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Genetic Diversity Studies in the Solanesol and Nicotine Lines of Tobacco using SCoT Polymorphism

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ABSTRACT

Solanesol($C_{45}H_{74}O$) is an organic compound with many applications in day- to- day Health of mankind. It is a white waxy solid first isolated from the solanaceous commercial crop *Nicotiana tabacum*. In a walk towards Green Chemistry an effort was made by the Scientists of ICAR-Central Tobacco Research Institute, Rajamahendravaram, AP, INDIA towards the enhancement in the production of Solanesol. This was accomplished by establishing Recombinant Inbred Lines and Di-Haploid Lines with several combinations to find out the better germplasm which could have an enhanced production of solanesol and meet the present demand. Biochemical analysis was done by Reverse Phase High Performance Liquid Chromatography.Genetic diversity studies were carried out by amplifying Genomic DNA isolated from the mature leaves by SCoT primers which target a Gene. A statistical Package iMEC was used to evaluate the efficiency of the primer. A statistical package NTSYSpc version 2.21w with the subprogram SIMQUAL is used to generate Jaccard Similarity coefficients. Phylogenetic tree was constructed using UPGMA statistical method with substitution model of Maximum composite likelihood, a sub sub program in NTSYS-pc version 2.21w.

Keywords:Di-haploid Lines, Recombinant Inbred Lines, Solanesol, Start Codon Targeted Polymorphism

1. INTRODUCTION

*Nicotiana tabacum*and its cultivars are known for a terpenoid derivative, Solanesol. The genome of *N tabacum*is having 24 chromosomes but the gene models of the chromosome 11 were only developed till today and are yet to be developed for other chromosomes.Uniqueness of Start Codon Targeted (SCoT) polymorphism which was developed by Collard and Mackill (2009) lies in targeting a gene. It targets short region flanking the "ATG" start codon at positions (+1, +2 and +3) "G" at position +4 and "ACC" at positions +7, +8 and +9 respectively. These were fixed. This method uses single 18-mer oligo nucleotide for amplification. Most oligos differ from one other by atleast 1 nucleotide with a prominence on variations at the 3^{l} end which has been shown to be prominent for oligo-template specificity.

2. MATERIALS AND METHODS

A. Plant material

Plant material was collected from the Experimental Farm of ICAR-Central Tobacco Research Institute at Katheru, AP, INDIA grown during the Rabi Season and harvested the produce in the month of Aprilon the onset of flowering and seed setting. Mature leaves were collected from the parents, RILs (Recombinant Inbred Lines) as well as Di-Haploid Lines bred for the reductionof the Heterozygosity and increase in the Homozygosity in the successive Filial generations of establishing Pure Lines with enhanced 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 and dissolved in 20ml of methanol or iso-propanoland, shakenthoroughly to mix the contents on an 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.[3]. The quality and quantity of DNA were measured using Nanodrop Spectrophotometer.

D. Amplification of genomic DNA

Table 3: The Oligos of SCoT used in amplification of genomic DNA

OLIGO NAME	OLIGO SEQUENCE (5^{l}…..3^{l})	Size of Nucleotides	T_m given	T_m done
SCOT12	ACGACATGGCGACCAAC	17	58.2^0C	50.0^0C
SCOT13	ACGACATGGCGACCATCG	18	58.2^0C	55.0^0C
SCOT18	ACCATGGCTACCACCGCC	18	60.5^0C	55.0^0C
SCOT19	ACCATGGCTACCACCGGC	18	60.5^0C	55.0^0C
SCOT20	ACCATGGCTACCACCGCG	18	60.5^0C	55.0^0C
SCOT22	AACCATGGCTACCACCAC	18	56.0^0C	51.0^0C
SCOT29	CCATGGCTACCACCGGCC	18	62.8^0C	58.0^0C
SCOT31	CCATGGCTACCACCGCCT	18	60.5^0C	55.0^0C
SCOT33	CCATGGCTACCACCGCAG	18	60.5^0C	55.0^0C

