja river system, Irian Jaya, Indonesia where information on *Oryza* species is lacking.

ii There is much that is not known about Australian *Oryza* genetic resources, particularly *O. australiensis* that has the largest genome in the genus *Oryza*. *O. australiensis* is widely distributed across northern Australia and its habitat is distinct from but often sympatric with AA genome species in Australia. *O. australiensis* occurs in more elevated and thus drier areas than AA genome species It appears to be a highly variable species with rhizomatous and non-rhizomatous forms Annual, biennial and perennial strategies are also reported for *O. australiensis*. It is high seed producing but reproductive isolation in its natural habitat seems to be ensured by its afternoon pollen shed (Oka, 1978, Second,

1987) The diversity of this species has been little studied and it may be worthwhile determining if within this species there is much variation in genome size.

iii There has been much speculation regarding the DD genome. The are three explanations regarding the yet undiscovered diploid DD *Oryza* genome species,

a. It is present in an *Oryza* accession already in germplasm collections There is currently no credible evidence that this is the case.

b. It occurs in a species that is now extinct

c. The DD genome species is yet to be found. Given the large areas of Latin America not yet explored for *Oryza* genetic resources this later seems most plausible explanation. For example, *Oryza* germplasm of the *O. officinalis* complex from the Pacific coast of Ecuador has not been studied.

iv. Variation within species and standard accessions

As indicated at the start of this paper some species are at the boundary of the genus with other genera. Similarly some accessions can be considered at the boundary of two species We have identified, based on genetic markers, one population of *O. rhizomatis* that is intermediate with *O. eichingeri* in Sri Lanka (Bautista et al , this conference) Tateoka (1965a,b) identified two populations based on morphological characters of *O. eichingeri* that were intermediate with *O. punctata*. Thus all accessions of a species are not equally representative of a species

Species often show clear genetic differentiation in relation to location where they grow. Thus an accession of *O. rufipogon* for Indonesia means something different for an accession of *O. rufipogon* from Sumatra, Indonesia and *O. rufipogon* from Irian Jaya, Indonesia.

These comments explain the need for what we have called a standard set of germplasm or accessions representative of species diversity. Our Asian *Vigna* research has repeatedly used, for a wide range of studies, the same set of accessions that from our collection best represent the species diversity of Asian *Vigna*. A similar approach based on international agreement to study species in the genus *Oryza* would help further our understanding of the genus Certainly the accessions used to create the BAC libraries for the genus *Oryza* would logically be included in the standard set Our distinction between standard germplasm set and core collection of germplasm is that the standard set is much more restricted in number and accessions represent as near as possible typical accessions of the species This necessarily requires analysis of intra-specific genetic diversity.

Nipponbare and Kasalath have become two of the main standard accessions for rice research, which accessions should be the standard accessions for *Oryza* research?

Some of the characteristics of a standard set of accessions should include:

a. Accessions that produce sufficient seeds for research purposes (this will mean some bias to high seed producing accessions),

b. To the extent possible choose accessions where the original population still exist and are accessible;

c. Accessions for which herbarium specimens have been widely distributed;

d. Coordination by the Rice Genetic Stock Committee of the International Rice Cooperative and Genetic Resources coordinators of countries where these populations originated and exists

v. What does *Oryza* research offer to rice research?

The usual merits given for research on the wild relatives of rice are their usefulness as a source of genes for resistance to pathogens, pests and eco-edaphic stresses Genes for resistance to pests and diseases might most successfully be sought in accessions from areas where wild rice grows in areas where rice has been grown since ancient times The zone south of the Asian mountain chains into which winds and rain are driven during the monsoon are likely places to find pest and disease resistance in wild rice. However, logic is not necessarily an approach to finding sources of resistance since both allopatric and sympatric resistance are know in rice germplasm. Useful sources of eco-edaphic stress resistance might be more widely found in the genus

Rice is a genome model for the monocots *Oryza* is a rather primitive, small genus in which species have widely diverged. *Oryza* may offer the best example among major cereals to understand what the domestication syndrome means The contrast offered by the *O. sativa* complex and *O. officinalis* complex may furnish answers not available with other cereals A full understanding of the genetics of domestication and inability to become domesticated are key research questions Contrasting Asian and African elephants can provide answers in relation to animal domestication. Perhaps contrasting the *O. sativa* complex and *O. officinalis* complex can provide some answers to these questions for plants

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The Oryza Map Alignment Project Toward a closed experimental system for the genus Oryza.

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With the approaching completion of the International Rice Genome Sequencing Project's penultimate goal of assembling a base-perfect finished rice genome sequence by December 2004, science must now set its sights on a complete functional characterization of rice. Such analyses will include functional annotation of the rice genome using full length cDNAs and mutant knockout lines, proteomic and metabolic profiling and comparative genomics Ultimately, the knowledge gained from this work will lead to superior rice cultivars that are essential to feed our ever expanding world population.

One important area in the functional characterization of rice is in the analysis of the wild relatives of rice. Wild rice species offer an enormous potential to expand the gene pool for cultivated rice as well as help in the identification of conserved regulatory elements To this end our consortia was just funded by the US National Science Foundation to develop and exploit a set of tools designed to physically align the genomes of representatives of 12 wild rice species (Table 1) to the sequenced *Oryza sativa* ssp. *japonica* cv. Nipponbare genome – The *Oryza* Map Alignment Project (OMAP www.omap.org)

The long term objective of OMAP is to create a genome-level closed experimental system for the genus Oryza that can be used as a research platform to study evolution, development, genome organization, polyploidy, domestication, comparative genomics, gene regulatory networks, positional cloning, and crop improvement

The specific objectives are to:

- Construct fingerprint/BAC end sequence physical maps of 12 wild rice species comprising the 10 genome types
- Align the 12 physical maps with the sequenced reference AA genome diploid *Oryza sativa* ssp. *japonica* cv. Nipponbare
- Reconstruct rice chromosomes 1, 3 and 10 across all 12 wild species
- Data mine BAC end sequence/positional information for SNPs, repetitive elements etc.

In my talk I will discuss OMAP, recent progress in BAC library construction of the wild species, and a proposal to determine if the wild species accessions that we are aligning to the sequenced japonica genome can be used as the standard reference species for experimental work throughout the world.

Table 1

Genus species	Genome type	Genome size	Clones	Genome coverage
Oryza rufipogon	AA (annual)	760	29,231	5X
Oryza rufipogon	AA (perennial)		29,231	5X
O. glaberrima	AA	809	31115	5X
O. punctata	BB	539	41,462	10X
O. officinalis	CC	1201	92,385	10X
O. minuta	BBCC	1691	130,077	10X
O. australiensis	EE	1054	81,077	10X
O. latifolia	CCDD	1127	86,692	10X
O. schlechteri	HHKK	1568	120,615	10X
O. ridleyi	HHJJ	1568	120,615	10X
O. brachyantha	FF	343	26,385	10X
O. granulata	GG	907	69.769	10X

Cytogenetical Studies on Alien Chromosome Addition Lines in Rice (*Oryza sativa* L.), Carrying Extra Chromosomes of *O. punctata* Kotschy

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Key words: *Oryza sativa* L., *Oryza punctata,* Kotschy, monosomic alien addition line (MAAL), introgression, insect resistance,

Abstract

Genome analysis of the genus *Oryza* based on chromosome pairing in interspecific hybrids has been carried out and A, B, C, D, E, and F genomes have been identified. The genus *Oryza* includes 22 species, two of which are cultivated and the others are wild relatives (Vaughan 1994) *O. sativa* and *O. glaberrima* which are A genome species consist of cultivated rice and the remaining 20 species including five A genome species are wild relatives Wild relatives are sources of useful genes for resistance to major diseases and insect pests (Heinrichs *et al* 1985) as well as to abiotic stresses (Swaminathan 1986) A comparison of the percentages of cultivated and wild rice accessions resistant to various pests revealed that the high proportion of wild accessions showed resistance to insect pests The wild relatives with such resistance to insect pests and pathogen are useful germplasm for rice breeding. Hybrids within A genome species can be easily obtained and alien gene from wild relatives, for instance, transferred to cultivated rice (Fujita *et al* 2003)

To transfer useful genes from more distantly related species is much more difficult

because of their poor crossability with cultivated rice. Wide hybridization between *O. sativa* and such wild relatives has been carried out (Jena and Khush 1984, Brar *et al* 1991) The first step in transferring alien genes is the development of monosomic alien addition lines (MAALs), which have an alien chromosome in addition to the diploid complement of the rice cultivar Jena and Khush (1989) first developed a complete set of MAALs for *O. officinalis* (2n=24, CC genome) The second step involves the screening of alien introgression lines with intact alien genes or alien chromosome segments in the recipient genetic background. Another approach in transferring alien genes to cultivated rice is through the integration or substitution of small chromosome fragments derived from wild relatives to the normal chromosome complement This research was conducted to study the cytogenetical basis of various kinds of alien chromosome addition lines each carrying an *O. punctata* chromosome(s) and to advance basic knowledge about transferring alien genes of wild rice to cultivated rice.

A complete set of MAALs was produced to determine alien traits specific to *O. punctata* chromosomes (Yasui and Iwata 1991) Fifty-three monosomic alien addition plants (2n=25) were isolated from the three original interspecific hybrids (2n=3x=AAB) obtained from a cross between autotetraploid *O. sativa* L. of Japonica cultivar Nipponbare (2n=4x=AAAA) and a diploid strain of *O. punctata* Kotschy, W1514 (2n=2x=BB) Among them, 39 can be grouped to 12 types on the basis of their morphological resemblance to the 12 primary trisomics of cultivar Nipponbare (Iwata and Omura 1984) Morphological features of the other 14 MAALs did not correspond to any of the primary trisomics, hence could not be classified under any groups Of the 12 morphological types, 10 were fertile and the alien chromosome can be transmitted to each progenies, MAAL 3 and MAAL 12, however, were completely sterile. Cytological analysis showed mainly $12_{II}+1_{I}$ association at meiosis in all the MAALs suggesting that gene transfer from *O. punctata* into *O. sativa* cv. Nipponbare through recombination is very low in frequency.

Several traits derived from a diploid strain of *O. punctata* were assigned to the specific chromosomes by comparing each primary trisomics with the respective MAALs, which have the same genetic background of japonica cultivar Nipponbare. The addition of *O. punctata* chromosome 12 to the genetic background of japonica cultivar Nipponbare with Nipponbare cytoplasm caused severe male sterility. This suggests that one of the gene(s) for F_1 sterility between *O. sativa* and *O. punctata* was assigned to chromosome 12 transferred from *O. punctata*. Thus it is necessary to eliminate these gene(s) on *O. punctata* chromosome 12 to transfer successfully alien genes from *O. punctata* to Japonica cultivar Nipponbare. Major genes for purple stigma, red pericarp pigmentation, spotted leaves at ripening stage and late heading were also assigned to chromosomes 3, 7, 8 and 11 of *O. punctata*, respectively. In addition, green leafhopper resistance transferred from *O. punctata* was also assigned to a specific alien chromosome of a MAAL in which alien chromosome could not be identified by karyotype analysis.

