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The evolution of sex chromosomes in the genus *Rumex* (Polygonaceae): Identification of a new species with heteromorphic sex chromosomes

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Abstract

The structural features and evolutionary state of the sex chromosomes of the XX/XY species of *Rumex* are unknown. Here, we report a study of the meiotic behaviour of the XY bivalent in *Rumex acetosella* and *R. suffruticosus*, a new species which we describe cytogenetically for the first time in this paper, and also that of the XY_1Y_2 trivalent of *R. acetosa* by both conventional cytogenetic techniques and analysis of synaptonemal complex formation. Fluorescent *in situ* hybridization with satellite DNA and rDNA sequences as probes was used to analyse the degree of cytogenetic differentiation between the X and Y chromosomes in order to depict their evolutionary stage in the three species. Contrasting with the advanced state of genetic differentiation between the X and the Y chromosomes in *R. acetosa*, we have found that *R. acetosella* and *R. suffruticosus* represent an early stage of genetic differentiation between sex chromosomes. Our findings further demonstrate the usefulness of the genus *Rumex* as a model for analysing the evolution of sex chromosomes in plants, since within this genus it is now possible to study the different levels of genetic differentiation between the sex chromosomes and to analyse their evolutionary history from their origin.

Introduction

The genus *Rumex* L. (Polygonaceae) constitutes a model system to study dioecy and sex chromosome evolution, being composed by hermaphroditic, polygamous, gynodioecious and dioecious species (Degraeve 1976, 1980). It has recently been demonstrated that dioecy has appeared once in this genus, gynodioecy constituting an intermediate stage from hermaphroditism (Navajas-Pérez *et al.* 2005a). Among the dioecious species, there are two monophyletic lineages (Navajas-Pérez *et al.* 2005a): an older one comprised of species with an XX/XY chromosome system and a Y-based sex-determining mechanism; and a younger one with species having an XX/XY_1Y_2 chromosome system together with a sex-determination mechanism based on the X:A ratio. The group with the complex sex-chromosome system includes species classified within the *Acetosa* section of the subgenus *Acetosa* such as *R. acetosa*, *R. papillaris* or *R. intermedius*. They are characterized by similar morphological and karyological features (Löve 1957, Smith 1969, Degraeve 1976, Wilby and Parker 1988, Ainsworth *et al.* 1999). Thus, the two Y chromosomes are almost heterochromatic and have accumulated two satellite-DNA families, RAE180 and RAYSI (Ruiz Rejón *et al.* 1994, Shibata *et al.* 1999, 2000, Navajas-Pérez *et al.* 2005b, 2006), and also different transposable elements (Mariotti *et al.* 2006). However, the XX/XY group is composed of species classified early in different sections of the subgenera *Acetosella* and *Acetosa* (Navajas-Pérez *et al.* 2005a), namely: *R. acetosella* and *R. graminifolius* (subgenus *Acetosella*), *R. hastatulus*, and *R. paucifolius* (section Americanae, subgenus *Acetosa*), and *R. suffruticosus*, an endemic of central, northern, and north-western mountains of the Iberian Peninsula (López González 1990). It is worth mentioning that this last species, which is dioecious, has traditionally been classified as a member of the *Scutati* section, subgenus *Acetosa*, in which only hermaphroditic-polygamous relatives have been reported (López González 1990).

The structural features and evolutionary state of the sex chromosomes of the XX/XY species are quite unknown. Notwithstanding this, classical cytogenetic studies were conducted in *R. acetosella* (Löve 1944, Singh 1971, Degraeve 1980), *R. paucifolius* (Smith 1968) and *R. hastatulus* (Smith 1964, 1969). These studies revealed the putative existence of a pair of heteromorphic sex chromosomes in these species, independently of their ploidy level (Degraeve 1980), and also that, in *R. hastatulus*, there is an additional chromosomal race XX/XY_1Y_2 (North Carolina race) that would have evolved secondarily from an XX/XY race (Texas race) (Smith 1964). Navajas-Pérez *et al.* (2005a) confirmed this finding by means of molecular systematic analyses and also suggested the possible existence of a heteromorphic bivalent in the male meiosis of *R. suffruticosus*, a species for which there are no previous chromosomal data. Taking into account these results, we here analyse the chromosome sets of *R. suffruticosus* (for the first time) and *R. acetosella* to test the existence of sex chromosomes and, if they exist, to shed light on their evolutionary stage of sex-chromosome differentiation.

