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Molecular and structural evolution of *Citrus* satellite DNA

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Abstract Highly repeated satellite DNA (stDNA) of citric plants was characterized by cloning and sequencing 10–14 repeats of each plant (*Citrus limon*, *C. sinensis*, *C. ichangensis*, *Poncirus trifoliata*). The monomers are mostly 181 bp in length with a GC-content between 60% and 68% (significantly higher than the average GC-content of the citrus group genomes). Similarity among the repeats indicates that they belong to a satellite family that underwent species-specific modifications, which are reflected in the phylogenetic relationships. Curvature provoked by dA-stretches of the repeats analyzed by gel shifts revealed structural conservation, even though the nucleotide sequences vary among species, thereby probably supporting the heterochromatic structure of stDNA. We show that the species-specific modification of the satellite consensus involves changes in the position and number of dA tracts. The molecule shapes of satellite oligomeres predicted by computer modelling indicate a superhelical structure of the tandem repeats which is in a good agreement with the satellite sequence dendrogram. The contribution of DNA bending elements to the evolution of plant satellite repeats is discussed.

Keywords Curvature · Evolution · Heterochromatin · Repetitive DNA · Rutaceae

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Introduction

Tandemly arranged, highly repeated satellite DNA (stDNA) represents an important fraction of the eucaryotic genome located mainly at centromeric and telomeric regions of the chromosomes (Beridze 1986). The bending and curvature of DNA (Trifonov 1985) may play a role in the formation of constitutive stDNA heterochromatin within the cell nucleus. For several species of plants stDNA has been characterized and shown to be the most variable component of the genome with a high rate of molecular evolution (Hemleben et al. 2000). The distribution and sequence of stDNA may therefore provide insight into phylogenetic relationships among species (Hemleben 1993; King et al. 1995; Stadler et al. 1995).

Satellite DNA of citric plants was first characterized using analytical ultracentrifugation in CsCl buyoant density gradients (Ingle et al. 1973; Bragvadze 1983; Bragvadze and Beridze 1983). In citric plants, stDNA content differs from species to species, occasionally reaching more than 20% of the genome; for example, large tandem arrays have been found in the wild species *Citrus ichangensis* with a relatively high GC-content ranging from 60% to 65%. This stDNA of *C. ichangensis* of the subtribus Citrinae was investigated with respect to sequence and structure (Beridze et al. 1992, 1994).

In eukaryotes, including yeast (Murphy et al. 1991), animal (Martinez-Balbas et al. 1990) and plant (Matyasek et al. 1997) species, satellite repeats forming constitutive heterochromatin often display DNA curvature. Intrinsic DNA curvature of centromeric DNA has been found at all 16 yeast chromosomes (Bechert et al. 1999), suggesting the involvement of "curved" DNA in the formation of the centromere complex. If the A-tracts are repeated in phase with the helix screw, total bend will grow to a considerable size, and static bend can be detected by physical methods (Marini et al. 1982; Trifonov 1985). Evidence for the role of dA-tracts in the curvature of tobacco satellites has been supported by site directed mutagenesis of bending elements (Královics et al. 1995). The repeating units of *C. ichangensis* stDNA are approximately 181 bp

in length and contain tracts of adenine residues. Computer analysis had shown that the bending observed in the monomer of stDNA of *C. ichangensis* promotes the formation of a coiled double helix (CDH) structure during the transition to the oligomeric form (hexamer and higher multimers; Beridze et al. 1992). Polyacrylamide gel electrophoresis demonstrated that the apparent size of a satellite hexamer differs from that of a linear control fragment at low temperatures, and an electron microscopic analysis revealed small-diameter circles and thick rod-like particles of the hexamer (Beridze et al. 1994).

The genus *Citrus* (subtribus: Citrinae; family: Rutaceae) occupies a wide habitat that extends from the Himalayas and northern China to Australia and New Caledonia. In northern China, wild species have been replaced by the related genus *Poncirus* (Zhukowski 1971). The origin of cultivated species of citric plants is not known. *Citrus sinensis* (orange) was used by man as early as the second millenium B.C. Despite the abundance of natural and man-made hybrids, none of the hybrids is closely related to present-day cultivated species. This suggests that species that participated in the formation of the cultivated plants no longer exist in nature.

