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ORIGINAL PAPER

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Abstract We developed SCAR primers based on isolated and sequenced male-specific fragments as identified in an AFLP analysis of the dioecious plant *Rumex nivalis*. PCR amplification using these primers on females and males resulted in fragments exclusively present in males. Co-amplification of the nuclear rDNA internal transcribed spacer 2 together with the male-specific fragment was applied as an internal control for successful PCR reactions to avoid false-negative sex scoring. With a length of about 164 bp, the AFLP fragment was of a similar size as the tandemly arranged, repetitive sequences of 180 bp located on the Y chromosomes of *Rumex acetosa*. The genetic distances between the Y-chromosomal sequences of *R. nivalis* and *R. acetosa*, both members of the section *Acetosa*, were substantial. We found intra-individual divergence among cloned sequences of the male-specific fragment in *R. nivalis*. The patterns of interspecific and intra-individual sequence variation found are in accordance with proposed modes of the evolution of sex chromosomes. Y chromosomes possibly arose only once in the genus *Rumex* and consist mainly of heterochromatic DNA. Due to the almost complete absence of selection on them, Y chromosomes are likely to accumulate large numbers of mutations.

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Introduction

In the animal kingdom, the separation of female and male functions among individuals is the common sexual system. In contrast, most flowering plants are hermaphroditic, with flowers bearing both female and male reproductive organs. Only about 6% of angiosperms are dioecious, where individuals either produce staminate or pistillate flowers (Renner and Ricklefs 1995). In a minority of dioecious plants, sex determination depends on sex chromosomes, usually an XY system, in which males are heterogametic (XY) and females are homogametic (XX; Ainsworth 2000; Matsunaga and Kawano 2001; Charlesworth 2002). There are two types of sex chromosomes (Matsunaga and Kawano 2001): homomorphic sex chromosomes, in which the sex chromosomes are morphologically indistinguishable from autosomes, and heteromorphic sex chromosomes, which can be discriminated in cytological analyses. Heteromorphic sex chromosomes have been reported in several families (e.g., *Cannabis* and *Humulus*, Cannabinaceae; *Silene*, Caryophyllaceae; Matsunaga and Kawano 2001), but our understanding of their evolution and genetics is still relatively poor.

Sex determination in the genus *Rumex* is heterogametic, characterised by heteromorphic sex chromosomes and an XY system different from the one described above (Matsunaga and Kawano 2001). Females possess two X chromosomes, and males one X and two Y chromosomes. In the section *Acetosa*, including the well-studied *Rumex acetosa*, sex is determined by a balance between female determinants located on the X chromosomes and female and male determinants on the autosomes (Kihara and Ono 1923; Löve 1969; Zuk 1970a, b). Y chromosomes affect only male fertility but not sex determination (Wilby and Parker 1988). Ruiz Rejón et al. (1994) and Shibata et al. (1999) independently detected a sequence of 180 bp located on both Y chromosomes of *R. acetosa* using two fundamentally different molecular methods. The approach used in the study of Ruiz Rejón et al. (1994) comprised a digestion of genomic DNA with restriction

enzymes separately for females and males, resulting in one band being more intense in male individuals. This band was cloned, in situ hybridised and inferred to be located in the heterochromatic region of the Y chromosomes (Ruiz Rejón et al. 1994). Shibata et al. (1999) used the heteromorphic property of Y chromosomes and manually isolated them from autosomes and X chromosomes by microdissection. DNA from these Y chromosomes was then amplified using the degenerate oligonucleotide primed-polymerase chain reaction (DOP-PCR; Telenius et al. 1992). Labelled PCR products were hybridised in situ, and the Y chromosomes were again stained. Shibata et al. (1999) further constructed a library of the Y-chromosomal DNA and found a sequence specific to the Y chromosomes. Both studies characterised this Y chromosome-specific sequence of 180 bp to be tandemly arranged and highly repetitive. This fits well with the cytological results of Wilby and Parker (1988) on several *Rumex* taxa, in that Y chromosomes are highly heterochromatic except for a minute terminal euchromatic region corresponding to the pairing segment.

In a phylogeographic study that aimed at resolving geographic variation of DNA polymorphisms in the dioecious European endemic *Rumex nivalis* Hegetschw. (Stehlik 2002), male-specific fragments were found using amplified fragment-length polymorphisms (AFLP; Vos et al. 1995). *R. nivalis* belongs to the section *Acetosa* (Wagenitz 1981) for which sex determination is outlined above. Thus, we hypothesized that these male-specific fragments are located in the heterochromatic region of the Y chromosomes as well.