Materials and methods

Plants of *R. suffruticosus* and *R. acetosella* (Puerto de Navacerrada, Segovia, Spain), and *R. acetosa* (Capileira, Granada, Spain) were used in this study. According to Löve (1944), diploid representatives of *R. acetosella* should be found both in central and southern Spanish regions. However, our sampling in several populations of these regions has failed to fulfil the prediction, indicating that diploids are quite rare in the Iberian Peninsula (López González, personal communication and our own observations).

Root tips were obtained from seeds germinated in Petri dishes at 25°C and then pretreated with 2 mM 8-hydroxiquinoleine for 1–2 h at germinating temperature, followed by 1–2 h at 4°C for metaphase accumulation. They were then fixed in ethanol–glacial acetic acid (3:1) until required. Floral buds were also fixed using the same procedure. Fresh anthers at zygotene-pachytene stages were prepared for synaptonemal complex isolation and silver staining as described by Cuñado *et al.* (1996). Nuclei of the first meiotic division were examined using a Jeol 1200EX electron microscope and photographed on Agfa-Scientia film. Colour figures and overlays were prepared using Adobe Photoshop 7.0 software.

Also, by Southern-blot hybridization (see Garrido-Ramos *et al.* 1999), we tested the presence of two *R. acetosa* satellite-DNA families, RAE180 and RAYSI (Shibata *et al.* 2000, Navajas-Pérez *et al.* 2005b, 2006), in *R. acetosella* and *R. suffruticosus*. Probe labelling, hybridization, and detection of hybridization sites was performed using the nonradioactive chemiluminescence method (ECL, Amersham), following the manufacturer's instructions. Hybond N+ nylon filters were hybridized for 12–16 h at 42°C with horseradish peroxidase-labelled probes at 10 ng/ml of ECL hybridization buffer containing 6 M urea, 0.5 M NaCl, and 5% blocking agent. After hybridization, the filters were washed twice, for 20 min each, in 6 M urea, $0.1 \times$ SSC and 0.4% SDS at 42°C (high-stringency conditions) or $1 \times$ SSC and 0.4% SDS at 55°C for 10 min (low-stringency conditions). The membranes were then washed twice in $2 \times$ SSC at room temperature for 5 min. The 6 M urea in the hybridization and wash buffers is equivalent to 50% formamide (Amersham).

For fluorescence *in situ* hybridization (FISH) experiments, we followed the procedure of Cuñado *et al.* (2000). Briefly, chromosome preparations were pretreated with DNase-free RNase (100 μg/ml) and pepsin (500 μg/ml), dehydrated in an ethanol series, and air-dried. The hybridization mixture, consisting of 50 ng of DNA probe and 500 ng of sheared salmon sperm DNA in 50% (v/v) formamide, 10% (w/v) dextran sulphate and $2 \times$ SSC (SSC is 50 mM NaCl, 15 mM sodium citrate), was denatured on a heating block at 90°C for 10 min and placed on ice for 5 min. The probe was applied to the slides (15 μl/slide), covered with a coverslip and sealed. The slides were incubated at 75°C for 3 min and then at 37°C overnight in a modified thermocycler. To locate the RAE180 satellite-DNA family in *R. acetosa*, we used plasmid inserts of the clone RAE180_ra_31 (EMBL/GenBank accession number AJ580332). RAE180 satellite-DNA species-specific probes for *R. suffruticosus* and *R. acetosella* were obtained by means of a set of specific primers and PCR settings described previously (Navajas-Pérez *et al.* 2005b). PCR products were purified, cloned, and analysed as described previously (Navajas-Pérez *et al.* 2005b). Among the recombinant clones obtained, we used RAE180_suff_71 from *R. suffruticosus* (EMBL/GenBank accession number AM397924) and RAE180_ace_22 from *R. acetosella* (accession number AM397925). To locate rDNA sequences, we used the following. (i) The clone pTa71 (Gerlach and Bedbrook 1979), containing a 9 kb EcoRI fragment of *Triticum aestivum* consisting of the 18S–5.8S–25S rRNA genes and the corresponding spacer regions. Digoxygenin-dUTP was incorporated by nick translation following the manufacturer's instructions (Roche) and it was detected by FITC-antibodies. (ii) Plasmid pCT4.2 containing the 5S rRNA gene from *A. thaliana* as a 500 bp insert cloned in pBlu. Biotin dUTP was also incorporated by nick translation and detected by avidin-Cy3 antibodies. Preparations were counterstained with propidium iodide (1 μg/ml) or with DAPI, 4′,6-diamidino-2-phenylindol, (1 μg/ml) and mounted with Vectashield (Vector Labs).