SCOT34	ACCATGGCTACCACCGCA	18	58.2^{0}C	55.0^{0}C

Amplification of genomic DNA was done taking a reaction mixture of 25µL containing 2µL of Genomic DNA (30ng/ µL) ;2µL of 0.2 µM Primer;2µL of 2mM dNTP's;0.3µl Taq DNA Polymerase; 0.5µLof 25mM $MgCl_2$; 2.5µl Taq Buffer E(1x) and 15.7µl of PCR waterand 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 35 cycles. Step1: An Initial Denaturation at 94^{0}C for 3min. and Denaturation at 94^{0}C for 1min., Step2: Annealing at 55^{0}C for 1min. (set as per given T_m). Step3: 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 Poly 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 ImageJ.

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 2.21w., followed by Phylogenetic tree constructiou using UPGMA statistical method with a substitution model of Maximum composite likelihood, a sub sub program in NTSYS-pc version 2.21w.

3. RESULTS AND DISCUSSION

The Amplicon profiles were well generated by the DNA markers developed based on the shortconserved region flanking the ATG start codon in plant genesof the experimental set of Germplasm

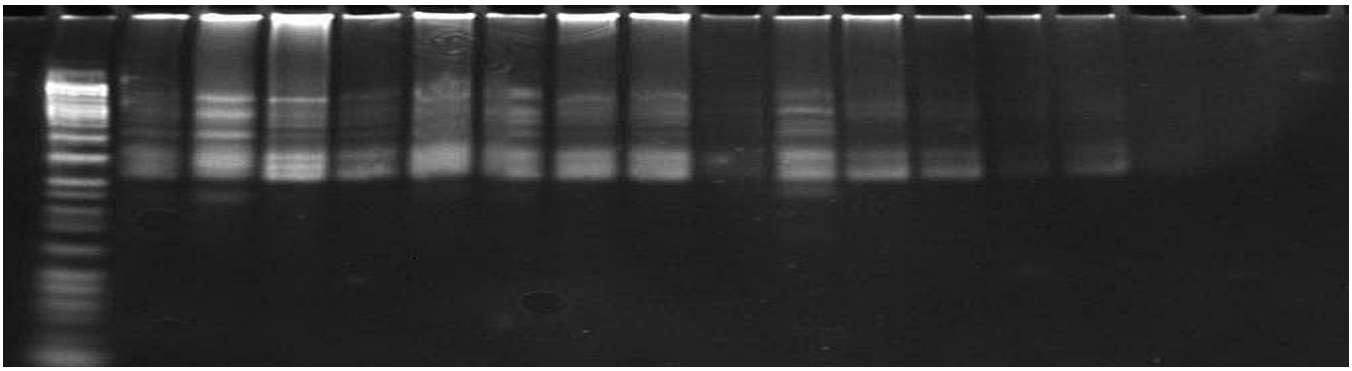

Figure 1: Amplicon generated by Poly Acrylamide Gel Electrophoresis of SCoT18 : M-20bp 1. HDBRG, 2. BY-53, 3. HDBRGxBY-53, 4. LOW SOLANESOLBULK, 5.HIGH SOLANESOL BULK, 6. GT-7, 7. HDBRG, 8. HDBRGxGT7, 9.CANDEL, 10. NIS-NICOTYNONY-121, 11. CANDELxNISNICOTYNONY-121, 12. LOW NICOTINE BULK, 13. HIGH NICOTINE BULK, 14. KUMKUMATHRI MATHRI, 15. NISNICOTYNONY121xKUMKUMATHRI MATHRI, 16. D H LINE3 (GT-7xNISNICOTYNONY121)

