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RESEARCH ARTICLE

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An accumulation of tandem DNA repeats on the Y chromosome in *Silene latifolia* during early stages of sex chromosome evolution

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Abstract Sex chromosomes in mammals are about 300 million years old and typically have a highly degenerated Y chromosome. The sex chromosomes in the dioecious plant *Silene latifolia* in contrast, represent an early stage of evolution in which functional X–Y gene pairs are still frequent. In this study, we characterize a novel tandem repeat called TRAYC, which has accumulated on the Y chromosome in *S. latifolia*. Its presence demonstrates that processes of satellite accumulation are at work even in this early stage of sex chromosome evolution. The presence of TRAYC in other species of the *Elisanthe* section suggests that this repeat had spread after the sex chromosomes evolved but before speciation within this section. TRAYC possesses a palindromic character and a strong potential to form secondary structures, which could play a role in satellite evolution. TRAYC accumulation is most prominent near the centromere of the Y chromosome. We propose a role for the centromere as a starting point for the cessation of recombination between the X and Y chromosomes.

Introduction

The structure and evolution of sex chromosomes are key questions in evolutionary and reproduction biology. Sex chromosomes are supposed to have evolved from a pair of ordinary autosomes (Ohno 1967). The Y chromosomes of both plants and animals are often made up of huge blocks of largely nonrecombining DNA. The lack of recombination is thought to lead to the degeneration of the Y chromosome. This degeneration is characterized by loss of functional genes, except those with male specific function, and the accumulation of repetitive DNA sequences, both transposable elements and tandem arrays of satellite DNA sequences (Charlesworth et al. 1994). In flowering dioecious plants, heteromorphic sex chromosomes have been reported only in a few species (Vyskot and Hobza 2004). In the case of *Rumex acetosa*, it has been shown that degeneracy in later stages of the evolution of sex chromosomes is accompanied by accumulation of Y chromosome-specific repetitive sequences (Shibata et al. 1999, 2000).

Silene latifolia, or white campion, is the dioecious plant with an X/Y sex determination system (Westergaard 1958). Recent studies show that for a majority of X-linked genes, it is possible to find their functional homologues (Delichere et al. 1999; Atanassov et al. 2001; Moore et al. 2003; Filatov 2005; Nicolas et al. 2005). However, Guttman and Charlesworth (1998) found that an X-linked gene *MROS3* has a degenerate homologue with nucleotide deletions and accumulation of repeats in the nonpairing region of Y chromosome. A question that has remained unexplained is why the Y chromosome in *S. latifolia* is about 1.4× larger than the X chromosome.

To construct a basic cytogenetic map of *S. latifolia*, we prepared a sample BAC library and isolated appropriate markers. Using five BAC clones and three other repetitive DNA sequences, we were able to characterize individual chromosomes (Lengerova et al. 2004). Two out of the five BACs (BAC7H5 and BAC9B7) yielded more abundant signals on the Y chromosome. BAC7H5 was analyzed in detail and we found it was of plastid origin (Kejnovsky et al. 2006). The other clone BAC9B7 has been subcloned to identify DNA elements responsible for its strong signal on the Y chromosome. In this study, we describe this novel tandem repeat unit called TRAYC (*T*andem *R*epeat *A*ccumulated on the *YC*hromosome) accumulated on the *S. latifolia* Y chromosome. The characterization of

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TRAYC is the first evidence of a large-scale accumulation of tandem repeats on the nonrecombining Y chromosome in early stages of its evolution.

Materials and methods

Plant material

S. latifolia Garcke (syn. *Melandrium album* Poiret) plants of the U9 genotype 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). All other plants used in this study (*S. latifolia* Brno ecotype, *Silene dioica*, *Silene diclinis*, *Silene viscosa*, *Silene vulgaris*, and *Silene otites*) come from the seed collection of the Institute of Biophysics, Brno. All these *Silene* species are diploid and possess the same number of chromosomes (2*n*=24).

