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Structural diversity and evolution of the *Rf-1* locus in the genus *Oryza*

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The *Rf-1* locus in rice is agriculturally important as it restores fertility in plants with BT-type cytoplasmic male sterility (CMS). The *Rf-1* locus contains several duplicated copies of the gene responsible for restoration of fertility. We analyzed the genomic structure of the *Rf*-1 locus in the genus *Oryza* to clarify the structural diversity and evolution of the locus. We identified six genes (*Rf-1A* to *Rf-1F*) with homology to *Rf-1* at this locus in *Oryza* species with an AA genome. The *Rf-1* locus structures in the rice accessions examined were very complex and fell into at least six classification types. The nucleotide sequences of the duplicated genes and their flanking regions were highly conserved suggesting that the complex *Rf-1* locus structures were produced by homologous recombination between the duplicated genes. The fact that complex *Rf-1* locus structures were common to *Oryza* species that have evolved independently indicates that a duplication of the ancestral *Rf-1* gene occurred early in rice evolution and that homologous recombination resulted in the diversification of *Rf-1* locus structures. Additionally, the amino acid sequences of each duplicated gene were conserved between species. This suggests that the duplicated genes in the *Rf-1* locus may have divergent functions and may act by controlling mitochondrial gene expression in rice as occurs in the restoration of CMS.
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Introduction

Cytoplasmic male sterility (CMS) is a common phenomenon in higher plants and is the result of incompatibility between nuclear and cytoplasmic gene products, which results in the failure of sporogenesis (Newton, 1988; Levings and Brown, 1989). The combination of CMS and a nuclear gene for restoration of fertility (*Rf*) are essential in self-fertilizing crop species such as rice for breeding hybrid varieties and for hybrid seed production.

In rice, the 'BT-type' of CMS occurs when the cytoplasm of Chinsurah Boro II (*indica*) is combined with the nuclear genome of Taichung 65 (*japonica*). The gene *Rf-1,* initially identified in Chinsurah Boro II, can restore fertility in plants with BT-type CMS (Shinjyo, 1975; Shinjyo, 1984). *Rf-1* was recently shown to encode a mitochondrial targeting, pentatricopeptide (PPR) protein (Kazama and Toriyama, 2003; Akagi *et al*., 2004; Komori *et al*., 2004). The *Rf-1* gene product is involved in controlling the expression of CMS by processing an abnormal chimeric transcript generated by a mitochondrial open reading frame, *orf79*, downstream of mitochondrial *atp6* (Iwabuchi *et al*., 1993; Akagi *et al*., 1994; Kazama and Toriyama, 2003; Wang *et al*., 2006).

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The *Rf-1* locus is complex and contains several duplicated copies of the *Rf-1* gene. In addition, the number of duplicated genes in the *Rf-1* locus also varies in different rice lines (Kazama and Toriyama, 2003; Akagi *et al*., 2004; Komori *et al*., 2004). The complex nature of the *Rf-1* locus may have been generated by gene duplication, and the functions of the duplicated gene may have diverged during rice evolution. The classical *Rf-1* locus consists of two closely linked genes, *Rf-1A* and *Rf-1b*. Both genes can recover Bt-type CMS in the different manners (Wang *et al*., 2006). Duplicated PPR genes have also been identified in the *Rf* locus region in *Petunia* and *Brassica* (Bentolila *et al*., 2002; Brown *et al*., 2003; Koizuka *et al*., 2003). Such duplication and mutation of *Rf* genes encoding PPR proteins is thought to be one of the strategies by which plants acquire new *Rf* gene functions (Shikanai, 2006).

Rice is one of the most important crops and feeds about 40% of the world population. Hybrid rice shows heterosis and gives higher yields (Yuan, 1994; Fujimura *et al*., 1996). The duplicated genes in the *Rf-1* locus may possibly play roles in restoration of CMS by controlling mitochondrial gene expression. Thus, the *Rf-1* locus is important for rice breeding, particularly for the production of hybrid rice, and consequently, it is necessary to clarify the functions of the *Rf-1* genes.

The genus *Oryza* is divided into four species complexes and two discrete species that evolved from a common ancestor (Wang *et al*., 1992; Aggarwal *et al*., 1999). The *Oryza* sativa complex contains all of the AA genome species, including two cultivated species, *O. sativa* and *O. glaberrima*, and five wild species,

O. rufipogon, *O. barthii*, *O. glumaepatula*, *O. longistaminata* and *O. meridionalis* (Vaughan and Morishima, 2003). The two cultivated species, *O. sativa* and *O. glaberrima*, were independently domesticated from *O. rufipogon* in Asia and *O. barthii* in Africa, respectively (Oka, 1988; Second, 1991). The evolutionary relationships between species in the genus *Oryza* have been thoroughly analyzed by comparison of their DNAs (Wang *et al*., 1992; Ishii *et al*., 1996; Cheng *et al*., 2002). Since the complex structure of the *Rf-1* locus may be derived from a single ancestral gene, genomic analysis of the locus in a range of rice lines should provide insight into the evolution of *Rf-1*.

In this study, we analyzed allelic variants of the *Rf-1* locus in AA genome species of the genus *Oryza* that had been collected from locations with a wide geographic distribution from Asia to Africa. A PCR analysis of *Rf-1* locus sequences revealed a high diversification of the structure of the *Rf-1* locus in these species. The ancestor of the *Rf-1* gene may have been duplicated early in rice evolution, and subsequent recombination may have created the present diversified structure of the *Rf-1* locus.

