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Genomic Variation of The Rice *Rim2/Hipa* Superfamily and Dendrogram and Fingerprinting Analysis of Rice Germplasm Based on *Rim2/Hipa* Paralog Display*

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Abstract The rice *Rim2*/*Hipa* is a stress-induced transposon superfamily recently identified in *Oryza* genomes. Genomic variation was found in the *Rim2* core region among rice genetic resources/genomes, indicative of high genomic divergence accumulated during the *Rim2* evolution. Based on the divergence and quiescent state of the *Rim2* elements, a *Rim2* paralog display-based fingerprinting approach was developed to effectively identify rice genetic resources and explore their genetic relationships within a set of rice germplasm including 45 accessions of *O. sativa* and 8 accessions of its wild relatives *O. rufipogon*. A dendrogram showed not only clear genetic diversity of rice germplasm, but also considerable genetic differentiation among wild rice resources. The wild rice relatives were either clustered as an independent group, or among the *japonica* varieties. This *Rim2*-based fingerprinting approach could also serve as a sensitive tool to identify rice hybrids from their parents, and variety stability, demonstrating its great potential in evolution study of rice genomes and in rice breeding and seed production.

Key words *Oryza*, *Rim2/Hipa*, genomic variation, dendrogram, fingerprint

Increasing evidence reiterates the fact that transposable elements (TEs) play a significant role in sculpting a genome leading to genomic variation [1]. Usually, TEs, including class 1 (retrotransposons) and class 2 (DNA transposons), harbor quite structural or sequence variation due to deletion, duplication or interelement recombination [2]. Variation in TEs has much impact on evolutional studies of genomes [3]. In addition to these evolutionary events, mutation induced by TE insertion, such as Ac/Ds transposition, has been widely used in plant functional genomics[4].

It has been demonstrated that rice, a monocotyledonous model plant, is a TE-rich species [5~8]. Interestingly, the rice retrotransposon *Tos17* transposes under tissue culture conditions therefore is used to create mutants for rice functional genomics[9,10]. More recently, Jiang *et al.*[11] found that the rice DNA transposon *mPing* was active in cell cultures. *Tos17* and *mPing* are the only TEs that have been found to possess the capacity of transposition in the rice genome so far. The rice *Rim2*/*Hipa* is a novel CACTA-like transposon superfamily recently identified as an ancient component of the *Oryza* genome with evolutionary divergence[7,12,13]. It is one of the most frequent TEs in this species, comprising 3 subgroups, RIM2-coding, RIM2-pseudogene and noncoding [13]. It has been documented that the *Rim2* core sequence (RIM2-coding and pseudogene sequences) is unique to the rice genome. In addition, the *Rim2* core sequence is highly variable within the *japonica* genome, suggesting that there might exist great polymorphism of the sequence among rice

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genetic resources including wild rice and the cultivated subspecies.

In the past decade, DNA markers such as RAPD, AFLP, RFLP and SSR have been extensively used for fingerprinting and exploring the genetic diversity and evolutionary relation of rice genetic resources[14~19]. TE display is also adopted in the fingerprinting practice of plant genetic resources[20,21]. Although these molecular techniques are constantly undergoing modification, few of highly effective and costly economic protocols have been put forward for differentiating closely related rice species or varieties.

This study aims to elucidate genomic variation of the *Rim2* family in a set of rice germplasm including wild rice, and to estimate the potential of the family in developing a novel *Rim2*-based rice DNA marker. We found that the *Rim2* core sequence exhibited wide variation among different rice genetic resources, leading to the development of a novel fingerprinting tool with advantages of simplicity and high resolution based on *Rim2* paralog display using internal primers, which could be used in differentiating rice genetic resources, evolutionary analysis, breeding program and seed production.

1 Materials and methods

1.1 Variation analysis of *Rim2* core region

PCR was conducted to assay the polymorphism of the *Rim2* core sequence (coding/pseudogene region) [12,13], by using DNA templates from IR29723-1438 (*indica*), IR38 (*indica*), H7R (*indica*), Hongtu 31 (*indica*), Taipei 309 (*japonica*), Liaojin151 (*japonica*), Nipponbare (*japonica*), and Shuiyuan 290 (intermediate), and the forward and reverse primers Rim2-11 (5′ GTGACAACGTACGAGGCTAAGGAG 3′), Rim2-12 (5′ ATTGGCACCCGGAACAGAG-GAAC 3′) to amplify main fragments of the *Rim2* core region (AF121139). PCR was performed as follows: 94℃, 1 min; 58℃, 1 min; 72℃, 1 min for 30 cycles followed by 72℃, 10 min for extension. PCR products were cloned into T-easy vector (Promega) and sequenced to confirm variation, by compared with the public Nipponbare sequences (http://rgp.dna.affrc.go.jp/IRGSP/index.html).

