Theor Appl Genet (1995) 90: 1000–1006

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DNA fingerprinting to detect genetic variation in rice using hypervariable DNA sequences

Received: 26 July 1994 / Accepted: 30 September 1994

Abstract The suitability of mini- and microsatellite related DNA sequences capable of detecting multiple loci was investigated for their ability to generate DNA fingerprints in rice. These included R18.1, a cattle-derived probe, the M13 repeat probe, pV47, a human minisatellite probe; and repeats in the *Per* gene, telomere, chi sequence and 3′ hypervariable region of apolipoprotein B. With the R18.1, pV47 and M13 repeat probes, the level of polymorphism was high enough to identify all of the cultivars and wild rice species used in this study. R18.1, which showed the highest level of polymorphism, was estimated to identify up to 2.5×10^{20} genotypes of rice. In a F_2 population of a 'Basmati-370' and 'Taichung-65' cross, loci detected by R18.1 segregated in a Mendelian fashion. DNA fingerprints were somatically stable and the hybridization patterns were identical among different plants of the same cultivar. Application of the above molecular genetic markers for identification of rice genotypes is reported here for the first time.

Key words DNA fingerprinting · Genetic variation · Hypervariable DNA sequences · Rice

Introduction

Minisatellite and microsatellite DNA sequences consist of a core sequence that is repeated in tandem and dispersed throughout the genome representing many loci, where each locus is comprised of alleles differing in the number of core repeats. These sequences hybridize to multiple loci and have been shown to be hypervariable in nature (Jeffreys et al. 1985; Tautz et al. 1986). In humans, in particular, it has been shown that markers best suited for tagging the entire genome are alleles of minisatellite and microsatellite loci. Through the use of these markers, complex DNA fingerprint patterns have been generated in animals, plants and microorganisms.

A 15 base pair (bp) repeat motif in the protein III gene of bacteriophage M13 appears to be extremely useful in detecting polymorphisms (Vassart et al. 1987) and serves as a universal marker for DNA fingerprinting (Ryskov et al. 1988). For example, it has been used to generate population-specific DNA fingerprints in a neotropical pseudoscorpion (Zeh et al. 1992) and to assess genetic variability in the solitary bee *Megachile rotundata* (Blanchetot 1992), mediterranean fruitfly, *Ceratitis capitata* (Haymer et al. 1992), and Koala (Taylor et al. 1991). It was used for the first time in plants by Ryskov et al. (1988) to identify polymorphic regions in barley. Subsequently, it has been used to detect minisatellite-like sequences in a few gymnosperms and angiosperms (Rogstad et al. 1988; Zimmerman et al. 1989). This probe has been further used for the identification and assessment of genetic variability in *Rosaceae* (Nybom et al. 1990) and ornamental plants (Tzuri et al. 1991).

Highly polymorphic DNA fingerprint patterns have been generated in farm animals and ornamental plants with a bovine genomic clone, R18.1, which contains six poly(GT) stretches (Haberfeld et al. 1991; Tzuri et al. 1991), and in a variety of vertebrates and horses with a simple $(TG)_n$ probe (Kashi et al. 1990; Ellegren et al. 1992). Further, sex-specific hybridization patterns have been obtained with the $(TG)_{10}$ synthetic oligonucleotide probe. The poly (TG) family has been shown to be the most abundant microsatellite in the human genome (Hamada et al. 1982). A novel human minisatellite probe, pV47, was found to generate individual specific DNA fingerprints in humans and cattle (Longmire et al. 1990; Dolf et al. 1992) and also sex-specific restriction fragments in brown skua (Miller et al. 1992).

An oligonucleotide with homology to the chi sequence of *E.coli* has been shown to be useful for DNA fingerprinting in humans (Ehtesham et al. 1992). Recently, a telo-

NCL Communication No. 5789

Communicated by P. M. A. Tigerstedt

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meric repeat which is conserved in a variety of eukaryotes has been shown to detect polymorphisms in tomato and maize (Brown et al. 1992; Burr et al. 1992). Repeats in the *Per* gene of Drosophila (Georges et al. 1987; Ben-shlomo et al. 1993) and the 3′ hypervariable region of apolipoprotein B (Huang and Breslow 1987) have also been shown to be highly polymorphic.

