Theor Appl Genet (2000) 100:361–367

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Cytological heterozygosity and the hybrid origin of sweet orange [*Citrus sinensis* (L.) Osbeck]

Received: 9 April 1999 / Accepted: 22 June 1999

Abstract *Citrus sinensis* chromosomes, although small in size, present a remarkable differentiation of bands with the fluorochromes CMA and DAPI. These bands suggest that some heteromorphisms are fixed in this species. To investigate the extension of these heteromorphisms, ten cultivars of *C. sinenesis* were analysed with CMA/DAPI staining and, in some of them, the 18S–5.8S–25S rRNA and 5S rRNA genes were located by in situ hybridization. CMA/DAPI staining showed exactly the same $CMA^+/DAPI^-$ banding pattern for all cultivars. In situ hybridization revealed three 18S–5.8S–25S rRNA gene sites, two proximally located on two similar chromosomes and one terminally located on a third non-related chromosome. Two 5S rRNA gene sites were observed in this species, with one located proximal to the telomeric 18S–5.8S–25S rDNA site. Both cytological approaches revealed an invariable, heterozygotic karyotype among sweet orange cultivars. Based on these data, the putative hybrid origin of the species is discussed.

Key words *Citrus sinensis* · CMA banding · FISH · rRNA genes · Heterochromatin · Heterozygosis

Introduction

Citrus sinensis (L.) Osbeck, sweet orange, is the main evergreen fruit-crop species, responsible for 75% of citrus production used both as fresh fruit and processed juice (Cameron and Soost 1986; Spiegel-Roy and Goldschmidt 1996). As in other citrus species, it presents two important reproductive characteristics: high fertility between interespecific hybrids and asexual reproduction by nucellar embryony. These characteristics, together with gene mutation, are considered responsible for the large variability present in the genus (Cameron and Frost 1968; Spiegel-Roy and Goldschmidt 1996).

The range of morphological variability between *C. sinensis* horticultural cultivars is as high as between some *Citrus* species (Barrett and Rhodes 1976). Although it has been treated as a taxonomic species, morphological, phytochemical and molecular data support the idea that the sweet orange originated by crossing between pummelo, *C. maxima* (Burm.) Merrill, and mandarin, *C. reticulata* Blanco (Scora 1975; Barrett and Rhodes 1976; Green et al. 1986; Yamamoto et al. 1993). The origin of the different cultivars has been attributed to repeated interbreeding between pummelo and mandarin (Sauer 1994) or to somatic changes fixed by asexual reproduction (Cameron and Frost 1968; Barrett and Rhodes 1976). Furthermore, at least some cultivars, such as Washington Navel, Shamouti and Lue Gim Gong, are known to have originated by gene mutation (Hodgson 1967; Cameron and Soost 1986).

CMA/DAPI staining has proved to be very useful for the cytogenetic characterisation of citrus species, revealing a well-differentiated pattern of $CMA^+/DAPI^-$ bands, which exhibit a large interspecific polymorphism as well as a high degree of heterozygosis (Guerra 1993; Miranda et al. 1997). However, in spite of this variability, three different samples of *C. sinensis*, analysed by Guerra (1993), Matsuyama et al. (1996) and Miranda et al. (1997), revealed an identical chromosome banding pattern. Noteworthy, all samples were heterozygotic for at least two chromosome pairs, giving further support to the putative hybrid origin of *C. sinensis.*

In an effort to better characterise the chromosomes of *C. sinensis*, sites of 18S–5.8S–25S and 5S rRNA genes and telomere-specific repeated sequences have been localised by in situ hybridization (Matsuyama et al. 1996; Roose et al. 1998). However, different numbers of 18S–5.8S–25S rRNA gene sites were reported by these authors.

In the present paper, we have investigated the heterozygotic condition in different *C. sinensis* cultivars

Communicated by F. Mechelke

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through two cytogenetic parameters: CMA banding pattern and the distribution of 45S and 5S rRNA gene sites. Based on these data, the possible hybrid origin of the species is discussed.