Results

The meiotic chromosome complement in pollen mother cells (PMCs) of male *R. suffruticosus* consists of seven similarly sized bivalents and a conspicuous monochiasmate heteromorphic bivalent in which the size of one of the chromosomes involved is roughly twice that of the other (Figure 1A, B, C). It can be concluded that *R. suffruticosus* would have a basic chromosome number of x = 8 and a sex chromosome system XX/XY. On the other hand, tetraploid *R. acetosella* and diploid *R. acetosa* have a basic autosomal chromosome number of x = 7, but whereas only a monochiasmate heteromorphic bivalent was observed in males of *R. acetosella* (XX/XY; Figure 2A, B), *R. acetosa* males showed six homomorphic bivalents and a sexual trivalent in which each Y chromosome was associated with one of the terminal regions of the X chromosome (XX/XY_1Y_2; Figure 2C). While the Y chromosomes of *R. acetosa* are heteropicnotic and show DAPI + and C + bands (Ruiz Rejón *et al.* 1994, see also Figure 2C, 2D), *R. suffruticosus* and *R. acetosella* lacked any contrastable DAPI + or C + bands in their chromosome complements (Figures 1A, 1C and 2A).

FISH using rDNA probes indicated that rDNA hybridization signals were not associated with the sex chromosomes in any of these species (Figures 1A, 2A and 2C). *R. suffruticosus* contained one 45S rDNA locus and one 5S rDNA locus located in different chromosomes (Figure 1A). The tetraploid *R. acetosella* had four 45S rDNA loci and two 5S rDNA loci in different chromosomes (Figure 2A). In *R. acetosa*, the 45S ribosomal unit was present in two

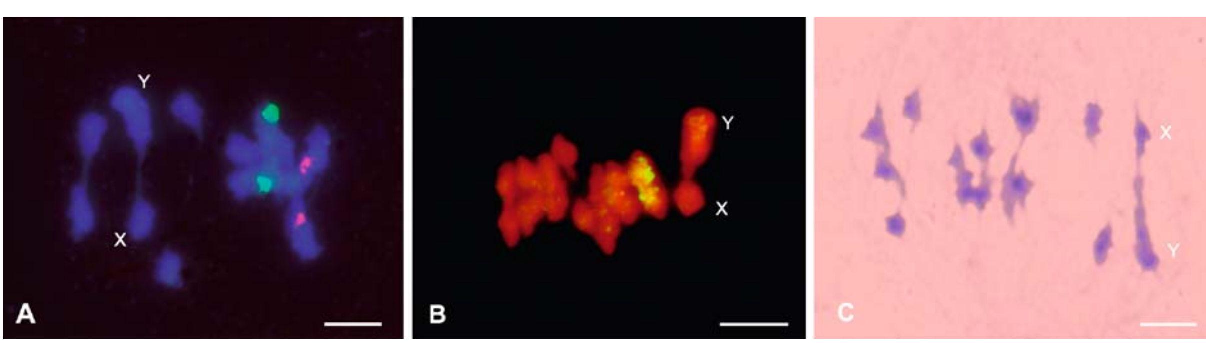

Figure 1. FISH (**A**, **B**) and C-banding (**C**) in metaphase I pollen mother cells of *R. suffruticosus*. (**A**) Location of 45S and 5S rDNA sequences indicated by green and red signals, respectively. (**B**) Location of the RAE180 repetitive family of sequences. (**C**) Contrastable C-bands are not apparent in the sex bivalent. Sex chromosomes are indicated. Bars represent 5 μm.

