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Molecular Systematics, GISH and the Origin of Hybrid Taxa in *Nicotiana* (Solanaceae)

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Phylogenetic relationships in the genus *Nicotiana* were investigated using parsimony analyses of the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (nrDNA). In addition, origins of some amphidiploid taxa in *Nicotiana* were investigated using the techniques of genomic *in situ* hybridization (GISH), and the results of both sets of analyses were used to evaluate previous hypotheses about the origins of these taxa. Phylogenetic analyses of the ITS nrDNA data were performed on the entire genus (66 of 77 naturally occurring species, plus three artificial hybrids), comprising both diploid and polyploid taxa, and on the diploid taxa only (35 species) to examine the effects of amphidiploids on estimates of relationships. All taxa, regardless of ploidy, produced clean, single copies of the ITS region, even though some taxa are hybrids. Results are compared with a published plastid (*matK*) phylogeny using fewer, but many of the same, taxa. The patterns of relationships in *Nicotiana*, as seen in both analyses, are largely congruent with each other and previous evolutionary ideas based on morphology and cytology, but some important differences are apparent. None of the currently recognized subgenera of *Nicotiana* is monophyletic and, although most of the currently recognized sections are coherent, others are clearly polyphyletic. Relying solely upon ITS nrDNA analysis to reveal phylogenetic patterns in a complex genus such as *Nicotiana* is insufficient, and it is clear that conventional analysis of single data sets, such as ITS, is likely to be misleading in at least some respects about evolutionary history. ITS sequences of natural and well-documented amphidiploids are similar or identical to one of their two parents—usually, but not always, the maternal parent—and are not in any sense themselves 'hybrid'. Knowing how ITS evolves in artificial amphidiploids gives insight into what ITS analysis might reveal about naturally occurring amphidiploids of unknown origin, and it is in this perspective that analysis of ITS sequences is highly informative.

Key words: *Nicotiana*, ITS, nuclear ribosomal DNA, cladistic analysis, cytology, hybridization, polyploidy.

INTRODUCTION

The family Solanaceae contains many taxa of importance, both agronomically (potatoes, tomatoes and peppers) and medicinally (mandrake, tobacco, deadly nightshade and henbane). Members of the family occur worldwide, but the highest species diversity is found in the Neotropics. *Nicotiana* L. is the fourth largest genus in the family after *Solanum* L., *Lycianthes* Hassl. and *Cestrum* Dunal, with 77 naturally occurring species (Table 1) distributed primarily in the Americas and Australia. Members of the genus are important in traditional medicine in both South America and Australia, and *N. tabacum* is one of the most widely used drug plants in the world. *Nicotiana* is one of the most comprehensively studied flowering plant genera with numerous studies having accumulated a large body of information concerning evolution, cytology, taxonomy and biogeography (East, 1928; Wheeler, 1935, 1945; Kostoff, 1943; Goodspeed, 1954; Horton, 1981; Purdie *et al*., 1982; Japan Tobacco Inc., 1994).

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Since the last authoritative monograph of *Nicotiana* (Goodspeed, 1954), no further taxonomic revisions have been undertaken. Goodspeed recognized 60 species in *Nicotiana*, and several new species from Australia, Africa and South America have been described since (see Table 1). Goodspeed's monograph was a comprehensive analysis of cytology, crossing relationships and morphology. He divided *Nicotiana* into three subgenera and 14 sections, based mostly on flower morphology, chromosome number and distribution (Table 1). In reconstructing the evolutionary history of the genus, Goodspeed postulated that two ancestral gene pools ('pre-petunioid' and 'pre-cestroid') had combined to give rise to two morphological lineages in modern *Nicotiana*: those resembling *Cestrum* on one hand and *Petunia* Juss. on the other. He emphasized the role of chromosome doubling and hybridization in the evolution of the genus and felt that his evidence supported the continued importance of genic rather than genomic (as suggested by Clausen *et al*., 1945) evolution in amphidiploid lineages. Although Goodspeed did not explicitly indicate sister-group relationships, he did hypothesize the derivation of modern groups from one another or unspecified ancestral taxa. Important relationships identified by Goodspeed are:

TABLE 1. *Classification of* Nicotiana *according to Goodspeed (1954)*

Taxon	*n*	Geographical distribution
Nicotiana subgenus *Rustica* (Don) Goodsp.		
Nicotiana section *Paniculatae* Goodsp.		
Nicotiana glauca Graham	12	NW and C Argentina
Nicotiana paniculata L.	12	W Peru
Nicotiana knightiana Goodsp.	12	S Peru (coast)
Nicotiana solanifolia Walp.	12	N Chile (coast)
Nicotiana benavidesii Goodsp.	12	Peru
Nicotiana cordifolia Phil.	12	Chile, Masafuera
Nicotiana raimondii J.F.Macbr.	12	Peru, Urubamba valley
Nicotiana cutleri D'Arcy	12	S Bolivia
Nicotiana section *Thyrsiflorae* Goodsp.		
Nicotiana thyrsiflora Bitter ex Goodsp.	12	Peru, Marañon valley
Nicotiana section *Rusticae* Goodsp.		
Nicotiana rustica L.	24	SW Ecuador to Bolivia
Nicotiana Subgenus *Tabacum* (Don) Goodsp.		
Nicotiana section *Tomentosae* Goodsp.		
Nicotiana tomentosa Ruiz & Pavón	12	S and C Peru, W Bolivia
Nicotiana tomentosiformis Goodsp.	12	Bolivia
Nicotiana otophora Griseb.	12	C–S Bolivia, NW Argentina
Nicotiana setchellii Goodsp.	12	N Peru (Chachapoyas)
Nicotiana glutinosa L.	12	N, C Peru, S Ecuador
Nicotiana kawakamii Y.Ohashi	12	Bolivia
Nicotiana section *Genuinae* Goodsp.		
Nicotiana tabacum L.	24	Cultivated
Nicotiana Subgenus *Petunioides* (Don) Goodsp.		
Nicotiana section *Undulatae* Goodsp.		
Nicotiana undulata Ruiz & Pavón	12	N Peru–NW Argentina
Nicotiana arentsii Goodsp.	24	SW Peru–NW Bolivia (Puno and La Paz)
Nicotiana wigandioides Koch & Fintelm.	12	Bolivia
Nicotiana section *Trigonophylleae* Goodsp.		
Nicotiana obtusifolia M. Martens & Galeotti (syn: *N. trigonophylla* Dunal)	12	SW USA, Mexico
Nicotiana palmeri A.Gray	12	SW USA
Nicotiana section *Alatae* Goodsp.		
Nicotiana sylvestris Speg. & Comes	12	NW Argentina, Bolivia
Nicotiana langsdorfii Weinm.	9	Brazil–Uruguay–Argentina
Nicotiana alata Link & Otto	9	Uruguay-Brazil and Argentina
Nicotiana forgetiana Hemsl.	9	SE Brazil
Nicotiana bonariensis Lehm.	9	SE Brazil, Argentina–Uruguay
Nicotiana longiflora Cav.	10	Uruguay-Brazil and Bolivia
Nicotiana plumbaginifolia Viv.	10	Andes–NW Argentina
Nicotiana azambujae L.B.Sm. & Downs	?	Santa Catarina, Brazil
Nicotiana mutabilis Stehmann & Semir	9	Rio Grande do Sul, Brazil
Nicotiana section *Repandae* Goodsp.		
Nicotiana repanda Willd.	24	Texas, Mexico, Cuba
Nicotiana stocktonii Brandegee	24	Mexico (Revillagigedo Isl.)
Nicotiana nesophila I.M.Johnst.	?	Mexico (Revillagigedo Isl.)
Nicotiana section *Noctiflorae* Goodsp.		
Nicotiana noctiflora Hooker	12	N Argentina–NW Chile
Nicotiana petunioides (Griseb.) Millán	12	W Argentina, N Chile
Nicotiana acaulis Speg.	12	Patagonia
Nicotiana ameghinoi Speg.	?	Patagonia
Nicotiana paa Martínez Crovedo	12	N Argentina
Nicotiana section *Acuminatae* Goodsp.		
Nicotiana acuminata (Graham) Hooker	12	Chile, Andes of Argentina
Nicotiana pauciflora Remy	12	Coastal Chile
Nicotiana attenuata Torr. ex S.Watson	12	W USA, Baja California
Nicotiana corymbosa Remy	12	Coastal ranges and Andes of C Chile and adjacent Argentina
Nicotiana longibracteata Phil.	?	Andes of N Argentina and Chile
Nicotiana miersii Remy	12	Chile
Nicotiana linearis Phil.	12	Argentina–Chile
Nicotiana spegazzinii Millán	12	CE Argentina
Nicotiana section *Bigelovianae* Goodsp.		
Nicotiana quadrivalvis Pursh (syn: *N. bigelovii* (Torr.) Wats.)	24	W USA and adjacent Mexico
Nicotiana clevelandii A.Gray	24	Baja California, California and Arizona

TABLE 1 *Continued*

Taxon	*n*	Geographical distribution
Nicotiana section *Nudicaules* Goodsp.		
Nicotiana nudicaulis S.Watson	24	NE Mexico
Nicotiana section *Suaveolentes* Goodsp.		
Nicotiana suaveolens Lehm.	16 (32)	SE Australia
Nicotiana maritima H.-M.Wheeler	16	SE Australia
Nicotiana velutina H.-M.Wheeler	16	SE, C Australia
Nicotiana gossei Domin	18	C Australia
Nicotiana excelsior (J.M.Black) J.M.Black	19	SW Australia
Nicotiana megalosiphon VanHuerck & Müll.Arg.	20	E Australia
Nicotiana exigua H.-M.Wheeler	16	S Queensland
Nicotiana rosulata (S. Moore) Domin	20	S and E Australia
Nicotiana goodspeedii H.-M.Wheeler	20	S Australia
Nicotiana ingulba J.M.Black	20	C, SW and W Australia
Nicotiana stenocarpa H.-M.Wheeler	20	SW Australia
Nicotiana occidentalis H.-M.Wheeler	21	NW and S Australia
Nicotiana rotundifolia Lindl.	22	SW Australia
Nicotiana debneyi Domin	24	Coast E Australia, New Caledonia
Nicotiana benthamiana Domin	19	NC and NW Australia
Nicotiana fragrans Hooker	24	S Pacific
Nicotiana umbratica N.T.Burb.	23	W Australia
Nicotiana cavicola N.T.Burb.	20, 23	W Australia
Nicotiana amplexicaulis N.T.Burb.	18	S Queensland, Australia
Nicotiana hesperis N.T.Burb.	21?	Coastal W Australia and islands
Nicotiana simulans N.T.Burb.	20	Coastal W Australia to New South Wales
Nicotiana burbidgeae Symon	21	S Australia
Nicotiana heterantha Kenneally & Symon	24	W Australia
Nicotiana wuttkei Clarkson & Symon	14	Queensland
Nicotiana truncata Symon ined.	?	W Australia
Nicotiana 'eastii' Kostoff	32	SE Australia
Nicotiana africana Merxm.	23	Namibia
Synthetic amphidiploid and known hybrid species of *Nicotiana*		
Nicotiana × *digluta*	24	*N. glutinosa* × *N. tabacum*
Nicotiana × *didepta*	24	*N. debneyi* × *N. tabacum*
Nicotiana × *sanderae* Hort. ex Wats.		*N. alata* × *N. forgetiana*

If nomenclatural changes have been made to species commonly encountered in the literature, the previous synonym name is given in parentheses.

Chromosome numbers and distributions are taken from Goodspeed (1954), Merxmüller and Buttler (1975), Purdie *et al*. (1982) and Japan Tobacco Inc. (1994).

N. section *Alatae* with the Australian taxa (section *Suaveolentes*); *N.* section *Rusticae* with *N.* section *Paniculatae*; and *N.* sections *Tomentosae* and *Undulatae* with *N.* section *Paniculatae*. He suggested that hybridization was frequent, thus making resolution of relationships difficult (Fig. 1A and B). He concluded that the entire genus was composed of species either on primary (n = 12) or secondary (n = 24) polyploid levels derived from an ancestral, extinct, six-paired (n = 6) taxon and believed that the combination of amphiploidy and gradual genetic differentiation was critical to explain present distribution. Thus n = 12 'diploid species' were hypothesized to be even more ancient amphiploids. Goodspeed even proposed a future scenario for evolution of *Nicotiana*, in which narrowly endemic diploid species would become extinct, and the number of species of higher ploidy would increase.

