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The origin and evolution of the variability in a Y-specific satellite-DNA of *Rumex acetosa* and its relatives

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Abstract

In this paper, we analyze a satellite-DNA family, the RAYSI family, which is specific of the Y chromosomes of *Rumex acetosa*, a dioecious plant species with a multiple sex-chromosome system in which the females are XX and the males are XY_1Y_2. Here, we demonstrate that this satellite DNA is common to other relatives of *R. acetosa*, including *Rumex papillaris, Rumex intermedius, Rumex thyrsoides* and *Rumex tuberosus* that are also dioecious species with a multiple system of sex chromosomes. This satellite-DNA family is absent from the genomes of other dioecious *Rumex* species having an XX/XY sex-chromosome system. Our data confirm recent molecular phylogenies that support a unique origin for all dioecious species of *Rumex* and two separate lineages for species with single or complex sex-chromosome systems. Our data also support an accelerated degeneration of Y-chromosome in XX/XY_1Y_2 species by the accumulation of satellite-DNA sequences. On the other hand, the particular non-recombining nature of the Y chromosomes of *R. acetosa* and their closest relatives lead to a particular mode of evolution of RAYSI sequences. Thus, mechanisms leading to the suppression of recombination between the Y chromosomes reduced the rate of concerted evolution and gave rise to the apparition of different RAYSI subfamilies. Thus, *R. acetosa* and *R. intermedius* have two subfamilies (the RAYSI-S and RAYSI-J subfamilies and the INT-A and INT-B subfamilies, respectively), while *R. papillaris* only has one, the RAYSI-J subfamily. The RAYSI-S and RAYSI-J subfamilies of *R. acetosa* differ in 83 fixed diagnostic sites and several diagnostic deletions while the INT-A and the INT-B of *R. intermedius* differ in 27 fixed diagnostic sites. Pairwise comparisons between RAYSI-S and RAYSI-J sequences or between INT-A and INT-B sequences revealed these sites to be shared mutations detectable in repeats of the same variant in same positions. Evolutionary comparisons suggest that the subfamily RAYSI-J has appeared in the common ancestor of *R. acetosa* and *R. papillaris,* in which RAYSI-J has replaced totally (*R. papillaris*) or almost totally the ancestral sequence (*R. acetosa*). This scenario assumes that RAYSI-S sequences should be considered ancestral sequences and that a secondary event of subfamily subdivision should be occurring in *R. intermedius*, with their RAYSI subfamilies more closely related to one another than with other RAYSI sequences. Our analysis suggests that the different subfamilies diverged by a gradual and cohesive way probably mediated by sister-chromatid interchanges while their expansion or contraction in number might be explained by alternating cycles of sudden mechanisms of amplification or elimination.

Keywords: *Rumex*; Satellite DNA; Sex chromosomes; Y-chromosome degeneration; Concerted evolution; Satellite-DNA subfamilies

1. Introduction

Although dioecious plants (with separate male and female individuals) account for an estimated 6% of flowering plant species (Renner and Ricklefs, 1995), heteromorphic sex chromosomes are rare in plant species (less than one dozen of species of plants have these types of chromosomes; Ruiz Rejón, 2003). Sex chromosomes, in general, are thought to

Abbreviations: A, adenosine; bp, base pair(s); C, cytidine; DAPI, 4′,6-diamino-2-phenylindole; dNTP, deoxyribonucleoside triphosphate; EDTA, ethylenediaminetetraacetic acid; EMBL, European Molecular Biology Laboratory; G, guanosine; Mya, million year ago; Myr, million year; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; T, thymidine.

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have arisen as a consequence of the establishment of a rarely-recombining region containing the genes involved in sex determination, followed by chromosome degeneration (Charlesworth, 1996). Where identified, differentiated plant sex chromosomes have evolved relatively recently from their autosome progenitors (Parker, 1990), but appear to be undergoing rapid changes in sequence composition. Classic knowledge of this topic comes mainly from *Silene* (family Caryophyllaceae) in which there have been many different attempts to shed light on the genetics of sex-determination systems (reviewed in Lengerova et al., 2003), from *Rumex* (family Polygonaceae) (Ainsworth et al., 1999), and more recently from species such as, *Cannabis sativa*, Cannabaceae (Peil et al., 2003; Sakamoto et al., 2000), *Marchantia polymorpha,* Marchantiaceae (Okada et al., 2001) and *Carica papaya*, Caricaceae (Liu et al., 2004; Charlesworth, 2004). However, little is known about the genomic nature of plant sex chromosomes, the DNA sequences they harbor or the mechanisms of sequences expansion and genetic degeneration. Evolutionary processes affecting sex chromosomes in different groups of organisms suggest that progressive suppression of recombination finally leads to the accumulation of diverse repetitive sequences such as mobile elements and satellite DNAs (Steinemann and Steinemann, 1997; Bachtrog, 2003a,b; Skaletsky et al., 2003). However, not many Y-linked repetitive sequences that could provide reference of Y-degeneration degree have been isolated in plants.

Exceptionally, several species of the genus *Rumex* represent a good example of Y-chromosome differentiation and degeneration. The section *Acetosa* of this genus comprises a group of closely related dioecious species that all have a complex sex-chromosome system. In *Rumex acetosa* and in its closely relatives including *Rumex papillaris, Rumex thyrsoides, Rumex intermedius, Rumex tuberosus*, etc., females have a karyotype composed of 14 chromosomes ($2n=12+XX$) while the males have 15 chromosomes ($2n=XY_1Y_2$). In *R. acetosa,* during male meiosis a sex trivalent occurs and both Y chromosomes pair only with one end of the X chromosome (Ruiz Rejón, 2003). These two Y chromosomes are heterochromatic and stain strongly with the fluorochrome DAPI. To date, two satellite-DNA families, RAYSI and RAE180, represent the principal components of the Y heterochromatin in *R. acetosa* (Shibata et al., 1999, 2000b). These two satellite-DNA families are distinct from the RAE730 sequences present in the autosomal heterochromatic segments of this species (Shibata et al., 2000a), since they show reduced rates of sequence evolution (Navajas-Pérez et al., 2005a) a feature that has been related to the lack of recombination between the Y chromosomes. Additionally, for RAYSI sequences, we have found two satellite-DNA subfamilies (RAYSI-S and RAYSI-J) within the genome of *R. acetosa* (Navajas-Pérez et al., 2005a). The evolutionary pathways leading to the appearance of the RAYSI-S and RAYSI-J subfamilies is analyzed here by two different approaches: (i) by analyzing the molecular bases of the genetic differentiation between the two RAYSI subfamilies in *R. acetosa* as well as their different location at the two Y-chromosomes and (ii) by comparing RAYSI sequences of this species with those of other closely related species such as *R. papillaris* and *R. intermedius*.

