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ORIGINAL PAPER

Accumulation of Y-specific satellite DNAs during the evolution of *Rumex acetosa* sex chromosomes

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Abstract The study of the molecular structure of young heteromorphic sex chromosomes of plants has shed light on the evolutionary forces that control the differentiation of the X and Y during the earlier stages of their evolution. We have used the model plant *Rumex acetosa*, a dioecious species with multiple sex chromosomes, $2n = 12 + XX$ female and $2n = 12 + XY_1Y_2$ male, to analyse the significance of repetitive DNA accumulation during the differentiation of the Y. A bulk segregant analysis (BSA) approach allowed us to identify and isolate random amplified polymorphic DNA (RAPD) markers linked to the sex chromosomes. From a total of 86 RAPD markers in the parents, 6 markers were found to be linked to the Ys and 1 to the X. Two of the Y-linked markers represent two AT-rich satellite DNAs (satDNAs), named RAYSII and RAYSIII, that share about 80% homology, as well as with RAYSI, another satDNA of *R. acetosa*. Fluorescent in situ hybridisation demonstrated that RAYSII is specific for Y_1, whilst RAYSIII is located in different clusters along Y_1 and Y_2. The two satDNAs were only detected in the genome of the dioecious species with XX/XY_1Y_2 multiple sex chromosome systems in the subgenus Acetosa, but were absent from other dioecious species with an XX/XY system of the subgenera Acetosa or Acetosella, as well as in gynodioecious or hermaphrodite species of the subgenera Acetosa, Rumex and Platypodium. Phylogenetic analysis with different cloned monomers of RAYSII and RAYSIII from both *R. acetosa* and *R. papillaris* indicate that these two satDNAs are completely separated from each other, and from RAYSI, in both species. The three Y-specific satDNAs, however, evolved from an ancestral satDNA with repeating units of 120 bp, through intermediate satDNAs of 360 bp. The data therefore support the idea that Y-chromosome differentiation and heterochromatinisation in the *Rumex* species having a multiple sex chromosome system have occurred by different amplification events from a common ancestral satDNA. Since dioecious species with multiple XX/XY_1Y_2 sex chromosome systems of the section Acetosa appear to have evolved from dioecious species with an XX/XY system, the amplification of tandemly repetitive elements in the Ys of the section Acetosa is a recent evolutionary process that has contributed to an increase in the size and differentiation of the already non-recombining Y chromosomes.

Keywords Sex chromosome evolution · Satellite DNA · Y-chromosome differentiation · *Rumex acetosa*

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Introduction

Although the separation of male and female sex organs in different individuals (dioecy) is a widespread mechanism that guarantees recombination and genetic diversity promoted by outcrossing in the animal kingdom, dioecious plants only account for 6% of flowering species (Renner and Ricklefs 1995). Dioecious plant species have arisen separately during the evolution of angiosperms, and have therefore adopted different mechanisms to control sexual

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dimorphism, from species in which sex is controlled by either a single gene or a number of non-linked genes to species in which sex determination depends on sex chromosomes (Ainsworth et al. 1999; Jamilena et al. 2008). Sex chromosomes have been found in a small number of species belonging to 15 families (Charlesworth and Guttman 1999; Matsunaga and Kawano 2001; Vyskot and Hobza 2004; Ming et al. 2007). Most of them are homomorphic and cannot be distinguished in size and shape from each other or from autosomes, and only few species belonging to five families, Hepaticeae, Cannabidaceae, Caryophillaceae, Cucurbitaceae and Polygonaceae, have distinctive sex chromosomes as heteromorphic as those found in mammals and insects. The sex chromosomes of plants are therefore much younger than those of animals, but the mechanisms that regulate sex determination in plants are quite similar to those of animals. In some species, such as *Silene latifolia* and *Carica papaya*, sexual dimorphisms are controlled by an active Y chromosome, the Y being necessary to activate male development, as in mammals. In others species, such as *Rumex acetosa* and *Cannabis sativa*, sex determination is not dependent on the presence or absence of the Y chromosomes, but is controlled, as in *Drosophila* and *Caenorhabditis elegans*, by the balance between the number of Xs and autosomes (X/A balance) (Jamilena et al. 2008). Despite the small number of dioecious plants with heteromorphic sex chromosomes, their recent origin makes them an ideal model species to analyse the evolutionary forces that control the differentiation of sex chromosomes during the earlier stages of their evolution.

R. acetosa has a multiple sex chromosome system in which females have two Xs ($2n = 12 + XX$), whilst males have one X and two distinctive Ys ($2n = 12 + XY_1Y_2$). The sex chromosomes are the largest of the karyotype, and although the X is slightly larger than each one of the Ys, the two Ys still account for about 26% of the male genome (Wilby and Parker 1988). The two Ys are both heterochromatic and differentiated from the euchromatic X chromosome (Clark et al. 1993; Ruiz Rejón et al. 1994; Lengerova and Vyskot 2001; Mosiolek et al. 2005). It has been estimated that the sex chromosomes of *R. acetosa* are not more than 15–20 million years old (Navajas-Pérez et al. 2005a). The differentiation between the X and Y is not due to the accumulation of retrotransposons, but appears to have occurred by the amplification of tandemly repetitive DNA sequences. In fact, we have isolated and characterised five transposon-like repetitive DNA families which are dispersed throughout the genome of both males and females, but no appreciable accumulation or differentiation of these types of sequences has been found in the Y chromosomes (Mariotti et al. 2006). Nevertheless, different families of satellite DNA (satDNA) have been found to be located in the two Ys of *R. acetosa*. The family RAE180, which is located at a single autosomal locus, is a major component of both Y_1 and Y_2 (Shibata et al. 2000a), whilst two different subfamilies of the RAYSI satDNA are specifically located in the Ys of *R. acetosa* (Shibata et al. 1999; Navajas-Pérez et al. 2005b, 2006).

