

Note

PCR-based INDEL markers co-dominant between *Oryza sativa*, *japonica* cultivars and closely-related wild *Oryza* species

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Wild relatives genetically close to cultivars are precious genetic resources for plant breeding. *Oryza rufipogon*, *O. barthii*, *O. glumaepatula*, *O. meridionalis* and *O. longistaminata* are such wild species, and are also categorized as AA genome species based on their structural similarities. Chromosome segment substitution lines (CSSLs) are a powerful resource in breeding and genetics, and numerous rice CSSLs have been produced. This study aimed to develop DNA markers for evaluation of CSSLs directly by PCR and subsequent gel electrophoresis. We confirmed that up to 155 of 188 markers developed for detection of *japonica-indica* INDELS could also detect INDELS between rice cultivars and wild AA-species accessions. Percentages of applicable markers were higher in *O. rufipogon* accessions (61.7 to 85.6%), and lower in accessions of other four AA species (39.8 to 51.4%). These markers were distributed throughout the rice chromosomes, and will be useful for genotyping of CSSLs and other genetic resources derived from crosses between rice cultivars and closely related wild species.

Key Words: insertion/deletion marker, AA genome, rice, wild species, genetic resource.

Introduction

Rice is one of three major crops alongside wheat and maize, and supports a large proportion of the world population's diet and calories. Therefore, cultivated rice and other crops have been extensively bred for a long time, resulting in development of numerous modern varieties with superior agronomical traits. Local varieties or landraces, widely adapted to various cultivation environments, have been traditionally used as breeding resources. However, recent genome structural analyses revealed that in the breeding lineage of Japanese rice cultivars, a restricted number of superior landraces and their descendants have been repeatedly used as breeding parents (Yamasaki and Ideta 2013). Consequently, the genetic diversity of modern varieties has become smaller, frequently resulting in serious damage to the grain harvest due to genetic breakdown of agronomical traits, such as resistance to plant diseases and insects. Thus, exploration of genetic resources other than landraces is an important and urgent issue in crop breeding. The use of wild plant species is a promising approach to extend

the pool of genetic resources.

Wild relatives of the genus *Oryza* are classified into 21 species (Vaughan and Morishima 2003). Five of them, *O. rufipogon*, *O. barthii*, *O. glumaepatula*, *O. meridionalis* and *O. longistaminata*, are genetically close relatives to the cultivated rice species *O. sativa* and *O. glaberrima*. Based on the similarity of their genome structures, these seven species are also called AA genome species. F₁ hybrids and their descendants can be obtained by the usual crossing and backcrossing in most combinations between AA species, and thus, the wild AA genome species are precious genetic resources for rice breeding. A trial to use these wild resources has already started. Chromosome segment substitution lines (CSSLs), which contain a particular chromosomal segment of a donor parent substituted with the genetic background of a recurrent parent, are a powerful breeding resource and a genetic tool for fine mapping and comprehensive analysis of quantitative trait loci (Yano 2001), and many rice CSSLs have been produced to date (Hirabayashi *et al.* 2010, Shim *et al.* 2010, Yoshimura *et al.* 2010, for example).

This study aimed to develop polymerase chain reaction (PCR)-based insertion/deletion (INDEL) markers to assist in production of rice CSSLs. One merit of co-dominant INDEL markers is easy detection directly by PCR and subsequent gel electrophoresis (Pacurar *et al.* 2012). Here, we

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Table 1. PCR amplification patterns between *japonica* cultivars and wild AA genome species using 181 INDEL markers

Accession No.	Species	Origin	No. of INDEL markers*				Total	Application rate (% Class I/Total)
			Class I	Class II	Class III	Others		
W0106	<i>O. rufipogon</i>	India	153	21	6	1	181	84.5
W0120	<i>O. rufipogon</i>	India	129	39	5	7	180	71.7
W0137	<i>O. rufipogon</i>	India	111	51	18	0	180	61.7
W1551	<i>O. rufipogon</i>	Thailand	155	18	8	0	181	85.6
W1681	<i>O. rufipogon</i>	India	147	18	6	10	181	81.2
W0049	<i>O. barthii</i>	unknown	89	54	30	0	173	51.4
W1605	<i>O. barthii</i>	Nigeria	91	58	32	0	181	50.3
W1169	<i>O. glumaepatula</i>	Cuba	89	52	40	0	181	49.2
W1171	<i>O. glumaepatula</i>	Cuba	90	51	40	0	181	49.7
W1297	<i>O. meridionalis</i>	Australia	72	23	85	1	181	39.8
W2103	<i>O. meridionalis</i>	Australia	77	22	81	1	181	42.5
W1413	<i>O. longistaminata</i>	Sierra Leone	73	37	63	8	181	40.3
W1508	<i>O. longistaminata</i>	Madagascar	68	28	61	6	163	41.7
W1624	<i>O. longistaminata</i>	Cameroun	69	31	60	3	163	42.3

*: See the text about the definition of markers in classes I, II, III and Others.

confirmed that 155 markers out of 188 used for detection of INDELs intraspecific between *japonica* and *indica* were also applicable to detect interspecific INDELs between *japonica* rice cultivars and AA genome wild species.

