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The Ups and Downs of Genome Size Evolution in Polyploid Species of *Nicotiana* (Solanaceae)

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• *Background* In studies looking at individual polyploid species, the most common patterns of genomic change are that either genome size in the polyploid is additive (i.e. the sum of parental genome donors) or there is evidence of genome downsizing. Reports showing an increase in genome size are rare. In a large-scale analysis of 3008 species, genome downsizing was shown to be a widespread biological response to polyploidy. Polyploidy in the genus *Nicotiana* (Solanaceae) is common with approx. 40 % of the approx. 75 species being allotetraploid. Recent advances in understanding phylogenetic relationships of *Nicotiana* species and dating polyploid formation enable a temporal dimension to be added to the analysis of genome size evolution in these polyploids.
• *Methods* Genome sizes were measured in 18 species of *Nicotiana* (nine diploids and nine polyploids) ranging in age from <200 000 years to approx. 4·5 Myr old, to determine the direction and extent of genome size change following polyploidy. These data were combined with data from genomic *in situ* hybridization and increasing amounts of information on sequence composition in *Nicotiana* to provide insights into the molecular basis of genome size changes.
• *Key Results and Conclusions* By comparing the expected genome size of the polyploid (based on summing the genome size of species identified as either a parent or most closely related to the diploid progenitors) with the observed genome size, four polyploids showed genome downsizing and five showed increases. There was no discernable pattern in the direction of genome size change with age of polyploids, although with increasing age the amount of genome size change increased. In older polyploids (approx. 4·5 million years old) the increase in genome size was associated with loss of detectable genomic *in situ* hybridization signal, whereas some hybridization signal was still detected in species exhibiting genome downsizing. The possible significance of these results is discussed.

Key words: Genome downsizing, genome size, *Nicotiana*, polyploidy, sequence elimination, Solanaceae.

INTRODUCTION

The genus *Nicotiana*, comprising approx. 75 species, displays a range of genomic changes including gene conversion, tandem and dispersed sequence evolution, intergenomic translocations, dysploidy, polyploidy, etc. (Kenton *et al.*, 1993; Matzke *et al.*, 2004; Melayah *et al.*, 2004; Dadejova *et al.*, 2007; Lim *et al.*, 2007; Kovarik *et al.*, 2008). Given the increasingly robust phylogenetic framework now available (Chase *et al.*, 2003; Clarkson *et al.*, 2004, 2005), *Nicotiana* is thus ideally suited to study patterns of genome evolution.

Polyploidy is common in the genus, with approx. 40 % of species being allotetraploid. They comprise (*a*) *N. tabacum* (section *Nicotianae*), (*b*) *N. rustica* (section *Rusticae*), (*c*) *N. arentsii* (section *Undulatae*), (*d*) *N. clevelandii* and *N. quadrivalvis* (section *Polydicliae*), (*e*) *N. nudicaulis*, *N. repanda*, *N. nesophila* and *N. stocktonii* (section *Repandae*) and (*f*) all approx. 23 species in section *Suaveolentes*. All polyploids, except some of those in section *Suaveolentes*, are $2n = 4x = 48$, representing a doubling of the diploid chromosome number for the genus ($2n = 2x = 24$). In section *Suaveolentes*, polyploid evolution has been accompanied by changes in chromosome number, probably through dysploid reductions via chromosome deletions or fusions ($2n$ ranges from 32 to 48).

Based on cytological and floral morphology, combined with sequence data from plastid and nuclear genes (Goodspeed, 1954; Aoki and Ito, 2000; Chase *et al.*, 2003; Clarkson *et al.*, 2004), the likely parentage of nearly all allotetraploid species has now been determined, together with estimates of their ages based on combining molecular clock analysis and calibration with the ages of oceanic volcanic islands (Clarkson *et al.*, 2005; Kovarik *et al.*, 2008) (Fig. 1). These data show that the polyploid species range considerably in age. The youngest (*N. tabacum*, *N. rustica* and *N. arentsii*) are each estimated to have arisen <200 000 years ago, followed by the two species in section *Polydicliae*, which are approx. 1 million years (Myr) old, and the five species in section *Repandae*, which are approx. 4·5 Myr old (Clarkson *et al.*, 2005). The oldest polyploids, in section *Suaveolentes*, are considered to have originated from a single polyploid event >10 Myr ago, followed by speciation to produce the approx. 23 species known today.

Nicotiana polyploids not only vary in age but also in the relatedness of the parental species contributing genomes to