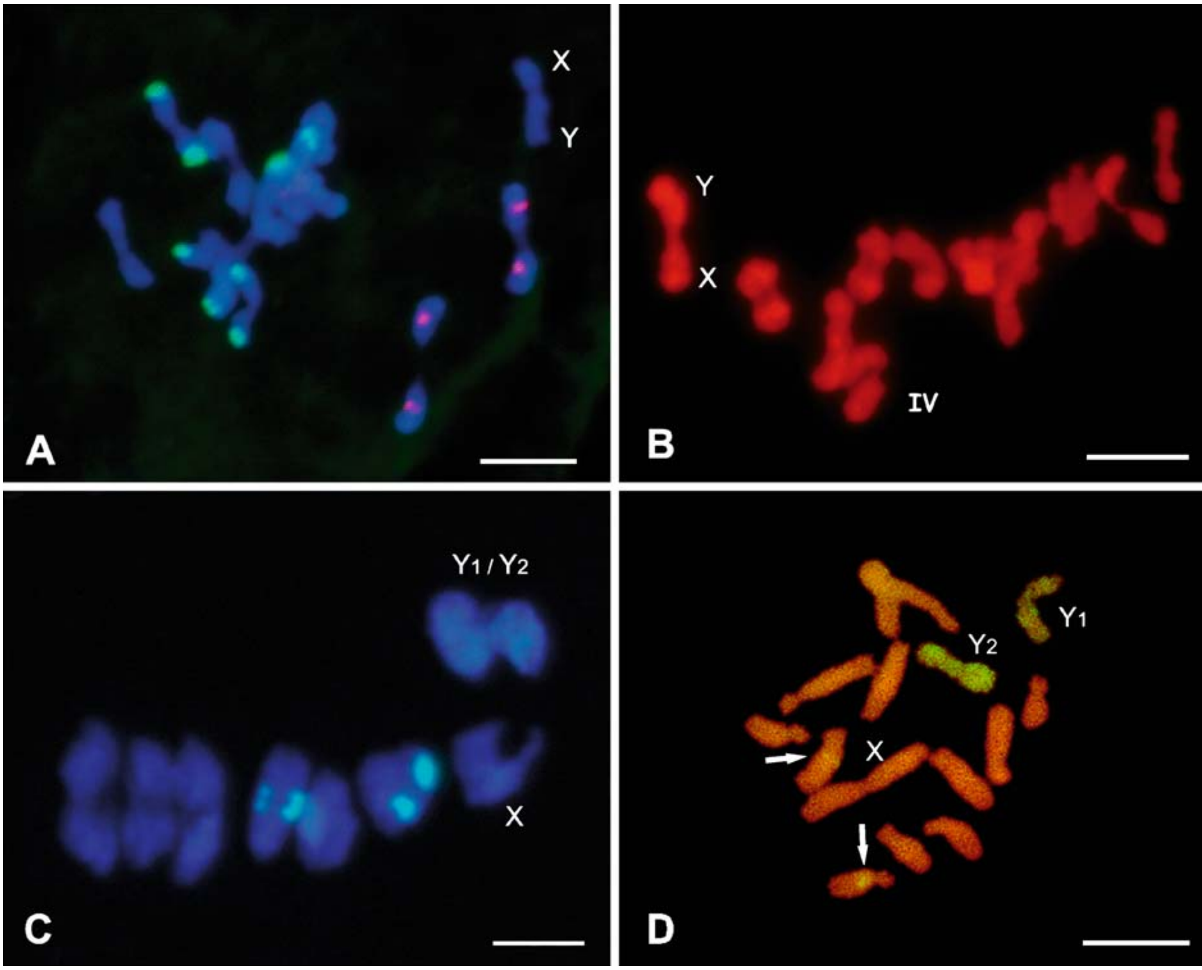

Figure 2. FISH in metaphase I pollen mother cells of *R. acetosella* (4×) (**A**, **B**) and *R. acetosa* (**C**), and in a mitotic metaphase of a *R. acetosa* male (**D**). (**A**) Location of 45S and 5S rDNA sequences indicated by green and red signals, respectively. (**C**) Location of the 45S rDNA sequence. (**B**, **D**) Location of the RAE180 repetitive family of sequences. Arrows in D indicate an additional punctual RAE180 site present in a pair of autosomes. Sex chromosomes are indicated. Bars represent 5 μm.

autosomal loci (Figure 2C), while there is one 5S rDNA locus (Koo *et al*. 2004).

Both Southern-blot hybridization and PCR amplification techniques demonstrated the presence of RAE180 satellite-DNA sequences in the three species analysed (see Figure 5). However, the Y-specific RAYSI satellite-DNA sequences were present only in the genome of *R. acetosa* but not in *R. suffruticosus* and *R. acetosella* (Navajas-Pérez *et al.* 2006, see also Figure 5). This result was coincident both after low-stringency and after high-stringency conditions. Then, for the three species, FISH using species-specific RAE180 satellite-DNA probes was employed. These sequences are located mainly in the Y chromosomes of *R. acetosa* (Figure 2D) but are restricted to a single autosomal bivalent in males of *R. suffruticosus* (Figure 1B) and apparently absent from *R. acetosella* chromosomes (Figure 2B).