Except for a few reports, a systematic study with reference to the inter- and intraspecific variability in plants using the above probes, which meet the requirement for a large number of polymorphic genetic markers in other systems, is missing. *Oryza* is an agronomically important genus containing *O. sativa*, cultivated rice, and many wild species. These wild species form a rich source of genes for resistance to diseases and insect pests. Some of these genes are those responsible for resistance to grassy stunt virus from O. *nivara*, brownplant hopper from O. *officinalis* and bacterial blight and blast resistance from O. *minuta* to cultivated rice (Jena and Khush 1990; Amante-Bordeos et al. 1992). Prior to the application of DNA fingerprinting in rice breeding programs, it is necessary to show that minisatellite and microsatellites used in the present study can detect intra- and interspecific variability in rice. Previously, we have shown that oligonucleotide probes specific for simple repetitive DNA sequences such as $(GATA)_4$, $(GGAT)_4$, $(GAA)_6$, $(CAC)_5$ and $(GACA)_4$ can generate DNA fingerprints in rice (Ramakrishna et al. 1994; Gupta et al. 1994). In this report, we demonstrate the use of several hypervariable DNA sequences as probes for identification of different rice genotypes.

Materials and methods

Plant material

Seeds of different cultivars of *Oryza sativa* were obtained from authentic sources such as the Directorate of Rice Research (DRR), Hyderabad, Central Rice Research Institute (CRRI), Cuttack, and Agricultural Research Station, Vadgaon, India. While cvs 'Indrayani', 'Basmati-370', 'Pranava' and 'Tambdarambhog' belong to the indica subspecies, 'Hakkoda', 'Norin-49' and 'Fujisaka' belong to the japonica subspecies. Accessions of wild rice species of *O. rufipogon* and *O. nivara* (AA genome), *O. punctata, O. minuta, O. malampuzhansis* (BBCC genome), *O. officinalis* (CC genome) and *O. alta* (CCDD genome) were procured from DRR and CRRI, India.

Genomic DNA extraction

DNA was extracted from the leaves of various cultivated and wild rice genotypes as well as from the roots and seedlings of cv 'Indrayani' as described by Rogers and Bendich (1988). Frozen leaf tissue was ground to a fine powder in liquid nitrogen. Thirty milliliters of 2×CTAB buffer (2% Cetyltriethyl ammonium bromide (CTAB), 100 m*M* TRIS-HCl pH 8.0, 20 m*M* EDTA pH 8.0, 1.4 *M* NaCl, 1% Polyvinyl pyrrolidone) was added to 10 g frozen tissue and the suspension was incubated at 60 °C for 15 min. An equal volume of a chloroform:isoamyl alcohol (24:1) mixture was added and mixed thoroughly to form an emulsion and then centrifuged for 10 min at 10,000 rpm in a SS34 rotor. The supernatant was transferred to a fresh tube containing one-tenth volume of 10% CTAB solution (10% CTAB, 0.7 *M* NaCl) and extracted with chloroform. To the supernatant, an equal volume of CTAB precipitation buffer (1% CTAB, 50 m*M* TRIS-HCl pH 8.0, 10 m*M* EDTA pH 8.0) was added, mixed gently and centrifuged at 10,000 rpm. The DNA pellet was dissolved in a high salt TE buffer (1 *M* NaCl, 10 m*M* TRIS-HCl pH 8.0, 1 m*M* EDTA pH 8.0) and was precipitated with twice the volume of chilled ethanol. The DNA precipitate was washed with 70% ethanol and redissolved in TE buffer. The DNA was incubated at 37 °C for 1 h with RNase, extracted with chloroform, precipitated with ethanol and washed with 70% ethanol. A 10 μg aliquot of DNA was dissolved in TE buffer and digested with restriction enzymes according to the manufacturer's instructions (Boehringer Mannheim Biochemicals and New England BioLabs). The complete digestion of DNA was ensured by adding excess enzyme and incubating for a longer time. DNA was electrophoresed on 1% agarose gels in 1×TAE buffer (40 m*M* TRIS acetate, 1 m*M* EDTA pH 8.0) and Southern-blotted using a nylon membrane (Schleicher and Schuell) as described by Sambrook et al. (1989).