Materials and methods

Plant materials

A total of 96 accessions of the genus *Oryza* were used in this study (see Table 2). The 63 accessions of the cultivated rice species *Oryza sativa* (AA) included 53 local varieties and 10 modern cultivars. The local varieties had been collected in Asia and were classified into three subspecies, *indica* (I:29 lines), *javanica* (V:*tropic-japonica*; 14 lines) and *japonica* (J:*temperate-japonica*; 10 lines) (Morishima and Oka, 1981). Six lines of the other cultivated species *O. glaberrima* were used. A total of 27 lines of wild rice were also used, including 4 lines of *O. rufipogon* (AA), 1 line of *O. barthii* (AA), 2 lines of *O. longistaminata* (AA), 2 lines of *O. glumaepatula* (AA), 2 lines of *O. meridionalis* (AA), 2 lines of *O. punctata* (BBCC), 2 lines of *O. minuta* (BBCC), 1 line of *O. officinalis* (CC), 2 lines of *latifolia* (CCDD), 2 lines of *O. grandiglumis* (CCDD), 2 lines of *O. alta* (CCDD), 2 lines of *O. brachyantha* (FF), 1 line of *O. longiglumis* (HHJJ), 1 line of *O. granulata* (GG) and 1 line of *O. meyeriana* (GG). With the exception of the 10 modern cultivars, rice lines were kindly provided by the National Institute of Genetics. The accession numbers of the original collections were used in this study.

Crude DNA extraction

Leaf tips (1 cm) were collected from young rice seedlings using 2 ml Eppendorf tubes. Leaves were completely dried at 70°C for 2 h and were, then, ground until it became powder with a stainless ball (φ3mm) in a vibrating 2 ml Eppendorf tube using a Micro Smash (MS-100 TOMY). Extraction buffer (1 ml; Edwards *et al*., 1991) was added to the leaf powder and the mixture was incubated at room temperature for 1 h. After centrifugation at 10 000 r.p.m. for 3 min, the supernatant was collected. Crude DNA was precipitated from the supernatant by adding an equal volume of 2-propanol.

PCR

PCR was performed with primers specific for the *Rf-1* locus (Table 1, Figure 1a) using *TAKARA Ex Taq* (Takara Bio Inc., Shiga, Japan) or *TAKARA LA Taq* with *GC I* buffer (Takara Bio Inc.). PCR was carried out in a 20 μl of reaction volume consisting of 1 U of *TAKARA Ex Taq* or *LA Taq* with the accompanying buffer, 4 nmol dNTP, 10 pmol of each set of primers and 10 ng of crude DNA using a Thermal Cycler 9600 or 9700 (Perkin-Elmer, Foster City, CA, USA). Thirty-five PCR cycles, each consisting of 10 s of denaturation at 94°C, 30 s of annealing at 55°C and 2 or 3 min of polymerization at 72°C, were performed using a Thermal Cycler 9600 or

Table 1 The primer sequences used here

Name	*Sequence*
a	5′-GTAAAGAACAAAGCTTCTTCAGAC-3′
b	5′-GCCTCGATCTACGGCTTGAA-3′
c	5′-CGATCTCTCATTCTCTCCAC-3′
d	5′-AGCTAAAGTTTCAGCAGAGG-3′
e	5′-GGCTCTCAAGGCTAAGCTTG-3′
f	5′-GTAACTAGTCTCAGTACCTG-3′
g	5′-CCCTTCTCTCCCACCGTAAA-3′
h	5′-GACAGGGGTTGCTAGAAGAA-3′
i	5′-TCCCTCCTCTAATAGGACTG-3′
j	5′-AGCTAAAGTTTCAGCAGAGG-3′
k	5′-AACTGCGCAAGAGATCGATC-3′
l	5′-GGGAGGGTTATAGAATCTGG-3′
m	5′-CCTCGAGATGCGTGATTA-3′
n	5′-GAGCCAAATAAGCAGTTGTC-3′
o	5′-CACAGATAGGAACACCAAGC-3′
p	5′-ATTAGAGGAGGGATCTTCTC-3′
q	5′-GATTGATACACACAAATGAATCC-3′
r	5′-CTCTCCGTATCAGCTGCGTC-3′
s	5′-GTAGGTCACAACATTTGGCG-3′

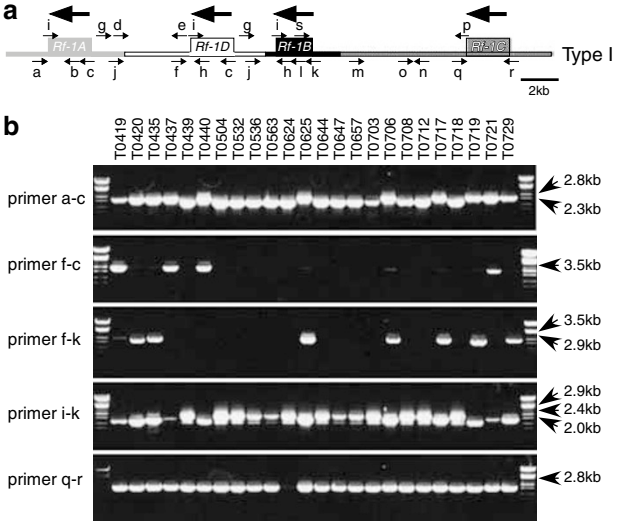

Figure 1 PCR determination of the structure of the *Rf-1* locus in the genus *Oryza* using various combinations of primer pairs. (**a**) The structure of the *Rf-1* locus of IR24 (Komori *et al*., 2004) is shown, and the arrows indicate the positions and direction of the primers used here. The region specific for each duplicated gene was represented by different patterns (see Figure 3). Directions of the duplicated genes are represented by arrows above the genes. (**b**) Examples of amplification profiles of 24 lines out of the 96 lines using pairs of the primers in (**a**) are shown. T0419 to T0729 are the accession names of 24 lines (see Table 2). The PCR products were electrophoresed on 1% agarose gels and then stained with ethidium bromide. DNA size-marker lanes contain λ/StyI. Approximate sizes of the amplicons are also indicated.