1.2 Dendrogram analysis of rice genetic resources / genomes through *Rim2* paralog display

A *Rim2* paralog display approach was adopted to establish a dendrogram for a set of rice genetic resources containing 53 accessions of cultivated rice

Table 1 Rice germplasm used in dendrogram analysis

Variety/Accession	Type	Origin	Variety/Accession	Type	Origin
IR29723-1438	*indica*	Philippines, IRRI	Hongxinnuo	*indica*	China, Yunnan
IR38	*indica*	Philippines, IRRI	Ruandao	*indica*	China, Yunnan
IR36	*indica*	Philippines, IRRI	Alunuo	*indica*	China, Yunnan
IR64	*indica*	Philippines, IRRI	Shuijia-99	*indica*	China, Yunnan
RP2235-48-54-6	*indica*	Philippines, IRRI	Shuiyuan-290	intermediate	Korea
Aixianzhan	*indica*	China, Guangdong	Liaojing-151	*japonica*	China, Liaoning
Changlunzhan	*indica*	China, Guangdong	Lemont	*japonica*	USA
Guiyu-100	*indica*	China, Guizhou	Mars	*japonica*	USA
Guiyu-330	*indica*	China, Guizhou	Baimaojing	*japonica*	China, Yunnan
Chengbao-2	*indica*	China, Jiangxi	Haogan	*japonica*	China, Yunnan
Hongtu-31	*indica*	China, Zhejiang	Jijing-62	*japonica*	China, Jilin
Jiayu-293	*indica*	China, Zhejiang	Nipponbare	*japonica*	Japan
Wanhui-9	*indica*	China, Anhui	Guichao	*japonica*	China, Yunnan
Wujienuo	*indica*	China, Guizhou	Jingdao-1	*japonica*	China, Yunnan
Dabeizigu	*indica*	China, Hubei	Taibai-8	*japonica*	China, Taiwan
Taizhongxianyu	*indica*	China, Taiwan	343	*japonica*	China, Yunnan
Taizhongyu-5	*indica*	China, Taiwan	Guangtougu	*japonica*	China, Yunnan
Sankecun	*indica*	China, Hubei	Chujingxiang-1	*japonica*	China, Yunnan
Maogunuo	*indica*	China, Yunnan	*O. rufipogon*-DX	wild	China, Jiangxi
Jiudao-6	*indica*	China, Jiangsu	*O. rufipogon*-BL	wild	China, Guangdong
Jinyougui-99	*indica*	China, Yunnan	*O. rufipogon*-SX	wild	China, Guangdong
Gangyou-225	*indica*	China, Yunnan	*O. rufipogon*-GZ	wild	China, Guangdong
Hongyou	*indica*	China, Yunnan	*O. rufipogon*-FG	wild	China, Guangdong
Xianggu	*indica*	China, Yunnan	*O. rufipogon*-HL	wild	China, Guangdong
Gangyou-22	*indica*	China, Yunnan	*O. rufipogon*-ZC	wild	China, Guangdong
Xiandaonuo	*indica*	China, Yunnan	*O. rufipogon*-CL	wild	China, Hunan
Xianyounuo	*indica*	China, Yunnan			

and wild rice *O. rufipogon* with different origins as shown in Table 1. Five pairs of internal PCR primers were selected to produce polymorphism fragments according to the *Rim2* core sequence (Table 2). PCR was performed in a 25 μl volume containing 1 U of Taq polymerase, 200 μmol/L (each) dNTPs, and 0.2 μmol/L (each) primers. PCR products were separated on 1.2% agarose gels. Each PCR was repeated at least three times, amplified bands were recorded for fingerprinting and cluster analysis. Ratios of commonly amplified fragments were calculated and a dendrogram showing genetic relationships of the 53 accessions was constructed, based on similarity coefficients between accessions quantified using the unweighted pair-group method with arithmetic mean (UPGMA) of the NTSYS-pc analytical software[22].

