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DNA Fingerprinting of Rice Cultivars using AFLP and RAPD Markers

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ABSTRACT

This experiment was conducted to evaluate genetic variation in 48 rice accessions (*Oryza sativa* L.) using AFLP and RAPD markers. For AFLP, a total of 928 bands were generated with 11 primer combinations and 327 bands (35.2%) of them were polymorphic among 48 accessions. In RAPD analyses using 22 random primers 145 bands were produced, and 121 (83.4%) were polymorphic among 48 accessions. Each accession revealed a distinct fingerprint by two DNA marker systems. Cluster analysis using AFLP-based genetic similarity tended to classify rice cultivars into different groups corresponding to their varietal types and breeding pedigrees, but not using RAPD-based genetic similarity. The AFLP marker system was more sensitive than RAPD in fingerprinting of rice cultivars with narrow genetic diversity.

Key words : rice, cultivar, *indica, japonica, javanica*, AFLP, RAPD, fingerprint, genetic similarity.

Several types of markers have been used to assess genetic diversity and phylogenetic relationship in plant germplasm. While restriction fragment length polymorphisms (RFLPs) has been employed in a number of systematic studies (Cho et al., 1995a; Wang & Tanksley, 1989), the procedure is laborious and expensive, and only a few loci are detected per assay. Random amplified polymorphic DNA (RAPD) analysis allows large number of markers to be assayed inexpensively using the polymerase chain reaction (PCR) technique and single oligonucleotide primers of arbitrary sequence and has been successfully used to fingerprint and to analyse phylogenetic relationships in rice cultivars (Ahn et al., 1996; Cho et al., 1995b; Kwon et al., 1998; Mackill, 1995). RAPD markers are so sensitive as to detect relatively low level of polymorphism within *japonica* pool (Ahn et al., 1996; Redona & Mackill, 1996). Another PCR-based marker system, microsatellite or simple sequence repeat (SSR) offers the potential for fingerprinting of plant species (Jeong et al., 1998; Powell et al., 1996; Russell et al., 1997).

Amplified fragment length polymorphism (AFLP) which combines the desirable characteristics of RFLP and RAPD was developed (Vos et al., 1995). As compared to RFLP, a PCR-based AFLP technique requires only minimal amount of template DNA and generates more polymorphism among even closely related species. Also, unlike RAPD analysis, AFLP markers are robust, reliable and reproducible. In addition, AFLP analysis requires no prior sequence knowledge of the target genome so that its cost is reduced for screening the genome with the highly polymorphic primers (Bates et al., 1996). An AFLP technique is composed of three major steps : (1) restriction endonuclease digestion of genomic DNA followed by ligation of adapters (2) PCR amplification of the restricted fragments and (3) size-fractionation of the radio-labelled PCR products on polyacrylamide gel. A large number of AFLP markers are available to be used for DNA fingerprinting of plant species (Hill et al., 1996).

In the present study, we compared the efficiency of two marker systems, AFLP and RAPD, for fingerprinting of varieties and evaluation of genetic diversity among 48 rice accessions (*Oryza sativa* L.).

MATERIALS AND METHODS

Plant materials and DNA isolation

Forty-eight cultivars (Table 1) of *indica, japonica, javanica* (tropical *japonica*), Korean Tongil-type (*indica/japonica*) rice pools originated from six countries and IRRI were used in this study. Accessions were grown in greenhouse at IRRI, Philippines. Total genomic DNA was isolated from fresh leaves of 5-week-old seedlings with a modification of the method described by Dellaporta et al. (1983).

AFLP analysis

AFLP analysis was conducted as described by Vos et al. (1995). About 500 ng of genomic DNA was digested with *EcoRI* and *MseI* and double-stranded adapters were ligated to the fragment ends. This was followed by a pre-amplification step using non-selective *EcoRI* primers and one selective *MseI* (+C) primers. Selective amplifications were performed on the pre-amplified fragment mixture using a total of eleven primer combinations. Only the *EcoRI* primer was radio-labelled with (γ-^{32}P)ATP (Sigma). Five *EcoRI* primers had two selective nucleotides (+2), and four *MseI* primers had three selective

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Table 1. Ecotypes and origins of 48 rice accessions used in AFLP and RAPD analyses.

