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RESEARCH ARTICLE

Differentiation of *Indica-Japonica* rice revealed by insertion/deletion (InDel) fragments obtained from the comparative genomic study of DNA sequences between 93-11 (*Indica*) and Nipponbare (*Japonica*)

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Abstract DNA polymorphisms from nucleotide insertion/deletions (InDels) in genomic sequences are the basis for developing InDel molecular markers. To validate the InDel primer pairs on the basis of the comparative genomic study on DNA sequences between an *Indica* rice 93-11 and a *Japonica* rice Nipponbare for identifying *Indica* and *Japonica* rice varieties and studying wild *Oryza* species, we studied 49 *Indica*, 43 *Japonica*, and 24 wild rice accessions collected from ten Asian countries using 45 InDel primer pairs. Results indicated that of the 45 InDel primer pairs, 41 can accurately identify *Indica* and *Japonica* rice varieties with a reliability of over 80%. The scatter plotting data of the principal component analysis (PCA) indicated that: (i) the InDel primer pairs can easily distinguish *Indica* from *Japonica* rice varieties, in addition to revealing their genetic differentiation; (ii) the AA-genome wild rice species showed a relatively close genetic relationship with the *Indica* rice varieties; and (iii) the non-AA genome wild rice species did not show evident differentiation into the *Indica* and *Japonica* types. It is concluded from the study that most of the InDel primer pairs obtained from DNA sequences of 93-11 and Nipponbare can be used for identifying *Indica* and *Japonica* rice varieties, and for studying genetic relationships of wild rice species, particularly in terms of the *Indica-Japonica* differentiation.

Keywords Asian cultivated rice, InDel primer pairs, *Indica-Japonica* differentiation, variety identification

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1 Introduction

Asian cultivated rice (*Oryza sativa* L.) is a very important food crop in the world (Chang, 1984). It is considered that cultivated rice was domesticated 11,500 years ago (Normile, 1997). Cultivated rice has accumulated abundant genetic diversity and undergone significant genetic differentiation during the domestication process to adapt to different environmental conditions (Morishima and Oka, 1981; Glaszmann, 1987; Wang and Tanksley, 1989; Blair et al., 1999). As a consequence, two subspecies, i.e. subsp. *indica* and subsp. *japonica*, representing two partially isolated gene pools, were formed and widely recognized. In eco-geographical terms, *indica* rice is known to be grown primarily throughout tropical Asia at low latitudes and low elevations, while *japonica* are typically found in temperate East Asia, Southeast Asia, and South Asia at high latitudes and high elevations. Considerable differentiation is observed between the two subspecies in terms of morphological, agronomical, and physiological characteristics. A strong hybrid vigor (heterosis) found in many *indica-japonica* hybrids has attracted the attention of rice geneticists and breeders targeting super-heterosis in hybrid breeding for the past several decades. However, serious problems of partial sterility from the hybrids between the *idica* and *japonica* subspecies have severely constrained the effective utilization of the inter-subspecific heterosis in *indica-japonica* hybrid rice breeding. Therefore, it is very important to understand genetic relationships of *indica* and *japonica* rice at the molecular level and to accurately identify *indica* and *japonica* cultivars in rice breeding programs (Jiang et al., 2003).

For a long time, the most effective methodology to classify rice varieties in agricultural application has been based on

