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ORIGINAL PAPER

MK17, a specific marker closely linked to the gynoecium suppression region on the Y chromosome in *Silene latifolia*

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Abstract The aim of this work was to isolate new DNA markers linked to the *Silene latifolia* Y chromosome. To do this we created a chromosome-specific plasmid library after DOP-PCR amplification of laser-microdissected Y-chromosomes. The library screening led to the isolation of several clones yielding mostly to exclusive male specific hybridization signals. Subsequent PCR confirmed the Y-unique linkage for one of the sequences. This DNA sequence called MK17 has no homology to any known DNA sequence and it is not expressed. Based on PCR and Southern analyses, MK17 is present only in dioecious species of the *Elisanthe* section of the genus *Silene* (*S. latifolia*, *S. dioica*, and *S. diclinis*) and it is absent in related gynodioecious and hermaphroditic species. The mapping analysis using a panel of deletion mutants showed that MK17 is closely linked to the region controlling suppression of gynoecium development. Hence MK17 represents a valuable marker to isolate genes controlling the gynoecium development suppression on the Y chromosome of *S. latifolia*.

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Introduction

Sex chromosomes have evolved independently in many animal and plant species (reviewed in Charlesworth et al. 2005). At present, the human sex chromosomes are the most intensively studied (reviewed in Vallender and Lahn 2004; Gvozdev et al. 2005). However, because they are evolutionary old and highly degenerated (reviewed in Charlesworth and Charlesworth 2000), they are not suitable to study early stages of sex chromosome evolution. As the basic mechanisms of sex chromosome evolution share some common features (Marin and Baker 1998), these early events can be studied using other model organisms possessing sex chromosomes of a more recent origin.

In order to study sex chromosome evolution in a given species, it is necessary to know the structure of both the X and Y (or Z and W) chromosomes. Even without a whole-genome sequencing, it is relatively easy to construct X chromosome maps because the order and relative distances of the known X-linked (and Z-linked) genes can be obtained by recombination mapping. Construction of the Y- (and W-) chromosome maps is more difficult because these chromosomes do not undergo recombination during meiosis. The most common approach involves radiation hybrid ordering (Raudsepp et al. 2004) or deletion mapping (Lebel-Hardenack et al. 2002). To do this, it is necessary to prepare a panel of radiation hybrids or organisms with deletions on different parts of the Y (or W) chromosome and to use not only Y (or W)

chromosome-linked genes to be mapped but also a high number of Y (or W) chromosome-linked markers.

With an aim to study the evolutionary young sex chromosomes, dioecious plant species are recently used as models (reviewed in Vyskot and Hobza 2004). Among them, *Silene latifolia* (syn. *Melandrium album* or white campion) has been most intensively studied (reviewed in Negrutiu et al. 2001). *S. latifolia* is a dioecious plant possessing a pair of heteromorphic sex chromosomes with the sex determination system based on the dominant role of the Y chromosome. The Y chromosome consists of four basic functional regions (Westergaard 1958): (1) a region causing suppression of gynoecium development (Lardon et al. 1999), (2) a region causing promotion of stamen development (Farbos et al. 1999), (3) a region containing gene(s) necessary for late anther development, and (4) a recombining pseudoautosomal region (Westergaard 1958). The recent results show that *S. latifolia* represents an appropriate model species to study evolutionary processes taking place on the Y chromosome (Filatov et al. 2000; Filatov and Charlesworth 2002; Nicolas et al. 2005). It is the only organism with evolutionary young heteromorphic sex chromosomes where maps of the X and Y chromosomes are available.

The X chromosome recombination map of *S. latifolia* has been reported recently (Nicolas et al. 2005). The map consists of a pseudoautosomal marker and five functional genes. The Y chromosome map is based on mapping of 44 X-ray-induced sexual phenotype mutants (Lebel-Hardenack et al. 2002). The map was originally built up using 27 AFLP markers, 2 Southern hybridization-based markers, and 1 PCR marker (Lebel-Hardenack et al. 2002). It was used by Moore et al. (2003) to order three genes on the Y chromosome possessing copies on the X chromosome—*SlY1* (Delichére et al. 1999), *SlY4* (Atanassov et al. 2001), and *DD44Y* (Moore et al. 2003). The map was further refined by Zluvova et al. (2005) by adding of several other markers including three Y-linked genes—*Slss* (Filatov 2005), *SlY3* (Nicolas et al. 2005), and *SlAP3Y* (Matsunaga et al. 2003). This map enabled comparison of gene orders on the X and Y chromosomes which revealed an inversion on the sex chromosomes that occurred after the recombination arrest (Zluvova et al. 2005).