9700 (Perkin-Elmer). The PCR products were mixed with bromophenol blue loading dye and were analyzed by electrophoresis on 1% agarose gels (Invitrogen, Calsbad, CA, USA) using 1 × TBE (tris-borate-ethylenediamine tetraacetic acid) buffer at room temperature.

Sequence analysis

The nucleotide sequences of the amplicons were determined by direct sequence analysis. After DNA amplification, PCR products were purified using a PCR Purification kit (Qiagen, Hilden, Germany). Purified fragments were sequenced with primers specific for the *Rf-1* locus by using BigDye Terminator Cycle Sequencing v1.1 Ready Reaction Kit (Applied Biosystems Inc., Foster City, CA, USA), and nucleotide sequences were determined using a DNA sequencing system (ABI 377, Applied Biosystems Inc.). DNA sequences were analyzed using GENETYX-Mac (Software Development Co., Tokyo, Japan). Nucleotide sequences of DNA fragments corresponding to duplicated genes of the *Rf-1* gene were aligned using the ClustalW program available from the web site of the DNA Data Bank of Japan (DDBJ) and a phylogenetic tree was produced using TREE VIEW software (Page, 1996).

Results and discussion

PCR analysis of the *Rf-1* locus of the genus *Oryza*

The structure of the *Rf-1* locus was determined by PCR, using primers specific for the *Rf-1* locus (Figure 1a, Table 2), in 69 lines from 2 cultivated *Oryza* species and 27 lines from 15 wild species.

PCR products of 2.3 and 2.8 kb, encompassing the entire *Rf-1A* gene, were amplified with primers 'a' and 'c' from the 67 lines of cultivated rice (Figure 1b, Table 2). These two lengths of amplicons corresponded to the truncated and complete *Rf-1A* gene, respectively (Akagi *et al*., 2004). The 3′ end of the *Rf-1A* gene was amplified with primers 'a' and 'b' in all lines of cultivated species (Table 2). The amplification products from five wild species with an AA genome (*O. rufipogon*, *O. barthii*, *O. logistaminata*, *O. glumaepatula* and *O. meridionalis*) indicated that they carried either a complete *Rf-1A* gene or a substantial part of it (Table 2). This result suggests that the *Rf-1A* gene is conserved within the AA genome species of the genus *Oryza*.

Primers 'f' and 'c' amplified a region corresponding to the *Rf-1D* gene only in 10 lines of *O. sativa*, 2 lines of *O. rufipogon* and 2 lines of *O. longistaminata* (Figure 1b, Table 2). In these lines, the region between the *Rf-1A* and *Rf-1D* genes was also amplified with primer 'd' and 'e' (Table 2). Therefore, the genomic structure from the *Rf-1A* gene to the *Rf-1D* gene was conserved in this group. However, the analysis also suggested that the majority of the tested lines did not carry the region that included the *Rf-1D* gene.

Three types of amplicons of 2.0, 2.4 and 2.9 kb, comprising the *Rf-1B* gene (this gene is different from the *Rf-1b* gene (Wang *et al*., 2006)), amplified in almost all lines with an AA genome; the exceptions were 2 lines of *O. sativa* and 2 lines of *O. meridionalis* using the primer combination 'k' (which is specific for the region upstream of *Rf-1B*) and 'i' (Figure 1b, Table 2). Furthermore, the region upstream from *Rf-1B*, which includes the *Rf-1C* gene, was detected in all lines of *O. sativa* and *O. rufipogon* (Figure 1b, Table 2). The African cultivated species, *O. glaberrima*, had a similar genomic structure as *O. sativa*, in terms of amplification profile, in 2 of the 5 lines tested (Table 2). The results indicate that the genomic structure upstream of *Rf-1B* gene was conserved between *O. sativa* and *O. glaberrima*. On the other hand, two amplicons of 1.1 and 2.0 kb, comprising the 3′ part of *Rf-1B* gene, were amplified only in 28 lines of *O. sativa* and 1 line of *O. rufipogon* using the primers 'j' and 'l' (Table 2). This suggested that the genomic structure or nucleotide sequence downstream of the *Rf-1B* gene was not conserved in the genus *Oryza*.

As genomic structures downstream of *Rf-1B* were unclear in several lines, we investigated these using primer 'k' (which is specific for the region upstream of *Rf-1B*) in combination with primer 'f' (which is specific for region downstream *Rf-1D*). Surprisingly, two amplicons of 2.9 and 3.5 kb were amplified from 26 lines of the AA genome species (Figure 1b, Table 2). This suggested that these lines carried a chimeric gene containing the 5′ region of *Rf-1B* and the 3′ region of *Rf-1D*.

Since no amplicon was observed in the wild species belonging to the BB and CC genome species with the primer combinations in Table 2, the target sequences of the primers may not be conserved in these wild species.

Nucleotide sequences of the amplicons

Sequence analysis showed that the amplicons contained *Rf-1A*, *Rf-1B*, *Rf-1C* or *Rf-1D*, and their flanking sequences (data not shown). Thus, the primer combinations used in the PCR specifically amplified their target regions in the *Rf-1* locus.

Two amplicons of 2.9 and 3.5 kb (Figure 1b, Table 2) were obtained using the primer pair 'f' and 'k'. These amplicons contained the flanking regions upstream of *Rf-1B* and downstream of *Rf-1D*. However, these amplicons contained sequences that differed from both the *Rf-1B* and *Rf-1D* genes. We, therefore, named the duplicated genes in the 2.9 and 3.5 kb fragments as *Rf-1E* and *Rf-1F*, respectively.

The nucleotide sequences of the duplicated *Rf-1* genes were compared using ClustalW (Figure 2). Phylogenetic analysis indicated that the duplicated genes were closely related (Figure 2), suggesting that the genes were generated by tandem duplication. The newly identified *Rf-1E* and *Rf-1F* genes belonged to the phylogenetic clade that contained *Rf-1D* (Figure 2).