morphological variations and the "six morphological characteristics index" in particular. The Cheng's method is essentially based on the score of six diagnostic traits: apiculus hair, leaf pubescence, length/width ratio of grains, phenol reaction, interval between first and second nodes of panicle axis, and color of hulls at heading time (Cheng et al., 1984). With the fast development of molecular technologies over the past twenty years, different approaches such as isozyme markers (Glaszmann, 1987), molecular markers and DNA sequences (Zhang et al., 1992; Zhuang et al., 1995; Sun et al., 2002; Long and Xu, 2002; Zhu and Xu, 2002; Zhu et al., 2004) were gradually applied for the classification of *indica* and *japonica* rice cultivars. This has not only significantly speeded up research into *indica* and *japonica* genetic diversity and differentiation, but also greatly facilitated rice breeders in various breeding programs. Because the domestication and diversification of *indica* and *japonica* rice varieties have taken a sufficiently long time, there were considerable magnitudes of them and so many different types of rice varieties differentiated. In other words, some *indica* or *japonica* rice cultivars have differentiated incompletely (intermediate types), whereas other cultivars had significant *indica-japonica* differentiation (typical *indica* or *japonica*). It is therefore difficult to accurately identify those incompletely differentiated rice cultivars with traditional morphological characteristics. Current modern tools such as isozyme markers and various molecular markers (e.g. RFLP, SSR, RAPD) can identify between *indica* and *japonica* cultivars to a certain extent, but the mechanism for such identification is still unclear (Huang et al., 2003). For that reason, it is urgent to develop a new type of molecular markers directly associated with the *indica-japonica* differentiation for the accurate and easy identification of *indica-japonica* rice cultivars.

Recently, with tremendous progress and development in genome sequencing and comparative genomic technologies, the genomic sequencing of *japonica* rice cv. Nipponbare and *indica* rice cv. 93-11 was completed in 2002 (Yu et al., 2001). The completion and global availability of total rice genome sequences have made the development of specific molecular markers possible using tools of comparative genomics that are essentially based on the differences of entire genomic sequences between *indica* and *japonica* rice. These molecular markers can significantly facilitate the identification of *indica* and *japonica* cultivars, promote studies of genetic diversity and differentiation of rice in depth, and help construct a genome-wide rice DNA polymorphism database using the genomes of Nipponbare and 93-11. The database included DNA fragments with 1,703,176 single nucleotide polymorphisms (SNPs) and 479,406 insertions or deletions (InDels). Differences of insertion and deletion in the total genomic DNA sequences between Nipponbare and 93-11 enabled the development of many InDel markers (Shen et al., 2004). Because Nipponbare is the typical *japonica* and 93-11 the typical *indica*, some InDels between the two genomic sequences may, to a large extent, represent the genetic differences between the *indica* and *japonica* subspecies. To validate whether InDel markers truly reflect the genetic differences of *indica* and *japonica* rice and are able to accurately identify the *indica* and *japonica* cultivars, ninety-two accessions of rice cultivars (including the typical *indica* and *japonica* cultivars identified by the Chen's index) and 24 accessions of wild rice species with different genomes were used for PCR analysis. Knowledge on these InDel markers will not only promote fast and accurate *indica-japonica* identification for breeding purposes, but also encourage deep studies of the evolutionary progress defining cultivated rice and its wild relatives in the genus *Oryza*.

2 Materials and methods

2.1 Plant materials

A total of 92 accessions of rice cultivars (including 49 accessions of typical *indica* and *japonica* cultivars) from nine Asian countries and 13 provinces of China, and 24 accessions of wild rice representing 15 species with different genomes in the genus *Oryza* were used in this study (Table 1). The seeds were donated respectively by the International Rice Research Institute (IRRI), Shanghai Agrobiological Gene Center, Zhejiang University, and Yunnan Agricultural University (Zhu et al., 2004). The donating institutions provided background information of the cultivated rice accessions with the indica or japonica determination.

Table 1 Parameters of the cultivated rice varieties and wild rice species used in this study (numbers in parentheses indicate the number of accessions of rice cultivars used)

