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A genetic linkage map of *Nicotiana plumbaginifolia/ Nicotiana longiflora* based on RFLP and RAPD markers

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Abstract We have constructed a genetic linkage map for *Nicotiana plumbaginifolia*/*Nicotiana longiflora* (2*n*=2*x*=20), based on the segregation of 69 RFLP and 102 RAPD loci in 99 F_2 plants from the cross *N. plumbaginifolia*×*N. longiflora.* The map consists of nine major linkage groups, each containing more than nine marker loci, and spans 1062 cM. Twenty of the RFLP markers were mapped previously to *Nicotiana sylvestris* (2*n*=2*x*=24) chromosomes using monosomic alien addition lines. Taxonomically, *N. plumbaginifolia* and *N. sylvestris* belong to the same section, namely the *Alatae*; however, cytogenetic evidence indicates that they are not closely related. Comparison of the distribution of markers common to both maps suggests that genome reorganization has occurred during the evolution of these two species. Evidence is also presented that genome reorganization may be accompanied by gain and loss of specific classes of DNA sequences in their genomes.

Keywords *Nicotiana plumbaginifolia* · *Nicotiana longiflora* · *Nicotiana sylvestris* · Genetic map · RFLP · RAPD

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Introduction

High-density genetic linkage maps are useful tools for studying genome organization, phylogeny and evolution, and for mapping and cloning genes of interest (Tanksley et al. 1989). Such maps have been constructed for important crops like rice (Harushima et al. 1998), maize (Gardiner et al. 1993) and tomato (Tanksley et al. 1992), but less commonly for non-crop species (Chang et al. 1988; Lin and Ritland 1996; Whitkus 1998).

The genus *Nicotiana* comprises 66 species, which are divided into three subgenera and 14 sections (Goodspeed 1954). In two sections, the *Alatae* and *Suaveolente*, chromosome numbers show aneuploid seriations, while in all other sections only counts of 2*n*=24 and 2*n*=48 occur. The seven species in the section *Alatae* could be classified into three groups according to karyotype. *Nicotiana alata*, *Nicotiana langsdorffii, Nicotiana forgetiana* and *Nicotiana bonariensis* have nine pairs of chromosomes, of which 5–6 pairs are meta- or submeta-centric and the remaining chromosomes are subtelocentric or acrocentric. The karyotypes of *Nicotiana longiflora* and *Nicotiana plumbaginifolia* are almost identical, both possessing ten pairs of acrocentric or telocentric chromosomes. *Nicotiana sylvestris*, the maternal progenitor of cultivated allotetraploid *Nicotiana tabacum* (Gray et al. 1974; Bland et al. 1985; Olmstead and Palmer 1991), is the only species with 12 pairs of chromosomes in the section *Alatae* (Goodspeed 1954).

Cytogenetic evidence indicates that the 2*n*=20 and 2*n*=18 species are closely related and formed a group the “alata”; relationships between the “alata” group and 2*n*=24 *N. sylvestris* are more remote (Avery 1938). It has been suggested that the 2*n*=24 species is the primitive form from which the 2*n*=18 and 2*n*=20 species arose (Goodspeed 1954; Aoki and Ito 2000); however, the mechanisms of these changes are not understood.

Comparative genome mapping (Bonierbale et al. 1988) would be a promising approach for elucidating chromosomal structural relationships among species in the section *Alatae*. In a previous paper, we reported

mapping of *N. plumbaginifolia* genomic DNA and the cDNA to *N. sylvestris* chromosomes using monosomic alien addition lines (Suen et al. 1997). In the present study, we constructed a tentative linkage genetic map for *N. plumbaginifolia*/*N. longiflora* based on these and additional RAPD markers. These markers and the map constructed form a framework for comparative genome analysis in the section *Alatae*. Furthermore, *N. plumbaginifolia* is a model species in somatic cell genetics (Maliga et al. 1982) and has been extensively used for mutant isolation (Negrutiu et al. 1984), somatic hybridization (Harms 1985), gene transfer (Horsch et al. 1984) and organelle genetics (Medgyesy et al. 1985). The genetic map of *N. plumbaginifolia*/*N. longiflora* provides a useful tool for investigating genome organization and strengthens the value of this species for genetic research.

