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Identification of a set of RFLP probes for subspecies differentiation in *Oryza sativa* L.

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Abstract Sixty-eight *indica-japonica* tester-differentiating RFLP probes were tested in seven *indica* and seven *japonica* varieties of rice (*Oryza sativa* L.) with four enzyme digestions (*Eco*RI, *Eco*RV, *Hin*dIII and *Dra*I). Twenty-one DNA clones were isolated as *indica-japonica* subspecies-differentiating probes. A set of 13 probes was established as core probes for subspecies differentiation and a pooled blotting analysis was carried out to facilitate the application of RFLP in rice genetics and breeding practice. A dendrogram of 12 wide-compatibility varieties was constructed based on RFLPs detected by 13 core probes with single enzyme digestions. It was speculated that most RFLPs of *indica-japonica* differentiating probes were generated by insertions/deletions, which may be of great significance for the origin and differentiation of subspecies in *Oryza sativa* L.

Key words Restriction fragment length polymorphism (RFLP) · Probe screening · Subspecies differentiation · Wide compatibility variety · *Oryza sativa* L.

Introduction

DNA restriction fragment length polymorphisms (RFLPs) are the most widely used molecular marker for reliability and reproducibility (Tanksley et al. 1989). New techniques have been incorporated into RFLP analysis to improve its sensitivity, to simplify the procedures and to avoid the inconvenience of using radioactive isotopes. RFLPs can be used widely in plant breeding. One of the most ready applications is in measuring genetic variation in natural populations in order to explore the evolutionary relationships among plant taxa (Saghai-Maroof et al. 1984).

Rice is one of the most important crops in China as well as in the other parts of the world, and is an ideal monocot species for molecular genetic studies because of its small genome and its low level of repetitive sequences. The classification and origins of subspecies have long bothered rice geneticists and breeders. Morishima and Oka (1981) and Glaszmann (1987) grouped various rice varieties based on morophological traits and isozymes respectively. A morphological index, based on the scores of six characters, was also proposed and evaluated for the classification of rice cultivars (Cheng 1985; Cheng et al. 1985). RFLP offers a new approach to plant taxonomy which is both more effective and more reliable (Wang and Tanksley 1989). However, a survey of RFLPs in the entire rice genome is a major undertaking requiring much time and money. At least 100 probes are needed to cover the rice genome at an average interval of 20 cM.

We have previously surveyed three *indica* and three *japonica* testers for wide compatibility, together with 21 wide-compatibility varieties (WCVs) using 160 RFLP probes combined with four enzymes (*Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I) in rice. One-hundred and twenty-five probes were found polymorphic among the 27 varieties; 68 of them were *indica-japonica* tester differentiating and produced indentical hybridization patterns for testers within subspecies but different patterns between subspecies. It was also possible to isolate probes more powerful in *indica-japonica* differentiation analysis (Zheng et al. 1994).

The study reported here is a continuation of the work described above. Sixty-eight *indica-japonica* tester-differentiating probes were further screened in seven *indica* and seven *japonica* varieties to distinguish subspecies-differentiating probes, and a set of core probes for subspecies differentiation was established and confirmed as practicable for the phylogenetic analysis of 12 WCVs in the exploitation of interspecies heterosis in rice.

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Materials and methods

Plant materials

Fourteen rice cultivars, whcih were established *indica* and *japonica* varieties, were adopted to screen *indica-japonica*-differentiating probes and core probes for subspecies differentiation (Table 1, nos. 1–14). These varieties were selected from various origins. Nos. 15–26 in Table 1 were 12 wide-compatibility varieties (WCVs), selected from the previously used 21 WCVs, which were employed to test the applicability of the core probes for subspecies differentiation (Zheng et al. 1994).

Rice plants were grown in the paddy field during a normal season in Hangzhou, China. Leaves were harvested for DNA extraction from a single plant of each variety.

RFLP markers

Three types of RFLP markers were used. RG# and G# clones are random rice genomic DNA fragments, RZ# are rice cDNA fragments, and CDO# are oat cDNA fragments, respectively. The two G# clones were a gift from Dr. Uchimiya of the Institute of Applied Microbiology, the University of Tokyo, Japan. All other markers were from Dr. Tanksley of Cornell University, USA. Most of these markers have been mapped on rice chromosomes (McCouch et al. 1988, 1991; Oba et al. 1991; Uchimiya, personal communication). Plasmids were isolated according to the procedure of Wilimizig (1985).