Entry no.	Accession	Group	Origin	Entry no.	Accession	Group	Origin
1	Sobaegbyeo	*Japonica*	Korea	25	Daeribbyeo 1	*Japonica*	Korea
2	Fuji 269	*Japonica*	Japan	26	S-201	*Japonica*	USA
3	Odaebyeo	*Japonica*	Korea	27	S-202	*Japonica*	USA
4	Akitsuho	*Japonica*	Japan	28	M-202	*Japonica*	USA
5	Samnambyeo	*Japonica*	Korea	29	M-401	*Japonica*	USA
6	Ilpumbyeo	*Japonica*	Korea	30	T(N) 1[‡]	*Indica*	Taiwan
7	Inabawase	*Japonica*	Japan	31	Tongil	Tongil	Korea
8	Jinmibyeo	*Japonica*	Korea	32	Milyang 23	Tongil	Korea
9	Cheonmabyeo	*Japonica*	Korea	33	Namcheonbyeo	Tongil	Korea
10	Fuji 280	*Japonica*	Japan	34	Taebaegbyeo	Tongil	Korea
11	Jinbubyeo	*Japonica*	Korea	35	Dasanbyeo	Tongil	Korea
12	Koshihikari	*Japonica*	Japan	36	IR65597-29-3-2-3	NPT[§]	IRRI
13	Nonganbyeo	Tongil[†]	Korea	37	IR65598-112-2	NPT	IRRI
14	Nongbaeg	*Japonica*	Korea	38	IR65600-1-2-3	NPT	IRRI
15	Dongjinbyeo	*Japonica*	Korea	39	L-202	*Javanica*	USA
16	Seomjinbyeo	*Japonica*	Korea	40	Labelle	*Javanica*	USA
17	Hwaseongbyeo	*Japonica*	Korea	41	BL 1	*Javanica*	Japan
18	Tamjinbyeo	*Japonica*	Korea	42	Gaok	*Javanica*	Indonesia
19	Hanyangjo	*Indica*	Korea	43	Gendjah Gempol	*Javanica*	Indonesia
20	Chucheongbyeo	*Japonica*	Korea	44	Gendjah Wangkal	*Javanica*	Indonesia
21	Sasanishiki	*Japonica*	Japan	45	Tetep	*Indica*	Vietnam
22	Kinuhikari	*Japonica*	Japan	46	IR 8	*Indica*	IRRI
23	Hyangmibyeo 1	Tongil	Korea	47	IR 36	*Indica*	IRRI
24	Yangjobyeo	*Japonica*	Korea	48	IR 64	*Indica*	IRRI

[†] Tongil-type : *indica*/*japonica*; [‡] T(N) 1 : Taichung Native 1
[§] NPT : The breeding lines of New Plant Type (*indica*/*javanica*)

Table 2. Oligonucleotide adapters and primers used for AFLP analysis.

EcoRI-adapter[†] CTCGTAGACTGCGTACC
CTGACGCATGGTTAA
MseI-adapter[†] GACGATGAGTCCTGAG
TACTCAGGACTCAT

AFLP primers[‡]

EcoRI+0 : GACTGCGTACCAATTC+0

EcoRI+2:
E1 GACTGCGTACCAATTC+**AA**
E2 GACTGCGTACCAATTC+**AC**
E3 GACTGCGTACCAATTC+**AT**
E4 GACTGCGTACCAATTC+**TC**
E5 GACTGCGTACCAATTC+**TG**

MseI+1 : GATGAGTCCTGAGTAA+C

MseI+3 :
M1 GATGAGTCCTGAGTAA+**CAG**
M2 GATGAGTCCTGAGTAA+**CAT**
M3 GATGAGTCCTGAGTAA+**CTC**
M4 GATGAGTCCTGAGTAA+**CTG**

Primer combinations analyzed in this experiment
E1/M2, E1/M3, E1/M4, E2/M1, E2/M4, E3/M3, E3/M4, E4/M1, E4/M3, E4/M4, E5/M2

[†] *EcoRI* and *MseI* adapters were ligated onto the ends of restricted fragments of template genomic DNAs.
[‡] *EcoRI*+0 and *MseI*+1 primers were used in the preamplification of template DNA. The AFLP fingerprint was generated using pairs *EcoRI*+2 and *MseI*+3 primers.

nucleotides (+C+2) (Table 2). Amplification products were separated with denaturing 6% polyacrylamide gel electrophoresis (PAGE), and visualised by autoradiography.