Many species of *Nicotiana* are polyploid with n = 24, having arisen from amphidiploidy (e.g. Parokonny *et al*., 1992*b*; Lim *et al*., 2000*a*). Goodspeed hypothesized probable parental gene pools of the existing amphidiploids based on analyses of karyotypes and morphology. Thus, in addition to the scenario of phylesis (see Fig. 1A and B), he postulated specific origins for polyploid taxa: section *Suaveolentes* involving one parent from *N.* section *Alatae*; *N. tabacum* from species within *N.* sections *Tomentosae* and *Alatae*; and *N. rustica* from *N. undulata* and a member of *N.* section *Paniculatae*.

Goodspeed (1954) identified several problematic taxa for which he was uncertain of placement or evolutionary history. He placed *N. glauca* in *N.* section *Paniculatae* emphasizing its 'separate evolution' (Goodspeed, 1954: 339) and *N. glutinosa* in *N.* section *Tomentosae*, although he noted its apparent mixture of traits characteristic of other sections. He also voiced doubt as to the exact parentage of the Australian taxa (*N.* section *Suaveolentes*, but see below), stating that they were obviously, in part, derived from an 'alatoid' line, but that both *N.* sections *Acuminatae* and *Noctiflorae* were likely sources of the other parental gene pools.

The geographical distribution of *Nicotiana* is intriguing, and for a long time it was thought to be confined to three continents: North and South America and Australia. The

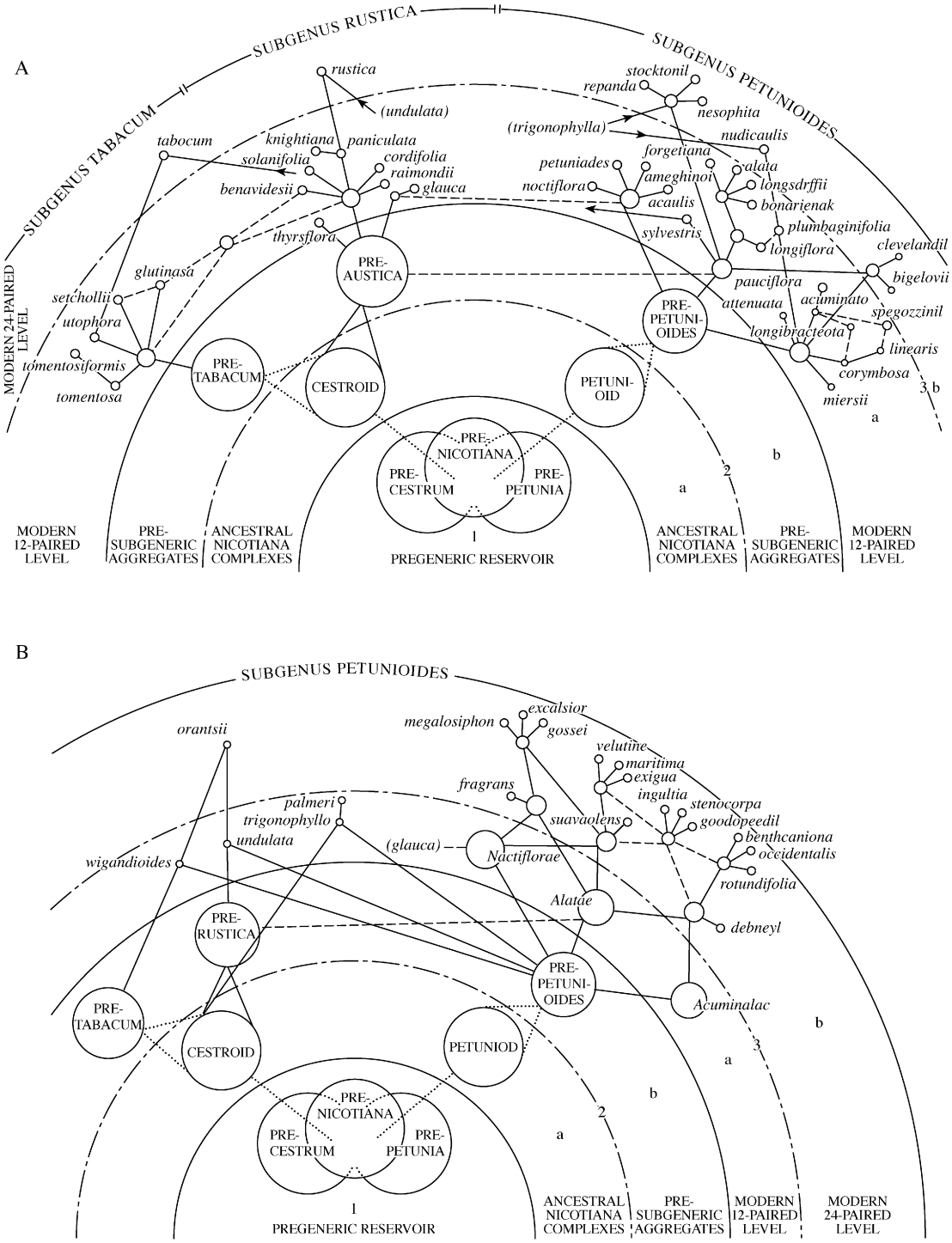


FIG. 1. Phyletic diagram of the genus *Nicotiana* (from Goodspeed, 1954). A, *Nicotiana* subgenera *Tabacum*, *Rustica* and *Petunioides*; B, *N.* subgenus *Petunioides*, expanded.

discovery of *N. africana* in Namibia (Merxmüller and Buttler, 1975) prompted a re-evaluation of biogeographic patterns. Morphological and cytological evidence point to an origin of the genus in South America (Goodspeed, 1954), followed by subsequent long-distance dispersals to explain current distribution patterns. Olmstead and Palmer (1991) suggested that the Australian species of *Nicotiana* (*N.* section *Suaveolentes*) are a recent radiation resulting

from a single colonization rather than vicariance. They based this conclusion on the lack of variation shown in plastid DNA restriction sites in the Australian species, which means that they could only have arrived recently. A vicariant pattern should involve much greater levels of variability and a less derivative phylogenetic placement.

The use of molecular systematic techniques in Solanaceae has been concentrated at the family level (e.g. Olmstead and Sweere, 1994; Fay *et al*., 1998; Olmstead *et al*., 1999) and in the genus *Solanum* (e.g. Spooner *et al*., 1993, Bohs and Olmstead, 1997, 1999; Olmstead and Palmer, 1997; Peralta and Spooner, 2001). Despite its economic importance and the use of *N. tabacum* as a model organism (for its complete plastid genome sequence see, Shinozaki *et al*., 1986), the species level phylogeny of *Nicotiana* has only been of interest recently (preliminary ITS sequences, Komarnitsky *et al*., 1998*a*; 5S nuclear ribosomal spacer sequences, Komarnitsky *et al*., 1998*b*, Kitamura *et al*., 2001; *matK* plastid DNA sequences, Aoki and Ito, 2000).

A similar study was undertaken, using sequences of the internal transcribed spacers of nuclear ribosomal DNA (ITS nrDNA) to evaluate species relationships and, hence, provide a phylogenetic framework from which to assess competing theories of speciation and geographical distribution. The analysis carried out is the most complete to date and includes 66 of the 74 naturally occurring species of *Nicotiana*, leaving no section unsampled. ITS is often a useful tool to investigate origins of amphidiploids because it can provide clear evidence of parentage (Kim and Jansen, 1994; Wendel *et al*., 1995; Franzke and Mummenhoff, 1999), but interpretation of results depends upon a clear understanding of how it is evolving (Mummenhoff *et al*., 1995; Wendel *et al*., 1995). Although part of the nuclear genome and thus, in theory, inherited biparentally, in many taxa including *Nicotiana* only one ITS copy is retained due to rapid gene conversion (see Lim *et al*., 2000*a*). For this reason, three artificial amplidiploids of known parentage were included in the analysis to determine how ITS is evolving within *Nicotiana*. If many taxa within *Nicotiana* are hybrids, as hypothesized by Goodspeed, then use of a region that evolves through conversion of one parental copy to that of the other is likely to be misleading if there is no other information available about parentage.

The molecular cytogenetic technique of genomic *in situ* hybridization (GISH) has shed new light on the origins and status of cryptic hybrid taxa (see Bennett, 1995). This technique employs fluorescently labelled DNA probes to 'paint' metaphase preparations of taxa of interest (Parokonny *et al*., 1992*a*, *b*; Kenton *et al*., 1993; Parokonny and Kenton, 1995). GISH has been used to answer a wide range of questions about genome relationships, origins of hybrid taxa and evolution. This technique has been used to elucidate the complex origins of *N. tabacum* (Kenton *et al*., 1993; Volkov *et al*., 1999) and construct a chromosomal phylogeny of *N.* section *Tomentosae* (Lim *et al*., 2000*a*), but has not been used broadly in the genus as a systematic tool in conjunction with phylogenetic techniques.