The nucleotide sequence of the complete *Rf-1A* gene of *O. glaberrima* (C0501) was identical to that of *O. sativa* (T0041, MTC-10R, IR24, Zhen-Shan 97). The *Rf-1E* gene showed 99.9% conservation at the nucleotide level and 100% at the amino acid level in these two cultivated species (between C0501 and Zhen-Shan 97). These results showed that the duplicated genes at the *Rf-1* locus were conserved in *O. sativa* and *O. glaberrima*, which were independently domesticated in Asia and Africa, respectively. Thus, *Rf-1* gene duplication appears to have predated the divergence of ancestral wild species of *O. sativa and O. glaberrima*, and their nucleotide sequences have been conserved since this divergence.

Structural diversification of the *Rf-1* locus in the Asian cultivated species, *O. sativa*

Based on the PCR and sequence analyses, the *Rf-1* locus was classified into six structural types, named here Types

Table 2 Summary of PCR amplification using primers specific for the *Rf-1* locus

Species	*Accession*[a] */ name*	*Origin*[b]	*Subspeceis*[c]	*Primer combination*[d]												*Classification*[e]
				a–c (A)	*a–b (A)*	*d–e (A–D)*	*f–c (D)*	*g–h (D–B)*	*i–k (B/F/E)*	*j–l (B)*	*s–k (B)*	*f–k (F/E)*	*m–n (B–C)*	*o–p (B–C)*	*q–r (C)*	
O. sativa	T0001	Vietanam	I	2.8	2.5	2.7	—	—	—	—	0.96	—	2.3	—	2.8	—
(local variety)	T0002	Vietanam	I	2.8	2.5	2.7	3.5	—	—	—	0.96	—	2.3	2.1	2.8	IV
	T0041	Vietanam	I	—	2.5	—	—	—	2.4	—	0.96	—	2.3	2.1	2.8	VI
	T0051	Vietanam	I	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	T0101	Taiwan	I	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	T0108	Taiwan	I	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	T0130	Taiwan	I	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	T0143	Taiwan	I	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	T0144	Taiwan	I	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	T0153	Taiwan	I	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	T0160	Taiwan	I	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	T0201	Philippines	V	2.8	2.5	2.7	—	—	—	—	0.96	—	2.3	2.1	2.8	IV
	T0206	Philippines	V	2.8	2.5	2.7	—	—	—	—	0.96	—	2.3	2.1	—	IV
	T0220	Philippines	V	2.8	2.5	2.7	—	—	—	—	—	—	2.3	2.1	—	—
	T0221	Philippines	V	2.8	2.5	2.7	—	—	—	—	0.96	—	2.3	2.1	—	IV
	T0224	Philippines	V	2.8	2.5	2.7	—	—	—	—	—	—	2.3	2.1	—	—
	T0230	Philippines	I	—	2.5	—	—	—	2.4	—	0.96	—	2.3	2.1	2.8	—
	T0318	Indonesia	V	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0321	Indonesia	J	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	T0325	Indonesia	J	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0347	Indonesia	J	2.8	2.5	2.7	3.5	—	—	—	0.96	—	2.3	2.1	—	—
	T0414	India	I	2.8	2.5	2.7	3.5	—	—	—	0.96	—	2.3	2.1	—	IV
	T0415	India	I	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	T0417	India	I	2.8	2.5	2.7	3.5	2.2	2.0	1.1	0.45	—	2.3	2.1	2.8	I/III[f]
	T0419	India	I	2.8	2.5	2.7	3.5	2.2	2.0	1.1	0.45	—	2.3	2.1	2.8	I
	T0420	India	I	2.8	2.5	2.7	—	—	2.0	—	0.45	2.9	2.3	2.1	2.8	II
	T0435	India	I	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	T0437	India	I	2.8	2.5	2.7	3.5	—	—	—	0.96	—	2.3	2.1	2.8	IV
	T0439	India	I	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0440	India	I	2.8	2.5	2.7	3.5	2.2	2.0	1.1	0.45	—	2.3	2.1	2.8	I
	T0504	Taiwan	J	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0532	Japan	V	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0536	Japan	V	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0563	Japan	J	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0624	Indonesia	V	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0625	Indonesia	I	2.8	2.5	2.7	—	—	2.0	—	0.96	3.5	2.3	2.1	2.8	V
	T0644	Indonesia	V	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0647	Indonesia	V	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0657	Indonesia	V	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0703	China	J	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0706	China	I	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	T0708	China	J	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0712	China	J	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0717	China	J	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	T0718	China	V	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0719	China	I	2.8	2.5	2.7	—	2.2	2.0	—	0.45	2.9	2.3	2.1	2.8	II
	T0721	China	I	2.8	2.5	2.7	3.5	—	—	—	0.96	—	2.3	2.1	2.8	IV
	T0729	China	I	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	T0731	China	I	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	T0757	China	V	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0853	Taiwan	I	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	T0869	Taiwan	J	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	T0868	Taiwan	I	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V