RFLP detection

DNA extraction, restriction endonuclease digestion, electrophoresis and Southern blotting were all based on mehtods described previously (Zheng et al. 1990; Lu and Zheng 1992).

Table 1 *Indica, japonica* and wide compatibility varieties of *Oryza sativa* L. used in this study

No.	Variety	Origin
	Indica	
1	Ai-zi-zhan	Guangxi, China
2	Di-jiao-wu-jian	Taiwan, China
3	Xian-feng 1	Zhejiang, China
4	Guang-lu-ai 4	Guangdong, China
5	Gui-chao 2	Guangdong, China
6	Zhe-fu 802	Zhejiang, China
7	Nanjing 14	Jiangsu, China
	Japonica	
8	Daikoku dwarf	Japan
9	Lao-lai-qing	Jiangsu, China
10	Xue-he-ai-zao	Yunnan, China
11	Tai-zhong 65	Taiwan, China
12	Reimei	Japan
13	Koshihikari	Japan
14	Nong-ken 58	Japan
	WCV	
15	02428	Jiangsu, China
16	CPSLO17	USA
17	CPSLO19	USA
18	Lun-hui 422	Hunan, China
19	Pei-ai 64	Hunan, China
20	Ketan Nangka	Indonesia
21	Akenohoshi	Japan
22	L201	USA
23	Suweon 283	Korea
24	Bellement	USA
25	Dourado precoce	Italy
26	Dular	India

DNAs were digested with the following four enzymes: *Eco*RI, *Eco*RV, *Hin*dIII and *Dra*I. Whole plasmids were hexamer labelled with ^{32}P-dCTP to high specific activities ($5–10 \times 10^8$ cpm/ug), and used as probes on filters of rice DNAs. All enzymes and DNA labelling kits were products from GIBCO-BRL and Genescreen-plus nylon membranes came from the DuPont Company.

Probe labelling and hybridization were carried out by two approaches. One was conventional single marker labelling. The other involved multiple marker labelling, where DNAs of 2–5 single-copy clones from the rice probe library were pooled based on their original concentrations; the final concentration was about 0.3 μg of DNA for each clone per reaction. The pooled clones then were treated as a single probe for labelling and hybridization.

Data analysis

Hybridization patterns were investigated for each probe-enzyme combination. Subspecies-differentiating probes were isolated based on their performance between *indica* and *japonica* subspecies. Shared fragments between 12 WCVs were scored based on single enzyme digestions only, since when polymorphisms were detected between DNAs of *indica* and *japonica* varieties digested by one enzyme they were also usually be detected by other enzymes. This indicated that these polymorphisms were produced by insertions/deletions of DNA sequences, so that they reflected similar changes at one locus.

When the ratios of shared fragments were calculated, each restriction fragment detected by Southern analysis was treated as one unit character for a comparision of the polymorphisms between varieties. Ratios of shared fragments and genetic distances between varieties were quantified according to Nei (1987, Formula 5.53–5.55). A dendrogram was constructed using the unweighted paired-group method (UPGMA) (Sokal and Michener 1958) based on 13 core probes with single enzyme digestions.

Results and discussions

Comparison of single and pooled probe hybridization

Most of the DNA clones used in this study have been tested in a RFLP survey in our previous work and the molecular weights of hybridization fragments of each probe-enzyme combination could be predicted. Single-copy RFLP probes, which generated one or two hybridization fragments of different lengths for different probes, were then mixed and treated as one probe during labelling and hybridizing to rice DNAs.

When rice DNAs were hybridized by pooled probes, each probe in the pool hybridized to its own homogeneous fragments; they did not interfere with each other. In Fig. 1, DNAs were hybridized by a five-probe pool, which produced the same hybridization patterns as did the individual hybridization fragments of each of the five pooled probes with no obvious reduction in the hybridization signal of each band. Probes for specific bands could be recognized by comparing the hybridization patterns of the pooled probes with those of each probe involved in the probe pool.

