African Journal of Biotechnology Vol. 6 (2), pp. 148-162, 18 January 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

Phylogenetic diversity and relationships among species of genus *Nicotiana* using RAPDs analysis

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Accepted 4 October, 2006

Fifty six *Nicotiana* species were used to construct phylogenetic trees and to asses the genetic relationships between them. Genetic distances estimated from RAPD analysis was used to construct phylogenetic trees using Phylogenetic Inference Package (PHYLIP). Since phylogenetic relationships estimated for closely related species are more reliable than that estimated for distantly related species, analyses were conducted separately for groups of taxonomically related species. The results of the present investigation are broadly similar to the inferences by Goodspeed (1954) on the divergence and evolution of *Nicotiana* species. As would be expected, species included in the same or related subgeneric sections showed greater genetic affinity than species included in distant subgeneric sections. Available information on the geographical distribution of species, their karyotype differentiation in evolution, genome size variation and chromosome affinity in interspecific hybrids were used to interpret the results. The study has confirmed that the RAPD analysis is suitable for studying phylogenetic relationships between related species.

Key words: *Nicotiana*, phylogenetic relationship, RAPD.

INTRODUCTION

Nicotiana is one of the 5 larger genera in *Solanaceae*. *Nicotiana* species show close genetic similarities to species of related genera *Cestrum* and *Petunia*. In describing the origin and evolution of this genus Goodspeed (1954) postulated that the three related genera have evolved from a pre generic gene pool with 3 major components pre-C*estrum,* pre-*Petunia* and pre-*Nicotiana*. He further postulated that pre-*Nicotiana* was composite in its genetic make up resembling pre-*Cestrum* and pre-*Petunia* giving rise to cestroid and petunioid ancestral complexes. Subgenera *Tabacum* and *Rustica* have descended from the cestroid complex and subgenus *Petunioides* from the petunioides complex. In terms of geographical distribution about 75% of the species are endemic to central and South America; the remainder are distributed naturally in Australia. The vast commercial interest in this genus is due to the Amphiploid species, *N. tabacum* and *N. rustica*; the two species are cultivated extensively in the tropical and subtropical regions.

The authoritative monograph of Goodspeed (1954) describes the phylogenetic relationships between *Nicotiana* species based on geographical distribution, chromosome morphology and the genetic affinity between species as revealed by chromosome pairing in interspecific hybrids. The genus was the basis for several classical studies in genetics such as amphiploidi in evolution, monosomic analysis and interspecific gene transfer. Fundamental studies on genetic tumours, tissue culture, radiation sensitivity, and quantitative inheritance (Mather, 1949; Anderson, 1949) were also reported in this genus.

Nicotiana has since become a model system to study the genetic, molecular and ecological factors that govern species differentiation in evolution (Narayan, 1987, 1988; Japan Tabacco Inc., 1994; Volkove et al., 1999; Lim et al., 2000; Chase et al., 2003). Among the 64 species of *Nicotiana*, 11 are amphidiploids (2n = 4x= 48). Subgenera *Rustica* and *Tabacum* have species with haploid chromosome numbers either 12 or 24. Species of subgenus *Petunioides* display, in addition, an aneuploid seriation of chromosome numbers n = 9, 10, 16, 18, 19, 20, 21

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and 22. It is estimated that the basic chromosome number for this genus is n = 6. Interspecific gene exchange between evolving species, amphiploidisation of interspecific hybrids and random genetic drift due to geographical isolation have played major roles in the evolution of this genus. From karyotype analyses and study of chromosome affinity in interspecific crosses (Goodspeed, 1954; Clausen et al., 1945) it was shown that phylogenetic differentiation was accompanied by significant changes in karyotype organisation in this genus; this is reflected in the large scale genome size variation between species. The range of DNA variation is fivefold between *Nicotiana* species. Among diploid species it varies from 2.6 pg in *N. trigonophylla* to 11.80 pg in *N. acaulis* both species with the same chromosome number (2n =24) Narayan (1987). DNA sequences encoding genetic information important to growth, development and reproductive fitness are conserved in evolution; much of the DNA changes implicated in genome evolution are due to changes in non coding repetitive sequences.

Molecular systematic studies in *Solanaceae* have concentrated mainly at the family level (Bohs and Olmstead, 1997; Olmstead et al., 1999). 5S ribosomal spacer sequences (Komarnitsky et al., 1998) and the *mtk* gene sequences (Aoki and Ito, 2000) were used for molecular phylogenetic studies of some *Nicotiana* species. Genomic *in situ* hybridization technique has been used to study genome relationship origin of hybrid taxa in evolution (Volkov et al., 1999; Lim et al., 2000).

Molecular phylogenetic analysis has not been used extensively in the genus as systematic tool to study species differentiation and evolution. Random amplified polymorphic DNA (RAPD) analysis is a useful molecular marker technique which uses polymerase chain reaction (William et al., 1990). The DNA sequence polymorphism as revealed in the electrophoretic profile results from genomic sequence differences in the primer binding sites. In interspecific or infraspecific comparisons they represent DNA sequence differentces that have occurred during evolution. Caetano-Anolles et al. (1991) have postulated that such polymorphisms are phylogenetically conserved and individual specific. In other words, differences or similarities estimated from RAPD profiles are a measure of the phylogenetic distances between species or populations that are investigated. Demeke et al. (1992) have shown that taxonomical relationships inferred through RAPD analyses closely paralleled the relatedness established through the classical taxonomy of species. Ever since, this analytical method has been used extensively to compare interspecific and intraspecific affinities between species and plant populations (Tulsiram et al., 1992; Cai et al., 1994; Virik et al., 1995; De Loose et al., 1995; Gustin and Sandeson, 2001).

The present investigation aims to compare the DNA sequence polymorphism between species and phylogenetic groups by RAPD analysis. The phylogenetic relationships as established by Goodspeed (1954) and the large-scale variation in genome size during species differentiation provide the basis for the present molecular comparison. Results of molecular phylogenetic compareson together with the extensive morphological and cytotaxonomical data available would provide a greater understanding of the evolutionary processes underlying genome differentiation in *Nicotiana* and help solve several unresolved problems in the evolution of this genus.

MATERIALS AND METHODS

Plant material

The 56 *Nicotiana* species and their haploid chromosome numbers are listed in Table 1. The seeds obtained from Agricultural research station, Beltsville Maryland, USA were grown and maintained in the University of Wales, Aberystwyth. The experiment was conducted in two batches under identical experimental conditions. The first batch included 15 species from subgenera *Tabacum* and *Rustica* and 14 species from subgenus *Petunioides*. Subgeneric sections *Undulatae*, *Trigonophyllae* and *Alatae* are known to have close genetic affinity with species of subgenera *Tabacum* and *Rustica*. *N. undulata* of subgeneric section *Undulatae* and *N sylvestris* of section *Alatae* are the putative ancestors of amphiploids *N. rustica* and *N. tabacum*. Phylogenetic analysis was done separately for species included in subgenera *Tabacum, Rustica* and *Petunioides*.

