Genetica (2007) 129:273–279
DOI 10.1007/s10709-006-0007-4

ORIGINAL PAPER

A simple chromosomal marker can reliably distinguishes *Poncirus* from *Citrus* species

A. C. Brasileiro-Vidal · J. A. dos Santos-Serejo · W. dos S. Soares Filho · M. Guerra

Received: 20 December 2005 / Accepted: 5 April 2006 / Published online: 4 August 2006

Abstract Several chromosome types have been recognized in *Citrus* and related genera by chromomycin A_3 (CMA) banding patterns and fluorescent in situ hybridization (FISH). They can be used to characterize cultivars and species or as markers in hybridization and backcrossing experiments. In the present work, characterization of six cultivars of *P. trifoliata* (''Barnes'', ''Fawcett'', ''Flying Dragon'', ''Pomeroy'', ''Rubidoux'', ''USDA'') and one *P. trifoliata* × *C. limonia* hybrid was performed by sequential analyses of CMA banding and FISH using 5S and 45S rDNA as probes. All six cultivars showed a similar CMA^+ banding pattern with the karyotype formula 4B + 8D + 6F. The capital letters indicate chromosomal types: B, a chromosome with one telomeric and one proximal band; D, with only one telomeric band; F, without bands. In situ hybridization labeling was also similar among cultivars. Three chromosome pairs displayed a closely linked set of 5S and 45S rDNA sites, two of them co-located with the proximal band of the B type chromosomes (B/5S-45S) and the third one co-located with the terminal band of a D pair (D/5S-45S). The B/5S-45S chromosome has never been found in any citrus accessions investigated so far. Therefore, this B chromosome can be used as a marker to recognize the intergeneric *Poncirus* × *Citrus* hybrids. The intergeneric hybrid analyzed here displayed the karyotype formula 4B + 8D + 6F, with two chromosome types B/5S-45S and two D/5S-45S. The karyotype formula and the presence of two B/5S-45S chromosomes clearly indicate that the plant investigated is a symmetric hybrid. It also demonstrates the suitability of karyotype analyses to differentiate zygotic embryos or somatic cell fusions involving trifoliate orange germplasm.

Keywords Chromosome · *Citrus* · CMA · FISH · *Poncirus* · rDNA

During the submission of this paper, we analyzed 25 other citrus cultivars with the same methodology and we found that the chromosome marker reported here can indeed distinguish *Poncirus trifoliata* from grapefruits, pummelos, and one variegated access of *Citrus*, besides the previously reported access of limes, limons, citrons, and sweet-oranges. However, among 14 mandarin cultivars, two of them displayed a single B/5S-45S chromosome, whereas in *Citrus hystrix* D.C., a far related species belonging to the *Papeda* subgenus, this chromosome type was found in homozygosis. Since these two mandarin cultivars are probably of hybrid origin, we assume that for almost all commercial cultivars and species of the subgenus *Citrus* this B type chromosome is a useful genetic marker.

A. C. Brasileiro-Vidal · M. Guerra
Departamento de Botânica, Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Rua Nelson Chaves s/n. Cidade Universitária, Recife, PE 50.670-420, Brazil

J. A. dos Santos-Serejo · W. dos S. Soares Filho
Embrapa Mandioca e Fruticultura, Cruz das Almas BA 44.380-000, Brazil

A. C. Brasileiro-Vidal (✉)
Departamento de Genética, Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Av. Prof. Moraes Rego s/n. Cidade Universitária, Recife, PE 50.732-970, Brazil
e-mail: brasileiro_vidal@hotmail.com

Introduction

Citrus trees are economically the most important fruit crop, with an annual production exceeding 108 million tons in 2004 (FAOSTAT 2005). Commercial citrus cultivars belong to several species, which are asexually

propagated by grafting onto rootstock cultivars propagated by seed. Among the several species indicated for rootstock, or for rootstock breeding programs, the Rangpur lime (*C. limonia* Osbeck) and trifoliate orange [*Poncirus trifoliata* (L.) Raf.] are often preferred for their tolerance to citrus tristeza virus (CTV) and some other adverse conditions. In Brazil, for example, about 80% of citrus varieties are grafted onto *C. limonia* (Schäfer et al. 2001). In China, Japan and other countries in the temperate zone the trifoliate orange is the most widely used rootstock whereas in the USA hybrids between trifoliate orange and citrus species are the most common rootstock (Fang et al. 1977).

