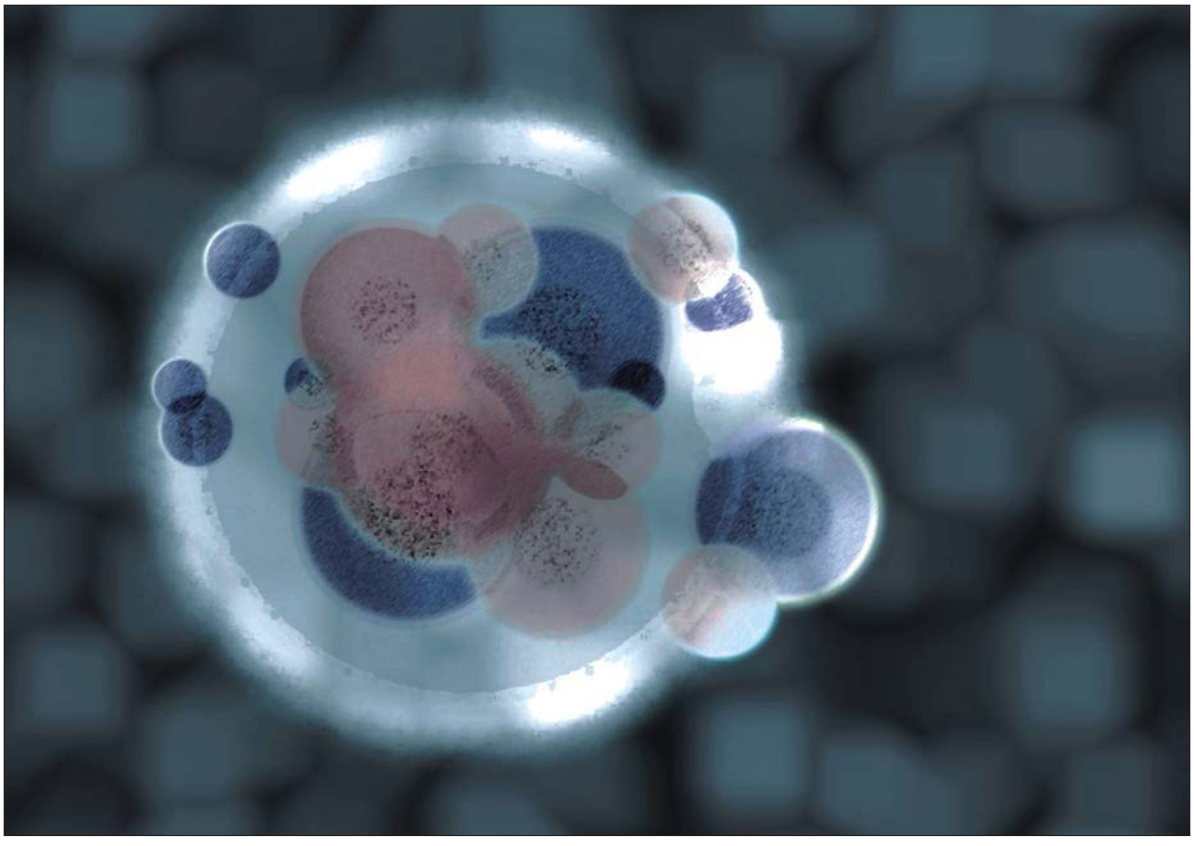

Journal of Biological Sciences

ISSN 1727-3048

science alert

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Journal of Biological Sciences 4 (2): 160-169, 2004
ISSN 1727-3048

AFLP Analysis of Genome Difference Between Male and Females in Dioecious Plant *Rumex acetosa*

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Abstract: Sorrel (*Rumex acetosa* L.) is dioecious model plant with heteromorphic sex chromosome system with 2n=12+XX in females and 2n=12+XY_1Y_2 in males. Sex is determined by the X:autosome balance. The major sex determining genes locate on X-chromosomes and the Y-chromosomes are not required for sex expression. Y-chromosomes, which contain genes for pollen fertility, constitute 26% of male genome but the structure and functions of the Ys are largely unknown. AFLP using *Mse*I/*Pst*I digestion reveals very low levels of male specific markers in *R. acetosa* and related species. Fifteen primer-combinations tested in *R. acetosa* generated 1085 bands, 4 of which were male specific. Two male associated DNA fragments, *MADR1* (583 bp) and *MADR2* (133 bp), were isolated and sequenced. These sequences show similarity to short stretches (up to 20-30 bp) of known sequences from several organisms. The deduced amino acid sequence of *MADR1* showed similarity to phosphatase genes from *Arabidopsis thaliana*, while *MADR2* showed similarity to retroelement sequences. The generation of very few male specific AFLP markers and their sequence similarity to the stretches of human DNA from autosomes and both the X and Y-chromosomes indicate the likely origin of the Ys from the X-chromosome.

Key words: Sorrel, *Rumex acetosa*, AFLP markers, male specific sequences

INTRODUCTION

The dioecious plant species sorrel (*Rumex acetosa* L.) has evolved a distinctive sex chromosome system with 2n=12+XX in females and 2n=12+XY_1Y_2 in males[1]. Sex is determined by the ratio between the number of X-chromosome and the number of autosome sets. The major sex determining genes locate on the X-chromosomes. While Y-chromosomes contain genes for pollen fertility and play important role in sex chromosome distribution in male meiosis, they are not required for sex expression[2]. Based on cytological estimate of metaphase chromosome length, it appears that the two Y-chromosomes constitute 26.44% of male genomic DNA which is absent in normal females[3]. The structure and functions of the huge DNA content of the Y-chromosomes are largely unknown. Sorrel thus represents a unique model plant for the study of sexual dimorphism.

