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Relationships among *Nicotiana* species revealed by the 5S rDNA spacer sequence and fluorescence in situ hybridization

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Abstract To investigate phylogenetic relationships in *Nicotiana*, the intergenic spacer sequences of 5S rDNA were analyzed in species with 2n=18, 20 or 24, and amphidiploid species with 2n=48. The chromosomal localization of the 5S rDNA was determined by fluorescence in situ hybridization (FISH). In species with 2n=24 and their descendants, a major 5S rDNA-specific PCR fragment of 400–650 bp was obtained. The amphidiploid species contained similar length of 5S rDNA units derived from putative diploid progenitors. Among the five clones from each representative PCR fragment, some nucleotide exchanges and length heterogeneity were observed. The latter was due to variation in the spacer region, such as differences in the length of poly A and/or poly T tracts as well as insertions/deletions. Interspecific comparisons of each 5S rDNA sequence demonstrated that the spacer sequence could be divided into three regions. Excluding gaps from the aligned spacer sequences of 5S rDNA, phylogenetic trees were constructed. Each phylogenetic tree showed an almost identical topology even if different algorithms were applied. The chromosomal locations of the 5S rDNA in each species correlated with the phylogenetic topology. The phylogenetic trees were generally in agreement with the current classification.

Keywords 5S rDNA spacer sequence · Fluorescence in situ hybridization (FISH) · Molecular phylogeny · *Nicotiana*

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Introduction

The genus *Nicotiana* comprises 66 species primarily endemic to South America, North America and Oceania. The classification of *Nicotiana* relies on the morphological and cytological investigations of Goodspeed (1954). The current classification comprises three subgenera, i.e. *Rustica*, *Tabacum* and *Petunioides*, and 14 sections (Smith 1979; Reed 1991). The majority of the *Nicotiana* species possess 12 (29 species) or 24 (12 species) chromosome pairs. The remaining species have 9–10 or 16–23 pairs.

Phylogenetic studies based on DNA and protein sequences provide new insights into interspecific relationships, and often lead to a modification in the traditional classification (Soltis and Soltis 2000). Molecular phylogenetic analyses of the genus *Nicotiana* are limited (Olmstead et al. 1990; Bogani et al. 1997; Aoki and Ito 2000), although there are several reports on the origin of cultivated amphidiploid tobacco (Bland et al. 1985; Koukalová et al. 1993; Gazdová et al. 1995; Volkov et al. 1996; Matyásek et al. 1997; Riechers and Timko 1999).

In most higher eukaryotes, the 5S ribosomal RNA genes (5S rDNA) are arranged in tandem arrays at one or more chromosomal loci, mostly separated from the 45S rDNA (Fedoroff 1979). The 5S rDNA consists of a coding sequence of approximately 120 bp and a non-transcribed spacer of several hundred bp. The coding sequences are highly conserved across a broad taxonomic range. This led to the expectation that coding sequences were valuable for phylogenetic analysis at higher taxonomic ranks. It was, however, reported that coding sequences of 5S rDNA were of little use in reconstructing phylogenetic relationships because of their short length and the presence of nucleotide sites that were free to vary (Halanych 1991; Steele et al. 1991). On the other hand, non-transcribed intergenic spacer sequences tend to be diverged between closely related species, and have been used for inferring phylogenetic relationships at lower taxonomic ranks (Xie et al. 1994; Kellogg and Appels 1995; Cronn et al. 1996).

Previously, the location of the 5S rDNA was studied in *Nicotiana tabacum* and its putative progenitors, showing that the T subgenome of *N. tabacum* was more similar to the *Nicotiana tomentosiformis* genome than to that of *Nicotiana otophora* (Kitamura et al. 2000). In the present study, 5S rDNA spacer regions in *Nicotiana* species with 12 chromosome pairs and their descendants with 9 and 10 pairs were sequenced. From these data and from the chromosomal location of the 5S rDNA, phylogenetic relationships were deduced.

Materials and methods

Plant materials and DNA extraction

The seventeen species listed in Table 1 were used in this experiment. The seeds of these species were obtained from the Japan Tobacco Inc., Japan. They were germinated and grown in a greenhouse at about 25°C with natural day light.

Using young leaves, total genomic DNA was extracted by the CTAB procedure (Murray and Thompson 1980).

Fluorescence in situ hybridization (FISH)

A part of the coding region of 5S rDNA (72 bp in length), amplified from the total genomic DNA of *N. tabacum* (Kitamura et al. 2000), was labeled with biotin-16-dUTP by PCR (Fukui et al. 1994). The 18S rDNA was labeled with digoxigenin-11-dUTP.

FISH was carried out on mitotic chromosomes according to Kitamura et al. (2000). Hybridization of biotinylated 5S rDNA and the digoxigenin-labeled 18S rDNA probe was visualized with FITC and rhodamine-Texas red, respectively. The chromosomal DNA was counterstained with DAPI. The rDNA signals were localized on chromosomes by integration with DAPI-counterstained images using Adobe Photoshop.

