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Molecular and cytogenetic characterization of an AT-rich satellite DNA family in *Urvillea chacoensis* Hunz. (Paullinieae, Sapindaceae)

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Abstract *Urvillea chacoensis* is a climber with $2n = 22$ and some terminal AT-rich heterochromatin blocks that differentiate it from other species of the genus. The AT-rich highly repeated satellite DNA was isolated from *U. chacoensis* by the digestion of total nuclear DNA with *Hin*dIII and *Xba*I and cloned in *Escherichia coli*. Satellite DNA structure and chromosomal distribution were investigated. DNA sequencing revealed that the repeat length of satDNA ranges between 721 and 728 bp, the percentage of AT-base pairs was about 72–73% and the studied clones showed an identity of 92.5–95.9%. Although this monomer has a tetranucleosomal size, direct imperfect repetitions of ~180 bp subdividing it in four nucleosomal subregions were observed. The results obtained with FISH indicate that this monomer usually appears distributed in the terminal regions of most chromosomes and is associated to heterochromatin blocks observed after DAPI staining. These observations are discussed in relation to the satellite DNA evolution and compared with other features observed in several plant groups.

Keywords DAPI · FISH · Heterochromatin · Karyotype · Satellite DNA · *Urvillea*

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Introduction

Urvillea Kunth (Paullinieae, Sapindaceae) comprises 17 species and is widely distributed in the neotropical regions of America, from southern United States to northern Argentina (Ferrucci 2006). *Urvillea* has two basic chromosome numbers, $x = 12$ (*Stenelytron*), with a single diploid level, and $x = 11$ in section *Urvillea*, with diploid, tetraploid, and octaploid levels (Lombello and Forni-Martins 1998; Ferrucci 2000; Urdampilleta et al. 2006). *Urvillea chacoensis* Hunz. is a climber that occurs in "chaqueña" regions from Argentina, Brazil and Paraguay. As the other species of the *Urvillea* section, its diploid number is $2n = 22$ (Ferrucci 2000; Urdampilleta et al. 2006), but it is cytogenetically differentiated by the presence of large terminal AT-rich heterochromatin blocks and three chromosome pairs bearing NOR (Urdampilleta et al. 2006).

Highly repetitive DNA appears to be one of the main components of plant genomes, and changes in these sequences may be responsible for the variations in genome size and karyotypical features (Flavell 1986; San Miguel and Bennetzen 1998). Different types of repetitive DNA exist within each genome, and the satellite DNA (satDNA), which is formed by tandemly arranged monomers of tens to thousands base pairs (Charlesworth et al. 1994), often constitutes heterochromatin blocks up to 100 Mb.

Additionally, the whole heterochromatin of a genome can be composed of different families of satellite DNA (Kurbis et al. 1998; Sharma and Raina 2005), which can also be located or accumulated in specific chromosomal positions (Flavell 1986). Some new sequences of satDNA have recently been reported and a database was created to understand the evolution and distribution of satDNA in plant genomes (Macas et al. 2002). Since satDNA can be species-specific or typical to some groups of species (Schmidt et al. 1991; Svitashev et al. 1994), it represents a useful tool in the field of taxonomy.

Different techniques can be used to isolate satellite DNA, as centrifugation in density gradients (Deumling 1981), screening of fractions referred to as low Cot DNA (Neumann et al. 2001; Ho and Leung 2002), purification and cloning of band fragments obtained by gel electrophoresis of enzyme-digested genomic DNA (Kato et al. 1984; Ganal and Hemleben 1986; Lakshmikumaran and Ranade 1990; Lorite et al. 2001) and genomic self-priming PCR (GSP-PCR) (Buntjer and Lenstra 1998; Macas et al. 2000). Since we intended to characterize and localize a satellite DNA family of *U. chacoensis* in situ, we isolated and characterized satDNA sequences by purifying and cloning fragments of enzyme-digested genomic DNA. To do so, this marker was FISH mapped and the results were matched with the distribution of 45S rDNA loci and the heterochromatin pattern obtained with DAPI.

Materials and methods

Plant material

Seeds of *U. chacoensis* obtained from the voucher specimen *U. chacoensis*: Bolivia. Dpto. Chuquisaca. Prov. Calvo, 22 km N de Tarairí camino a Boyuibe, 18VII 2003, Ferrucci et al. N° 1763, deposited at the herbarium of the Instituto de Botánica del Nordeste (CTES), Argentina, were collected and cultivated in pots under greenhouse conditions.