DNA probes and Southern hybridizations

pV47 is a human minisatellite sequence containing tandem repeats isolated from a human chromosome 16-specific library, cloned in pUC8 and propagated in JM 101. R18.1 is a cloned bovine DNA fragment containing six poly (GT) stretches, cloned in pIBI30 and propagated in DH5α. A 282-bp fragment containing nine tandem repeats of a 15-bp core sequence from M13mp18RF was obtained upon digestion with *Hae*III and *Cla*I. All these probes were [^{32}P]-labelled as described by Sambrook et al. (1989) and were used for hybridization in formamide, 5×SSPE, 0.1% SDS, 5×Denhardt's solution, 0.1×BLOTTO at 42 °C. The filters were washed in 1×SSPE, 0.1% SDS for 15 min at room temperature twice and then at 55 °C for 10 min for M13 and pV47. For R18.1, the filters were washed in 0.1× SSPE, 0.1% SDS as described above except that the hot wash was given at 60 °C for 15 min. Oligonucleotides were synthesized on gene assembler plus (Pharmacia), desalted on a NAP-5 column and purified on a 20% denaturing polyacrylamide gel. Oligonucleotide probes were end-labelled as described by Sambrook et al. (1989). Hybridizations were performed at Tm-5 °C and were first washed with 5×SSPE, 0.1% SDS twice for 15 min at room temperature and then at the hybridization temperature for 2 min, after which they were exposed to X-ray films at −70 °C with intensifying screens.

Analysis of DNA fingerprint patterns

Fingerprinting analysis was carried out on DNA fragments over 2 kb in length by scoring differences in band patterns on the basis of absence or presence of bands. A similarity index D expressing the probability that a fragment in one variety is also found in another for all pairwise comparisons was calculated (X_D represents the average similarity index for all pairwise comparisons). The probability that the DNA fingerprints of two varieties of rice will be identical by chance was then estimated as $(X_D)^n$ (Wetton et al. 1987).

Results

Abundance and polymorphism of mini- and microsatellite related hypervariable DNA sequences in the rice genome

To investigate the presence and polymorphic behavior of different minisatellites, microsatellites and other hypervariable DNA sequences in the rice genome, rice DNA was initially digested with several restriction enzymes such as *Alu*I, *Hae*III, *Hin*fI, *Taq*I, *Bam*HI, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Rsa*I, *Xba*I and hybridized to eight differ-

ent probes. Restriction enzymes with four base pair specificities gave rise to a background smear in the low-molecular-weight-range, whereas restriction enzymes with six base-pair specificities, such as *Dra*I, gave the highest level of polymorphism with good resolution. Two tandem repeats, namely, GGTGGTGG, corresponding to the consensus core repeat of the *chi* sequence of *E. coli*, detected limited variability in rice cultivars, while interspecific variability was more pronounced. The sequence ACCGGGACAGGAACTGGA, corresponding to the repeat in the *Per* gene of Drosophila (Georges et al. 1987), showed less polymorphism in rice. Similarly, repeats in the 3′ hypervariable region of apolipoprotein B TTTTATAATTAATATTTT could detect relatively weak signals. Three tandem repeats of the sequence TTAGGG, that constitute telomeric repeats, previously shown to be conserved in eukaryotes, were, however, observed to be remarkably polymorphic. Strong hybridization signals appeared above 30 kb in most cases. These could not be resolved on agarose gel, suggesting a lack of restriction enzyme site in telomeres. In one cultivar, 'Adt-27', an interesting observation was made in the *Dra*I digest of DNA, where the average length of the telomeric sequences appeared to be shorter than that in the other rice cultivars (data not shown). This is not the result of DNA degradation because the patterns of restriction enzyme-digested DNAs on ethidium bromide-stained gel were similar in all cultivars. Similar results and conclusions were made in maize (Burr et al. 1992).