In order to investigate stDNA of these citric species in more detail, we determined the primary structure of stDNAs of two cultivated (lemon, orange) and two wild species of the subtribus Citrinae in order to reveal phylogenetic relationships among species using stDNA as the molecular marker. Constraints in primary sequence on the curvature of plant satellite repeats were elucidated.

Materials and methods

Plant material and DNA characterization

Plant material of *Citrus sinensis* (orange), *C. limon* (lemon), *C. ichangensis* and *Poncirus trifoliata* (subtribus Citrinae, Rutaceae) was obtained from the Sukhumi Branch of the All-Union Institute of Plant Research Institute, Leningrad, GUS.

Total DNA was isolated from leaves, and stDNA was obtained by CsCl gradient centrifugation (Beridze 1980), cleaved by restriction with *Sty*I and electrophoretically separated on agarose gels; Southern blot hybridization with a satellite element of *C. limon* (Beridze et al. 1992) and washing procedure of the filter was carried out at 65°C according to Ganal et al. (1986). Enriched stDNA of all four plants digested with *Sty*I was ligated into pUC18 and cloned in *Escherichia coli*; the satellite elements containing clones were detected by colony filter hybridization at 60°C, and 10–14 clones of each species carrying 180-bp inserts approximately were sequenced using the methods described by King et al. (1995). Sequences were evaluated with the computer program "Align/sequence alignment program" (Myers and Miller 1988) and the neighbour-joining method (Saitu and Nei 1987). Sequences are available in EMBL databank under the accession numbers Z77674–Z77687 for CI1 to CI14, Z77688–Z77699 for CL1 to CL12, Z77700–Z77710 for CS1 to CS11 and Z77711–Z77722 for PT1 to PT12.

For structural analyses, oligomers of stDNA were cloned, partially digested with *Sty*I and separated on 5% polyacrylamide gels at 4° and 60°C, respectively, with the 123-bp ladder (Gibco, BRL) and *Eco*RI/*Hin*dIII-digested lambda DNA as size markers. The deviation from standard mobility is expressed as the k-factor (ratio between apparent mobility and the expected mobility deduced from the sequence length).

Computation analysis of DNA curvature

The nearest-neighbour wedge model of intrinsic DNA curvature (Bolshoy et al. 1991) was implemented to calculate the overall DNA path of the analyzed sequences. The wedge angle between neighboring base pairs is assumed to be different for all dinucleotides with dAA/dTT being the largest. The dA4–6 blocks arranged in phase with a helix turn result in deflection of the DNA helix, and the latter is detected macroscopically along a longer sequence as DNA curvature. DNA paths were calculated and drawn with the CURVATURE software (Shpiegelman et al. 1993), kindly provided by Dr. Shpiegelman (College of Judea and Samaria, Research Institute, Gene Structure Research Centre, Ariel, Israel).

Results and discussion

Satellite sequence analysis

Four species of the subtribus Citrinae were chosen for investigation of stDNA: two cultivated species, *C. limon* (lemon) and *C. sinensis* (orange), the wild species *C. ichangensis* and a species of the related genus *Poncirus*, *P. trifoliata*. The latter also belongs to the subtribus *Citrinae*, it can be crossed with citric plants and is often used as root stock for grafting with *Citrus* species. A satellite element of *C. limon* (Beridze et al. 1992) was used as a hybridization probe for Southern blots with *Sty*I-digested DNA previously enriched by CsCl buyoant density centrifugation (Fig. 1). DNA of all four plants exhibited a ladder-like hybridization pattern typical of

