Non-Mendelian Single-Nucleotide Polymorphism Inheritance and Atypical Meiotic Configurations are Prevalent in Hop

Dong Zhang, Katherine A. Easterling, Nicholi J. Pitra, Mark C. Coles, Edward S. Buckler, Hank W. Bass, and Paul D. Matthews*

ABSTRACT

Hop (*Humulus lupulus* L.) breeding programs seek to exploit genetic resources for bitter flavor, aroma, and disease resistance. However, these efforts have been thwarted by segregation distortion including female-biased sex ratios. To better understand the transmission genetics of hop, we genotyped 4512 worldwide accessions of hop, including cultivars, landraces, and over 100 wild accessions using a genotyping-by-sequencing (GBS) approach. From the resulting ~1.2 million single-nucleotide polymorphisms (SNPs), prequalified GBS markers were validated by inferences in population structures and phylogeny. Analysis of pseudo-testcross (Pt) mapping data from F_1 families revealed mixed patterns of Mendelian and non-Mendelian segregation. Three-dimensional (3D) cytogenetic analysis of late meiotic prophase nuclei from two wild and two cultivated hop revealed conspicuous and prevalent occurrences of multiple, atypical, nondisomic chromosome complexes including autosomes. We used genome-wide association studies (GWAS) and fixation index (F_{st}) analysis to demonstrate selection mapping of genetic loci for key traits including sex, bitter acids, and drought tolerance. Among the possible mechanisms underlying the observed segregation distortion from the genomic data analysis, the cytogenetic analysis points to meiotic chromosome behavior as one of the contributing factors. The findings shed light on long-standing questions on the unusual transmission genetics and phenotypic variation in hop, with major implications for breeding, cultivation, and the natural history of *Humulus*.

Core Ideas

- GBS pseudo-testcross data from F_1 families reveal extensive segregation distortion.
- Cytogenetic analyses reveal atypical, nondisomic, meiotic configurations.
- Genetic loci associated with sex determination are mapped to LG 4.
- Hot spots exhibiting unusual F_{st} variance provide clues about signatures of selection in hops.
- Combined analyses implicate meiotic chromosome behavior in segregation distortion.

THE Cannabaceae family of flowering plants has a rich history of contributions to humanity, with the promise of still greater contributions as a result of new commercial values and invigorated research in two members, hop ($2n = 2x = 20$) and *Cannabis sativa* L. (hemp, marijuana) ($2n = 2x = 20$) (van Bakel et al., 2011), which diverged ~27.8 million yr ago (Laursen, 2015). The hop plant is a high-climbing dioecious bine and an

D. Zhang, K.A. Easterling, N.J. Pitra, M.C. Coles, P.D. Matthews, Hopsteiner, S.S. Steiner, Inc., New York, NY, 10065; D. Zhang, E.S. Buckler, Institute for Genomic Diversity, Cornell Univ., Ithaca, NY, 14853; E.S. Buckler, Agricultural Research Service, USDA, Ithaca, NY, 14853; K.A. Easterling, H.W. Bass, Dep. of Biological Science, Florida State Univ., Tallahassee, FL, 32306-4295. D. Zhang, K.A. Easterling, and N.J. Pitra contributed equally to this work. Received 4 Apr. 2017. Accepted 31 Aug. 2017. *Corresponding author (pmatthews@hopsteiner.com).

Abbreviations: 3D, three-dimensional; CV, modern cultivar; FISH, fluorescence in situ hybridization; F_{st}, fixation index; GBS, genotyping-by-sequencing; GWAS, genome-wide association studies; IBS, identity-by-state; LG, linkage group; LLE, locally linear embedding; MAF, minor allele frequency; MBA, meiocyte Buffer A; MLM, mixed linear model; MMC, modulated modularity clustering; NGS, next-generation sequencing; SD, segregation distortion; SNP, single-nucleotide polymorphism; SW, Shinshu Wase.

herbaceous perennial with historic uses in brewing and nutraceutical medicine and modern uses as biofuel and animal fodder (Siragusa et al., 2008). Metabolic engineering and marker-directed breeding in hop recently increased as clinical studies identified hop-derived prenylflavonoids as therapeutic agents for treatment of cancer, dyslipidemia, and postmenopausal symptoms (Ososki and Kennelly, 2003; Stevens and Page, 2004; Nagel et al., 2008; Miranda et al., 2016). Despite the value of these traits and products, the hop plant has proven refractory to traditional breeding and conventional genomic strategies for genetic dissection of complex, quantitative traits. Several factors contribute to this difficulty including aspects of its reproductive system such as dioecy and obligate outcrossing, high degree of heterozygosity, large genome size, and a poorly understood sex-determination system (Neve, 1958).

Wild hop is represented by at least five extant taxonomic varieties: (i) *H. lupulus* L. var. *lupulus* for European wild hop, (ii) *H. lupulus* L. var. *cordifolius* (Miq.) Maxim. mainly distributed in Japan, (iii) *H. lupulus* L. var. *neomexicanus* A. Nelson & Cockerell in the US Southwest, (iv) *H. lupulus* L. var. *pubescens* E. Small in the eastern and midwestern United States, and (v) *H. lupulus* L. var. *lupuloides* E. Small throughout the northern Great Plains and spreading into other parts of North America. Asian and North American wild hop resemble each other morphologically, suggesting a genetically close relationship, while they differ more so from European hop (Murakami et al., 2006). Many contemporary cultivars are hybrids of North American and European genetic materials, in which North American hop have been characterized by their higher bitterness and aroma (Reeves and Richards, 2011) than European cultivars. In other crops, breeding programs have successfully exploited novel genetic variations from wild exotic germplasms into modern cultivars (Tanksley and McCouch, 1997; Bradshaw, 2016) to gain desirable traits such as desired flavors, drought tolerance, and disease resistance. Successes with wild resources and predictions of climate change have spurred resurgence in conservation biology of plant genetic resources (Castañeda-Álvarez et al., 2016; Gruber, 2016).

Molecular marker systems including nonreferenced GBS markers (Matthews et al., 2013) and GWAS (Henning et al., 2015; Hill et al., 2016) have been developed and used for genetic mapping of disease resistance and sex determination. Despite these advances, understanding the genetic inheritance patterns in hop remains a major challenge. For example, significant distortion from Mendelian segregation expectations has been repeatedly reported in mapping populations, indicating that the segregation bias was due to genetic properties rather than genotyping errors (Seefelder et al., 2000; McAdam et al., 2013). Relatedly, female-biased sex ratios have been observed in most families (Neve, 1991; Jakse et al., 2008). The segregation data for hop resemble to some extent those from segregation distortion systems that are well described in certain plants known to exhibit

chromosomal rearrangements or meiotic drive (reviewed by Taylor and Ingvarsson, 2003). For instance, in *Clarkia*, *Oenothera*, *Viscum*, and *Calycadenia*, translocation heterozygosity and other chromosomal abnormalities can modify Mendelian segregation patterns and impact intraspecies fertility (Snow, 1960; Wiens and Barlow, 1975; Carr and Carr, 1983; Rauwolf et al., 2008; Golczyk et al., 2014).

With regard to the chromosomal composition of hop, classical cytogenetics has established that the species has heteromorphic sex chromosomes and occasional meiotic quadrivalents of unknown chromosomal composition (Sinotô, 1929; Neve, 1958; Haunold, 1991; Shephard and Parker, 2000). More recently, somatic hop karyotypes have been developed for several varieties, including FISH mapping of the locations of the NOR, 5S rDNA and the abundant *Humulus* subtelomeric repeats, HSR1 (Karlov et al., 2003; Divashuk et al., 2011). Functional genomics in hop has been advanced by detailed linkage analysis (Henning et al., 2017) and whole-genome sequencing (Natsume et al., 2015), yet these data are not integrated into a single annotated reference genome nor connected to the chromosome numbers of the published karyotypes.

To further characterize the genome of hop, we performed next-generation sequencing (NGS) of 4512 accessions, including 22 F_1 families, genotyped with GBS SNP marker system, comprising 1.2 million SNPs. This study greatly extends the previous NGS GBS studies in hop (Matthews et al., 2013; Henning et al., 2015; Hill et al., 2016) with much larger association panels and marker sets, providing new population structure information. Instead of filtering out SNPs that show segregation distortion (SD), we included and exploited them in our analysis, strengthening the size and quality of candidate gene lists. We also examined several plants at the cytological level and found peculiarities consistent with the marker segregation irregularities. These new findings advance our working knowledge of the genome of hop and point to chromosome structure and recombination constraints as important aspects guiding future breeding strategies.

