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Molecular Cytogenetics (FISH, GISH) of *Coccinia grandis*: A ca. 3 myr-Old Species of Cucurbitaceae with the Largest Y/Autosome Divergence in Flowering Plants

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Key Words

5S and 45S rDNA · C-Banding · FISH · GISH · Sex chromosome · Telomeres

Abstract

The independent evolution of heteromorphic sex chromosomes in 19 species from 4 families of flowering plants permits studying X/Y divergence after the initial recombination suppression. Here, we document autosome/Y divergence in the tropical Cucurbitaceae *Coccinia grandis*, which is ca. 3 myr old. Karyotyping and C-value measurements show that the *C. grandis* Y chromosome has twice the size of any of the other chromosomes, with a male/female C-value difference of 0.094 pg or 10% of the total genome. FISH staining revealed 5S and 45S rDNA sites on autosomes but not on the Y chromosome, making it unlikely that rDNA contributed to the elongation of the Y chromosome; recent end-to-end fusion also seems unlikely given the lack of interstitial telomeric signals. GISH with different concentrations of female blocking DNA detected a possible pseudo-autosomal region on the Y chromosome, and C-banding suggests that the entire Y chromosome in *C. grandis* is heterochromatic. During meiosis, there is an end-to-end connection between the X and the Y chromosome, but the X does not otherwise differ from the remaining chromosomes. These findings and a re-

view of plants with heteromorphic sex chromosomes reveal no relationship between species age and degree of sex chromosome dimorphism. Its relatively small genome size (0.943 pg/2C in males), large Y chromosome, and phylogenetic proximity to the fully sequenced *Cucumis sativus* make *C. grandis* a promising model to study sex chromosome evolution.

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Sex chromosomes in land plants are known from 48 species in 20 families of liverworts, gymnosperms, and flowering plants, where they evolved independently and over widely different time spans [Ming et al., 2011]. Indeed, the sex chromosomes of liverworts differ so fundamentally from those of vascular plants in functioning during the haploid phase of the life cycle, that they might better be considered a third chromosomal sex-determining system, besides X/Y and W/Z systems [Bachtrog et al., 2011]. These independent origins offer the opportunity to compare incipient sex chromosomes, such as those of *Papaya* and *Fragaria*, which are just 0.5–2.2 myr old [Liu et al., 2004; Spigler et al., 2008, 2010; Yu et al., 2008], with older ones, such as those of *Silene* or *Rumex*, which are thought to be over 10 myr old [Moore et al., 2003; Navajas-Pérez et al., 2005; but see Discussion section]. So far,

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heteromorphic sex chromosomes are reliably known from 19 species of Cannabaceae, Caryophyllaceae, Cucurbitaceae, and Polygonaceae [Ming et al., 2011]. About half of the 19 species have been studied with molecular-genetic tools (e.g. Sakamoto et al. [2000]: *Cannabis* (Cannabaceae); Karlov et al. [2003], Divashuk et al. [2011], Grabowska-Joachimiak et al. [2011]: *Humulus* (Cannabaceae); Ruiz Rejón et al. [1994], Shibata et al. [1999, 2000], Mariotti et al. [2006, 2009], Cuñado et al. [2007]: *Rumex* (Polygonaceae); Uchida et al. [2002], Lengerova et al. [2004], Hobza et al. [2006], Cermak et al. [2008], Kejnovsky et al. [2009]: *Silene* (Caryophyllaceae)).

Conspicuously neglected among the plants with heteromorphic sex chromosomes is the Cucurbitaceae *Coccinia grandis*. Classic cytogenetic work established that the Y chromosome in this species is much larger than the other chromosomes [Kumar and Deodikar, 1940; Bhaduri and Bose, 1947; Chakravorti, 1948; Kumar and Vishveshwaraiah, 1952], and experimental work in the 1970s confirmed the sex-determining role of the single Y chromosome [Roy and Roy, 1971]. In spite of the growing interest in plant sex chromosomes [Ming et al., 2011], modern cytogenetic methods have not been applied to *C. grandis* nor has the size of its genome been determined. *C. grandis* belongs to a small genus (25 species) that is phylogenetically close to *Cucumis*, the genus containing cucumber and melon [Schaefer and Renner, 2011]. A dated molecular phylogeny for 24 *Coccinia* species including *C. grandis* is available [Holstein and Renner, 2011].

Here, we characterize the karyotype of male and female *C. grandis* using mitotic and meiotic cell preparations, flow cytometry, FISH with telomere and 5S and 45S rDNA probes, and GISH to evaluate differences between the sexes. We also review XY chromosome size differences in land plants (including the haploid-dominant bryophytes), relating the differences to species ages inferred from molecular-clock studies. The questions we wanted to answer were (i) if rDNA or end-to-end fusions likely have contributed to the elongation of the Y chromosome in *C. grandis* and (ii) if there is a relationship between the age of vascular plant sex chromosomes and the extent of X/Y or Y/autosome morphological divergence.

Materials and Methods

Plant Material

C. grandis (L.) Voigt (including the illegitimate name *C. indica* Wight & Arn.) ranges from tropical Africa to subtropical and tropical Asia and is an invasive weed on Hawaii, other Pacific is-

lands, and in tropical Australia. It belongs to *Coccinia*, a genus of 25 species in sub-Saharan Africa, all of them dioecious climbers. A recent revision of the genus has clarified the boundaries among the species [Holstein, 2012], and a dated molecular phylogeny that includes all but one of the species indicates that the entire genus evolved over just 7 myr [Holstein and Renner, 2011].

