Tobacco Research 38 (2) : 64-71, 2012

EVALUATION OF MOLECULAR GENETIC VARIABILITY AMONG IDENTIFIED *NATU* TOBACCO (*NICOTIANA TABACUM*) GERMPLASM

T.G.K. MURTHY AND K. SIVA RAJU

Central Tobacco Research Institute, Rajahmundry - 533 105

(Received on 30th November, 2011)

Genetic diversity among *Natu* tobacco varieties and landraces was studied by RAPD, SSR and isozyme markers. A total of 78 amplified fragments were produced by 10 RAPD primers with 42.3% polymorphism. A total of 45 amplicons were common to all accessions indicating the conserved nature of genome among *Natu* tobacco. Sixteen SSR primers produced 83 amplified fragments with 44.5% polymorphism. Isoenzymes of polyphenol oxidase showed 7 loci with maximum variability among the accessions. The genetic similarity among the cultivars and land- races of *Natu* tobacco based on RAPD, SSR and isozymes varied from 73 to 97%, 58 to 93% and 50 to 92%, respectively. The clustering pattern based on UPGMA was nearly similar with three types of markers with respect to the varieties but differed in sub-clustering. The RAPD markers showed higher per cent of average genetic similarity when compared to SSR markers. SSR markers showed more variety/land race specific markers. The higher genetic variability existing among the *Natu* tobacco genotypes can be utilized in the breeding programme for the development of varieties with required yield and quality traits.

INTRODUCTION

The genus *Nicotiana* is a member of the family *Solanaceae*. Out of 72 recognised species (Goodspeed, 1954, Narayan, 1987), only two species *viz.*, *N. tabacum* and *N. rustica* are grown commercially in India and other countries. Different types of tobaccos grown commercially are defined to a large extent by region of production, method of curing and intended use in the manufacturing as well as some distinct morphological characters and chemical differences. Hence, breeding and selection of tobacco varieties is location specific right from the choice of parents to the final stages of evaluation and selection.

Natu (*N. tabacum*) is a unique type of tobacco grown in India and used in the manufacture of cheroot, a hand made smoking product of rural folk especially for East Coast belt of Andhra Pradesh and Odisha. The *Natu* tobacco is air-cured and has nicotine levels ranging from 2.5 to 4.0% and reducing sugars ranging from 1 to 3%. In addition to cheroots, *Natu* tobacco is also used for chewing, snuff and for preparation of low cost cigarettes. *Natu* tobacco grown in Guntur, Kurnool and Mahabubnagar districts of Andhra Pradesh is popularly known as rainfed/ cigarette *Natu* tobacco. Irrigated *Natu* tobacco, another type used for preparation of cheaper cigarettes and country cheroots is cultivated under irrigation in Krishna, West Godavari, East Godavari, Khammam and Warangal districts of Andhra Pradesh. *Natu* tobacco, locally known as *pikka* tobacco in Odisha is cultivated in Ganjam, Parlakhemundhi and Gajapati districts. Though the varietal diversification is apparent among commercial *Natu* cultivars, an assessment of their genetic diversity useful for further cultivar improvement is lacking.

Isozymes have been used for assessment of varietal, spatial and temporal differences in varieties but these markers are superseded by DNA markers as the latter are not affected by biotic, abiotic or growth conditions. Information regarding DNA polymorphism is more elaborative than polymorphism at phenotypic level to properly explain the genetic diversity and to find out more diverse genotypes for crossing programme. DNA fingerprinting in different tobacco types has been done through RAPD and AFLP markers (Ren and Timko, 2001; Siva Raju *et al.*, 2008). Among different molecular markers, SSRs are more abundant, ubiquitous in presence, hyper variable in nature and have high polymorphic information (Gupta *et al.*, 1996).