PCR amplification

A pair of primers was designed within the coding region of the 5S rDNA of tobacco (Fulnecek et al. 1998) to amplify fragments including the full length of the intergenic spacer sequences. 5SrDNA-3 (5′-GTG CTT GGG CGA GAG TAG TA-3′) and 5SrDNA-4 (5′-GGT GCG TTA GTG CTG GTA TG-3′) primers were set between positions +58 to +77 on the sense strand, and +10 to +29 on the antisense strand, respectively (Fig. 1). Amplification was done in a total volume of 50 µl including 10 ng of template DNA, 1× PCR buffer (Perkin Elmer), 1.5 mM $MgCl_2$, 1 µM of each primer, 200 µM of each dNTP, and 1.25 U of *Taq* DNA polymerase (AmpliTaq Gold, Perkin Elmer). The PCR conditions were as follows: an initial denaturation step at 94°C for 10 min, 30 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 3 min, and a final extension step at 72°C for 10 min. The PCR products were fractionated by electrophoresis in 1.5% agarose gel with TAE buffer, stained with ethidium bromide, and visualized under an ultraviolet transilluminator.

Cloning and sequencing

The major PCR fragment(s) of each species were recovered from agarose gels (Qiagen, Germany), and cloned by TA-cloning according to the manufacturer's protocol (Promega, USA). Positive clones were identified by the white-blue color system and by colony PCR with M13 universal primers. Sequencing was conducted using an ABI PRISM 377 automated sequencer with a BigDye Terminator Cycle Sequencing Kit (ABI PRISM, Perkin Elmer). Each clone was sequenced in sense and antisense direction. At least five clones were completely sequenced for each fragment.

Phylogenetic analysis

Multiple alignment was done using Clustal W ver. 1.7 (Thompson et al. 1994). Maximum parsimony analysis was carried out with heuristic search strategies using PAUP ver. 3.1 (Swofford 1993). The neighbor-joining method (Saitou and Nei 1987) was also applied using the package PHYLIP ver. 3.57 (Felsenstein 1995). Dendrograms obtained with the neighbor-joining algorithm were drawn by TreeView (Page 1996).

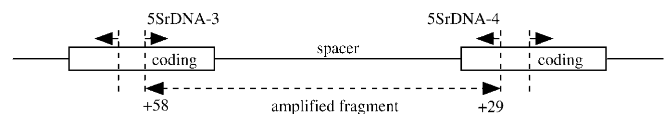

Fig. 1 PCR amplification product for sequencing the 5S rDNA spacer region. Specific primers (5SrDNA-3 and 5SrDNA-4) are labeled by arrows

Table 1 Species used in this study. The classification of *Nicotiana* is according to Reed (1991)

Subgenus	Section	Species	Chromosome number (2n)	Genome designation
Rustica	Paniculatae	*N. glauca* Graham*[a]	24	–[b]
		N. knightiana Goodspeed*	24	–
		N. paniculata L.	24	P
	Rusticae	*N. rustica* L.*	48	PU
Tabacum	Tomentosae	*N. tomentosiformis* Goodspeed*	24	T
		N. otophora Grisebach*	24	T
		N. kawakamii Y. Ohashi	24	–
		N. glutinosa L.*	24	–
	Genuinae	*N. tabacum* L. cv Bright Yellow 4*	48	ST
Petunioides	Undulatae	*N. undulata* Ruiz & Pavon*	24	U
	Trigonophyllae	*N. trigonophylla* Dunal*	24	–
	Alatae	*N. sylvestris* Speg. & Comes	24	S
		N. langsdorffii Weinmann*	18	–
		N. alata Link & Otto	18	–
		N. forgetiana Hort. & Hemsley	18	–
		N.longiflora Cavanilles	20	–
		N. plumbaginifolia Viviani*	20	–

[a] *, Species used for cloning and sequencing of 5S rDNA spacer regions
[b] –, Unidentified