Materials and methods

Plant material

A total of 99 F_2 plants, derived from a single plant from the cross *N. plumbaginifolia*× *N. longiflora*, were used as the mapping population. Seeds of the parental species were provided by the US Department of Agriculture, Beltsville, Md. These two species have the same chromosome number ($2n=2x=20$) and their F_1 hybrids have normal meiotic chromosome behavior and are highly fertile (Goodspeed 1954; Chen, unpublished results).

DNA isolation

Genomic DNA was extracted from single plants of the parental species, as well as F_1 and F_2 hybrids, according to the method of Suen et al. (1997).

RFLP analysis

The clones used in this study are listed in Table 1. These include: (1) 129 single and low-copy *Pst*I genomic clones from *N. plumbaginifolia* (designated g18 or g19, Suen et al. 1997), (2) 50 uncharacterized clones from a *N. plumbaginifolia* cDNA library (designated c, provided by Dr. J.C. Chen, Institute of Molecular Biology, Academia Sinica), (3) 14 single and low-copy clones derived from the ends of *N. plumbaginifolia* YAC clones (designated Y) (Chen et al. 1996), and (4) a cDNA clone for the nitrate reductase apoenzyme (pNR) from *N. tabacum* (Suen et al. 1997). These clones were labelled with digoxigenin-11-dUTP (Roche Molecular Biochemicals, Mannhein, Germany) by the polymerase chain reaction (PCR) as previously described (Suen et al. 1997).

Total genomic DNA from the parental species, the F_1 and F_2 plants was digested with *Eco*RI, *Eco*RV, *Hin*dIII and *Dra*I and separated on an 0.8% agarose gel. Blotting, hybridization, washing and signal detection were carried out as described by Suen et al. (1997). Luminograms were used for RFLP analysis.

RAPD analysis

Four-hundred random decamers (from the University of British Columbia, Vancouver, B.C., Canada) were screened against the genomic DNA of the parental species and their F_1 hybrid for the selection of RAPD markers. DNA amplification was performed according to the method of Williams et al. (1993) with some modifications. Each reaction (25 µl) contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 µM of dNTPs (Amersham Pharmacia Biotech, Piscataway, NJ), 0.2 µM of primer, 40 ng of genomic DNA and 2 units of Pro-Zyme DNA polymerase (PROtech, Taipei, Taiwan). DNA was amplified with a thermal cycler (Model 480, Perkin Elmer, Norwalk, Conn.) programmed for two cycles of 2 min at 94°C, 1 min at 36°C and 2 min at 72°C, followed by 43 cycles of 30 s at 94°C, 30 s at 36°C and 1 min at 72°C. After all cycles were completed, the reactions were held at 72°C for 10 min and slowly cooled to 4°C. The amplified DNA fragments were separated in 1.7% agarose gels. All amplifications were conducted at least twice for reproducibility

Photographs from ethidium bromide-stained gels were used for RAPD analysis. RAPD markers were designated according to the manufacturer's primer code followed by an alphabetical suffix indicating the number of the fragment amplified. For example, marker L110a stands for fragment "a" amplified by primer L110. Segregation of markers was scored as the presence or absence of the amplified band.

Segregation and linkage analysis

A χ^2 test was performed to test the null hypothesis of 1:2:1 segregation of RFLP markers and the 3:1 segregation of RAPD markers. If the calculated χ^2 value exceeded the theoretical values given under $P=0.05$, the differences were considered to be significant.

Linkage analysis was processed through the MAPMAKER program (Lander et al. 1987; Lincoln et al. 1992) employing a minimum LOD score of 3.0 and a maximum distance of 30 cM. Markers were placed into linkage groups using ORDER, COMPARE and TRY commands. The final order of loci on a linkage group was checked with the RIPPLE command.

Results

Screening of RFLP markers

Clones of various sources (Table 1) were surveyed for polymorphism by Southern hybridization to genomic DNA from *N. plumbaginifolia* and *N. longiflora* digested with *Eco*RI, *Eco*RV, *Hin*dIII and *Dra*I. The four restriction enzymes differed in their ability to produce polymorphism. *Dra*I revealed significantly less polymorphisms (31% clones) than the other enzymes (41% to 49% clones). Clones detecting polymorphisms between the two parents with at least one of the enzymes were used as markers for segregation and linkage analysis (Table 1).