2. Materials and methods

Three different species of the section *Acetosa* of the subgenus *Acetosa* of the genus *Rumex*: *R. acetosa*, *R. papillaris* and *R. intermedius*, were collected from natural populations in Sierra Nevada and Sierra de Baza, both in Granada (Spain), and Volubilis (Morocco), respectively. Collected seeds were kept in a cold and dry place until germination and leave material sampled from five males from every location, was stored at −80 °C for further analysis. DNA extraction was performed using the Plant DNAzol kit (Invitrogen) following the manufacturer's recommendations. Additionally, the following species were checked for the presence in their genomes of the RAYSI sequences: *R. thyrsoides, R. tuberosus, Rumex suffruticosus*, *Rumex acetosella*, *Rumex hastatulus* (Texas race and North Carolina race), *Rumex bucephalophorus*, *Rumex scutatus, Rumex induratus, Rumex lunaria*, *Rumex maderensis*, *Rumex roseus, Rumex vesicarius, Rumex conglomeratus*, *Rumex crispus*, *Rumex patientia*, *Rumex obtusifolius* and *Rumex pulcher*, collected from different sources (for locations see Navajas-Pérez et al., 2005b).

Southern blot and dot-blot hybridization analyses were made following Garrido-Ramos et al. (1999), using different DNAs from the different species.

The primers RAYSI-A, 5′-ATGTAAGCATTTGGTCCTAA-3′, and RAYSI-D, 5′-TCGAGTACTACACGATTGT-3′ (Navajas-Pérez et al., 2005a), were used in this paper for the amplification of RAYSI sequences from the genome of *R. papillaris* and *R. intermedius*. The three species (*R. acetosa, R. papillaris* and *R. intermedius*) were checked also in this paper for the presence of the two RAYSI subfamilies previously described in *R. acetosa*. Primers RAYSI-J, 5′-GAGAGTCAATAGAGTG-GAAG-3′, and RAYSI-S, 5′-ACGTAGTCTTTTAGAGGATC-3′, were used in combination with the primer RAYSI-D, 5′-TCGAGTACTACACGATTGT-3′ for the specific amplification of monomer sequences of each of the two subfamilies, RAYSI-J and RAYSI-S, as described before (Navajas-Pérez et al., 2005a). PCR amplifications were made 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 reaction buffer. Thermal cycles consisted of 1min at 94°, 1min at 55° and 1 min at 72°. 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. The clones from each marker were sequenced by the dideoxy-sequencing method using the automatic ABI-Prism 377 sequencer (Applied Biosystems). In this study we have obtained a total of seven new *R. papillaris* RAYSI repeat units and twenty-five *R. intermedius* RAYSI sequences. These new sequences were analyzed in conjunction with other sixty-eight obtained previously from *R. acetosa* and *R. papillaris.* The EMBL accession numbers for all the sequences analyzed in this paper are: AJ580382 to AJ580385, AJ634548 to AJ634566, AJ580386 to AJ580392 and

AJ639929 to AJ639940, AM051234 to AM051258 and AM055595 to AM055601.

For sequence analysis, multiple alignments were performed using Clustal X (Thompson et al., 1997) followed by manual adjustments. We computed basic sequence statistics with the program DnaSP v.3 (Rozas and Rozas, 1999). Phylogenetic and molecular evolutionary analyses were conducted using MEGA 2.1 (Kumar et al., 2001). Distances were calculated according to the Jukes-Cantor method and trees constructed by the neighbour-joining method (Saito and Nei, 1987).

For fluorescent in situ hybridization (FISH), root tips were obtained from seeds germinated in Petri dishes at 25 °C and then pre-treated with 2 mM 8-hydroxiquinoleine for 1–2 h at germinating temperature, followed by 1–2 h at 4 °C for metaphase accumulation. The roots were then fixed in 3:1 ethanol/glacial acetic acid for at least 4 h. The root tips were treated with cellulase and pectinase enzymes mixture for 1 h at 37 °C and were transferred subsequently to 45% and 60% acetic acid, and chromosome preparations were made following Schwarzacher and Heslop-Harrison (2000). Probes were labelled with digoxigenine-dUTP or biotin-dUTP (Roche) by random priming according to the specifications of the Random Primer Labelling System (Invitrogen). Labelled probes (25–50 ng) were added to the hybridization mixture (50% formamide, 2×SSC (saline sodium citrate), 20% dextran sulphate, 0.125% SDS (sodium dodecil sulphate) and 0.125 mM EDTA (ethylenediaminetetraacetic acid)) (see Schwarzacher and Heslop-Harrison, 2000). Combined denaturing of the probe and chromosomal DNA were performed at 80 °C for 8 min using a ThermoHybaid HyPro-20 and re-annealed at 37 °C overnight. Stringent washes (20% formamide and 0.1×SSC at 42 °C) were carried out prior to detection. Biotin and digoxigenine probes were detected with Alexa594/streptavidin (Molecular Probes, 0.5 ng/ml) and FITC/anti-digoxigenine (Roche, 1 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-diamino-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 by using Adobe Photoshop 7.0 software, using only those processing functions that are applied to all pixels of the image.