Pooled-probe hybridization is thus similar to single-probe hybridization in most aspects except that all the fragments generated were not homogeneous and produced more hybridization bands, and accordingly

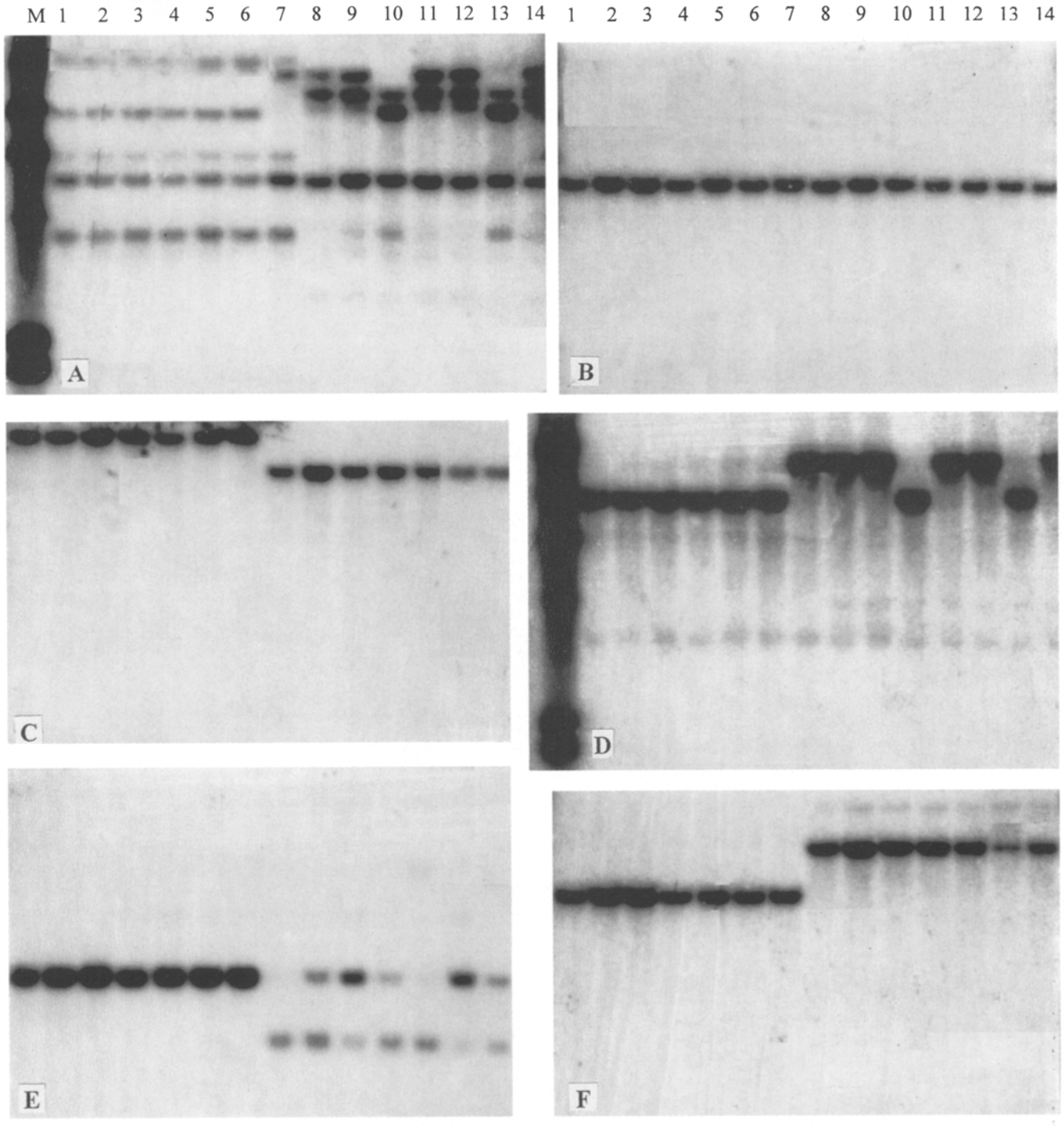

Fig. 1A–F Blotting hybridization by pooled and single probes. Rice total DNAs were digested by *Eco*RI; *M* = λ DNA/*Hin*dIII, *lanes 1–7* were *indica* varieties and *8–14 japonica* varieties numbered as in Table 1. **A** A probe pool of RG329, RG345, RG346, RG351 and RG462; **B** Single probe RG329; **C** RG345; **D** RG346; **E** RG351; **F** RG462

provided more polymorphisms and more information on the genome than did any single hybridization.