MATERIALS AND METHODS

Plant Materials

The hop plants used in this study were grown under standard agronomic conditions at the Golden Gate Ranches, S.S. Steiner, Inc., Yakima, WA. The undomesticated, exotic hop plants are from the National Clonal Germplasm Repository in Corvallis, OR (accession details in Supplemental Table S1–S3). Fifty milligrams of young leaf tissues were extracted in a 96-well block using Qiagen Plant DNeasy Kits and was tested for quality, quantity, and purity prior to library preparations using an Agilent 2100 Bioanalyzer (Applied Biosystems) and Life Technologies Qubit 3.0 Fluorometer. The GBS libraries were prepared using the *Ape*K1 enzyme according to Elshire et al. (2011). Pools of 96 accessions were sequenced on one lane of an Illumina HighSeq 2000 (Illumina)

Three-Dimensional Cytogenetic Analysis of Male Meiotic Prophase Nuclei

Hop panicles were harvested from the Hopsteiner male yard (Yakima, WA) throughout the day, fixed in Carnoy's solution (3:1 ethanol:acetic acid) overnight, and exchanged into 70% ethanol for storage at –20°C. For 3D microscopy, buds were equilibrated in meiocyte Buffer A [MBA, (Bass et al., 1997)] for 15 min at room temperature, repeated twice, then fixed in 2% formaldehyde in MBA at RT for 2 h. After fixation, buds were washed twice in MBA, 15 min each, at room temperature, and stored in MBA at 4°C. Anther lengths were recorded and meiotic cells were microdissected onto glass slides and mounted in VectaShield + DAPI (Vector Laboratories). Three-dimensional images were collected on a DeltaVision deconvolution microscope, using a 60X lens and 0.2 μm Z-step optical sections (as summarized by Howe et al., 2013). Three-dimensional datasets capturing entire nuclei at various stages of meiosis were collected. Deconvolved images were further processed using linear scaling of intensity and software programs (Volume Viewer, Copy Region, Projection, 3D Model) to allow for inspection from various angles.

Classification and quantification of meiotic chromosome configurations were made on diakinesis stage nuclei using a combination of visual inspection methods including paging back and forth through individual optical sections of the 3D data stacks along with inspection of through focus projections made from multiple angles as well as viewing of cropped subvolumes. For this study, a nucleus determined to be in diakinesis had at least two bivalents <5 μ in length. The number of bivalents and nonbivalent complexes were counted for each plant using at least 20 diakinesis nuclei. The nonbivalent complexes were split into two subcategories: quadrivalents (two bivalents joined into a ring of four, or interlocked chain link structure) or other complexes (nonquadrivalents with variable number of chromosomes).

Single-Nucleotide Polymorphism Calling and Quality Control

The reference sequence refers to a draft haploid genome sequence of 'Shinshu Wase' (SW) (Natsume et al., 2015), which is a modern cultivar bred from a seedling selection cross between Saazer and White Vine-OP. The draft genome, with a total size of 2.05 Gb, consists of ~130,000 scaffolds covering ~80% of the estimated genome size of hop (2.57 Gb).

Tassel 5 GBS v2 Pipeline (Glaubitz et al., 2014) was applied to identify tags with at least 10x total coverage and to call SNPs. Tag sequences were mapped to the reference genome using BWA aligner.

One main source of erroneous SNP calling is misalignment caused by incomplete reference genome, gene duplication, and low-complexity regions. To filter out erroneous SNPs from misalignment, we used two criteria: (i) SNPs with an excessive coverage can be false positives; we observed that heterozygosity rates and minor allele frequency (MAF) are significantly increased when read coverage exceeds 127 (Supplemental Fig. S1), and (ii) the orientation of paired reads of the cultivar Apollo (unpublished data), a highly used maternal line in our F_1 families, was used to detect false positive SNPs caused by gene duplications. Paired-end alignment was generated by BWA Sampe. Identification of correctly aligned regions was based on SAM flags indicating reads mapped in proper pairs. Using criterion (ii), we were able to detect ~73% SNPs with the excessive coverage.

Pseudo Testcross

Three F_1 families were used to conduct Pt recombination mappings including (i) 144 ($N = 179$) derived from a cross between Nugget (maternal line) and Male50 (paternal line), (ii) 247 ($N = 364$) derived from two parental lines, Super Galena and Male15, and (iii) 265 ($N = 95$) derived from a cross between Chinook and Male57. Using markers heterozygous in the maternal line and homozygous in the paternal line, three genetic map sets were constructed, consisting of 3551 SNPs for 144, 2369 SNPs for 247, and 4506 SNPs for 265.

Our analyses followed the main steps in HetMappS pipelines (Hyma et al., 2015), specifically: (i) to remove contaminants, identity-by-state (IBS)-based distance matrices calculated by TASSEL (Bradbury et al., 2007) were used to identify outliers for each family; (ii) SNPs having both parental genotypes (e.g., AA×Aa) with read depth ≥ 4 were retained for the next step; (iii) in progeny, SNPs with average read depth ≥ 4 and with site coverage $\geq 50\%$ were retained for the next step; (iv) to eliminate the effect of undercalling heterozygotes and sequencing errors, we masked progeny genotypes with depth = 1 and converted genotypes aa to Aa because genotype aa cannot exist for parental genotypes AA×Aa in Pt; (v) after correction, SNPs with 15% $\leq$ MAF $\leq$ 35% were selected to create linkage groups (LGs), and SNPs with 5% $\leq$ MAF < 15% were deemed the pronounced SD markers; (vi) to cluster and order markers, an adjacency matrix with Spearman's correlation (ρ) were derived from the remaining SNPs; and (vii) on the basis of absolute values of ρ, the Louvain method (Blondel et al., 2008) implemented in NetworkX (http://networkx.github.io/) was applied to detect communities (clusters). The Louvain method is an efficient algorithm for community detection in large networks. A similar method, modulated modularity clustering (MMC) (Stone and Ayroles, 2009), has been successfully applied to construct LGs. The clustering patterns of markers were cross-checked by the locally linear embedding (LLE) method (Roweis et al., 2000), a nonlinear dimensionality reduction method, implemented in Python scikit-learn; (viii) to identify coupling phase from each absolute ρ cluster, negative values of ρ were set to zero, and the Louvain method was applied to positive values of ρ (Hyma et al., 2015); (ix) MSTmap (Wu et al., 2008) was used to provide a solution of genetic ordering within each LG.
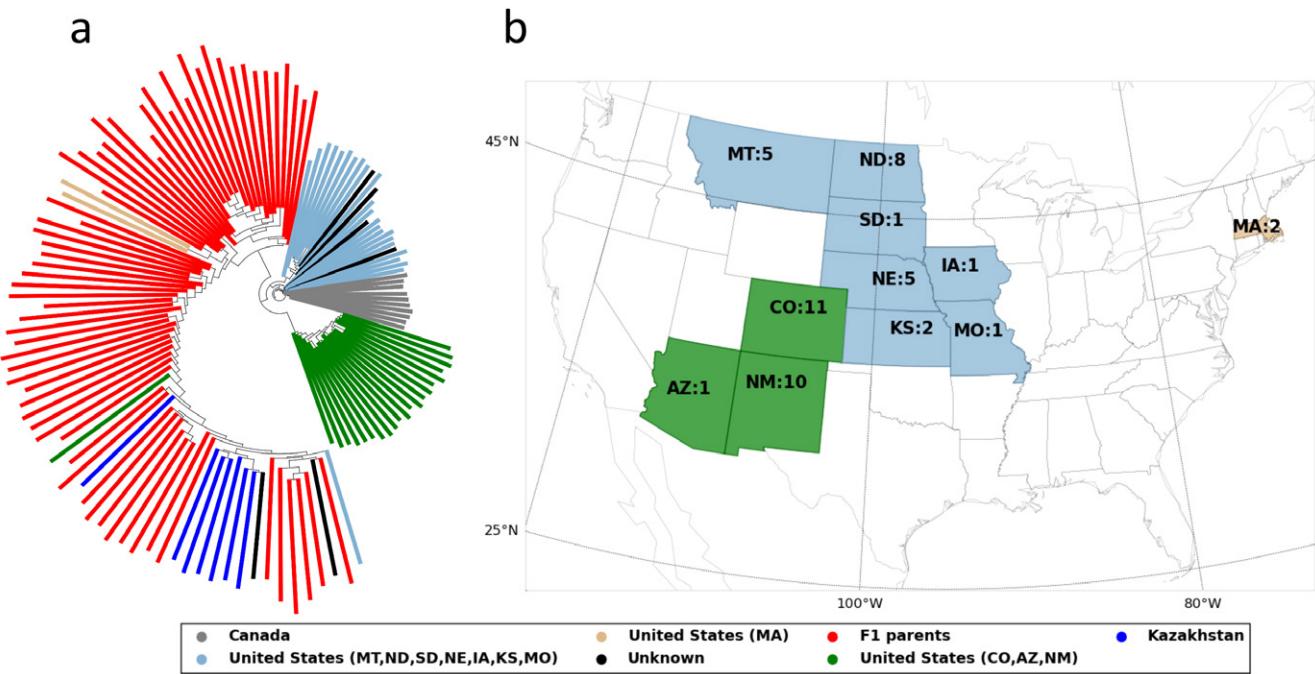
Fig. 1. Population structure of 251 hop accessions and geographic origins of the US wild hop: 183 modern cultivars are indicated by red color and 68 wild hop are color-coded by geographic origins. (a) Neighbor-joining tree of the 251 hop accessions. (b) The state names are followed by sample counts. Three state groups (MT, ND, SD, NE, IA, KS, MO; CO, AZ, NM; and MA) are color-coded to distinguish from one another.