3. Results

3.1. Survey of RAYSI sequences in Rumex species

The presence of RAYSI sequences within the species of the genus *Rumex* was analyzed by means of Southern-blot and dot-blot hybridizations (Fig. 1). We found these types of sequences

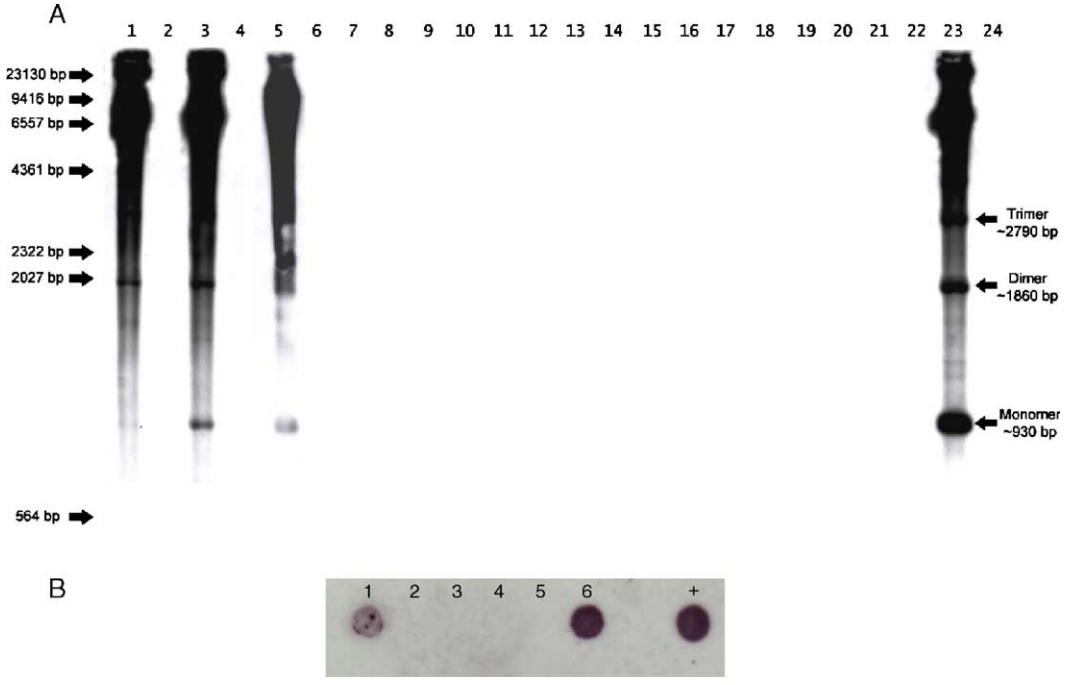


Fig. 1. (A) Southern blot hybridization on *Eco*RI-digested DNA from different representative species of *Rumex* using as a probe a RAYSI repeating unit. 1. *R. papillaris*, male individual; 2. *R. papillaris*, female individual; 3. *R. intermedius*, male; 4. *R. intermedius*, female; 5. *R. thyrsoides*, male; 6. *R. thyrsoides*, female; 7. *R. hastatulus* (Texas race), male; 8. *R. hastatulus* (Texas race), female; 9. *R. hastatulus* (North Carolina race), male; 10. *R. hastatulus* (North Carolina race), female; 11. *R. acetosella*, male; 12. *R. acetosella*, female; 13. *R. suffruticosus*, male; 14. *R. suffruticosus*, female; 15. *R. bucephalophorus*; 16. *R. scutatus*; 17. *R. induratus*; 18. *R. maderensis*; 19. *R. roseus*; 20. *R. conglomeratus*; 21. *R. patientia*; 22. *R. pulcher*; 23. *R. acetosa*, male; 24. *R. acetosa*, female. Hybridization patterns are typical of satellite DNAs, with arrows indicating the hybridized monomeric, dimeric and trimeric units. (B) Species not analyzed by Southern blot hybridization were studied by means of dot-blot hybridization. In the figure, positive hybridization of a RAYSI probe on *R. tuberosus* DNA (1), on *R. acetosa* DNA (6) and on the positive control DNA (+, RAYSI sequences). *R. lunaria* (2), *R. vesicarius* (3), *R. crispus*(4) and *R. obtusifolius*(5) did not hybridize.

in all five dioecious species of the section *Acetosa* analyzed (*R. acetosa, R. papillaris, R. intermedius, R. thyrsoides,*and *R. tuberosus*) having a multiple sex chromosome system, XX/XY_1Y_2, but not in other dioecious species having a XX/XY chromosome system of sex determination (*R. acetosella, R. suffruticosus* and the XX/XY chromosomal race of *R. hastatulus*, the Texas race). The sequences were also absent in the XX/XY_1Y_2 chromosomal race of *R. hastatulus* (the North Carolina race) or in the genomes of hermaphroditic and/or polygamous species of different subgenera such as *R. bucephalophorus*, *R. scutatus*, *R. induratus, R. lunaria*, *R. maderensis*, *R. roseus, R. vesicarius, R. conglomeratus*, *R. crispus*, *R. patientia, R. obtusifolius*and *R. pulcher*. Among the species having RAYSI sequences, *R. papillaris* and *R. intermedius* were selected for further analysis, the former being the closest relative to *R. acetosa*, and the latter being related to *R. acetosa* and *R.*