The citrus breeding rootstock programs aim at obtaining not only new cultivars resistant to disease, but also cultivars, which can adapt better to adverse growing conditions in order to diversify the limited choice of currently available rootstocks. In spite of the tolerance observed in some citrus species, the resistance to several diseases has been introduced from other related genera, like *Poncirus*, *Swinglea*, and *Severinia* (Schäfer et al. 2001). Since the development of disease resistant cultivars has become an objective of high priority, much effort has been dedicated to introgressing these characters into citrus cultivars by sexual hybridization. However, trifoliate orange, like many citrus taxa, are highly polyembryonic, producing seeds with two types of embryos: nucellar (apomictic) embryos, genetically identical to the mother tree, and zygotic (sexual) embryos, which are less frequent and often less vigorous than the former ones (Spiegel-Roy and Goldschmidt 1996). The nucellar embryony largely complicates breeding efforts, since in some trifoliate orange cultivars up to 90% of the embryos are of nucellar origin (Khan and Roose 1998). Several biochemical, enzymatic and molecular techniques have been proposed to distinguish zygotic seedlings from nucellar ones (Spiegel-Roy and Goldschmidt 1996) but very little is known about cytogenetic markers that could distinguish the chromosomes from *P. trifoliata* incorporated into hybrids of *Poncirus* with *Citrus* or with other related genera.

Until recently, the cytogenetics of *Citrus* species have often been misunderstood because of their nearly identical karyotypes, small chromosomes, same chromosome number ($2n = 18$), and very similar chromosome size and morphology (Guerra et al. 1997). Nevertheless, the introduction of chromosome staining with chromomycin A_3 (CMA), a GC base-specific fluorochrome, counterstained with the AT-specific 4′-6-diamidino-2-phenyl-indole (DAPI) fluorochrome, revealed that most species and many *Citrus* cultivars are karyotypically distinct (Guerra 1993). Nowadays, over 50 *Citrus* accessions have been characterized by CMA chromosome bands (Miranda et al. 1997; Befu et al. 2001, 2002; Cornélio et al. 2003; Yamamoto and Tominaga 2003; Carvalho et al. 2005). The distribution of bands along the chromosomes produced different chromosome types, the most conspicuous of them are type A (with three CMA^+ bands), B (with a proximal and a terminal band), C (with two terminal bands), D (with a single terminal band) and F (without bands). Furthermore, Guerra et al. (2000) have extended the analyses of CMA banding to 13 other *Citrus*-related genera, demonstrating that all those genera exhibit different combinations of these chromosome types.

Further chromosomal differentiation has been obtained by combining the CMA band patterns with the number and position of 5S and 45S rDNA sites revealed by fluorescent in situ hybridization (FISH). Thus, the *C. limonia* karyotype, for instance, can be described by the formula 1B + 11D + 6F, according to the chromosome types identified by CMA banding. Among these chromosomes, one can distinguish by FISH two 45S rDNA sites, one on the proximal band of the B chromosome and the other on the terminal band of a D chromosome, and two 5S rDNA sites, one of them near the 45S rDNA site of the D chromosome (identified as D/5S-45S chromosome) and the other on the opposite end of another D chromosome (identified as D/5S) (Carvalho et al. 2005).

In this paper, the karyotype of six cultivars of trifoliate orange was investigated with CMA/DAPI and FISH, in order to identify chromosome tags that distinguish trifoliate orange chromosomes from citrus ones. It was found that this species has not only a CMA banding pattern different from the *Citrus* species but it also has an exclusive B chromosome type with both 5S and 45S rDNA sites located at the proximal region. In addition to that, a hybrid from the crossing *P. trifoliata* × *C. limonia* was analyzed so as to confirm whether intergeneric hybrids can be unequivocally identified by these chromosome markers.