Table 2 Continued

Species	*Accession*[a] */ name*	*Origin*[b]	*Subspeceis*[c]	*Primer combination*[d]												*Classification*[e]
				a–c (A)	*a–b (A)*	*d–e (A–D)*	*f–c (D)*	*g–h (D–B)*	*i–k (B/F/E)*	*j–l (B)*	*s–k (B)*	*f–k (F/E)*	*m–n (B–C)*	*o–p (B–C)*	*q–r (C)*	
Modern cultivar	Akitakomachi	Japan	J	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	Akayanagi	Japan	J	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	Sekaiichi	Japan	J	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	Sensyo	Japan	I	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	Italica Livorno	Italy	V	2.3	2.0	—	—	2.2	2.9	2.0	0.45	—	2.3	2.1	2.8	III
	IR36	Philippines	I	2.8	2.5	2.7	3.5	2.2	2.0	1.1	0.45	—	2.3	2.1	2.8	I
	MTC-10R[g]		—	2.8	2.5	2.7	3.5	2.2	2.0	1.1	0.45	—	2.3	2.1	2.8	I/III[f]
	Zhen-Shan 97	China	I	2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	Dular	India	I	2.8	2.5	—	—	2.2	2.4	—	0.45	—	2.3	2.1	2.8	—
	Basmati	India	I	2.8	2.5	—	—	2.2	—	1.1	0.96	—	2.3	2.1	2.8	—
O. rufipogon	W0120	India		—	—	2.7	3.5	2.2	2.0	1.1	0.45	—	2.3	2.1	2.8	I
	W1294	Philippines		2.8	2.5	2.7	—	—	2.0	—	0.45	2.9	2.3	2.1	2.8	II
	W1866	Thailand		—	2.5	2.7	3.5	—	—	—	0.96	—	2.3	2.1	2.8	—
	W2003	India		—	—	2.7	—	—	2.4	—	0.45	3.5	2.3	2.1	2.8	V
O. glaberrima	C0401	French Guinea		2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	—	—	—	—
	C0487	French Guinea		2.8	2.5	2.7	—	—	—	—	0.96	—	—	—	—	—
	C0501	Senegal		2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	C0541	French Guinea		2.8	2.5	2.7	—	—	—	—	0.96	—	—	—	—	—
	C0650	Gambia		2.8	2.5	2.7	—	—	2.4	—	0.96	3.5	2.3	2.1	2.8	V
	C0658	Guinea		2.8	2.5	2.7	—	—	2.9	—	0.96	—	—	—	—	—
O. barthii	W0652	Sierra Leone		2.8	2.5	2.7	—	—	2.4	—	0.96	—	—	—	—	—
O. longistaminata	W1413	Sierra Leone		—	2.5	2.7	3.5	—	2.9	—	0.96	—	—	—	—	—
	W1508	Madagascar		—	2.5	—	3.5	—	2.9	—	0.96	—	—	—	—	—
O. glumaepatule	W1169	Cuba		2.8	2.5	—	—	—	2.4	—	0.96	—	—	—	—	—
	W2199	Brazil		2.8	2.5	—	—	—	2.9	—	0.96	—	—	—	—	—
O. meridionalis	W1625	Australia		—	2.5	—	—	—	—	—	—	—	—	—	—	—
	W1635	Australia		—	2.5	—	—	—	—	—	—	—	—	—	—	—
O. latifolia	W1197	Colombia		—	—	2.7	—	—	—	—	—	—	—	—	—	—
	W2200	Brazil		—	—	2.7	—	—	—	—	—	—	—	—	—	—
O. grandiglumis	W0613	Brazil		—	—	2.7	—	—	—	—	—	—	—	—	—	—
	W1194	Brazil		—	—	2.7	—	—	—	—	—	—	—	—	—	—
O. officinalis	W1361	Malaysia		—	—	2.7	—	—	—	—	—	—	—	—	—	—
O. punctata($\times 2$)	W1514	Kenya		—	—	—	—	—	—	—	—	—	—	—	—	—
O. punctata($\times 4$)	W1924	Thailand		—	—	—	—	—	—	—	—	—	—	—	—	—
O. minuta	W1213	Philippines		—	—	—	—	—	—	—	—	—	—	—	—	—
	W1331	Philippines		—	—	—	—	—	—	—	—	—	—	—	—	—
O. brachyantha	W1401	Sierra Leone		—	—	—	—	—	—	—	—	—	—	—	—	—
	W0656	Guinea		—	—	—	—	—	—	—	—	—	—	—	—	—
O. longiglumis	W1220	Dutch New Guinea		—	—	—	—	—	—	—	—	—	—	—	—	—
O. alta	W0017	Surinam		—	—	—	—	—	—	—	—	—	—	—	—	—
O. alta or O. latifolia	W1182	Guinea		—	—	—	—	—	—	—	—	—	—	—	—	—
O. granulata	W0067	Thailand		—	—	—	—	—	—	—	—	—	—	—	—	—
O. meyeriana	W1356	Malaysia		—	—	—	—	—	—	—	—	—	—	—	—	—

[a]Accsession numbers follow the original number of Oryzabase.
[b]Origins of the local varieties were derived from the data of Oryzabase.
[c]According to Morishima and Oka (1981), the accessions were classified into three subspecies. I, J and V indicated *indica*, *japonica* and *javanica* subspecies, respectively.
[d]() indicate the target sequences. Approximate sizes (kb) of amplicons are indicated. — indicates that no amplicon was observed.
[e]Unidentified structure.
[f]The lines carried both Type I and variant of Type III. The variant of Type III carried the complete *Rf-1A* and *Rf-1B* gene.
[g]MTC-10R had been bred by recurrent backcrossing of Chinsurah Boro II and Taichung 65. MTC-10R has the nuclear genome from Taichung 65 except for the Rf-1 locus.

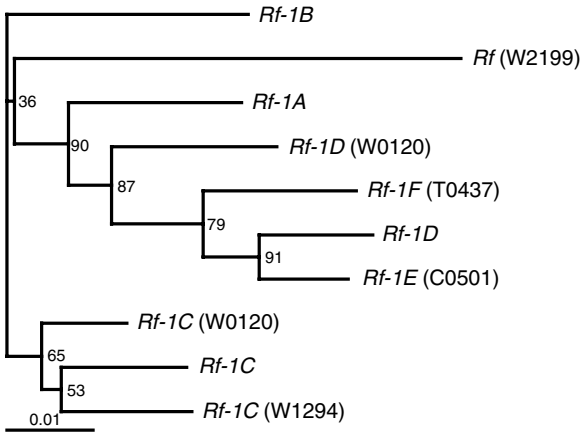

Figure 2 Phylogenetic tree of the duplicated genes of the *Rf-1* locus in the AA genome species of the genus *Oryza*. Nucleotide sequences of *Rf-1F* of T0437 and *Rf-1E* of C0501 were compared with those of the *Rf-1A*, *Rf-1B*, *Rf-1C* and *Rf-1D* genes from MTC-10R. To investigate phylogenic relationships of the *Rf-1C* and *Rf-1D* gene from *O. rufipogon*, nucleotide sequences of *Rf-1C* and *Rf-1D* of *O. rufipogon* (W0120, W1294) were also aligned with these sequences using the ClustalW program. A phylogenetic tree was created from these distances with the TreeView program using the neighbor-joining method. Bootstrap values are shown on the tree. The position of the amplicon corresponding to the *Rf-1A* gene from *O. glumaepatule* is also indicated as *Rf* (W2199).