Table 2 The primers selected for PCR

Primer pairs	Sequences	Anneal temperatures/℃
A	Rim2-11:GTGACAACGTACGAGGCTAAGGAG	61
	PRM-4:AATTACGGCATTCCATCGGC	
B	PRM-7:CAAGCAACCTGGTAACGACA	56
	PRM-4:AATTACGGCATTCCATCGGC	
C	Rim2-14:TAAGTGGATAAATCGTAATAAGCAAAT	58
	PRM-7:CAAGCAACCTGGTAACGACA	
D	ZH19-1:ACCCGGAACCAAAGGAGAAAG	60
	PRM-4:AATTACGGCATTCCATCGGC	
E	PRM-5:ATCGTCGATTCCTTGCAGCA	56
	Rim2-14:TAAGTGGATAAATCGTAATAAGCAAAT	

1.3 Differentiation of hybrids from the parents and variety purity

For the utilization of the *Rim2*-based fingerprinting technique in rice breeding and seed production, genomic DNA was extracted from individual plants of the varieties Nipponbare (*japonica*) and H7R (*indica*), the early breeding generation of Sheng10/SWR22 (genetically unstable), the advanced breeding generation of Zhenshan 97/eui stock (nearly stable), hybrids (F_1) 101A/H9816, FengA/H1, XieA/H6, and their corresponding parents. PCR was performed with the 5 pairs of primers as described above to detect varietal purity and distinguish hybrids from their parents.

2 Results

2.1 Genomic divergence of the *Rim2* core region

Our previous finding of numerous *Rim2* copies with variable internal regions in the Nipponbare genome suggested that there might exist a great genomic variation of the *Rim2* core region including coding and pseudogene sequences[13], among different *Oryza* genomes or genetic resources. Therefore, we used PCR to detect polymorphism in the *Rim2* core region as described above. The primers Rim2-11 and Rim2-12 amplified a 2.4 kb *Rim2* core fragment (Figure 1), along with about 1 kb and 0.7 kb fragments that showed polymorphic within the 8 rice varieties tested. Cloning and sequencing of the PCR products proved that the smaller bands were amplified from the *Rim2* core sequence with deletions (accession No. AY423426, 957 bp and AY423427, 726 bp). Furthermore, we conducted a detailed analysis of the *Rim2* sequences from the international *japonica* genome program (http://rgp.dna.affrc.go.jp/IRGSP/

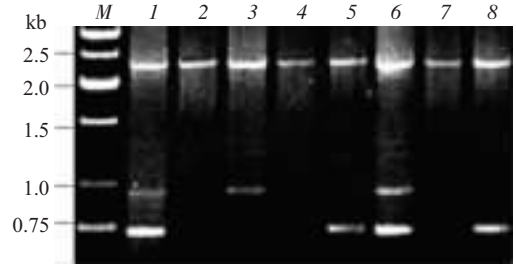
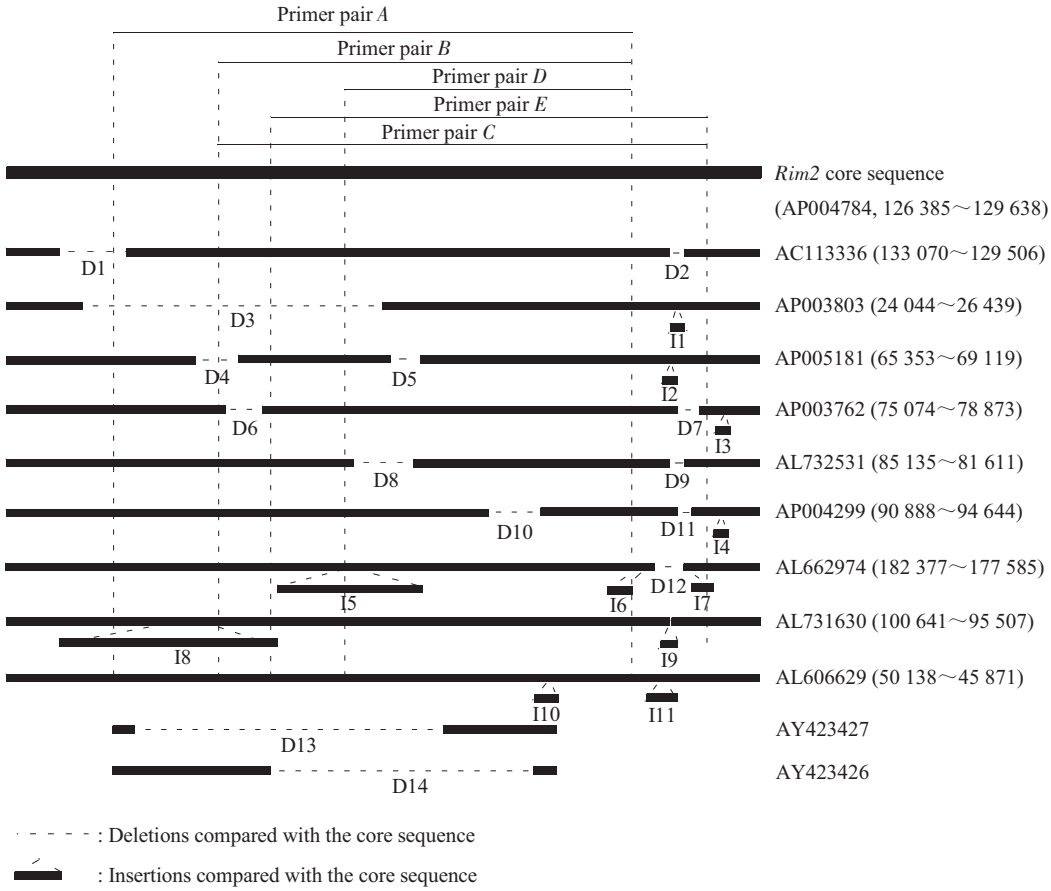


