International Journal of Enhanced Research in Science, Technology & Engineering
ISSN: 2319-7463, Vol. 11 Issue 6, June-2022, Impact Factor: 7.957

Genetic diversity studies in the tobacco germplasm using SSR markers

Siva Lakshmi T.V[1], Sarala K[2], Prabhakara Rao K[3], Reddy A.M[4]

Murthy T.G.K[5]

[1.2,3,5]ICAR-Central Tobacco Research Institute, Rajamahendravaram, AP, INDIA
[1.4]Adikavi Nannaya University, Rajamahendravaram, AP, INDIA

ABSTRACT

Solanesol is an anti-carcinogenic secondary metabolite first isolated from *Nicotiana tabacum*. Till date none other solanaceous crops have reports of existence of Solanesol more than that which was present in either *N. tobacumor* most of its *cultivars. N. tabacum* (common tobacco) is one of the most widely cultivated commercial crop world-wide in approximately 120 countries. The three most commonly used Tobacco types are Flue-cured (or Virginia), Burley and Oriental. Tobacco being a model plant for studying the biological processes a comparative study was undertaken within the experimental set of germplasm consisting of Flue-cured varieties Gowtami andSiri. a burley variety HDBRG and an Oriental variety *Abirami. A* Recombinant Inbred Line having a Maternal parent as HDBRG but varying in the paternal parents were also taken as per the levels of Solanesol. They are {1/1. 1/29. 1/50, 1/41. 1/25]. Estimation of Solanesol is done by Reverse phase HPLC and Genetic diversity studies were conducted using the package NTSYSpc 2.21w. In the present study maximum jacquard similarity coefficient is 0.5000. It is shown by It reveals equal molecular similarity and divergence between Gowtami. Abirami and 1/29.

Keywords: HDBRG- Harvel De Bouxo Rio Grande, HPLC- High Performance Liquid Chromatography, Recombinant in-bred Line, Solanesol.

1. INTRODUCTION

Large scale development of Microsatellite markers was done by Z Tong et al, in.2012 from a population of 207 double Haploid (DH) Lines derived from a cross between two Flue cured Tobacco varieties Honghua Dajinyuan and Hicks Broad Leaf. A genetic map consisting of 611 SSR loci which has their distribution on 24 tentative linkage groups was constructed. In the present study Micro satellites which were selected after screening for polymorphism were TM10163 of Linkage group HH21. TM10654 of Linkage Group HH18 and TM11175 of Linkage Group HH22. Along with these a Tobacco Microsatellite TbM36 developed from a clone "C8-PL-4"generated from the genomic DNA Chromosome 1 of *N. tabacum* cultivar “JAYASRI” since the reports of HPLC revealed significant presence of solanesol in comparison to other *Nicotiana sps.* was taken. It has a Tagged genomic DNA is of 1493 bp and a repeat region between 270...293 bp. It is a tandem Repeat of motif (gaa)8. This was developed by the Scientists of Central Tobacco Research Institute.

2. MATERIALS AND METHODS

A. *Plant material*

Mature Leaves known to possess more solanesol in comparison to other parts of plant in terms of expression of genes related to Solanesol Biosynthesis and also presence of Solanesol. Somature leaves were collected from the Experimental Farm of ICAR-Central Tobacco Research Institute at Katheru, AP, INDIA grown in black soils during the Rabi Season on the onset of flowering and seed setting. Freshly harvested Leaves were used in DNA extraction for molecular marker studies. Flue cured or air cured leaves were powdered and the dried biomass is down-stream processed and analyzed biochemically by reverse phase HPLC (High Performance Liquid Chromatography.

B. *Biochemical Analysis :*

Weighed 100mg of Tobacco Powder and transferred into conical flask Added 20 ml of Iso-propanol. Shaken it for 30min, on an orbital shaker. Filtered.and the Filtrate collected in Cuvette is fed to HPLC.

