Rapid neo-sex chromosome evolution and incipient speciation in a major forest pest

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Genome evolution is predicted to be rapid following the establishment of new (neo) sex chromosomes, but it is not known if neo-sex chromosome evolution plays an important role in speciation. Here we combine extensive crossing experiments with population and functional genomic data to examine neo-XY chromosome evolution and incipient speciation in the mountain pine beetle. We find a broad continuum of intrinsic incompatibilities in hybrid males that increase in strength with geographic distance between reproductively isolated populations. This striking progression of reproductive isolation is coupled with extensive gene specialization, natural selection, and elevated genetic differentiation on both sex chromosomes. Closely related populations isolated by hybrid male sterility also show fixation of alternative neo-Y haplotypes that differ in structure and male-specific gene content. Our results suggest that neo-sex chromosome evolution can drive rapid functional divergence between closely related populations irrespective of ecological drivers of divergence.
Understanding how reproductive isolation evolves between populations is essential to understanding the origin of biodiversity. Nearly a century of research has established that hybrid incompatibilities usually manifest in the heterogametic (male) sex first (i.e., Haldane’s Rule) and that the early stages of hybrid male sterility (HMS) and inviability (HMI) are almost always asymmetric in reciprocal genetic crosses. These patterns reflect the fundamental role that heteromorphic sex chromosomes play in the evolution of deleterious epistatic interactions underlying intrinsic reproductive isolation, but the importance of these processes to the early stages of speciation has been debated. For example, while sex-linked hybrid sterility generally evolves much more rapidly than inviability, both are thought to accumulate more slowly than other forms of ecological or behavioural reproductive isolation. However, most speciation research has focused on animal systems with well-established and highly heteromorphic sex chromosomes, resulting in a limited view of the contribution of sex chromosome evolution to the origin of species.

It is now apparent that transitions to new (neo) sex chromosomes are common over evolutionary timescales, with some groups showing high levels of turnover in chromosomal systems of sex determination. Neo-sex chromosomes can originate through diverse mechanisms, such as the de novo evolution of a sex determining factor on otherwise homomorphic autosomal chromosomes (e.g., wild strawberry) or through the fusion of an ancestral sex chromosome with an autosome (e.g., Drosophila miranda). Given the surprisingly fluid nature of sex chromosome systems in some groups, there are at least three general stages of sex chromosome evolution—each with distinct evolutionary dynamics—that might contribute to the process of speciation (Fig. 1). First, the establishment of a new sex chromosome system within a population could lead to reproductive isolation between species with different sex chromosome systems. Second, once established, the functional conversion of autosomes to sex chromosomes results in dynamic changes in chromosome structure, gene content, and expression underlying the evolution of sex-specific functions. The extensive genic specialization and structural degeneration accompanying such chromosomal transformations provide some of the most extreme examples of long-term genome evolution. In XY systems, the rate of sex chromosome differentiation and neo-Y degeneration is dependent on the suppression of recombination between the neo-XY pair. In principle, sex chromosome transitions could also result in rapid functional divergence and the accumulation of genetic incompatibilities between populations, but the tempo of these genomic changes and their contribution to broader patterns of species diversification remains largely unknown. For example, genes involved in reproductive isolation have been linked to recently established neo-sex chromosomes in threespine sticklebacks and butterflies, but it is unclear if or how these hybrid incompatibilities relate to neo-sex chromosome evolutionary dynamics per se. Finally, it is well established that older, highly heteromorphic sex chromosomes often play a disproportionately large role in speciation through the exposure of recessive genetic incompatibilities in hybrid males (i.e., dominance theory) and various evolutionary dynamics (e.g., faster-X, meiotic drive) that can drive rapid sex-linked divergence.