In a number of agriculturally important plants such as kiwi fruit, date palm, hops, papaya and pistachio, the females produce the commercial harvest, while in some others, such as *Asparagus*, males provide the better quality produce. Identification of the sex of such plants at their early stage of growth can be of great economic potential. Moreover, studies on marker technology regarding dioecy in general would render better understanding of the developmental[4] as well as evolutionary pathways of dimorphism[5-7].

As a powerful technique of generating large numbers of molecular markers, amplified fragment length polymorphism (AFLP, Vos *et al.*[8]) provides a rapid method for scanning the genomes of different individuals for sequence variation. In this article, we report AFLP in *R. acetosa* and analyse the difference between male and female genomes to understand the origin of Y-chromosomes.

MATERIALS AND METHODS

Ten male and ten female plants of *R. acetosa* selected from the progenies of five genetic crosses (kindly provided by Professor John Parker of Cambridge Botanic Garden) were analysed for AFLP. The male plant accessions were 22.22, 22.25, 57.1, 57.2, 57.11, 57.12, 58.2, 59.2, 59.24 and 65.20 and the female plant accessions were 57.9, 57.10, 58.1, 58.4, 58.6, 58.7, 58.8, 59.1, 59.4 and 59.17. The AFLP technique followed is detailed in Rahman[9].

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DNA from leaf samples were isolated according to Ainsworth *et al.*[10] or using Nucleon Phytopure plant DNA extraction kit (Amersham Life Science) and digested with two restriction enzymes: *Mse*I, a frequent cutter and *Pst*I, a rare cutter. *Mse*I and *Pst*I (biotinylated) adapters were ligated and the *Pst*I-cut fragments were selected linking with Steptavidin Dynabeads. The restriction fragments were selectively PCR amplified using the following *Mse*I (end-labelled with [γ-33P] ATP) and *Pst*I primers with 3 and 2 selective nucleotides, respectively.

Primers	Sequences
*Mse*I primers	19-mer
*Mse*I.1	5′-GAT GAG TCC TGA GTA Aga a-3′
*Mse*I.2	5′-GAT GAG TCC TGA GTA Aac a-3′
*Mse*I.3	5′-GAT GAG TCC TGA GTA Aac c-3′
*Mse*I.4	5′-GAT GAG TCC TGA GTA Acc g-3′
*Pst*I primers	18-mer
*Pst*I.1	5′-GAC TGC GTA CAT GCA Gac-3′
*Pst*I.2	5′-GAC TGC GTA CAT GCA Gaa-3′
*Pst*I.3	5′-GAC TGC GTA CAT GCA Gca-3′
*Pst*I.4	5′-GAC TGC GTA CAT GCA Gcc-3′

* Sequences in lower case are the selective nucleotides at the 3' end of the primers

The thermal cycles in PCR were as below 12 cycles: denaturation 94°C for 30 sec; annealing (lower annealing temperature by 0.7°C each cycle i.e. 65, 64.3, 63.6, 62.9, 62.2, 61.5, 60.8, 60.1, 59.4, 58.7, 57.3°C) for 30 sec; extension 72°C for 60 sec. 23 cycles: denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 60 sec.

Following electrophoresis of the PCR products in 5% denaturing polyacrylamide gel and autoradiographic visualisation, polymorphism in the AFLP banding patterns was scored. Each primer set generated a large number of bands of which 60 to100 could easily be scored. Bands that were clear and distinguished and which were either common to all samples or showed polymorphism were scored. Additional bands could not be scored either because of faint, inconsistent amplification or the inability to differentiate two or more fragments of a similar molecular mass.

Fifteen combinations of primer sets were tested and the AFLP scores were recorded as 1 or 0 for the presence or absence of a specific band in corresponding samples. These data were analysed based on their symmetry using Genstat Version 5.

RESULTS

AFLPs in *Rumex acetosa*

Polymorphism within each sex: Fifteen combinations of primer sets were tested generating a total of 1085 scorable bands (Table 1).