Putative 10-by-2 LGs in coupling were obtained in each F_1 family. As the karyotype has not been fully understood in hop, the LG ID numbers were arbitrarily assigned in 144. Using the genetic map in 144 as a central reference, we assigned the ID numbers to LGs in other crosses. Linkage groups deriving from two grandparents are distinguished by suffix .1 and .2. Linkage groups may or may not represent one chromosome because of pseudo linkage resulting from chromosomal rearrangement as discussed in the Results section.

Genome-Wide Association Studies

An association population includes 850 individuals, in which 837 (116 males and 721 females) are progeny in six F_1 families and 13 are paternal lines. Male and female were encoded as 1 and 0 individually. A total of 356,527 SNPs with coverage ≥50% and MAF ≥5% were retained. The mixed linear model (MLM) (Bradbury et al., 2007; Lipka et al., 2012) was used to assess genotype–phenotype association. The Bonferroni method was used to adjust the significance cutoff for an overall probability of 0.05 for type I error.

RESULTS

Phylogenetic Relationships of Modern Cultivars and North American Indigenous Exotics

European *H. lupulus* L. var. *lupulus* is the ancestor of most commercial hop used today; thereby, commercial cultivars retain a large proportion this genome. In addition, the genetic diversity of hop crop has been contributed by mostly male donors from North America and Asia. To understand the phylogenetic relatedness in hop races, we focused on a subset of 251 accessions comprised of 183 modern cultivars (CVs) including all progenitors of F_1 families in this study and 68 wild hop as summarized in Fig. 1. The neighbor-joining tree (Fig. 1a) shows three distinct clusters. The modern cultivars were clustered together, indicating a common derivation in domestication of hop. The other two clusters reflect geographical origins of North American wild hop (Fig. 1b), in which one group (SW_wild) includes 22 southwestern United States wild hop (represented by var. *neomexicanus*), and the other group contains 20 wild hop (represented by *H. lupulus* L. var. *lupuloides*) from northern United States and Canada (N_wild) and three (represented by *H. lupulus* L. var. *pubescens*) from the US Midwest (MW_wild). Seven wild individuals from Kazakhstan are intermediate among the modern cultivars, consistent with a previous inference (Murakami et al., 2006) of a close genetic relationship between wild hop from Europe and the Altai region (close to western China, located on boundaries of Russia, Mongolia, Kazakhstan, and China).

The level of population differentiation, F_{st}, was measured across the three clusters. Group SW_wild exhibits relatively close genetic relationship (F_{st} = 0.1663) with N_wild, apparently supporting relatively close ancestry and geographical origins of the two wild populations. Genetic distinction between the modern cultivars and the North American wild hop is evident: [F_{st} (CV vs. SW_wild) = 0.31; F_{st} (CV vs. N_wild) = 0.295].

To demonstrate the population structure of F_1 families and varieties clones ($N \geq 60$) (Supplemental Fig. S2a) in our dataset, we used a nonlinear algorithm (implemented in Python scikit-learn), t-distributed stochastic neighbor embedding (Van der Maaten and Hinton, 2008), for dimension reduction of the IBS-based distance matrix. The F_1 families derived from genetically divergent progenitors can be easily distinguished from one another, while the half-sibling families exhibit ambiguous clustering patterns (Supplemental Fig. S2).

Three-Dimensional Cytogenetic Analysis of Meiotic Chromosomes

Cytological analysis was performed using 3D imaging of nuclei from four different male *H. lupulus* hop plants that were obtained from either wild seed (var. *lupuloides* from Crooked Lake or var. *neomexicanus* from Chimney Rock) or produced as progeny from crosses within the Hopsteiner breeding program (cross 265, cross 255). Late meiotic prophase nuclei were stained with DAPI and imaged using 3D microscopy to survey the chromosome configurations. The hop meiocytes used in this study should have a chromosome constitution of $2n = 2x = 20$, including sex chromosomes (Sinotô, 1929; Winge, 1929) of unresolved constitution.

Typically, diploid nuclei from organisms with normal disomic inheritance exhibit diakinesis chromosomes in which each bivalent is distinct and spatially separate from other bivalents and distributed around the nuclear periphery. In striking contrast, hop diakinesis described here showed considerable deviation from a typical pattern of 10 well-separated bivalents, as summarized in Fig. 2 for diakinesis-stage nuclei. A notable diversity of chromosome configurations was observed including canonical bivalents (arrows, Fig. 2a) and various other complexes. The average number of bivalents per nucleus is depicted for each plant (Fig. 2a). None of the plants averaged more than six bivalents per nucleus, leaving at least four homolog pairs of chromosomes on average per nucleus that could be involved in other configurations. Chimney Rock (var. *neomexicanus*) contained an average of 2.2 bivalents per nucleus, by far the fewest of the four plants examined. The other wild plant, Crooked Lake (var. *lupuloides*) contained an average of 5.7 bivalents per nucleus. The F_1 progeny from cross 265 and 255 contained an average of 4.7 and 6.0 bivalents, respectively. Nuclei with 10 bivalents were observed at a low frequency (~5%) in Crooked Lake and crosses 265 and 255, but, so far, not at all in Chimney Rock. Taken together, the findings from this 3D analysis reveal that complexes are not limited to heteromorphic sex chromosomes, but instead are both prevalent and heterogeneous within and among different plants.

To further classify the chromosome configurations, we performed detailed analysis of subnuclear regions cropped in 3D from the full datasets (Fig. 2b–d). Individual chromosomes or complexes were classified on the basis of their morphology and proximity into several categories, bivalents, quadrivalents, and other complexes. The bivalents (Fig. 2b) were classified as three types: ring, which appeared as pairs of chromosomes frequently in a ring configuration; sex (XY), which appeared as the only heteromorphic pair in the set; or NOR-linked, which appeared to be attached to a nucleolus. The quadrivalents (Fig. 2c) were defined as two pairs of nonhomologous chromosomes joined together by presumed chiasmata. The quadrivalents were classified as three types: ring of four, which appeared as two bivalents in an open ring; a double ring, which appeared as two bivalents in a chain-link pattern; or NOR-linked plus X, which appeared as connected to both the nucleolus and the X chromosome of the sex bivalent. Quadrivalents of any type were found to occur with an average per-cell frequency of 1.4 for Crooked Lake, 0.2 for Chimney Rock, 1.3 for cross 265 hybrid, and 1.0 for cross 255 hybrid. The other complexes (Fig. 2d) were heterogeneous and less readily classified but referred to as multiple, which included nonquadrivalent complexes of variable composition, or long chain, which appeared as numerous interconnected series of chromosomes. The most common multiple complexes involved more than two pairs of chromosomes, but occasional complexes of one bivalent plus one univalent were also observed. Combining all types, the average per-cell frequencies of complexes were 0.55 for Crooked Lake, 2.08 for Chimney Rock, 0.38 for cross 265 hybrid, and 0.85 for cross 255 hybrid.