A 282-bp M13 repeat probe, an oligonucleotide GAGGGTGGCGGTTCT consisting of the consensus core repeat of M13 and the human minisatellite probe, pV47, revealed complex hybridization patterns with a good polymorphism among rice varieties. Diffuse background regions were reported when DNA fingerprinting in plants was compared to that in human DNA (Rogstad et al. 1988). Background cross-hybridizing sequences indicate that either they are divergent from mini- or microsatellite repeats or fewer subrepeats are present. R18.1, containing six poly(GT) stretches, hybridized to several restriction fragments and could generate highly polymorphic DNA fingerprints among rice genotypes, thereby indicating the presence of a limited number of loci with poly (GT) stretches. We have previously shown that a simple $(TG)_{10}$ probe results in a heavy background smear which indicates the presence of large number of loci containing $(TG)_{10}$ repeat (Ramakrishna et al. 1994). Thus, the results showed that all of the tested DNA sequences, although present in rice genome, occur at different abundances. In view of these results, we will now describe the data obtained mainly with the R18.1, pV47 and M13 probes.

Intra- and interspecific variation revealed by the R18.1, pV47 and M13 probes

Figures 1, 2 and 3 represent hybridization patterns detected by the R18.1, pV47 and M13 repeat probes, respectively, in *Dra*I digests of *O. sativa* cultivars from subspecies indica (lanes 1–4), japonica (lanes 5–7) and seven wild rice

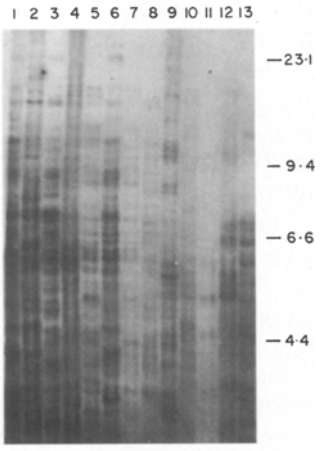

Fig. 1 DNA fingerprints of *Dra*I-digested DNA from several rice genotypes probed with R18.1. *Lane 1* 'Indrayani', *2* 'Basmati-370', *3* 'Pranava', *4* 'Tambdarambhog', belonging to indica subspecies, *5* 'Fujisaka', *6* 'Hakkoda', *7* 'Norin 49', belonging to japonica subspecies of O. *sativa*, accessions of *8 O. nivara*, *9 O. rufipogon*, *10 O. minuta*, *11 O. punctata*, *12 O. officinalis*, *13 O. alta*. Molecular size markers are indicated (kb) in the *right margin*

genotypes (lanes 8–14) representing different genomes of rice. Some hybridization signals were weak but still visible on the original x-ray film. The M13 oligonucleotide probe detected a greater number of loci than the M13 repeat probe since the former has one copy of the consensus core sequence compared to the latter, which has nine copies of a slightly diverged sequence. Hence, the background smear was more intense in case of the M13 oligonucleotide probe (data not shown). Lower molecular-weight fragments could not be resolved with pV47 and M13 repeat probes and were mostly monomorphic. Intraspecific variability among the indicas and japonicas as well as extensive interspecific variability were detected between the cultivated and wild rices with R18.1, pV47 and M13 repeat probes. However, probe R18.1 produced the best resolution of scorable bands as compared to pV47 and M13. Different fingerprinting parameters calculated from the hybridization patterns in Figs. 1, 2 and 3 have been depicted in Table 1. When probe R18.1 was used, the number of bands detected was higher (38.8) and the level of band sharing between rice genotypes was lower (0.31), indicating a higher level of polymorphism. A proper combination of probe and enzyme is thus the key to efficient DNA fingerprinting, which in turn finds application in both varietal identification and breeding.

A probe suitable for DNA fingerprinting should distinguish genotypes to such an extent that the probability is very low that two genotypes will show identical fragment profiles. We have estimated the probability of identical fingerprints by chance to be 2.5×10^{-20}, 1.5×10^{-11} and 2.0×10^{-10} for genus *Oryza* and 1.0×10^{-17}, 1.1×10^{-8} and 4.4×10^{-7} for rice cultivars with R18.1, pV47 and M13 repeat probes, respectively.

The mean probability that two different rice genotypes have identical fingerprints for probes R18.1, pV47 and M13 is $2.5 \times 10^{-20} \times 1.5 \times 10^{-11} \times 2.0 \times 10^{-10} = 7.5 \times 10^{-41}$. This estimation is based on the assumption that the DNA fingerprint bands identified by different probes do not overlap with each other. On the basis of the above assumption, up to 10^{41} rice genotypes can be distinguished using R18.1, pV47 and M13 probes.

