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Characterization of RUSI, a telomere-associated satellite DNA, in the genus *Rumex* (Polygonaceae)

R. Navajas-Pérez[a] T. Schwarzacher[b] M. Ruiz Rejón[c] M.A. Garrido-Ramos[c]

[a]Plant Genome Mapping Laboratory, University of Georgia, Athens, GA (USA); [b]Department of Biology, University of Leicester, Leicester (UK); [c]Departamento de Genética, Facultad de Ciencias, Universidad de Granada, Granada (Spain)

Abstract

A satellite-DNA family (RUSI) has been isolated and characterized in *Rumex induratus* Boiss and Reuter (Polygonaceae), an Iberian endemic polygamous sorrel. The RUSI repeats are 170 bp in length and ~68% AT-rich containing different variants of degenerate telomere motifs – $(TT)_nAN(GG)_n$ –, a typical feature of subtelomeric DNA repeats adjacent to telomeres, which have been referred to as telomere-associated sequences or TASs. In fact, fluorescent in situ hybridization showed that this satellite DNA is located in subtelomeric positions of most of the chromosomes of *R. induratus*, with some centromeric loci. PCR and Southern-blot hybridization assays for sequence conservation in the genus *Rumex*, indicated that the RUSI sequences are restricted to the genomes of *R. induratus* and *R. scutatus*, both species of the section *Scutati*, suggesting that they are recently evolved. Sequence variation within the two species is high (mean value of sequence differences between repeats of 15% for *R. induratus* and 7.5% for *R. scutatus*) and the degree of sequence differentiation between species is low with no species-specific variants, postulated to be due to slowed rates of spreading of sequence variants by molecular homogenizing mechanisms. Characteristics of RUSI sequences are discussed in the light of their chromosomal location and analyzed for their evolutionary and phylogenetic implications.

Rumex (Polygonaceae) is a plant genus containing dioecious, gynodioecious, polygamous and hermaphroditic species (Rechinger, 1964). Many scientific programs have focused on this genus because of its biological and evolutionary significance in sexual dimorphism. Within the genus *Rumex* three phylogenetic clades can be defined (Navajas-Pérez et al., 2005a; Fig. 1). The basal clade is composed of hermaphroditic docks, while the second comprises polygamous/gynodioecious sorrels and the third dioecious sorrels except for one hermaphrodite, this latter clade containing species with different sex-determination systems (XX/XY and XX/XY_1Y_2). Polygamous and gynodioecious sorrels have important evolutionary significance because they are intermediate forms on the way toward the evolution of separate sexes (Navajas-Pérez et al., 2005a) and could help unravel the origin of dioecy and sex chromosomes in *Rumex* species. Furthermore, these species have applied interests in medicine (Rivera and Obón, 1995), as a dietary source of potential bioactive compounds (Ferreres et al., 2006) or have been proposed as candidates for Hg phytoremedia-

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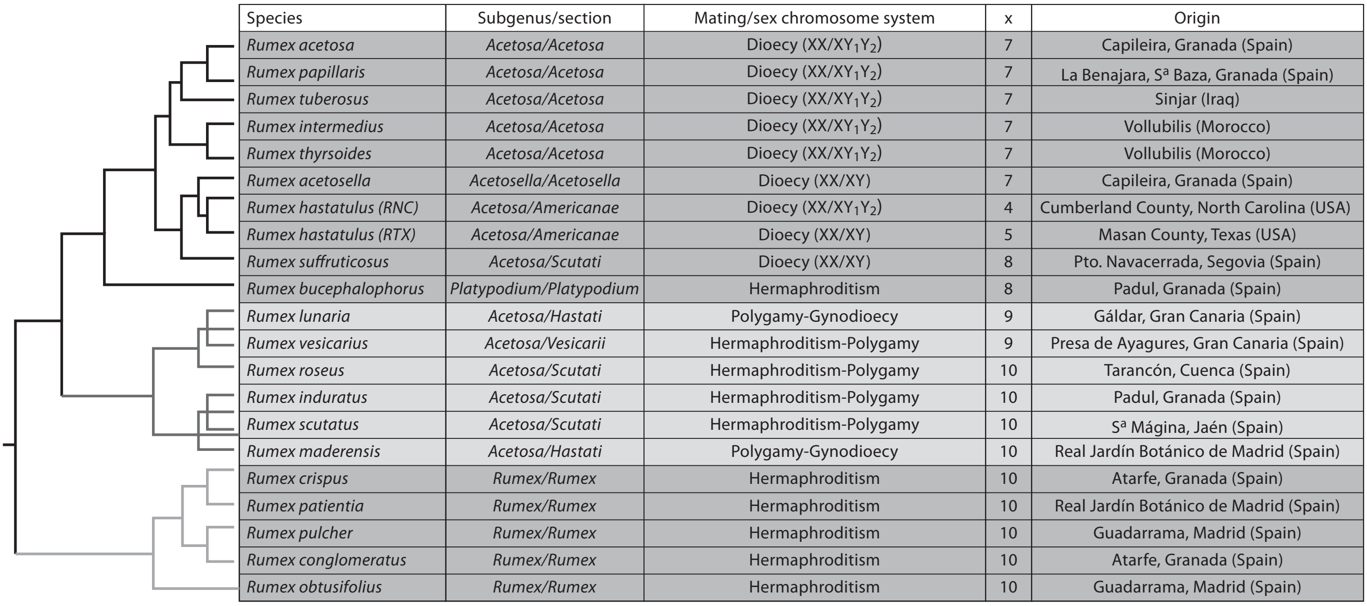
Rafael Navajas-Pérez
Current address: Departamento de Genética, Facultad de Ciencias
Universidad de Granada, Granada (Spain)
telephone: +34 958 24 30 80; fax: +34 958 24 40 73; e–mail: rnavajas@ugr.es