Materials and methods

Plant materials

Seeds of ten cultivars of *C. sinensis* (L.) Osbeck. [Early Oblong (CRC267), Olivelands (BRA 005126), Comuna (reference code unavailable), Kona (BRA 005185), Valencia (BRA 000213), Pera (BRA 005380), Ruby (BRA 000183), Hamlin (BRA 005347), Parson Brown (BRA 000141) and Gardner (reference code unavailable)] were kindly supplied by Dr. Walter Soares-Filho from the germplasm bank of the National Research Centre of Cassava and Tropical Fruticulture (CNPMF-EMBRAPA), Cruz das Almas, BA, Brazil.

Cytological procedure

Root tips from germinating seeds were pre-treated with 8-hydroxyquinoline (2 mM) for 1 h at room temperature and for a further 20 h–23 h at 10°C before being fixed in Carnoy (3:1 of ethanol:acetic acid). Seeds with at least three seedlings each were preferentially used in order to reduce the chance of working with zygotic embryos. Thus, a banding pattern found in more than one seedling would probably represent the nucellar or maternal karyotype.

Root tips were macerated in an enzyme solution [2% cellulase (Sigma) plus 20% pectinase (Sigma) in 0.01 M citric acid-sodium citrate pH 4.8 buffer] at 37°C (1 h) and squashed in a drop of 45% acetic acid. After coverslip removal, slides were mounted in a mixture of 1:1 (v/v) DAPI (2 µg/ml)-glycerol, for selection, and washed in 3:1 (v/v) ethanol-acetic acid (30 min) and absolute ethanol (overnight).

CMA/DAPI staining

Fluorochrome staining was performed in all cultivars according to Schweizer (1976) and Deumling and Greilhuber (1982). Slides aged for 3 days were double-stained with 0.5 mg/ml of Chromomycin A3 (CMA) (1 h) and 2 µg/ml of diamidino-2-phenyl-indole (DAPI) (30 min) and mounted in 1:1 (v/v) McIlvaine's pH 7.0 buffer-glycerol. Slides were aged for a further 3 days before analysis. Afterwards, some of these slides were de-stained and sequentially stained with hematoxylin 1% (Guerra 1999).

DNA probes

Four DNA probes were used: SK 18S+25S, containing two separately re-cloned fragments of 18S and 25S rDNA from *Arabidopsis thaliana* (Unfried et al. 1989; Unfried and Gruendler 1990), pXV 1–2, containing two separately cloned 5S rRNA genes from *Beta vulgaris* with a length of 349 bp and 351 bp respectively (Schmidt et al. 1994); pTa71, a 9-kb fragment from *Triticum aestivum* containing the 18S–5.8S–25S rDNA and intergenic spacers (Gerlach and Bedbrook 1979); and pTa794, a 410-bp fragment, also from wheat, containing the 5S rRNA-gene repeated unit (Gerlach and Dyer 1980). The former two probes were directly labelled with Cy3-dCTP by nick translation (Amersham). pTa71 was labelled with digoxigenin-11-dUTP by nick translation (Life Technologies) and pTa794 was directly labelled with rhodamine-4-dUTP by PCR.

Fluorescence in situ hybridization

Cytological preparations were made and selected as described above, but some modifications were included to reduce background. Root tips were washed in the enzyme buffer before and after enzyme digestion, left in 45% acetic acid for at least 30 min and flamed before squashing. Slide preparations were treated with RNase, post-fixed in 4% (w/v) paraformaldehyde, dehydrated in an ethanol series and air dried, as performed by Cuadrado and Jouve (1994).

Chromosome and probe denaturation, in situ hybridization, post-hybridization washing and detection were all performed according to Heslop-Harrison et al. (1991). The hybridization mix consisted of: 50% (v/v) formamide, 10% (w/v) dextran sulphate, 0.3% (w/v) SDS, 0.3 ng/µl of autoclaved salmon sperm DNA, 2×SSC and 2–5 ng/µl of probe. The slides were either denatured for 10 min at 90°C in an oven or at 75°C in a programmable thermal controller. The digoxigenin-labelled probe was detected using an anti-digoxigenin FITC conjugate (Boehringer) in 5% (w/v) BSA. All preparations were counterstained with DAPI. For sequential staining CMA/DAPI-FISH, the CMA/DAPI procedure was performed before or after FISH.