Table 1 Analysis of DNA fingerprints of rice

	M13 repeat probe		pV47		R18.1	
	Cultivated genotypes	Cultivated and wild genotypes	Cultivated genotypes	Cultivated and wild genotpyes	Cultivated genotypes	Cultivated and wild genotypes
Average number of bands (n) ± SD	29.6 ± 2.1	29.6 ± 1.8	29.7 ± 2.0	27.2 ± 3.6	40.4 ± 5.1	38.8 ± 4.5
Number of comparisons	21	91	21	91	21	78
Average number of specific bands between pairs ± SD	23.1 ± 6.9	31.4 ± 7.5	27.3 ± 7.3	32.6 ± 7.3	50.5 ± 8.2	53.5 ± 9.1
Average similarity index[a] $[(\bar{X})]_D$ ± SD	0.61 ± 0.12	0.47 ± 0.14	0.54 ± 0.13	0.4 ± 0.13	0.38 ± 0.07	0.31 ± 0.08
Probability of identical match by chance	4.4×10^{-7}	2.0×10^{-10}	1.1×10^{-8}	1.5×10^{-11}	1.0×10^{-17}	2.5×10^{-20}

[a] Similarity index was calculated as $X_D = 2\frac{N_{AB}}{[N_A + N_B]}$, where N_{AB} is the number of bands present in both lanes, N_A is the total number of bands in Lane A and N_B is the total number of bands in lane B

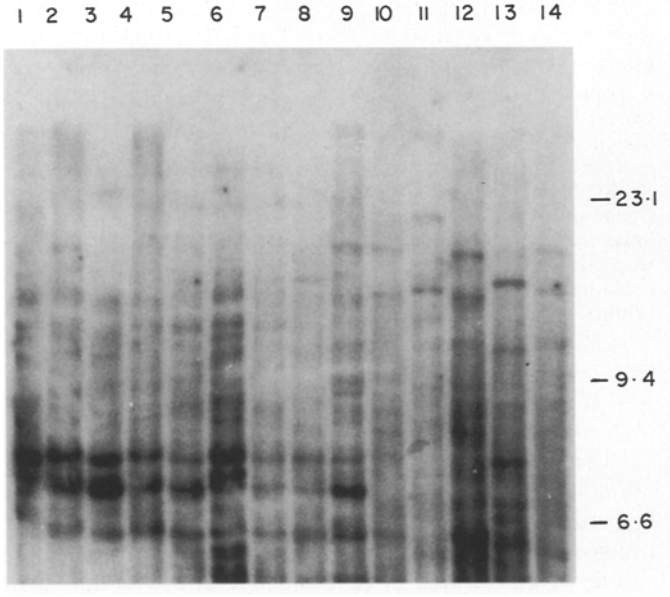

Fig. 2 DNA fingerprints of *Dra*I-digested DNA from rice genotypes probed with pV47. *Lanes 1–13* are as described in Fig. 1. *Lane 14* contains DNA from *O. malampuzhansis*. Molecular size markers are indicated (kb) in the *right margin*

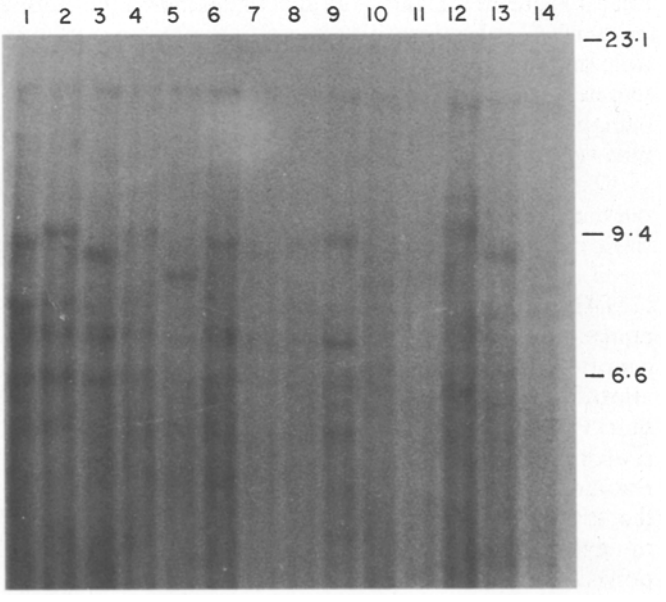

Fig. 3 DNA fingerprints of *Dra*I-digested DNA from rice genotypes probed with the M13 repeat probe. *Lanes 1–14* are as described in Fig. 2. Molecular size markers are indicated (kb) in the *right margin*