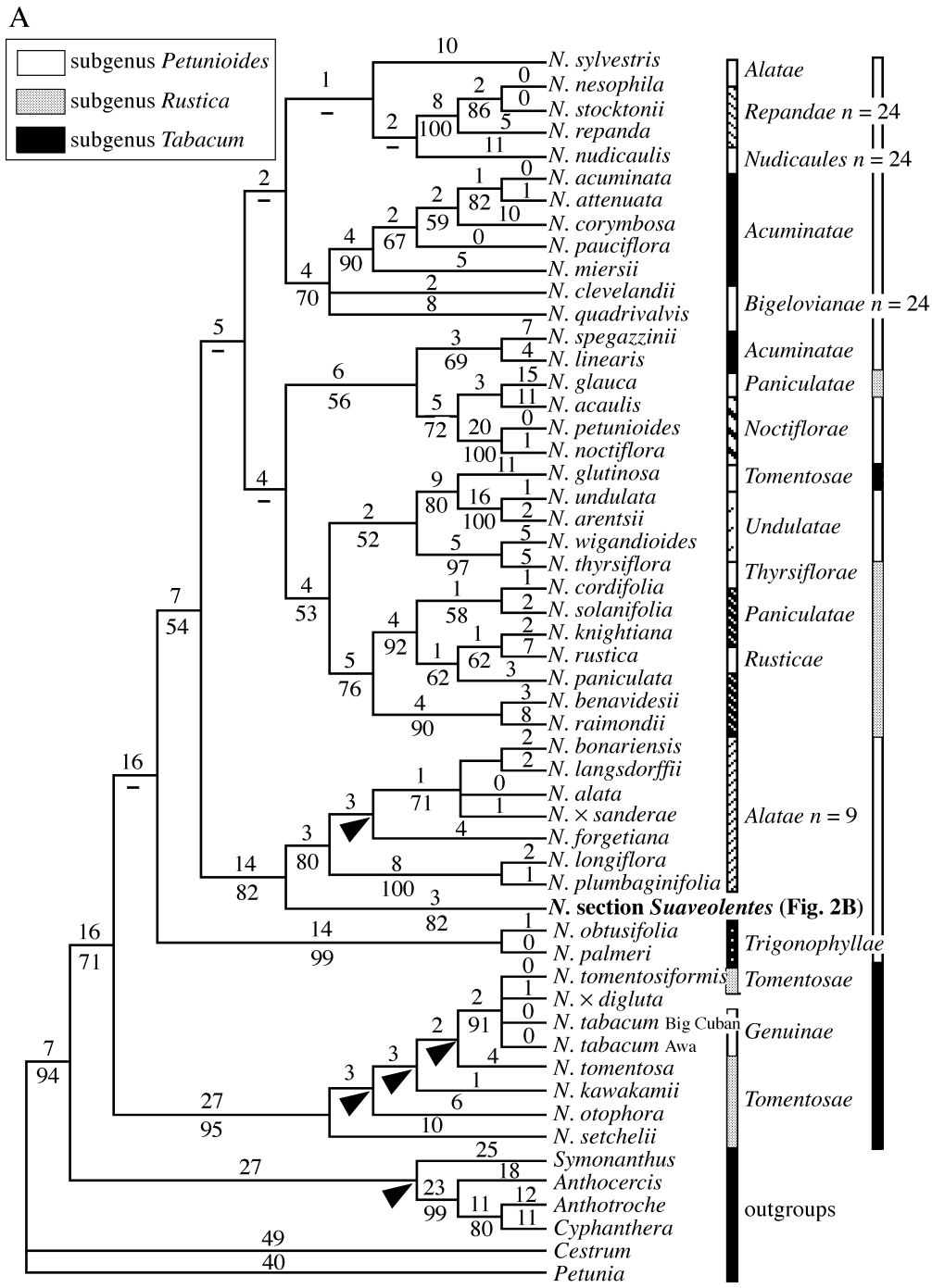
The breadth of Goodspeed's (1954) monograph allows clear and unambiguous evaluation of hypotheses of species relationships, speciation and geographical distribution. The application of new data to problems, highlighted by Goodspeed, provides a good opportunity to assess whether molecular techniques may resolve some of these ambiguous areas. Using ITS nrDNA sequences and comparison with a published plastid phylogeny (Aoki and Ito, 2000) and preliminary GISH experiments, the focus of this study was on (*a*) relationships of the species in *Nicotiana* and composition of sections or monophyletic groups and (*b*) origins of the amphidiploid taxa. Pairwise evaluations of genomic relationships among all *Nicotiana* species are not practical at present, so a restricted set of Goodspeed's hypotheses, concerning origins of some putative amphidiploid taxa, was examined: (*a*) *N. rustica* is a simple amphidiploid of *N. undulata* and *N. paniculata* (Goodspeed, 1954: 288); (*b*) *N. arentsii* is a simple amphidiploid of *N. undulata* and *N. wigandioides* (Goodspeed, 1944, 1954: 290); (*c*) *N. tabacum* is an complex amphidiploid involving *N. sylvestris* and *N. tomentosa* genomes (Goodspeed, 1954: 290; see also Kenton *et al*., 1993); (*d*) the members of *N.* section *Bigelovianae* originated from amphidiploidy involving *N. attenuata* and an 'alatoid' progenitor (Goodspeed, 1954: 293); (*e*) 'alatoid' and 'acuminatoid' progenitors (i.e. ancestral members of *N.* sections *Alatae* and *Acuminatae*) were involved in the origin of the entirely polyploid *N.* section *Suaveolentes* (Goodspeed, 1954: 294); and (*f*) amphiploidy involving an 'alatoid' progenitor gave rise to the members of *N.* section *Repandae* (Goodspeed, 1954: 291).

MATERIALS AND METHODS

Plants were grown in the glasshouses at the Royal Botanic Gardens, Kew, from which herbarium vouchers and DNA samples were prepared (Appendix). DNA sequences of the nuclear ribosomal spacers ITS1 and ITS2, together with the 5.8S ribosomal gene, were determined for 70 accessions of *Nicotiana* and four species in four genera of the Australian endemic tribe, Anthocercidae, which has been shown to be the taxon closest to *Nicotiana* (Olmstead *et al*., 1999). Species from *Cestrum* and *Petunia* were used as the ultimate outgroup, based on currently available plastid DNA phylogenetic studies of Solanaceae (Olmstead *et al*., 1999). In addition to 66 of the 74 naturally occurring species (Table 1), the synthetic amphidiploids *N. didepta* and *N. digluta* (Clausen and Goodspeed, 1925; Clausen, 1928) and the cultivar *N. sanderae* (Table 1) were included in our analyses. It was not possible to obtain material of *N. ameghinoi*, which is not known in cultivation and which has an extremely limited range in its native habitat, or other recently described species (Table 1; Appendix). Accessions of two different strains of *N. tabacum* were included to look for differences in ITS within cultivated material (there were none, but both are included in Fig. 2A). Two accessions of *N. sylvestris* and *N. tomentosiformis* (the latter from both the valleys from which it is known) were also examined, but again there were no differences in the ITS sequences produced (only one was included in the figures). Instead of

using accessions from gene banks, as much material as possible was collected in the wild (Table 1), which should be preferred over accessions of unknown wild origin that have been maintained for a long period of time in cultivation.

DNA was extracted according to the modified 2× CTAB method of Doyle and Doyle (1987). The ITS DNA region was amplified in one piece using the primers described by Baldwin (Baldwin, 1993; Baldwin *et al*., 1995) or Sun *et al*. (1994). $MgCl_2$ was used at 25 mM with 0·4 % bovine serum albumin. Many different ITS copies were amplified from some species unless 2·0–4·0 % DMSO was included. The PCR protocol followed was: 94 °C pre-melt for 3 min, followed by 28 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 3 min, followed by a single 7 min extension. Amplified DNA was purified using 'Wizard' mini-columns (Promega, West Crawley, UK) according to the manufacturer's protocols and sequenced directly on an ABI 377 automated sequencer (Applied Biosystems, Inc., Warrington, UK) using standard fluorescent dye-terminator



chemistry, also according to the manufacturer's protocols. In the cycle sequencing reactions, 2 % DMSO was included because GC-rich regions of these ITS sequences resulted in premature termination of most strands within 100–150 base pairs (bp) of the initiation point. Sequences were determined for both DNA strands, and each base position was individually examined for agreement of the two strands. DNA sequences have been submitted to GenBank; accession numbers are given in the Appendix.

DNA sequences were aligned by eye after an initial alignment was created with ClustalW for Power Macintosh (Thompson *et al*., 1995). Gaps were coded as missing. Regions of insertion/deletion ('indel') activity were few within *Nicotiana*, and all sequence data were included in the analysis except for the 120 bases (for those using the Sun *et al*. primers, which amplify a longer fragment including more of the 18S and 26S rDNA genes) or 20 bases (for those amplified with the Baldwin primers) at the beginning and end of the matrix (these were not present in all taxa and so were excluded from the analysis). The aligned matrix is available electronically from the first author (m.chase@rbgkew.org.uk).

All parsimony analyses were undertaken using PAUP version 4.0b8 (Swofford, 2001). The complete data matrix was analysed initially using 1000 replicates of random taxon-addition order, tree-bisection-reconnection (TBR) branch swapping, MulTrees (keeping multiple, equally parsimonious trees), and with all character transformations treated as equally likely and unordered (Fitch parsimony; Fitch, 1971). Ten trees only were saved from each replicate to minimize the time searching on sub-optimal 'islands' (Maddison, 1991) with potentially thousands of trees. All trees thus collected were combined and used as starting trees, with MulTrees on and no tree limit (these trees were then swapped to completion). Internal support was assessed using 500 bootstrap replicates (Felsenstein, 1985) with TBR swapping but permitting only ten trees per replicate to be held. A second analysis using the same methodology was conducted with only the diploid species of *Nicotiana* included; this was done to examine the effects of removing known hybrids, natural as well as artificial.

In situ hybridization experiments were carried out according to conditions and protocols described by Parokonny *et al*. (1992*b*). Owing to problems with permanency of the chromosome prepartions, each could only be used for one hybridization and, hence, although it is considered that each putative parent hybridizes to a complementary set of chromosomes, it is only through careful examination of chromosome morphology that this can be determined. Our preparations are not as technically excellent as would be desired and thus can only be tentatively used to confirm parentages proposed by Goodspeed (1954) and the results of the ITS presented here and *matK* produced by Aoki and Ito (2000).

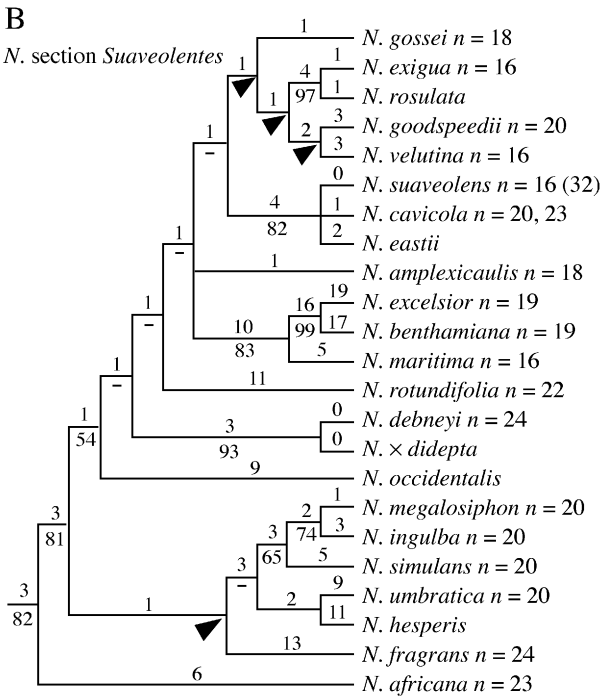


FIG. 2. One of the most parsimonious, all-taxon trees showing cladistic relationships in *Nicotiana*. Shaded bars indicate Goodspeed's (1954) taxonomic categories. Branch lengths (ACCTRAN optimization) are indicated above the branches and bootstrap percentages below (any clade with a hyphen has BP <50). An arrowhead indicates nodes collapsing in the strict consensus of all most-parsimonious trees. A, All taxa excluding *N.* section *Suaveolentes*; B, *N.* section *Suaveolentes*.

RESULTS

PCR amplifications

PCR-amplified DNA fragments from these *Nicotiana* species and hybrids showed a clean, single band when examined on 1·4 % agarose gels. The artificial hybrids in particular were closely examined, and no evidence for additional or polymorphic bands was detected. The ITS nrDNA sequences varied in length from 647 to 696 bp.

Cladistic analyses

Alignment of all 76 DNA sequences yielded 670, included nucleotide positions of which 294 positions (44 %) were variable and 181 (27 %) were potentially parsimony informative (some would end up being found to be parallelisms in two or more species and thus not informative). Analysis produced more than 27 000 equally most parsimonious trees of 767 steps with a consistency index (CI) (autapomorphies are included throughout the paper) = 0·54 and a retention index (RI) = 0·72. One of these trees is shown in Fig. 2. Sidebars indicate subgeneric groups defined by Goodspeed (1954). Numbers above branches indicate estimated numbers of substitutions (ACCTRAN optimization); bootstrap percentages are indicated below branches. Arrowheads indicate nodes collapsing in the strict consensus of all most parsimonious trees. A single tree is shown so that relative levels of sequence divergence can be observed; this is not meant to imply that this topology is favoured over others of the same tree length.

All shortest trees, regardless of the analysis, indicate that *Nicotiana* is monophyletic, but this result receives a

bootstrap percentage (BP) of only 71. The genera of Anthocercidae, *Anthocercis* Labill., *Anthotroche* Endl., *Cyphanthera* Miers and *Symonanthus* Haegi are clearly the least diverged taxa from *Nicotiana*, but they are considerably more divergent from any species of *Nicotiana* than any of the latter is from other congeneric species, thus also supporting the idea that *Nicotiana* is monophyletic.