By using RAPD markers and a bulk segregant analysis (BSA) approach we have identified different X- and Y-linked DNA sequences. Here we describe the cloning and characterisation of two of the isolated Y-specific markers. They represent new Y-specific satDNAs from the *R. acetosa* genome. The sequence homology between these two new satDNAs and also the Y-specific RAYSI, as well as their specific conservation in the *Rumex* species with multiple XX/XY_1Y_2 sex chromosome systems, has shed light on the evolution of Y chromosomes in this group of species.

Materials and methods

Plant material, DNA extraction and RAPD reactions

Plants of *R. acetosa* were derived from seeds of two natural populations in Capileira and Puerto de la Ragua, both in the province of Granada, Spain. Plants were grown individually in pots in a greenhouse at the University of Almería, Spain; once they had developed 10–15 leaves the sex of each one was determined by Southern hybridisation with a probe derived from the Y-specific satDNA RAYSI (Shibata et al. 2000a). The sex was later confirmed when plants flowered during the first or the second year after planting. The rest of the *Rumex* species used in this paper also originate from different natural populations and represent dioecious, hermaphrodite, polygamous, and gynodioecious species from different subgenera (Table 1). DNA from male and female plants of *Rumex* species was isolated from leaves, following the protocol of the Plant DNAzol kit (Invitrogen).

RAPDs markers linked to the sex chromosome of *R. acetosa* were detected by using BSA. Two separate bulks were generated by mixing equal amounts of DNA derived from seven female or seven male plants of the offspring from a controlled cross between two plants of *R. acetosa*. The RAPD analysis was then carried out with DNA from each of the parental plants, as well as with the male and female DNA bulks from the offspring. The Quiagen-Operon RAPDs' 10-mer primers were used. The optimised RAPDs reactions contained 15 ng of genomic DNA, 37.5 ng of primer, 2.5 mM of dNTPs, 2.5 mM of $MgCl_2$ and 1.5 units of *Taq* polymerase (Sigma) in a final volume of 15 µl. PCR conditions were as follows: 94°C for 4 min, followed by 3 cycles of 94°C/15 s, 35°C/15 s and 72°C/75 s, 40 cycles of 94°C/15 s, 40°C/15 s and 72°C/75 s, and a final extension period of 7 min at 72°C. After completion,

Table 1 Mating systems and location of the populations of *Rumex* species analysed

Subgenus/section	Species	Mating systems (sex chromosomes)	Population
Acetosa/Acetosa	*Rumex acetosa*	D (XX/XY_1Y_2)	Capileira, Puerto de la Ragua, Granada (Spain)
	Rumex papillaris	D (XX/XY_1Y_2)	La Benajara, S^a Baza, Granada (Spain)
Acetosa/Americanae	*Rumex hastatulus*	D (XX/XY_1Y_2)	Cumberland County, North Carolina (USA)
			Duke University Herbarium, M. 73318
Acetosa/Scutati	*Rumex induratus*	H	Padul, Granada (Spain)
	Rumex suffruticosus	D (XX/XY)	Pto. Navacerrada, Segovia (Spain)
Acetosa/Hastati	*Rumex lunaria*	G	Gáldar, Gran Canaria (Spain)
Acetosella	*Rumex acetosella*	D (XX/XY)	Capileira, Granada (Spain)
Rumex	*Rumex conglomeratus*	H	Atarfe, Granada (Spain)
	Rumex crispus	H	Atarfe, Granada (Spain)
Platypodium	*Rumex bucephalophorus*	H	Padul, Granada (Spain)

D *dioecious*, *G* gynodioecious, *H* hermaphrodite

PCR samples were electrophoresed in 1.5% agarose gels. Some 110 primers of 10-mer (Operon series A-N) were initially used with the DNA from female and male parental plants, as well as the bulks. Individual plants of the offspring were also analysed with selected primers using the same method, to confirm the linkage to sex chromosomes of certain identified markers.

Cloning, Southern hybridisations and sequence analysis

Two of the RAPD-PCR products that appeared linked to the Y chromosomes of *R. acetosa* were extracted from the gel and cloned. The recovered PCR fragments were ligated into linearised pGEMT-easy vector (Promega) and transformed in DH5α *E. coli* competent cells. Recombinant clones were selected by their insert size, as determined by PCR with universal forward and reverse primers, and also by their Southern hybridisation patterns on DNA from the same RAPD-PCR reactions from which they were derived. Clones whose inserts only hybridised with the Y-linked polymorphic band were selected for further analysis.

For Southern hybridisation, genomic DNA from male and female plants of *R. acetosa* as well as from other species of *Rumex* was isolated from leaves, following the protocol of the Plant DNAzol kit (Invitrogen). For probe labelling, hybridisation and detection, the ECL direct labelling and detection system (Amersham) were used following the manufacturer's recommendations.

Primer URG08-F (CCAATTGGTCTCAACTAGAACA) and URG08-R (TGTTATAGGTTTTGGACTGCCA), and ACE17-R (AATCGATCAAAATGGT CAG) and ACE17-F (TTAGTCTACTAAGGAGTTT) were used to amplify the repeating units of RAYSII and RAYSIII satDNAs in both *R. acetosa* and *R. papillaris*, respectively. The PCR products were also cloned into pGEMT-easy vector (Promega). Plasmid DNAs were extracted from recombinant clones by the *Perfect preparation plasmid mini kit* from Eppendorf, following the recommendations of the manufacturer. The cloned DNA fragments were sequenced by the dideoxy sequencing method using the ABI-Prism 377 sequencer system of Applied Biosystems, and the DNA sequences deposited in the EMBL database under the accession numbers EU644727 to EU644747. Sequence comparison was performed using the BLAST software in the website of the National Centre for Biotechnology Information (NCBI). Multiple alignments were performed using ClustalW program and the phylogenetic analysis conducted with MEGA3 (Kumar et al. 2004). Phylogenetic trees were constructed by the neighbour-joining method (Saitou and Nei 1987).