Estimation of Solanesol by HPLC :

HPLC:HPLC System composed of two LC10 AT VP pumps, an SPD-10A VP diode array detector, a SIL 10AD VP auto injector, a DGU 12A de-gasser and SCL-10 AVP system controller (Shimadju, Japan). A reverse phase Kromasil C8(Eka Chemicals, Sweden) Column(250x4.6mmx5µm) was used for separation. The chromatographic and integrated data were recorded using HP-Vectra computer system (Hewlett Packed, Germany) computer system. The HPLC system consisting of two LC-20AT pumps, an SPD-M20A diode array detector, a SIL-20AC auto sampler, a DGU-20A3 de-gasser and CBM-20A communication bus module (Shimadju, Japan) was used. A reverse phase Kromasil C8(Eka Chemicals, Sweden) column(250x4.6mm x 5µm) was used for separation. The chromatographic and the integrated data were recorded using HP-Vectra computer system using LC-solution data acquiring software (Shimadju, Japan).

Reagents:

All reagents used were of analytical reagent grade. Glass-distilled and de-ionized water (Nona pure, USA), Acetonitrile (HPLC-grade), Isopropyl alcohol (S.D. Fine Chem, India), Solanesol (prepared at CTRI) were used

HPLC Analysis:

Separation was carried out on Kromasil C18 column maintained at 500C with an iso-cratic elution using ACN : IPA as mobile phase and PDA detector set at 210nm.The mobile phase was aceto-nitrile and iso-propyl alcohol (80:20 v/v). Before delivering into the system, it was filtered through 0.45µm PTFE filter and de-gassed using vacuum. The analysis was carried out under isocratic conditions using a flow rate of 1.0ml/min. at (50 0C). The chromatograms were recorded at 210nm using an SPD-M10 AVP diode array detector.

Based on the results obtained from the HPLC analysis depicting the concentrations of Solanesol Experimental set of Germplasm for the molecular studies was designed

Table 1: Experimental set of Germplasm

Genotype	Tobacco type	Concentration of Solanesol
HDBRG	Dark Burley	2.15
BY-53		1.34
HDBRGxBY-53{1/1}		0.91
Siri	Flue-cured	0.40
Abirami	Oriental	0.90
Gowtami	Flue-cured	2.45
1/29		1.82
1/50		1.39
1/41		2.08
1/25		0.93

C. *Extraction of the genomic DNA:*

Genomic DNA was extracted from mature leaves of the selected experimental set of genotypes based on the presence of Solanesol using a modified Cetyl tri-methyl ammonium bromide (CTAB) method.[2]. The quality and quantity of DNA were measured using Nanodrop Spectrophotometer.

D. *Amplification of genomic DNA:*

Genomic DNA was amplified by the following selected set of primers after Screening of several primers.

Table 3: The Oligos used in amplification of genomic DNA

Linkage Group/Chromosome number	Name of the Oligo Nucleotide	OLIGO SEQUENCE(5l…..3l)	Size of Oligo Nucleotides	Tm given
HH21	TM10163-F	TGTAGCTTTGGGTTTTCATCC	21	55.90C
	TM10163-R	CCATCCTCACGAAGATGTGA	20	57.30C
HH18	TM10654-F	ATGGGGCCCACATAGTGTAT	20	57.30C
	TM10654-R	GGTCTTGGATCATGAGAGAACC	22	60.30C
HH22	TM11175-F	CAATGGAGTCGAGACGAGGT	20	55.90C
	TM11175-R	TTTCTCGCGGCTCGATATTA	20	57.30C
Chr 1	TbM36-F	ATGTCGGTATCAGCACTTTTGAC	23	64.00C
	TbM36-R	TATTCTAACTCCTCGACCATTGACT	25	62.90C

.

Reagents for Amplification:

A reaction mixture of 25µL containing 2µL of Genomic DNA (30ng/ µL) as a template, 1 µL of 0.2µM Primer (Forward), 1 µL of 0.2µM Primer (Reverse). 2 µL of 2mM dNTP's, 0.3µl Taq DNA Polymerase 0.3µl, 0.5µl of 25mM MgCl2, 2.5µl Taq Buffer E(1x) and 15.7µl of PCR water.