More fertile alien chromosome addition lines were developed with stable transmission of the gametes carrying an alien telocentric or acrocentric chromosome (Yasui and Iwata 1998a) These plants were designated as monotelosomic alien addition lines (MtAALs: 2n=2x+1t) 2, 7 and 9S (short arm of *O. punctata* chromosome 9) and monoacrosomic alien addition line (MaAAL) $4S^{4L}$, respectively. Most of the PMCs in the MtAALs and the MaAAL showed $12_{II}+1_{I}$ configuration at diakinesis and first metaphase. This suggests that the extra telocentric or acrocentric chromosomes originated from a misdivision of an alien univalent at anaphase I or anaphase II followed by chromosome breakage in the respective MAALs Their seed fertility was higher than the respective MAALs, suggesting that the addition lines carrying a small chromosome fragment such as telocentric or acrocentic chromosomes can produce functional

gametes Ditelocentric alien addition lines (DtAALs 2n=2x+2t) of rice each carrying a pair of telocentric chromosomes of *O. punctata* were isolated from the progenies of respective MtAALs 7, 11 and one unidentified MtAAL (Yasui and Iwata 1998b) During the meiosis, the alien telocentric chromosome of the three DtAALs completely paired at the pachytene and usually separated to each daughter cell at anaphase I, giving rise to viable gametes with an alien telocentric chromosome at high frequencies The transmission rates of the alien chromosome were considerably high in the self-pollinated progeny of DtAALs The DtAALs showed stable transmission of alien telocentric chromosome(s), where gametes with an extra telocentric chromosome are functional in both female and male germ cells This suggests that a telocentric chromosome, that is a small chromosome fragment with functional centromere, can be transmitted to the progenies and stable in the next generation. These alien telocentric chromosomes may be transferred to any cultivated genetic background through hybridization.

Genomic *in situ* hybridization (GISH) using total *O. punctata* DNA as a probe was applied to detect alien chromosomes transferred from *O. punctata* to the Japonica cultivar, Nipponbare (Yasui *et al* 1997) GISH using *O. punctata* total DNA allowed the detection of a specific chromosome transferred into cultivated rice. This method may be useful to identify introgressed segments or translocated chromosomes in the progenies of the MAALs each carrying an alien chromosome of wild relatives

Introgression has been extremely limited in the disomic progeny of MAALs because single homoeologous chromosomes from *O. punctata* usually form univalent during meiosis of MAALs Thus, it may be impossible that homoeologous recombination occurs during the meiosis of MAALs To transfer alien chromosome segments to cultivated rice, monitoring disomic derivatives in the BC_2 progeny derived from the hybrids between wild and cultivated rice will be proposed. The F_1 plants have a complete set of chromosomes of cultivated and wild species

where the homoeologous chromosomes may rarely pair and exchange each other Several genes for favorable traits had been successfully introgressed from wild relatives to cultivated rice in the disomic progenies of BC_2 plants (Jena and Khush 1990, Ishii *et al* 1994) Another approach for alien introgression will be gamma-ray irradiation of MAALs carrying limited amount of alien chromosome segment(s) conferring favorable agronomic traits

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POSTER PRESENTATIONS

PROCEEDINGS OF INTERNATIONAL GENETIC RESOURCES WORKSHOP ON THE GENUS *ORYZA*

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Genetic relationships among the three diploid CC genome species of *Oryza*

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Keywords: Oryza; CC genome species, molecular markers

There are three diploid CC genome species, *O. eichingeri*, *O. officinalis* and *O. rhizomatis*. The distribution of diploid CC genome species is from West Africa to Papua New Guinea. However, only in southern South Asia can all three species be found. The objective of this study was to analyze the genetic diversity of the three CC genome species from South Asia.

Materials and Methods

Materials used consisted of 19 accessions of *O. rhizomatis*, 7 of *O. eichingeri* (all from Sri Lanka), 4 of *O. officinalis* (from Kerala and Assam, India and China) Outgroup accessions of AA genome species were consisting of 8 accessions of *O. nivara* and 5 of *O. rufipogon* (all from southern South Asia) and 1 accession of *O. sativa* (Nipponbare- used only in SSR analysis) Three molecular techniques, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSR) were employed in the analysis of diploid CC genome species, *O. rhizomatis*, *O. eichingeri* and *O. officinalis* accessions Since there are no available SSR primers derived from CC genome species, the AA genome derived SSR primers from Cornell University were surveyed to find out which primers can be useful in detecting polymorphism in the diploid CC genome species Of these primers, 17.2% revealed polymorphism among the three diploid CC genome accessions These primers are distributed on the 12 linkage groups of rice, but relatively fewer primers were found in chromosomes 4, 9 and 10.

Results

1 All three molecular techniques revealed that *O. rhizomatis* is genetically more closely related to *O. eichingeri* than *O. officinalis* (Table 1) One accession of *O. rhizomatis* clusters with *O. eichingeri* accessions (Fig. 1) This accession has all the morphological characteristics of *O. rhizomatis* but was collected within 5 km of populations of *O. eichingeri* in Sri Lanka. Both *O.*

rhizomatis and *O. eichingeri* occur in Sri Lanka whereas *O. officinalis* is found in other parts of Asia.

2. AFLP and RAPD analyses revealed eco-geographic differentiation in *O. rhizomatis*. Cluster analysis grouped the accessions of *O. rhizomatis* from northern and southern Sri Lanka separately. This was not revealed by SSR analysis and that may be due to the larger number of polymorphic bands revealed by the dominant markers RAPD and AFLP compared to those of the co-dominant SSR markers

3 AFLP and RAPD analyses revealed that inter-population genetic diversity in diploid CC genome species was greater than AA genome species (*O. nivara* and *O. rufipogon*) from the same region (Table 1) This suggests that the CC genome species may be a rich gene source for rice improvement

4. Intra species genetic diversity among the CC genome species analysed was greatest for *O. officinalis* and least for *O. eichingeri* This may be a reflection of habitat stability of *O. eichingeri* compared with the other two CC genome species

Table 1. Average genetic distance between pairs of the 3 diploid CC genome species and among AA and CC genome species of rice based on RAPD, AFLP and SSR analyses

Comparison	RAPD		AFLP		SSR	
	N	GDAve	N	GDAve	N	GDAve
Between *O. rhizomatis* and *O. eichingeri*	102	0.1901	133	0.1369	133	0.0908
Between *O. rhizomatis* and *O. officinalis*	68	0.1930	76	0.1633	76	0.1146
Between *O. eichingeri* and *O. officinalis*	24	0.1954	28	0.1701	28	0.1102
Among CC genome accessions	351	0.1493	435	0.1063	435	0.0807
Among AA genome accessions	78	0.1096	78	0.0639	91	0.0892

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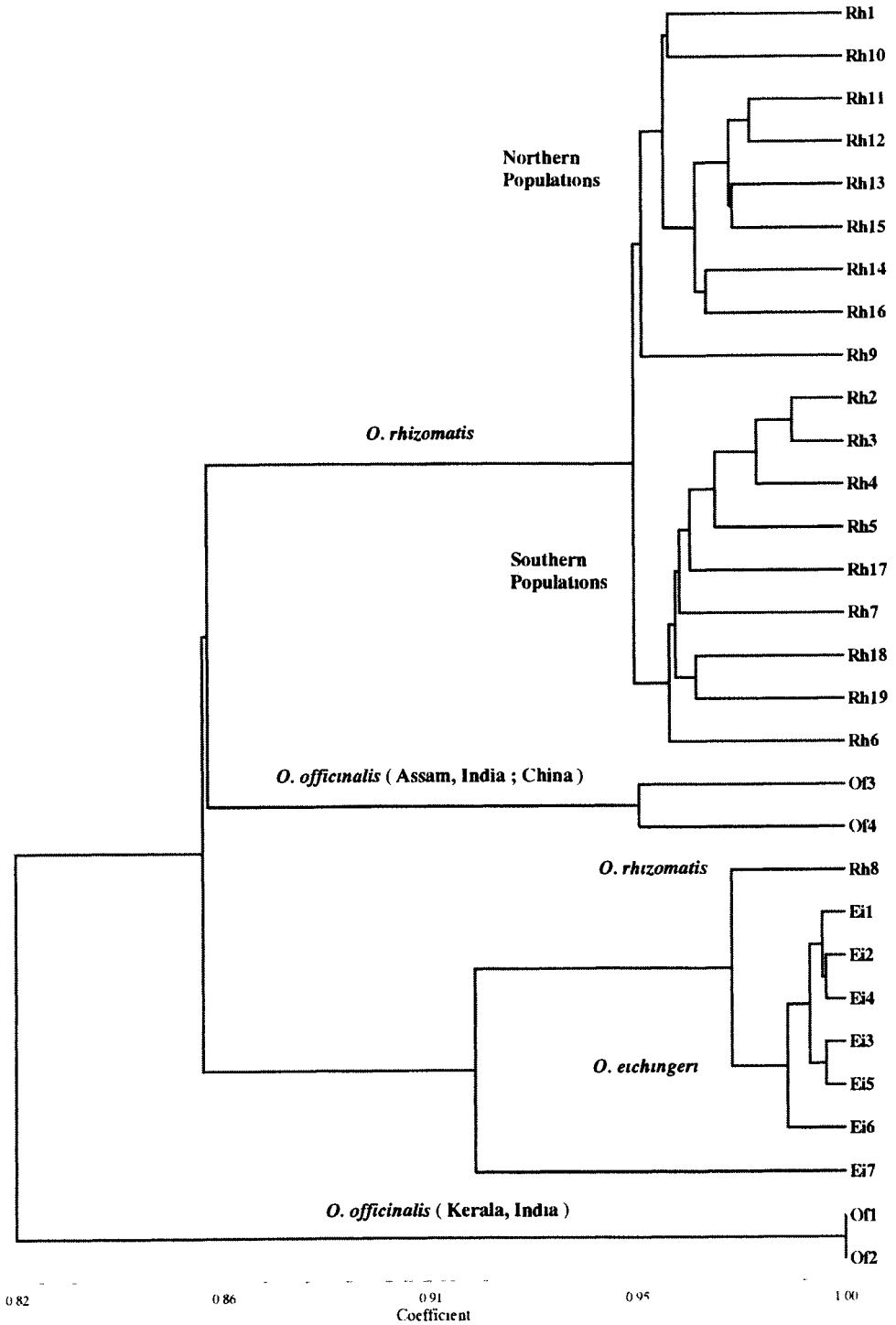

Fig. 1. Dendrogram of diploid CC genome species diversity based on polymorphism revealed by AFLP analysis. Rh – *O. rhizomatis*; Ei – *O. eichingeri*; Of – *O. officinalis*.

Biased distribution of pyrimidine-rich microsatellites in Rice and Arabidopsis Full-length cDNAs

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Keywords: microsatellite, tandem repeat, full-length cDNA

Summary: Repeats, such as microsatellites, are ubiquitously present in eukaryotic genomes. Microsatellites located within or near transcribed regions can potentially affect the transcriptional or translational activities. In this research, we conducted a computational analysis to assess the variations of microsatellites in transcribed regions using full-length cDNAs of rice (*Oryza sativa* ssp. japonica c.v. Nipponbare) (Kikuchi S. *et al.* 2003) and *Arabidopsis thaliana*. In addition, human and mouse (Okazaki Y. *et al.* 2002) were simultaneously analyzed for a comparative analysis. Our analyses confirmed that the densities of microsatellites near transcription start sites (TSSs) in plant genes were higher than those in mammals (Fig. 1). It was also confirmed that microsatellites containing CpT dinucleotides, such as (CT)n, and (CCTT)n are found intensively near the TSSs, specifically in the two plants, but not in the mammals. Our additional finding is that microsatellites containing CpG dinucleotides (e.g., (CG)n, (CGCT)n, and (CGTCT)n) are preferentially located in TSS-flanking regions only in rice. Our results suggest that microsatellites located with high frequency in 5'-flanking regions can potentially act as factors in transcriptional and/or translational regulation, possibly repressing transcription in response to methylation, controlling transcription efficiency through forming unusual DNA structures, or being *cis*-regulatory element to control translation efficiency.