Despite the large efforts, the current Y chromosome map still contains several gaps for which markers are not available. In this work, the construction and screening of Y-chromosome-specific library was used to search for DNA markers localized on the Y chromosome. We demonstrate that this approach facilitated recovery of new Y-specific DNA sequences. A new marker closely associated with the gynoecium suppression region is described in detail.

Materials and methods

Plant material

Silene latifolia Garcke (syn. *Melandrium album* Poiret) plants were derived from an inbred line generated by eight generations of brother–sister mating (a gift from Dr. J van Brederode, State University, Utrecht, The Netherlands). *S. dioica* seeds were collected at Tisnov (Czech Republic), *S. otites* and *S. vulgaris* at Brno surroundings (Czech Republic), and *S. viscosa* was collected at Veseli/Morava (Czech Republic). *S. diclinis* plants were obtained from the seed collection of the Institute of Biophysics, Brno.

Metaphase chromosomes preparation and microdissection

To accumulate cells in metaphase, root tips of germinating seeds were treated as described in Lengerova et al. (2004). The root tips were subsequently cut off and protoplasts were isolated as described by Hladilova et al. (1998). Slides were prepared by dropping the protoplast suspension in Farmer's fixative (ethanol: acetic acid, 3:1) on slides covered with polyethylene naphthalate membrane (P.A.L.M. GmbH, Bernried, Germany). The PALM MicroLaser system (P.A.L.M. GmbH) was used to dissect and collect X, Y, and autosomes. All procedures were performed as described in Kubickova et al. (2001).

DOP-PCR amplification of microdissected chromosomes

DOP-PCR was performed in an Eppendorf tube with ten microdissected chromosomes, either X or Y, as a template in a PTC-200 thermocycler (MJ Research, Watertown, USA). PCR was carried out according to Telenius et al. (1992) with minor modifications. Microdissected chromosomes were directly used as a PCR template without any special pretreatment. Amplification reaction contained 1 × *Taq* polymerase buffer (PCR Blue buffer, Top-Bio, Czech Republic), 0.2 mM each of four dNTPs (Promega, USA), 2 μM DOP primer 5′-CCG ACT CGA GNN NNN NAT GTG G-3′ (Telenius et al. 1992), and 0.04 U/μl *Taq* polymerase (Top-Bio, Czech Republic). After initial denaturation step at 95°C for 10 min, *Taq* polymerase was added and samples were subjected to eight cycles for 1 min at

95°C, 1.5 min at 30°C, 1.5 min transition to 72°C and 3 min at 72°C, followed by 35 cycles of 30 s at 95°C, 30 s transition to 56°C, 1 min at 56°C, 1 min transition to 72°C, and 72°C for 2 min. The final extension was carried out at 72°C for 10 min.

Southern hybridization-based test of DOP-PCR complexity

Aliquots (20 µl) of DOP-PCR products were subjected to electrophoresis on 1% agarose gel and blotted onto Hybond N membrane (Amersham, UK) using capillary transfer. Hybridization was carried out with *S. latifolia* male genomic DNA isolated from leaves (Vallejos et al. 1992). Genomic DNA (25 ng) was radiolabelled by random priming (Prime-It II Random Primer Labeling Kit, Stratagene, USA) incorporating 50 µCi of [α-^{32}P]-dATP. Hybridization was carried out for 16 h at 65°C in HYBSOL hybridization solution (Yang et al. 1993). A high stringency wash was done in 0.1 × SSC and 0.1% SDS at 65°C.