Image analysis

At least five to ten metaphase cells of each cultivar, from different individuals, were sequentially photographed with either CMA/DAPI and FISH to allow an effective comparison of band and probe locations. Photographs were taken in T-Max Kodak 400 and Fuji Superia 400 ASA in a DMRB Leica epifluorescence microscope or in a Zeiss Axioplan equipped with a mono cool-view CCD camera (Photometrics). Colour images from the camera were combined using the Iplab software.

Results

Fluorochrome staining resulted in a very clear $CMA^+/DAPI^-$ banding pattern, which was invariably the same in the ten cultivars of *C. sinensis* analysed. Only in two individuals (one from 'Early Oblong' and one from 'Olivelands'), out of 47 seedlings analysed with CMA/DAPI staining, were different CMA-banding patterns observed. As they were not confirmed in other individuals from those cultivars, they were considered of zygotic origin and excluded.

The 18 chromosomes could be easily separated into four groups (based on the classification of Guerra 1993): two chromosomes with a larger telomeric band and a smaller proximal one (type B); two chromosomes with terminal bands at both telomeres, heteromorphic either in band and chromosome sizes (type C); seven chromosomes with a single telomeric band, variable in both band and chromosome sizes (type D/E); and seven chromosomes of different sizes without bands or with a very small, faint and terminal band (type F). Among the type-F chromosomes, only the largest pair was easily distinguishable from the remainder. All CMA^+ blocks were $DAPI^-$, but three of them were remarkably duller with DAPI ($CMA^+/DAPI^{--}$) and often more distended than the others. One of them was located on a single-banded small chromosome (type D/E) and the other two on the proximal band of both type-B chromosomes (Fig. 1a, b). The two divergent individuals presented different karyotypes. The seedling of 'Early Oblong' showed a karyotype formula 2B–4C–6D/E–6F, while the seedling of 'Olivelands' showed one chromosome of type A (with one proximal and two terminal $CMA^+/DAPI^-$ bands)