I–VI, (Figure 3). Type I carried *Rf-1A*, *Rf-1D*, *Rf-1B* and *Rf-1C*. This type has been previously described by Komori *et al*. (2004). Both Type II and Type III lacked the region containing the *Rf-1D* gene and, therefore, carried three duplicated genes, *Rf-1A*, *Rf-1B* and *Rf-1C* (Figures 3a and b). In addition, Type III carried truncated *Rf-1A* and *Rf-1B* genes (Figure 3b, Akagi *et al*., 2004). Type IV and Type V carried *Rf-1F* or *Rf-1E*, respectively, instead of *Rf-1D* and *Rf-1B* (Figures 3c and d). Only one of the rice lines had Type VI, which was characterized by a large deletion leaving only the *Rf-1A* and *Rf-1C* genes (Figure 3e). Sixty of the 69 *O. sativa* lines could be classified into one of these six types (Table 3). However, the structure of the *Rf-1* locus in the remaining nine lines could not be determined in this study (Table 3).

Nucleotide sequence conservation was not limited to the duplicated genes but was also present in their flanking regions. It is possible, therefore, that homologous recombination in the 3′ flanking regions of *Rf-1D* and *Rf-1B* may have generated Type II from Type I (Figure 3a). Similarly, Type III may have been produced by homologous recombination in the upstream region of a Type I *Rf-1A* and *Rf-1D* structure that resulted in the loss of the *Rf-1D* gene (Figure 3b). In Type VI, the region from the *Rf-1D* to *Rf-1B* gene may have been lost by recombination between the 5′ regions of the *Rf-1A* and *Rf-1B* genes (Figure 3e). Thus, the highly conserved nucleotide sequences in the duplicated genes and their flanking regions suggest that the complex structure of the *Rf-1* locus may have had been generated by homologous recombination (Figure 3).

Evolution of the *Rf-1* locus in different subspecies of *O. sativa*

The accessions of *O. sativa* used here were collected from widely dispersed geographical sites throughout Asia, and were classified into three subspecies (Morishima and Oka, 1981). It was anticipated that these would cover a large part of the genetic variation present in *O. sativa*. Most of the *indica* subspecies were classified as Type V, whereas both *japonica* and *javanica* (*tropic* and *temperate japonica*) subspecies were mainly classified as Type III (Table 3). Moreover, Type I, Type II and Type VI were found only in *indica* subspecies (Table 3). Introgression of the *Rf-1* locus between the *javanica* and *indica* subspecies may have produced the exceptional genotypes in the two lines of *javanica* and one line of *indica* that had Type V and Type III loci, respectively (Second, 1982). The consistency of the *Rf-1* structure in the different subspecies suggested that the structural variation at the *Rf-1* locus may have arisen before the divergence of these subspecies.

Geographical distribution of *Rf-1* locus genotypes in Asian cultivars

The geographical distribution of the *Rf-1* locus genotypes of *O. sativa* in Asia is illustrated in Figure 4. It was clear that the genotypes showed geographical variation in their frequencies (Figure 4). Type I was rare and found only in India, whereas Types II–V were distributed throughout Asia (Figure 4). In India, almost all types were represented, the exception being Type VI (Figure 4). India is known to be one of the secondary centers of rice origin (Khush, 1997) and, consequently, the diversity of *Rf-1* locus structures may represent the distribution of genetically divergent varieties throughout India. In contrast, Types II and III predominated in Taiwan and Japan, respectively (Figure 4). The local varieties collected in Taiwan and Japan belonged to the *indica* and *japonica* subspecies, respectively. Therefore, the geographic distributions of the *Rf-1* locus genotypes represent the distribution of subspecies of *O. sativa*.

Genomic structure of the *Rf-1* locus in wild species

The *Rf-1* locus structures found in *O. rufipogon*, the wild ancestral species of the Asian cultivar of *O. sativa*, are shown in Figure 5. The *Rf-1* locus of W0120, W1299 and W2003 was classified as Type I, Type II and Type V, respectively (Figure 5a). However, W0120 and W2003 also had a deletion downstream of the *Rf-1A* gene and partial deletions in both the 5′ and 3′ regions of the *Rf-1A* gene (Figure 5a). The remaining accession, W1866, carried the *Rf-1A*, *Rf-1D* and *Rf-1C* genes; however, the genomic structure between the *Rf-1D* and *Rf-1C* genes was not clarified (Figure 5a).

We also examined the structure of the *Rf-1* locus in the African cultivar, *O. glaberrima*, and its wild relative, *O. barthii* (Figure 5b). Two lines of *O. glaberrima*, C0501 and C0650, had Type V structures (Figure 5b). In the remaining four lines, the region upstream of *Rf-1E* gene, including the *Rf-1C* gene, was not detected by the PCR analysis. These lines probably carried a variant of Type V (Figure 5b). One line of *O. barthii*, W0652, gave the same amplification profile as these four lines, suggesting that W0652 also carried the same variant of Type V (Figure 5b).

