Microsatellite accumulation on the Y chromosome in *Silene latifolia*

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Abstract: The dioecious plant *Silene latifolia* possesses evolutionarily young sex chromosomes, and so serves as a model system to study the early stages of sex chromosome evolution. Sex chromosomes often differ distinctly from autosomes in both their structure and their patterns of evolution. The *S. latifolia* Y chromosome is particularly unique owing to its large size, which contrasts with the size of smaller, degenerate mammalian Y chromosomes. It is thought that the suppression of recombination on the *S. latifolia* Y chromosome could have resulted in the accumulation of repetitive sequences that account for its large size. Here we used fluorescence in situ hybridization (FISH) to study the chromosomal distribution of various microsatellites in *S. latifolia* including all possible mono-, di-, and tri-nucleotides. Our results demonstrate that a majority of microsatellites are accumulated on the q arm of the Y chromosome, which stopped recombining relatively recently and has had less time to accumulate repetitive DNA sequences compared with the p arm. Based on these results we can speculate that microsatellites have accumulated in regions that predate the genome expansion, supporting the view that the accumulation of repetitive DNA sequences occurred prior to, not because of, the degeneration of genes.

Key words: plant sex chromosomes, microsatellites, *Silene latifolia*.

Résumé : La plante dioïque *Silene latifolia* possède des chromosomes sexuels d'origine récente et elle sert donc de modèle pour l'étude des premiers stades de l'évolution des chromosomes sexuels. Les chromosomes sexuels diffèrent souvent de manière marquée des autosomes tant pour ce qui est de leur structure que de leur évolution. Le chromosome Y du *S. latifolia* est particulièrement unique en raison de sa grande taille, ce qui contraste avec les chromosomes Y dégénérés et de petite taille chez les mammifères. On pense que la suppression de la recombinaison sur le chromosome Y du *S. latifolia* pourrait avoir conduit à l'accumulation de séquences répétées et expliquerait ainsi leur grande taille. Les auteurs ont employé l'hybridation in situ en fluorescence (« FISH ») pour étudier la distribution de divers microsatellites chez le *S. latifolia* à l'aide de tous les mono-, di- et trinucléotides possibles. Les résultats montrent qu'une majorité des microsatellites se sont accumulés sur le bras q du chromosome Y, lequel a cessé la recombinaison assez récemment et aurait eu moins de temps pour accumuler des séquences répétées que le bras p. Sur la base de ces observations, les auteurs spéculent que les microsatellites se seraient accumulés dans des régions avant l'expansion du génome. Cela vient appuyer l'hypothèse voulant que l'accumulation de séquences répétées se serait produite avant, et non pas en raison de, la dégénérescence des gènes.

Mots-clés : chromosomes sexuels de plantes, microsatellites, *Silene latifolia*.

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Introduction

While sex chromosomes in mammals are ancient, sex chromosomes in dioecious plants are evolutionarily young (for review see Vyskot and Hobza 2004; Charlesworth et al. 2005). In contrast to the typically small mammalian Y chromosome, heteromorphic Y chromosomes in angiosperms are often the largest chromosomes in the male genome, e.g., in *Silene latifolia* (Ciupercescu 1990), *Cannabis sativa* (Sakamoto et al. 1998), and *Rumex acetosa* (Shibata et al. 1999). The most probable cause of this size increase is that a cessation of recombination between large parts of the sex chromosomes has allowed for the degeneration of the Y chromosome. The non-recombining part of a genome is subject to several evolutionary processes including the degeneration of the majority of genes, the addition of genes or chromosomal parts, and the accumulation of repetitive DNA sequences (Charlesworth and Charlesworth 2000). Experimental studies have documented the degeneration of genes in *S. latifolia* (Guttman and Charlesworth 1998) as well as the acquisition of new genes (Matsunaga et al. 2003). It appears that processes forming the sex chromosomes are more dynamic (Bachtrog 2006*a*) than previously thought.