Isolation of genomic DNA, cloning and sequencing of repetitive DNA fragments

Leaf genomic DNA was isolated according to the method described by Hoisington et al. (1994) and digested with *Hin*dIII and *Xba*I. The selected bands of about 750 bp were eluted and purified from the agarose gel. The fragments of repetitive DNA were cloned using *Escherichia coli* DH5α as host and pBluescript KS plus (Stratagene) as vector. Colonies containing recombinant plasmids were identified for selection on LB agar medium supplemented with X-gal and IPTG. Recombinant plasmid was isolated using alkaline minipreparation procedure and the insert nucleotide sequences were determined with an ABI377 automated DNA sequencer (Applied Biosystems). Sequences were analysed with Lasergene 7 (DNAStar, Madison, WI, USA) and aligned by using the ClustalW option of the MegAlign program.

Preparation of mitotic chromosomes

Root tips were pretreated with 2 mM 8-hydroxyquinolin for 4–5 h at 15°C, fixed in ethanol–acetic acid (3:1, v:v) and digested at 37°C in a solution composed of 2% cellulase and 20% pectinase. After squashing the meristems in a drop of 45% acetic acid, the preparations were frozen in liquid nitrogen and the coverslips were removed. To identify AT-rich heterochromatin blocks, slides were stained with 2 mg ml^{-1} DAPI for 30 min and mounted with glycerol/McIlvaine buffer pH 7.0, 1:1 (v:v), plus 2.5 mM $MgCl_2$.

Fluorescent in situ hybridization

To compare the distribution of satDNA sequences in relation to genes of rDNA and to DAPI heterochromatin pattern, we used probes marked with nick translation. Recombinant plasmids isolated by alkaline minipreparation containing 18S-5.8S-26S rDNA of wheat (p*Ta*71) (Gerlach and Bedbrook 1979) was labeled with biotin-14-dUTP (Bionick, Invitrogen) and the cloned fragments of satDNA were labeled with DIG (DIG Nick translation mix, Roche). In situ hybridization followed the protocols of Heslop-Harrison et al. (1991) and Schwarzacher and Heslop-Harrison (2000). Slides were incubated in 100 μg ml^{-1} RNAse, post-fixed in 4% (w/v) paraformaldehyde, dehydrated in a 70–100% graded ethanol series and air-dried. Later, 30 μl of the hybridization mixture (4–6 ng μl^{-1} of probe, 50% (v/v) formamide, 10% (w/v) dextran sulfate, 3.3 ng μl^{-1} of calf thymus DNA, 2× SSC and 0.3% (w/v) SDS), previously denatured at 70°C for 10 min, were applied. Samples were denatured/hybridized at 90°C for 10 min, 48°C for 10 min and 38°C for 5 min, using a thermal cycler (Mastercycler, Eppendorf) and the slides were kept overnight in a humid chamber at 37°C. Hybridization signals were detected with avidin-FITC (Sigma) for p*Ta*71 and anti-DIG-Rhodamine (Roche) for satDNA. The slides were counterstained with DAPI and mounted with 25 μl of VectaShield (Vector Laboratories). Photomicrographies were obtained with a BX51 Olympus coupled to an Evolution MT CCD photosystem and Image ProPlus v6 software was used to capture the images.

Results

Isolation of *Uch*725 satellite DNA

The genomic DNA of *U. chacoensis* digested with *Hin*dIII and *Xba*I showed clear electrophoretic bands at ~750 and 1,500 bp (Fig. 1), indicating the presence of tandemly arranged repetitive elements in the genome. Such band pattern suggested the presence of satDNA with a site for *Hin*dIII and another one for *Xba*I in the same repetitive units. The 750 bp repetitive DNA fragments cloned and sequenced represent monomers of satDNA with ~725 bp, since they ranged between 721 and 728 bp in all the studied clones. The p*Uch*1, p*Uch*6 and p*Uch*8 clones contained the monomer element digested with *Hin*dIII, and the p*Uch*11, p*Uch*13 and p*Uch*15 clones contained the monomer element digested with *Xba*I. Figure 2 shows the aligned nucleotide sequences of the studied clones and a sequence analysis revealed some differential characteristics of these sequences. The satDNA family was thus named *Uch*725. This repetition unit possesses a restriction site for *Hin*dIII and another one for *Xba*I, separated by 53–54 bp, which allows to obtain fragments of equal size after the digestion of the genomic DNA. The analyzed sequences were highly AT-rich, with 72.1–73.2% of A or T, and an identity of 92.5–95.9% was observed in the studied clones.

