International Journal of Enhanced Research in Science, Technology & Engineering
ISSN: 2319-7463, Vol. 11 Issue 5, May-2022, Impact Factor: 7.957

Genetic Diversity Studies in the Solanesol and Nicotine Lines of Tobacco Using UBC (ISSR) 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 a phytochemical first to be isolated from *Nicotianatabacum* and was subsequently reported from other solanaceous plants. But among all reports of Quantification Tobacco has the highest solanesol content. An effort is being put by the Scientists of ICAR-Central Tobacco Research Institute, INDIA to improve the production of Solanesol by raising the Recombinant Inbred Lines as well as the Di-Haploid Lines. Besides estimation of the Solanesol content along with other primary and secondary metabolites of tobacco and tobacco Inbred Lines efforts were made to understand the influence of coding or non- coding sequences of Genome in the Biosynthesis of Solanesol, in enhancement of production and Marker Assisted Selection. So, a study was undertaken to examine the intra- and inter specific variations by amplification of the genomic segments between Inversely Oriented Repeats. Amplification profiles of ISSR markers UBC-807, UBC-809, UBC-813, UBC-823, UBC-836 were generated. A statistical package NTSYSpc version 2.21w with the subprogram SIMQUAL is used to generate Jaccard S*imi*larity coefficient. Interestingly, a value of 0.0000 similarity is found between Low solanesol Bulk and HDBRG x BY53; GT7 and HDBRG x BY53; Nisnicotinony121 and HDBRG x BY53; Nisnicotinony121 x Kumkumatri matri and Low solanesol Bulk; Di-Haploid Line (GT7x Kumkumatri matri) and Low solanesol Bulk; Candel and GT7. Nisnicotinony121 and HDBRG; Nisnicotinony121 x Kumkumatri matri and GT7; Di-Haploid Line3(GT7 x Kumkumatri matri) and GT7. This implies that multiple polymorphic loci markers, the Inter Simple Sequence Repeats can well be used as a tool to study the diversity among the selected set of Solanesol, Nicotine and Di-haploid Lines.

Keywords:Di-haploid Lines, Inter Simple sequence repeats, Recombinant Inbred Lines, Solanesol

1. INTRODUCTION

Nicotiana tabacum and its varieties or Inbred Lines are known for a terpenoid derivative, Solanesol. The genome of *N. tabacum* is still having undetermined nucleotides, scaffolds, Super scaffolds, Super scaffold linkers coding and noncoding sequenced which are yet to be identified and assembled. Inter Simple Sequence Repeats (ISSRs) are advantageous than SSRs since not any prior knowledge of genomic sequence is required. They make use of single primers that are anchored at the 5^{l} or 3^{l} end of a repeat region and extend into the flanking region and allows amplification of genomic segments between inversely oriented repeats. The advantages of this technique include multiple polymorphic loci, high through put, inexpensive and are utilized to examine intra- and inter-specific variation.

2. MATERIALS AND METHODS

A. Plant material

Plant material was chosen from the Experimental Farm of ICAR-Central Tobacco Research Institute at Katheru, AP, INDIA grown during the Rabi Season and harvested the produce on Maturity. Mature leaves were collected from the parents, RILs (Recombinant Inbred Lines) as well as Di-Haploid Lines bred for the trait -enhancement in production of Solanesol.

B. Estimation of Solanesol by HPLC

Flue- cured or air- cured matured leaves are made into powder . Taken 100mg of the powder was dissolved in 20ml of methanol or iso-propanoland shakedthoroughly and mixed the contents on Orbital shaker. Collected the filtrate into cuvette and fed to HPLC. Separation was carried out on Kromasil C_{18} column with an iso-cratic elution using ACN : IPA as mobile phase and PDA detector set at 210nm. Solanesol (prepared at CTRI) was taken as a Standard. The mobile phase was aceto-nitrile **:** 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 iso-cratic conditions using

a flow rate of 1.0ml/min. at 50^{0}C. The chromatograms were recorded at 210nm using an SPD-M10 AVP diode array detector, Based on the presence of Solanesol the experimental set of germplasm was selected.

