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DNA fingerprinting in rice using oligonucleotide probes specific for simple repetitive DNA sequences

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Abstract In this report we describe the use of five oligonucleotide probes, namely $(GATA)_4$, $(GACA)_4$, $(GGAT)_4$, $(GAA)_6$ and $(CAC)_5$, to reveal highly polymorphic DNA regions in rice. With each of the oligonucleotide probes, the level of polymorphism was high enough to distinguish several rice genotypes. Moreover, individual plants of one cultivar showed the same cultivar-specific DNA fingerprint. The multilocus fingerprint patterns were somatically stable. Our study demonstrates that microsatellite-derived DNA fingerprints are ideally suited for the identification of rice genotypes. As the majority of the probes detected a high level of polymorphism, they can be very useful in monitoring and aiding gene introgression from wild rice into cultivars.

Key words DNA fingerprinting · Genotype identification · Oligo probes · Rice

Introduction

Repetitive DNA sequences constitute a large fraction of the eukaryotic genome. VNTR (variable number of tandem repeats) or minisatellites is one class of repeat sequences that are comprised of tandem repeats of short, core sequences dispersed throughout the genome. When these core sequences are hybridized to restriction enzyme digested genomic DNAs, they detect several hypervariable loci simulataneously (Jeffreys et al. 1985a). Oligonucleotides specific for simple sequence repeats or "microsatellites" are another class of repeats that can also detect high levels of polymorphism at multiple loci and which can serve as a major source of genetic variation (Tautz et al. 1986; Tautz 1989). This has led to the individual identification in humans, animals, plants and microorganisms (Jeffreys et al. 1985b; Dallas 1988; Rogstad et al. 1988; Ryskov et al. 1988). Complex and highly variable hybridization patterns have been obtained with $(GATA)_4$, $(GACA)_4$, $(TG)_n$, $(CAC)_5$ and several other oligonucleotide probes used for DNA fingerprinting (Ali et al. 1986; Schafer et al. 1988; Turner et al. 1992; Haberfeld et al. 1991; Ellegren et al. 1992). Slot-blot hybridizations with simple repeats have been demonstrated in a variety of eukaryotic genomes (Epplen 1988). This finding was especially promising since unlimited numbers of these simple repeats can be used for DNA fingerprinting in genomes other than humans. Recently, oligonucleotide probes have been successfully used for DNA fingerprinting in higher plants, especially in chick pea, barley and tomato (Weising et al. 1991, 1992; Beyermann et al. 1992; Vosman et al. 1992), but except for these few reports, a systematic study with reference to the inter- and intraspecific variability in plants using oligonucleotide probes is lacking.

Oryza is an agronomically important genus, and many of the wild species grouped therein contain genes for resistance to biotic and abiotic stresses, especially to diseases and insect pests. Wild species of *Oryza* therefore provide a rich source for alien gene transfer to cultivated rice. The transfer of genes for resistance to grassy stunt virus from *O. nivara*, brown plant hopper and white backed plant hopper from *O. officinalis* and bacterial blight and blast resistance from *O. minuta* to cultivated rice has been reported (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 simple repetitive sequences can detect intra- and inter-specific variability. In this article, we report on the use of several oligonucleotide probes for identification of different rice genotypes.

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Materials and methods

Plant material

Seeds of different cultivars of *O. sativa* were obtained from the Directorate of Rice Research (DRR), Hyderabad and Central Rice Research Institute (CRRI), Cuttack, India. *O. sativa* cvs 'Indrayani', 'Basmati-370', 'Pranava' and 'Tambdarambhog' belong to the Indica subspecies, whereas 'Hakkoda', 'Norin-49' and 'Fujisaka' belong to the Japonica subspecies. 'Adt-27' is an Indica-Japonica derivative. Accessions of wild rice species of *O. rufipogon* and *O. nivara* (AA genome), *O. punctata*, *O. minuta* (BBCC genome), *O. officinalis* (CC genome) and *O. alta* (CCDD genome) were obtained from DRR, India.