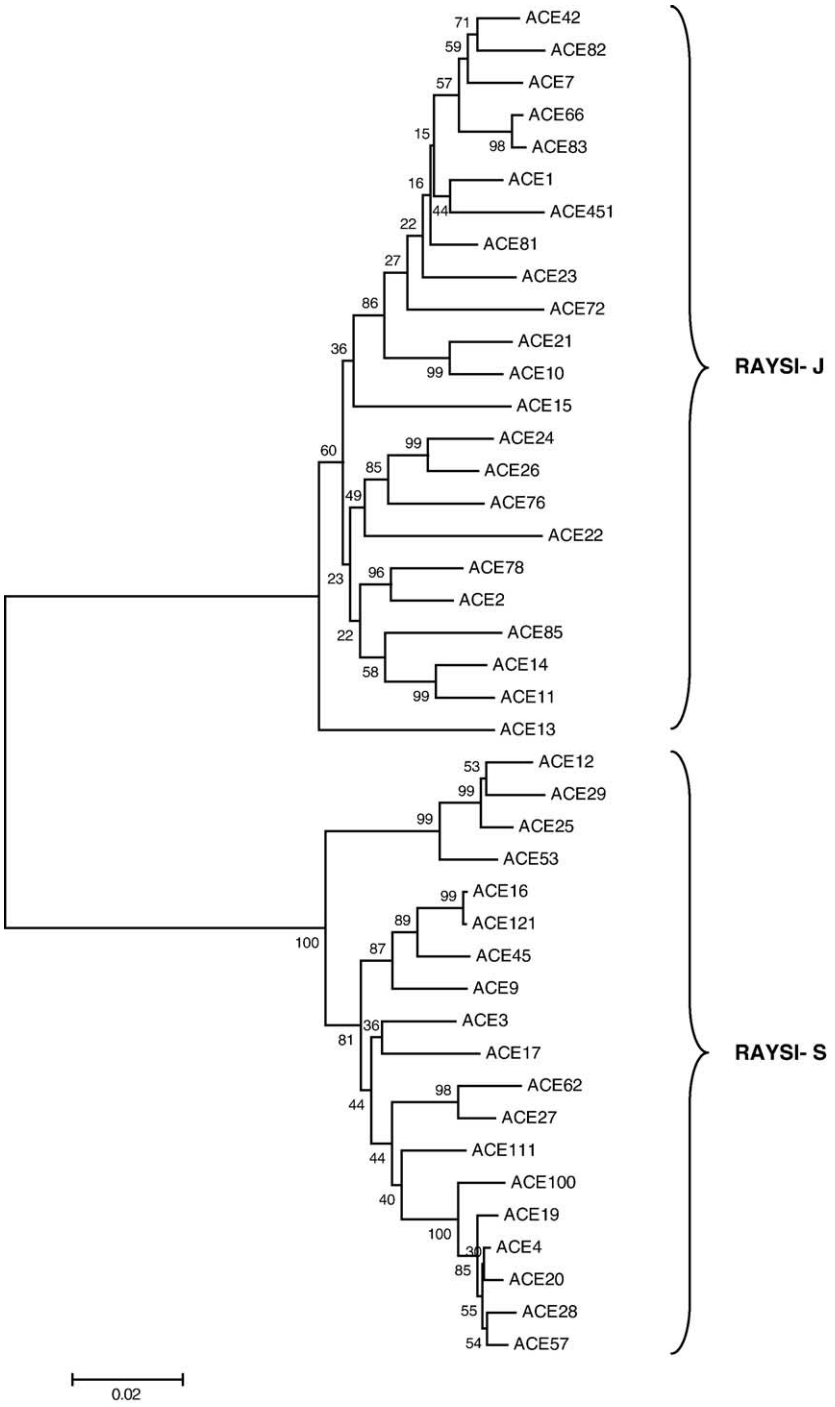


Fig. 2. Neighbour-joining tree showing the RAYSI sequences of *R. acetosa* in two separate clades (RAYSI-J and RAYSI-S). Numbers at each node indicate the percentage of trees representing the particular node out of 1000 bootstrap replicates. Bootstrap values under 50 are not indicated. In this tree, the labels of the sequences correspond to the first three letters of the species name (ACE) and a number representing the repeat analyzed.

papillaris. We followed a PCR assay in order to test the presence/absence of the two different RAYSI subfamilies (S and J) in the three species (*R. acetosa, R. papillaris*and *R. intermedius*). The three combinations of primers (RAYSI-A/RAYSI-D, RAYSI-J/RAYSI-D and RAYSI-S/RAYSI-D) led to the amplification of PCR product of expected size (roughly 930 bp) in *R. acetosa*. The primers RAYSI-A/RAYSI-D and RAYSI-J/RAYSI-D gave 930 bp PCR product in *R. papillaris* but not the RAYSI-S/RAYSI-D set of primers, while in *R. intermedius* only the RAYSI-A/RAYSI-D combination gave the expected 930 bp PCR product. In fact, we did not obtain amplified product with the RAYSI-J/RAYSI-D or the RAYSI-S/RAYSI-D set of primers.

3.2. Sequence analysis

According to the results of the PCR assay and after sequencing the cloned products we found two types of sequences in *R. acetosa*, namely the RAYSI-S and the RAYSI-J sequences. The two types of RAYSI sequences (the RAYSI-S and RAYSI-J subfamilies) found in *R. acetosa* were established according to 83 diagnostic sites, each representing a particular mutation shared by all the sequences of one group, while at the same sites all the sequences of the other group had a different nucleotide (Figs. 2 and 4). The mean inter-family divergence between them was 17.3%. However, the mean intra-family percentage of differences was 4.3% for RAYSI-S (19 sequences analyzed in total) and 4.8% for RAYSI-J (23 sequences analyzed in total). Both types of sequences have diagnostic deletions found at different positions in the RAYSI monomers (Fig. 4). RAYSI-S sequences were not amplified in *R. papillaris* either by using the RAYSI-A/RAYSI-D combination of primers or by using the subfamily-specific primers (RAYSI-S/RAYSI-D). Either from the amplification with RAYSI-A/RAYSI-D or with RAYSI-J/RAYSI-D, all the sequences obtained from *R. papillaris* belonged to the RAYSI-J subfamily. We analyzed a total of 33 RAYSI-J sequences from *R. papillaris* showing 5.4% of mean sequence differences. Finally, in the case of *R. intermedius* we found differentiated sequences with respect to both the RAYSI-S and the RAYSI-J subfamilies. However, in this species we also found two types of sequences (INT-A and INT-B) that can be grouped into two separate groups differentiated in this case only by 27 diagnostic sites. As in *R. acetosa*, each site represents a particular mutation shared by all the sequences of one group, while at the same sites all the sequences of the other group had a different nucleotide (Figs. 3 and 4). The mean inter-family divergence between them was 16.4% while the mean intra-family percentage of differences was 7.0% for INT-A sequences (11 sequences analyzed in total) and 5.4% for INT-B (14 sequences analyzed in total).

Fig. 4 shows the alignment of the consensus RAYSI sequences derived for *R. acetosa, R. papillaris* and *R. intermedius*. For a correct inter-specific comparison, this figure includes the consensus sequences derived for each RAYSI subfamily in *R. acetosa* and in *R. intermedius*. The alignment of the consensus sequences of the three species analyzed gives

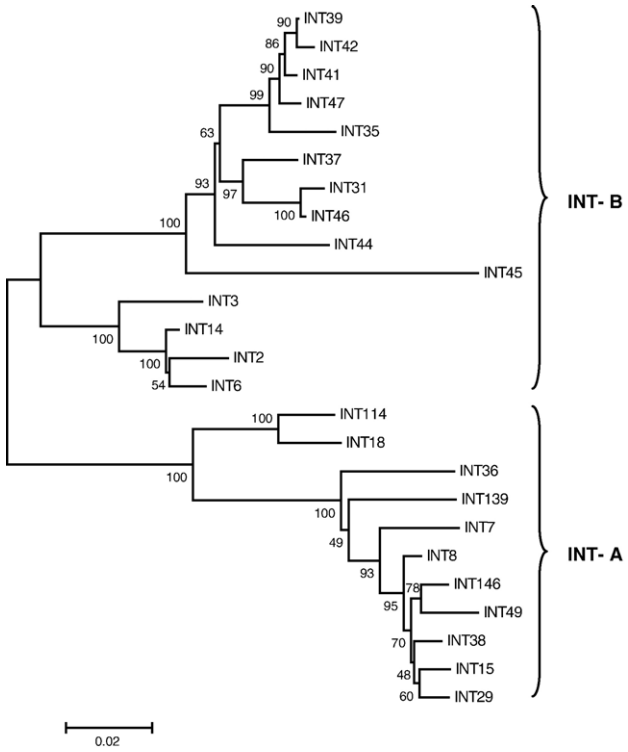


Fig. 3. Neighbour-joining tree showing the RAYSI sequences of *R. intermedius* in two separate clades (INT-A and INT-B). Numbers at each node indicate the percentage of trees representing the particular node out of 1000 bootstrap replicates. Bootstrap values under 50 are not indicated. In this tree, the labels of the sequences correspond to the first three letters of the species name (INT) and a number representing the repeat analyzed.

