frontiers in Plant Science

ORIGINAL RESEARCH
published: 01 November 2018
doi: 10.3389/fpls.2018.01501

3D Molecular Cytology of Hop (*Humulus lupulus*) Meiotic Chromosomes Reveals Non-disomic Pairing and Segregation, Aneuploidy, and Genomic Structural Variation

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Hop (*Humulus lupulus* L.) is an important crop worldwide, known as the main flavoring ingredient in beer. The diversifying brewing industry demands variation in flavors, superior process properties, and sustainable agronomics, which are the focus of advanced molecular breeding efforts in hops. Hop breeders have been limited in their ability to create strains with desirable traits, however, because of the unusual and unpredictable inheritance patterns and associated non-Mendelian genetic marker segregation. Cytogenetic analysis of meiotic chromosome behavior has also revealed conspicuous and prevalent occurrences of multiple, atypical, non-disomic chromosome complexes, including those involving autosomes in late prophase. To explore the role of meiosis in segregation distortion, we undertook 3D cytogenetic analysis of hop pollen mother cells stained with DAPI and FISH. We used telomere FISH to demonstrate that hop exhibits a normal telomere clustering bouquet. We also identified and characterized a new sub-terminal 180 bp satellite DNA tandem repeat family called HSR0, located proximal to telomeres. Highly variable 5S rDNA FISH patterns within and between plants, together with the detection of anaphase chromosome bridges, reflect extensive departures from normal disomic signal composition and distribution. Subsequent FACS analysis revealed variable DNA content in a cultivated pedigree. Together, these findings implicate multiple phenomena, including aneuploidy, segmental aneuploidy, or chromosome rearrangements, as contributing factors to segregation distortion in hop.

Keywords: cytogenetics, FISH, non-Mendelian inheritance, segregation distortion, telomere bouquet

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science

Received: *28 June 2018*
Accepted: *25 September 2018*
Published: *01 November 2018*

Citation:
Easterling KA, Pitra NJ, Jones RJ, Lopes LG, Aquino JR, Zhang D, Matthews PD and Bass HW (2018) 3D Molecular Cytology of Hop (Humulus lupulus) Meiotic Chromosomes Reveals Non-disomic Pairing and Segregation, Aneuploidy, and Genomic Structural Variation. Front. Plant Sci. 9:1501. doi: 10.3389/fpls.2018.01501

INTRODUCTION

Humulus lupulus (hop), a member of the Cannabaceae family of flowering plants, is a dioecious, high-climbing, herbaceous bine that is best known as a flavoring agent in beer. Hop cultivation for this purpose has been traced back to Germany, 736 AD (Neve, 1991). For hop, despite its long successful history of domestication, modern breeding practices are associated with a number of challenges. For instance, although hop is generally cultivated vegetatively by rhizomes, sexual crosses are necessary in order to breed for new disease-resistant and chemically

desirable varieties. The long history of cultivation includes colchicine-induced polyploidization (Roborgh, 1969) and the introgression of genetically distinct wild populations (Small, 1980; Reeves and Richards, 2011). Recent genetic, genomic, and quantitative trait analyses have demonstrated that the genome of hop is complex and structurally diverse (Zhang et al., 2017). Irregularities in hop transmission genetics are reflected in non-Mendelian segregation distortion and sex ratio bias (Seefelder et al., 2000; Jakse et al., 2008, 2011; McAdam et al., 2013, 2016).

Meiotic chromosome behavior is implicated as a cause for the unusual transmission genetics that are found in hop (Zhang et al., 2017). In sexually reproducing organisms, meiosis reduces the genomes from diploid to haploid (John, 1990) and homologous chromosomes from each parental genome undergo pairing, synapsis, recombination (John, 1990). These events ensure proper segregation of chromosomes into balanced gametes for subsequent fertilization and transmission to the progeny of the next generation (Stebbins, 1935; John, 1990; Murphy and Bass, 2012). Deviations from such normal disomic pairing and disjunction can lead to a variety of genetic inheritance problems including gene dosage imbalance, aneuploidy, and chromosomal rearrangements.