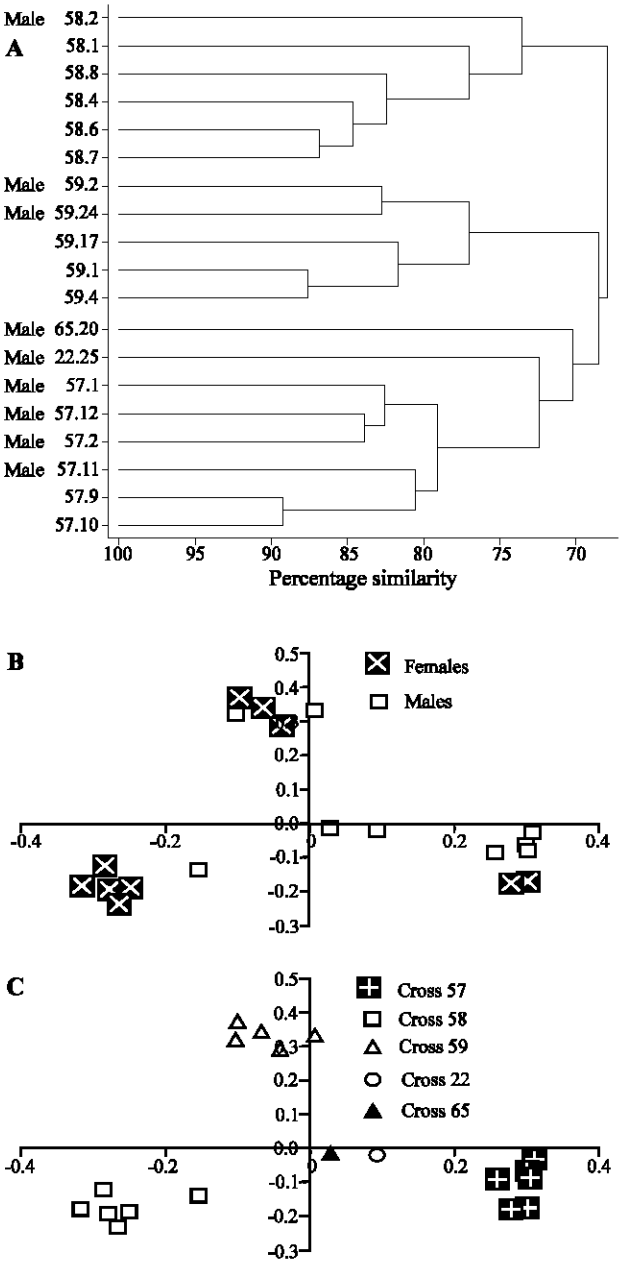

Fig. 1: AFLP in *R. acetosa* generated by four primer combinations (*Mse*I.1/*Pst*I.1, *Mse*I.1/*Pst*I.3, *Mse*I.3/*Pst*I.2.and *Mse*I.3/*Pst*I.3) classify the male and female plants according to their pedigree. Dendrogram shows closeness of the plants (male plants are marked, the others are females) generated from the same cross (A). PCO plot presentations (1st versus 2nd co-ordinate) show random distribution of male and female plants irrespective of their sex (B) and cluster distribution in relation to their origin (C)

Table 1: Sex specific AFLP markers in *R. acetosa* as generated by fifteen primer combinations Figures in parentheses show the percentages of total bands scored

Primer combinations	Male specific monomorphic bands	Male specific polymorphic bands	Female specific polymorphic bands	Total number of scorable bands
*Mse*I.1/*Pst*I.1	1	5	-	58
*Mse*I.1/*Pst*I.2	-	-	-	89
*Mse*I.1/*Pst*I.3	2	-	-	89
*Mse*I.1/*Pst*I.4	-	-	-	63
*Mse*I.2/*Pst*I.1	-	-	-	63
*Mse*I.2/*Pst*I.2	-	-	-	91
*Mse*I.2/*Pst*I.3	-	-	-	88
*Mse*I.2/*Pst*I.4	-	3	-	67
*Mse*I.3/*Pst*I.1	-	-	-	65
*Mse*I.3/*Pst*I.2	2	6	1	94
*Mse*I.3/*Pst*I.3	-	5	-	60
*Mse*I.3/*Pst*I.4	-	-	-	76
*Mse*I.4/*Pst*I.1	-	5	-	68
*Mse*I.4/*Pst*I.2	-	-	-	59
*Mse*I.4/*Pst*I.3	-	3	-	55
Total score	4 (0.37%)	27 (2.49%)	1 (0.092%)	1085

AFLP band patterns in *R. acetosa* shows high level of variation between individuals, which were unrelated to sex. Four AFLP gels were scored in detail to estimate the level of polymorphism in this species. There were 34 polymorphic bands (58.6%) out of 58 bands scored on the autoradiograph generated by *Mse*I.1/*Pst*I.1 primer combination. The *Mse*I.1/*Pst*I.3 combination produced 51 polymorphic bands (57.3%) out of 89 scored. *Mse*I.3/*Pst*I.2 produced 67 polymorphic bands (71.3%) out of 94 scored. *Mse*I.3/*Pst*I.3 produced 36 polymorphic bands (60%) out of 60 scored. On summation, 188 AFLP bands were found to be polymorphic out of 301 bands scored from the gels generated by the four primer combinations, producing an average band polymorphism of 62.5%.

Principle coordinate analysis (PCO, Gower[35]) of the scored AFLP data shows a large amount of within population similarity ranging from 75-80%. The combined data obtained from four primer combinations classify the plant samples into five groups according to their origin without any bias to their sex (Fig. 1A). The PCO plot presentation of the 1st coordinate versus 2nd coordinate components of the AFLP band scores also reveals no cluster distribution of the female and male samples in respect of sex; rather they form clusters as per the cross populations from where they originated (Fig. 1B-C).

Male specific AFLP markers in *R. acetosa*: AFLP in *R. acetosa* generated very low level (0.37% of total scorable bands) of male specific markers (Table 1). Of the 15 primer combinations tested in this species only two combinations produced male specific bands. Each of the primer combinations *Mse*I.1/*Pst*I.3 and *Mse*I.3/*Pst*I.2 generated in two male specific monomorphic bands (Fig. 2).

