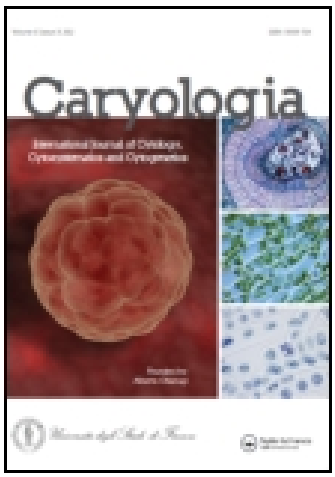

Caryologia: International Journal of Cytology, Cytosystematics and Cytogenetics
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tcar20

On the hybrid origin of Narcissus biflorus (Amaryllidaceae): analysis of C-banding and rDNA structure

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Published online: 18 Dec 2012.

To cite this article: R.I. De Dominicis , G. D'Amato & G.F. Tucci (2002) On the hybrid origin of Narcissus biflorus (Amaryllidaceae): analysis of C-banding and rDNA structure, Caryologia: International Journal of Cytology, Cytosystematics and Cytogenetics, 55:2, 129-134, DOI: 10.1080/00087114.2002.10589268

To link to this article: http://dx.doi.org/10.1080/00087114.2002.10589268

CARYOLOGIA Vol. 55, no. 2: 129-134, 2002

On the hybrid origin of *Narcissus biflorus* (Amaryllidaceae): analysis of C-banding and rDNA structure

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Abstract - Giemsa and fluorochrome banding with DAPI and chromomycin A_3, were utilized to assess karyological details which correlate *N. biflorus* with the parental species: *N. tazetta* and *N. poëticus.* The banding profile in *N. biflorus* clearly reproduced the model of its progenitors. The EcoR1 restriction pattern of rDNA obtained by Southern blot hybridization indicated, in our material, that each species has more than one ribosomal gene type and in *N. biflorus* both the ribosomal repeat units of the progenitor species are present.

Key words: C-banding, hybrids, *Narcissus biflorus,* rDNA.

INTRODUCTION

Studies on somatic chromosome complement and meiotic behaviour of the pollen mother cells in *Narcissus,* have been extensively carried out in wild populations, in cultivated plants and in hybrids. Basic works on the subject were published by FERNANDES (1934, 1951, 1966, 1975), MAUGINI (1953), BRANDHAM and KIRTON (1987).

N. biflorus Curtis is a hybrid taxon derived by the crossing between *N. tazetta* and *N. poëticus*; the chromosome count reports $2n$=17 (FERNANDES 1934) and $2n$=24 (NAGAO 1933; GARBARI 1973). According to FERNANDES (l.c.), the chromosome morphology elucidates well its hybrid origin; the plants with $2n$=17 found by the Author are the result of a crossing between *N. tazetta* and *N. poëticus* with normally reduced gametes, while the specimens with $2n$=24 derived by an unreduced gamete of *N. poëticus* ($2n$=14) with a reduced one of *N. tazetta* (n=10).

N. biflorus is sterile, but it can reproduce vegetatively easily; morphologically resembling *N. poëticus*, it differs for the pale yellow corona without a reddish edge and for the scape generally double flowered. The plant originated probably in the South of France and is now largely cultivated and naturalized elsewhere (WEBB 1980). However, as it was found, in some regions, in mixed populations with the parental species, it may have been risen independently in different localities. In *Narcissus*, indeed, hybridization occurs frequently in natural populations, within members of a different section or also of a different subgenus, as it is the case of *N. biflorus* where the parental species belong to the two subgenera, Hermione and Narcissus, each one characterized by distinctive basic chromosome number.

As a result of our previous studies in *Helleborus* (Ranunculaceae), the combined use of C-banding and ribosomal DNA analysis proved to be valuable tool in discriminating within members of the different sections in which the genus is divided (D'AMATO and BIANCHI 1989; DE DOMINICIS and D'AMATO 1995).

In eukaryotes the nuclear ribosomal RNA genes are clustered in multiple copies at nucleolar organizer regions (NORs). The copy number is highly variable: in plants it can vary from 500 to 40.000 copies per diploid cell (ROGERS and BENDICH 1987). Nevertheless the single copies do not evolve independently, they become nearly homogeneous, undergoing to concerted evo-

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lution. This phenomenon is due to unequal crossing over and gene conversion. The rDNA copies contain a transcription unit, and an intergenic spacer (IGS), which exhibits length and sequences heterogeneity.