One of the dominant processes shaping the Y chromosome in general is the accumulation of repetitive DNA sequences (Charlesworth et al. 1994). On the *S. latifolia* Y chromosome, we recently demonstrated an accumulation of tandem repeats (Hobza et al. 2006), chloroplast DNA (Kej-

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novsky et al. 2006*a*), and retrotransposons (E. Kejnovsky, unpublished data). The divergence of the X and Y chromosomes in *S. latifolia* at the level of repetitive DNA enabled us to paint sex chromosomes using FAST-FISH (Hobza et al. 2004). Similarly, the accumulation of various types of repetitive DNA sequences on the Y chromosome has been demonstrated in other dioecious plant species such as *R. acetosa* (Shibata et al. 1999), *C. sativa* (Sakamoto et al. 2000), and *Marchantia polymorpha* (Okada et al. 2001). In papaya, the plant with the youngest studied Y chromosome, the male-specific region of the primitive Y chromosome is enriched with repetitive sequences (Liu et al. 2004). The same processes have also been observed in young fish sex chromosomes of threespine sticklebacks, *Gasterosteus aculeatus* (Peichel et al. 2004), and medaka, *Oryzias latipes* (Nanda et al. 2002). These observations suggest that repetitive DNA sequence accumulation is probably one of the earliest events in Y chromosome evolution, occurring even before genes start to degenerate (Charlesworth et al. 2005).

The major repetitive component of eukaryotic genomes is microsatellites, short DNA sequence stretches in which motifs of 1 to 6 nucleotides are tandemly repeated (for review see Ellegren 2004; Schlotterer 2000). They are among the fastest evolving DNA sequences. It has been shown that most microsatellites reside in regions predating the recent genome expansion in many plants (Morgante et al. 2002). Here, we studied the chromosomal distribution of various microsatellites in *S. latifolia*; this is the first such study of plant sex chromosomes. We found that a majority of microsatellites are accumulated on the Y chromosome, largely on its q arm, where the pseudoautosomal region is also located.

Materials and methods

Silene latifolia seeds were obtained from the seed collection of the Institute of Biophysics, Brno, Czech Republic. To synchronize germinating seeds, the DNA polymerase inhibitor aphidicolin (15 μmol/L) was added for 12 h, and mitoses were then accumulated with an application of oryzalin (30 μmol/L) for 4 h. Slides were prepared from root tips and treated as described in Lengerova et al. (2004) with slight modifications. Slide denaturation was performed in 7:3 (*v*/*v*) formamide : 2× SSC for 2 min at 72 °C. Slides were immediately dehydrated through 50%, 70%, and 100% ethanol (–20 °C) and air-dried. The probe (100 ng per slide) was denatured at 70 °C for 10 min in a mix containing 50% formamide (*v*/*v*), 2× SSC, and 10% dextran sulphate (*w*/*v*). Oligonucleotides containing microsatellite sequences were directly labeled with Cy3 at the 5′ end during synthesis by VBC-Biotech (Vienna, Austria). The denatured probe was applied to the slide, covered with a plastic cover slip, and hybridized for 18 h at 37 °C. Slides were washed at room temperature twice for 5 min in 2× SSC and twice for 5 min in 1× SSC. Slides were analyzed using an Olympus Provis microscope, and image analysis was performed using ISIS software (Metasystems). The longitudinal distribution of signals along the chromosomes was studied using Image-Pro software (Media Cybernetics) when both chromatides were analyzed together (Fig. 3*a*), while ISIS software was used for analysis of separate chromatides (Fig. 3*b*). We scored 20 metaphases for each oligonucleotide and measured the distribution of signals along sex chromosomes in one typical metaphase. To differentiate the arms of the Y chromosome, a cytogenetic FISH marker, X-43.1, was used (Buzek et al. 1997). It is accumulated at subtelomeric regions of a majority of chromosomes in *S. latifolia*, including the q arm of the Y chromosome, but is absent on the p arm. X-43.1 was labeled with SpectrumGreen-conjugated dUTP using the Nick Translation Kit (both Vysis).