The second batch included 29 species in subgenus *Petunioides*. The 29 species showed wider geographical distribution and numerical chromosomes variation. Species included in sections *Rapandae*, *Noctiflorae*, *Acuminatae*, *Bigelovianae* and *Nudicaules* have the same chromosome number 2n=24 or 4X= 48 and are native to America. Species included in section *Suaveolentes* are indigenous to Australia and show a seriation of diploid chromosome numbers. It is established that in RAPD analysis phylogenetic relationship is more reliable between closely related species than between species that are distantly related (Stammer et al., 1994). Phylogenetic analysis was carried out separately for the 17 species included in sugeneric sections (*Rapandae*, *Noctiflorae*, *Acuminatae*, *Bigelovianae* and *Nudicaules*) and 12 species included in subgeneric section Suaveolentes.

Surface sterilised seeds were grown in glass houses under axenic conditions. Young leaves and shoots were collected when plants were two weeks old. They were washed in sterile distilled water, dried and stored frozen in liquid nitrogen.

Random amplified polymorphic DNA (RAPD) analysis

The total genomic DNA was extracted using the method suggested by Saghai-Maroof et al. (1984). The purified DNA was measured by spectrophotometry. Four different 10- mer random primer sequences selected from preliminary experiments were used. They were, IM6 (5`-GGTACAGTCA-3`), IM5 (5`-GAGCTACTGT-3`), IM2 (5`-GGTATCCTCA-3`) and SB3 (5`-GGATCGAGCC-3`). In each reaction the DNA concentration was adjusted to 12.5 ng/µl and the concentration of random primer sequences to 10 picomoles/µl. The method used for PCR amplification was similar to that reported by William et al. (1990). The reaction mixture (final volume 50 µl) contained 5 µl 10X PCR buffer; 2.5 µl, 2 mM dNTPs mixture; 4 µl, 25 mM $MgCl_2$, 2 µl decanucleotide primer; 0.2 µl (5 units/µl) Taq

Table 1. Geographical distribution and haploid chromosome numbers of the 56 *Nicotiana* species used in the present investigation

Taxon	n	Geographical distribution
Subgenus *Rustica* (Don) Goodsp. **Section *Paniculatae*** Goodsp.		
N. knightiana Goodsp.	12	South Peru (Coast)
N. paniculata L.	12	W Peru
N. raimondii J.F.Macbr	12	Peru, Urubamba valley
N. cordifolia Phil.	12	Chile, Masafuera
N. benavidesii Goodspeed	12	Peru
N. solanifolia Walp	12	N Chile (Coast)
N. glauca Graham	12	NW and C Argentina
Section *Thyrsiflorae* Goodsp.		
N. thyrsiflora Bitter ex Goodsp.	12	Peru, Maranon valley
Section *Rusticae* Goodsp.		
N. rustica L.	24	SW Ecuador to Bolivia
Subgenus *Tabacum* (Don) Goodsp **Section *Tomentosae*** Goodsp.		
N. tomentosiformis Goodsp.	12	Bolivia
N. otophora Griseb..	12	C-S Bolivia, NW Argentina
N. tomentosa Ruin & Pavon	12	S and C Peru, W Bolivia
N. setchellii Goodsp.	12	N Peru (Cjhachapoyas)
N. glutinosa L	12	N, C Peru, S Ecuador
Section *Genuinae* Goodsp.		
N. tabacum L.	24	Cultivated
Subgenus *Petunioides* (Don)Goodsp **Section *Undulatae*** Goodsp**.**		
N. wigandioides Koch & Fintelm	12	Bolivia
N. undulata Ruin & Pavon	12	N Peru-NW Argentina
N. arentsii Goodsp.	24	SW Peru-NW Bolivia (Puno and La Paz)
Section *Trigonophyllae* Goodsp.		
N. trigonophylla Dunal	12	SW USA, Mexico
N. palmerii A. Gray	12	SW USA
Section *Alatae* Goodsp.		
N. alata Link &Otto	9	Uruguay-Brazil and Argentina
N. langsdorffii Weinm	9	Brazil-Uruguay-Argentina
N. bonariensis Lehm	9	SE Brazil, Argentina-Uruguay
N. forgetiana Hemsl	9	SE Brazil
N. longiflora Cav.	10	Uruguay-Brazil and Bolivia
N. plumbaginifolia Viv.	10	Andes-NW Argentina
N. sylvestris Speg & Comes	12	NW Argentina, Bolivia
Section *Repandae* Goodsp**.**		

Table 1. Contd.

N. repanda Willd.	24	Texas, Mexico, Cuba
N. stocktonii Brandegee	24	Mexico (Revillagigedo Isl.)
N. nesophila I.M.Johnst.	?	Mexico (Revillagigedo Isl.
Section *Notctiflorae* Goodsp.		
N. acaulis Speg.	12	Patagonia
N. noctiflora Hooker	12	N Argentina-NW Chile
N. petunioides (Griseb) Millan	12	W Argentina, N Chile
Section *Acuminatae* Goodsp.		
N. acuminata (Graham) Hooker	12	Chile, Andes of Argentina
N. pauciflora Remy	12	Coastal Chile
N. corymbosa Remy	12	Coastal ranges and Andes of Chile
N. miersii Remy	12	Chile
N. linearis Phil.	12	Argentina-Chile
N. attenuata Torr.ex S. Watson	12	W USA, Baja California
N. spegazzinii Millan	12	CE Argentina
Section *Bigelovianae* Goodsp.		
N. bigelovii (Torr) Wats.	24	W USA and adjacent Mexico
N. clevelandii A. Gray	24	Baja California, California and Arizona
Section *Nudicaules* Goodsp.		
N. nudicaulis S. Watson		NE Mexico
Section *Suaveolentes* Goodsp		
N. velutina H.-M. Wheeler	16	SE, C Australia
N. suaveolens Lehm.	16(32)	SE Australia
N. maritima H.-M. Wheeler	16	SE Australia
N. exigua H.-M. Wheeler	16	S Queensland
N. gossei Domin	18	C Australia
N. excelsior J.M.Black) J.M. Black	19	SW Australia
N. benthamiana Domin	19	NC and NW Australia
N. goodspeedii H.-M. Wheeler	20	S Australia
N. occidentalis H.-M. Wheeler	21	NW and S Australia
N. rotundifolia Lndil.	22	SW Australia
N. debneyi Domin	24	Coast E Australia, New Caledonia
N. fragrans Hooker	24	S Pacific
N. megalosiphon VanHuerck & MÜll.Arg.	20	E Australia

The haploid chromosome (n) and geographical distributions of species are from Goodspeed (1954), MerxmÜller and Buttler (1975), Purdie et al. (1982) and Japan Tobacco Inc. (1994).

polymerase (Advanced Biotechnology) and 2 µl genomic DNA. The final volume was made up with HPLC purified distilled water. 45 repeats of PCR amplifications were done in a Perkin Elmer-Cetus thermal cycler as follows: at 94°C for 1min, at 35°C for 2 min, at 72°C for 2 min and final extension at 72°C for 7 min. Amplified DNA sequences were separated in 2% agarose gels immersed in 1XTBE (Tris borate) buffer. After staining with ethidium bromide the sequences were photographed under 313 nm UV.

