ELSEVIER

Forensic Science International 116 (2001) 63–68

Forensic Science International

www.elsevier.com/locate/forsciint

Case report

DNA profiling of disputed chilli samples (*Capsicum annum*) using ISSR-PCR and FISSR-PCR marker assays

Lekha D. Kumar, M. Kathirvel, G.V. Rao, J. Nagaraju*

Laboratory of Molecular Genetics, Centre for DNA Fingerprinting and Diagnostics, 4-87/1, ECIL Road, Nacharam, Hyderabad 500076, India

Received 22 January 2000; received in revised form 18 July 2000; accepted 20 July 2000

Abstract

A case of marketing of spurious seeds of chilli, *Capsicum annum* in the brand name of an elite variety referred to us from an Indian court of law, for identification is described here. The highly reproducible molecular marker assays, inter simple sequence repeat polymerase chain reaction [ISSR-PCR] and FISSR-PCR (for fluorescent ISSR-PCR) were used for differentiating the four disputed chilli samples. A total number of 17 ISSR anchored primers, which included nine di-, and eight tri-nucleotide primers were used for the analysis. The ISSR-PCR products were separated on a 2% agarose gel. A total of 212 and 288 bands were resolved by seven di- and eight tri-nucleotide primers, respectively, with an average of 30 bands per primer. Five out of nine dinucleotide primers and four out of eight trinucleotide primers could unambiguously differentiate all the four disputed chilli samples. The sensitivity and informativeness of the ISSR-PCR assay were further enhanced by the use of FISSR-PCR technique. The FISSR-PCR assay revealed a total number of 566 bands using three tri- and one di-nucleotide primers with an average of 141 bands per primer. These four primers could reliably distinguish all the four disputed samples unambiguously. In developing countries like India, violation of Plant Breeder's Rights is a major concern of law. The present report is, therefore, a step to protect the Plant Breeder's Rights by making use of reliable and modern DNA technologies.

Keywords: Chillies; PBR; *Capsicum annum*; Forensic; ISSR-PCR; FISSR-PCR

1. Introduction

The *sui generis* system for the protection of plant varieties (*Sui generis* protection implies a system different from other categories of intellectual property protection such as patents and is in a class by itself) is the system for the protection of Plant Breeder's Rights (PBR). Dunkel text also gives an effective *sui generis* system for plant protection. There is an international convention for protection of new varieties of plants known by its French acronym the UPOV convention (*Union Pour Le Protection Des Obtentions Vegetals*) established and signed on December 2 1961 and modified in 1991 imparts plant variety protection relating to the product as such, seed or other propagating material of a specific plant variety. The effect of the right granted to the breeder is that the prior authorisation of the variety is required for a large scale production for purposes of commercial marketing, the offer for sale or the marketing of the reproductive or vegetative propagating material of the variety. This version of PBR contains a specific provision relating to ''farmers privilege'' but in a considerably tightened form which can be extended to his

* Corresponding author. Tel.: +91-40-715-1612; fax: +91-40-715-5610.
E-mail address: jnagaraju@www.edfd.org.in (J. Nagaraju).

0379-0738/01/$ – see front matter
PII: S0379-0738(00)00350-9

using saved seeds obtained from the harvest of his own holdings. As long as the farmer remains a ''grain producer'' and does not turn himself into a commercial seed seller of the protected variety, the PBR system will not come in his way. In many developing countries, an independent patent system does not work efficiently because of lack of local knowledge, trained manpower, inadequate documentation and paucity of funds. It would be impossible for individual farmers to fight cases of theft of the materials raised and protected by IPRs (Intellectual Property Rights) or PBRs, unless public institutions bear the legal costs of litigation on behalf of the affected parties.

The case investigated here is related to the unauthorised commercial sale of spurious chilli seeds raised by a particular person and marketed in the brand name of an elite variety. The spurious seeds sold in the same brand name as that of the elite variety mocking the trade mark of the seed owner, with the help of duplicate screen printer, were caught from three different sacs from two accused persons independently and a case was registered with the court of law by the breeder who had raised the registered variety. The seeds of the registered variety bearing well defined and protectable trade marks along with the disputed samples were sent to Centre for DNA Fingerprinting and Diagnostics, for DNA analysis.