Although not entirely consistent, the strict concensus tree contained many clades that are highly similar to the subgeneric groups originally identified by Goodspeed (1954). *Nicotiana* sections *Trigonophyllae* and *Undulatae* were monophyletic in all the shortest trees. Clades in the ITS trees correspond directly to *N.* sections *Suaveolentes* (BP 82; including *N. africana* and the artificial hybrid *N. didepta*), to which *N.* section *Alatae* (BP 80, but excluding *N. sylvestris*) is sister (BP 82), *Repandae* (BP 100) to which *N. nudicaulis* and *N. sylvestris* are successive sister species (but each BP <50), *Noctiflorae* (BP 72) in which *N. glauca* (*N.* section *Paniculatae sensu* Goodspeed) is embedded (BP 51), *Paniculatae* (BP 76; including *N. rustica*, but excluding *N. glauca*) and *Tomentosae* (excluding *N. glutinosa*, but including *N. tabacum* and the synthetic amphidiploid *N. digluta*; BP 91). The two species of *N.* section *Bigelovianae*, *N. clevelandii* and *N. quadrivalvis*, are unresolved in most trees, but their monophyly is not refuted. *Nicotiana* section *Tomentosae* (excluding *N. glutinosa*) are supported (BP 99) as the sister of the remaining sections, but most of the rest of the spine of the tree receives BP <54. None of the three subgenera (*sensu* Goodspeed, 1954) is monophyletic (Fig. 2A), although a single misplaced species accounts for this in all cases. *Nicotiana thyrsiflora*, the sole member of Goodspeed's *N.* section *Thyrsiflorae*, falls within *N.* section *Undulatae* as sister to *N. wigandioides*. *Nicotiana thyrsiflora* and *N. glauca* were considered members of *N.* subgenus *Rusticae*, but both as well as the rest of *N.* subgenus *Rusticae* are embedded within *N.* subgenus *Petunioides* (Fig. 2A). *Nicotiana* subgenus *Tabacum* is monophyletic, except for *N. glutinosa*, which falls in *N.* subgenus *Petunioides*. Bootstrap percentages within the major clades are low, which is caused by the generally low levels of divergence detected (e.g. a hard polytomy is present in *N.* section *Alatae*).

Removal of the polyploids (which are hypothesized to be amphidiploids) as well as the artificial hybrids had little effect on the clades of diploids found in the shortest trees (Fig. 3). In this analysis, only 283 positions were variable, of which 144 (21 %) were potentially parsimony informative. The 140 shortest Fitch trees found in this analysis had 533 steps with CI = 0·64 and RI = 0·61. Overall, removal of hybrids and polyploids changed estimates of relationships little; the spine of the tree is largely resolved but still with BP <50 due to the short branches there.

Patterns of bootstrap support differed in some respects between the full and diploid cladograms. Support for *N.* section *Alatae* (*sensu* Goodspeed, 1954, but excluding *N. sylvestris*) was much higher in the diploid tree (BP 82 *vs.* 95). *Nicotiana* section *Acuminatae* (without *N. linearis* and *N. spegazzinii*) has also somewhat higher levels of support in the diploid tree (BP 90 *vs.* 98). *Nicotiana* section *Tomentosae* was clearly separated from the other species in both analyses (BP 95).

In all analyses, species of Goodspeed's *N.* section *Acuminatae* fell into two separate clades. The first, composed of *N. spegazzinii* and *N. linearis* (BP 69 in the all-species tree), is sister (BP 50) to *N. glauca* plus *N.* section *Noctiflorae,* and the clade (BP 70) with the rest of the section additionally contains *N. clevelandii* and *N. quadrivalis* of Goodspeed's *N.* section *Bigelovianae*. Most of the species of *N.* section *Paniculatae* form a clade (BP 77) in which *N. rustica* is embedded; *N. arentsii*, *N. undulata* and *N. wigandioides* of *N.* section *Undulatae, N. thyrsiflora* of *N.* section *Thyrsiflorae* and *N. glutinosa* of *N.* section *Tomentosae* are sister to *N.* section *Paniculatae* (BP 56).

To begin with, it was imagined that there could be several scenarios for how ITS might be evolving in hybrids, and it was hoped that by looking at artificial and known natural hybrids some possibilities could be eliminated. The patterns of substitution in artificial and natural hybrids (e.g. *N. digluta*, *N. tabacum*, etc.) were examined and it was found that, in all cases, hybrids produced ITS sequences that were identical, or nearly so, to one of their putative parental species. For example, the ITS of *N. tabacum* was identical to that of *N. tomentosiformis* of *N.* section *Tomentosae*, which had been thought to be a parent (see below). Likewise, the artificial hybrid *N. digluta* [*N. glutinosa* (maternal) × *N. tabacum* (paternal); see Clausen and Goodspeed, 1925; Clausen, 1928)] has an ITS sequence identical to that of the two accessions of *N. tabacum*, its paternal parent with which it is strongly supported to have a relationship (BP 91). *Nicotiana didepta* is a cross between *N. tabacum* (maternal) and *N. debneyi* (paternal), and it also has an ITS sequence identical to that of its paternal parent (Fig. 2B). All hybrid taxa, for which parents are known, exhibit a similar pattern of falling with one parent in a derived position and not near the base of a clade, in what could be considered a hybrid or intermediate position. Gene conversion is apparently homogenizing ITS in hybrids, artificial as well as natural, and this process involves the loss of one parental copy, at least as detected with PCR, and not the creation of a mosaic or hybrid sequence.

GISH

The results of all GISH experiments are summarized in Table 2 and some of these are shown in Fig. 4. These experiments are preliminary and included here purely as pictorial corroboration of some of Goodspeed's hypotheses. These were also completed before the ITS sequencing was finished and long before the results of the *matK* study were published (Aoki and Ito, 2000), so there are some obvious GISH experiments that have not been undertaken because the GISH phase of the work had been concluded by then. Much more detailed cytogenetic analysis of chromosome compliments will be undertaken in future studies.

Simple amphidiploids: N. rustica*,* N. arentsii *and* N. tabacum. GISH provides evidence that supports the

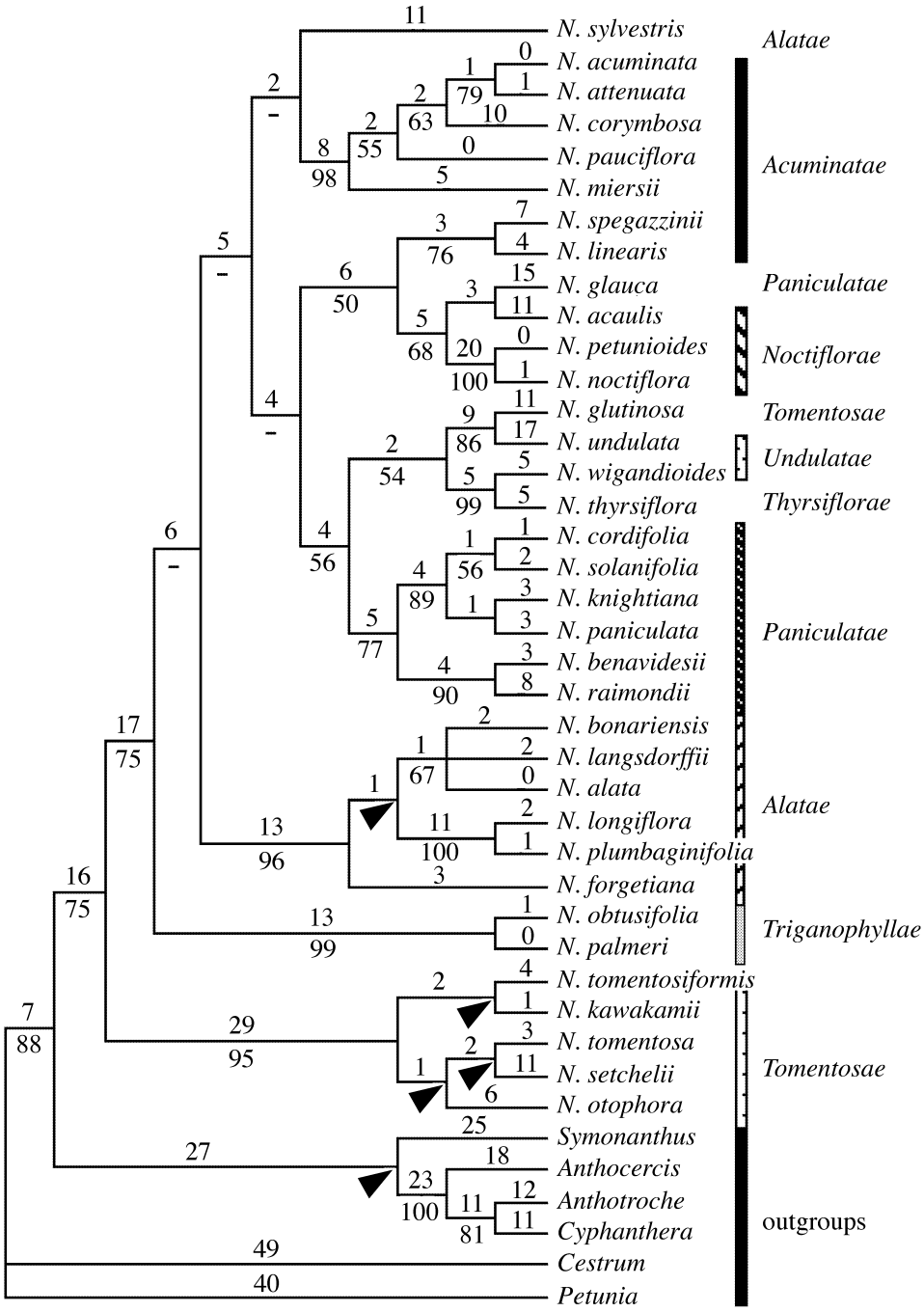


FIG. 3. One of the most parsimonious diploid-only trees showing relationships in *Nicotiana*. Shaded bars indicate Goodspeed's (1954) taxonomic categories, subgeneric membership as in Fig. 2. Branch lengths (ACCTRAN optimization) are indicated above the branches and bootstrap percentages below (any clade with a hyphen has BP <50). An arrowhead indicates nodes collapsing in the strict consensus of all most-parsimonious trees.

hypothesis that *N. rustica* is an amphidiploid resulting from hybridization of *N. undulata* and *N. paniculata*. Genomic DNA probes of both *N. undulata* and *N. paniculata* labelled putatively complimentary chromosome sets in *N. rustica* (Fig. 4A and B).

Nicotiana arentsii ($n = 24$) is morphologically intermediate between the diploids, *N. undulata* and *N. wigandioides* (Goodspeed, 1954), and GISH fully supports Goodspeed's (1954) hypothesis that it is an amphidiploid derived from these two species. Genomic DNA of *N. undulata* and *N. wigandioides* labelled apparently complementary chromosome sets in this taxon (Fig. 4C and D).

The hypothesis that *N. tabacum* is an amphidiploid of *N. tomentosiformis* and *N. sylvestris*, with additional genomic contributions from *N. otophora* (Kenton *et al.*, 1993), is supported by our GISH results (not shown;

TABLE 2. *Summarized results of genomic* in situ *hybridization (GISH) experiments*

	N. paniculata	*N. raimondii*	*N. tomentosa*	*N. tomentosiformis*	*N. otophora*	*N. undulata*	*N. wigandioides*	*N. obtusifolia*	*N. palmeri*	*N. sylvestris*	*N. alata*	*N. longiflora*	*N. plumbaginifolia*	*N. attenuata*	*N. linearis*
N. rustica	+					+									
N. tabacum			+	+	+					+					
N. arentsii		–				+	+								
N. repanda								+	+	–		–	–	–	
N. stocktonii								+	+	–		–	–	–	
N. bigelovii										+				+	
N. clevelandii										+				+	
N. nudicaulis								+	+	+		–	–	–	
N. africana												+	+	–	
N. cavicola										–				–	
N. debneyi										–	–	+	+	–	–
N. gossei											–	+	+		
N. maritima												+/?	+/?	–	
N. velutina										–	–	+/?	+/?	–	
N. excelsior										–	–	+	+		–

+, Probe DNA binds differentially; –, no differential probe labelling; '?', poorly defined or equivocal probe DNA binding.

Empty cells indicate test not performed.

Rows are metaphase preparations; columns are probe DNA.

Table 2). Genomic DNA of *N. otophora* and *N. tomentosa* (both *N.* section *Tomentosae*) also labelled chromosomes of *N. tabacum* but to a lesser degree than that of *N. tomentosiformis* (Table 2).

Amphidiploid species complexes: N. *sections* Bigelovianae*,* Suaveolentes *and* Repandae. Labelled genomic DNA of *N. attenuata* hybridized to one of the genomes of both *N. clevelandii* and *N. quadrivalvis* (Fig. 4E and F). Labelled genomic DNA of *N. sylvestris* (*N.* section *Alatae sensu* Goodspeed but not placed there by the ITS analyses) hybridized to the other genome in *N. clevelandii* and *N. quadrivalvis* (Table 2).