Electron-microscopic observations of whole-mount preparations of synaptonemal complexes (SCs) at pachytene in these three species confirm the results mentioned above with respect to their basic chromosome number, and also to the tetraploid condition of the *R. acetosella* males (Figure 3A–C). In addition, they also provide the possibility of performing a more accurate analysis of the meiotic

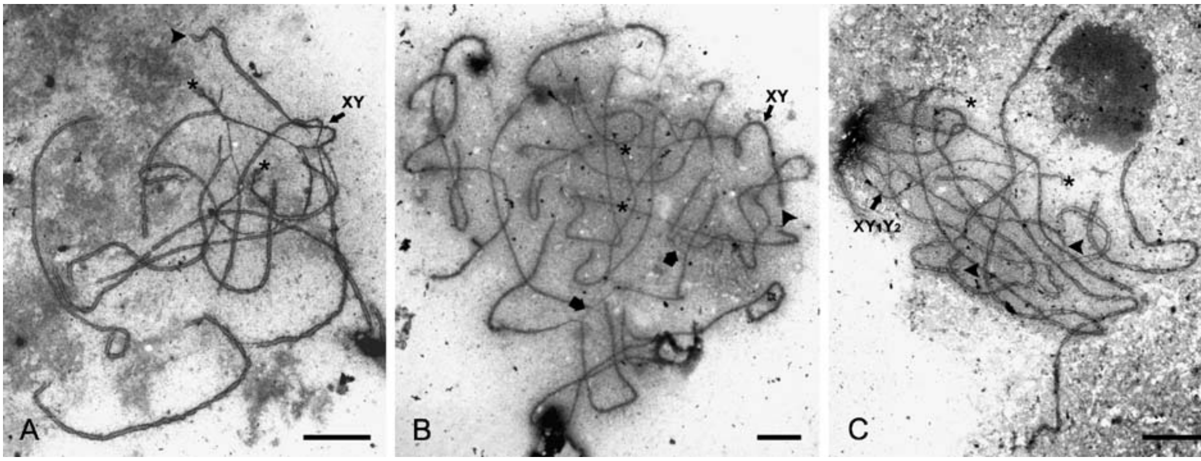

Figure 3. Electron micrographs of silver-stained pachytene nuclei in pollen mother cells of *Rumex: R. suffruticosus* (**A**), *R. acetosella* (4×) (**B**), and *R. acetosa* (**C**). The partially synapsed XY bivalents (**A**, **B**) and the Y_1XY_2 trivalent (**C**) are arrowed. Asterisks indicate the ends of the asynapsed chromosome ends, while arrowheads indicate the ends of the synaptonemal complex (SC). Wide arrows in B mark pairing-partner switches in two autosomal tetravalents, one in each. Bars represent 5 μm.

sex-chromosome behaviour. In pachytene nuclei of *R. suffruticosus* (Figures 3A, 4A, 4B) and *R. acetosella* males (Figures 3B, 4C, 4D), we found a partially synapsed bivalent in which the axial elements of the chromosomes differed markedly in length. In both cases, we found only one synaptic initiation point, located in a distal chromosome region. The length of the synapsed region varied among the 10 nuclei analysed in each species, being longer in late pachytene nuclei than in those with shorter autosomal SC lengths. Therefore, the possibility of some synapsis between non-homologous regions of the X and Y chromosomes cannot be ruled out. It is clear, however, that those regions involved earliest in synapsis must be homologous because the single chiasma formed between sex chromosomes, as observed at metaphase I, was invariably located there (Figure 4A–D). In all 10 sexual trivalents of *R. acetosa* analysed, homologous synapsis between the ends of the X chromosome and one end of each Y_1 and Y_2 chromosome always takes place (Figures 3C, 4E, 4F)—just those regions in which chiasmata have formed (Figure 4E). Only in one additional late pachytene nucleus did we detect a fully synapsed trivalent (Figure 4G, H), which implies the existence of non-homologous synapsis. In the three species analysed, the remaining chromosomes (autosomes) showed regular synapsis with the lateral elements of equal length (Figure 3).

Discussion

The taxonomic distribution of dioecy and sex-chromosome determination systems in flowering plants indicates that sex chromosomes have evolved recently through replicated, independent events. Plant sex chromosomes, therefore, offer opportunities to study the most interesting early stages of the evolution of sex chromosomes (Charlesworth 2002). In this sense, it is generally accepted that sex-chromosome evolution includes roughly three consecutive stages (Charlesworth 2002, Ruiz Rejón 2004): (i) the establishment of a pair of proto-undifferentiated sex chromosomes and not heteromorphism; (ii) an early stage of genetic differentiation between sex chromosomes with a short region in which recombination is suppressed and some heteromorphism; and (iii) a further state of differentiation with a larger region of non-recombination between sex chromosomes, with heteromorphism and with Y chromosome degeneration by gradual accumulation of deleterious mutations (Filatov 2005) and, subsequently or simultaneously, by the accumulation of a set of diverse repetitive sequences such as mobile elements and satellite DNAs (Bachtrog 2003, Skaletsky *et al.* 2003).