Oryza species	Genome	Accessions	Origin
O. sativa subsp. indica	AA	49	Japan(1), Korea(1), China(33), Laos(2), Thailand(2), Philippines(1), Indonesia(1), Bengal(2), India(5), Sri Lanka(1)
O. sativa subsp. Japonica	AA	43	Japan(3), Korea(2), China (36), Indonesia(1), Philippines(1)
O. barthii	AA	1	Cameroon
O. glaberrima	AA	1	Liberia
O. glumaepatula	AA	1	Brazil
O. longistaminata	AA	2	Tanzania, Ethiopia
O. meridionalis	AA	1	Australia
O. nivara	AA	5	Papua New Guinea, Burma, Bangladesh, Nepal
O. rufipogon	AA	5	India, Malaysia, Thailand, China
O. punctata (2x)	BB	1	Chad
O. punctata (4x)	BBCC	2	Kenya, Sri Lanka
O. officinalis	CC	2	Papua New Guinea, Indica
O. alta	CCDD	1	Suriname
O. granulata	GG	1	China
O. ridleyi	HHJJ	1	Thailand

2.2 DNA extraction

Rice seeds of each accession were germinated in a growth cabinet with constant temperature at 37°C. For DNA extraction, leaf samples of the rice materials were collected from seedlings of one individual (representing each accession) planted in a green house. Total genomic DNA was isolated from green leaves by the cetyltrimethyl ammonium bromide (CTBA) method following the procedures of Song (Song et al., 2003), with minor modifications described by Murray (Murray and Thompson, 1980).

2.3 PCR amplification and electrophoretic analysis

A total of 45 InDel primer pairs (Table 2) were selected on the basis of the previous study to assay genetic polymorphisms in all the materials (Shen et al., 2004). The standard of primers selection was based on the reliability of the electrophoretic results of PCR amplification on polyacrylamide gel. The PCR reactions were performed in a PTC-200 thermocycler (Bio-Rad laboratories, Inc.). A denaturation period of 4 min at 94°C was followed by 36 cycles of 40 s at 94°C, 30 s at 55°C and 40 s at 72°C, and then 10 min at 72°C for final extension. Reactions were carried out in a 20-μl volume containing 1 mmol/L buffer, 1 mmol/L each of dNTP, 10 mmol/L of InDel primer (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.), 50 ng of genomic DNA and 0.6 units of *Taq* polymerase (TaKaRa Inc.). PCR products were resolved on a 4% denaturing polyacrylamide gel. After electrophoresis, bands were revealed using the silver-staining procedure described by Song et al., (2003). The null alleles were confirmed after several repetitions with different amplification conditions and electrophoresis to ensure that no reaction failure existed.

2.4 Data analysis

Based on the banding patterns of 93-11 and Nipponbare, all banding patterns of the studied samples were scored as co-dominant patterns (ii and jj for homozygous genotypes, ij for heterozygous genotype). The banding pattern of 93-11 was used as the typical reference for *indica* rice, whereas that of Nipponbare was used as the typical reference for *japonica* rice. The banding patterns that were different from 93-11 and Nipponbare were scored according to molecular weight. To verify the accuracy of each InDel primer pair, the frequency of the *indica* specific allele (*i*) or the *japonica* specific allele (*j*) was calculated by the formula: $F_i = N_i / N_{ti}$ or $F_j = N_j / N_{tj}$, where N_i is the number of cultivars with the same banding patterns as 93-11; N_j indicated the number of cultivars with the same banding patterns as Nipponbare; and N_{ti} or N_{tj} represented the total number of *indica* cultivars or *japonica* cultivars, respectively.

To analyze genetic relationships among *indica*, *japonica* and wild rice, all the banding patterns were transferred into a 0 (absent) or 1 (present) data matrix on the basis of the electrophoretic patters of each DNA sample. The data matrices constituted by the 0, 1 pattern were subjected to principal component analysis (PCA) using the program STATISTICA 6.0 (Petrie, 2002). Program SigmaPlot 8.0 (Hilbe, 2003) was undertaken to establish the scatter plots according to the eigenvector values of the first and the second component.