PCR, cloning, and sequence analysis

The reactions were performed in a volume of 50 μl, and the final concentration of reagents was 0.2 mM dNTP, 0.2 μM primers (Rep1 AGCGGTCCGCCTAATGAAATCCT), 1×buffer (TopBio) containing 1.5 mM $MgCl_2$, and 0.6 U of *Taq* polymerase (TopBio). The initial denaturation was followed by 25 cycles of 50 s at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final extension of 7 min. The PTC-200 thermal cycler (MJ Research) was used. Polymerase chain reaction (PCR) products were purified using the Qiagen PCR purification kit, cloned using the pGEM-T Easy cloning system (Invitrogen), and sequenced by VBC-Genomics (Vienna, Austria). Basic sequence analysis was done with Staden Package software (Staden 1996). Multiple sequence comparisons including phylogenetic tree construction were performed with ClustalW (Thompson et al. 1994), plots of sequences were done with JDotter (Brodie et al. 2004; http://athena.bioc.uvic.ca/pbr/jdotter/), and secondary structure prediction was done by MFold program (Zuker 2003).

Southern hybridization and copy number estimation

Genomic DNA was isolated from young leaves using a DNeasy Plant Mini kit (Qiagen). Hybridizations were carried out with DNA (25 ng) radio-labeled 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), and washed with a high stringency wash containing 0.1×SSC and 0.1% sodium dodecyl sulfate (SDS) at 65°C. In copy number estimation, serial dilutions of both male and female genomic DNA were spotted onto a nylon membrane and hybridized with radio-labeled monomer sequence as a probe. Finally, the spots were cut out and measured by scintillation counting.

Slide preparation and laser manipulation

Mitotic slides were prepared according to Hladilova et al. (1998). Briefly, seedlings were synchronized with aphidicolin (30 μM, Sigma, for 16 h) and metaphases accumulated with oryzalin (15 μM, Sigma, for 4 h). Root tips were cut off, converted into protoplasts, and fixed in Farmer's fixative (ethanol to acetic acid, 3:1). The protoplast suspension was dropped on slides to be used for fluorescence in situ hybridization (FISH) analysis. Alternatively, the protoplasts were applied on slides covered with polyethylene naphthalate membrane (P.A.L.M. GmbH, Bernried, Germany) where naked chromosomes were released and used for laser microdissection and collection, and later amplified by degenerate oligonucleotide primer-PCR (for details, see Hobza et al. 2004).

Fluorescence in situ hybridization

Probes were purified using Nucleotide Removal Kit (Qiagen) and labeled by nick translation using Nick Translation Mix (Roche) with Fluorolink Cy3-dUTP (Amersham Pharmacia Biotech) or SpectrumGreen (Vysis). Slides were preheated at 60°C for 30 min, treated with 100 μg/ml RNase A (Sigma) in 2×SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7) for 1 h at 37°C, washed three times for 5 min in 2×SSC, treated with 5 mg/ml pepsin (Sigma) in 0.01 N HCl for 12 min at 37°C, washed as before, postfixed in 3.7% formaldehyde (Merck) in 1×PBS for 10 min, washed again, and dehydrated in increasing ethanol series (70, 90, 96% ethanol, 5 min each). The hybridization mixture contained 50% formamide (*v*/*v*, SigmaULTRA), 10% dextran sulphate (*w*/*v*, Sigma), 2×SSC, and 10–100 ng of probe. The mixture was predenaturated by incubation at 75°C for 10 min and immediately placed on ice. Typically, 20 μl was applied per slide and covered with plastic coverslip. Controlled denaturation and annealing was done on thermal cycler with heated platform by step-by-step incubation of slides in the following manner (75°C for 5 min → 65°C for 2 min → 55°C for 2 min → 45°C for 2 min → 37°C for 2 min). Slides were then incubated at 37°C in a moist chamber. Posthybridization washing was done at 76% stringency by following these steps: 2×SSC (42°C, twice for 5 min), 0.1×SSC (42°C, twice for 5 min), 2×SSC (42°C, twice for 5 min), 4×SSC + 0.1% Tween 20 (RT, 7 min). Slides were counterstained with 4′, 6 diamidino-2-phenylindole (DAPI, 0.5 μg/ml) in Vectashield (Vector). To orient 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 of the *Elisanthe* section, including one arm (Yq) of the Y chromosome. Images were captured using a charge-coupled device (CCD) camera and ISIS software (MetaSystems). The

longitudinal distribution of signals along the chromosomes was studied using ImagePro software (Media Cybernetics).