The first objective of our study was to develop a method that uses sex-specific fragments as identified by AFLPs to generate male-specific PCR primers based on sequence-characterised amplified-regions analysis (SCAR; Paran and Michelmore 1993). Sex chromosome specific markers were developed for several plants (e.g., Mulcahy et al. 1992 and Zhang et al. 1998: *Silene latifolia*; Hormaza et al. 1994: *Pistacia vera*; Harvey et al. 1997: *Actinidia* spp.; Mandolino et al. 1999: *Cannabis sativa*; Korpelainen 2002: *R. acetosa*), mostly by combining bulked segregant analysis (Michelmore et al. 1991) with random amplified polymorphic DNA (RAPD; Williams et al. 1990). The procedure of the RAPD protocol is less time-consuming than AFLPs (Griffiths and Orr 1999). Moreover, AFLPs are much more costly in terms of laboratory equipment. Despite these disadvantages, AFLPs are more powerful in potentially detecting polymorphisms, as they usually produce 50 to 100 bands per primer per individual (Vos et al. 1995) as compared to RAPDs with only five to ten bands (Griffiths and Tiwary 1993).

The second objective of our study was to compare the sequence variation in SCAR regions of *R. nivalis* with the Y chromosome-specific sequences of *R. acetosa* and, thereby, to infer whether these are in accordance with the proposed mode of the evolution of plant sex chromosomes (Charlesworth 2002).

Materials and methods

Plant material, DNA extraction and AFLPs

R. nivalis is a diminutive wind-pollinated, perennial herb growing in snow-beds or along creeks above timber-line at 1,600 to 2,900 m a.s.l. in Central and southeastern Europe (Wagenitz 1981). In a previous AFLP analysis (Stehlik 2002), some fragments were tentatively identified as sex-specific. One of these fragments was especially intense and present in all male plants (N=80). This fragment was the amplification product of the selective primer pair EA+CC/M0+CTC (for sequences see below) and sized by 192 bp. To determine the effectiveness of this fragment as a male-specific marker, we collected leaf material of flowering females and males in Flims (in both cases N=60), Canton of Grisons, Switzerland (2,100 m a.s.l., 9°13'32.6''N/46°53'5.8''E).

We extracted genomic DNA using the DNeasy extraction kit (QIAGEN), according to the manufacturer's instructions. As an additional purification step, all samples were cleaned via precipitation with Na-acetate and cold ethanol. This latter purification step was performed routinely in our *Rumex* accessions due to the sensitivity of the ligation to secondary compounds. We carried out the AFLP protocol following the procedure described by Vos et al. (1995) with minor modifications as given in Stehlik et al. (2000). The ligate was pre-amplified with the one-base selective primer EA (*5′-GACTGCGTACCAATTCA-3′*) and the non-selective M0 (*5′-GATGAGTCCTGAGTAAG-3′*), and subsequently selectively amplified using the primer pair EA+CC/M0+CTC.

Fragment isolation, cloning, sequence analysis and SCAR primer design

We separated the total AFLP amplification product of one male of *R. nivalis* on an Elchrom-spreadex-600 gel (Elchrom Scientific) at 20°C and 120 V/200 mA, using the SEA 2000 electrophoresis apparatus (Elchrom Scientific) for 225 min and stained in ethidium-bromide for 1 h. A small piece of the gel containing the targeted fragment was excised and directly re-amplified without prior cleaning using the same PCR conditions as for pre-selective AFLP amplification (Stehlik et al. 2000), but with a shorter elongation (30 s instead of 60 s). The resulting fragment was electrophoretically purified in a 1.5% agarose gel, eluted with a QIAquick Gel Extraction Kit (QIAGEN) and TA-cloned in the pGEM-T Easy vector (Promega). After plasmid purification with the QIAGEN Plasmid Mini Kit, we sequenced ten clones with the Rhodamine Dye Terminator technology (PE Biosystems) according to the manufacturer's recommendations. Sequence detection was performed on an ABI 377 DNA sequencer (PE Biosystems). We manually aligned the seven identified different sequences of *R. nivalis* to published Y chromosome sequences of *R. acetosa* and used them to design two *R. nivalis*-specific SCAR primers (RnivY-F: *5′-GTTAGAATAATCTATTTCATTTGCC-3′*; RnivY-R: *5′-TTCACCTATATCGATGACC-3′*). We calculated pairwise maximum-likelihood distances and a phenetic tree with the neighbour-joining algorithm in PAUP* 4.0 (Swofford 2002) to investigate genetic relationships among these sequences.