Species	Subgenus/section	Mating/sex chromosome system	x	Origin
Rumex acetosa	*Acetosa/Acetosa*	Dioecy (XX/XY$_1$Y$_2$)	7	Capileira, Granada (Spain)
Rumex papillaris	*Acetosa/Acetosa*	Dioecy (XX/XY$_1$Y$_2$)	7	La Benajara, Sª Baza, Granada (Spain)
Rumex tuberosus	*Acetosa/Acetosa*	Dioecy (XX/XY$_1$Y$_2$)	7	Sinjar (Iraq)
Rumex intermedius	*Acetosa/Acetosa*	Dioecy (XX/XY$_1$Y$_2$)	7	Vollubilis (Morocco)
Rumex thyrsoides	*Acetosa/Acetosa*	Dioecy (XX/XY$_1$Y$_2$)	7	Vollubilis (Morocco)
Rumex acetosella	*Acetosella/Acetosella*	Dioecy (XX/XY)	7	Capileira, Granada (Spain)
Rumex hastatulus (RNC)	*Acetosa/Americanae*	Dioecy (XX/XY$_1$Y$_2$)	4	Cumberland County, North Carolina (USA)
Rumex hastatulus (RTX)	*Acetosa/Americanae*	Dioecy (XX/XY)	5	Masan County, Texas (USA)
Rumex suffruticosus	*Acetosa/Scutati*	Dioecy (XX/XY)	8	Pto. Navacerrada, Segovia (Spain)
Rumex bucephalophorus	*Platypodium/Platypodium*	Hermaphroditism	8	Padul, Granada (Spain)
Rumex lunaria	*Acetosa/Hastati*	Polygamy-Gynodioecy	9	Gáldar, Gran Canaria (Spain)
Rumex vesicarius	*Acetosa/Vesicarii*	Hermaphroditism-Polygamy	9	Presa de Ayagures, Gran Canaria (Spain)
Rumex roseus	*Acetosa/Scutati*	Hermaphroditism-Polygamy	10	Tarancón, Cuenca (Spain)
Rumex induratus	*Acetosa/Scutati*	Hermaphroditism-Polygamy	10	Padul, Granada (Spain)
Rumex scutatus	*Acetosa/Scutati*	Hermaphroditism-Polygamy	10	Sª Mágina, Jaén (Spain)
Rumex maderensis	*Acetosa/Hastati*	Polygamy-Gynodioecy	10	Real Jardín Botánico de Madrid (Spain)
Rumex crispus	*Rumex/Rumex*	Hermaphroditism	10	Atarfe, Granada (Spain)
Rumex patientia	*Rumex/Rumex*	Hermaphroditism	10	Real Jardín Botánico de Madrid (Spain)
Rumex pulcher	*Rumex/Rumex*	Hermaphroditism	10	Guadarrama, Madrid (Spain)
Rumex conglomeratus	*Rumex/Rumex*	Hermaphroditism	10	Atarfe, Granada (Spain)
Rumex obtusifolius	*Rumex/Rumex*	Hermaphroditism	10	Guadarrama, Madrid (Spain)

Fig. 1. List *Rumex* species analyzed in this paper, indicating their affiliations, mating/sex chromosome system, basic chromosome number (x), and their phylogenetic relationship (left – based on Navajas-Pérez et al., 2005a).

tion of contaminated soils (Moreno-Jiménez et al., 2006). In this context, we are interested here in the cytogenetic and molecular analysis of *Rumex induratus* Boiss and Reuter (Polygonaceae), an Iberian endemic polygamous sorrel. Ultimately, we aim to understand the phylogenetic aspects of sequence and chromosomal evolution in *Rumex* using DNA markers to study the processes involved in karyotype and sex-chromosome evolution. In particular, satellite-DNA and other repetitive-DNA sequences have proved to be powerful markers for these types of evolutionary studies (Heslop-Harrison, 2000; Navajas-Pérez et al., 2005b, 2006; Mariotti et al., 2006; Cuñado et al., 2007).

Satellite-DNA sequences are non-coding highly repetitive tandem arrayed sequences originally so named because they form shoulders – satellites – in density buoyant gradients if their AT versus GC content is significantly different from the bulk DNA, but later extended to all highly repeated tandemly organized sequences (Beridze, 1986). Together with retrotransposon-like dispersed sequences, they represent most of the DNA content in plants (Heslop-Harrison, 2000). Satellite-DNA sequences are located at heterochromatin which is found mostly in centromeric and subtelomeric regions in the chromosomes, but also at intercalary positions (reviewed in Sharma and Raina, 2005). Within satellite-DNA families, subtelomeric satellites are repetitive sequences adjacent to telomeres that have been referred to as telomere-associated sequences or TASs (Louis and Vershinin, 2005) which in addition to location have several specific characteristics such as the presence of telomere degenerated motifs within the repeat sequence and the ineffectiveness of sequence homogenization events (Contento et al., 2005).

In the present study, we analyze a new satellite-DNA family isolated from *R. induratus*, RUSI, comprised of 170-bp repeats. RUSI sequences are tandemly located in subtelomeric positions of most of the chromosomes of the species, with centromeric sites in some of them, and have main features to be regarded as TASs.

Materials and methods

Seeds and leaves of *R. induratus* were collected from a natural population in Padul, Granada (Spain). Seeds were kept in a cold and dry place until germination and leaves sampled from up to ten individuals were stored at –80°C for further analysis. DNA isolation was performed using the Plant DNAzol kit (Invitrogen) following the manufacturer's recommendations. DNA from the following species was isolated previously and includes *R. acetosa*, *R. intermedius*, *R. thyrsoides*, *R. tuberosus*, *R. suffruticosus*, *R. acetosella*, *R. hastatulus* (Texas race and North Carolina race), *R. bu-*

cephalophorus, *R. scutatus*, *R. lunaria*, *R. maderensis*, *R. roseus*, *R. vesicarius*, *R. conglomeratus*, *R. crispus*, *R. patientia*, *R. obtusifolius* and *R. pulcher*, collected from different sources (for systematics and locations, see Fig. 1 and Navajas-Pérez et al., 2005a).

RUSI satellite-DNA was isolated after restriction analysis from *R. induratus* total genomic DNA using *Bgl*I restriction endonuclease and electrophoresis in agarose gel with ethidium bromide. The most prominent bands visible under UV light were excised from the gel and purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences). Purified *Bgl*I fragments were then ligated to the *Bam*HI compatible ends of the pUC18 vector and cloned in competent DH5-α cells (Gibco BRL). Minipreparations were made using Perfectprep® Plasmid Mini (Eppendorf) and recombinant plasmids carrying the monomeric sequences were identified after screening with the purified band by dot-blot hybridization.