Overall, complexes were found in all plants, wilds, and F_1 progeny. The wild plant from Chimney rock (var. *neomexicanus*) exhibited an unexpectedly large number and variety of complexed chromosomes including long-chain arrangements (e.g., Fig. 2d) and atypical configurations with more than one nucleolus. To the extent that the complexes are held together by crossovers, these findings may reflect translocation heterozygosity, segmental aneuploidy, or other atypical pairing regions resulting in the segregation distortion reported here and previously (Seefelder et al., 2000; McAdam et al., 2013).

Segregation Distortion in Progeny from F_1 Crosses

Genetic markers that exhibit non-Mendelian inheritance frequencies can result from biological processes or technical errors. While genotyping errors are random, the biologically distorted markers typically exhibit pronounced correlation with Mendelian segregation markers. On the basis of clustering of pairwise Spearman's correlation in Pt markers (exemplified in Fig. 3) in three F_1 families, we observed that the loci with 5 to 15% MAF, deviated significantly from the 25% allele frequency expected for Pt markers. These MAFs account for 28.3, 49 and 48.3% in families 144, 247, and 265, respectively, in which proportions of the distorted loci correlated ($\rho \geq 0.3$) to the Mendelian segregation markers (15–35% MAF) are 78.3, 48.9, and 71.8% (Supplemental Fig. S4). This finding is consistent with a previous QTL study in hop using DArT markers (McAdam et al., 2013). These
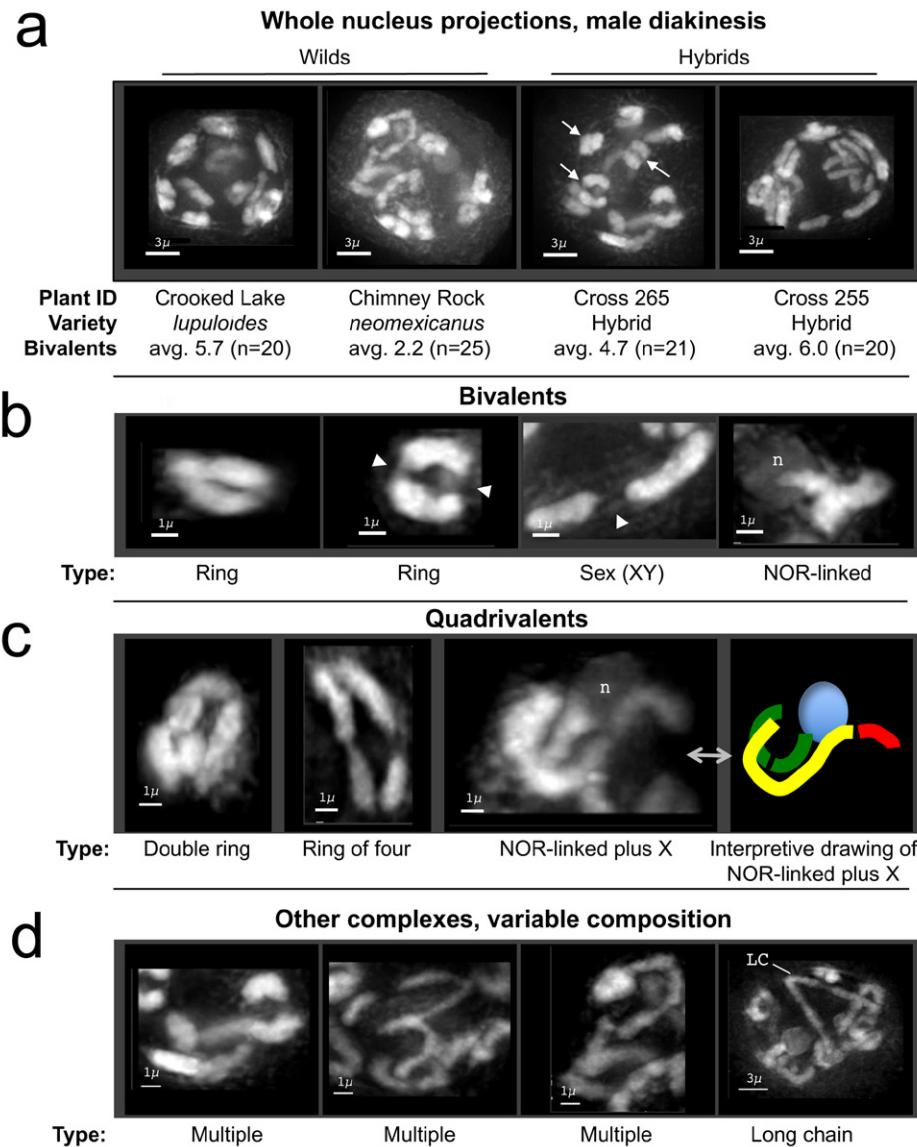
Fig. 2. Three-dimensional (3D) cytology of hop chromosomes from pollen mother cells at diakinesis. For cytogenetic analysis of hop meiotic chromosomes, male panicles were fixed in Carnoy's solution then formaldehyde. Meiocytes were extruded from anthers, placed on glass slides, stained with DAPI, and imaged by 3D deconvolution microscopy. Through-focus maximum intensity projections are shown for whole nuclei in (a) or 3D-cropped chromosomes in (b–d). (a) Representative diakinesis nuclei are shown for two wild and two hybrid plants. Plant IDs and bivalent frequencies per nucleus are shown under each panel along with the total number of full 3D nuclei analyzed. Examples of ring bivalents are shown (arrows) and scale bars are indicated in microns. (b) Bivalent examples are shown and classified into types (Ring, Sex (XY), or NOR-linked) listed under each panel and the nucleolus (n) is indicated in the NOR-linked example. Examples of presumed chiasmata (crossovers) are indicated (arrowheads) and they show the typical appearance as small gaps or spaces. (c) Quadrivalent examples are shown and classified into types (double ring, ring of four, or NOR-linked plus X) listed under each panel. An interpretive tracing of the NOR-linked plus X quadrivalent shows the nucleolus (blue), NOR-linked bivalent (green), the sex chromosome X (yellow), and the sex chromosome Y (red). (d) Examples of other complexes involving multiple chromosomes of unknown composition are shown along with general descriptions (multiple or long chain) under each panel. The first three images show 3D-cropped regions that capture entire complexes. The last panel shows an entire nucleus with a long chain (LC) configuration of complex that winds around in space along with two separate nucleoli.

observations are consistent with two resulting hypotheses: (i) that large-scale, genome-wide, and atypical meiotic chromosomal interactions occur in the progenitors of the three populations and (ii) that patterns of linkage can differ across the three populations.

Analyses of Pt data from families 144 and 247 show multiple super LGs in terms of their size and intermarker correlation (Fig. 4a; Supplemental Fig. S3a). In family 265, LGs tend to have equal size (Supplemental Fig. S3b) but exhibit relatively high correlation to one another. Alignments across the three sets of maternal linkage maps, before phasing coupling groups (Fig. 4b,c), show that most of the common or anchor markers were distinctly clustered. The clustering patterns of markers
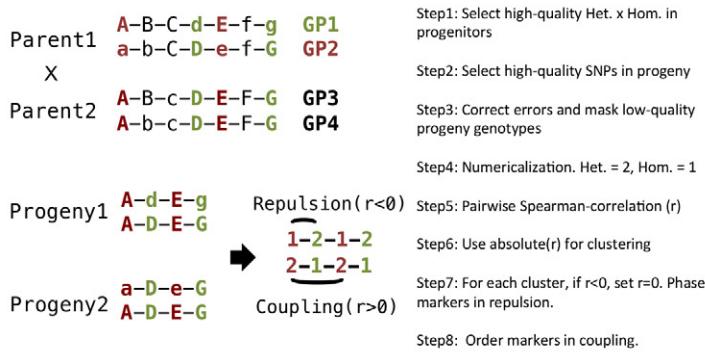
(a) Flow-chart of Pseudo-testcross core steps
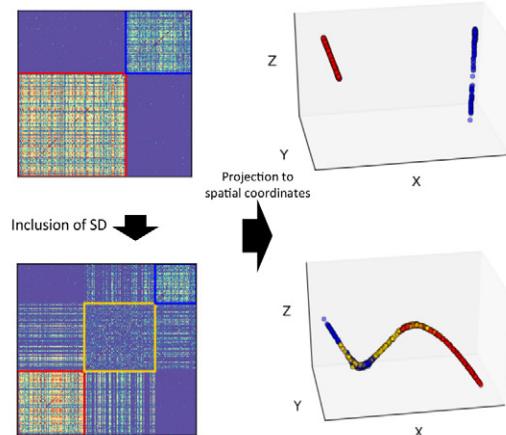
(b) Clustering without and with inclusion of SD markers

