GENETIC AND EVOLUTIONARY RELATIONSHIP AMONG *NICOTIANA* SPECIES AS ELUCIDATED BY AFLP

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ABSTRACT

Twenty two species representing three subgenera of the genus Nicotiana and two interspecific hybrids were analysed by AFLP. A total of 554 bands were amplified by 6 primer pairs, of which 541 (97.65%) were polymorphic and 13 (2.34%) monomorphic. The primer combination E-AAC/M-CTG gave the highest polymorphic information content (PIC) across all species, whereas E-AAG/M-CAC gave the lowest PIC score. A total of 59 species specific markers were generated, of which 54, 4 and 1 were specific to subgenera Petunioides, Tabacum and Rustica respectively. The pair-wise similarity measure in the species of subgenus Rustica was 0.35 whereas it was 0.29-0.50 in subgenus Tabacum, suggesting that there was significant diversity among the species of these subgenera. In the species of subgenus Petunioides, the range of pair-wise similarity measure was 0.25 to 0.95. The high genetic similarity (95%) was due close relationship among the species belonging to the sections Rependae and Trigonophyllae. Sub-grouping of the species within the main clusters of dendrogram was largely based on the chromosome number. AFLP was found efficient in determining the extent of interspecific genetic diversity existing in genus Nicotiana. The subgenus and species specific AFLP markers identified in this study would be useful in introgression breeding programs of tobacco. N. tabacum shared 174/198 AFLP fragments with the two progenitor species, thus providing molecular evidence that these two wild species contributed genetic information to N. tabacum.

***Keywords**: AFLP, evolution, genetic diversity, Nicotiana species,*

INTRODUCTION

The genus *Nicotiana* is one of the five major genera of the family Solanaceae, consisting of 64 species (Goodspeed 1954). All the representative species are annual herbs and form a distinct polyploidy series. *N. tabacum* and *N. rustica* are the only two cultivated species in this genus and several commercial varieties of them are cultivated through out the world. The genus is divided into three sub-genera viz. *Rustica*, *Tabacum* and *Petunioides* mainly based on morphology, chromosomal pairing 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 (Goodspeed, 1954). It was assumed that with a higher survival value, the alloploids had masked the older 6 paired types. The 24-paired species including *N. tabacum* and *N. rustica* are modern descendents of the 12 paired progenitors with amphidiploid origin. Because of its economic importance and value as a biological research tool, numerous investigations have been undertaken in tobacco to examine its evolution, genomic structure and organization. Wild *Nicotiana* species are store houses of resistant genes for several diseases and pests, in addition to genes for several important quality traits and phytochemicals, which are not present in cultivated varieties. Molecular techniques could play a major role in confirmation of

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the conventional classification of the genus *Nicotiana.*

Nicotiana tabacum is a natural amphidiploid (2*n*=48) thought to have arisen by hybridization of wild progenitor species (Gerstel, 1963). Studies on karyotypes, chromosome pairing and segregation of phenotypic characteristics in hybrids derived by crossing various diploid species, suggested that *N. sylvestris* (subgenus *Petunioides*, section *Alatae*) was the contributor of the maternal genome and the paternal genome is coming from either *N. tomentosiformis* or *N. otophora* (subgenus *Tabacum*, section *Tomentosae*) (Godspeed, 1954). Many of the modern molecular studies supported *N. sylvestris* as the maternal parent (Shinshi *et al.*,; 1988, Sperisen *et al.,* 1991). But as the paternal parent, some studies proposed *N. tomentosiformis* (Komarnitsky *et al*., 1998; Lim *et al*., 2000a, 2000b) and others proposed an introgressed hybrid between *N. tomentosiformis* and *N. otophora* (Hashimoto *et al*., 1998; Riechers and Timko, 1999).

Species relationships in genus *Nicotiana* were largely confirmed with various molecular markers such as RFLP (Yang *et al*., 1992). RAPD (Yung and Tsai, 1997) and AFLP (Ren and Timko, 2001). However, these assessments are largely limited to a small set of different *Nicotiana* species. AFLP technique (Vos *et al*., 1995) is a robust, highly effective method of DNA finger printing that can be used to assess molecular genetic variability. The AFLP markers, although scored dominantly (*i.e.,* without distinction between homozygotes and heterozygotes) and not always homologus (Mechanda et al. 2004), have shown to be effective in detecting phylogenetic signals in many plant species (Koopman, 2005; Althoff *et al*., 2007), including *Nicotiana* species (Ren and Timko, 2002). However, there were no comprehensive reports on genetic relationships among all major species of genus Nicotiana. The objectives of this study were to assess the genetic diversity in 22 *Nicotiana* species and two interspecific hybrids using AFLP technique and to infer the genetic relationships among *Nicotiana* species.

