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Genetic Diversity Studies in Solanesol and Nicotine Lines of Tobacco using tobacco Microsatellite 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

Nicotiana tabacum is an amphidiploid species (2n=4x=48,SSTT) evolved from its progenitors N. sylvestris (2x=24,SS) and N. tomentosiformis (2x=24,TT), The genome size of N.tabacum is approx. 4.5 GB. with an approximate inclusion of 2.6 GB genome from maternal parent N. sylvestris and 2-7GB genome from its paternal parent N. tomentosiformis. The basic chromosome number of tobacco(n=12) as of many other solanaceous species such as tomato, tobacco, pepper and egg plant which are known for a nona-isoprenol, Solanesol (C45H74O). Approx.>70% of the genome is said to contain the repeat regions. So, an effort was made to analyse the Nicotianasps which are rich sources of Solanesol. In the present study Tobacco Microsatellite TbM36 is said to have a maximum Polymorphism Information Content (PIC) value 0.374167 and TbM56 showing a minimum PIC value of 0.278961. D value of TbM12 is 0.951582, value being highest and nearing to 1 evaluates the efficiency of microsatellite in identification of Tobacco accessions. TbM30 is found to be best for DH Line3 derived from a cross between GT7 and Kumkumatri Mathri. Jaccard Similarity value 0.1379 implies Candel and BY53 and also RIL Candel x Nisnicotinony121 and By53 are more diversified. With a least value 0.1000 Nis-nicotinony121 and BY53 are most diversified among the Experimental set of Germplasm.

Keywords: Solanesol, Tobacco Microsatellite Markers.

INTRODUCTION

Tobacco Microsatellites as other Simple Sequence repeats are DNA stretches consisting of short, tandemly repeated di-, tri-.tetra-, pent- or hexa- nucleotide motifs. These were developed by the Scientists of Central Tobacco Research Institute, India to find out which Simple Sequence Repeats can differentiate the Low Solanesol and High Solanesol Lines and assist in Marker assisted Breeding. Clones were generated from the genomic DNA of N. tabacum cultivar "JAYASRI" since HPLC analysis reveal significant presence of solanesol in comparison to other Nicotiana sps. All the microsatellites generated were linked to Chromosome 1 of Nicotiana sps.

Table1: Tobacco Microsatellites and their motifs

	Microsatellite	Clone	Genomic DNA(bp)	repeat region	repeat type	motif
1	TbM12	"B9-PL-2"	1..649	477..520	Tandem	$(tc)_{22}$
2	TbM30	"A9-PL-4"	1..564	234..269	Tandem	$(ggagaa)_6$
3	TbM31	"C2-PL-4"	1..548	387..423	Tandem	$(ag)_{13}$
4	TbM56				Tandem	$(gt)_6$
5	TbM36	"C8-PL-4"	1..493	270..293	Tandem	$(gaa)_8$

MATERIALS AND METHODS

Plant material: It was chosen from the experimental Farm of Central Tobacco Research Institute at Katheru, Rajamahendravaram, INDIA grown during the Rabi Season. Mature leaves were collected from the parents, RIL's (Recombinant Inbred Lines)as well as Di-Haploid Lines bred for the enhancement in the production of Solanesol.

HPLC Analysis: Separation was carried out on Kromasil C18 column with an isocratic 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 isocratic conditions using a flow rate of 1.0ml/min. at (500C). 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 2: Experimental Set of Germplasm selected 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
Population2	RIL	HDBRGxGT-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

Extraction of 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.[1]. The quality and quantity of DNA were measured using Nanodrop Spectrophotometer.

Amplification of genomic DNA: From 70N.tabacum microsatellite sequencesidentified from Chromosome 1 of N.tabacum "JAYASRI" variety Primers were designed by Primer3 software and few were used in present study after screening.