The primer combination *Mse*I.1/*Pst*I.1 produced a generally male specific band, but in repeated experiments this band could be amplified with low intensity from female samples (Fig. 3). Twenty seven polymorphic male specific bands, covering only 2.49% of total 1085 bands, were also generated by different primer combinations (Table 1). Here 'monomorphic band' refers to a sex specific marker that is present in all the samples of one sex and is absent in the samples of other sex, while 'polymorphic band' refers to a sex specific marker found in one or few samples of one sex or the other.

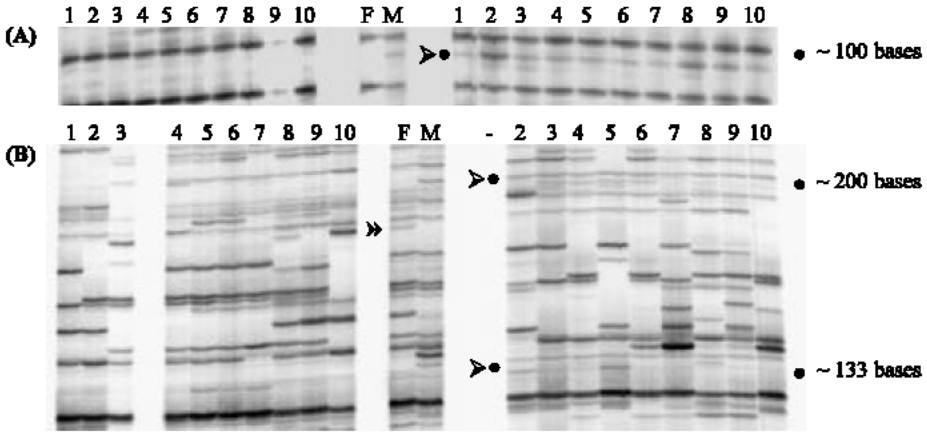

Fig. 2: AFLP generates male specific markers in *R. acetosa*. The *Mse* I.1/*Pst* I.3 primer combination generates two male specific bands including one (arrowed) with nearly 100 bases (A). The *Mse* I.3/*Pst* I.2 primer combination also generates two (nearly 200 and 133-base long) male specific bands (arrowed) and a polymorphic female specific band (double arrowed) (B). Ten lanes on the left are female samples and ten lanes on the right are male samples. F and M are bulked female and male samples run in the middle of the gel for easy comparison of sex specific bands

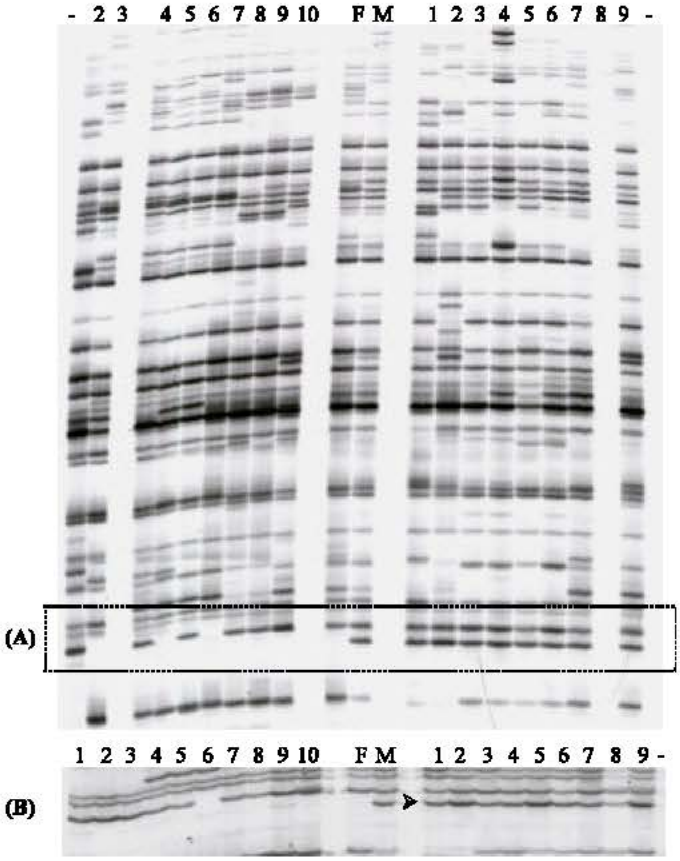

Fig. 3: AFLP in *R. acetosa* generated by the *Mse*I.1/*Pst*I.1 primer combination shows a generally male specific band as arrowed (A). In repeated experiments, this band rarely appears with low intensity in female samples (B). Ten lanes on the left are female samples and ten lanes on the right are male samples. F and M are bulked female and male samples run in the middle of the gel for easy comparison of sex specific bands

MADR1

```
gctagaaaat  ctgctacctg  attccttggg  ccacggcatt  ttcatggctt  atatcagaat  aaatatctcc  atttgctgga  80
ggagcaacag  gacttcccaa  gcacactgca  ttaggtgaag  cagcttcaga  aatagattgc  ccattcctgt  catctgtgaa  160
gccatgtctc  ccaggcaagc  gccctagaga  tgaagcagct  gcaattgtct  cagcagaaga  catgtcttcg  gcaagcagta  240
gatcatcgag  caaaacccct  gtagtaatgt  tattagaata  tcagcaagat  ccccaacatt  caacaaaaag  ttccaatact  320
actgctaata  caactaactg  cccccaataa  acctcacata  taagcaaatc  agagataaaa  cctacacaat  ggcacaaatt  400
tagtatttta  gccttgcatg  tatcaaagct  tattcaacaa  atcatatgta  gcacaaaagc  cgacaaagag  gaaccaagca  480
attcatatat  ccccaaagga  gcgtaatttg  aagaaataaa  gtgaaccaag  catcaagtct  taccaccacg  taaaccacca  560
tacatataaa  tcaaatcgcc  gat 583
```