Evolution of the *Rf-1* locus in the AA genome species

The molecular structure of the *Rf-1* locus of three AA genome species, *O. sativa*, *O. rufipogon* and *O. glaberrima*,

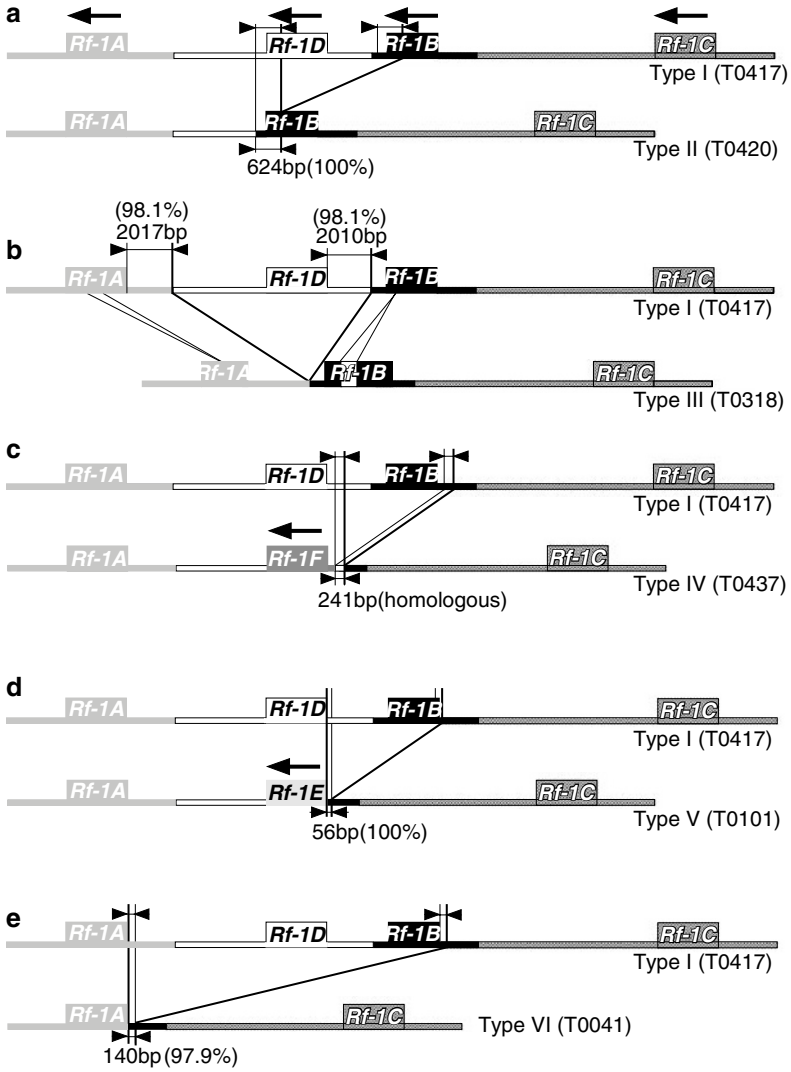

Figure 3 Genomic structure of the *Rf-1* locus in *O. sativa*. The six types of *Rf-1* locus structure are represented here as Types I–VI. The structural features of each genotype are indicated under Type I. The region specific for each duplicated gene was represented by different patterns. Borders of the specific regions were determined by comparison of nucleotide sequences around putative recombination points of Types I–VI. Boxes with gene name represent the position of the duplicated genes. Nucleotide sequence similarities around putative recombination positions between each genotype and Type I are also indicated. Directions of the *Rf-1F* and *Rf-1E* gene are indicated by arrow over each gene. Type III carried the truncated *Rf-1A* and *Rf-1B* gene as described previously (Akagi *et al*., 2004).

Table 3 The distribution of the genotypes of the *Rf-1* locus among subspecies of local cultivars

Genotype of the Rf-1 locus	O. sativa			O. glaberrima	*Total*
	Indica	Javanica	Japonica		
Type I	3	0	0	0	3
Type II	2	0	0	0	2
Type III	1	7	9	0	17
Type IV	4	0	3	0	7
Type V	16	2	0	2	20
Type VI	1	0	0	0	1
Unidentified	2	1	2	4	9
Total	29	10	14	6	59

The local varieties were classified into three subspecies by Morishima *et al*. (1963).

was revealed by both, PCR and nucleotide sequence analysis (Figures 2 and 5). The *Rf-1* locus genotypes of these species are indicated in the dendrogram (Figure 6). Type I, Type II and Type V were found in *O. rufipogon*, an ancestor of *O. sativa* (Figures 5 and 6), indicating that these three genotypes had formed before the divergence of *O. sativa* and *O. rufipogon* (Figure 6). Previous reports have suggested that the *japonica* and *indica* subspecies differentiated before their domestication from different *O. rufipogon* populations (Second, 1982; Wang *et al*., 1992; Chen *et al*., 1993). In this study, only four lines of *O. rufipogon* from three regions (India, Philippines and Thailand) were analyzed. Because *O. rufipogon* is distributed in many regions of world, there is the possibility that other *Rf-1* locus genotypes might be present within *O. rufipogon*. The Type V genotype was common to both *O. sativa* and *O. glaberrima*. The two cultivated species, *O. glaberrima* and *O. sativa*, originated from a common AA genome ancestor; they have evolved in parallel from *O. barthii* in Africa and *O. rufipogon* in Asia (Morishima *et al*., 1963; Second, 1982). Thus, Type V must have formed before the divergence of the ancestors

of *O. sativa* and *O. glaberrima*. These results suggest that duplication of the *Rf-1* gene occurred at an early stage of AA genome evolution and that this was followed by structural diversification of the *Rf-1* locus.

Conclusion

We have shown here that the *Rf-1* locus has a highly diversified structure and contains several duplicated genes. Our results indicated that duplication of the *Rf-1* gene occurred early in rice revolution, and that subsequent diversification produced the complex *Rf-1* locus structure found in the AA genome species of the genus *Oryza*.