Materials and Methods

Plant materials

Fourteen accessions of five wild *Oryza* species were used: *O. rufipogon* (accession Nos. W0106, W0120, W0137, W1551, W1681), *O. barthii* (W0049, W1605), *O. glumaepatula* (W1169, W1171), *O. meridionalis* (W1297, W2103), and *O. longistaminata* (W1413, W1508, W1624) (Table 1). Seeds of all accessions were prepared from the wild *Oryza* collection conserved at the National Institute of Genetics (NIG) (Nonomura *et al.* 2010). The *japonica* cultivars Nipponbare and Taichung 65 (T65), and the *indica* cv. Kasalath were used as standards of cultivated species (Fig. 1, Supplemental Table 1). All plants were grown in the field of NIG, Mishima, Japan.

Preparation of PCR primers for INDEL detection

The INDEL sites polymorphic between the *japonica* cv. Nipponbare and the *indica* cv. 93-11 were first detected by the genome browser of the MSU Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu>). We selected 785 sites, each of which includes a putative INDEL 15-500 base pairs (bp) in length. PCR primers 20-bp long were designed and confirmed to be usable as sequence-tagged site markers by e-PCR (<http://www.ncbi.nlm.nih.gov/tools/epcr/>). Subsequent PCR analyses revealed that 595 of the 785 sites could be used as co-dominant INDEL markers between Nipponbare and 93-11. We selected 181 INDEL markers, 160 of which were used in a previous study (Mizuta *et al.* 2010), that covered all of the rice chromosomes, and used them in this study.

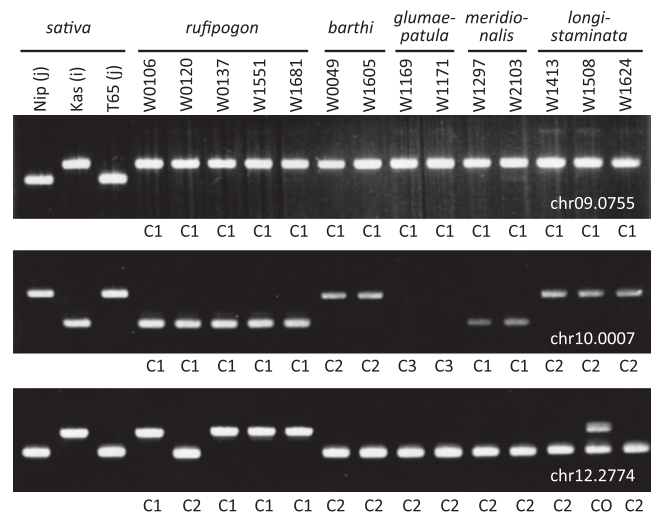


Fig. 1. Different INDEL patterns among cultivars and AA genome wild rice species. Three independent INDEL markers, chr09.0755, chr10.0007 and chr12.2774, were used for example. C1; class I marker, C2; class II marker, C3; class III marker, CO; class “Others” (See the text and Table 1). Nip (j), Kas (i) and T65 (j) indicate standard rice cultivars, Nipponbare (*japonica*), Kasalath (*indica*) and Taichung 65 (*japonica*), respectively.

DNA extraction and PCR analysis

Genomic DNA was extracted from freeze-dried leaf samples according to the CTAB method (Rogers and Bendich 1988). PCR was performed in a 10 µL reaction mixture containing 5 µL GoTaq Green Master Mix (Promega), 5 µM each of a PCR-primer pair, and 2 ng of template DNA. The PCR program used was 98°C for 3 min for initial denaturation, followed by 35 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min, with 72°C for 3 min for a final extension. PCR products were run in 1.5% agarose gels (Certified low range ultra agarose; Bio-Rad) in 0.5x TAE buffer. In Supplemental Tables 1, 2, the length predicted

by e-PCR was adopted to determine the size of PCR products; if they were identical between wild species and cultivars on the electrophoretic image, we estimated the approximate size manually by comparison with a size marker, 100 bp DNA Ladder One (Nacalai Tesque).

Results and Discussion

We examined how many of the 181 markers, all of which contain INDELS between cvs. Nipponbare and 93-11 (see Methods), were applicable for detecting polymorphisms between *japonica* cultivars and each of 14 accessions classified into any of 5 wild species: *O. rufipogon*, *O. barthii*, *O. glumaepatula*, *O. meridionalis* and *O. longistaminata* (Table 1).