MADR2

```
cctgtaatca  acgcacaagc  gccaagaacc  gtctttttc  ttcaccagaa  taatgggaga  ggcatatggg  ctagagctgt  80
actgaattgt  accctggcta  agcatctcct  gtatcatttt  atcgatgata  tct 133
```

Fig. 4: Nucleotide sequences of the male specific genomic DNA fragments isolated from differential AFLP bands in *R. acetosa* (*MADR1 = male associated DNA in Rumex 1; MADR2 = male associated DNA in Rumex 2*)

Female specific AFLP markers in *R. acetosa:* Of the total of 1085 AFLP markers generated by the 15 sets of primers, no monomorphic female specific band was found in *R. acetosa*. However, the primer combination *Mse*I.3/*Pst*I.2 generated one polymorphic female specific band (Fig. 2B).

Sequence analysis of male specific AFLP markers: Among the few male specific AFLP markers in *R. acetosa*, two fragments of approximately 600 and 100-bp, were generated by the primer combination *Mse*I.1/*Pst*I.3 and two fragments of approximately 200 and 150-bp were generated by the primer combination *Mse*I.3/*Pst*I.2. The

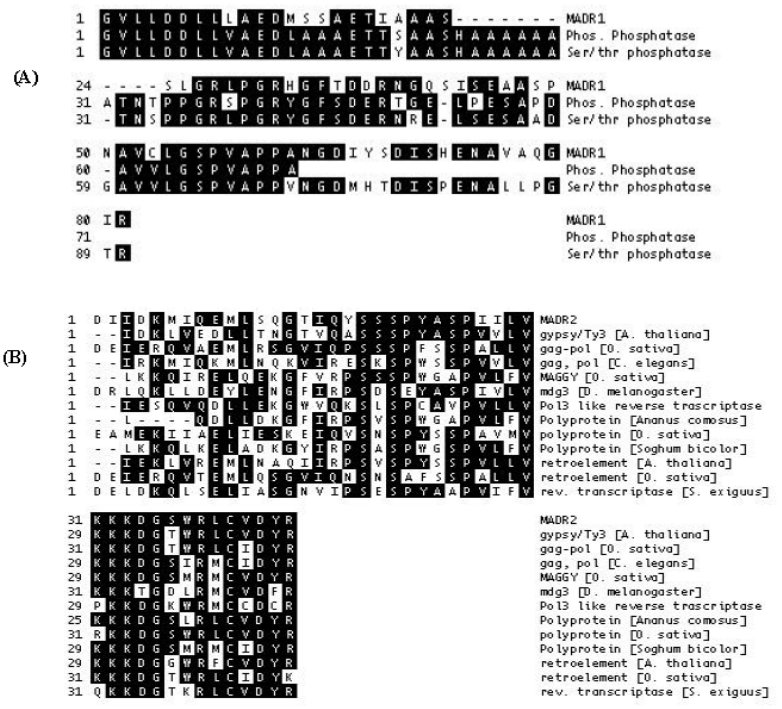

Fig. 5: Multiple alignments of the deduced amino acid sequence of male associated DNA in *R. acetosa* show similarity with known sequences from diverse organisms: (A) *MADR1* with protein serine/threonine phosphatase alpha and putative phosphoprotein phosphatase from *Arabidopsis thaliana*; (B) *MADR2* with seven homologous protein sequences: gypsy/Ty3 retroelement polyprotein (*A. thaliana*), a putative gag-pol polyprotein (*Oryza sativa*), gag pol env protein precursor (*Caenorhabditis elegans*), transposon MAGGY gag and pol gene homologues (*O. sativa*), retrotransposon mdg3 (*Drosophila melanogaster*), a putative POL3-like reverse transcriptase (*Phaseolus coccineus*), a polyprotein (*Ananas comosus*), a putative polyprotein (*O. sativa*), polyprotein (*Sorghum bicolor*), a putative retroelement pol polyprotein (*A. thaliana*), a putative retroelement (*O. sativa*) and a reverse transcriptase (*Saccharomyces exiguus*)

600 and 150-bp DNA fragments, named *MADR1* and *MADR2* (male-associated DNA sequence in *Rumex acetosa*), respectively were cloned into the plasmid vector, pGEM-T. On sequencing, *MADR1* and *MADR2* were found to be 583 bp and 133 bp long, respectively (Fig. 4). The A+T and C+G contents of *MADR1* are 59.01 and 40.99%, while those in *MADR2* are 56.39 and 43%, respectively. BLASTn (Basic Local Alignment Search Tool) data search for similar sequences has shown sequence similarity to short stretches of genomic DNA (10-30 bp) from several organisms.