Table 1: Experimental Set Of Germplasm Lines As Per The Levels Of Solanesol After Hplc Analysis

Population	Type of Inbred line	Tobacco accessions/ Germplasm	Presence of Solanesol
		SOLANESOL set of Germplasm	
Parent		HDBRG	High
Parent		BY53	Low
Population 1	RIL	HDBRG x BY53 [(1/1)]	Medium
	RIL	Low Solanesol Bulk [(1/22, 1/25.1/46, 1/50)]	Low
Population 1	RIL	High Solanesol Bulk [(1/2,1/12, 1/26, 1/29,1/52)]	High
Parent		GT-7	Low
Parent		HDBRG	High
Population 2	RIL	HDBRG x GT-7 [(2/1)]	Medium
		NICOTINE set of Germplasm	
Parent		Candel	Low
Parent		Nisnicotinony-121	High
Population 4	RIL	Candel x Nisnicotinony-121 [(4/1)]	Medium
Population 4	RIL	Low Nicotine bulk [(4/64, 4/71, 4/97, 4/99, 4/102)]	High
Population 4	RIL	High Nicotine bulk [(4/8, 4/65, 4/74, 4/100, 4/110)]	Low
Parent		Kumkumatri matri	High
Population 9	RIL	Nisnicotinony121x Kumkumatri matri [(9/1)]	Low
	Di-Haploid Line	**DH Line3(GT-7 x Nisnicotinony-121)**	Medium

C. Extraction of the genomic DNA

Genomic DNA was extracted from mature leaves of the selected experimental set of solanesol and Nicotine lines 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

Table 2: The Oligos of UBC used in amplification of genomic DNA

S. No	Name of the Oligo Nucleotide	OLIGO SEQUENCE(5^l…..3^l)	Size of Nucleotides	T_m given	T_m done
1	UBC-807	AGAGAGAGAGAGAGAGT	17	50.4^{0}C	42.0^{0}C
2	UBC-809	AGAGAGAGAGAGAGAGG	17	52.8^{0}C	42.0^{0}C
3	UBC-813	CTCTCTCTCTCTCTCTT	17	50.4^{0}C	42.0^{0}C
4	UBC-823	TCTCTCTCTCTCTCTCC	17	52.8^{0}C	50.0^{0}C
5	UBC-836	AGAGAGAGAGAGAGAGTA	18	52.6^{0}C	50.0^{0}C

Amplification of genomic DNA was done taking a reaction mixture of 25µL containing 2µL of Genomic DNA (30ng/ µL). 2 µL of 0.2 µM Primer (2µL of 2mM dNTP's, 0.3µl Taq DNA Polymerase; 0.5µLof 25mM MgCl2; 2.5µl Taq Buffer E(1x) and 15.7 µl of PCR water. and gently mixed all the contents. The reaction mixture was placed in chilled conditions to prevent any unusual reaction before placing in Thermo-Cycler.

Amplification of Genomic DNA taken as Template was done in Eppendorf master cycler®X50 in 3steps of 45 cycles. Step 1: An Initial Denaturation at 94^{0}C for 5min. and Denaturation at 94^{0}C for 1min., Step 2: Annealing at 52^{0}C for 1min. (set as per given Tm). Step 3: An Extension of 72^{0}C for 2min. and a Final Extension at 72^{0}C for 5minutes.

Amplicon profiles were generated by running the PCR products in Acrylamide Gel electrophoresis. Amplified DNA is intercalated by ethidium bromide and the fluorescent DNA is visualized in UV transilluminator It is documented in Bio-Rad Doc 2000, a gel documentation system and then further analyzed by the program Image J.

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 NTSYS-pc version 2.2w., 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 given into account.[3],[4].