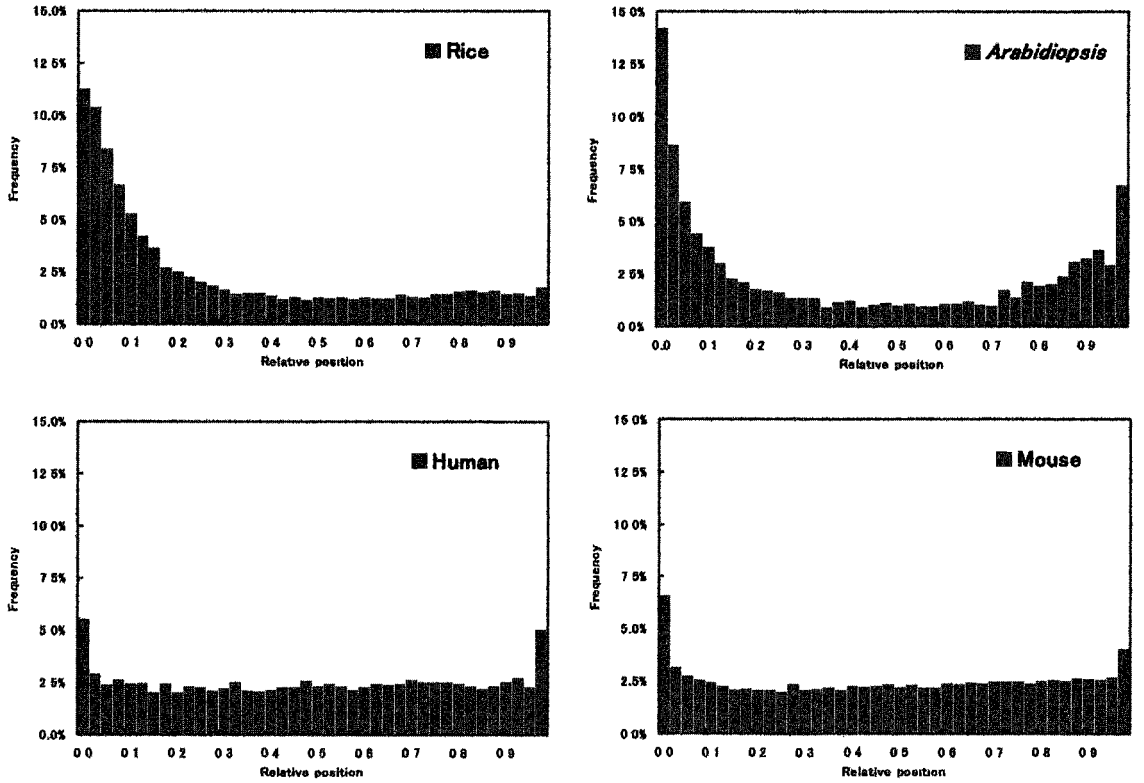

Figure 1. Relative frequencies of microsatellites in full-length cDNAs. Histograms illustrate percentages of repeats on each position, in cDNAs. The relative position of the microsatellite in each cDNA was calculated as the sequence length of the upstream of the microsatellite divided by the length of each cDNA excluding the microsatellite.

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Mapping of a new resistance gene for green rice leafhopper introgressed from *Oryza rufipogon* Griff. to cultivated rice, *Oryza sativa* L.

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Keywords, *Oryza sativa*, Green rice leafhopper (GRH), resistance gene, introgression, *Oryza rufipogon*

Wild relatives are sources of useful genes for resistance to major diseases and insect pests The *Nephotettix cincticeps* Uhler (Green rice leafhopper GRH) is one of the serious insect pests of rice in temperate Asia. An accession of wild relative, *Oryza rufipogon* Griff. (W1962) was found to be highly resistant to GRH by testing antibiosis (Kishino and Ando 1978) In the present study, introgression lines of rice each carrying *O. rufipogon* chromosome segments were used to analyze the GRH resistance introduced from *O. rufipogon*. A resistant accession of *O. rufipogon* (W1962) as female was crossed with a Japonica variety Taichung 65 as male parent The hybrids was obtained and continuously backcrossed by Taichung 65 to BC_4F_1

To evaluate nymph mortality as GRH resistance, leaf blades of 98 BC_4F_2 plants were excised and infested with 7-10 first- or second-instar nymphs The insect population was collected in Fukuoka Prefecture in 1991, and have been rearing at 25℃ and 16h light 8h dark condition. The nymph mortality in BC_4F_2 showed a discrete distribution, segregating into 69 resistant and 29 susceptible plants The segregation ratio fitted to a 3 1 ratio (χ^2=1.35), indicating that the GRH resistance was controlled by a single dominant gene designated as *Grh5* (Green rice leafhopper resistance 5), tentatively. To determine the chromosomal location of *Grh5*, BC_1F_1 plants were analyzed using 767 Simple Sequence Repeats (SSR) markers (McCouch *et al* 2002) by bulked segregant analysis The linkage between *Grh5* and one SSR marker, MRG3154 was detected. Further SSR analysis using BC_4F_2 revealed that *Grh5* was located between MRG0615 and MRG5845 and tightly linked to MRG2754 and MRG2761 on the distal region in long arm of chromosome 8 (Fig. 1) There have been no GRH resistance genes located on chromosome 8. We finally identified a new GRH resistance gene located on chromosome 8 using *O. rufipogon* introgression lines Nearly isogenic line for *Grh5* was selected in BC_4F_2 population. The nymph mortality was more than 90% that is higher than that of variety carrying single GRH resistance genes reported previously. Thus, *Grh5* introgressed from *O. rufipogon* is a favorable gene resource for rice improvement

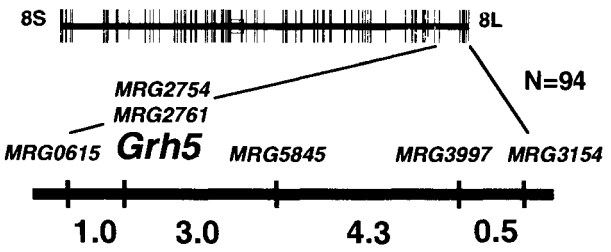

Fig. 1 Linkage map of *Grh5*, a gene for resistance to green rice leafhopper, on chromosome 8. Framework map on the upper was quoted from Harushima *et al* (1998)

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Variation in the spacer length of the ribosomal RNA gene cluster in *Oryza sativa*.

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Keywords ribosomal RNA gene, non-transcribed spacer, heterogeneity, *Oryza sativa*

Genes encoding 17S, 5.8S and 25S ribosomal RNA (rRNA) are clustered in tandem at one or few loci in the genome and form nucleolus, that is a structurally distinct entity in nucleus and the site for biogenesis of ribosome. RNA polymerase I transcribes the precursor RNA that contains above three rRNA sequences and the transcript is processed to generate each rRNA species Transcription start and termination sites locate within the non-transcribed spacer region that separates 25S and 18S rRNA coding sequences In general, the non-transcribed spacer region consists of a set of complex repeats and, in some organisms, additional promoters that generate short transcripts with unknown function have been identified in the non-transcribed spacer region. DNA methylation in the non-transcribed spacer region has been shown to be involved in the epigenetic regulation of the rRNA gene activity in inter-specific hybrids of *Brassica*, known as nucleolar dominance.

Previous studies have shown that there is a considerable variation in the length of the non-transcribed spacer in the genus *Oryza*. The length heterogeneity has often been observed even within individuals in a cultivar and the length variants were inherited independently, indicating that the rRNA gene clusters carrying different spacer variants locate at unliked loci in the genome.

Towards understanding the epigenetic regulation of rRNA genes in *Oryza sativa*, we re-examined the length heterogeneity in the non-transcribed spacer length in twelve cultivars of *Oryza sativa*, including three *japonica*, two *javanica* and seven *indica* cultivars. The length heterogeneity within individuals was observed all of the cultivars of *javanica* and *indica*, but not in those of *japonica*. The differences in the length of the variants are often slight and intensity of one of the signals is weak in many cases Since it has been shown that Kasalath has two rRNA loci each on chromosome 9

and 10, respectively, we analyzed the Nipponbare-Kasalath chromosome segment substitution lines carrying the Kasalath sequence of the short arm of chromosome 9 in the Nipponbare background and the results indicate that the major variant of the non-transcribed spacer in Kasalath locates in the short arm of the chromosome 9.

The results presented here show that the length heterogeneity in the non-transcribed spacer of rRNA gene cluster is common in *javanica* and *indica* cultivars The presence of the length heterogeneity in *javanica* and *indica*, but not *japonica*, cultivars coincides with the previous data of *in situ* hybridization showing that *javanica* and *indica*, but not *japonica*, cultivars tend to have two rRNA loci in the genome. The representative data of the Nipponbare-Kasalath chromosome segment substitution lines suggest that most of the *javanica* and *indica* cultivars possibly carry a distinct but homogeneous sequence of the non-transcribed spacer in each cluster of the rRNA genes

Development of DNA markers close to the wheat *Wx-B1* locus using rice genomic sequences

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Keywords: *Triticum aestivum*, *Wx-B1* locus, DNA marker, genetic colinearity

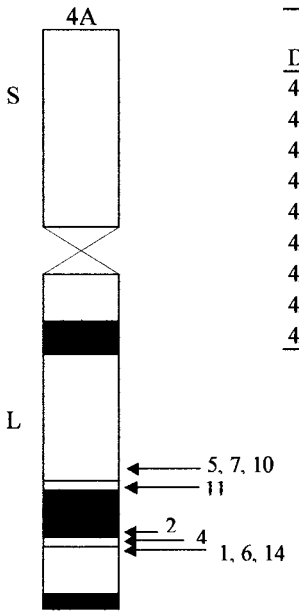
In hexaploid wheat (*Triticum aestivum* L.), there are three granule-bound starch synthase genes (*GBSS*) genes, also known as *waxy* genes, which synthesize amylose in endosperm tissue. Recently, null *Wx* alleles for each Wx protein have been identified in wheat varieties from around the world by several groups. Each null allele has an effect on the amylose content in starch, thereby greatly influencing the quality of the Japanese noodle, Udon. Zhao et al. (1998) reported that all of the Australian cultivars preferred for making white salted noodle carried the null *Wx-B1* (*Wx-B1b*) allele. However, the superior noodle making quality of these cultivars could not be explained simply by a decrease in amylose content caused by the null *Wx-B1* allele.