The results reveal clear participation of an 'alatoid' genome in the amphidiploid ancestor of *N.* section *Suaveolentes* (including *N. africana*). GISH using *N. plumbaginifolia* and *N. longiflora* genomic DNA clearly distinguishes one of the two ancestral genomes present in a range of Australian species and *N. africana*, but probes consisting of DNA from two species in *N.* section *Acuminatae* (*N. attenuata* and *N. linearis*) failed to label chromosomes in any of the amphidiploid genomes examined (Table 2).

Extensive GISH experiments employing genomic probes from members of *N.* section *Alatae sensu* Goodspeed (see Table 2) fail to support the hypothesis that a species from this section was involved in the origins of section *Repandae*. Instead, genomic DNA of both *N. palmeri* and *N. obtusifolia* (chosen because of their North American distribution) showed diffuse labelling throughout the genome of all three species of *N.* section *Repandae* (Table 2).

DISCUSSION

Goodspeed (1954) based his taxonomic scheme for *Nicotiana* upon a good deal of cytological information (i.e. genome organization as could best be inferred at that time), and thus it should not be too surprising that his classification and the ITS trees are in fairly close agreement. However, ITS is following this process in a rather idiosyncratic manner. It seems that ITS in the amphidiploid species in this study is evolving as if it were not part of the diploid nuclear genome; after hybridization and subsequent episodes of meiosis, ITS is clearly like that of one of its two parents in the same way that organellar markers are, except that the parent favoured varies. Organellar markers

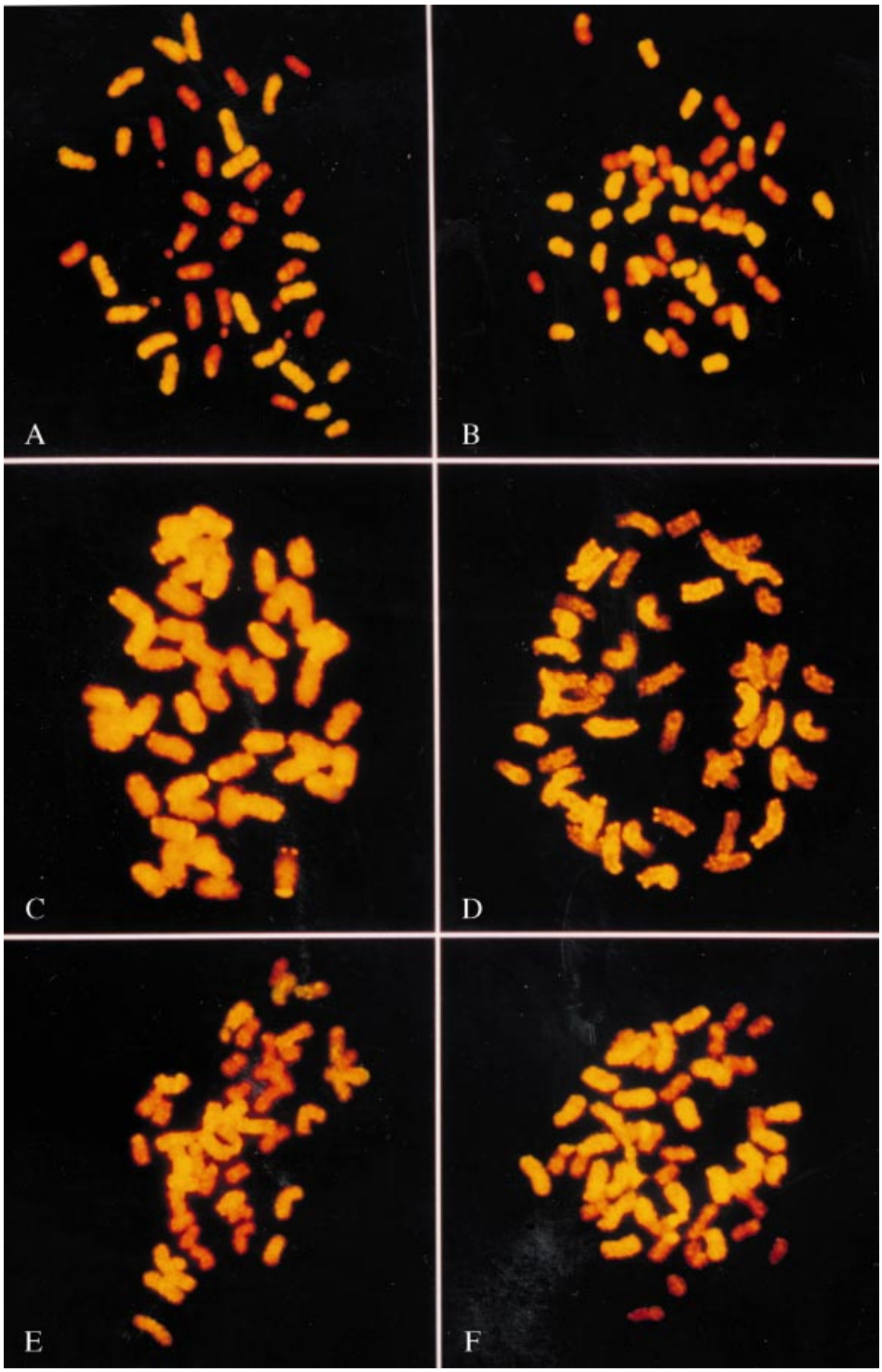


FIG. 4. Genomic *in situ* hybridization to metaphases of *Nicotiana* species. In all parts, yellow fluorescence indicates hybridization to the probe, whereas unlabelled chromatin fluoresces red with propidium iodide counterstain. In each of the three pairs of GISH experiments, putatively complementary sets of chromosomes (12) are labelled by each of the parental diploids suggested by Goodspeed (1954). A, *Nicotiana rustica* probed with *N. paniculata* DNA; B, *N. rustica* probed with *N. undulata* DNA; C, *N. arentsii* probed with *N. undulata* DNA; D, *N. arentsii* probed with *N. wigandioides* DNA; E, *N. quadrivalvis* probed with *N. attenuata* DNA; F, *N. clevelandii* probed with *N. attenuata* DNA.

most commonly match consistently those of one parent, usually the maternal. In the artificial amphidiploids, the ITS in one, *N. digluta*, is like that of its paternal parent, while in another, *N. didepta*, it matches its probable maternal parent; in the third, *N. sanderae*, ITS is also like that of its maternal parent, *N. alata*.

Here we discuss (1) the molecular evolution of ITS and its relationship with phylogenetic analysis; (2) the phylo-

genetic relationships among species of *Nicotiana*; and (3) the origins of amphidiploid species and species complexes in the genus, comparing and contrasting the results from the ITS tree described here, the *matK* tree of Aoki and Ito (2000) and the preliminary GISH experiments also described here.

Molecular evolution of ITS

Since the publication of Baldwin (1992), ITS nrDNA sequences have become widely used in flowering plant phylogenetics to infer species trees, but several problems with duplications (paralogous copies) limiting its utility have been detected (McDade, 1990, 1992, 1995; Buckler *et al*., 1997). Upon amplifying a single ITS copy type, most workers assume that the phylogenetic patterns they obtain from analyses of this region represent a potential species tree because ITS is from the biparentally inherited nuclear genome (see Doyle, 1992). For *Nicotiana*, in which interspecific hybridization is well documented and a single copy of ITS was recovered from all taxa, an ITS tree could clearly not be representative of the species phylogeny, but instead must be tracking some aspect of genomic organization in the hybrids. The ITS region is thus subject to being 'captured' by hybridization, just as plastid or mitochondrial markers can transverse species boundaries through hybridization and introgression. If a researcher sequences ITS from a plant that he does not know is a hybrid and produces a single clear ITS sequence, then it is possible that the 'phylogenetic' analysis of the ITS sequences will produce a tree in agreement with that from an analysis of plastid markers, leading to an assumption that the species tree has been identified when in fact this is not the case. ITS is thus not a generally reliable tool for the detection of hybrids, but rather a potential indicator of parentage of taxa known from other evidence to be hybrids.

Since ITS repeats are located at nucleolar organizer regions (NOR) on chromosomes, it is relatively easy to assess whether taxa are likely to have multiple ITS sequences by counting the number of chromosomes possessing secondary constrictions at metaphase (Flavell, 1980). The numbers of satellited chromosomes in the majority of *Nicotiana* species have been documented in detail (Reed, 1991). However, *in situ* hybridization experiments have shown that additional minor sites (probably incapable of organizing their own nucleoli) are present in the genome of *N. tabacum* (Kenton *et al*., 1993; Lim *et al*., 2000*a*, *b*). Kenton *et al*. (1993) documented eight chromosomal locations displaying sequence identity with the NOR-specific DNA probe, pTA71 (Gerlach and Bedbrook, 1979). Hence, a traditional cytological approach of counting secondary constrictions may underestimate the true number of ITS copies present.

Inactivation of a NOR region (possibly by suppression due to methylation; see Lim *et al*., 2000*b*) may release it from functional constraints. This may lead to more rapid patterns of DNA substitution across both coding and non-coding portions of the NOR region if concerted evolution continues to homogenize both types of sites. Sequence divergence may lead to the abolition of the conserved secondary structure formed by ITS products. Buckler *et al*. (1997) have identified putative 'pseudo-gene' sequences in five species of *N.* section *Alatae*. They suggested that paralogous ITS copies freed from functional constraints are more likely to be recovered during amplification reactions, and therefore 'pseudo-genes' are likely to predominate. There is no evidence that the ITS sequences described here are pseudo-gene copies; they all have an intact and highly conserved 5·8S gene region, and they appear to have normal secondary structure (A. Coleman, Brown University, pers. comm.). A further caveat could be added that as long as the NOR-copy type can be annealed to efficiently by the PCR primers, then that copy type, which is present in much greater numbers, will predominate in the PCR to such a high degree that pseudo-gene versions are not observed in the sequencing reactions. In the absence of cloning, addition of DMSO or other agents that reduce secondary structure would seem to be important for efficient amplification of the cytologically active version of what could be many different ITS sequences within a given species. To evaluate further the validity of the speculations described above, it will be necessary to perform *in situ* PCR using primers that will only amplify one repeat type, but that is outside the scope of this paper.

The existence of multiple ITS loci in hybrid taxa, possibly diverging with respect to their collective sequences, could lead to recovery of multiple ITS products during DNA amplification. Such products have been identified as additive DNA sequence signals present in a single sequencing reaction (Baldwin *et al*., 1995). For example, in *Paeonia* L. (Paeoniaceae), hybrid taxa had multiple ITS sequences (Sang *et al*., 1995), and concerted evolution had not homogenized parental sequences. Sang *et al*. (1995) suggested vegetative reproduction as the likely reason for maintenance of distinct parental sequences in hybrids, but similar results were obtained in *Krigia* (Asteraceae; Kim and Jansen, 1994) and *Arabidopsis* (Brassicaceae; O'Kane *et al*., 1996), neither of which reproduces vegetatively. Additivity and the presence of multiple ITS sequences appear not to be recovered from the *Nicotiana* amphidiploid hybrids sampled, even from recently constituted hybrids (Lim *et al*., 2000*a*, *b*). Franzke and Mummenhoff (1999) showed a similarly rapid rate of gene conversion in amphidiploids in Brassicaceae. This perhaps indicates that either these taxa do not possess divergent sequences at rDNA loci, or that competition during PCR amplification is sufficiently high to ensure only a single molecular 'species' is selected (see Fig. 2B, in which *N. didepta* falls out within the strongly supported *N.* section *Suaveolentes* despite having *N. tabacum* as one of its parents).

Several studies have reported that amphidiploids tend to favour the ITS copy type of their maternal parent, perhaps due to selection for cytoplasmic and nuclear compatibility (Soltis and Soltis, 1995; Franzke and Mummenhoff, 1999). The evidence for *Nicotiana* demonstrates no strong bias; for the artificial hybrids, there are examples of both maternal and paternal patterns being retained, and in the amphidiploids for which there is clear evidence, there are instances of both patterns: *N. tabacum* with a paternal ITS allele and *N. rustica* with the maternal allele (see below).