Using several random single-copy clones together as a single hybridization probe is very useful in many areas. In molecular typing, testing several clones together is a efficient way of obtaining information for analyzing differences between the genomes of two varieties. This technique can also be applied in gene mapping for isolating probes which are positively linked to target loci. When polymorphisms between two parents of a population is low, e.g., between near iso-genic lines, searching for positive (or polymorphic) probes is very difficult. Testing the clones in groups, and then testing each clone in the polymorphic groups to isolate the polymorphic one will accelerate such a screening.

This technique should also greatly reduce the number of hybridizations, resulting in reduced time and expense.

Screening of subspecies-differentiating probes

Sixty-eight previously tested *indica-japonica* tester-differentiating probes were further screened in seven *indica* and seven *japonica* varieties. About two-thirds of them could not consistently distinguish *indica* from *japonica* varieties, and generated identical hybridization patterns between *indica* and *japonica* varieties or between parts of them.

Twenty-one probes were confirmed to be subspecies-differentiating probes and showed different hybridization patterns between *indica* and *japonica* subspecies with at least one enzyme digestion (Table 2). Most of these showed identical patterns within subspecies. RG64 generated identical patterns within *indica*, but gave different ones within *japonica* varieties. RG81 showed different patterns within both *indica* and *japonica* varieties, though they also detected differentiation between *indica* and *japonica* varieties (Fig. 2).

RG358, RG375 and G318 were *indica*-specific probes; they hybridized to DNAs from *indica* varieties readily, while hardly at all to DNAs from *japonica* varieties. Strictly, RG358 and RG375 were not subspecies-differentiating probes according to our definition, for they showed little homogeneity to DNAs of some *indica* varieties and could not distinguish them from *japonica* varieties. Thus, their *indica*-specificity was not complete (Fig. 3). Subspecies-specific probes may be extremely useful in classification and are of great significance in the evolution of cultivated rice; therefore we included them in the subspecies-differentiating probes.

For 19 of the 21 *indica-japonica* differentiating probes, polymorphisms between *indica* and *japonica* varieties were detected by more than one enzyme (Table 2), indicating that most of the polymorphisms between the two subspecies were due to insertions/deletions (McCouch et al. 1988). This implied that rearrangements in the genome have played an important role in the evolution of cultivated rice.

Indica-specific probes hybridized only to DNAs of *indica* varieties. This indicated that DNA sequences homogeneous in these clones were deleted in *japonica* varieties. However, RG358 hardly hybridized to the *indica* varieties Xian-feng 1, Guang-lu-ai 4 and Gui-chao 2, and RG375 hardly to Zhe-fu 802, implying that *indica* and *japonica* subspecies do not have an absolute boundary (Oka 1992). Thus, it is difficult to distinguish them from each other definitively without the use by several probes.

Table 2 Probes differentiating between rice subspecies

Probe	Chr[a]	Enzyme[b]	No. of enzymes
RG64	6	EV	1
RG81	12	EI, EV, Hd, DI	4
RG96	3	EI, EV, Hd, DI	4
RG101	3	EI, EV, Hd	3
RG256	2	EI, EV, DI	3
RG345	1	EI, EV, Hd	3
RG351	7	EI, EV, Hd	3
RG358	9	EI, EV, Hd, DI	4
RG375	4	EI, EV, Hd, DI	4
RG437	2	EI, EV, Hd	3
RG462	1	EI, EV, Hd, DI	4
RG482	3	EI, EV, Hd, DI	4
RG553	9	EI, EV, Hd, DI	4
RG570	9	EI, EV	2
RG620	4	EI, EV, Hd, DI	4
RG667	9	EI, Hd, DI	3
RG684	?	EV, Hd, DI	3
RG869	12	EI, Hd, DI	3
RG944	3	EI	1
G318	12	EI, EV, Hd, DI	4
CDO281	1	EI, DI	2