Seeds for this study were collected in spring 2011 on the campus of Kakatiya University in Vidyaranya, located in the northern part of Bangalore, state of Warangal, India. In Munich, they were germinated on moist filter paper and then transferred to plastic pots with standard potting soil. Female and male plants were identified by chromosome preparations, and later verified by checking if their flowers were male or female. Plants are still in cultivation in the greenhouses of the Botanical Garden Munich, and a voucher has been deposited in the herbarium of Munich (Sousa and Silber 1 and 2).

Flow Cytometric Genome Size Measurement

Nuclei were isolated from young leaves of 1 male and 1 female *C. grandis*, the sex of which was known since the plants had flowered. Measurements were made on 2 leaves per sex, with each measurement repeated 6 times on 2 different days. Roughly 50 mm² of leaf tissue were co-chopped with equal amounts of young leaf tissue of *Glycine max*, cv. Cina 5202 'Vorán' (IPK gene bank accession number SOJA 392; 2C = 2.23 pg; Borchert et al. [2007]) as an internal reference standard using a razor blade in a Petri dish containing 0.7 ml of nuclei isolation buffer [Galbraith et al., 1983] supplemented with 1% polyvinylpyrrolidone 25, 0.1% Tween 20[®], DNase-free RNase (50 µg/ml) and propidium iodide (50 µg/ml). The nuclei suspension was filtered through a 35-µm-mesh cell strainer cap into a 5-ml polystyrene falcon tube. After at least 15 min of incubation, DNA content measurement was performed on the FACStar^{PLUS} cell sorter (BD Biosciences) equipped with an argon ion laser INNOVA 90C (Coherent). Approximately 10,000 particles per sample were analyzed, and fluorescence intensities of nuclei were measured using the software CELL Quest ver. 3.3 (BD Biosciences). The absolute DNA amounts were calculated based on the values of the G1 peak means.

Chromosome Preparation

Mitotic metaphase chromosomes were prepared from root tips pre-treated in 2 mM 8-hydroxyquinoline for 20 h at 4°C, fixed in freshly prepared 3:1 (v/v) ethanol/glacial acetic acid at room temperature overnight and kept at -20°C. Fixed root tips were washed 3 times for 5 min in distilled water, digested with 1% cellulase (w/v; Onozuka RS, Serva), 0.4% pectolyase (w/v; Sigma), 0.4% cytohelicase (w/v; Sigma) in citric buffer, pH 4.8 for 30 min at 37°C, dissected in a drop of 45% acetic acid and squashed. Coverslips were removed after freezing in dry ice and preparations were air-dried at room temperature. The quality of spreads was checked microscopically using phase-contrast, and only preparations with at least 10 well-spread metaphases were used for FISH/GISH.

Meiotic preparations were made from anthers of young buds. Anthers were fixed in 3:1 (v/v) ethanol/glacial acetic acid at room temperature overnight and stored at -20°C. Fixed anthers were quickly washed in distilled water, dissected in a drop of 45% acetic acid and squashed. Coverslips were removed after freezing, air-dried at room temperature, and the best slides were stained with DAPI (2 µg/ml). After taking pictures, slides were destained

in 3:1 (v/v) ethanol/glacial acetic acid at room temperature for 30 min, kept overnight at 10°C in 100% ethanol, air-dried and kept at room temperature until they were used for C-banding.

DNA Probes

The heterologous ribosomal DNA sequences used as FISH probes were the 18S-5.8S-25S rDNA repeat unit of *Arabidopsis thaliana* in the pBSK+ plasmid, labeled with digoxigenin-11-dUTP (Roche) by nick translation, and a 349-bp fragment of the 5S rRNA gene repeated unit from *Beta vulgaris* cloned into pBSK+ [Schmidt et al., 1994], labeled with biotin-16-dUTP (Roche) by PCR. An *Arabidopsis*-like telomeric probe was amplified by PCR according to Ijdo et al. [1991] using the oligomer primers (5'-TTTAGGG-3')₅ and (5'-CCCTAAA-3')₅ and labeled with digoxigenin-11-dUTP by nick translation.

For GISH, genomic DNA from *C. grandis* male and female plants was isolated using the DNeasy Plant Maxi Kit (QIAGEN). Genomic DNA (1 µg) was autoclaved for 2 min to a fragment size range of 200–400 bp and labeled with digoxigenin-11-dUTP or biotin-16-dUTP (Roche) by nick translation. Blocking DNA was obtained by autoclaving total genomic DNA for 5 min, yielding fragments of approximately 100–200 bp. In GISH experiments, the probe/block ratio was 1:47, 1:70 and 1:100. Digoxigenin-labeled probes were detected with anti-digoxigenin conjugated with FITC (Roche) and biotin-labeled probes with ExtrAvidin conjugated with Cy3 (Sigma).

FISH

FISH was carried out using the method of Schwarzach and Heslop-Harrison [2000] with minor modifications. Slides were pre-treated with 100 µg/ml of RNase A in 2× SSC buffer for 1 h at 37°C and washed 3 times for 5 min in 2× SSC. They were then treated with 10 µg/ml Pepsin (Sigma) in 0.01 N HCl for 20 min at 37°C, washed twice for 5 min in 2× SSC, post-fixed in 4% formaldehyde solution (Roth) for 5 min at room temperature, washed again 3 times for 5 min in 2× SSC, dehydrated for 5 min in a 70 and 100% ethanol series and air-dried for at least 1 h at room temperature. Hybridization mixtures consisted of 50% formamide (w/v), 2× SSC, 10% dextran sulfate (w/v) and 70–200 ng of labeled probe. The hybridization mix was denatured at 75°C for 10 min and immediately cooled on ice for 10 min; 10–15 µl of the mix was then added to each slide and covered with a glass coverslip. For hybridization, the chromosomes, together with the hybridization mixture, were denatured for 5 min at 75°C. Hybridization was carried out in a humid chamber at 37°C for 20 h. After hybridization, the slides were washed 3 times for 5 min in 2× SSC at 42°C, 5 min in 2× SSC at room temperature and 5 min in 2× SSC/0.1% (v/v) Tween 20 at room temperature. For digoxigenin and biotin detection, slides were incubated in blocking buffer (2% BSA in 2× SSC) in a humid chamber for 30 min at 37°C, followed by incubation with anti-DIG-FITC conjugate (Roche) and streptavidin-Cy3 conjugate (Sigma) at 37°C for 1 h. Excess of antibody was removed by washing the slides twice for 7 min in 2× SSC and for 7 min in 2× SSC/0.1% (v/v) Tween 20 at 42°C. The chromosomes were counterstained with DAPI (2 µg/ml) and mounted in Vectashield (Vector).