Systematic relationships of the species of Nicotiana

Goodspeed devoted much of his botanical career to the study of *Nicotiana*, and the results of many of his detailed and insightful analyses have stood the test of time. Although he was not trying to create a taxonomy based entirely on the concept of monophyly, his syntheses of many sources and types of data allowed him to recognize groups that, using a phylogenetic framework, are now found to be monophyletic. Although results described here do not completely mirror Goodspeed's taxonomy, the congruence is remarkable. Modern molecular techniques provide access to more evolutionary characters (e.g. the use of GISH to elucidate the distribution of diploid genomes in hybrid taxa) and permit more detailed insights into phylogenetic history. A reclassification of *Nicotiana* is not provided because there is ample evidence that the evolution of the genus is substantially more complicated than has been thought previously. The generally low levels of internal support (Fig. 2) for the topology also warrant this conservative approach.

Before examining species relationships, a comment will be given on Goodspeed's (1954) ideas that 'pre-petunioid' and 'pre-cestroid' ancestors gave rise to the extant diversity of floral formats. Exactly what was meant by this idea is not entirely clear. There are two general flower types in *Nicotiana*, one that superficially resembles *Petunia* and the other *Cestrum*, which could simply be due to convergence on the same pollinating insects and have nothing to do with the evolutionary origins of *Nicotiana*. On the other hand, Goodspeed could have meant that the extant diploids had been produced by crosses between a taxon that was *Petunia*-like and another that was *Cestrum*-like, which is a radically different concept. In the Olmstead *et al*. (1999) analysis of *rbcL* and *ndhF* as well as the ones presented here with ITS, neither *Petunia* nor *Cestrum* is sister to *Nicotiana* (indicating that neither of these was an exclusive maternal parent), but it could be that Goodspeed's concept was not exclusive to *Nicotiana* and applied to the related genera of the tribe Anthocercidae as well (although these taxa were never thought to be closely related to *Nicotiana* by Goodspeed or others). If extant diploids of *Nicotiana* and related genera in Anthocercidae are the product of hybridization between a *Petunia*-like and *Cestrum*-like progenitor, then it is likely that evidence of this would be found in the two different patterns of gene arrangements, corresponding to those of the two progenitors. This might be possible, but it would be made difficult by subsequent inter-genomic translocations. If evidence is to be found to support such a hypothesis, it will be by examining patterns of genomic organization. Goodspeed's (1954) hypothesis that the entire genus *Nicotiana* comprises amphidiploid species derived from an extinct $n = 6$ progenitor may have some bearing on the Andean taxa of *N.* sections *Tomentosae*, *Paniculatae* and *Undulatae* (including *N. thyrsiflora*). Of course, this may not be what Goodspeed was trying to hypothesize; it could be that his idea of 'pre-petunioid' and 'pre-cestroid' progenitors was a simple, pre-cladistic concept completely lacking a structure clear enough to permit more explicit evaluation.

Using the plastid genes *rbcL* and *ndhF*, Olmstead *et al*. (1999) identified the endemic Australian tribe Anthocercidae (including *Cyphanthera*, *Duboisia* R.Br., *Anthocercis* and *Symonanthus*; see Purdie *et al*., 1982) as the sister to their two sampled species of *Nicotiana*. This result raised the possibility that the genus *Nicotiana* as currently defined is paraphyletic because neither of the species of *Nicotiana* sampled is Australian. These results, with a great deal more sampling, support *Nicotiana* as the sister of Anthocercidae (Fig. 2A).

Although *N.* section *Trigonophyllae* was included in *N.* subgenus *Petunioides* by Goodspeed (1954), the two species of this section, *N. obtusifolia* and *N. palmeri*, fell in an isolated position in the analyses. The results of the plastid *matK* analysis (Fig. 5) recovered a similar position for *N. obtusifolia* (as *N. trigonophylla*), but placed it with one of the amphidiploid species, *N. quadrivalvis* (as *N. bigelovii*), which it was suspected at first might be due to interspecific hybridization resulting in that accession of *N. quadrivalvis* having a foreign plastid genome (see below). The similarity of the plastid and ITS trees indicates that *N.* section *Trigonophyllae* is not related to the rest of *N.* subgenus *Petunioides*, although neither alone represents a strong argument against Goodspeed's classification.

Goodspeed (1954) identified *N. glauca* and *N. glutinosa* as strongly divergent taxa that were difficult to place. His emphasis on floral characters led him to place *N. glauca* in *N.* section *Paniculatae*, with which it shares 'the corolla shape and ovate-cordate leaf blade common to all members of *N.* section *Paniculatae*' (Goodspeed, 1954: 339). However, in his discussion of *N. noctiflora*, similarities of inflorescence structure with *N. glauca* are mentioned. Both the analysis described here and that of Aoki and Ito (2000) placed *N. glauca* as sister to the clade containing the members of *N.* section *Noctiflorae* (Figs 2A and 5). Both *N.* sections *Paniculatae* and *Noctiflorae* are from southern South America, so it is also possible that *N. glauca* has experienced introgression from a species in the latter, perhaps *N. acaulis*, and that its ITS and plastid sequences have both been 'captured'. Given what was observed for the ITS sequences of the artificial hybrids, and its position as sister to *N.* section *Noctiflorae*, either *N. glauca* really is a member of *N.* section *Noctiflorae* with an unusual yellow, tubular flower, or introgression occurred relatively early in the evolution of this clade (it is assumed that if hybridization were a recent event, then the ITS sequence of *N. glauca* would more closely resemble that of another species, unless that parent is the unsampled Patagonian endemic *N. ameghinoi*). That Goodspeed noted morphological similarities between *N. glauca* and *N.* section *Noctiflorae* seems an unlikely coincidence considering the position of *N. glauca* in the ITS and plastid trees.

In his discussion of *N. glutinosa* Goodspeed (1954: 371) emphasized its ambiguous affinities, stating that its 'habit and leaf character . . . approximate those of *N. benavidesii* Goodsp. of section *Paniculatae* whereas flower characters are those common to other members of section *Tomentosae*'. The analysis indicates that *N. glutinosa* is related to the members of *N.* section *Undulatae*, particularly *N. undulata* (Figs 2A and 3), which is sister to *N.* section

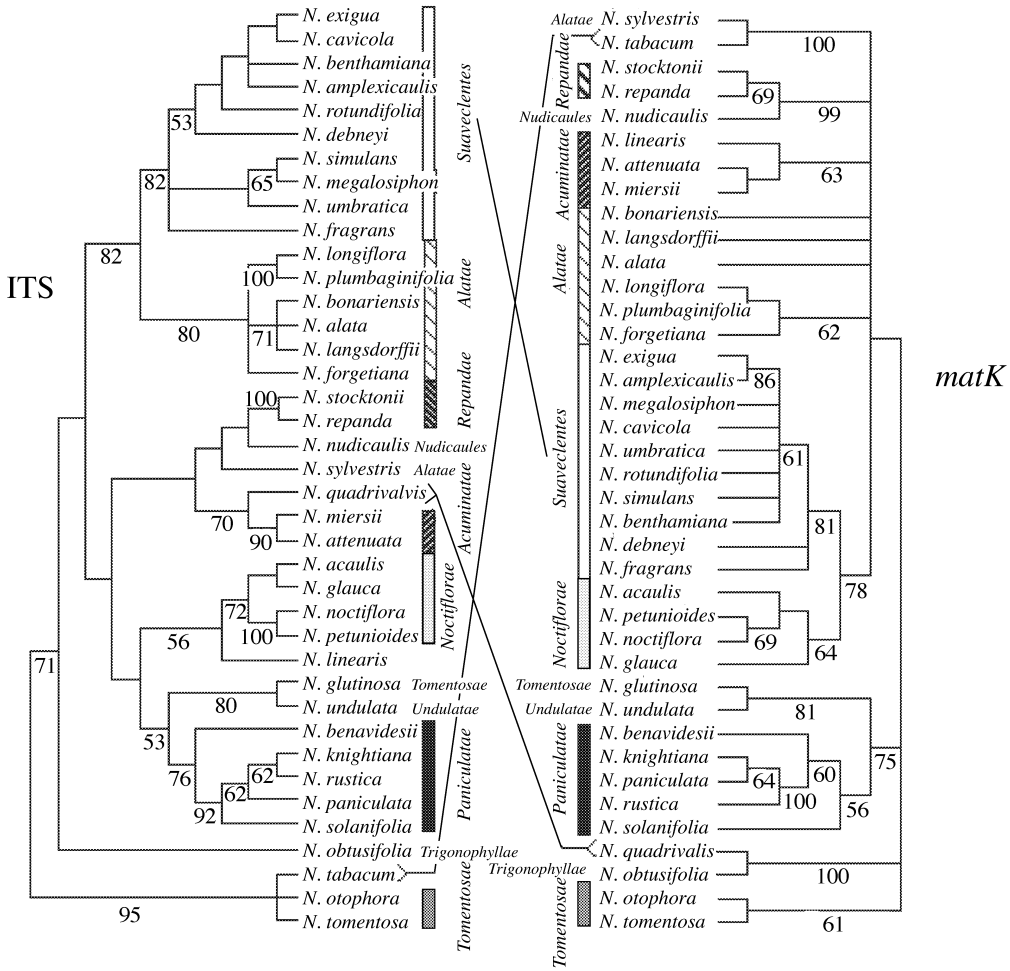


FIG. 5. Comparison of results (strict consensus tree) of ITS with those produced by the *matK* data (Aoki and Ito, 2000) for the same set of taxa analysed by the latter. Side bars indicate the sections of Goodspeed (1954); arrows show positions of taxa that differ in placements between the plastid and ITS trees.

Paniculatae. The *matK* analysis (Fig. 5) clearly placed *N. glutinosa* with *N. undulata*. Other studies (Lim *et al*., 2000*a*) have also indicated that genomic organization of *N. glutinosa* is completely unlike that of other members of *N.* section *Tomentosae*; it would appear that the leaf and habit dissimilarities to *N.* section *Tomentosae* were a more accurate indicator of its relationships than its (convergent) floral morphology.

Goodspeed (1954) created *N.* section *Thyrsiflorae* for the morphologically aberrant *N. thyrsiflora*; with its spike-like inflorescence and unbranched habit, it is unlike any other *Nicotiana*. The greenish flowers indicated to Goodspeed that it was related to *N. rustica* and the members of *N.* section *Paniculatae*. Floral morphology, however, supports the result that *N. thyrsiflora* is a member of the clade containing *N. wigandioides*, highlighting the need to examine a broad suite of characters in assessing relationships in this complex genus.

That the diploid tree differs little from the all-taxon tree indicates that the removal of the amphidiploid species does not result in a reduction of internal character conflict. In morphological analyses, such conflict would be an expected result of hybridization. This lack of change is in agreement with the observation that the ITS sequences of hybrid taxa are not hybrid (mosaic) sequences, so their removal would not be expected to alter overall patterns of relationships or levels of bootstrap support. It has been suggested that removal of hybrid taxa is a prerequisite to cladistic analysis (Humphries, 1983; Nelson, 1983; Wagner, 1983). This, however, is based on the supposition that hybrids are intermediate or mixtures of the characters exhibited by their parents, and this is clearly not applicable to ITS sequences of hybrid taxa of *Nicotiana*.

Farris *et al*. (1997) have shown that as more taxa are included in an analysis it becomes more stable and perhaps more accurate. In this context, the species that gave rise to the amphidiploid taxa are likely to have gone extinct [Goodspeed (1954) supposed that they were driven extinct by their more competitive, polyploid progeny], and thus their progeny now represent ITS lineages that were derived from several now extinct taxa. For example, no extant members of *N.* section *Alatae* gave rise to *N.* section *Suaveolentes*, so collectively the sequences of the latter are sister to the former and break the long branch (13 steps;

Fig. 3) into this section observed in the diploid-only analysis. Thus the presence of *N.* section *Suaveolentes*, all members of which are amphidiploids, ironically stabilizes the patterns in this portion of the tree by breaking up longer branches (three steps into *N.* section *Alatae*; Fig. 2A).