Table 2 The number of DNA bands of different *Oryza* samples generated by the 45 InDel primer pairs and the efficiency of these InDel primer pairs in identifying *indica* and *japonica* (*I*, *J* and *W* indicate the number of amplified bands in *indica, japonica* and wild rice; F_i and F_j indicate the efficiency of each InDel primer pair in the identification of *indica* and *japonica* rice varieties)

Primer	*I*	*J*	*W*	F_i	F_j
R1M7	2	2	4	0.959	0.930
R1M20	5	4	3	0.633	0.674
R1M30	3	2	6	0.878	0.977
R1M37	3	4	5	0.959	0.907
R1M47	3	2	4	0.898	0.977
R2M10	3	2	4	0.857	0.977
R2M24	2	5	5	0.918	0.860
R2M26	3	2	4	0.878	0.977
R2M37	4	3	5	0.429	0.814
R2M50	2	2	4	0.857	1.000
R3M10	3	2	3	0.918	0.837
R3M23	3	2	4	0.878	0.884
R3M30	3	3	4	0.816	0.907
R3M37	5	3	4	0.571	0.721
R3M53	3	2	4	0.653	0.907
R4M13	6	2	4	0.857	0.884
R4M17	2	2	4	0.857	0.953
R4M30	4	3	4	0.367	0.977
R4M43	3	1	2	0.837	1.000
R4M50	3	4	3	0.755	0.930
R5M13	3	2	3	0.755	0.791
R5M30	3	3	5	0.755	0.837
R6M14	3	2	4	0.714	0.907
R6M30	3	3	4	0.878	0.419
R6M44	3	2	5	0.837	0.977
R7M7	4	1	4	0.612	1.000
R7M20	3	2	3	0.898	0.535
R7M37	3	3	4	0.878	0.884
R8M23	2	3	2	0.959	0.651
R8M33	3	2	3	0.796	0.930
R8M46	4	4	8	0.857	0.837
R9M10	6	2	5	0.612	0.907
R9M20	4	4	10	0.857	0.558
R9M30	7	5	8	0.571	0.884
R9M42	2	2	3	0.898	0.953
R10M10	3	5	4	0.429	0.884
R10M17	4	4	4	0.878	0.837
R10M30	3	2	5	0.735	0.977
R10M40	3	2	4	0.857	0.977
R11M23	2	3	2	0.959	0.744
R11M40	2	3	4	0.776	0.884
R12M10	2	3	4	0.980	0.721
R12M27	5	2	6	0.531	0.721
R12M33	4	2	4	0.633	0.977
R12M43	3	4	5	0.837	0.767

3 Results

3.1 The accuracy of the InDel primers in identifying *indica* and *japonica*

There were 27 of the 45 InDel primer pairs that could ideally identify *indica* cultivars with accuracy of over 80% (including seven primer pairs with accuracy of over 90%). There were 34 pairs that could ideally identify *japonica* cultivars with accuracy of over 80% (including 22 primer pairs with accuracy of over 90% and three pairs with 100% accuracy, Table 2). These results indicated that *indica* cultivars had more variation than *japonica* cultivars.

PCR analysis revealed that all the 45 InDel loci were polymorphic among nearly all the *O. sativa* accessions. The number of alleles per locus ranged from 2 to 10. The null alleles appeared in some wild rice samples and only a few rice cultivar samples demonstrated polymorphism in 40 InDel loci. Considerable variation in the flanking sequences of InDel may lead to PCR failure when primers were designed from a sequence derived from a genotype different from that used in the DNA amplification experiment. This could partially explain "null" alleles seen for some samples. The frequency of null alleles detected in the wild rice samples with non-AA genome was obviously higher than that in the rice cultivar samples, which may indicate significant differentiation between cultivated and wild rice species with other genomes. A considerable portion (about 4%) of heterozygous alleles was detected in the wild rice accessions compared with about 0.1% in the cultivated rice accessions, indicating high frequencies of outcrossing in wild rice species. Our experiment validated the 45 InDel primer pairs in estimating genetic relationships and differentiation of *Oryza* species.