Fluorescence in situ hybridisation (FISH)

Chromosome spreads were prepared from root tips of germinated seeds and treated as described in Lengerova et al. (2004) with slight modifications. Slide denaturation was performed in 7:3 (v/v) formamide: 2× SSC for 2 min at 72°C. Slides were immediately dehydrated through ice cold 50, 70 and 100% ethanol, and air dried. The probes were denatured at 75°C for 10 min in 50% formamide, 2× SSC, 10% dextran sulphate, and 100 ng of denatured probe was added and hybridised at 37°C for 16 h in a wet chamber. DNA was labelled with Fluorolink Cy3-dUTP (Amersham Pharmacia Biotech) (red labelling) in combination with the nick translation mix (Roche) or with SpectrumGreen direct-labelled dUTP (green labelling) and the Nick Translation kit (both Vysis).

Results

Identification of RAPD markers linked to the Y chromosomes of *R. acetosa*

Bulked segregant analysis was used to identify DNA markers linked to the sex chromosomes of *R. acetosa*. DNAs from seven female and seven male offspring plants, derived from an experimental cross between an individual male and female of *R. acetosa*, were mixed to form two bulks. DNA from female and male parental plants as well as the bulked DNA from female and male offspring was used in RAPD-PCR reactions with different 10-mer primers. PCR reactions were repeated three times, scoring only markers that appeared consistently in the three reactions. After a preliminary screening with 110 primers, 40 primers that produced polymorphic and reproducible bands were selected. Table 1 summarises the results obtained with the selected primers. A total of 274 RAPDs markers were scored, of which 86 (31.4%) were polymorphic. Only seven bands (2.7%) were identified as being linked to the sex chromosomes (Table 2). Only one polymorphic band was detected that was present in the male parent and in the female offspring, which indicates that it is X-linked. Six polymorphic bands were found to be linked to the Y chromosomes and were therefore present in both the male parent and the male offspring, but absent in females (Table 2).

The Y-linked markers of about 600 and 650 bp detected with primers OPG08 and OPN18, named Y_1 and Y_2, respectively (Fig. 1), were further characterised. DNA from 10 males and 10 females of the offspring was separately used to confirm that the polymorphic fragments obtained with these primers were indeed Y-linked. The polymorphic markers of 600 and 650 bp were only present in the male parent and in the male offspring, which demonstrate their linkage to the Y chromosomes (data not shown). The two RAPD markers were purified from agarose gels and cloned into plasmid vectors, and the resulting recombinant plasmids were selected by their size but by the hybridisation patterns on the RAPD products obtained with primers OPG08 and OPN18 (Fig. 2). Recombinant plasmids that hybridised specifically with the Y-linked RAPD markers were selected for sequencing. Y_1 and Y_2 fragments corresponded to sequences of 579 and 659 bp, and shared 58% homology in a 335 bp fragment.

Y_1 and Y_2 RAPD markers represent two related Y-specific satDNAs

The organisation of the Y_1 and Y_2 fragments was analysed by Southern hybridisation. DNA from males and females was restricted with different restriction enzymes and hybridised with the cloned Y_1 and Y_2 fragments. The hybridisation patterns shown by Y_1 and Y_2 markers indicated that they form part of two different satDNAs, named RAYSII and RAYSIII, that are specifically located in the Y chromosomes

Table 2 Relation of polymorphic and sex-linked RAPD markers generated with the different 10-mer primers

Primer	Sequence	Total markers	Polymorphic markers	Sex-linked markers (bp)
OPA03	AGTCGACCAC	9	2	
OPB15	GGAGGGTGTT	5	2	
OPC01	GTGAGGCGTC	5	2	Y-linked (700) Y-linked (500)
OPC05	GATGACCGCC	10	2	
OPC07	GTCCCGACGA	6	2	
OPC08	TGGACCGGTG	5	3	
OPC11	AAAGCTGCGG	8	3	
OPD02	GGACCCAACC	6	2	
OPD07	TTGGCACGGG	6	1	
OPD11	AGCGCCATTG	8	3	
OPD13	GGGGTGACGA	9	5	
OPD15	CATCCGTGCT	4	1	
OPD18	GAGAGCCAAC	11	2	
OPD20	ACCCGGTCAC	7	2	
OPG08	TCACGTCCAC	7	4	Y-linked (1,500) Y-linked (600)
OPG09	CTGACGTCAC	5	0	
OPH05	AGTCGTCCCC	9	2	
OPH12	ACGCGCATGT	8	3	
OPH20	GGGAGACATC	6	2	
OPJ04	CCGAACACGG	6	2	
OPJ06	TCGTTCCGCA	4	1	
OPJ09	TGAGCCTCAC	6	3	
OPJ10	AAGCCCGAGG	7	3	
OPJ18	TGGTCGCAGA	5	2	
OPJ19	GGACACCACT	5	1	
OPK10	GTGCAACGTG	3	0	
OPK14	CCCGCTACAC	6	1	
OPL16	AGGTTGCAGG	8	3	
OPL18	ACCACCCACC	4	1	
OPM01	GTTGGTGGCT	10	2	
OPM05	GGGAACGTGT	8	2	
OPM07	CCGTGACTCA	6	4	
OPN02	ACCAGGGGCA	12	5	
OPN03	GACCGACCCA	11	3	X-linked (1,200) Y-linked (500)
OPN05	ACTGAACGCC	3	0	
OPN06	GAGACGCACA	5	1	
OPN08	ACCTCAGCTC	4	2	
OPN12	CACAGACACC	8	2	
OPN15	CAGCGACTGT	9	2	
OPN18	GGTGAGGTCA	10	3	Y-linked (650)
Total		274	86 (31.4%)	7 (2.6%)