In higher plants each trascription unit consists of coding sequences corresponding to 18S, 5.8S and 25S rRNAs. These sequences are highly conserved in the plant kingdom and their arrangement and the presence of different restriction sites can be useful in taxonomic and evolutionary studies, even if not too informative for phylogenetic reconstruction among closely related taxa. On the other hand, the internal transcribed spacers between 18S and 5.8S (ITS 1) and between 5.8S and 25S (ITS 2) evolve more rapidly and their analysis is phylogenetically informative at the species and generic levels (BALDWIN 1992; BALDWIN *et al.* 1995; SUSANNA *et al.* 1995).

The aim of the present investigation is to assess more precisely the origin of *N. biflorus* and to verify relationships with the parental species utilizing the following diagnostic characters: 1) karyological data, such as the amount and distribution of constitutive heterochromatin, the number of nucleoli and nucleolar organizing regions (NORs); and 2) molecular data inferred from the analysis of the rDNA Eco R1 restriction pattern, also verifying in the hybrid the inheritance of ribosomal genes.

MATERIALS AND METHODS

Material

The plants of *Narcissus* utilized in the present investigation were collected in different localities, mostly in the neighbourhood of Rome.

Bulbs of *N. biflorus* were collected at Monte Soratte, *N. poëticus* at Monte Cavo and at Piano Cinque Miglia (L'Aquila). The samples of *N. tazetta* were of garden origin cultivated in the Botanical Garden of the University of Rome, moreover two bulbs were collected at Castelporziano (Rome).

Karyology

For chromosome count and banding, the root tips were treated with a solution of colchicine (0.5%) for about five hours, fixed overnight in etilic alcool/ acetic acid solution (3:1). C-banding followed the technique described in a previous report (D'AMATO and DE DOMINICIS 1996), fluorescence staining with DAPI and Chromomicine A3 was performed according to SCHWEIZER (1976). Nucleoli were stained according to MEHRA *et al.* (1984).

DNA extraction, labelled probes preparation, and Southern hybridization

DNA extraction was carried out according to the CTAB method by ROGERS and BENDICH (1994). The restriction pattern of rDNA was obtained by single digestion of total nuclear DNA with endonuclease EcoRI, (Gibco BRL, Life Technology, Italy), and Southern hybridization with 18S and 25S homologous rDNA probes PCR labelled with dUTP digoxigenin (Boehringer Mannheim). Chemioluminescent signals of the restriction fragments were developed with Tropix procedure for CDP substrate (Perkin Elmer Italy) and detected by means of autoradiography.

RESULTS AND DISCUSSION

Karyological data

The chromosome number, karyotype structure and banding pattern in the parental species were the following: *N. tazetta* was characterized by $2n=20$ chromosomes in all the examined bulbs. In one of the garden samples, we found $2n=22$ with two small accessory chromosomes. The taxon *N. tazetta* (sensu amplo) represents a polymorphic species displaying remarkable variability and different chromosome numbers. In general, $2n=20$ is the diploid number of Fernandes series Hermione which comprises *N. tazetta* (sensu strictu), while $2n=22$ is found in the series Albiflori and Luteiflori (FERNANDES 1975). The Author, however, considers that the basic number of all the subgenus should be $n=5$ while $2n=20, 22$, may represent tetraploid numbers (the latter an amphiploid) which were stabilized as functional diploids during the evolution of the genus (FERNANDES 1975). The chromosome formula for our specimens, following LEVAN *et al.* (1964), was: 8(L) st + 4(L) sm/m +6(S)st +2(S) st sat., which is in agreement with previous data from plants of Israel (WEITZ and FEINBRUN 1972) and Greece (HONG 1982). C-banded haploid sets are represented in Fig.1; as visible, the banding pattern is very simple, consisting of a pair of long st chromosomes with a subtelomeric band on the long and short arm and a pair of satellited chromosomes with the heterochromatin flanking the NOR. The whole satellite is characteristically heterochromatic.

The fluorescence pattern gave CMA_3^+/DAPI$^-$ bands strictly corresponding to the C-bands (not shown). The intensity of the fluorescence in the st pairs was variable, however always weaker than that found associated to the NORs in the satellite pair.