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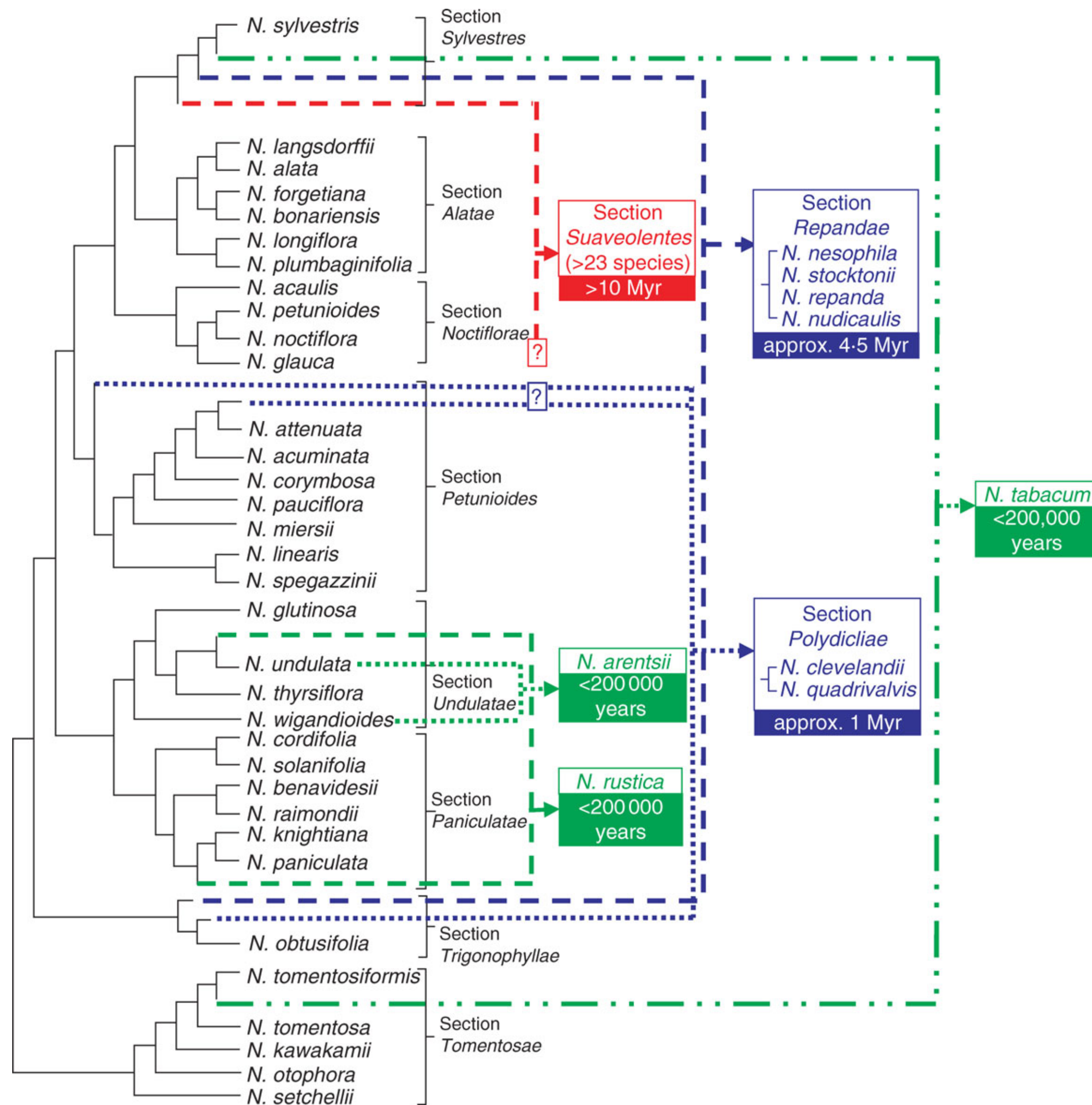

FIG. 1. Summary of phylogenetic relationships of *Nicotiana* species with proposed origins of polyploids. Data used in analyses include plastid and internal transcribed spacer loci. Figure modified and adapted from Knapp *et al.* (2004) using more recent phylogenetic information taken from glutamine synthase (J. J. Clarkson *et al.*, unpubl. res.). Uncertainty concerning one of the parental genome donors for sections *Polydicliae* and *Suaveolentes* is indicated by question marks.

the polyploid nucleus. For example, *N. arentsii* is an intrasectional polyploid, the hybrids and diploid progenitors all belonging to section *Undulatae*. In *N. rustica* (section *Rusticae*) the parental species are in closely related sections (*Paniculatae* and *Undulatae*) (Clarkson *et al.*, 2004; Kovarik *et al.*, 2008). In contrast, the diploid species giving rise to *N. tabacum* and polyploids in section *Repandae*, *Polydicliae* and *Suaveolentes* are from distantly related sections (Figs 1 and 2).

Insights into contrasting patterns of molecular evolution in *Nicotiana* polyploids have been gained through the study of natural and synthetic species. These have involved analysing both specific DNA sequences [e.g. ribosomal DNA (rDNA), non-coding tandem repeats and retrotransposons] and global genome organization using fluorescent *in situ* hybridization (FISH) [including genomic *in situ* hybridization (GISH); Kovarik *et al.*, 1996, 2004; Chase *et al.*, 2003; Lim *et al.*, 2004, 2005, 2006*b*, 2007; Clarkson *et al.*, 2005; Skalicka *et al.*, 2005; Petit *et al.*, 2007]. Such studies have shown that allopolyploidy in *Nicotiana* has been accompanied by numerous genetic changes including (depending on the polyploid species in question) rDNA homogenization and loss of loci (Matyasek *et al.*, 2003; Kovarik *et al.*, 2004; Clarkson *et al.*, 2005), intergenomic translocations (Kenton *et al.*, 1993; Chase *et al.*, 2003; Lim *et al.*, 2004) and changes in copy number and organization of both tandem and dispersed repeats (Melayah *et al.*, 2004; Petit *et al.*, 2007). By combining these data with the known ages of the polyploids, a temporal perspective on polyploidy evolution has been obtained. Lim *et al.* (2007) showed that during early evolution of *Nicotiana* polyploids (i.e. those <200 000