On Blastx search, *MADR1* and *MADR2* showed similarity to known protein sequences from diverse organisms. Deduced amino acid sequences of *MADR1* showed 47.9 and 56.8% similarity to protein serine/threonine alpha and a putative phosphoprotein phosphatase, respectively, from *Arabidopsis thaliana* (Fig. 5A). Deduced amino acid sequences of *MADR2* showed significant similarity to many retroelement sequences including gypsy/Ty3 retoelement (71.4%) from *A. thaliana*, a putative gag-pol polyprotein (63.6%) and transposon MAGGY gag and pol gene homologue (52.4%) from *Oryza sativa*, gag pol env protein precursor (64.3%) from *Caenorhabditis elegans* (Fig. 5B).

AFLP markers in related *Rumex* spp: AFLP was also investigated in the male and female plants of *R. rothschildianus*, *R. hastatulus* and *R. acetosella*. AFLP in four *Rumex* species including *R. acetosa* shows high levels of polymorphism in this genus (Fig. 6).

Rumex rothschildianus: *R. rothschildianus* is one of the ten dioecious species of the subgenus *Acetosa* of the genus *Rumex*, characterised by a distinctive sex chromosome system with 2n=12+XX in females and 2n=12+XYY in males. This very rare species is restricted to the light soils of the Sharon coastal plain in Israel and North Africa[4,11].

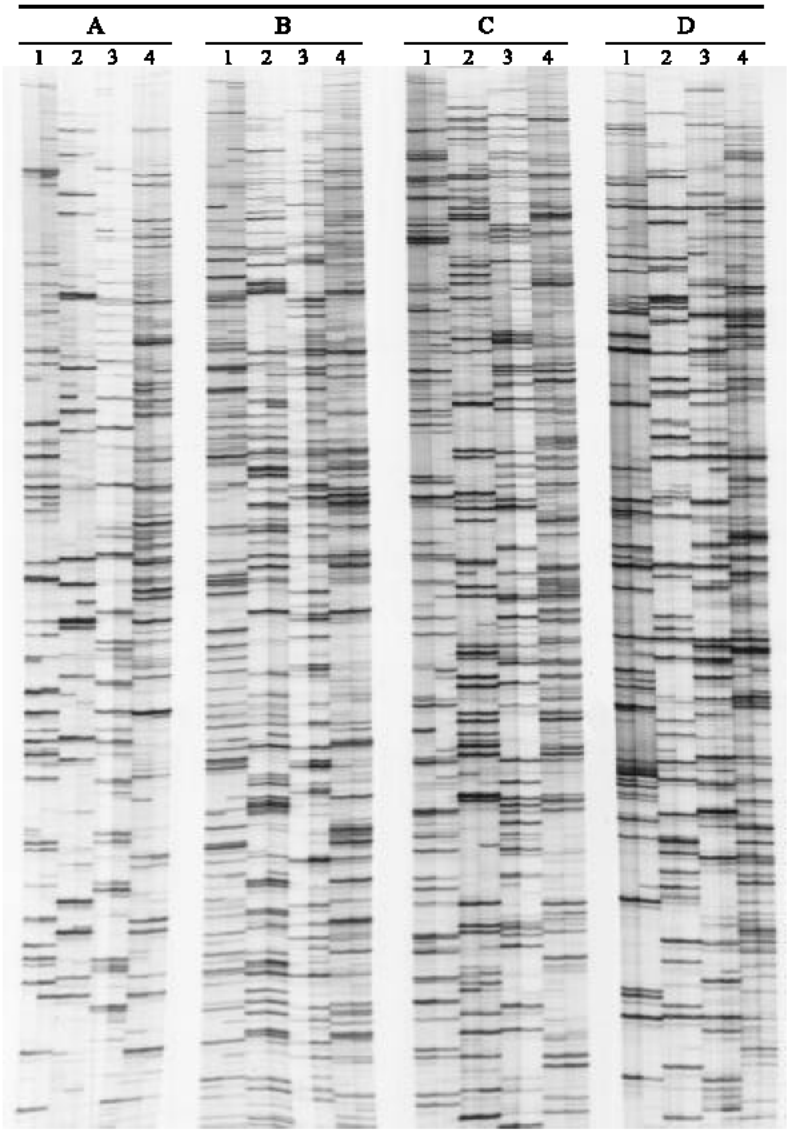

Fig. 6: AFLP generated from the bulked male and female DNA samples of four *Rumex* species using four primer combinations shows high levels of polymorphism in this genus. (A) *Mse*I.1/*Pst*I.1, (B) *Mse*I.1/*Pst*I.3, (C) *Mse*I.3/*Pst*I.2, (D) *Mse*I.3/*Pst*I.3, (1) *R. acetosa* bulked female/male, (2) *R. hastatulus* bulked female/male, (3) *R. rothschildianus* bulked female/male, (4) *R. acetosella* bulked female/male

AFLP in *R. rothschildianus* generated by eight primer combinations showed a very low level of sex related polymorphism as found in *R. acetosa*. Out of total 464 bands scored in *R. rothschildianus* only two (0.43%) were monomorphic male specific bands generated by the primer combination *Mse*I.1/*Pst*I.1 (Table 2). Four polymorphic male specific bands (0.86%) were also scored one from the combination *Mse*I.1/*Pst*I.1, two from *Mse*I.3/*Pst*I.3 and one from *Mse*I.3/*Pst*I.4 (Table 2). No female specific AFLP band was identified.