Plant DNA extraction, restriction enzyme digestion and gel electrophoresis

DNA was isolated from leaves, seeds, seedlings and roots using cetyltrimethyl ammonium bromide as described by Rogers and Bendich (1988). Ten micrograms DNA was digested according to the suppliers' recommendations with the restriction enzymes *Alu*I, *Hae*III, *Hin*fI, *Taq*I, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I. Electrophoresis was carried out on 1% agarose gels in TAE buffer (40 m*M* TRIS-acetate, 1 m*M* EDTA, pH 8.0), and gels were dried on a vacuum gel dryer (Tsao et al. 1983). The complete digestion of DNAs with the restriction enzyme was confirmed as fingerprint patterns were reproducible even when enzyme concentrations and digestion times were increased.

Oligonucleotide probes and hybridizations

The oligonucleotides $(CAC)_5$, $(GAA)_6$, $(GATA)_4$, $(GACA)_4$, $(GGAT)_4$ and $(TG)_{10}$ were synthesized on gene assembler plus (Pharmacia) using phosphoramidite chemistry, desalted on a NAP-5 column and purified on a 20% denaturing polyacrylamide gel. Oligonucleotides were 5'-end-labeled using gamma [^{32}P]-ATP with T4 polynucleotide kinase, and the labeled probes were purified through a DE52 column (Whatman). Dry gels were denatured, neutralized and rinsed in 6× SSC prior to hybridization with oligonucleotide probes, and stringent washes were performed as described by Weising et al. (1992) with slight modifications. The gels were autoradiographed at −70 °C with intensifying screens.

Analysis of DNA fingerprint patterns

Differences in band patterns were scored on the basis of the absence or presence of bands. Analysis was carried out on DNA fragments over 2 kb in length. A similarity index D expressing the probability that a fragment in one variety is also found in another for all pairwise comparisons was calculated. ($\bar{X}_D$ 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 $(\bar{X}_D)^n$ (Wetton et al. 1987).

Results and discussion

In the first set of experiments, several restriction enzymes, *Alu*I, *Hae*III, *Hin*fI, *Taq*I, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I, and six oligonucleotide probes, $(GATA)_4$, $(GACA)_4$, $(GGAT)_4$, $(CAC)_5$, $(GAA)_6$ and $(TG)_{10}$, were explored for DNA fingerprinting in rice. All of these simple sequence motifs were present in the rice genome, although the level of informativeness and fingerprint profiles revealed by different probes and enzymes varied.

Restriction enzymes with four base-pair specificities are widely used in DNA fingerprinting. In our study, the use of such enzymes gave rise to a background smear with faint bands in the low-molecular-weight range that were not scorable (data not shown). This suggests that these repeat motifs are not present in long uninterrupted stretches that lack tetracutting restriction enzyme sites. This observation is interesting since much larger and distinct fragments have been obtained using restriction enzymes with four-base pair recognition site in humans and animals suggesting the clustering of tandemly repeated micro/minisatellites (Kashi et al. 1990; Georges et al. 1988; Zischler et al. 1989).

All of the rice genotypes used in the present study could be distinguished with most of the probes when restriction enzymes with six base-pair specificities, such as *Cla*I, *Dra*I, *Eco*RI, and *Hin*dIII, were used. In most cases, distinct bands could not be detected in the low-molecular-weight region (less than 2 kb), and consequently the gels were run until the 2 kb band of the lambda-*Hin*dIII marker reached the end of the gel. Figure 1a–f represents hybridization patterns detected in *Dra*I digests of four cultivars of *O. sativa* and one accession each of *O. rufipogon* (AA) and *O. officinalis* (CC) with different oligonucleotide probes. Hybridization with $(GATA)_4$, $(CAC)_5$, $(GACA)_4$, $(GAA)_6$ and $(GGAT)_4$ resulted in highly polymorphic strong bands, whereas hybridization with $(TG)_{10}$ resulted in a heavy background smear indicating a large number of loci containing TG repeats. Different fingerprinting parameters calculated from the hybridization patterns in Fig. 1 are shown in Table 1.