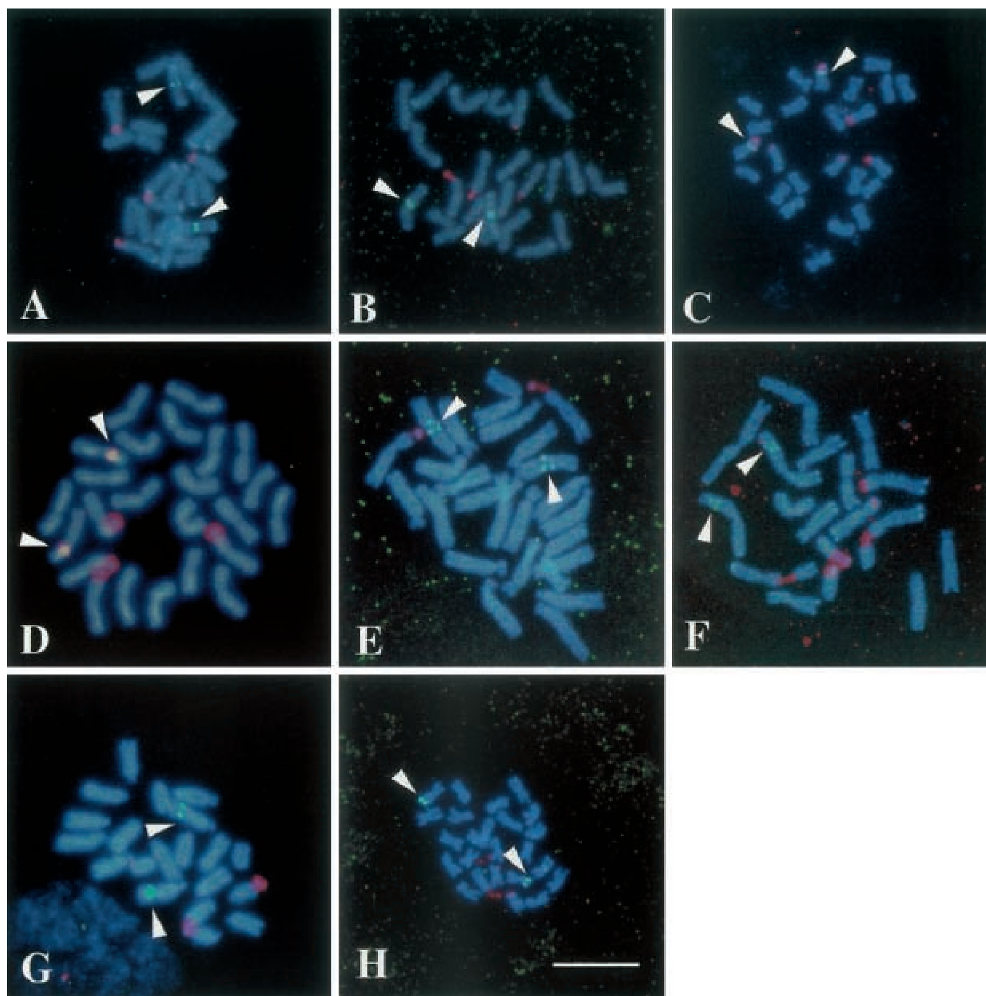

Fig. 2A–H FISH localization of 5S (*green signals*, *arrowheads*) and 18S (*red signals*) rDNA sequences on mitotic chromosomes. **A** *N. paniculata*; **B** *N. knightiana*; **C** *N. undulata*; **D** *N. glutinosa*; **E** *N. glauca*; **F** *N. langsdorffii*; **G** *N. plumbaginifolia*; **H** *N. trigonophylla*. Two *yellowish signals* in **D** (*arrowheads*) indicate overlapping signals of 5S and 18S rDNA probes; *bar*=10 μm

Results

Chromosomal localization of 5S rDNA arrays

5S and 18S rDNA sites were localized simultaneously on the chromosomes by FISH. One 5S rDNA locus each was detected for *Nicotiana paniculata* and *Nicotiana knightiana* within the proximal region of the long arm of a submetacentric pair (Fig. 2A, B). For *Nicotiana undulata*, 5S rDNA occurred in interstitial region on the short arm of a submetacentric pair (Fig. 2C). In *Nicotiana glutinosa*, it was co-localized with one of the 18S rDNA sites at the interstitial region of the short arm (Fig. 2D). For *Nicotiana glauca* (Fig. 2E) and *Nicotiana langsdorffii* (Fig. 2F), two adjacent sites of 5S rDNA were detected in intercalary position within the long arms of an acrocentric (*N. glauca*) or a submetacentric pair (*N. langsdorffii*). In addition, the physical location of the 5S rDNA in *N. langsdorffii* appeared to be somewhat more terminal than that in *N. glauca*. For *Nicotiana alata* and *Nicotiana forgetiana*, the localization of 5S rDNA was identical to that in *N. langsdorffii* (data not shown). One 5S rDNA site was visualized in *Nicotiana plumbaginifolia* (Fig. 2G), and its chromosomal location was similar to that in *N. langsdorffii* but the chromosome pair with that array was acrocentric. In *Nicotiana trigonophylla*, a major 5S rDNA signal was observed subterminally on the long arm of a small submetacentric pair (Fig. 2H). For the amphidiploid *Nicotiana rustica*, the positions of the 5S rDNA corresponding to those in *N. paniculata* and *N. undulata* were confirmed (data not shown). The distribution of rDNA sites in *N. tabacum* and its relatives, *N. otophora*, *N. tomentosiformis*, *Nicotiana kawakamii* and *Nicotiana sylvestris*, is reported elsewhere (Kitamura et al. 2000; Nakamura et al. 2001).

Amplification of the spacer sequence of the 5S rDNA

The spacer regions of 5S rDNA were amplified and fractionated by 1.5% agarose-gel electrophoresis (Fig. 3). One major band was identified in species with 12 chromosome pairs except for *N. paniculata*, *N. knightiana* and *N. kawakamii*. In *N. paniculata*, three bands were detected. Preliminary Southern analysis demonstrated that the major 5S rDNA unit of *N. paniculata* corresponded to a 500-bp PCR band (data not shown). For *N. knightiana*, a few clear bands were amplified, but a