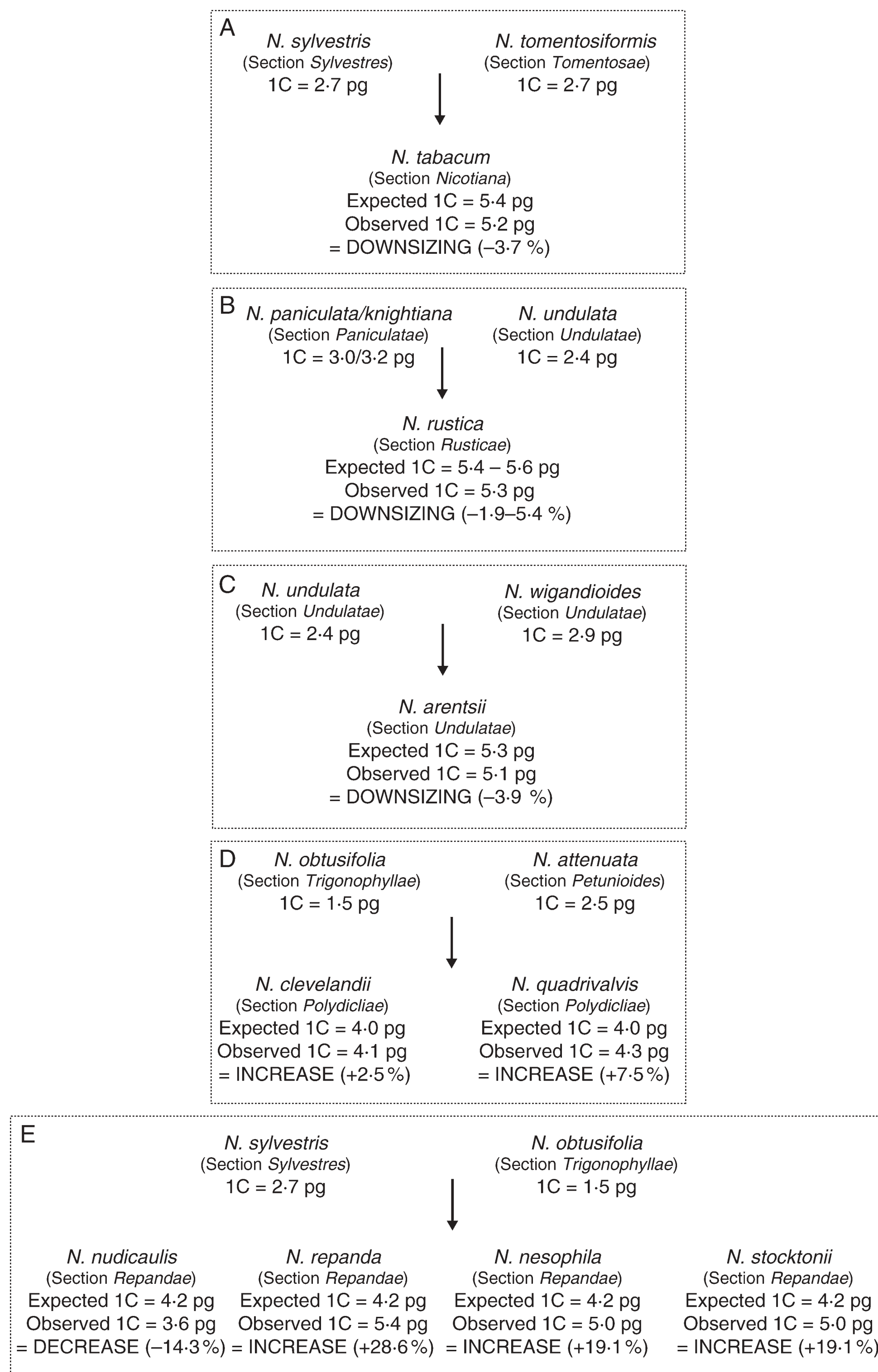

FIG. 2. Direction of genome size evolution in *Nicotiana* polyploids varying in age from $<$200 000 years (A–C), approx. 1 Myr (D) to approx. 4·5 Myr (E) old. The observed versus expected 1C DNA amounts in nine polyploids were based on comparison between genome sizes determined for diploid and polyploid species using genome size data taken from Table 1. Putative diploid parental genome donors used for comparison of the various polyploids are based on a range of molecular and cytogenetic results (see text).

TABLE 1. *List of* Nicotiana *species studied together with chromosome number, 1C and 4C DNA amount, calibration standard and method of estimating genome size*