GISH

The GISH procedure resembled the FISH procedure except that blocking DNA was added to the hybridization mixture. The

latter thus consisted of 50% formamide (w/v), 2× SSC, 10% dextran sulfate (w/v), 83 ng of digoxigenin-labeled *C. grandis* male DNA probe, and 3,500–8,500 ng of non-labeled genomic DNA of a *C. grandis* female. To achieve a 1:47, 1:70 or 1:100 ratio between probe and blocking DNA we used *C. grandis* female DNA at concentrations of 3,928, 6,017 and 8,300 ng.

C-Banding

C-banding was performed according to Schwarzach et al. [1980] with minor modifications. Slides were left for 3 d at room temperature and then incubated in 45% acetic acid at 60°C for 10 min, washed for 1 min in running tap water, dried using an air pump, and incubated in barium hydroxide (Roth) at room temperature for 10 min. The crystals of barium hydroxide were removed by briefly washing the slides in running tap water, followed by a rinse in 45% acetic acid, another 2 min in running tap water and a final rinse in distilled water. The slides were dried using an air pump, and incubated in 2× SSC at 60°C for 1 h 20 min. After the incubation, the slides were washed in distilled water, dried, counterstained with DAPI (2 µg/ml), and mounted in Vectashield (Vector).

Image Analysis

Images were taken with a Leica DMR microscope equipped with a KAPPA-CCD camera and the KAPPA software. They were optimized for best contrast and brightness using Adobe Photoshop CS3 version 10.0.

Karyotype Analysis

Chromosomes and positions of rDNA sites were measured using Adobe Photoshop CS3, and idiograms were constructed based on the analysis of 4 well-spread metaphases, with chromosomes ordered from the largest to the shortest pair, except for the Y chromosome. The X chromosome was assumed to be the smallest chromosome not pairing with an equal-sized autosome; no specific X probes are so far known for *C. grandis*. The chromosome arm ratio (AR, defined as length of the long arm/length of the short arm) was used to classify chromosomes as metacentric (AR = 1–1.4), submetacentric (AR = 1.5–2.9), or acrocentric (AR ≥ 3.0) following Guerra [1986].

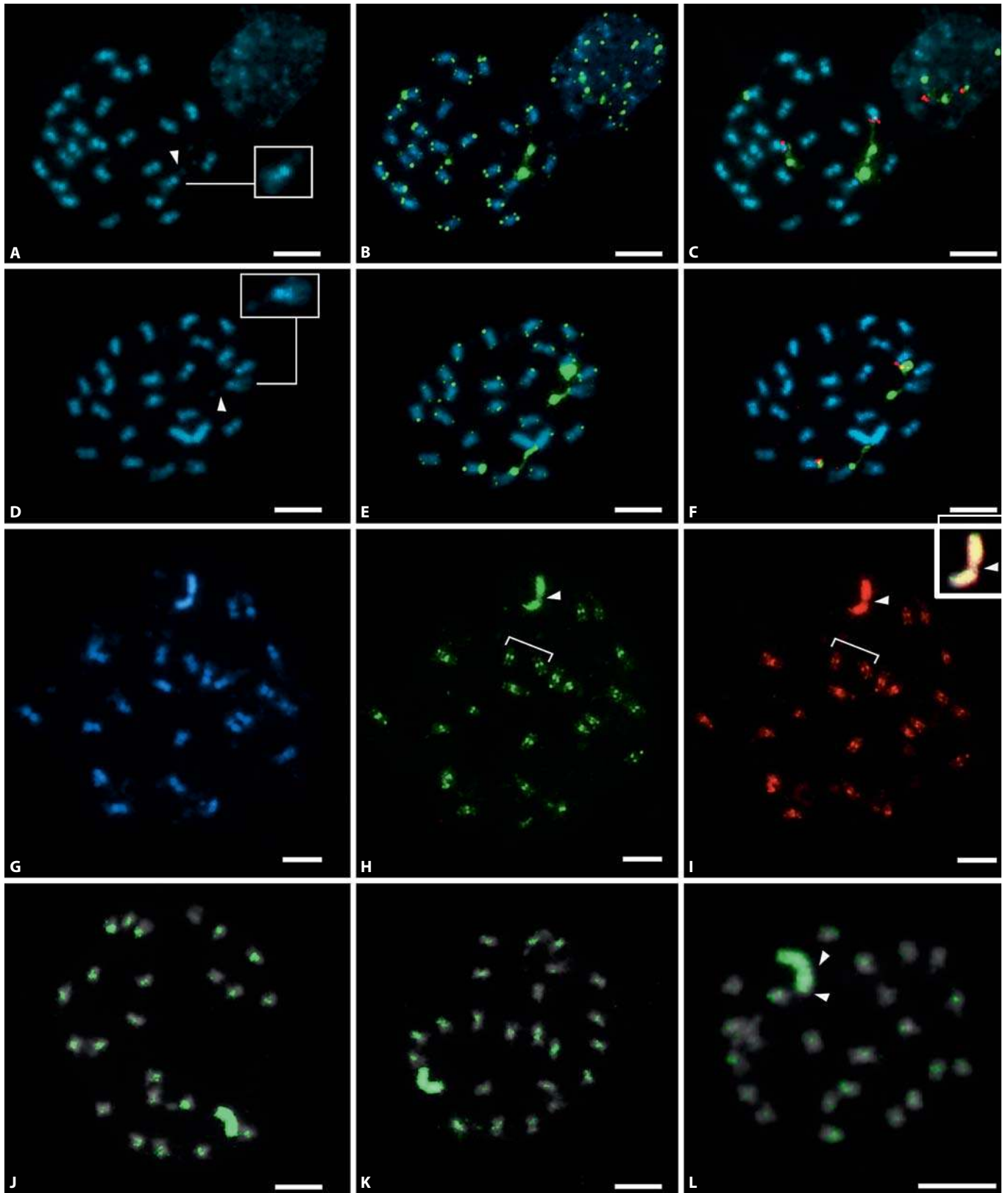
Review of X/Y or Y/Autosome Size Differences in Land Plants