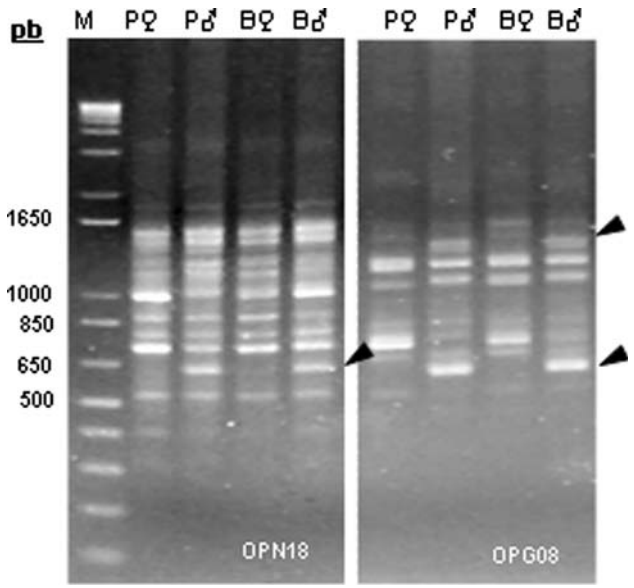

Fig. 1 RAPDs markers obtained with primers OPN18 and OPG08. RAPD reaction were carried out in parental male (P♂) and female (P♀) plants as well as in the bulks from male (B♂) and female (B♀) offspring. *Arrowheads* point to the Y-linked polymorphic bands. *M* = 1 kb ladder

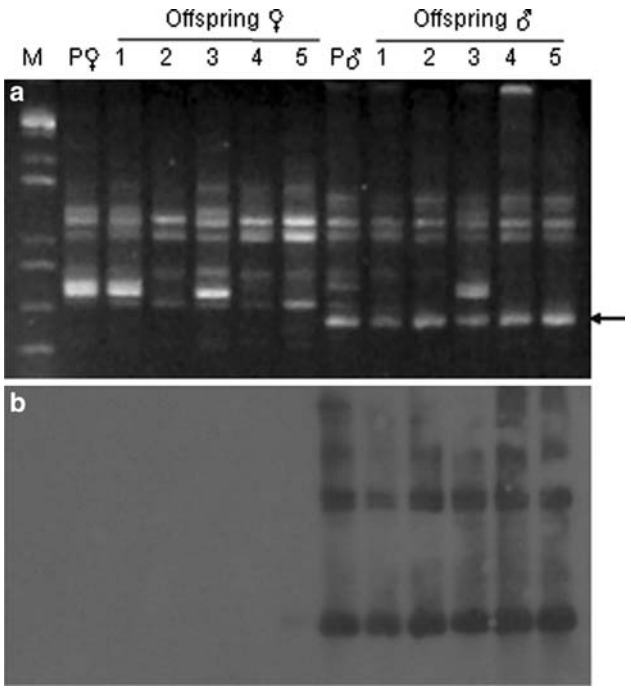

Fig. 2 **a** RAPD markers obtained with primer OPG08 in *Rumex acetosa* parental plants as well as in five female and five male individual plants of the offspring. *Arrows* point to a male specific 600 bp fragments. **b** Southern hybridisation pattern of the cloned 600 bp fragment on RAPD bands in Fig. 1a. The hybridisation pattern confirmed that the cloned DNA corresponded to the Y-linked polymorphic marker, hybridising not only with the 600 bp band but also with other Y-linked markers of higher molecular weight. *M* = 1 kb ladder

of *R. acetosa* (Fig. 3). Both Y_1 and Y_2 only hybridised to male genomic DNA (Fig. 3). Enzymes having no restriction site in the monomers of the satDNAs (*Bam*HI in RAYSII, and *Hin*dIII, *Eco*RI and *Kpn*I in RAYSIII) left the satDNAs unrestricted. Enzymes having more than one restriction site in the monomer (*Hin*fI in RAYSII and RAYSIII) cut the satDNAs into small fragments. Those enzymes having only one restriction site in monomers of the satDNAs (*Rsa*I and *Hin*dIII in RAYSII and *Bam*HI in RAYSIII) produced a ladder pattern in which each band corresponded to multiples of the monomeric unit. Digestions with these latter enzymes indicated that RAYSII and RAYSIII satDNAs comprised repeating units of 700 and 1,100 bp, respectively. The hybridisation patterns of RAYSII and RAYSIII differed from that produced by RAYSI (data not shown), another *R. acetosa* Y-specific satDNA with repeating units of 930 bp (Shibata et al. 2000a, b).

Two sets of primers were designed from Y_1 and Y_2 sequences in order to amplify the complete monomers of each of the two satDNAs. Primers in each set were spaced by only one nucleotide, but had an inverse orientation which allowed specific amplification of sequences that were arranged in tandem when one of the primers anneals to one repeating unit and the other to the adjacent one. PCR reactions produced the expected fragments of approximately 700 and 1,100 bp, corresponding to the monomers of RAYSII and RAYSIII satDNAs, respectively. The resulting PCR fragments were cloned and five independent repeats of each satDNA were sequenced and analysed. The sequences of RAYSII and RAYSIII are AT rich, and have 68 and 69% of AT content, respectively.

FISH confirmed that RAYSII and RAYSIII are Y specific, although they are differentially located in the two Y chromosomes of *R. acetosa* (Fig. 4). Whilst RAYSIII is distributed in different loci along the two heterochromatic Ys, the RAYSII satDNA was found to be in two big clusters in the Y_1 chromosome, one of which maps around the centromere. The FISH patterns of these two satDNAs also differ from that showed by Y-specific satDNA RAYSI (Fig. 4).

A study of the internal organisation of RAYSII and RAYSIII indicated that monomers of both satDNAs could be subdivided into six and nine sub-repetitions of 120 bp, respectively. Neighbour-joining trees generated with the 120 bp repeats from either RAYSII or RAYSIII also suggest that repetitions in both satDNA are likely to have evolved through duplications of an intermediate repeat of 360 bp. Thus, the six 120 bp subunits in RAYSII were grouped in pairs that denote the existence of two repetitions of 360 bp (Fig. 5), whilst in RAYSIII the tree showed the occurrence of two duplications of 360 bp in the centre of the monomer and three separate 120 bp subrepeats at the end of the monomer (Fig. 5).