The probability of identical match by chance was found to be 2.5×10^{-9} for $(GATA)_4$, 5.5×10^{-9} for $(GAA)_6$, 1.1×10^{-11} for $(GGAT)_4$, 7.5×10^{-7} for $(CAC)_5$ and 2.1×10^{-5} for $(GACA)_4$. The mean probability that two different rice genotypes have identical fingerprints for probes $(GACA)_4$, $(GATA)_4$, $(CAC)_5$, $(GAA)_6$ and $(GGAT)_4$ is $2.1 \times 10^{-5} \times 2.5 \times 10^{-9} \times 7.5 \times 10^{-7} \times 5.5 \times 10^{-9} \times 1.1 \times 10^{-11} = 2.4 \times 10^{-39}$. This estimation is based on the assumption that the DNA fingerprint bands identified by different oligonucleotide probes do not overlap each other, and if this assumption is accepted, up to 10^{39} rice genotypes can be distinguished using these five oligonucleotide probes. The proper combination of probe and enzyme is probably the key to the efficient application of DNA fingerprinting in both varietal identification and breeding. Based on the maximum number of specific bands detected, $(GGAT)_4$ would be the best choice. However, probes $(GATA)_4$, $(GAA)_6$ and $(CAC)_5$ are preferred because they gave better band resolution.

The M13 probe has already had been used to distinguish several genera in Rosaceae and *Rubus* cultivars (Nybom et al. 1990; Nybom and Hall 1991), and Jeffreys

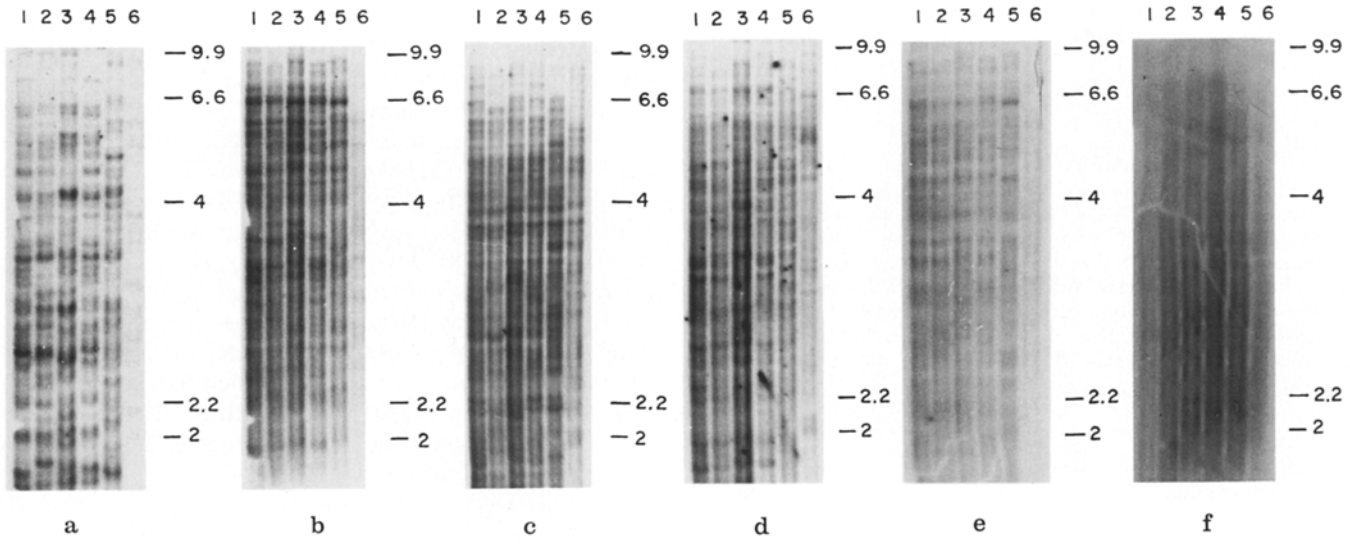

Fig. 1a–f DNA fingerprints of rice genotypes using oligonucleotide probes **a** $(GATA)_4$, **b** $(CAC)_5$, **c** $(GAA)_6$, **d** $(GGAT)_4$, **e** $(GACA)_4$ and **f** $(TG)_{10}$. DNA was digested with *DraI*. *Lanes 1–6* contain DNA from *O. sativa* cvs 'Indrayani' (*1*), 'Adt-27' (*2*), 'Basmati-370 (*3*), 'Hakkoda' (*4*) and accessions of *O. rufipogon* (*5*) and *O. officinalis* (*6*). Molecular size markers in kb are indicated in the *right* margin

