Tobacco Research 38 (1) : 29-33, 2012

APPLICATION OF SIMPLE SEQUENCE REPEAT (SSR) MARKERS IN DIVERSITY ANALYSIS OF TOBACCO TYPES

K. PRABHAKARARAO, K. SARALA, T.G.K. MURTHY, P.V. VENUGOPALA RAO, V. KRISHNAMURTHY AND M. SUMA

Central Tobacco Research Institute, Rajahmundry – 533 105

(Received on 21st November 2011)

Tobacco is a high value cash crop with variety of end uses and also serving as a model plant in biotechnology with its broad genetic base. Assessment of genetic variation among different tobacco types and varieties will form the basis for various crop improvement programmes and genetic studies. Molecular markers in biological studies opened new possibilities for analyzing genetic diversity and determining the genetic relationships among and within the species. Microsatellite or Simple Sequence Repeat (SSR) markers are known for the reliable detection of genetic diversity, for their co-dominant nature and repeatability. The present study revealed the efficacy of 37 SSR markers in identifying the polymorphism among 48 varieties covering different tobacco types. The results revealed that 25 of them are potential polymorphic markers and were able to resolve the diversity among tobacco varieties. Analysis of genetic similarity coefficients based on SSR amplification data using clustering tools (UPGMA) grouped them into different clusters. Microsatellite markers provide sufficient resolution to distinguish among closely related tobacco types and it also projects their applicability in DNA fingerprinting.

INTRODCUTION

Tobacco is an economically important agricultural product processed from the leaves of genus *Nicotiana* plants. It is a high value cash crop with variety of end uses and also serving as a model system in biotechnology with its broad genetic base. Different types of tobacco *viz.*, burley, *natu, lanka,* cigar wrapper, cigar filler, chewing, hookah, country cheroot, *bidi* and FCV tobacco are available for different end uses. Majority of these cultivated tobacco types differ from each other morphologically with little variation at genome level. Assessment of genetic diversity among these cultivated varieties is imperative for genetic investigations and crop improvement on long term basis. Molecular genetic markers have become useful tools to provide relatively unbiased estimation of genetic diversity in plants.

Simple sequence repeats (SSR), also known as microsatellites markers targeting the repeat regions of the genome of plant material were developed for better understanding of some crops. These repeat regions are abundant and dispersed throughout the plant genomes (Morgante *et al.*, 2002). The frequency of SSRs in plant genomes is estimated as one in every 6-7 kb, and the abundance in the genome suggests a high level of genetic diversity (Powell *et al.*, 1996). SSR markers exhibit high levels of polymorphism among various crops, varieties and and can be used for the discrimination among various varieties and germplasm accessions. Previously Random Amplified Polymorphic DNA (RAPD) has been used in tobacco mainly to identify markers linked to genes for resistance to pathogens (Bogani *et al.*, 1997; Rufty *et al.*, 1997; Yi *et al.*, 1998). Later genetic diversity studies were carriedout in *N. tabacum* and *N. rustica* using RAPD (Del Piano *et al.*, 2000; Sarala and Rao, 2008), Amplified Fragment Length Polymorphism (Sivaraju *et al.*, 2008). The development of microsatellite, or simple sequence repeat (SSR), markers for tobacco has offered increased possibilities for characterizing and utilizing genetic variation in *N. tabacum* (Bindler *et al.*, 2007). They reported 637 functional microsatellite markers in tobacco, of which 282 were highly polymorphic and are useful for identification of closely related varieties of tobacco. Microsatellite markers have been used in comparative studies of the genetic relatedness of cultivated tobacco varieties from USA, Central and South America (Moon *et al.*, 2009). In the present investigation genetic diversity among genotypes of different tobacco types were studied using microsatellite based SSR markers.

MATERIALS AND METHODS

A total of 48 non FCV varieties (Table1) from *N. tabacum* and *N. Rustica* were used in this study.

Genomic DNA was isolated from 40 days old seedlings by CTAB method, purified DNA was quantified by spectrophotometer (Nanodrop) and analyzed in agarose gel electrophoresis. The DNA isolated was amplified using 37 SSR primers *viz.*, PT30043, PT40035, PT30277, PT30160, PT30057, PT30044, PT30339, PT30168, PT30259, PT30242, PT30250, PT30257, PT30392, PT30403, PT30028, PT30361, PT30077, PT30408, PT30470, PT30005, PT30399, PT30114, PT30351, PT30281, PT30307, PT1037, PT11244, PT20286, PT20287, PT30411, PT30123, PT30419, PT20401, PT3O146, PT20391, PT30368, and PT30485 selected from the list published by Bindler *et al.* (2007) in thermal cycler (Eppendorf). For each PCR reaction 50 ng of DNA was taken as template. The PCR was performed in 25 µL final volume containing 1 x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each forward and reverse primers and 1U of Taq DNA polymerase. PCR programme consisted of initial denaturation at 94°C for 5 minutes, 34 cycles consisting of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension 72°C for 1 minute followed by final extension at 72°C for 10 minutes. The PCR products were analyzed in 5% polyacrylamide gel and subsequent scoring was done for the monomorphic and polymorphic bands. The data was analyzed with NTSYS-pc software version 2.02 (Rohlf, 1998) for calculation of Jaccards similarity coefficients followed by Sequential Agglomerative Hierarchial Nesting (SAHN) and Unweighted Pair Group Method with Arithmetic Means (UPGMA) clustering to generate the dendrogram.