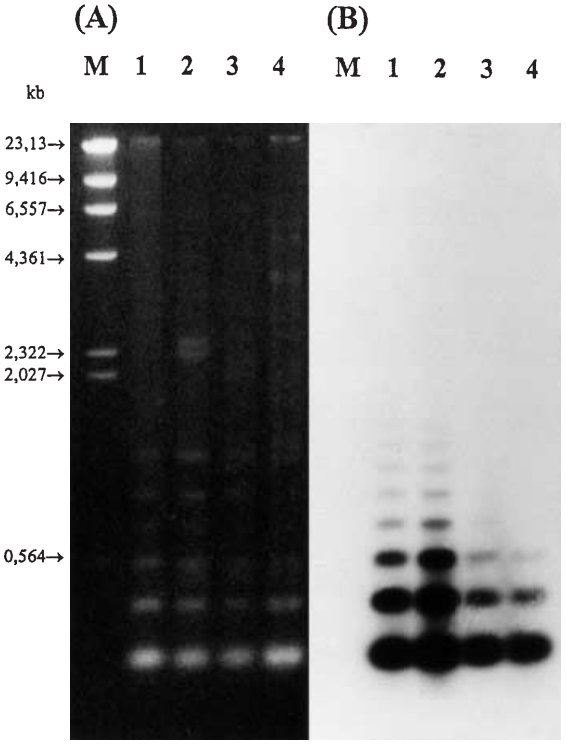

Fig. 1A, B Southern hybridization of DNA from citric plants. Enriched stDNA digested with the restriction enzyme *Sty*I was separated on a 1% agarose gel (**A**), Southern-blotted and hybridized to a radioactively labelled satellite element of *Citrus limon* (**B**). *Lanes*: *1 C. ichangensis*, *2 C. limon*, *3 C. sinensis*, *4 Poncirus trifoliata*. *M* 123-bp ladder marker

Table 1 Compilation of stDNA sequences of four citric plants: *Citrus ichangensis* (CI), *C. limon* (CL), *C. sinensis* (CS), and *Poncirus trifoliata* (PT)

Reference molecule:	CI_1	Length (bp) 1–181 (181)	Homology[a] (%) 100	GC (%) 63.0
Sequence 2:	CI_2	1–181 (181)	94	62.4
Sequence 3:	CI_3	1–170 (170)	89	64.7
Sequence 4:	CI_4	1–180 (180)	94	63.3
Sequence 5:	CI_5	1–179 (179)	92	62.0
Sequence 6:	CI_6	1–180 (180)	93	62.8
Sequence 7:	CI_7	1–180 (180)	94	63.3
Sequence 8:	CI_8	1–179 (179)	94	62.6
Sequence 9:	CI_9	1–181 (181)	90	61.9
Sequence 10:	CI_10	1–181 (181)	92	63.5
Sequence 11:	CI_11	1–181 (181)	93	64.6
Sequence 12:	CI_12	1–182 (182)	92	64.3
Sequence 13:	CI_13	1–181 (181)	91	61.9
Sequence 14:	CI_14	1–181 (181)	79	65.2
Sequence 15:	CL_1	1–181 (181)	80	64.6
Sequence 16:	CL_2	1–181 (181)	80	64.6
Sequence 17:	CL-3	1–182 (182)	79	63.2
Sequence 18:	CL_4	1–182 (182)	79	63.7
Sequence 19:	CL_5	1–181 (181)	79	63.5
Sequence 20:	CL_6	1–181 (181)	80	64.6
Sequence 21:	CL_7	1–179 (179)	78	63.7
Sequence 22:	CL_8	1–181 (181)	78	62.4
Sequence 23:	CL_9	1–181 (181)	81	63.0
Sequence 24:	CL_10	1–181 (181)	83	59.1
Sequence 25:	CL_11	1–181 (181)	83	58.6
Sequence 26:	CL_12	1–181 (181)	83	63.0
Sequence 27:	CS_1	1–181 (181)	83	61.9
Sequence 28:	CS_2	1–169 (169)	78	60.9
Sequence 29:	CS_3	1–180 (180)	83	63.3
Sequence 30:	CS_4	1–181 (181)	83	63.5
Sequence 31:	CS_5	1–181 (181)	83	63.0
Sequence 32:	CS_6	1–181 (181)	81	63.5
Sequence 33:	CS_7	1–175 (175)	78	61.7
Sequence 34:	CS_8	1–181 (181)	79	64.1
Sequence 35:	CS_9	1–180 (180)	79	63.9
Sequence 36:	CS_10	1–184 (184)	93	64.7
Sequence 37:	CS_11	1–181 (181)	91	61.3
Sequence 38:	PT_1	1–181 (181)	70	66.3
Sequence 39:	PT_2	1–181 (181)	70	68.0
Sequence 40:	PT_3	1–181 (181)	71	65.2
Sequence 41:	PT_4	1–181 (181)	74	67.4
Sequence 42:	PT_5	1–181 (181)	73	66.9
Sequence 43:	PT_6	1–181 (181)	75	65.2
Sequence 44:	PT_7	1–186 (186)	75	66.7
Sequence 45:	PT_8	1–180 (180)	71	64.4
Sequence 46:	PT_9	1–180 (180)	76	62.2
Sequence 47:	PT_10	1–181 (181)	83	63.0
Sequence 48:	PT_11	1–181 (181)	78	62.4
Sequence 49:	PT_12	1–182 (182)	78	63.2