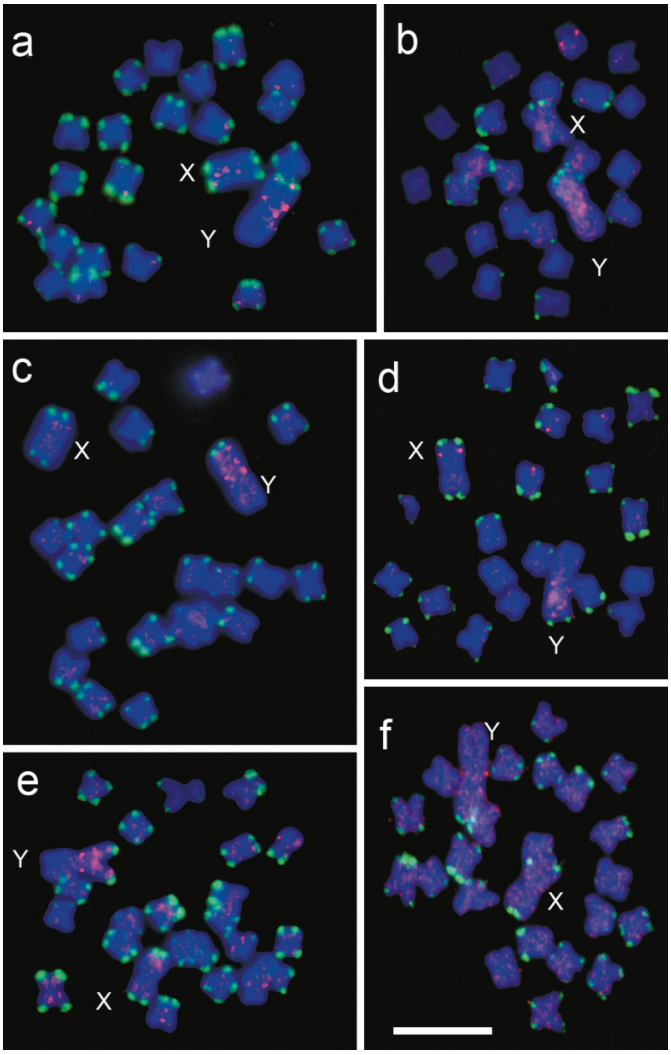
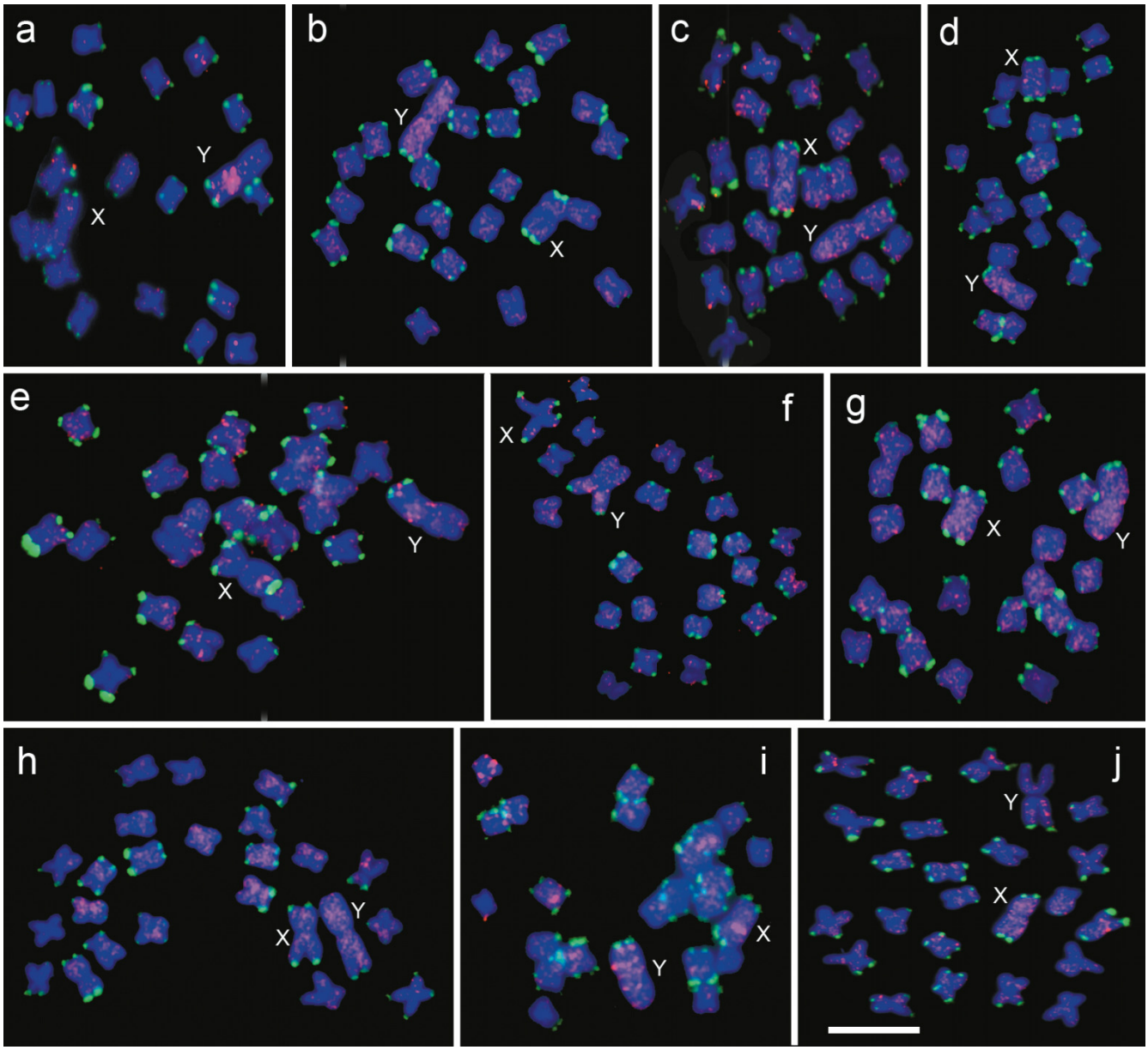