Amplification of Template Genomic DNA:

It was done in Eppendorf master cycler ®X50. Polymerase chain Reaction for the Amplification of template DNA was done in 3 steps with an Initial Denaturation at 94 0C for 5 min. Denaturation at 94 0C for 1 min, annealing at 550C for 1 min. (set as per given Tm) Extension 72 0C for 1 min. Final Extension at 72 0C for 10 min. Computed the program of amplification for 35 cycles and Amplicon profiles were generated by Poly acrylamide gel electrophoresis. DNA is intercalated by ethidium bromide and the fluorescent DNA is visualized in UV transilluminator (Bio-Rad Doc 2000) gel documentation system. And further analyzed in Program ImageJ.

DATA Analysis after Amplicon profiling:

Data analyzed by NTSYS (pc version 2.21w (Numerical Taxonomy and Multivariate Analysis system) package. The allele for its presence at the locus is taken as "1" and "0" for its absence to calculate the Jacquards coefficient for genetic similarity, Using SHAN a sub program for clustering and a sub-sub program UPGMA (Un- weighted Pair Group method with arithmetic method) a Phylogram was generated.

E. ***Calculation of the Polymorphism statistics:***

For the calculation of Polymorphism statistics web application iMEC (Online Marker Efficiency Calculator) was used[1]. It is available at https://irscope.shinyapps.io/iMEC/. Input data was given in Binary form coded (0, 1). where '0' is for absence of the band and '1' for the presence of band. Calculated the Heterozygosity Index(H), Polymorphism Information Content(PIC), Discriminating power (D), Effective Multiplex ratio(E), Marker Index (MI), arithmetic mean Heterozygosity(H avp), and Resolving power (R)avp.

F. ***Data analysis for assessing the Genetic Diversity***

Data analysed by a statistical package NTSYS(pc version 2.21w). Numerical Taxonomy and Multivariate Analysis system. The allelic data was marked as "1" for presence and "0" for absence and used as an input data for calculation of Jaccard coefficients for similarity. Genetic Similarity among the Recombinant Inbred lines and Di-haploid lines of Solanesol trait improvement were deduced by Jaccard Similarity coefficient within the SIMQUAL, a subpackage of NTSYSpc version 2.21w., followed by Phylogenetic tree constructiou using UPGMA statistical method with substitution model of Maximum composite likelihood, a sub sub program in NTSYS-pc version 2.21w.

3. RESULTS AND DISCUSSION

The Amplicon profiles of the Germplasm found to be polymorphic were considered for diversity analysis and which were found to be monomorphic were not taken into account.[3],[4].