In the larger analysis (Fig. 2A), *N. sylvestris* was sister to *N.* sections *Nudicaules* and *Repandae*, but without the hybrids it was sister to *N.* section *Acuminatae* (with less than 50 BP in both cases). In the *matK* analysis, it occupied a similarly isolated position (except that *N. tabacum* shared its plastid genome; Fig. 5). Lin *et al*. (2001) suggested that *N. sylvestris* is a member of *N.* section *Alatae* based on RFLP and RAPD markers, but they only analysed one other species of the group (*N. plumbaginifolia*). It is unclear if *N. sylvestris,* the only species of *N.* section *Alatae* that is $n = 12$ (the rest are $n = 9$), is related to the rest. The separation of *N. sylvestris* ($n = 12$) from *N.* section *Alatae* in the ITS analysis indicates that its lineage (and that leading as well to *N.* sections *Acuminatae*, *Noctiflorae*, *Paniculatae* and *Undulatae*) diverged before the hybridization event that resulted in the formation of the *N.* section *Suaveolentes*. Chromosome fusions in *N.* section *Suaveolentes* resulting in reduction (to $n = 9$) took place after this hybridization, and thus species of *N.* section *Suaveolentes* have chromosome numbers based on reductions from $n = 24$ because both parental entities from *N.* sections *Alatae* and *Noctiflorae* (see below) were now extinct, $n = 12$ species.

The role of *N. sylvestris* in the origins of several of the amphidiploid taxa is also intriguing to us. The *N. sylvestris* genome shows some degree of sequence identity with the genomes of *N. nudicaulis*, *N. tabacum*, *N. quadrivalvis* and *N. clevelandii* (Table 2). Goodspeed (1954: 308) placed *N. sylvestris* in *N.* section *Alatae* based on floral morphology, although its chromosomes physically resemble those of *N.* section *Acuminatae*, with which it falls in the diploid-only analysis (Fig. 3). He noted that '*N. sylvestris* may be thought of as a derivative of a stock incorporating elements of all three subgenera'. A '*sylvestris*-type' genome could thus be ancestral in *Nicotiana*, and this facilitates wide crosses within the genus.

Cytogenetic evolution of single amphidiploids: origins of N. rustica*,* N. arentsii *and* N. tabacum

The results from the GISH experiments described here revealed that genome evolution in *Nicotiana* is significantly more complicated than previously thought. Each specific hypothesis evaluated is discussed here in relation to the phylogenetic data. In the natural hybrids of *Nicotiana*, the parentage for many can often be inferred by comparing the ITS presented here and the plastid *matK* trees of Aoki and Ito (2000).

The ITS cladogram shows a sister relationship between *N. rustica* and *N. knightiana*, a member of *N.* section *Paniculatae* (Fig. 2A). This indicates that PCR is recovering a 'paniculatoid' genome sequence from *N. rustica*. Results of both ITS and *matK* indicated that in this case either *N. paniculata* or *N. knightiana* could be one of the parents, but the DNA of the latter was not examined in the GISH experiments.

The situation of *N. rustica* is one in which reliance upon an incongruent result in plastid *vs.* nuclear (putatively biparental) pattern to indicate hybridization would be misleading. *Nicotiana rustica* was supported as a member of the section including what is assumed to be its maternal parent, *N.* section *Paniculatae*, in both the *matK* and ITS analyses. If it had not been known from other data (cytological, in this case) that *N. rustica* was a hybrid, its status as an amphidiploid could not be determined by incongruent ITS and *matK* results, as was the case with *N. tabacum* below. As stated earlier, ITS evolution precludes its use as a reliable indicator of hybridization. Nonetheless, ITS is an additional, useful piece of information in sorting out the complicated evolutionary patterns in *Nicotiana*, but only in the context of the extraordinary, pre-existing work by Goodspeed (1954).

Nicotiana arentsii is another simple amphidiploid, and GISH results indicated that *N. wigandioides* and *N. undulata* contributed genomes. These three taxa form *N.* section *Undulatae sensu* Goodspeed (1954), which, together with the members of *N.* section *Paniculatae*, form a clade in the tree shown here (Fig. 2a). That the putatively parental genomes label different chromosome sets in *N. arentsii* leads to the belief that the hybridization event leading to its formation occurred after differentiation of *N. wigandioides* and *N. undulata*. The ITS sequence of *N. arentsii* is strongly supported (BP 96) as the sister to, and like that of, *N. undulata*, but *N. arentsii* was not included in the *matK* analysis of Aoki and Ito (2000), so it is not possible to say which species was maternal/paternal. Data from plastid *matK* and *ndhF* sequences (J. J. Clarkson and M. W. Chase, unpubl. res.) also place *N. arentsii* with *N. undulata*, so this is another case in which ITS has converted to the maternal type.

The genomic relationships of *N. tabacum* have been the subject of detailed investigations at the molecular level (Borisjuk *et al*., 1997; Volkov *et al*., 1999; Kitamura *et al*., 2000; Lim *et al*., 2000*a*, *b*). GISH results show multiple, chromosome translocations between S (*N. sylvestris*-like) and T (*N. tomentosiformis*-like) genomes (Kenton *et al*., 1993; Parokonny and Kenton, 1995; Kitamura *et al*., 2000; Lim *et al*., 2000*a*). The relationships of *N. tabacum* reflected in the ITS trees indicate that PCR is recovering an ITS sequence of the T genome rather than the more distantly related S genome (Fig. 2A). *Nicotiana tabacum* falls with *N. sylvestris* in the plastid RFLP and *matK* trees (Olmstead and Palmer, 1991; Aoki and Ito, 2000; Fig. 5), which is thus its maternal parent, whereas it has an ITS sequence identical to that of what can be assumed to be its paternal parent, *N. tomentosiformis* (Lim *et al*., 2000*a*). This result is confirmed in general by GISH results, which demonstrated genomic similarity to *N. sylvestris* and several species of *N.* section *Tomentosae*. DNA of *N. tomentosiformis* demonstrated the strongest hybridization signal of those examined, which confirms the pattern observed for ITS.

Cytogenetic evolution of amphidiploid species complexes: N. *sections* Bigelovianae*,* Suaveolentes *and* Repandae

The situation for sections of *Nicotiana* in which speciation has taken place subsequent to the hybridization

events are more complex and not as easily addressed by GISH. These are presumably older events than those giving rise to single species, as in the case with simple amphidiploids, and the parental species are extinct and perhaps replaced by several, more modern descendent species. In a number of cases, as with *N.* sections *Bigelovianae* and *Repandae*, the extant species occur in regions widely separated from sections hypothesized by Goodspeed to have been the parents; for example *N.* section *Bigelovianae* occurs only in western North America [Table 1; California and adjacent parts of Baja California and Arizona, although it has been transported widely due to its use by native Americans, see Goodspeed (1954) and references therein], whereas most of its putative parents, except for *N. attenuata*, are now found only in temperate parts of South America. These differences in geography would seem to imply that the circumstances surrounding the origins of these polyploid taxa are different from those today, whereas simple hybrids now sympatric with their parents are more recent and have more readily documented patterns with GISH. At the least, the disjunctions between putative parents and hybrids imply long-distance migrations or large-scale extinction within certain parts of the overall range of *Nicotiana*.

It is clear that Goodspeed accurately predicted at least one of the component genomes (that of *N. attenuata*) of the amphidiploid taxa comprising *N.* section *Bigelovianae*. Whether his contention that an 'alatoid' element was involved is correct is still not clear. Although Goodspeed (1954) placed *N. sylvestris* in *N.* section *Alatae* (see above), it is karyotypically similar to the members of *N.* section *Acuminatae* of which *N. attenuata* is a member, and its ITS sequence is also similar to sequences of this section (Fig. 3). This karyotypic similarity and the fact that genomic DNA of *N. sylvestris* and *N. attenuata* hybridizes to the same chromosome set in *N. clevelandii* and *N. quadrivalvis* (Table 2) indicate that another species must also have been involved.

In the ITS results, *N. clevelandii* and *N. quadrivalvis* (of *N.* section *Bigelovianae*) were supported (but with only BP 68) as members of the clade containing *N.* section *Acuminatae*, whereas with *matK*, *N. quadrivalvis* (labelled as *N. bigelovii*; Aoki and Ito, 2000) is strongly supported as sister to *N. obtusifolia* (labelled as *N. trigonophylla*; Aoki and Ito, 2000; Fig. 5). This unpredicted result could have two causes: Goodspeed's hypothesis and the GISH results that indicated involvement of an alatoid element were wrong, and the other parental genome involved was from something like *N. obtusifolia*; or the sample of *N. quadrivalvis* used in the *matK* analysis acquired a *N. obtusifolia*-like plastid genome through hybridization, which is perhaps likely given their geographic distribution (see Table 1). *Nicotiana quadrivalvis* was widely transported around the western United States by indigenous peoples, and several cultivars exist, so provenance of samples is critical. Additional samples of *N. obtusifolia* as well as *N. clevelandii* have been sequenced for *matK*, but they have the same plastid genome as that studied by Aoki and Ito (2000). In any case, the ITS results indicate that one parent was indeed acuminoid but that none of the extant species from this section nor *N. sylvestris* is the actual parent of *N.* section *Bigelovianae*. This event predates the evolution of any of the extant species of *N.* sections *Acuminatae* and *Alatae*.

Goodspeed (1954) considered the origin of the clade containing the Australian species (*N.* section *Suaveolentes*) to be found in an ancestor of the present-day *N.* section *Alatae*. He hypothesized extensive, subsequent chromosome fusions to explain the range of chromosome numbers present in this group (Table 1). A member of *N.* section *Acuminatae* was considered to be the other parental element in the amphidiploid origin of at least *N. debneyi* (Goodspeed, 1954), but the other parental element for *N. fragrans* (the other $n = 24$ species) was considered to be 'less obvious' (Goodspeed, 1954: 295). The GISH results presented here fully support Goodspeed's hypothesis of an alatoid parent being involved in the production of the Australian clade, including *N. africana*. Within *N.* section *Suaveolentes*, Goodspeed (1954) hypothesized more than one origin (i.e. the section is polyphyletic) and identified two separate hybridization events, one giving rise to *N. debneyi* between alatoid and acuminatoid progenitors and the other giving rise to *N. fragrans* between alatoid and noctifloroid elements. It was not possible to investigate the latter, but no sequence identity exists between *N. debneyi* and *N. attenuata*, the latter a member of *N.* section *Acuminatae*, indicating that a different donor genome may be involved in this amphidiploid event (Table 2).

The fact that *N.* section *Suaveolentes* (see Fig. 2A and B) is monophyletic in the ITS tree indicates that only the alatoid ITS sequence is being recovered by PCR. Several other species were investigated as possible donor genomes (Table 2), but none showed labelling. Olmstead and Palmer (1991), using plastid RFLP data, found that *N. glauca* was sister to the Australian clade and in the *matK* results *N. glauca* plus *N.* section *Noctiflorae* were sister (BP 78; Fig. 5). This is an indication that this type of genome is likely to have been the maternal one involved in the hybridization that produced *N.* section *Suaveolentes*, but the exact nature of this will require further investigation. No species of *N.* section *Noctiflorae* were examined with GISH (which was completed long before the *matK* results were published), but in future experiments they should be. Although a bootstrap of 78 BP is not high enough to be reliable, it is a plausible alternative to the hypothesis that this event involved an acuminatoid element.

The origin of *N.* section *Suaveolentes* presents several paradoxes. Two members of *N.* section *Alatae*, *N. alata* and *N. sylvestris*, failed to show hybridization, but two others, the closely related *N. longiflora* and *N. plumbaginifolia*, did exhibit a reaction to one of the sets of chromosomes in a range of species from *N.* section *Suaveolentes*, which would seem to confirm that a species from *N.* section *Alatae* was involved. This potentially old, putatively single event (see below) subsequently resulted in the evolution of many species; they are supported by the bootstrap as monophyletic in both the ITS and *matK* analyses (Fig. 5). However, none of them occurs in South America where both putative parental lineages now occur. The derivative positions of *N.* section *Suaveolentes* in both the ITS and

matK analyses and the relatively low levels of divergence in both DNA regions indicate that their present distribution is the result of long-distance dispersal rather than vicariance (Olmstead and Palmer, 1991; Aoki and Ito, 2000). Failure of sequence hybridization to *N. alata* and *N. sylvestris* indicates that substantial genome evolution has gone on since that dispersal event.

