Molecular diversity in the genus *Nicotiana* as revealed by randomly amplified polymorphic DNA

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ABSTRACT

The genus *Nicotiana* consists of 64 recognized species of which, only two species, *tabacum* and *rustica* are cultivated extensively. Wild *Nicotiana* species are storehouses of genes for several diseases and pests, besides genes for several important phytochemicals and quality traits, which are not present in cultivated varieties. Randomly amplified polymorphic DNA (RAPD) analysis was used to determine the degree of genetic variation in the genus *Nicotiana* and to develop species specific markers. Twenty two species and two interspecific hybrids were analyzed by using 18 random decamer primers. Genetic polymorphism abounds among the wild species of genus *Nicotiana* (99.5 %) as evidenced by the high degree of polymorphism in RAPD profiles. The pairwise similarity measures in the species of subgenus *Rustica* was 0.252 whereas in the subgenus *Tabacum* was 0.189, suggesting that there was significant diversity among the species of these subgenera. In the species of subgenus *Petunioides*, the range of pairwise similarity measures was 0.128 to 0.941. The clustering pattern coincided with the traditional classification of *Nicotiana* species. All the primers generated specific bands in the various species. Thirty six species-specific markers identified in the present study will be useful in interspecific breeding programs. [Physiol. Mol. Biol. Plants 2008; 14(4) : 377-382] E-mail : rajuks2002@yahoo.co.in

Key words : *Nicotiana*, Genetic diversity, RAPD markers

The genus *Nicotiana* is one of the five major genera of the family *Solanaceae*. Of the total 64 species in the genus, 44 were indigenous to North or South America and 20 were native of Australia (Goodspeed, 1954). The genus is divided into three sub-genera viz. *Rustica*, *Tabacum* and *Petunioides* mainly based on morphology, crossability relationships, chromosomal number and behavior in interspecific hybrids. Although 6 chromosome paired species in *Nicotiana* were known, the predominance of 12 chromosome paired species indicates that 6 was the basic chromosome number for *Nicotiana* and both 12 paired, and 24 paired species were derived numbers. It was assumed that with a higher survival value, the allopolyploids have eliminated the older 6 paired types. The 9- and 10-paired species are considered to have evolved as a result of hybridization between the ancestral 6-paired and the present day 12-paired members. The 24-paired species, including *N. tabacum* and *N. rustica* are modern descendants of the 12 paired progenitors entered into amphidiploid origin.

Molecular techniques can play a major role in confirmation of the conventional classification of the genus. Two species *N. tabacum* and *N. rustica*, which are natural amphidiploids ($2n=48$) are grown commercially in the world. Although tropical in origin, tobacco can now be found growing from about 60 °N to 45 °S (Akehurst, 1981). Because of its economic importance and value as a biological research tool, numerous investigations have been undertaken in tobacco to examine its evolutionary and genomic structure and organization. Wild *Nicotiana* species are store houses of genes for several diseases and pests, besides genes for several important quality traits and phytochemicals, which are not present in cultivated varieties. In India, Central Tobacco Research Institute (CTRI), Rajahmundry is actively engaged in acquisition, maintenance and evaluation of *Nicotiana* species. The wild species of *Nicotiana* are being used in interspecific hybridization programs at CTRI for transfer of aphid and caterpillar resistance from *N. gossei*, *N. repanda*, *N. umbratica-nesophila*, *N. benthamiana-repanda*, black shank resistance from *N. plumbaginifolia* and root knot

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resistance from *N. longiflora* to the commercial varieties of *N. tabacum*.