Species	Accession number (where available) and source of material*	2*n*	4C DNA (± s.d.) (pg)	1C DNA (± s.e.) (pg)	Standard used[†]	Method used[‡]
Section *Nicotiana*						
N. tabacum L.	Nee *et al.* 51789[a]	48	20·70 (1·34)	5·2 (0·060)	*Pisum*	Fe
Section *Petunioides*						
N. attenuata Torrey ex S.Watson	TW13[b]	24	9·93 (0·05)	2·5 (0·002)	*Pisum*	FC
Section *Paniculatae*						
N. knightiana Goodsp.	CPG[e]	24	12·64 (0·06)	3·2 (0·002)	*Pisum*	FC
N. paniculata L.	TW99[b]	24	11·78 (0·08)	3·0 (0·007)	*Pisum*	FC
Section *Polydicliae*						
N. clevelandii A.Gray	TW30[b]	48	16·56 (0·21)	4·1 (0·018)	*Pisum*	FC
N. quadrivalvis Pursh.	TW18[b]	48	17·01 (0·06)	4·3 (0·005)	*Hordeum*	FC
Section *Repandae*						
N. repanda Willd.	TW110[b]	48	21·76 (0·35)	5·4 (0·005)	*Zea*	FC
N. nesophila I.M.Johnston	964750097[d]	48	20·13 (0·07)	5·0 (0·005)	*Pisum*	FC
N. nudicaulis S.Watson	964750051[d]	48	14·22 (0·07)	3·6 (0·008)	*Pisum*	FC
N. stocktonii Brandegee	974750101[d]	48	19·99 (0·06)	5·0 (0·005)	*Pisum*	FC
Section *Rusticae*						
N. rustica L.	NIC 616147[c]	48	21·19 (0·08)	5·3 (0·040)	*Pisum*	FC
Section *Sylvestres*						
N. sylvestris Speg. & Comes	TW127[b]	24	10·78 (0·10)	2·7 (0·002)	*Pisum*	FC
Section *Tomentosae*						
N. tomentosiformis Goodsp.	Nee *et al.* 51771[a]	24	10·97 (0·19)	2·7 (0·020)	*Pisum*	FC
Section *Trigonophyllae*						
N. obtusifolia M.Martens & Galeotti	894750176[d]	24	6·18 (0·03)	1·5 (0·002)	*Solanum*	FC
Section *Undulatae*						
N. arentsii Goodsp.	NIC 445/82[c]	48	20·22 (0·15)	5·1 (0·060)	*Pisum*	FC
N. glutinosa L.	Wood 11732[a]	24	8·94 (0·10)	2·2 (0·010)	*Solanum*	FC
N. undulata Ruiz & Pav.	RBG, Kew[f]	24	9·66 (0·04)	2·4 (0·006)	*Pisum*	FC
N. wigandioides Koch & Fintelm.	Nee *et al.* 51764[a]	24	11·38 (0·50)	2·9 (0·020)	*Pisum*	Fe

* Source of material: a = New York Botanic Garden, New York, USA; b = USDA, North Carolina State University, Raleigh, NC, USA; c = IPK, Gatersleben, Germany; d = Botanical and Experimental Garden, Radboud University Nijmegen,The Netherlands; e = Chelsea Physic Garden, London, UK; f = Royal Botanic Gardens, Kew, UK.

[†] Species and 4C values used for calibration standards are as follows: *Pisum* = *Pisum sativum* cv. Minerva Maple, 4C = 17·52 pg; *Solanum* = *Solanum lycopersicum*, 4C = 4·00 pg; *Hordeum* = *Hordeum vulgare* cv. Sultan, 4C = 22·24 pg; *Zea* = *Zea mays* CE-777, 4C = 11·34 pg.

[‡] FC = flow cytometry; Fe = Feulgen microdensitometry.

years old) intergenomic translocations and rearrangement and loss of repeated DNA sequences may take place. Over the next 1–2 Myr, considerable exchange of repeats between the parental genomes becomes apparent, and by 5 Myr there can be near complete genomic turnover including evolution of new repeats not present in the parental species.

Studies of genome size evolution in polyploids in a diverse range of angiosperms have been conducted at a number of levels. For specific polyploid species, there are many reports showing no change or a decrease in DNA amount relative to the proposed progenitor species (see review in Leitch and Bennett, 2004); those showing an increase are much rarer but include a few naturally occurring species of *Hordeum* (Jakob *et al.*, 2004) and artificially induced dihaploids of *Nicotiana* (Dhillon *et al.*, 1983). At the other end of the spectrum, large-scale analyses combining available genome size data for 3008 angiosperms have led to the proposal that genome downsizing is a widespread biological response to polyploidization leading to diploidization of the polyploid genome (Leitch and Bennett, 2004). Such conclusions have been supported by comparative molecular studies. For example, nearly 80 % of the duplicated genes have been lost from rice (*Oryza sativa*) since the polyploid event that gave rise to it approx. 70 Myr ago (Wang *et al.*, 2005), and >50 % of the duplicated genes in maize (*Zea mays*) have been lost since its polyploid origin approx. 3–11 Myr ago (Messing *et al.*, 2004). In addition, comparative analyses of DNA sequences surrounding the *AdhA* locus in diploid and polyploid *Gossypium* genomes suggest that polyploidization may lead to increased rates of illegitimate recombination resulting in a greater loss of DNA from polyploids than related diploids (Grover *et al.*, 2007*b*). Rapid loss of DNA (based both on genome size measurements or loss of AFLP and/or RFLP bands or specific sequences) has also been reported following synthesis of various artificial hybrids and polyploids of *Brassica* (Song *et al.*, 1995), *Nicotiana* (Skalicka *et al.*, 2003, 2005; Petit *et al.*, 2007) and *Aegilops* and *Triticum* (Ozkan *et al.*, 2001, 2003; Kashkush *et al.*, 2002; Levy and Feldman, 2004).

Given contrasting ages of *Nicotiana* polyploids (<200 000 years to >10 Myr), differences in the extent of genome divergence in diploid progenitors and the diversity of molecular changes that have been documented to occur following polyploidization, it seems timely to examine patterns of

genome size evolution in *Nicotiana* polyploids of different ages. Although there have been several previous studies reporting genome sizes for species of *Nicotiana*, the most extensive has been by Narayan (1987) who listed C-values for 51 *Nicotiana* taxa, including all but two of the species studied here. However, in his discussion, genome size evolution was only assessed in the three 'young' polyploids (*N. tabacum*, *N. rustica* and *N. arentsii*), and there were considerable discrepancies between some of his genome size estimates compared with those previously published by himself (Narayan and Rees, 1974), other authors (e.g. Ingle *et al.*, 1975; Galbraith *et al.*, 1983) and here (see Discussion). Thus, genome sizes were estimated in nine diploid and nine polyploid species. Unfortunately, taxonomic relationships and parental genome donors of polyploid *Suaveolentes* species are not yet sufficiently understood to be investigated in this way.