Although no significant inverted repetition was detected within the repetition units, we observed direct imperfect repetitions of ~180 bp. These subrepetitions allow to divide the monomer in four sub-regions (Fig. 2). The alignment of these sub-regions (Fig. 3a) showed an identity of about 70–80% (Fig. 3b).

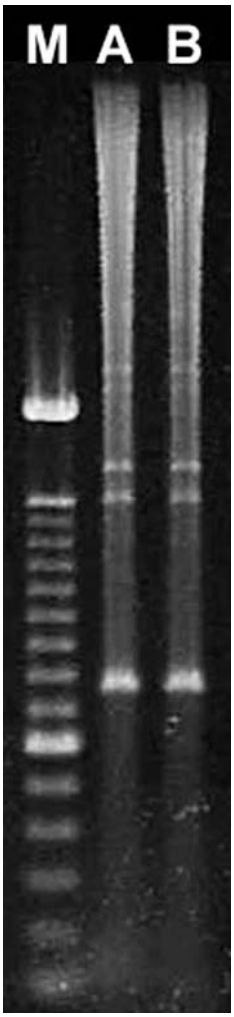


Fig. 1 Restriction enzyme analysis of total genomic DNA of *U. chacoensis*. Genomic nuclear DNA digested with *Hin*dIII (A) and *Xba*I (B) and restriction fragments resolved on 1% agarose gel. M, 100 bp leader

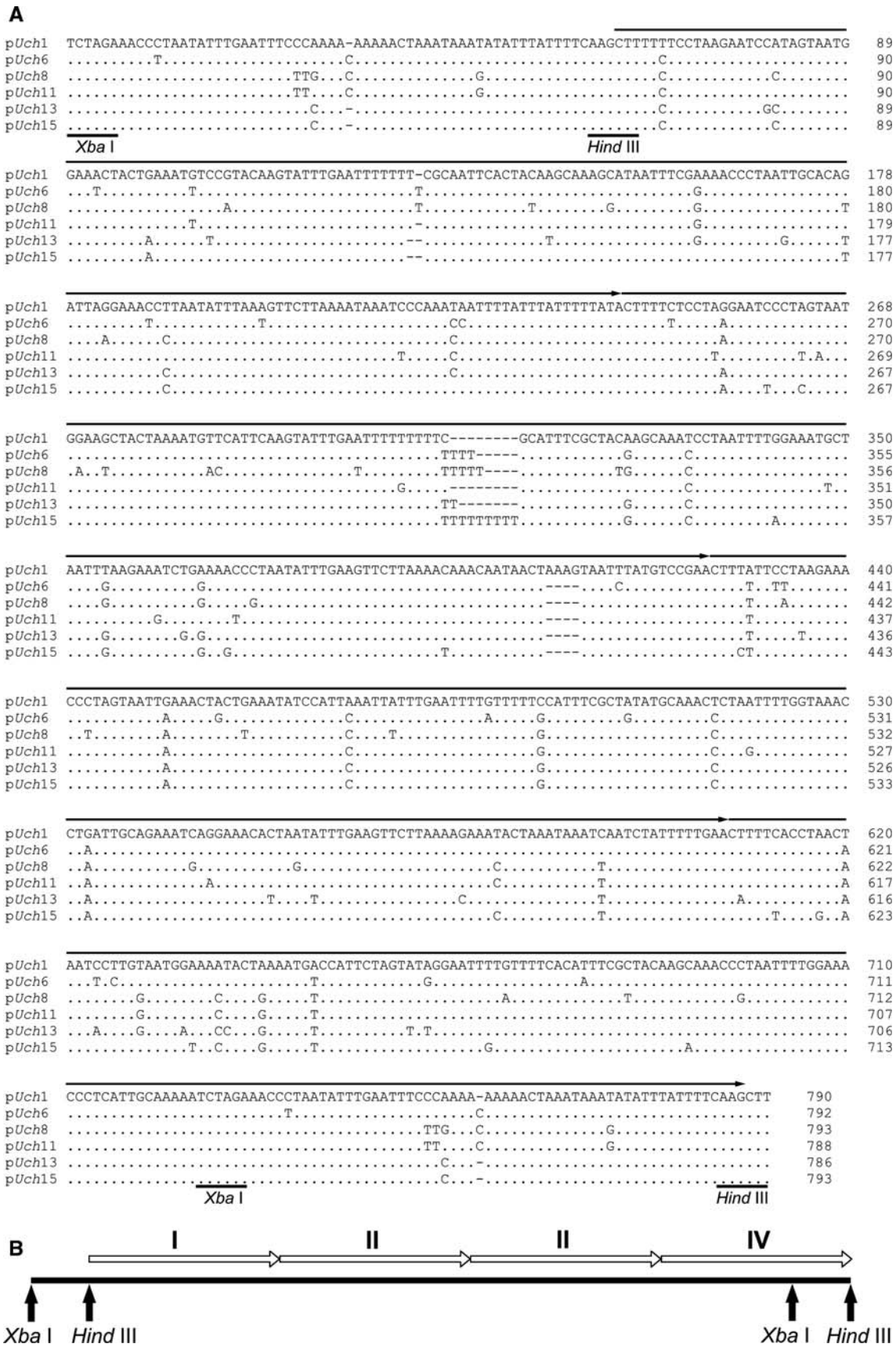
Karyotypical features in *U. chacoensis*