Of the 129 *Pst*I genomic clones (Suen et al. 1997) tested, 37 detected polymorphisms with one enzyme, 30 with two enzymes, 21 with three enzymes and 5 with four enzymes. The detection of polymorphism by a high frequency of clones with multiple enzymes suggests that DNA rearrangements such as deletion and insertion are major causes of detectable differences between the two parental species (Apuya et al. 1988).

A survey of 50 random *N. plumbaginifolia* cDNA clones revealed that 31 were single or low-copy sequences, and among these 17 detected polymorphisms between the two parental species (Table 1). The remaining 19 clones were repetitive and multiple-copy sequences.

Of the 14 YAC-end clones assayed, only four detected polymorphisms between the two parents (Table 1).

Table 1 Summary of screening and mapping of RFLP markers

Source of clones	No. of clones	Polymorphic clones	No. of clones mapped	No. of loci
*Pst*I genomic library	129	93	49	49
cDNA library	50	17	13	16
YAC ends	14	4	3	3
pNR	1	1	1	1
Total	194	115	66	69

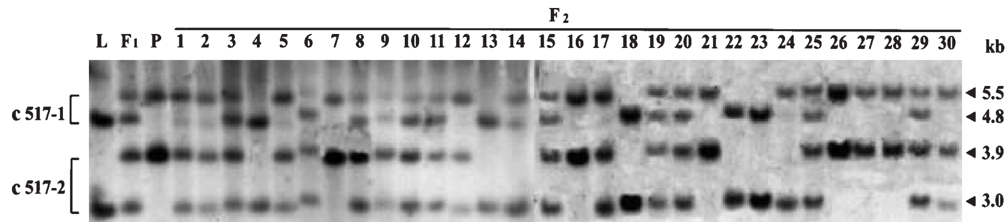

Fig. 1 Luminograph showing Southern hybridization of clone c517 to *Hin*dIII-digested genomic DNA from *N. plumbaginifolia* (*P*), *N. longiflora* (*L*), F_1 and F_2 plants. The banding pattern indicates hybridization of the clone to two segregating loci (*c517–1,2*)

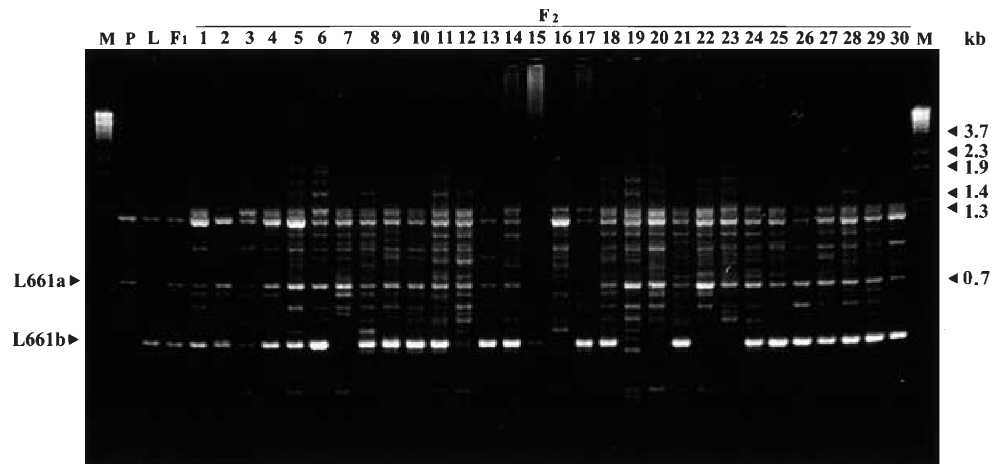

Fig. 2 Ethidium bromide-stained amplification products from genomic DNA of *N. plumbaginifolia* (*P*), *N. longiflora* (*L*), F_1 and F_2 plants using primer L661. Molecular weight markers (*M*) are λ/*Bst*EII DNA fragments. Two segregating polymorphic loci (*L661a,b*) are scored

Screening of RAPD markers

Fifty four decamers were selected as primers to amplify genomic DNA from the two parents and their F_1 hybrid. Polymorphic markers were identified by the presence of a fragment in one parent and the F_1 hybrid, but absence in the other parent. A total of 104 RAPD markers were identified. The number of polymorphic fragments amplified from one primer varied between 1 and 5, with an average of 1.9.