3.2 Results of principal component analysis (PCA)

The 0, 1 data matrixes from all the 116 samples in groups (Indica, Japonica, AA-genome wild rice, and non-AA genome wild rice) in this study were constituted and subjected to PCA, with polymorphism detected by each pair of InDel primers treated as the variables. The space distance between samples in the scatter plotting showed genetic relationships among the accessions. The first and second principal components accounted for 42.91% and 7.28% of the total genetic diversity respectively. In the scatter plot, four relatively weak groups were identified from the synthesis of distribution of the accessions representing *indica*, *japonica*, AA-genome wild rice, and non-AA genome wild rice, respectively. Obvious genetic differentiation was detected between *indica* group and *japonica* group, as they were clustered mostly into two major groups along the first principal component. The distribution of the *indica* group was more scattered than that of the *japonica* group, which *indica*ted that the *indica* varieties had higher genetic diversity than the *japonica* varieties. Those *indica* samples scattered near *japonica* group showed close genetic relationships with the *japonica* group at the molecular level. Wild rice accessions were noticeably distributed between the *indica* group and the *japonica* group along the first principal component, suggesting insufficient *indica-japonica* differentiation. However, the wild *Oryza* species with different genomes had a variable relationship with the cultivated *O. sativa*. The wild rice species with AA-genome shared more similarity with *indica* than *japonica* because of their considerable overlaps with the cultivated rice varieties in distributions in the plot. The wild *Oryza* species were clustered into the AA-genome group and the non-AA genome group (containing BB, BBCC, CC and CCDD). Based on these results, the InDel primer pairs designed from the differences in insertion/deletion fragments obtained from comparative genomic study of DNA sequences between 93-11 and Nipponbare were valuable not only in identifying *indica* or *japonica* varieties, but also in revealing the genetic relationships among cultivated rice varieties and wild *Oryza* species.

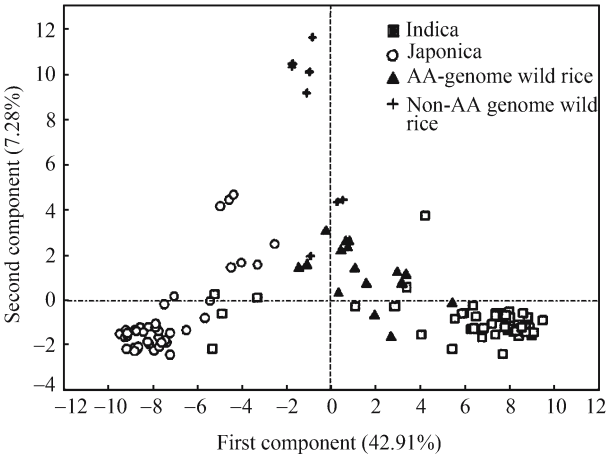

Fig. 1 The scatter plotting showing the association among the *indica-japonica* rice varieties and wild rice samples based on PCA

4 Discussion

This experiment validated 45 InDel primer pairs on the basis of the differences in insertion/deletion fragments from the comparative genomic study on DNA sequences between 93-11 and Nipponbare, involving typical *indica* and *japonica* varieties. A total of 27 *indica*-specific alleles (*i*) and 34 *japonica*-specific alleles (*j*) were detected by the 45 InDel primer pairs, with identifying efficiency of over 80%. The result confirmed that differences in the DNA sequences between 93-11 and Nipponbare may represent the genomic differences between *indica* and *japonica* subspecies. Therefore, these InDel primer pairs will be very useful for identifying *indica* or *japonica* varieties in rice breeding programs.