The DNA markers are used for many diverse purposes ranging from forensic sciences to identifying genes responsible for genetic diseases and inferring evolutionary relationships among species [1]. The first DNA marker applied was restriction fragment length polymorphism (RFLP) analysis in plants [2]. However, with the development of polymerase chain reaction (PCR), marker systems based on PCR have emerged as major molecular tools for various genetic analyses. These marker systems include random amplified polymorphic DNA (RAPD) [3], simple sequence repeats (SSRs) [4–6], inter simple sequence repeats-PCR (ISSR-PCR) [7], and amplified length polymorphisms (AFLP) analysis [8]. The applications of DNA markers include DNA fingerprinting for cultivar identification and protection of plant variety rights, phylogenetic and diversity analysis, hybrid confirmation, genome mapping and gene tagging for marker assisted selections. These marker assays especially, RAPD and ISSR-PCR markers have been shown to be useful in genetic fingerprinting of raspberry cultivars [9], maize [10], genetic diversity analysis in *Brassica* [11], grapevine [12], *Azolla-Anabaena* [13], peanut [14], and celery cultivars [15]. These techniques have also been used for the genetic mapping of *Capsicum* species, pepper [16]. The ISSR-PCR markers have also been shown to be useful in fingerprinting diverse species of animals [7,17]. There are very few published reports of forensic cases involving DNA analysis of plants and animal varieties using molecular markers other than SSRs; for example, identification of *Cannabis sativa* in the illicit forensic sample has been done using *Cannabis* specific DNA primers [18]. RAPD analysis has been used to differentiate different forensic plant samples, which could not be differentiated by HPLC analysis [19]. In spite of its extensive use in various genetic analyses, the RAPD assay in forensic studies has to be approached with caution since inconsistencies in reproducibility have been encountered. Analysis of SSRs provides co-dominant, highly reproducible and genetically informative markers [4–6]. But this method requires sequence information of the species being analysed to design primers for the sequences flanking the SSRs motifs. As of today the information on SSR loci is available only for a few crop and animal species. The ISSR-PCR method provides an alternative choice to obtain highly reproducible markers without any necessity for prior sequence information for various genetic analyses. The ISSR-PCR method takes advantage of the ubiquitously distributed SSRs in the eukaryotic genomes. The oligonucleotide primers based on SSRs anchored at either the 5′ or 3′ end with two to four purine or pyrimidine residues are used to initiate PCR amplification of genomic segments flanked by inversely oriented, closely spaced repeats [8]. The PCR products thus generated reveal multilocus profiles, which could be revealed on agarose or polyacrylamide gels. The ISSR-PCR method is considered especially suitable for forensic investigations where high reproducibility of the results is required to settle the disputes. In the present study, the chilli plants were raised carefully under controlled conditions from the seeds confiscated from the accused and the elite variety marked I, II, III and IV. DNA was extracted from all the four samples and they were subjected to ISSR-PCR and very recently developed FISSR-PCR (for fluorescent ISSR-PCR — Nagaraju et al., manuscript

under preparation) analyses to determine whether all the four sets of seeds belonged to the same or different samples. To the best of our knowledge this is the first report of such an analysis on chilli varieties.

2. Materials and methods

2.1. Plant materials

Four sets of chilli seed samples marked I, II, III, and IV forwarded by the Indian Judicial Court.

2.2. Chemicals

All chemicals used for the experiments were of molecular biology grade and were procured from Sigma Chemical Company, USA. All glasswares and plasticwares and the buffers prepared in Milli Q water were sterilised by autoclaving at 15 lbs for 15 min before using.

2.3. Primers

The primers used for ISSR-PCR and FISSR-PCR analyses were in-house designed and synthesised at the Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India (Table 1).