Fig. 3. Pseudo-testcross (Pt) schema. (a) Single-nucleotide polymorphism sites used in the testcross are color-coded. Minor alleles are segregated either from grandparent1 (GP1) (green), or from GP2 (red). In other words, linkage groups of grandparents are joined with their phases in repulsion. Two phases are indicated by colors of green and red individually. Markers in coupling and repulsion are distinguished by positive and negative correlation individually. (b) Correlation coefficient-based clustering and spatial coordinates of Pt markers. We used two methods, Louvain modularity and locally linear embedding, to cross-check the clustering patterns of markers without and with inclusion of segregation distortion (SD). Mendelian segregation markers are enclosed by blue and red frames, and SD markers are enclosed in a yellow frame. See Methods section for more details.
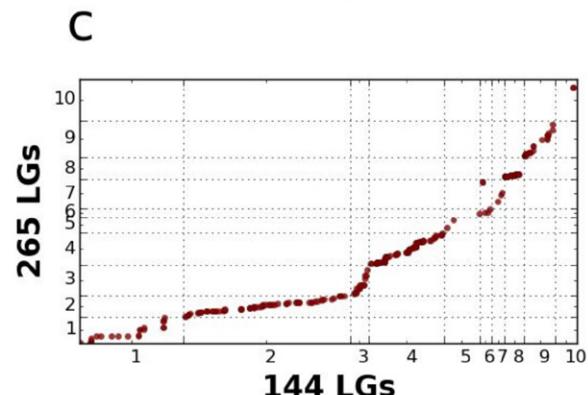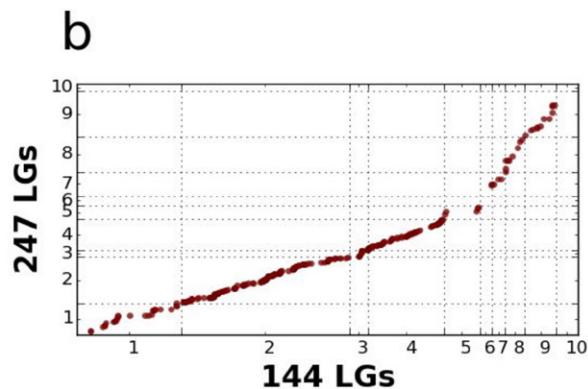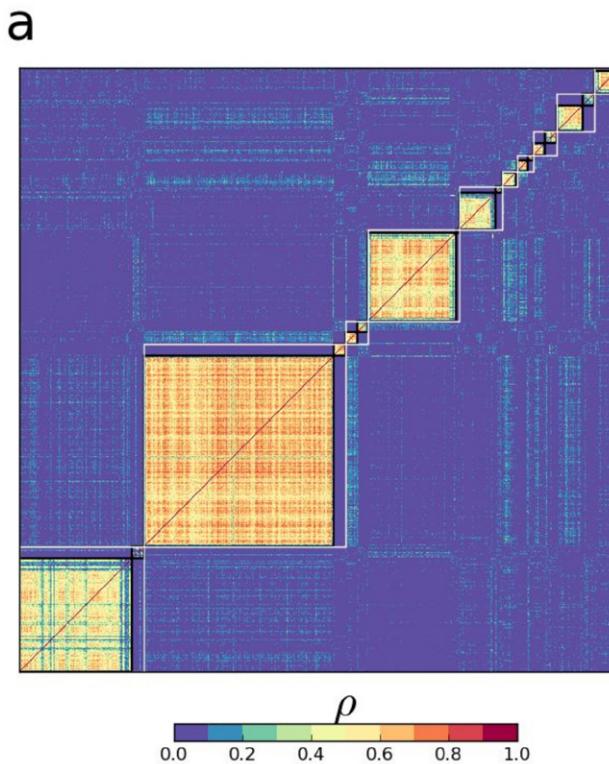

Fig. 4. Linkage groups for the maternal line of family 144 and correspondence across three genetic map sets. The degrees of Spearman's correlation (rho) are color-coded. (a) Unphased and phased (linkage for grandparents) groups are bounded by white and black frames individually. Alignment of unphased groups (b) between 144 and 247 and (c) between 144 and 265. The markers in alignments are indexed by pseudo-chromosomal positions. The alignments demonstrate the consistency of clustering patterns of the common markers across 144, 247, and 265.
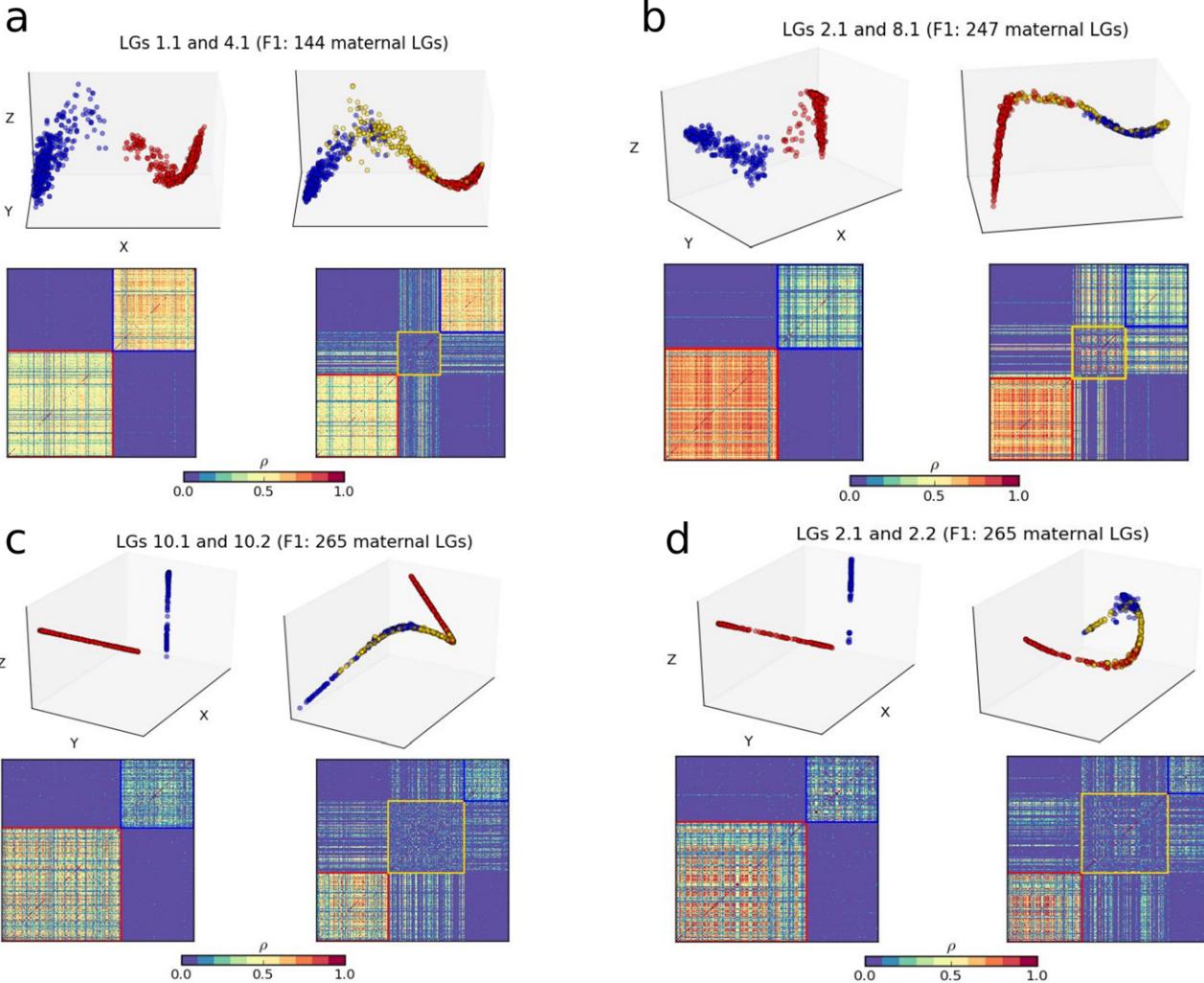
Fig. 5. Linkage of Mendelian (15% ≤ MAF ≤ 30%) and non-Mendelian pseudo-testcross (Pt) markers (5% ≤ MAF < 15%) based on Spearman's correlation (rho). In each subfigure, clustering patterns without (left) and with (right) inclusion of segregation distortion are presented by locally linear embedding method (LLE) (top) and the Louvain Modularity (bottom). Mendelian markers in two linkage groups (LGs) are indicated by blue and red colors individually. Segregation distortion (SD) markers are indicated by yellow color. Correlation map (a) of LG1.1 and LG4.1 in maternal linkage of cross 144, (b) of LG2.1 and LG8.1 in maternal linkage of cross 247, (c) of LG10.1 and LG10.2 in maternal linkage of cross 265, and (d) of LG2.1 and LG2.2 in maternal linkage of cross 265.