Primed in situ DNA labeling

The primed in situ DNA labeling (PRINS) reaction was performed according to Kubalakova et al. (1997) using Frame-Seal chambers and a PTC-200 thermal cycler equipped with a Twin Tower block (MJ Research). The reaction mix consisted of 0.1 mM dATP, dCTP, and dGTP and 0.01 mM Cy-3dUTP, 1×PCR buffer (Top-Bio) and 5U/50 µl *Taq* polymerase (Top-Bio), and TRAYC-Y primer (GTCCGCCTAATGAAATCCTTACACGGAT). The temperature profile consisted of denaturation step (94°C, 3 min), primer annealing (42°C, 5 min), and primer extension (72°C, 30 min). The heating rate between the primer annealing and extension steps was 0.1°C/s. The reaction was stopped by rinsing the slides in 0.5 M Tris–HCl, and 0.05 M EDTA (pH 8) at room temperature.

Results

Isolation and characterization of TRAYC

In our analysis of the structure of the Y chromosome, we investigated BAC clone 9B7 which had been previously identified as a DNA motif responsible for strong FISH signals on the Y chromosome (Lengerova et al. 2004). It was hypothesized that this signal is caused by intensity of some repetitive DNA sequences within the BAC clone rather than by a specific signal of the whole clone. We constructed a library of subclones from BAC 9B7 by cleaving it with *Hind*III and *Taq*I, and by subsequent cloning of the DNA fragments. The subclone library was screened with the male genomic DNA probe to distinguish highly repetitive inserts. The subclones yielding the strongest hybridization signals were chosen for sequencing. All of them contained a 172-bp long DNA unit in tandem arrangement (up to 8 units in longer inserts; Fig. 1a). This repetitive DNA sequence was named TRAYC (*T*andem *R*epeat *A*ccumulated on the *YC*hromosome, access number GenBank DQ386857). The monomer unit shows no similarity to any known sequence. A phylogenetic analysis using the set of eight monomers from one array gave a neighbor-joining tree showing the existence of two clades represented by the monomers 1–4 and 5–8 (Fig. 1b). It suggests that the first four and the last four monomers of the array originate from different ancestral units.

A study of the internal organization of the TRAYC monomer revealed a further structural peculiarity; we found the presence of inverted repeats within the TRAYC monomer. We used the Mfold program to predict the secondary structure of the TRAYC sequence. It revealed the potential of TRAYC to form a large stable hairpin structure (Fig. 1c) under physiological conditions (150 mM