For Southern-blot (all the species listed in the first paragraph of this section were checked for the presence of RUSI sequences by the Southern-blot hybridization technique) and dot-blot hybridization and fluorescent in situ hybridization (FISH), a plasmid insert of clone RUSI_19 was used as a probe. Southern-blot and dot-blot hybridizations were carried out following Garrido-Ramos et al. (1999). For FISH, chromosome preparations were made following Schwarzacher and Heslop-Harrison (2000). Probe was labeled with biotin-dUTP by random priming according to the specifications of the Random Primer Labelling System (Invitrogen). Labeled probe (25–50 ng) was added to the hybridization mixture (50% formamide, 2× SSC, 20% dextran sulphate, 0.125% SDS and 0.125 mM EDTA) (see Schwarzacher and Heslop-Harrison, 2000). Combined denaturation of the probe and chromosomal DNA was performed at 80°C for 8 min using a Thermo-Hybaid HyPro-20 and re-annealed at 37°C overnight. Stringent washes (20% formamide and 0.1× SSC at 42°C) were made prior to detection. Probe was detected with Alexa594/streptavidin (Molecular Probes, 0.5 ng/ml) in 5% (w/v) Bovine Serum Albumin (BSA) in 4× SSC, 0.2% Tween 20 following Schwarzacher and Heslop-Harrison (2000). Preparations were counterstained with 4,6-diamidino-2-phenylindole (DAPI, 2 μg/ml) and mounted in antifade solution. Preparations were analyzed with a Zeiss Axioplan 2 epifluorescence microscope (Oberkochen, Germany) with suitable filters and photographed with a CCD camera (Optronics, model s97790). Colour figures and overlays were prepared with Adobe Photoshop 7.0 software, using only those processing functions that are applied to all pixels of the image. Karyotyping was performed according to a classical orcein staining method for 4–5 h followed by mounting preparations in 45% acetic acid.

For amplification of RUSI sequences in *R. scutatus*, we designed the pair of specific primers RUSI-A, 5′-CGAGTTTCACTTATTTGTCC-3′, and RUSI-B, 5′-AAGTATACAAATTCAACCTT-3′, from the sequences isolated in *R. induratus*. Presence/absence status in the rest of species was also screened using the same primer combination. PCR amplifications were carried out in 50 μl reactions containing 10 ng of purified DNA, 2 mM of dNTPs, 2 mM of each primer and 1.25 units of Taq polymerase in 10 mM Tris HCl at pH 8.3, 5 mM KCl, 2 mM $MgCl_2$ reaction buffer. Thermal cycles consisted of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. The PCR products were electrophoresed in agarose gels, thereafter the bands were cut out of the gel, purified and ligated to the cloning plasmid pGEM-Teasy (Promega) and cloned in *Escherichia coli* JM109 competent cells (Promega) following the manufacturer's instructions.

Recombinant clones belonging to both a pUC18 and a pGEM library were sequenced by the dideoxy-sequencing method using the automatic ABI-Prism 377 sequencer (Applied Biosystems). The EMBL accession numbers for all the sequences analyzed in this paper are: AM398567 to AM398599.

For sequence analysis, multiple alignments were performed using Clustal X (Thompson et al., 1997) followed by manual adjustments. Basic sequence evolutionary calculations, as well as the detection of transition stages of satellite-DNA evolution (Strachan et al., 1985) and shared and non-shared polymorphisms were performed by the software satDNA Analyzer (Navajas-Pérez et al., 2007). Additionally, a Perl script able to recognize regular expressions in both strands was written to search specifically for degenerated telomere motifs.

Results

Isolation and organization of a new satellite-DNA family in R. induratus

When total genomic DNA of *R. induratus* was digested with *Bgl*I and electrophoresed in agarose gels stained with ethidium bromide, several prominent bands of 170-bp fold units were observed (not shown). We isolated and cloned these 170-bp fragments as candidate repeat units of a satellite-DNA family and 16 clones were selected for sequencing. One of these clones (RUSI_19) was used as a probe for Southern blot hybridization against the genomic DNA of *R. induratus* digested with different restriction enzymes. A typical ladder pattern with a repeat unit of 170 bp was detected for enzymes *Dra*I and *Bgl*I suggesting tandem repeat organization typical for satellite-DNA. In the case of enzymes *Eco*RI and *Hin*dIII a type B ladder-pattern was detected (Fig. 2, lanes 1–4).

DNA sequence analysis, showed that the 16 *Bgl*I repeats analyzed ranged from 167 to 182 bp in length, with an AT content of 67%. The mean percentage of variation among these sequences is 15%. Significantly, degenerated variants of the *Arabidopsis*-like TTTAGGG telomere repetitive motif matching the formula (TT)nAN(GG)n, were detected in both forward and reverse sense in most of the monomeric sequences (Fig. 3). No significant positive matches were detected when sequences were contrasted with the NCBI/EMBL database (July, 2008 release). Thus, they were considered to be a new satellite-DNA family, that we named RUSI (**RU**mex **S**cutati section **I**nduratus species sequence).

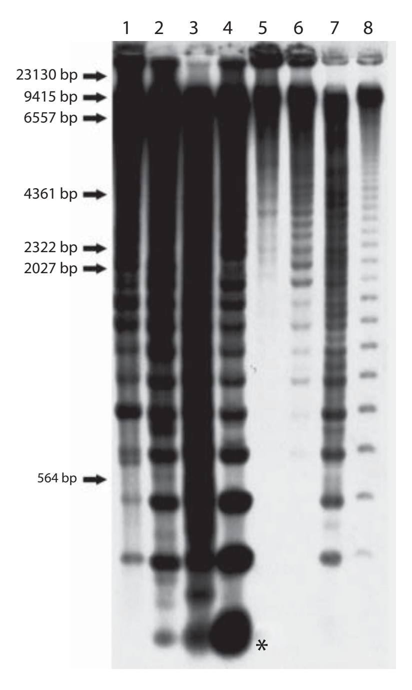


Fig. 2. Southern blot hybridization of RUSI sequences against total genomic DNA of *Rumex induratus* and *Rumex scutatus* digested with *Eco*RI (1, 5), *Hin*dIII (2, 6), *Dra*I (3, 7) and *Bgl*I (4, 8) restriction enzymes. Size markers are indicated on the left. (*) indicate monomeric repetitive unit (170 bp).