Dot-blot-based test of DOP-PCR complexity

A set of six pairs of primers was used to amplify gene sequences from *S. latifolia* male genomic DNA: DD44F1 and DD44R1 to amplify a part of *DD44X/Y* gene (Moore et al. 2003), R20 and U20 to amplify the Y-specific sequence ORF285 (Nakao et al. 2002), MROS2-F1 and MROS2-R1 to amplify autosomal sequence of *MROS2* gene (Kejnovsky et al. 2001), MROS4-F1 and MROS4-R1 to amplify autosomal sequence of *MROS4* gene (Kejnovsky et al. 2001), SlY4-F and SlY4-R to amplify a part of the *SlY4* gene (Atanassov et al. 2001), SlX4-F and SlX4-R to amplify a part of the *SlX4* gene (Atanassov et al. 2001); and S1, AS9, and AS10 to amplify a part of the *SlX1/Y1* gene (Delichére et al. 1999). PCR products were cut off the gel, purified (Gel Extraction Kit, Qiagen) and dot-blotted onto Hybond N membrane (Amersham, UK), immobilized by UV-crosslinking and hybridized with DOP-PCR amplified DNA of the X (DOP-X) and Y (DOP-Y) chromosomes. Hybridization procedure was performed as described above.

Partial library preparation and screening

The DOP-PCR amplified DNA of X and Y chromosomes was purified by PCR purification kit (Qiagen) and cloned into pGEM-*T* easy vector (Promega, USA). Bacterial colonies were doubly spotted (2 × 10,000) onto 22 × 22 cm Hybond N + filters (AP Biotech, Buckinghamshire, UK) with the GeneTAC^{TM} G3 workstation. This library was screened using DOP-X, DOP-Y, and a pool of DOP-PCR amplified autosomes. Southern hybridization was performed as described above. Chromosome Y-positive clones were sequenced and their exclusive presence on the Y chromosome was checked using PCR on genomic DNA prepared from male and female plants. One of the clones, called MK17, was selected for further characterization.

Mapping of MK17 using Y deletion mutants

To determine the physical position of MK17 on the Y chromosome, PCR with two different pairs of primers, MK17f (GAT TTC GGT CGG TAT CTG GA)/MK17r (TGT GGG ATG ATT ACA AAC CTC A), and MK17-longf (GGC AGA TGT GGG TAA TTG CT)/MK17-longr (GGA CTA GAA GGT AAC ACG GGA AG) was done on a panel of *S. latifolia* Y chromosome deletion mutants, which were described by Lebel-Hardenack et al. (2002). Primers amplifying the sequence Bgl10 (Donnison et al. 1996), which has been already mapped (Lebel-Hardenack et al. 2002), and primers amplifying the autosomal sequence *MROS2* (Matsunaga et al. 1996), served as controls of the quality of template. Position of MK17 on the Y chromosome was based on the map constructed by Zluvova et al. (2005). Fisher's exact test analysis was performed using the SAS statistics package. Logarithm of the odds of linkage (LOD) scores and position of the MK17 marker on the map were estimated as described by Lebel-Hardenack et al. (2002).

Results

The first step in construction of the Y chromosome specific DNA library was the microdissection of the Y chromosome and its amplification by DOP-PCR. We improved the DOP-PCR reaction by adding three reaction cycles during the low annealing temperature step to avoid any preferential amplification, which would be detected as prominent DNA bands visible after gel electrophoresis. The size of the amplified DNA molecules varied from 200 to 3,000 bp, when the X or Y chromosomes (Fig. 1a), or autosomes were used as a template. To test a complexity of the products, the gel was blotted onto a nylon membrane and hybridized with radioactively labelled male genomic DNA and confirmed the plant origin of amplified DNA. We also observed a strong homogenous hybridization pattern with both the labelled X and Y DOP-PCR products without any prominent bands which

