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Phylogenetic relationships in *Nicotiana* (Solanaceae) inferred from multiple plastid DNA regions

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Abstract

For *Nicotiana*, with 75 naturally occurring species (40 diploids and 35 allopolyploids), we produced 4656 bp of plastid DNA sequence for 87 accessions and various outgroups. The loci sequenced were *trnL* intron and *trnL-F* spacer, *trnS-G* spacer and two genes, *ndhF* and *matK*. Parsimony and Bayesian analyses yielded identical relationships for the diploids, and these are consistent with other data, producing the best-supported phylogenetic assessment currently available for the genus. For the allopolyploids, the line of maternal inheritance is traced via the plastid tree. *Nicotiana* and the Australian endemic tribe Anthocercideae form a sister pair. *Symonanthus* is sister to the rest of Anthocercideae. *Nicotiana* sect. *Tomentosae* is sister to the rest of the genus. The maternal parent of the allopolyploid species of *N.* sect. *Polydicliae* were ancestors of the same species, but the allopolyploids were produced at different times, thus making such sections paraphyletic to their extant diploid relatives. *Nicotiana* is likely to have evolved in southern South America east of the Andes and later dispersed to Africa, Australia, and southwestern North America.

Keywords: Phylogeny; Allopolyploidy; *trnL-F*; *trnS-G*; *matK*; *ndhF*

1. Introduction

1.1. General overview

Solanaceae are a large angiosperm family including many economically important crop plants. Named in honor of the French diplomat Jean Nicot, who brought plants to France from Portugal around 1559, *Nicotiana* has 75 species and is the fifth largest genus in Solanaceae after *Solanum*, *Cestrum*, *Physalis*, and *Lycium*. Species in the genus have a wide range of floral and vegetative morphology and can vary in height at maturity from a few centimeters to four or more meters; for illustrations of most of the recognized species see Goodspeed (1954); Japan Tobacco Inc. (1994), and Knapp et al. (2004).

Linnaeus (1753) recognized four species of *Nicotiana* (*N. glutinosa*, *N. paniculata*, *N. rustica*, and *N. tabacum*), all from tropical America. Goodspeed (1954) provides a detailed history of the taxonomy of the genus, in which he considered evidence from morphology, cytology, biogeography, and crossing experiments. He expressed phylogeny as overall affinities in "phyletic" diagrams (see Chase et al., 2003). Goodspeed hypothesized that two ancestral gene pools of 'pre-petunioid' and 'pre-cestroid' plants gave rise to two distinct lineages in *Nicotiana*. He hypothesized that the base chromosome number of the genus was $n = 12$ and emphasized the role of doubling and hybridization in the evolution of the genus. Goodspeed split *Nicotiana* into three subgenera and 14 sections, but his nomenclature did not in all cases correspond to requirements of the *International*

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Code of Botanical Nomenclature (Greuter, 2000). A new taxonomic scheme based on these rules and incorporating the results of several recent phylogenetic studies (Aoki and Ito, 2000; Chase et al., 2003, and this paper) has been published elsewhere (Knapp et al., 2004), and we follow this classification here. For a summary of the new sectional classification see Table 1.

In Goodspeed's monograph (1954), a number of important relationships were proposed. He provided insights into the parentage of the two most commonly cultivated amphidiploid species, *N. tabacum* and *N. rustica*. He hypothesized that *N. tabacum* was a cross between an unnamed member of *N.* section *Tomentosae* and another unnamed species from *N.* section *Alatae*. In the case of *N. rustica* he went even further and stated that one parent was *N. undulata* and the other a species from section *Paniculatae*. The close relationship between *N.* sections *Rusticae* and *Paniculatae* was emphasized, as were affinities between *Paniculatae* and both *Tomentosae* and *Undulatae*. He suggested the Australian amphidiploids in section *Suaveolentes* had origins in an ancient hybridization event between a member of *Alatae* and another parent that was either a member of *N.* section *Acuminatae* (which should be called *Petunioides*, following Knapp et al., 2004) or *N.* sect. *Noctiflorae*.

Nicotiana has a disjunct (largely Neotropical) distribution that can be broken down into approximately 75% of species occurring in the Americas and 25% of species occurring in Australia. The discovery of *N. africana* by Merxmüller and Buttler (1975) was perplexing, because it is the first (and only) species to be discovered in Africa (Namibia). This taxon has been shown to be sister to the Australian section *Suaveolentes* and both *N. africana* and the rest of *N.* section *Suaveolentes* probably arrived in their respective continents only relatively recently (Chase et al., 2003; Olmstead and Palmer, 1991). There have been few new species described in the genus since Goodspeed's monograph; a complete list of all species and the sections of *Nicotiana* to which they are assigned is shown in Table 1.

1.2. Allopolyploids

Recent molecular biological techniques such as FISH (fluorescent in situ hybridization) and GISH (genomic in situ hybridization) have shed much light on the origin of allopolyploids in many groups (Bennett, 1995). This has proved particularly enlightening in *Nicotiana* since allopolyploids amount to about 47% of the naturally occurring species (Goodspeed, 1954). GISH was first used

Table 1
A summary of the sectional classification of *Nicotiana* according to Knapp et al. (2004)

Nicotiana section	Included species (with authorities)
Alatae	*Nicotiana alata* Link and Otto; *Nicotiana azambujae* L.B. Smith and Downs; *Nicotiana bonariensis* Lehm.; *Nicotiana forgetiana* Hemsl.; *Nicotiana langsdorffii* Weinm.; *Nicotiana longiflora* Cav.; *Nicotiana mutabilis* Stehmann and Samir; *Nicotiana plumbaginifolia* L.
Nicotiana	*Nicotiana tabacum* L.
Noctiflorae	*Nicotiana acaulis* Speg.; *Nicotiana ameghinoi* Speg.; *Nicotiana glauca* Graham; *Nicotiana noctiflora* Hook; *Nicotiana paa* Mart. Crov.; *Nicotiana petuniodes* (Griseb.) Millán
Paniculatae	*Nicotiana benavidesii* Goodsp.; *Nicotiana cordifolia* Phil.; *Nicotiana cutleri* D'Arcy; *Nicotiana knightiana* Goodsp.; *Nicotiana paniculata* L.; *Nicotiana raimondii* J.F. Macbr.; *Nicotiana solanifolia* Walp.
Petunioides	*Nicotiana acuminata* (Graham) Hook.; *Nicotiana attenuata* Torrey ex S. Watson (Fig. 1F); *Nicotiana corymbosa* J. Rémy; *Nicotiana linearis* Phil; *Nicotiana longibracteata* Phil.; *Nicotiana miersii* J. Rémy; *Nicotiana pauciflora* J. Rémy; *Nicotiana spegazzinii* Millán
Polydicliae	*Nicotiana clevelandii* A. Gray; *Nicotiana quadrivalvis* Pursh
Repandae	*Nicotiana nesophila* I.M. Johnston; *Nicotiana nudicaulis* S. Watson; *Nicotiana repanda* Willd.; *Nicotiana stocktonii* Brandegee
Rusticae	*Nicotiana rustica* L.
Suaveolentes	*Nicotiana africana* Merxm.; *Nicotiana amplexicaulis* N.T.Burb.; *Nicotiana benthamiana* Domin; *Nicotiana burbidgeae* Symon; *Nicotiana cavicola* N.T.Burb.; *Nicotiana debneyi* Domin; *Nicotiana excelsior* (J.M.Black) J.M.Black; *Nicotiana exigua* H.-M.Wheeler; *Nicotiana fragrans* Hooker; *Nicotiana goodspeedii* H.-M.Wheeler; *Nicotiana gossei* Domin; *Nicotiana hesperis* N.T.Burb.; *Nicotiana heterantha* Kenneally and Symon; *Nicotiana ingulba* J.M.Black; *Nicotiana maritima* H.-M.Wheeler; *Nicotiana megalosiphon* Van Huerck and Müll.Arg.; *Nicotiana occidentalis* H.-M.Wheeler; *Nicotiana rosulata* (S. Moore) Domin; *Nicotiana rotundifolia* Lindl.; *Nicotiana simulans* N.T.Burb.; *Nicotiana stenocarpa* H.-M.Wheeler; *Nicotiana suaveolens* Lehm; *Nicotiana truncata* D.E. Symon; *Nicotiana umbratica* N.T.Burb.; *Nicotiana velutina* H.-M.Wheeler; *Nicotiana wuttkei* Clarkson and Symon
Sylvestres	*Nicotiana sylvestris* Speg. and Comes
Tomentosae	*Nicotiana kawakamii* Y. Ohashi; *Nicotiana otophora* Griseb.; *Nicotiana setchellii* Goodsp.; *Nicotiana tomentosa* Ruiz and Pav.; *Nicotiana tomentosiformis* Goodsp.
Trigonophyllae	*Nicotiana obtusifolia* M. Martens and Galeotti; *Nicotiana palmeri* A. Gray
Undulatae	*Nicotiana arentsii* Goodsp.; *Nicotiana glutinosa* L.; *Nicotiana thrysiflora* Bitter ex Goodsp.; *Nicotiana undulata* Ruiz and Pav.; *Nicotiana wigandioides* Koch and Fintelm.

in *Nicotiana* to determine the parental species of *N. tabacum* (Kenton et al., 1993). The nature of the *N. tabacum* paternal genome was further traced to a particular *N. tomentosiformis* lineage (Murad et al., 2002). FISH has been used to physically map the chromosomes of *N. tabacum* (Parokonny and Kenton, 1995). The chromosomal distribution of ten repetitive sequences has been traced across *N.* section *Tomentosae* (Lim et al., 2000). Chase et al. (2003) demonstrated using GISH which of the diploid taxa were involved in the production of 15 *Nicotiana* allopolyploids.