The mountain pine beetle (Dendroctonus ponderosae) is the most important forest pest in western North America, and is the primary contributor to recent tree mortality on 6.6 million hectares in the western US, exceeding tree mortality caused by wildfire. Previous studies have found that populations at the southern reaches of the beetle’s range (southern California and Arizona) are the most genetically divergent and genetic variation follows an isolation-by-distance pattern around the Great Basin.}

![Fig. 1 Neo-sex chromosome evolution. The predicted stages of heteromorphic differentiation are shown left to right for a neo-XY pair formed from a fusion between an X chromosome and an autosome. The relative timing and intensity of sex-specific gene specialization and structural evolution are depicted for the neo-X and neo-Y separately (shaded boxes, bottom). Following establishment, the neo-sex chromosomes are expected to undergo sex-biased gene specialization and at least some structural evolution dependent on the cessation of recombination. Both processes should occur more rapidly and be more extensive on the male-specific neo-Y chromosome. As seen in highly heteromorphic sex chromosomes, the evolution of heteromorphic neo-sex chromosomes is also predicted to further contribute to reproductive isolation through the exposure of recessive genetic incompatibilities and more rapid sex-linked divergence in the heterogametic sex. The fate of the highly degenerate ancestral Y chromosome is not shown and is often unclear in X-autosome fusions. Figure adapted from Bachtrog.](image-url)
Desert 22 (although small isolated populations do occur across the Great Basin 23). Despite very low levels of genetic differentiation between populations 22, hybrid male sterility (HMS) has been detected between some mountain pine beetle populations consistent with cryptic speciation 24. In addition, the mountain pine beetle shares a neo-XY chromosome system with its closest relative, the Jeffrey pine beetle (Dendroctonus jeffreyi), descending from a fusion between the ancestral X chromosome (anc-X) and the largest autosome in the common ancestor of these species 25. There is no known karyotypic variation within either species 25 and the degree of genetic, structural, or functional divergence between these nascent sex chromosomes has not been evaluated. However, several Dendroctonus species and many other beetles have distance-pairing heteromorphic sex chromosomes that do not synapse and are fully non-recombinant 12. In these systems, the Y chromosome appears highly degenerate and is a small fraction of the size of the X. If distance-pairing and the associated loss of recombination carries over following an autosomal X

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Fig. 2 Reproductive isolation and genomic differentiation. 

a The mountain pine beetle occurs in close association with host trees in western North America (dark grey) and gene flow is thought to be restricted across the Great Basin Desert. We combined extensive genetic crossing data with beetle genomes sequenced from ten localities (circles and squares; all relevant crossing shown in Supplementary Fig. 1). Reproductive isolation was observed between localities connected with lines, increasing in severity (top left inset) with distance from a central boundary (dashed line; See also Supplementary Fig. 2 and Supplementary Table 1). Sex symbols denote the direction of the cross when any asymmetric isolation (HMS or HMI) was observed (top left inset). Sterile males show reduced quantities of sperm in the seminal vesicle (bottom left inset; see also Supplementary Fig. 3).

b Model of neo-sex chromosome evolution in the mountain pine beetle (and D. jeffreyi) from an ancestral X-autosome fusion. The ancestral X_Y notation describes systems where the Y chromosome is small and highly degenerate and the X forms a 'parachute' like structure while pairing at a distance from the Y during meiosis 12.

c Results from principal component (left) and admixture analyses (f3 statistics, right) for autosomal SNPs across all population comparisons. Significantly admixed populations are positioned by geography and symbol size scaled relative to the number of significant three-population tests (Supplementary Table 3) supporting admixture of the focal population (z-score < -10).

d SplitsTree networks for mtDNA and four nuclear genomic partitions (autosomes = 60 K SNPs, anc-X = 87 K, neo-X = 75 K, neo-Y = 544). For additional analyses of population structure see Supplementary Fig. 7 and Supplementary Table 2.
chromosome fusion event, then neo-sex chromosomes may be immediately predisposed to the rapid evolution of heteromorphy in this system.

Here we use extensive genetic crossing experiments coupled with population and functional genomics to examine neo-sex chromosome evolution and incipient speciation in the mountain pine beetle system. We detected extraordinary variation in the strength and pattern of reproductive isolation between very closely related populations. The distinct genetic architecture of F1 reproductive isolation in these crosses combined with genomic patterns of population differentiation and admixture on and off the sex chromosomes supports a central role for sex-linked reproductive isolation in mountain pine beetle populations. In parallel, we also document extensive variation in neo-Y structure and male-specific gene content between populations isolated by hybrid male sterility. Overall, our results reveal that extensive genomic specialization and structural degeneration on the neo-sex chromosomes can be sufficiently rapid to drive intrinsic functional differentiation between closely related populations, which may in turn play an important role in incipient speciation.