probes 33.15 and 33.6 have generated individual specific fingerprints in humans and detected genetic variation in birds (Jeffreys et al. 1985b; Wetton et al. 1987). The use of oligonucleotide probes to generate individual specific fingerprints has been reported in humans, horses and cattle (Ali et al. 1986; Schafer et al. 1988; Buitkamp et al. 1991; Ellegren et al. 1992). The potential of the oligonucleotide probes, even individually to distinguish rice genotypes, is comparable with the probability of obtaining identical fingerprints by chance using the M13 probe, Jeffreys probes and oligonucleotide probes in other eukaryotes reported so far. In an attempt to ascertain whether the oligoprobes can distinguish even more genotypes of rice, we extended our study to include more wild rice genotypes as well as cultivars representing both Indica and Japonica subspecies. Hybridization with probe $(GATA)_4$ was carried out as it resulted in DNA fingerprints with the highest resolution. Figure 2 represents hybridization patterns detected by $(GATA)_4$ in *Hin*dIII digests of *O. sativa* cultivars from subspecies Indica (lanes 1–4), Japonica (lanes 5–7) and six wild rice genotypes (lanes 8–13) representing different genomes of rice as described in the Materials and methods. With $(GATA)_4$ as probe, all 13 rice genotypes could be distinguished. In the present study, with respect to GATA-containing loci, Indica and Japonica subspecies appear to form two distinct groups. Secondly, there is a clear distinction between the wild and cultivated rice genotypes. In addition, the level of variation observed between the cultivated rice and wild species having the AA genome, especially *O. rufipogon*, is less than has been observed in other genomes of rice. This supports the view that *O. rufipogon* is the ancestor of *O. sativa* as has been suggested by Kochko et al. (1991).

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 the oligonucleotides used in this study. No differences were detected among the individual plants (data not shown). Next we examined the fingerprint patterns of different tissues (seed, seedling, leaf and root) obtained from cv 'Indrayani'. Again no differences were observed with the various oligonucleotide probes tested, suggesting that the DNA fingerprint patterns were somatically stable (data not shown).

The results presented in this report demonstrate that oligonucleotide fingerprinting in rice can serve as a powerful tool for the identification of different rice genotypes as most of the genome can be investigated using a collection of different oligonucleotide probes. This type of analysis will assist in the characterization and evaluation of rice genetic resources, in the testing of the homogeneity of inbred lines and in the identification

Table 1 Analysis of DNA fingerprints using different oligonucleotide probes (Similarity index was calculated as $X_D = (2N_{AB})/(N_A + N_B)$, 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*)

	$(GATA)_4$	$(GAA)_6$	$(GGAT)_4$	$(CAC)_5$	$(GACA)_4$
Average number of bands (n) ± SD	32.2 ± 3.4	33.8 ± 2.9	36.7 ± 2.1	31.2 ± 2.3	24.2 ± 1.7
Average number of polymorphic bands between pairs ± SD	29.5 ± 8.3	28.7 ± 10.1	36.4 ± 12.9	22.5 ± 10.0	17.4 ± 9.1
Average similarity index ($\bar{X}_D$) ± SD	0.54 ± 0.14	0.57 ± 0.17	0.5 ± 0.17	0.64 ± 0.29	0.64 ± 0.18
Probability of identical match by chance $(\bar{X}_D)^n$	2.5×10^{-9}	5.5×10^{-9}	1.1×10^{-11}	7.5×10^{-7}	2.1×10^{-5}