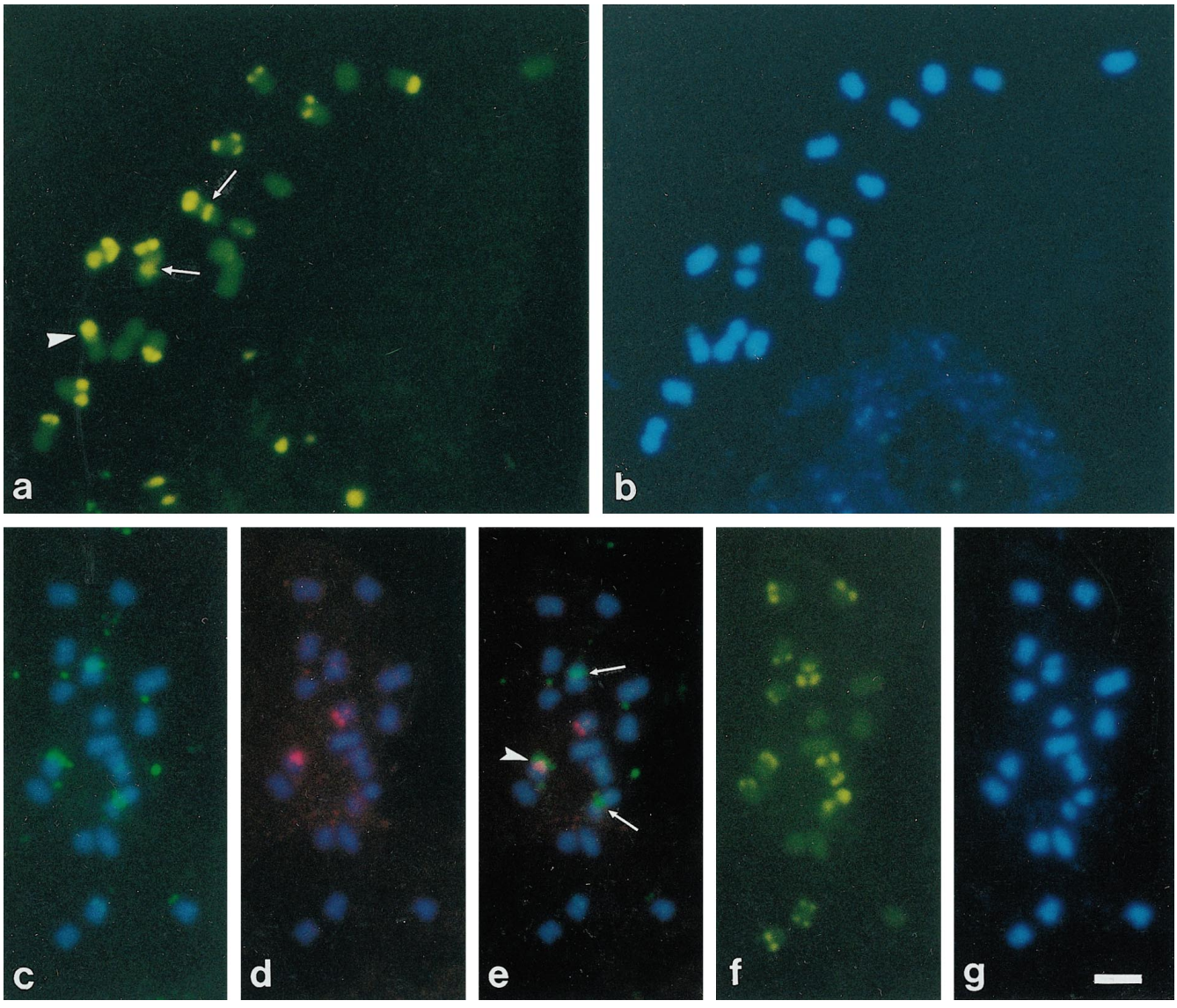

Fig. 1 Metaphase cells of *C. sinensis* cv Valencia (**a, b**) double-stained with CMA (**a**)/DAPI (**b**) and cv Pera (**c–g**) hybridized in situ with pTa71 (**c, e**) and pTa794 (**d, e**) and sequentially double-stained with CMA (**f**)/DAPI (**g**). pTa71 was labelled with digoxigenin and detected with anti-digoxigenin-FITC (*green*), pTa794 was labelled with rhodamin (*red*). Chromosomes were counterstained in **c, d** and **e** with DAPI (*blue*). The two type-B chromosomes are indicated by *arrows* and the type-D/E chromosome by *arrowheads*. The bar in **g** corresponds to 2.5 µm

not existing in *C. sinensis* and a karyotype formula 1A–2B–2C–4D/E–9F.

CMA/DAPI-hematoxylin sequential staining was performed to check if the $CMA^{+}/DAPI^{--}$ blocks were associated with the nucleolus. Thirteen prometaphase cells of six cultivars were analysed, in which both the $CMA^{+}/DAPI^{--}$ blocks and the nucleolus were distinguishable. In one of them, no chromosome was found associated with the nucleolus. In the remaining 12, one type-D/E chromosome and one type-B chromosome, both bearing a $CMA^{+}/DAPI^{--}$ band, were associated with the nucleolus (Fig. 2). In only two of them, the second type-B chromosome was close to it.

The chromosomal position of the 45S rDNA sites was located in 53 metaphase cells of six cultivars: ‘Comuna’, ‘Hamlin’, ‘Olivelands’, ‘Parson Brown’, ‘Pera’, and ‘Valencia’. In the last five of these, the 5S rRNA gene clusters were also located either separately (‘Hamlin’ and ‘Parson Brown’) or simultaneously with the 45S rDNA (‘Olivelands’, ‘Pera’ and ‘Valencia’) in a total of 32 metaphase cells. The location and size of the signals were the same with either pTa71 and SK 18S+25S or pTa794 and pXV 1–2. The different labelling systems used also did not produce any detectable variation, though the antibody detection produced a larger background.

The 45S rDNA probes hybridized on two chromosomes proximally, and terminally on another one. The terminal site was twice as large as the proximal ones and sometimes it was as distended as one of the proximal sites. No difference in position or size of the 45S rDNA clusters was observed among cultivars. Two sites of the