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

	N.tabacum Microsatellites from which Oligos were designed	OLIGO NAME	OLIGO SEQUENCE (5^1…..3^1)	Temp. Given	Temp. done
1	>DQ865416|DQ865416.1 Nicotiana tabacum microsatellite A12 sequence	TbM12-F	ATTGTCTGTCTTGTACAGTCTTTGG	63.0^{0}C	55.0^{0}C
		TbM12-F	CCATTCTCCCAGAATAGCTCTAAGT	64.0^{0}C	
2	>DQ865434|DQ865434.1 Nicotiana tabacum microsatellite A30 sequence	TbM30-F	AGAGGAAGAGTAGAGATCGGGATAG	63.0^{0}C	55.0^{0}C
		TbM30-R	AAGAGTGTTGTCACCTGCTGTCT	64.2^{0}C	
3	>DQ865435|DQ865435.1 Nicotiana tabacum microsatellite A31 sequence	TbM31-F	GACACAGTATGAGATGGGATTTTCT	63.2^{0}C	55.0^{0}C
		TbM31-R	ATGTCGACAACCTCATCAAAAGTAG	64.1^{0}C	
4	>EF375958|EF375958.1 Nicotiana tabacum microsatellite A36 sequence	TbM36-F	ATGTCGGTATCAGCACTTTTGAC	64.0^{0}C	57.0^{0}C
		TbM36-R	TATTCTAACTCCTCGACCATTGACT	62.0^{0}C	
5	>EF375978|EF375978.1 Nicotiana tabacum microsatellite A56 sequence	TbM56-F	GTGACTCAGAAGCCCAGATTATCC	65.9^{0}C	57.0^{0}C
		TbM56-R	TCCTTCACTTCCCTATCTTCTAACC	63,5^{0}C	

Reaction mixture of 25µL was prepared taking 2µL of Genomic DNA (30ng/µL);1µL of 0.2µMPrimer (Forward); 1µL of 0.2µM Primer(Reverse); 2µL of 2mM dNTP's; 0.3µl Taq DNA Polymerase; 0.5µL of 25mM MgCl2; 2.5µl Taq BufferE (1x) and 15.7µl of PCR water and gently mixed all the contents. The reaction mixture was placed in chill conditions to prevent unusual reaction before placing in Thermo-Cycler.

Amplification of Genomic DNA taken as Template was done in Eppendorf master cycler®X50in 3steps of 35 cycles.Step1: An Initial Denaturation at 940C for 5 min. and Denaturation at 940C for 1min., Step2: Annealing at 550C for 1min. (set as per given Tm). Step3: An Extension of 720C for 1min. and a Final Extension at 720C for 10min. Poly acrylamide Gel electrophoresis was run and later 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 analysed with program Image J.

Calculation of the Polymorphism Statistics:

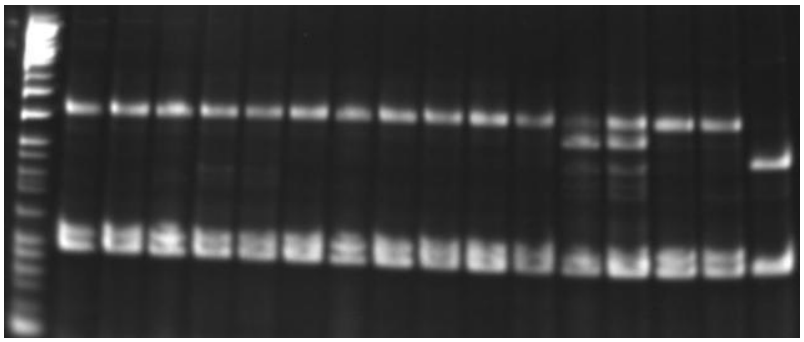
For the calculation of Polymorphism statistics web application iMEC (Online Marker Efficiency Calculator) was used. It is available at https://irscope.shinyapps.io/iMEC/.Input data was given in Binary form coded (0, 1) where '0' is for absence of the band and '1' for the presence of band. Calculated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

Data analysis for assessing the Genetic Diversity: Data analysed by a statistical package NTSYS (pc version 2.2w). 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. Using SHAN(Sequential Agglomerative Heirarchial and Nested Clustering),a sub program cluster analysis was done.By UPGMA (Un-weighted Pair Group method with arithmetic method) a Phylogenetic tree was generated.

RESULTS AND DISCUSSION

The Amplicon profiles of the Tobacco microsatellites found to be polymorphic were considered for diversity analysis and which were found to be monomorphic were not given into account.[2],[3].

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16



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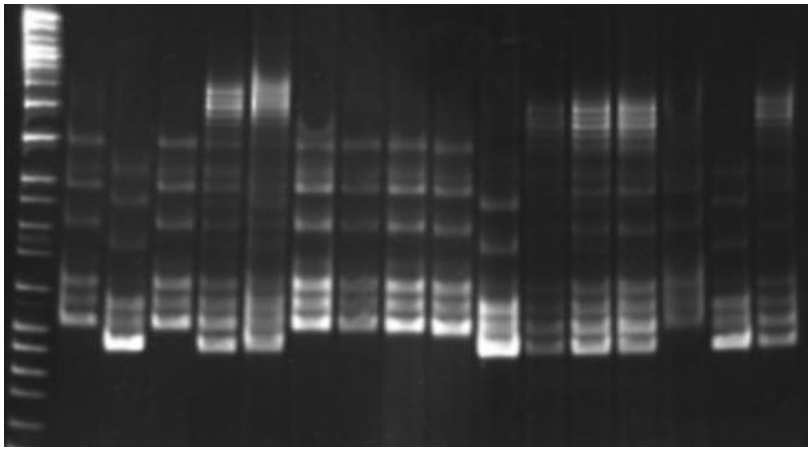