[a] Parameters set: Mismatch=2, open gap=4, extended gap=1

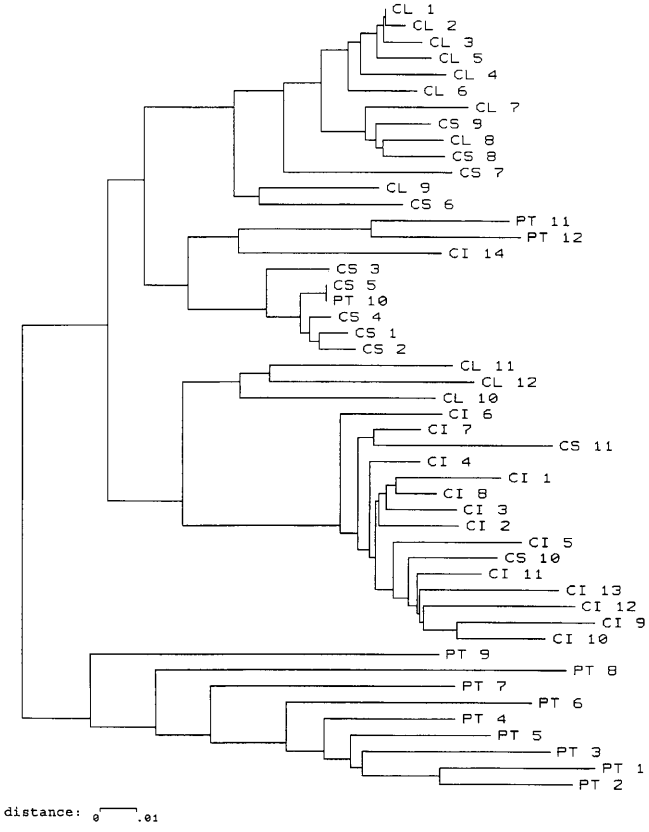

Fig. 2 Neighbor-joining dendogram of stDNA sequences of four citric plants: *CI Citrus ichangensis, CL C. limon, CS C. sinensis, PT Poncirus trifoliata*

tandemly organized stDNA with a monomer length of approximately 180 bp.

*Sty*I-digested stDNA was cloned for each plant species, and 10–14 stDNA-containing clones were sequenced and compared by computer analysis. The majority of the cloned repeats were 181 bp in length although some were slightly longer or shorter. The G+C content varied between 60% and 68% (Table 1). A phylogenetic dendrogram was constructed on the basis of the sequences where *Citrus* sequences are separated from most of the *Poncirus* stDNA sequences, thereby reflecting the taxonomic relationships (Fig. 2). Within in the *Citrus* branch, nine *C. limon* (CL) elements group with some of the *C. sinensis* (CS) repeats, whereas three other CL and two CS repeats group closer to the rather uniformly distributed *C. ichangensis* (CI) elements. Some CS sequences appear to be interspersed into either the CL or the CI group. Most of the PT (*Poncirus trifoliata*) repeats form an independent branch of the dendrogram; three others (PT_10, 11 and 12) appear in the CS group.

Consequently, we were able to observe satellite repeat type differentiation within one species. Clearly, most of the repeats of the two cultivated plants (lemon and orange) group more closely to each other than to the wild species *C. ichangensis*, but some of the repeats are included in the CI group, which means that the repeats are not completely homogenized; arrays of the respective type are also present in the other plants. This suggests that the wild progenitor of *C. limon* and *C. sinensis* was related to *C. ichangenis*, but possibly became extinct during domestication. Within the more distantly related species *P. trifoliata* most of the PT-stDNA repeats appear to be distinctly differentiated but still show 70–83% similarity to the other citric satellites. Large sets of stDNA sequences may thus be used as valuable molecular markers with high resolution.