These markers have been used for genotype identification in many crop plants such as soybean (Song *et al.*, 1999), potato (Barandalla *et al.*, 2006), brinjal (Behera *et al.*, 2006) and rice (Sheetal Yadav *et al.*, 2008), chewing tobacco (Siva Raju, 2011) among several others. Microsatellites have been shown to be almost twice as informative as dominant markers (RAPD and AFLP) and much more informative than RFLPs in soybean (Powell *et al.*, 1996). Thus, these markers are ideal for discriminating individuals and for parentage determinations. Such systematic information on the existing diversity pattern in *Natu* tobacco varieties, which is crucial to define future breeding strategy in this important crop, is not available. Characterization of germplasm is imperative for best utilization in breeding programme aimed at development of varieties with various required traits. Also, in the changing context of agriculture and various kinds of rights of plant material, it is necessary to characterize the indigenously developed plant material at molecular level. This paper reports the genetic diversity based on RAPD, SSR and isoenzyme analysis among 12 *Natu* tobacco cultivated varieties and landraces.

MATERIALS AND METHODS

Plant material and DNA isolation

Twelve genotypes of *Natu* tobacco (Table 1) comprising released varieties and identified land races were subjected to RAPD, SSR and isoenzyme analysis. This collection represents different commercial *Natu* tobacco varieties defined by their morphological and biochemical characteristics. Thirty-day-old seedlings of each variety were collected, bulked, frozen in liquid nitrogen, and stored at -80 C until used for DNA extraction. DNA was extracted from pulverized frozen seedlings as per the modified protocol by Doyle and Doyle (1990). The DNA concentration was estimated by comparing with known concentration of lambda DNA as standard in agarose gel electrophoresis (0.8% gel containing 0.5 µg/ml ethidium bromide).

Polymerase chain reaction

A total of 15 decamer primers belonging to plant RAPD primer series (Bangalore Genei) were screened for RAPD analysis. Of these, 10 primers (GPP1 to GPP10) were selected for analysis based on repeatability. The PCR amplification conditions were as described by Williams *et al.* (1990) with minor modifications. Amplifications were carried out in a 25 ml reaction mixture containing 25 ng template DNA, 0.5 units of Taq DNA polymerase, 0.2 mM of each dNTP and 20 ng of each primer. Amplification was carried out in a DNA thermal cycler (PTC-100, Peltier thermal cycler, M.J. Biotech, USA) programmed to run the following thermal profile: initial denaturation at 94°C for 5 min, 40 cycles of 94°C x 1 min, 37°C x 1 min, 72°C x 2 min, followed by final extension at 72°C for 10 min. Amplification products were resolved on 1.4% agarose gel at 50 V and gels were documented in gel documentation system (Gene genius, Syngene Bio-imaging System, U.K). The size of the fragments was estimated using Gene Ruler 100 bp DNA ladder plus (MBI Fermentas, Lithuania) marker.

Sixteen SSR primers out of 25 were used in the present study based on reproducibility and their NCBI accession numbers are DQ865416, DQ865430, DQ865431, DQ865433, DQ865434, DQ865435, DQ865437, DQ865439, DQ865442, DQ865444, DQ865445, DQ86546, EF375960, EF375967, EF375968 and EF375969. Amplifications were carried out in a 25 ml reaction mixture containing 15 ng template DNA, 0.5 units of Taq DNA polymerase, 0.2 mM of each dNTP and 10 ng of each of forward and reverse primers, 2.5 ml of 10 x buffer and 2.5 mM $MgCl_2$. PCR cycles consisted of one cycle at 94 C for 5 min, followed by 35 cycles at 94 C for 1 min, 55 C for 1 min and 72 C for 2 min. Final primer extension for 7 min was carried out at 72 C. The PCR products were electrophoresed on 10% polyacrylamide gels to achieve better resolution of the bands. The gels were stained in ethidium bromide for 20 min and documented. The size of the fragments was estimated using Gene Ruler as in RAPD.

For isoenzyme analysis, 0.5 g leaf material was homogenized with 5 ml of cold extraction buffer (phosphate buffer pH 7, 0.1M) and centrifuged at 18,000 rpm for 15 min at 4°C. The supernatant was used as enzyme source. Polyacrylamide gel electrophoresis (PAGE) was performed by using 4% stacking gel and 8%

Table 1: List of *Natu* tobacco varieties and landraces selected for evaluation of genetic variability