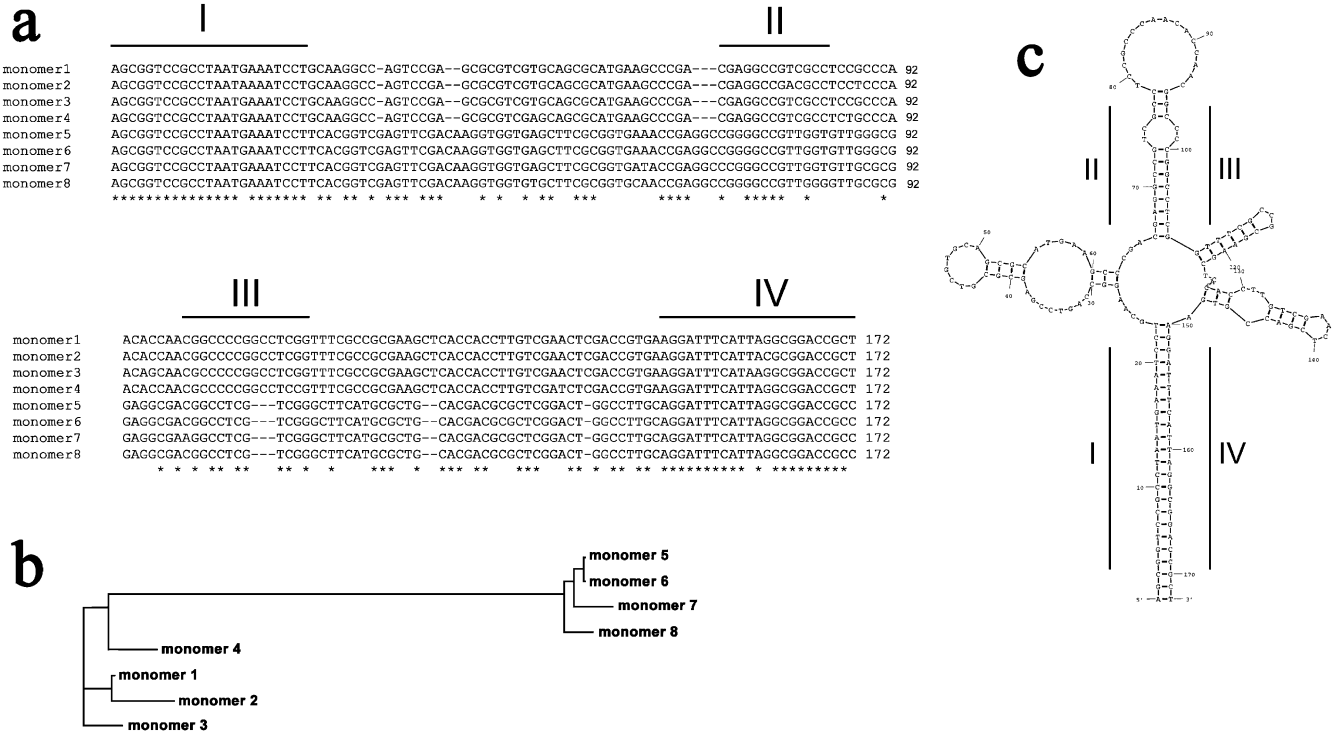

Fig. 1 **a** Multiple alignments (by ClustalW) of eight monomers of the tandem repeat TRAYC. Missing nucleotides are represented by *dash*, *asterisks* indicate positions where nucleotides are identical in all monomers. **b** Neighbor-joining tree showing the eight TRAYC monomers of one array isolated from *S. latifolia* in two separate clades (monomers 1–4 and monomers 5–8). **c** A DNA secondary structure prediction for the TRAYC monomer (monomer 1 was used) generated by MFold program. The *solid lines numbered I–IV* mark the corresponding regions in multiple alignment and in the predicted secondary structure

NaCl and 37°C). The most stable structure has a 34-bp long main stem with three smaller branching stems. The corresponding free energy of this structure is −32.6 kcal and the melting temperature is T_m=61.9°C. The multiple alignment also demonstrates the progressive degeneration of monomers with the most degenerated segment in the middle, forming the loop(s), and almost the most conserved segments at the ends where the stem is formed (Fig. 1a,c).

TRAYC is tandemly arranged and specific for the *Elisanthe* section

Southern hybridization with the genomic DNA (both male and female), digested with *Taq*I enzyme, which has a restriction site within the TRAYC monomer, confirmed the tandem arrangement of TRAYC in the species of section *Elisanthe*—*S. latifolia*, *S. dioica*, and *S. diclinis* (Fig. 2a, lanes 1–8). The other *Silene* species tested in this study yielded only very weak hybridization signal (Fig. 2a, lanes 9–11). PCR carried out with the monomer specific primer on both female genomic template and microdissected Y chromosome revealed a ladder of products suggesting that large tandem arrays of monomer sequence are present on the autosomes as well as on the X and Y chromosomes of *S. latifolia* (Fig. 2b).