Chromosomal analysis and location of RUSI in R. induratus karyotype

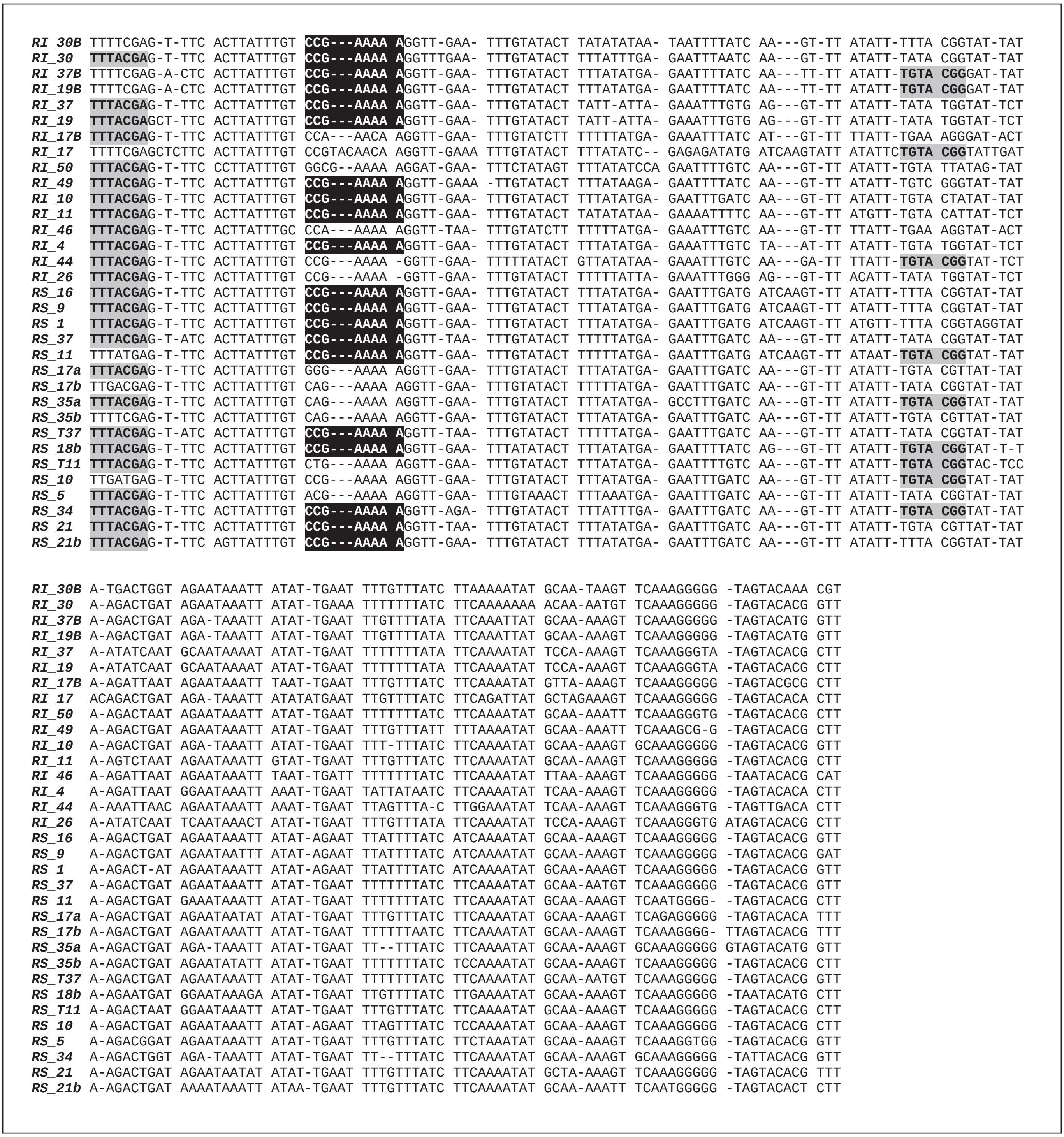
R. induratus is tetraploid, with a karyotype composed of 2n = 40. According to the chromosomal morphology and the fluorescent in situ hybridization (FISH) pattern of RUSI sequences, the karyotype of *R. induratus* can be grouped in two complements of ten chromosome pairs, six meta-/submetacentric and four subtelocentric pairs each confirming the basic chromosome number of the group, x = 10 (Figs. 4 and 5) (Navajas-Pérez et al., 2005a). Clusters of RUSI sequences are located in most of the larger chromosomes of *R. induratus,* mainly in subtelomeric positions. Subtelomeric/distal FISH signals were found either in one arm or in both arms depending on the chromosome. Additionally, a few chromosomes show hybridization signals indicating the presence of RUSI sequences in the centromeric region, exclusively or in addition to the subtelomeric sites. We then found subtelomeric/distal FISH signal of: (1) both arms in the two homeologous chromosome pairs I; (2) in the short arm of the two homeologous pairs III and IV; (3) in the long arm of the two homeologous pairs VI and VII. Pairs II and V in both sets and pair III of one set show paracentromeric hybridization signal indicating the presence of RUSI sequences in the centromeric region exclusively, but strongly (pairs II) or weaker and in addition to the subtelomeric sites (pairs III and V). Homeologous chromosome pairs VIII to X have no evidence of hybridization (Fig. 5).

Interspecific analysis of RUSI sequences

We screened the distribution of this new satellite-DNA family in representative species of the four subgenera of genus *Rumex* (López González, 1990). For that, we blotted onto a nylon membrane total genomic DNA digested with *Eco*RI of *Rumex* species listed in Fig. 1. Southern-blot hybridization with the RUSI_19 clone gave a positive signal only in *R. scutatus* and in the positive control *R. induratus*. These results agree with additional PCR experiments using specific primers for RUSI sequences (not shown). The pattern of hybridization in *R. scutatus* was slightly different from that of *R. induratus* since all restriction enzymes used in Southern-blot hybridization experiments gave a type B ladder pattern (Fig. 2, lanes 5–8).

From PCR experiments, a total of 17 RUSI monomeric sequences were obtained from *R. scutatus*. These sequences showed the same features described for *R. induratus*, including an average size of 170 bp and a high AT content (66.7%). The degree of intraspecific identity was 7.5%. As in *R. induratus*, we also detected the presence of (TT)nAN(GG)n degenerated telomere motifs (Fig. 3).

For interspecific sequence comparison monomeric sequences isolated from *R. induratus* and *R. scutatus* were aligned requiring a 188-character dataset due to the inclusion of some indels as a consequence of insertions/deletions. The degree of interspecific divergence among sequences belonging to both species was 12%. A tree generated using an NJ approach does not reveal the existence of highly supported clades of sequences, which do not appear clustered according to taxonomic affinity (not shown). The study of the alignment position per position for the analysis of the different stages toward the homogenization according to Strachan's model (Strachan et al., 1985; Navajas-Pérez et al., 2007) revealed the lack of positions between transition stages IV to VI. Up to 65 (35%) positions were detected to be among II and III transition stages, while 35 (19%) of the positions represented shared polymorphic positions between the two species (Table 1). Therefore, no species-specific variants were detected and, in fact, consensus sequences were identical between the two species.