Table 1
List of ISSR primers used in disputed chilli samples

S. no.	Primer code	Primer sequence
1	$(TG)_7$	5′ RYA CRY RCA R $(TG)_7$ 3′
2	$(TG)_7T$	5′ YAY GYA CAY $(TG)_7$ T 3′
3	C $(GA)_7$	5′ RAY RAT AY $(GA)_7$ 3′
4	T $(GA)_8$	5′ YGY RAY $(GA)_8$ 3′
5	$(GA)_8$ C	5′ $(GA)_8$ RGY 3′
6	$(CA)_7$	5′ GRT RCY GRT R $(CA)_7$ 3′
7	T $(GT)_9$	5′ CRT AY $(GT)_9$ 3′
8	GC $(GCC)_4$	5′ YGR GY $(GCC)_4$ 3′
9	TA $(CAG)_4$	5′ ARR TY $(CAG)_4$ 3′
10	CT $(AAT)_4$	5′ RYR CY $(AAT)_4$ 3′
11	T_3 $(ATT)_4$	5′ RA TYT $(ATT)_4$ 3′
12	TC $(ATT)_4$	5′ GAR TY $(ATT)_4$ 3′
13	CGA $(ATT)_4$	5′ AGC RR $(ATT)_4$ 3′
14	AA $(AAT)_4$	5′ TR AAR $(AAT)_4$ 3′
15	A_3 $(AAT)_4$	5′ ACR ARA $(AAT)_4$ 3′
16	GA $(ATT)_4$	5′AGY GR $(ATT)_4$ 3′

2.4. DNA extraction

The seeds of four chilli samples were pre-soaked and sown under controlled conditions of temperature and humidity. Fresh young and disease free leaves were collected from the plants and immediately frozen in liquid nitrogen and stored at −70°C till further use. The DNA was extracted using the modified method described earlier [21]. 5 g of frozen leaves from each of the samples was separately ground on sterile and pre-chilled pestle and mortar prior to DNA isolation. The purity of the extracted DNA was determined by taking the ratio of absorbance at 260 nm and 280 nm. The DNA was quantified on 0.8% agarose gel and diluted to uniform concentration of 50 ng/μl and 7 ng/μl for ISSR-PCR and FISSR-PCR analyses, respectively.

2.5. ISSR-PCR and FISSR-PCR amplifications

ISSR-PCR amplifications were carried out using in-house designed and synthesised di- and tri-nucleotide microsatellite motifs, anchored with a stretch of degenerate nucleotides either at 5′ or 3′ ends. The PCR reactions were set up in a 10 μl reaction containing 50 ng genomic DNA, 1X PCR reaction buffer containing 1.5 mM $MgCl_2$, 0.8 μM primer, 25 μM each of dCTP, dGTP, dTTP and dATP and 0.5 unit of Taq Gold DNA polymerase (Perkin Elmer) whenever dinucleotide primers were used. For trinucleotide primers Taq DNA polymerase from MBI Fermentas was used. Amplification was performed on a thermal cycler (Model 2 400, Perkin Elmer) with a programme of initial denaturation at 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min followed by final extension at 72°C for 10 min and finally stored at 4°C. The products were electrophoresed on a 2% agarose gel at 60 V for 10 h, stained with ethidium bromide (0.5 μg/ml) and photographed.

FISSR-PCR amplifications were carried out using the primers, which revealed polymorphisms in agarose gel. The PCR reactions were set up in 7 μl volume containing 7 ng genomic DNA, 1XPCR buffer (1.5 mM $MgCl_2$, 0.8 μM primer, 25 μM each of dCTP, dGTP, dTTP and dATP), fluorescent 0.4 μM dUTP (Tamara, Perkin Elmer) and 0.4 units of Taq Gold DNA Polymerase [Perkin Elmer] and Taq DNA polymerase (MBI Fermentas) for di- and tri-nucleotide

anchored primers, respectively. The amplifications were carried out as in ISSR-PCR reactions and the products were run on 5% polyacrylamide gel with 7 M urea, on an ABI automated sequencer at a constant voltage of 3 000 kV for 7 h. The data were analysed using genescan analysis software.

3. Results and discussion

High molecular weight DNA extracted from the leaf material was found to be pure and free of RNA. The DNA was diluted at a concentration of 50 ng/μl and was amplified using 17 ISSR-PCR primers which included nine di- and eight tri-nucleotide repeat primers and were analysed on 2% agarose gels. In both the cases, the experiments were replicated and only the consistently reproducible bands were taken for the analysis. Out of nine dinucleotide repeat primers, two primers did not yield discrete bands and two primers did not show any discernible polymorphisms. The remaining five dinucleotide primers distinguished the four chilli samples. Out of eight trinucleotide repeat primers, four revealed polymorphisms between the samples. A total of 212 bands ranging from 0.82 to 0.12 kB were amplified by dinucleotide primers, while trinucleotide primers amplified a total of 288 bands ranging from 0.87 to 0.2 kB. The average number of bands were 30 and 36 per di- and tri-nucleotide primers, respectively.