The molecular analysis of *Wx-B1b* indicated a deletion of the entire *Wx-B1* coding unit (Vrinten et al. 1999), but the deletion breakpoints of the *Wx-B1b* allele are still unknown. Thus, there is a possibility that gene(s) related to noodle making quality were lost along with the *Wx-B1* gene when the deletion event occurred. Development of DNA markers around the *Wx-B1* locus is one way to discover the deletion breakpoints of *Wx-B1b*, but there is little sequence information around the *waxy* loci of wheat, and any reported DNA makers reported are distant from the loci. Therefore, we tried to develop new DNA markers tightly linked to the *Wx-B1* locus using the colinearity between the rice and wheat genome.

The *Wx-A1* and *Wx-D1* genes of wheat are located on distal parts of the short arms of respective group 7 chromosomes, while the *Wx-B1* gene is located on the long arm of 4AL because of a translocation of the 7B short arm (Gale and Devos 1998). The positions of common DNA markers for cereals, including the *waxy* gene, indicate a broad level of colinearity between wheat group 7 chromosomes and rice chromosome 6. We are now able to access all rice genome sequence data provided by the National Institute of Agrobiological Sciences DNA bank. We used wheat EST clone sequences as

queries and searched for rice genomic sequences showing high homology using BLAST. Several wheat EST clones showing high homology scores to sequences near the rice *waxy* gene were identified. A number of primer sets were constructed using sequence information from the EST clones.

From these primer sets, we were able to select two sets of primers which only generated the expected PCR fragment from the 4A chromosome. Subsequent analyses with deletion lines of 4AL (Yamamori et al. 1994) showed that the amplified fragments could be used as markers. These are the closest markers to the *Wx-B1* locus that have been identified to date. Based on a rice physical map, one marker locates on a position about 6.5 kbp distal to the centromere from the *waxy* gene and the other one locates about 40 kbp proximal to the centromere. However, the marker fragments were amplified by the PCR primer sets when DNA samples from Australian cultivars carrying the *Wx-B1b* allele were used as templates. This indicated that that the deletion breakpoints of the *Wx-B1b* allele are inside of these markers.



Deletion line	*Wx-B1*	DNA maker A	DNA maker B
4AL-5	-	-	-
4AL-7	-	-	-
4AL-10	-	-	-
4AL-11	-	-	-
4AL-2	-	-	-
4AL-4	-	-	+
4AL-1	-	-	+
4AL-6	+	+	+
4AL-14	+	+	+

Fig. 1. Breakpoints of deletion lines are indicated by arrows beside a 4A chromosome ideogram (Yamamori et al., 1994) Presence (+) or absence (-) of *Wx-B1* gene and amplified products with the generated DNA markers are shown in tabular form. Based on a rice physical map, the marker (A) locates on a position about 6 5 kbp distal to the centromere from the *waxy* gene and the (B) locates about 40 kbp proximal to the centromere

Acknowledgments

The deletion lines were provided from Laboratory of Plant Genetics, Graduate School of Agriculture, Kyoto University, which supported by the National Bio Resource Project from the Ministry of Education, Science and Culture, Japan.

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Rice Growth Imaging System for Phenotypic Functional Analysis from Middle Seedling to Mature Plant

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Key words: phenotype, image, measurement, leaf growth, long-day

We have been developing an automatic digital imaging system (Hitachi Rice Imaging System; HI-RIS) for assigning rice gene functions by measurement of detailed visible phenotypes in mutants and transgenic plants (1). For the purpose discussed here, we made "Type junior" for analyzing the growth of rice plants and tillering. This system is composed of an image capturer and an image analyzer. The image capturer acquires growth images of 24 plants at 60-minute intervals. The image analyzer visualizes the growth of plants and measures forms of organs continuously and comprehensively using sequential images.

Firstly, we measured the growth of *Oryza sativa* L. cv. Nourin-8 under a long-day condition (14-h light/10-h dark) from the 14th day to the 50th day after seeding using HI-RIS

system. The temperature was 28℃ in the light and 23℃ in the dark. From each plant we acquired approximately 4000 images from 8 different angles. These sequential images made it possible to identify each leaf of main culm and tillers for measuring the rate of plant height growth. Plant height was measured as the distance from the ground to the highest point of leaves of main culm. Similarly, the heights of two primary tillers were examined. New leaves appeared within a cycle of 3 to 5 days in both main culm and tillers. Each leaf of the primary tillers elongated synchronously with the corresponding leaf of the main culm (2) The fifth to tenth leaves subsequently grew at a maximum growth rate (3-5mm an hour) for 2 days, then their growth rate decreased within 4 days

Next, we compared the growth of the same material under a short-day condition (10-h light/14-h dark) and the long-day condition in combination with two different temperature controls (light 28℃/dark 23℃ and light 30℃/dark 25℃) We found that the plants grown under long-day condition in higher temperature grew faster than those grown under the other conditions. Under the long-day/higher temperature condition, the periodicity of leaf appearance was shortest (3.1-3.8days), the maximum leaf growth rate was greatest (9.6-12.9cm/day) and the growing period of each leaf was shortest (3.2-4.3 days). This suggests that photoperiod and temperature affect their phenotypes. HI-RIS images effectively covered these visible growth and development procedures quantitatively.

This work was partly supported by grants from the Ministry of Agriculture, Forestry and Fisheries of Japan (Rice Genome Project SY-1108 and IP-1006).

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Large-scale Analysis of Alternative Splicing Regulation using Full-length cDNA of *Oryza sativa*

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Keywords: alternative splicing, exonic splicing enhancer (ESE), full-length cDNA.

1 Introduction

Although an extensive number of genes have been reported to be alternatively spliced, most of the information obtained by this process has not allowed for general understanding of alternative splicing regulation, especially in plants. The basic requirements for plant splicing is similar to those for vertebrate splicing; the consensus sequences of the 5'-splice sites and 3'-splice sites and the branch point region at the ~30 nt distance from 3'-splice sites are similar in plants and vertebrates (Lurhrsen, 1994). On the other hand, there are plant-specific requirements for pre-mRNA splicing; plant introns have a strong compositional bias for UA- or U-rich sequences and plant exons contains more GC-rich sequences than vertebrates (Lorkovic, 2000). Prior studies have indicated that the 3'-splice sites and 5'-splice site located at transition regions from UA- to GC-rich sequence is prefentially selected for splicing (Lou, 1993). Also, the mechanisms of splice-site recognition may differ between dicot and monocot plants; it has been indicated that the sequence composition of both introns and exons was clearly different between dicot and monocot plants (White, 1992). Here we compared alternative splicing regulation in four eukaryotes, *O. sativa, A. thaliana, H. sapiens,* and *M musculus,* and constructed models for alternative splicing regulation in *O. sativa.*

2 Results

First, we computationally detected putative splice variants by mapping full-length cDNA clones to complete genomic sequences; clones were then grouped into clusters if all the internal regions were correctly mapped. Putative splice variants were then classified according to alternative splicing patterns. We then compared the distribution of known splicing regulatory sequences, which tended to be exonic in all four species, suggesting that

the basic mechanism of pre-mRNA splicing is similar in all eukaryotes. Higher O/E (observed/expected) values of regulatory sequences in plants indicated that there may be plant-specific regulatory features other than known regulatory elements in metazoans. Based on these results, we constructed models for alternative splicing regulation in *Oryza sativa.* Figure 1 shows one of the constructed models; all of the constructed models are available at http://www.bioinfo.sfc.keio.ac.jp/research/intron/.

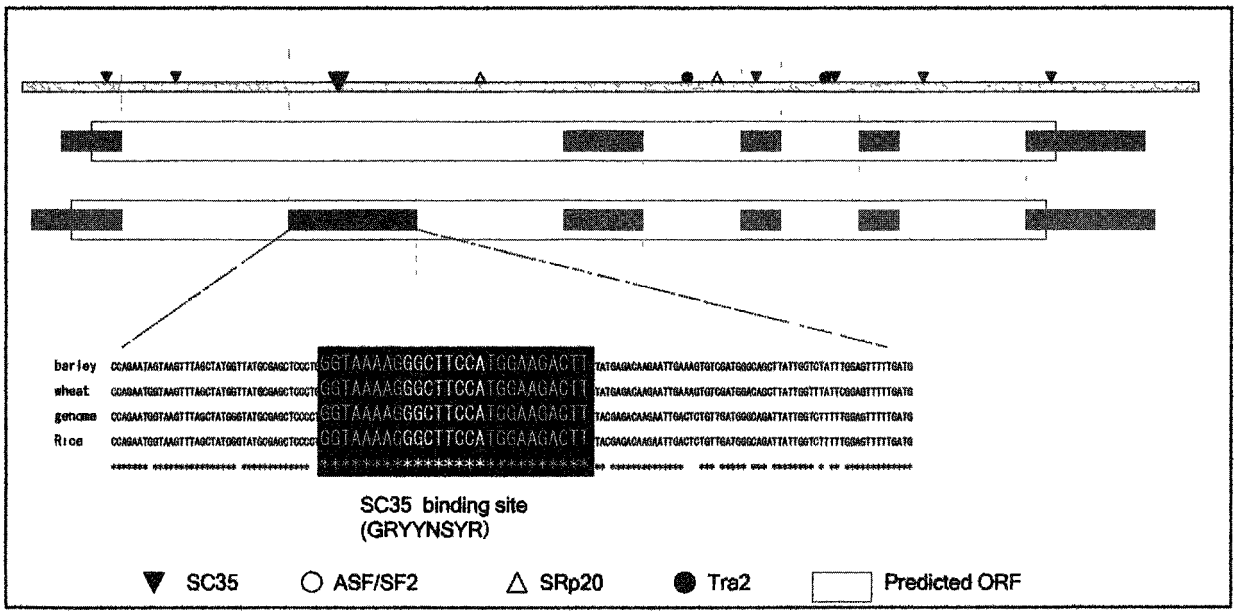

Figure 1. Cross-species comparison of putative alternate exon in transcripts homologous to *A. thaliana* unknown protein. Shaded box represent conserved regions across rice, barley, and wheat.

Acknowledgement

We would also like to thank TMRI for offering *Oryza sativa* genomic sequence data. This work was supported by the Ministry of Agriculture, Forestry, and Fisheries of Japan (Rice Genome Project SY-1104).

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all of the constructed models for Alternative Splicing.