S.No.	Variety	Parentage	Intend use	Growing state
1	Natu Eluru	Landrace	Cheroot and low cost cigarettes	Andhra Pradesh
2	Natu Singarayakonda	Landrace	Cheroot and low cost cigarettes	Andhra Pradesh
3	Rayala DK	Landrace	Cheroot and low cost cigarettes	Andhra Pradesh
4	Rayala Farm	Landrace	Cheroot and low cost cigarettes	Andhra Pradesh
5	Tokaku Guntur	Landrace	Cheroot and low cost cigarettes	Andhra Pradesh
6	Tokaku Farm	Landrace	Cheroot and low cost cigarettes	Andhra Pradesh
7	Vishwanath	Karuvazhai x Rayala	Cheroot and low cost cigarettes	Andhra Pradesh
8	WAF	Exotic Introduction	Cheroot and low cost cigarettes	Andhra Pradesh
9	Bhairavi	Vishwanath x WAF	Cheroot and low cost cigarettes	Andhra Pradesh
10	Natu Special	(Botcha Vithanam x KVT) x Mutant (Tokaku)	Cheroot and low cost cigarettes	Andhra Pradesh
11	Prabhat	Tokaku x Harrison Special	Cheroot and low cost cigarettes	Andhra Pradesh
12	Pyruvithanam	Pure line selection in local population	Cheroot, low cost cigarette, Kharamasala and Snuff	Andhra Pradesh and Orissa

resolving gel in Tris-glycine buffer (Laemmli, 1991). For isoenzymes, 25 ml of enzyme extract was mixed with 25 ml of loading dye (0.1% bromophenol blue and 30% sucrose in Tris-HCl pH 6.8, 0.1 M) and loaded the gel. The gel was run at 4°C with 1 m Amp per well. After the run, gels were stained in appropriate staining solutions for superoxide dismutase, peroxidase, catalase, esterase and polyphenol oxidase (Sadasivam and Manikam, 1992).

Data analysis

All the genotypes were scored for presence or absence of the RAPD, SSR and isoenzyme bands. Only intense bands and those showing repeated amplification were scored visually. The data were entered into a binary matrix as discrete variables, 1 for presence and 0 for absence of the character and this data matrix was subjected to further analysis. The 'Microsoft Excel' file containing the binary data was imported into NT Edit of NTSYS-pc software version 2.02 (Rohlf, 1998). The 0/1 matrix was used to calculate similarity as DICE coefficient using SIMQUAL subroutine in similarity routine. The resultant similarity matrix was employed to construct dendrogram using Sequential Agglomerative Hierarchical Nesting (SAHN) based Unweighted Pair Group Method with Arithmetic Means (UPGMA) to infer genetic relationships and phylogeny.

RESULTS AND DISCUSSION

Extent of genetic variation elucidated by molecular markers

Out of 15 primers used in RAPD to characterize *Natu* tobacco (*Nicotiana tabacum* L.) varieties and landraces, reproducible patterns were obtained with 10 primers, which generated a total of 78 highly reproducible bands in the size ranged from 0.226 to 2.6 kb. The total number of fragments amplified per primer ranged from 4 to 14 with an average of 7.8 (Table 2). Nine primers (90%) were found to be polymorphic while the primer GPP8 was monomorphic. The level of polymorphism detected with different primers ranged from 0 to 71.4%. Out of all the 78 amplified fragments, 33 (42.3%) were polymorphic. The observed level of polymorphism in *Natu* tobacco was very much higher than that reported by Del Piano *et al.* (2000) and Yang *et al.* (2006) in tobacco but it was less than that was reported by Siva Raju *et al.* (2008), who reported 59.4% polymorphism in 10 types of tobacco by RAPD, may be due to difference in genetic architecture. Besides, the primers used in the present study being different in sequence, possibly assayed

Table 2: Data on RAPD amplification

Primer	Total no. of bands	No. of polymorphic bands	Polymorphism (%)	Specific bands
GPP1	6	2	33.3	
GPP2	8	3	37.5	580 bp to Pyruvithanam
GPP3	8	5	62.5	450 bp to Vishwanath
GPP4	7	5	71.4	
GPP5	9	5	55.5	700 bp to Vishwanath, 410 bp to RayalaDK
GPP6	4	2	50	
GPP7	9	4	44.4	
GPP8	9	0	0	
GPP9	14	6	42.8	640 bp to Bhairavi
GPP10	5	1	20	

different genome regions. A total of 45 bands were common to all the cultivars indicating the close relationship among *Natu* tobacco genotypes. The primer GPP8 produced fragments which are common to all the cultivars and size of the amplified fragments varied between 286 and 2020 bp. The amplified fragments with size 580, 450 and 640 bp by the primers GPP2, GPP3 and GPP9 were specific to the accessions Pyruvithanam, Vishwanath and Natu Eluru, respectively. The primer GPP5 produced amplicons with 700 and 2410 bp specific to the landrace Rayala DK. The combined profiles based on the polymorphic primers, however, provide variety-specific patterns and thus some of the varieties could be distinguished. Photograph of the gel by RAPD primer GPP10 is given in Fig. 1.