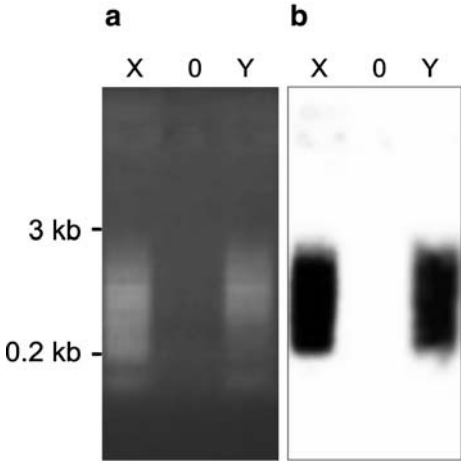

Fig. 1 Amplification of microdissected chromosomes by DOP-PCR followed by hybridization of PCR products with the male genomic probe. *X* amplified X chromosomes, *Y* amplified Y chromosomes, *0* no DNA template (DOP-PCR reaction on a small part of polyethylene naphthalate membrane, dissected from a slide from which the Y chromosome was microdissected—negative control). **a** DOP-PCR products were separated on a 1% agarose gel and stained with ethidium bromide. The size of PCR products ranged from 200 to 3,000 bp. **b** Southern hybridization carried out with the male genomic DNA as a probe. Both the DOP-X and DOP-Y PCR products hybridized strongly to the genomic DNA

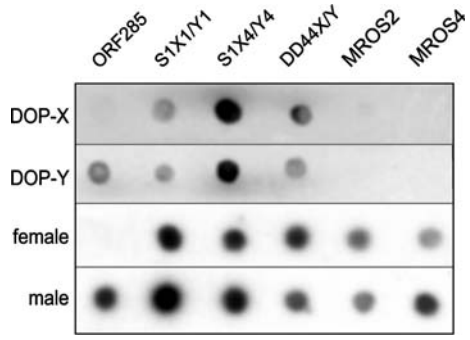

Fig. 2 Dot-blot hybridization of six gene sequences, amplified from the *S. latifolia* male genomic DNA, with the radiolabelled DOP-X, DOP-Y, female and male genomic probes. Only the Y chromosome-specific sequence (ORF285) hybridized exclusively with both DOP-Y and the male genomic probe. As expected, the genes present on the X and Y chromosomes (*SlX1/Y1*, *SlX4/Y4*, *DD44X/Y*) hybridized with all probes. The autosomal genes *MROS2* and *MROS4* gave no signal with DOP-X and DOP-Y probes but strongly hybridized with the female and male genomic probes. Equal amounts (20 ng) of PCR products were spotted

could have indicated a repeat-biased amplification (Fig. 1b).

In order to check a sequence complexity of DOP-PCR products, we have chosen several specific DNA sequences (genes), either from the sex chromosomes or from the autosomes of *S. latifolia*. The amplified sequences were of three types: (1) sequences which are localized only on both the X and Y chromosomes—*DD44X/Y*, *SlX1/Y1*, and *SlX4/Y4*, (2) a sequence localized exclusively on the Y chromosome—*ORF285*, and (3) sequences localized only on the autosomes—*MROS2* and *MROS4*. PCR products of expected size were dot-blotted on nylon membrane and hybridized with the labelled DOP-X or DOP-Y PCR products, and male or female genomic DNA (Fig. 2). The unique signal with the DOP-Y DNA was clear only in the case of *ORF285*, as expected. The sequences of the X and Y chromosome origin hybridized to both the DOP-X and DOP-Y samples, as expected. There was no hybridization signal with genes of the autosomal origin. These results thus present a clear evidence of complexity and specificity of the DOP-PCR derived sex chromosome specific DNA.

The DOP-PCR products from the amplified Y chromosome were cloned and 10,000 bacterial colonies were spotted onto Hybond N + filters with the GeneTAC™ G3 workstation. The partial library was screened using the DOP-PCR amplified X and Y chromosomes, and autosomes. Based on the hybridization intensity, nine clones showing a strong signal with the DOP-Y probe and a weak signal with the DOP-X and DOP-A probes were isolated. Their exclusive presence on the Y chromosome was checked by PCR on the genomic DNA from male and female individuals. Out of these clones, one clone (named MK17; GenBank database number DQ376008) was found to be located exclusively on the Y chromosome.

This male specific MK17 was characterized in detail. Its 320 nucleotide-long sequence showed no homology to any sequence in the EMBL database. To verify the localization of MK17 on the Y chromosome, we performed PCR directly on microdissected X and Y chromosomes and the pool of autosomes with two sets of MK17 primers. The PCR product was found exclusively in the microdissected Y chromosomes (Fig. 3) and the *S. latifolia* male genomic DNA (Fig. 4). A similar result was obtained in males of the other closely related dioecious species *S. dioica* and *S. diclinis*. The amplification of MK17 on gynodioecious and hermaphrodite species yielded no PCR products (Fig. 4).