As a result of PCR analyses, the 181 INDEL markers were classified into three classes: a class I marker that gave co-dominant, polymorphic PCR amplicons between *japonica* cultivated and wild species (C1 in Fig. 1, for example), class II that gave amplicons, but identical between the two species (C2 in Fig. 1), and class III that gave no amplicon in wild species (C3 in Fig. 1). A class I marker is assumed to contain an INDEL in the internal sequence between a PCR primer pair, with the sequences largely conserved among cultivated and wild species. In case of class II, the internal sequence contains no interspecific INDEL, while sequences corresponding to the primer pair are conserved. A class III marker includes primer sequence polymorphisms deleterious for PCR amplification, and gives no information about the internal sequence polymorphism.

Some markers that give multiple or smeared amplicons are inappropriate for genotyping (CO in Fig. 1), and are categorized as “Others” in Table 1. The results of PCR-assisted classification of 181 INDEL markers are summarized in Table 1, and the complete data are shown in Supplemental Table 1. In this study, the class I markers were only defined as “applicable” for genotyping of CSSLs, and the application rate (% of class I out of total markers) was calculated for each wild accession (Table 1).

The applicable marker rates were higher in *O. rufipogon* accessions. The rates differed intraspecifically among the four accessions we used; the highest was 155 markers (85.6%) in W1551 and the lowest was 111 markers (61.7%) in W0137 (Table 1). The high application rate in this species was largely because it had the fewest class III markers. This is not surprising because *O. rufipogon* is widely accepted as an ancestral progenitor of *O. sativa* cultivars (Vaughan and Morishima 2003). These applicable markers were distributed through all rice chromosomes (Fig. 2A), and were sufficient for genotyping of CSSLs and other AA genome-derived genetic resources.

The application rates declined in the order *O. barthii* and *O. glumaepatula*, with the lowest for *O. meridionalis* and *O. longistaminata* (Table 1). *O. barthii* is thought to be an ancestral progenitor of the African cultivated species, *O. glaberrima* (Vaughan and Morishima 2003), and *O. meridionalis* and *O. longistaminata* each have been phylogenetically distinct from other AA genome species (Doi *et al.* 1995, Wang *et al.* 1992, Xu *et al.* 2005). These results were also consistent with our previous report on other