(exemplified in Fig. 5) are cross-checked using the Louvain method (Blondel et al., 2008) and the LLE method (Roweis et al., 2000) (see details in Methods section).

Translocation heterozygosity can extend linkage beyond the limits of a single chromosome, resulting in segregation ratios distorted from Mendelian expectations. Severe SD is known to result from altered recombination and linkage that occurs near breakpoints, creating pseudolinkage, or suppressing crossovers, and complicating marker ordering efforts in these regions (Taylor and Ingvarsson, 2003; Rauwolf et al., 2008; Farré et al., 2011). We used spatial coordinates calculated from LLE of Pt markers, in agreement with correlation heatmaps, to visualize genetic linkage patterns that emerge with and without inclusion of SD markers, as shown in Fig. 5. The markers showing segregation distortion (yellow dots in Fig. 5) appear to bridge the otherwise distinct LGs (red or blue dots, Fig. 5). These intriguing marker behavior

patterns could be related to the chromosome interactions observed at late prophase by 3D cytology (Fig. 2). Together, these observations suggest that chromosome structural variation impacts hop transmission genetics.

The largest LG is from family 265, as shown in Fig. 6. It appears as a major linkage complex that is derived from five interacting groups of well-linked markers with 15% ≤ MAF ≤ 35% (Fig. 6a). By plotting the normal and distorted markers in separate colors (Fig. 6b, gray vs. cyan, respectively), a clear pattern emerges in which the SD markers predominate in the space bridging the nondistorted markers. Chromosome markers appear to change in their degree of distortion as they approach and enter the area of convergence. This may reflect a multitude of chromosomal phenomena superimposed over multiple individuals. Indeed, our cytogenetic analysis shows variable chromosomal interaction patterns for multiple nuclei from individual plants.
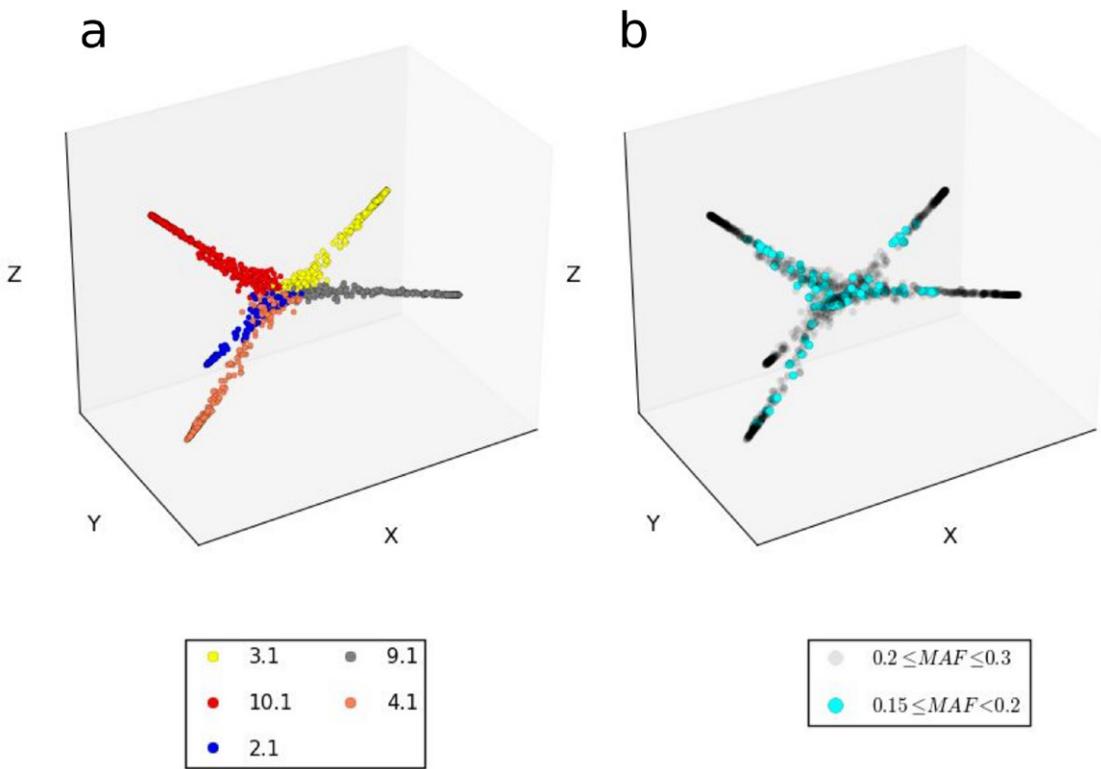
Fig. 6. Linkage patterns of the five largest linkage groups in family 265, based on spatial coordinates defined by LLE. (a) Linkage groups are color-coded. (b) Markers with non-Mendelian frequencies (cyan, for $0.15 \leq MAF < 0.2$) versus Mendelian frequencies (gray, for $0.2 \leq MAF \leq 0.3$) are coplotted.

One LG in one family corresponding to multiple groups in the other family suggests loci in common involved in recombination suppression and linkage disequilibrium, which is most likely influenced by the presence of chromosome rearrangements in the progenitor of the former family. One striking case (Fig. 7; Supplemental Fig. S5) in LG2.1 of family 144 corresponding to two coupling LGs (2.1 and 2.2) in 265. Two additional correspondences (LG1.1–LG1.2 and LG3.1–LG3.1) were used as positive control of the clustering approaches. However, such one-to-multiple correspondence was seldom observed across the three families. This may reflect the conservation of normally segregating chromosomal parts positioning in the heterozygotes complex and invariable occurrence of the translocation heterozygotes in the progenitors of the three families.

Genome-Wide Association Studies for Sex Determination

Despite the prevalence of segregation distortion, the GBS linkage data should still be amenable to genetic analysis linking genotype to phenotype. To test this idea, we examined markers for sex determination in hop, a dioecious species with a chromosomal sex determination system (Shephard and Parker, 2000; Ming et al., 2011). We used a MLM to assess evidence of phenotype–genotype association as shown in Fig. 8. In families 247 ($N = 364$, $N_{male} = 30$) and 265 ($N = 95$, $N_{male} = 13$), LG4 consistently shows the most striking association with sex (Fig.

8a; Supplemental Fig. S6), even though 265 has a small effective population size. This signal was additionally supported by F_{st} mapping in 247 (Fig. 8b), but Pt only accounts for part of association signals. To extend the analysis genome wide, we assessed association between 356,527 markers and 850 individuals ($N_{male} = 129$, $N_{female} = 721$). A total of 588 SNPs with $P \leq 10^{-7}$ were identified (Fig. 8c; Supplemental Table S4), with LG4 and other LGs accounting for 38.6 and 0.0% of the association markers, respectively. The 588 SNPs were highly correlated (Fig. 8d), as would be expected if the association markers derive primarily from one linkage disequilibrium block. Adding up scaffolds showing association approximates ~9.75 Mb of the mapping resolution accounting for ~0.38% of the hop genome. These results confirm the importance of our LG4 in sex determination in hop, suggesting that LG4 may be a sex chromosome. These findings establish the utility of the GBS data for linkage mapping and provide clues about specific genes and families involved in sex determination system in hop.