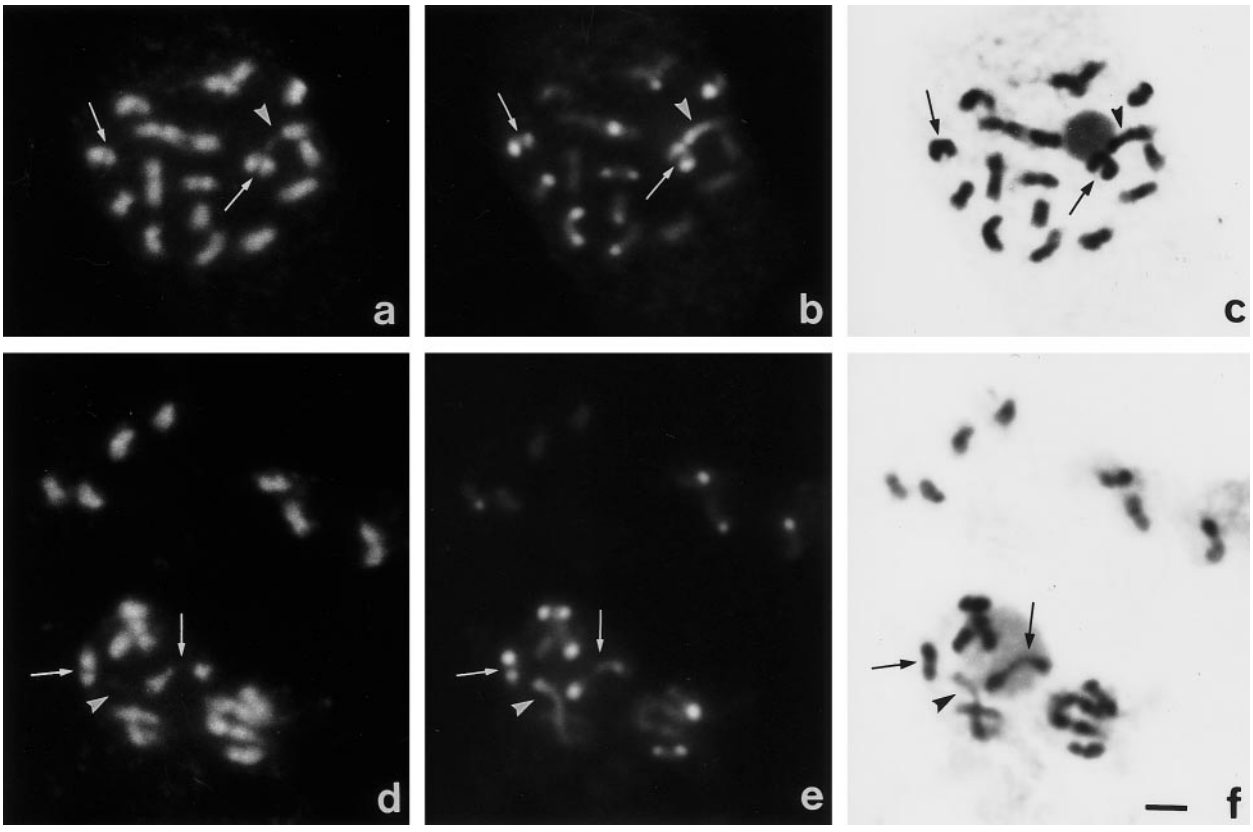

Fig. 2 Metaphase cells of *C. sinensis* cv Ruby (**a–c**) and cv Parson Brown (**d–f**) double-stained with DAPI (**a** and **d**) and CMA (**b** and **e**) and sequentially stained with hematoxylin (**c** and **f**). The two type-B chromosomes are indicated by *arrows* and the type-D/E chromosome by *arrowheads*. Note the association of two of them with the nucleolus. The bar in **f** corresponds to 2.5 μm

5S rRNA gene were found in the five cultivars analysed, one on the terminal and the other on the subterminal region of two small chromosomes.

Sequential CMA/DAPI staining, performed before or after double in situ hybridization, gave identical results. The three 45S rDNA sites were located in the three $CMA^+/DAPI^{--}$ bands; therefore terminally on one type-D/E chromosome and proximally on both type-B chromosomes. One of the 5S rRNA gene sites was terminally located on a small chromosome without bands (type F). The other 5S cluster was located on a type-D/E chromosome, proximal to the telomeric 45S rDNA site (Fig. 1c–g). Figure 3 shows a schematic representation of the complement of *C. sinensis* with the relative positions of the CMA^+ bands and rRNA genes sites. Chromosome and band size are only approximately represented and are based on the idiogram presented by Guerra (1993).