Segregation analysis

One hundred and fifteen RFLP clones were analyzed for segregation in the F_2 population. Of these, only 66 clones produced unambiguously scorable patterns (Table 1). Three cDNA clones, c243, c504 and c517, each disclosed two polymorphisms with one restriction enzyme, which segregated independently as two loci in F_2 plants (Fig. 1); the remaining 63 clones each detected a single locus. The two alleles of all loci were inherited codominantly, typical of RFLP markers.

Segregation of all the 104 polymorphic fragments amplified from the 54 decamers could be scored in the F_2 population, and these fragments were transmitted as dominant markers (Fig. 2).

χ^2 analysis showed that segregation of 25 (36.2%) RFLP loci and 46 (44.2%) RAPD loci deviated from the expected Mendelian ratios at P=0.05. In both cases, skewness was toward the *N. plumbaginifolia* parent (84% RFLP loci and 63% RAPD loci). Among the RAPD markers violating the expected 3:1 ratio, two (L497a and L116a) were found to be co-migrating markers by testing the goodness of fit to a 15:1 ratio. These markers were excluded from further analysis.

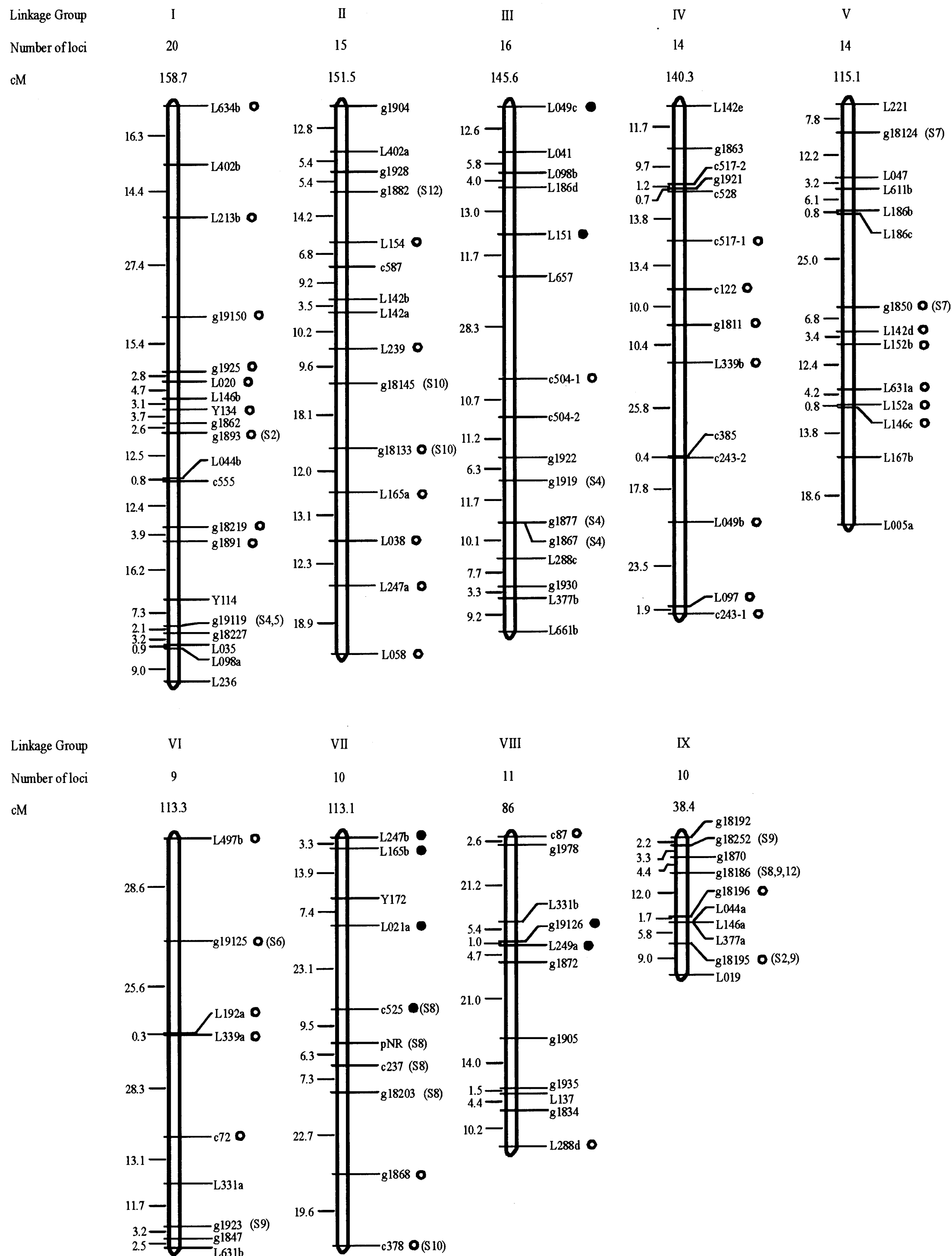

Fig. 3 The nine major linkage groups of *N. plumbaginifolia*/*N. longiflora*. *Open circles* indicate skewness in favor of *N. plumbaginifolia* alleles. *Solid circles* indicate skewness in favor of *N. longiflora* alleles. Loci also detected on *N. sylvestris* chromosomes (Suen et al. 1997) are indicated in *parenthesis*

Linkage analysis

Nineteen tentative linkage groups were inferred from a two-point analysis of allelic segregation of 171 loci in the F_2 population. The map consists of 151 loci (2 RFLP markers and 18 RAPD markers were unlinked) and covers a total of 1385.6 cM. Linkage groups 1 to 9 range from 38.4 cM to 158.7 cM and consist of 9 to 20 loci (Fig. 3). The remaining ten linkage groups contain only two to four markers and are not shown on the map.