MATERIALS AND METHODS

Plant material

Seeds of twenty two species representing three subgenera of the genus *Nicotiana* and two interspecific hybrids were obtained from the division of crop improvement, Central Tobacco Research Institute, Rajahmundry, Andhra Pradesh, India. Summary of this material presented in Table 01.

DNA isolation and AFLP analysis

Total genomic DNA was isolated (Doyle and Doyle, 1990) from thirty-day-old seedlings and quantity and concentration of the extracted DNA were estimated on 0.8% agarose gel using diluted uncut lambda DNA as the standard. AFLP fingerprints were generated based on the protocol (Vos *et al*., 1995) with slight modifications as described in the manufacturers protocol (Life Technologies, Inc.) with six primer pairs (E-AAG/M-CAC, E-AGC/M-CAG, E-AAG/M-CAA, E-AAG/M-CAA, E-AAC/M-CTG and E-ACT/M-CAG). Genomic DNA (250 ng) was restricted with *Eco*RI and *Mse*I (1.5 U/µl each) in a restriction buffer (50 mM Tris-HCl, pH 7.5, 50 mM Magnesium acetate, 250 mM Potassium acetate) in a total volume of 25 µl. *Mse*I and *Eco*RI adapters were subsequently ligated to digested DNA fragments. The adapter ligated DNA was pre-amplified using the following cyclic parameters; 20 cycles of 30 s at 94°C, 60 s at 72°C. The pre-amplified DNA was diluted with Tris-EDTA buffer (Tris 10 mM and EDTA 1 mM) in a ratio of 1:50 and was used as a template for the selective amplification using + 3 primers (*Eco*RI and *Mse*I). The *Eco*RI primers were labeled with γ ^{33}p ATP. The cycling parameters were: 1 cycle of 30 s at 94°C, 30 s at 65°C and 60 s at 72°C. The annealing temperature was lowered by 0.7^{o}C per cycle during the first 12 cycles followed by 23 cycles at 94°C for 30 s, 56°C for 30 s

and 72°C for 60 s. The samples were resolved on 5% polyacrylamide gel containing 7M urea, dried and autoradiographed (Sambrook *et al*., 1989). The size of the fragments was estimated using 20 bp DNA ladder (MBI Fermantas, Lithuania).

Data analysis

Clearly resolved AFLP fragments in the size range of 100-700 bp were scored for presence (1) or absence (0). The data on 554 fragments generated by the 6 pair-wise combinations of primers were used for the analysis of genetic diversity. The NTSYS pc software version 2.02 (Rohlf, 1998) was used to calculate similarity coefficients among species. Based on Unweighted Paired Group Method of Arithmetic mean (UPGMA) and SAHN clustering, a dendrogram depicting the genetic relationship among the species 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 used to identify primers that would distinguish species most efficiently using formula PIC = $2xP_iQ_j$, where P_i is the frequency of presence of band and Q_j is the frequency of absence of band (Botstein et al. 1980). PIC values for all the polymorphic fragments for a primer-pair were averaged to provide PIC value for primer-pair.

Table 01: Characters of the wild *Nicotiana* species used in the present study

Sub-genus	Section	Species	Somatic Chromosome no.
Rustica	Paniculatae	1. *gluaca*	24
		2. knightiana	24
Tabacum	Tomentosae	3. *glutinosa*	24
		4. *otophora.*	24
		5.tomentosiformis	24
	Genuinae	6. MC12	48
Petunioides	Undulatae	7. *undulata*	24
	Trigonophyllae	8. *palmeri*	24
		9. *trigonophylla*	24
	Alatae	10. *longiflora*	20
		11.*plumbaginifolia*	20
	Repandae	12. *repanda*	48
		13. *nesophila*	48
		14. *umbratica-nes.*	48
		15.*stocktonii*	48
	Nudicaules	16.*nudicaulis*	48
	Suaveolentes	17.*benthamiana*	38
		18. *benthamiana- repanda*	-
		19.*gossei*	36
		20 *amplexicaulis*	36
		21. *velutina*	32
		22. *hesperis*	42
		23. *occidentales*	42
		24. *megalosiphon*	40