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RI_30  TTTACGAG-T-TTC ACTTATTTGT CCG---AAAA AGGTTTGAA- TTTGTATACT TTTATTTGA- GAATTTAATC AA---GT-TT ATATT-TATA CGGTAT-TAT
RI_37B TTTTCGAG-A-CTC ACTTATTTGT CCG---AAAA AGGTT-GAA- TTTGTATACT TTTATATGA- GAATTTTATC AA---TT-TT ATATT-TGTA CGGGAT-TAT
RI_19B TTTTCGAG-A-CTC ACTTATTTGT CCG---AAAA AGGTT-GAA- TTTGTATACT TTTATATGA- GAATTTTATC AA---TT-TT ATATT-TGTA CGGGAT-TAT
RI_37  TTTACGAG-T-TTC ACTTATTTGT CCG---AAAA AGGTT-GAA- TTTGTATACT TATT-ATTA- GAAATTTGTG AG---GT-TT ATATT-TATA TGGTAT-TCT
RI_19  TTTACGAGCT-TTC ACTTATTTGT CCG---AAAA AGGTT-GAA- TTTGTATACT TATT-ATTA- GAAATTTGTG AG---GT-TT ATATT-TATA TGGTAT-TCT
RI_17B TTTACGAG-T-TTC ACTTATTTGT CCA---AACA AGGTT-GAA- TTTGTATCTT TTTTTATGA- GAAATTTATC AT---GT-TT TTATT-TGAA AGGGAT-ACT
RI_17  TTTTCGAGCTCTTC ACTTATTTGT CCGTACAACA AGGTT-GAAA TTTGTATACT TTTATATC-- GAGAGATATG ATCAAGTATT ATATTCTGTA CGGTATTGAT
RI_50  TTTACGAG-T-TTC CCTTATTTGT GGCG--AAAA AGGAT-GAA- TTTCTATAGT TTTATATCCA GAATTTTGTC AA---GT-TT ATATT-TGTA TTATAG-TAT
RI_49  TTTACGAG-T-TTC ACTTATTTGT CCG---AAAA AGGTT-GAAA -TTGTATACT TTTATAAGA- GAATTTTATC AA---GT-TT ATATT-TGTC GGGTAT-TAT
RI_10  TTTACGAG-T-TTC ACTTATTTGT CCG---AAAA AGGTT-GAA- TTTGTATACT TTTATATGA- GAATTTGATC AA---GT-TT ATATT-TGTA CTATAT-TAT
RI_11  TTTACGAG-T-TTC ACTTATTTGT CCG---AAAA AGGTT-GAA- TTTGTATACT TATATATAA- GAAAATTTTC AA---GT-TT ATGTT-TGTA CATTAT-TCT
RI_46  TTTACGAG-T-TTC ACTTATTTGC CCA---AAAA AGGTT-TAA- TTTGTATCTT TTTTTATGA- GAAATTTGTC AA---GT-TT TTATT-TGAA AGGTAT-ACT
RI_4   TTTACGAG-T-TTC ACTTATTTGT CCG---AAAA AGGTT-GAA- TTTGTATACT TTTATATGA- GAAATTTGTC TA---AT-TT ATATT-TGTA TGGTAT-TCT
RI_44  TTTACGAG-T-TTC ACTTATTTGT CCG---AAAA -GGTT-GAA- TTTTTATACT GTTATATAA- GAAATTTGTC AA---GA-TT TTATT-TGTA CGGTAT-TCT
RI_26  TTTACGAG-T-TTC ACTTATTTGT CCG---AAAA -GGTT-GAA- TTTGTATACT TTTTTATTA- GAAATTTGGG AG---GT-TT ACATT-TATA TGGTAT-TCT
RS_16  TTTACGAG-T-TTC ACTTATTTGT CCG---AAAA AGGTT-GAA- TTTGTATACT TTTATATGA- GAATTTGATG ATCAAGT-TT ATATT-TTTA CGGTAT-TAT
RS_9   TTTACGAG-T-TTC ACTTATTTGT CCG---AAAA AGGTT-GAA- TTTGTATACT TTTATATGA- GAATTTGATG ATCAAGT-TT ATATT-TTTA CGGTAT-TAT
RS_1   TTTACGAG-T-TTC ACTTATTTGT CCG---AAAA AGGTT-GAA- TTTGTATACT TTTATATGA- GAATTTGATG ATCAAGT-TT ATGTT-TTTA CGGTAGGTAT
RS_37  TTTACGAG-T-ATC ACTTATTTGT CCG---AAAA AGGTT-TAA- TTTGTATACT TTTTTATGA- GAATTTGATC AA---GT-TT ATATT-TATA CGGTAT-TAT
RS_11  TTTATGAG-T-TTC ACTTATTTGT CCG---AAAA AGGTT-GAA- TTTGTATACT TTTTTATGA- GAATTTGATG ATCAAGT-TT ATAAT-TGTA CGGTAT-TAT
RS_17a TTTACGAG-T-TTC ACTTATTTGT GGG---AAAA AGGTT-GAA- TTTGTATACT TTTATATGA- GAATTTGATC AA---GT-TT ATATT-TGTA CGTTAT-TAT
RS_17b TTGACGAG-T-TTC ACTTATTTGT CAG---AAAA AGGTT-GAA- TTTGTATACT TTTTTATGA- GAATTTGATC AA---GT-TT ATATT-TATA CGGTAT-TAT
RS_35a TTTACGAG-T-TTC ACTTATTTGT CAG---AAAA AGGTT-GAA- TTTGTATACT TTTATATGA- GCCTTTGATC AA---GT-TT ATATT-TGTA CGGTAT-TAT
RS_35b TTTTCGAG-T-TTC ACTTATTTGT CAG---AAAA AGGTT-GAA- TTTGTATACT TTTATATGA- GAATTTGATC AA---GT-TT ATATT-TGTA CGTTAT-TAT
RS_T37 TTTACGAG-T-ATC ACTTATTTGT CCG---AAAA AGGTT-TAA- TTTGTATACT TTTTTATGA- GAATTTGATC AA---GT-TT ATATT-TATA CGGTAT-TAT
RS_18b TTTACGAG-T-TTC ACTTATTTGT CCG---AAAA AGGTT-GAA- TTTATATACT TTTATATGA- GAATTTTATC AG---GT-TT ATATT-TGTA CGGTAT-T-T
RS_T11 TTTACGAG-T-TTC ACTTATTTGT CTG---AAAA AGGTT-GAA- TTTGTATACT TTTATATGA- GAATTTTGTC AA---GT-TT ATATT-TGTA CGGTAC-TCC
RS_10  TTGATGAG-T-TTC ACTTATTTGT CCG---AAAA AGGTT-GAA- TTTGTATACT TTTATATGA- GAATTTGATC AA---GT-TT ATATT-TGTA CGGTAT-TAT
RS_5   TTTACGAG-T-TTC ACTTATTTGT ACG---AAAA AGGTT-GAA- TTTGTAAACT TTTAAATGA- GAATTTGATC AA---GT-TT ATATT-TATA CGGTAT-TAT
RS_34  TTTACGAG-T-TTC ACTTATTTGT CCG---AAAA AGGTT-AGA- TTTGTATACT TTTATTTGA- GAATTTGATC AA---GT-TT ATATT-TGTA CGGTAT-TAT
RS_21  TTTACGAG-T-TTC ACTTATTTGT CCG---AAAA AGGTT-TAA- TTTGTATACT TTTATATGA- GAATTTGATC AA---GT-TT ATATT-TGTA CGTTAT-TAT
RS_21b TTTACGAG-T-TTC AGTTATTTGT CCG---AAAA AGGTT-GAA- TTTGTATACT TTTATATGA- GAATTTGATC AA---GT-TT ATATT-TTTA CGGTAT-TAT