Urvillea chacoensis, with $2n = 22$ chromosomes, which are mostly metacentrics, showed several large terminal heterochromatic blocks after DAPI staining (Fig. 4a). The six longest chromosome pairs showed heterochromatin blocks in both terminal regions, while the smallest chromosomes showed small bands at their tips or no band at all. This banding pattern indicates the presence of a high proportion of AT-rich heterochromatin, usually located in the subtelomeric regions. FISH using the p*Ta*71 probe (18-5.8-26S rDNA) located terminal signals in the short arms of three chromosome pairs (five sites were observed in Fig. 4b, d). $DAPI^+$ bands were not observed in the short arms in NOR carrier chromosomes.

The DAPI banding pattern coincided with the hybridization sites of the p*Uch*6 probe (Fig. 4). The hybridization signals with the p*Uch*6 probe were detected in the two terminal regions of the six largest chromosome pairs. Two chromosome pairs exhibited p*Uch*6 signals in the terminal region of the long arm, and of three small chromosome pairs, only one showed reliable hybridization signals in both terminal regions. Some chromosomes presented hybridization signals with both the p*Uch*6 and p*Ta*71 probes, but p*Ta*71 hybridized at terminal regions of short arms, while p*Uch*6 hybridized at long arms (Fig. 4b–d).

Discussion

The results obtained with DAPI banding and in situ hybridization with the p*Ta*71 probe coincide with the observations by Urdampilleta et al. (2006) for *U. chacoensis*. In addition, in our study, the coincident DAPI banding pattern and FISH with satDNA probes suggest that this satellite DNA represents a structural component of the heterochromatin blocks within the *U. chacoensis* genome. Our results confirmed the presence of a high proportion of subterminal AT-rich heterochromatin with an equilocal distribution.