RESULTS AND DISCUSSION

Level of polymorphism

AFLP analysis gave a large number of distinct fragments per primer-pair (Figure 01). All bands that could be reliably read within the size range of 100- 700 bp were treated as individual dominant loci. A total of 554 bands were obtained from genus *Nicotiana* with 6 primer pairs, of which 541 (97.65%) were polymorphic and 13 (2.34%) monomorphic (Table 02). The presence of 2.34% monomorphic bands indicated that these markers have remained conserved in evolution in these species. Sequence analysis of multiple nuclear fragments from such a potentially conserved regions of the genome would be useful for establishing phenetic trees and characterizing patterns of divergence. The number of fragments amplified by each primer ranged from 81 to 108 with an average of 92.33 fragments/primer combinations. The number of fragments/primer-pair obtained in the present study were similar to the AFLP fragments obtained in the cultivated tobacco (Siva Raju *et al*., 2008)

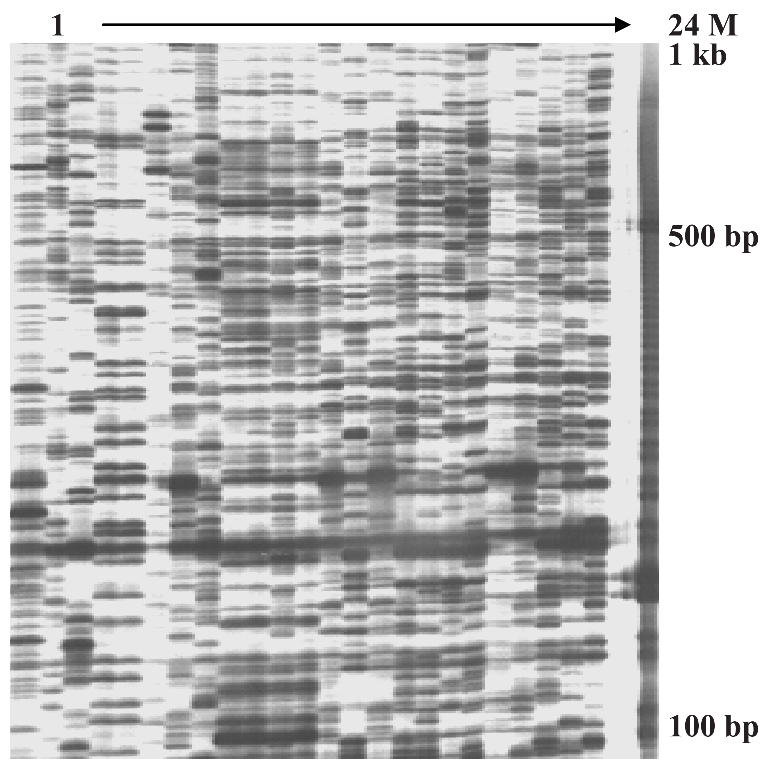

Figure 01: AFLP profile showing the genetic polymorphism detected among the wild species of Nicotiana using selective primers combinations EAAG-MCAA. The numbers1-24 above each lane corresponds to the species listed in the Table 01.

Table 02: Data on AFLP amplification

Primer	Total no. of bands	Polymorphic bands	% of Polymophism	PIC	EMR
E-AAG/M-CAC	108	105	97.22	0.544	101.85
E-AGC/M-CAG	80	78	97.5	0.581	75.66
E-AAG/M-CAA	106	105	99.05	0.589	103.95
E-AAG/M-CAA	90	87	96.66	0.589	103.95
E-AAC/M-CTG	89	88	98.87	0.606	86.94
E-ACT/M-CAG	81	78	96.29	0.595	56.028