Copy number of the TRAYC element was determined by dot-blot hybridization of serial dilutions of genomic DNA using a monomer unit as a probe (data not shown). TRAYC represents a moderately abundant repetitive sequence forming about 1% of the *S. latifolia* male genome and 0.6% of the female genome. According to the *S. latifolia* genome size of 5.85×10^9 bp/C (Vagera et al. 1994), there are about 3.6×10^5 copies per male haploid genome complement and 2.2×10^5 copies per female haploid complement. Chromosomal localization of the TRAYC sequence FISH with labeled TRAYC revealed strong hybridization signals located at pericentromeric regions of several autosomes and the X chromosome (Fig. 3a). However, the signal on the Y chromosome covers both arms with a specific satellite-like pattern. Similar results were obtained using PRINS technique with the Y chromosome-derived primer (Fig. 3b). This primer was designed according to the sequence of 15 TRAYC monomers amplified on the microdissected Y chromosome to specifically amplify only the Y-linked copies of TRAYC. The similarity of the PRINS labeling pattern with the FISH pattern indicates the identity of units. Other species of the section *Elisanthe* (e.g., *S. dioica*, Fig. 3c) also accumulated the TRAYC signals on the sex chromosomes, while hermaphroditic species outside this section yielded no reproducible FISH signal (e.g., *S. viscosa*, Fig. 3d). If we compare the hybridization patterns on *S. latifolia* (Fig. 3a) and *S. dioica* (Fig. 3c) it is clear that the signals in the former species is much stronger. While in both species, the sex chromosomes are labeled to similar extent, there are only about a half of autosomes with the TRAYC signal in *S. dioica* compared with *S. latifolia*. We can speculate that the TRAYC repetitive sequence, largely originating at the pericentromeric regions of the *Elisanthe* species, has been spread in *S. latifolia* more quickly than in *S. dioica*.

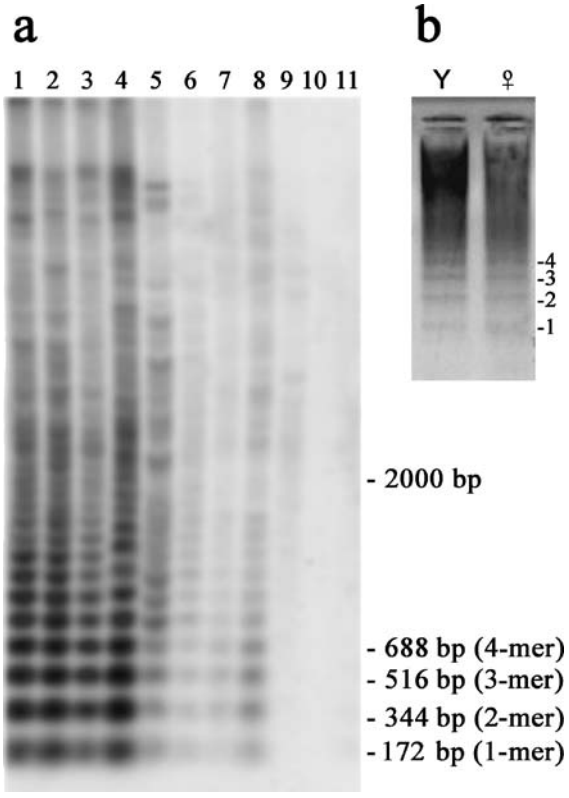

Fig. 2 **a** Southern analysis of genomic DNA digested with *Taq*I and hybridized with the TRAYC tandem repeat as a probe. *Lanes 1–4*, *S. latifolia*: *1* male, U9 ecotype; *2* female, U9 ecotype; *3* male, Brno ecotype; *4* female, Brno ecotype. *Lanes 5–6*, *S. dioica*: *5* male; *6* female. *Lanes 7–8*, *S. diclinis*: *7* male; *8* female; *lane 9S. viscosa*; *lane 10S. vulgaris*; *lane 11S. otites* (male plus female). **b** PCR with the tandem repeat specific primer Rep1 on the microdissected Y chromosomes and the female genomic DNA. Numbers of repeat units are indicated