**Results**

A geographic progression of intrinsic reproductive isolation. We have completed an extensive genetic crossing experiment to characterize patterns of reproductive isolation between populations sampled from across the US range of the mountain pine beetle (Fig. 2a). Combining extensive new (Supplementary Table 1) and published data24 (9 populations, 18 cross-types, 1109 crosses, Supplementary Fig. 1), we uncovered a geographic continuum of intrinsic hybrid incompatibilities. Crosses between some populations in the central part of the range resulted in HMS (Supplementary Fig. 2) due to disrupted spermatogenesis (Supplementary Fig. 3). Although we have not described hybrid male fertility in all possible population pairs, the severity and F1 architecture of HMS consistently changed with geographic distance of hybridizing population pairs found on either side of a central East-West boundary of reproductive isolation. Crosses between proximate populations on either side of this boundary resulted in hybrid males with reduced fertility (Supplementary Fig. 2) in one direction of the cross (i.e., weak asymmetric HMS; female ID × male OR), while reciprocal crosses between more distant localities resulted in severe or complete HMS (i.e., strong symmetric HMS; Fig. 2a).

We also detected asymmetric delayed hybrid male development in crosses between beetles from Arizona (AZ) and Southern California (female AZ × male sCA; Supplementary Fig. 4), which is the most geographically distant cross type based on the distribution of host trees (Fig. 2a). This transgressive hybrid phenotype likely reflects intrinsic developmental incompatibilities (i.e., HMI), but the fitness consequences depend on population ecology (i.e., extrinsic). Mountain pine beetles are univoltine (one generation per year) and the timing of their development is likely under strong selection in nature26. Following emergence, beetles colonize heavily defended host pine trees by staging highly coordinated attacks and development time and emergence synchrony are crucial for beetle success. Thus, delayed development time would be highly maladaptive, thereby providing an ecologically relevant measure of hybrid viability. Information on hybrid development time was not available for all crosses, but available data (Supplementary Fig. 1) suggest that abnormal hybrid male development may be restricted to crosses between the most geographically distant and genetically divergent populations. These populations (AZ and sCA) do not come into contact, thus delayed development may not currently contribute to reproductive isolation in nature but it does reveal another important component of evolutionary divergence leading to intrinsic incompatibilities between beetle populations.

These cryptic patterns of reproductive isolation (Fig. 2a) did not coincide with described phenotypic differences between populations27 or follow any obvious ecological gradients (e.g., host use), as might be expected if hybrid incompatibilities are an indirect consequence of ecological divergence. For example, beetles from populations bordering the HMS boundary (OR and ID) were collected from the same host tree species (Pinus contorta), were morphologically indistinguishable, and do not differ in body size or development time26. Phenotype profiles were also found to be very similar between reproductively isolated OR and ID populations (Supplementary Fig. 5). Although we did observe some slight clustering of female pheromone blends by population (Supplementary Fig. 5), the partial separation that we observed was due to two compounds (acetophenone and 1-phenylethanol) that do not attract either sex to traps or alter responses to an attractive pheromone trap lure28. Cumulatively, there is no evidence of ecological divergence between populations near the HMS boundary and the minor phenomere differences that we detected are unlikely to have a meaningful effect on behaviour or mate recognition. Thus, hybrid male sterility and delayed development are the only known phenotypes isolating populations of mountain pine beetles, and the geographic pattern of these intrinsic incompatibilities suggests a fairly complex and polymorphic genetic basis9.