1.3. Molecular phylogenetics—Solanaceae

At higher levels in Solanaceae, Olmstead and Sweere (1994) and Olmstead et al. (1999) used a combined data set of plastid DNA restriction site variation and both *ndhF* and *rbcL* plastid DNA sequences to explore intergeneric relationships. A number of monophyletic groups were demonstrated to exist and of particular interest here is the group "Nicotianoideae" (sensu Olmstead et al., 1999), which consists of *Nicotiana* and an endemic Australian group of genera, tribe Anthocercideae. At lower taxonomic levels, a number of DNA sequence based species-level phylogenetic studies of large genera in the Solanaceae have been published. Bohs and Olmstead (1997) explored relationships in *Solanum* using analyses of *ndhF* sequences. Species in *Lycium* have also been sequenced in a combined plastid phylogenetic study using *matK*, *trnT-L* spacer, and *trnL* intron and *trnL-F* spacer (Fukuda et al., 2001).

1.4. Molecular phylogenetics—"Nicotianoideae"–Anthocercideae

A tribe of Australian Solanaceae, Anthocercideae, were sister to *Nicotiana* in plastid DNA restriction site studies (Olmstead and Palmer, 1991), which was later confirmed by a combination of plastid DNA restriction site data, *ndhF* and *rbcL* sequences (Olmstead and Sweere, 1994; Olmstead et al., 1999). Anthocercideae are not exclusively related to the Australian species of *Nicotiana*, but represent a separate colonization event of the continent (Olmstead and Palmer, 1991). These analyses also demonstrated that the sister group of the Anthocercideae-*Nicotiana* clade ("Nicotianoideae"; Olmstead et al., 1999) was subfamily Solanoideae, together comprising the "$x = 12$" clade. Anthocercideae consist of seven small genera, all endemic to Australia except *Duboisia myoporoides*, which also occurs in New Caledonia (Purdie et al., 1982). Garcia and Olmstead (2003), analyzing a matrix composed of plastid *ndhF*, the *trnL* intron, and the *trnL-F* spacer (hereafter the *trnL-F* region), were unable to elucidate the relationships among *Symonanthus*, the rest of Anthocercideae, and *Nicotiana*, leading to the possibility that relationships were not as they had been assumed to be. Knapp et al. (2000) had shown pollen morphology in Anthocercideae, including *Symonanthus*, to be uniform and different from that of *Nicotiana*, which could be interpreted to support the monophyly of Anthocercideae relative to *Nicotiana*, but the study of Garcia and Olmstead (2003) could not confirm this relationship.

1.5. Molecular phylogenetics—"Nicotianoideae"–Nicotiana

Nicotiana tabacum was the first angiosperm to have its complete plastid genome sequenced (Shinozaki et al., 1986), which has proven valuable in showing the relative physical positions of its exons and introns. The first molecular phylogenetic analysis of many of the species in the genus (21 species) was performed by Olmstead and Palmer (1991), who used data from variation in plastid DNA restriction sites. They found that *N. tabacum* is identical in these patterns to its putative maternal parent, *N. sylvestris*. They also formulated a recent, long distance dispersal hypothesis to explain the distribution of the Australian *N.* sect. *Suaveolentes*, which was based on the monophyly of the section and the low levels of variation among the component species.

Komarnitsky et al. (1998a,b) used the internal transcribed spacer (ITS) of ribosomal DNA (rDNA) for phylogenetic analysis of the genus. However this study produced some sequences that were unusually divergent, and it is now thought that they represent mixtures (created by naïve editing of electropherograms) of active and pseudogene copies (paralogues) of ITS and should not be used in further studies of the genus (Chase et al., 2003). More recently, a small-scale 5S rDNA phylogenetic analysis was published, but taxon sampling was too sparse to make major conclusions about evolution of many groups (Kitamura et al., 2001).

Aoki and Ito (2000) sequenced the plastid gene *matK* for 39 *Nicotiana* species, which produced a set of relationships for the diploids and identified the maternal parent of many of the hybrids. Many portions of the trees were highly unresolved due to too few informative characters in *matK* alone, and little could be stated with confidence about the relationships of the sections, although the major clades identified by Aoki and Ito (2000) corresponded well to the sections in Goodspeed's monograph (1954). Chase et al. (2003) published a phylogenetic analysis with almost complete species sampling based on a newly collected set of sequences (see Section 2) from the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA). In the case of certain allopolyploids, these trees provided evidence of a different history of inheritance than were observed in those of Aoki and Ito (2000) based on plastid *matK*. The hybrid taxa in no case produced 'hybrid' sequences

due to the effect of gene conversion (Wendel et al., 1995). Only in cases for which gene conversion favors the maternal type would taxa be resolved in the same position as in the plastid *matK* trees. ITS nrDNA data are useful, but they need to be carefully interpreted along with other evidence. Neither plastid *matK* nor ITS nrDNA sequences alone can produce a "species tree" for *Nicotiana* with all of its putative allopolyploids. However the high levels of congruence between the patterns of relationships found for the diploids in these two data sets and the sections circumscribed by Goodspeed (1954) indicate that the species tree may have been recovered for the diploid species.

This study aims to produce a well-resolved maternal phylogenetic assessment for *Nicotiana* and therefore requires more plastid regions to be sequenced per taxon than Aoki and Ito (2000). Additionally, more taxon sampling is required to assess overall relationships, and more outgroup sampling is required because the monophyly of the genus and its relationship to Anthocercideae, particularly *Symonanthus*, have not previously been resolved (Garcia and Olmstead, 2003).

This study constructed of a four-locus plastid matrix that builds on the work of the other groups to produce a much more extensive data set (around 4650 bp per taxon). The *trnL-F* and *ndhF* data sets build upon the 24 taxa of Anthocercideae sequenced by Garcia and Olmstead (2003), and the *matK* data set builds upon the 39 species of *Nicotiana* already sequenced by Aoki and Ito (2000). The *trnS-G* spacer data are totally new, and this region was chosen because it has more variable characters relative to its length than any other region evaluated.

2. Materials and methods

Most of the *Nicotiana* plant material was cultivated at the Royal Botanic Gardens, Kew, and extracted directly from fresh tissue, but some taxa were available only from dried (herbarium or field dried in silica gel) sources. We used 56 of the 75 naturally occurring species of *Nicotiana* in our analyses, plus 2 artificial hybrids and 24 of the 32 species of Anthocercideae. Collections made in the field were both dried and stored in silica gel (Chase and Hills, 1991). For extractions, 1 g of fresh tissue or 0.3 g of silica-dried material was used. All accessions of *Nicotiana* were vouchered at The Natural History Museum, London (BM; Table 2). Vouchers for Anthocercideae were provided in Garcia and Olmstead (2003). Table 2 lists all voucher material; herbarium acronyms follow Holmgren et al. (1990).

DNA was extracted using a modified Doyle and Doyle (1987) 2× CTAB method. DNA was precipitated in chilled ethanol (−20 °C) for at least 24 h and then resuspended in 1.55 g/ml cesium chloride/ethidium bromide. Samples were then purified using a density gradient, followed by removal of the ethidium with butanol/dialysis and storage in TE.

Target regions were amplified in a Gene Amp 9700 PCR system (ABI, Applied Biosystems, Warrington, Cheshire, UK) using ReddyMix PCR Mastermix at 2.5 mM $MgCl_2$ concentration (ABGene, Epsom, Surrey, UK). See Table 3 for details of primers used. A new internal primer was designed for *matK* and designated *matK* 1350R (5′-GTA CTT TTG TGT TTA CGA GCC A-3′). PCR programs varied for each region. The shortest regions, *trnL-F* and *trnS-G*, were amplified using the following program: 4 min at 94 °C followed by 28 cycles of 1 min at 94 °C, 1 min at 48 °C, and 1 min at 72 °C. For *matK* and *ndhF*, we used the following: 4 min at 94 °C followed by 28 cycles of 1 min at 94 °C, 1 min at 50 °C, and 3 min at 72 °C. PCR products were cleaned using Concert miniprep columns (Life technologies, Paisley, Strathclyde, UK) following the manufacturer's protocols. Samples were sequenced on a Prism 377 automated sequencer (ABI) and, more recently, an ABI 3100 capillary DNA sequencer using Big Dye terminator v3.1 chemistry, following the manufacturer's protocols (ABI). For cleaning of cycle sequencing products, we used precipitation in ethanol.

The raw sequences were edited and assembled using Sequencher version 4.1 (Gene Codes, Ann Arbor, Michigan, USA). They were aligned by eye in the matrix following the guidelines provided by Kelchner (2000). Previous phylogenetic analyses of Solanaceae (Olmstead and Palmer, 1992; Olmstead and Sweere, 1994; Olmstead et al., 1999) identified the Solanoideae as sister of Olmstead et al.'s (1999) "Nicotianoideae" (*Nicotiana* and Anthocercideae). Therefore we selected *Atropa* and *Mandragora* from different parts of this clade to use as outgroups. *Cestrum* and *Petunia* were also included to evaluate Goodspeed's hypothesis that some sort of "prepetunioid" and "pre-cestroid" groups were involved in the evolution of *Nicotiana*. Gaps were coded as missing data in the analysis and were thus excluded from the analysis. We did however survey their distribution after the analysis to see if their inclusion would provide improved resolution or bootstrap percentages (BP), but in no case were they useful in this way; they routinely marked clades for which bootstrap support was already high (greater than BP 90) based simply on analyses of the sequences themselves. Table 2 includes GenBank accession numbers for all plastid sequences including those from previously published studies.