On these grounds, *R. acetosa* might represent the third evolutionary stage (Guttman and Charlesworth 1998) because the Y chromosomes of the males are

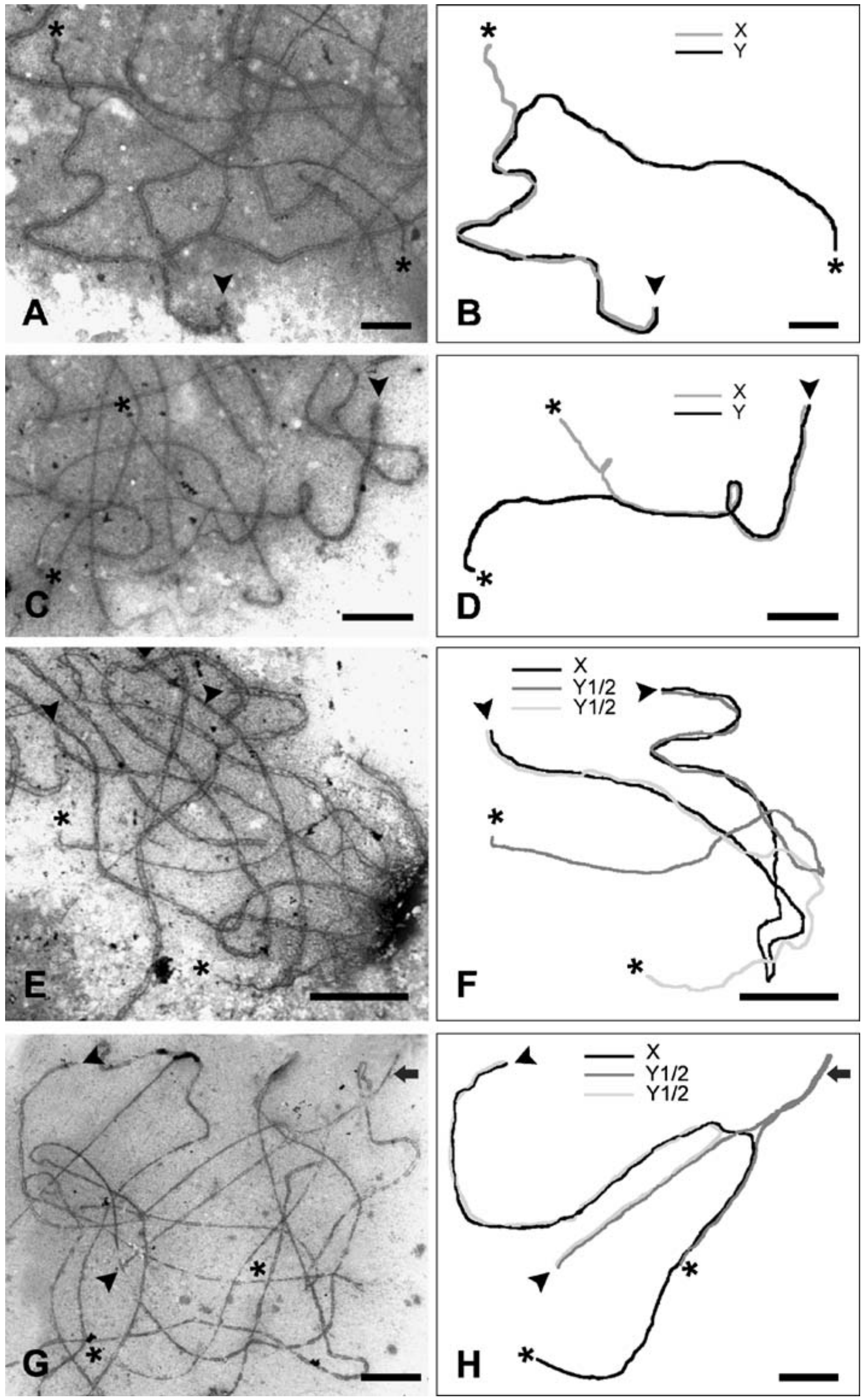

Figure 4. Electron micrographs of pachytene synaptonemal complex configurations formed by sex chromosomes in males of three *Rumex* species (**A**, **C**, **E**, **G**) and their corresponding diagrammatic representations (**B**, **D**, **F**, **H**). XY bivalent in *R. suffruticosus* (**A**, **B**) and in *R. acetosella* (**C**, **D**). Y_1XY_2 trivalent in *R. acetosa* (**E**, **F** and **G**, **H**). Asterisks indicate the ends of the asynapsed chromosome ends, while arrowheads indicate the ends of the synaptonemal complex. Arrow in G, H indicates a self-synapsed region in one of the Y chromosomes. Bars represent 5 μm.