Molecular genetic markers have become useful tools to provide a relatively unbiased estimation of genetic diversity and phylogeny in plants (Clegg, 1990). PCR based markers like Randomly amplified polymorphic DNA (RAPD) are being used in the analysis of genetic diversity in crop plants because of the relative ease with which PCR assays can be carried out compared to the other markers. Besides, prior knowledge about the genome is also not a pre-requisite, which makes RAPD a common method for such studies in different crops. In tobacco, RAPD has been used mainly to identify markers linked to genes for resistance to pathogens (Bai *et al.*, 1995; Rufty *et al.*, 1997; Yi *et al.*, 1998). RAPD procedure was developed for tobacco by using 12 species of *Nicotiana* and 12 varieties of *N. tabacum* using 3 random primers (Del Piano *et al.*, 2000). Evolutionary relationship among seven species of *Nicotiana* was analyzed by Amplified fragment length polymorphism (AFLP) markers was reported earlier (Ren and Timko, 2002). However, there were no comprehensive reports on molecular evolutionary relationships among all the major species of genus *Nicotiana*, which will be helpful in understanding the genetic diversity among the species as well as the derived species-specific markers will be useful in the identification of introgressed interspecific derivatives. This paper reports the results of a study on the genetic diversity based on RAPD among twenty two wild species representing three sub genera and two interspecific hybrids, which have been used as donors of biotic stress tolerance genes.

MATERIALS AND METHODS

Plant material

Seeds of 22 species of genus *Nicotiana* and 2 interspecific hybrids were obtained from Central Tobacco Research Institute (CTRI), Rajahmundry, India (Table 1).

DNA extraction and RAPD assay

Thirty day old seedlings of each variety were collected, bulked, frozen in liquid nitrogen, and stored at -80 °C until they were used for DNA extraction. DNA was extracted from pulverized frozen seedlings (Doyle and Doyle, 1990) and quantified on 0.8 % agarose gel. The following PCR amplification (Williams *et al.*, 1990) conditions were followed with minor modifications. 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 20 ng of each primer. Sixty random primers (Operon Technologies, Alameda, CA, USA) were used for amplification. PCR cycles consisted of one cycle at 94 °C for 1 min, 37 °C for 1 min and 72 °C for 2 min followed by 44 cycles at 94 °C for 1 min, 37 °C for 1 min and 72 °C for 2 min. Final primer extension for 7 min was carried out at 72 °C. The amplification product was electrophoresed on 1.2 % agarose gel in 1x TBE buffer and stained using ethidium bromide. The size of the fragments was estimated using Gene Ruler 100 bp DNA ladder plus (MBI Fermentas, Lithuania) marker.

Data analysis

Clearly resolved bands were scored for presence (1) or absence (0). The data on 439 bands generated by 40 primers were selected for the analysis of genetic diversity. The NTSYS-pc software version 2.02 (Rohlf, 1998) was used to calculate similarity coefficients (Jaccard, 1908). Based on UPGMA and SAHN clustering, a dendrogram depicting the genetic relationship among the varieties was prepared. Mean similarity of individual species with the rest and among the species within a particular cluster was computed from the similarity matrix table. Polymorphism information content (PIC) was calculated using formula $PIC = 2 \times P_i Q_j$, where P_i is the frequency of the presence of band and Q_j is the frequency of its absence (Botstein *et al.*, 1980). PIC values for all the polymorphic fragments for a primer were averaged to provide PIC value for a primer.

RESULTS AND DISCUSSION

Level of polymorphism

Twenty eight random decamer primers were used in this study, out of which 18 were chosen for analysis based on clear and well resolved RAPD pattern. The number of fragments amplified by each primer ranged between 9-17 with an average of 12.7 fragments/primer across all three subgenera of genus *Nicotiana*. The fragment polymorphism was higher than that reported in case of egg plant (10.28 bands per primer), which also belongs to *Solanaeaceae* (Singh *et al.*, 2005). Maximum number of bands (17) was produced by the primer OPP18, whereas least number of bands (9) was produced by the primer OPP12 in the genus *Nicotiana*. A representative RAPD profile obtained with the primer OPAB1 and OPAC1 was given in Fig. 1.

The primers OPAC10 and OPAC1 produced maximum number of bands (8) in the subgenus *Rustica*, whereas