Twenty two of the 25 RFLP loci and 27 of the 44 RAPD loci showing a distorted segregation ratio at P=0.05 were mapped to the nine major linkage groups (Fig. 3). Two trends of distribution of these loci on the map were observed. First, a majority of the skewed loci are clustered at specific regions of some linkage groups; for example, the g18133-L058 (five loci) region in linkage group II, the c517–1-L339b (four loci) region in linkage group IV, and the g1850-L146c (six loci) region in linkage group V. Second, loci in the same region or linkage group tend to skew to the same direction. For example, loci in the L247b-c525 region of linkage group VII skewed in favor of *N. longiflora* alleles, while loci in most other regions skewed in favor of *N. plumbaginifolia* alleles.

Discussion

We have constructed a tentative genetic linkage map for *N. plumbaginifolia*/*N. longiflora*. This map consists of 60 RFLP and 59 RAPD loci distributed on nine major linkage groups spanning 1062 cM. Owing to the shortage of markers, the map has not coalesced into ten linkage groups, corresponding to the haploid chromosome number of *N. plumbaginifolia*. However, each individual F_2 plant in the mapping population was self-fertilized and F_3 seeds were harvested. The F_3 families, therefore, can be used as a mapping population for the addition of more markers to the map. We are particularly interested in mapping genes and cDNA sequences registered in GenBank.

In this study, four restriction enzymes, *Eco*RI, *Eco*RV, *Hin*dIII and *Dra*I, were employed to reveal RFLP between the *N. plumbaginifolia* and *N. longiflora* genomes. Of the 160 random genomic and cDNA clones tested, about half of them could detect polymorphism with more than one enzyme. *N. plumbaginifolia* and *N. longiflora* have been considered very closely related, as they have almost identical karyotypes (Goodspeed 1954; Chang 1996) and nuclear DNA content (Narayan 1987), a similar 45S rDNA structure (Borisjuk et al. 1997) and *matK* gene sequence (Aoki and Ito 2000), and their F_1 and F_2 hybrids show normal meiotic chromosome pairing and segregation, and are highly fertile (Goodspeed 1954; Chen, unpublished results). The detection of polymorphism by a high frequency of clones with multiple enzymes suggests that DNA rearrangements not detectable at the microscopic level have taken place during genome differentiation (Apuya et al. 1988).