RAPD analysis

RAPD analysis was performed with some modifications of the method described by Williams et al. (1990). Amplified PCR products were separated in 1.5% agarose gel containing 0.5μg/ml EtBr in 1 x TAE buffer (40 mM Tris base, 20 mM glacial acetic acid, 2mM Na_2-EDTA · $2H_2O$) at 80~100 volts for 2~3 hours. Separated PCR products were visualized under UV light and photographed with Polaroid film 667 to examine the band patterns. The 22 primers used in this study were OPA-01, OPA-18, OPB-19, OPD-06, OPE-01, OPF-18, OPG-05, OPN-01, OPN-05, OPN-08, OPN-12, OPN-16, OPN-18, OPAB-13, OPAJ-01, OPAJ-02, OPAJ-05, OPAJ-06, OPAJ-09, OPAJ-10, OPAJ-11, and OPAJ-13. These primers were selected based on the results of the preliminary experiment, and their sequences are available from the manufacturer's information.

AFLP and RAPD markers scoring and data analysis

Only clear and unambiguous bands were scored. Band profiles generated by AFLP and RAPD analyses were scored with 1 indicating the presence and 0 indicating the absence of the corresponding band. Each band was then treated as a unit character and pair-wise genetic similarity coefficients among cultivars were quantified based on Nei's formular [$GS=2N_{ab}/(N_a+N_b)$, (N_a : the number of band produced for accession A; N_b : the number of band produced for accession B; N_{ab} : the number of common band produced for accessions A and B)] (1987) using NTSYS-pc software (Rohlf, 1992). The cluster analysis was carried out by the unweighted pair-group method with arithmetic mean (Sokal & Michener, 1958).

RESULTS

Characteristics of DNA markers

A typical AFLP fingerprint using the primer combination E1(E+AA)/M3(M+CTC) is shown in Figure 1. A few major bands were polymorphic among 26 rice accessions. Different results were obtained when only a selective sequence in two primer combinations E2 (E+*AC*)/M1(M+CAG) and E4(E+*TC*)/M1(M+CAG) was changed (results not shown). A number of bands produced by 11 primer combinations ranged from 43 to 121 with an average of 84.4 per primer combination. A total of 928 AFLP markers were generated and 327 bands (35.2%) were informative in differentiating at least one cultivar from the others. The AFLP bands unique to *japonica* (black arrow head 2 in Fig. 1) and *indica* (black arrow head 1 in Fig. 1) were 17 and 14, respectively. RAPD profile of 48 accessions using a primer OPN-05 is shown with polymorphic bands (Fig. 2). A total of 145 bands were produced by 22 RAPD primers among 48 accessions, and 121 bands (83.4%) were polymorphic. The number of bands for each primer ranged from 2 to 11 with an average of 5.5. The bands unique to *japonica* or *indica* (white arrow heads in Fig. 2) rice pools were 8 and 7 in RAPD analysis, respectively. Within closely related *japonica* cultivars of Korea and Japan, the average percent polymorphisms were 53.8% for RAPD and 29.7% for AFLP, respectively.

Variety fingerprint

Forty-eight rice accessions could be differentiated using two DNA marker systems. Any single primer combination of AFLP analysis could not successfully fingerprint all the accessions analyzed. The results of single unit by five AFLP primer combinations E1/M3, E1/M4, E2/M1, E3/M4 and E4/M3 could discriminate other *japonica* rice accessions except for four to six *japonica* cultivars. Thirteen *indica* cultivars including six Korean Tongil-type cultivars were differentiated from each other by single AFLP primer except for E2/M4, E4/M1 and E4/M4. Differentiation of 48 rice accessions was possible by 22 kinds of the combined results of two assay units in