Southern hybridization data confirmed the linkage of MK17 to the Y chromosome (Fig. 5). Our results indicate that MK17 is exclusively localized on the Y chromosome of *S. latifolia* (two copies) and *S. dioica* (one copy). In *S. diclinis*, there is, except of the two Y copies, at least another one copy of MK17 localized on the X chromosome or autosomes. From the presented data it is not clear if a copy of MK17 in *S. diclinis*, not linked to the Y chromosome, is a new allele of MK17

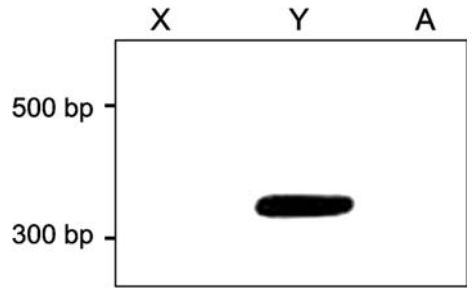

Fig. 3 PCR-amplification of MK17 on the laser microdissected sex chromosomes X and Y, and autosomes (A) of *S. latifolia*. Product was obtained only when the Y chromosome was used as a template

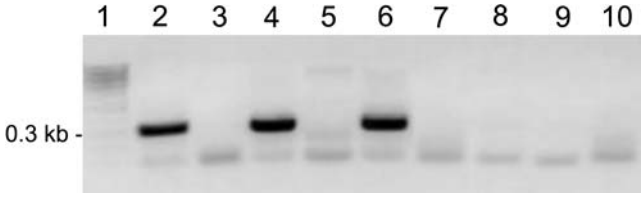

Fig. 4 PCR amplification of MK17 on genomic DNA of dioecious plants from the *Elisanthe* section and related gynodioecios and hermaphrodite species. *Lane 1* size ladder, *Lane 2 S. latifolia* male, *Lane 3 S. latifolia* female, *Lane 4 S. dioica* male, *Lane 5 S. dioica* female, *Lane 6 S. diclinis* male, *Lane 7 S. diclinis* female, *Lane 8 S. vulgaris*, *Lane 9 S. viscosa* (hermaphrodite), and *Lane 10 S. otites* (male plus female DNA genomic samples)

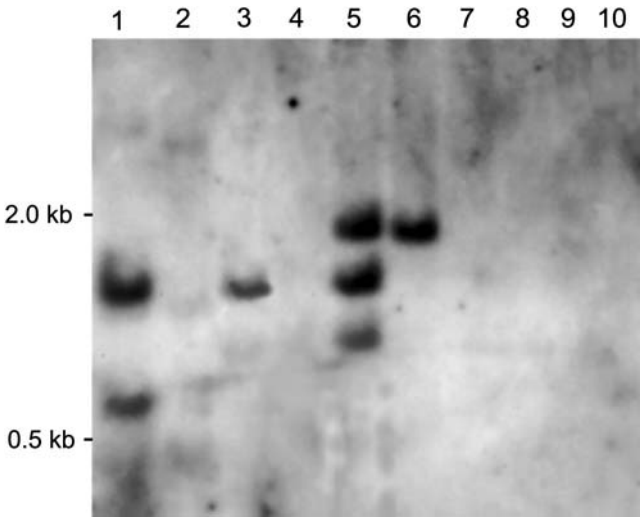

Fig. 5 Genomic Southern hybridization of MK17 in a panel of *Silene* species (20 µg of genomic DNA was digested with *Mbo*I). *Lane 1 S. latifolia* male, *Lane 2 S. latifolia* female, *Lane 3 S. dioica* male, *Lane 4 S. dioica* female, *Lane 5 S. diclinis* male, *Lane 6 S. diclinis* female, *Lane 7 S. vulgaris*, *Lane 8 S. viscosa*, *Lane 9 S. otites* male, and *Lane 10 S. otites* female

or if this allele was lost in *S. latifolia* and *S. dioica.* The Southern blot pattern with MK17 as the hybridization probe at least suggests that *S. latifolia* is more closely related to *S. dioica* than to *S. diclinis.* The results also confirm the absence of this marker in the species without sex chromosomes as well as in the dioecious *S. otites*, which does not belong to the *Elisanthe* section. RT-PCR and northern analyses revealed no expression of MK17 in any of tissues studied (flowers, buds, leaves, and seedlings; data not shown).