Genetic Differences and Phenotypic Variation Across Populations

To assess genetic contributions to between-population phenotypic differences, we used F_{st} analysis (Supplemental Table S5), plotted as LG-based pairwise F_{st} heatmaps, for the population differentiation across var. *neomexicanus*, var. *lupuloides*, and CV (Supplemental Fig. S7). The F_{st} values are a measure of allele frequency
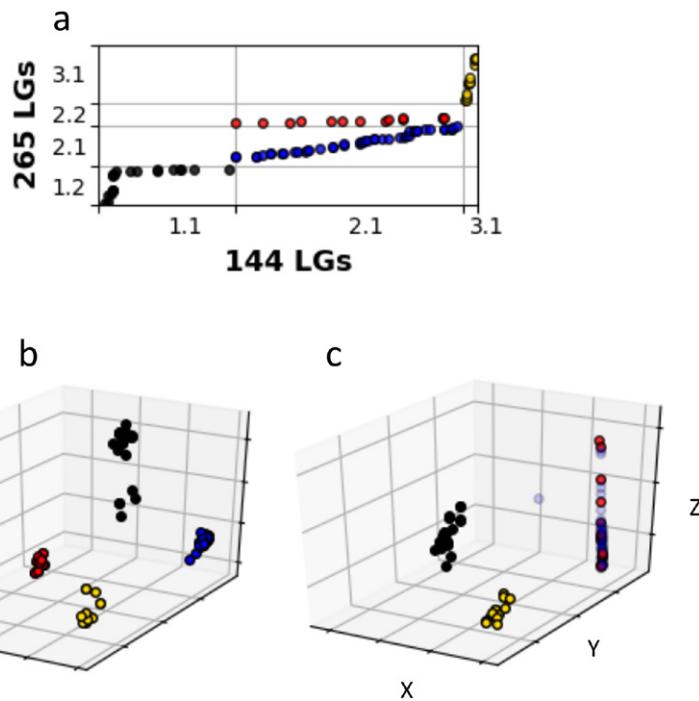
Fig. 7. One-to-two genetic correspondence between families 144 and 265. (a) LG2.1 in 144 corresponds to LG2.1 and LG2.2 in 265. Two instances of one-to-one correspondence (LG1.1-LG1.2 and LG3.1-LG3.1) are added for control. Spatial representations (XYZ coordinates) of linkage groups in (b) 265 and (c) 144 were derived from LLE.
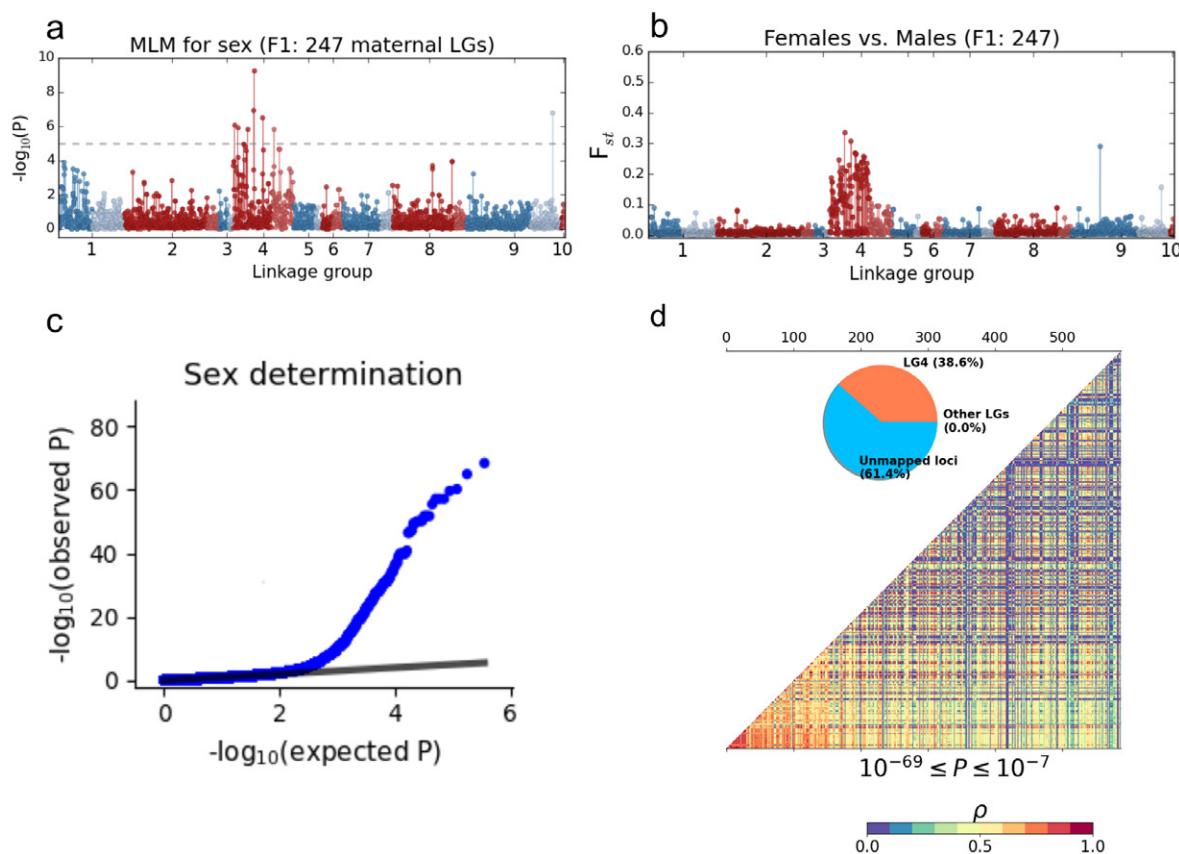

Fig. 8. Association studies and F_{st} mapping of sex determination in hop. (a) Linkage group-based Manhattan plot of mixed linear model (MLM) for sex determination in family 247 ($N = 364$, $N_{male} = 30$). Light and deep colors are used to distinguish two phases (linkage for grandparents) in coupling. (b) Manhattan plot of F_{st} in females versus males in 247. (c) Log quantile-quantile (QQ) plot of 356,526 association tests (SNPs) for sex determination in 850 individuals ($N_{male} = 129$, $N_{female} = 721$). (d) Correlation among 588 association ($P \leq 10^{-7}$) markers, the proportions of 588 markers in LG4, other LGs, and unmapped data set.

variance between populations, and they can be used to identify regions of domestication or targets for breeding. From this analysis, two notable patterns emerged. First, the degree of genetic variation, as expected, is much greater in CV versus either of the wilds, *neomexicanus* or *lupuloides,* than in the wilds, *neomexicanus* vs. *lupuloides*. Regions of high F_{st} in CV versus *neomexicanus* are also found to exhibit high F_{st} in CV versus *lupuloides*. Second, the five largest LGs account for a large proportion of genetic variation between populations. Taken together, these results confirmed our suspicion that domestication traits should result in unusual F_{st} values when comparing wilds to cultivars, but not between wilds, which have undergone different degrees of natural versus domestication-based selection for certain traits. The hotspots with unusually high F_{st} values can be prioritized to identify genetic loci affecting certain traits especially for chemical composition and drought tolerance.

DISCUSSION

Hop crop acreage and usage is rapidly expanding and diversifying because of a burgeoning craft brewing industry. Hop breeding programs have a long history of attempting to exploit genetic resources for bitter flavor, aroma, and disease resistance. However, a worsening drought and unseasonably hot weather pose major challenges to these efforts. For example, in Europe and the United States, most hop farms experienced severe water shortage in 2015. Like many other crops, exploitation of novel genetic variation in response to drought stress is of paramount importance for a sustainable hop production system.