Vascular plants with heteromorphic sex chromosomes were tabulated based on Ming et al. [2011] and the most recent available data on their karyotypes, chromosome lengths, and male/female C-value differences were compiled from the literature. Divergence times for the relevant species inferred with molecular clocks were compiled from phylogenetic studies.

Results

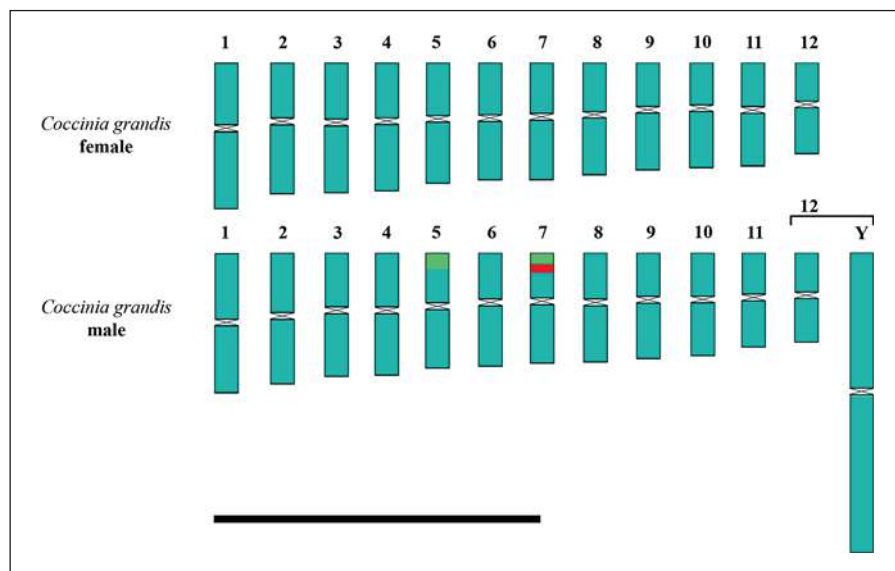
Karyotype, Idiogram, Meiosis and FISH Results

C. grandis females have a karyotype of $2n = 22 + XX$ and males have $2n = 22 + XY$. As the unpaired large chromosome correlates with maleness in the phenotype, *C. grandis* has heteromorphic sex chromosomes. On an agarose gel (online suppl. fig. 1, for all online suppl. materi-



1

Fig. 2. Idiograms of the haploid chromosome complement of *C. grandis*, including 5S (red) and 45S (green) rDNA sites (although only shown in the male, they are equally distributed in the female). Chromosome pairs were put together by similarity. The X chromosome is shown beside the Y chromosome in the male. Telomere sites were detected in all chromosome ends and are not represented in this idiogram. Bar corresponds to 5 μ m.



als, see www.karger.com/doi/10.1159/000345370), auto-claved *C. grandis* female DNA was more stable than male DNA.

Two 45S rDNA sites and one 5S rDNA site were detected in both male and female individuals. The two 45S rDNA sites were always located at the terminal regions of the chromosomes, and the 5S rDNA site was adjacent to one 45S rDNA site (fig. 1C, F). Secondary constrictions were observed in at least 1 chromosome per karyotype in both sexes (see arrowheads in fig. 1A, D and their insets). The *Arabidopsis*-like telomeric probe revealed telomere sequences at the ends of all chromosomes in both females and males (fig. 1B, E), but no interstitial telomere sites.

Fig. 1. FISH (A–F) and GISH (G–L) on mitotic metaphase chromosomes of *C. grandis*. **A, D** DAPI stained chromosomes ($2n = 24$) with 24 homomorphic chromosomes in a female plant, and 23 homomorphic chromosomes and a large heteromorphic Y chromosome in a male plant, respectively. **Insets** show magnified chromosomes with arrowheads marking satellites. **B, E** Distribution of telomeric sequences (small green dots located at the end of the chromosomes) and 45S rDNA (4 strong green signals). **C–F** Bicolor FISH with 45S rDNA (green) and 5S rDNA probe (red). DAPI male metaphase (**G**), and GISH using male genomic probe (**H**) and female genomic probe (**I**). Arrowheads in **H** and **I** show the Y centromere region; the **inset (I)** shows an enlarged Y chromosome with its centromeric region not strongly labeled by either genomic probe. **J–L** GISH using 47 \times , 70 \times , and 100 \times excess of female blocking DNA, respectively. Arrowheads in **L** show small hybridization gaps. Scale bars correspond to 5 μ m.

Figure 2 shows idiograms of *C. grandis* male and female individuals. rDNA sites are presented in figure 2 only in males; females had the same numbers and positions of rDNA.

In meiosis, 12 bivalents could be seen in late prophase I (diakinesis) and in the metaphase plate (fig. 3). Clear end-to-end connections between the X and the Y chromosome were observed (fig. 3A, C, E; as also reported by Bhaduri and Bose [1947]).