Fig. 1 The variation of the *Rim2* core region reveled by PCR using the primers Rim2-11 and Rim2-12 among eight rice varieties

1: IR29723-1438; *2*: IR38; *3*: H7R; *4*: Hongtu 31; *5*: Taipei 309; *6*: Shuiyuan 290; *7*: Liaojin151; *8*: Nipponbare. The amplified fragments were cloned and sequenced. *M*: DNA markers.

index.html), and found many deletions and insertions in the core region dispersed in different *Rim2* members (Figure 2). These results demonstrated that the *Rim2* core region exhibited a great variation during the genome evolution.



D1: 245 bp	D2: 111 bp	D3: 1 636 bp	D4: 104 bp	D5: 160 bp	D6: 139 bp
D7: 108 bp	D8: 314 bp	D9: 91 bp	D10: 210 bp	D11: 108 bp	D12: 107 bp
D13: 1 592 bp	D14: 1 374 bp				
I1: 108 bp	I2: 112 bp	I3: 106 bp	I4: 103 bp	I5: 751 bp	I6: 112 bp
I7: 107 bp	I8: 1 089 bp	I9: 90 bp	I10: 145 bp	I11: 221 bp	

Fig. 2 Schematic representation of divergence of the *Rim2* core region

Examples for different deletions (D1～14) and insertions (I1～17) were mined from BACs (indicated on right) of the *japonica* genome, compared with the core sequence in the BAC AP004784 (highlighted). The lengths of the deletions and insertions are showed. The beginning and end sites of PCR primers for fingerprinting are indicated.

2.2 Genetic relationships of rice resources / genomes based on *Rim2* paralog display

Since PCR of *Rim2* sequences demonstrated a clear polymorphism among the rice genetic resources (Figure 1), we conducted a wide *Rim2* variation analysis and estimated genetic relationships of a set of rice germplasm, including 45 accessions of rice varieties and 8 accessions of wild rice (*O. rufipogon*) with different origins (Table 1), using 5 PCR primer pairs designed based on the *Rim2* core sequence that amplified internal fragments (Figure 2). All the 5 primer pairs (*A* to *E*) yielded informative bands with obvious polymorphisms among the *Oryza* accessions (Figure 3). A total of 63 polymorphic fragments with lengths from 300 bp to 2 900 bp were generated by the 5 primer pairs, with an average of about 12 polymorphic fragments for each primer pair. We sequenced some of the PCR products and confirmed their derivation of *Rim2* sequences.