Analysis of RAPD data

The electrophoretic profiles of amplified sequences were analysed for the presence or absence of shared sequences. The number of shared fragments were counted and recorded for each species and for each primer. The banding pattern for the 56 species for each primer was compared and the number of common bands was recorded as a matrix. The total number of bands produced by each species for different primers was summed up. The number of bands common for a set of species for each primer was also added up. The proportion of shared bands (F) was calculated using the formula given by Nei and Li (1979). F = 2nXY/nX+nY, where nX is the number of common bands in species X and nY the number of common bands in species Y and and nXY, the number of common bands to both species. The F value was used to compute the genetic distances between species. The estimated genetic distances were used as the input data for the Phylogenetic Inference Package (PHYLIP) of Felenstein (1993). The Least Square method of Fitch and Margoliash (1967) was used to construct the phylogenetic tree.

RESULTS AND DISCUSSION

The 2C nuclear DNA amounts and karyotype arrangements of the species investigated are given in Table 2. The electrophoretic distribution of fragment lengths for primer sequences IM6, IM5, IM2 and SB3 showed significant differences. Most species however showed several DNA bands that are of similar molecular weights. In rare instances where species did not reveal resolvable amplified sequences, they were included in the analysis as missing values. The data for different species within each subgenus were combined and used to calculate the proportion of shared fragments (F). The phylogenetic inference package (PHYLIP) was used and the best fitting phylogenetic trees generated by the least-square method are shown in Figures, 1a, 1b and 1c. The genetic distances between different species are given on the branches of the phylogenetic tree.

Subgenus *Rustica*

The 9 species included in subgenus *Rustica* are classified into three subgeneric sections, *Paniculatae*, *Thyrsiflorae* and *Rusticae*. Eight species are diploids with 2n=24; *N. rustica* is an amphidiploid with 2n= 4X=48. The 9 species are assumed to have diverged from a common ancestral genetic source, the pre-Rustica aggregate (Goodspeed, 1954)

Section *Paniculatae*

Among the seven species included in this section five (*N. raimondii, N. cordifolia, N. benvidessii, N. knightiana,* and *N. paniculata*) are very similar in external morphology and have similar karyotype organisation. Goodspeed (1954) classified the five species together as the “core species” from which the marginal species *N. glauca* and *N. solanifolia* have evolved. *N. paniculata*, less specialised in external morphology and with a larger geographical distribution, is considered as the most primitive species in *Paniculatae.* In external morphology and karyotype arrangements *N. knightiana* is most closely related to *N. paniculata* than to any other species in this subgenus. The karyotypes of *N. raimondii* and *N. cordifolia* show close similarity to each other. Despite the differences between them the five core species form a closely related group.

The marginal species are significantly diverged from each other for most morphological characters. Moreover they differ from the core species in plant habits, shape of the inflorescence and karyotype organisation. *N. solanifolia* shows greater specialisation in morphological characters and in its greater adaptation to higher atmospheric humidity. The karyotype of this species is similar to that of the core species *N. raimondii* and *N. cordifolia*.

Section *Thyrsiflorae*

This section is monotypic; the single species *N. thyrsiflora* resembles, in external morphology, the amphidiploid *N. rustica*. Goodspeed (1954) has proposed that interspecific hybridisation with an ancestral species of *N. undulata* of section *Alatae* might be a possible factor in the evolution of this species. It bears varying degrees of similarity to members of section *Paniculatae*.

Section *Rusticae*

The single species included in this section *N. rustica,* is an amphidiploid with 2n= 4x=48 chromosomes. It is postulated to have originated by the doubling of the chromosome complement of an interspecific hybrid between the ancestral species of *N. paniculata* of section *Paniculatae* and of *N. undulata* of section *Alatae* (subgenus *Petunioides*). The karyotype of *N. rustica* resembles the combined karyotypes of present day *N. paniculata* and *N. undulata.* The nature of homeologous chromosome pairing in interspecific hybrids between *N. rustica* and its putative ancestors supports this hypothesis.

The phylogenetic tree for subgenus *Rustica* is shown in Figure 1a. *N. knightiana* that failed to amplify DNA sequences by PCR was not included in the analysis. The phylogenetic tree shows species relationships comparable to that previously deduced from morphological, cytotaxonomical and molecular studies. Karyotype comparison and the study of genome size variations in *Nicotiana* have shown that phylogenetic differentiation in this genus was accompanied by substantial differentiation in genome organisation (Narayan 1987, 1988). The “core

Table 2. Nuclear DNA amounts (2C) and karyotype arrangements of species in subgenera *Rustica*, *Tabacum* and *Petunioides* in *Nicotiana*.

Section/ species	Karyotype m sm st	2C nuclear DNA (pg)
Subgenus *Rustica*		
Section *Paniculatae*		
Core species		
N. knightiana	6 6	7.72 ± 0.17
N. paniculata	6 6	7.78 ± 0.13
N. raimondii	4 8	8.67 ± 0.09
N. cordifolia	4 8	8.93 ± 0.12
N. benavidesii	6 4 2	9.03 ± 0.18
Marginal species		
N. solanifolia	4 8	10.45 ± 0.16
N. glauca	1 1 10	10.65 ± 0.16
Section *Thyrsiflorae*		
N. thyrsiflora	12	7.49 ± 0.11
Section *Rusticae*		
N. rustica	12 12	14.57 ± 0.17
Subgenus *Tabacum*		
Section *Tomentosae*		
Core species		
N. tomentosiformis	7 5	5.66 ± 0.09
N. otophora	7 5	6.54 ± 0.09
N. tomentosa	3 4 5	6.58 ± 0.15
Marginal species		
N. glutinosa	5 7	7.72 ± 0.15
N. setchellii	7 5	4.38 ± 0.07
Section *Genuinae*		
N. tabacum	10 5 9	11.71 ± 0.21
Subgenus Petunioides		
Section *Undulatae*		
N. wigandioides	5 3 4	7.56 ± 0.11
N. undulata	6 6	6.46 ± 0 13
N. arentsii	11 9 4	12.18 ± 0.14
Section *Trigonophyllae*		
N. trigonophylla	4 5 3	2.86 ± 0.04
N. palmerii	4 5 3	4.76 ± 0.06
Section *Alatae*		
N. alata	4 2 3	4.87 ± 0.04
N. langsdorffii	2 4 3	8.68 ± 0.19
N. bonariensis	2 3 4	8.17 ± 0.07
N. longiflora	10	8.10 ± 0.15
N. forgetiana	4 2 3	6.41 ± 0.14
N. plumbaginifolia	10	8.29 ± 0.14
N. sylvestris	5 3 4	5.74 ± 0.08
Section *Repandae*		
N. stocktonii	10 11 3	5.58 ± 0.05
N. nesophila	10 11 3	10.98 ± 0.10

Table 2. Contd.