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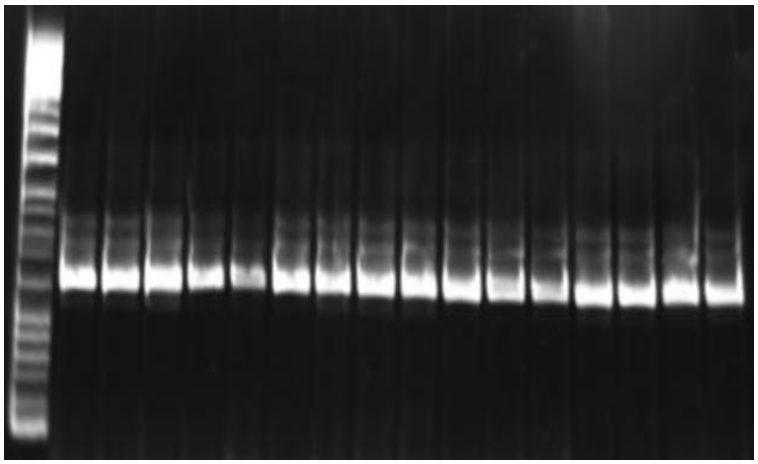

Figs 1, 2, 3: Page of Tbm30, Tbm36, Tbm56: M. Marker 20bp 1. Hdbrg, 2. By 53, 3. Hdbrgxby53, 4. Low Solanesol Bulk, 5. High Solanesol Bulk, 6. Gt-7, 7. Hdbrg, 8. Hdbrgxgt7, 9. Candel, 10. Nis-Nicotinony-121, 11. Candelxnis-Nicotinony121, 12. Low Nicotine Bulk, 13. High Nicotine Bulk, 14. Kumkumathri Mathri, 15. Nis- Nicotynony121 X Kumkumathri Mathri, 16. Dhline3 (Gt-7xnis-Nicotynony121)

Table 4: Polymorphism Statistics calculation by iMEC

	H_0	PIC_0	E_0	H_{avp} _0	MI_0	D_0	R_0
TbM12	0.34875	0.287937	1.125	0.004359	0.004904	0.951582	2.25
TBM30	0.48875	0.369312	4.25	0.003055	0.012982	0.820912	5.25
TBM36	0.498336	0.374167	6.875	0.002396	0.016471	0.721525	7.25
TbM56	0.300292	0.278961	1	0.300292	0.300292	0.067901	

Among the Microsatellite markers taken for study maximum PIC value of 0.374167 is shown by TbM36 and a minimum PIC value of 0.278961 is shown by TbM56. D parameter evaluates the efficiency of microsatellites in identification of Tobacco accessions. D value of TbM12 is 0.951582, (Value being highest and closest to 1) implies a lower probability of confusion between Tobacco accessions.

	HDBRG	BY-53	HDBRGxBY	LowSolan	HighSola
HDBRG	1.0000				
BY-53	0.7500	1.0000			
HDBRGxBY-53	0.6667	0.4783	1.0000		
LowSolanesolBulk	0.5200	0.3704	0.6087	1.0000	
HighSolanesolBulk	0.6667	0.4783	0.6190	0.5417	1.0000
GT7	0.5455	0.3750	0.5714	0.6364	0.5714
HDBRG-F	0.4800	0.4400	0.5000	0.5000	0.4400
HDBRGxGT-7	0.3846	0.3462	0.4583	0.4615	0.4000
Candel	0.2593	0.1379	0.3750	0.3846	0.2692
Nisnicotinony-121	0.2143	0.1000	0.2692	0.3333	0.2222
CandelxNisnicotynony	0.1724	0.1379	0.3200	0.2857	0.2222
LowNicotineBulk	0.1765	0.1471	0.2188	0.3125	0.2188
HighNicotineBulk	0.2500	0.2188	0.3000	0.3548	0.3000
Kumkumadrimatri	0.3333	0.2963	0.3462	0.3103	0.3462
Nisnicotinony121xKumkumadri	0.3333	0.2963	0.2963	0.2258	0.4000
DiHaploidLine(GT-7xKumkumadri)	0.2143	0.1786	0.2692	0.2000	0.2222