PIC: Polymorphic information content; EMR: Effective multiplex ratio

The maximum number of fragments was amplified by the primer pairs E-AAG/M-CAC (108), E-AAG/M-CAA (106), whereas minimum was by the primer pair E-AGC/M-CAG (80). In species of subgenus *rustica*, a total of 275 fragments were amplified, of which 179 (65.09%) were polymorphic and 96 were monomorphic (34.90%). In the species of subgenus *Tabacum*, 443 fragments were amplified of which 352 (79.29%) were polymorphic and 92 were monomorphic. In the species of subgenus *Petunioides*, a total of 531 fragments were amplified, of which 517 (97.36%) were polymorphic. Maximum fragments were amplified by the primer combinations E-AAG/M-CAC in the species of *N. rustica* whereas the primer combination E-AAG/M-CAA generated maximum in the species of subgenus *Petunioides*. The primer combination E-ACT/M-CAG amplified minimum number of fragments in all subgenera. All the primer pairs showed high PIC values (0.544 to 0.606) which were a measure to know the usefulness of the primer for diversity analysis. The primer combination E-AAC/M-CTG gave the highest PIC across all species, whereas E-AAG/M-CAC gave the lowest PIC scores (Table 02).

Species specific markers

A total of 59 species specific markers were identified, of which 54 were specific to the subgenus *Petunioides*, 4 to subgenus *Tabacum* and 1 to subgenus *Rustica*. 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 had 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 segregates particularly with regard to the genomic contribution of the wild species.

AFLP based genetic relationship

The genetic similarity among the three subgenera of genus *Nicotiana* was low (0.25). However, the number of bands shared between subgenera *Tabacum* and *Rustica*, *Rustica* and *Petunioides*, and *Tabacum* and *Petunioides* were 231, 266 and 420 respectively. The subgenus *Rustica* shared more amplified fragments (49.90%) with subgenus *Petunioides* than with subgenus *Tabacum* (43.33%). The subgenus *Rustica* was close to the subgenus *Petunioides* because the pre-*Rustica* (pre-subgeneric component) contributed to the evolution of pre-*Petunioides* (pre-subgeneric component).

All the species of genus *Nicotiana* were separated into five major clusters (1-5) based on morphology and number of chromosomes (Figure 02). The pair-wise similarity measure in the species of subgenus *Rustica* was 0.35 whereas in the subgenus *Tabacum* was 0.29 to 0.50, suggesting that there was a significant diversity among the species. In the species of subgenus *Petunioides*, the range of pair-wise similarity measure was 0.25 to 0.95. The observed high genetic similarity (95%) was due to close relationship in morphological as well as cytological characters among the species belonging to the sections *Rependae* and *Trigonophyllae* (Goodspeed, 1954).

Sub-grouping of the species within the main clusters of dendrogram was largely based on the chromosome number. Two species of *Rustica* with 24 chromosome each remained together in the cluster -1. In cluster -2, the cultivar of *N. tabacum* (CM-12) with 48 chromosomes, species *N. otophora* and *N. tomentosiformis* each with 24 chromosomes were grouped together. The species *N. glutinosa* beongs to section *Tomentosae* of subgenus *Tabacum* with 24 chromosomes was not linked to cluster-2 along with other members of this section. According to Goodspeed (1954), the section *Tomentosae* consists of five species of which *tomentosa, tomentosiformis* and

otophora are considered as core group and the other two species *glutinosa* and *setchellii* were distinct due to their morphological (for example, inflorescence expression in section *Tomentosae* is thyrse except in *N. glutinosa*) and cytological characters. Thus *N. glutinosa* was a marginal species in section *Tomentosae* which could be the reason for the *glutinosa* to cluster separately from the subgenus *Tabacum* but the position of it was justified in the dendrogram by positioning just above the *Tabacum*. The third cluster was divided into two sub-clusters (3a and 3b) based on the sections of the traditional classification. The sub-cluster 3a consists of the species *N.* r*ependa*, *N. nesophila*, and *N. stocktonii* of section *Rependae*. The interspecific derivative *N. umbratica* x *N. nesophila* was also clustered in the same group since *nesophila* was one of the parent to this interspecific hybrid, and all of them had same number of chromosomes ($2n$=48). In the cluster 3b, species *N. occidentalis* and *N. megalosiphon* of section *Suaveolentes* were grouped together and the species *N. nudicaulis* of the section *Nudicaules* and interspecific hybrid *N. benthamiana* x *N. rependa* were independently linked to this group. The fourth cluster consists of two sub-clusters with the species of subgenus *Petunioides*. In the sub-cluster 4a, the species *N. gossei* and *N. amlplexicaulis* with 36 chromosomes in each were grouped together and the species *N. hesperis* (42 chromosomes) and *N. velutina* (32 chromosomes) were linked independently to this group. The sub-cluster 4b was formed by 2 species of section *Triganophyllae* and one species of section *Undulatae*, each with same number of chromosomes ($2n$=24). The species *N. benthamiana* with 38 chromosomes linked independently to the fourth cluster; this can be justified based on the correlations between numerical and morphological variations in the chromosomes was consistent throughout the section *Suaveolantes* of sub-genus *Petunioides* with the exception of 19 paired *N. benthamiana* (Goodspeed 1954). The fifth cluster was formed by the two species *N.longiflora* and *N. plumbagenifolia* of section *Alatae* each with $2n$=20 chromosomes. In the present study, the groupings of species were in accordance with the traditional classification, which was mainly based on the chromosome number. Though the subgenus *Petunioides* consists of species with varied number of chromosomes, they largely clustered in agreement with the traditional taxonomical classification.