RESULTS AND DISCUSSION

Molecular markers in biological studies opened new possibilities for analyzing genetic diversity and determining the genetic relationships among and within the species. Among the markers, Microsatellite or Simple Sequence Repeat (SSR) markers are known for the reliable detection of genetic diversity, for their co-dominant nature and repeatability. In the present study DNA isolated from 48 cultivars belong to different tobacco types were amplified using 37 SSR primers. Analysis of PCR products revealed that 25 SSR primers *viz.*, PT30043, PT40035, PT30277, PT30057, PT30044, PT30339, PT30168, PT30259, PT30242, PT30250, PT30257, PT30392, PT30403, PT30361, PT30077, PT30005, PT30399, PT30114, PT30351, PT20286, PT30419, PT20401, PT20391, PT30368 and PT30485 were polymorphic and others were monomorphic. Amplification profile of primers PT30044, PT30392, PT30277 and

Table 1: List of tobacco types and varieties used in the study

S. No	Tobacco type	Varieties
1.	Burley	Burley 21, Banket-A1, HDBRG
2.	*Natu*	*Natu* special, Gajapati, Prabhath, Viswanath
3.	Lanka	Lanka special, DR1
4.	Cigar wrapper	Dixie shade, S-5
5.	Cigar filler	Krishna, Olor
6.	Chewing & Hookah	Dharla , Manasi, *Jati, podali*, Hemti, Torsa, DCT-4
7.	Chewing Bihar	Vaishali special, Lichhavi, DP401, Gandakbahar, Prabha, Sona, PT-76
8.	Chewing Tamil Nadu	Abhirami, Meenakshi (CR), Thangam, Bhagyalakshmi, Maragadam, Vairam, Meenakshi,
9.	Chewing Gujarat	Anand 145, GT-6, GT-8
10.	Country cheroot	Bhavani special, Sendrapatti special, Sangami,
11.	*Bidi*-Karnataka	NBD-43, Spoorhty, NPN 190, Bhavyasree
12.	*Bidi*-Gujarat	Anand-119, GT-4, GT-5, GT-7, GT-9

PT40035 with selected 19 tobacco genotypes was given (Fig 1). The SSR primer PT30044 specifically produced a unique banding pattern with respect to Dharla variety. The PCR amplified products were ranged between 140 and 450 bp and the number of alleles per locus ranged from one to six with an average of three alleles per locus. Majority of the SSR markers analyzed are polymorphic and these can be used further in varietal developmental programmes.

Genetic relationships among these varieties were studied using Jaccards similarity coefficients and were found in the range of 0.08 to 1.0, PT-76 a chewing (Bihar) variety sharing least similarity (0.08) with burley, Banket A1 and *Lanka* special. Highest similarity (1.0) was observed (Fig.2) between Viswanath (*natu* type) and DR1 (*lanka* type) and also between S-5 (cigar wrapper) and Olor (cigar filler), this may be due to similarities in geographical conditions of these tobacco types and also the SSR markers analyzed are monopmorphic for these varieties. Similarity coefficient matrices were used to generate a dendrogram based on UPGMA method and SAHN clustering. All the 48 genotypes grouped into two major clusters and the variety PT-76 independently linked to the main cluster (Fig.2). One major cluster is with burley and majority of hookah varieties and the other with *bidi* type and majority of chewing varieties. Each major cluster further divided into small sub-clusters consisting of major varieties from different types of non-FCV tobacco which includes burley (Burley 21, Banket A1 and HDBRG), cigar filler& wrapper (S-5, Olor), lanka type (Viswanath and DR1), chewing tobacco from Bihar (Manasi, Lichhavi and Vaishali special), chewing tobacco from Tamil Nadu (Thangam, Bhagyalakshmi and Maragadam), chewing tobacco from Gujarat (GT6 and GT8), *bidi* from