The situation here resembles the behavior of stDNA in the genus *Beta* (Schmidt and Heslop-Harrison 1994)

Table 2 Electrophoretic migration properties of citric satellite monomers (181 bp) and hexamers (1081 bp) at different temperatures (4° and 60°C, respectively). The deviation from standard mobility is expressed as a k-factor (ratio between apparent mobility and the expected mobility deduced from the sequence length)

	4°C		60°C	
	lambda	123 bp ladder	lambda	123 bp ladder
181-bp CL_5	0.88	0.94	1.00	1.08
181-bp CS_1	0.86	0.92	1.00	1.08
1086-bp CL_5	1.09	1.09	1.13	1.06
1086-bp CS_1	1.07	1.04	1.15	1.06

or *Solanum* (Stadler et al. 1995) and also that of the 350-bp stDNA in species of the genus *Cucurbita* in reflecting the relatively close taxonomic relationship among the species investigated (King et al. 1995), whereas with an additional 170-bp stDNA type no clear separation of the *Cucurbita* species is possible. In contrast, cultivated species of the genus *Cucumis* (Cucurbitaceae) each amplified a prominent satellite DNA component that showed only 62–74% similarity among the species. The point in time of domestication and cultivation possibly influences the molecular evolution of satellite sequences (Helm and Hemleben 1997).

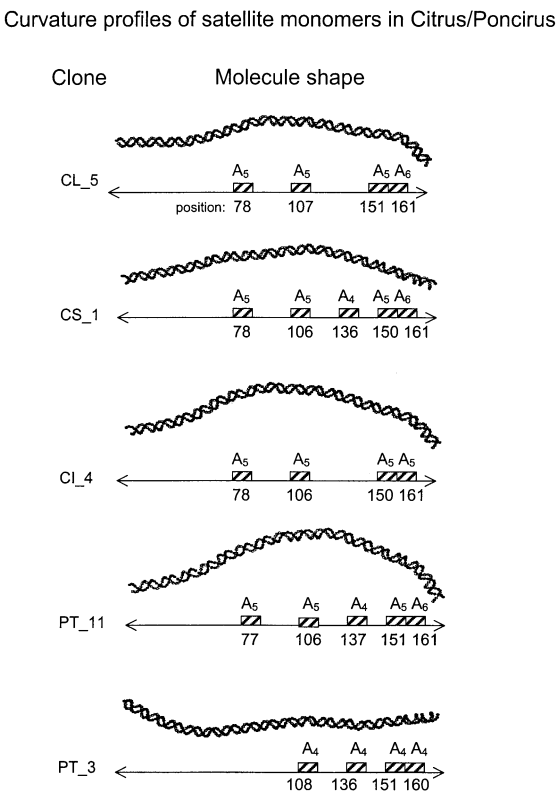

Fig. 3 Profiles of bending and curvature of representative stDNA repeats of citric plants. One complete repeat (1–181 or 180 bp, respectively) is presented: *CL_5 C. limon*, *CS_1 C. sinensis*, *CI_4 C. ichangensis*, *PT_3 Poncirus trifoliata*. *Boxed squares* indicate positions of dA4–6 tracts along individual sequences

Electrophoretic behavior of citric satellite monomers and oligomers

Oligomers of *C. limon* (CL) and *C. sinensis* (CS) stDNA were cloned, and partially digested with *Sty*I, the temperature-dependent changes in mobility were investigated by separating the fragments on 5% non-denaturing polyacrylamide gels at 4° and 60°C, respectively. Plotting the standard data points for the $\log_{10}$ of each fragment size and its electrophoretic mobility always resulted in smooth lines almost irrespective of the changes in temperature conditions. Clearly, both hexamers of the *C. limon* and *C. sinensis* satellites migrated slower than the standard DNA fragments (*Eco*RI/*Hin*dIII-digested lambda DNA and 123 bp-ladder DNA; Table 2). This is in accord with a corresponding analysis of the *C. ichangensis* satellite performed previously by Beridze et al. (1994) suggesting that all citric satellites contain intrinsic curvature.