GISH and C-Banding Results

GISH experiments were performed with males, using male and female genomic probes. Figure 1H shows that the male genomic probe labeled the (peri-)centromeric and some subterminal regions plus the complete Y chromosome. When the same metaphase preparation was hybridized with the female genomic probe (fig. 1I), the centromeric regions and the Y chromosome again were intensely labeled. The overlap of male and female probes (fig. 1I, inset using DAPI in gray) on the Y chromosome shows that the centromeric region was not well-labeled in comparison to the other chromosomes (arrowheads fig. 1H, I), suggesting that the centromere sequences of the autosomes/X chromosome and the Y chromosome differ in DNA composition. In a few chromosomes, including the Y chromosome, the subterminal regions were predominantly labeled with male genomic probe (these chromosomes are marked by brackets in fig. 1H, I), indicating that subterminal repetitive sequences may have accumulated on the Y chromosome.

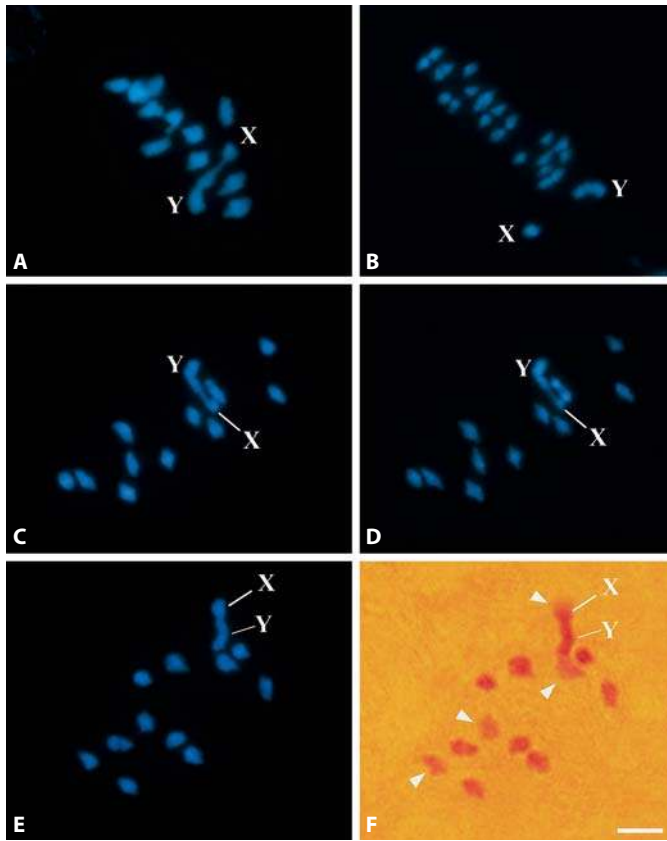


Fig. 3. Meiosis I in a *C. grandis* male, with the position of the sex chromosomes (X/Y) indicated in all cells. **A** Twelve bivalents in metaphase I moving together along the metaphase plate. **B** Early anaphase I with the migration of bivalents and X/Y chromosomes to opposite poles of the cell. **C, E** Cells stained with DAPI before C-banding (**D, F**). **D** Bivalents stained with DAPI showing no detectable morphological distinction between the X and autosomes. **F** Cell stained with Giemsa showing weakly stained bivalents (arrowheads) including the terminal region of X chromosome. Bar corresponds to 5 μm .

When male genomic probes were used with female blocking DNA in different concentrations, the intensity of the hybridization signals in the centromeric region of the chromosomes decreased or disappeared entirely with increasing concentration of female DNA. With 47 \times excess of blocking DNA, the Y chromosome was well-labeled as were most of the chromosomes (fig. 1J); with 70 \times excess of blocking DNA, the Y chromosome still was well-labeled, but a few chromosomes exhibited no or weak hybridization signals (fig. 1K); with 100 \times excess of blocking DNA, the Y chromosome started to present hybridization gaps not labeled by the male genomic probe, and 1 terminal region did not show any detectable hy-

bridization signal (fig. 1L, arrowheads). The reduction of the signal strength presumably is associated with similar repetitive sequences shared by male and female *C. grandis*.

C-banded heterochromatic regions in females were mostly concentrated in centromeric/pericentromeric regions (fig. 4B, C) while in males (fig. 4E, F) they were diffusely pericentromeric/subterminal. The Y chromosome showed the same DAPI intensity before and after C-banding, again suggesting that the Y chromosome in *C. grandis* is heterochromatic. Using Giemsa staining (fig. 4C, F), female pre-metaphase chromosomes were all more or less well-stained while male pre-metaphases showed only few chromosomes, including the Y chromosome, with strong Giemsa-labeling.

In meiotic cells stained with DAPI, bivalents in metaphase I displayed few differences before and after C-banding (see fig. 3C, D). The terminal region of the X chromosome, but not the Y chromosome, was DAPI-positive, implying that the pseudoautosomal region is mainly euchromatic (fig. 3D). The autosomes were more intensely stained in the internal region of each bivalent, and no detectable morphological distinction could be observed between the X and the autosomes (fig. 3D). With Giemsa-staining (fig. 3F), some bivalents in late prophase I exhibited less labeling than others (see arrowheads) after C-banding, and the free terminal region of the X chromosome was less strongly labeled than its other end, connected to the Y chromosome.

Chromosome Measurements, C-Values and Comparison with Other Vascular Plant Sex Chromosomes

Chromosome lengths in the female varied from 1.35 to 2.26 μm and in the male from 1.33 to 4.71 μm . The largest autosome in males was 2.28 μm long, meaning that the Y chromosome, with 4.71 μm , is around twice as long as the largest chromosome. On the basis of their centromere position, all *C. grandis* chromosomes have an AR index of 1–1.4, making them metacentric (see table 1). Based on measurements on nuclei isolated from young leaf tissue, female individuals have a C-value of 0.849 pg/2C and male individuals of 0.943 pg/2C (table 2).