Amplifications to test for sex-specificity were conducted under standard PCR conditions using 3 ng of genomic DNA. To avoid false-positive scoring of individuals as females due to failures of PCR, i.e., an absence of the male-specific amplification product, we co-amplified the nuclear ribosomal DNA (nrDNA) internal transcribed spacer 2 (ITS2) together with the male-specific fragment as an internal PCR control. Therefore, we included both RnivY primers together with primers ITS-B and ITS-D (Blattner 1999) in one PCR reaction under the following cycling conditions: 4 min at 94°C followed by 35 cycles of 45 s at 92°C, 45 s at 54°C and 30 s at 72°C, and a final extension time of 10 min at 72°C. PCR products were visualised on 1.6% agarose gels stained with ethidium-bromide.

Results and Discussion

Molecular analysis of sex chromosomes in *R. nivalis*

PCR with the AFLP-derived SCAR primers resulted in an exclusively male-specific fragment of 150-bp length in all *R. nivalis* individuals tested (Fig. 1). Thus, AFLPs indeed proved to be a powerful method to detect male-specific fragments in this sexually heterogametic and heteromorphic species and, at the same time, they were a useful starting point for the development of SCAR primers. The potential of AFLPs to produce a high number of fragments (Vos et al. 1995) and, therefore, an increased chance to detect character-linked markers, is reflected in the fact that the source of the present development of male-specific markers in *R. nivalis* was a side-product of its phylogeographic investigation (Stehlik 2002).

The co-amplification of ITS2 together with male-specific SCAR-primers proved to be an effective method to prevent erroneous scorings of males as females, the latter being possibly due to a disfunction of PCR (i.e., no amplification product). Basically, the sole amplification of ITS2 resulted in one band present in both females and males (Fig. 1). In the co-amplification of ITS2 with SCAR-primers in females, as expected, the ITS2 fragment was present, whereas the male-specific fragment was absent. Using both sets of primers in males, the Y-chromosomal band was present but, surprisingly, the ITS2 fragment was absent or faint (Fig. 1). This is most-likely due to the competitive nature of PCR, where the shorter Y-specific fragment outnumbers the longer ITS2 amplification product. Possibly also differences in copy numbers might influence this result: rDNA-repeats normally occur in numbers from 100 to a few thousand in the genome whereas the Y-chromosomal sequence might even be present in much higher numbers (see below; Ruiz Rejón et al. 1994; Shibata et al. 1999). Therefore, a larger chance for a sole amplification of the Y-chromosomal sequence as compared to the ITS2 in males of *R. nivalis* occurs, despite the successful amplification when using the ITS-primers only (Fig. 1). This PCR control system with the co-amplification of ITS2 and male-specific SCAR-primers is now successfully used in a study on *R. nivalis* to infer unbiased sex ratios at different life stages (Stehlik, unpublished). *R. nivalis* belongs to the few known cases in dioecious plant species in which populations are strongly female-biased (Delph 1999; Stehlik, unpublished).

The length of 192 bp (164 bp after subtraction of the AFLP adapters on both ends of the fragment) of the male-specific AFLP band used as a starting point for SCAR primer development, was of similar size as the tandemly arranged, repetitive sequence of 180 bp located on the Y chromosomes of *R. acetosa* (Ruiz Rejón et al. 1994; Shibata et al. 1999; Korpelainen 2002). Within ten sequenced clones of this AFLP fragment in *R. nivalis*, we detected seven different sequences. These sequences (EMBL database numbers AJ544227–AJ544233) matched with 67 to 71% identity published sequences of the *R.*

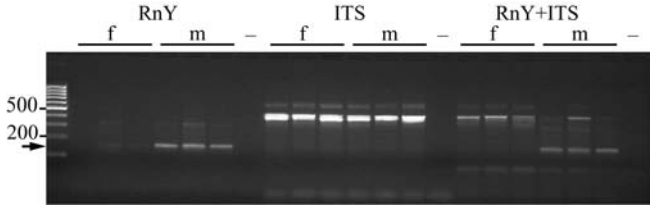

Fig. 1 Sex-specific PCR products (SCAR markers) of six individuals in *R. nivalis*. Males (m) are characterized by a single fragment of 150-bp length (*arrow*) that is absent in females (f). Co-amplification of the internal transcribed spacer 2 (ITS) together with the male-specific fragment (RnY) serves as an internal control for successful PCR reactions to avoid false-negative sex scoring.