Fig. 1. Mitotic metaphase chromosomes of male *S. latifolia* were hybridized with various labeled microsatellite-containing oligonucleotides: (*a*) $(A)_{30}$, (*b*) $(C)_{30}$, (*c*) $(CA)_{15}$, (*d*) $(GA)_{15}$, (*e*) $(GC)_{15}$, and (*f*) $(TA)_{15}$. Chromosomes were counterstained with DAPI (blue); microsatellite probes were directly labeled with Cy3 during synthesis (red signals). The subtelomeric repeat X-43.1, which marks the q arm of the Y chromosome, was labeled with SpectrumGreen-conjugated nucleotides (green signals). The X and Y chromosomes are indicated; bar = 10 μm.

Results

We studied the chromosomal distribution of microsatellites in *S. latifolia* using fluorescence in situ hybridization. We used the following labeled oligonucleotides: $d(A)_{30}$, $d(C)_{30}$, $d(CA)_{15}$, $d(GA)_{15}$, $d(GC)_{15}$, $d(TA)_{15}$, $d(CAA)_{10}$, $d(CAG)_{10}$, $d(CGG)_{10}$, $d(GAA)_{10}$, $d(CAC)_{10}$, $d(CAT)_{10}$,

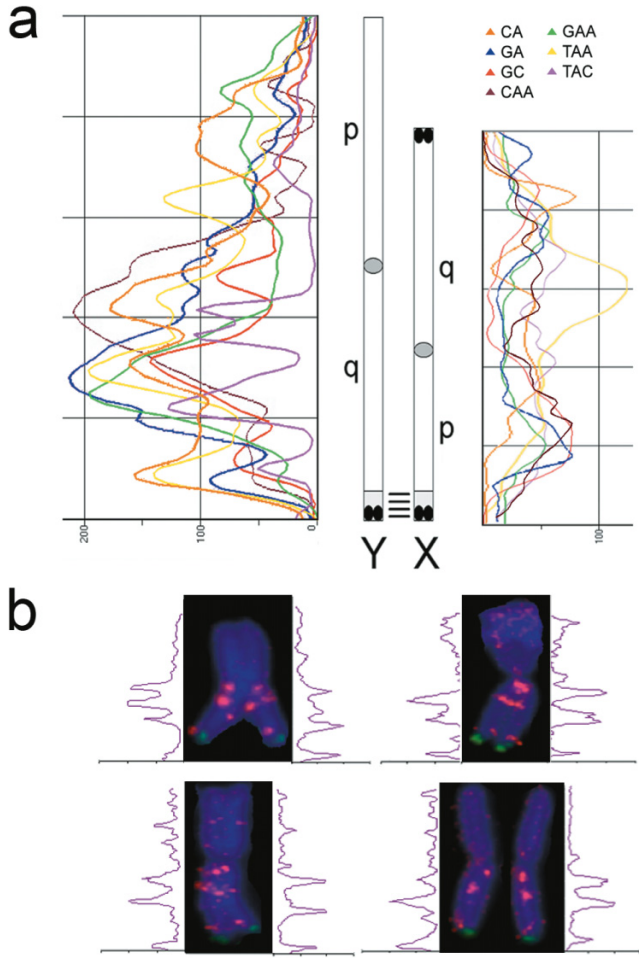
Fig. 2. Mitotic metaphase chromosomes of male *S. latifolia* were hybridized with various labeled microsatellite-containing oligonucleotides: (*a*) $(CAA)_{10}$, (*b*) $(CAG)_{10}$, (*c*) $(CAC)_{10}$, (*d*) $(CAT)_{10}$, (*e*) $(CGG)_{10}$, (*f*) $(GAA)_{10}$, (*g*) $(GAC)_{10}$, (*h*) $(GAG)_{10}$, (*i*) $(TAA)_{10}$, and (*j*) $(TAC)_{10}$. Chromosomes were counterstained with DAPI (blue); microsatellite probes were directly labeled with Cy3 during synthesis (red signals). The subtelomeric repeat X-43.1, which marks the q arm of the Y chromosome, was labeled with SpectrumGreen-conjugated nucleotides (green signals). The X and Y chromosomes are indicated; bar = 10 μm.