	GT7	HDBRG-F	HDBRGxGT	Candel	Nisnicot
GT7	1.0000				
HDBRG-F	0.5909	1.0000			
HDBRGxGT-7	0.6190	0.7619	1.0000		
Candel	0.5238	0.5217	0.6190	1.0000	
Nisnicotinony-121	0.4545	0.4583	0.5455	0.6842	1.0000
CandelxNisnicotynony	0.3333	0.4583	0.4783	0.4545	0.5238
LowNicotineBulk	0.2667	0.2813	0.3333	0.2667	0.3103
HighNicotineBulk	0.3571	0.4138	0.4815	0.2667	0.4074
Kumkumadrimatri	0.3600	0.6818	0.5000	0.3077	0.4783
Nisnicotinony121xKumkumadri	0.2143	0.3214	0.3846	0.1724	0.3077
DiHaploidLine(GT-7xKumkumadri)	0.2308	0.3462	0.4167	0.2800	0.3333

	CandelxN	LowNicot	HighNico	Kumkumad	Nisnicot
CandelxNisnicotynony	1.0000				
LowNicotineBulk	0.5200	1.0000			
HighNicotineBulk	0.5200	0.7600	1.0000		
Kumkumadrimatri	0.6190	0.3793	0.5385	1.0000	
Nisnicotinony121xKumkumadri	0.3600	0.4286	0.5385	0.5000	1.0000
DiHaploidLine(GT-7xKumkumadri)	0.2800	0.4074	0.4615	0.4167	0.4783

	DiHaploi
DiHaploidLine(GT-7xKumkumadri)	1.0000

Fig 4: Jaccard similarity coefficients among solanesol and Nicotine Lines of Tobacco

Jaccard Similarity value of 0.7619implies a RIL of Population 4 i.e., HDBRG x GT7 and HDBRG are most similar and awith a value of 0.7600 High Nicotine Bulk and Low Nicotine Bulk are more similar. Jaccard Similarity value 0.1379 implies Candel and BY53 and also RIL of population 9 i.e., Candel x Nisnicotinony121 and By53 are more diversified. With a least value 0.1000 Nisnicotinony121 and BY53 are most diversified.

Phylogram Construction: Phylogramis constructed using UPGMA cluster analysis depicting genetic relationships among the given tobacco lines are shown below. Two major clusters were obtained one being set of Germplasm rich in Solanesol and the other being rich in Solanesol. HDBRG is said to be rich in Solanesol as well as Nicotine.

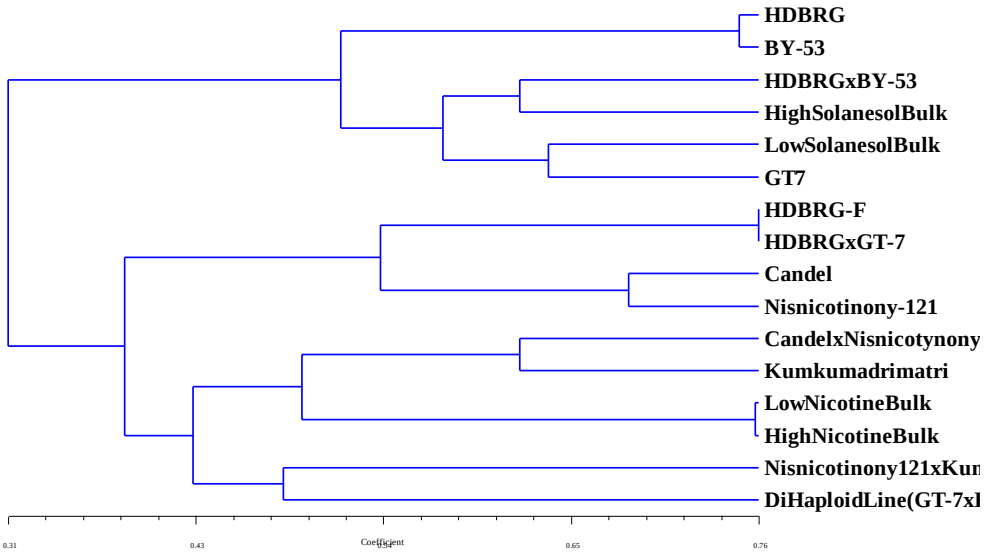

Fig. 5: Dendrogram depicting the Genetic diversity among Solanesol and Nicotine Lines.

CONCLUSION

Depending upon genetic similarity coefficients based on Jacquard's similarity coefficient it is known that more the value of Jaccard similarity coefficient more is the genetic similarity and less the value more are they diversified. Tobacco micro satellites can well be used as a versatile tool because of their high ability of showing diversity.

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