RI_30B A-TGACTGGT AGAATAAATT ATAT-TGAAT TTTGTTTATC TTAAAAATAT GCAA-TAAGT TCAAAGGGGG -TAGTACAAA CGT
RI_30  A-AGACTGAT AGAATAAATT ATAT-TGAAA TTTTTTTATC TTCAAAAAAA ACAA-AATGT TCAAAGGGGG -TAGTACACG GTT
RI_37B A-AGACTGAT AGA-TAAATT ATAT-TGAAT TTGTTTTATA TTCAAATTAT GCAA-AAAGT TCAAAGGGGG -TAGTACATG GTT
RI_19B A-AGACTGAT AGA-TAAATT ATAT-TGAAT TTGTTTTATA TTCAAATTAT GCAA-AAAGT TCAAAGGGGG -TAGTACATG GTT
RI_37  A-ATATCAAT GCAATAAAAT ATAT-TGAAT TTTTTTTATA TTCAAAATAT TCCA-AAAGT TCAAAGGGTA -TAGTACACG CTT
RI_19  A-ATATCAAT GCAATAAAAT ATAT-TGAAT TTTTTTTATA TTCAAAATAT TCCA-AAAGT TCAAAGGGTA -TAGTACACG CTT
RI_17B A-AGATTAAT AGAATAAATT TAAT-TGAAT TTTGTTTATC TTCAAAATAT GTTA-AAAGT TCAAAGGGGG -TAGTACGCG CTT
RI_17  ACAGACTGAT AGA-TAAATT ATATATGAAT TTGTTTTATC TTCAGATTAT GCTAGAAAGT TCAAAGGGGG -TAGTACACA CTT
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RI_11  A-AGTCTAAT AGAATAAATT GTAT-TGAAT TTTGTTTATC TTCAAAATAT GCAA-AAAGT TCAAAGGGGG -TAGTACACG CTT
RI_46  A-AGATTAAT AGAATAAATT TAAT-TGATT TTTTTTTATC TTCAAAATAT TTAA-AAAGT TCAAAGGGGG -TAATACACG CAT
RI_4   A-AGATTAAT GGAATAAATT AAAT-TGAAT TATTATAATC TTCAAAATAT TCAA-AAAGT TCAAAGGGGG -TAGTACACA CTT
RI_44  A-AAATTAAC AGAATAAATT AAAT-TGAAT TTAGTTTA-C TTGGAAATAT TCAA-AAAGT TCAAAGGGTG -TAGTTGACA CTT
RI_26  A-ATATCAAT TCAATAAACT ATAT-TGAAT TTTGTTTATA TTCAAAATAT TCCA-AAAGT TCAAAGGGTG ATAGTACACG CTT
RS_16  A-AGACTGAT AGAATAAATT ATAT-AGAAT TTATTTTATC ATCAAAATAT GCAA-AAAGT TCAAAGGGGG -TAGTACACG GTT
RS_9   A-AGACTGAT AGAATAATTT ATAT-AGAAT TTATTTTATC ATCAAAATAT GCAA-AAAGT TCAAAGGGGG -TAGTACACG GAT
RS_1   A-AGACT-AT AGAATAAATT ATAT-AGAAT TTATTTTATC ATCAAAATAT GCAA-AAAGT TCAAAGGGGG -TAGTACACG GTT
RS_37  A-AGACTGAT AGAATAAATT ATAT-TGAAT TTTTTTTATC TTCAAAATAT GCAA-AATGT TCAAAGGGGG -TAGTACACG GTT
RS_11  A-AGACTGAT GAAATAAATT ATAT-TGAAT TTTTTTTATC TTCAAAATAT GCAA-AAAGT TCAATGGGG- -TAGTACACG CTT
RS_17a A-AGACTGAT AGAATAATAT ATAT-TGAAT TTTGTTTATC TTCAAAATAT GCAA-AAAGT TCAGAGGGGG -TAGTACACA TTT
RS_17b A-AGACTGAT AGAATAAATT ATAT-TGAAT TTTTTTAATC TTCAAAATAT GCAA-AAAGT TCAAAGGGG- TTAGTACACG TTT
RS_35a A-AGACTGAT AGA-TAAATT ATAT-TGAAT TT--TTTATC TTCAAAATAT GCAA-AAAGT GCAAAGGGGG GTAGTACATG GTT
RS_35b A-AGACTGAT AGAATATATT ATAT-TGAAT TTTTTTTATC TCCAAAATAT GCAA-AAAGT TCAAAGGGGG -TAGTACACG CTT
RS_T37 A-AGACTGAT AGAATAAATT ATAT-TGAAT TTTTTTTATC TTCAAAATAT GCAA-AATGT TCAAAGGGGG -TAGTACACG GTT
RS_18b A-AGAATGAT GGAATAAAGA ATAT-TGAAT TTGTTTTATC TTGAAAATAT GCAA-AAAGT TCAAAGGGGG -TAATACATG CTT
RS_T11 A-AGACTAAT GGAATAAATT ATAT-TGAAT TTTGTTTATC TTCAAAATAT GCAA-AAAGT TCAAAGGGGG -TAGTACACG CTT
RS_10  A-AGACTGAT AGAATAAATT ATAT-AGAAT TTAGTTTATC TCCAAAATAT GCAA-AAAGT TCAAAGGGGG -TAGTACACG CTT
RS_5   A-AGACGGAT AGAATAAATT ATAT-TGAAT TTTGTTTATC TTCTAAATAT GCAA-AAAGT TCAAAGGTGG -TAGTACACG GTT
RS_34  A-AGACTGGT AGA-TAAATT ATAT-TGAAT TT--TTTATC TTCAAAATAT GCAA-AAAGT GCAAAGGGGG -TATTACACG GTT
RS_21  A-AGACTGAT AGAATAATAT ATAT-TGAAT TTTGTTTATC TTCAAAATAT GCTA-AAAGT TCAAAGGGGG -TAGTACACG TTT
RS_21b A-AGACTGAT AAAATAAATT ATAA-TGAAT TTTGTTTATC TTCAAAATAT GCAA-AAATT TCAATGGGGG -TAGTACACT CTT
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Fig. 3. Alignment of *R. induratus* and *R. scutatus* RUSI sequences. Shaded nucleotides indicate degenerated variants of the *Arabidopsis*-like TTTAGGG telomere repetitive motif in both forward (grey) and reverse (black) senses.