2.1. Parsimony analysis

PAUP version 4.0b (Swofford, 2001) was used for parsimony analysis. For full searches, 86 terminals were included, 35 of which were used also for a "diploids only" analysis. Heuristic searches were performed using

Table 2
Vouchers (herbarium acronyms follow Holmgren et al., 1990 and www.nybg.org/ih)

Species	Voucher details	DNA bank number	GenBank accession number for designated region			
			trnL-F	*trnS-G*	*matK*	*ndhF*
Nicotiana						
Nicotiana acaulis Speg.	Helgason and Monro 600 BM	12642	AJ577422	AJ584973	AB039985	AJ585918
Nicotiana acuminata (Graham) Hook.	Lim 015 BM	12643	AJ577428	AJ584979	AJ585849	AJ585924
Nicotiana africana Merxm.	Clarkson 020 BM	12685	AJ577448	AJ585022	AJ585881	AJ585943
Nicotiana alata Link and Otto	Helgason and Monro 501, 518 BM	12609	AJ577436	AJ584987	AB040000	AJ585932
Nicotiana amplexicaulis N.T. Burb.	Clarkson 010 BM	14219	AJ577444	AJ584995	AB040019	AJ585940
Nicotiana arentsii Goodsp.	Clarkson 001 BM	12675	AJ577415	AJ584966	AJ585844	AJ585911
Nicotiana attenuata Torrey ex S. Wats.	Helgason and Monro 621 BM	12610	AJ577401	AJ584953	AJ585837	AJ585898
Nicotiana benavidesii Goodsp.	Helgason and Monro 601 BM	12720	AJ577402	AJ584954	AB039991	AJ585899
Nicotiana bonariensis Lehm.	Helgason and Monro 622 BM	12611	AJ577434	AJ584985	AB039986	AJ585930
Nicotiana cavicola N.T. Burb.	Saikia 006 BM	12688	AJ577442	AJ584993	AB040016	AJ585938
Nicotiana clevelandii A. Gray	Lim 019 BM	12682	AJ577429	AJ584980	AJ585850	AJ585925
Nicotiana cordifolia Phil.	Saikia 008 BM	12639	AJ577430	AJ584981	AJ585851	AJ585926
Nicotiana corymbosa J. Rémy	Lim s.n. BM	12683	AJ577431	AJ584982	AJ585852	AJ585927
Nicotiana debneyi Domin	Saikia 001 BM	12693	AJ577403	AJ584955	AB040017	AJ585900
Nicotiana × *digluta*	Saikia 021 BM	12680	AJ577432	AJ584983	AJ585853	AJ585928
Nicotiana exigua H.-M. Wheeler	Clarkson 011 BM	14220	AJ577445	AJ584996	AJ585856	AJ585941
Nicotiana forgetiana Hemsl.	Helgason and Monro 500, 512 BM	12614	AJ577400	AJ584952	AB040001	AJ585897
Nicotiana glauca Graham	Nee et al. 51725 BM	12673	AJ577414	AJ584965	AB039987	AJ585910
Nicotiana glutinosa L.	Helgason and Monro 514 BM	12615	AJ577404	AJ584956	AB039995	AJ585901
Nicotiana goodspeedii H.-M. Wheeler	Saikia 009 BM	12695	AJ577457	AJ585031	AJ585890	AJ585951
Nicotiana gossei Domin	Olmstead S-48 WTU	s.n.	AY098700	—	AB040018	AY098727
Nicotiana kawakamii Y. Oyashi	Helgason and Monro 632 BM	12674	AJ577416	AJ584967	AJ585845	AJ585912
Nicotiana knightiana Goodsp.	Saikia 017 BM	12677	AJ577417	AJ584968	AB039989	AJ585913
Nicotiana langsdorfii Weinm.	Helgason and Monro 528 BM	12616	AJ577440	AJ584991	AB039999	AJ585936
Nicotiana linearis Phil.	Helgason and Monro 609 BM	12617	AJ577405	AJ584957	AB040011	AJ585902
Nicotiana longiflora Cav.	Helgason and Monro 510 BM	12618	AJ577399	AJ584951	AB040002	AJ585896
Nicotiana maritime H.-M. Wheeler	Clarkson 014 BM	14223	AJ577451	AJ585025	AJ585884	AJ585945
Nicotiana megalosiphon Van Heurck and Müll.Arg.	Saikia 002 BM	12692	AJ577443	AJ584994	AJ585855	AJ585939
Nicotiana miersii J. Rémy	Clarkson 003 BM	12641	AJ577423	AJ584974	AJ585848	AJ585919
Nicotiana nesophila I.M. Johnst.	Saikia 011 BM	12660	AJ577441	AJ584992	AJ585854	AJ585937
Nicotiana noctiflora Hook.	Lim 005 BM	12640	AJ577424	AJ584975	AB040006	AJ585920
Nicotiana nudicaulis S. Wats.	Saikia 004 BM	12662	AJ577412	AJ584964	AB040013	AJ585909
Nicotiana obtusifolia M. Martens and Galeotii	Lim 004 BM	12661	AJ577438	AJ584989	AB039997	AJ585934
Nicotiana occidentalis H.-M. Wheeler	Saikia 012 BM	12691	AJ577456	AJ585030	AJ585889	AJ585950
Nicotiana otophora Griseb.	Nee et al. 51739 BM	12266	AJ577418	AJ584969	AJ585846	AJ585915
Nicotiana palmeri A. Gray	Helgason and Monro 631 BM	12619	AJ577406	AJ584958	AJ585838	AJ585903
Nicotiana paniculata L.	Helgason and Monro 502 BM	12620	AJ577398	AJ584950	AB039988	AJ585895
Nicotiana pauciflora J. Rémy	Helgason and Monro 635 BM	12621	AJ577407	AJ584959	AJ585839	AJ585904
Nicotiana petunioides (Griseb.) Millán	Lim 001 BM	12645	AJ577425	AJ584976	AB040008	AJ585921
Nicotiana plumbaginifolia Viv..	Helgason and Monro 505 BM	12623	AJ577397	AJ584949	AB040003	AJ585894
Nicotiana quadrivalvis Pursh	Chase 11944 K	11944	AJ577437	AJ584988	AB040012	AJ585933
Nicotiana raimondii J.F. Macbr.	Helgason and Monro 603 BM	12625	AJ577408	AJ584960	AJ585840	AJ585905
Nicotiana repanda Willd.	Clarkson 019 BM	12681	AJ577427	AJ584978	AB040004	AJ585923
Nicotiana rosulata (S. Moore) Domin	Clarkson 015 BM	14224	AJ577452	AJ585026	AJ585885	AJ585946
Nicotiana rotundifolia Lindl.	Clarkson 016 BM	14225	AJ577453	AJ585027	AJ585886	AJ585947
Nicotiana rustica L.	Helgason and Monro 626 BM	12626	AJ577439	AJ584990	AB039992	AJ585935
Nicotiana × *sanderae*	Helgason and Monro 616 BM	12627	AJ577409	AJ584961	AJ585841	AJ585906
Nicotiana solanifolia Walp.	Clarkson 004 BM	12679	AJ577433	AJ584984	AB039990	AJ585929
Nicotiana stocktonii Brandegee	Lim 003 BM	12644	AJ577426	AJ584977	AB040005	AJ585922
Nicotiana suaveolens Lehm.	Helgason and Monro 517 BM	12628	AJ577410	AJ584962	AJ585842	AJ585907
Nicotiana sylvestris Speg. and Comes	Helgason and Monro 628 BM	12629	AJ577396	AJ584948	AB039998	AJ585893
Nicotiana tabacum L. 'Big Cuban'	Clarkson 005 BM	12650	AJ577435	AJ584986	Z00044	AJ585931
Nicotiana thyrsiflora Goodsp.	Clarkson 009 BM	12690	AJ577455	AJ585029	AJ585888	AJ585949
Nicotiana tomentosa Ruiz and Pav.	Saikia 020 BM	12684	AJ577421	AJ584972	AB039993	AJ585917
Nicotiana tomentosiformis Goodsp.	Clarkson 007 BM	12671	AJ577420	AJ584971	AJ585847	AJ585916
Nicotiana undulata Ruiz and Pav.	Saikia 015 BM	12678	AJ577419	AJ584970	AB039996	AJ585915

Table 2 (*continued*)