A
pUch1 TCTAGAAACCCTAATATTTGAATTTCCCAAAA-AAAAACTAAATAAATATATTTATTTTCAAGCTTTTTTCCTAAGAATCCATAGTAATG 89
pUch6 .....T.....C.....C..... 90
pUch8 .....TTG...C.....G.....C.....C..... 90
pUch11 .....TT....C.....G.....C..... 90
pUch13 .....C...-.....C.....GC..... 89
pUch15 .....C...-.....C.....C..... 89
Xba I
Hind III
pUch1 GAAACTACTGAAATGTCCGTACAAGTATTTGAATTTTTTT-CGCAATTCACTACAAGCAAAGCATAATTTCGAAAACCCTAATTGCACAG 178
pUch6 ...T.....T.....T.....G..... 180
pUch8 .....A.....T.....T.....T.....G.....G.....T 180
pUch11 .....T.....-.....G..... 179
pUch13 .....A.....T.....--.....T.....G.....G.....T 177
pUch15 .....A.....--.....T 177
pUch1 ATTAGGAAACCTTAATATTTAAAGTTCTTAAAATAAATCCCAAATAATTTTATTTATTTTTATACTTTTCTCCTAGGAATCCCTAGTAAT 268
pUch6 .....T.....T.....CC.....T.....A..... 270
pUch8 ....A.....C.....C.....A..... 270
pUch11 .....T.....C.....T.....T.A... 269
pUch13 .....C.....C.....A..... 267
pUch15 .....C.....A....T...C..... 267
pUch1 GGAAGCTACTAAAATGTTCATTCAAGTATTTGAATTTTTTTTTC--------GCATTTCGCTACAAGCAAATCCTAATTTTGGAAATGCT 350
pUch6 .....TTTT-----.....G.....C..... 355
pUch8 .A..T.....AC.....T.....TTTTT----.....TG.....C..... 356
pUch11 .....G.....--------.....C.....T.. 351
pUch13 .....TT-------.....G.....C..... 350
pUch15 .....TTTTTTTTT.....G.....C.....A..... 357
pUch1 AATTTAAGAAATCTGAAAACCCTAATATTTGAAGTTCTTAAAACAAACAATAACTAAAGTAATTTATGTCCGAACTTTATTCCTAAGAAA 440
pUch6 ....G.....G.....----....C.....T..TT..... 441
pUch8 ....G.....G.....G.....----.....T...A..... 442
pUch11 .....G.....T.....----.....T..... 437
pUch13 ....G.....G.G.....----.....T.....T..... 436
pUch15 ....G.....G..G.....T.....----.....CT..... 443
pUch1 CCCTAGTAATTGAAACTACTGAAATATCCATTAAATTATTTGAATTTTGTTTTTCCATTTCGCTATATGCAAACTCTAATTTTGGTAAAC 530
pUch6 .....A.....G.....C.....A.....G.....G.....C..... 531
pUch8 ..T.....A.....T.....C....T.....G.....C..... 532
pUch11 .....A.....C.....G.....C...G..... 527
pUch13 .....A.....C.....G.....C..... 526
pUch15 .....A.....C.....G.....C..... 533
pUch1 CTGATTGCAGAAATCAGGAAACACTAATATTTGAAGTTCTTAAAAGAAATACTAAATAAATCAATCTATTTTTGAACTTTTCACCTAACT 620
pUch6 ..A.....A 621
pUch8 ..A.....G.....G.....C.....T.....A 622
pUch11 ..A.....A.....C.....T.....A 617
pUch13 ..A.....T....T.....C.....T.....A.....A 616
pUch15 ..A.....C.....T.....T....G..A 623
pUch1 AATCCTTGTAATGGAAAATACTAAAATGACCATTCTAGTATAGGAATTTTGTTTTCACATTTCGCTACAAGCAAACCCTAATTTTGGAAA 710
pUch6 ...T.C.....T.....G.....A..... 711
pUch8 .....G.....C....G.....T.....A.....T.....G..... 712
pUch11 .....G.....C....G.....T..... 707
pUch13 ...A....G....A...CC...G.....T.....T.T..... 706
pUch15 .....T..C....G.....T.....G.....A..... 713
pUch1 CCCTCATTGCAAAAATCTAGAAACCCTAATATTTGAATTTCCCAAAA-AAAAACTAAATAAATATATTTATTTTCAAGCTT 790
pUch6 .....T.....C..... 792
pUch8 .....TTG...C.....G..... 793
pUch11 .....TT....C.....G..... 788
pUch13 .....C...-..... 786
pUch15 .....C...-..... 793
Xba I
Hind III
B
I
II
II
IV
Xba I
Hind III
Xba I
Hind III

◀ **Fig. 2** Sequence analysis of the studied clones. **a** Alignment of the six clones, p*Uch*1, p*Uch*6, p*Uch*8, p*Uch*11, p*Uch*13 and p*Uch*15. Imperfect direct repeats are indicated by *arrows*. **b** satDNA monomer scheme showing subunit with *arrows*

According to Urdampilleta et al. (2006), some karyotypical features in *U. chacoensis* are important and allow to distinguish this species from other species of the *Urvillea* genus (Urdampilleta et al. 2006). *Urvillea laevis* and *U. filipes*, for example, do not present C-bands, but these authors reported small, scarce $DAPI^+$ sites in *U. laevis*. On the other hand, *U. ulmacea* showed abundant terminal C-bands constituted by CMA_3^+, and $DAPI^+$ bands in several chromosomes. GC-rich terminal regions (CMA_3^+) may be associated to NORs in *U. chacoensis*, but not in *U. ulmacea* (Urdampilleta et al. 2006). These results also confirm that the heterochromatin is not homogeneous and can vary both qualitatively and quantitatively among the