Contrary to expectation the CL and CS monomeric fragments showed increased migration relative to the standard DNA fragments at 4°C. At 60°C the migration anomaly was lost, and mobility was close to "normal" compared to the DNA size markers, suggesting that the rapid mobility of monomers was caused by an altered conformation of DNA.

Increased mobilities of the monomers parallel findings from electrophoretic studies of other animal G+C-rich satellites for example, e.g. cow, box turtle, Komodo dragon and vulture, and also some non-satellite sequences (Fitzerald et al. 1994). It has been proposed that decreased temperatures might cause stiffness and increased rigidity resulting in a rod-like shape of a G+C-rich molecule (Anderson et al. 1986). In this scenario the effective diameter of the molecule would decrease and its electrophoretic mobility increase. It is likely that the high G+C content of the citric satellites could be a cause for the faster migration behavior found for the monomers. However, with increasing length of the DNA chain the "coiling" effect (possibly induced by a phased dA-tract) becomes highly significant (see the comparison of the curvature paths of monomers and hexamers; Figs. 3 and 4). The coiled double helix (CDH) structure of tandemly repeated units can compensate for the contribution of increased DNA rigidity to the electrophoretic mobility over a long range. Consequently, hexamers and possibly longer chains show retardation, a hallmark of curved molecules.

Computation analysis of *Citrus* and *Poncirus* satellite monomers

For the analysis of local curvature the monomeric units of the respective repeats were analyzed using the CURVATURE program (Shpiegelman et al. 1993).

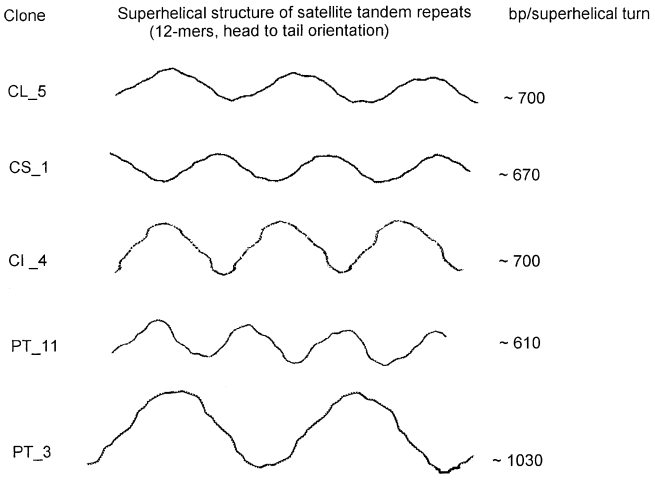

Fig. 4 Curvature profiles of citric satellite oligomers. DNA paths calculated by the CURVATURE program for oligomers containing 12 tandemly arranged units are shown

In Fig. 3 the profiles of curvature for five stDNA repeats are shown. In all repeats the computer analysis of the sequences revealed a stable intrinsic DNA curvature. Curved DNA has been frequently correlated with the presence of multiple dA-tracts in both synthetic (Diekmann 1986) and naturally (Marini et al. 1982) occurring DNA molecules. Indeed, all citric stDNA clones contained several stretches of adenine residues despite the overall high G+C content of these sequences. The two dA-tracts located at the 3′-end appeared to be spaced at one helix turn (10–11 bp). This arrangement is typical of the most curved naturally occurring molecules found in mitochondrial DNA of *Leishmania tarantolae* (kinetoplast DNA; Marini et al. 1982). In accordance with this observation the CURVATURE program revealed a maximum helix axis bending (about 2.0 degrees per dinucleotide step) in the 3′-region of most units. The magnitude of curvature along the whole 180- to 181-bp sequence was highest in the CL_1, CI_4 and PT_11 elements. It might not be coincidental that these highly curved sequences contain multiple tracts with five to six consecutive adenine nucleotides (dA5–6; Fig. 3). In experimental models the maximal curvature was obtained with DNA containing five to six consecutive adenine residues adopting a B′ structure (Koo et al. 1986). The curvature of PT_3 was relatively low, which may be related to the shorter adenine (dA4) tracts in this sequence. In addition, this molecule had a rather convex curvature profile, which may be caused by the absence of an adenine tract at position 78. In general, the distribution of curvature correlated well with conserved dA-motifs in all of the molecules analyzed. The most monomeric units have at least two, in most cases three, dA5-tracts, which is in accordance with known clustering of dA in satellites (Martinez-Balbaz et al. 1990). These characteristic adenine tracts can be also observed in stDNA of *Cucumis* species (Ganal et al. 1986).