Evidence for Tissue-specific Transcription regulated by Alternative First Exons in *Oryza sativa*

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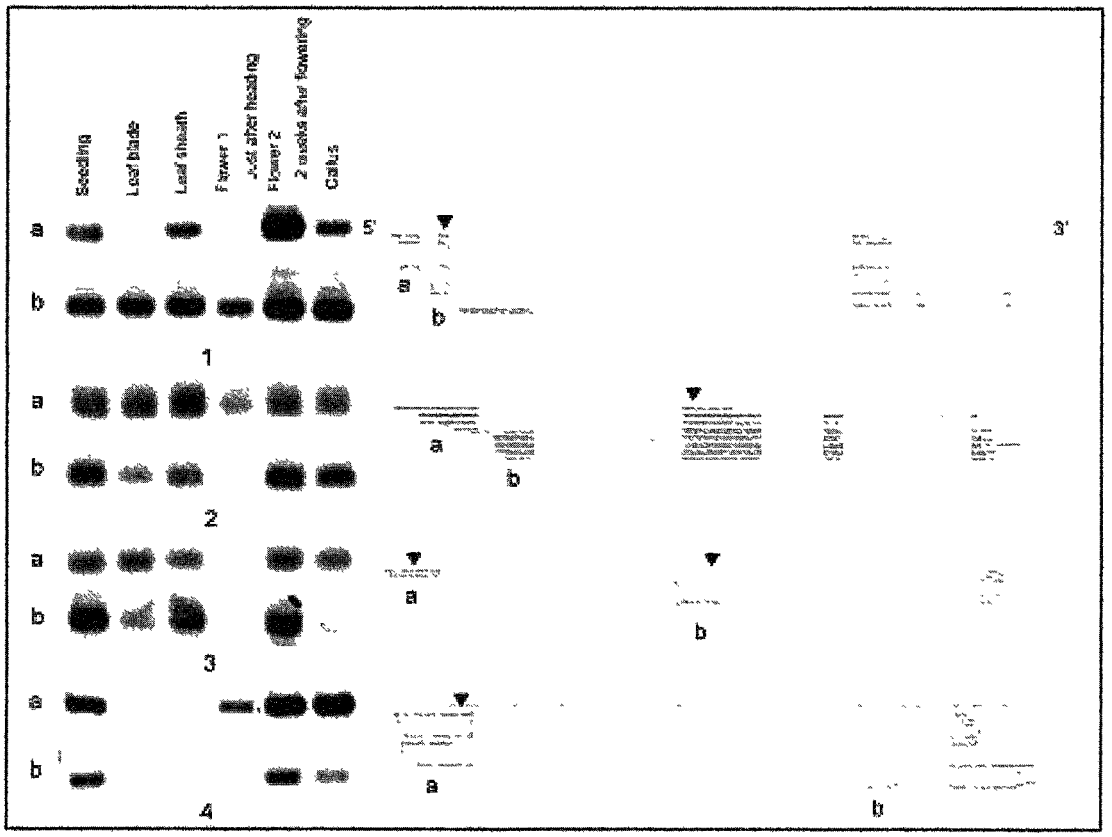
Keywords: alternative first exons, transcription start site, reverse transcription-polymerase chain reaction (RT-PCR), 5'-end EST.

Abstract

The diversity of transcription start-site and alternative splicing greatly contribute to expansion of protein diversity, but their regulations are still not clear, especially in plant. Although recent studies have revealed the existence of an alternative first exon (Hugnot *et al* 1992; Kelner *et al.* 2000; Zavolan *et al 2002*), most of them have been carried out in individual genes; as such, the mechanism regulating the alternative first exon has not yet to be understood. In the present study we detected a putative alternative first exon in *Oryza sativa*, verified the candidates using reverse transcription-polymerase chain reaction (RT-PCR), and searched for core promoters that might regulate the alternative first exon. As a result, we have predicted a number of unreported alternative first exons, some of which are regulated in a tissue-specific manner.

Figure 1. RT-PCR results for each cluster, indicating tissue-specific expression. Tissue names are given above each lane (Seedling, Leaf blade, Leaf sheath, Flower1 just after heading, Flower2 two weeks after flowering, and Callus). The transcript from exon 1a is shown in a, and that from exon 1b in b. Figures on the right are structures of 5'-end EST. Filled triangles indicate predicted start codons on full-length cDNA, corresponding to 5'-end EST of each cluster.



Acknowledgement

We would also like to thank TMRI for offering *Oryza sativa* genomic sequence data. This work was supported by the Ministry of Agriculture, Forestry, and Fisheries of Japan (Rice Genome Project SY-1104).

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Differential heterosis in a natural population of Asian wild rice (*Oryza rufipogon*) due to reproductive strategy and edge effect

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Key words: heterosis, SSR (Sinple Sequence Repeat), *Oryza rufipogon*, reproductive systems, sampling locations

Abstract

Asian common wild rice (*Oryza rufipogon*) is a polycarpic perennial plant with a mixed inbreeder/outbreeder strategy. Seeds and clones are both reproductive components of the population. However, when collected in the flowering stage, the two differ substantially as to whether they have experienced natural selection directly or not This study is aimed at evaluating mechanisms for the survival of a population by comparing genetic structure among

subpopulations that are classified in terms of 1) reproductive systems (clones or seeds) and also 2) location (fringe or inside; **Fig. 1**) First, the genotypes determined at seven SSR (Simple Sequence Repeat) loci showed that parameters of observed heterozygosity (H_O) and outcrossing rate (t) were clearly higher in clones (H_O = 0.609, t = 82.4%) than in seeds (H_O = 0.346, t = 35.5%) although the two had approximately the same values for the number of alleles (A) and expected heterozygosity (H_E) (A = 4.43 in both clones and seeds, H_E = 0.709, 0.692 in clones and seeds, respectively; **Table 1**) This result indicates that the individuals showing "heterosis", with high numbers of heterozygous loci and outcrossing rates, are more likely to survive in the natural habitat Secondly, parameters of observed heterozygosity and outcrossing rates were lower for seeds from the fringe area (H_O = 0.238, t = 24.6%) compared to seeds from the inside area (H_O = 0.443, t = 60.7%), although values were similar in clones of both the fringe and inside areas (**Table 1**). This result suggests that the "edge effect" might be due mainly to the restriction of wind strength in fringe area of the forested swamp.

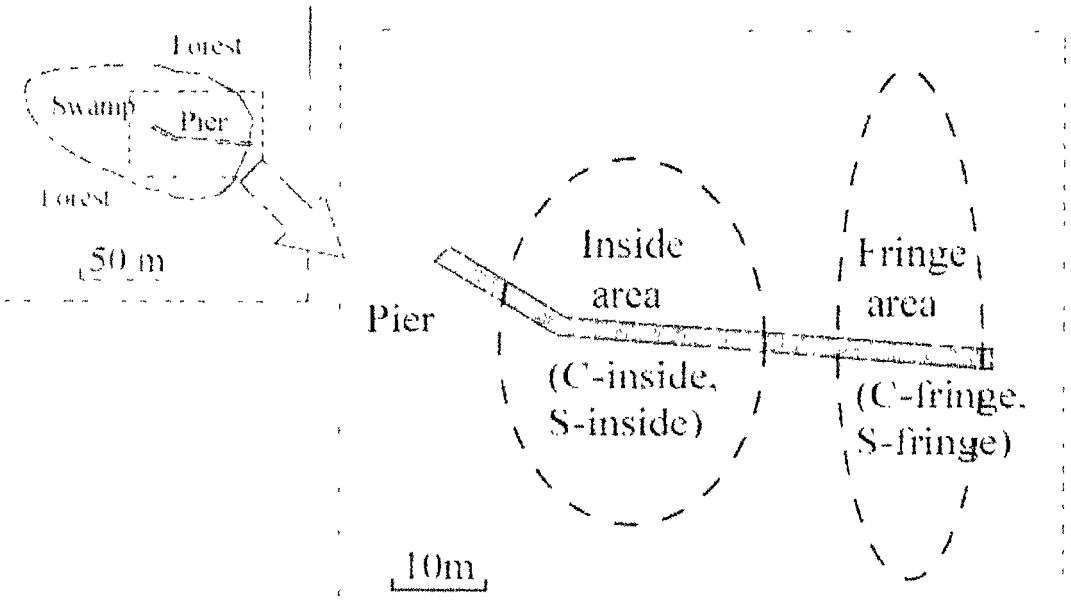

Fig. 1. Sampling areas along the pier in the *in-situ* conservation area of *O. rufipogon* of the four subpopulations S-fringe, C-fringe, S-inside, and C-inside.

Table 1. Expected and observed heterozygosity, fixation index and outcrossing rate for subpopulations of *O. rufipogon* in the conservation area, classified in terms of reproductive component (clones or seeds) and location (fringe or inside).

Population Subpopulation	H_E[a]	H_O[b]	t[c]
Clone[1]	0.709	0.609	0.824
C-fringe	0.694	0.603	0.833
C-inside	0.688	0.614	0.884
Seed[2]	0.692	0.346	0.355
S-fringe	0.536	0.238	0.246
S-inside	0.627	0.443	0.607

[1]Clone = C-fringe + C-inside; [2]Seed = S-fringe + S-inside; [a]Expected heterozygosity (Nei, 1973); [b]Observed heterozygosity; [c]Outcrossing rate (Weir, 1990).

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Crossing barrier within the primary genepool of rice uncovered by means of molecular dissection

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Keywords: Post-fertilization barrier, Hidden variation, Near-isogenic line, Sex-specific reaction, Primary genepool

Crossing barrier is one of the most effective isolating barriers which restrict gene flow between diverged populations. Sexual affinity or cross-compatibility has been widely surveyed in crops and their wild relatives since knowledge about the primary gene pool is a prerequisite for hybridization breeding (Harlan 1975), however, our present understanding of the genes involved in these phenomena and their distribution within the primary genepool is limited plant species including rice.

The present study was carried out to examine the genetic basis of the unidirectional cross-incompatibility observed in hybrid derivatives between cultivated (*Oryza sativa*) and wild (*O. rufipogon*) rice strains A domesticated plant and its progenitor generally belong to the same biological species, which consists of groups of potentially interbreeding populations, and the corresponding cultivated and wild forms of rice are regarded to be the *O. sativa* - *O. rufipogon* complex (Harlan 1975; Oka 1988) The unidirectional cross-incompatibility was detected in advanced generations of backcrossing between wild (*Oryza rufipogon*) and cultivated (*O. sativa*) rice strains The near-isogenic line of T65*wx* (Japonica type) carrying an alien segment of chromosome 6 from a wild strain gave a reduced seed setting only when crossed with T65*wx* as the male (Sano 1992) This provides a unique example in which genes for crossing barriers were present within the primary genepool as hidden variation, and a distinct isolating barrier resulted from hybridization and recombination, although no distinct crossing barrier has been reported within the rice species complex (Chu *et al* 1969; Sitch *et al* 1989)

The genetic basis of this cross-incompatibility reactions in the female and male were investigated by testcrosses using recombinant inbred lines that were established through dissecting the introgressed segments of wild and cultivated (Indica type) strains The results revealed that the cross-incompatibility reaction was controlled by *Cif* in the female and by *cim* in the male. When the female plant with *Cif* was crossed with the male plant with *cim*, a failure of early endosperm development was observed in the hybrid zygotes Among cultivars of *O. sativa*, *cim* was

predominantly distributed in the Japonica type but not in Indica type. In addition, a dominant suppressor, *Su-Cif*, which changes the reaction in the female from incompatible to compatible was proposed to present near the centromere of chromosome 6 of the Indica type. We propose a model for the genic interactions responsible for the cross-incompatibility reactions in the female and male as shown in Figure 1 The gene block detected on chromosome 6 predicts that it might maintain the established sexual reactions against a breakdown due to recombination, or the recombined genes might generate the diversified sexual affinities actually observed in nature.