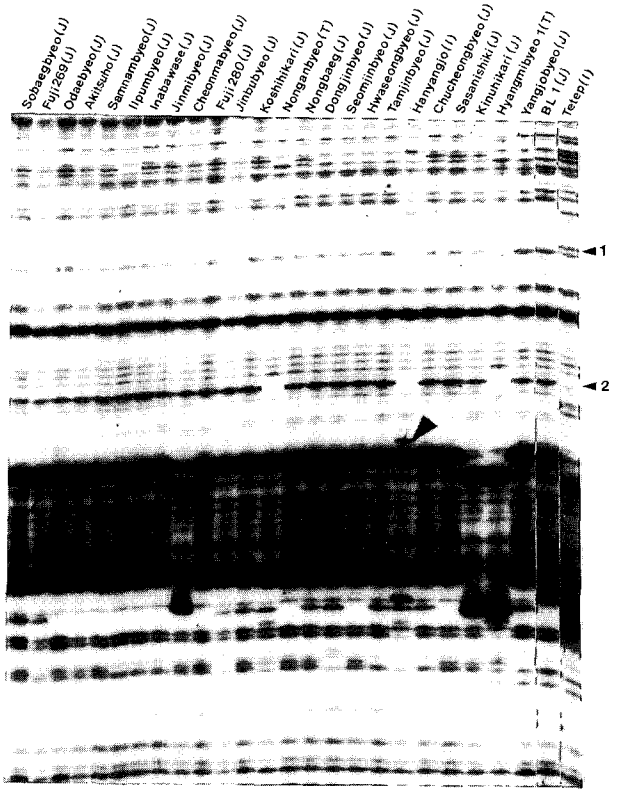

Fig. 1. A portion of AFLP fingerprint patterns in rice cultivars using a primer combination E1(E+AA)/M3(M+CTC).
( ) : J, *japonica*; T, Korean Tongil-type; I, *indica*. The black arrow heads indicate the subspecies-specific unique band to *japonica* rices (◀2) and *indica* (◀1), respectively. The white arrow head (◁) indicates the unique band to Hanyangjo (Korean native rice).

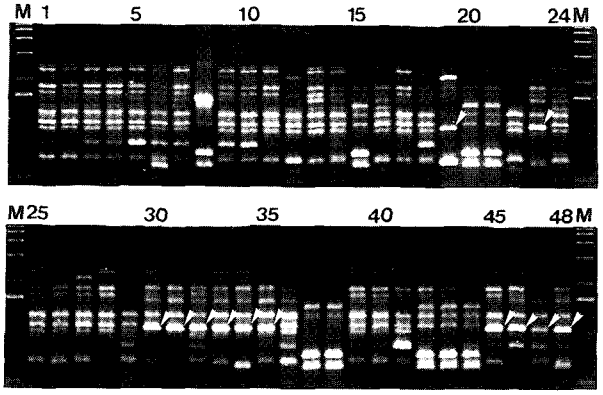

Fig. 2. RAPD profiles of 48 rice accessions. Genomic DNAs were amplified using an arbitrary primer OPN-05 (5'-ACTGAACGCC-3'). See Table 1 for lane number. The white arrow head (◁) indicates the unique band to *indica*. M is 1-kb ladder.

Table 3. List of primer combinations that could uniquely differentiate among 48 rice accessions by AFLP analysis.

Primer combinations	Primer combinations
E1/M2 and E1/M4	E1/M4 and E3/M4
E1/M2 and E2/M1	E2/M1 and E1/M4
E1/M2 and E3/M3	E2/M1 and E2/M4
E1/M2 and E3/M4	E2/M1 and E3/M3
E1/M2 and E4/M3	E2/M1 and E3/M4
E1/M3 and E1/M4	E2/M1 and E4/M1
E1/M3 and E2/M1	E2/M1 and E4/M3
E1/M3 and E2/M4	E2/M4 and E3/M4
E1/M3 and E3/M3	E2/M4 and E4/M3
E1/M3 and E3/M4	E4/M3 and E3/M4
E1/M3 and E5/M2	E4/M3 and E5/M2

AFLP analysis, and five primer combinations E1/M2, E1/M3, E2/M1, E3/M4 and E4/M3 were effective (Table 3). Nine AFLP primer combinations except for E1/M4, E3/M4 and E4/M4 produced the accession-specific bands that could fingerprint one to five specific rice accessions like the specific band produced only for Hanyangjo (white arrow head in Fig. 1). The individual 22 RAPD primers were not able to discriminate 48 accessions because RAPD markers were limited with the number of bands than AFLP. The fingerprinting of 48 accessions was possible with seven kinds of the combined results by ten RAPD assay units. Ten RAPD primers OPB19, OPF18, OPG5, OPN1, OPN5, OPN12, OPN18, OPAB 13, OPAJ6, and OPAJ13 were effective to fingerprint among rice cultivars in this study.