Multiple origins for the now many species of *N.* section *Suaveolentes* would seem to be ruled out by their monophyly in both the plastid and ITS results (Fig. 5), but it is important to remember that this event took place before any of the extant species in *N.* sections *Acuminatae*, *Alatae* and *Noctiflorae* evolved. These sections form a monophyletic clade (with less than 50 BP) in the diploids-only analysis (Fig. 3). If the hybridizations took place before the accumulation of the present higher levels of divergence, or were followed by ITS or plastid capture, then it would not be possible to detect the polyphyletic origin of *N.* section *Suaveolentes* hypothesized by Goodspeed (1954). Furthermore, if more than one paternal parent crossed with the same maternal parent and both ITS were converted to that of the maternal ITS, then ITS alone would be incapable of revealing this polyphyletic origin. Polyphyly could now only be established by examining aspects of genomic organization, as is the case for testing the palaeo-amphidiploid origin of the genus as a whole. ITS and plastid DNA sequences are likely to be imperfect tools for uncovering such complex patterns.

Goodspeed (1954) suggested that the closest relative of the members of *N.* section *Repandae* (*N. stocktonii*, *N. repanda* and *N. nesophila*) was a now extinct, *N. plumbaginifolia*-like, $n = 12$, alatoid taxon, but hybridization experiments do not support this hypothesis (Table 2). The diffuse labelling of chromosomes of members of *N.* section *Repandae* by genomic DNA of both *N. palmeri* and *N. obtusifolia* (Table 2) may indicate that the members of *N.* section *Repandae* have an autopolyploid origin, in contrast to the allopolyploid pattern in *Nicotiana*. The diffuse labelling of *N.* section *Repandae* with DNA from *N. palmeri* and *N. obtusifolia*, distantly placed in the ITS tree, is even more difficult to explain given that ITS indicates relationships to at least one parent of polyploid taxa. The position of the members of *N.* section *Repandae* in the ITS tree is indicative of involvement of another species (i.e. members of *N.* sections *Acuminatae* or *Alatae*) from this portion of the ITS tree in the ancestry of these taxa. The same can be said of *N. nudicaulis*, which is nearby in the ITS tree and from the same part of North America, but which was not evaluated with GISH here.

ITS sequences demonstrate a weakly supported association of *N.* section *Repandae* with *N. sylvestris*, which may be indicative of an ancient event. Neighbour-joining analysis of the *matK* sequences also shows a weakly supported (BP <50) relationship with *N. sylvestris* (the parsimony analysis demonstrated no clear affinities), which again may be an indication of an alatoid involvement in the production of *N.* section *Repandae*. None of the extant species of *N.* section *Alatae* now grows anywhere near western North America, which is where these allopolyploid, putative derivatives of *N.* section *Alatae* now occur (*N.* sections *Bigelovianae* and *Repandae*), again leaving the impression that much has changed since the origin of these groups.

CONCLUSIONS

The separate analyses of diploid and polyploid species of *Nicotiana* have produced congruent trees; inclusion of amphidiploid species clearly does not obscure the patterns although the precise effects are difficult to quantify. These results, in combination with the GISH results detailed above, indicate that hybridization leading to the formation of at least some of the amphidiploid taxa of *Nicotiana* is relatively recent. However, molecular cytogenetic techniques may be unable to detect the products of ancient hybridization events responsible for many of the unsupported internal nodes in the *Nicotiana* tree. If in fact the genus *Nicotiana* is the result of ancient amphidiploidy between two $n = 6$ progenitors (Goodspeed, 1954), evolutionary patterns in the genus may prove intractable to standard cladistic analysis. Amphidiploidy appears to introduce an additional layer of complexity from which the species tree may never be recovered by analyses of just ITS and plastid DNA. It is known that the ITS tree is definitely not the species tree for *Nicotiana*, but there is a pattern in these data, as evidenced by the consistent groupings in both the all-taxon and diploid trees. The overall low levels of divergence found within *Nicotiana* produced a fairly weak assessment of relationships within the genus, but these results clearly are highly congruent with many of Goodspeed's ideas about relationships and his classification of the genus. Thus, it is believed that the analysis of these ITS sequences is useful because congruence with other kinds of evidence is clear, and this much similarity could not be due merely to chance. Therefore, the ITS results are considered to be more robust and useful, although not necessarily in a phylogenetic context, than the low levels of bootstrap support would indicate. The results described here show that, in *Nicotiana*, ITS of a hybrid will associate with one of its two parents, so this pattern of association for amphidiploids should aid in corroboration of hypotheses based on GISH results and other lines of evidence. From those cases in which parentage is well established and ITS fits the established pattern, it is possible then to extrapolate to those taxa for which little else is known and make predictions that should aid in future research. It is clear that despite the potential complicating effects that hybrids of amphidiploid origin introduce to phylogeny reconstruction, they should not preclude its use if the evolution of the region being analysed is understood.

For groups such as *Nicotiana*, in which reticulate evolution has often occurred, no single data set will be able to resolve relationships adequately. Due to the ubiquity or near-ubiquity of hybridization and introgression in plant groups (see Stebbins, 1950; Grant, 1981; Funk, 1985; Reiseberg and Morefield, 1995; Reiseberg and Wendel, 1995; Arnold, 1997; Wendel, 2000) multiple approaches are essential, involving data from as many sources and techniques as possible. A more in-depth, synthetic approach (Kluge, 1989; Eernisse and Kluge, 1993; Patterson *et al.*,

1993; Bruneau *et al*., 1995; Bremer, 1996; Nixon and Carpenter, 1996) including additional molecular, morphological and cytological data will be required to explain adequately the complex patterns of species relationships in *Nicotiana*. In the absence of molecular cytogenetic data, one could easily be led to incorrect conclusions about evolutionary relationships in *Nicotiana*. It has been argued that the reticulating evolutionary patterns displayed by ribosomal DNA loci should limit their utility in phylogenetic studies. This is clearly the case in *Nicotiana*, and it is argued that studies of ITS sequences must be used in conjunction with a broad range of other techniques if an accurate picture of phylogenetic relationships is to emerge.

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APPENDIX

Plant material used in this study

All plant specimens are housed at BM (The Natural History Museum, London). Vouchers were collected by T. Helgason and A. Monro, unless noted otherwise.

APPENDIX I

Taxon	Voucher no.	GenBank no.
Anthocercis gracilis Benth.	*Stace s.n.* KPBG	AJ492457
Anthotroche pannosa Endl.	*Stace s.n.* KPBG	AJ492458
Cestrum elegans Schltdl.	*Chase 12217* K	AJ492459
Nicotiana acaulis Speg.	600	AJ492389
Nicotiana acuminata (Graham) Hook.	*Lim 015*	AJ492426
Nicotiana africana Merxm.	613	AJ492393
Nicotiana alata Link & Otto	501, 518	AJ492424
Nicotiana amplexicaulis N.T.Burb.	503	AJ492394
Nicotiana arentsii Goodsp.	*Clarkson 001*	AJ492437
Nicotiana attenuata Torr. ex S.Watson	621	AJ492427
Nicotiana benavidesii Goodsp.	601	AJ492411
Nicotiana benthamiana Domin	516	AJ492409
Nicotiana bonariensis Lehm.	622	AJ492382
Nicotiana cavicola N.T.Burb.	525	AJ492395
Nicotiana clevelandii A.Gray	*Lim 019*	AJ492444
Nicotiana cordifolia Phil.	*Saikia 008*	AJ492440
Nicotiana corymbosa Remy	Unknown	AJ492388
Nicotiana debneyi Domin	506	AJ492439
Nicotiana eastii Kostoff	527	AJ492396
Nicotiana excelsior (J.M.Black) J.M.Black	521	AJ492399
Nicotiana exigua H.-M.Wheeler	530	AJ492391
Nicotiana forgetiana Hemsl.	500, 512	AJ492419
Nicotiana fragrans Hook.	630	AJ492397
Nicotiana glauca Graham	640	AJ492410
Nicotiana glutinosa L.	514	AJ492433
Nicotiana goodspeedii H.-M.Wheeler	526	AJ492401
Nicotiana gossei Domin	523	AJ492390
Nicotiana hesperis N.T.Burb.	515	AJ492402
Nicotiana ingulba J.M.Black	641	AJ492403
Nicotiana kawakamii Y.Ohashi	632	AJ492445
Nicotiana knightiana Goodsp.	607	AJ492412
Nicotiana langsdorfii Weinm.	528	AJ492384
Nicotiana linearis Phil.	609	AJ492425
Nicotiana longiflora Cav.	510	AJ492385
Nicotiana maritima H.-M.Wheeler	511	AJ492404
Nicotiana megalosiphon Van Huerck & Müll.Arg.	532	AJ492392
Nicotiana miersii Remy in Gay	*Clarkson 003*	AJ492429
Nicotiana nesophila I.M.Johnst.	*Saikia 011*	AJ492442
Nicotiana noctiflora Hook.	*Lim 005*	AJ492432
Nicotiana nudicaulis S.Watson	508	AJ492416
Nicotiana occidentalis H.-M.Wheeler	531	AJ492417
Nicotiana otophora Griseb.	*Nee et al. 51739*	AJ492454
Nicotiana palmeri A.Gray	631	AJ492451
Nicotiana paniculata L.	502	AJ492413
Nicotiana pauciflora Remy in Gay	635	AJ492428
Nicotiana petunioides (Griseb.) Millán	*Lim 001*	AJ492431
Nicotiana plumbaginifolia Viv.	505	AJ492386
Nicotiana quadrivalvis Pursh	*Chase 11944K*	AJ492452
Nicotiana raimondii J.F.Macbr.	603	AJ492414
Nicotiana repanda Willd.	641	AJ492418
Nicotiana rosulata (S. Moore) Domin	Unknown	AJ492405
Nicotiana rotundifolia Lindl.	622	AJ492406

APPENDIX I *Continued*

Taxon	Voucher no.	GenBank no.
Nicotiana rustica L.	626	AJ492415
Nicotiana setchellii Goodsp.	636	AJ492421
Nicotiana simulans N.T.Burb.	524	AJ492407
Nicotiana solanifolia Walp.	*Clarkson 004*	AJ492441
Nicotiana spegazzinii Millán	648	AJ492387
Nicotiana stocktonii Brandegee	*Lim 003*	AJ492443
Nicotiana suaveolens Lehm.	517	AJ492438
Nicotiana sylvestris Speg. & Comes	628	AJ492423
Nicotiana tabacum L.	*Clarkson 005*	AJ492447
Nicotiana tabacum L.	*Saikia 023*	AJ492448
Nicotiana thyrsiflora Bitter ex Goodsp.	*Clarkson 009*	AJ492436
Nicotiana tomentosa Ruiz & Pavón	*Saikia 020*	AJ492449
Nicotiana tomentosiformis Goodsp.	624	AJ492420
Nicotiana tomentosiformis Goodsp.	*Clarkson 007*	AJ492450
Nicotiana obtusifolia M.Martens & Galeotti	504, 529	AJ492430
Nicotiana umbratica N.T.Burb.	617	AJ492400
Nicotiana undulata Ruiz & Pavón	533	AJ492434
Nicotiana velutina H.-M.Wheeler	509	AJ492408
Nicotiana wigandioides Koch & Fintelm.	658	AJ492435
Nicotiana × *didepta*	604	AJ492398
Nicotiana × *digluta*	*Clarkson 002*	AJ492446
Nicotiana × *sanderae* Hort. ex Wats.	616	AJ492383
Petunia axillaris (Lam.) Britton	*Chase 2371* K	AJ492460
Symonanthus bancroftii (F.Muell.) L.Haegi	*Stace s.n.* KPBG	AJ492456