Among the hallmarks of meiosis that are cytologically evident are (1) the presence of a telomere bouquet in early prophase that guides homology search and subsequent synapsis, (2) the presence of bivalents at diakinesis during late prophase, (3) complete and equal separation of homologs at meiosis I, and (4) the production of 4 haploid nuclei with 1:1:1:1 segregation, as evidenced from tetrad analysis. For hop, non-disomic and heteromorphic sex chromosome figures have been noted and speculated to impact segregation patterns (Sinotô, 1929; Jacobsen, 1957; Neve, 1958; Haunold, 1991; Shephard et al., 2000; Zhang et al., 2017). However, neither the early prophase telomere bouquet nor the post-meiotic segregation has been characterized cytogenetically.

Here we set out to further examine the cytogenetics of male hop meiosis. To date, several somatic karyotypes for hop have been produced (Sinotô, 1929; Winge, 1929; Shephard et al., 2000). The more recent hop karyotypes include FISH-mapped loci for the Humulus lupulus-specific subtelomeric repeat-1 (HSR1), the Humulus japonicus-specific subtelomeric repeat (HSJR), the nucleolus organizer region (NOR), and 5S rDNA (Karlov et al., 2003; Divashuk et al., 2011; Alexandrov et al., 2012). Telomere FISH has also been used in hop, but not to test for the presence of the telomere bouquet at early prophase. In this study, we employed 3D acrylamide FISH (Howe et al., 2013) to show that hop has a classical zygotene telomere bouquet, but non-uniform segregation of 5S rDNA loci. Detection of anaphase bridges and variable DNA content further implicate genome structure variability in the unusual inheritance patterns of hop.

METHODS

Plant Materials and Fixation

Plants were collected and fixed according to previously described methods (Zhang et al., 2017), with exceptions on timing and paraformaldehyde concentration during the meiocyte buffer A (MBA) steps. Briefly, hop panicles were field collected and immediately fixed in Farmer's fluid (3:1 ethanol:acetic acid) overnight, replaced with Farmer's fluid for a second overnight period, and exchanged into 70% ethanol for storage at 4°C. Hopsteiner varieties were collected on site in the company germplasm yard, and were grown under standard agronomic conditions at the Golden Gate Ranches, S.S. Steiner, Inc, Yakima, WA. The H. lupulus var. neomexicanus plant SH2, was collected in the Coronado National Forest in Arizona.

Bioinformatic Identification of Tandem Repeat Family, H. lupulus Subterminal Repeat 0, HSR0

DNA from Apollo was used to make a library of large DNA fragments using the RSII technology. PacBio Single Molecule, Real-Time (SMRT) DNA sequences were manually and randomly screened for tandem repeats using the dot plot function of GenomeMatcher (Ohtsubo et al., 2008). Tandem repeats in the clones were detected as parallel stripes exhibiting a striping pattern evenly spaced and parallel to the main diagonal of identity. This striping pattern is diagnostic for DNA containing tandemly repeated sequences, easily detected by dot plot inspection, and was used to find new candidate loci for FISH probe development. Potential hits were further analyzed by dot plotting against other hits to detect "allelism" among the repeats found within individual clones. Computational tandem repeat searching was done using "ksift" (https://github.com/dvera/ksift) Jelly sh-2 (https://github.com/zippav/Jelly sh-2) and YASS dot-plotter (https://github.com/laurentnoe/yass).