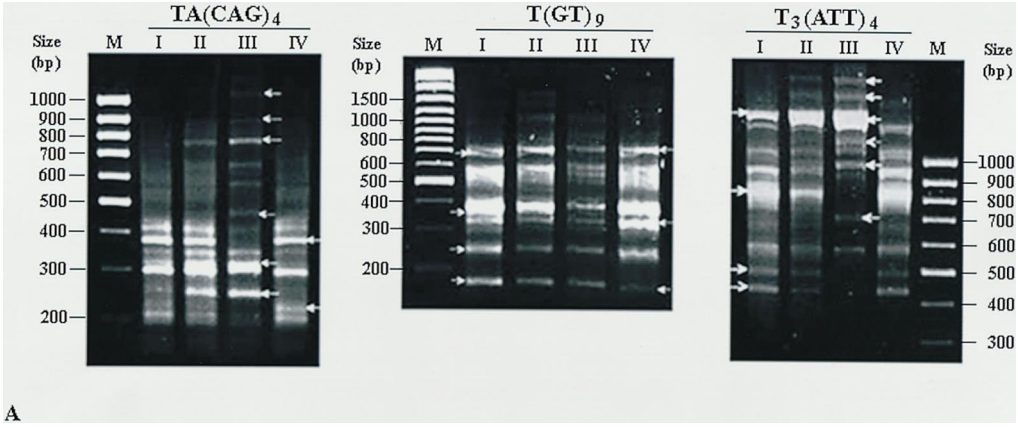
Out of five di-nucleotide primers which showed polymorphisms when analysed on agarose gel, $(TG)_7$ T distinguished chilli samples I, II, and IV, from III, C $(GA)_7$ could distinguish I and II from III and IV, T $(GA)_8$ and $(GA)_8$ C distinguished I and IV from II and III samples and T $(GT)_9$ differentiated I and IV from II and III (Fig. 1A).

Out of eight tri-nucleotides primers used, TA $(CAG)_4$ differentiated IV from I, II and III and III from I, II and IV samples (Fig. 1A), T_3 $(ATT)_4$ primer could distinguish all the four samples (Fig. 1A), and GC $(GCC)_4$ differentiated II from III and IV, CT $(AAT)_4$ differentiated I and II from III and IV and TC $(ATT)_4$ distinguished IV from I, II and III samples.

As a further confirmation of these results, we carried out experiments using recently developed FISSR-PCR technique. FISSR-PCR method gave rise to a total of 566 bands ranging from 0.15 to 0.85 kB with an average of 141 bands per primer when three tri- and one di-nucleotide primers were used (Fig. 1B). All the four primers used gave very clear polymorphisms between the disputed samples. These results suggest that the FISSR-PCR is a very sensitive technique to reveal polymorphisms between highly conserved and even closely related genomes.

The results obtained in the present study have unambiguously proved that the four disputed chilli samples, are different from each other although were being marketed in the name of an elite variety. ISSR-PCR assay is more reliable and sensitive method since higher annealing temperature (50°C) and longer primers are used. By virtue of the greater number of bands amplified per primer, ISSR markers have been used as markers of choice as compared to RFLP and RAPD markers for DNA fingerprinting and diversity analysis in *eleucine*, rice and maize and various other plant and animal species [7,17,20,22–24]. Most of the forensic studies which are being carried out are either based on SSRs [25,26] or minisatellites [27]. The former requires sequence information of the particular SSR loci thus restricting the forensic investigations only to the species where sequencing of the relevant microsatellite loci have been carried out while the latter involves southern blotting and hybridisation requiring large quantities of the template DNA (>5 μg) which is quite often a major limiting factor in the forensic investigations. In this regard, ISSR-PCR would be highly advantageous since no prior sequence information is required and only small quantity of DNA (<50 ng) is sufficient to carry out the investigation.