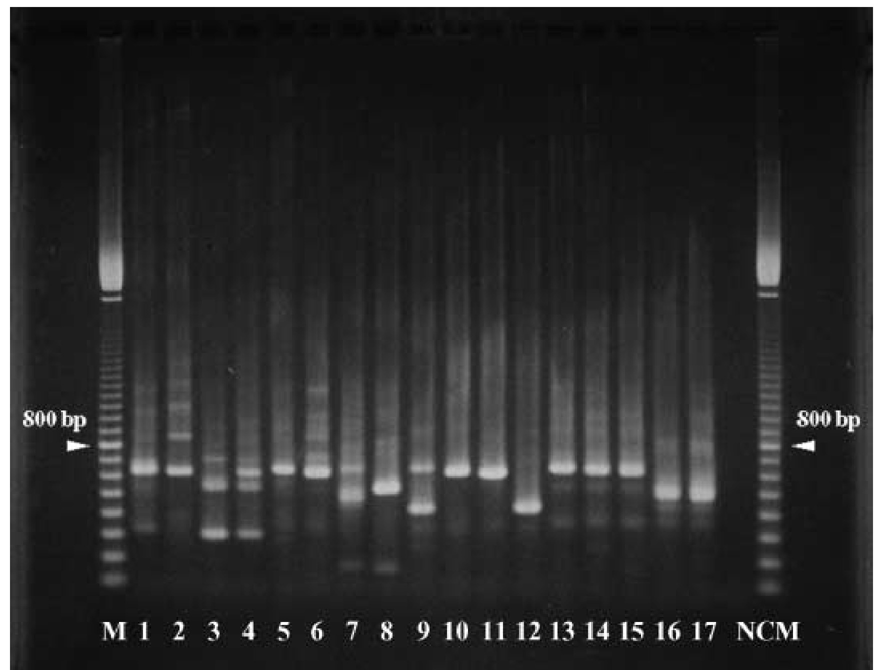

Fig. 3 Agarose-gel electrophoresis of PCR products for the 5S rDNA spacer region. *1 N. glauca*; *2 N. knightiana*; *3 N. paniculata*; *4 N. rustica*; *5 N. tomentosiformis*; *6 N. otophora*; *7 N. kawakamii*; *8 N. glutinosa*; *9 N. tabacum* cv Bright Yellow 4; *10 N. undulata*; *11 N. trigonophylla*; *12 N. sylvestris*; *13 N. langsdorffii*; *14 N. alata*; *15 N. forgetiana*; *16 N. longiflora*; *17 N. plumbaginifolia*; *NC* negative control; *M* 100-bp ladder marker

600-bp band showed the most intense signal and was selected for subsequent analysis. For *N. kawakamii*, only a smear around 500 bp and faint bands were observed. Therefore no 5S rDNA fragment was isolated. In *N. tabacum*, a 400-bp and a 600-bp band were clearly detected, corresponding to that of the putative progenitors (*N. sylvestris* and the core species of Tomentosae, Fulnecek et al. 1998). Another amphidiploid species, *N. rustica*, showed four bands, one identical to that in *N. undulata* and three to those in *N. paniculata*.

Three species with nine chromosome pairs exhibited almost identical major bands of approximately 600 bp. Since identical restriction patterns were observed after digestion of these PCR products with the restriction endonucleases *Rsa*I and *Sau*3AI (data not shown), that of *N. langsdorffii* was selected for sequence analysis. A similarity was also observed for the two species with ten chromosome pairs. That of *N. plumbaginifolia* was used for sequencing.

Characterization of the spacer sequence of 5S rDNA

Five cloned sequences derived from each selected fragment including part of the coding region (91/119 bp) were compared. Nucleotide substitutions among five clones occurred in the spacer and the coding region, as reported for the 5S rDNA of other plants (Kellogg and Appels 1995; Cronn et al. 1996). For several species, a slight length heterogeneity was also detected mainly due to differences in the length of poly T and/or poly A tracts in the spacer region. In these cases, the consensus sequence was easily determined by the majority rule at each nucleotide site and used in the subsequent phylogenetic analysis. For *N. glauca*, *N. tomentosiformis* and *N. langsdorffii*, the variation in length was more than 10 bp among each of the five clones. One clone of each species was discriminated by the presence of several insertions/deletions (indels) within the spacer region. The corresponding clones were treated as 'non-consensus' sequences compared to the other four clones. For *N. otophora*, the five clones were divided into two groups: two had 614 bp and the other three had shorter fragments ranging from 599 to 605 bp. Consequently, two consensus sequences were used for *N. otophora*. For the 'non-consensus' sequences of *N. glauca* and *N. tomentosiformis*, nucleotide substitutions within "box C", which is highly conserved and represents the primary binding site of transcription factor III A (Pieler et al. 1987; Hayes and Tullius 1992), were detected (Fig. 4). For the 'non-consensus' sequence of *N. langsdorffii*, nucleotide substitution was observed in the vicinity of box C. Southern analysis confirmed that the 'non-consensus' sequence of *N. tomentosiformis* (550 bp) corresponded to the minor unit of the 5S rDNA in this species, while this could not be demonstrated for the other 'non-consensus' sequences due to their similar size (data not shown).

Multiple alignment was carried out using respective consensus and 'non-consensus' sequences (Fig. 4). Consensus sequences from *N. undulata* and the 600-bp band of *N. rustica* were identical. The coding region (91/119 bp) showed no gaps and a high degree of conservation across species. The spacer region was highly divergent and showed many nucleotide exchanges. However, the 5′- and 3′-flanking regions of the coding sequence were considerably invariable among the respective sequences. The spacer sequence could be divided into three regions on the basis of nucleotide similarity:

The 3′-flanking sequence of the coding region of approximately 100 bp is defined as *Region I*. It is relative-