Meiotic Chromosome Pairing Interactions in Wild and Hybrid Hop

Previous cytogenetic and genetic studies, together with the current genomic findings, prompted cytogenetic analysis for evidence of nondisomic meiotic chromosome configurations. Analysis of more than 100 diakinesis stage nuclei confirmed the presence of atypical meiotic chromosomal configurations in hop, revealing additional complexities (Fig. 2d). This study confirms the tendency for sex chromosomes to be involved in quadrivalent or multiple associations (Sinotô, 1929; Winge, 1929). In addition, these new findings clearly implicate autosomes and possible structural heterozygosity as prevalent in hop. This idea is consistent with early speculations from Winge regarding autosomes being involved in tetrapartite–quadrivalent associations (reviewed by Vyskot and Hobza, 2004). Here, only one set of heteromorphic sex chromosomes were observed in all plants, but this cannot be stated as certain without chromosome-specific fluorescence in situ hybridization (FISH) probes. However, sex-bivalent and NOR-bearing chromosomes are the only chromosomes that are morphologically distinct, and therefore, their interactions with each other, alone, or with other chromosomes were noted. Specifically, sex bivalents were observed to interact directly with the NOR-bearing chromosome in approximately two in 20

nuclei for each plant except Crooked Lake. In contrast to previous studies, the current study clearly documents autosome-only complexes in both wild and cultivated hybrid hop plants. For example, the ring of four (Fig. 2c), double ring (Fig. 2c), and the multiple complex (Fig. 2d, first image) provide examples of nonsex chromosome multiples.

In considering why such observations may not have been reported, we considered several possible reasons. First, previous studies (as reported and reviewed by Shephard and Parker, 2000) focused primarily on somatic karyotypic analyses rather than meiotic pairing configurations in late prophase. Second, the 3D cytological analyses reported here likely affords a greater opportunity to detect interactions, given the ability to visualize single nuclei and subnuclear regions from multiple perspectives after imaging. We note that the nature of the interactions observed are not defined at the molecular level but likely represent crossovers. This interpretation is consistent with classical and modern cytology of chiasmata in cell staining preparations and is supported by the GBS-based segregation data reported here.

Variable Segregation Patterns Revealed by Linkage Analysis of Genotyping-By-Sequencing Data

The lack of detailed cytological evidence hinders the correspondence of our LGs with the exact meiotic configuration. Moreover, we cannot rule out the possibility that rather than a single meiotic configuration, the clustering of markers may depict meiotic events occurring in many nuclei, which were captured by our GBS data. Nondistorted markers in one super LG may originate in a translocation complex derived from multiple chromosomes. Such complexes could lead directly to several segregation distortion patterns that involve SNP marker groups of variable sizes from small to large. For a multivalent with two terminal crossovers per chromosome, small LGs could reflect regions distal to subterminal crossovers. Medium LGs may reflect normal chromosomes or even partial cosegregation of groups of subterminal regions. Large LGs could reflect normal large chromosomes or even cosegregation of groups of chromosomes. For example, if chromosomes were arranged in a Renner complex, or something comparable, then translocation heterozyogous multivalents could assort by copolar cosegregation of every other centromere and the linked loci therein. And by extension, if multivalents are forming in different ways, as our cytogenetics show (Fig. 2), then the cosegregation signals would be weaker but detectable. One way that heterogeneity in complex formation could occur is that common chromosomal regions, such as the abundant subtelomere repeats HSR1, could mediate synapsis. If they did, and also could recombine, that could shuffle the distal segments with loci expected to display some degree of cosegregation instead of independent assortment.

We have observed diverse meiotic configurations in two F[1] progeny and two wild hop plants. This raises the possibility that a complex involves a large number of chromosomes and perhaps the whole genome. Indeed, a complex of at least four pairs of chromosomes has been observed in Chimney Rock hop. In addition, a large LG was simulated in F[1] family 265 by our clustering model. There is a need for additional cytogenetic studies to answer the intriguing question of the largest chromosomal complexes in hop.

Notable in these findings is the fact that we seldom observed one-to-multiple correspondence across the three families. That may reflect the conservation of normally segregating chromosomal parts positioning in the chromosomal complexes and invariable occurrence of the structural translocation heterozygosity in the progenitors of the three families.

Structural Polymorphism and Variable Meiotic Chromosome Interactions May Contribute to Segregation Distortion in Hop

At least 57 species of flowering plants are characterized by permanent translocation heterozygotes (Holsinger and Ellstrand, 1984). For instance, in *Clarkia* (2*n* = 18) chromosomal polymorphisms, such as structural heterozygosity, has been observed in nearly half of the 34 known species (Snow, 1960). Similarly, natural and distinct cytotypes or chromosome races have been well described in Asteraceae family (Carr and Carr, 1983). In that study, structurally heterozygous individuals were found to occur within natural populations with various cytotypes or chromosome races being characterized on the basis of meiotic pairing configurations (Carr, 1977). Given that segregation distortion is a ubiquitous phenomenon in hop [Seefelder et al. (2000), McAdam et al., (2013), and extended by our study], together with multiple examples of naturally occurring chromosomal polymorphism in other plant genera, we favor the idea that phenomena such as structural heterozygosity and segmental aneuploidy might play important roles in the population dynamics of hop.

Translocation heterozygosity may have an important connection to the significantly distorted sex ratio in favor of females in hop. Likewise, female-biased sex ratios have been found in mistletoe (*Viscum album* L.), another notable dioecious case of translocation heterozygosity. To maintain heterozygosity, *Oenothera*, a notable case of translocation heterozygosity, uses a system of balanced lethal to purge the lethal homozygotes (Steiner, 1956; Harte, 1994), which is referred to as recessive lethals. In the context of XY system, heteromorphism of sex chromosomes dictates that males are more severely affected than females by X-linked recessive lethals, because males only have one copy of the X chromosome. Hence, hop may use a system of balanced lethals at the expense of male offspring to preserve genetic heterozygosity.

Our results are compelling for translocation heterozygosity studies in light of high-density molecular markers in many other biota. For example, such large-scale recombination suppression is also presented in at least 10 species of termite (Isoptera), some types of centipede (Chilopoda), and perhaps all of the monotremes (Holsinger and Ellstrand, 1984; Rowell, 1987; Rens et al., 2004). Beyond homologous crossover, translocation heterozygosity has shown considerable evolutionary interest and selective advantage in its own right.

In future studies, it will be important to further characterize these genomes for evidence of structural polymorphisms and to explore the mechanistic underpinnings and biological consequences of these phenomena in hop. Investigations should include FISH to track specific loci through meiosis and into both postmeiotic daughter cells and the next generation; pollen-based assays expected to reveal aspects of grain viability and fertility; and continued structural and comparative genomics to directly resolve presumed points of chromosomal breakage, which could uncover specific deletions, duplications, inversions, or translocations. Given the potential genetic and genomic complexities within and between hop species, future progress and investigation of questions from this and prior studies is a significant challenge requiring the integration of multiple disciplines and lines of evidence from a variety of different experiments in domestic and wild hops.

Perspectives of Breeding Strategies in Hop

Understanding genetic recombination is essential for speed and accuracy of plant breeding. Indeed, it is generally difficult to breed new commercial hop varieties through mass selection and crossing. Our findings show that a large-scale, perhaps genome-wide, atypical meiotic chromosome behavior may be common in hop. Translocation heterozygosity can extend linkage to nonhomologous chromosomes and favor severe segregation distortion accumulated near the translocation breakpoints (Taylor and Ingvarsson, 2003; Farré et al., 2011). Such a high degree of recombination suppression may hinder effective selection of desired allele combinations that make use of marker-assisted selection based on Mendelian segregation patterns.

Hence, targeted resequencing and mapping the consensus genomic regions that segregate appropriately may deserve emphasis in hop. A "normal" reference genome may be essential to elucidate structural differences arising from rearrangement events. In silico screening of primers and enzymes to avoid the regions with the tendency of segregation distortion may fulfill the purpose of cost-effective genotyping platforms in hop breeding programs.

Supplemental Information Available

Supplemental Figures. The file contains Supplemental Fig. S1–S7.

Supplemental Table S1: Pedigrees of genotyped F[1] populations.

Supplemental Table S2: Cultivar and landrace accessions.

Supplemental Table S3: Wild exotic accessions.

Supplemental Table S4: 588 sex association ($P \leq 10^{-10}$) SNPs. Scaffold, position, P-value and MAF are indicated.

Supplemental Table S5: SNPs with $F_{st} \geq 0.5$ in pairwise comparisons of var. *neomexicanus*, var. *lupuloides*, and CV.

HapMap SNPs can be accessed at https://hopsteiner. app.box.com/s/r0tzqpdzcagvmxtxducy21lrdykuhbdl

Conflict of Interest Disclosure

The authors declare that there is no conflict of interest.

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