The two most frequent tandem repeat families found in this way were the 385 bp tandemly repeated sequence previously defined as the Humulus-specific subtelomeric repeat-1, HSR1, GenBank Accession GU831574 (Divashuk et al., 2014), and a new 180 bp tandemly-repeated sequence designated here as H. lupulus Subterminal Repeat 0, HSR0. The names of five HSR0-containing Apollo genomic DNA sequences from PacBio SMRT and their length and GenBank Accession numbers are HuluTR180-120 (6,005 bp, Acc. MH188533), HuluTR180-69 (11,761 bp, Acc. MH188534), HuluTR180TEL-954 (10,443 bp, Acc. MH188536), HuluTR180TEL-316 (5,717 bp, Acc. MH188537), and HuluTR180-270 (10,794 bp, Acc. MH188538). Two of these, HuluTR180TEL-316 and HuluTR180TEL-316, contain both HSR0 and telomeric repeats. For comparison, a clone with telomeric DNA repeats but lacking HSR0 has also been deposited as HuluTEL-347 (5,551 bp, Acc. MH188535).

Fluorescence in-situ Hybridization, FISH

For 3D FISH, whole Farmer's-fixed buds attached to peduncles were equilibrated in meiocyte Buffer A (Bass et al., 1997) for 30 min at RT, repeated twice, followed by fixation in 1% formaldehyde in MBA for 1 h at RT. After fixation, the tissue was washed three times in MBA for 15-min each at RT and stored in MBA at 4C. Meiotic cells were micro-dissected from buds of various sizes spanning meiosis (from 1.5 to 2.5 mm in length) and embedded in acrylamide as described for the 3D acrylamide FISH technique (Howe et al., 2013).

FISH probes used in this study were synthetic oligonucleotides co-synthetically coupled to fluorescent dyes and designed to detect telomeres ("MTLF-29," 5′-[FITC]-(CCCTAAA)$_4$-3′ or "MTLY-28-16" 5′-[ATTO647N]-(CCCTAAA)$_4$-3′), HSR0 tandem repeats ("ZERO-Y," 5′-[ATTO647N]-AGAAATATG AGTGAATTACGAAATCGC-3′), HSR1 tandem repeats ("HSR1a_22," 5′-Alexa488-GGTACCCCTCTGGTGAAT TGGA-3′), or 5S rDNA (pool of three oligos: "5SBOB1F," 5′-[Alexa488]-GCACCGGATCCCATCAGAACTCC-3′, "5SBOB2F," 5′-[Alexa488]-AGTTAAGCGTGCTTGGGCGAG AG-3′, and "5SBOB3F," 5′-[Alexa488]-GTGACCTCCTGGGAA GTCCTCGTG-3′). The three 5S rDNA probes were selected on the basis of their 100% identity, according to the 5S rRNA Database (Szymanski et al., 2002), for *Cannabis sativa* (5sRNAdb Record ID E00464), *Gossypium hirsutum* (5sRNAdb Record ID E00193), and *Zea mays* (5sRNAdb Record ID E00011). Prehybridization, hybridization, post-hybridization washes, DAPI counterstaining, and slide mounting were done as described (Howe et al., 2013) using denaturation temperatures ranging from 88 to 92°C.

Collection, Analysis, and Display of 3D Deconvolution Microscopy Images

Three-dimensional images were collected on a DeltaVision deconvolution microscope, using a 60X lens and 0.2 micron Z-step optical sections as described (Zhang et al., 2017) for DAPI imaging, but also including FITC, TRITC, or CY5 imaging for FISH probes that fluoresce green, red, or far-red, respectively. Distance measurements between the 5S rDNA FISH signals were obtained as point-to-point Euclidean distances using the Measure Distances program, manually selecting the brightest voxel centered in the X, Y, and Z dimensions for any given FISH signal.

FACS Analysis of DNA Content

A two-step protocol (Pellicer and Leitch, 2014) was followed to isolate hop nuclei from 20 mg of fresh leaf tissue chopped and stained in LB01 buffer. The homogenate was filtered through a 30–42 um nylon mesh filter, centrifuged, and resuspended in LB01 buffer. Samples were vortexed before being analyzed at the Iowa State University (Ames, IA) Flow Cytometry Facility using an unmodified BD Biosciences FACSCanto (San Jose, CA) flow cytometer.