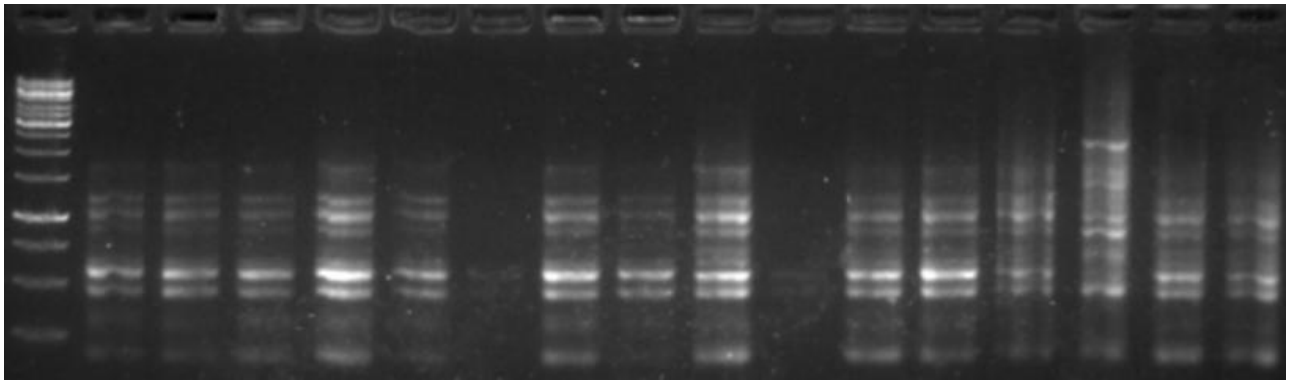

Figure 2: Amplicon generated by Acrylamide Gel Electrophoresis ofSCoT19 : M-100bp 1. HDBRG, 2. BY-53, 3. HDBRGxBY-53, 4. LOW SOLANESOLBULK, 5..HIGH SOLANESOL BULK, 6. GT-7, 7. HDBRG, 8. HDBRGxGT7, 9.CANDEL, 10. NIS-NICOTYNONY-121, 11. CANDELxNISNICOTYNONY-121, 12. LOW NICOTINE BULK, 13. HIGH NICOTINE BULK, 14. KUMKUMATHRI MATHRI, 15. NISNICOTYNONY121xKUMKUMATHRI MATHRI, 16. D H LINE3 (GT-7xNISNICOTYNONY121)