[a] Chr = Chromosome;? not yet mapped

[b] Enzymes with which a probe detected different hybridization patterns between seven *indica* and seven *japonica* varieties. EI = *Eco*RI, EV = *Eco*RI, Hd = *Hin*dIII, DI = *Dra*I

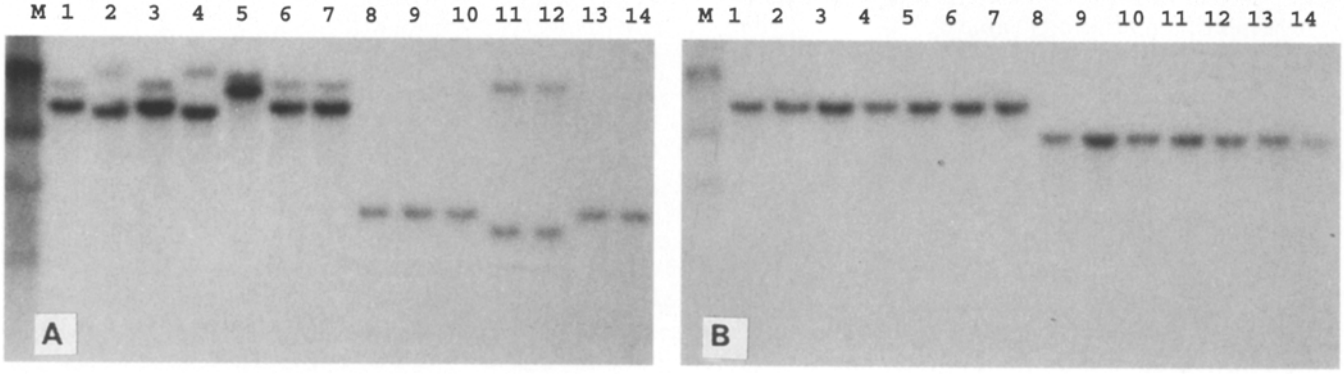

Fig. 2A,B Hybridization patterns detected by subspecies differentiating probes. $M = \lambda$ DNA/*Hin*dIII, *lanes 1–14* were DNAs from *indica* and *japonica* varieties numbered as in Table 1. **A** By RG81/*Eco*RV, different fragments within both *indica* and *japonica* varieties. **B** By RG570/*Eco*RV, identical fragments within both *indica* and *japonica* varieties