$d(GAC)_{10}$, $d(GAG)_{10}$, $d(TAA)_{10}$, and $d(TAC)_{10}$, which represent all possible mono-, di-, and tri-nucleotides (taking into account permutations of these microsatellites and their complementary strands). Our results demonstrate that most microsatellites in the *S. latifolia* genome are accumulated on the Y chromosome, especially on its q arm, where the pseudoautosomal region is located. The q arm is always distinguished by the presence of subtelomeric marker X-43.1 (Buzek et al. 1997).

Among mononucleotides, both $d(A)_{30}$ and $d(C)_{30}$ are accumulated on the Y chromosome, especially on the q arm and centromere. For both of these microsatellites some signals are also present on the X chromosome (Figs. 1*a*, 1*b*). Dinucleotides show a variable extent of accumulation on the Y chromosome. A strong accumulation was observed in the case of $d(CA)_{15}$, $d(GA)_{15}$, and $d(GC)_{15}$, and these microsatellites are always more abundant on the q arm of the Y chromosome (Figs. 1*c*–1*e*). Again, some weaker signals are found also on other chromosomes, especially the X chromosome. The microsatellite $d(TA)_{15}$ is more or less evenly distributed on a majority of chromosomes, with only slight accumulation on the Y chromosome (Fig. 1*f*). This could be explained by the potential of $d(TA)_{15}$ to dimerize with itself, which can decrease the amount of single-strand oligonucleotide available for binding to chromosomes. Among all 10 possible trinucleotide microsatellites, the probes $d(CAA)_{10}$, $d(CAG)_{10}$, $d(GAA)_{10}$, and $d(TAA)_{10}$ exhibit the strongest accumulation on the Y chromosome (Figs. 2*a*, 2*b*, 2*f*, 2*i*),

Fig. 3. (*a*) Longitudinal distribution of the 7 most strongly accumulated microsatellites along the Y chromosome (left) and X chromosome (right) of *S. latifolia* (chromosomes from Figs. 1 and 2 were analyzed) alongside a schematic of *S. latifolia* sex chromosomes (middle) with indicated centromeres (gray circles), the subtelomeric repeat probe X-43.1 (●), and marked arms. Recombining pseudoautosomal regions are marked by short horizontal lines. Intensity of fluorescence is expressed in arbitrary units. (*b*) The Y chromosome from 4 independent metaphases hybridized with $(CAA)_{10}$, with profiles for each chromatide shown separately.



where especially $d(CAA)_{10}$ and $d(GAA)_{10}$ are accumulated on its q arm. The microsatellites $d(CAT)_{10}$ and $d(TAC)_{10}$ are only slightly accumulated on the Y chromosome (Figs. 2*d*, 2*j*), while $d(CGG)_{10}$, $d(CAC)_{10}$, $d(GAC)_{10}$, and $d(GAG)_{10}$ are uniformly spread along all the chromosomes without any significant accumulation (Figs. 2*c*, 2*e*, 2*g*, 2*h*). We quantified the longitudinal distribution of signals along the Y and X chromosomes for the 7 di- and tri-nucleotide microsatellites with the highest accumulation on the Y chromosome (Fig. 3*a*). The profiles alongside the scheme of sex chromosomes show both the stronger signal on the Y chromosome compared with the X chromosome and the accumulation of microsatellites on the q arm of the Y chromosome. The differences in FISH patterns between individual metaphases are illustrated for 4 Y chromosomes from different metaphases hybridized with $d(CAA)_{10}$ (Fig. 3*b*). Based on our data, we cannot exclude the possibility that some microsatellites are co-localized and have thus co-evolved.