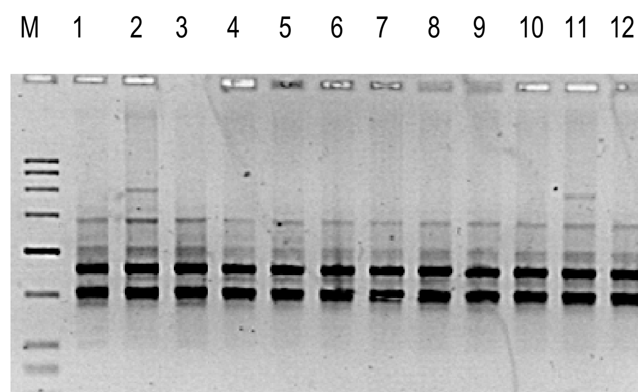

Fig. 1: Amplifications by RAPD primer GPP10. M= DNA ladder. Lanes 1. Bhairavi, 2. Vishwanath, 3. Natu Eluru, 4. Natu Singarayakonda, 5. Natu Speciall, 6.. Rayala DK, 7. Rayala Farm, 8. WAF, 9. Pyruvithanam, 10. Prabhat, 11. Tokaku Guntur and 12. Tokaku Farm

Tobacco specific SSR marker analysis was carried out using 20 primers, out of which 16 primers were chosen for analysis based on reproducibility. The number of fragments amplified with these primers ranged from 2 to 11 with an average of 5.18. Out of 83 fragments amplified by 16 primers, 37 (44.5%) were polymorphic (Table 3). The primer DQ865435 and EF375969 gave the highest level of polymorphism (100%) followed by DQ865431 and EF375967. The number of polymorphic bands ranged from 1 to 8 with an average of 2.31. The observed level of polymorphism was very much higher than that reported by Yang *et al.* (2006), who reported 24.43% and 38.56% polymorphic loci in different tobacco types collected from different countries based on inter simple sequence repeats (ISSR) and inter retrotransposon amplification polymorphism (IRAP) markers. The primer DQ865435 produced variety-specific markers to Natu Special. No single primer individually differentiated all the genotypes but genotype specific profile was observed with some primers. The combined profiles based on the polymorphic primers, however provide variety-specific patterns and thus some of the varieties could be distinguished.

Table 3: Data on SSR amplification

Primer	Total no. of bands	No. of polymorphic bands	Polymorphism (%)	Specific bands
DQ865416	6	3	50	
DQ865430	6	0	0	
DQ865431	8	6	75	
DQ865433	4	1	25	
DQ865434	2	0	0	
DQ865435	7	7	100	700 bp to Natu Special
DQ865437	4	2	50	550 bp to Tokaku Guntur, 180 bp to Pyruvithanam
DQ865439	3	0	0	
DQ865442	4	1	25	
DQ865444	4	0	0	
DQ865445	11	4	36.3	
DQ865450	2	0	0	
EF375960	4	1	25	
EF375967	5	2	66.6	480 bp and 240 bp to WAF
EF375968	5	0	0	
EF375969	8	8	100	

Isozyme variation

Isoenzymes of polyphenol oxidase, peroxidase, catalase and malate dehydrogenase were used to study the genetic diversity among the test genotypes. Isoenzymes of malate dehydrogenase showed 4 isozyme loci. The isoenzyme with Rf value 0.3 was present in all the varieties and land races. The band with Rf value 0.41 was present in 8 accessions and absent in others. The band with Rf value 0.45 was specifically present in the variety Bhairavi. Isoenzymes of catalase showed 4 isozyme loci. The isoenzyme with Rf value 0.16 was present only in the varieties Vishwanath and Natu Special whereas the band with Rf value 0.17 was present in Tokaku Guntur and Tokaku Farm. The isoenzyme with Rf value 0.18 was specifically present in the variety Prabhat. The isoenzyme with Rf value 0.2 was present in 8 accessions.