Evolutionary relationship in Nicotiana

The amplified fragments from the combined AFLP profiles of tobacco cultivar (CM-12) and presumed paternal progenitor species of cultivated tobacco (*N. otophora* and *N. tomentosiformis*) were compared. A total of 398 amplified products were identified among the combined profiles of cultivated tobacco, *N. otophora* and *N. tomentosiformis*. Only 22 of total 198 amplified fragments in tobacco cultivar CM-12 were not found in any of the two species. In pair-wise comparison, 46.61% (124/266) of fragments present in *N. otophora* and 51.21% (147/289) of bands present in *N. tomentosiformis* were represented in tobacco cultivar profile. These data indicate that the two progenitor species contributed information to the evolution of the *N. tabacum* genome. As noted *N. otophora*, *N. tomentosiformis* and *N. tabacum* were clustered together. A significant amount of morphological and cytological information is available on the evolutionary relationship among the species of *Nicotiana*, a little information is available between cultivated *N. tabacum* and wild *Nicotiana* species. In the present AFLP study, with specific combinations of selective primers, it is possible to produce a sufficient number of polymorphic bands to infer meaningful comparison among the cultivated tobacco and species.

Twenty two species included in the present study were representatives of three subgenera,

Tabacum, *Rustica* and *Petunioides* of genus *Nicotiana*. When compared with the low level of genetic variation observed among the cultivated N. *tabacum* accessions (Siva Raju *et al.,* 2008), interspecific variations were significantly higher. The high degree of genetic polymorphism observed among the species in the present AFLP analysis was consistent with the results from the analysis of interspecific variations using RAPD (Bogani *et al*., 1997) and AFLP analysis in 8 *Nicotiana* species (Ren and Timko, 2001). In addition to detection of similar trends in interspecific variation, this approach gave very similar phylogenitic groupings which were consistent with the proposed clustering of species based on cytological and morphological characteristics (Narayan, 1987). For example, Goodpseed (1954) postulated that the present day assemblage of species was derived from a pregeneric genetic reservoir with three major components that had been designated pre-*Nicotiana*, pre-*Cestrum* and pre-*Petunia*. The *Cestroid* complex was thought to be ancestral to the subgenera *Tabacum* and *Rustica* whereas *Petunioid* complex ancestral to the subgenus *Petunioides*. Based on the genetic distance and dendrogram (Figure 02) generated by analysis of AFLP data, *N. tomentosiformis*, *N. otophora* and *N. tabacum* were clustered together. The AFLP markers present in all members of three subgenera, as well as markers associated with subgenera specific were identified. These subgenus-specific markers represent highly conserved sequences that have remained unaltered throughout the course of evolution and may be useful in subsequent taxonomic studies.