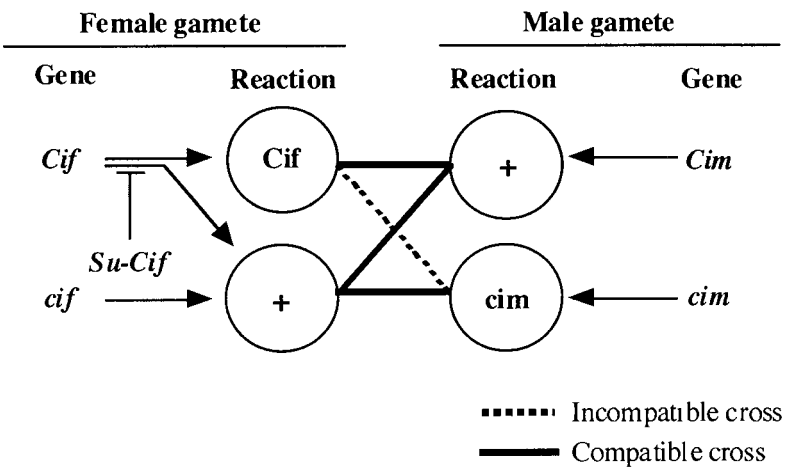

Figure 1 Putative genetic model by which the three genes *Cif*, *cim* and *Su-Cif* are involved in unidirectional cross-incompatibility (Matsubara *et al* 2003) Cross-incompatibility occurs when gametes with the cross-incompatibility reactions in the female (Cif) and the male (cim) are used for fertilization. + indicates the cross-compatibility reaction.

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Genomic differentiation in rice using recombinant inbred lines between an annual type of wild rice and a landrace in the northern-most region for rice cultivation

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Keywords: Adaptive traits, MITEs, QTL cluster, Rice, RIL

The objective of the present study is to investigate genetic differentiation associated with adaptive changes reflecting the domestication as well as varying environments within the primary gene pool of Asian rice. The wild progenitor of the cultivated rice species is *O. rufipogon* which is mostly habits in tropical areas while cultivated forms of *O. sativa* are widely distributed from tropic to temperate regions In Hokkaido, the northern area of Japan, intensive improvements made rice to be successfully cultivated after the beginning of the last century To examine the genetic bases involved, we compared a landrace (A58) from Hokkaido and an annual form (W107) of wild rice from India by means of QTL mapping since the two strains are different in latitudinal distribution.

We raised 79 recombinant inbred lines (RILs) from the F5 population from the cross between A58 and W107. In addition to PCR-based markers, miniature inverted repeat transposable

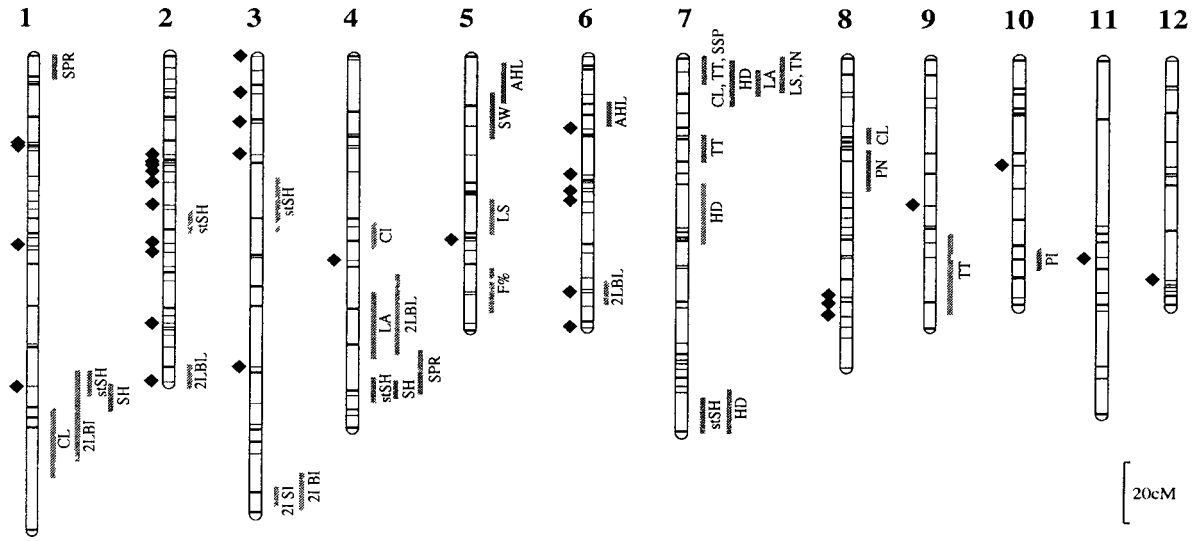

Figure 1 A constructed map showing the effectiveness for discrimination between Indica and Japonica types and positions of QTLs
Position of MITE and PCR-based markers are indicated by thin and thick lines along chromosomes ◆ shows effective MITE markers for discrimination between Indica and Japonica types Putative QTL is represented as one-LOD support interval A LOD threshold of 2 4 was selected for declaring the significance of a putative QTL 2LBL 2LSL Leaf blade and leaf sheath length of 2nd leaf LS Leaf sheath length at vegetative stage TN Tiller number TI Tiller type. LA Leaf angle CL Culm length PN Panicle number PL Panicle length SPP Spikelets per panicle HD Heading date SH Shattering of fertile seed stSH Shattering of sterile spikelet SPR Spreading of panicle SW Seed width AHL Apiculus hair length F% Seed fertility

elements (MITEs) were used to construct a molecular map. MITEs are a major component of interspersed repetitive sequences and a number of polymorphic changes are effectively detected among rice taxa using the MITE transposon display method (Takagi *et al*. 2003) The constructed map included a total of 268 markers (155 MITEs and 113 PCR-based markers) which were distributed over 12 chromosomes The total length of the map was 1376.8 cM and the average distance between two markers was 5.4 cM, showing that the constructed map enables us to analyze a whole region in rice genome (Figure 1)

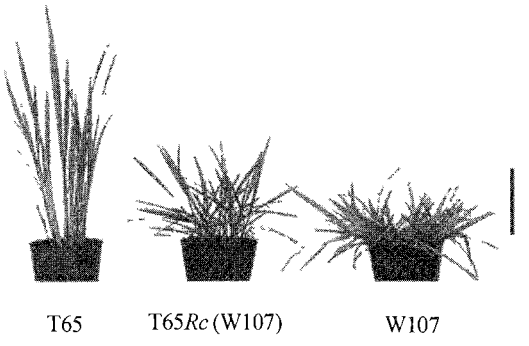

Figure 2 Comparison of plant type at the vegetative stage among T65*Rc*(W107) and its parental lines Bar = 20 cm

Genomic differentiation was examined based on polymorphic patterns of MITEs in 9 cultivated and 7 wild forms In each MITE marker, the degree of discrimination was estimated to show to what extent it discriminates different taxa (Figure 1) The value of the degree ranges from 0 to 1, and the value of 1 indicates that it perfectly discriminates different taxa. For discrimination between Indica and Japonica types, the high value was observed in 35 MITE markers among the 155 examined and they were frequently distributed on chromosomes 1, 2, 3 and 6. However, for discrimination between wild and cultivated forms, most of the markers showed an intermediate value although the reason is uncertain. This suggests that genomic differentiation may have occurred in different regions of chromosomes depending on different taxa.

To survey the genomic region associated with adaptive changes, QTL analysis was performed in 24 traits which were different between the wild and cultivated forms Among 33 QTLs detected over the genome, 7 QTLs forming a cluster were found on the short arm of chromosome 7 (Figure 1) This cluster included various traits associated with plant type such as plant height, tiller and leaf angles and so on. To confirm the presence of the QTL cluster, the short arm of chromosome 7 was introduced into T65 from W107 by backcrosses The NIL of T65*Rc*(W107) showed a rosette-like habit at the early stage of development (Figure 2), showing that the region might carry various genes responsible for weedy or wild forms Further studies are in progress for understanding the genetic bases of adaptive changes including cold tolerances at various stages of development

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Identification and mapping of seed shattering genes using introgression lines from wild rice species

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Keywords: Seed shattering, *Oryza glumaepatula*, *Oryza meridionalis*, Introgression lines

Seed shattering is an important agronomic trait in rice because it affects yield. Two species of wild rice, *Oryza glumaepatula* Steud. (IRGC Acc. No. 105668) and *Oryza meridionalis* Ng (W1625), were used to identify and map genes controlling seed shattering. Backcrossed populations (BC_4F_2's) were derived from a cross between Taichung 65 and *O. glumaepatula* (Sobrizal *et al* 1999), and a cross between Taichung 65 and *O. meridionalis* (Kurakazu *et al* 2001) BC_4F_2 24 population from *O. glumaepatula* (N=65) and BC_4F_2 108 from *O. meridionalis* (N=71) exhibiting wide variation in seed shattering were planted. After heading, the leaves were collected from each plant for DNA extraction. One hundred and six RFLP markers showing polymorphism in the two crosses were used. Whole genome surveys were performed in BC_3F_1 generation. The retained heterozygous regions were further evaluated in BC_4F_1 generation (Fig. 1) Shattering genes were mapped using phenotyping and genotyping data, and were analyzed using MAPMAKER/EXP ver 3.0.

Grains of *O. glumaepatula* and *O. meridionalis* shatter at ripening stage. Segregation was observed in the population derived from both crosses Forty nine shattering and 16 normal plants were observed in BC_4F_2 24-glum population; 37

shattering and 34 normal plants were observed in BC_4F_2 108 in BC_4F_2 108-mer population. Segregation ratio fitted the 3 1 ratio in BC_4F_2 24-glum, suggesting that shattering was controlled by a dominant *O. glumaepatula* allele (Table 1) In contrast, segregation of BC_4F_2 108-mer did not fit the 3 1 ratio due to segregation distortion observed in this population. Shattering was controlled by a dominant allele from *O. meridionalis*.

Linkage analyses showed that genes controlling seed shattering in both populations were located on chromosome 5 The gene controlling seed shattering in BC_4F_2 24-glum was tightly linked to RFLP marker *C1268*, whereas the seed shattering gene in BC_4F_2 108-mer was tightly linked to RFLP marker *R2232* (Fig. 2) These newly detected shattering genes were designated as *Sh5-glum* and *Sh6-mer*, respectively. These genes could be used for further genetic studies to fully analyze the biological nature and mechanisms of seed shattering in rice.

This study was supported, in part, by Bio-oriented Technology Research Advancement Institution (BRAIN), Japan.

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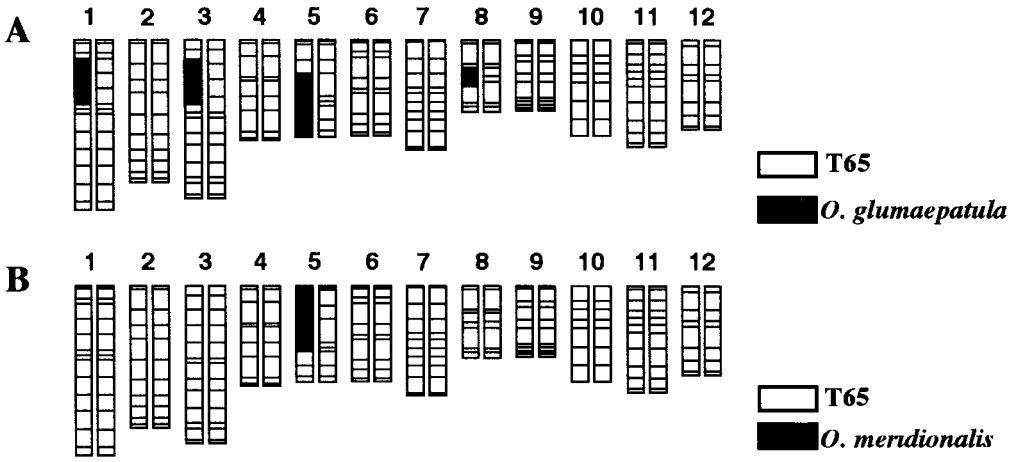

Fig. 1 Graphical genotypes showing the retained heterozygous regions in BC4F1 24 (A) and BC4F1 108 (B).