Material and methods

Plant material

Seedlings or adult plants of six cultivars of *Poncirus trifoliata* (''Barnes'', ''Fawcett'', ''Flying Dragon'', ''Pomeroy'', ''Rubidoux'', ''USDA'') and one *P. trifoliata* × *C. limonia* hybrid from the *Citrus* Germplasm Collection of the Embrapa Cassava and Fruit, Cruz das Almas, Bahia, Brazil, were analyzed.

Chromosome preparation

For mitotic analysis, root tips from germinating seeds (''Barnes'', ''Fawcett'', ''Pomeroy'', ''Rubidoux'') or apical meristems from adult plants (''Flying Dragon'', ''USDA'', *P. trifoliata* × *C. limonia*) were pretreated with 8-hydroxyquinoline (2 mM) for 18–20 h at 10°C before fixation in ethanol:acetic acid (3:1, v/v) for 2–24 h at room temperature and stored at −20°C.

CMA banding procedure

Slide preparation and CMA/DAPI double staining followed Guerra (1993). The meristems were washed twice in distilled water, 5 min each, digested with a 2% cellulase–20% pectinase solution for 60–90 min and squashed in 45% acetic acid. Only one root tip was used in each slide, in order to avoid mixing roots of nucellar and zygotic seedlings. After coverslip removal in liquid nitrogen the slides were stored for 3 days at room temperature, stained with CMA for 60 min, counterstained with DAPI for 30 min, and mounted in McIlvaine's buffer (pH 7.0): glycerol (1:1, v/v).

For each cultivar, 3–6 slides were checked, and good cells at metaphase were photographed for karyotype analysis. Mitotic cells were analyzed with an epifluorescence Leica DMLB microscope and images of the best cells were captured with a Cohu CCD camera, using the QFISH program of Leica. Afterwards, the slides were destained in ethanol: acetic acid (3:1, v/v) for 30 min followed by immersion in absolute ethanol at room temperature for 2 h and stored at −20°C.

DNA probes and labeling

A 6.5-kb fragment (R2) of an 18S-5.8S-25S repeat unit from *Arabidopsis thaliana* (Wanzenböck et al. 1997) was used for chromosome identification. The probe was labeled with digoxigenin-11-dUTP (Boerinhger) by nick translation. The 5S rDNA probe was generated by PCR from genomic DNA of *P. trifoliata* cv. Pomeroy, using the primers 5′-GTGCGATCATACCAGC(A/G)(G/T)TAATGCACCGG-3′ and 5′-GAG GTGCAACACGAGGACTTCCCAGGAGG-3′ at an annealing temperature of 55°C, and simultaneously labeled with biotin-11-dUTP (Sigma).

In situ hybridization

The procedure and conditions for in situ hybridization followed basically Moscone et al. (1996), at 83% stringency. Each probe was diluted in a hybridization mixture to a final concentration of 1.7–5.0 ng/μl, containing 60% formamide, 2× SSC, 5% dextran sulphate and 0.01% salmon sperm DNA. The hybridization mixture and the cytological preparations were denatured at 75°C for 10 min and hybridized for 18–20 h at 37°C. The 45S rDNA probe was detected with sheep anti-digoxigenin-FITC (Boehringer) and the signal amplified with FITC-conjugated anti-sheep secondary antibody (DAKO). Biotin-labeled probes were detected with mouse anti-biotin (DAKO) followed by TRITC-conjugated anti-mouse secondary antibody (DAKO). The slides were counterstained with DAPI 2 μg/ml and mounted in Vectashield H-1000 (Vector). The cells previously acquired with CMA/DAPI staining were captured again using the same microscope and software described before. The images were optimized for better contrast and brightness with Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA).

Results

All cultivars analyzed had $2n = 18$ chromosomes. No variation in the CMA banding pattern was found among seedlings of the same cultivar. The CMA stained chromosomes displayed a large number of highly reproducible and clearly identified CMA^+/$DAPI^-$ bands, preferentially located at the end of the long arms. Metaphase cells of four of the six cultivars investigated are illustrated in Fig. 1. Proximal bands were sometimes weakly or neutrally stained with CMA, but unambiguously recognized as $DAPI^-$ bands. A satellite was only observed in one D chromosome of some cells (Fig. 1a, b, j, k, m, n).