**Reproductive isolation and population differentiation.** Our extensive crossing experiments revealed a broad range of intrinsic F1 incompatibilities, establishing that reproductive isolation unequivocally proceeds through asymmetric postzygotic isolation (Fig. 2a). Furthermore, there is no evidence for variable endosymbiont infections (e.g., Wolbachia) that might contribute to asymmetric HMS in this system based on standard genetic and molecular diagnostics24. Thus, this distinct genetic architecture requires epistatic interactions involving genetic factors with uniparental inheritance29. In mountain pine beetles this equates to nuclear-cytoplasmic interactions involving mitochondrial DNA (mtDNA) and/or sex chromosome-linked interactions. To test this hypothesis and to evaluate the overall genomic context of these hybrid incompatibilities we first generated a range-wide population genomic data set by combining two published genomes29 (CAN) with 27 additional whole genomes from nine populations (two males/one female sequenced per population, 18x average coverage per genome; Fig. 2a), and two Jeffrey pine beetle genomes (16x average coverage per genome). The published male and female reference genomes identified part of the ancestral X (anc-X) chromosome based on synteny with flour beetles (Tribolium), but did not differentiate the autosomes from the neo-sex chromosomes29 which are thought to descend from an ancestral X-autosome fusion25 (Fig. 2b). To overcome these limitations, we assembled individual mtDNA genomes and used sex-specific patterns of whole genome sequencing (CAN) to identify autosomal (70.5% of the male genome build), X-linked (16% anc + neo-X; 664 scaffolds), and neo-Y linked (6.6%, 2272 scaffolds) regions of the genome (Supplementary Fig. 6).

These analyses revealed three important attributes of neo-sex chromosome evolution in this system. First, the sex chromosomes comprise ~22% of the assembled genome. This estimate is broadly consistent with karyotypic data25 and excludes any residual pseudoautosomal regions, which would be indistinguishable from autosomes with respect to sex-specific coverage. Second, the neo-Y appears smaller, contains fewer genes, and is more fragmented when compared to the neo-X chromosome (Table 1, Supplementary Fig. 6). Third, retained homologous neo-
Table 1 Genome assembly statistics by genomic partition

| Partition | N50 (kb) | Largest scaffold (kb) | Number of scaffolds | Mean phred-scaled quality score (male) | Number of predicted genes |
|-----------|----------|-----------------------|---------------------|----------------------------------------|---------------------------|
| neo-Y     | 11       | 67                    | 2272                | 35.0                                   | 781                        |
| neo-X     | 164      | 688                   | 658                 | 45.4                                   | 1227                       |
| anc-X     | 3493     | 3885                  | 6                   | 55.3                                   | 666                        |
| autosomes | 1024     | 4163                  | 1178                | 54.5                                   | 9308                       |

*a*Mean mapping qualities of CAN male reads mapped to male reference genome

*b*On the basis of the predicted annotations from the published assembly

X and neo-Y genic regions show moderate levels of sequence divergence (119 pairwise X–Y gametologs; mean $K_s = 5.7\%$, median $K_s = 2.2\%$). These estimates of synonymous gene divergence are about two-fold higher than those found in $D. miranda$, an early neo-XY system ($K_s = 1.5\%$; ~1 million years old, ~10 generations per year) where signatures of specialization and degeneration are already apparent. The age of the $Dendroctonus$ neo-sex chromosomes are not clear, but the Jeffrey pine beetle and mountain pine beetle share the same neo-XY configuration. Assuming a simple mtDNA molecular clock, the neo-sex chromosomes in the mountain pine beetle are at least 2–3 million years old (1 generation per year).

We found very low levels of sequence divergence across the genome punctuated by strikingly different patterns of genetic variation within and between populations across different nuclear partitions (autosomes, anc-X, neo-X, and neo-Y) and individual mtDNA genomes (Fig. 2c, d, Supplementary Fig. 7). On the basis of 5.3 million autosomal single nucleotide polymorphisms (SNPs), we found no evidence for a genetic split coincident with the HMS boundary (OR vs. West $F_{st} = 0.01$). Instead, genetic variation largely grouped populations based on geographic proximity (Fig. 2c) with low overall genetic divergence ($D_{xy}$ average = 0.72%, min = 0.62%, max = 0.83%) and low between-population genetic differentiation ($F_{st}$ average = 0.17; min = 0.00, max = 0.45; Supplementary Table 2). Autosomal phylogenetic networks showed extensive reticulation (Fig. 2d) and estimates of population structure indicate a broad transition in population assignment across the HMS boundary (Supplementary Fig. 7). The mtDNA genomes also showed low sequence divergence between populations with no phylogeographic structure associated with populations isolated by HMS (Fig. 2d). These autosomal and mtDNA results are consistent with previous studies in the mountain pine beetle which found no evidence for a genetic split near the HMS boundary and described isolation-by-distance around the Great Basin Desert with populations at the southern extreme of the distribution being the most genetically divergent.