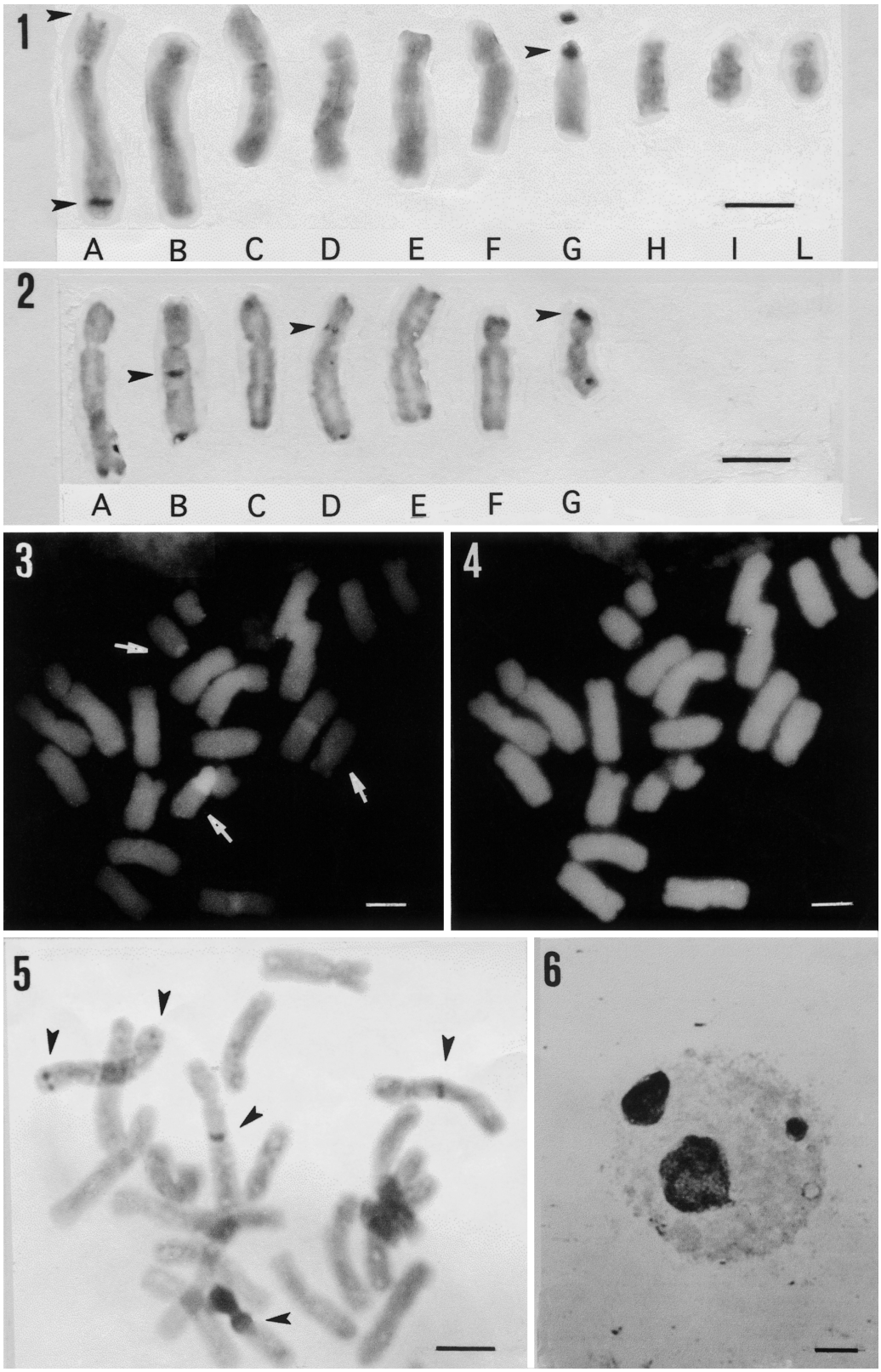

Fig. 1 – *N. tazetta* chromosomes stained by C-banding method (haploid set). Fig. 2 – *N. poëticus*, chromosomes stained as above (haploid set). Figs. 3-5 – Metaphase chromosomes of *N. biflorus*, after CMA^{+}_{3} staining (Fig. 3), after DAPI staining (Fig. 4), and C-banded (Fig. 5). Fig. 6 – Telophase nucleus of *N. biflorus* stained by silver nitrate. Bar = 5 μm. Arrow heads indicate C-band; arrows point to satellite chromosomes.