Species	Voucher details	DNA bank number	GenBank accession number for designated region			
			trnL-F	*trnS-G*	*matK*	*ndhF*
Nicotiana velutina H.-M. Wheeler	Clarkson 017 BM	14226	AJ577454	AJ585028	AJ585887	AJ585948
Nicotiana wigandioides Koch and Fintelm.	Helgason and Monro 658 BM	12632	AJ577411	AJ584963	AJ585843	AJ585908
Anthocercidae						
Anthocercis angustifola F. Muell.	Olmstead 94-05 WTU	12696	AY098671	AJ584997	AJ585857	AY098704
Anthocercis gracilis Benth.	Stace s.n. 12/1/98 (Herb. unknown)	12697	AY098672	AJ584998	AJ585858	AY098705
Anthocercis ilicifolia Hook.	Stace s.n. 3/12/98 UWA	12698	AY098673	AJ584999	AJ585859	AY098706
Anthocercis intricata F. Muell.	Stace s.n. 4/12/98 KPBG	12699	AY098674	AJ585001	AJ585861	AY098707
Anthocercis littorea Labill.	Stace s.n. 12/1/98 (Herb. unknown)	12700	AY098675	AJ585000	AJ585860	AY098708
Anthocercis sylvicola Macfar. and Ward.	Middleton s.n. 12/21/98 PERTH	12701	AY098676	AJ585002	AJ585862	AY098709
Anthocercis viscosa R. Br.	Symon 14835 AD	12702	AY098677	AJ585003	AJ585863	U08914
Anthotroche blackii F. Muell.	Stace s.n. 4/12/98 KPBG	12703	AY098678	AJ585004	AJ585864	AY098711
Anthotroche myoporoides C.A. Gardner	Stace s.n. 4/12/98 KPBG	12704	AY098679	AJ585005	AJ585865	AY098810
Anthotroche pannosa Endl.	Stace s.n. 4/12/98 KPBG	12705	AY098680	AJ585006	AJ585866	AY098712
Anthotroche walcottii F. Muell.	Bellairs and Bellairs 2035 PERTH	12706	AY098681	AJ585007	AJ585867	AY098713
Crenidium spinescens Haegi	Lepschi and Lally 1672 CANB	12707	AY098682	AJ585008	AJ585868	AY098714
Cyphanthera albicans (A. Cunn.) Miers	Lepschi and Lally 1722 CANB	12708	AY098683	AJ585010	AJ585870	AY098715
Cyphanthera anthocercidea (F. Muell.)	Haegi 1456 AD	12709	AY098684	AJ585009	AJ585869	AY098716
Cyphanthera microphylla Miers	Lepschi 2170 PERTH	12710	AY098685	AJ585013	AJ585873	AY098717
Cyphanthera myosotidea (F. Muell.) Haegi	Alcock 9117 AD	12711	AY098686	AJ585014	AJ585874	—
Cyphanthera odgersii (F. Muell.) Haegi	Chinnock 3100 AD	12712	AY098687	AJ585016	AJ585875	AY098718
Cyphanthera racemosa (F. Muell.) Haegi	Haegi 1959 AD	12713	AY098688	AJ585015	AJ585867	—
Duboisia leichhardtii (F. Muell.) Haegi	Haegi 2056 AD	12714	AY098689	AJ585012	AJ585872	AY098719
Duboisia myoporoides R. Br.	Symon 14832 AD	12715	AY098690	AJ585011	AJ585871	AY098720
Grammosolen dixonii Muell. and Tate	Symon 14833 AD	12716	AY098691	AJ585017	AJ585876	AY098721
Grammosolen truncatus (Ising) Haegi	Canty 2429 AD	12717	AY098692	AJ585018	AJ585877	AY098722
Symonanthus aromaticus (C.A. Gardner) Haegi	McKinney s.n. 12/14/98 (Herb. unknown)	12718	AY098693	AJ585019	AJ585878	AY098723
Symonanthus bancroftii (F. Muell.) Haegi	Stace s.n. 4/12/98 KPBG	12719	AY098694	AJ585020	AJ585879	AY098724
Outgroups						
Mandragora officinarum L.	Chase 13338 K	13338	AJ577450	AJ585024	AJ585883	U08922
Petunia axillaris (Lam.) Britton, Stern and Poggenb.	Chase 2371 K	2371	AJ577447	AJ585021	AJ585880	AJ585942
Cestrum elegans Schltdl.	Chase 12217 K	12217	AJ577458	AJ585032	AJ585891	AJ585952
Atropa belladonna L.	Sheahan 015 K	11048	AJ577449	AJ585023	AJ585882	AJ585944

tree bisection-reconnection (TBR) branch swapping and 1000 replicates of random taxon addition were performed with 10 trees held at each step to reduce time searching suboptimal 'islands' of trees. All character transformations are treated as equally likely and unordered (Fitch, 1971). This was followed by successive approximation weighting, according to Farris (1989), to select the most stable trees (see also 'choosing parsimony trees' in Carpenter, 1988). DELTRAN character optimization was used to illustrate branch lengths throughout (due to reported errors with ACCTRAN optimization in PAUP version 4.0b). To asses internal support, 1000 bootstrap replicates (Felsenstein, 1985) were performed with equal weights using TBR branch swapping with 10 trees held at each step and simple taxon addition. We did not analyze each of the plastid regions separately because they all exhibit low levels of sequence divergence (Garcia and Olmstead, 2003) and there is no reason to suspect incongruence among different regions of a uniparentally inherited, non-recombining genome. Plastid and ITS data were analyzed separately. Hybrid accessions were removed before directly combining these data, and none of the "tests of combinability" were performed. Instead we prefer to look for highly supported incongruent patterns in the separate analyses (BP > 90) and see if other groups appear or bootstrap percentages decrease in the combined analysis (Reeves et al., 2000; Whitten et al., 2000).

Table 3
Sources of primers used in this study

Primer region name	Primer sequences first published	Primers used in this study
trnL-F	Taberlet et al. (1991)	c (forward) f (reverse)
ndhF	Olmstead and Sweere (1994)	972F (forward) 2110R (reverse)
matK (& *trnK*)	Aoki and Ito (2000)	Start (forward) TF (forward) 1350R[a](reverse) *trn*K01[b] (reverse)
trnS-G	Hamilton (1999)	S (forward) G (reverse)

[a] Internal primer designed for this work (sequence provided in text).
[b] *trnK* primer designed from sequence provided by Neuhaus and Link (1987).

2.2. Bayesian analyses

Bayesian analysis was performed using MrBayes (Huelsenbeck and Ronquist, 2001). This considers the many possible histories of substitution, weighted by their probability of occurring in a specific model of evolution (Huelsenbeck et al., 2001). An HKY85 model was specified in which all transitions and transversions have potentially different rates. More complex models were also used, but these provided the same tree with similar posterior probabilities (PP). The analysis was performed with 500,000 generations of Monte Carlo Markov chains with equal rates and a sampling frequency of 10. Microsoft Excel was used to plot generation number against lnL to find the 'burn in.' Trees of low PP were deleted, and all remaining trees were imported into PAUP 4.0b. A majority rule consensus tree was produced showing the frequencies (i.e., posterior probabilities) of all observed bi-partitions.

3. Results

3.1. Plastid parsimony analysis

The total number of characters was 4649 of which 817 were variable (17.6%) and 410 (8.8%) were potentially parsimony informative. The number of characters

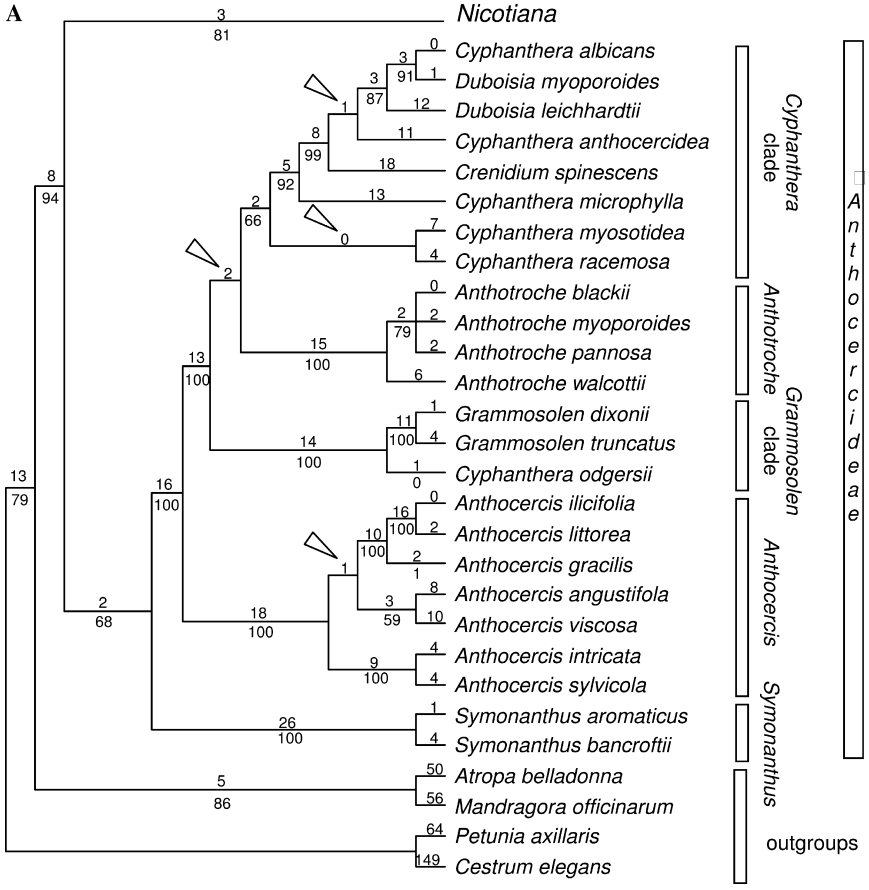

Fig. 1. One of the most parsimonous trees from the all taxon plastid analysis. Branch lengths (DELTRAN optimization) are shown above branches and all BP above 50 are shown below branches. An open arrowhead indicates groups not in all SW trees. A shaded arrowhead indicates groups not in all SW trees and all Fitch trees. (A) Anthocercideae and outgroups (B) *Nicotiana* excluding section *Suaveolentes* (C) *N.* section *Suaveolentes*. Bars indicate, in (A) Anthocercideae genera/informal groupings of Garcia et al. (2004) and (B). *Nicotiana* sections according to Knapp et al. (2004).

contributed by each individual region is 988 from *trnL-F* (intron and spacer), 1633 from *matK*, 953 from *trnS-G*, and 1075 from *ndhF*. Analysis produced 1620 equally most-parsimonious trees (length = 1141 steps; consistency index, CI, (including autapomorphies here and below) = 0.81; retention index, RI, = 0.90). Successive weighting (SW) yielded 972 equally most-parsimonious trees each with a SW score of 837.00 (1141 Fitch length—these were a subset of the Fitch trees; SW CI = 0.81; SW RI = 0.90). One of the SW trees with its Fitch branch lengths (DELTRAN optimization) is shown in Figs. 1A–C. Groups not found in all SW trees are marked with an open arrowhead, whereas those not found in all Fitch trees as well are marked with a solid arrowhead. Bootstrap percentages (BP) consistent with the strict consensus tree are shown below each branch, but groups with BP less than 50 are not indicated.