Discussion

The CMA/DAPI banding pattern observed in ten cultivars of *C. sinensis* analysed in present paper was similar to the pattern previously reported in other cultivars (Guerra 1993; Matsuyama et al. 1996; Miranda et al. 1997). This strongly suggests that, despite its high morphological variability, this species has a single, invariable banding-pattern karyotype. Iwamasa and Nito (1988), analysing 13 *C. sinensis* cultivars, reported meiotic irregularities only in 'Valencia' and 'Lue Gim Gong' oranges, which they attributed to a heterozygous recipro-

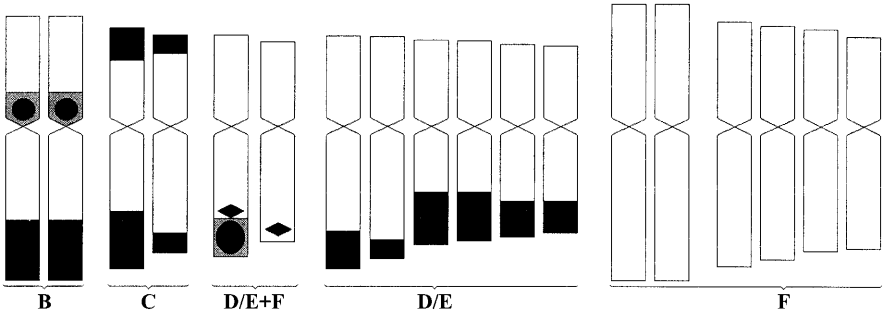

Fig. 3 Schematic representation of the 18 chromosomes of *C. sinensis* divided into four groups according to their CMA^+ banding pattern. *Black areas* correspond to $CMA^+/DAPI^-$ blocks, *grey areas* to $CMA^+/DAPI^{--}$ blocks, *circles* to 45S rDNA sites and *lozenges* to 5S rRNA gene sites

cal translation. However, in our sample, 'Valencia' did not differ from the other cultivars. Therefore, if there are rearrangements in some *C. sinensis* cultivars, they are probably restricted to genome areas in which CMA^+ bands or rRNA gene-clusters do not occur.

In the present work, three sites of 45S rDNA, two proximally located on both type-B chromosomes and one terminally located on one type-D/E chromosome, were observed in six cultivars of *C. sinensis.* Furthermore, two sites of the 5S rRNA gene were observed in five cultivars: one proximally on a type-D/E chromosome, adjacent to the terminal 45S rDNA site, and one terminally on a type-F chromosome. These results confirmed the number and location of 45S rDNA sites found by Matsuyama et al. (1996), but differed from the description of Roose et al. (1998), who, in 'Argentina' sweet orange, reported five 45S rDNA and at least four 5S rRNA gene clusters, with some of them close to the 45S rDNA sites. Although it is possible that variability within the species might be responsible for such differences, the high conservation of the CMA/DAPI banding pattern observed in 13 cultivars (Guerra 1993; Matsuyama et al. 1996; Miranda et al. 1997; present paper), and the identical location of 45S rDNA sites in the seven cultivars observed here and by Matsuyama et al. (1996), suggest that sweet orange cultivars should not diverge to such an extent. The results of Roose et al. (1998) seem to be distorted by technical difficulties. They reported two 45S rDNA clusters in a very characteristic chromosome pair with two terminal CMA^+ bands (type C), which did not present any rDNA cluster according to our analysis. Furthermore, they concluded that at least four 5S rRNA gene clusters exist in the variety, although they only observed three 5S rRNA gene sites in only two cells and one or two sites in others.

The three 45S rDNA sites of *C. sinensis* were $CMA^+/DAPI^{--}$, as already observed in some species (see e.g. Deumling and Greilhuber 1982). The sequential CMA/DAPI-hematoxylin staining also showed that the $CMA^+/DAPI^{--}$ blocks corresponded to the NOR, since two of them, one on a type-D/E chromosome and another on a type-B chromosome, were usually associated with the nucleolus. The NOR located on the other type-B chromosome was rarely observed near the nucleolus, as expected in a hierarchy of activation of NORs previously reported in this species, with only one proximal site and the subterminal one usually being expressed (Pedrosa et al. 1997). Since no visible difference in size or CMA/DAPI reactivity was detected between the two proximal NORs, other factors responsible for the activation of transcription may be involved (Zurita et al. 1997).