some clues about the evolutionary history of RAYSI sequences in *Rumex* species. According to the alignment of the Fig. 4, the consensus length for the RAYSI sequences is 943 bp. However, the lengths of the *R. acetosa* and *R. papillaris* RAYSI consensus sequences are shorter (RAYSI-S of *R. acetosa*: 911 bp; RAYSI-J of *R. acetosa* and *R. papillaris*: 926 bp). The shorter RAYSI-S monomers are due to diagnostic deletions found between positions 497 and 511 and between positions 579 and 594 of the alignment, while in the case of RAYSI-J sequences the cause is two deletions between positions 873 and 880 and between positions 901 and 910.

3.3. Phylogenetic analysis of RAYSI sequences

A phylogenetic analysis using the whole set of sequences gave a neighbor-joining tree showing the existence of two main clades supported by 100% of bootstraps replicates (Fig. 5): one including RAYSI-J sequences of *R. acetosa* and *R. papillaris*, while the other included the RAYSI-S sequences of *R. acetosa* and all the sequences of *R. intermedius*. Within the RAYSI-J clade, sequences of *R. acetosa* and *R. papillaris* did not group by taxonomic affinity. In the second clade, however, three differentiated subclades were detected, on one hand the RAYSI-S sequences of *R. acetosa* and, on the other hand, the sequences of *R. intemedius* separated into two groups of sequences, the INT-A and the INT-B sequences.

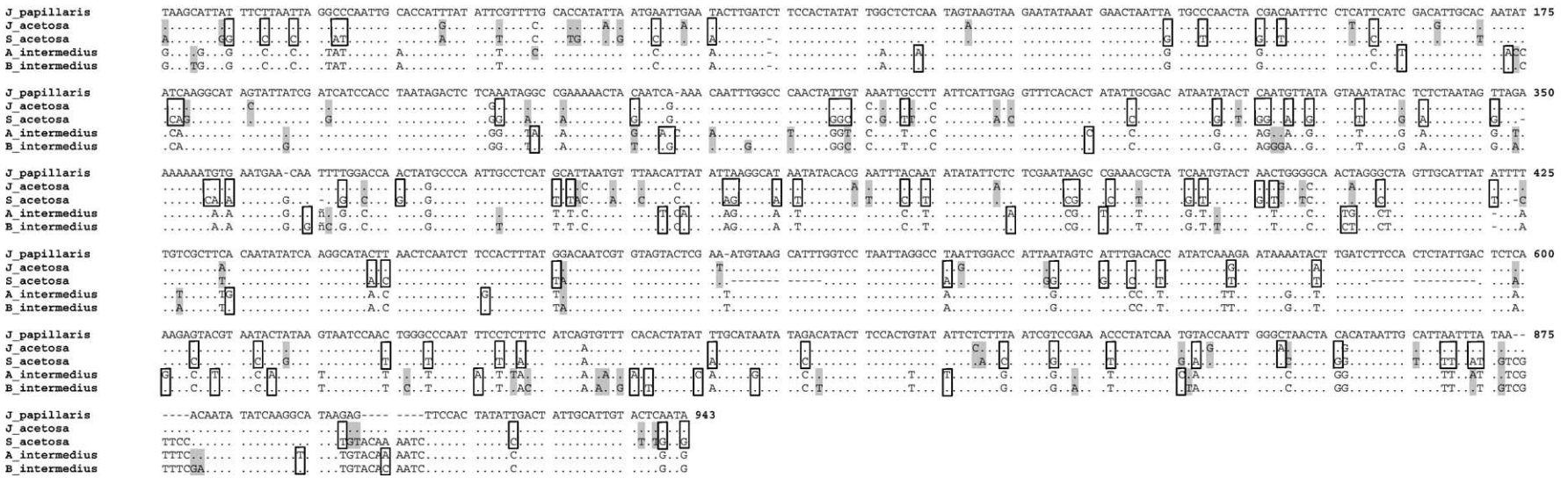


Fig. 4. Nucleotide-sequence alignment between the consensus sequences of RAYSI-J and RAYSI-S monomers of *R. acetosa*, RAYSI-J monomers of *R. papillaris* and INT-A and INT-B sequences of *R. intermedius*. Fixed differences between subfamilies analyzed two to two (RAYSI-J vs. RAYSI-S and INT-A vs. INT-B) are boxed. Shaded nucleotides indicate intra-subfamily variable sites.

3.4. Fluorescent in situ hybridization

We have analyzed by fluorescent in situ hybridization (FISH) the chromosomal location of RAYSI-S and RAYSI-J subfamilies in *R. acetosa*. For this, we have used two probes: one isolated from the clone RAYSI5-10 belonging to a RAYSI-S enriched library of *R. acetosa* and other isolated from the clone RAYSI5-7 belonging to a RAYSI-J enriched library of *R. acetosa*. With the RAYSI-S probe, the hybridization signals were found as one band in the centromeric region of the Y_1 chromosome and two faint bands at each sides of the centromere (Fig. 6A, C1, C3). With the RAYSI-J probe, the hybridization signals were found as two bands located in the short arm of the Y_1 chromosome and two weak bands at each sides of the centromere (Fig. 6B, C1, C2). Additionally, the RAYSI-J probe hybridized to the Y_2 chromosome as a band in the telomeric region of the short arm (Fig. 6D1, D2). No RAYSI-S sequences were visible on the Y_2 chromosome (Fig. 6D3). Finally, double-target in situ hybridization with both probes (Fig. 6C, D) confirmed that the RAYSI-S is juxtaposed with sequences from the RAYSI-J subfamily.