In the present study, chilli being a self-pollinated crop and the disputed samples received were in all probability being local chilli varieties where polymorphisms are hard to detect, FISSR-PCR with four anchored primers, could distinguish all the four disputed chilli samples. Expectedly, FISSR-PCR method, as compared to ISSR-PCR, revealed more number of bands per primer due to its high sensitivity and resolution. Besides as little as 2–5 ng of template DNA is sufficient to carry out the experiments. We feel that in laboratories where automated sequencer facility is available, the plant samples where it is hard to detect DNA polymorphisms by using agarose gel electrophoresis and other manual PAGE based methods, the FISSR-PCR could be a method of choice.



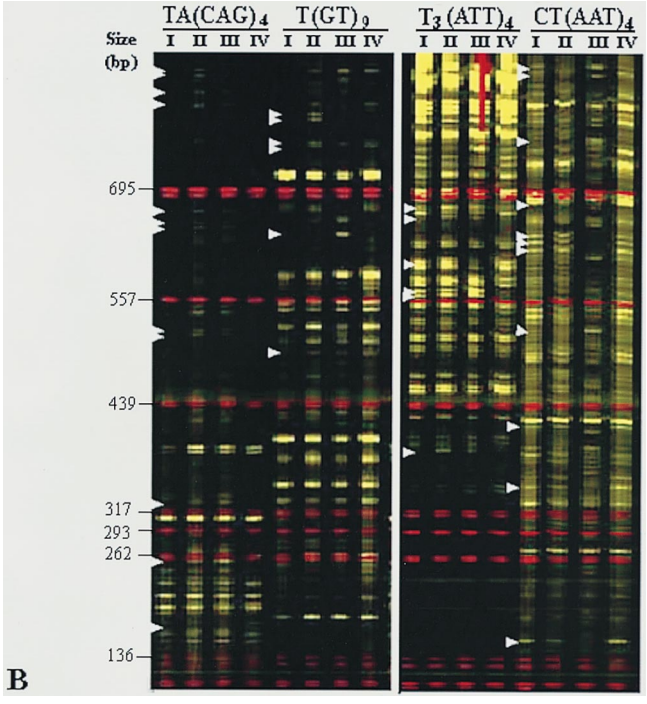


Fig. 1. (A) ISSR-PCR and (B) FISSR-PCR profiles of four chilli samples, obtained using different 5′-anchored ISSR primers. Arrows in (A) and arrowheads in (B) indicate samples specific amplification products.

Hence we strongly recommend the use of FISSR-PCR for quick and reproducible forensic analysis.

Acknowledgements

The authors gratefully acknowledge A.V. Rao, Centre for Cellular and Molecular Biology, for raising the chilli plants under controlled conditions.

References

[1] A.H. Paterson, Genome Mapping in Plants, Academic press, Austin, USA, 1996.
[2] J.S. Beckman, M. Soller, Restriction fragment length polymorphisms in genetic improvement: methodologies, mapping and costs, Theor. Appl. Genet. 67 (1983) 36–43.
[3] J.G.K. Williams, A.R. Kubelik, K.J. Livak, J.A. Rafalski, S.V. Tingey, DNA polymorphisms amplified by arbitrary primers