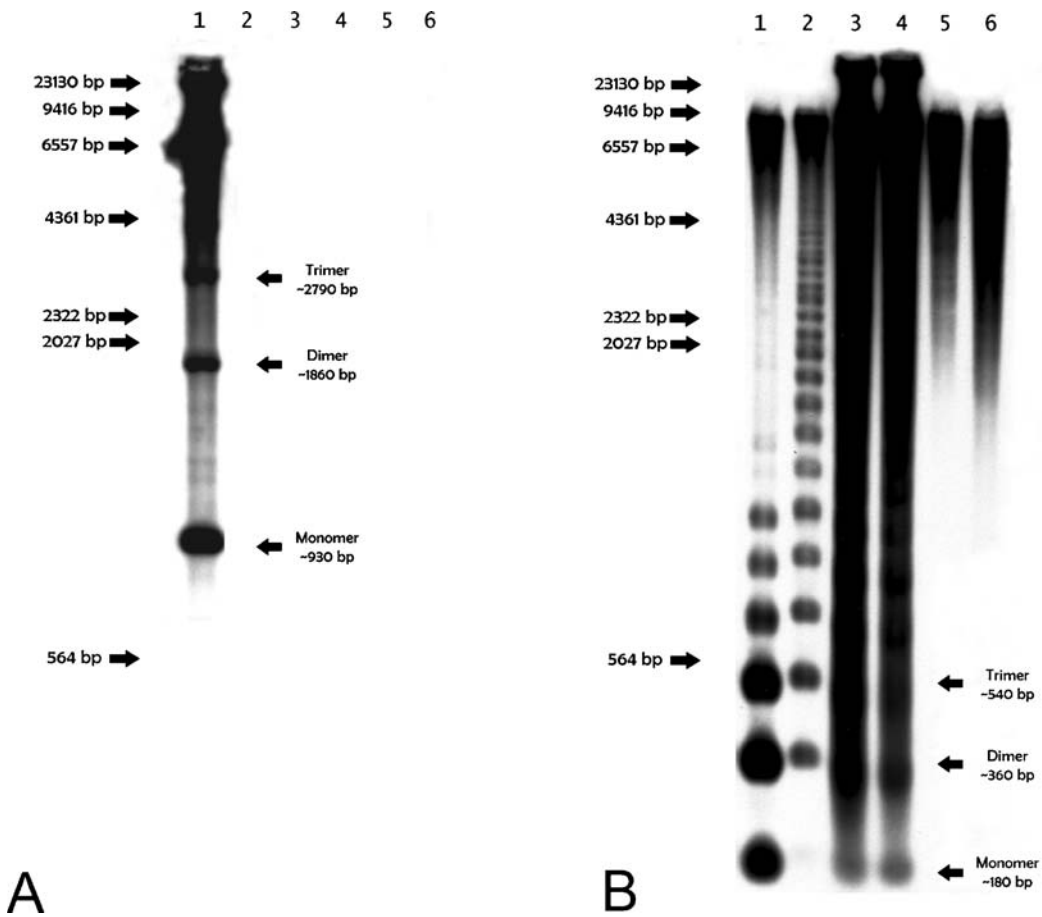

Figure 5. Southern blot hybridization using the monomeric RAYSI (**A**) and RAE180 (**B**) satellite DNA sequences. Species: (1–2) ♂♀ *R. acetosa*, (3–4) ♂♀ *R. suffruticosus*, (5–6) ♂♀ *R. acetosella*.

heterochromatinized and contain many satellite-DNA sequences (Ruiz Rejón *et al.* 1994, Shibata *et al.* 1999, 2000, Navajas-Pérez *et al.* 2006, this paper, see Figure 2D) and mobile elements (Mariotti *et al.* 2006). The satellite-DNA families were RAYSI, a Y-specific repetitive family restricted to the genomes of species having the complex XX/XY_1Y_2 chromosome system (Navajas-Pérez *et al.* 2006) and the RAE180 family found both in the XX/XY_1Y_2 and in the XX/XY species (Navajas-Pérez *et al.* 2005b; this paper). The cytogenetic differentiation between the X and the Y chromosomes is also confirmed here by the SC analysis.