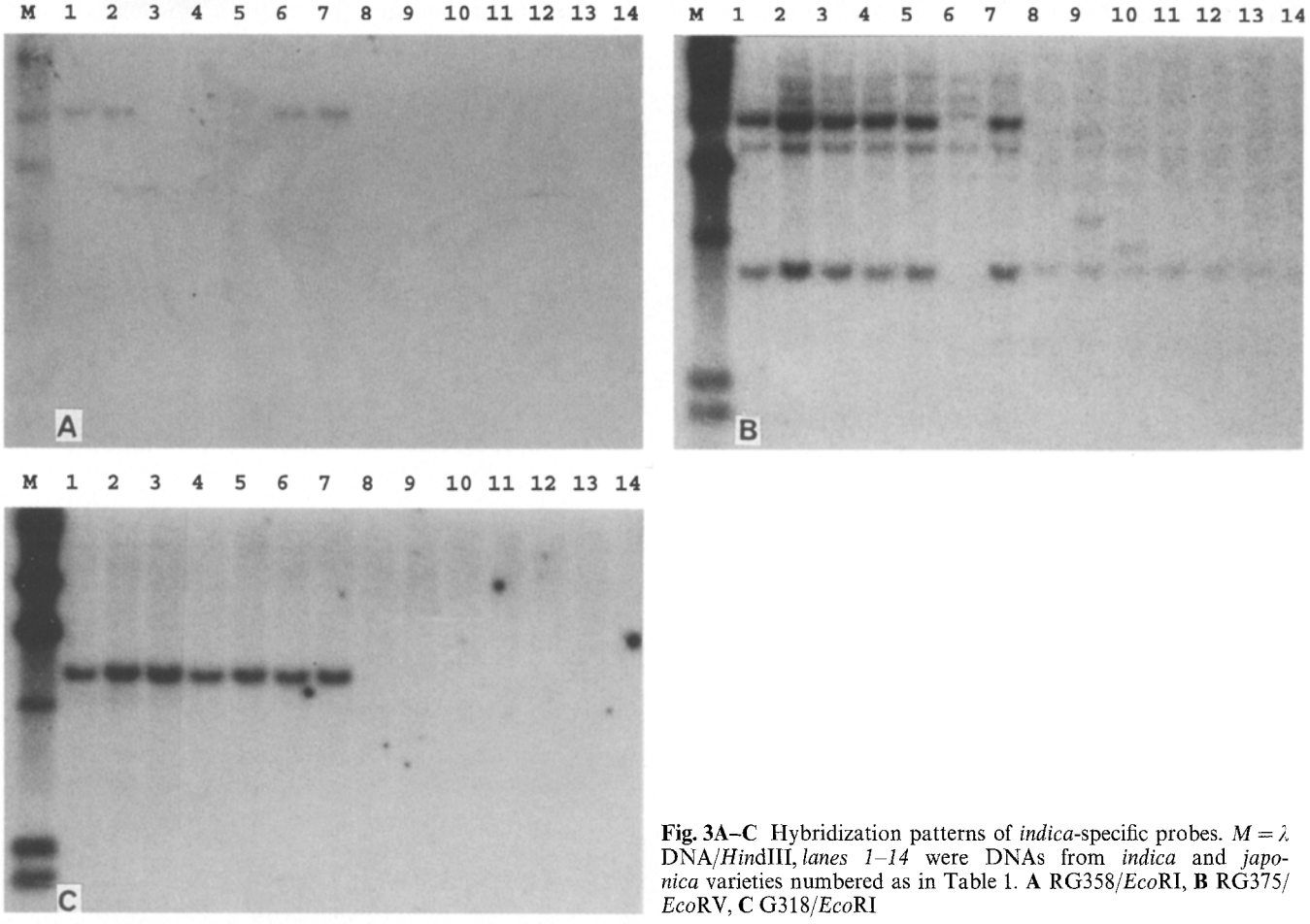

Fig. 3A–C Hybridization patterns of *indica*-specific probes. $M = \lambda$ DNA/*Hin*dIII, *lanes 1–14* were DNAs from *indica* and *japonica* varieties numbered as in Table 1. **A** RG358/*Eco*RI, **B** RG375/*Eco*RV, **C** G318/*Eco*RI

Core probe for subspecies differentiation

In addition to three *indica*-specific probes a set of ten subspecies-differentiating probes was isolated, from the 21 subspecies-differentiating probes, together with the preferred digestion enzymes, to facilitate the utilization of such probes in rice breeding. Probe-enzyme combinations were selected based essentially on: (1) identical hybridization patterns within subspecies, (2) with no more than two on one chromosome, (3) with single-copy and distinguishable hybridization bands, and (4) when more than one enzyme generated similar hybridization patterns, *Eco*RI or *Hin*dIII was selected for their cheapness. This set of probes was defined as core probes for subspecies differentiation (Table 3).

Twelve of the core probes for subspecies differentiation were distributed on seven chromosomes, namely 1, 2, 3, 4, 7, 9 and 12; RG684 has not yet been mapped. Most of these probes gnerated only one restriction fragment in rice, and the hybridization bands between *indica* and *japonica* varieties can be scored easily for their obvious differences in length.

Ratios of shared fragments between the 12 WCVs were estimated and a dendrogram showing their phylogenetic relationship was constructed based on these 13 core probes with their preferred enzymes. *Indica* and *japonica* varieties were also included as controls (Fig. 4). The twelve WCVs were clearly divided into two groups in which *indica* and *japonica* controls were respectively distributed. Varieties within each group

Table 3 Core probes with recommended enzymes for subspecies differentiation and their hybridization fragments in *indica* and *japonica* varieties of rice