RESULTS

Telomere FISH Reveals Bouquet Formation in Hop

Given the pervasive irregularities noted in Zhang et al. (2017) for meiotic chromosome behavior together with SD, we first wanted to ask which, if any, of the cytological hallmarks of normal meiotic prophase are found in hop using molecular cytology. The unusual chromosome interactions and genetic segregation defects previously observed in hop could result from missing or faulty bouquet, given the importance of that structure in efficient pairing and recombination. For comparison, a normal bouquet involves the clustering of telomeres on the nuclear envelope in early meiotic prophase. Specifically, the bouquet stage actually spans from late leptotene, through all of zygotene and into early pachytene (Bass et al., 1997; Bass, 2003; Scherthan, 2007). Using 3D telomere FISH, we document the presence of a telomere bouquet, as summarized in Figure 1. Of the hop nuclei exhibiting a telomere FISH bouquet (*n* = 59 from plants 255C, 243C, 243D, Male 15), 36 nuclei showed tightly clustered signals and 23 nuclei showed less tightly clustered FISH signals at the nuclear periphery. For two representative hop nuclei at early meiotic prophase, the telomere FISH signals are clustered in one area of the nuclear periphery, forming a normal-looking bouquet (Figures 1B,E, labeled "BQ") (Bass et al., 1997). The first nucleus (Figures 1A–C) shows a common pattern in which the nuclear volume (Figure 1A, circle traces at the nucleus-cytoplasm boundary) is larger than that occupied by the chromatin mass. This clumping of chromatin is a zygotene stage-specific, coagulative- fix-dependent artifact, producing the so-called the synizetic knot, as described for *Oenothera* and other plant species (Golczyk et al., 2008). A second nucleus (Figures 1D–F) shows that the bouquet is clearly present in a nucleus that appears to be a later stage than the first nucleus, but still in early prophase, as evidenced by the presence of discrete chromosome fibers. These experiments showed that the telomere bouquet does indeed occur in hop male meiocytes, and was observed in both modern cultivars (Figure 1) and wild-collected plants (not shown). From these analyses, we find no compelling evidence to implicate a faulty or missing bouquet as causal for segregation distortion in hop. These analyses only establish that the bouquet appears normal in both structure and timing.

HSR0 Is an Abundant Subtelomeric 180 bp Satellite DNA Tandem Repeat

In order to further characterize the behavior of hop meiotic chromosomes, we set out develop new FISH probes for cytological tracking of multiple and specific regions of chromosomes. New probes would help solve the problem that hop chromosomes are very similar in centromere location and size but linkage groups remain largely unassigned to chromosomes. We focused on tandem repeats as a class of sequences that make for ideal FISH probes suitable for karyotype development (Albert et al., 2010; Divashuk et al., 2014). They yield bright, discrete signals that can be seen to pair and segregate as reporter loci in meiosis (Bass et al., 2000, 2003).

We used dot plot and kmer analysis of long-read PacBio genomic sequence data to find tandem repeat candidates for new FISH probes. The two most frequent tandem repeat families found were the 385 bp tandemly repeated sequence previously described as the *Humulus*-specific subtelomeric repeat-1, HSR1 (Divashuk et al., 2014), and a new 180 bp tandemly-repeated satellite DNA sequence designated HSR0 (*Humulus* Subterminal Repeat 0). As shown in Figure 2, the HSR0 repeat sequence arrays can span an entire sequencing read (Figure 2A), part of a PacBio sequence (Figure 2B), or arranged as blocks of inverted polarity within a single sequence (Figure 2F). The HSR0 repeats also showed an intriguing pattern, occasionally appearing adjacent