N. repanda	-	-	-	- -
Section *Notctiflorae*				
N. acaulis		12		11.80 ± 0.08
N. noctiflora		12		6.32 ± 0.10
N. petunioides	1	11		4.53 ± 0.05
Section *Acuminatae*				
N. acuminata	2	6	4	6.61 ± 0.05
N. pauciflora	2	6	4	6.16 ± 0.08
N. corymbosa	2	6	4	5.91 ± 0.07
N. miersii	2	6	4	5.74 ± 0.11
N. linearis	1	3	8	4.66 ± 0.09
Section *Bigelovianae*				
N. bigelovii	8	10	6	8.35 ± 0.07
N. clevelandii	8	10	6	8.10 ± 0.08
Section *Nudicaules*				
N. nudicaulis	5	12	7	6.14 ± 0.09
Section *Suaveolentes*				
N. suaveolens	8	4	4	12.74 ± 0.13
N. maritima	8	4	4	6.51 ± 0.12
N. exigua	6	5	5	6.00 ± 0.12
N. gossei	5	4	9	6.28 ± 0.10
N. excelsior	5	4	10	6.73 ± 0.07
N. benthamiana	1	4	14	6.40 ± 0.08
N. goodspeedii	6	4	10	7.80 ± 0.12
N. occidentalis	4	3	14	6.09 ± 0.07
N. rotundifolia	4	3	15	4.86 ± 0.08
N. debneyi	4	1	19	12.01 ± 0.11
N. fragrans	2	6	16	6.67 ± 0.12

2C nuclear DNA amounts are from Narayan (1987) and karyotypes from Goodspeed (1954), m = median, sm = submedian and st = subterminal chromosomes.

species" of section *Panicula-tae*, (*N. cordifolia, N. paniculata, N. benavidessi, N. raim-ondii* and *N. Knightiana*) share closely similar genome sizes with an average value of 8.42 pg. the marginal species *N. glauca* and *N. solanifolia* have larger genome sizes with a mean value of 10.5 pg. The two marginal species themselves are much diverged from each other. *N. glauca* has a geographical distribution quite different from that of all other species in subgenus *Rustica*. Its karyotype contrasts with the karyotypes of other diploid species (Table 2). In the phylogenetic tree the "core species" *N. cordifolia*, *N. paniculata*, *N. benavidessii* and *N. raimondii* show close genetic relationships to one another. The genetic distances between these species are smaller when compared to the genetic distances between other species in subgenus *Rustica*.

The two marginal species show much divergence between them as shown by the greater genetic distances in the phylogenetic tree. This is comparable with their diverged geographical distributions and the greater specialisations shown by *N. solanifolia* in morphological characters, particularly in its adaptation to environmental factors such as high humidity. The core species *N. cordifolia* and marginal species *N. solanifolia* show a closer genetic

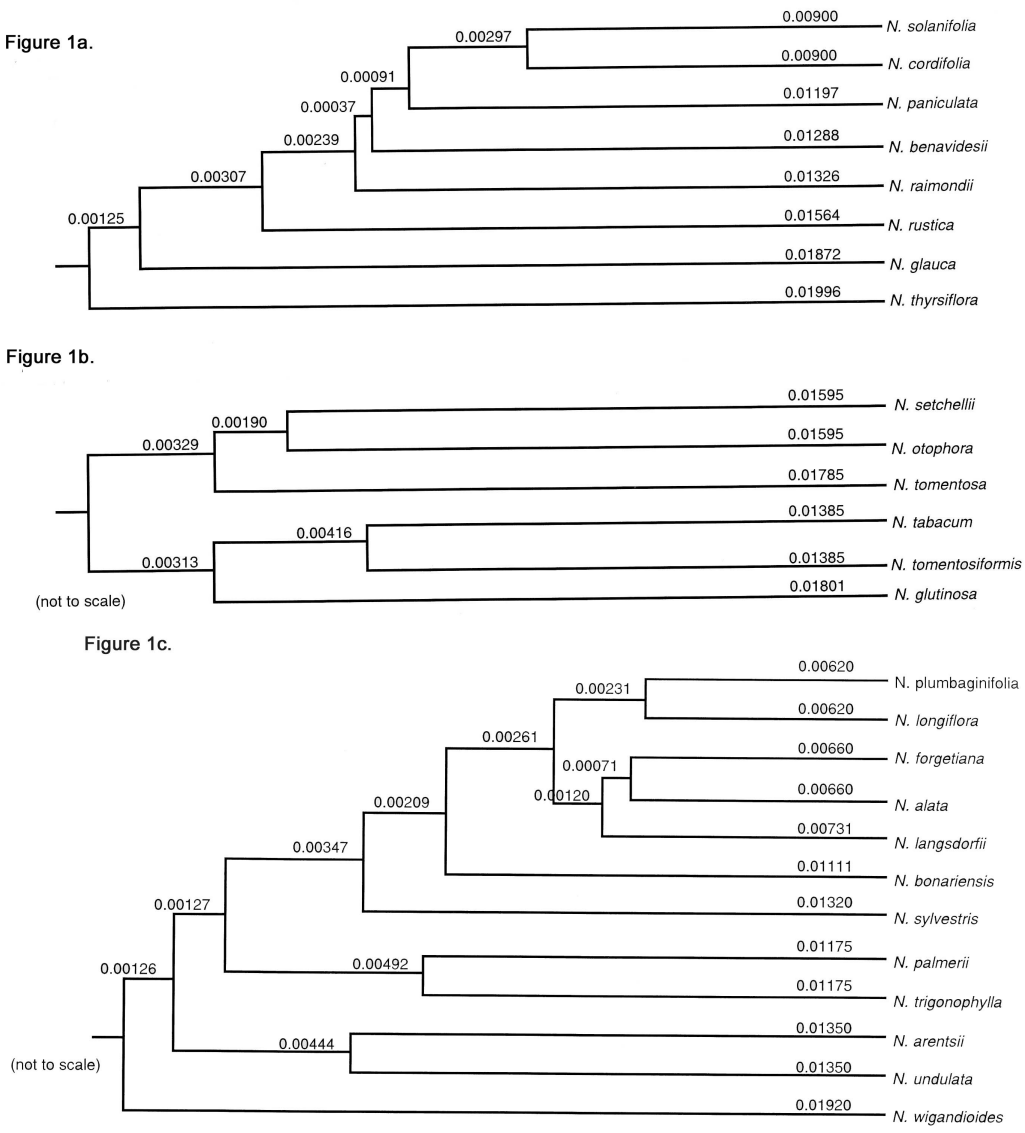

Figure 1. Phylogenetic trees of *Nicotiana* species included in the subgenera (**a**) *Rustica*, (**b**) *Tabacum* and (**c**) *Petunioides* (sections *Undulatae*, *Trigonophyllae*, and *Alatae*). Genetic distances are indicated on each arm of the tree.