In contrast, we detected markedly increased genetic differentiation on the anc-X and neo-X and extreme differentiation of the neo-Y between reproductively isolated populations (Fig. 2d, Supplementary Fig. 7). On the neo-Y, we found exceptionally low population-level variation in 'East' (CO, UT, ID, and MT), 'West' (sCA, mCA, nCA, and OR) and 'Southeast' (AZ) groups on the neo-Y such that the vast majority of SNPs corresponded to fixed differences between these three neo-Y haplotypes. While comparisons between partitions are complicated by inherent differences in sequence complexities and assembly qualities, general population genomic patterns presented here and below were consistent across a range of SNP filtering regimes (Methods section).

Combining these broad population genomic patterns with our extensive crossing data provides two key insights into speciation. First, hybrid incompatibilities are accumulating extremely rapidly in this system. Intrinsic reproductive isolation tends to follow predictable patterns that are recapitulated in our crosses: incompatibilities manifest in the heterogametic sex first, sterility evolves more rapidly than inviability, and asymmetry precedes reciprocal isolation. These evolutionary transitions have mostly been established through comparative analyses of crosses between different species pairs spanning a broad range of genetic divergences. The existence of population-level variation in hybrid incompatibilities is now well established, however, the striking progression in the strength and pattern of intrinsic reproductive isolation occurring between populations separated by such low levels of genomic divergence is highly unusual. Second, sex-linked incompatibilities are playing a central role in the evolution of reproductive isolation. Based on overall patterns of population genetic differentiation and HMS in reciprocal crosses, involvement of mtDNA can be unambiguously dismissed while the X and neo-Y chromosomes show moderate to complete differentiation between populations that are also isolated by hybrid male sterility (Fig. 2a). In particular, we have detected some degree of hybrid male sterility in all crosses between populations with highly divergent West vs. East or Southeast neo-Y haplotypes, and no reproductive isolation between populations bearing the same neo-Y haplotypes (Fig. 2d, Supplementary Fig. 1). Male fertility has not been thoroughly characterized between East and Southeast neo-Y beetles (see below). Given this limitation, we focus subsequent results and discussion on the East vs. West reproductive isolation boundary.

Population history and natural selection. The progression of reproductive isolation coupled with low autosomal differentiation around the Great Basin Desert could be produced by two alternative population histories. First, genetic divergence may have accumulated along a series of interbreeding populations in the absence of reduced gene flow (i.e., primary divergence-with-gene-flow). Alternatively, reproductive isolation may have evolved during an initial period of geographic isolation or allopatry followed by secondary contact. The low population differentiation and extensive network reticulation observed at autosomal loci (Fig. 2c, d, Supplementary Fig. 7) could reflect the persistence of shared variation between recently diverged populations (i.e., incomplete lineage sorting independent of secondary gene flow) or secondary contact and introgressive hybridization. Distinguishing between these alternatives is crucial to understanding the evolutionary history and genetic architecture of reproductive isolation in this system.

Interbreeding between two previously isolated populations results in discernible patterns of admixed genetic ancestry, allowing us to differentiate these primary vs. secondary models of divergence-with-gene-flow. We first calculated the $f_2$ statistic for all possible three-way population comparisons using 1,269,066 high quality autosomal SNPs. This approach uses allele frequency correlations between populations to provide a formal test of admixture that is robust across a broad range of population histories. We detected strongly negative skews in the $f_2$
statistic in the centre of the range, indicative of secondary contact followed by extensive autosomal admixture between populations adjacent to the HMS boundary with signatures of admixture tailing off with geographic distance from the boundary (Fig. 2c, Supplementary Table 3). These strong signatures of autosomal admixture establish a history of secondary contact near the HMS boundary with subsequent gene flow.

The population-frequency based $f_2$ statistics are not appropriate for sex-linked SNPs given our sampling, so we next tested for introgression at both autosomal and X-linked SNPs using the ABBA-BABA framework. Consistent with the $f_2$ results, we again found evidence of autosomal gene flow near the HMS contact zone (Supplementary Table 4). We detected some gene flow occurring on both the neo-X and anc-X (Supplementary Table 4). These results are in stark contrast to what we observed on the neo-Y chromosome where alternative neo-Y haplotypes appear fixed across the HMS contact zone (i.e., $FST \sim 1$ and 97% of sites are BBAA).