Isozymes of esterase showed 5 isozyme loci. The isozyme with Rf values 0.04, 0.25 and 0.29 were present in all the accessions. The bands with Rf values 0.13 and 0.2 were present in the land race Natu Eluru only. Isoenzymes of peroxidase showed a maximum of 4 loci. All 4 isoenzyme loci were present in Pyruvithanam, Vishwanath, WAF and Natu Eluru. The isozyme with Rf value 0.04 was present in all the accessions whereas isozyme with Rf value 0.41 was present in all the accessions with various intensity. The isozyme with Rf value 0.45 was present in all the accessions with exception in the land race Rayala Farm. The isozyme with Rf value 0.5 present in the varieties Pyruvithanam, Vishwanath, WAF and Natu Eluru. The landrace Rayala Farm showed only 2 isozyme loci. Isoenzymes of polyphenol oxidase showed 7 loci. The isozyme with Rf value 0.5 was present in all the accessions. The variety Pyruvithanam showed a maximum of five isozyme loci whereas the landrace Rayala DK showed a minimum of 2 isozyme loci. The isozyme band with Rf value 0.73 was specifically present in the variety Pyruvithanam. The band with Rf value 0.52 was present with high intensity in the variety Bhairavi whereas with low intensity in the variety Vishwanath. The band with Rf value 0.48 was present in the land race WAF and the variety Pyruvithanam.

Genetic relationship among the *Natu* genotypes

The genetic relationship among *Natu* tobacco genotypes was established separately

with the two DNA and isoenzyme marker systems. In case of RAPD, the Jaccard's coefficient of similarity for all the varieties and land races, pair-wise comparisons ranged from 0.73 to 0.97 with an average of 0.85. The maximum genetic similarity (97%) was observed between the land races Rayala Farm and Tokaku Farm whereas minimum genetic similarity (73%) was between the pairs Bhairavi-Natu Eluru and Prabhat-Pyruvithanam. In the dendrogram constructed based on RAPD (Fig. 2), 12 accessions were divided into two main clusters (1 and 2) and the varieties Natu Special and Pyruvithanam linked to the main cluster independently (Fig 2). The cluster 1 was formed by two sub-clusters. Sub-cluster 1a included the varieties Bhairavi, Vishwanath and WAF. The varieties Vishwanath and WAF happened to be the parents of the variety Bhairavi. The sub-cluster 1b was formed by the variety Prabhat and Tokaku Guntur. The land race Tokaku Guntur was one of the parents to the variety Prabhat. The cluster 2 was formed by five landraces.

Based on the SSR profiles, the pair-wise similarity coefficients ranged from 0.58 to 0.93 with an average 0.75. The varieties Pyruvithanam and WAF and Tokaku Guntur and Natu Eluru showed minimum genetic similarity (58%) whereas the landraces Tokaku Guntur and Tokaku Farm showed the maximum genetic

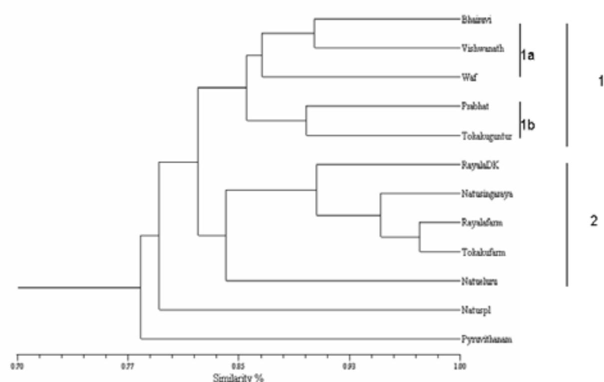

Fig. 2: Dendrogram constructed using an UPGMA and SAHN algorithm from the Jaccard's similarity matrix data from all the RAPD marker profiles consisting of amplified fragments, revealing genetic relationship among the varieties and landraces.