Table 1. Segregation of shattering plants in introgression lines with *O. glumaepatula* and *O. meridionalis* cytoplasm.

Population	Segregation		Total	χ^2 for 3:1
	Shattering	Normal		
BC4F2 24 (Glum.)	50	17	67	0.005^{ns}
BC4F2 108 (Mer.)	37	34	71	19.835***

ns not significant, *** significant at 5% level

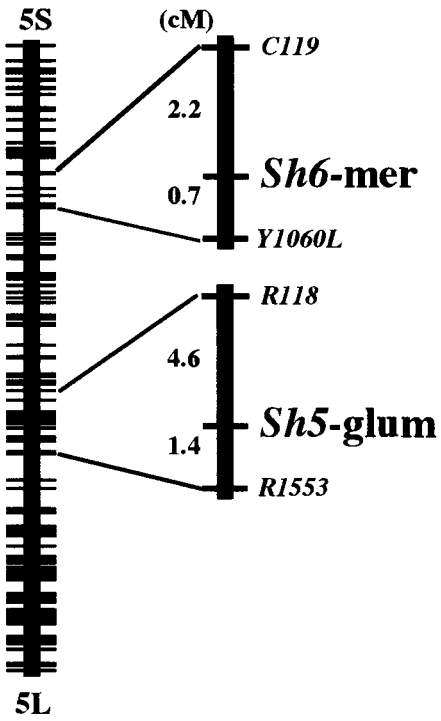

Fig. 2. Linkage map showing the locations of *Sh5*-glum and *Sh6*-mer on chromosome 5.

Identification and mapping of heading genes in rice using *Oryza glumaepatula* introgression lines

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Keywords: Days to heading, Early heading, Late heading, *Oryza glumaepatula*, Introgression lines

Days to heading (DTH, the number of days from sowing to emergence of the first panicle) is an important agronomic trait because it determines maturity in rice. To identify and map genes controlling DTH, backcrossed populations (BC_4F_2) from a cross between Taichung 65 (T65) and *Oryza glumaepatula* Steud. (Sobrizal *et al* 1999) were used. BC_4F_2 254 (N=72), 222(N=78), 204 (N=74) and 204 (N=72) populations which exhibited wide variation in late and early DTH were planted under natural daylength conditions Each plant was monitored for DTH. After heading, leaves were collected for DNA extraction. One hundred six RFLP markers showing polymorphism between *O. glumaepatula* and T65 were used for the whole genome survey in BC_3F_1 generation. The retained heterozygous regions were further evaluated in BC_4F_1 and BC_4F_2 generations (Fig. 1A) DTH genes were mapped using phenotyping and genotyping data, and were analyzed using MAPMAKER/EXP ver 3.0 and MAPMAKER/QTL v.1 1

The frequency distribution for DTH of selected BC_4F_2 populations are shown (Fig. 1B) *O. glumaepatula* did not flower under natural daylength conditions Segregation in BC_4F_2 254 plants (20 early and 52 late heading individuals) agreed with 1 (early) 3 (late) ratio, indicating that late heading was controlled by a single dominant gene

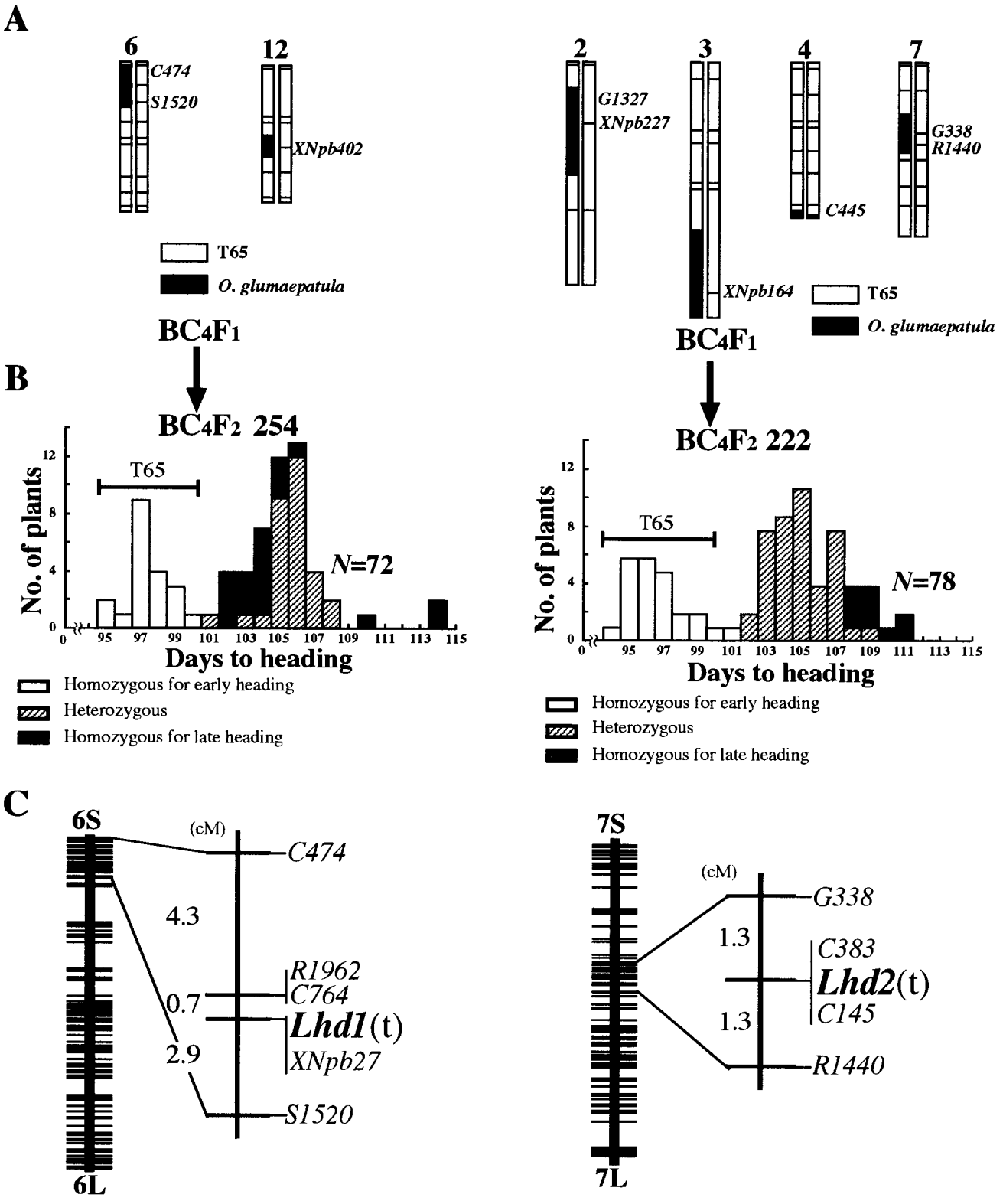
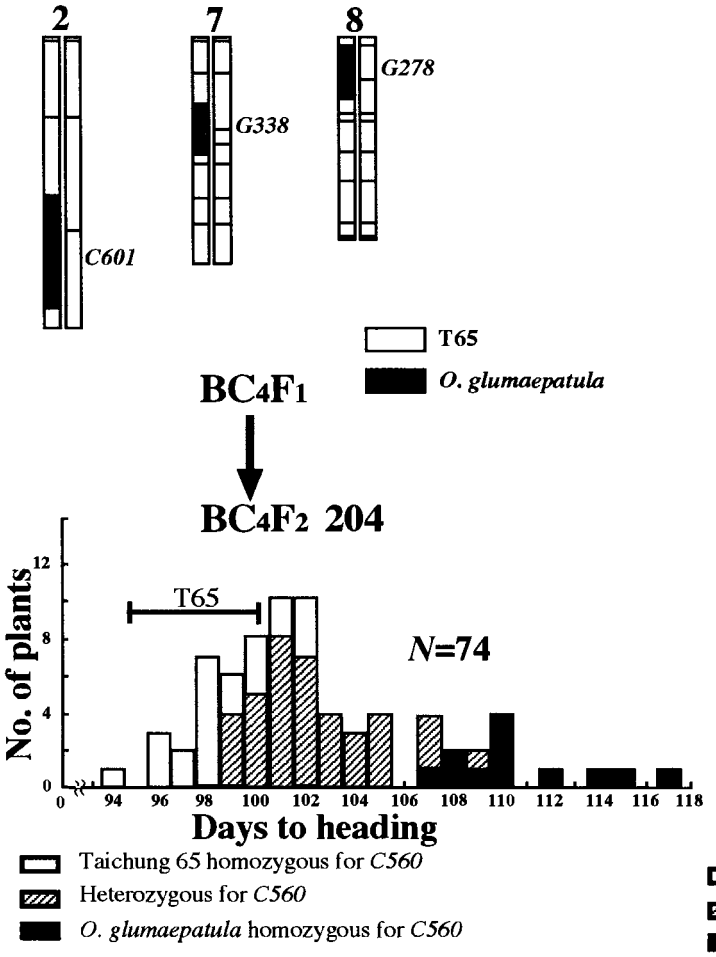
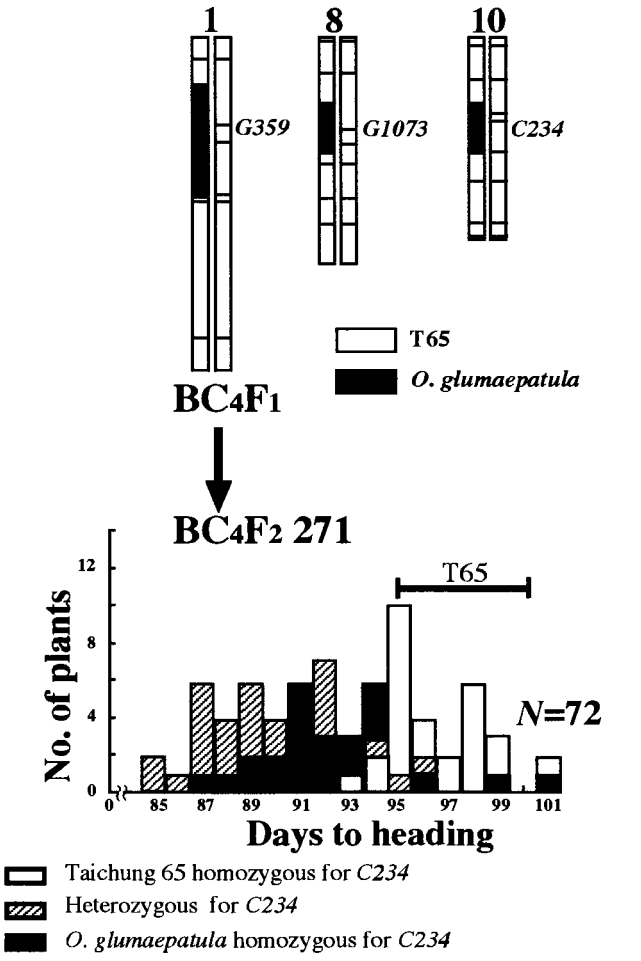
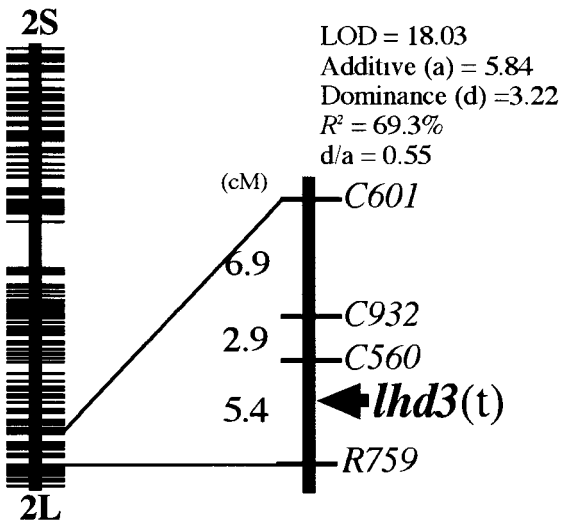
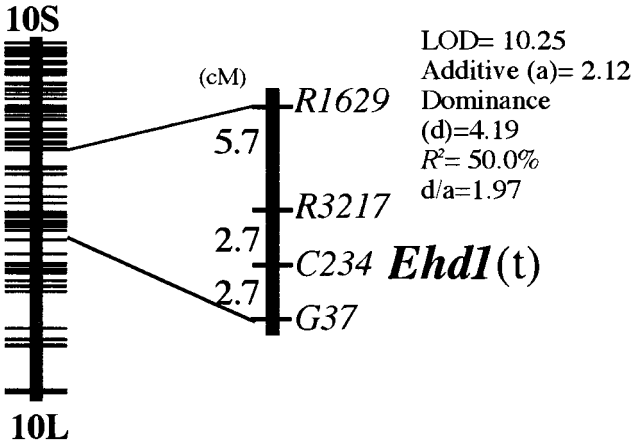