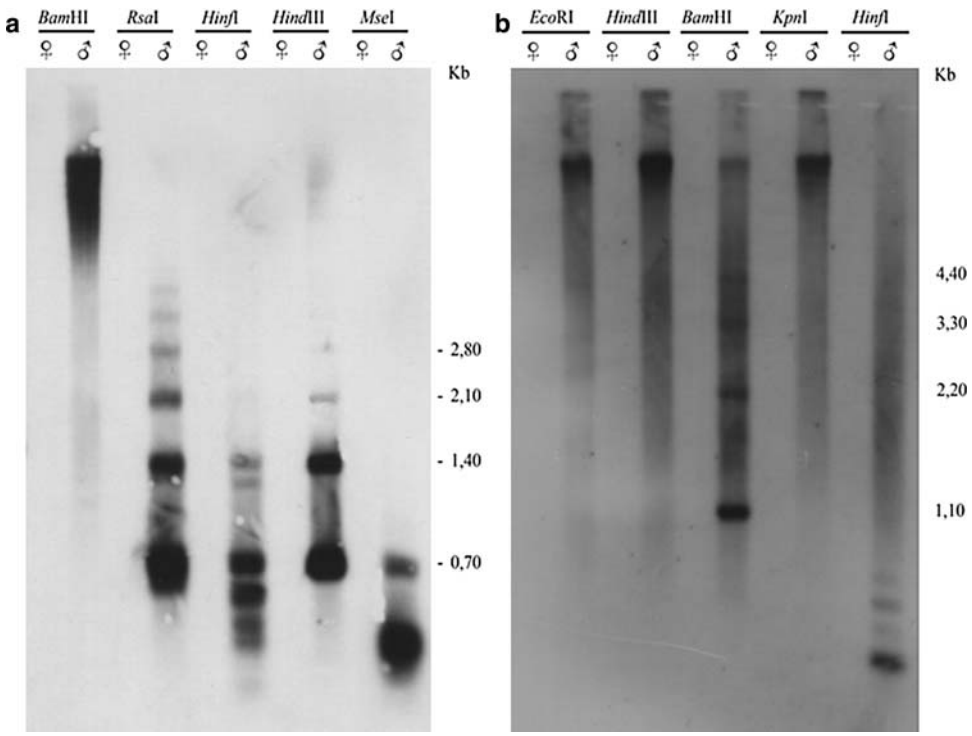

Fig. 3 Genomic organisation of RAYSII and RAYSIII (**b**) satellite DNAs. Genomic DNA from male and female plants of *R. acetosa* was restricted with different enzymes and hybridised with cloned Y_1 (**a**) and Y_2 (**b**) RAPD markers

A search of homology with existing sequences in the databases indicated that RAYSII and RAYSIII monomers maintain a certain degree of identity with monomers of RAYSI, another Y-specific satDNA of *R. acetosa* (Shibata et al. 1999), as well as with those of RAE730, a family of repeated DNA specifically located in an autosomal supernumerary segments of *R. acetosa* (Shibata et al. 2000b). The consensus sequence of RAYSII monomers has 61% homology with RAYSI in a fragment of 557 bp and 58% homology with the complete monomer of the RAE730 satDNA. On the other hand, RAYSIII consensus sequence shared 58% homology with a 778 bp fragment of RAYSI monomer and 59% homology with the complete sequence of RAE730 monomer (data not shown).

Evolution of RAYSII and RAYSIII satDNAs

The conservation of RAYSII and RAYSIII satDNAs in the different species of the genus *Rumex* was determined by Southern hybridisations and PCR. Both satDNAs were detected only in the male genome of the dioecious species of the subgenus Acetosa, section Acetosa: *R. acetosa* and *R. papillaris*, both having a complex XX/XY_1Y_2 sex chromosomes system. No hybridisation signal and no PCR amplification fragments were detected in either male or female plants of other dioecious species with the same XX/XY_1Y_2 system; neither from the section Americanae (*R. hastatulus*, North Carolina race) nor in dioecious species with an XX/XY system, such as those in subgenus Acetosa, section Scutati (*R. suffruticosus*) and in the subgenus Acetosella (*R. acetosella*). The two satDNAs were not identified in gynodioecious species of the subgenus Acetosa, section Hastati (*R. lunaria*), or in hermaphrodite species of the subgenera Acetosa (*R. induratus*), Rumex (*R. crispus* and *R. conglomeratus*) and Platypodium (*R. bucephalophorus*).

Given that the satDNAs were only detected in *R. papillaris*, the 700 and 1,100 bp PCR products representing the monomeric units of RAYSII and RAYSIII in this species were cloned in *E. coli*, and six independent monomers of each satDNA were sequenced and analysed. The monomers of RAYSII and RAYSIII satDNAs in *R. acetosa* and *R. papillaris* showed 95–97% homology. A phylogenetic analysis based on the neighbour-joining method, using 22 monomeric sequences of RAYSII and RAYSIII from *R. acetosa* and *R. papillaris*, and also 13 monomers of the RAYSI satDNA from *R. acetosa*, *R. papillaris* and *R. intermedius*, is shown in Fig. 6. RAYSI, RAYSII and RAYSIII are perfectly separated into three different clusters, indicating that the three satDNAs, although having a common origin and location, differentiated from each other before the separation of *R. acetosa* and *R. papillaris*. Although in the RAYSI satDNA it is possible to establish the existence of two subfamilies, named RAYSI-J and RAYSI-S (Navajas-Pérez et al. 2005a), the phylogenetic tree obtained with RAYSII and RAYSIII sequences does not reflect the existence of different subfamilies in these satDNAs (Fig. 6).