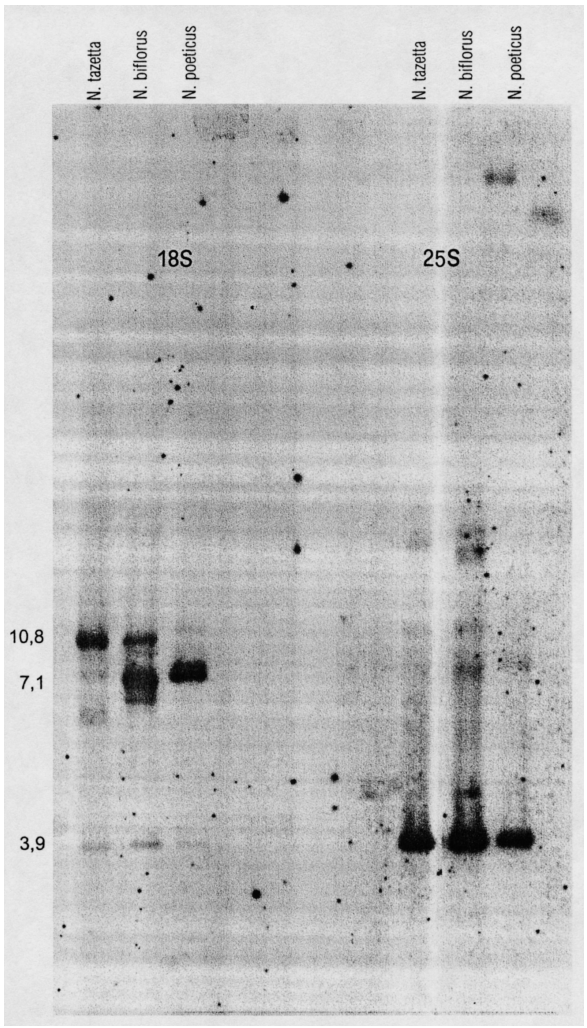

Fig. 7 – Restriction pattern of rDNA 18S and 25S after EcoR1 digestion.

N. poëticus was characterized by 2*n*=14 chromosomes, the banding profile, after Giemsa and DAPI/CMA3 double staining, included an interstitial band on the long arm of a pair of sm (pair B), a thin interstitial band on the short arm of another pair of sm (pair D, not visible by fluorochromes staining); the satellited pair showed a band near the secondary constriction (Fig. 2). As found in *N. tazetta* the CMA_3 fluorescence of interstitial band resulted less intense than that of the nucleolar heterochromatin. The chromosomes pairs B, C, D, resulted very similar in external morphology, and the C-banding allowed to discriminate within them.

The chromosome count in all the specimens of *N. biflorus* examined was 2*n*= 24; the banding pattern (Figs. 3-5), consisted of a pair of sm chromosomes with an interstitial band on the long arm, another pair with the band on the short arm (sometimes not visible), a single st chromosome with a subtelomeric band both on the long and short arm and three chromosomes with a band adjacent to the NOR, one of which characterized by a large heterochromatic satellite; all the bands were DAPI-/CMA3+. Scoring of the number of nucleoli, in interphase cells (fig. 6), confirmed that all three secondary constrictions were active in the hybrid. Considering the hypotesis that 2*n*=24 is originated from a reduced chromosome set of *N. tazetta* and an unreduced set of *N. poëticus*, the banding profile we found in *N. biflorus*, well reproduces the model of its putative progenitors.

rDNA restriction analysis

For our study we used genomic DNA from single individuals. DNA from *N. tazetta* was extracted from an individual with 2*n*= 20. The EcoR1 restriction pattern of ribosomal genes is shown in Fig. 7. The Eco R1 digestion generated, in all the examined species, a fragment about 3.9 kb long that hybridizes weakly to 18S and more abundantly to 25 S probe, indicating that the EcoR1 restriction sites are located near the 3' end