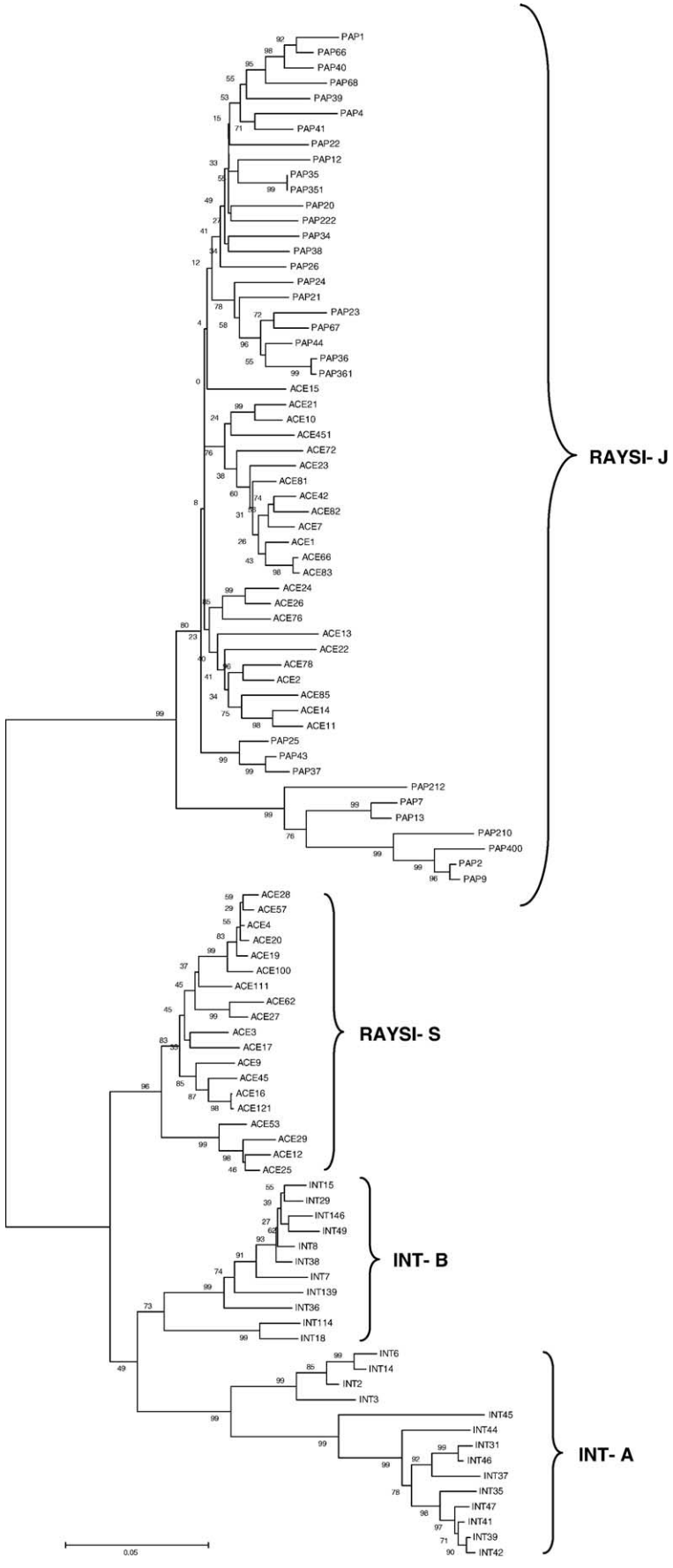
4. Discussion

4.1. Y chromosomes degeneration

The Y-specific RAYSI satellite-DNA family of *R. acetosa* is composed of highly repetitive sequences present only within the genome of its closest relatives—that is, dioecious species with a multiple chromosome system (XX/XY_1Y_2) such as *R. papillaris* and *R. intermedius*. Theories about sex-chromosome evolution predict that gradual suppression of recombination between the X and Y chromosomes lead to the progressive degeneration of the Y by the loss of function of most genes (Filatov et al., 2000) and the expansion of tandemly repetitive DNA families (Charlesworth, 1996; Jobling and Tyler-Smith, 2003). This is what has occurred in some dioecious species of the genus *Rumex*. The origin of dioecy and the appearance of sex chromosomes in *Rumex* have been estimated to be about 15–16 Myr (Navajas-Pérez et al., 2005b). This corresponds to the approximate time estimated for the appearance of sex chromosomes in other plant groups such as in *Silene*, leaving only a short time in evolutionary terms for the degeneration of the Y chromosomes (Scutt et al., 1997; Garrido-Ramos et al., 1999; Kazama et al., 2003). In fact, within the dioecious *Rumex* species, there are two phylogenetically differentiated groups, one composed of species having a single XX/XY sex-chromosome system and another composed of species having the complex XX/XY_1Y_2 sex-chromosome system (Navajas-Pérez et al., 2005b). We here demonstrate that RAYSI sequences have accumulated only in the Y chromosomes of these species having a multiple sex-chromosome system. Thus, it appears that the degeneration of the Y chromosomes in *Rumex* has been accelerated by the accumulation of RAYSI sequences, among other repeats. This acceleration was presumably assisted by the rearrangements giving rise to the multiple system of sex chromosomes in *Rumex*.