Genetic similarity

Genetic similarities among 48 rice accessions calculated by Nei's formular (1987) ranged from 0.755 to 0.986 by AFLP and from 0.503 to 0.979 by RAPD, respectively. *Japonica* rice pool had lower genetic variation than *indica* and *javanica* rice pools in both marker systems. Genetic similarities among twenty-one *japonica* cultivars with high grain quality of Korea and Japan origins ranged from 0.873 to 0.986 with an average of 0.951 by AFLP and from 0.807 to 0.979 with an average of 0.897 by RAPD. Genetic similarities among 13 *indica* rice cultivars including Korean Tongil-type ranged from 0.840 to 0.969 (0. 908 on average) by AFLP and from 0.593 to 0.966 (0.770 on average) by RAPD. Genetic similarities among *javanica* cultivars including three NPT (New Plant Type) breeding lines developed from the crosses between *indica* and *javanica* were similar to *indica*.

Cluster analysis

Two dendrograms were constructed among 48 rice accessions based on genetic similarities of AFLP and RAPD analyses respectively (Fig. 3). Forty-eight accessions were separated into two main clusters of *indica* and *japonica* groups at 80.4% genetic similarity by AFLP and

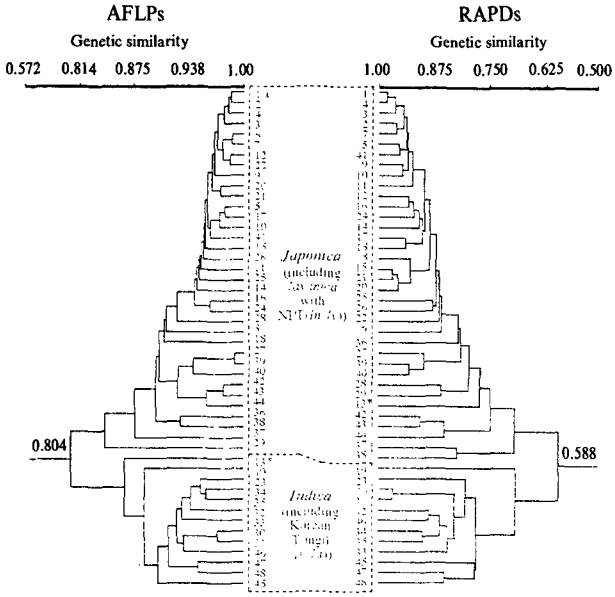

Fig. 3. Cluster diagrams for 48 rice accessions (*Oryza sativa* L.) are classified based on AFLPs(left) and RAPDs(right) analyses, respectively.
a/The numbers are the entry no. of Table 1.
*#13 : Nonganbyeo developed from the cross between *japonica* and Tongil-type was clustered into *indica* and *japonica* by AFLPs and RAPDs, respectively.

58.8% by RAPD, respectively. Korea-originated *japonica* cultivars had a trend to be clustered with Japanese *japonica* cultivars which were used as parents in the AFLP -based dendrogram, but not in the RAPD-based dendrogram. Korean Tongil-type cultivars were clustered within *indica* group by both AFLP and RAPD analyses. A Korean cultivar Nonganbyeo (*japonica*/Tongil-type) was clustered into *indica* by AFLP and into *javanica* by RAPD, respectively. *Javanica* cultivars belonged to *japonica* group, but clear distinction was not found between *japonica* and *javanica* groups based on both AFLP and RAPD markers. Six U.S.A. cultivars with short-, medium- and long-grain types were clustered into *japonica* group including *javanica*.