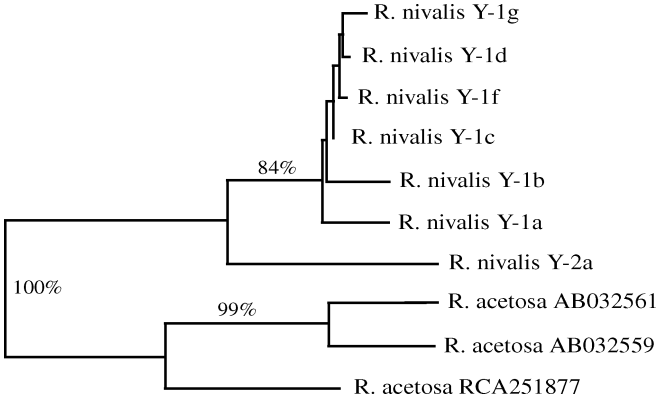

Fig. 2 Neighbor-joining tree of the aligned Y chromosome-specific sequences of *R. nivalis* and *R. acetosa*, calculated from maximum-likelihood distances (GTR+Γ). Bootstrap values from 500 bootstrap re-samples are given above the branches

acetosa Y chromosome (Shibata et al. 1999, 2000: EMBL database numbers AB032559–AB032561; Ruiz Rejón et al. 1994: RAC251877) in FastA database searches. Phenetic analysis of these sequences together with the easily alignable and probably homologous Y-chromosomal sequences of *R. acetosa*, revealed two different classes of Y chromosome sequences in *R. nivalis* (here named Y-1 and Y-2; Fig. 2). Within the Y-1 group (six sequences), genetic distances were about 1 to 11%, whereas between Y-1 and Y-2 sequences, genetic distances ranged from 29 to 33%. The *R. acetosa* sequences were clearly separated (bootstrap support: 100%) from the *R. nivalis* sequences with genetic distances between 55% and 75% (Fig. 2).

Implications for the evolution of sex chromosomes

The among-clone genetic differentiation within one individual of *R. nivalis* is in accordance with results based on theoretical studies on the evolution of sex chromosomes (Charlesworth 2002). Sex-chromosome evolution involves the cessation of genetic exchange between the proto-X and the proto-Y chromosomes.

Mutation accumulation by Muller's ratchet, hitch-hiking and fixation of mutations due to non-recombination, are proposed to trigger differentiation between the emerging sex chromosomes (Charlesworth 2002). This accumulation of mutations on Y chromosomes is compatible with our finding of multiple copies of Y chromosome-specific sequences in *R. nivalis* and the relatively large genetic differences between species from the same section within *Rumex* (Fig. 2). The accumulation of mutations on Y chromosomes should coincide with Y chromosome degeneration (Charlesworth 2002) which is especially evident in all studied species of *Rumex*, in which Y chromosomes consist of a massive heterochromatic block except for a minute terminal euchromatic region corresponding to the pairing segment (Wilby and Parker 1988). Hence, our markers most likely represent sections of this tandemly repeated non-coding DNA, that are probably not under selection (Shibata et al. 1999; Charlesworth 2002).

The straightforward alignment of Y-chromosomal sequences of *R. nivalis* with those of *R. acetosa* (despite the high rate of accumulation of mutations) supports the generally accepted hypothesis of a single origin of Y chromosomes and, hence, the heterogametic sex determination in *Rumex* (Löve 1969; Zuk 1970a, b; Wilby and Parker 1988; Shibata et al. 2000). The higher divergence between the sequences of the Y-1 and Y-2 classes in *R. nivalis* and the existence of two comparatively diverse groups of Y chromosome-specific sequences in *R. acetosa* (with genetic distances about 40%; Fig. 2) is consistent with the hypothesis of an early duplication of the proto-Y chromosome in (at least) the section *Acetosa*, though a certain amount of recombination and homogenisation between both Y chromosomes seems necessary to explain the topology shown in Fig. 2.

Nevertheless, our results should be interpreted with caution due to the limited clone sample-size and because neither Ruiz Rejón et al. (1994) nor Shibata et al. (2000) were able to discriminate the two Y chromosomes in *R. acetosa*. Therefore, it should be more thoroughly tested; whether the occurrence of two different classes of Y chromosome-specific sequences is indicative for a divergence of Y chromosomes in *Rumex*. Such differences among cloned sequences could solely be due to the existence of different elements of repetitive DNA within single Y chromosomes, irrespective of whether they are located on the Y1 or Y2 chromosome.

Given the substantial sequence homology among the two species of the section *Acetosa* examined in the present investigation, a thorough systematic investigation of multiple representatives of all sections of *Rumex* using Y chromosome-specific primers would open the possibility to trace the evolution of this rare heteromorphic sex determination system. This would be of special interest since different modes of sex determination occur within the genus (Löve 1969). In *R. acetosella*, females are characterized by XX and males by XY. The Y chromosome is male-determining and sufficient to confer maleness even in artificial dodecaploids. This contrasts with the situation in section *Acetosa*, where sex is determined by a balance between female determinants on the X and male *and* female determinants on the autosomes (Löve 1969). In the latter case, the Y chromosomes only affect male fertility, i.e., loss of parts of Y chromosomes results in sterile pollen (Löve 1969; Zuk 1970a, b).

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