Discussion

Patterns of microsatellite accumulation on the Y chromosome in *S. latifolia*

In this work we demonstrate that the majority of accumulated microsatellite sequences in *S. latifolia* are on the Y chromosome. This finding is in agreement with models predicting accumulation of repetitive DNA sequences in regions with very low recombination (Charlesworth et al. 1994; Stephan and Cho 1994). Recombination is often suppressed near centromeres and telomeres, where typically satellites are accumulated (Charlesworth et al. 1986), forming heterochromatic parts of chromosomes. In contrast, minisatellites and microsatellites are often located in euchromatic portions of chromosomes. This is consistent with our observations: the Y chromosome of *S. latifolia* is largely euchromatic, having only short centromeric and subtelomeric heterochromatic regions revealed by DAPI banding. Microsatellite signals were present only within these euchromatic regions.

While most of the Y chromosome is non-recombining, a small distal part of the q arm, the pseudoautosomal region (PAR), recombines with the X chromosome. The q arm contains regions that have stopped recombining only relatively recently, while the p arm, which is far from the PAR, carries regions where recombination ceased at the beginning of sex chromosome evolution (Nicolas et al. 2005; Bergero et al. 2007). It could be a consequence of the large inversions on the Y chromosome. The existence of large inversions on the Y chromosome is supported by physical mapping results (Hobza et al. 2007). Thus, the q arm has had less time to accumulate repetitive DNA sequences and to expand compared with the p arm. Surprisingly, we found a stronger accumulation of some microsatellites on the q arm as compared with the p arm. Based on these facts we can speculate that microsatellites have accumulated in regions that predate the genome expansion. A similar conclusion was made by Morgante et al. (2002), who showed for several plant species that microsatellites are more abundant in single-copy or low-copy DNA than in repetitive DNA, as also shown by Tero et al. (2006) for *S. tatarica*. However, the forces governing the evolution of the small clusters studied by Morgante et al. (2002) are likely to be different from the mechanisms of evolution of the large clusters studied here by FISH.

Our recent finding of chloroplast DNA accumulation (Kejnovsky et al. 2006*a*) further supports the idea that dynamic processes are at work on the q arm of the Y chromosome. We suggested that the low divergence of chloroplast DNA sequences located in the nucleus is the result of recent gene transfer events, indicating that there is probably a high rate of turnover of organelle sequences in the nucleus, as suggested by Timmis et al. (2004). Chloroplast DNA could therefore represent another class of rapidly evolving sequences that have accumulated in regions predating expansions. In contrast, the p arm appears to display an older expansion of repetitive DNA. Genes located further from the PAR contain more repetitive sequences in their introns than genes

closer to the PAR (Marais et al. 2008). However, microsatellites do not co-localize with gene-rich regions, which in *S. latifolia* are predominantly located in distal regions of chromosomes, as was shown by cDNA hybridization and histone acetylation studies (Vyskot et al. 1999).

Accumulation of repetitive DNA on the Y chromosome is an early event

The role of various types of repetitive DNA with respect to their accumulation on the Y chromosome in *S. latifolia* has recently been systematically studied. The accumulation of tandem repeats (Hobza et al. 2006) as well as retrotransposons (E. Kejnovsky et al., unpublished data) on the Y chromosome was revealed during these studies. Recently, a new retrotransposon containing a tandem repeat was described in *S. latifolia*, and it is also located on the sex chromosomes (Kejnovsky et al. 2006*b*). To characterize repetitive elements we used libraries of both small inserts (Hobza et al. 2007) and large inserts (Lengerova et al. 2004) and X or Y chromosome-specific libraries (Hobza et al. 2004). Surprisingly, during screening of these libraries we found only a small number of clones containing microsatellite sequences. This could be explained either by the resistance of microsatellites to cloning (Nichol and Pearson 2002) or by a lower representation of these sequences in the genome.