METHODS AND MATERIALS

Plant material

Table 1 lists the 18 species analysed in the current work together with their origin.

Genome size estimation

Two methods were used to estimate genome size in *Nicotiana* species: flow cytometry and Feulgen microdensitometry. Both methods have been shown to produce comparable results (Doležel *et al.*, 1998).

Feulgen microdensitometry using a Vickers M85a microdensitometer followed the methods described in Hanson *et al.* (2001), and flow cytometry was conducted using a Partec CyFlow or PAII as described in Hanson *et al.* (2005). The calibration standard and method used for each species are listed in Table 1. Expected genome sizes of the polyploids were determined by adding the genome sizes of the putative diploid progenitor species.

GISH

GISH was carried out as described in Clarkson *et al.* (2005). Briefly, slides were pretreated with RNase A (100 μg mL^{-1}, 1 h) and pepsin (0·25 μg mL^{-1}, 5 min), followed by denaturation in 70 % formamide in 2× SSC (0·3 M sodium chloride, 0·03 M sodium citrate) at 70 °C for 2 min. The hybridization mixture included 8 μg mL^{-1} digoxigenin-labelled *N. sylvestris* DNA and 8 μg mL^{-1} biotin-labelled *N. obtusifolia* DNA. *In situ* hybridization was carried out overnight at 37 °C. Post-hybridization washes included formamide [20 % (v/v) in 0·1× SSC, 42 °C] giving an estimated hybridization stringency of 80–85 %. Sites of probe hybridization were detected with fluorescein-conjugated anti-digoxigenin IgG (Roche Biochemicals) (20 μg mL^{-1}) and Cy3-conjugated avidin (Amersham Biosciences; 5 μg mL^{-1}). Chromosomes were counterstained with DAPI (4',6-diamidino-2-phenylindole; 2 μg mL^{-1} in 4× SSC,) and mounted in Vectashield medium (Vector Laboratories). Metaphases were photographed on a Leica DMRA2 epifluorescence microscope with an Orca ER camera. Images were processed for colour balance, contrast and brightness uniformly.

RESULTS

Genome size estimates in Nicotiana

Table 1 lists genome size estimates obtained for the species analysed. Figure 2 shows the genome size of the diploid species considered to be most closely related to the species that gave rise to the polyploids, together with the expected versus the observed genome size estimates for these polyploids. Genome downsizing was observed in four of the polyploids studied and genome size increases were observed in the remaining five polyploids.

Comparisons between genome size estimates obtained in the present work for 16 species with those reported by Narayan (1987) are shown diagrammatically in Fig. 3. For 11 species Narayan's estimates were larger than ours whereas in the remaining five species our estimates were greater than Narayan's.

GISH in section Repandae

When GISH was applied to chromosome preparations of the polyploid species *N. nudicaulis* and *N. nesophila* (both in section *Repandae*) the labelling patterns were quite different (Fig. 4). In *N. nudicaulis* both probes (i.e. genomic DNA from *N. sylvestris* and *N. obtusifolia*) hybridized weakly across all chromosomes, although some chromosomes were more strongly labelled with *N. sylvestris* probe (green signal) and others were more strongly labelled with the *N. obtusifolia* probe (red signal). Given the amount of cross-hybridization it was not possible to distinguish the genomic origin of each chromosome in *N. nudicaulis*.

In contrast, hybridization of both probes to chromosomes of *N. nesophila* was weak, with only scattered signal

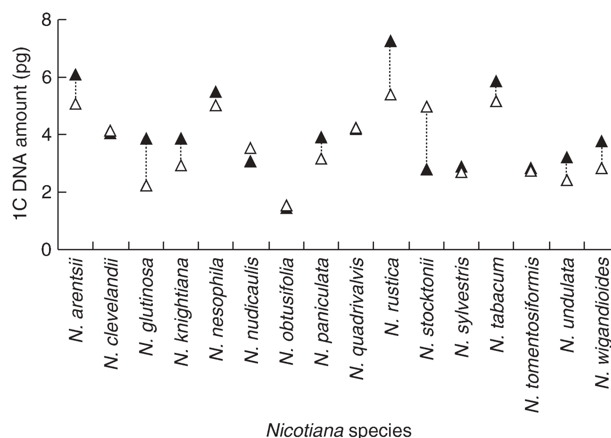

FIG. 3. Comparison between 1C DNA amounts reported by Narayan (1987) (closed triangles) and those given in Table 1 of this paper (open triangles).