4.2. Origin and evolution of RAYSI sequences

RAYSI sequences constitute a Y-linked satellite-DNA family that originated through repetitive cycles of duplication, unequal crossing-over and divergence from a basic 120-bp repeat unit (Navajas-Pérez et al., 2005a). Specifically, this satellite-DNA family is composed of the repetition of eight 120-bp basic units. The consensus length for the RAYSI sequences of the three species analyzed in the present paper is 943 bp (Fig. 4). This length is close to the proposed 120 bp 8-mer (960 bp) after some rearrangements. However, there are two types of RAYSI sequences according to 83 fixed differences and several diagnostic deletions, leading to the shortening of 31 bp (RAYSI-S sequences) and 16 bp (RAYSI-J sequences) from the consensus monomer length. Both subfamilies have been found in *R. acetosa*, but only RAYSI-J was found in *R. papillaris*, the closest relative (Navajas-Pérez et al., 2005b). On the other hand, two other subfamilies have been found in *R. intermedius* (INT-A and INT-B). These latter subfamilies are differentiated by point substitutions but not by characteristic deletions or insertions as in RAYSI-S and RAYSI-J sequences. There are different possibilities for explaining the evolution of RAYSI sequences in this group of species, but only two alternative hypotheses support the phylogenetic tree shown in the Fig. 5 (see Fig. 7). One possibility is that the subfamilies RAYSI-S and RAYSI-J were present in the genome of the common ancestor of the three species with the former disappearing in the genome of *R. papillaris* and the second in the genome of *R. intermedius*. Another hypothesis with equivalent results is that the subfamily RAYSI-J has appeared in the common ancestor of *R. acetosa* and *R. papillaris,* and that RAYSI-J has replaced totally, in *R. papillaris*, or almost totally, in *R. acetosa*, the ancestral sequence (in this case, the RAYSI-S subfamily). The difference with the first scenario is that, in this second case, RAYSI-S sequences should be considered ancestral sequences and that the differences between RAYSI sequences of *R. intermedius* and RAYSI-S sequences of *R. acetosa* should mark the divergence between extant RAYSI-S sequences and ancestral RAYSI sequences. This second hypothesis appears most plausible from a parsimonious position and it is supported by the diagnostic deletions found in RAYSI-S and RAYSI-J subfamilies that are not found in RAYSI sequences of *R. intermedius*, assuming then that those deletions are derived states with respect to the ancestral length represented by the RAYSI sequences of *R. intermedius* (INT-A and INT-B). Furthermore, if we assume the evolutionary rate of RAYSI-J sequences–11.74×10^{-9} per site and year– (Navajas-Pérez et al., 2005a) to estimate the divergence time between RAYSI-S and RAYSI-J, we might expect these two subfamilies to have split some 7.5 Mya. Also, although we must exercise caution in accepting the divergence times between some *Rumex* species because the existence of rate variation across lineages (Navajas-Pérez et al., 2005b), the separation between these two RAYSI subfamilies should have occurred after the splitting of the lineages leading to *R. acetosa–R. papillaris* on one hand and to *R. intermedius*, on the other (10 Myr according to Navajas-Pérez et al., 2005b).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PAP38
PAP26
PAP24
PAP21
PAP23
PAP67
PAP44
PAP36
PAP361
ACE15
ACE21
ACE10
ACE451
ACE72
ACE23
ACE81
ACE42
ACE82
ACE7
ACE1
ACE66
ACE83
ACE24
ACE26
ACE76
ACE13
ACE22
ACE78
ACE2
ACE85
ACE14
ACE11
PAP25
PAP43
PAP37
PAP212
PAP7
PAP13
PAP210
PAP400
PAP2
PAP9
RAYSI- J
ACE28
ACE57
ACE4
ACE20
ACE19
ACE100
ACE111
ACE62
ACE27
ACE3
ACE17
ACE9
ACE45
ACE16
ACE121
ACE53
ACE29
ACE12
ACE25
RAYSI- S
INT15
INT29
INT146
INT49
INT8
INT38
INT7
INT139
INT36
INT114
INT18
INT- B
INT6
INT14
INT2
INT3
INT45
INT44
INT31
INT46
INT37
INT35
INT47
INT41
INT39
INT42
INT- A
0.05

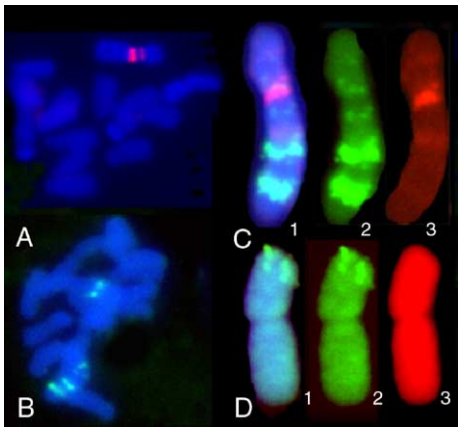

Fig. 6. *R. acetosa* male metaphases showing positive hybridization with RAYSI-S (detected with Alexa594/streptavidin-red) probes (A) and RAYSI-J (detected with FITC-green) (B). (The chromosomes were counterstained with DAPI in each case). Double target in situ hybridization with both RAYSI subfamilies in the Y_1 chromosome (C). Visualized simultaneously (C.1), RAYSI-J subfamily (C.2), RAYSI-S subfamily (C.3). Location of RAYSI sequences in the Y_2 chromosome (D), visualized simultaneously (D.1), RAYSI-J subfamily (D.2), negative hybridization with RAYSI-S probe (D.3).

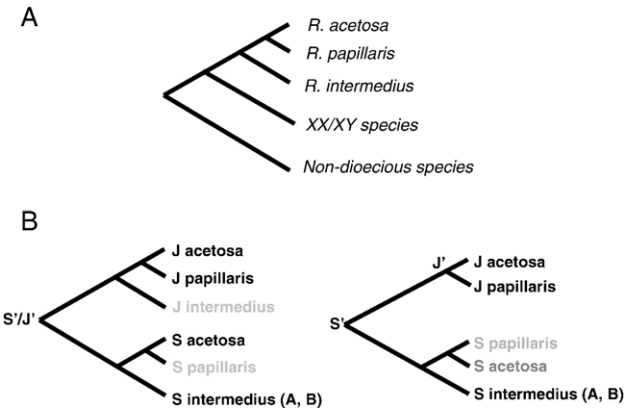

Fig. 7. (A) Phylogenetic relationships between the three XX/XY_1Y_2 *Rumex*-species analyzed here and between them and other dioecious and non-dioecious species of this genus. Taken from Navajas-Pérez et al. (2005a). (B) Two alternative pathways explaining the origin and evolution of RAYSI subfamilies. S′ and J′: ancestral RAYSI-S and RAYSI-J sequences. S and J: extant RAYSI sequences of *R. acetosa*, *R. papillaris* and *R. intermedius*. Variants in gray colour are extinct (clearer) or almost extinct sequences. See text for details.

Thus, this was also after the split (12–13 Myr) between species having the RAYSI sequences (those dioecious species with XX/XY_1Y_2 sex chromosomes) and species not having RAYSI sequences (those dioecious species with XX/XY sex chromosomes) (Navajas-Pérez et al., 2005b). A secondary event of subfamily subdivision is postulated to have occurred in *R. intermedius* that has two RAYSI subfamilies more closely related to each other than to other RAYSI sequences.