The two genera from the Solanoideae form a clade (BP 86). Anthocercideae and *Nicotiana* form a well-supported clade (BP 94) and are sister groups with BP 81 for *Nicotiana* and BP 68 for Anthocercideae (Fig. 1A).

Anthocercis (BP 100), *Anthotroche* (BP 100), *Grammosolen* (BP 100), and *Symonanthus* (BP 100) all

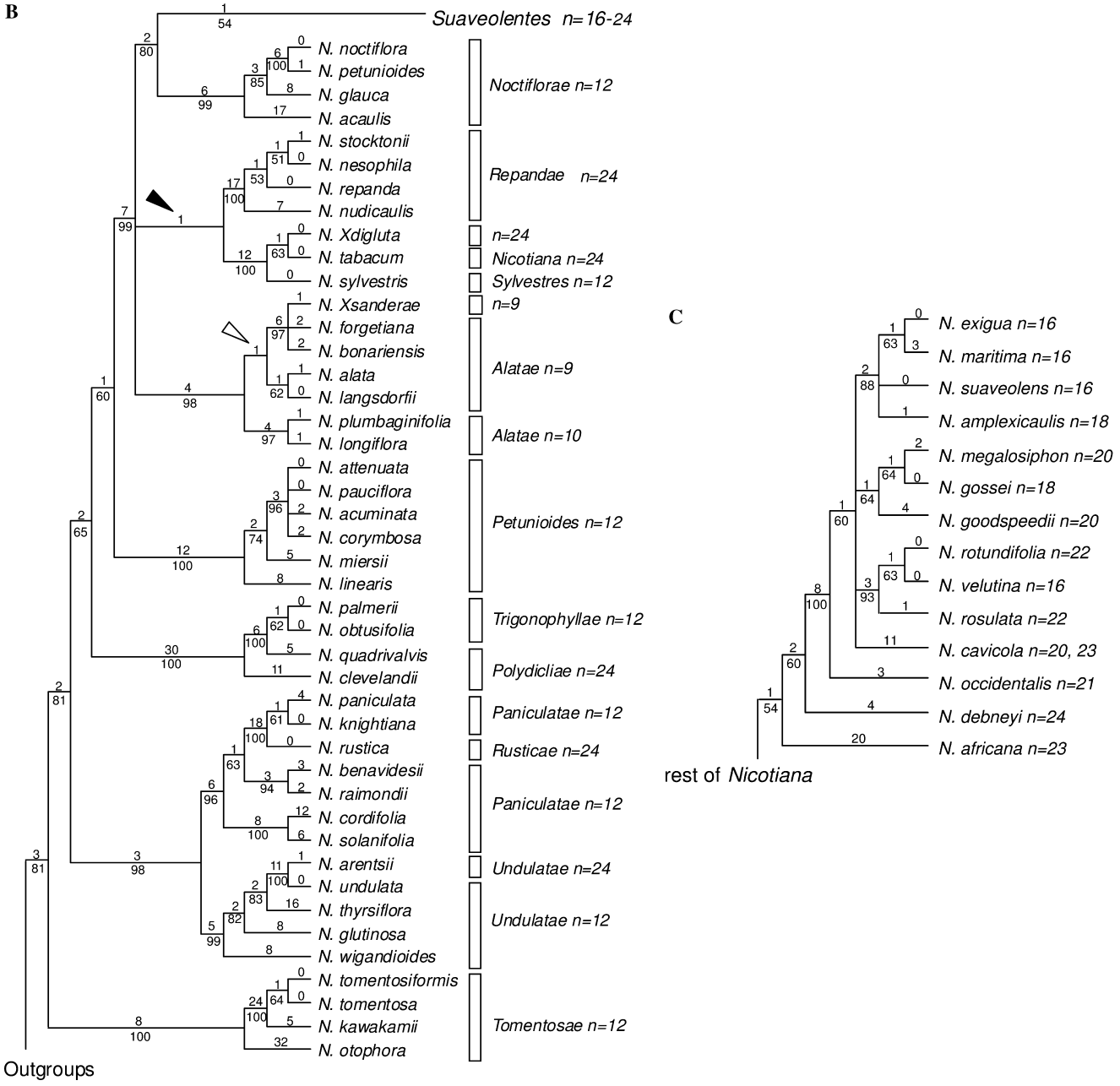

Fig. 1. (*continued*)

form well-supported monophyletic groups. *Cyphanthera* and *Duboisia* are not monophyletic. *Cyphanthera odgersii* is sister to *Grammosolen* (BP 100). *Cyphanthera albicans* is resolved within the *Duboisia* clade. Among all five regions sequenced, *Cyphanthera albicans* has only one difference from *Duboisia myoporoides* (Fig. 1A).

In *Nicotiana* (Fig. 1B), *N*. sections *Tomentosae* (BP 100), *Undulatae* (BP 99), *Paniculatae* (BP 96), *Trigonophyllae* (BP 62), and *Petunioides* (BP 100). *Alatae* (BP 98), *Repandae* (BP 100), *Noctiflorae* (BP 99), and *Suaveolentes* (BP 54) all form monophyletic groups. *Polydicliae* are not monophyletic due to having *Trigonophyllae* embedded within them (Fig. 1B).

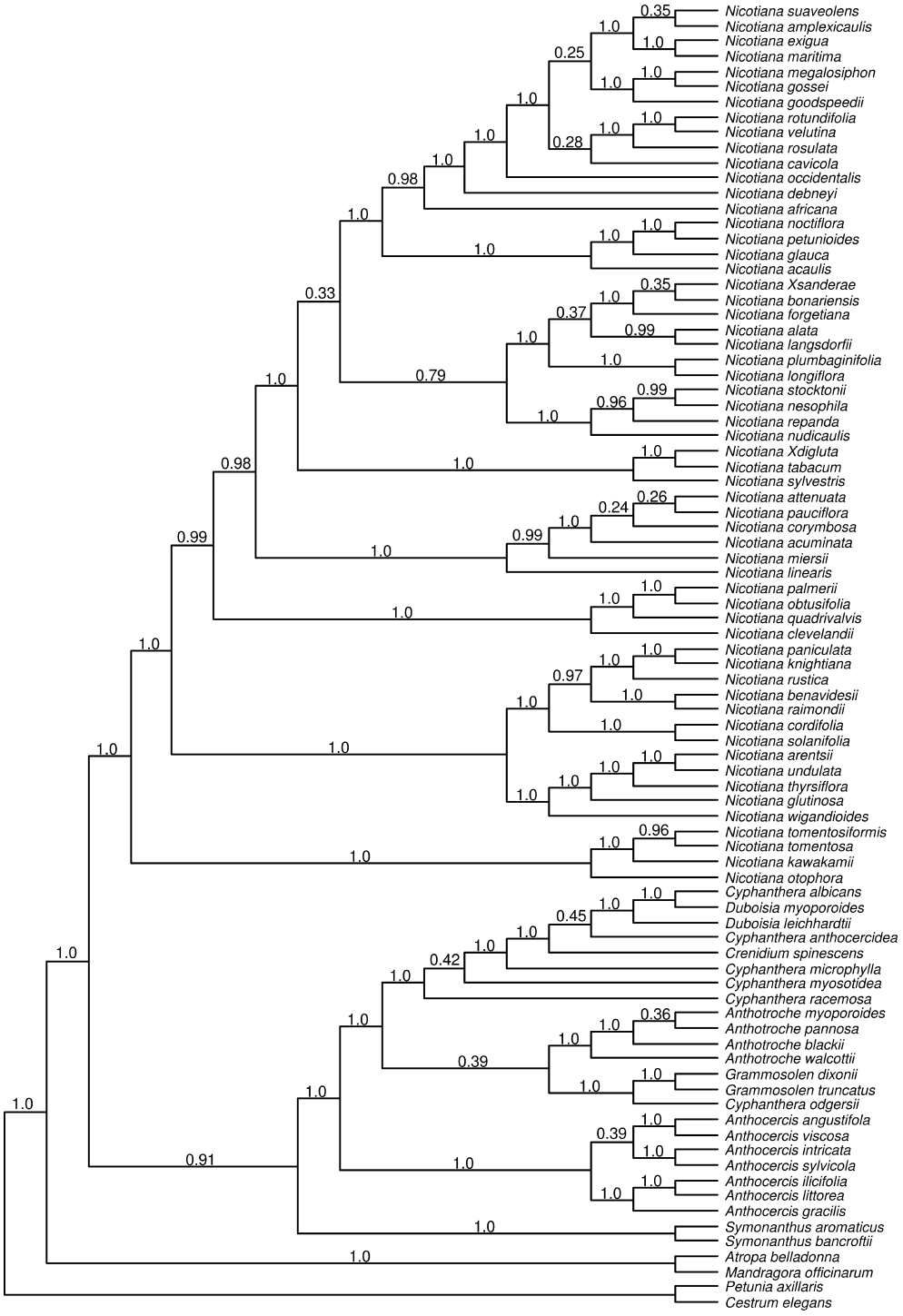

Fig. 2. Bayesian analysis of all taxon plastid dataset. Consensus of 33001 trees with posterior probabilities shown above branches.

3.2. Plastid Bayesian analysis

The "burn-in," 16999 trees of low likelihood, were omitted from the consensus tree. Bayesian analysis produced the same overall topology as did parsimony (Fig. 2). All of the clades that differ have low BP in parsimony analysis and low PP in the Bayesian tree, although the general trend is that the Bayesian posterior probabilities are higher than bootstrap percentages.