are useful as genetic markers, Nucl. Acids Res. 18 (1990) 6531–6535.
[4] D. Tautz, Hypervariability of simple sequences as a general source of polymorphic DNA markers, Nucl. Acids Res. 17 (1989) 6463–6471.
[5] J.L. Weber, P.E. May, Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction, Am. J. Hum. Genet. 44 (1989) 388–396.
[6] P. Broun, S.D. Tanksley, Characterisation and genetic mapping of simple sequences in the tomato genome, Mol. Gen. Genet. 250 (1996) 39–49.
[7] E. Zietkienicz, A. Rafalksi, D. Labuda, Genome fingerprinting by simple sequence repeats (SSR) anchored polymerase chain reaction amplification, Genomics 20 (1994) 176–183.
[8] P. Vos, R. Hogers, M. Bleeker, M. Reijans, T. Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, M. Zabeau, AFLP, a new technique for DNA fingerprinting, Nucl. Acids Res. 23 (1995) 4407–4414.
[9] J. Graham, R.J. McNicol, K. Grieg, W.T.G. Vaan de Ven, Identification of red raspberry cultivars and assessments of their relatedness using fingerprints produced by random primers, J. Hortic. Sci. 69 (1994) 123–130.
[10] J. Welsh, R.J. Hoercutt, M. McClelland, B.W.S. Sobral, Parentage determination in maize hybrids using the arbitrarily primed polymerase chain reaction (AP-PCR), Theor. Appl. Genet. 82 (1991) 473–476.
[11] C.F. Quiros, J. Hu, P. This, A.M. Chevre, M. Deiseny, Development and chromosomal localisation of genome-specific markers by polymerase chain reaction in *Brassica*, Theor. Appl. Genet. 82 (1991) 627–632.
[12] G.G. Collins, R.H. Symons, Polymorphism in grapevine detected by the RAPD PCR technique, Plant Mol. Biol. Rep. 11 (1993) 105–112.
[13] D.L. Eskew, G. Caetano-Anolles, B.J. Bassam, P.M. Gresshoff, DNA amplification fingerprinting of the *Azolla-Anabaena* symbiosis, Plant Mol. Biol. 21 (1993) 363–373.
[14] T. Halward, T. Stalker, E. Larue, G. Kochert, Use of single primer DNA amplifications in genetic studies of peanut (*Arachis hypogaea*), Plant Mol. Biol. 18 (1992) 315–325.
[15] X. Yang, C. Quiros, Identification and classification of celery cultivars with RAPD markers, Theor. Appl. Genet. 86 (1993) 205–212.
[16] K.D. Livingstone, V.K. Lackney, J.R. Blauth, R. van Wijk, M.K. Jahn, Genome mapping in *Capsicum* and the evolution of genome structure in the solanaceae, Genetics 152 (1999) 1183–1202.
[17] K.D. Reddy, J. Nagaraju, E.G. Abraham, Genetic characterisation of the silkworm *Bombyx mori* by simple sequence repeat (SSR)-anchored PCR, Heredity 83 (1999) 681–687.
[18] A. Linacre, J. Thorpe, Detection and identification of *Canabis* by DNA, Forensic Sci. Int. 91 (1998) 71–76.
[19] R. Gillan, M.D. Cole, A. Linacre, J.W. Thorpe, N.D. Watson, Comparison of *Cannabis sativa* by random amplification of polymorphic DNA (RAPD) and HPLC of Cannabinoids: a preliminary study, Sci. Justice 35 (1995) 169–177.
[20] I.D. Godwin, E.A.B. Aitken, L.W. Smith, Application of inter simple sequence repeat (ISSR) markers to plant genetics, Electrophoresis 18 (1997) 1524–1528.
[21] S.L. Dellaporta, J. Wood, J.B. Hicks, A plant DNA preparation version II, Plant Mol. Biol. Rep. 4 (1983) 19–21.
[22] S.S. Salimath, A.C. de Olivera, I.D. Godwin, J.L. Bennetzen, Assesment of genome origins and genetic diversity in the genus *Eleucine* with DNA markers, Genome 38 (1995) 757–763.
[23] B.J. Parsons, H.J. Newbury, M.T. Jackson, B.V. Ford-Lloyd, Contrasting genetic diversity relationships are revealed in rice (*Oryza sativa* L.) using different marker types, Mol. Breed 3 (1997) 115–125.
[24] R.V. Kantety, X.P. Zeng, J.L. Bennetzen, B.E. Zehr, Assesment of genetic diversity in dent and popcorn (*Zea mays* L.) inbred lines, Mol. Breed 1 (1995) 365–373.
[25] R. Chakraborty, D.N. Stivers, B. Su, Y. Zhong, B. Budowle, The utility of short tandem repeat loci beyond human identification: implications for development of new DNA typing systems (Review), Electrophoresis 20 (1999) 1682–1696.
[26] J. Burger, S. Hummel, B. Hermann, DNA preservation: a microsatellite DNA based study on ancient skeletal remains, Electrophoresis 20 (1999) 1728–1729.
[27] A.J. Jeffreys, et al., Minisatellite repeat coding as a digital approach to DNA typing, Nature 354 (1991) 204–209.