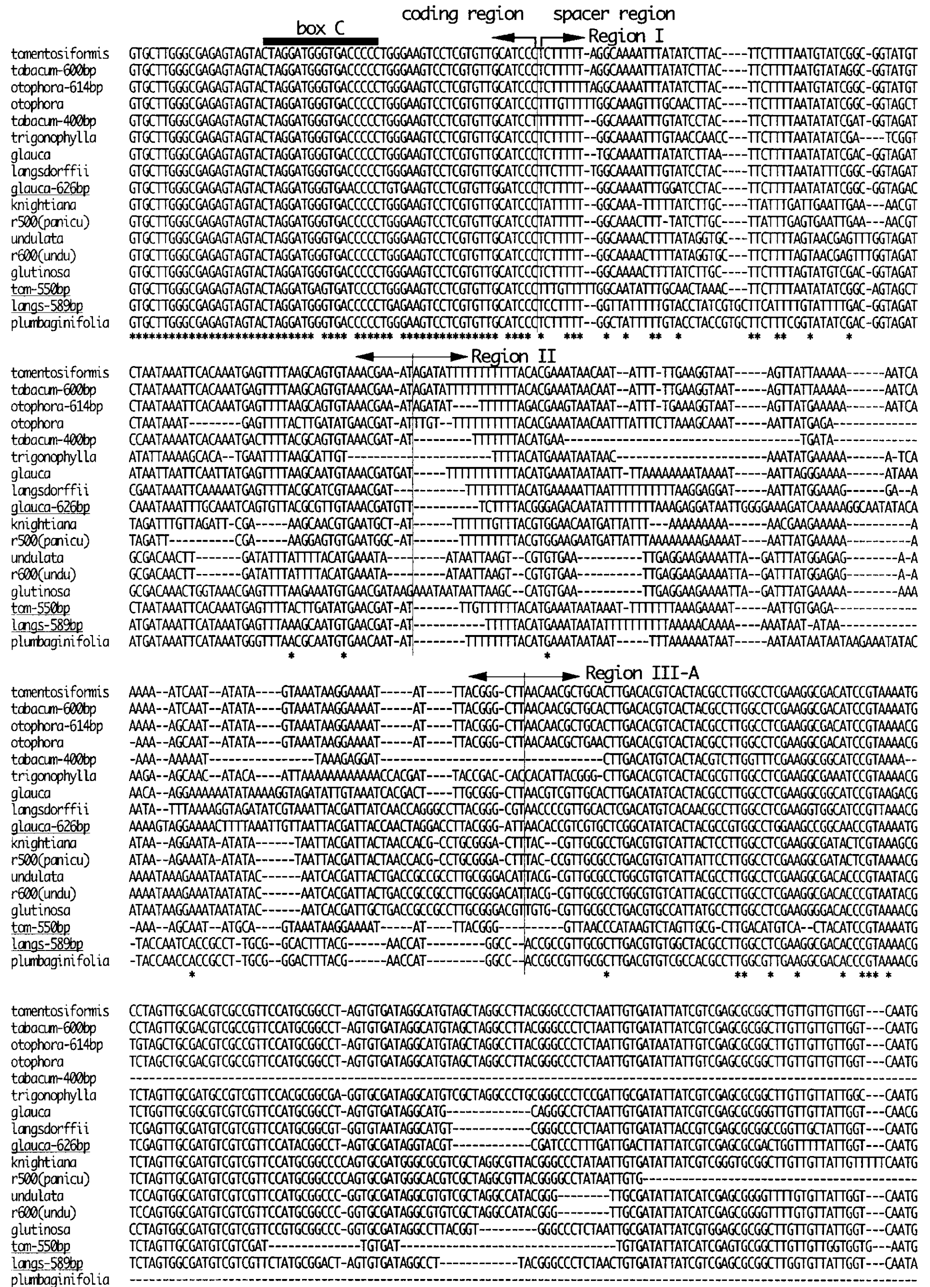

Fig. 4 Aligned nucleotide sequences of the spacer regions of 5S rDNA in *Nicotiana*. *Dashes* indicate gaps. *Asterisks* indicate positions of invariable nucleotides. The boundaries between the coding and spacer regions are according to Kellogg and Appels (1995). The 'non-consensus' sequences are *underlined*. *r500*(*panicu*)= 500-bp sequence of *N. rustica* (*N. paniculata* subgenome component); *r600*(*undu*)= 600-bp sequence of *N. rustica* (*N. undulata* subgenome component); *tom-550bp*=550-bp sequence of *N. tomentosiformis*; *langs-589bp*=589-bp sequence of *N. langsdorffii*

ly conserved and possesses some common features across the species, e.g., a 4–5 T tract downstream from the coding region and a TTMTTT sequence further downstream.

Region II is the most variable region within the spacer and corresponds to about 150 bp immediately downstream from Region I. Poly T and poly A tracts are scattered throughout this region. Most of these tracts differed in length and in position among the respective sequences. Like Region I, this region was AT-rich with values from 66.0% in *N. undulata* to 84.2% in the 400-bp sequence of *N. tabacum*.

The rest of the spacer sequence is defined as *Region III*, subdivided into Region III-A and Region III-B. Region III-A corresponds to about 300 bp immediately downstream from Region II and is characterized by the presence of large indels. The most remarkable deletions were placed in consensus sequences of *N. plumbaginifolia* (146 bp), *N. glutinosa* (107 bp), the 400-bp band of *N. tabacum* (138 bp), and the 500-bp band of *N. rustica* (94 bp). Many of the large deletions in Region III-A were species-specific, but one deletion was shared by *N. langsdorffii* and *N. glauca*. Region III-B (about 40 bp) corresponds to the 5′-flanking sequences of the coding sequence. About 65% of nucleotide sites in this region were completely conserved among the 17 aligned sequences, and a sequence element of $^{-32}$TATTAAG^{-25} was observed in 11 sequences.