relationship (Figure 1a). Although the two species are similar in their karyotypes (Goodspeed, 1954) heteromorphic bivalents in their F1 hybrids have suggested substantial divergence in genome organisation. Moreover there is significant genome size difference between the two species. All species included in a genus have descended from a common genetic stock. In the early stages of evolution of a genus genetic affinity between species was greater than it is now. Genetic exchange through inter-specific hybridization between the ancestral species of *N. cordifolia* and *N. solanifolia* might be a possible cause of the close genetic affinity between the two species. *N. thyrsiflora* a single species of section *Thyrsiflorae* shows greater genetic divergence from core and marginal species in the phylogenetic tree. The genetic affinity of *N. rustica* with the core species in the dendrogram supports the hypothesis that the amphiploid species is derived from the interspecific hybridisation between the putative ancestors of *N. paniculata* and *N. undulata* of subgenus Petunioides. Half the chromosome complement of *N. rustica* is homoeologous to the chromosome complement of *N. paniculata*.

Subgenus *Tabacum*

Five diploid (2n=24) and one amphiploid species (2n=48) make up this subgenus. All diplod species are included in section *Tomentosae* and the cultivated amphiploid species *N. tabacum* in section *Genuinae*.

Section *Tomentosae*

Based on external morphology karyotype arrangement and geographical distributions Goodspeed (1954) classified the five diploid species into "core" and "marginal" species. The core species *N. tomentosa*, *N. tomentosiformis* and *N. otophora* are closely similar to each other although the karyotype of *N. tomentosa* differs from the karyotypes of *N. otophora* and *N. tomentosiformis* (Table 2). Goodspeed (1954) views that *N. tomentosa* represented, more than other species, the primitive stock from which the section presumably arose. In the phylogenetic tree (Figure 1b) the core species *N. tomentosiformis* and the amphidiploid *N. tabacum* show close genetic relationship to one another. *N. tomentosa* is situated apart from the remaining core species of section *Tomentosae*.

The marginal species *N. setchelli* and *N. glutinosa*, are distinct from each other in external morphology, but they resemble the core species of *Tomentosae* in different characters to different degrees. They are separated from each other to a greater degree than other species in the dendrogram. As regards the origin of *N. setchelli,* Goodspeed (1954) has proposed that hybridisation between the ancestral species of *N. glutinosa* and an ancestor of *N. tomentosae* followed by reorganisation of the chromosome complement must be a possible evolutionary mechanism. But *N. setchelli* does not show any morphological or karyotype similarity to *N. glutinosa*. In the dendrogram (Figure 1b) it shows greater affinity to *N. otophora* than to *N. glutinosa*. Moreover the karyotype of *N. setchelli* is similar to the karyotype of *N. otophora*. It is quite likely that an ancestral species closely related to *N. otophora* might be involved in the evolution of *N. setchelli*.

Section *Genuinae*

This section contains the most widely cultivated amphiploid species *N. tabacum.* It is postulated to have evolved as an amphiploid derivative of the F_1 hybrid between the ancestor of *N. sylvestris* (subgenus *Petunioides*) and an ancestral species of section *Tomentosae.* The "S" genome of *N. tabacum* is derived from the ancestor of modern *N. sylvestris* and the "T" genome from the section *Tomentosae.* The karyotype arrangement of the "T" genome of *N. tabacum* closely resembles that of modern *N. otophora*. None the less, chromosome affinity in artificial polyploids combining *N. tabacum* and different core species of section *Tomentosae* suggests that *N. tomentosiformis* is the more likely progenitor of *N. tabacum* (Gerstel, 1960, 1966). Evidence in support of *N. tomentosiformis* has also come from isozyme analysis (Sheen, 1972) and electrofocussing of the fraction-1 proteins (Gray et al., 1974). The sum of the 2C DNA values of modern *N. sylvestris* and *N. tomentosiformis* is closer to the 2C DNA value of *N. tabacum* than are the sums obtained when *N. tomentosa* or *N. otophora* were used (Narayan, 1987, 1988). The "S" genome donor *N. sylvestris* bears strong resemblance to species of section *Tomentosae.* Goodspeed (1954) has postulated that tomentosoid elements were introgressed into the ancestral "S" genome through early interspecific hybridisations.

The close genetic affinity between *N. tomentosiformis* and *N. tabacum* in the dendogram (Figure 1b) is further proof that the "T" genome of *N. tabacum* presumably have originated from an ancestral species of *N. tomentosiformis*.

Subgenus *Petunioides*

Subgenus Petunioides contains the largest number of species among the three subgenera of *Nicotiana*. The 45 species in this subgenus, classified into nine subgeneric sections, show greater variation in chromosome number and chromosome morphology than species included in subgenera, *Rustica* and *Tabacum*. The twelve species included in sections *Undulatae*, *Trigonophyllae* and *Alatae* are taxonomically closely related to each other (Goodspeed, 1954). Ancestral species of these subgeneric sections have also provided the putative ancestors of amphiploids *N. rustica* and *N. tabacum.* The twelve species were included in the first batch of RAPD analysis as described under Material and Methods.

Section *Undulatae*

This section includes two diploid species *N. undulata* and *N. wigandioides* (2n=24) and an amphiploid species *N. arentsii* with 2n=4x = 48. *N. arentsii* is thought to have evolved by the doubling of the chromosome number of the F_1 hybrid between the two diploid species. The ancestry of *N. arentsii* is traced to the modern diploid species *N. undulata and N. wigandioides* rather than to their remote ancestors. The karyotypes of the present-day diploid species *N. undulata* (6 m+6 sm) and *N. wigandioides* (5m + 3sm + 4st) add up to the karyotype of *N. arentsii* (5m + 3sm + 4st). Further confirmation comes from the scheme of chromosome pairing in the F_1 hybrids between *N. arentsii* and the two diploid progenitors. A comparison of the genome sizes of the three species have shown that the 2C nuclear DNA amount of *N. arentsii* is not equal to the sum of the 2C DNA values of its diploid progenitors. Evolution of *N. arentsii* and the associated genome reorganisation appears to have involved a loss of about 1.84 pg of nuclear DNA (Narayan, 1987). Meiotic imbalance seen in the many polymorphic races of *N. arentsii* suggests genome imbalance and continuing karyotype rearrangements in the evolution of the species. Goodspeed (1954) has suggested that the many polymorphic races, given sufficient reproductive isolation, might evolve into new

species through gene mutations and karyotype reorganisations. In the RAPD generated phylogenetic tree (Figure 1c) *N. undulata*, *N. wigandioides* and *N. arentsii* form a closely related group. The close genetic affinity between the three species confirms the phylogenetic relationships as established from external morphology, karyotype arrangement, chromosome affinity in interspecific hybrids, and nuclear DNA amounts.