Based on the number and position of CMA^+ bands three chromosomes types were identified: type B, one telomeric band at the long arm and one proximal band at the short arm; D, one telomeric band at the long arm; and F, without bands (Fig. 2A). The chromosome compositions of all *P. trifoliata* cultivars investigated displayed the same karyotype formula (4B + 8D + 6F). They always exhibited small variations in chromosome size or band size, especially for one B chromosome type, which displayed a duller and smaller terminal CMA band, indicated by an asterisk in Fig. 1a, d, g, j. It was difficult to ascertain heteromorphism in D chromosomes because such chromosomes were the most numerous and the bands varied gradually in size. Furthermore, the intensity of the staining often influenced the visibility of small bands. In all cultivars investigated the largest F chromosome pair could be easily identified and never exhibited the fine terminal band observed in some *Citrus* accessions (Cornélio et al. 2003; Carvalho et al. 2005).

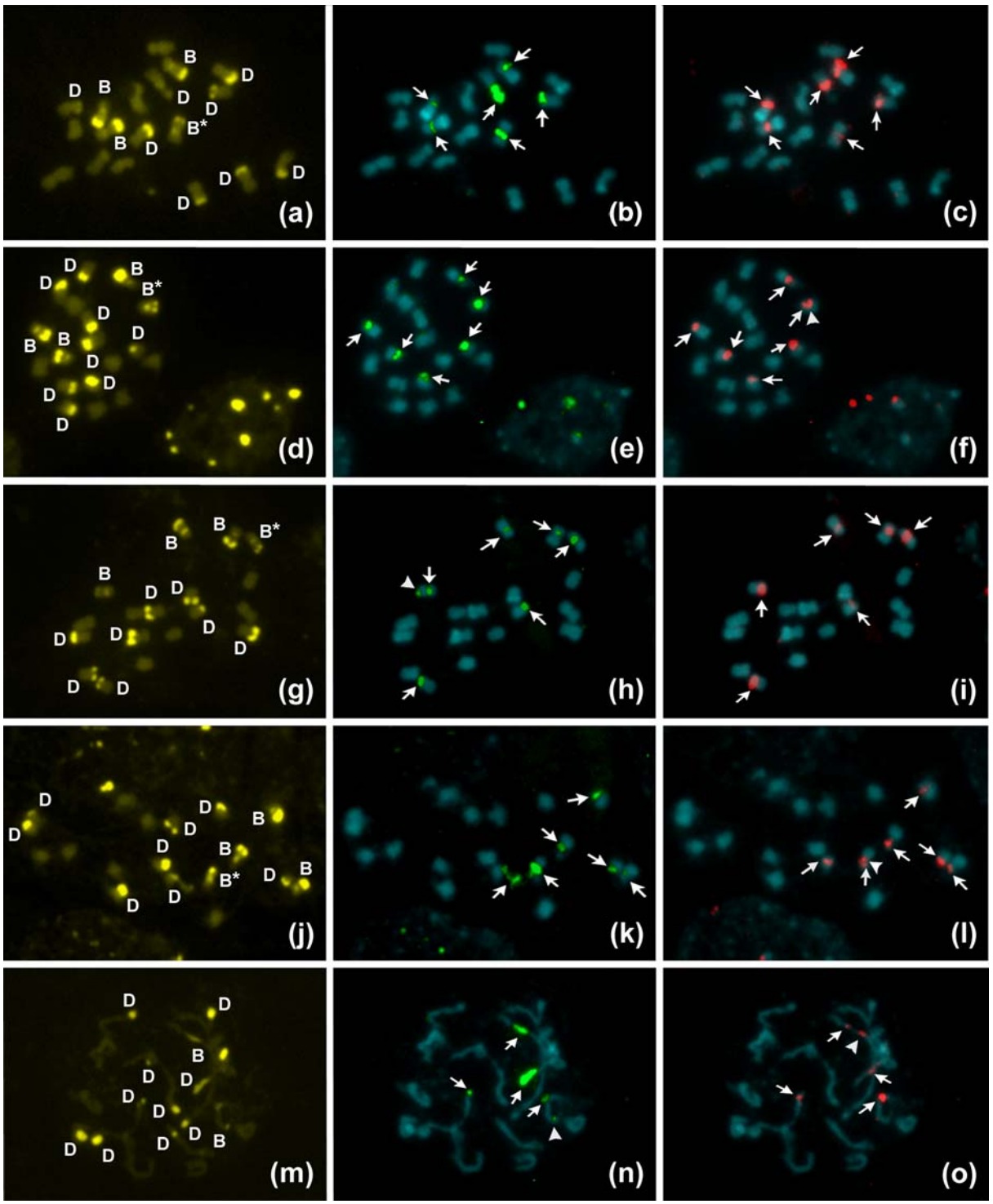

Fig. 1 Distribution of the CMA^{+} bands and 5S and 45S rDNA sites in four cultivars of *Poncirus trifoliata* (**a**–**l**) and in the *P. trifoliata* × *C. limonia* hybrid (**m**–**o**). Mitotic metaphases of ''Fawcett'' (**a**–**c**), ''Flying Dragon'' (**d**–**f**), ''Pomeroy'' (**g**–**i**), ''Rubidoux'' (**j**–**l**) and prophase of the hybrid (**m**–**o**) stained with CMA (**a**, **d**, **g**, **j**, **m**). The same cells were destained and in situ hybridized with 45S (**b**, **e**, **h**, **k**, **n**) and 5S (**c**, **f**, **i**, **l**, **o**) rDNA probes. Asterisk in **a**, **d**, **g** and **j** indicate a B chromosome type with smaller and faint terminal band. Arrows point at 5S (red) and 45S (green) rDNA sites. Arrowheads point at the extra 45S rDNA sites (H, N) or the extra 5S rDNA sites (**f**, **l**, **o**). Observe that the proximal bands of B chromosomes are usually much smaller than the terminal ones

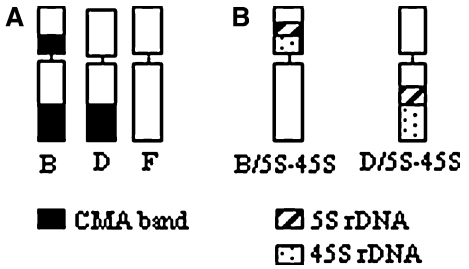

Fig. 2 Chromosome types identified in *Poncirus trifoliata*, after CMA/DAPI staining (**A**) and after FISH with rDNA probes (**B**). All B chromosomes co-localized the proximal band with the rDNA sites but only one among several D chromosomes co-localized its CMA band with the rDNA sites

In situ hybridization patterns were also similar among cultivars. There were six 5S and six 45S rDNA sites at each diploid complement. Most remarkable, each 5S rDNA site was closely linked to a 45S rDNA site. Four 5S-45S rDNA sites were located at the proximal region of the four B chromosomes or B/5S-45S, whereas two other sets of rDNA sites were located at the terminal band of a D pair (or D/5S-45S). In all cases, the 45S rDNA site co-localized with the CMA band of the respective chromosomes (Figs. 1, 2). Extra and small sites of 5S or 45S rDNA were sometimes observed. An extra 45S rDNA site was found at the terminal band of one B chromosome type (Fig. 1h), whereas an extra 5S rDNA site was observed in the proximal band of another B chromosome, in a more proximal position in relation to the 45S rDNA site, generating a complex 5S-45S-5S rDNA site (Fig. 1f, l).