similarity. The landrace Natu Eluru showed more genetic difference with other accessions ranging from 34 to 42%. The average genetic similarity obtained in the present study was similar to the average genetic similarity in chewing tobacco varieties grown in Tamil Nadu and Bihar (Siva Raju, 2011). In the dendrogram constructed based on SSR, 12 accessions were divided into two main clusters (1 and 2) and the variety Pyruvithanam linked independently to the cluster 1 whereas the land race Natu Eluru linked independently to the main cluster. Cluster 1 was divided into three sub-clusters. Sub-cluster 1a was formed by variety Natu Special and Natu Singarayakonda. The sub-cluster 1b was formed by two landraces and variety Prabhat. Tokaku Guntur was one of the parents to the variety Prabhat and both were included in the same sub-cluster 1b with genetic similarity of 81%. The sub-cluster 1c was formed by two land races. The cluster 2 was formed by Bhairavi and its two parents Vishwanath and WAF. The variety Bhairavi showed 78 and 77% of genetic similarity with Vishwanath and WAF, respectively.

The genetic similarity based on isoenzymes among the *Natu* tobacco accessions varied from 50 to 92% with an average genetic similarity 71%. The maximum genetic similarity was shown by the varieties Prabhat and Tokaku Guntur whereas minimum genetic similarity (50%) was shown by Natu Eluru with Natu Singarayakonda and Natu Special. In the dendrogram constructed based on isoenzymes, the 12 accessions were grouped into 2 main clusters. Cluster 1 was formed by 3 sub-clusters. Sub-cluster 1a included Bhairavi and its two parents. The sub-cluster 1b composed of variety Natu Special and land race Rayala DK and Rayala Farm. The sub-cluster 1c was formed by landraces Natu Eluru and Natu Singarayakonda with genetic similarity of 83%. The second cluster was formed by the variety Prabhat and the land races Tokaku Farm and Tokaku Guntur. Tokaku Guntur happens to be one of the parents of variety Prabhat. The variety Pyruvithanam was linked independently to the main cluster.

The landraces Rayala DK and Rayala Farm showed 79, 83 and 90% genetic similarity between them in SSR, isoenzyme and RAPD, respectively indicating the close relationship between them. The landraces Tokaku Farm and

Tokaku Guntur showed 93, 85 and 85% genetic similarity between them in SSR, RAPD and isoenzymes, respectively, again indicating close genetic similarity. These might have collected and considered as two different land races due to a few phenotypic variations. The landrace Natu Eluru showed more genetic differences with other landraces ranging from 33 to 41% in SSR markers showing its genetic variation with other *Natu* tobacco accessions. The variety Pyruvithanam, which was a local selection showed genetic differences with other *Natu* accessions ranging from 26 to 42% and 27 to 50% in SSR and isoenzyme markers, respectively indicating its genetic difference with *Natu* accessions and also justified by its position in clustering pattern. The landrace Natu Singarayakonda showed 21 to 41% and 31 to 49% (with exception with Natu Eluru) genetic differences with other landraces in SSR and isoenzymes, respectively but very low genetic differences in RAPD markers.

The average of amplified fragments/primer was higher in RAPD (7.8) than SSR markers (5.18), it could be due to the fact that SSR primers used in this study were tobacco specific. Among SSRs, the primer DQ865445 amplified more number of amplicans (11), otherwise the average fragments for SSR primer will be 4.8. The cluster analysis from RAPD, SSR and isoenzymes data produced similar but not identical phylogenetic relationships; it could be due to the fact that probability of scoring of non-homologous RAPD fragments as identical is higher in RAPD than SSR. The isoenzyme markers also showed nearly similar pattern of cluster formation and high genetic differences when compared to SSR and RAPD, it may be due to use of only five enzymes.

It has clearly emerged from the cluster analysis based on RAPD, SSR and isoenzyme markers that the genetic variation in *Natu* tobacco was not very narrow as reported by Siva Raju *et al.* (2008) in different tobacco types. The genetic similarity among the cultivars and landraces was not high indicating that there is a possibility to select landraces as parents in breeding programme to develop improved *Natu* varieties. The clustering pattern of the cultivars corresponded more or less similar based on parentage in the three marker systems.