The persistence of genomic divergence in the face of gene flow is often used to infer the genetic basis of reproductive isolation, especially in systems where reproductively isolating phenotypes and their underlying genetic architectures are unknown. In mountain pine beetles, the distinct asymmetric architecture of HMS (Fig. 2a) coupled with heterogeneous patterns of differentiation and admixture suggests that the neo-Y and the neo-X chromosomes are involved in the evolution of HMS. Both sex chromosomes also stand out in population comparisons for showing highly elevated $FST$, however, it would be premature to interpret these patterns as entirely products of reduced gene flow. Measures of relative divergence, such as $FST$, are sensitive to levels of variation within and between populations and both sex chromosomes showed reduced population-level diversity ($\pi$) and lower absolute sequence divergence ($D_{xy}$) between populations (Table 2). Thus, elevated sex-linked $FST$ between East and West populations could reflect the combined effects of reduced gene flow as well as any process that reduces variation within these populations (e.g., linked natural selection). Genetic drift is generally predicted to be stronger on the sex chromosomes due to differences in their effective population sizes. Assuming equal sex ratios, genetic diversity on the X and Y should be ~75% and ~25% of autosomal variation. However, we found that the anc-X, neo-X, and neo-Y all showed significantly reduced levels of genetic variation relative to these theoretical predictions (~60, ~23 and 2% of autosomal diversity, respectively) after accounting for variation in mutation rate (Table 2).

Skews in mating sex ratios cannot account for reduced variation on both sex chromosomes, or for significant reductions in X-linked but not mtDNA diversity. Alternatively, recurrent positive and purifying natural selection can reduce chromosome-levels of genetic diversity, especially on non-recombinant Y chromosomes. Absolute sequence divergence (e.g., $D_{xy}$) should be robust to the influence of selection within contemporary populations, but is sensitive to chromosomal differences in effective population sizes including the effects of selection within ancestral populations. We observed lower absolute X and Y-linked divergence ($D_{xy}$) between reproductively isolated populations of beetles (Table 2) and these patterns persisted when $D_{xy}$ was normalized by divergence to the Jeffrey pine beetle to account for variation in substitution rates between chromosomes (i.e., relative node depth or RND). Thus, patterns of diversity and divergence both indicate significant long-term reductions in the effective population sizes of the sex chromosomes in mountain pine beetles. Moreover, while more efficacious selection is common on sex chromosomes, dramatic long-term reductions of neo-Y and neo-X diversity relative to the anc-X and the autosomes suggests that natural selection has been particularly intense during the ongoing evolution of the mountain pine beetle neo-sex chromosomes.

Given the potential for stronger genetic drift and more intense linked natural selection acting on the sex chromosomes, we decided to conduct more thorough population-level analyses of the X and Y chromosomes. We performed reduced representation sequencing (RADseq) of an additional 30 beetles from the neighbouring OR and ID populations (15 per population, 21× per beetle), which showed very low autosomal differentiation and extensive admixture in our range-wide whole genome sequencing (Fig. 2c). Dispersal in the mountain pine beetle is high and not strongly sex-biased; therefore, we would only expect the persistence of strong X and/or Y differentiation over this fine geographic scale if HMS involves sex-linked interactions. Consistent with this, only the sex chromosomes clearly partitioned genetic variation between these neighbouring populations (Fig. 3a, b). Differentiation was very low for the autosomes ($FST = 0.03$), elevated on the anc-X (0.15), and even higher for the neo-X (0.51) (Fig. 3c). The neo-Y again showed complete fixation ($FST = 0.97$; Fig. 3a, c) of the alternative haplotypes identified using whole genome sequencing (Fig. 2d).