The hybrid *P. trifoliata* × *C. limonia* displayed the karyotype formula 2B + 10D + 6F. In one of the D chromosomes the CMA^+ band was subterminally located (Fig. 1m). Four chromosomes exhibited both 5S and 45S rDNA sites. Two of them were of the type B/5S-45S and two others were of the type D/5S-45S. In both chromosome types, 45S rDNA sites were co-localized with a CMA^+ band. One of the two B/5S-45S chromosomes showed an extra 45S rDNA site co-localized with the terminal band, while the other one displayed an extra proximal 5S rDNA site (Fig. 1m–o), similar to the one found in *P. trifoliata.*

Discussion

The six analyzed cultivars of *Poncirus trifoliata* conserved the same CMA banding pattern, confirming the low genetic diversity reported by Fang et al. (1977) in 48 trifoliate orange accessions. Small heteromorphisms found for the banding pattern reflect mainly the natural variability of the heterochromatin, as observed in several other species. In wheat and related species, for example, Shang et al. (1989) reported heterochromatic band size polymorphisms for all accessions investigated. Similar results were verified in maize and barley (Mastenbroek and de Wet 1983; Kakeda et al. 1991).

The karyotype formula of these accessions (4B + 8D + 6F), with two pairs of B type chromosomes, differs from all other *Citrus* accessions so far reported. *Citrus* accessions may display only one B pair, a single B or no B type chromosome at all. The banding pattern described here is identical to the one showed by Befu et al. (2000), but differs from the karyotype described by Miranda et al. (1997) with a single pair of B chromosomes. The difference between the present results and those reported by Miranda et al. (1997) may be due to the difficulty in identifying the proximal CMA band, which sometimes can be small and not bright enough to be recognized as a CMA^+ band. In this case, the B chromosome would look like a D chromosome type. If a B chromosome pair of the karyotype reported by Miranda et al. (1997) had pale proximal bands, they might have been misidentified as a D chromosome. This could explain why these authors reported 2B + 10D + 6F for *P. trifoliata*, instead of 4B + 8D + 6F as observed by Befu et al. (2000) and in the present work. Guerra et al. (2000) also reported a different karyotype for *P. trifoliata*. However, this report was based on seedlings from a single individual growing in a botanical garden and of unknown provenance and, based on its karyotype, it is an intergeneric hybrid of *P. trifoliata.* On the other hand, Befu et al. (2000) analyzed young leaves of an adult plant, which seems to be a true trifoliate orange.

The CMA banding pattern observed in the *P. trifoliata* × *C. limonia* hybrid was exactly one of the two possible formulae expected from this cross, since *C. limonia* has a heteromorphic BD chromosome pair (Carvalho et al. 2005). The other chromosome types of this hybrid (2B + 10D + 6F) could be inferred from the karyotype formula of its parents. *Citrus limonia*, like some other lemons and limes, has one or two D chromosomes with a subterminal band instead of a terminal one (Carvalho et al. 2005). Such a D chromosome type was not observed in *P. trifoliata* but was found in the hybrid.

Beside the singular karyotype formula of *P. trifoliata*, the most conspicuous characteristic of this karyotype is the occurrence of 5S and 45S rDNA sites closely linked at the proximal CMA band of all B chromosomes and in one of the D pairs. The linkage between these two rDNA sites have been previously reported in *P. trifoliata* by Roose et al. (1998) who recognized six major sites of 45S rDNA (a seventh minor site was sometimes detected) and four 5S rDNA sites. These authors were able to

identify three 45S rDNA sites adjacent to 5S rDNA sites but they do not indicate the chromosomal types bearing the rDNA sites. Carvalho et al. (2005) observed that all B chromosomes of nine *Citrus* species investigated, including *C. limonia*, had a 45S rDNA site at the proximal CMA^+ band but no 5S rDNA site was found on B chromosomes. Similar results were observed in *C. sinensis* (Pedrosa et al. 2000) and several mandarin and grapefruit accessions investigated in our laboratory (unpublished data). On the other hand, isolated 5S rDNA sites were always observed on D or F chromosomes of *Citrus* species (Pedrosa et al. 2000; Carvalho et al. 2005). Since *C. limonia* has a D/5S-45S chromosome, the two chromosomes of this type observed in the hybrid were inherited, one from each parent. Therefore, no *Citrus* species seems to exhibit B/5S-45S chromosomes making this particular B chromosome type a reliable marker for F_1 hybrids between *P. trifoliata* and *Citrus* species. The reliability of this marker was successfully confirmed here in a *Poncirus* × *Citrus* hybrid, which had the two expected B/5S-45S.