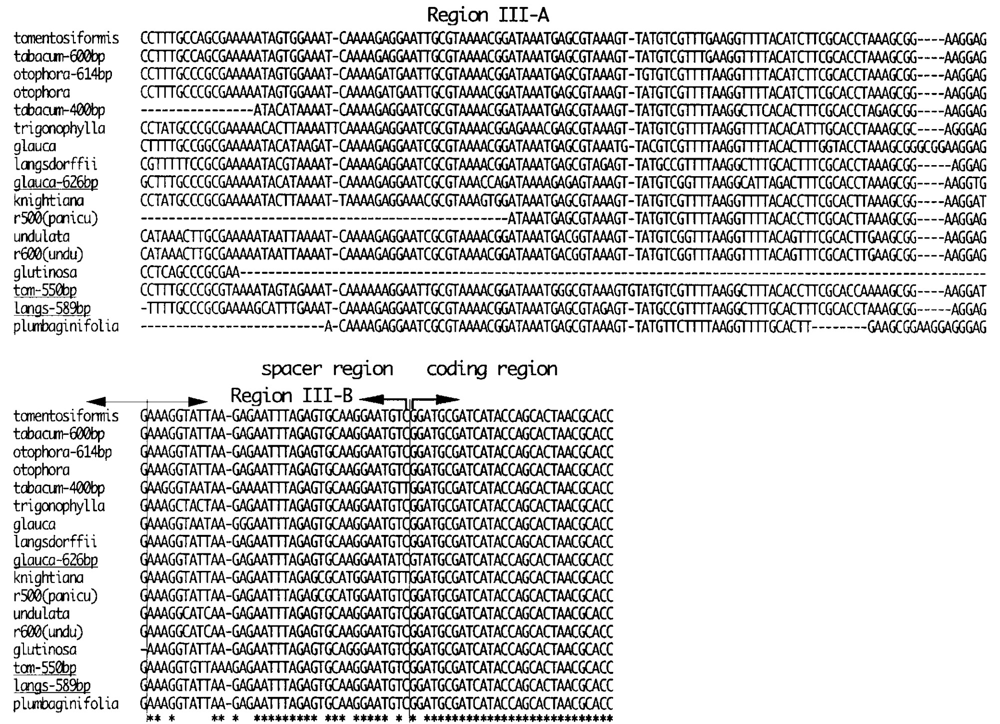

Fig. 4 (continued)

Phylogenetic analysis based on the spacer sequences

Because the evolutionary rate of nucleotide substitution was different from that of indels or gaps (Saitou and Ueda 1994), for phylogenetic analysis the gaps of the spacer sequences were excluded. Considering all 17 sequences (Fig. 4), only about 1/3 of the nucleotide sites of the aligned spacer region could be evaluated due to the distribution pattern of gaps. In order to evaluate larger numbers of nucleotide sites, comparisons were made in different combinations of sequences in Fig. 4.

Figure 5A and B show the maximum parsimony tree and the neighbor-joining tree, respectively, which were obtained when the two consensus sequences with the largest deletions (the 400-bp sequence in *N. tabacum* and *N. plumbaginifolia*) were excluded. In this case, the left side of Region III-A can be compared. The topologies of the two trees were quite similar. The T-genome species *N. otophora* and *N. tomentosiformis*, and the T-subgenome component of *N. tabacum* (600-bp sequence) were clustered. *N. knightiana* and the P subgenome component of *N. rustica* (500-bp sequence), and *N. undulata* and *N. glutinosa* were also aggregated as monophyletic groups, with nearly complete bootstrap values (99–100%). These two clusters grouped with each other with a bootstrap value of about 90%. In addition, a consensus sequence of *N. langsdorffii* and a 'non-consensus' sequence of *N. glauca* were placed into a cluster with a high bootstrap value.

When *N. plumbaginifolia* instead of *N. glutinosa* was included in the phylogenetic analysis, we could compare the right side of Region III-A. Then, a new parsimony tree (Fig. 5C) and a neighbor-joining tree (Fig. 5D) were constructed. The topologies of these two trees were not only almost identical to each other but also similar to those of Fig. 5A and B. In this case, a consensus sequence of *N. plumbaginifolia* was clustered with a 'non-consensus' sequence of *N. langsdorffii* with a 100% bootstrap value.

Discussion

5S rDNA spacer sequences in *Nicotiana*

Multiple alignment of the 5S rDNA sequences from the listed *Nicotiana* species (Table 1) demonstrates that Region I (3′-downstream) and Region III (5′-upstream) are more conserved than Region II (Fig. 4), suggesting that Region I and Region III are meaningful for the function of the 5S rRNA genes. This is supported by our observations of the conserved features in the *Nicotiana* 5S rDNA spacer sequences, such as a poly T motif immediately downstream from the coding region, which is implicated in transcription termination (Capesius 1991; Campell et al. 1992; Sastri et al. 1992), an AT-rich motif further downstream that is considered to play a secondary termination role (Scoles et al. 1988), and a TATTAAG motif about 30 bp upstream of the coding region, which is similar to a putative protein-binding motif (Gerlach and Dyer 1980; Goldsbrough et al. 1982). Large indels within the spacer were also found in the monocot tribe Triticeae (Scoles et al. 1988), and considered to be due to the occurrence of DNA replication slippage by large loop formation (Efstradiatis et al. 1980; Nevers et al. 1986). Scoles et al. (1988) have divided the