The amino acid sequences of the duplicated genes were highly conserved. This outcome is consistent with our previous study in which we found that the *Rf-1C* gene encoded a PPR protein with 88.6% homology to the Rf-1A protein (Akagi *et al*., 2004). The *Rf-1A* gene, but not the *Rf-1C* gene, can recover BT-type CMS (Akagi *et al*., 2004). The newly identified *Rf-1b* recovers BT-type CMS by modification of mRNA from the mitochondrial *atp6* gene, but does so in a different manner to the *Rf-1A* gene (Wang *et al*., 2006). This indicates that the molecular functions of the duplicated genes have also diversified during rice evolution.

Moreover, the deduced amino acid sequences of the duplicated *Rf-1* locus genes were highly conserved across the species barrier in the AA genome species. This suggests that these duplicated genes could be functional. The fertility restorer genes in *Petunia* and *Brassica* are similar to those in rice in that they are duplicated and encode a PPR protein (Bentolila *et al*., 2002, Brown *et al*., 2003, Koizuka *et al*., 2003). These duplicated genes are thought to have arisen from a restorer gene in response to the appearance of new forms of CMS during evolution (Brown *et al*., 2003). The PPR proteins are involved in controlling organelle gene expression by the processing of transcripts (Small and Peeters, 2000). Therefore, the duplicated genes in the *Oryza Rf-1* locus may encode PPR proteins that play diverse roles in the regulation of mitochondrial genes, although, currently, the details of their molecular function are unclear.

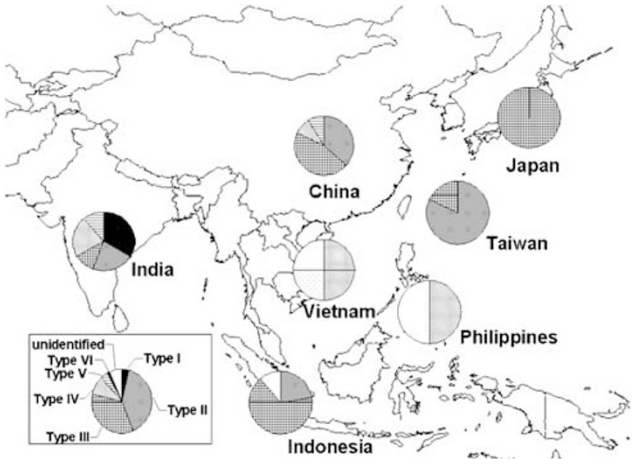

Figure 4 Geographical distribution of *Rf-1* locus genotypes in Asian cultivars. The geographical locations of the genotypes were classified according to the countries or areas in which the local varieties were initially collected. Numbers of accessions for each area are 9 (India), 4 (Vietnam), 9 (Indonesia), 6 (Philippines), 11 (China), 11 (Taiwan) and 3 (Japan). The relative proportions of accessions carrying each genotype, including unclassified genotypes, are indicated.

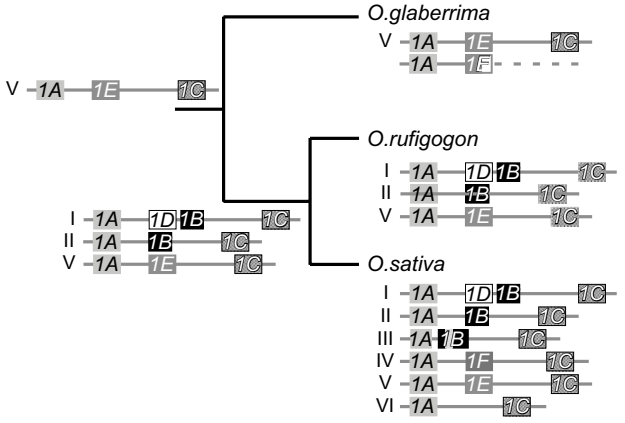

Figure 6 Evolutionary relationships among different *Rf-1* locus variants in the genus *Oryza*. The genomic structure of the *Rf-1* locus identified in each taxon, *O. sativa*, *O. rufipogon* and *O. glaberrima*, is indicated below each taxon name. Commonalities between species are highlighted.

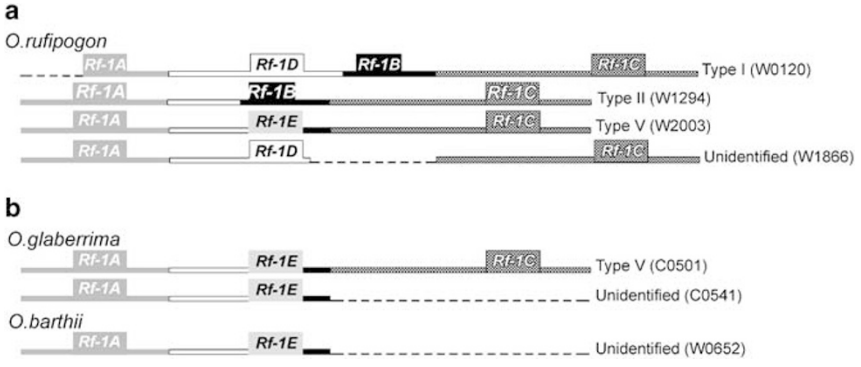

Figure 5 The genomic structure of the *Rf-1* locus of the AA genome species. (**a**) The genotypes of the *Rf-1* locus in four accessions of *O. rufipogon*, the ancestor of the Asian cultivated species, *O. sativa* are shown. (**b**) The genotypes of three accessions of the African cultivar, *O. glaberrima*, and a wild relative, *O. barthii* are shown. The region specific for each duplicated gene was represented by different patterns (see Figure 3). Dotted lines indicate that structure was unidentified because of no amplicon was amplified.

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