High frequencies of duplicated loci have been found in the genomes of maize (Helentjaris et al. 1988), *Brassica oleracea* (McGrath et al. 1990) and *Brassica rapa* (Song et al. 1991; Chyi et al. 1992), indicative of the polyploid origin of these species. There have been controversies about the basic chromosome number of the genus *Nicotiana*. Based on the presence of bivalents and secondary associations in haploids and interspecific hybrids between distantly related diploid species, Kostoff (1942) and Goodspeed (1954) proposed that the original basic chromosome number of *Nicotiana* was six. However, a comparison of the mode of inheritance of biochemical mutants in 2n=48 *N. tabacum* (Müller 1983; Bourgin et al. 1985) and 2n=20 *N. plumbaginifolia* (Gabard et al. 1987; Marion-Poll et al. 1988), the study of meiotic chromosome association in anther-derived haploid plants of several *Nicotiana* species (Lin and Chen 1990), and phylogenetic analysis of chloroplast DNA restriction sites and gene sequences in Solanaceae and *Nicotiana* (Olmstead and Sweere 1994; Aoki and Ito 2000) all suggest that the ancestral basic chromosome number of *Nicotiana* should be 12. In *N. sylvestris* and *N. plumbaginifolia*, in which RFLP markers have been mapped, the frequencies of duplicated loci were found to be much lower (Suen et al. 1997; Table 1 and Fig. 3 of this study) than in maize (Helentjaris et al. 1988) and *Brassica* species (McGrath et al. 1990; Song et al. 1991; Chyi et al. 1992). The paucity of duplicated loci in these two species supports the findings of more recent investigations.

Twenty of the RFLP markers previously localized on *N. sylvestris* chromosomes using monosomic alien addition lines (Suen et al. 1997) were mapped to the nine major linkage groups of *N. plumbaginifolia*. This provides an opportunity to compare the distribution of these markers, although the number is small, in the two maps. We found that the synteny groups of *N. sylvestris* and the linkage groups of *N. plumbaginifolia* do not correspond well. For example, three markers on chromosome 10 of *N. sylvestris*, g18133, g18145 and c378, were mapped to two different linkage groups of *N. plumbaginifolia*; and, additionally, linkage groups I, II, VI and VII of *N. plumbaginifolia* each contained markers belonging to two *N. sylvestris* chromosomes (Fig. 3). This is not unexpected, since karyotypes of these two *Nicotiana* species differ significantly (Suen et al. 1997) and chromosome reorganization may have taken place during evolution.

We also observed that three duplicated loci and one triplicated locus in the genome of *N. sylvestris* (Suen et al. 1997) appeared to be single loci in the genetic map of *N. plumbaginifolia* (Fig. 3). It has been hypothesized that in the section *Alatae* the evolutionary trend of changes in basic chromosome number is from 12 to 9 and 10 (Goodspeed 1954; Aoki and Ito 2000). If this is true, then the above result seems to suggest that karyotype reorganization during evolution from 2n=24 *N. sylvestris* or its progenitors to 2n=20 *N. plumbaginifolia* may be accompanied by the loss of nuclear DNA, probably sequences belonging to multigene families. However, according to Narayan (1987) the nuclear DNA content of *N. plumbaginifolia* (8.29 pg/2 C) is much higher than that of *N. sylvestris* (5.74 pg/2 C). This inconsistency may be reconciled by the fact that *N. plumbaginifolia* has abundant dispersed repetitive sequences which are absent or present in only minute amounts in the genome of *N. sylvestris* (Speeckaert and Jacobs 1988; Piastush and Bates 1990; Kovtun et al. 1993; Chen et al., unpublished results). Acquisition and amplification of repeti-

tive sequences has long been recognized to be important mechanisms for the increase of genome size in higher plants (Moore 1995).

The orders of RFLP markers on *N. sylvestris* chromosomes are not known owing to the method used for mapping (Suen et al. 1997). However, it has been shown in several groups of plants that the orders of genes in linkage blocks are generally conserved between related species, in spite of karyotype reshuffling during evolution (Ahn and Tanksley 1993; Kowalski et al. 1994). Thus, from the orders of markers in the linkage blocks of *N. plumbaginifolia* (Fig. 3), we may be able to predict the relative positions of these markers on the *N. sylvestris* chromosomes. However, in order to obtain a clear picture of the chromosomal relationships between these two species, more markers on both maps are needed. Recently, we have constructed *N. plumbaginifolia-sylvestris* addition lines, each containing a telocentric or deficient *N. sylvestris* chromosome, and are using these lines to map RFLP markers to chromosome arms and regions.

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