Fig. 3 Alignment of all four subrepetitions of p*Uch*6 (**a**) and percent identity of sequences of each sub-region (**b**)

A

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pUch6 I   CTTTTCTCCTAAGAATCCATAGTAATGGAATCTACTGAAATTTCCGTACAAGTATTTGAATT 62
pUch6 II  .....T............C...........G.....A....G.T.A.T.............. 62
pUch6 III .....T.TT......A..C.......TA..A..G.......A...A.T...T.......... 62
pUch6 IV  ......A.....C..ATTCCT.........AA....A....G...A.T.T.....GG..... 62

pUch6 I   TT-TTTT---CGCAATTCACTACAAGCAAAGCATAATTTCGGAAACCCTAATTGCACAGATT 120
pUch6 II  ..T....TTTT...T...G....G......C.C......T.....TG.......A.G.A..C 124
pUch6 III ..A....---T...T...G..GT.T.....C.C......T..T..A..........G.A..C 121
pUch6 IV  ..G....---.A......G...........C.C......T.........C......A.A..C 121

pUch6 I   AGGAAATCTTAATATTTAATGTTCTTAAAATAAATCCCAAACCATTTTATTTATTTTTATA  181
pUch6 II  T.....C.C........G.A..........C...CA----.TA.C..A..C...G.CCGA.  181
pUch6 III ......CAC........G.A..........G....A.T...TA.A.CA..C.......GA.  182
pUch6 IV  TA....C..........G.AT...CC....C...AA.T...TA.A.A..........C.AG  182
```

B

	I	II	III	IV
I	***	70.2	75.1	76.2
II		***	79.6	76.2
III			***	74.7
IV				***

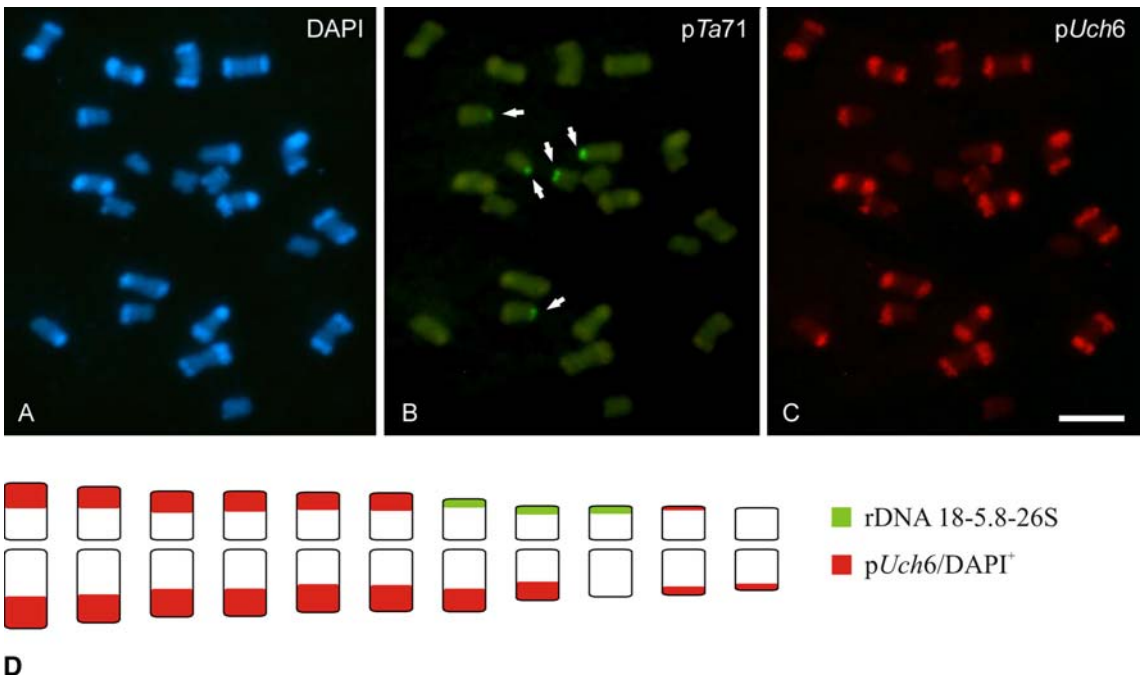

Fig. 4 Chromosome banding and fluorescence in situ hybridization in metaphasic chromosome of *U. chacoensis*. **a** AT-rich heterochromatin terminal blocks observed with DAPI staining; **b** localization of rDNA 18-5.8-26S using p*Ta*71 probe; **c** terminal distribution of *Uch*725 family observed for FISH with p*Uch*6 probe; and **d** idiograms by indicating the localization of rDNA and satDNA. *Bar* = 5 μm

species, within a same group (Guerra 2000), valuing these features as taxonomic markers.

The occurrence of AT-rich terminal repeated sequences is a frequent characteristic in plant genomes (Flavell 1986; Kurbis et al. 1998; San Miguel and Bennetzen 1998). The sequence analysis of plant genomes also emphasizes that ~60% of the satDNA families studied are AT-rich sequences (Macas et al. 2002) and the comparison of banding patterns in different plant groups suggests that the proportion of species with AT-rich heterochromatin is even higher (Guerra 2000). These data thus suggest that the distribution and chemical composition of these sequences are not casual and might be related to still unknown functions of the genome structure.

In plants, the size of the satellite DNA usually varies between 135–195 and 315–375 bp, corresponding to the length of mono and dinucleosomes (Macas et al. 2002; Sharma and Raina 2005). However, some plants have satellite DNA with monomers longer that 600 bp. *Secale cereale*, for example, presents satDNA with units of repetition of 3,900 bp (Langdon et al. 2000). In *U. chacoensis*, monomers of the *Uch*725 family show a size similar to that of the satDNA found in *Aegilops speltoides* (Anamthawat-Jonsson and Heslop-Harrison 1993), *Allium fistulosum* (Seo et al. 2007), *Rumex acetosa* (Shibata et al. 2000), *Sinapis arvensis* (Kapila et al. 1996). Nevertheless, no homology was found in the GenBank and EMBL databases for *Uch*725, indicating that these sequence may be typical of *Urvillea* group.