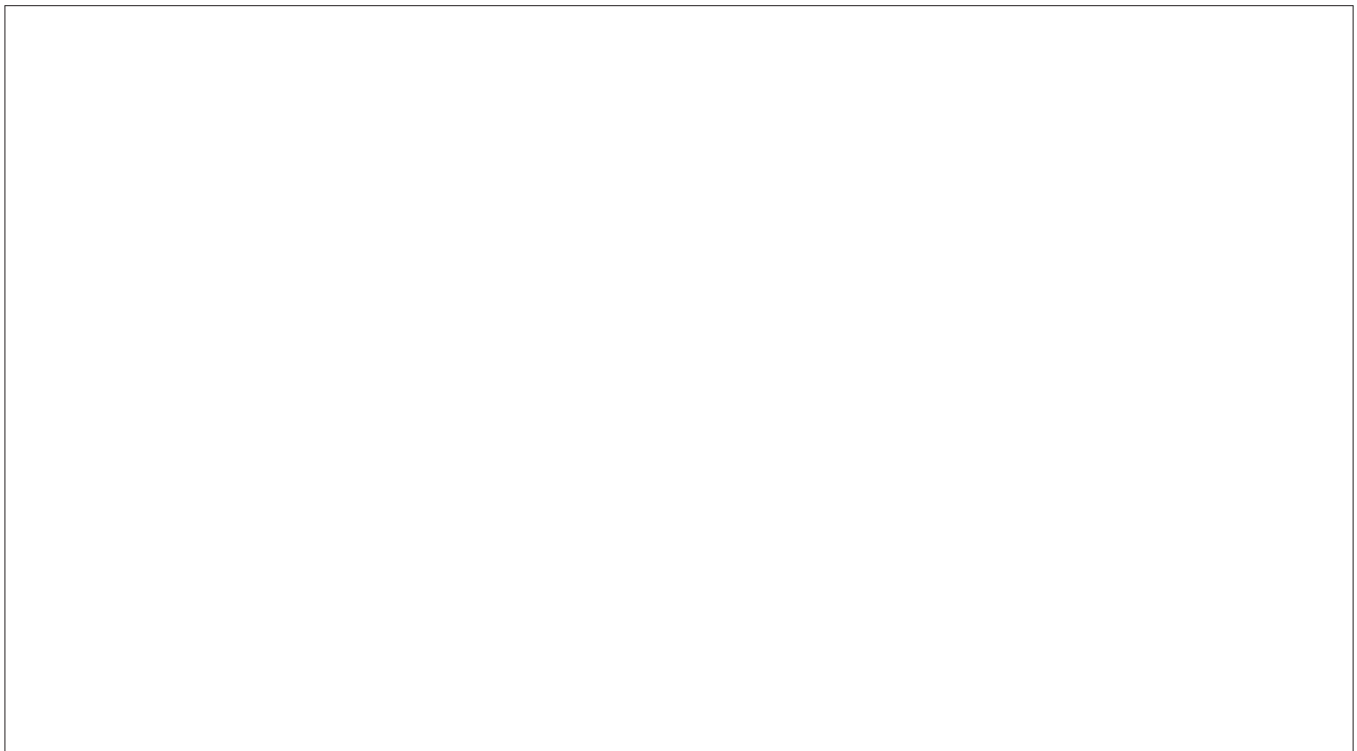

FIGURE 1 | Telomere bouquet in pollen mother cells of hop. Male owers were harvested and xed in Farmer's Fluid, then exchanged into Buffer A and formaldehyde xed before microdissecting pollen mother cells from anthers for 3D acrylamide FISH. Through-focus projections of nuclei, one nucleus per row, are shown as through-focus maximum-intensity projections in gray-scale for individual wavelengths or in color for overlay images, as labeled across the top. (A–C) Hop nucleus from plant 255C at early prophase showing the nuclear periphery (A- white circle) and the telomere cluster region (bracketed, BQ). (D–F) Hop nucleus from plant 243C at early-middle prophase showing the bouquet (BQ) with three examples of single telomere FISH signals (TEL) indicated.

to telomere repeat DNA (Figure 2D) or even interspersed with telomere repeats (Figure 2E). These observations suggest that HSR0 should make a good FISH probe that is predicted to be near telomeres. The HSR0 sequence is di erent than HSR1, and none of the HSR0 pac-bio clones examined included HSR1 repeats.