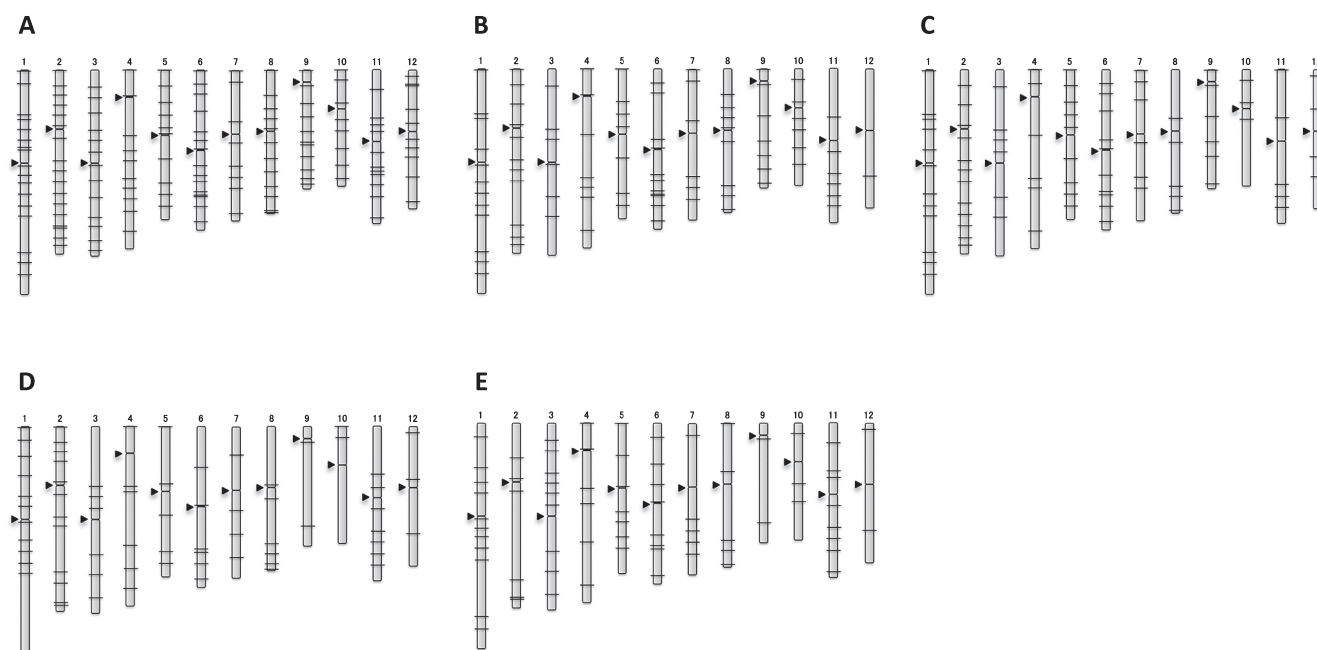


Fig. 2. Distribution of applicable INDEL markers, polymorphic between cv. T65 and closely-related wild rice species; (A) *O. rufipogon* (acc. No. W0106), (B) *O. barthii* (W0049), (C) *O. glumaepatula* (W1169), (D) *O. meridionalis* (W1297), and (E) *O. longistaminata* (W1413). The marker names were omitted, but corresponded to those registered in Supplemental Table 2 in the order from the top to the bottom of each chromosome. Centromere positions were indicated by arrowheads.

Table 2. The number of INDEL markers that give *indica*-type amplicon in wild species used in this study

Accession No.	Species	No. of markers		I/C ratio (%)
		co-dominant with <i>japonica</i> (C)	<i>indica</i> -type amplicon in wild species (I)	
W0106	<i>O. rufipogon</i>	153	139	90.8
W0120	<i>O. rufipogon</i>	129	112	86.8
W0137	<i>O. rufipogon</i>	111	96	86.5
W1551	<i>O. rufipogon</i>	155	140	90.3
W1681	<i>O. rufipogon</i>	147	134	91.2
W0049	<i>O. barthii</i>	89	76	85.4
W1605	<i>O. barthii</i>	91	79	86.8
W1169	<i>O. glumaepatula</i>	89	71	79.8
W1171	<i>O. glumaepatula</i>	90	70	77.8
W1297	<i>O. meridionalis</i>	72	43	59.7
W2103	<i>O. meridionalis</i>	77	47	61.0
W1413	<i>O. longistaminata</i>	73	48	65.8
W1508	<i>O. longistaminata</i>	68	46	67.6
W1624	<i>O. longistaminata</i>	69	48	69.6

INDEL markers (Yamaki *et al.* 2013). Thus, we concluded that these results represent generally accepted phylogenetic relationships of AA genome species. In the four wild species other than *O. rufipogon*, the application rates of INDEL markers were lower, ranging from 39.8 to 51.4%, about half or less than the rates for *O. rufipogon* accessions (Table 1). However, in each of the four species, at least one or more markers were applicable on each of all chromosomes (Fig. 2B–2D). Thus, these marker sets are also usable for genotyping of CSSLs derived from AA species other than *O. rufipogon*.

It was noteworthy that a considerable number of wild species-derived amplicons were identical to their corresponding *indica*-type amplicons in length (Table 2, chr09.0755 in Fig. 1, for example). In particular, more than 85% of the markers applicable for *O. rufipogon* accessions were of this type. This was consistent with a previous result, in which the level of genetic differentiation of *indica* cultivars from *O. rufipogon* species was more modest than that of *japonica* cultivars (Huang *et al.* 2012). Actually, all five *O. rufipogon* accessions in this study were also used in that report (Huang *et al.* 2012). The results of this and previous studies indicate that DNA markers polymorphic for *O. rufipogon* are obtained more easily in *japonica* than in *indica* cultivars. However, we cannot rule out the possibility that the modest differentiation between *indica* cultivars and *O. rufipogon* accessions is attributable to mutual introgression at their habitats of origin, such as a paddy, before collection, or introgression during *ex situ* conservation in germplasm centers. Chromosomal regions tagged with such markers should be handled carefully, particularly when using wild accessions for evolutionary or species differentiation studies. In addition, in CSSL evaluation by INDELS of this study, note that the order of marker alignments not always conserved among the species. Genome rearrangement, such as translocation, inversion and duplication, frequently takes

place among *Oryza* species (Tian *et al.* 2011). This study does not take any linkage relation among INDEL markers into consideration.

As a concluding remark, this study showed that up to 155 INDEL markers were co-dominant between *japonica* rice cultivars and five wild species having AA genome chromosomes (Supplemental Table 1). To make them more convenient for evaluation of CSSLs, the markers were also compiled for each accession (Supplemental Table 2). We are now producing CSSLs of each of the five wild species as donor parents, using cv. T65 as a recurrent parent. All wild accessions used in this study have been confirmed to have strong resistance to bacterial blight diseases (Yoshimura, Nonomura *et al.*, unpublished), and/or to brown plant-hopper, green rice leafhopper, or both (Yasui, Nonomura *et al.*, unpublished). Thus, it is expected that some of the CSSL descendants bear resistance to diseases or insects inherent in the donor accessions. The INDEL marker sets developed in this study will be a powerful tool for effective selection and evaluation of these CSSLs.

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