Table 1. Characters of the wild *Nicotiana* species used in the present study

Sub-genus	Section	Species	Somatic Chromosome no.	Resistance to diseases
<i>Rustica</i>	Paniculatae	<i>glauca</i>	24	PM, An, RV,TE
		<i>knightiana</i>	24	PM,RK,TE
<i>Tabacum</i>	Tomentosae	<i>glutinosa</i>	24	PM, TMV
	Genuinae	<i>CM12</i>	48	BS
<i>Petunioides</i>	Undulatae	<i>undulate</i>	24	RV,TE,TMV,WF
	Trigonophyllae	<i>palmeri</i>	24	An,PM
		<i>trigonophylla</i>	24	An,PM
	Alatae	<i>longiflora</i>	20	An,RR,RK,WF,PM
		<i>plumbagenifolia</i>	20	BS,PM,WF
	Repandae	<i>repanda</i>	48	An,CN,PM,RK,TMV
		<i>nisophila</i>	48	An
		<i>umbratica-nes.</i>	48	—
		<i>stocketonii</i>	48	—
	Nudicaules	<i>nudicaulis</i>	48	An,PM,RK,WF
	Suaveolentes	<i>benthamiana</i>	38	PM,TMV
		<i>benthamiana-repanda</i>	48	—
		<i>gossei</i>	36	RR,PM,TMV,TS
		<i>amplexicaulis</i>	36	BM,SP,PM,TMV
		<i>velutina</i>	32	—
		<i>hesperis</i>	42	BM
		<i>occidentales</i>	42	An,SP,PM
		<i>megalosiphon</i>	40	An,PM
		<i>excelsior</i>	38	PM
		<i>debneyi</i>	48	An,PR,BM,PM,RV
		<i>stenocarpa</i>	—	—

An : Anthracnose, BM : Blue mold, BS : Black shank, SP : Brown spot, Cn : Cyst nematode, TMV : Tobacco mosaic virus, PM : Powdery mildew, RK : Root –Knot nematode, RV : Rattle virus, WF : Wild fire

Fig. 1. RAPD profile showing the genetic polymorphism among the wild species of *Nicotiana* detected using OP primers OPAB1 OPAC1. Lane 1. *N. glauca*, 2. *N. knightiana*, 3. *N. glutinosa*, 4. Cultivar *CM12*, 5. *N. palmeri*, 6. *N. trigonophylla*, 7. *N. longiflora*, 8. *N. plumbaginifolia*, 9. *N. undulata*, 10. *N. repanda*, 11. *N. nesophila*, 12. *N. um-nes*, 13. *N. stocktonii*, 14. *N. nudicaulis*, 15. *N. benthamiana*, 16. *N. ben-rep*, 17. *N. gossei*, 18. *N. amplexicaulis*, 19. *N. velutina*, 20. *N. hesperis*, 21. *N. occidentalis*, 22. *N. megalosiphon*, 23. *N. excelsior* and 24. *N. debneyi*. M = DNA marker

Table 2. Analysis of polymorphism obtained with random primers among various species of the genus *Nicotiana*. (Figures in the parentheses represent % of polymorphism)

Primer	Genus <i>Nicotiana</i>		Sub genus <i>Rustica</i>		Sub genus <i>Tabacum</i>		Sub genus <i>Petunioides</i>	
	Total number of bands	Number of polymorphic bands	Total number of bands	Number of polymorphic bands	Total number of bands	Number of polymorphic bands	Total number of bands	Number of polymorphic bands
OPAB	15	15(100 %)	6	4(66.6 %)	4	2(50 %)	15	15(100 %)
OPAC1	14	14(100 %)	8	5(62.5)	7	7(100 %)	14	14(100 %)
OPC11	10	9(90 %)	4	1(25 %)	6	5(83.3 %)	9	7(77.7 %)
OPC12	14	14(100 %)	4	4(100 %)	4	3(75 %)	14	14(100 %)
OPC13	11	11(100 %)	4	4(100 %)	3	2(66.6 %)	11	11(100 %)
OPC15	11	11(100 %)	4	4(100 %)	6	2(33.3 %)	9	9(100 %)
OPC4	16	16(100 %)	7	6(85.7 %)	6	6(100 %)	15	15(100 %)
OPC8	13	13(100 %)	5	4(80 %)	4	3(75 %)	12	12(100 %)
OPAB12	15	15(100 %)	5	5(100 %)	2	2(100 %)	14	14(100 %)
OPAC2	9	9(100 %)	2	2(100 %)	3	2(66.6 %)	9	9(100 %)
OPAC10	14	14(100 %)	8	4(50 %)	6	5(83.3 %)	13	13(100 %)
OPD11	9	9(100 %)	4	4(100 %)	1	1(100 %)	7	7(100 %)
OPD18	13	13(100 %)	4	3(75 %)	4	3(75 %)	12	12(100 %)
OPC16	12	12(100 %)	7	6(85 %)	3	3(100 %)	12	12(100 %)
OPC18	13	13(100 %)	5	4(80 %)	4	3(75 %)	13	13(100 %)
OPC5	10	10(100 %)	6	5(83 %)	5	5(100 %)	9	9(100 %)
OPC14	13	13(100 %)	5	5(100 %)	6	5(83.3 %)	13	13(100 %)
OPD13	17	17(100 %)	7	1(14.2 %)	8	7(87.5 %)	16	16(100 %)