Analyses of population structure in OR and ID revealed further important differences between the anc-X and neo-X in individual ancestry. While the neo-X showed a clear split between OR and...
Incompatibilities (Fig. 2a) often reexpanded northward as the climate warmed. Mountain refugia during the Pleistocene and subsequently Pinus persisted at only a subset of the genome. Asymmetric hybrid neo-Y = neo-X = neo-Y = 203. For example, pollen and molecular data show that retractions and secondary contact within the same geographic patterns of population isolation during Pleistocene range centre of the mountain pine beetle range. Consistent with this model, multiple host tree species show broadly concordant divergence followed by secondary contact and gene flow near the centre of the mountain pine beetle range. Consistent with this model, multiple host tree species show broadly concordant patterns of population isolation during Pleistocene range retraction and secondary contact within the same geographic region. For example, pollen and molecular data show that several Pinus host trees were isolated in distinct coastal and Rocky Mountain refugia during the Pleistocene and subsequently expanded northward as the climate warmed.

Collectively, these results indicate a period of allopatry leading to genetic differentiation and functional divergence between some populations followed by secondary contact and gene flow near the centre of the mountain pine beetle range. Consistent with this model, multiple host tree species show broadly concordant patterns of population isolation during Pleistocene range retraction and secondary contact within the same geographic region. For example, pollen and molecular data show that several Pinus host trees were isolated in distinct coastal and Rocky Mountain refugia during the Pleistocene and subsequently expanded northward as the climate warmed.

Our findings parallel recent cases where species boundaries persist at only a subset of the genome. Asymmetric hybrid incompatibilities (Fig. 2a) often reflect negative epistatic interactions that include sex-linked loci. Reproductive barriers are expected to quickly break down in the face of gene flow under a classic epistatic model of hybrid incompatibilities, but may persist within co-adapted genetic pathways and/or in subsets of the genome harbouring many linked incompatibilities. In mountain pine beetles, sex-linked reproductive incompatibilities persist between these nascent species despite extensive autosomal admixture (Fig. 2c), but the overall strength of isolation may have partially attenuated due to introgression resulting in a distinct ring-like pattern of reproductive isolation around the Great Basin Desert (Fig. 2a). This model of allopatric population divergence followed by secondary contact and gene flow stands as a viable model for other species that present a geographic continuum of reproductive isolation. Given the rapid pace of neo-sex chromosome evolution, it also is possible that the complex geographic progression of reproductive isolation in this system (Fig. 2a) also partially reflects the ongoing accumulation of hybrid incompatibilities across parts of the mountain pine beetle range. Differentiating between these scenarios will require a much more detailed understanding of how both the genetic basis and architecture of HMS and HMI varies by geography.

**Neo-sex chromosome structural and functional evolution.** The genetic architecture of HMS combined with patterns of population genomic variation, differentiation, and admixture suggests a central role for neo-Y evolution in reproductive isolation. Male-limited sterility and limitations of the mountain pine beetle system restrict our ability to further dissect incompatibilities on the non-recombinant neo-Y chromosome. Moreover, as with most
population genomic analyses of speciation, these proposed connections to reproductive isolation are indirect and removed from the molecular evolution of the neo-sex chromosomes and the functional underpinnings of relevant hybrid phenotypes. Neo-sex chromosome evolution can result in rapid functional specialization and, under some conditions, the structural degeneration of the sex-limited (Y) chromosome. Here we are particularly interested in understanding if these long-term dynamic genomic changes occur on a timescale that is relevant to the evolution of male sterility between populations.