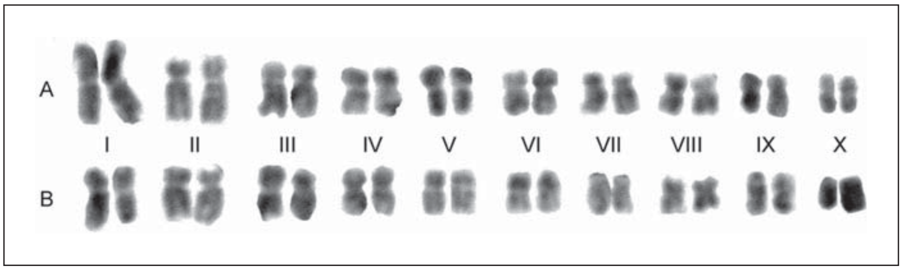

Fig. 4. Karyotype of *R. induratus*. Note the presence of 20 chromosome pairs ordered in two ten-pair complements (A, B), corresponding to the basic chromosome number of the group, x = 10.

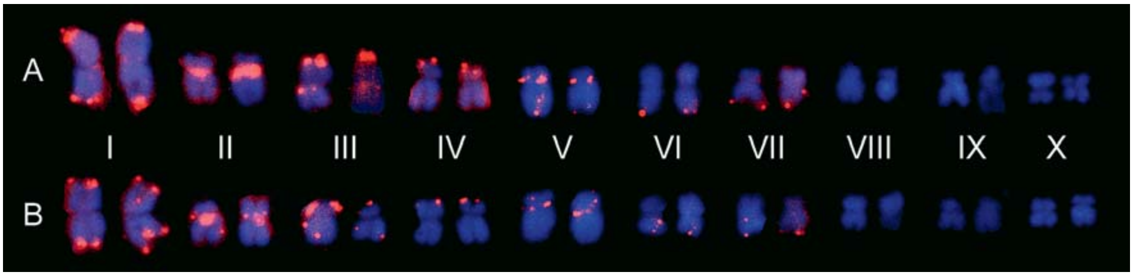

Fig. 5. Composite karyotype of *R. induratus* metaphase chromosomes (A, B) analyzed after FISH with RUSI sequences.

Table 1. Statistics for RUSI satellite-DNA sequences, including intraspecific and interspecific mean distances between monomeric sequences. The differences between species are stated as Fixed positions for fixed or nearly fixed polymorphisms while Transitional positions represent intermediate stages in the process of homogenization toward the fixation, according to the Strachan et al. (1985) method (Navajas-Pérez et al., 2007).

Mean distance			Differences between species	
Intraspecific *(R. induratus/R. scutatus)*		Inter-specific	Fixed (IV–V)	Transitional (II–III)
0.15	0.075	0.12	0	65

Discussion

R. induratus is an endemic polygamous species of the Iberian Peninsula. This species is tetraploid (Fernández-Casas, 1977) while the widespread closely related *R. scutatus* is diploid (Rechinger, 1964). In the present study, we have confirmed a karyotype of 2n = 4x = 40 for *R. induratus*, with a basic chromosome number of x = 10 (Figs. 4 and 5). This basic chromosome number has been found to be the ancestral number in all docks and in most of the hermaphroditic and polygamous species of sorrels (Degraeve, 1975; Navajas-Pérez et al., 2005a). Recently, we have identified that, within the clade of polygamous and hermaphroditic sorrels, there are two major subclades, one of them contains the species *R. scutatus* and *R. induratus* in the *Scutati* section (subgenus *Acetosa*) and *R. maderensis* in the *Hastati* section (*Acetosa*) (Navajas-Pérez et al., 2005a). The macaronesian endemic *R. maderensis* is diploid and a basal species of the phylogenetic clade containing these three species. Thus, the tetraploidy of *R. induratus* can be interpreted as a derived character state that evolved in this clade independently of other polyploidization events occurring in other *Rumex* lineages.