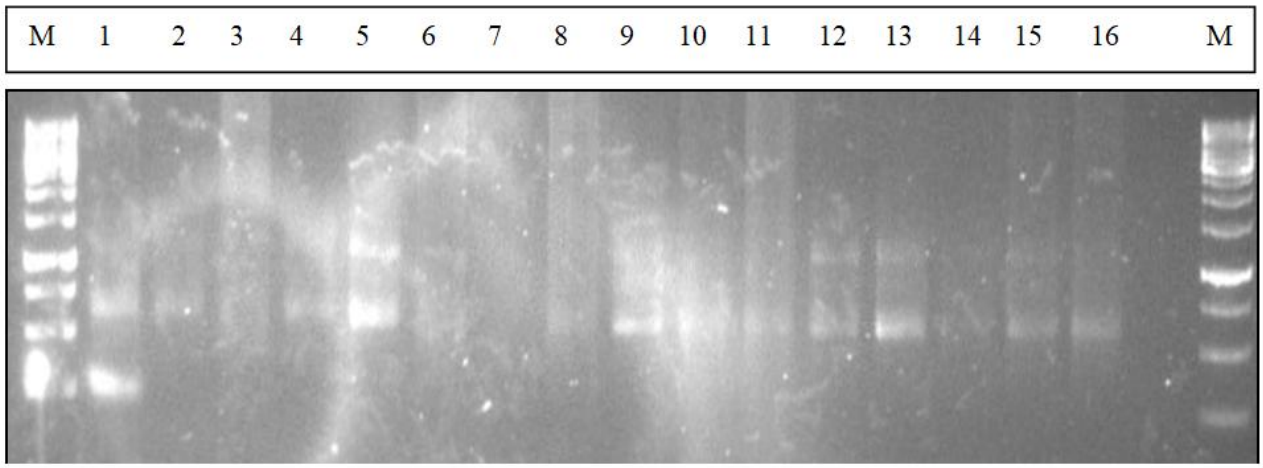

Figure 1 Age of UBC 823: M-100bp 1. HDBRG, 2. BY-53, 3. HDBRGxBY-53, 4. LOW SOLANESOLBULK, 5..HIGH SOLANESOL BULK, 6. GT-7, 7. HDBRG, 8. HDBRGxGT7, 9.CANDEL, 10. NIS-NICOTYNONY-121, 11. CANDELxNISNICOTYNONY-121, 12. LOW NICOTINE BULK, 13. HIGH NICOTINE BULK, 14. KUMKUMATHRI MATHRI, 15. NISNICOTYNONY121xKUMKUMATHRI MATHRI, 16. D H LINE3 (GT-7xNISNICOTYNONY121)

Table 3: Polymorphism Statistics calculation by iMEC

S.No.		H_0	PIC_0	E_0	H.av_0	MI_0	D_0	R_0
1	**UBC-807**	0.375	0.304688	1.25	0.004688	0.005859	0.939873	1.75
2	**UBC-809**	0.2032	0.190169	1	0.2032	0.2032	0.033133	
3	**UBC-813**	0.486966	0.425093	1	0.486966	0.486966	0.112153	
4	**UBC-823**	0.240329	0.218521	1	0.240329	0.240329	0.029123	
5	**UBC-836**	0.210611	0.198355	1	0.210611	0.210611	0.042271	

Among the UBC markers taken for study maximum PIC value of 0.425093 is shown by UBC-813 and a minimum PIC value of 0.190169 is shown by UBC-809. D parameter {discriminating power of primer) evaluates the efficiency of the

primer in identification of Tobacco accessions. D value of UBC-807 is 0.9398, (Value being highest and closest to 1) implies its efficiency in discriminating the Experimental set of Germplasm Lines.

	HDBRG	BY-53	HDBRGxBY	LowSolan	HighSola
HDBRG	1.0000				
BY-53	0.7083	1.0000			
HDBRGxBY-53	0.6875	0.7292	1.0000		
LowSolanesolBulk	0.6250	0.6667	0.7292	1.0000	
HighSolanesolBulk	0.6667	0.6250	0.8125	0.7917	1.0000
GT7	0.6458	0.6875	0.7500	0.8958	0.8125
HDBRG-F	0.6667	0.7083	0.9375	0.7500	0.8333
HDBRGxGT-7	0.7083	0.6667	0.7292	0.7083	0.7500
Candel	0.6458	0.6042	0.7500	0.6458	0.8125
Nisnicotinony-121	0.7292	0.6875	0.7083	0.7292	0.7292
CandelxNisnicotynony	0.6250	0.6667	0.6875	0.7500	0.7500
LowNicotineBulk	0.5000	0.6250	0.5208	0.5000	0.5417
HighNicotineBulk	0.5833	0.6667	0.6042	0.5833	0.6250
Kumkumadrimatri	0.6458	0.6458	0.7083	0.7292	0.6875
Nisnicotinony121xKumkumadri	0.7500	0.7500	0.8958	0.7083	0.7917
DiHaploidLine(GT-7xKumkumadri)	0.7292	0.7292	0.8750	0.6875	0.7708

	GT7	HDBRG-F	HDBRGxGT	Candel	Nisnicot
GT7	1.0000				
HDBRG-F	0.7708	1.0000			
HDBRGxGT-7	0.6875	0.7500	1.0000		
Candel	0.6250	0.7292	0.7292	1.0000	
Nisnicotinony-121	0.7083	0.6875	0.8125	0.8750	1.0000
CandelxNisnicotynony	0.7292	0.7083	0.7917	0.7708	0.8542
LowNicotineBulk	0.4792	0.5000	0.6250	0.6875	0.7292
HighNicotineBulk	0.5625	0.5833	0.7083	0.7292	0.7292
Kumkumadrimatri	0.7083	0.7292	0.8542	0.6667	0.7500
Nisnicotinony121xKumkumadri	0.7292	0.8750	0.7083	0.8542	0.8125
DiHaploidLine(GT-7xKumkumadri)	0.7083	0.8542	0.6875	0.8333	0.7917