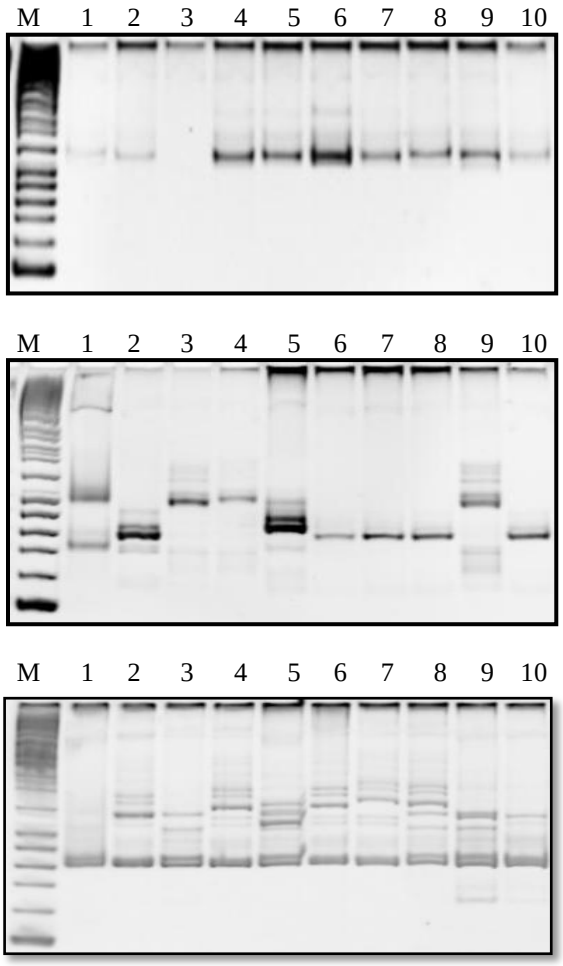

Fig. 1: Amplicons of PrimerTbM36, TM101654, TM10163; M- 20 bp, 1. HDBRG, 2. BY-53, 3. HDBRG X BY53, 4. SIRI,5. ABIRAMI, 6. GOWTAMI, 7. 1/29, 8. 1/50, 9. 1/41, 10. 1/25generated by Poly Acrylamide Gel Electrophoresis

Table 4: Polymorphism Statistics calculation by iMEC

	H_0	PIC_0	E_0	H.av_0	MI_0	D_0	R_0
TbM36	0.4928	0.371374	2.2	0.009856	0.021683	0.811429	4
TM11175	0.32	0.2688	1.6	0.004	0.0064	0.962025	3.2
TM10654	0.311327	0.262864	2.7	0.002224	0.006004	0.963926	5
TM10163	0.447811	0.347543	4.4	0.003445	0.015157	0.887179	6.4

In the comparative studies of the molecular markers maximum PIC value is exhibited by Tobacco Microsatellite marker TbM36 and maximum D value is exhibited by TM10654.

Among the SSR markers developed by Z.Tong *et al*.,2012and taken in present study maximum PIC value of 0.347543 is shown by TM10163 and a minimum PIC value of 0.262864 is shown by TM10654. D parameter {discriminating power of primer) evaluates the efficiency of the primer in identification of Tobacco accessions. D value of TM10654 is 0.963926 (Value being highest and closest to 1) implies its efficiency in discriminating the Experimental set of Germplasm.

	HDBRGT	BY53	HDBRG^BY	SIRI	ABHIRAMI	GOWTHAMI	1/29
HDBRGT	1.0000						
BY53	0.2143	1.0000					
HDBRG^BY53	0.1333	0.1429	1.0000				
SIRI	0.3077	0.3333	0.2000	1.0000			
ABHIRAMI	0.2000	0.2500	0.1905	0.4706	1.0000		
GOWTHAMI	0.0667	0.1500	0.1500	0.3529	0.5000	1.0000	
1/29	0.1538	0.2941	0.0476	0.2222	0.2778	0.5000	1.0000
1/50	0.1429	0.2778	0.1500	0.1500	0.2000	0.2222	0.3125
1/41	0.2500	0.1739	0.1739	0.1250	0.1667	0.0833	0.1364
1/25	0.3000	0.2500	0.1765	0.1111	0.2353	0.2667	0.2000

	1/50	1/41	1/25
1/50	1.0000		
1/41	0.1818	1.0000	
1/25	0.3571	0.2105	1.0000

Figure 2 : Jaccard Similarity Coefficients matrix of Experimental Set of Tobacco

Depending upon Jaccard similarity coefficients it is known that more the genetic similarity value the more they related, the less the value they are diversified. Based on the genetic similarity values, it is observed that the maximum genetic similarity values is found to be 0.5000 between Gowtami and Abirami and also 1/29 and Gowtami; with a value of 0.4706 between Abirami and Siri.

With a Jaccard Similarity coefficient value of 0.0667Gowtami and HDBRG are more diversified and with a least value 0.0476 1/29 and HDBRG x BY53 are most diversified.

Construction of the Phylogenetic Tree

Phylogenetic tree is constructed using UPGMA cluster analysis depicting genetic relationships among the Experimental set of tobacco lines and accessions is shown below.