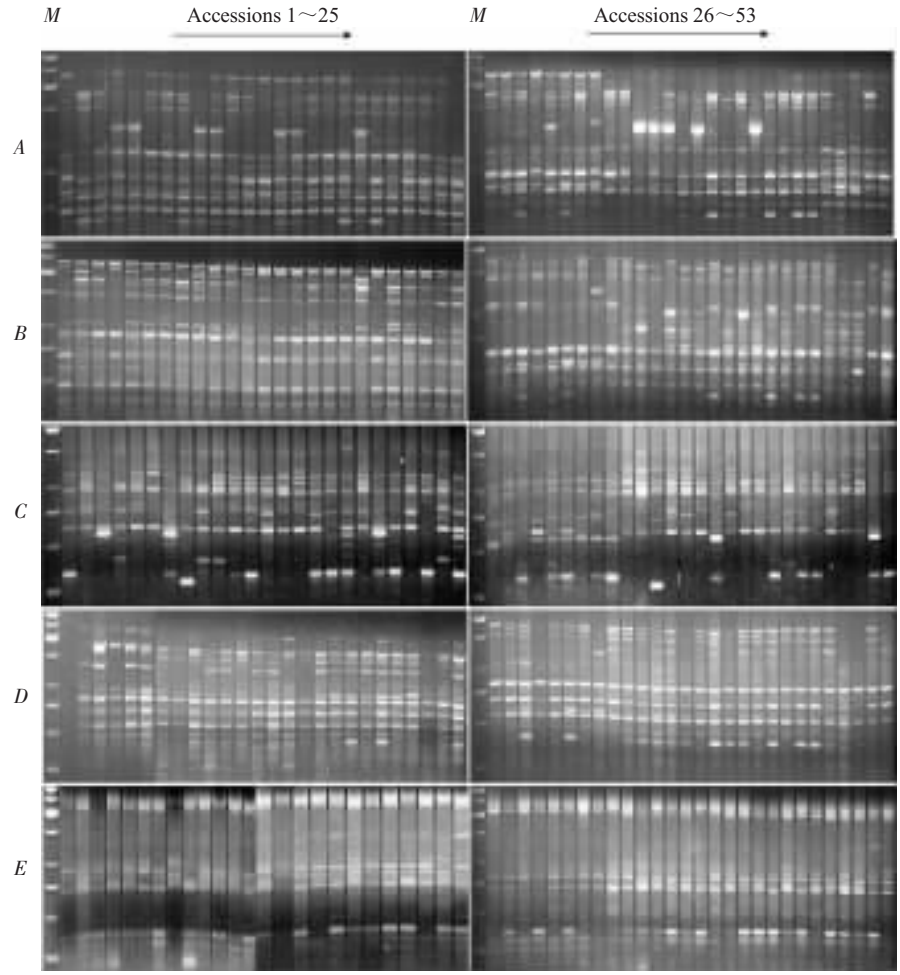


Fig. 3 *Rim2* paralog display for fingerprinting the 53 rice accessions

1～53: IR29723-1438, IR38, Aixianzhan, Changlunzhan, Guiyu-100, Guiyu-330, Chengbao-2, RP2235-48-54-6, Hongtu-31, Jiayu-293, Wanhui-9, Wujienuo, Dabeizigu, Taizhongxianyu, Taizhongyu-5, Sankecun, Maogunuo, Jiudao-6, Shuiyuan-290, Liaojing-151, Lemont, Mars, Baimaojing, Haogan, Jijing-62, *O. rufipogen*-DX, *O. rufipogen*-BL, *O. rufipogen*-SX, *O. rufipogen*-GZ, *O. rufipogen*-FG, *O. rufipogen*-HL, *O. rufipogen*-ZC, *O. rufipogen*-CL, Nipponbare, IR36, IR64, Jinyougui-99, Gangyou-225, Hongyou, Guichao, Jingdao-1, Xianggu, Gangyou-22, Xiangdaonuo, Taibei-8, Xiangyounuo, 343, Guangtugu, Shuijia-99, Hongxinnuo, Ruandao, Chujingxiang-1, Alunuo (I, *indica*; J, *japonica*; M, *intermediate*). PCR reactions were conducted with the primer pairs *A*～*E*. *M*: DNA markers.

With all the characteristic bands generated from each rice accession, an UPGMA dendrogram was constructed that showed genetic diversity of the included rice germplasm, with similarity coefficients ranging from 0.42 to 0.88 (Figure 4). The result indicated the power of the simple fingerprinting technique derived from the *Rim2* sequence variation, which could effectively distinguish the diverse set of rice germplasm with only a few primer pairs. Interestingly, there was a clear tendency that nearly all the *indica* varieties were clustered into one group and the *japonica* varieties into another group, with some exceptions. The single accession of the intermediate type (Shuiyuan 290) was included in the *indica* group, indicating its close evolutionary linkage with the *indica* rice genome. The rice wild relative *O. rufipogon* accessions were either clustered independently from the cultivated rice varieties, or among the *japonica* varieties. The independent wild rice group included accessions from Guangdong Province (*O. rufipogon*- GZ and *O. rufipogon*-ZC) and Jiangxi Province (*O. rufipogon*-DX), whereas the accessions that clustered with *japonica* varieties were from Guangdong Province (*O. rufipogon*-SX, *O. rufipogon*-HL, *O. rufipogon*-FG and *O. rufipogon*-BL) and Hunan Province (*O. rufipogon*-CL). There was no apparent association of the wild rice accessions with their geographical origins.