Computation analysis of *Citrus* and *Poncirus* satellite oligomers

Beridze et al. (1994) reported the formation of small-diameter circles and thick rod-like particles of the hexamer using electron microscopic analysis. Since DNA curvature has been shown to be responsible for circulatization of oligomeric kinetoplast and synthetic DNA molecules, we were interested in whether intrinsic curvature exhibited by monomeric satellite units potentially influence the formation of higher-order structures in longer oligomeric DNAs. The repetitive arrays of 12 artificially “head-to-tail” ligated monomeric satellite units were analyzed using the CURVATURE program, since this is the most frequently observed genomic organization of satellite repeats. The projection showed that all tandem repeats tend to adopt regularly coiled solenoidal structures (Fig. 4). The periodicity of the superhelical repeat defined by the length of the superhelical turn (bp/turn) ranged from 600 bp for PT_11 to 1000 bp for PT_3. The low value of periodicity (higher degree of supercoiling) in PT_11, CL_5, CI_4 and CS_1 12-mers correlated with the curvature patterns of the monomeric units (see Fig. 3). Thus, it seems that theoretical predictions of a superhelical structure of *Citrus* and *Poncirus* repeats were in a good agreement with electron microscopical observations. Moreover, DNA contours of PT_11, CS, CL and CI tandems had a similar degree of right-handed supercoiling, confirming their evolutionary relationships (see Fig. 2), whereas the evolutionary diverged PT_2/PT_3 tandem repeats adopted a left-handed solenoid with a longer higher-order repeat length (Fig. 4).

The level of superhelix coiling may be an important structural characteristic of satellite repeats. The sequencing of multiple members of a satellite repeat family often reveals a considerable degree of heterogeneity. Computer analysis could help to solve important questions, namely which mutations affect the tertiary structure of DNA, possibly important for chromatin folding (Vogt et al. 1990), and which mutations are possibly selectively neutral, representing merely a mutation noise. Curvature in the monomer means that long arrays of stDNA will form a specific tertiary structure – a coiled double helix (CDH-form; Beridze et al. 1992). It may be that the tertiary structure of stDNAs and the existence of specific stDNA binding proteins are closely interrelated (Levinger and Varshavsky 1982; Fischer et al. 1994). These proteins could fix distant regions of stDNA, resulting in a compact tertiary structure that may form the compact state of constitutive heterochromatin.

DNA curvature and evolution of repeats

In plant genomes there exists a strong trend towards homogenization of repeats (Schmidt and Heslop-Harrison 1998). The same type of repeats are often found at different chromosomes. The mechanism of rapid sequence homogenization is not yet fully clarified and in other or-

ganisms, including mammals, such extensive interlocus homogenization does not seem to occur. In *Poncirus*, we have identified two satellite types: the one (represented by PT_3 clone) being unique for *Poncirus* and forming a clearly separated branch in the evolutionary tree (see Fig. 2) and the second type (represented by PT_11 clone) that did not exhibit a species-specific character. It is likely that these satellites escaped homogenization processes, although we do not yet know whether they occupy distinct chromosome domains. Here, we show that sequence divergence between both satellite groups is accompanied by dramatic changes in the structural properties of DNA reflected by DNA curvature. The search for centers of local curvature revealed that while some dA-tracts retained their position, number and spacing of other dA-motifs were significantly altered. This may suggest that relatively simple reshuffling of dA-tracts and/or increasing/decreasing its numbers could lead to profound changes in the molecule shapes of relatively homologous sequences, especially when these are arranged in long tandems (see oligomer curvature profiles). It is conceivable that alterations in three-dimensional chromatin structure determined by, for example, the angles between individual nucleosomes could influence protein interactions, resulting in altered higher-order structure of heterochromatin (Vogt 1990). These new variants may be either fixed (possibly leading to species-specific restriction satellites) or lost within the species due to recombination events. Hence, the balance between heterochromatin diversity and homogeneity is maintained.

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