the primer OPD13 produced maximum number of bands (8 and 16) in *Tabacum* and *Petunioides* respectively (Table 2). The minimum number bands were observed with the primers OPAC2, OPC5 and OPD11 (2, 1 and 7) in the subgenera *Rustica*, *Tabacum* and *Petunioides* respectively. The number of fragments amplified in *N. tabacum* was significantly higher than that reported earlier (Del piano *et al.*, 2000).

All the primers except OPC11 were polymorphic among the genus *Nicotiana*. Seventeen, six, and five primers among the 18 tested, showed complete polymorphism in the subgenera *Petunioides*, *Rustica* and *Tabacum* respectively. The PIC scores per primer across the genus ranged from 0.134–0.366 with an average of 0.274. The primer OPC16 gave highest PIC (0.366) and the primer OPC11 gave the lowest PIC (0.134). Six primers gave PIC value more than 0.3 whereas 12 primers showed PIC value more than the average PIC. The high PIC primers will be highly useful in further characterization of *Nicotiana* species.

A total of 229 bands were amplified of which 228 (99.5 %) were polymorphic across the genus *Nicotiana*. The level of polymorphism among the species of the subgenus *Rustica* and *Tabacum* was 74.73 % and 76.82 % whereas in the subgenus *Petunioides*, much higher level of polymorphism (99.07 %) was observed. The subgenus *Petunioides* was one of the largest genera, which contains species having chromosome number between 20 and 48. High degree of genetic polymorphism among seven species of *Nicotiana* was also reported by using AFLP (Ren and Timko, 2002). In contrast, genetic diversity analysis of the genus *Lycopersicon* by RAPD markers revealed 79 % polymorphism in *Lycopersicon peruvianum* and 9 % in *Lycopersicon parviflorum* (Kochieva *et al.*, 2002).

Species specific markers

A total of 39 species-specific markers were generated, of which, 6 were specific to subgenus *Rustica*, 2 to subgenus *Tabacum*, and 31 to subgenus *petunioides*.

Many wild species carry useful genes including resistance for tobacco mosaic virus (TMV), powdery mildew, blue mold and aphid. Since some of the species were crossable with cultivated species, the target genes are being transferred to desirable agronomic base through backcross breeding. The identification of interspecific hybrids and assessment of gene introgression have been based on phenotype, that was highly subjective. The species-specific markers identified in this study would aid unambiguous identification of the true hybrids, monitoring introgression of the target gene(s) and estimation of the genetic background of the desirable segregants, particularly with regard to the genomic contribution of the wild species.

Among 22 species, 19 species were clearly differentiated by producing one or two specific bands with all the 18 primers used in this present study (Table 2). The primer OPAC2 gave two specific bands to three species namely; *N. trigonophylla*, *N. benthamiana* and *N. repanda* (Table 2). Species-specific RAPD patterns has been developed and used to confirm hybridity in potato (Takemori *et al.*, 1994) and intergeneric hybrids of *Sacharum* and *Eraihthus* (Nair *et al.*, 2002).