Rumex hastatulus: *R. hastatulus* is an annual member of the family Polygonaceae and a distantly related member of the subgenus *Acetosa*[12] *R. hastatulus* is polytypic for sex chromosomes and has features both of X/autosome balance and active Y systems[13]. The two alternative chromosome constitutions of *R. hastatulus* are 2n=8+XX/XY (2n=10) in the Texas race and 2n=6+XX/XY$_1$Y$_2$ (2n=8/9) in the North California race[12].

In *R. hastatulus*, AFLP generated by five primer combinations produced a total of 254 scorable bands.

Table 2: Sex specific AFLP markers in *R. rothschildianus*, *R. hastatulus* and *R. acetosella* as generated by different primer combinations. Figures in parentheses show percentages of total band scored

Primer combinations	Male specific Monomorphic bands	Male specific polymorphic bands	Female specific polymorphic bands	Total number of scorable bands
R. rothschildianus				
*Mse*I.1/*Pst*I.1	2	1	-	51
*Mse*I.1/*Pst*I.2	-	-	-	63
*Mse*I.1/*Pst*I.3	-	-	-	57
*Mse*I.1/*Pst*I.4	-	-	-	63
*Mse*I.3/*Pst*I.1	-	-	-	45
*Mse*I.3/*Pst*I.2	-	-	-	62
*Mse*I.3/*Pst*I.3	-	2	-	75
*Mse*I.3/*Pst*I.4	-	1	-	48
Total score	2 (0.43%)	4 (0.86%)	-	464
R. hastatulus				
*Mse*I.1/*Pst*I.2	-	-	-	40
*Mse*I.1/*Pst*I.3	2	2	2	77
*Mse*I.1/*Pst*I.4	1	-	-	32
*Mse*I.3/*Pst*I.1	-	3	1	51
*Mse*I.3/*Pst*I.3	-	2	-	54
Total score	3 (1.18%)	7 (2.76%)	3 (1.18%)	254
R. acetosella				
*Mse*I.1/*Pst*I.1	-	-	-	121
*Mse*I.1/*Pst*I.3	-	-	-	134
*Mse*I.3/*Pst*I.2	-	-	-	141
*Mse*I.3/*Pst*I.3	-	-	-	138
Total score	-	-	-	534

Only three monomorphic male specific bands (1.18%) were scored, two generated by the primer combination *Mse*I.1/*Pst*I.3 and one by the primer combination *Mse*I.1/*Pst*I.4. Seven polymorphic male specific bands (2.76%) were also scored, two from either of the combinations *Mse*I.1/*Pst*I.3 and *Mse*I.3/*Pst*I.3 and three from the combination *Mse*I.3/*Pst*I.1. Three female specific polymorphic AFLP bands (1.18%) were identified, two generated by the primer combination *Mse*I.1/*Pst*I.3 and one by *Mse*I.3/*Pst*I.1 (Table 2).

Rumex acetosella: *R. acetosella*, also known as sheep sorrel, is one of the common European species that belongs to a taxonomically difficult species aggregate which includes several taxa of uncertain status[14,15]. This a polyploid species with chromosome constitutions reported as 2n=36+(XXXX)XX in females and 2n=36+(XXXX)XY in males[16]. The presence of heterochromatic Y-chromosome in *Rumex acetosella* has not been authenticated. It is, however, considered that sex in this plant may be controlled by the differential role of Y-chromosomes rather than by an X/autosome dosage system[36].

A remarkably high level of polymorphism was found within the male and female samples of *R. acetosella*. Out of total 534 bands scored, primer combinations *Mse*I.1/*Pst*I.1, *Mse*I.1/*Pst*I.3, *Mse*I.3/*Pst*I.2 and *Mse*I.3/*Pst*I.3 generated 121, 134, 141 and 138 bands, respectively (Table 2). None of these primer combinations generated any sex specific bands. In other *Rumex* species under investigation, either one or more of these four primer combinations tested in *R. acetosella*, generated male specific band(s). There remains a dispute whether *R. acetosella* has evolved with heteromorphic sex chromosome system like *R. aceotsa*. The absence of male specific band in *R. acetosella* supports the view of non existence of well-differentiated heteromorphic sex chromosome in this species. This might also indicate a system of sex determination that is different from the dominant Y-chromosome system.

DISCUSSION

Sexual dimorphism in *R. acetosa* group is regulated by the X: autosome dosage system where the aneuploid condition for a particular autosome can also lead to unexpected sex expression, often with the production of intersex phenotypes. Except for the variation of sex chromosomes as XX in females or XYY in males, normal male and females both carry six pairs of autosomes. The present AFLP investigation in sorrel addresses the DNA represented by the two Y-chromosomes (26.4%) of the male genome. Our study using 15 sets of primer combinations reveals a total of 1085 scorable bands of which only 4 were male specific (0.37%) in *R. acetosa*. Very low level of male specific bands was also observed in other *Rumex* spp having heteromorphic sex chromosomes. Small number of male-specific band suggests that most of the sequences of the Ys are similar to those of X-chromosomes supporting the view of the recent origin of the Ys.

Although AFLP generates more markers and is regarded as 10-times as powerful as RAPD[8] differences in AFLP banding pattern may occur between template DNA extracted from different plant organs[17] and also in duplicate accessions[18]. In *R. acetosa*, a similar variation was found. In several repeated experiments using *Mse*I.1/*Pst*I.1, a generally male specific band could occasionally be amplified from some female samples.