The 45S rDNA sites commonly stain positively with CMA and negatively with DAPI ($CMA^+/DAPI^-$ bands), as was observed in the present work. In several *Citrus* species, some 45S rDNA sites are $CMA^+/DAPI^-$ while others stain as $CMA^0/DAPI^-$ bands (Carvalho et al., 2005). This uneven reaction may be due to changes in the GC content of these sites, as expected from the higher affinity of chromomycin A3 by GC-rich sequences (Schweizer 1981). Alternatively, the rDNA may exist in distinct chromatin conformations determined by specific epigenetic codes, such as cytosine methylation and post-translational changes in histones (Neves et al. 2005) that could affect its reactivity with fluorochromes.

A few minor 5S and 45S rDNA sites were observed in some but not all cells of *P. trifoliata*, because they were small and hardly detectable. Carvalho et al. (2005) also reported the occurrence of minor 45S rDNA sites in some lemon species, such as *Citrus jambhiri* and *C. volkameriana*. Minor rDNA sites have often been described in several Triticeae species, including wheat (Mukai et al. 1991), barley (Leitch and Heslop-Harrison 1992; Pedersen and Linde-Laursen 1994), and *Thinopyrum ponticum* (Li and Zhang 2002; Li et al. 2004). All these reports confirm the instability of this site, which apparently can jump from one chromosome locus to another in a relatively short time (Dubcovsky and Dvořák, 1995; Schubert and Wobus 1996). Further progress in the molecular cytogenetics of citrus species using DNA analyses of microdissected chromosomes (Huang et al. 2004) should shed more light on this phenomenon.

Acknowledgment This work was supported by the Brazilian agencies FACEPE and CNPq.

References

Befu M, Kitajima A, Hasegawa K (2001) Chromosome composition of some *Citrus* species and cultivars based on the chromomycin A (3) (CMA) banding patterns. J Hort Sci 70:83–88

Befu M, Kitajima A, Hasegawa K (2002) Classification of the *Citrus* chromosomes with same types of chromomycin A banding patterns. J Hort Sci 71:394–400

Befu M, Kitajima A, Ling YX, Hasegawa K (2000) Classification of 'Tosa – Buntan' pummelo (*Citrus grandis* [L.] Osb.), 'Washington' navel orange (*C. sinensis* [L.] Osb.) and trifoliate orange (*Poncirus trifoliata* [L.] Raf.) chromosomes using young leaves. J Hort Sci 69:22–28

Carvalho R, Soares Filho WS, Brasileiro-Vidal AC, Guerra M (2005) The relationships among lemons, limes and citron: a chromosomal comparison. Cytogenet Genome Res 109:276–282

Cornélio MTMN, Figueirôa ARS, Santos KGB, Carvalho R, Soares Filho WS, Guerra M (2003) Chromosomal relationships among cultivars of *Citrus reticulata* Blanco, its hybrids and related species. Plant Syst Evol 240:149–161

Dubcovsky J, Dvořák J (1995) Ribosomal-RNA multigene loci-nomads of the Triticeae genomes. Genetics 140:1367–1377

Fang DQ, Roose ML, Krueger RR, Federici CT (1977) Fingerprinting trifoliate orange germ plasm accessions with isoenzymes, RFLPs, and inter-simple sequence repeat markers. Theor Appl Genet 95:211–219

FAOSTAT (2005) Agriculture data. Available at http://www.faostat.fao.org. Accessed: 27 June 2005

Guerra M (1993) Cytogenetics of Rutaceae. V. High chromosomal variability in *Citrus* species revealed by CMA/DAPI staining. Heredity 71:234–241

Guerra M, Pedrosa A, Silva AEB, Cornélio MTM, Santos KGB, Soares Filho WS (1997) Chromosome number and secondary constriction variation in 51 accessions of a *Citrus* germplasm bank. Brazil J Genet 20:489–496

Guerra M, Santos KGB, Silva AEB, Ehrendorfer F (2000) Heterochromatin banding patterns in Rutaceae-Aurantioideae – a case of parallel chromosomal evolution. Am J Bot 87:735–747