	CandelxN	LowNicot	HighNico	Kumkumad	Nisnicot
CandelxNisnicotynony	1.0000				
LowNicotineBulk	0.6250	1.0000			
HighNicotineBulk	0.6667	0.8333	1.0000		
Kumkumadrimatri	0.7708	0.6042	0.6875	1.0000	
Nisnicotinony121xKumkumadri	0.7083	0.5833	0.6250	0.6875	1.0000
DiHaploidLine(GT-7xKumkumadri)	0.6875	0.5625	0.6042	0.6667	0.9792

	DiHaploi
DiHaploidLine(GT-7xKumkumadri)	1.0000

Figure 2 : Jaccard Similarity Coefficients matrix of Solanesol and Nicotine Lines of Tobacco

Jaccard Similarity coefficient value of 0.6522 is shown between High Nicotine Bulk and Low Nicotine Bulk are said to be most similar and with a value of 0.5455 GT7 and Low solanesol Bulk are more similar.

Jaccard Similarity coefficient value 0.0400 implies Low Nicotine Bulk and HDBRGare more diversified. A Jaccard similarity coefficient of 0.0000 is found between Low solanesol Bulk and HDBRG x BY53; GT7 and HDBRG x BY53; Nisnicotinony121 and HDBRG x BY53; Nisnicotinony121 x Kumkumatri matri and Low solanesol Bulk; Di-Haploid Line (GT7x Kumkumatri matri) and Low solanesol Bulk; Candel and GT7. Nisnicotinony121 and HDBRG; Nisnicotinony121 x Kumkumatri matri and GT7; Di-Haploid Line3 (GT7 x Kumkumatri matri) and GT7. With a least value 0.0000 all of the above are most diversified.

Construction of the Phylogenetic Tree

Phylogenetic tree is constructed using UPGMA cluster analysis depicting genetic relationships among the given tobacco lines is shown below.

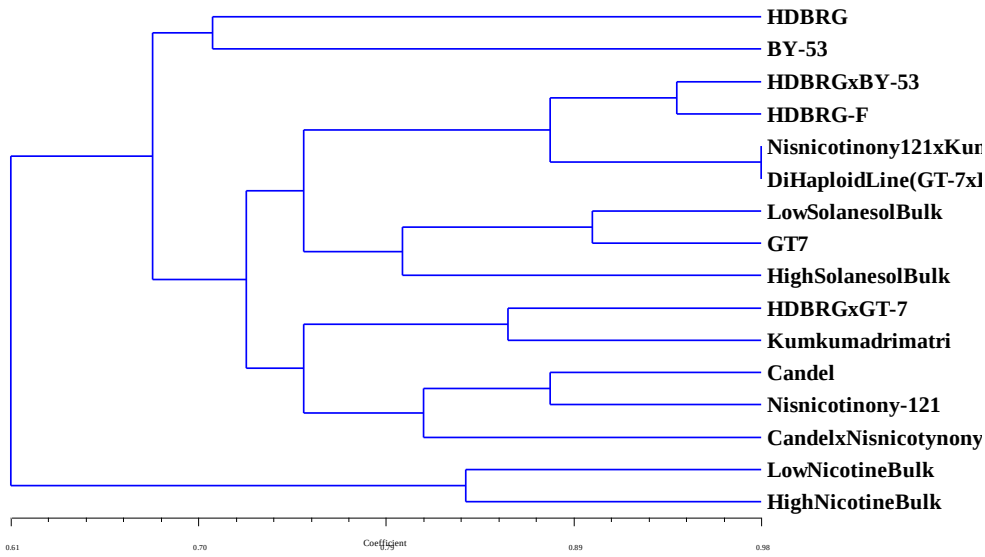

Figure 3: Dendrogram depicting the Genetic diversity among Solanesol and Nicotine Lines.

CONCLUSION

More the value of Jaccard similarity coefficient more is the genetic similarity and less the value more are they diversified. Amplicon profiling by Inter Simple Sequence Repeats can well be used as a promising tool to study the diversity among the selected Experimental set of Solanesol, Nicotine and Di-haploid Lines.

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 especially 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 Sciencesvol. 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.