Fig. 1. (A) The graphical genotypes showing the retained heterozygous regions in BC4F1 generation; (B) The frequency distributions of days to heading of BC4F2 254, 222, 204 and 271 populations; and (C) The linkage maps showing the locations of *Lhd1*(t), *Lhd2*(t), *lhd3*(t), and *Ehd1*(t)on rice chromosomes 6, 7, 2 and 10. Each left vertical bar shows the RFLP linkage map (Harushima *et al.* 1998).


2
7
8
G278
G338
C601
T65
O. glumaepatula
BC4F1
BC4F2 204
No. of plants
T65
N=74
Days to heading
Taichung 65 homozygous for C560
Heterozygous for C560
O. glumaepatula homozygous for C560


1
8
10
G359
G1073
C234
T65
O. glumaepatula
BC4F1
BC4F2 271
No. of plants
T65
N=72
Days to heading
Taichung 65 homozygous for C234
Heterozygous for C234
O. glumaepatula homozygous for C234


2S
LOD = 18.03
Additive (a) = 5.84
Dominance (d) =3.22
R² = 69.3%
d/a = 0.55
(cM)
C601
6.9
C932
2.9
C560
5.4
lhd3(t)
R759
2L


10S
LOD= 10.25
Additive (a)= 2.12
Dominance
(d)=4.19
R²= 50.0%
d/a=1.97
(cM)
R1629
5.7
R3217
2.7
C234 Ehd1(t)
2.7
G37
10L

(*Lhd1*(t)) from *O. glumaepatula*. A segregation of 23 early and 55 late heading plants in BC_4F_2 222 agreed with 1 3 ratio, indicates that late heading was governed by a single partially dominant gene (*Lhd2*(t)) from *O. glumaepatula*. BC_4F_2 204 plants, classified into 27 lines for early and 47 lines for late heading did not fit the1 3 ratio due to segregation distortion. Variation for DTH in BC_4F_2 204 was controlled by a recessive late heading gene from *O. glumaepatula*, designated as *lhd3*(t) BC_4F_2 271 plants headed earlier than T65 The gene controlling early heading in these plants was designated as *Ehd1*(t)

Chromosomal locations of the identified DTH genes are shown (Fig. 1C) *Lhd1*(t) was tightly linked to RFLP marker *XNpb27* on chromosome 6. *Lhd2*(t) was linked to RFLP markers *C383* and *C145* on chromosome 7. *lhd3*(t) was mapped between RFLP markers *C560* and *R759* on chromosome 2 (LOD 18.0) *Ehd1*(t) gene was linked to RFLP marker *C234* on chromosome 10. *Ehd1*(t) promotes early heading by about 10 days under natural daylength conditions compared to T65

This study was supported, in part, by Bio-oriented Technology Research Advancement Institution (BRAIN), Japan.

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Sobrizal, Ikeda, K., Sanchez, P.L. et al (1999) Development of *Oryza glumaepatula* introgression lines in rice, *O. sativa* L. Rice Genet Newsl 16. 107-108

Screening for a candidate gene of QTL influencing tissue culture trait using rice DNA microarray

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Keywords DNA microarray, tissue culture trait, QTL, candidate gene, *Oryza sativa*

Candidate genes for quantitative trait loci (QTL) controlling tissue culture traits in rice were screened using DNA microarray analysis Through QTL analysis using a population derived from two varieties, Koshihikari and Kasalath, six QTL were detected. In two QTL on chromosome 1, Kasalath allele gave improved tissue culture ability in subculture and regeneration. Four near-isogenic lines for these two QTL were developed, mostly with a Koshihikari background, and the putative region of the QTL had homozygous Kasalath allele, and tissue culture ability was improved over Koshihikari mRNAs from these four lines were compared to those of Koshihikari using rice 8987 ESTs arrayed on slide glasses mRNA was prepared from callus at two time-points (1) one week after subculture in medium containing 2,4-D, and (2) 1 hr after transfer to regeneration medium containing cytokinins ESTs in which expression ratios were more than three fold were screened. Through a search for homology in the htgs and nr database using the NCBI BLAST program, ESTs were located on

the linkage map. In (1), 54 ESTs were screened, out of which four clones were found to be located in the region for QTL on chromosome 1 In (2), 153 ESTs were screened, among which there were

eleven ESTs also found in (1) Nine of 153 ESTs were located in the region for QTL. Two ESTs were screened by Northern blot analysis of ESTs and located in the region for QTL.

Mapping the quantitative trait loci responsible for differences in floral morphology between *Oryza sativa* L. and *O. rufipogon* Griff.

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Keywords: floral morphology, *Oryza sativa* L., *Oryza rufipogon* Griff., quantitative trait loci, differentiation

Asian cultivated rice (*Oryza sativa* L.) was domesticated from its wild progenitor (*O. rufipogon* Griff.). This domestication process resulted in substantial changes in the morphological and physiological characteristics of Asian rice. In fact, the floral morphology of Asian rice varies widely between that of cultivated and wild rice. Measurements of eleven traits related to the pistil, stamen, and glume of 128 cultivated and 53 wild accessions showed that cultivated rice had a less exserted stigma, shorter stigma, shorter anther, and thicker and wider glume than wild rice. Moreover, the degree of stigma exsertion, stigma length, and anther length were negatively correlated with the thickness and width of the glume. To clarify the genetic basis of these interspecific variations, we analyzed 16 traits related to the three organs using recombinant inbred lines, derived from a cross between *O. sativa*, Pei-kuh, and *O. rufipogon*, W1944. The quantitative trait loci (QTLs) responsible for floral morphology were detected by composite interval mapping using a linkage map constructed using 147 markers, mostly RFLPs. A total of 7, 4, 14, and 6 QTLs were detected for traits related to the pistil, stamen, and glume size and shape, respectively. A comparison of 31 QTLs affecting these organs revealed 10 QTLs that affected the different organs in four adjacent regions on chromosomes 2, 4, 5, and 10, but most of the QTLs (68%) were located separately on various chromosomes. Although 4 QTLs for stigma breadth, anther length, and thickness of the lemma and palea explained more than 25% of the total phenotypic variance, most of the QTLs (87%) had smaller effects. Our data indicate that such genes play a more important role than genes with large effects in the differences in floral morphology between cultivated and wild rice. For example, the anther length of W1944 was four times that of Pei-kuh, and 4 QTLs that had small effects on anther length were detected.

Acknowledgments

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Dual origin of the cultivated rice based on molecular markers of newly collected annual and perennial strains of wild rice species, *Oryza nivara* and *O. rufipogon*

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.Key words annual and perennial types, *indica-japonica* differentiation, p-SINE1-r2, sampling method

Asian common wild rice (*Oryza rufipogon* sensu lato), the wild progenitor of Asian cultivated rice (*O. sativa*), tends to be differentiated into annual (*O. nivara*) and perennial (*O. rufipogon* sensu srticto) types Previous studies supposed that *O. sativa* diverged from the intermediate type of *O. rufipogon* (e.g. Sano et al , 1980), and it has been widely accepted that *O. sativa* has a monophyletic origin (Oka, 1974; Chang, 1976) To elucidate whether *O. sativa* has monophyletic or diphyletic origin(s), and if diphyletic origins, whether the annual and perennial types are the ancestors of *indica* and *japonica*, the distribution of the retrotransposon p-SINE1-r2, a short interspersed nuclear element (SINE) at the waxy locus (e.g. Umeda et al 1991) was analyzed in 46 strains (23 each of *O. nivara* and *O. rufipogon*) of wild relatives of rice. Results indicated that most annual types of *O. nivara* possessed p-SINE1-r2, while most perennial types of *O. rufipogon* did not have, suggesting that p-SINE1-r2 polymorphism corresponds to annual-perennial differentiation in wild relatives

To clarify the relationship between annual-perennial and *indica-japonica* differentiations, we also applied two DNA markers discriminating *indica* and *japonica* of *O. sativa* effectively at both chloroplast and nuclear DNAs (Yamanaka et al 2003) Most of *O. rufipogon* showed non-deletion (ND) in the ORF100 region of chloroplast DNA and *japonica* (J) type of nuclear DNA; alternately, most of *O. nivara* showed deletion (D) and *indica* (I) type. From the results, annual-perennial habit (A, P), p-SINE1-r2 polymorphism (+, -), chloroplast DNA (D, ND) and nuclear DNA (I, J) were nonrandomly associated with each other indicating typical *O. rufipogon* (P, -, ND, J) and typical *O. nivara* (A, +, D, I)

In this study, the wild relatives of *O. sativa* had clearly differentiated into annual and perennial groups by p-SINE1-r2 polymorphisms, and these two groups are corresponding to *indica* type of *O. nivara* and *japonica* type of *O. rufipogon*, contradicting results to the previous studies We considered some of the materials used in the previous studies are intermediate types rather than typical perennial types, because of seed-derived collection and through several seed propagation for multiplication. The perennial types used in this study were collected in the form of living clones and maintained by vegetative propagation. It is probable that collection and multiplication methods (i e. seed or clone) could affect the genetic structure of the materials, particularly in the partially outbreeding perennial strains It was strongly suggested that *O. nivara* and *O. rufipogon* are wild ancestors of *indica* and *japonica*, respectively and the origin of *O. sativa* seems to be diphyletic.

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