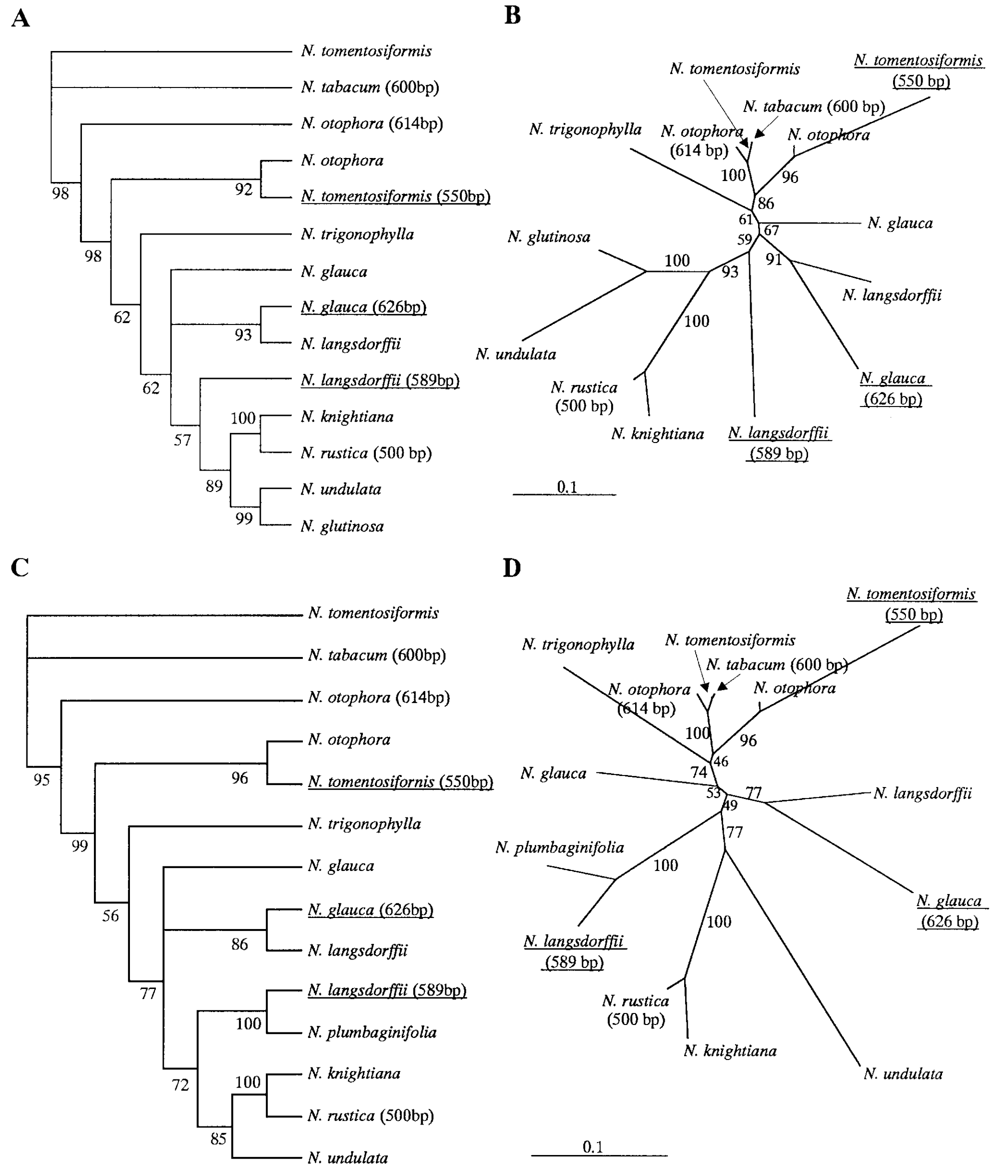

Fig. 5 Phylogenetic trees obtained by maximum parsimony methods (**A**, **C**) and neighbor-joining methods (**B**, **D**). From **A** and **B** the sequences of *N. plumbaginifolia* and 400 bp of *N. tabacum* were excluded. From **C** and **D** the sequence of *N. plumbaginifolia* was included instead of that of *N. glutinosa*. Bootstrap values are indicated at each fork. The 'non-consensus' sequences are *underlined*

5S rDNA spacer sequences of the Triticeae into three regions, based on the frequencies of nucleotide exchange and gaps. According to their definition, Region III-A in the present study would be assigned to Region II, the most divergent region of the spacer. However, the level of nucleotide identity of Region III-A is high enough to be assigned into the most-conserved region, if the large deletions are not taken into consideration (Fig. 4). The biological significance of Region III-A is not known, though it is characteristic of the 5S rDNA spacer sequence in *Nicotiana*.

Construction of phylogenetic trees

Although all gaps were excluded from evaluation, some showed identical patterns within at least two sequences, which might affect the topology of the tree (Fig. 4). As shown in Figs. 4 and 5, the patterns sharing these informative gaps were apparently concordant with branching patterns. Therefore, we assume that exclusion of gap sites does not affect the phylogenetic tree, although it may cause an underestimation of the length of branches in a distance-based tree.

In order to increase the number of nucleotides for evaluation, comparisons were made for different combinations of sequences, but this did not yield topological differences (Fig. 5). Almost identical topologies were obtained by using maximum parsimony and neighbor-joining methods (Fig. 5). Therefore, it is conceivable that the phylogenetic trees of Fig. 5 show possible relationships of the 5S rDNA spacer sequences in *Nicotiana*.