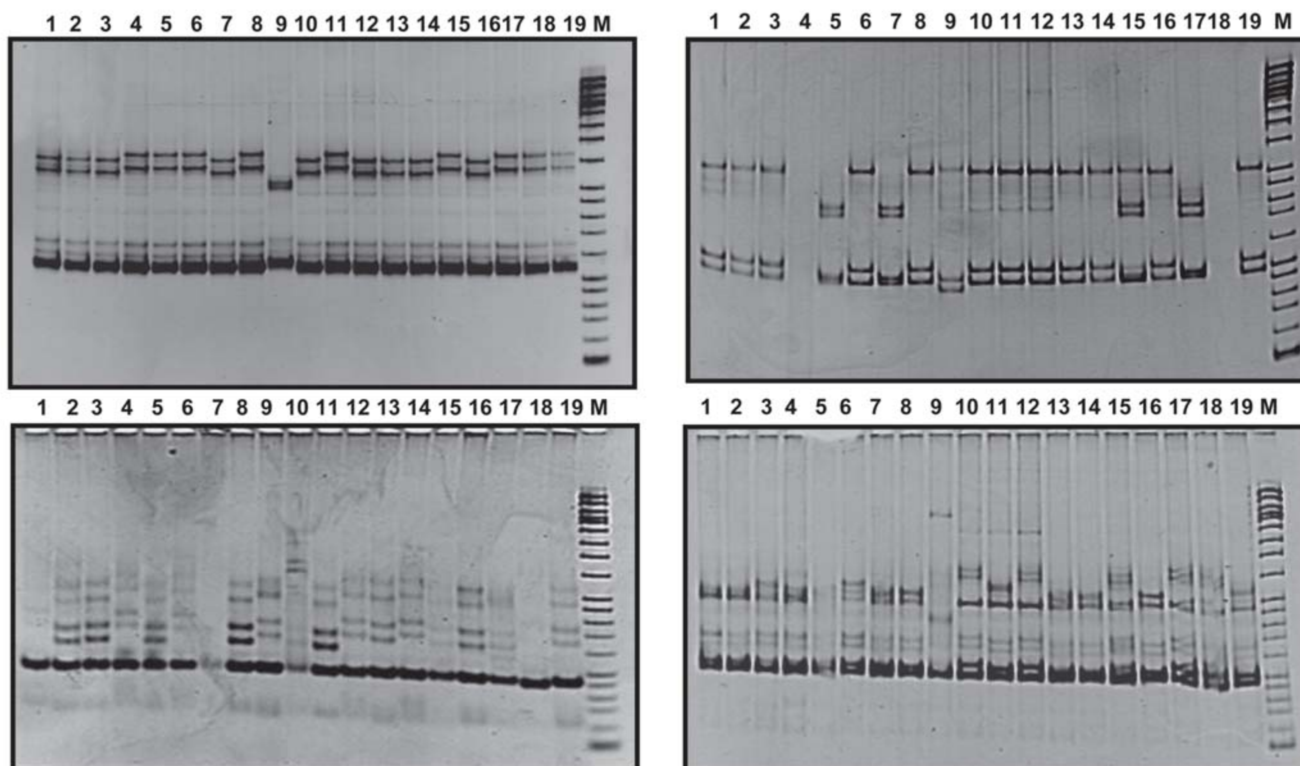

Fig.1: Amplification profile of different tobacco genotypes with SSR markers PT 30044, PT 30392, PT 30277 and PT 40035 where lane 1 to 19 represents as follows 1. Burley-21, 2. Banket-A1, 3.HDBRG, 4. Natuspl, 5. Gajapati, 6. Lanka spl, 7. Dixie shade, 8. Krishna, 9. Dharla, 10. Manasi, 11.Vaishali spl, 12. Lichhavi, 13. Abirami, 14. Meenakshi, 15. Anand-145, 16. Bavanispl, 17. NBD-43, 18. Anand-119, 19. GT-9 and M. 20bp DNA ladder