R. acetosella constitutes a different situation because it forms a polyploid series with populations ranging from $2n = 2x$ to $2n = 8x$. Notwithstanding this, apparently only one pair of sex chromosomes remains after the polyploidization processes while the rest of the sex-chromosome pairs should have de-differentiated into autosomes (Degraeve 1980). We found support here for this hypothesis because only one heteromorphic sex chromosome pair has been detected in tetraploid males of *R. acetosella* (Figures 2A, 2B, 3B, 4C, 4D). *R. acetosella* sex chromosomes consistently formed a monochiasmate heteromorphic bivalent (Figure 2A, B) indicative of the existence of a pseudoautosomal region between the X and the Y chromosomes. Unfortunately, the size of such a region could not be ascertained because late pachytene nuclei displayed a longer synapsed region in the sex bivalent than did the early ones, implying that the existence of some non-homologous synapsis, a common feature in mid–late pachytene of animals and plants (von Wettstein *et al.* 1984, Santos *et al.* 1993, 1995), cannot be ruled out. As opposed to that found in *R. acetosa*, no evidence for satellite-DNA accumulation in the Y chromosomes of *R. acetosella* was found. In fact, neither RAE180 nor RAYSI sequences, the satellite-DNA families found in the Y chromosomes of the former species, appear to be present (at least in significant quantities) within the genome of *R. acetosella* (Figure 2B), which is consonant with the absence of contrastable DAPI + or C + bands (Figure 2A) in its chromosome complement. The FISH result contrasts with those corresponding to PCR and Southern-blot hybridization techniques and might be explained by the fact that RAE180 sequences in *R. acetosella* are under-represented or non-tandemly organized at a level below the resolution of the FISH technique.

The case of *R. suffruticosus* is especially interesting because it is a Spanish dioecious endemic species not previously analysed. We have found here that the basic chromosome number of this diploid species is x = 8, a number that appears to be ancestral to the monophyletic group of dioecious *Rumex* species (Navajas-Pérez *et al.* 2005a). Also, we have detected the presence of a pair of heteromorphic sex chromosomes that forms a monochiasmate bivalent in meiosis (Figure 1A, B, C). Southern-blot hybridization (Navajas-Pérez *et al.* 2006, Figure 5 of this paper) and PCR amplification (this paper) demonstrated the absence of RAYSI satellite-DNA sequences within the genome of this species. This was not the case for the RAE180 satellite DNA because, although it was located in a pair of chromosomes, they were autosomes (Figure 1B). Absence of satellite-DNA sequences in the Y chromosome was consonant with the absence of contrastable DAPI + or C + regions in that chromosome (Figure 1A, C).

Dioecy appeared in *Rumex* between 15 and 16 million years ago (Mya), while the divergence time between the *R. acetosella*-*R. suffruticosus* clade (XX/XY species) and the *Acetosa* clade (XX/XY_1Y_2 species) should be 12–13 Mya (Navajas-Pérez *et al.* 2005a). However, though dioecy emerged at a similar time in *Rumex* and *Silene* (Guttman and Charlesworth 1998, Filatov *et al.* 2000), dioecious species of the latter genus have not accumulated a quantitatively important amount of repetitive-DNA sequences in the Y chromosomes (Buzek *et al.* 1997, Scutt *et al.* 1997, Garrido-Ramos *et al.* 1999, Hobza *et al.* 2006). According to the data gathered in the present paper, *R. acetosella* and *R. suffruticosus* appear to be species with sex chromosomes less cytogenetically differentiated than those of *R. acetosa* and more similar to the dioecious species of the genus *Silene* that are still in the earliest steps of sex-chromosome differentiation (Lengerova *et al.* 2003), although a certain process of Y-chromosome degeneration could have been initiated, as has recently been found in *Silene latifolia* (Hobza *et al.* 2006), something that should be tested in *R. suffruticosus* and *R. acetosella* only after genomic strategies of looking for satellite-DNA sequences (Hobza *et al.* 2006). In any case, the apparent accelerated process of Y-chromosome differentiation within the *Acetosa* group might be involved in, or be a consequence of, the chromosomal rearrangements leading to the multiple sex-chromosome system. In addition, our observations suggest that the chromosomes bearing the ribosomal DNA loci are not implicated in these rearrangements.

One of the most important outstanding issues within evolutionary biology concerns the study of the origin and the evolution of sex-determining mechanisms and of sex chromosomes. Recently evolved sex chromosome systems constitute excellent study models for the advancement of knowledge in this respect. On these grounds, dioecious plant species with heteromorphic sex chromosomes represent a unique opportunity to investigate the very early stages of sex-chromosome evolution. There are few examples of dioecious plants with heteromorphic sex chromosomes. The discovery of new species harbouring differentiated sex chromosomes can open new promising opportunities to shed light on sex-chromosome evolution. In this respect, the analysis developed in this paper concerning the species *R. suffruticosus* is valuable and promotes the genus *Rumex* as a model for further studies on sex-chromosome evolution in plants, since within this genus it is now possible to study the different levels of genetic differentiation between the sex chromosomes as well as to analyse their evolutionary history from their origin.

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