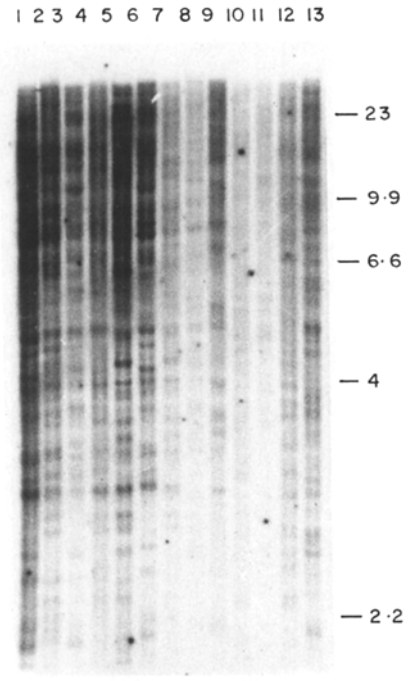

Fig. 2 Hybridization patterns of *Hin*dIII-digested DNA from several rice genotypes probed with $(GATA)_4$. *Lanes 1–13* are *O. sativa* cvs 'Indrayani' (*1*), 'Basmati-370' (*2*), 'Pranava' (*3*), 'Tambda rambhog' (*4*), 'Fujisaka' (*5*), 'Hakkoda' (*6*), 'Norin-49' (*7*) and accessions of *O. nivara* (*8*), *O. rufipogon* (*9*), *O. minuta* (*10*), *O. punctata* (*11*), *O. officinalis* (*12*) and *O. alta* (*13*). Molecular size markers in kb are indicated in the *right margin*

of somatic hybrids and chromosome addition lines. DNA fingerprinting can be of great value in monitoring and aiding gene introgression from wild rices into modern cultivars. As DNA fingerprint loci (DFP) are extremely polymorphic, genotypes having maximum similarity to the recipient line and minimal similarity to the donor line can be selected in crosses involving wild and cultivated rice (Hillel et al. 1990). This ensures a large number of heterozygous DNA fingerprint loci, thereby reducing the required number of backcross generations in gene introgression breeding programs. Such DNA fingerprint loci can also aid in the removal of both marker-linked donor DNA flanking the gene of interest (to minimize the linkage drag) and unlinked DNA (Tanksley et al. 1989). A correlation between variability at the DNA level (measured by restriction fragment length polymorphism) and heterosis has proved to be of great value in allocating maize genotypes to different heterotic groups (Lee et al. 1989; Godshalk et al. 1990). Similarly, by means of DNA fingerprinting rice varieties with good combining ability can be identified without the evaluation of F_1 hybrids in the field to predict heterotic combinations. Linkage between RFLP markers and quantitative trait loci (QTL) for improvement of an elite hybrid has been reported in maize (Zehr et al. 1992). DNA fingerprinting technology can serve as an efficient method for such an analysis.

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References

Ali S, Muller CR, Epplen JT (1986) DNA fingerprinting by oligonucleotide probes specific for simple repeats. Hum Genet 74:239–248

Amante-Bordeos A, Sitch LA, Nelson R, Dalmacio RD, Oliva NP, Aswidinnoor H, Leung H (1992) Transfer of bacterial blight and blast resistance from *Oryza minuta* to cultivated rice, *Oryza sativa*. Theor Appl Genet 84:345–354

Beyermann B, Nurnberg P, Weihe A, Meixner M, Epplen JT, Borner T (1992) Fingerprinting plant genomes with oligonucleotide probes specific for simple repetitive DNA sequences. Theor Appl Genet 83:691–694

Buitkamp J, Kuhn C, Zischler H, Epplen JT, Geldermann H (1991) DNA fingerprinting in cattle using oligonucleotide probes. Anim Genet 12:169–174

Dallas JF (1988) Detection of DNA "fingerprints" of cultivated rice by hybridization with a human minisatellite probe. Proc Natl Acad Sci USA 85:8631–6835

Ellegren H, Andersson L, Johansson M, Sandberg K (1992) DNA fingerprinting using a simple $(TG)_n$ probe and its application to population comparisons. Anim Genet 23:1–9

Epplen JT (1988) On simple repetitive GATA/GACA sequences: a critical reappraisal. J Hered 79:409–417

George M, Lequarre AS, Catelli M, Hanset R, Vassart G (1988) DNA fingerprinting in domestic animals using four different minisatellite probes. Cytogenet Cell Genet 47:127–131

Goldshalk EB, Lee M, Lamkey KR (1990) Relationship of restriction fragment length polymorphisms to single cross hybrid performance of maize. Theor Appl Genet 80:273–280

Haberfeld A, Cahaner A, Yoffe O, Plotsky Y, Hillel J (1991) DNA fingerprints of farm animals generated by microsatellite and minisatellite DNA probes. Anim Genet 22:299–305

Hillel J, Schaap T, Haberfeld A, Jeffreys AJ, Plotzky Y, Cahaner A, Lavi U (1990) DNA fingerprints applied to gene introgression in breeding programs. Genetics 124:783–789

Jeffreys AJ, Wilson V, Thein SL (1985a) Hypervariable "minisatellite" regions in human DNA. Nature 314:67–73