One of the 5S rRNA gene sites of the species was located very close to one of the most active 45S rDNA sites (type D/E). Linkage between at least one 45S rDNA and one 5S rRNA gene site has been identified by in situ hybridization in several species, including wheat, barley, *Avena*, *Arabidopsis thaliana*, *Cicer, Helianthus annuus, Lolium, Vicia faba* and *Zingeria biebersteiniana* (Mukai et al. 1990; Leitch and Heslop-Harrison 1993; Bennett et al. 1995; Galasso et al. 1996; Thomas et al. 1996; Katsiotis et al. 1997; Murata et al. 1997; Schrader et al. 1997; Fuchs et al. 1998). Thus, since both sites play a fundamental role in nucleolus formation, it is possible that this association is not random but rather favoured by selection, placing two functional gene clusters together in the same domain.

The CMA/DAPI banding pattern together with the FISH results showed that at least four chromosomes have no clear counterpart. Two of them were of type C, which were different either in size and band length, one was of type F, with a 5S rRNA gene site, and one was of type D/E, bearing a 5S and a 45S rRNA genes site. This heterozygosis seems to be maintained by asexual reproduction (apomixis) since in most *C. sinensis* cultivars a high proportion of nucellar embryos has been observed (Frost and Soost 1968; Cameron and Soost 1986; Ashari et al. 1988). In our sample, only two individuals presented different CMA^+ banding patterns. As they were quite different, it is reasonable to suppose that they were hybrids between *C. sinensis* and other citrus species from the germplasm bank.

The heterozygosis observed may be attributed to the putative hybrid origin of *C. sinensis*. Scora (1975), Barrett and Rhodes (1976), Green et al. (1986) and Yamamoto et al. (1993), based on phytochemical, morphological and molecular data, suggested that *C. sinensis* is a hybrid between *C. maxima* and *C. reticulata*. However, *C. reticulata* and *C. maxima* did not present any type-B chromosome, like *C. sinensis*, and *C. maxima* presented three type-A chromosomes, which did not occur in sweet orange (Cornélio e Guerra 1993; Guerra 1993; Miranda et al. 1997). It is possible that the banding patterns of the sweet orange parents were different from those observed in the cultivars of *C. reticulata* and *C. maxima* so far analysed. Both species are quire polymorphic for isozymes and RFLPs (Roose 1988), and different accessions of each species presented a different heterozygosity index (Federici et al. 1998). Furthermore, *C. maxima* is a monoembryonic species and different banding patterns were observed among its seedlings (Miranda et al. 1997). On the other hand, *C. reticulata* is rather variable, considered either as one species with many hybrids and cultivars (Swingle and Reece 1967) or as a group of different species (Hodgson 1967; Tanaka 1969), which may present different banding patterns (Santos et al. 1993).

The complex and stable karyotype observed among *C. sinensis* cultivars fits with the hypothesis of a monophyletic origin followed by somatic mutation and the selection of asexually reproduced clones (Cameron and Frost 1968; Barrett and Rhodes 1976). This hypothesis is also supported by the great genetic similarity observed among *C. sinensis* cultivars by isozyme and RAPD analysis (Roose 1988; Luro et al. 1995) and, on the other hand, by the high genetic variability of its proposed parents. Only if one accepts that the parental cultivars were both homozygous in the past, would it be possible that different crosses could have given rise to such genetically homogeneous species, as admitted by Sauer (1994).

Acknowledgements The authors are grateful to Dr. Walter Soares-Filho for providing the plant material, Dr. Angeles Cuadrado for kindly supplying the pTa71 and pTa794 probes, Dr. Andreas Bachmair and Dr. J.S. Heslop-Harrison for kindly supplying the SK 18S+25S and pXV 1–2 probes respectively, Mag. Karin Garber and Dr. André Vanzela for probes labelling, Dr. Franz Klein for image-processing assistance and Dr. Jörg Fuchs for critical reading of the manuscript. This work was supported by the Brazilian agencies FACEPE, CNP_q and BNB and partially supported by ÖNB P7172 (D.S.). These experiments comply with the current laws of Brazil and Austria.

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