3.3. Combined parsimony analysis (diploids only)

The total number of characters was 5049 of which 495 were variable (9.8%) and 316 (6.3%) were potentially parsimony informative. The number of character contributed by each individual region is 926 from *trnL-F* (intron and spacer), 1560 from *matK*, 813 from *trnS-G*, 1074 from *ndhF*, and 676 from ITS. Analysis produced a single most parsimonious tree (length = 731 steps, CI = 0.76; RI = 0.83) which is shown in Fig. 3 (DELTRAN optimization). *Nicotiana* sections *Tomentosae* (BP 100), *Trigonophyllae* (BP 100), *Undulatae* (BP 98), *Paniculatae* (BP 97), *Petunioides* (BP 100), *Noctiflorae* (BP 100), and *Alatae* (BP 99) are all well supported.

3.4. Combined Bayesian analysis

In the Bayesian analysis, 9999 trees of low likelihood were eliminated before producing the consensus tree (Fig. 4). *Nicotiana* sections *Tomentosae* (PP 1.0), *Trigonophyllae* (PP 1.0), *Undulatae* (PP 1.0), *Paniculatae* (PP 1.0), *Petunioides* (PP 1.0), *Noctiflorae* (PP 1.0), and *Alatae* (PP 1.0) are all well supported. For the most part, the spine of the tree also has high PP (1.0), except for the position of *N. sylvestris* (PP 0.91) relative to *N.* sects. *Alatae* and *Noctiflorae* and the position of the clade composed of *N.* sects. *Undulatae* and *Paniculatae* (PP 0.99) relative to *N.* sect. *Trigonophyllae* and a large clade composed of the remaining sections except for *N.* sect. *Tomentosae* (Fig. 4).

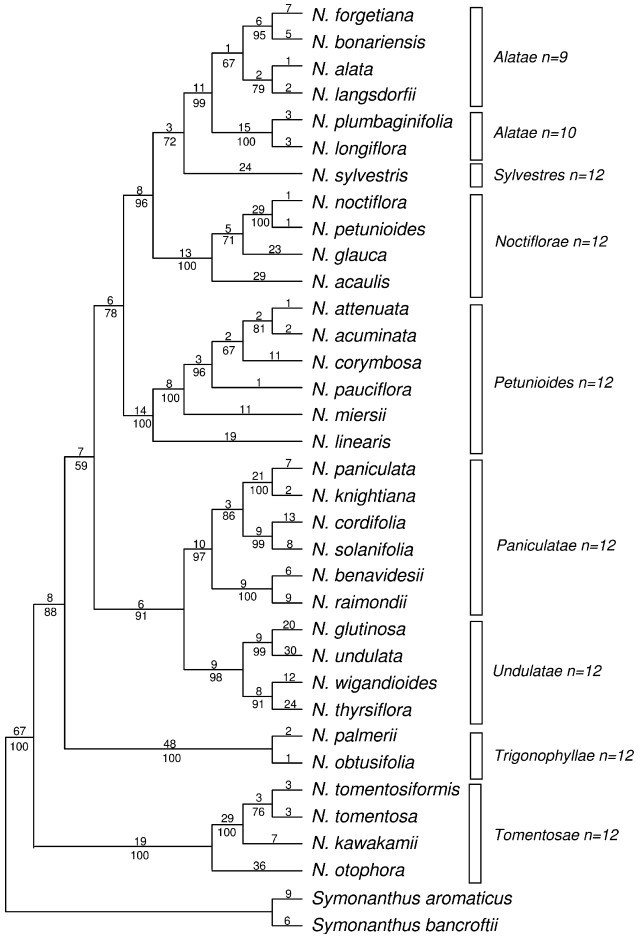

Fig. 3. The single most parsimonous trees from the diploids only combined (plastid and ITS) parsimony analysis. Branch lengths (DELTRAN optimization) are shown above branches and all BP above 50 are shown below branches. Bars indicate *Nicotiana* sections according to Knapp et al. (2004).

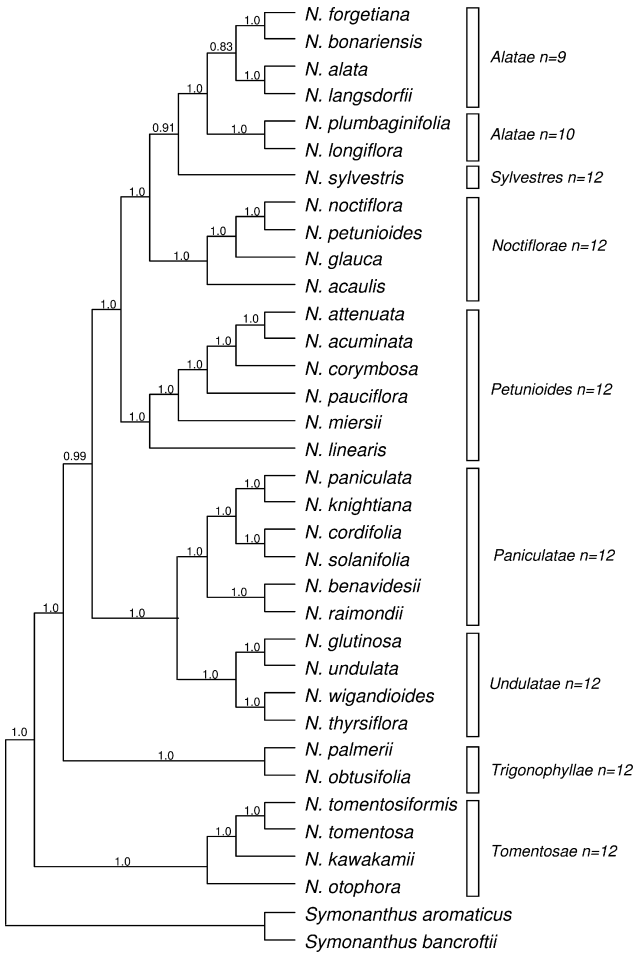

Fig. 4. Bayesian analysis of diploids only combined dataset (plastid and ITS). Consensus of 40001 trees with posterior probabilities shown above branches. Bars indicate *Nicotiana* sections according to Knapp et al. (2004).

4. Discussion

Previous analyses that have included more genera (Fay et al., 1997; Olmstead and Palmer, 1992; Olmstead and Sweere, 1994; Olmstead et al., 1999) support *Petunia* and *Cestrum* as being distantly related to *Nicotiana*. As other authors have pointed out (Chase et al., 2003), it is difficult to know what Goodspeed (1954) meant by his idea of *Nicotiana* having "pre-petunioid" and "pre-cestroid" progenitors. It is clear from molecular trees that the sister group of *Nicotiana* is the Australian tribe Anthocercideae (Fig. 1A) and that any involvement of *Cestrum*, *Petunia*, or their ancestors is unlikely.

4.1. Phylogenetic relationships in Anthocercideae

The monophyly of Anthocercideae, including *Symonanthus*, which was unclear in the previous study of Garcia and Olmstead (2003), is here confirmed, albeit with low support in the plastid analyses (BP 68, PP 0.91). Excluding *Symonanthus*, the support for the monophyly of Anthocercidae is strong (BP 100; PP 1.0). Their monophyly is in agreement with the distribution of other types of data available for the tribe, such as their unique combination of morphological characters (Armstrong, 1986; Haegi, 1986; Knapp et al., 2000). Much of the overall topology of our tree resembles the those presented by Garcia and Olmstead (2003), but many nodes have higher BP. Increased BP (100 vs. 75 in Garcia and Olmstead) can be seen for the monophyly of the large clade consisting of the *Grammosolen* clade, *Anthotroche*, and the *Cyphanthera* clade. The two species of *Grammosolen* are more firmly confirmed as sisters (BP 100 vs. 95). Increased support for the *Grammosolen* clade supports *Cyphanthera odgersii* as their sister. Within *Anthotroche*, the three species, *A. blackii*, *A. myoporoides*, and *A. pannosa*, form a moderately supported clade (BP 79 vs. 65). The *Duboisia* group, which contains *Cyphanthera albicans*, has increased support (BP 87 vs. 77). This relationship further complicates the use of fruit type as a generic character in Anthocercideae; most of the tribe have capsular fruits, with the exception of the species currently recognized as *Duboisia*, which have berries. As in the previous study (Garcia and Olmstead, 2003), *Cyphanthera* and *Duboisia* do not form monophyletic groups. Re-circumscription of these genera should be undertaken when the remaining species from Anthocercideae have been included (particularly representatives of the two, non-monophyletic genera).

4.2. Phylogenetic relationships in Nicotiana

Both the plastid tree and the combined tree (diploids only) have a well-supported spine. Their topologies are largely in agreement, and the only substantive point at which they differ corresponds to the placement of sect. *Trigonophyllae*, which has low support in the plastid and combined trees. As first noted by Olmstead and Palmer (1991) and confirmed by the studies of Aoki and Ito (2000), and Chase et al. (2003), the subgenera of *Nicotiana* as proposed by Goodspeed (1954) are not monophyletic. However Goodspeed's sections, to a large extent, are natural groups. The formal classification of the genus has been recently refined to reflect the growing body of evidence on *Nicotiana* (Knapp et al., 2004), but these changes are as much nomenclatural as phylogenetic.

Members of *N.* section *Tomentosae* are sister to the rest of *Nicotiana* (BP 88; PP 1.0). This pattern has been observed in all previous DNA studies, but has never had strong support. Within the section, *N. otophora* is isolated and sister to the rest. *Nicotiana kawakamii* is resolved as sister to the species pair, *N. tomentosa* and *N. tomentosiformis* (BP 76, PP 1.0). These last two taxa have identical plastid sequences (Fig. 1B) but are slightly different for ITS (six substitutions; Fig. 3). *Nicotiana tomentosa* and *N. tomentosiformis* are similar morphologically with whitish pink flowers with curved floral tubes and exerted anthers, but it is clear that they differ genetically; the ITS sequence found in accessions of *N. tabacum* (allotetraploid) are identical to those found in *N. tomentosiformis* and differ consistently from those in *N. tomentosa*. Furthermore, the virus-derived inserts found in some accessions of cultivated tobacco are much more similar to those found in some accessions of *N. tomentosiformis* than to those found in *N. tomentosa* (Murad et al., 2002).