Based on cytological, morphological and growth characters, Gerstel (1963) proposed that *N. tabacum*, a natural allotetraploid originated by the synthesis of two ancestral genomes, *N. sylvestris* (2*n* = 24) and either *N. otophora* (2*n* = 24) or *N. tomentosiformis* (2*n* = 24). Various researchers based on molecular studies (Bland et al. 1985; Olmstead and Palmer 1991) proposed that a species similar to *N. sylvestris* donated the maternal genome of tobacco and *N. tomentosiformis* as paternal genome donor (Kuhrova et al. 1991; Volkov *et al*., 1999b; Lim *et al*., 2000b). Based on *in situ* hybridization studies of individual chromosomes in cultivated tobacco (Kenton et al. 1993) and analysis of the organization and structure of the gene family encoding putrescine-N-methyltransferase (Riechers and Timko, 1999), it was proposed that the paternal genome of tobacco may be derived from an introgressive hybrid between *N. tomentosiformis* and *N. otophora*. Earlier it was reported that there was no single copy of DNA divergence between *N. tabacum* and its diploid progenitors (Okamura and Golgberg 1985; Reaman - Buttner *et al*., 1999; Volkov *et al*., 1999a), but the AFLP profiles generated among *N. tabacum* and its two proposed paternal progenitor species *N. otophora* and *N. tomentosiformis* were highly informative. *N. tabacum* shared 114 AFLP fragments with both *N. otophora* and *N. tomentosiformis* and 174/198 fragments present in *N. tabacum* were represented in one of the two progenitor species, thus providing molecular evidence that these two wild species contributed genetic information to *N. tabacum* and this is inconsistent with the cytological information (Kenton *et al*., 1993). During the evolution, there is a possibility of loss or retention of ancestral alleles, since it was reported that the genomes of allotetraploids show a tendency towards rearrangement and loss of genetic information from one or both parental species (Volkov *et al*., 1999a)

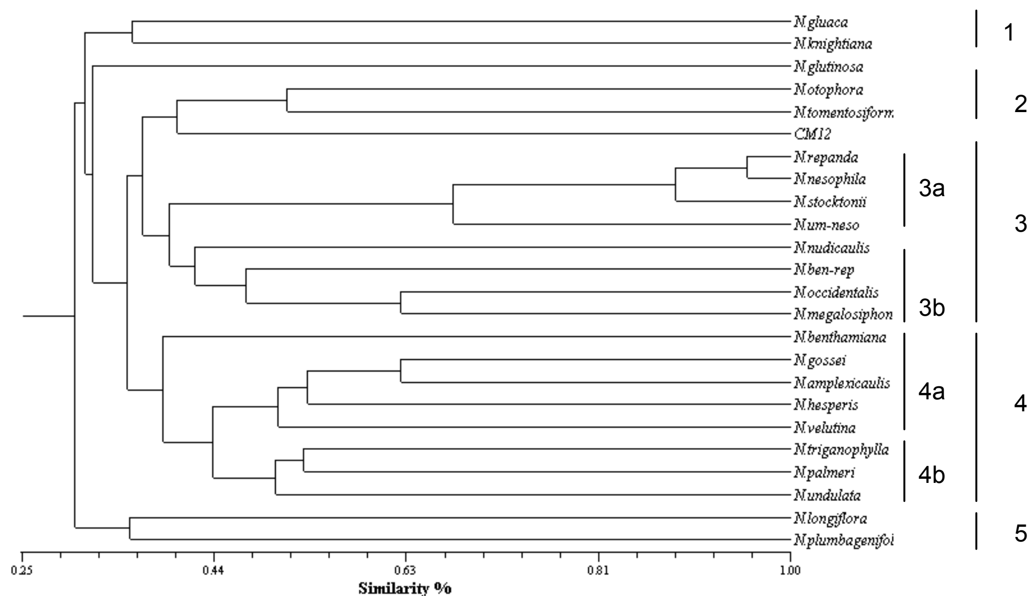

Figure 02: Dendrogram showing the phenetic relationships among 22 *Nicotiana* species and two interspecific hybrids

CONCLUSIONS

In the present studies, the AFLP analysis using 6 primer pair combinations showed sufficient resolution to distinguish among the closely related species of *Nicotiana* which allowed forming distinct sub-groups in consistence with the traditional classification and it would be useful for establishing molecular phylogeny in the species of *Nicotiana*. AFLP was also found efficient in determining the extent of interspecific genetic diversity existing in genus *Nicotiana*. The subgenus and species specific AFLP markers identified in this study would be useful in introgression breeding programs of tobacco.

ACKNOWLEDGEMENTS

The work was carried out at the National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi, under the National Agricultural Technology Project (NATP) of the Indian Council of Agricultural Research (ICAR), New Delhi. The authors are thankful to Dr. T. Mahopatra, Pr. Scientist and Dr. K. R. Koundal, Director, NRCPB, and New Delhi for giving facilities and to the Director, Central Tobacco Research Institute (CTRI), Rajahmundry, Andhra Pradesh, India for providing the experimental material.

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