Thus, in general RAPD, SSR and isoenzyme markers are efficient in detecting polymorphism even among closely related *Natu* tobacco cultivars and landraces. However, SSR markers were found to be more efficient than RAPD in estimation of parentage of the *Natu* tobacco cultivars. The variety-specific markers identified in the study will benefit the Plant Breeders to choose appropriate parents for genetic improvement of *Natu* tobacco.

REFERENCES

Barandalla, L., Ruiz de Galarreta, D. Rios and E. Ritter. 2006. Molecular analysis of local potato cultivars from Tenerife Island using microsatellite markers. **Euphytica** 152: 283-91.

Behera, T.K., P. Sharma, B.K. Singh, G. Kumar, R. Kumar, T. Mohapatra and N.K. Singh. 2006. Assessment of genetic diversity and species relationship in eggplant (*Solanum melongena* L.) using STMS markers. **Scientia Hort.** 107: 352-7.

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 Tabac Inter.** 19: 1-15.

Doyle, J.J. and J.L. Doyle. 1990. Isolation of plant DNA from fresh tissue. **Focus** 12: 13-5.

Goodspeed, T.H. 1954: The genus *Nicotiana*. Chronica Bot Wallham, Mass.USA.

Gupta, P.K., H.S. Balyan, P.C. Sharma and B. Ramesh. 1996. Microsatellites in plants: A new class of molecular markers. **Curr. Sci.** 70: 45-54.

Laemmli, U.K. 1970. Clevage of structural proteins during the assembly of the head of the bacteriophage T4. **Nature** 227: 685-9.

Narayan, R.K. 1987. Nuclear DNA changes, genome differentiation and evolution in

Nicotiana (*Solanaceae*). **Plant Syst. Evol**. 157: 161-80.

Nielsen, G. 1985. The use of isozymes as a probe to identify and lable plant varieties and cultivars. In: *Isozymes: Current topics in Biological and Medical Research* (M.C. Rattazzi, J.G. Scandalios and G.S. Whitt, Eds.), Vol.12. Alan R. Liss, New York, pp. 1-32.

Powell, W., M. Morgante, C. Andre, M. Hanafey, J. Vogel, S. Tingey and A. Rafalski. 1996. The comparision of RFLP, RAPD, AFLP and SSR markers for germplasm analysis. **Mol. Breeding** 2: 225-38.

Ren, N. and M.P. Timko. 2001. AFLP analysis of genetic polymorphism and evolutionary relationships among cultivated and wild *Nicotiana* species. **Genome** 44: 559-71.

Rohlf, F.J. 1998. NTSYS-pc: Numerical taxonomy and multivariate analysis system. Version 2.02, Exter

Sadasivam, S. and A. Manikam. 1992. *Biochemical methods for Agricultural Sciences*. 2nd edn. Newage International Publishers, New Delhi, Pp. 246.

Sheetal Yadav, Poonum Rana, Navinder Saini, Sunita Jain and Rajinder Jain. 2008. Assessment of genetic diversity among rice genotypes with different adaptations to salinity using physio-morphological and molecular markers. **J. Plant Biochem. Biotech**. 17: 1-8.

Siva Raju, K., M.S. Madhav, R.K. Sharma, T.G.K. Murthy, N.K. Singh, K.C. Bansal, K.R. Koundal and T. Mohapatra. 2008. Molecular diversity in Indian tobacco types as revealed by Randomly Amplified DNA polymorphism. **J. Plant Biochem. Biotech.** 17: 51-6.

Siva Raju, K. 2011. Genetic diversity in Indian chewing tobacco (*Nicotiana tabacum*) as revealed by RAPD and SSR markers. **Indian J. Agric. Sci**. 81: 12-7.

Song, Q.J., C.V. Quigley, R.L. Nelson, T.E. Carter, H.R. Boerma and J.R. Strachan. 1999. A selected set of trinuclotide simple sequence repeat markers for soybean cultivar identification. **Plant Var. Seeds** 12: 207-20.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. **Nucleic Acids Res.** 18: 6531-5.

Yang, B.C., B.G. Xiao, X.J. Chen and C.H. Shi. 2006. Assessing the genetic diversity of tobacco germplasm using intersimple sequence repeat and inter-retrotransposon amplification polymorphism markers. **Annals of Applied Biol**. 150: 393-401.