Phylogenetic relationships in *Nicotiana*

Our dendrograms (Fig. 5) were supported by the chromosomal location of 5S rDNA (Fig. 2) and generally

agree with the current classification of the genus (Smith 1979; Reed 1991). Close relationships were suggested between *N. paniculata*, *N. knightiana*, *N. undulata* and *N. glutinosa* (Fig. 5). The former two species are the Paniculatae species of *Rustica*, whereas *N. glutinosa* and *N. undulata* are classified into the Tomentosae of *Tabacum* and the Undulatae of *Petunioides*, respectively. Goodspeed (1954) reported that some morphological characteristics of the Paniculatae were exhibited by the latter two species, that the chromosome complement of *N. glutinosa* had high affinity to that of *Nicotiana benavidesii* Goodspeed of Paniculatae species, and that the karyotype of *N. undulata* is similar to that of Paniculatae species. At the molecular level, some similarities were found between *N. undulata* and *N. knightiana* by RAPD patterns (Bogani et al. 1997) and between Panuculatae species and *N. glutinosa* by Southern analysis of chloroplast DNA (Olmstead et al. 1990). These observations support the close relationships among those four species recognized in this study. However, it is surprising that *N. glutinosa* and *N. undulata* formed a specific cluster with a nearly complete bootstrap value (Fig. 5A, B). There were similarities between these two species in the chromosomal location of 5S rDNA based on chromosome morphology (Fig. 2C, D). Furthermore, three pairs of 18S rDNA loci were detected and one of them was localized on the 5S rDNA-bearing chromosome pair in both species (Fig. 2C, D). Recently, Aoki and Ito (2000) analyzed the *matK* gene sequences of the chloroplast genome and also found a cluster consisting of *N. glutinosa* and *N. undulata*. These results suggest a relatively recent divergence of the two species. Although the difference in overall chromosomal size is prominent (Fig. 2C, D), this might be due to a difference in the degree of chromosome condensation rather than in nuclear DNA content (Narayan 1987).

The origin of the cultivated amphidiploid tobacco, *N. tabacum*, has been studied by various approaches (Goodspeed and Clausen 1928; Gerstel 1960, 1963; Gray et al. 1974; Bland et al. 1985; Okamuro and Goldberg 1985; Kenton et al. 1993; Kitamura et al. 2000). It is obvious that the S subgenome progenitor of *N. tabacum* is closely related to the present-day *N. sylvestris*. Although *N. tomentosiformis* was thought to contribute the T subgenome of *N. tabacum* (Reed 1991), it was also proposed that the T subgenome of *N. tabacum* may have derived from an introgressive hybrid between two putative candidates, *N. tomentosiformis* and *N. otophora* (Kenton et al. 1993; Riechers and Timko 1999). Previously, we have reported that in *N. tabacum*, *N. otophora* and *N. tomentosiformis* the common 5S rDNA locus was located in the proximal region of a small submetacentric pair in each T (sub)genome; an additional locus was detected only in *N. otophora* (Kitamura et al. 2000). A close relationship between consensus sequences from *N. tomentosiformis* and the T-subgenome component of *N. tabacum* was observed in the present analysis (Fig. 5), ascertaining our previous finding that the T subgenome of *N. tabacum* is comparable to the *N. tomentosiformis* genome (Kitamura et al. 2000). This genomic relationship has been also suggested by another recent molecular cytogenetical study in Tomentosae species using several repetitive sequences (Lim et al. 2000). On the other hand, the 'non-consensus' sequence (550 bp) in *N. tomentosiformis* and the 600-bp sequence in *N. otophora* were clustered as a sister branch (Fig. 5). The 'non-consensus' sequence in *N. tomentosiformis* is a minor unit of 5S rDNA (data not shown) with nucleotide substitutions in box C (Fig. 4), probably representing a pseudogene (Kellogg and Appels 1995), which was not previously detected by FISH (Kitamura et al. 2000) and corresponds to an additional locus in *N. otophora*.

N. glauca is classified into the Paniculatae of *Rustica*. However, this species is distinct from other Paniculatae species in some morphological and karyotypic features and is related to the Noctiflorae species in *Petunioides* (Goodspeed 1954). In this study, the chromosomal location of 5S rDNA (Fig. 2) and its position within the dendrograms (Fig. 5) were obviously different between *N. glauca* and the other two Paniculatae species. The 5S rDNA localization suggests that *N. glauca* is related to 2n=18 Alatae species (Fig. 2E, F). In addition, a 'non-consensus' sequence of *N. glauca* was clustered with a consensus sequence of *N. langsdorffii*, a representative 2n=18 species (Fig. 5). Bogani et al. (1997) reported on relationships of *N. glauca* with Paniculatae species rather than with Alatae and Noctiflorae species on the basis of RAPD analysis with total genomic DNA. Hence, it is tempting to speculate that a transfer of chromosomal segments including the 5S rDNA loci has occurred between the ancestor of *N. glauca* and that of 2n=18 Alatae species, and thereafter these 5S rDNA sequences have evolved independently.

Finally, the phylogenetic trees based on the spacer sequences of 5S rDNA were reflected by the chromosomal location of these genes. Further analysis using other marker genes will be helpful to clarify the evolutionary processes and genome reorganization during speciation within the genus *Nicotiana*.

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