Section *Trigonophyllae*

Species ***N**. trigonophilla* and *N. palmerii* are the two species included in this section. Both species are diploids with the same chromosome number 2n = 24. The two species have the smallest chromosomes among diploid species of *Nicotiana* and their karyotypes are similar. The two species show similarities to members of most other sections of *Petunioides* and several morphological characters are in common with species of section *Undulatae*. The nature of chromosome pairing in the F_1 hybrids between species of sections *Tomentosae* and *Paniculatae* with species of section *Trigonopyllae* give further evidence to the substantial genetic relatedness between the three sections that are included in subgenera *Tabacum*, *Rustica* and *Petunioides respectively*. Based on this evidence, Goodspeed (1954) postulated that this section is of considerable age. The two species represent the most basic pattern of genome organisation in *Nicotiana. N. trigonophylla* has the smallest genome size (2C=2.86 pg) among *Nicotiana* species investigated. In the phylogenetic tree derived from RAPD analysis (Figure 1c) *N. trigonophylla* and *N. palmerii* show close genetic relatedness. Moreover, the species of section *Undulatae* show close genetic affinity to the two species.

Section *Alatae*

The seven species included in this section share several Petunioid morphological traits. Based on external morphology and changes in karyotype arrangement the seven species are classified into three groups. *N. alata*, *N. langsdorffi*, *N. forgetiana* and *N. bonariensis* share the same haploid number (n = 9) which is basic to the genus *Petunia.* Their karyotype arrangements and 2C nuclear DNA amounts are significantly different (Table 2) from other members of *Alatae*. *N. longiflora* and *N. pumbaginifolia* (n=10) form a second group in this section. The two species not only have identical karyotypes but also share similar 2C nuclear DNA amounts. The third group comprise a single species *N. sylvestris* with 12 pairs of chromosomes. As described earlier, an ancestor of *N. sylvestris* is considered to have provided the 'S'genome of *N. tabacum*. Its strong resemblance to species of section *Tomentosae* presumably is due to the intro duction of tomentosoid elements through interspecific hybridisation.

In the dendogram (Figure 1c) *N. forgetiana*, *N. alata*, *N. langsdorfii* and *N. bonariensis* show close genetic proximity. Among the four species however, two species *N. forgetiana* and *N. alata* show much closer genetic affinity than others. Their genome sizes (6.41 and 4.87 pg of 2C nuclear DNA respectively) are smaller than that of *N. bonariensis* and *N. langsdorfii* (mean 2C DNA=8.42 pg). In the dendogram *N. bonariensis* and *N. langsdorfii* are separated from the two related species *N. forgetiana* and *N. alata*. *N. plumbaginifolia* and *N. longiflora* with identical chromosome numbers (n = 10) show no karyotypic distinctions and have closely similar 2C nuclear DNA amounts (mean 2C DNA= 8.20 pg). Reciprocal chromosome exchanges, gene mutations and reproductive isolation must have driven their evolutionary divergence. The two species show close genetic affinity in the phylogenetic tree. *N. sylvestris* (n=12) has a narrow geographical distribution. The geographical races of this species show very little variation in external morphology. It has chromosome number and chromosome organisation different from that of other species included in section *Alatae*. The narrow geographical distribution must have promoted a greater genetic differentiation of this species from other species of section *Alatae*. In the phylogenetic tree *N. sylvestris* show significant genetic divergence from related species in *Alatae*. In general, species included in different subgeneric sections show close genetic relatedness in the dendrogram.

The remaining 29 species of subgenus *Petunioides* included 6 subgeneric sections *Repandae*, *Noctiflorae*, *Acuminatae*, *Bigelovianae*, *Nudicaules* and *Suaveolentes* (Table 1). They were analysed, as mentioned in material and methods, in the second batch of the experiment. Species included in sections *Repandae*, *Noctiflorae*, *Acuminatae*, *Bigelovianae* and *Nudicaules* have chromosome numbers either 2n = 24 or 2n= 4X= 48. They are native to central and south Americas. Species included in section *Suaveolentes* are indigenous to Australia and show a seriation of diploid chromosome numbers. On the basis of these fundamental differences phylogenetic analyses were carried out separately for the 17 species included in subgeneric sections *Repandae*, *Noctiflorae*, *Acuminatae*, *Bigelovianae* and *Nudicaules* and 12 species included in subgeneric section *Suaveolentes.*

Section *Repandae*

Three species included in this section *N. repanda*, *N. nesophila* and *N. stocktonii* are amphidiploids (2n = 4X = 48). Goodspeed (1954) has proposed that these species might be derivatives of a common amphidiploid ancestor although their genetic backgrounds are not well defined. *N. stocktonii* and *N. nesophila* have similar karyotypes and external morphology. But they

Figure 2a.

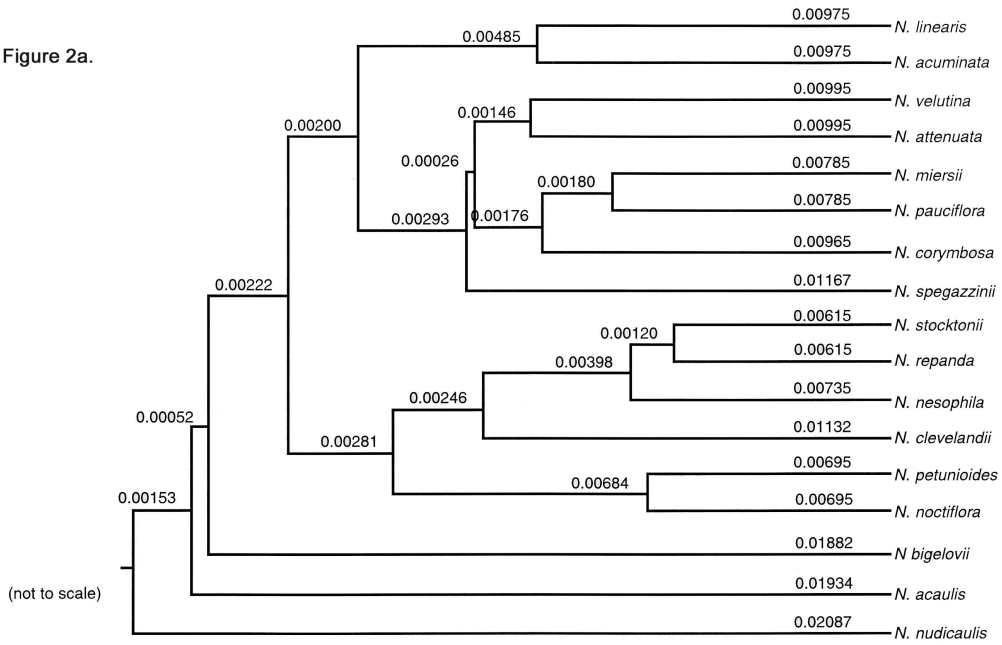

Figure 2b.