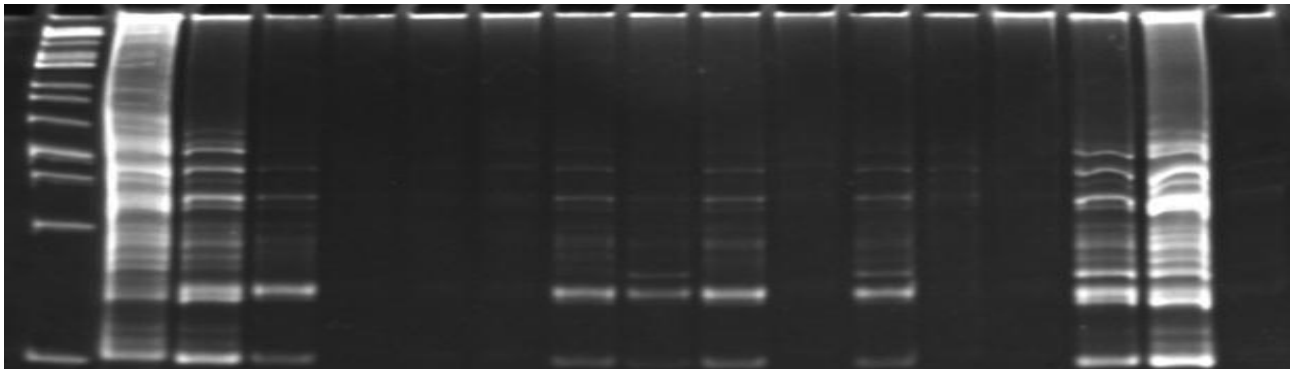

Figure 3: Amplicon generated by Poly Acrylamide Gel Electrophoresis ofSCoT31: M-20bp 1. HDBRG, 2. BY-53,3. HDBRGxBY-53, 4. LOW SOLANESOLBULK, 5.HIGH SOLANESOL BULK, 6. GT-7, 7. HDBRG, 8. HDBRGxGT7, 9.CANDEL, 10. NIS-NICOTYNONY-121, 11. CANDELxNISNICOTYNONY-121, 12. LOW NICOTINE BULK, 13. HIGH NICOTINE BULK, 14. KUMKUMATHRI MATHRI, 15. NISNICOTYNONY-121xKUMKUMATHRI MATHRI, 16. D H LINE3 (GT-7xNISNICOTYNONY121)

Table 4: Polymorphism Statistics calculation by iMEC

	H_0	PIC_0	E_0	H.av_0	MI_0	D_0	R_0
SCoT18	0.586792	0.512416	1	0.586792	0.586792	0.155208	
SCoT19	0.392219	0.315301	3.75	0.001751	0.006566	0.929132	6.25
SCoT20	0.482422	0.366056	1.625	0.007538	0.012249	0.83879	1.5
SCoT22	0.394965	0.316966	4.0625	0.001646	0.006686	0.927476	7.625
SCoT29	0.475088	0.362234	5.4375	0.002121	0.011533	0.850216	7.375
SCoT31	0.643889	0.568907	1	0.643889	0.643889	0.238043	
SCoT33	0.546078	0.458681	1	0.546078	0.546078	0.06087	
SCoT34	0.393768	0.316242	4.3125	0.001538	0.006633	0.928125	7.125

Among the SCoT markers taken for study maximum PIC value of0.512416is shown by SCoT18 and a minimum PIC value of 0.315301 is shown by SCoT19. D value of SCoT19 is 0.929132. D parameter {discriminating power of primer) evaluates the efficiency of the primer in identification of Tobacco accessions. D value of SCoT19 is 0.929132, (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	0.4386	1.0000			
HDBRGxBY-53	0.4038	0.3607	1.0000		
LowSolanesolBulk	0.2500	0.3462	0.4524	1.0000	
HighSolanesolBulk	0.2609	0.2593	0.2292	0.3939	1.0000
GT7	0.2115	0.2807	0.2800	0.4571	0.5313
HDBRG-F	0.1915	0.2222	0.2128	0.3750	0.6154
HDBRGxGT-7	0.1731	0.2241	0.2653	0.3243	0.3824
Candel	0.2115	0.1774	0.2308	0.2439	0.2564
Nisnicotinony-121	0.2037	0.1905	0.3750	0.2619	0.2750
CandelxNisnicotynony	0.3265	0.3393	0.4043	0.3250	0.2439
LowNicotineBulk	0.2745	0.3636	0.3469	0.3947	0.2143
HighNicotineBulk	0.1765	0.1667	0.2449	0.2308	0.2105
Kumkumadrimatri	0.3448	0.3538	0.3390	0.2941	0.2075
Nisnicotinony121xKumkumadri	0.1607	0.1364	0.2000	0.1277	0.1333
DiHaploidLine(GT-7xKumkumadri)	0.1731	0.1639	0.2400	0.1951	0.1463

	GT7	HDBRG-F	HDBRGxGT	Candel	Nisnicot
GT7	1.0000				
HDBRG-F	0.4242	1.0000			
HDBRGxGT-7	0.3333	0.3235	1.0000		
Candel	0.3500	0.2368	0.3000	1.0000	
Nisnicotinony-121	0.2444	0.2895	0.3500	0.3333	1.0000
CandelxNisnicotynony	0.3659	0.1951	0.2558	0.2444	0.3488
LowNicotineBulk	0.2444	0.1951	0.4595	0.2727	0.4146
HighNicotineBulk	0.2143	0.1892	0.1951	0.4167	0.4324
Kumkumadrimatri	0.3019	0.1923	0.2642	0.4375	0.3922
Nisnicotinony121xKumkumadri	0.1667	0.1951	0.2273	0.2444	0.3488
DiHaploidLine(GT-7xKumkumadri)	0.2093	0.1250	0.1628	0.2381	0.3500