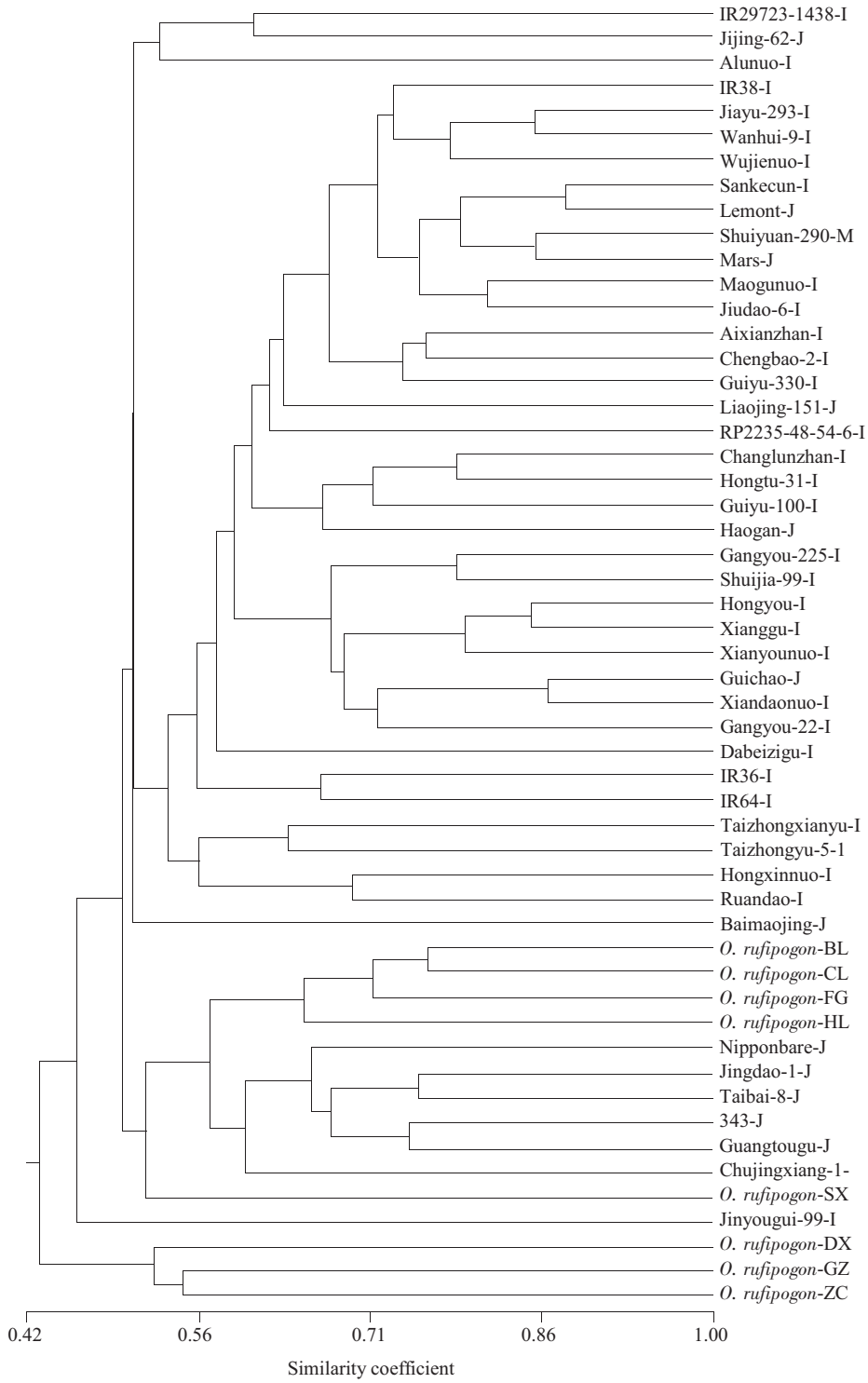


Fig. 4 A UPGMA dendrogram generated from cluster analysis of the amplified polymorphic bands of the *Rim2* core region, showing genetic diversity of the included rice genetic resources and a clear differentiation pattern of *indica* and *japonica* varieties