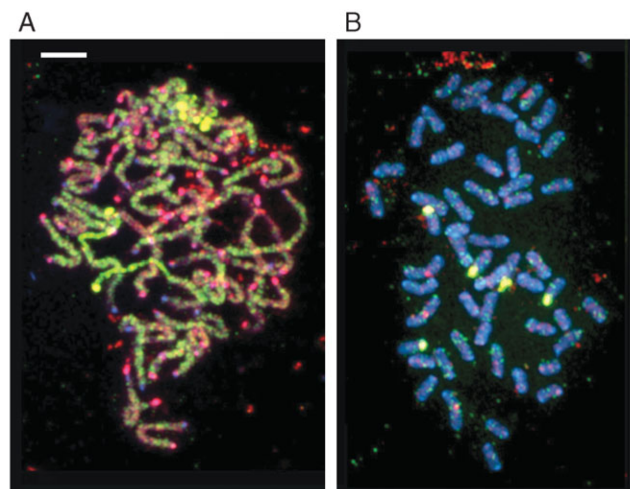

FIG. 4. GISH to (A) *Nicotiana nudicaulis* and (B) *N. nesophila* root-tip metaphase probed with *N. sylvestris* genomic DNA (digoxigenin labelled, FITC detected, yellow/green) and *N. obtusifolia* (biotin labelled, Cy3 detected, red). Chromosomes were counterstained with DAPI (blue). Scale bar = 10 μm.

detected from each probe. The only exception to this was the strong yellow hybridization signals corresponding to the location of ribosomal DNA loci.

DISCUSSION

Comparisons with previous genome size estimates

The most extensive survey of genome sizes in species of *Nicotiana* prior to the present one was that of Narayan (1987) who estimated DNA amounts in 51 species. However, as illustrated in Fig. 3, there are considerable discrepancies between some of his values and those estimated here (Table 1). The most notable are those for *N. rustica* for which Narayan's estimate is nearly one-third larger than ours (1C = 7·2 versus 5·3 pg), and *N. stocktonii* for which our estimate is nearly double that of Narayan's (1C = 5·0 versus 2·8 pg). Further discrepancies are noted between Narayan's estimates and those published by other workers. For example, whereas Galbraith *et al.* (1983) and we (Table 1) reported 1C values of 2·1 and 2·2 pg, respectively, for *N. glutinosa*, Narayan gave an estimate of 1C = 3·9 pg. The reasons for these discrepancies remain unknown, but the observation that the differences are not always in the same direction (i.e. sometimes Narayan's estimates are larger or smaller than ours and previous workers) suggests that there is not a simple, methodological basis behind the differences. Instead the discrepancies may be due to taxonomic issues (e.g. incorrect identification).

Evolution of genome size in Nicotiana *polyploids*

It is noted that the observations and discussions presented below assume that (*a*) the diploid species used are indeed closely related to the actual progenitors that gave rise to the polyploids, and that (*b*) since polyploid formation the genome sizes of these diploids have remained largely unchanged. Any violation of these two assumptions will affect interpretation of the results, but at present it is the best approximation available. Choice of diploid progenitor is based on extensive molecular and morphological analyses of the majority of species in the genus and is thus as comprehensive as possible. The possibility that the incorrect species have been selected is low but cannot be ruled out completely, particularly for the older polyploids. The second assumption is impossible to confirm as it requires estimating genome sizes in the diploid progenitor and polyploid species at the time of polyploidization – thousands to millions of years ago (depending on the polyploid). With these caveats, the following results and discussion on the evolution of genome size in polyploids of *Nicotiana* are presented.

Polyploids <200 000 years old. Three independently produced allotetraploids of *Nicotiana* (all $2n = 4x = 48$), *N. tabacum*, *N. rustica* and *N. arentsii*, are estimated to be approx. 200 000 years old or less. Based on flower and chromosome morphology (Goodspeed, 1954) and plastid and nuclear DNA sequence data (Aoki and Ito, 2000; Chase *et al.*, 2003) the parental genomes of these polyploids are considered to be derived from ancestors of the extant species shown in Fig. 2A–C. All three polyploids appear to have undergone a limited amount of genome downsizing, losing between 3·7 % and 5·4 % over approx. 200 000 years. These results agree with the lower numbers of certain repeats reported in these polyploids compared with their diploid progenitors. For example, in *N. tabacum* there are lower numbers of several repeat sequences, including NTRS, A1/A2 (Lim *et al.*, 2004), a pararetroviral repeat sequence NtoEPRV (Gregor *et al.*, 2004) and various retrotransposons (Melayah *et al.*, 2004; Petit *et al.*, 2007), than in the diploids. In *N. rustica*, an examination of satellite repeats showed they too differed in abundance and distribution relative to the diploid progenitor species (Lim *et al.*, 2005). One repeat (NUNSSP) was similar in organization and copy number to the diploid progenitors, whereas a comparison of a second repeat (NPAMBO) revealed minor changes in its chromosomal distribution but copy number was reduced by at least 10-fold compared with *N. paniculata* (the maternal donor species used for comparison). Such sequence elimination probably occurred since *N. rustica* evolved and could have contributed to the observed 2–5% reduction in DNA amount (Fig. 2B). Alternatively these sequences may have increased in the diploids after polyploid formation.

In all three polyploids the number of 35S rDNA loci is additive, yet within this framework considerable sequence elimination of individual copies has taken place with only 30 %, 50 % and 80 % of the expected copy numbers (based on estimates from the diploid species) observed in *N. tabacum*, *N. arentsii* and *N. rustica*, respectively. In *N. tabacum* and *N. rustica*, most of the remaining repeats from the maternal genome donor have been replaced, via gene conversion, with those originating from the paternal genome donor (Kovarik *et al.*, 2004, 2008). Similar observations have been reported for *N. arentsii*, although here the

direction of homogenization is different, with the 35S rDNA repeats being homogenized to the maternal repeat type (Kovarik *et al.*, 2004, 2008).