DISCUSSION

Efficiency of molecular biological techniques is important with the development of DNA markers for plant genome analysis. Comparisons of various tools are necessary in order to decide which technique is best suited to fingerprint rice cultivars and to evaluate genetic diversity and phylogenetic relationships of rice accessions. Although the polymorphism level revealed by AFLP was lower than that by RAPD, the number of polymorphic bands were much higher, producing the average of fourteen bands per assay unit. This result is similar to those of Mackill et al. (1996) and Russell et al. (1997). Powell et al. (1996) introduced the concept of Marker Index as an overall measure of marker efficiency, and demonstrated that AFLP had the highest Marker Index compared to other available marker systems in *Glycine*. The high Marker Index or diversity index reflects the efficiency of AFLP in detecting a large number of bands simultaneously. The major advantage of AFLP analysis is relatively rapid assay for a large number of loci. The 327 AFLP polymorphic loci by eleven assay units were generated in about 1 week after DNA preparation, whereas 121 polymorphic markers by 22 RAPD primers were generated in about ten days. This represents an almost 5 fold decrease in production time of AFLP markers compared with that required to generate the same number of polymorphic RAPD bands. AFLP analysis detected about 12 times more polymorphic loci per assay than that of RAPD in soybean (Vogel et al., 1994, recited in Hill et al., 1996).

Genetic diversity of modern rice cultivars has been reduced due to intensive breeding efforts (Mackill, 1995; Wang et al., 1992). Especially, most Korean commercial *japonica* cultivars were developed from the crosses among closely related *japonica* cultivars (Kim et al., 1994). Recently, many researchers carried out to discriminate very closely related commercial rice cultivars by several DNA marker systems. The feasibility of RFLP and microsatellite DNA markers for cultivar identification has been previously discussed in rice (Cho et al., 1995a; Jeong et al., 1998; Russell et al., 1997; Wang & Tanksley, 1989; Zhou & Gustafson, 1995). However, the RFLP approach was not successful in fingerprinting individuals because of low polymorphism among the populations used (Cho et al., 1995a). DNA fingerprinting with microsatellite DNA markers was more sensitive and informative than other types of DNA markers (Zhou & Gustafson, 1995). Vos et al. (1995) reported that the ideal fingerprinting assay should require no prior sequence knowledge. Microsatellites require prior sequence information of primer whereas AFLP and RAPD do not have this requirement. RAPD was useful for classification of rice cultivars, but a combination of several primers would be needed to fingerprint closely related *japonica* rice cultivars (Ahn et al., 1996; Kwon et al., 1998; Mackill, 1995). Our results indicated that at least ten assay units were required to successfully discriminate among 48 rice accessions in RAPD analysis. The advantage of AFLP analysis is the unlimited number of loci that can be assayed with different combinations of a relatively small number of oligonucleotide primers. Also, AFLP analysis can produce much higher number of polymorphic bands per assay unit than other marker systems. Fingerprinting of 48 rice accessions was possible by 22 kinds of the combined results of two assay units in AFLP analysis (Table 3) and nine AFLP primer combinations except for E1/M4, E3/M4 and E4/M4 produced the accession-specific bands that could fingerprint one to five specific rice accessions in single assay unit (white arrow head in Fig. 1). These results demonstrate that AFLP can be effectively used in fingerprinting of *japonica* rice cultivars with similar genetic background.

Classification of rice cultivars based on AFLP and RAPD analyses gave similar results (Fig. 3). Two marker systems classified most cultivars into two main groups (*japonica* vs. *indica*). Minor variation within subgroups was observed for the two types of markers, however, no firm boundary was observed between *japonica* and *javanica*. Most Korean *japonica* cultivars tended to group into different subunits corresponding to their breeding pedigrees by AFLP. Korean Tongil-type cultivars assayed were grouped as a subunit with their first parents IR 8 and Taichung native 1 within *indica* by AFLP. We suggest Korean Tongil-type (*indica*/*japonica*) cultivars have somewhat different genetic compositions with typical *indica*. The discrepancy between the AFLP- and RAPD-based dendrograms was the classification of Nonganbyeo; Nonganbyeo was grouped into *indica* by AFLP but into *javanica* by RAPD analysis. Although Nonganbyeo (*japonica*/Tongil-type) is phenotypically closer to the Tongil-type (*indica*) than to *japonica* or *javanica*, it has different genetic compositions to the Tongil-type. The U.S.-originated rice cultivars didn't usually cluster into a specific subgroup based on two marker systems, and this result was different from that of Mackill (1995) where short- and medium-grain type rices were classified into *japonica* (temprate type) and long-grain types into *javanica* (tropical type). Further studies are required to know whether this result stems from the different combination of primers or other rice

accession sources.

Based on the results from this study, we suggest that AFLP analysis is more effective than RAPD to fingerprint closely related rice cultivars and to assign rice accessions into different ecotypes corresponding to their varietal types and breeding pedigrees.

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