2.3 *Rim2* paralog display for detecting variety purity, hybrids and parents

For any fingerprinting analysis, it is important that a fingerprint is unique to a given genetically stable variety. To examine the sensitivity of the *Rim2*-based fingerprinting technique in identifying stable or unstable rice lines, we analyzed each 10 individuals from Nipponbare (*japonica*) and H7R (*indica*), the early breeding generation of Sheng10/SWR22 (BC_1F_3), and the advanced generation of Zhenshan 97/eui stock

(BC_2F_5). The results showed that all individuals of the purified varieties Nipponbare and H7R had identical bands amplified by the five primer pairs (Figure 5). As expected, individuals from the early generation of Sheng10/SWR22 showed variable fingerprints, whereas the advanced generation of Zhenshan 97/eui showed nearly identical amplification bands among the individuals. Interestingly, this fingerprinting technique could clearly differentiate hybrids and their parents (Figure 5). Moreover, the fingerprints of the hybrids appeared to be mixtures of the both parents. These results attested the practicability of the fingerprinting tool in not only detecting seed purity/variety stability, but also differentiating hybrid rice and parents.

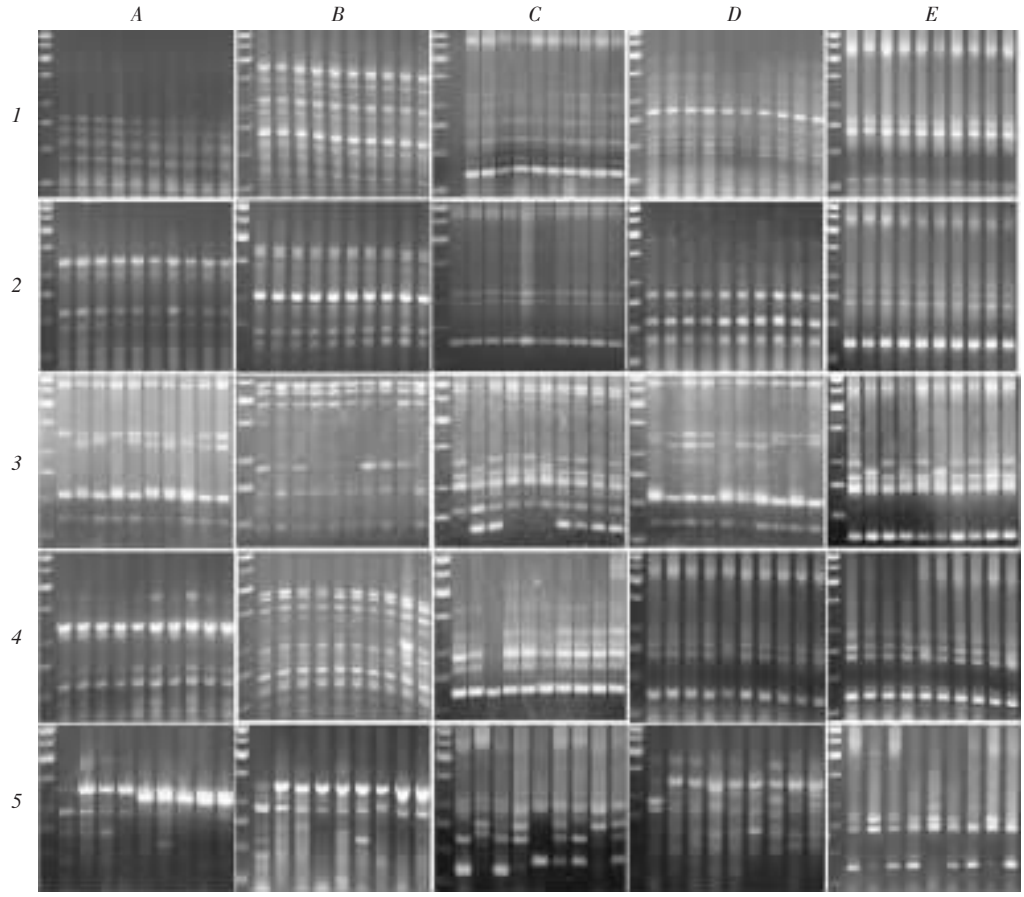


Fig. 5 *Rim2*-based fingerprinting for detecting seed purity, hybrids and their parents

Each 10 individuals from Nipponbare (*1*), H7R (*2*), the early breeding population (*3*), and the advanced breeding population (*4*) were subjected to PCR analysis with the primer pairs *A* to *E*. PCR was also performed to distinguish hybrids from their parents (*5*), with 101A, H9816, 101A/H9816 (F_1), FengA, H1, FengA/H1 (F_1), XieA, H6 and XieA/H6 (F_1) from left to right on each electrophoresis gel with DNA markers in the first lane.