Despite these observations, extensive sequence elimination is not apparently a universal response throughout the genomes of these young *Nicotiana* polyploids. In all three, the number of loci and copies of the 5S rDNA sequences are the sum of the diploid progenitors (i.e. additive), with no evidence for sequence loss or homogenization (Fulnecek *et al.*, 2002). Further, the copy number of a satellite repeat isolated from *N. paniculata* (NPAMBE) was greater in *N. rustica*, although the number of extra copies varied 1·7-fold between the seven accessions analysed (Lim *et al.*, 2005).

Polyploids approx. 1 million years old. The two polyploid species comprising section *Polydicliae*, *N. quadrivalvis* and *N. clevelandii*, are estimated to have formed approx. 1 Myr ago. Sequencing of both nuclear and plastid DNA indicates that they most likely arose from two different polyploidization events but involved the same diploid parents; an ancestor of *N. obtusifolia* (section *Trigonophyllae*) as the maternal genome donor and a progenitor of the lineage that later gave rise to *N. attenuata* (section *Petunioides*) as the paternal genome donor (Chase *et al.*, 2003; Clarkson *et al.*, 2004; Knapp *et al.*, 2004; Qu *et al.*, 2004). Analysis of genome size shows that in contrast to the younger polyploids discussed above, these two polyploids have undergone an increase in genome size. In *N. clevelandii*, genome upsizing is small – approx. 2·5 % – but in *N. quadrivalvis* it is more substantial (+7·5 %; Fig. 2D). NB These changes are based on using the genome size of *N. attenuata* as the species most closely related to the paternal genome donor of *Polydicliae*. However, given the lingering controversy concerning the choice of paternal genome donor (i.e. whether an ancestor of *N. attenuata* or an ancestor of all species in section *Petunioides*, Chase *et al.*, 2003), an additional estimate of the observed versus expected genome size in *Polydicliae* was determined by taking the mean of six of the eight species recognized in this section (Knapp *et al.*, 2004) as the genome size for the paternal genome donor (mean 1C = 2·57 pg; I. J. Leitch; L. Hanson, unpubl. res.). Using this approach differences in genome size were still detected for *N. clevelandii* and *N. quadrivalvis* but were smaller than just using the value for *N. attenuata* (+0·5 % and +6 %, respectively).

Currently there is little information on specific molecular changes that have accompanied evolution of species in section *Polydicliae*, although a reduction in genome size might be expected based on the chromosomal distribution of 35S and 5S rDNA sites using FISH. Kovarik *et al.* (2008) showed a loss of loci; both polyploids had just three 35S and one 5S loci compared with the expected five 35S and two 5S loci based on the numbers of loci observed in *N. attenuata* and *N. obtusifolia* (Kovarik *et al.*, 2008). In addition, Wu *et al.* (2006) analysed low-copy genes encoding a family of trypsin-proteinase inhibitors. From cDNA, intron and promoter sequence analysis and Southern blotting, they deduced that only the maternally inherited genes of *N. obtusifolia* were retained in both *N. clevelandii* and *N. quadrivalvis* whereas those of *N. attenuata* were deleted. Thus there is apparent incongruence between the sequence data showing mostly uniparental eliminations and the increased genome sizes. An explanation may stem from the GISH results on *N. quadrivalvis* by Lim *et al.* (2007) showing (*a*) intergenomic mixing of DNA between the two parental genomes and (*b*) the invasion of *N. attenuata* subtelomeric repeat sequences onto *N. obtusifolia* chromosomes followed by their replacement. Adding the genome size data to these observations suggests that genome evolution in these polyploids has been accompanied by increases in the number of existing repeats. Perhaps increase in genome size arises from copy and paste mechanisms that also blur the distinction between the two parental genomes, as revealed by GISH (Lim *et al.*, 2007). Further work is needed to characterize the nature and number of a representative sample of repeats in these polyploids.

Intergenomic sequence invasions involving both rDNA (Wendel *et al.*, 1995) and certain classes of transposable elements (Zhao *et al.*, 1998) have been observed in *Gossypium* polyploids that are estimated to be between 1 and 2 Myr old and thus similar in age to species in section *Polydicliae*. However, whether these rates of intergenomic sequence invasions in polyploid nuclei are ubiquitous or widespread in other polyploid groups of these ages needs to be determined.

Polyploids approx. 4·5 million years old. Section *Repandae* comprises four polyploid species, *N. nudicaulis*, *N. repanda*, *N. stocktonii* and *N. nesophila* (Knapp *et al.*, 2004). Based on DNA sequence data from both the internal transcribed spacer of nuclear rDNA (Chase *et al.*, 2003) and plastid DNA (Clarkson *et al.*, 2004) the group has been shown to be monophyletic with *N. nudicaulis* sister to and distinct (both morphologically and genetically) from the remaining three species (which differ minimally). Clarkson *et al.* (2005) suggested that the original allopolyploidization event occurred approx. 4·5 Myr ago. This was followed by subsequent speciation with *N. nudicaulis* diverging from the rest approx. 2–3 Myr ago and *N. stocktonii* and *N. nesophila* diverging from *N. repanda* more recently, approx. 1 Myr ago. Sequence data from glutamine synthase (Clarkson *et al.*, 2005) and plastid loci (Clarkson *et al.*, 2004) indicate that the maternal genome donor of these four polyploids was an ancestor of section *Sylvestres*, which today comprises a single species *N. sylvestris* (1C = 2·7 pg). The paternal genome donor is an ancestor of section *Trigonophyllae*, which also now comprises a single extant species *N. obtusifolia* (1C = 1·5 pg). If genome size evolution in the four polyploids were additive then one would expect each to have a 1C value of 4·2 pg. However, this is not the case (Table 1, Fig. 2). Instead, the following were observed: (*a*) genome decreases in *N. nudicaulis* (1C = 3·6 pg) with a loss of approx. 14·3 % of DNA compared with the diploid progenitors; (*b*) genome upsizing of 28·6 % in *N. repanda* (1C = 5·4pg) and 19·1 % in *N. nesophila* and *N. stocktonii* (both with 1C = 5·0 pg).