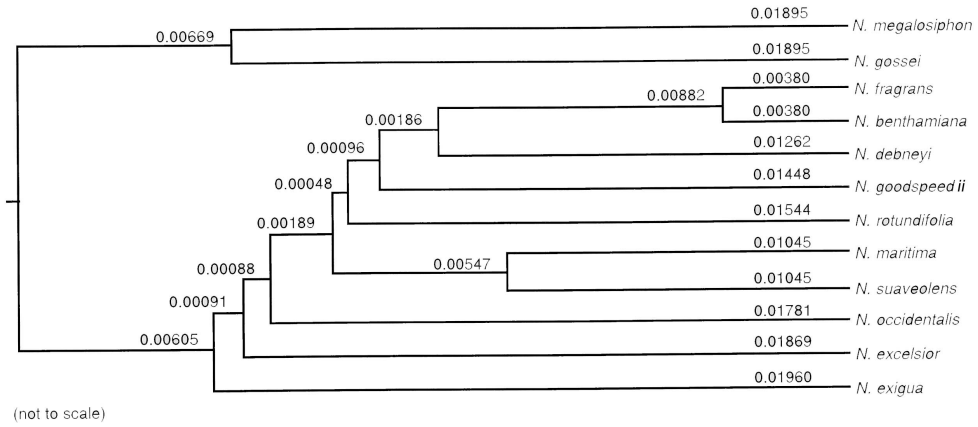

Figure 2. Phylogenetic trees of *Nicotiana* species included in the subgenus Petunioides. **a.** Sections *Repandae, Noctiflorae, Acuminatae, Bigelovianae* and *Nudicaulis*. **b.** Section *Suaveolentes.* Genetic distances are indicated on each arm of the tree.

have different 2C DNA amounts (Table 2). The 2C nuclear DNA amount for *N. repanda* is not available.

In Figure 2a species of *Repandae* show close phylogenetic relationship to each other. *N. stocktonii* and *N. repanda* show very close genetic affinity. *N. nesophila* has approximately twice as much nuclear DNA in its genome as in *N. stocktonii* (Table 2) although the two species have identical karyotypes. Phylogenetic differentiation has involved substantial differences in genome size (Table 2). In the dendrogram *N. nesophila* shows greater divergence from *N. stocktonii* and *N. repanda*.

Section *Noctiflorae*

The three diploid species of section *Noctiflorae* (*N. acaulis*, *N. noctiflora*, and *N. petunioides*) have the same chromosome number (n = 12). This section has presumably originated from the pre-Petunioid aggregate. Paniculatoid elements have been incorporated into this section through interspecific hybridisation. The genetic introgression is most clearly expressed in *N. noctiflora* and *N. petunioides* and this could be traced to *N. glauca* of section *Paniculatae*. It is viewed that *N. acaulis* has evolved recently. The three species have similar karyotype arrangements but the DNA

amounts vary significantly (Table 2).

In the phylogenetic tree (Figure 2a) species *N. noctiflora* and *N. petunioides* show very close genetic relationship. Both species have a wide geographical distribution. In contrast *N. acaulis* has a very narrow geographical distribution with a high degree of specialisation in morphological characters. It has outstandingly larger chromosomes than *N. noctiflora* and *N. petunioides*. The increase in chromosome size is accompanied by two to three-fold increases in the amount of 2C nuclear DNA (Table 2). The increase in nuclear DNA amount in *N. acaulis* further supports the view that its divergence recently from the primitive species *N. noctiflora* and *N. petunioides* is relatively recent. In the phylogenetic tree, although the 3 species show close genetic affinity, *N. acaulis* is separated from *N. noctiflora* and *N. petunioides.*

Section *Acuminatae*

The 8 species included in this section are wholly petunioid in character and there is no evidence to suggest that their origin and evolution involved outcrossing with species of other subgenera. The phylogenetic proximity of the 8 species is deduced from their morphological similarity for several characters and from the nature and extent of pairing at meiosis in F1 interspecific hybrids combining different species. Meiotic chromosome pairing is complete in F1 interspecific hybrids between *Acuminatae* species. In terms of external morphology the 8 species are interrelated, some species are related by similarity in one character while others by resemblance in another. *N. acuminata*, *N. attenuata*, *N. miersii N. pauciflora* and *N. corymbosa* have identical karyotypes (2 m + 6 sm + 4st (Table 2). Goodspeed (1954) suggested that these 5 species of section *Acuminatae* have originated from a common genetic source. The 2C nuclear DNA amount of *N. acuminata*, *N. pauciflora*, *N. corymbosa* and *N. miersii* are closely similar and this supports Goodspeed (1954) hypothesis of a common evolutionary origin. Their divergence was promoted by gene mutation and reproductive isolation rather than chromosome reorganisation. Goodspeed (1954) suggested that *N. linearis* and *N. spegazzinii* are recent products of evolution involving chromosome rearrangements. When chromosome complements are arranged in the order of size, *N. pauciflora*, *N. corymbosa* and *N. miersii* show close similarity. The chromosome complement of *N. linearis* is different not only in karyotype arrangement (1 m + 3 sm + 8 st) but also in chromosome size. The 2C nuclear DNA amount (Table 2) of *N. linearis* is smaller than that of other species. Genome size and karyotype of *N. spegazzinii* is not available.

In Figure 2a the 8 species of *Acuminatae* together show close genetic proximity to each other. In the phylogenetic tree 3 pairs of species, *N. velutina* and *N. attenuata*, *N. miersii* and *N. pauciflora*, *N. linearis* and *N. acuminata* show close genetic relationship. *N. corymbosa* is more closely related to *N. miersii* and *N. pauciflora* than to any other species in *Acuminatae*. This is in accordance with the close similarity they share in chromosome size and karyotype arrangements. *N. spegazzinii*, a recent product of speciation and evolution shows maximum genetic distance from all other *Acuminatae* species.

Sections *Bigelovianae and Nudicaules*

The three species included in the two sections are amphidiploids with 48 chromosomes. *N. bigelovii* and *N. clevelandii* of section *Bigelovianae* and *N. nudicaulis* of section *Nudicaules* show morphological affinity to members of section *Acuminatae*. The ancestry of these amphiploids is unresolved. Goodspeed (1954) speculated that the three species have originated from a common ancestral genetic source or from one another. It is suggested that the three species have in common one sub-genome of 12 chromosomes from section *Acuminatae*. The other parental line involved in their amphidiploid origin is not clearly understood although it is proposed that an ancestral species from section *Alatae* is involved in the origin of *N. bigelovii* and *N. clevelandii*. Similarly the second progenitor involved in the origin of *N. nudicaulis* has probably come from section *Trigonophyllae*. Unlike amphiploids *N. rustica*, *N. tabacum* and *N. wigandioides,* there is much uncertainty regarding the origin and phylogeny of these three amphiploid species. The ambiguity of their phylogenetic origin is reflected in the dendrogram. In Fig 2a species *N. bigelovii* and *N. clevelandii* of section *Bigelovianae* are separated from each other. *N. nudicaulis* of section *Nudicaules* is phylogenetically removed from species of section *Bigelovianae*. The scattered distribution of these species in the dendrogram is attributable to their unknown origins as amphidiploids.