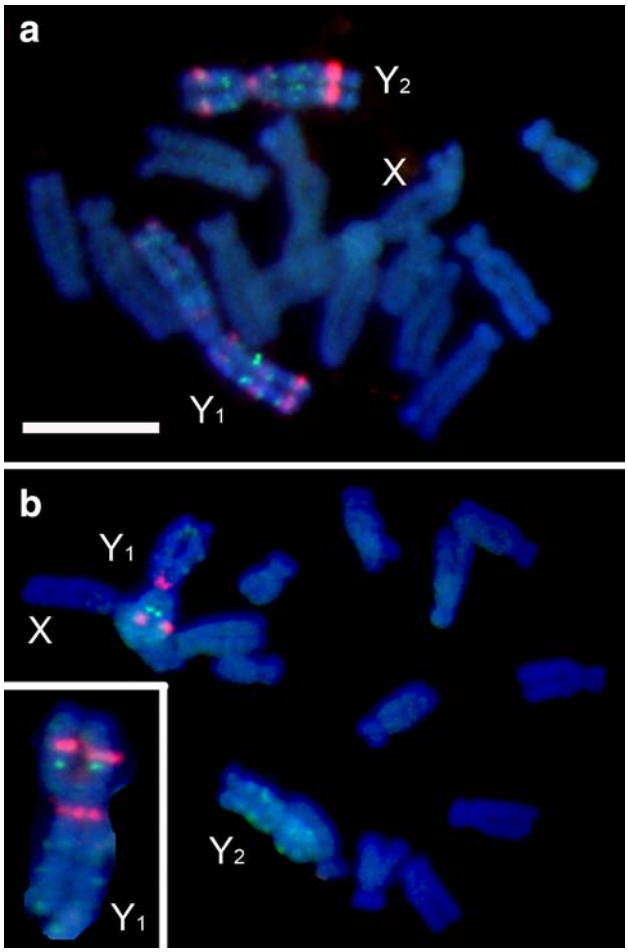

Fig. 4 Chromosomal distribution of RAYS tandem repeats in *R. acetosa* analysed with bicolour FISH. Mitotic metaphase chromosomes of male *R. acetosa* (counterstained by DAPI, blue) were hybridised with **a** RAYSI (*red signals*, Cy3 labelled) and RAYSIII (*green signals*, SpectrumGreen labelled), **b** RAYSII (*red signals*) and RAYSIII (*green signals*). The X, Y_1 and Y_2 chromosomes are indicated, bar = 10 µm (colour available in online version only)

Discussion

By using F1 populations derived from a controlled cross and a BSA analysis, we have identified a number of RAPD markers linked to the sex chromosomes of *R. acetosa*. RAPDs, RFLPs, AFLPs and microsatellite Y-linked markers have been previously detected in different dioecious plants (Jiang and Sink 1997; Mulcahy et al. 1992; Di Stilio et al. 1998; Zhang et al. 1998; Sakamoto et al. 1995); but the use of a BSA approach in male and female offspring from a controlled cross has allowed us to identify RAPD markers that were linked not only to the Y but also to the X chromosomes of this *R. acetosa*. Given the dominant nature of these markers, the BSA approach does not allow the recognition of X-linked markers that are heterozygous in the female parents (Peil et al. 2003), and therefore the number of the identified X-linked markers was low.

Although the parental plants were derived from the same natural population, the level of polymorphism detected by RAPD markers in this work (31% of polymorphic markers) is comparable with that obtained with these types of markers in other species (Shirkot et al. 2002; Khadka et al. 2002; Obara et al. 2002). The level of polymorphisms was lower than that obtained by Rahman and Ainsworth (2004) with AFLP markers in *R. acetosa*, which is indicative of the high level of genetic variability in the natural populations of this species. Despite the high level of polymorphism, the number of polymorphic markers linked to the Y chromosomes was relatively very low (2.6%). Given that the two Y chromosomes of *R. acetosa* are the largest of the karyotype, representing about 26% of the male genome (Wilby and Parker 1988), the low level of the observed Y-linked markers could indicate that the differentiation of the X and Y chromosomes of *R. acetosa* is still weak. Nevertheless, since previous cytogenetic and molecular studies have indicated that the X and Y chromosomes of this species are enriched in repetitive DNA (Ruiz Rejón et al. 1994; Shibata et al. 1999, 2000b), it is more likely that the low number of polymorphic Y-linked markers obtained is caused by a low complexity in Y-chromosome sequence composition. The low number of Y-linked AFLP markers detected by Rahman and Ainsworth (2004) in *R. acetosa* and Stehlik and Blattner (2004) in *Rumex nivalis*, also supports our conclusion for a low complexity of *Rumex* Y-chromosome sequence composition.

Different cytogenetic studies have revealed that the Y chromosomes of *R. acetosa* are DAPI positive and are therefore composed of AT-rich sequences (Ruiz Rejón et al. 1994; Shibata et al. 1999; Lengerova and Vyskot 2001). Up to now, two AT-rich satDNAs were identified in the Y chromosomes, the RAE180 satDNA that is located in the Ys and also in an autosomal locus (Ruiz Rejón et al. 1994; Shibata et al. 2000b), and the RAYSI satDNA that is specifically located in both Y_1 and Y_2 chromosomes (Shibata et al. 1999). The two Y-linked RAPD markers identified and cloned in this work represent two new satDNAs that are specifically located in the Ys of *R. acetosa*: RAYSII and RAYSIII. The sequences of these two satDNAs are AT rich and show about 60% homology with both the Y-specific satDNA RAYSI and the autosomal satDNA RAE730 (Shibata et al. 1999, 2000a). Given that the monomers of RAYSII and RAYSIII satDNAs, as occurs in the RAYSI and RAE730 (Navajas-Pérez et al. 2005a), can be divided into six and nine subrepeats of about 120 bp (Fig. 4), it is likely that the four satDNAs originated from a common ancestral satDNA with a repeating unit of 120 bp (Fig. 7). Moreover, since the homology between each three adjacent repetitions of 120 bp is higher, the actual satDNAs