The DNA fingerprint profiles in Figs. 1, 2 and 3 indicate 'Basmati-370' to be more closely related to the indicas than to japonicas and wilds. This is in contrast to the isozyme analysis by Glaszmann (1987) where 'Basmati-370' is the only variety with indica morphology placed in Group V and closer to the japonicas. Secondly, there is a clear distinction between the wild and cultivated rice genotypes.

Stability and inheritance of DNA fingerprints

In order to investigate differences between individual plants of the same cultivar, DNAs were isolated from individual plants of the cv 'Indrayani', digested with *Hin*dIII and probed with R18.1, pV47 and M13. No differences were detected among the individual plants mentioned above (data not shown). We also examined the fingerprint patterns of different tissues, root, seedling and leaf, of cv 'Indrayani'. Here again, no differences were observed with R18.1, pV47 and M13, indicating the somatic stability of fingerprints (data not shown).

To gain insight into the mode of inheritance of DNA fingerprints detected by R18.1, the most polymorphic probe in the present study, 20 plants of a F_2 population of a cross between 'Basmati-370' (indica) and 'Taichung-65' (japonica) were analyzed. The parents differed in many bands with different restriction enzymes as shown in Fig.

4. All of the bands in the offspring could be traced back to the parents. Representative DNA fingerprints are shown in Fig. 5. The segregation ratios did not deviate significantly from 3:1, indicating a Mendelian pattern of inheritance.

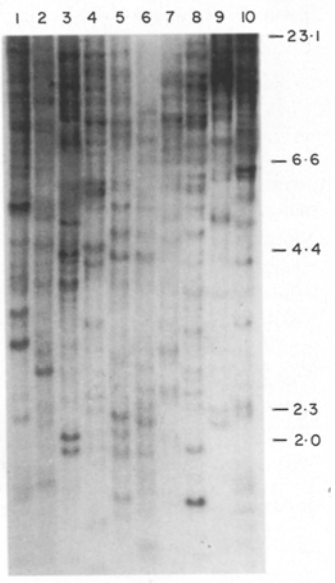

Fig. 4 DNA fingerprints of 'Basmati-370' (*lanes 1, 3, 5, 7, 9*) and 'Taichung-65' (*lanes 2, 4, 6, 8, 10*). DNAs were digested with *Bam*HI (*lanes 1, 2*), *Bgl*I (*lanes 3, 4*), *Eco*RI (*lanes 5, 6*), *Eco*RV (*lanes 7, 8*) and *Pst*I (*lanes 9, 10*) and probed with R18.1

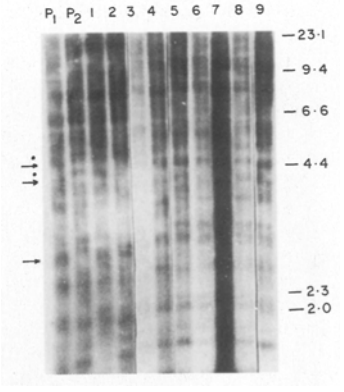

Fig. 5 DNA fingerprints of a F_2 population of a cross between 'Basmati-370' (P_1) and 'Taichung-65' (P_2) with R18.1 as a probe. *Lanes 1–9* contain *Eco*RV-digested F_2 DNAs. Bands specific to P_1 and P_2 are indicated by *arrows* and *dots*, respectively. Segregating bands could be reliably scored on the original X-ray film

Discussion

We have shown for the first time that minisatellites and microsatellites complementary to R18.1, pV47, M13 and several other multilocus probes are present in the rice genome and further support the concept of the ubiquitous appearance of these DNA sequences in eukaryotes. Although the occurrence of sequences homologous to the M13 and R18.1 probes has been shown in plants, there has been no report on the use of human minisatellite probe pV47 in the detection of polymorphism in plants with special reference to rice. It is clear from our work that the human minisatellite probe pV47 hybridizes to many restriction fragments (multiple loci) and reveals DNA fingerprints to detect genetic variability in rice. The ability of the chi sequence repeat to decipher polymorphism in rice is comparable to that in humans (Ehtesham et al. 1992).