Table 3 summarizes data on X and Y chromosome lengths, C-values, and inferred ages for vascular plant species with heteromorphic sex chromosomes. Species with sex chromosomes are usually characterized by ARs (p/q) and relative, not absolute lengths because length to some extent depends on the preparation protocol and environmental factors. The data available so far reveal no relation-

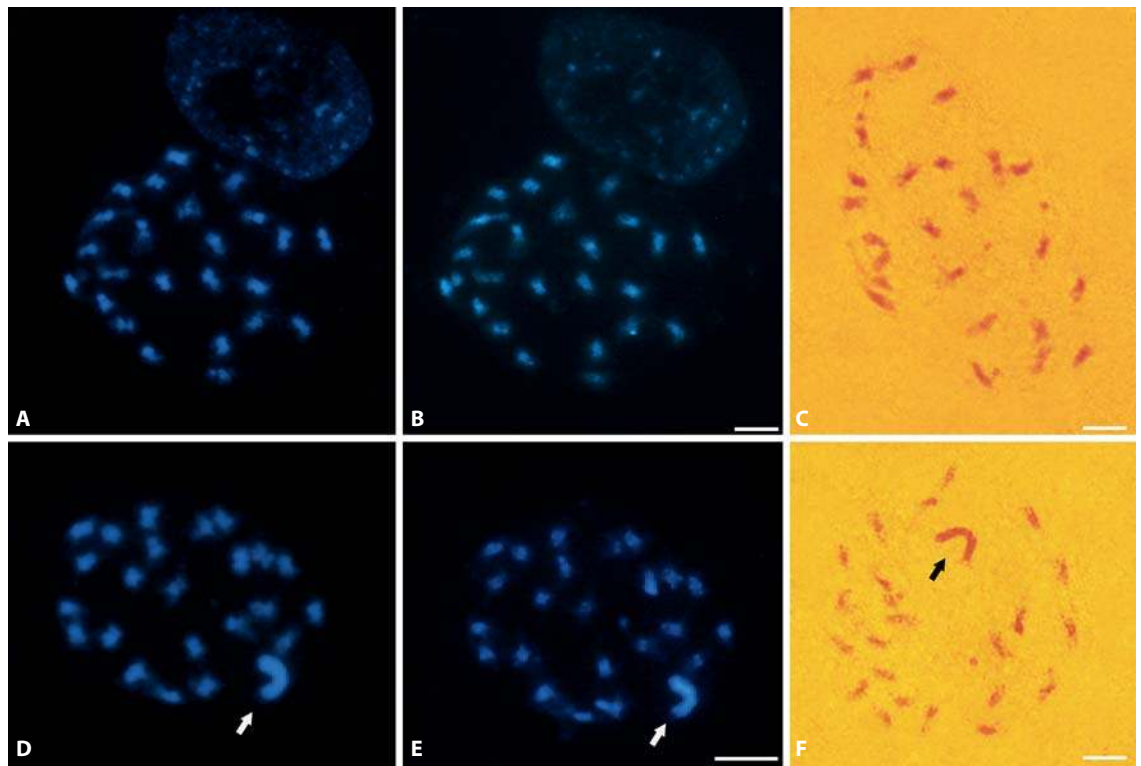


Fig. 4. C-banding in a *C. grandis* female (A–C) and male (D–F). A, D Metaphases stained with DAPI before C-banding. Chromosomes are stained along their entire length. B, E Metaphases showing centromeric and subterminal DAPI-positive regions, hence heterochromatic. The Y chromosome in D and E shows similar DAPI intensity before and after C-banding. C, F Pre-metaphases

stained with Giemsa after C-banding. The heterochromatin is well-distributed on all chromosomes in C while it is concentrated on the Y chromosome in F. Arrows in D–F indicate the Y chromosome. Bar in B valid for A, and in E valid for D. Bars correspond to 5 μ m.

Table 1. Morphology of *C. grandis* male and female chromosomes

Chromosome pairs	Chromosome size, μ m		AR		Chromosome type	
	male	female	male	female	male	female
1	2.28 \pm 0.25	2.26 \pm 0.06	1.13	1.21	m	m
2	2.01 \pm 0.14	2.03 \pm 0.13	1.12	1.24	m	m
3	1.87 \pm 0.14	2.01 \pm 0.06	1.17	1.22	m	m
4	1.87 \pm 0.14	1.96 \pm 0.05	1.15	1.19	m	m
5 ^a	1.75 \pm 0.21	1.85 \pm 0.03	1.19	1.16	m	m
6	1.73 \pm 0.11	1.78 \pm 0.02	1.28	1.14	m	m
7 ^b	1.66 \pm 0.14	1.75 \pm 0.04	1.29	1.23	m	m
8	1.66 \pm 0.14	1.69 \pm 0.05	1.23	1.14	m	m
9	1.60 \pm 0.06	1.65 \pm 0.04	1.28	1.36	m	m
10	1.56 \pm 0.03	1.60 \pm 0.06	1.22	1.32	m	m
11	1.44 \pm 0.04	1.58 \pm 0.08	1.14	1.26	m	m
12X ^c	1.33 \pm 0.05	1.35 \pm 0.12	1.09	1.21	m	m
12Y	4.71 \pm 0.34		1.18		m	

^a Chromosome pairs with only 45S. ^b Chromosome pairs with 45S and 5S rDNA. ^c Likely X chromosome/X chromosome pair. m = Metacentric. The length of satellites is not included in the chromosome length.