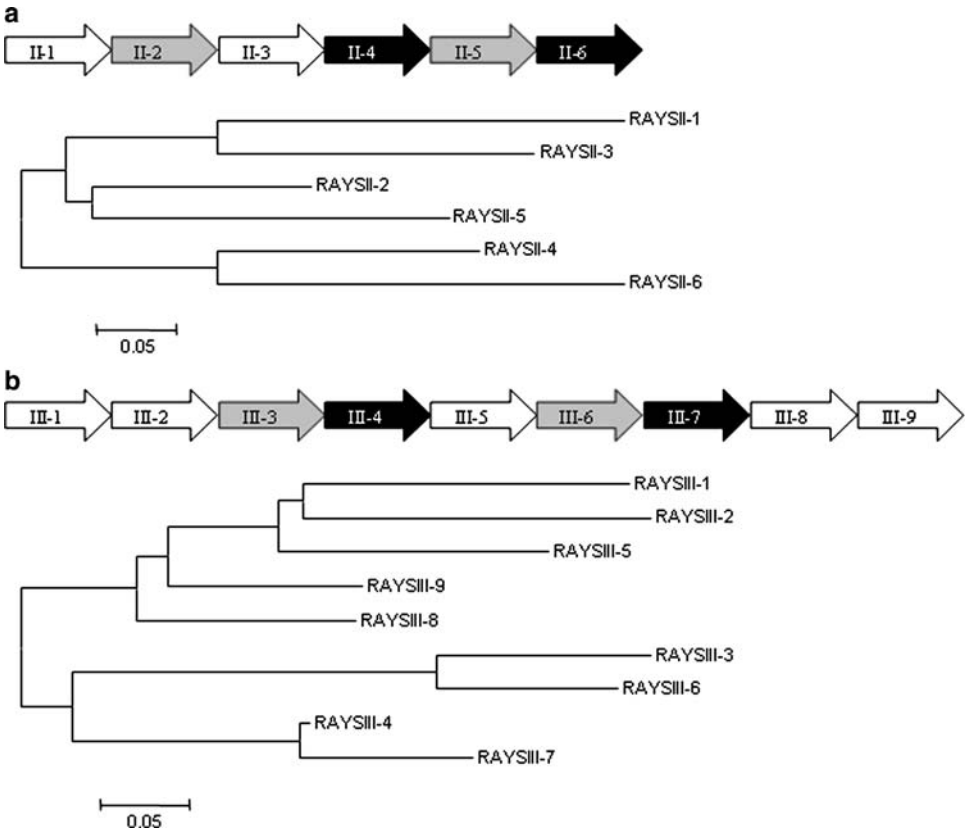

Fig. 5 Internal repetitions in the RAYSII (**a**) and RAYSIII (**b**) monomers. The repeating units of the two satDNAs can be divided into six and nine subrepeats of 120 bp. The neighbour-joining trees of the different subrepeats in each satDNA monomer suggest that the present monomers in RAYSII and RAYSIII satDNAs could have evolved from repeats of 120 bp throughout satDNAs of 360 bp

in the Y chromosomes of *R. acetosa* could have evolved throughout an intermediate satDNA of 360 bp repeats (Fig. 7). Phylogenetic analysis of the 360 bp subrepeats in the four satDNAs (Fig. 7) indicates that they did not originate from the same 360 satDNA, but evolved throughout different duplication events of the ancestral 120 bp satDNA, giving rise to different intermediates of 360 bp (Fig. 7). The evolution of all these satDNAs has therefore resulted in an increase in the length of the repeating units, an evolutionary process that could be promoted in those genomic regions that, as occurs in the *R. acetosa* Y chromosome, have low rates of recombination (Charlesworth et al. 1994).

The coexistence of different related satDNAs and subfamilies in different clusters of the Y chromosomes of *R. acetosa* could indicate that the intraspecific homogenisation mechanisms or concerted evolution of the Y-specific satDNAs are not very efficient. Loss of recombination between X and Y chromosomes would reduce the evolution rate of Y-specific satDNAs (Navajas-Pérez et al. 2005a) and promotes the appearance of different subfamilies and related satDNAs in different loci of the Y chromosomes. The concerted evolution observed amongst units of the same satDNA suggest, however, the occurrence of other mechanisms of homogenisation and amplification, such as the intrachromatid recombination (Skaletsky et al. 2003) and amplification of satDNA units by means of the rolling circle replication model (Feliciello et al. 2005). High intrachromosomal similarity has also been demonstrated for transposable elements in *S. latifolia* (Kejnovsky et al. 2007) and tandem repeats in *Anopheles gambiae* (Krzywinski et al. 2004). The rolling circle amplification mechanism could explain why different RAYS satellites do not coexist within the same chromosome cluster, but are located separately in different clusters of the Y_1 and/or Y_2 chromosomes. A variant of an ancestral satDNA could replicate and integrate in different loci of the Y chromosomes, followed by the amplification of the number of repeats in each cluster, to give rise to a new related satDNA. Nevertheless, the specific location of RAYSII in the Y_1 chromosome could indicate that RAYS satellites could also have originated following the amplification of a specific variant of an existing satellite in one specific locus, followed by the dispersion of this new satDNA to other locations in the Y chromosomes. As proposed by Cermak et al. (2008), the specific distribution of these satDNAs in the Ys indicates the existence of exclusive evolutionary forces governing the amplification of tandemly repetitive DNA in these non-recombining chromosomes.