Sister to Sect. *Tomentosae* is the rest of the genus (Figs. 1B, 2; BP 81, PP 1.0) and within this clade sections *Paniculatae*/*Undulatae* form a clade (BP 98; Fig. 1B) that is sister to the rest of the genus (BP 65), excluding *N.* sect. *Tomentosae*. In this clade, *Trigonophylleae*/*Polydicliae* (BP 100) are sister to the remainder, which form a clade with only low support (BP 60; Fig. 1B). However, when the ITS data are combined with the plastid data (Figs. 3, 4), *Trigonophyllae* (BP 100; PP 1.0) are sister to a larger clade (BP 59; PP 0.99) that includes the *Paniculatae*/*Undulatae* clade and the rest of the genus (BP 78; PP 1.0). For each data set, the Bayesian analysis puts these clades in the same positions as does parsimony, with similar levels of BP and PP support. In the Bayesian analysis of the combined matrix, the posterior probabilities are all high, which may be due in part to the reduced sampling of diploids only. This same pattern or something similar has been recovered consistently in most of the separate analyses as well, so this may be considered a reliable basis for further research on these taxa, but we have reservations about the level of confidence in these measures as indicators of reliability, because posterior probabilities have been shown to overestimate support (Suzuki et al., 2002), particularly when multiple data sets are combined as they are here.

Nicotiana palmeri and *N. obtusifolia* (*N.* sect. *Trigonophyllae*) have identical plastid sequences and only slightly different ITS sequences (three substitutions). Wells (1960) considered *N. palmeri* to be a subspecies of *N. obtusifolia*, but Goodspeed (1954) had treated them as distinct species. The line between species and subspecies cannot be defined in terms of number of substitutions, and therefore our data cannot be used to determine which is the most appropriate treatment.

Nicotiana sect. *Polydicliae* are paraphyletic to *N.* sect. *Trigonophylleae* in the plastid tree (Fig. 1B), because the same ancestral lineage of *N. obtusifolia*/*N. palmeri* was at different times the maternal progenitor of the two species in *N.* sect. *Polydicliae.* Chase et al. (2003) showed using analyses of ITS nrDNA sequences that the paternal parent is a progenitor species of the lineage that later gave rise to the extant species of *N.* section *Acuminatae*. Although the formation of the two species of *N.* section *Polydicliae* did not take place at the same time, thus making the section paraphyletic, the same two parental lineages gave rise to these taxa. It is perhaps, therefore, not appropriate to place these species in the same section, because they are not strictly monophyletic (i.e., did not have exactly the same parents), but because they are the products of reticulation and their parents appear to have given rise to morphologically similar extant species, we have for practical reasons placed them in the same section.

The next large clade consists of *N.* sects. *Undulatae* and *Paniculatae* (BP 98; PP 1.0). In section *Undulatae* (BP 99; PP 1.0), the plastid topology differs from the combined result. Groups with lower bootstrap support in the plastid analysis give way to a different topology with higher support when ITS is added. In the plastid Bayesian analysis, *N. wigandioides* is sister to the rest of the section (PP 1.0), followed successively by *N. glutinosa* (PP 1.0), *N. thrysiflora* (PP 1.0), and *N. undulata*/*N. arentsii* (PP 1.0), the last an allotetraploid with *N. undulata* its maternal parent. Conversely, in the combined analysis there are two pairs of sister species, *N. thyrsiflora*/*N. wigandioides* (PP 1.0) and *N. undulata*/*N. glutinosa* (PP 1.0) consistent with results of ITS alone (Chase et al., 2003). Thus in the Bayesian analyses of the plastid data alone, there are "strongly supported" (PP 1.0) and incongruent relationships. Bayesian and parsimony analyses of the plastid data recover the same topology, but in the latter the relationships are only moderately supported (BP 82-83). We prefer the combined estimate of relationships, because in the parsimony results the number of steps on each of the critical branches is only two substitutions. In the case of the plastid data set the Bayesian analysis appears to be overestimating support. Morphology of these species could be used to support either pattern, and there is considerable character conflict. The combined analysis topology places *N. wigandioides* and *N. thyrsiflora* as sister species (BP 91); they are similar in having flowers with short, relatively broad tubes that are inflated at the apex, and spreading corolla lobes. They differ, however, in habitat, with *N. wigandioides* growing in cloud forest and *N. thrysiflora* in dry forests, leaf morphology (*N. wigandioides* with petiolate leaves; *N. thyrisiflora* with sessile leaves) and flower colour (*N. wigandioides* white; *N. thyrsiflora* bright yellow). Goodspeed referred them to separate sections (*N.* sects. *Undulatae* and monotypic *Thyrsiflorae*, respectively) largely based on the unusual congested inflorescence and yellow flowers of *N. thrysiflora*. *Nicotiana glutinosa* does not share obvious morphological features with *N. undulata*; it was thought to be related to *N. tomentosa* by Goodspeed (Chase et al., 2003) due largely to its similar curved pinkish white flowers. It shares long-petiolate leaves with *N. wigandioides*. *Nicotiana undulata* shares sessile leaves and somewhat congested inflorescences with *N. thrysiflora*, and a slightly zygomorphic corolla with *N. wigandioides*. The pattern of morphological character distribution in the section is complex and unclear. Geography complicates matters further as *N. thrysiflora* occurs only within the range of *N. glutinosa* in northern Peru, whereas *N. undulata* and *N. glutinosa* are sympatric, though separated elevationally, in northern Bolivia. *Nicotiana wigandioides* is sympatric with *N. undulata* but occurs in lower-elevation cloud forests. Further analysis of these and other morphological characters in these species will help resolve the differences we see in the combined versus plastid analyses.

In *N.* section *Paniculatae*, three pairs of geographically close, sister species are identified: *N. raimondii* and *N. benavidesii* (BP 94 plastid; BP 100 combined); *N. solanifolia* and *N. cordifolia* (BP 100, 99); and *N. knightiana* and *N. paniculata* (BP 61, 100). All of these species pairs share long tubular, yellow or greenish yellow flowers, long-petiolate leaves with cordate or truncate bases, and soft white pubescence, further supporting their sister relationships. These species pairs are difficult to distinguish from each other both vegetatively and florally but are not found sympatrically. *Nicotiana cutleri* is similar to *N. benavidesii* and *N. raimondii* both morphologically (large somewhat inflated flowers with a small limb and strongly cordate leaves) and in terms of habitat (dry, high elevation forests), and we expect it to be a member of that clade. *Nicotiana knightiana* is closer genetically (one substitution in the plastid data) to the amphidiploid species *N. rustica* than its putative (Goodspeed, 1954) progenitor *N. paniculata* (five substitutions, one shared with *N. knightiana*; Fig. 1B). *Nicotiana knightiana* has never been used to 'paint' *N. rustica* chromosomes (Chase et al., 2003), although on the basis of our data it should be. It could be that the common ancestor of the sister pair, *N. knightiana*/*N. paniculata*, was the parent of *N. rustica* instead of either of the extant species.

The next clade is *N.* section *Petunioides* (BP 100; PP 1.0), in which *Nicotiana linearis* and *N. miersii* are successively sister to the rest. Goodspeed wrote that 'herbage of *N. linearis*, like that of *N. miersii*, is sweet-smelling,' indicating that they possibly may have chemical similarities. The remaining four species in the section are closely related according to the plastid data (BP 96; PP 1.0). This was suggested by Goodspeed (1954): "*N. acuminata* shows extremely close affinity with *N. pauciflora* ..." and "Crosses between *N. acuminata* and *N. corymbosa* have a high degree of fertility." *Nicotiana attenuata* and *N. pauciflora* produced identical plastid DNA sequences and differed only slightly in the ITS sequences. Goodspeed emphasized their similarities but distinguished them on the basis of differing floral morphology.

The last strongly supported clade (BP 99; PP 1.0) is composed of *N.* sections *Alatae*, *Sylvestres*, *Nicotiana*, *Repandae*, *Noctiflorae*, and *Suaveolentes*, among which relationships are unresolved in the plastid tree. *Nicotiana* sect. *Alatae* is monophyletic (BP 99; PP 1.0) and consists of three clusters of closely related species. A previous RAPD analysis also demonstrated this lack of variation between *Alatae* species (Bogani et al., 1997). *Nicotiana longiflora* and *N. plumbaginifolia* are supported as sister species (BP 100; PP 1.0). Genetic linkage maps were easy to produce between these sister species suggesting their close affinities (Lin et al., 2001). This is consistent with them being the only species of *Nicotiana* having $n = 10$, which is probably cytologically most like the ancestral *N.* sect. *Alatae* type with $n = 12$. There would then have been a subsequent further dysploid reduction to $n = 9$ for rest of the section, although since the two cytological groups are sisters we cannot say that they did not evolve independently from $n = 12$ or that $n = 10$ did not evolve from $n = 9$. It makes more sense for there to have been a descending dysploid series.

Nicotiana alata and *N. langsdorfii* are sister species (BP 79; PP 1.0) but have markedly different floral morphology; the flowers of *N. alata* are large and white with a long floral tube, whereas those of *N. langsdorfii* are green and with a much shorter and more open tube. There would appear to have been a switch in pollinator associated with the evolution of *N. langsdorfii*. The artificial hybrid *N.* × *sanderae* (*N. forgetiana* × *N. alata*) is part of a trichotomy with *N. forgetiana* and *N. bonariensis* (BP 97), which is consistent with its maternal parent being *N. forgetiana*.