In this species we describe here for the first time a new satellite-DNA family, RUSI. This satellite-DNA is located at the subtelomeric regions of most chromosomes of the karyotype (Fig. 5). Within the distal regions of the chromosomes in most eukaryotic species we can distinguish two differentiated parts. The more distal regions represent the true telomere and, except in rare exceptions, are formed of a variable number of short tandem repeats (Blackburn and Greider, 1995). In vertebrate species, the sequence TTAGGG is conserved at telomeres while in invertebrate species-specific variations of the sequence TTGGGG first described in *Tetrahymena* are present. In *Arabidopsis*, as well as in the vast majority of flowering plants analyzed up to date, TTTAGGG is the most common repetitive telomere sequence (Richards and Ausubel, 1988). The subterminal sequences adjacent to the telomeres have been referred to as telomere-associated sequences or TASs (Louis and Vershinin, 2005) and are usually composed of a variety of highly tandem repetitive DNA sequences or satellite DNAs. In plants, TASs are often structurally more variable and polymorphic than any other regions of the genome even in species where they were described as not highly repeated, such as in rice and maize for example (Burr et al., 1992; Ashikawa et al., 1994). These subtelomeric sequences do not necessarily participate in telomere function but, for example, can facilitate meiotic pairing or protect terminal genes against the loss and gain processes at the chromosome ends (Henderson, 1995; Kipling, 1995). They could also be involved in the regulation of subtelomeric genes (Sykorova et al., 2003). On the other hand, these sequences have been postulated to acquire vital importance when telomere sequences disappear. In fact, chromosomes with depleted telomeres have a propensity to be lost (Sandell and Zakian, 1993). In this sense, several cases of a satellite DNA adopting the telomere-repeat role have been reported, as for instance the substitution of most telomere repeats of Alliaceae by rDNA sequences, retrotransposable elements and satellite-DNA sequences (Barnes et al., 1985; Pich and Schubert, 1998; Do et al., 2001). Subtelomeric sequences are not conserved, but are often species specific with a variety of lengths and degrees of repetitiveness (Kipling, 1995; Pryde et al., 1997), even existing as different satellite-DNA families (Brown, 1989; Brown et al., 1990; Weber et al., 1990, 1991) or subfamilies of the same satellite DNA (Kazama et al., 2006) in different subsets of chromosomes within a species. A common characteristic of subtelomeric satellite DNAs is the direct proximity to telomere repeats as in tobacco (Fajkus et al., 1995), *Silene* (Garrido-Ramos et al., 1999), or Triticeae (Contento et al., 2005) which lead to the intermixing of subtelomeric repeats with telomere sequence that are often degenerate. Also, subtelomeric repeats often show inverted organization with head to head orientation (Vershinin et al., 1995; Contento et al., 2005). The intimate contact of RUSI repeats with the telomere is corroborated by the presence of degenerated telomere motifs intercalated with them and might represent a common feature that occurs as a consequence of dynamic processes taking place at chromosome ends.

Interestingly, the RUSI repeats are not only located at the telomeres but also at centromeric positions of some chromosomes (Fig. 5). Also interestingly, there are differences between chromosomes having subtelomeric RUSI repeats on one or both arms. These observations lead to the consideration of chromosome reorganization implying that some loss/gain processes may have occurred. This report represents an important exception to the equilocality principle of satellite-DNA distribution (Thomas, 1971). Presence of the same type of satellite-DNA sequences at both centromeric and subtelomeric sequences can be explained by chromosome reorganization such as Robertsonian translocations (Garagna et al., 2002; Castiglia et al., 2006) or by transposition (Abad and Villasante, 2000; Tek and Jiang, 2004; Bao et al., 2006). Reported cases of alteration of the equilocal distribution of satellite DNAs also included satellite-DNA amplifications in specific regions of particular chromosomes such as sex chromosomes (Cuñado et al., 2007), B chromosomes (Alfenito and Birchler, 1993) or microchromosomes (Kuhn et al., 2007) or exceptional cases of independent evolution of interstitial heterochromatic regions of one chromosome pair with respect to the rest of the heterochromatic regions of the karyotype, leading to the characteristic asymmetries found in the genus *Muscari* (de la Herrán et al., 2001). In the case described here, the appearance of RUSI sequences in two non-equilocal different regions of the chromosomes of *R. induratus* could imply chromosome reorganization such as inversions and/or transpositions, but also Robertsonian translocations.

Satellite-DNA families have been found to be irregularly distributed within the different groups of plants, including chromosome-specific ones, such as the WE35 family of *Triticum aestivum* (Ueng et al., 2000), species-specific families, as RAYSI Y-specific satellite DNA in several *Rumex* species (Navajas-Pérez et al., 2006) or satellite DNA borne by species from a whole tribe, such as the subtelomeric 120-bp repeat family of Triticeae (Contento et al., 2005). Assuming that two species bearing the same sequence would be more related to each other than those lacking these sequences, satellite DNA can be used as a cladistic marker by merely analysing the presence/absence status. Here, we have detected that the RUSI satellite-DNA family is exclusively distributed in two species of the genus *Rumex*, *R. induratus* and *R. scutatus*. Both classical and more novel systematics of the genus (López González, 2000; Navajas-Pérez et al., 2005a) describe these two species as closely related to each other as part of the section *Scutati* of the subgenus *Acetosa*. Since we have not found evidence of RUSI sequences in any other *Rumex* species analyzed (not shown), including those more phylogenetically related as *R. maderensis*, the present study demonstrates that this satellite DNA originated very recently (between 2.5 and 4.5 mya – Navajas-Pérez et al., 2005a). This is not an uncommon situation due to the high turnover rate of this part of the eukaryotic genome. Studies on satellite DNA dynamics explain loss or increase in copy number through not always well-known amplification mechanisms in a relatively short evolutionary time (Charlesworth et al., 1994).

Repetitive-DNA families are influenced by several molecular mechanisms of non-reciprocal exchange such as unequal crossing-over or gene conversion (Ohta and Dover, 1984) that can gradually spread a variant sequence throughout a family within a sexual population in a population-genetic process called molecular drive (Dover, 2002). Molecular drive leads to high homogeneity levels in a repetitive DNA family for species-diagnostic mutations, which subsequently is the origin of inter-species genetic divergence, an evolutionary pattern of repetitive sequences known as concerted evolution (Dover, 2002). Many empirical observations indicate that the rate of production of new sequence variants (mutations) in satellite-DNA sequences is a slower process than their rate of spread, while the general paucity of transition stages indicates also that the replacement is relatively fast (Ugarković and Plohl, 2002). However, this is not occurring for RUSI sequences since we detected high levels of intraspecific sequence variation (i.e., low rates of intraspecific sequence homogenization; Table 1). The intraspecific sequence variation has two sources: on one hand, a high percentage of shared polymorphisms between *R. induratus* and *R. scutatus* (19%), which might represent ancestral polymorphisms; on the other hand, a much higher percentage of new species-specific mutations (35% of transition stages II+III) representing an initial process of sequence divergence between the two species (mean sequence differences of 12%). However, spreading rates do not appear to operate at higher rates than mutation rates, a fact that could be explained as a consequence of the multichromosomal and multiloci distribution (i.e., chromosomal rearrangements) of RUSI sequences. Multichromosomal distribution and chromosomal rearrangements together with polyploidy (Krieger and Fuerst, 2002) might be among the possible causes for extensive intraspecific variability of repetitive sequences of various organisms. Polyploidization is also a fact to be considered here since we have found higher levels of variation in the tetraploid *R. induratus* than in the diploid *R. scutatus*.

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