Jeffreys AJ, Wilson V, Thein SL (1985a) Individual specific "fingerprints" of human DNA. Nature 316:76–79

Jena KK, Khush GS (1990) Introgression of genes from *Oryza officinalis* Well Ex. Watt to cultivated rice, *O. sativa* L. Theor Appl Genet 80:737–745

Kashi Y, Tikochinsky Y, Genislav E, Traqi F, Nave A, Beckmann JS, Gruenbaum Y, Soller M (1990) Large restriction fragments containing poly-TG are highly polymorphic in a variety of vertebrates. Nucleic Acids Res 18:1129–1132

Kochko AD, Keifer MC, Cordesse F, Reddy AS, Delseny M (1991) Distribution and organization of a 352-bp tandem repeat in *Oryzae* family. Theor Appl Genet 82:57–64

Lee M, Godshalk EB, Lamkey KR, Woodman WW (1989) Association of restriction fragment length polymorphisms among maize inbreds with agronomic performance of their crosses. Crop Sci 29:1067–1071

Nybom H, Hall HK (1991) Minisatellite DNA "fingerprints" can distinguish *Rubus* cultivars and estimate their degree of relatedness. Euphytica 53:107–114

Nybom H, Rogstad SH, Schaal BA (1990) Genetic variation detected by use of M13 "DNA fingerprint" probe in Malus, Prunus, and Rubus (Rosaceae). Theor Appl Genet 79:153–156

Rogers SO, Bendich AJ (1988) Extraction of DNA from plant tissues. In: Gelvin SB, Schilperoort RA (eds) Plant molecular biology manual A6:1. Kluwer Academic Publishers, Dordrecht

Rogstad SH, Patton JC, Schaal BA (1988) M13 repeat probe detects DNA minisatellite like sequences in gymnosperms and angiosperms. Proc Natl Acad USA 85:9176–9178

Ryskov AP, Jincharadze AG, Prosnayak MT, Tvanov PL, Limborska SA (1988) M13 phage DNA as a universal marker for DNA fingerprinting of animals, plants and microorganisms. FEBS Lett 233:388–392

Schafer R, Zischler H, Birsner U, Becker A, Epplen JT (1988) Optimized oligonucleotide probes for DNA fingerprinting. Electrophoresis 9:369–374

Tanskley SD, Young ND, Paterson AH, Bonierbale MW (1989) RFLP mapping in plant breeding – new tools for an old science. Bio/technology 7:257–264

Tautz D (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucleic Acids Res 17:6463–6471

Tautz D, Trick M, Dover GA (1986) Cryptic simplicity in DNA is a major source of genetic variation. Nature 322:652–656

Tsao SGS, Brunk CF, Pearlman RE (1983) Anal Biochem 131: 365–372

Turner BC, Elder JF, Laughlin TF, Davis WP, Taylor DS (1992) Extreme clonal diversity and divergence in populations of a selfing hermaphroditic fish. Proc Natl Acad Sci USA 89:10643–10647

Vosman B, Avens P, Rus-Kortekaas W, Smulders MJM (1992) Identification of highly polymorphic DNA regions in tomato. Theor Appl Genet 85:239–244

Weising K, Beyermann B, Ramser J, Kahl G (1991) Plant DNA fingerprinting with radioactive and digoxy geneted oligonucleotide probes complementary to simple repectitive DNA sequences. Electrophoresis 12:159–169

Weising K, Kaemmer D, Weigand F, Epplen JT, Kahl G (1992) Oligonucleotide fingerprinting reveals various probe dependent levels of informativeness in chick pea (*Cicer arietinum*). Genome 35:436–442

Wetton JH, Carter RE, Parkin DT, Walters D (1987) Demographic study of a wild house sparrow population by DNA fingerprinting. Nature 327:147–149

Zehr BE, Dudley JW, Chojecki J, Saghai Maroof MA, Mowers RP (1992) Use of RFLP markers to search for alleles in a maize population for improvement of an elite hybrid. Theor Appl Genet 83:903–911

Zischler H, Nanda T, Schafer R, Schmid M, Epplen JT (1989) Digoxygenated oligonucleotide probes specific for simple repeats in DNA fingerprinting and hybridization in situ. Hum Genet 82:227–233