Section *Suaveolentes*

The 15 species included in this section are indigenous to Australia. Species in this section show much variation in diploid chromosome number (n = 16, 18, 22, 24). Goodspeed (1954) has proposed that the numerical chromosome variation has originated by aneuploidy from ancestral amphidiploids with 48 chromosomes. *N. debneyi* and *N. fragrans*, the two amphidiploids included in this section are postulated to have originated from ancestral species with 12 pairs of chromosomes. From external morphology it is sugg-

ested that the ancestral species possibly are related to sections *Acuminatae*, *Noctiflorae* and *Alatae*. Species of section *Suaveolentes* show significant variation in nuclear DNA amounts (Table 2). Extensive chromosome reorganisation, aneuploidy, amphidiploidisation, accretion, or deletion of DNA base sequences without numerical chromosome changes all have played a significant role in the evolution of this section.

In the present study 12 of the 15 species in section *Suaveolentes* was included. The dendrogram in Figure 2b shows a scattered phylogenetic group of species. Three pairs of species show close genetic affinity. They are; *N. maritima* and *N. suaveolens*, *N. fragrans* and *N. benthamiana* and *N. megalosiphon* and *N. gossei*. The latter two species show much differentiation from the remainder of the species of subgeneric section *Suaveolentes*. The results point to the complex evolutionary relationship between species of section *Suaveolentes*.

Conclusion

In the study of plant speciation and evolution emphasis is generally given to external morphology, chromosome affinity in interspecific hybrids and the mode of reproduction and propagation. Many plant species are characterized by the differences in their breeding systems. In natural populations however, sexually reproducing plant species often switch to vegetative propagation and vice-versa. This leads to genetic changes at the population level and classification based on breeding systems and morphological traits alone becomes unreliable.

Genome specifies the genetic make up of a species. Species within a genus have diverged presumably from a common ancestral genetic stock. In the early stages of evolution of a genus species shared closer genetic affinity and gene exchanges between evolving species was more frequent. Subsequent differentiation of genomes in evolution has resulted in reproductive isolation between species. Within a stable species 2C nuclear DNA amount, haploid chromosome number and karyotype arrangement are constant. Comparative studies on the architecture of genomes have shown that the eukaryotic genome is in a state of flux undergoing genetic changes. The molecular processses determining the rate of genomic changes in evolution include saltatory amplification, deletion and conversion of DNA sequences. These changes are further compounded by nucleotide sequence divergence due to random mutations and inherent replication errors of the genomic machinery (Schopf, 1981). Transposable genetic elements can also alter genes and genomes by causing gross structural rearrangements which may result in a changed pattern of gene expression and there by alter linkage equilibrium in populations (Finnegen, 1989). At the chromosome level significant differences in total chromosome volume and karyotype organisation are found even among closely related species. In the long range evolution of a genus changes in haploid chromosome number, karyotype arrangement and genome size play a significant role.

A major development in plant genome analysis was the development of molecular phylogenetic maps. Comparative genome mapping using combinations of repeated sequences as molecular probes has provided insights into the organisation and evolution of the eukaryotic genome. Some ordered physical features shared by genomes of taxonomically distant species may have a common functional cause; other shared features with a genetic basis might reflect a common phylogeny. Recent developments in gene mapping have revealed astonishing similarities between species over surprisingly long taxonomic distances (Moore et al., 1993).

PCR techniques provide an efficient method for the amplification of genomic and cloned sequences. RAPD is based on the amplification of genomic DNA using short synthetic arbitrary primer sequences. Each amplification product is derived from a region of the genome in between two short DNA segments with some homology to the primer sequences used. The polymorphism between individuals results from the DNA sequence differences between the primer binding sites.

In this paper we compared RAPD generated phylogenetic trees of 56 *Nicotiana* species with the cytotaxonomical and morphological data reported previously by Goodspeed (1954) and other workers and the pattern of quantitative DNA changes associated with speciation and evolution of the genus. As described earlier RAPD generated phylogenetic relationship is more reliable between closely related species than between distantly related species (Stammers et al., 1994).

In classifying species of genus *Nicotiana* Goodspeed (1954) used morphological characters, geographical distribution of species as well as karyotypic similarities between chromosome complements. More importantly a vast array of interspecific hybrids was made and the extent of chromosome pairing in the F1 hybrids was studied. The data provide valuable information about the residual chromosome homology between related species. While morphological traits in general signify adaptation to specific environments, nuclear characters such as genome size, haploid chromosome number, karyotype arrangement and chromosome affinity in interspecific hybrids are relatively stable parameters and they provide reliable criteria for phylogenetic comparisons. Evolution and

speciation in *Nicotiana* has resulted in significant changes in organisation and at the molecular level divergence in nuclear DNA sequences. With the exception of section of S*uaveolentes* (Subgenus *Petunioides*) all species are native to central and south America. Except for sections *Alatae* and *Suaveolentes* of subgenus *Petunioides*, species are either diploids with 24 chromosomes or amphidiploids with 2n = 4X = 48. Even among diploid species with the same chromosome number there is large variation in chromosome size karyotype arrangements and 2C nuclear DNA amounts. Amphidiploids are derived from the spontaneous doubling of the chromosome complement of sterile F_1 hybrids between diploid species. This restores their fertility and their meiosis becomes regular as in their diploid progenitors. Cultivated *Nicotiana* species *N. tabacum* and *N. rustica* and the wild species *N. arentsii* are examples of amphiploidisation. The ancestry of these amphidiploid species, as described earlier, is well established. Aneuploidy has also been important in genome differentiation. Section Suaveolentes of subgenus Petunioides is exceptional. Species in this section are native to Australia and it displays an aneuploid seriation of chromosome numbers n = 16, 18, 19, 20, 21 and 22. Presumably they are derived from an amphidiploid ancestor with 48 chromosomes through aneuploidy.

Phylogenetic relationships deduced from the RAPD analysis closely parallel the conclusions derived using morphological and karyotype comparisons by Good speed and other investigators. The study has demonstrated that RAPD analysis is a suitable for the detection and analysis of genetic diversity between species and to deduce their phylogenetic relationships. As would be expected species included in the same subgeneric section show a greater genetic affinity than those included in different sections. The relationships between species in the dendrograms largely match the existing knowledge on the origin and differentiation of species in genus *Nicotiana*. A species relationship in section Suaveolentes is not clearly understood. RAPD analysis using a larger selection of DNA primers might have provided a more accurate picture of the relationships between species included in this section.

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