Nicotiana sylvestris has been removed (Knapp et al., 2004) from *N.* section *Alatae*, in which it was included by Goodspeed (1954) to the monotypic *N.* section *Sylvestres* due to its isolated position as sister to *N.* section *Alatae* (Fig. 3; BP 72; PP 0.91). It possesses many autapomorphies (24 substitutions in the combined analysis), reinforcing its distinctness and isolated position. Additionally, *N. sylvestris* is cytologically distinct from rest of *N.* section *Alatae* ($n = 12$; Lin et al., 2001). *Nicotiana sylvestris* is the maternal parent of amphidiploid *N. tabacum* and *N. tabacum* was the maternal parent of *N.* × *digluta* (*N. glutinosa* was the paternal parent). These amphidiploids have identical plastid sequences and they differ by a single substitution from *N. sylvestris* (Fig. 1B) and have identical ITS sequences (Chase et al., 2003).

Nicotiana nudicaulis (previously recognized as the sole member of *N.* section *Nudicaules*) is resolved as sister to the rest of *N.* section *Repandae* (BP 53; PP 0.96) (see Knapp et al., 2004, for these changes to the taxonomy of *Nicotiana*). The rest of the species of *N.* section *Repandae*, *N. repanda*, *N. nesophila*, and *N. stocktonii*, are all extremely closely related, and their relationships to each other are only weakly supported, reflecting the group's morphological uniformity. *Nicotiana nudicaulis*, however, has short-tubular diurnal flowers more similar to those of *N. obtusifolia* (see Figs. 3B and G in Knapp et al., 2004), the reason Goodspeed (1954) maintained it as a monotypic section distinct from section *Repandae*. These four species occur around the northern edge of the Gulf of Mexico in Texas and northeastern Mexico (*N. repanda*, *N. nudicaulis*) and the isolated Revillagigedo islands (*N. stocktonii* and *N. nesophila*). Unlike the other American allopolyploid section, *Polydicliae*, the species of *N.* section *Repandae* are all descended from a single common ancestal allopolyploid species (i.e., they are monophyletic, whereas the former section is paraphyletic because they were produced sequentially by the same parental lineages).

Nicotiana section *Noctiflorae* is well supported (BP 99; PP 1.0), with *N. acaulis* sister to the rest of the section. This species has many (17) autapomorphies, mirroring its morphological divergence from the rest of the species of this section; for example, it has the ability to spread vegetatively by producing underground stems (Goodspeed, 1954). As was found with ITS (Chase et al., 2003), *N. glauca* is clearly embedded in *Noctiflorae*; it had been a species of problematic affinities for Goodspeed (1954). He referred it to *N.* section *Paniculatae* based on flower color, but stressed its morphological and cytological distinctness. *Nicotiana glauca* has been transferred to *N.* sect. *Noctiflorae* by Knapp et al. (2004).

Goodspeed (1954) stressed the affinities between *N. noctiflora* and *N. petunioides* and went as far as saying that 'certain small-flowered races of the former species appear intermediate between the two.' This association is clearly seen with these data; the two taxa are separated by just two substitutions in their plastid and ITS sequences (Fig. 3). They are supported as sister to *N. glauca* (BP 71; PP 1.0), to which they are similar vegetatively, although their flowers are markedly different in form and color (see Fig. 2C and D, in Knapp et al., 2004).

Nicotiana section *Suaveolentes* is an almost exclusively Australian clade (the exception being *N. africana* of Namibia) of amphidiploids (BP 54; PP 98), which supports their singular origin. Their position, sister to *N.* section *Noctiflorae*, could indicate that the group's maternal parent was an ancestor of section *Noctiflorae*. Also, as concluded by Olmstead and Palmer (1991), the general lack of variation between members of the section indicates their diversification is a recent event. *Nicotiana africana* is weakly supported by the plastid data as sister to the clade comprising the Australian species (BP 60; PP 1.0), which is consistent with the ITS nrDNA analysis of Chase et al. (2003). When the two data sets are combined, this placement is strongly supported (BP 92; tree not shown). It seems likely that the amphidiploid ancestor of *N.* section *Suaveolentes* occurred in South America, where its parental species are found, and then the progenitor species dispersed to Africa and Australia separately at about the same time or first to one continent and then relatively quickly to the other. Only in Australia has an explosive radiation of taxa occurred, largely accompanied by dysploid reductions probably due to fusions of chromosomes.

Goodspeed (1954) stated that '*N. debneyi* is the modern representative of an earlier progenitor of section *Suaveolentes*.' He based this statement on its morphology, chromosome number, eastern coast (Australia) distribution, and preference for a humid environment. This general view is supported by our analysis because *N. debneyi* is sister to the rest of the Australian species. After this, *N. occidentalis* is sister to all other species in the plastid and combined data sets. Goodspeed (1954) noted that *N. debneyi* and *N. occidentalis* share 'somewhat auriculate cauline leaves.' The rest of *Suaveolentes* are much more closely related.

4.3. Biogeography of the sections of Nicotiana

Nicotiana section *Tomentosae*, which is sister to the rest of *Nicotiana* (BP 88, PP 1.0) is eastern Andean, ranging from northern Peru to Argentina where the species grow at high elevations in open and disturbed sites. *Nicotiana* section *Trigonophyllae* occurs in western North America, where its ancestor must have arrived by long distance dispersal from the Andes (only *N. plumbaginifolia* occurs in Central America today). The single or perhaps two species occur in arid habitats (Death Valley in California, for example) over a wide range of elevations. *Nicotiana* section *Undulatae* is principally eastern Andean from southern Ecuador to southern Bolivia, where the species grow at high elevation in a variety of habitats. Its sister group, *N.* sect. *Paniculatae*, is western Andean from Peru to Bolivia, generally in low elevation fog-influenced habitats (''lomas'') or high elevation, dry forests. One species is on Masafuera (Juan Fernández Islands), with its sister species on the adjacent Peruvian/Chilean coast. This distribution could have been achieved by a jump westward over the Andes and then dispersal to the coast with a final move out to Masafuera.

Nicotiana sect. *Petuniodes* is a disjunct section but is mainly southern Andean. *Nicotiana attenuata* is found in western North America and must have arrived there relatively recently by long distance dispersal, based on its close relationship to *N. acuminata* (BP 81, PP 1.0; only three substitutions different in the combined analysis; Fig. 3). The rest of the species are found in the southwestern Andes from northern Chile into Patagonia from sea level upwards. *Nicotiana* sect. *Noctiflorae* is also southern Andean from Argentina and Chile into Patagonia at 40°S, where they occur at sea level but range as high as 4300 m on both sides of the Andes. *Nicotiana glauca* is an invasive weed worldwide, but apparently it originated in northern Argentina or southern Bolivia, only somewhat disjunct from the rest of the members of the section.

Nicotiana sect. *Sylvestres* (sole member, *N. sylvestris*) is eastern Andean from southern Bolivia to northern Argentina at moderate elevations. *Nicotiana* sect. *Alatae* are plants of disturbed sites predominantly from the eastern Andes to southeastern Brazil; *N. plumbaginifolia* is found from the S United States to Argentina and Brazil, it is the most widespread diploid species of *Nicotiana*. *Nicotiana* sect. *Polydicliae* is found in the deserts of western North America. *Nicotiana quadrivalvis* was the native Indian tobacco of the western USA and was widespread in cultivation north to British Columbia on the Pacific coast; it is unclear where it is native. The species of *N.* sect. *Repandae* are sympatric on the southeastern coast of North America and around the Caribbean (Gulf of Mexico) or on the isolated, volcanic Revillagigedo Islands off the coast of Baja California; nearly all species are from low elevation.

The bimodal distribution of the diploid species (no species natively found in Central America, except perhaps *N. plumbaginifolia*, which may be introduced in the region) is perplexing, but several species clearly arrived in Mexico and North America by long distance dispersals from the Andes, where the rest of their relatives are distributed, so it may be that all of the North American distribution is the result of dispersal not vicariance. Alternatively, the genus was in the past broadly distributed throughout the Americas and its current disjunct distribution is the result of extinction in Central America. The greatest species diversity is found in the eastern Andes, and it is simplest to hypothesize that it was here that the genus evolved and then spread in a series of short and long distance moves to achieve its current distribution, including at least once to each of the western Andes (*N.* sect. *Paniculatae*) and Brazil (*N.* sect. *Alatae*) and from the Andes to North America on several occasions. Some species occur far enough south

in South America (Patagonia) to use the coastal route to reach the western slopes of the Andes (*N.* sects. *Noctiflorae* and *Petunioides*).

The allotetraploids have also shown vagility, the most spectacular of which was dispersal of *N.* sect. *Suaveolentes* to Africa and Australia; in the latter case radiating into numerous species. Few allotetraploids overlap in range with either of their parental diploids, making it appear that either the ranges of these diploids have shrunk or that hybridization and changes in ploidy were associated with dispersal, sometimes clearly over great distances. Human dispersal of some species has complicated understanding the underlying patterns in a few cases.

Establishing when these events took place would be desirable because it might provide more insight into whether dispersal or geological forces were responsible for the current bimodal pattern, but until more variable loci are sequenced this effort could only be tentative. There are some species on oceanic islands for which dates of emergence have been established (e. g., Masafuera and the Revillagigedo Islands), but the species restricted to these sites share identical or nearly identical sequences with their sister species on the mainland, which suggests very recent arrival on these islands, but does not permit us to calibrate a molecular clock.

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