Discussion

The data presented in this study show for the first time that degenerative processes of tandem repeat accumulation in the nonrecombining part of the Y chromosome are already present in the early stages of sex chromosomes evolution. An interesting feature of the tandem repeat TRAYC is its large pericentromeric localization. However, the Y chromosome accumulated this sequence also in some interstitial parts of both its arms. This unique pattern of signals suggests the process by which the initial stages of recombination may have occurred during the early stages of sex chromosome evolution. We speculate that the original sex determining locus in *S. latifolia* evolved close to the centromeric region and that the cessation of recombination in this region enabled TRAYC to spread within the nonrecombining parts of the Y chromosome. This spread could have been facilitated by a large inversion in the centromeric region of the Y chromosome, which itself may have been a consequence of arrested recombi-

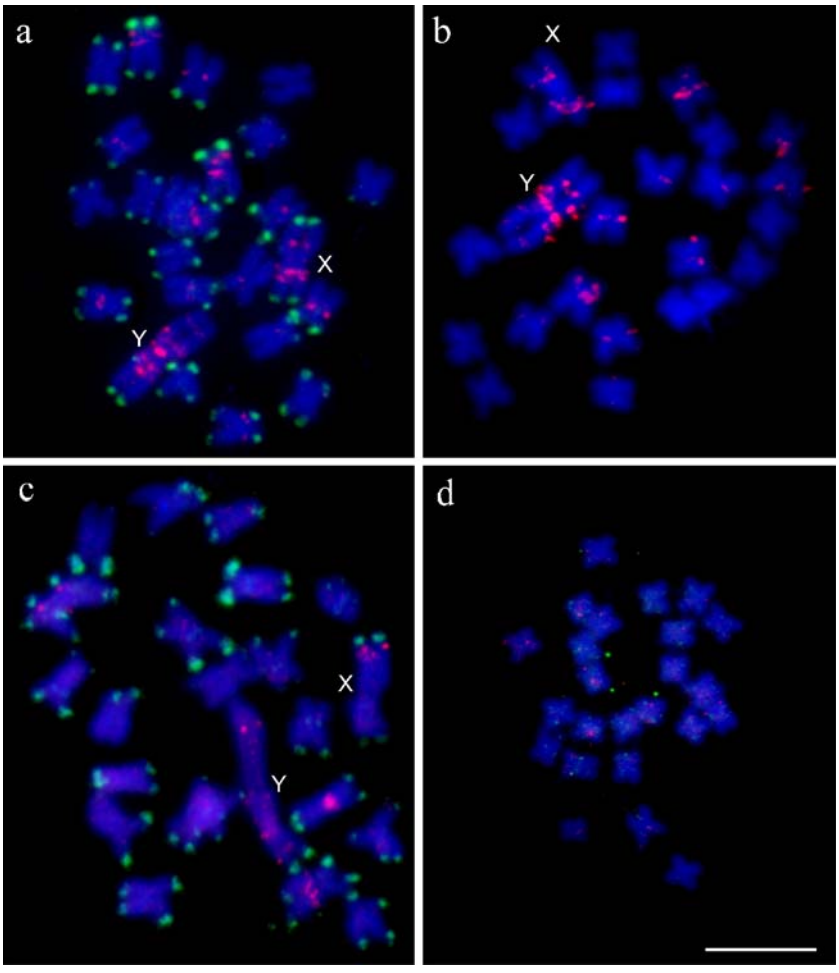

Fig. 3 Chromosomal distribution of the TRAYC sequences on *Silene* chromosomes, counterstained with DAPI (*blue*). **a** Metaphase chromosomes of *S. latifolia* male were hybridized with the TRAYC monomer unit (*red signals*) and X43.1 (*green signals*). **b** Localization of the TRAYC repetitive DNA on *S. latifolia* male chromosomes using PRINS. PRINS was carried out with primer Rep1-Y specific for the Y chromosome derived monomer units. **c** Male metaphase chromosomes of *S. dioica* hybridized with the TRAYC probe (*red*) and X43.1 (*green*) displayed a similar hybridization pattern as *S. latifolia*. **d***S. viscosa* metaphase plate, hybridized with the same probes, did not yield any reproducible hybridization signals. The X and Y chromosome are indicated, *bar* indicates 10 μm in all cases