Theories about the evolution of Y chromosomes predict that the gradual suppression of recombination between X and Y promotes the degeneration of the Y by the loss of gene function (Filatov et al. 2000) and by the expansion of tandemly repetitive DNA sequences (Charlesworth 1996;

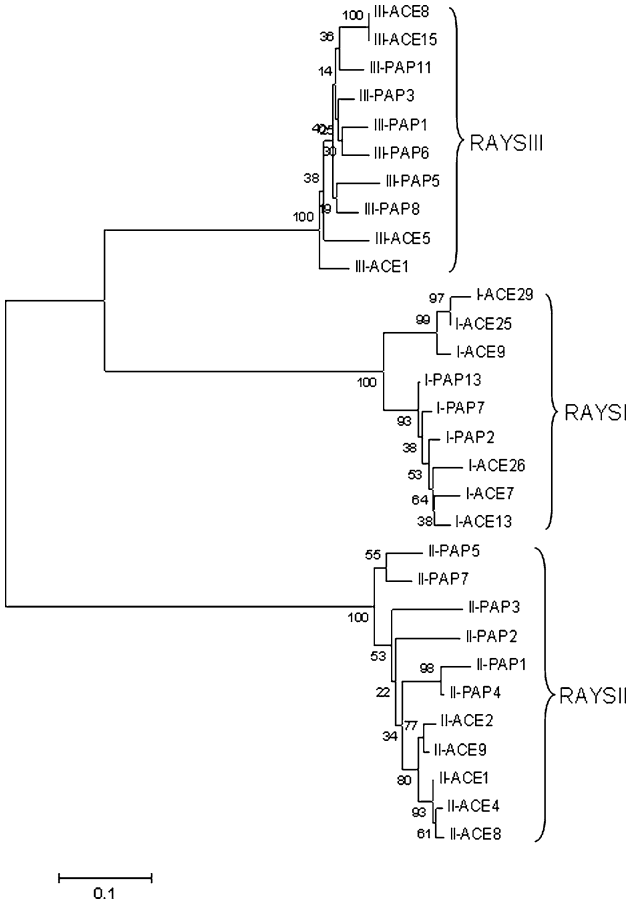

Fig. 6 Neighbor-joining tree from the aligned Y-chromosome-specific sequences of the satellite DNAs (RAYSI, RAYSII and RAYSIII) in *R. acetosa* (ACE) and *R. papillaris* (PAP). Bootstrap values from 1,000 bootstrap re-samples are given for each branch

Jobling and Tyler-Smith 2003). Our data demonstrates that in the *Rumex* species with complex sex chromosome systems, such as *R. acetosa* and *R. papillaris*, the Y chromosomes are especially enriched in satDNAs. With the exception of RAE180, which is located in the Ys and an autosomal locus (Shibata et al. 2000b), the three related satDNAs, RAYSI, RAYSII and RAYSIII, are specifically located in the Y chromosomes. The amplification and expansion of satDNA sequences in the Ys of *Rumex* could have accelerated the differentiation of the Ys in *Rumex* species with multiple sex chromosome systems in the section Acetosa. In fact, these satDNAs are absent in dioecious *Rumex* species with XX/XY sex chromosomes systems, such as *R. suffruticosus* and *R. acetosella*, species from which multiple sex chromosomes systems seem to have been derived (Navajas-Pérez et al. 2005b). The Y chromosomes of the dioecious species of the genus *Silene*, although originating about the same time as those of *Rumex* (Filatov and Charlesworth 2002; Navajas-Pérez et al. 2005a), and are neither heterochromatic nor have they accumulated so many satDNA sequences as those of *Rumex*, although recent studies have indicated that the *Silene* Y has accumulated certain Y-specific tandem repetitive elements (Hobza et al. 2006; Kejnovsky et al. 2006; Kubat et al. 2008; Marais et al. 2008). Therefore, the high level of accumulation of satDNA sequences in the recent sex chromosomes of plants, as occurs in some species of *Rumex*, are not only signals of advanced stages of degeneration of Y-chromosome evolution, but may also contribute to the expansion of the non-recombining region and Y-chromosome size in the early stages of sex chromosome evolution (Jamilena et al. 2008).

Taken in the round, our data indicate that the two heterochromatic Y chromosomes of *R. acetosa* are composed of high levels of tandemly repetitive DNA sequences and are organised in different Y-specific satDNAs, although occupying separated locations along the chromosome, they all have a common origin. The distribution of Y satDNAs in different hermaphrodite and dioecious species of the genus *Rumex* suggests that the amplification of tandemly repetitive DNA sequences in the Ys is not necessary to suppress recombination between Xs and Ys, but could be a rapid evolutionary force that accelerates the differentiation of the Y since the early stages of its evolution.

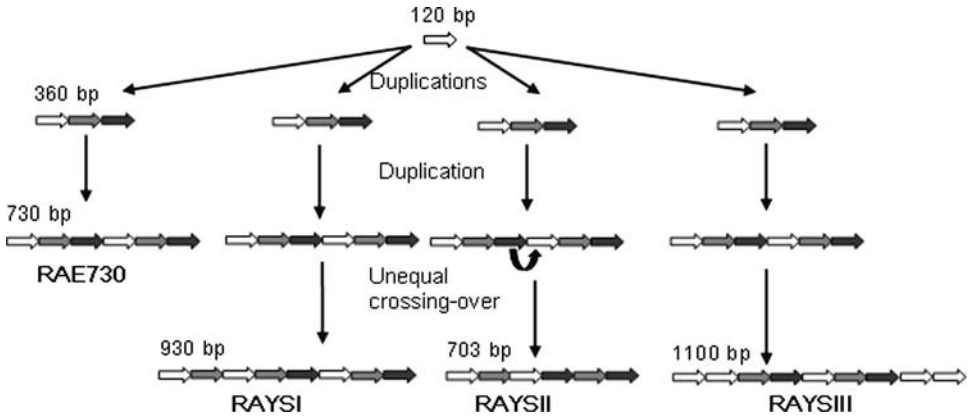

Fig. 7 Model for the evolution of the RAE730, RAYSI, RAYSII and RAYSIII satDNAs in the genome of *R. acetosa*. The repeating units of the four satDNAs are evolved from the same 120 bp unit throughout four different duplication events that originated intermediate satDNAs of 360 bp

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