Table 2. Flow cytometric measurements for male and female *C. grandis*

	Leaves	Samples	DNA content, pg/2C	SD
Male	2	12	0.943	0.005
Female	2	12	0.849	0.005

Table 3. Chromosome numbers, lengths, 2C-values, and inferred age of sex chromosomes in vascular plant species with heteromorphic sex chromosomes

Species	Chromosome number, 2n	Chromosomal sex determination	X chromosome length μm	Y chromosome length μm	DNA content (2C) pg	Age of species or clade, myr	References (studies with age estimates)
<i>Podocarpus macrophyllus</i>	37, 38	X ₁ X ₂ /Y	unknown	9.1	unknown	unknown	Hizume et al. [1988]
<i>Coccinia grandis</i>	24	XX/XY	indistinguishable from autosomes	4.71 (10% of male genome weight)	M = 0.943 F = 0.849	3–6	Holstein and Renner [2011]; Holstein [2012]
<i>Humulus japonicus</i>	16, 17	XX/XY ₁ Y ₂	3.11	Y ₁ = 2.98 Y ₂ = 2.75	M = 3.522	unknown	Grabowska-Joachimciak et al. [2006]
<i>Humulus lupulus</i>	20	XX/XY	2.39 ^a	1.63	M = 5.523	unknown	Grabowska-Joachimciak et al. [2006]
<i>Rumex acetosa</i>	14, 15	XX/XY ₁ Y ₂	3% of female genome weight	Y ₁ = 7.5 Y ₂ = 6.9 (20% of male genome weight)	M = 7.498 F = 7	15–16	Kurita and Kuroki [1970]; Blocka-Wandas et al. [2007]
<i>Silene latifolia</i>	24	XX/XY	slightly smaller than autosomes (8% of female genome weight)	much longer than autosomes (9% of male genome weight)	M = 5.85 F = 5.73	3.5–24	Siroky et al. [2001] (Moore et al. [2003]; Nicolas et al. [2005]; Rautenberg et al. [2012])

^a Putative X chromosome.

ship between the ages of sex chromosomes and the extent of Y/autosome or X/Y divergence. In terms of total genome size, *C. grandis* has the smallest genomes of all vascular plants with heteromorphic sex chromosomes (table 3).

Discussion

The Extent of Y/Autosome Divergence in C. grandis

Our results show that *C. grandis* has the greatest Y/autosome size difference documented in vascular plants (2.43 μm ; table 1): The Y chromosome of *C. grandis* is 2.06 times larger than the largest chromosome (in contrast to previous reports of it being 2.5 \times or 3–4 \times longer than the largest autosome; Bhaduri and Bose [1947]; Guha et al. [2004]). Experimental work on *C. grandis*, using diploid individuals and artificial polyploids, has established the male-determining effect of the presence of the Y chromosome; individual tetraploid plants with a karyotype of XXXY still were normal males [Roy and Roy, 1971]. As previously reported, the chromosome number

of *C. grandis* is $2n = 22 + XX$ or $22 + XY$ [Kumar and Deodikar, 1940 probably by mistake reported $2n = 26$ for both sexes; Bhaduri and Bose, 1947; Chakravorti, 1948; Kumar and Vishveshwaraiah, 1952].

The degree of divergence of the male and female genome in *C. grandis* is evident also from the C-values: The difference between the male and female genomes is almost 0.1 pg of DNA, which is in the range of an entire small plant genome (*Genlisea margaretae*, $1C = 0.065$ pg; Greilhuber et al. [2006]) and amounts to ca. 10% of the *C. grandis* genome (0.094 pg/2C). In *Silene latifolia*, the male genome weighs 5.85 pg/2C, the female 5.73 pg/2C, with the Y chromosome making up ca. 9% of the male genome and the X chromosome ca. 8% of the female genome [Siroky et al., 2001].

Autosome sizes in *C. grandis* vary from 2.28 to 1.44 μm in males and from 2.26 to 1.58 μm in females (table 1), both sexes having exclusively metacentric chromosomes (fig. 2), with the X chromosome probably the smallest chromosome of the complement, an assumption that needs testing. Both sexes also have the same number

and distribution of rDNA sites on the autosomes (fig. 1C, F) while no rDNA site was detected on the Y chromosome. At least one of the chromosome pairs of *C. grandis* labeled with 45S rDNA bears a secondary constriction, but in contrast to previous reports [Bhaduri and Bose, 1947; Agarwal and Roy, 1984; Chattopadhyay and Sharma, 1991] no secondary constriction was seen on the Y chromosome. In species of *Silene*, *Rumex* and *Humulus* with heteromorphic sex chromosomes, the rDNA sites are also restricted to autosomes [Siroky et al., 2001; Karlov et al., 2003; Cuñado et al., 2007; Grabowska-Joachimiak et al., 2011], but *Spinacia oleracea* has a 45S rDNA site on the X chromosome [Lan et al., 2006]. It thus appears that rDNA does not greatly or regularly contribute to the morphological divergence of plant Y chromosomes.

Of the heteromorphic sex chromosomes that have been studied, most have undergone extensive rearrangements or end-to-end fusions. Thus, in *Podocarpus macrophyllus* ($2n = 34 + X_1X_2Y$; table 3), females have 38 telocentric chromosomes while males have 36 telocentric and 1 large submetacentric Y chromosome. In meiosis I, the *P. macrophyllus* Y chromosome pairs with 2 telocentric chromosomes to form a trivalent, suggesting it may have originated from a telocentric fusion of 2 telocentric chromosomes [Hizume et al., 1988]. In *Humulus japonicus*, a species with an XY_1Y_2 sex chromosome system (table 3), interstitial telomeric sites on 1 autosome pair also point to a fusion event having led to the reduction of the chromosome number from 18 to $14 + XY_1Y_2$ [Grabowska-Joachimiak et al., 2011]. And in *S. latifolia* telomere-homologous sequences on the sex chromosomes provide evidence of a translocation of subtelomeric sites [Uchida et al., 2002]. In *C. grandis*, however, we did not find any telomeric sequences at interstitial sites (fig. 1B, E), suggesting that such fusions have not contributed, at least not recently, to the elongation of this species' Y chromosome.