Therefore, we systematically studied the chromosomal distribution of all possible mono-, di-, and tri-nucleotide microsatellites in *S. latifolia* by FISH. In plants, microsatellites were systematically investigated using FISH by Schmidt and Heslop-Harrison (1996) and Cuadrado and Schwarzacher (1998). We applied this approach for the first time to plant sex chromosomes. Its methodological novelty consists in the end-labeling of oligonucleotides by Cy3 during synthesis.

Our data on the accumulation of microsatellites on the Y chromosome in *S. latifolia* and findings of the accumulation of other repetitive DNA on the Y chromosome (Hobza et al. 2006, 2007; Kejnovsky et al. 2006*a*) in combination with the fact that genes on the Y chromosome are not largely degenerated (Matsunaga 2006; Bergero et al. 2007) indicate that *S. latifolia* represents a stage in evolution when repetitive DNA has just started to accumulate, but before most genes have lost their function or are deleted. These findings support the view of Steinemann and Steinemann (2005) that accumulation of repetitive DNA is an event that predates genetic degeneration of genes and is not its consequence.

Causes and consequences of microsatellite expansion on the Y chromosome

Which mechanisms stand behind microsatellite expansion on the Y chromosome? General mechanisms of microsatellite evolution including replication slippage are probably in action here, but longer microsatellite arrays, which were detected by FISH in our experiments, can also be subject to recombination-based processes such as unequal crossing-over or gene conversion. It has been demonstrated that unequal crossing-over does not act on very short tandem arrays (Stephan and Cho 1994). Levinson and Gutman (1987) proposed that slippage is a major factor in the initial expansion of microsatellites, while larger arrays are prone to further expansion by unequal crossing-over. It is surprising that microsatellites that are also dominant in the human genome — $d(CA)_n$, $d(GA)_n$, $d(CAA)_n$, $d(GAA)_n$, and $d(TAA)_n$ — show strong accumulation on the human Y chromosome (Subramanian et al. 2003).

It is also possible that microsatellites have played an important role in sex chromosome evolution in their relation to non-B DNA conformations and recombination. Microsatellite sequences can adopt various unusual DNA conformations including hairpins and triplex or tetraplex structures (Kejnovská et al. 2003; Wells et al. 2005). For example, $d(GAA)_n$ sequences have been recognized as recombination hot spots (Napierala et al. 2004). Their large tracts present on the same molecule can form a new type of non-B DNA conformation called "sticky DNA" (Sakamoto et al. 1999). We speculate that sticky DNA can bring two distant regions located on the same chromosome into contact and thus help gene conversion (a non-reciprocal recombination event) to act on them, especially in newly X–Y diverging regions. The role of gene conversion in microsatellite instability was demonstrated for the microsatellite $d(CAG)_n$ (Jakupciak and Wells 2000). Furthermore, recombination was proposed to be a primary force behind microsatellite evolution (Wells et al. 2005). The two above-mentioned microsatellites, $d(GAA)_n$ and $d(CAG)_n$, belong to the class of strongly amplified microsatellites on the Y chromosome in our experiments. Recently we also suggested that intrachromosomal gene conversion is more intensive on the Y chromosome than on other chromosomes in *S. latifolia* (Kejnovsky et al. 2007). Gene conversion is also responsible for the high identity of large palindromes on the human Y chromosome and thus seems to protect essential genes from degeneration (Skaletsky et al. 2003).

Sticky DNA adopted by microsatellite sequences can also inhibit transcription (Sakamoto et al. 2001). It is known that lower expression levels can result in faster evolution of proteins (Drummond et al. 2005), as observed recently in the degenerating neo-Y chromosome of *Drosophila miranda* (Bachtrog 2006*b*). Based on these facts we can speculate that the accumulation of microsatellite sequences on the Y chromosome could have triggered its diversification from the X chromosome and subsequent degeneration. In addition, the non-B DNA conformations of microsatellites serve as breakpoints for gross rearrangements such as deletions, insertions, inversions, and duplications (Bacolla and Wells 2004). These rearrangements are thought to have played a central role in the evolution of sex chromosomes, both in humans (Lahn and Page 1999) and in *S. latifolia* (Zluvova et al. 2005).

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