Heterochromatin was first identified by Heitz (1928) as a cytological event to describe condensed regions in the interphase. Although these structures have long been considered to be DNA without any function, different functions have been attributed to them these last decades (Yunis and Jasmineh 1971; Sýkorová et al. 2001), which guarantees their maintenance. Both in mammalian and plant cells, bulk chromatin presents a nucleosome periodicity of 180 ± 5 bp (Fajkus et al. 1995; Vershinin and Heslop-Harrison 1998) and the satDNA monomers often correspond to mono or dinucleosomes (135 and 195 or 315 and 375 bp) (Macas et al. 2000; Sharma and Raina 2005). Therefore, the satDNA could provide structural genetic codes for the chromatin packing (Trifonov 1989). Sýkorová et al. (2001) found that satDNA sequences favor the transition between telomeric domains and internal chromosomal regions, acting directly in the telomeric stabilization and regulation of genes from subterminal regions. As in *U. chacoensis*, various families of repetitive DNA occur in the subterminal regions of plants and constitute the so called telomere-associated sequences or TASs (Sharma and Raina 2005). In *U. chacoensis*, the presence of four subrepetitions of ~180 bp (tetranucleosome) within the satDNA monomer suggests that this substructure may be directly related to the establishment and packing of chromatin in terminal regions.

The phylogeny within Sapindaceae is not well resolved, since the tribal classification of many genera is confuse (Harrington et al. 2005). Studies on the presence and distribution of satellite DNA can contribute to understand the evolutionary aspects of the genome and thus to establish the taxonomy of some groups (Schmidt et al. 1991; Svitashev et al. 1994). The p*Uch*6 probe, isolated from *U. chacoensis*, has being previously tested in other species of *Urvillea* and genera of Sapindaceae, tribe Paullinieae. For the time being no signals were displayed after of hybridization with p*Uch*6 probe in *Cardiospermum grandiflorum* Sw., *Paullinia elegans* Cambess. and *U. ulmacea* Kunth. However by mean of PCR and by using primers designed from p*Uch*6 were detected some products possibly related to *Uch*725 (Urdampilleta et al. in preparation). As the genomic studies on this family are scarce, the isolation of *Uch725* satellite DNA in *U. chacoensis* offers an important chromosomal marker, whose presence and distribution in related species and genera might contribute to the systematic of Paullinieae.

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