Given that HSR0 and telomere repeats occur together in clones, we predicted that HSR0 satellite DNA may be subtelomeric, and if so should stain bouquet and co-localize with HSR1. To test this, we carried out FISH using HSR0 together with either telomere (n D 21) or HSR1 (n D 13). Most of the HSR0 FISH signals did indeed colocalize with telomere FISH signals (Figures 3A–H). Most, but not all, of the HSR0 FISH signals also clearly co-localized with HSR1 FISH signals (Figures 3I–L). Despite the tendency for HSR0 and HSR1 to co-localize, we did nd a few cases (Tk) where only one was detected (arrowheads, Figures 2J,K). These solo signals from combined FISH probes could be useful for distinguishing otherwise similar chromosomes. Together, the molecular and cytogenetic analyses indicate that the 180 bp satellite repeat family HSR0 comprises a newly characterized and abundant subtelomeric tandem repeat sequence family. The HRS0 oligo FISH probe, ZERO-Y, represents a valuable new reagent for karyotyping and analysis of meiosis.

Meiotic Chromosome Abnormalities Are Evident at Mid-prophase, Meiosis I, and II

Having shown that hop has a normal bouquet (Figure 1), we wanted to examine in more detail the middle prophase/pachytene stage, when long thick bers should appear and telomere distributions typically transition from clustered in early pachytene to dispersed in middle and late pachytene (Bass et al., 1997). We observed evidence of meiotic irregularities in pachytene, as shown in Figure 4. Speci cally, all of the meiotic nuclei imaged at mid-prophase (n D 72 total from 7 di erent plants) showed conspicuous lack of uniformity of ber appearance, a pattern resembling that of some meiotic mutants with disruption or loss of synchronous progression (Bass et al., 2003). For instance, in the example shown in Figure 4A, plant 243D and wild collected var. neomexicanus plant SH2, thick ber (Tk) cross sections were measured and averaged 670–850 nm whereas thin (Tn) bers averaged 320–460 nm. The fact that th telomeres are dispersed in this nucleus (Figure 4A) indicates that the cell has progressed beyond the bouquet stage which ends in early pachytene, at which point there should be no unpaired or unsynapsed chromosomes in a normal diploid cell. This bimodal ber thickness is a conspicuous and invariant recurring phenotype in hop that appears to persist through all pachytene. In addition to this conspicuous non-uniformity i ber morphology, nuclei at this stage also showed heteropycnot

FIGURE 2 | HSR0 dot plot outputs of tandemly repetitive genomic regions. PacBio Single Molecule, Real-Time (SMRT) DNA sequences were screened for tandem repeats (Ohtsubo et al., 2008). For each clone, a self dot-plot is shown along with the top 12 12mers for that clone using kmer analysis. The stripes represent internal tandem repeats of HSR0 (blue stripes) or telomere repeats (tightly packed blue stripe blocks). (A) The HuluTR180-120 clone showing HSR0 repeat occupies the entire 6 Kbp clone. (B) The HuluTR180-69 clone showing HSR0 repeat occupies around one half of the 6 Kbp clone, whereas the single diagonal represents sequences that are not repeated within the clone. (C) The HuluTRTEL-347 clone showing with unique sequences in the first 3 kbp followed by telomere repeat DNA ($CCCTAA_n$) in the last 3 kbp. (D) The HuluTR180TEL-954 clone showing a large block of HSR0 repeats immediately followed by more than 5 Kbp of telomere repeat DNA ($TTTAGGG_n$). (E) The HuluTR180TEL-316 clone showing interspersed blocks of HSR0 and telomere repeat DNA ($TTTAGGG_n$). (F) The HuluTR180–270 clone showing a block of HSR0 repeats in one direction (blue) followed by blocks of inverted repeat polarity (red) in another.