Genetic relationship in the genus *Nicotiana*

The genetic similarity among the three subgenera of *Nicotiana* was low (19.2 %). However, the percentage of bands shared between subgenus *Tabacum* and *Rustica* was more (43.9 %) which shows the evolutionary closeness of these two subgenera. Sequence analysis of multiple nuclear fragments from such potentially conserved regions of the genome would be useful for understanding the phylogenetic relationship and characterizing the pattern of divergence. Goodspeed (1954) has postulated that the present day assemblage of species is derived from a pregeneric genetic reservoir with three major components that have been designated pre-*Nicotiana*, pre-*Cestrum* and pre-*Petunia*. The *Cestroid* complex was thought to be ancestral to the subgenera *Tabacum* and *Rustica* where as Petunioid complex ancestral to the subgenus *Petunioides*. Grouping of these species was clearly in accordance with the earlier classification based on the traditional analysis of cytological and morphological characteristics. The subgenera *Tabacum* shared 34.10 % of amplified fragments with *Petunioides* whereas *Rustica* shared more (39.63 %) amplified fragments. The subgenus *Petunioides* was close to the subgenus *Rustica* because the pre *Rustica* (pre subgeneric component) contributed to the evolution of species from pre *Petunioides* (pre subgeneric component).

RAPD based genetic relationship

All the Species of genus of *Nicotiana* were separated into five main clusters (1-5) based on subgenera specificity and number of chromosome (Fig. 2). The pairwise similarity measures in the species of subgenus *Rustica* was 0.252 whereas in the subgenus *Tabacum* was 0.189, suggesting that there exists significant diversity among the species of these subgenera. In the species of subgenus *Petunioides*, the range of pairwise similarity measures was 0.128 to 0.941, which was due to the high genetic similarity among the species belongs to the sections of *Rependae* and *Trigonophyllae*. The three species of *Rependae* and two species of *Trigonophyllae* are very closely related even in morphological as well as cytological characters (Goodspeed, 1954).

Subgrouping of the species within in the main clusters was largely based on the chromosome number and sections of the traditional classification to which they belong. Two species of *Rustica* remained together in the cluster-1, both the species have equal chromosomal number (2n=24). In cluster -2, the cultivar of *N. tabacum*, CM-12 and inter specific hybrid *N. benthamiana-repanda* which have 48 chromosomes remained together, whereas the species *megalosiphon* (2n=40), which belongs to the subgenus *Petunioides* was independently linked to this group. The cluster 3 was divided into two sub-clusters (a and b) based on the sections of the traditional classification. The sub-cluster 3a formed with the species *repanda*, *nesophila*, and *stectonii* of section *Rependae*. The interspecific derivative *N. umbratica* × *N. nesophila* was also

Fig. 2. Dendrogram constructed using an UPGMA and SAHN algorithm from the Jaccard's similarity matrix data from all the marker profiles consisting of 229 polymorphic fragments, revealing genetic relationship among the species used in the study.

clustered in the same group, since *nesophila* was one of the parent to this inter specific hybrid. All of them have the same number of chromosomes ($2n=48$). The species *nudicalis* and *benthamiana* are formed a group and linked to the sub-cluster 3a. The sub-cluster 3b formed with 7 species of section *Suaveolentes*. The cluster 4 formed with the species of the section *Triganophyllae* and the species of section *Undulate* and all of these species have $2n=24$ chromosomes. The cluster 5 was formed by the two species *longiflora* and *plumbaginifolia* of section *Alatae*, these two species have $2n=20$ chromosomes. The species *glutinosa* of the section *Tomentosae* of subgenus *Tabacum* with 24 chromosomes was not linked to the any of the clusters. According to Goodspeed (1954), in the subgenus *Tabacum*, section *Tomentosae* consists of five species, of which *tomentosae*, *tomentosiformis* and *otophora* are considered as a core group and the other two species *glutinosa* and *setchellii* were distinct from the core group due to their morphological and cytological characters, which may be responsible for the *glutinosa* to cluster separately from the subgenus *tabacum*.

Grouping of species considered under this present study was in accordance with the traditional classification, which is mainly based on the chromosome number. Though the subgenus *petunoides* consists of species with varied number of chromosomes, they largely clustered based on the sections of the taxonomical classification. However, the RAPD analysis of *Carthamus* modified the existing taxonomic and karyotypic classification (Vilatersana *et al.*, 2005).

In conclusion, present study will be useful for establishing molecular phylogeny in the species of *Nicotiana*. RAPD markers were found to be effective in analyzing polymorphism at the subspecies level in *Nicotiana*. The species specific markers identified will be useful in inter specific breeding programs.

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