There are considerable differences in the GISH-labelling patterns between *N. nudicaulis* (Fig. 4A) *and N. nesophila* (Fig. 4B). GISH to *N. nudicaulis* gives substantial labelling

of the chromosomes, with some chromosomes that predominantly label with *N. sylvestris* genomic DNA and others predominantly with *N. obtusifolia* genomic DNA. However, due to cross hybridization of the probes, it is not possible to distinguish the parental origin of most chromosomes of the complement. In contrast, GISH to *N. nesophila* generates little signal at all, with only rDNA (large yellow signal in Fig. 4B) and some minor signals from both GISH probes. Lim *et al.* (2007) described the loss of signal as 'near-complete genome turnover' (see also Grover *et al.*, 2007*a*).

Comparing GISH signals with genome size data is informative. The presence of some GISH signal to *N. nudicaulis* is associated with genome downsizing, whereas the near absence of GISH signal to *N. nesophila* is associated with genome size increases. Although mobility of repeats and homogenization mechanisms would act to reduce GISH discrimination between the two parental chromosome sets, it would not almost completely eliminate GISH signal, as seen in *N. nesophila*. Genome downsizing is thought to involve illegitimate recombination leading to the production of small indels and unequal intrastrand homologous recombination in, for example, long terminal repeat retroelements (e.g. Devos *et al.*, 2002; Vitte and Panaud, 2003; Bennetzen *et al.*, 2005). If these mechanisms are occurring in *N. nudicaulis* then the overall similarity of the repeat elements must be retained sufficiently to enable GISH to work partially. In contrast, near absence of GISH signal on *N. nesophila* is associated with genome upsizing. Mechanisms responsible for this phenomenon include amplification, transposition and insertion of retroelements (Vitte and Bennetzen, 2006) and evolution and amplification of satellite repeats (Lim *et al.*, 2000, 2006*a*). Presumably these mechanisms, including evolution of the new satellite repeat NNE in *N. nesophila*, are occurring so rapidly that almost the entire repetitive portion of the genome has 'turned over', and little of the original character of the chromosomes remains (Lim *et al.*, 2007). Thus, these results suggest that genome downsizing leads to a less dramatic alteration of genome characteristics than genome upsizing. It is likely that evolution and amplification of new sequences in association with genome upsizing replace many of the original sequences that were in the ancestral polyploid genome.

CONCLUDING REMARKS

Genomic responses to polyploidy in *Nicotiana* are complex, variable and determined by many factors, with age and genomic similarity of the parental genome donors potentially playing a role. Age is important in determining the extent of DNA sequence divergence encountered in polyploids, with genome turnover becoming extensive after approx. 4·5 Myr (Lim *et al.*, 2007). Genomic relatedness of the parental genome donors may also be important in determining the extent of genome evolution in the polyploid genome. In *Nicotiana* the only polyploid shown to have undergone unequivocal intergenomic translocations is *N. tabacum*, which combines genomes from phylogenetically widely separated sections (Fig. 1; Kenton *et al.*, 1993; Lim *et al.*, 2004). The next most highly diverged genomes brought together are found in section *Repandae*, although genomic turnover and homogenization that has taken place since the four polyploids formed prevents GISH from effectively identifying any ancestral intergenomic translocations. No intergenomic translocations have been found in any of the other polyploids examined (Chase *et al.*, 2003; Lim *et al.*, 2004, 2007). These observations are similar to those of Song *et al.* (1995), who noted that, in synthetic polyploids of *Brassica*, the most extensive genomic changes occurred in polyploids with the most widely diverged parental genomes.

In terms of genome size evolution, no trends with increasing age of the polyploids or increasing distance between diploid progenitors of the polyploids were discernable, with four *Nicotiana* polyploids showing decreases and five showing increases. Even different polyploids originating from the same parental genome donors (i.e. section *Repandae*) responded differently with one species (*N. nudicaulis*) exhibiting decreases and the other three (*N. nesophila*, *N. stocktonii* and *N. repanda*) showing increases. The only noticeable trend was an increase in the extent of DNA amount change with increasing age of the polyploids. Thus, whereas 'young' polyploids (<200 000 years old) showed only small losses of DNA (2–5 %), after approx. 4·5 Myr of evolution DNA amount changes ranged from 14 % to 29 % (Fig. 2). The potential relationship between genome size increases and turnover (replacement of ancestral repeats with new repeats over time) and genome decreases with maintenance of ancestral repeats will need to be explored.

Studying this relationship in other systems may, however, be difficult because the only other reliable examples of genome increases in naturally occurring polyploids are found in a few species of *Hordeum* (Jakob *et al.*, 2004). The scarcity of genome size increases in polyploids in general suggests strong selection against this process. Perhaps the reason is that a newly formed polyploid already has a large genome compared with its diploid parental competitors so selection favours early polyploids which have undergone genome size decreases rather than increases. Nevertheless, the identification of genome size increases in five *Nicotiana* polyploids suggests that here either the additional DNA does confer some competitive advantage or, perhaps more likely, it has no function but there is no strong selection against it.

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