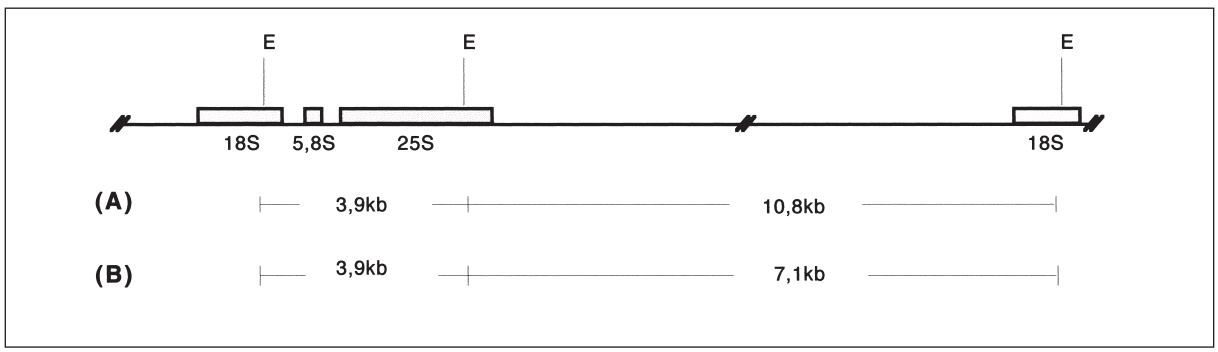

Fig. 8 – EcoR1 restriction map of the most represented rDNA repeat unit in *N. tazetta* (A) and in *N. poëticus* (B). Both are present in *N. biflorus*.

of both the 18S and 25S regions. This is similar to what is found in most plant species in which these sites are highly conserved, though monocots show some exceptions, such as species of *Brachypodium* (Pooideae), which lack of the Eco R1 restriction site near the 3' end of the 18S region. (SHI *et al*. 1993). Moreover, the rDNA restriction profile shows, in each species of *Narcissus*, a different number of other fragments of different intensity and size, as follows: *N. tazetta,* three weak fragments of about 7.0, 6.2 and 5.5 kb respectively, and a strong one of about 10.8 kb; *N. poëticus*, two bands, a weak one of about 7.1 kb and a strong one of 10.8 kb; *N. biflorus*, two strong bands of 10.8 and 7.1 kb respectively. Since all these rDNA fragments hybridize more abundantly to 18S than to 25S, they include the intergenic spacer (IGS). On these bases we can state that each species of *Narcissus* has more than one ribosomal repeat type, more or less represented, which exhibit a length heterogeneity caused by different size of the IGS. Two repeat units of different length are predominant: one, about 14.7 kb long in *N. tazetta*, the other one, about 11.0 kb long in *N. poëticus*. A restriction map of the two genes is reported in fig. 8. These two genes are both present in *N. biflorus* whose rDNA restriction profile combines in an additive pattern the rDNA fragments which are present in the progenitor species.

Conclusions

The present study has shown, in our species of *Narcissus*, the presence of constitutive heterochromatic segments, characterized by a high content of G/C base pairs, as revealed by CMA_3/DAPI staining, which represent a kind of heterochromatin preferentially associated with the NORs of satellited chromosomes in almost all organisms. It is noteworthy that in *N. biflorus* the pattern of heterochromatic segments reproduces the model of its progenitors in respect to the number, size and position. The ribosomal loci are highly variable regions of the genome and this variability affects the number and the size of the NORs. In *N. biflorus* they conserve the number of the chromosomal sets of the progenitors and the presence of three nucleoli indicates that all the three ribosomal loci are active.

The EcoR1 rDNA restriction pattern, in each species exhibits a polymorphism due to IGS length variation and therefore each individual possesses more than one repeat unit within its rDNA array, even though each parental species is characterized by a main length variant (10.8 kb in *N. tazetta* and 7.1 kb in *N. poëticus,* for the rDNA fragments containing IGS). The major abundance of these two different rDNA repeats is apparent from the differences in hybridization signal intensities, since the other fragments hybridized only weakly to the rDNA probes. The 18-25S rDNA shows, in our material, biparental gene inheritance, since additive pattern of both parental taxa is observed in *N. biflorus*. This is similar to what is typically found in hybrids (DOYLE *et al*. 1985). Moreover it is to observe that the occurrence of two size classes of rDNA unit length indicates little or no gene conversion which would homogenize repeat lengths, and this may be related to the fact that *N. biflorus* is sterile and can reproduce only vegetatively. It is known that concerted evolution can fail to homogenize rDNA repeat units in some cases, including the occurrence of asexual reproduction (CAMPBELL *et al.* 1997).

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Received November 23, 2001; accepted January 24, 2002