nation, as suggested by Zluvova et al. (2005). The prominent role of the centromeric region as a starting point for X–Y recombination arrest is also supported by the finding of an accumulation of RAYSI satellite sequences in the centromeric region of the Y chromosome in *Rumex acetosa* (Shibata et al. 1999, 2000; Navajas-Perez et al. 2006) as well as the centromeric localization of male-specific region of the Y chromosome (MSY) in papaya (R. Ming, personal communication). Recombination is well known to be reduced at centromeres and pericentromeres (e.g., Haupt et al. 2001). Concerning the mechanisms of amplification of repetitive DNA on the Y chromosome, we suggest that somatic unequal crossing-over could be the primary mechanism, while unequal meiotic exchange is less probable because amplified sequences are located inside the nonrecombining region of the Y chromosome. Other amplification mechanisms, such as sister chromatid exchanges, repair mechanisms or replication slippage are also less probable explanations.

TRAYC is accumulated not only in the Y chromosome but also in the X chromosome. Recently, it was shown that the X chromosome contains a higher number of functional retrotransposed genes in mammals (Emerson et al. 2004) as a result of high retrotransposition both on and off this chromosome, thus representing a genome's intersection (Khil et al. 2004). This phenomenon is explained by meiotic sex chromosome inactivation (Handel 2004) which occurs when "sex bodies" are formed during meiosis, chromatin is remodeled, and sex chromosomes are transcriptionally repressed. Another process, X chromosome inactivation in mammals, is shown to be associated with retrotransposon L1 accumulation. These elements serve as DNA signals to propagate the X inactivation along the chromosome (Bailey et al. 2000). Similar processes may also drive the accumulation of repetitive DNA on the X chromosome of *S. latifolia*.

Another interesting feature of TRAYC is its palindromic character and the potential to form a secondary structure. Palindromic sequences are important DNA motifs involved in the regulation of different cellular processes, but they are also a potential source of genetic instability (Lisnic et al. 2005). The palindromic character of TRAYC is interesting especially in light of the recent discovery of huge palindromic (ampliconic) sequences in the human and chimpanzee Y chromosome (Skaletsky et al. 2003; Rozen et al. 2003). A higher density of palindromic sequences was also observed in the male-specific region of the incipient Y chromosome in papaya that appears to have formed in the last few million years (Liu et al. 2004) suggesting that common processes associated with the evolution of the Y chromosome of plants and animals are in action.

We speculate that conformational polymorphism of centromeric satellites could be also important in initiating recombination arrest. An example of the importance of DNA conformation is the intrinsic curvature of all centromeric DNA sequences in *Saccharomyces cerevisiae* (Bechert et al. 1999) as well as other unusual DNA structures identified in the centromeric region of various higher eukaryotes (Ferrer et al. 1995). Moreover, DNA conformation could also play a role in mechanisms of satellite spreading. These mechanisms probably include processes like transposition, unequal crossing-over and gene conversion, amplification mediated by rolling-circle replication and reinsertion but the relative contribution of these processes is not clear (Charlesworth et al. 1994). Satellites are the fastest evolving components of genome, and therefore, there is no conservation of centromeric DNA sequences although their function is highly conserved among all eukaryotes. In plants, centromeric heterochromatin is a typical genome niche where satellite repeats as well as retrotransposons are accumulated (for review, see Jiang et al. 2003).

Another important question is dating the spread of tandem repeat TRAYS with respect to sex chromosome evolution and speciation. Our data indicate that the amplification of TRAYC on the Y chromosome occurred after sex chromosomes had evolved in the *Elisanthe* section but definitively before their speciation. This observation is supported by similar genomic arrangements of TRAYC in *S. latifolia*, *S. dioica*, and *S. diclinis*. In other species within the genus *Silene*, this sequence is nearly absent. This is consistent with the phylogenetic analysis by Nicolas et al. (2005) showing that three of four studied genes that have functional pairs on the X and Y chromosome ceased recombining before the speciation in the *Elisanthe* section. In addition, the absence of TRAYC in other dioecious species, *S. otites*, supports the idea of two independent evolutionary events leading to dioecy in the genus *Silene*.

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