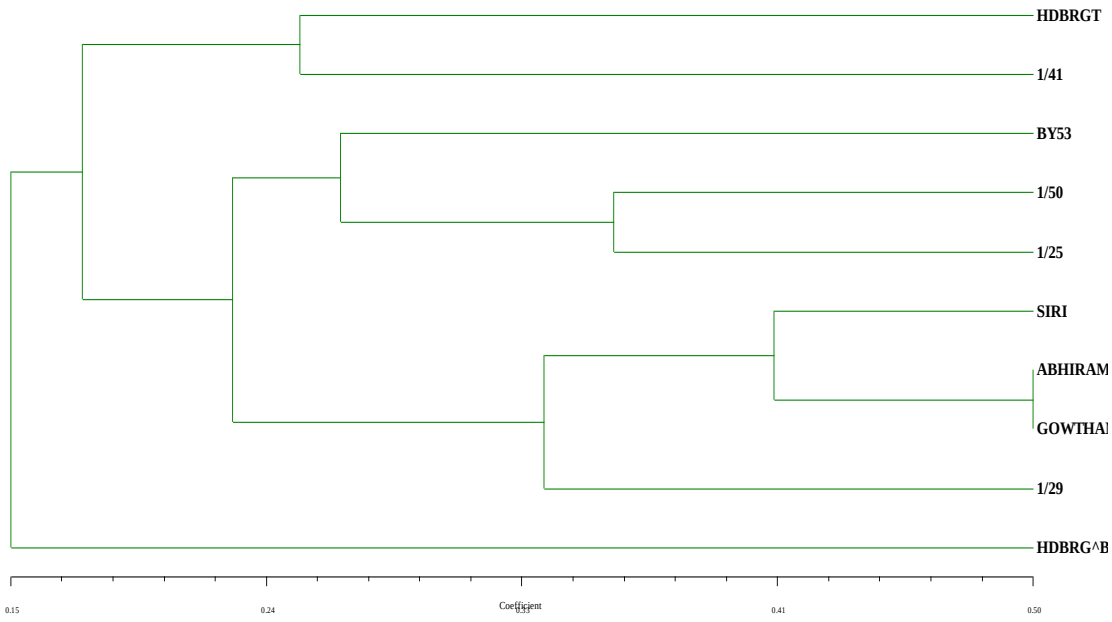

Figure 3: Dendrogram of the tobacco cultivars used in this study

CONCLUSION

More the value of Jaccard similarity coefficient more is the genetic similarity and less the value more are they diversified. Among the accessions taken for study Gowtami and Abirami were equally similar and diversified,

ACKNOWLEDGMENT

I would extend my Heart-felt Gratitude to Central Tobacco Research Institute and Adikavi Nannaya University for their valuable Guidance and Resources to work as well as who stood along with me in my work. Special thanks to T.V.S. Rama Krishna.

.

REFERENCES

[1]. Ali Amiryousefi, Jaakko Hyvonen and Peter, "iMEC: Online Marker Efficiency Calculator". Applications in Plants sciences, vol. 6(6), e01159, june 24, 2018.
[2]. M Ahmed, W. Islam, A. Arshad, W. Mannan, Ahmed and B. Mirza, "High Quality plant DNA extraction for PCR. An easy approach"Journal of Applied Genetics, vol. 50, pp. 105-107, 2009.
[3]. K. Phani Kiran, K. Siva Raju and C. V. Narasimha Rao*, "Genetic diversity in tobacco having different levels of solanesol using SSR markers", Journal of Medicinal and Aromatic Plant Sciences vol. 32(2), pp. 116-119, 2010.
[4]. K. Sarala and R. V. S. Rao. "Genetic diversity in Indian FCV and burley tobacco cultivars." Journal of Genetics, vol. 87(2), pp.159-163, 2008.
[5]. Zhizun Tong. Zemao Yang, Xuejun Chen, Fangchan Jiao, Xuying Li, Xiangfu Wu, Yulong Gao, Bingguang Xiao, Weiren Wu. "Large-scale development of microsatellite markers in Nicotiana tabacum and construction of a genetic map of flue-cured Tobacco." Plant Breeding, vol. 131(5), pp.674-680, 2012.