During the domestication process under stringent selective pressure by human and natural environments, Asian cultivated rice has had considerable genetic diversity and differentiation. The differentiation between *indica* and *japonica*

subspecies was the result of such an adaptive evolution in various ecological habitats where the cultivated rice was grown. The differences in morphological and physiological traits, including apiculus hair, leaf color, plant height, grain shape, tolerance to cold, growth rates, and physiological responses of seeds, are the accumulation of genetic variation across the rice genome during domestication and selection by humans. It has been recognized that a variation in DNA sequences such as DNA rearrangement, base substitution, insertion and deletion was the fundamental cause of *indica-japonica* differentiation and, which is reflected the morphological differences in this study (Wang et al., 2003). A lot of InDel fragments were found on the basis of the two genomic sequence comparison of Nipponbare and 93-11. It was shown from our experiment that polymorphisms of these InDel fragments existed in *indica* and *japonica* subspecies to a large extent. InDel represents insertion/deletion differences between two genome sequences, where one genome has an insertion of a number of nucleotides relative to the others (Jander et al., 2002). The function of the gene will change when the InDel locates in the open reading frame (ORF) of the functional genes, which will lead to substantial variation between the two types of rice varieties. Based on the fact that *indica* and *japonica* varieties exist, we question whether the differentiation into *indica* and *japonica* types has already occurred in the wild species before domestication. Although it is still difficult to satisfactorily answer this question with sound evidence at this moment, there is no doubt that human selection and rice adaptation to various ecological conditions have greatly speeded up the *indica-japonica* differentiation of *O. sativa*.

All the *indica*-specific and *japonica*-specific InDel alleles in this study were distributed evenly along the twelve rice chromosomes (2–5 InDel primers for each). Based on results from this study, we deduced that the processes relating to the *indica-japonica* differentiation involved many molecular evolutionary events associated with DNA sequence variation in most rice chromosomes. Thus, these molecular markers based on InDel fragments should be very useful in the study of *indica-japonica* differentiation and the origin of *O. sativa*.

To investigate the relationships among the cultivated rice varieties and wild rice species with different genomes, data collected from all the rice samples in this study were subjected to PCA. The results illustrated a clear genetic variation pattern of the included *Oryza* accessions as different groups, i.e. the *indica* group, *japonica* group, and wild rice group. The *indica* rice varieties showed relatively greater genetic diversity than the *japonica* rice varieties—a result supported by PCA and allelic polymorphism data. This probably suggests a longer domestication history and more extensive cultivation regions of *indica* rice. Results from the PCA further demonstrated that the wild *Oryza* species (especially the wild progenitor of Asian cultivated rice) were scattered randomly with the *indica* rice accessions, although the *japonica* varieties were grouped more or less independently from all other rice accessions included in this study. This result supports the conventional conclusion that the *indica* cultivated rice was domesticated directly from its ancestral wild species and the *japonica* type derived from the *indica* type later by adapting to particular environments at high elevations and latitudes (Chang, 1976; Lu et al., 2002; Song et al., 2003). There were four *indica* rice varieties from Jiangxi Province in China, South Korea, Laos and Indonesia, respectively, scattering around the *japonica* group. The results either indicate misidentification of these accessions by morphological characteristics, or reflect frequent introgression between the two rice subspecies during the cultivation process because both *indica* and *japonica* types of rice are cultivated in these countries. Some *japonica*-specific alleles may still exist in the *indica* rice varieties, although morphologically they look similar to *indica* rice varieties. The InDel primer pairs detected a high level of DNA polymorphisms in wild rice accessions used in this study, which indicates that these markers have great potential in studies on species relationships and evolutionary processes of genus *Oryza*.

Asian cultivated rice is an important model plant in studies on plant genomics. The origin and genetic differentiation processes are important questions to be addressed for a wide range of scientists, including geneticists, evolutionary biologists, and rice breeders. This study demonstrated the utility of the InDel primer pairs as new molecular markers based on the insertion/deletion fragments from comparative genomics of DNA sequences between 93-11 and Nipponbare. With high accuracy in identifying *indica* and *japonica* varieties, ease of analysis as length differences of PCR products, and clear position on the rice genomes, the InDel molecular markers will play an important role in revealing the molecular mechanism of rice genetic differentiation, map-based cloning of functioning genes, marker-assisted breeding, and studies on the origin of rice and evolution of the genus *Oryza*.

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