	CandelxN	LowNicot	HighNico	Kumkumad	Nisnicot
CandelxNisnicotynony	1.0000				
LowNicotineBulk	0.4872	1.0000			
HighNicotineBulk	0.2927	0.3590	1.0000		
Kumkumadrimatri	0.4490	0.4792	0.4348	1.0000	
Nisnicotinony121xKumkumadri	0.2609	0.3810	0.2927	0.3396	1.0000
DiHaploidLine(GT-7xKumkumadri)	0.3500	0.3500	0.3611	0.3958	0.3846

	DiHaploi
DiHaploidLine(GT-7xKumkumadri)	1.0000

Figure 4 : Jaccard Similarity Coefficients matrix of Solanesol and Nicotine Lines of Tobacco

Jaccard Similarity coefficient value of 0.6154 is shown between HDBRG and High solanesol Bulk so are said to be most similar and with a value of 0.5313 GT7 and High solanesol Bulk are more similar.

Jaccard Similarity coefficient value 0.1250 implies Di-Haploid Line3(GT7 x Kumkumadri matri) and HDBRGare more diversified. A Jaccard similarity coefficient of 0.1095 is found between Nisnicotinony121 and BY53With a least value 0.1095 Nisnicotinony121 and BY53are 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.

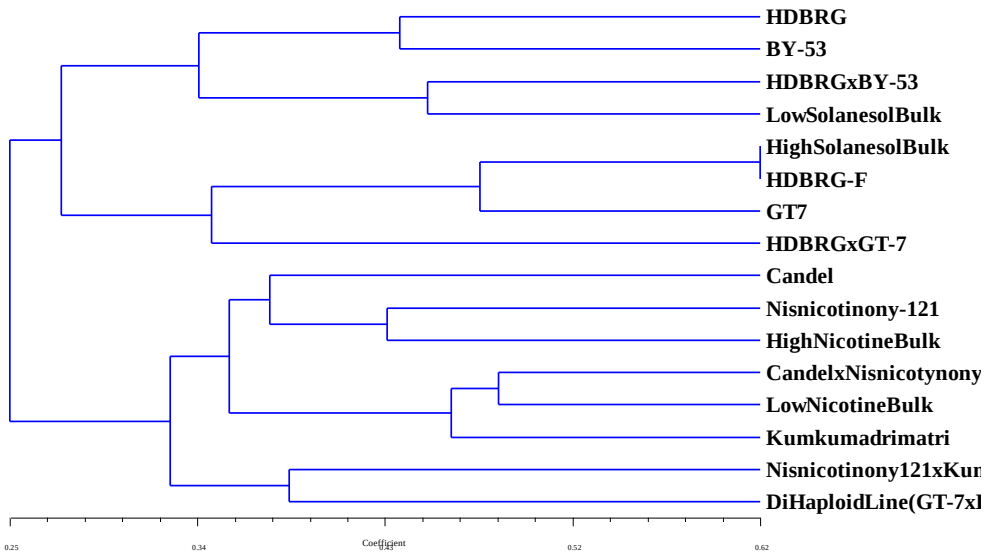

Figure 5: Dendrogram depicting the Genetic diversity among Solanesol and Nicotine Lines.

4. CONCLUSION

More the value of Jaccard similarity coefficient more is the genetic similarity and less the value more are they diversified. Amplicon profiling by Start Codon Targeted Polymorphism can well be used as a promising tool to study the genetic diversity.

5. ACKNOWLEDGMENT

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