Sequence analysis of the isolated male specific AFLP markers, *MADR1* (583-bp) and *MADR2* (133-bp), shows identity of short stretches of DNA with variety of known sequences from several organisms. As most of the Y sequences are duplications and repeated sequences[6,19,20] these male specific sequences seem to represent the accumulation of DNA with some similarity to short stretches of sequences of different human autosomes and sex chromosomes. These male-associated sequences are rich in AT content (56-59%) and are more likely to contain terminating codons. Indeed, these sequences do not

possess any well-defined open reading frames (ORF) of any length with transcription initiation (ATG) or termination codons (TAA, TAG or TGA). They appear to represent random sequences and may not correspond to any transcribable genes. However, the possibility of transcriptional activity of these sequences along with other sequences of the Y-chromosomes can not be excluded. The significant similarity of deduced amino acids of *MADR1* with stretches of phosphatase protein might indicate a likely functional role of this sequence on the Y-chromosome of *R. acetosa*.

The deduced amino acid sequences of *MADR1* showed low levels of similarity (28%) with zinc finger protein from *D. melanogaster*. In *Arabidopsis thaliana*, the floral homeotic gene *SUPERMAN* (*SUP*) encodes a C2H2-type zinc finger protein, which has a role in the maintenance of the whorl 3 to 4 boundary[21]. SUP in *Arabidopsis* is thought to function by repressing B function gene expression in the central carpel whorl of the flower. *MADR1* sequences may, therefore, be of potential importance to further analyse the structure of Y-chromosome DNA.

Male associated DNA fragments have previously been identified by random amplification of polymorphic DNA (RAPD) in many dioecious plants. Sakamoto and his colleagues cloned a 730-bp long DNA fragment named *MADC1* (male-associated DNA sequence in *Cannabis sativa*). However, *MADC1* does not include a long ORF and is not likely to correspond to a transcribed gene[22]. Using representational difference analysis, several male sex-specific restriction fragments in *Silene latifolia* have been isolated and cloned. These male-specific restriction fragments were found to be homologous to other sequences shared between male and female plants[23]. Y-chromosome DNA sequences were also isolated from *S. latifolia* using Y-chromosome microdissection and selective chromosome laser ablation techniques. These sequences were found to be repetitive and hybridised to DNA from both sexes[24,25]. Nine AFLP markers linked to the sex locus in asparagus have been identified using a non-radioactive AFLP technique and bulked segregation analysis[26].

Deduced amino acid sequences of *MADR2* showed significant similarity to many retroelement sequences from diverse organisms. While transposable elements and repeated sequences in many genomes are hypermethylated[27] methylation also plays a central role in sex chromosome inactivation as found in one of the X-chromosomes in the females of eutherian mammals. Likewise, it is also possible that most of the Y-chromosome DNA in *R. acetosa* is methylated. Although *Pst*I is sensitive to DNA methylation *Mse*I/*Pst*I digestion has been successfully used for AFLP study of genetic variation in a number of plant species including rice[28] black poplar[29] and moringa[30]. Further analysis of sorrel genomes using more enzyme combinations including that are not sensitive to methylation, such as *Eco*RI and more primer sets may provide valuable information.

In a separate study, Clark *et al.*[31] isolated seven non-homologous repeat sequences from *R. acetosa* that were estimated to represent 18% of the genome. However, when these clones were *in situ* hybridised separately to chromosomes of *R. acetosa*, they were found dispersed along all chromosomes with no bias to X, Y or autosomes[31]. Later, a repeated sequence of 180 bp in tandem arrays was isolated from *R. acetosa*, which was found to be localised to the X- and both Y-chromosomes by *in situ* hybridisation[32]. Based on molecular data, a hypothesis was proposed regarding the origin of Y-chromosome that the Ys have evolved from the X-chromosome by centric fission followed by isochromosome formation[32]. Thus, the Ys would have much the same sequences as the X-chromosome. An alternative pathway for the evolution of Y-chromosome is by X/autosome interchange[4]. The male associated DNA sequences in *R. acetosa* show identity to the stretches of human DNA from autosomes and both the X and Y-chromosomes. This indicates that the Y sequences may have originated from the X-chromosome or by X-autosome exchange. Some reports suggest that the heteromorphic sex chromosomes were derived from one pair of autosomes, different species adopting different ones[7,33,34]. More molecular data would depict the contributions of each route of Y-chromosome evolution. The major sex determining genes of *R. acetosa* must reside on the X-chromosomes or autosomes; they remain outside the target of this AFLP approach since X-chromosomes and autosomes are present in both the sexes. As the male associated sequences identified using genomic AFLP are the indicators of presence of the Ys, they do not play a direct role in sex determination. Further investigations using methylation insensitive enzymes may identify more male associated sequences with potential roles in pollen fertility.

ACKNOWLEDGMENTS

The award received from the Commonwealth Scholarship Commission to carry out this research is gratefully acknowledged. The authors are grateful to Professor John Parker of Cambridge Botanic Garden for kindly providing the *R. acetosa* plant samples and to Dr Mark Winfield of Cory Laboratory, Cambridge Botanic Garden for assistance with the AFLP analysis.

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