The subsequent analysis on the Y deletion mutants revealed that MK17 is absent in all hermaphroditic mutants, with the exception of UH13, and is present in all male-sterile deletion mutants. The use of two different approaches (by estimating LOD score, and by marker ordering according to the minimum obligate breaks criterion) place the MK17 marker to the proximity of the locus controlling the suppression of gynoecium development (LOD = 3.37; Fig. 6). Fisher's exact test confirmed the linkage ($P < 0.001$).

Discussion

The knowledge of basic mechanisms of sex determination in *S. latifolia* has provoked an interest of plant molecular biologists in this species. Several sex-specifically expressed or sex chromosome-linked genes and other DNA sequences were isolated in a search for sex determining genes. The first method used for isolation of Y-linked sequences was based on RAPD (Mulcahy et al. 1992; Zhang et al. 1998; Nakao et al. 2002). A disadvantage of this method is that screening of numerous primers and reaction conditions yields only a few Y-chromosome linked sequences, which are usually not transcribed because they often represent Y-specific units of repetitive sequences. Alternatively, they involve transposable elements. Later on, several other methods have been used in this effort including cDNA and genomic library subtraction techniques (Donnison et al. 1996; Matsunaga et al. 1996; Barbacar et al. 1997; Buzek et al. 1997; Robertson et al. 1997; Scutt et al. 1997; Pritham et al. 2003), fluorescence differential display (Scutt et al. 2002) and methods based on manually microdissected and DOP-PCR amplified sex chromosomes as probes for cDNA screening (Delichére et al. 1999; Atanassov et al. 2001; Nicolas et al. 2005). Although these attempts often resulted in isolation of repetitive DNA sequences (Donnison et al. 1996; Buzek et al. 1997) or transposable elements (Pritham et al. 2003), several male-specifically expressed genes were discovered (Matsunaga et al. 1996; Barbacar et al. 1997; Robertson et al. 1997; Scutt et al. 2002). One of these genes has been mapped to the X chromosome (Kejnovsky et al. 2001) and it was shown to have a degenerated homologue on the Y chromosome (Guttman