The differences in the band patterns, as observed from our data, represent variations in repetitive DNA sequences in rice. It is yet unclear as to why some repeats are organized differently and are more polymorphic than others. R18.1 provides distinct polymorphic banding patterns as compared to other probes. Individual stretches of poly-TG are often characterized by highly polymorphic length variation due to a variable number of tandem repeats of the TG unit. Poly-TG has a number of unique physico-chemical properties (Haniford and Pulleyblank 1983; Htun and Dahlberg 1989) and may serve regulatory functions (Braaten et al. 1988). Mini- and microsatellite variability is thought to result from unequal crossingover and replication slippage (Jarman and Wells 1989). The biological function and the conserved nature of the minisatellite core sequences, both in eukaryotes and even in prokaryotes where repeats constitute a small portion of the genome, remain largely unknown. However, their homology to the chi sequence of *E. coli* and bacteriophage lambda has led to speculation that minisatellites may be involved in homologous recombination as has been shown in humans (Wahls et al. 1990; Jeffreys et al. 1990). Most of the probes used in our study have homology with the chi sequence of *E. coli* and the breakpoints of oncogene translocations (Krowczynska et al. 1990).

The average similarity index value ($\overline{X}_D$) shows that a relatively high level of polymorphism (in terms of fingerprint identity) has been maintained in cultivated rice. However, the fact that R18.1-, pV47- and M13-derived DNA fingerprints do not show variations between individuals of the same cultivar or between different tissues indicates a rather slow turnover rate of these repeats. This makes these repeat probes suitable for application in rice breeding programs, such as for the testing of the homogeneity of inbred lines and analysis of linkage to agronomically important traits. Minisatellite and microsatellite DNA fingerprints can be of great value in gene introgression breeding programs to monitor the transfer of valuable genes from wild rice into cultivated rice by substantially reducing the number of backcross generations. In this aspect, our study is of significant importance as most of the probes show extensive interspecific variability. Alleles of minisatellite loci are best suited for tagging the entire genome as they are scattered throughout the genome (Beckman and Soller 1988; Hillel et al. 1990). Minisatellite and microsatellite DNA fingerprints detected by multi-locus DNA fingerprinting probes can also be used to identify hybrids and predict the success of crosses based on evolutionary distances. Linkage between DNA fingerprint bands and quantitative trait loci (QTL) has been reported in chickens (Plotsky et al. 1993) and hence DNA fingerprinting can serve as an efficient method for such analysis.

The probability of identical fragment profiles by chance using R18.1, pV47 and M13 compares quite favorably with the chance occurrence of identical fragment patterns in rice cultivars when a Jeffreys probe 33.6 is considered, which is about 10^{-11} (Dallas 1988). Therefore, we show a

new range of molecular genetic markers that can be efficiently used in cultivar identification and rice breeding programs in addition to the restriction fragment length polymorphic markers presently available. The molecular analysis of minisatellite and microsatellite containing loci in rice is presently under investigation.

Acknowledgements We would like to thank Prof. J. Hillel, Hebrew University of Jerusalem, for supplying probe R18.1 and Prof. J. L. Longmire, Los Alamos National Laboratory, for supplying probe pV47. WRK, MDL and KVC thank the Council of Scientific and Industrial Research, New Delhi for the award of Senior Research Fellowship, Research Associateship and Junior Research Fellowship, respectively. We acknowledge Dr. K. N. Ganesh and his group from Bio-organic Division, National Chemical Laboratory for synthesis of oligonucleotides and Dr. D. K. Kulkarni, Maharashtra Association for the Cultivation of Science for supply of land races. This work was supported in part by the Rockefeller Foundation, USA and Department of Biotechnology, New Delhi.

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