3 Discussion

The *Rim2* family is the biggest family of class 2 transposons with big size reported to date in plant kingdom [13]. Our current study provides tangible evidence that this family is really divergent. Genomic variation of the *Rim2* core region among the rice resources indicates that this family has undergone long persistence of amplification and mutation during its evolution. Furthermore, polymorphic bands stacking in the hybrid rice implied that interelement recombination could happen in progenies if they locate at the same chromosome regions (Figure 5), this kind of recombination certainly could be one of sources of its divergence. On the other hand, identical *Rim2* fragments were detected both among 10 individuals of *japonica* and *indica* varieties respectively (Figure 5). This fact illustrated that *Rim2* elements could have lapsed into relatively stable state, because the transposition of the elements would give rise to new recombinants or derivatives. This observation explains our finding of no active *Rim2* element detected yet

(Shi and He unpublished data). In deed, most of so many TE families identified in the past decade have been known silenced.

The high abundance and rich variation of *Rim2* allow us to develop a novel fingerprinting tool to study rice genetic resources through *Rim2* paralog display method (Figure 3 and 4). The remarkable polymorphism of the amplified bands sufficiently revealed the discrepancy of the core region among the 53 rice accessions. The *Rim2*-based fingerprinting technique described in this study certainly is a powerful tool not only for distinguishing a diverse set of rice accessions with different origins, but also for identifying rice hybrids from their parents and individuals from a breeding program. These findings demonstrated the potential of developing a more powerful molecular tool based on the tremendous variability of the *Rim2* superfamily for the administration of seed production, variety licensing, marker-aid selection in breeding programs, as well as genetic and evolutional studies, compared with other predominantly recruited fingerprinting methods such as RAPD, AFLP, RFLP and SSR which usually need more amount of assay work [14 ~19]. To our best knowledge, this is the first fingerprinting tool based on the variation of a single class 2 TE family in rice with highly discriminatory power.

Through dendrogram analysis based on the *Rim2* core sequence, identities of a diverse set of rice germplasm were determined with high resolution. Exhilaratingly, the *Rim2*-based dendrogram showed a well separation of *indica* and *japonica* rice varieties with only a few exceptions (Figure 3 and 4). It is in general very difficult to accurately identify the closely related *indica* and *japonica* rice varieties in germplasm conservation and rice breeding. Nevertheless, the correct determination of the two subspecies has significant value for selecting the desired rice germplasm in various genetic studies and rice breeding programs. The potential to develop more accurate *Rim2*-based markers for *indica* and *japonica* identification can be fully explored, given the advantage of a particularly high variability of the *Rim2*. In addition, these *O. rufipogon* accessions were scattered in the dendrogram (Figure 4), suggesting their considerable genetic diversity. The association of particular *O. rufipogon* accessions with *japonica* varieties may be attributed to the natural introgression between the cultivated and wild rice species during their evolutionary process. In fact, some *O. rufipogon* accessions from Guangdong province were found to strongly associated with cultivated rice by using other molecular markers (Lu unpublished data), confirming a significant introgression between wild and cultivated *Oryza* species through natural hybridization. It would be interesting to look for the original / active (autonomous) *Rim2* member and to track its evolutional pathway in rice species.

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水稻 *Rim2/Hipa* 超级家族的基因组变异和基于 *Rim2/Hipa* 展示的水稻资源的系统进化和指纹分析 *

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摘要 水稻 *Rim2*/*Hipa* 是最近鉴定的一个受逆境诱导的转座因子超级家族. 研究表明，*Rim2* 的核心序列在不同来源的水稻材料中存在显著的差异，暗示 *Rim2* 家族的长期进化历程. 基于 *Rim2* 因子间的差异性以及该因子的静止状态，开发出一种利用 *Rim2* 因子展示的新的分子指纹技术，可以灵敏地区分不同水稻资源以及它们的遗传关系. 仅用 5 对引物就可以清楚地将 53 个栽培稻和普通野生稻材料鉴定出来，并可将它们分为不同的系统进化组. 研究表明不仅在水稻资源而且在野生稻种质间均存在明显的多样性. 野生稻可以被单独分组，或者分散在粳稻中间. 这种新的指纹技术还可以将水稻的杂交子代和它们的亲本区分出来，并可用于种子纯度的鉴定，在水稻基因组进化研究、水稻育种和种子生产中有很好的应用前景.

关键词 水稻，*Rim2*/*Hipa*，基因组差异，系统树，指纹
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