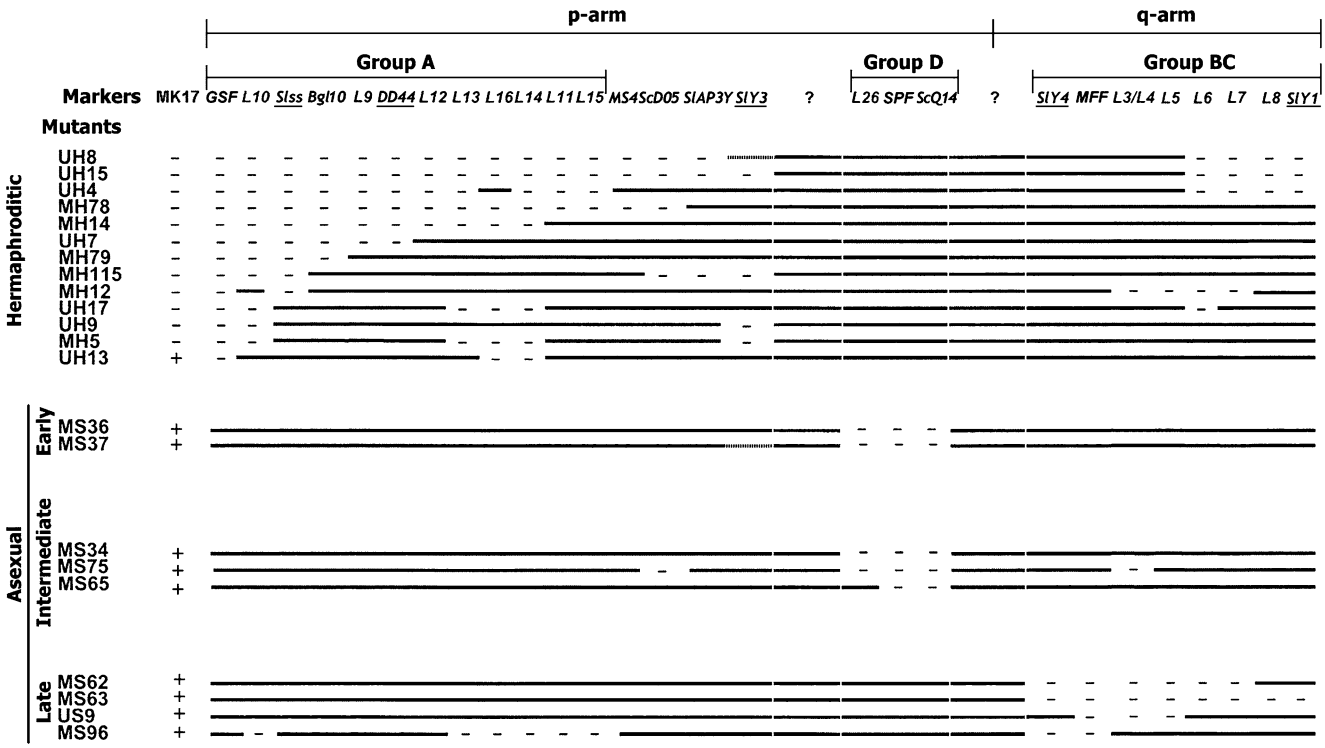

Fig. 6 Deletion map of the *S. latifolia* Y chromosome showing relative positions of MK17 as determined by PCR analysis on genomic DNA of Y-deletion mutants. The map is arranged according to Zluvova et al. (2005), with mutant identifiers on the left vertical axis, and marker loci on the *top horizontal axis*. The left side of the map represents the PCR result obtained with MK17; Y deletion mutants were scored for the presence (+) or absence (−) of MK17 PCR product. MK17 is absent in all but one hermaphrodite mutant and present in all asexual mutants, and maps in linkage group A close to the gynoecium suppressor

and Charlesworth 1998). At least five genes found on the Y chromosome—*SlY1* (Delichére et al. 1999), *SlY3* (Nicolas et al. 2005), *SlY4* (Atanassov et al. 2001), *SlssY* (Filatov 2005), and *DD44Y* (Moore et al. 2003)—have homologues on the X chromosome. In at least three pairs of the X-Y linked genes, both the Y and X homologues are expressed (*SlX/Y1*—Delichére et al. 1999; *SlX/Y4*—Atanassov et al. 2001; *DD44*—Moore et al. 2003). In spite of a large number of DNA sequences isolated, there is no link yet connecting the molecular data with the role of sex chromosome gene complexes in sex determination.

Here we re-examined possibility to search for DNA markers linked to the *S. latifolia* Y chromosome based on the construction of Y-chromosome specific library and screening this library by hybridization with male and female genomic DNA as a probe (previously used by Buzek et al. 1997). To prepare this library, we used the Y chromosome microdissection as a feasible tool used in *S. latifolia* for FISH analysis (Scutt et al. 1997; Matsunaga et al. 1999; Hobza et al. 2004). Our results obtained after screening the partial library of DOP-PCR products consisting of 10,000 clones indicates that our approach facilitate a discovery of new Y-linked markers and genes. The results reveal that MK17 is localized on the Y chromosome in three examined species from the *Elisanthe* section. Because we did not find this sequence in closely related species not possessing the sex chromosomes, we hypothesize that it evolved after the evolutionary separation of dioecies species within the section *Elisanthe*. MK17, thus represents a useful tool to study the evolution of Y-specific sequences and population dynamics.

It is expected that the availability of large-insert genomic DNA libraries cloned in a BAC (Bacterial artificial chromosome) vector will facilitate development of local BAC contigs spanning functionally important regions and positional cloning of the functionally important genes from the Y-chromosome. The contig development will depend critically on the availability of markers linked to genes involved in the sex determination. Our analyses demonstrate that the MK17 marker is closely linked to the region controlling suppression of gynoecium development. At present, MK17 is the most closely linked marker to this region. This marker can be detected by PCR and Southern hybridization, and hence its detection is, contrary to the AFLP markers published by Lebel-Hardenack

et al. (2002), population-independent. Due to its low-copy nature (see the results of Southern hybridization, Fig. 5), this marker could be used for library screening-based search for the gene(s) controlling the gynoecium development suppression.

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