Huang D, Wu W, Lu L (2004) Microdissection and molecular manipulation of single chromosomes in woody fruit trees with small chromosomes using pomelo (*Citrus grandis*) as a model. II. Cloning of resistance gene analogs from single chromosomes. Theor Appl Genet 108:1371–1377

Kakeda K, Fukui K, Yamagata H (1991) Heterochromatic differentiation in barley chromosomes revealed by C- and N-banding techniques. Theor Appl Genet 81:144–150

Khan IA, Roose ML (1998) Frequency and characteristics of nucellar and zygotic seedlings in three cultivars of trifoliate orange. J Am Soc Hort Sci 113:105–110

Leitch IJ, Heslop-Harrison JS (1992) Physical mapping of the 18S-5.8S-26S rRNA genes in barley by *in situ* hybridization. Genome 35:1013–1018

Li D, Zhang X (2002) Physical localization of the 18S-5.8S-26S rDNA and sequence analysis of ITS regions in *Thinopyrum ponticum* (Poaceae: Triticeae): implications for concerted evolution. Ann Bot 90:445–452

Li D, Yanyan RY, Zhang X (2004) Distribution of the 18S-5.8S-26S rDNA loci and heterogeneity of nuclear ITS regions in *Thinopyrum intermedium* (Poaceae: Triticeae). Acta Bot Sin 46:1234–1241

Mastenbroek I, de Wet JMJ (1983) Chromosome C-banding of *Zea mays* and its closest relatives. Can J Genet Cytol 25:203–209

Miranda M, Ikeda F, Endo T, Moriguchi T, Omura M (1997) Comparative analysis on the distribution of heterochromatin in *Citrus*, *Poncirus* and *Fortunella* chromosome. Chromosome Res 5:86–92

Moscone EA, Matzke MA, Matzke AJM (1996) The use of combined FISH/GISH in conjunction with DAPI counterstaining to identify chromosomes containing transgenes inserts in amphidiploid tobacco. Chromosoma 105:231–236

Mukai Y, Endo TR, Gill BS (1991) Physical mapping of the 18S.26S rRNA multigene family in common wheat: identification of a new locus. Chromosoma 100:71–78

Neves N, Delgado M, Silva M, Caperta A, Morais-Cecílio L, Viegas W (2005) Ribosomal DNA heterochromatin in plants. Cytogenet Genome Res 109:104–111

Pedersen C, Linde-Laursen IB (1994) Chromosomal locations of four minor rDNA loci and a marker microsatellite sequence in barley. Chromosome Res 2:65–71

Pedrosa A, Schweizer D, Guerra M (2000) Cytological heterozygosity and the hybrid origin of sweet orange [*Citrus sinensis* (L.) Osbeck. Theor Appl Genet 100:361–367

Roose ML, Schwarzacher T, Heslop-Harrison JS (1998) The chromosomes of *Citrus* and *Poncirus* species and hybrids: identification of characteristic chromosomes and physical mapping of rDNA loci using *in situ* hybridization and fluorochrome banding. J Hered 89:83–86

Schäfer G, Bastianel M, Dornelles ALC (2001) Citriculture rootstocks in use. Ci Rural 31:723–733

Schweizer D (1981). Counterstain-enhanced chromosome banding. Hum Genet 57:1–14

Shang XM, Nguyen HT, Jackson RC (1989) Heterochromatin differentiation and phylogenetic relationship of the A genomes in diploid and polyploidy wheats. Theor Appl Genet 77:84–94

Shubert I, Wobus U (1996) *In situ* hybridization confirms jumping nucleolus organizing regions in *Allium*. Chromosoma 92:143–148

Spiegel-Roy P, Goldschmidt EE (1996) Biology of citrus. Cambridge University Press, Cambridge

Wanzenböck E-M, Schöfer C, Schweizer D, Bachmair A (1997) Ribosomal transcription units integrated via T-DNA transformation associate with nucleolus and do not require upstream repeat sequences for activity in *Arabidopsis thaliana*. Plant J 11:1007–1016

Yamamoto M., Tominaga S (2003) High chromosomal variability of mandarins (*Citrus* spp.) revealed by CMA banding. Euphytica 129:267–274