Probe	Chr[a]	Enzyme	Length of hybridization fragment (kb)	
			indica	*japonica*
RG101	3	*Hin*dIII	21	12
RG256	2	*Eco*RV	9.4	7.2
RG345	1	*Hin*dIII	14	8.8
RG351	7	*Eco*RI	4.0	4.0
				3.0
RG358	9	*Hin*dIII	9.0[b]	/
RG375	4	*Eco*RI	8.5[b]	/
RG462	1	*Eco*RV	5.0	3.0
RG482	3	*Eco*RI	6.2	6.0
RG620	4	*Eco*RI	9.0	5.8
RG667	9	*Eco*RI	23	13
RG684	?	*Hin*dIII	9.4	3.0
RG869	12	*Hin*dIII	7.5	5.8
G318	12	*Eco*RI	5.8	/

[a] Chr = Chromosome;? not yet mapped
[b] Absence of this band in some *indica* varieties (see Fig. 3)

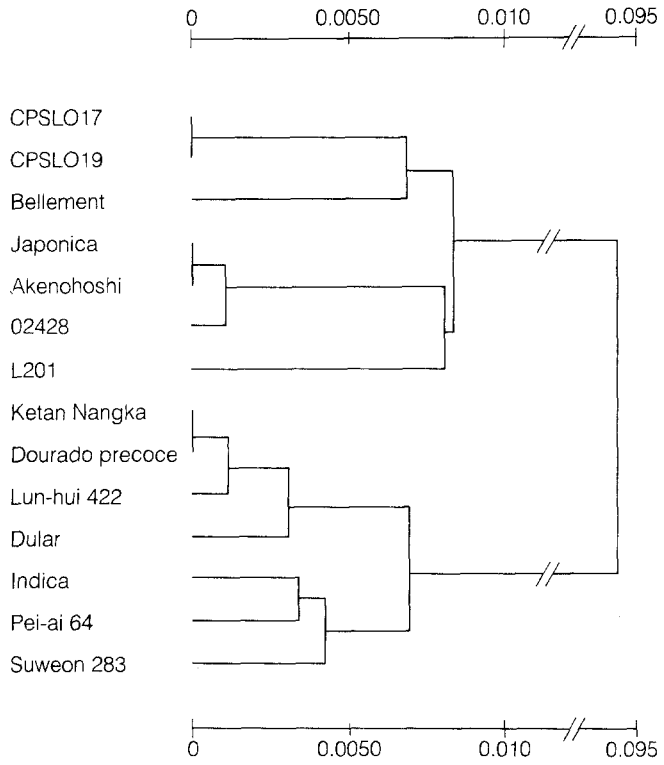

Fig. 4 A dendrogram of 12 WCVs and *indica, japonica* controls of *Oryza sativa* L. based on data of 13 core probes for subspecies differentiation. The numbers below and above dendrogram are genetic distance scales according to Nei (1987)

clustered more closely; however, the genetic distance between the two groups was much greater than those in the dendrograms of WCVs based on 125 polymorphic probes and 68 subspecies tester-differentiating probes (genetic distance 0.0085 v 0.016 within groups, and 0.093 v 0.022 between groups; Zheng et al. 1994). Generally, these three dendrograms matched each other very nicely, indicating that this set of probes was indeed practicable.

Hybrid rice has been widely planted in China and has gained great success. It is believed that stronger heterosis will be produced when two cultivars more remote in phylogenetic relationship are crossed (Zeng et al. 1980). In maize, a positive correlation of grain yield and genetic distance, measured by RFLPs between parent inbred lines, has been observed (Lee et al. 1989). The utilization of inter-subspecies heterosis was expected to produce a breakthrough in rice production (Yuan 1987). Rice wide-compatibility varieties (WCVs) produce fertile F_1 plants when crossed to *indica* as well as to *japonica* varieties, and thus open a way to solve the semisterility of interspecies crosses in rice (Ikehashi and Araki 1986). Moreover, a wide compatibility locus S_5 has been mapped via an RFLP approach (Zheng et al. 1992) and will be of great significance both in the genetic study of rice and in the practical application of rice production. RFLP analysis provides effective and accurate information on variation at the DNA level between rice varieties, so it will be very useful in selecting appropriate rice varieties to obtain high specific combining ability.

RFLPs have a wide application in crop improvement. The screening of *indica-japonica* differentiating and core probes for subspecies differentiation is helpful to reduce both the time and the resources needed for a rice RFLP survey. Moreover, by pooled-probe blotting, we can obtain much more information from one hybridization, so that the laboratory work will also be reduced. This should prove of great significance in applying RFLPs to breeding practice.

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