Satellite-DNA repeats are thought to spread horizontally within the repeat family by means of transposition, amplification mediated by rolling-circle replication and reinsertion, unequal crossing-over and gene conversion, although the relative contribution of each process is not clear (Charlesworth et al., 1994). These mechanisms usually lead to a high intra-specific similarity of tandem repeats and inter-specific dissimilarity and some, to the expansion or contraction of the tandem arrays. The probability and time necessary for intra-specific homogenization are dependent on population size, satellite DNA copy number, and rates as well as biases of nonreciprocal transfers (Dover, 1982; Ohta and Dover, 1984). However, within the RAYSI sequences this intra-specific homogeneity of a satellite-DNA family is not detected within a species, and different subfamilies are found within *R. acetosa* and *R. intermedius*. In *Arabidopsis thaliana*, restricted sequence variation at specific sites of the centromeric AtCEN sequences have been shown to be more abundant in some chromosomes postulating chromosome specific sequence variants (Harrison and Heslop-Harrison 1995) similar to those described for the centromeric satellite DNA in mammals (Willard and Waye, 1987a; Alexandrov et al., 1988). These subfamilies appear to have resulted from the absence of chromosomal exchanges between non-homologous chromosomes. This lack of chromosomal transfer gave rise to chromosome-specific subfamilies within the mammalian centromeric alpha satellites (Willard and Waye, 1987a,b; Alexandrov et al., 1988). An extreme case of such a lack of exchange might occur in the non-recombining Y chromosomes of *Rumex* species. Thus mechanisms impeding chromosomal interchanges, i.e. recombination, should reduce the rate of concerted evolution (Navajas-Pérez et al., 2005a), and should give rise to chromosome-specific satellite-DNA subfamilies (Pons and Gillespie, 2003). We postulate this mechanism for RAYSI sequences of the non-recombining Y chromosomes in the complex XX/XY_1Y_2 chromosomal system of *R. acetosa* and *R. intermedius*. The localization of the RAYSI-S and the RAYSI-J subfamilies of *R. acetosa* at different loci within the two Y chromosomes (Fig. 6) is a further feature that might be explained as a consequence of the lack of recombination between the two Y chromosomes.

Two additional considerations are due. The first consideration is related to the fact of the sequence divergence between RAYSI subfamilies within species. This fact resembles the concerted evolution process. As shown, the RAYSI-S and RAYSI-J subfamilies of *R. acetosa* differ in 83 fixed diagnostic sites while the INT-A and the INT-B of *R. intermedius* differ in 27 fixed diagnostic sites (Fig. 4). Pairwise comparisons between RAYSI-S and RAYSI-J sequences or between INT-A and INT-B sequences revealed these sites as shared mutations unambiguously observed in repeats of the same variant in exactly the same positions (Fig. 4). Any one of the two hypotheses proposed above to explain the timing of origin of the different subfamilies could support a pattern of gradual sequence divergence between RAYSI-S and RAYSI-J or between INT-A and INT-B subfamilies according to the RAYSI

Fig. 5. Neighbour-joining tree showing the relationships between all the RAYSI sequences analyzed in this paper. Numbers at each node indicate the percentage of trees representing the particular node out of 1000 bootstrap replicates. Bootstrap values under 50 are not indicated. In this tree, the labels of the sequences correspond to the first three letters of the species name (ACE, PAP, INT) and a number representing the repeat analyzed.

evolutionary rate (Navajas-Pérez et al., 2005a) and to their sequence differentiation. Assuming that the Y chromosomes of *R. acetosa* do not recombine, we might suspect sister-chromatid interchanges to explain gene conversion homogenizing events such as occur in the human Y chromosome (Skaletsky et al., 2003), which should lead to concerted evolution between subfamilies. Alternatively, some sort of sequence homogenization events could be driven by amplification mediated by rolling-circle replication and reintegration (Feliciello et al., 2005) or other related mechanisms. This connects with the second consideration, which is related to the expansion of the RAYSI-J subfamily and the tendency towards the disappearance of the RAYSI-S subfamily in *R. acetosa* and *R. papillaris*. In fact, the RAYSI-S subfamily has disappeared from the genome of *R. papillaris* and it has almost disappeared from the genome of *R. acetosa* (Fig. 6), something that could be explained as a mere consequence of the proper dynamic of satellite DNA evolution by unequal crossing-over (Charlesworth et al., 1994). On the contrary, RAYSI-J sequences have colonized different loci of the two Y chromosomes of *R. acetosa* (Fig. 6), suggesting a mechanism of sequence expansion mediated by such mentioned mechanisms as those mediated by rolling-circle replication and reintegration (Rossi et al., 1990; Feliciello et al., 2005).

Connecting the present data with previous findings (Navajas-Pérez et al., 2005a), we can conclude that the particular non-recombining nature of the Y chromosomes of *R. acetosa* and their closest relatives lead to a particular mode of evolution of RAYSI sequences. On one hand, the suppression of recombination between the Y chromosomes led to the appearance of different RAYSI subfamilies in which the sequences could diverge in a gradual and cohesive way while their expansion or contraction in number might be explained by alternating cycles of sudden mechanisms of amplification or elimination. On the other hand, the suppression of recombination led to a reduced rate of evolutionary change and of concerted evolution between species.

4.3. Phylogenetic implications

Finally, we want to emphasize the cladistic value from a phylogenetic standpoint of the RAYSI sequences. We have recently established that dioecy has a single origin in *Rumex* about 15–16 Myr and that two separate lineages split up about 12–13 Myr, one giving to XX/XY species and one, leading to XX/XY_1Y_2 species (Navajas-Pérez et al., 2005b). Our presence/absence analysis of RAYSI sequences within the genomes of different *Rumex* species support this view, since all XX/XY_1Y_2 have these sequences and, hence, the XX/XY_1Y_2 species share a common ancestor in which the RAYSI satellite-DNA has been amplified. Furthermore, within the XX/XY lineage, there is a species, *R. hastatulus*, for which a chromosomal race with the XX/XY_1Y_2 system has secondarily appeared. Neither the XX/XY nor the XX/XY_1Y_2 race of *R. hastatulus* appears to have the RAYSI sequences. This data not only support that the appearance of the multiple sex-chromosome system in this race of *R. hastatulus* is a different process occurring in a lineage different from that leading to *R. acetosa*and its relatives but also might be related to the different processes leading to the complex sex-chromosome system in the two lineages (Smith, 1969; Ruiz Rejón et al., 1994).

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