Our GISH experiments revealed the preferential distribution of repetitive sequences in male and female individuals of *C. grandis*. In plants with small genomes, GISH signals tend to be unclear and restricted to pericentromeric heterochromatin blocks [Ali et al., 2004]. In *C. grandis* males, however, male and female genomic probes clearly differed in spite of the small genome size of the species (female individuals 0.849 pg/2C; male individuals 0.943 pg/2C). Male genomic DNA (fig. 1H) hybridized to centromeric and some subterminal regions of the chromosomes, while female genomic DNA (fig. 1I) hybridized mainly to centromeric regions. Both genomic probes hybridized to the Y chromosome, and C-banding results

indicate that the Y chromosome is indeed mostly heterochromatic (fig. 4D, F). This fits with repetitive sequences forming large clusters in the centromeric and subterminal regions of the autosomes and having accumulated on the Y.

The types of repetitive DNA in the centromere of the *C. grandis* Y appear to be different from those in the centromeres of the autosomes and X chromosome (fig. 1I, inset). The situation might resemble that found in *S. latifolia*, where the centromeres of the autosomes and X chromosome are rich in *Silene* tandem repeat centromeric sequences and transposable elements, while the Y centromere contains *Silene* tandem repeat Y sequences [Cermak et al., 2008; Kejnovsky et al., 2009]. In *C. grandis* Y chromosomes, male-specific regions became progressively more visible with increasing concentrations of female blocking DNA (fig. 1J–L), and terminal regions that failed to label with either male or female DNA probably are pseudoautosomal regions, still engaged in recombination. In meiosis, there is an end-to-end connection between the X and the Y chromosome, but the X does not otherwise differ from the remaining chromosomes.

Ages of Plant Y Chromosomes and Their Size Change over Time

An increase in the size of some, but not all (table 3), vascular plant Y chromosomes has been attributed to the accumulation of repetitive DNA, especially transposable elements [Bergero et al. [2008], Cermak et al. [2008], Kejnovsky et al. [2009]: *Silene latifolia*; Mariotti et al. [2006, 2009], Cuñado et al. [2007]: *Rumex acetosa*). Such accumulation is thought to occur because of inefficient selection in non-recombining regions [Charlesworth and Charlesworth, 2000]. The best studied plant Y chromosome, that of *S. latifolia*, indeed does show signs of degeneration, including reduced levels of polymorphism, reduced gene expression levels, and transposable element insertion in Y genes [Filatov et al., 2000; Marais et al., 2008]. The degeneration, however, is less pronounced than that documented from animal sex chromosomes, perhaps because they are older or because of purifying selection during the haploid stage of the embryophyte life cycle [Armstrong and Filatov, 2008; Bergero and Charlesworth, 2011; Chibalina and Filatov, 2011]. An estimated 62% of the genes of *A. thaliana* are expressed in its haploid pollen tubes [Honys and Twell, 2003]. In liverworts, in which the haploid gametophyte is the predominant stage and in which there is no XX recombination, sex chromosome dimorphism may follow a different trajectory from that in vascular plants, where the diploid spo-

rophyte is the predominant life phase (Yamato et al. [2007]; Bachtrog et al. [2011]; but see Jamilena et al. [2008] for the opposite view that the *Marchantia polymorpha* Y is in an advanced stage of degeneration, caused by the accumulation of a large amount of unique repetitive DNA sequences).

Comparing the speed of X/Y divergence is complicated by our poor understanding of the absolute ages of plant sex chromosomes. Thus, the sex chromosomes in the *S. latifolia* species group are approximately between 3.5 and 24 myr old. Synonymous site divergence values suggest ages of 8–24 myr [Moore et al., 2003] or 5–10 myr [Nicolas et al., 2005], while a phylogenetic study that used a relaxed molecular-clock approach instead inferred an age of the *Silene* clade with sex chromosomes of ca. 3.5 myr (Rautenberg et al. [2012] fig. 4: the node in question is the divergence of *S. latifolia* from *S. samia*). Molecular-clock work in liverworts is scarce, but judging from genetic branch lengths *Frullania* species are >2 myr old (Pleistocene; Bombosch et al. [2010]).

It has been hypothesized that the evolution of plant sex chromosomes may proceed from the initial recombination suppression and the expansion of the male-specific region through increasing heteromorphy between the X and Y chromosomes, followed by severe degeneration of the Y to its eventual loss (Ming et al. [2011] fig. 2). So far, there is no evidence for such a trajectory (table 3), and the limited data rather suggest that transposon accumulation

and chromosome rearrangements occur idiosyncratically. It is clear, however, that plant sex chromosomes are all relatively young.

The sequencing and assembly of plant Y chromosomes is technically not yet feasible, and it is therefore unclear which transposon families they may accumulate. Nor is it clear in general how fast plant repetitive DNA is turned over [Renny-Byfield et al., 2011; Piednoel et al., 2012]. The only assembled Y chromosomes so far are those of *Homo sapiens* and chimpanzee [Skaletsky et al., 2003; Hughes et al., 2010]. However, once next-generation sequencing techniques yield longer read lengths, the relatively small genome of *C. grandis* compared to *S. latifolia* (c. 0.94 pg/2C vs. 5.85 pg; Costich et al. [1991]) and its phylogenetic proximity to the fully assembled crop species *C. sativus* [Huang et al., 2009] may make it a potentially useful additional system for the study of plant sex chromosomes.

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