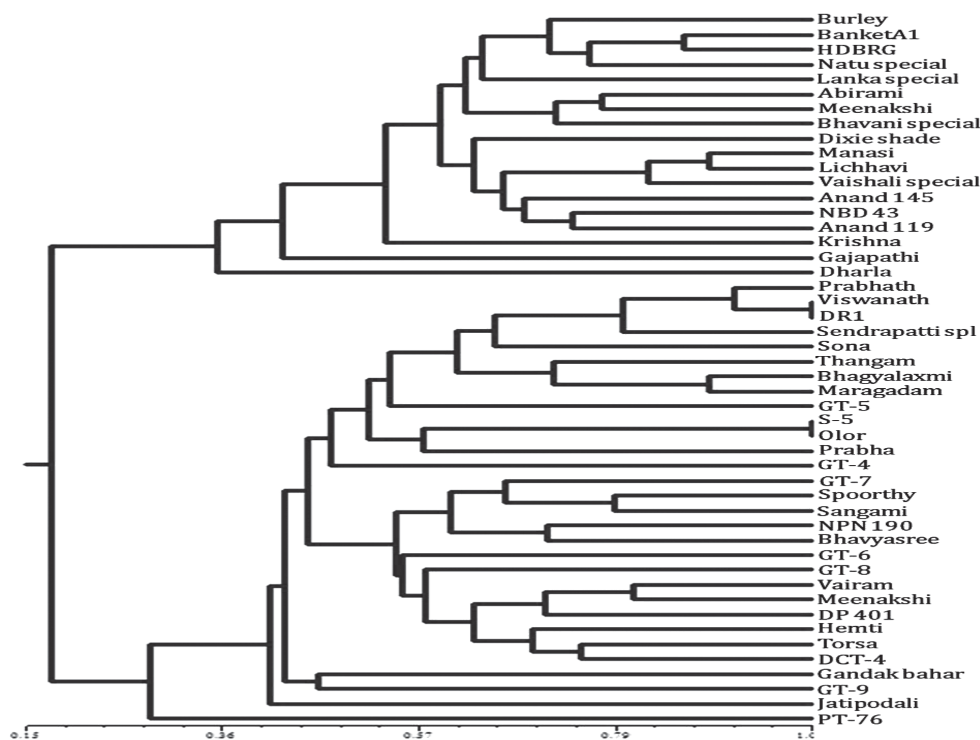

Fig. 2: Dendrogram representing the genetic relationships among the tobacco genotypes based on SSR markers

Karnataka and Gujarat. Eventhough majority of the sub-clusters were formed according to the tobacco types, certain deviations were observed, it may be due to usage of common parents in varietal development, and variations in geographical conditions which can be addressed by including more number of polymorphic SSR markers in analysis. The present investigation reveals that microsatellite markers are polymorphic and has the potential to distinguish closely related tobacco types. Genetic diversity was studied in FCV and non-FCV tobacco types using AFLP by Sivaraju *et al.* (2008), and using RAPD by Sarala and Rao (2008) and estimated genetic similarities among tobacco types. With the ease, reliability and recent developments in identification of functional SSR markers in tobacco one can clearly distinguish tobacco types by screening and incorporating more number of polymorphic SSR markers which can be further used in Varietal Fingerprinting. The polymorphic markers identified can be used in crop improvement programme for calculating the genetic relatedness and diversity among the parents.

REFERENCES

Bindler, G., R. Van der Hoeven, I. Gunduz, J. Plieske, M. Ganal, L. Rossi, F. Gadani and P. Donini. 2007. A microsatellite marker based linkage map of tobacco. **Theor. Appl. Genet.** 114: 341–9.

Bogani, P., P. Lio, M.C. Intrieri and M.A. Buiatti. 1997. Physiological and molecular analysis of genus *Nicotiana*. **Mol. Phylogenet. Evol**. 7: 62–70.

Del Piano, L., M. Abet, C. Sorrenlino, F. Acanfora, E. Cozzolino, F. Acanfora, E. Cozzolino and A. DiMuro. 2000. Genetic variability in *Nicotiana tabacum* and *Nicotiana* species as revealed by RAPD procedure. **Beitrege Zur. Tab. Int.** 19: 1-15.

Moon, H.S., J.S. Nicholson, A. Heineman, K. Lion, R. Van der Hoeven, A.J. Hayes and R.S. Lewis. 2009. Changes in genetic diversity of U.S. flue-cured tobacco germplasm over seven decades of cultivar development. **Crop Sci.** 49: 498-508.

Morgante, M., M. Hanafey and W. Powell. 2002. Microsatellites are preferentially associated with nonrepetitive DNA in plant genomes. **Nat. Genet.** 30: 194-200.

Powell, W., G.C. Machray and J. Provan. 1996. Polymorphism revealed by simple sequence repeats. **Trends Plant Sci.** 18: 215-22.

Rohlf, F.J. 1998. NTSYS-pc. Numerical taxonomy and multivariate analysis system, version 2.02. Exter Software, Setauket, New York,

Rufty, R.C., H.Y. Yi and E.A. Wernsman. 1997. RAPD markers linked to wild fire resistance in tobacco identified through bulked segregant analysis. In Bulletin CORESTA, Montréal, **Abstr**. AP 63, p.50.

Sarla, K. and R.V.S. Rao. 2008. Genetic diversity in Indian FCV and burley tobacco cultivars. **J. Genet.** 87: 159-63.

Siva Raju, K., M.S. Madhav, R.K. Sharma, T.G.K. Murthy and T. Mohapatra. 2008. Genetic polymorphism of Indian tobacco types as revealed by amplified fragment length polymorphism **Curr. Sci**. 94(5): 633-8.

Yi, H.Y., R.C. Rufty and E.A. Wernsman. 1998. Mapping root-knot nematode resistance gene (*RK*) in tobacco with RAPD markers. **Plant Dis**. 82: 319–22.