We first tested if sex-specific functional differences have evolved on the neo-sex chromosomes. Established X chromosomes tend to be enriched for female-specific functions (i.e., feminized) in insects and Y chromosomes are masculinized, but the evolutionary tempo of this specialization remains unclear. We characterized genome-wide expression patterns of multiple tissues (male head, female head, ovary, and testis) from beetles collected in Montana (i.e., East beetles). The anc-X was enriched for ovarian expression (i.e., feminized) and depleted of testis-specific genes (i.e., de-masculinized; Fig. 4 and Supplementary Table 5 and Supplementary Fig. 8). In contrast, the neo-X was slightly enriched for ovary-specific expression, but undifferentiated from the autosomes in testis-specific expression (Fig. 4 and Supplementary Table 5 and Supplementary Fig. 8) and in expression of genes detected in both reproductive tissues (Supplementary Fig. 8). The neo-Y was highly enriched for male-specific genes (Fig. 4 and Supplementary Table 5). Therefore, the anc-X and neo-Y are highly enriched for sex-specific functions but the neo-X appears to be still in the early stages of feminization. This intermediate stage of gene specialization, combined with sex-linked signatures of recurrent natural selection (Table 2), suggests that functional divergence is still actively evolving on the neo-sex chromosomes. Relatively few studies have evaluated sex-biased functional specialization of genes on neo-sex chromosomes at these early stages. Our results have evaluated sex-biased functional specialization of genes (i.e., de-masculinized; Fig. 4 and Supplementary Table 5 and Supplementary Fig. 8). In contrast, our expression analyses (Fig. 5b) show significant differences in expression of genes between West and Southeast beetles (Supplementary Fig. 10) due primarily to large deletions in West beetles. These deletions were also fixed in our finer scale sampling of beetles from the OR and ID populations (Supplementary Fig. 11). We also detected several deletions that were private to the Southeast population (Fig. 5a), consistent with the occurrence of three divergent Y haplotypes (Fig. 2d). Full enumeration of the number of deletions was not possible given the current genome assembly, but the distribution of full and partial deletions across many scaffolds suggests widespread neo-Y degeneration through several independent mutational events. The rate of neo-Y degeneration should depend on the rate and extent to which recombination is lost between the neo-XY pair. *Dendroctonus* and many other beetles appear to have fully non-recombining sex chromosomes, thus neo-sex chromosomes may be immediately predisposed to the rapid evolution of heteromorphism in this system and could account for the apparently rapid pace of structural degeneration.

Widespread specialization of male-specific genes on the neo-Y combined with extensive insertion-deletion variation suggests that crosses between populations with different neo-Y haplotypes may yield hybrid males that are missing genes that are essential to spermatogenesis. We observed HMS in hybrid males with West fathers, which are therefore missing ~1 Mb of the East neo-Y chromosome. We identified 11 predicted genes within these deletions that were present in both East and Southeast beetles, five of which were highly expressed in testis or were male-specific in our expression analyses (Fig. 5b). One particularly strong candidate HMS gene was the peptidase family M2 Angiotensin converting enzyme (M2-ACE), which belongs to a fertility-essential gene family. We also observed six putative neo-Y gene deletions between East and Southeastern beetles, and four deletions shared by the West and Southeast haplotypes (Supplementary Table 6). Only two of these genes were at least moderately expressed in male tissues and therefore these deletions may have less impact on male fertility (Supplementary Table 6). Nevertheless, Southeast neo-Y deletions may have phenotypic consequences in hybrid males that have yet to be uncovered.

Overall, gene deletions of highly expressed male-specific genes are likely to have functional consequences in hybrid males, providing an important conceptual link between the evolution of HMS and neo-XY evolution. But deletions cannot entirely explain the range of HMS that we observed. Loss of essential genes should also incur strong fertility defects within West beetles in the absence of compensatory evolution. Furthermore, additional negative epistatic interactions must be occurring in hybrid males since the neo-Y deletions are largely fixed across West populations that vary in HMS strength (Fig. 2a). The neo-X is the most likely location for interacting hybrid incompatibilities given patterns of gene flow near the contact zone (Fig. 3), but autosomal interactions also cannot be ruled out. Many homologous neo-XY gene pairs have likely been retained since these chromosomes are in the early stages of becoming heteromorphic,
Fig. 5 Large-scale neo-Y deletions. a Clustered heatmaps of sequencing coverage of neo-Y scaffolds (columns) in individual males (rows). See Supplementary Fig. 9 for X and autosome partitions. Population symbols are as in Fig. 2a and clustered deletions indicated with red boxes. b At least five genes were located within neo-Y deletions in West beetles. Normalized whole genome sequencing coverage and tissue expression profile in East beetles are shown (black bars $\geq 1$ FPKM). See also Supplementary Table 6.
including the putative neo-X M2-ACE homolog. Independent functional divergence of homologous neo-XY gene copies between allopatriotic populations could facilitate both neo-Y gene loss and the accumulation of incompatible hybrid interactions with other genomic partitions.

Discussion
Our results suggest that sex chromosome-autosome fusions can initiate rapid divergence between populations and that this divergence is likely to have fitness consequences in hybrids. Gene movement on and off of the sex chromosomes can play an important role in speciation and neo-sex chromosomes are known to rapidly evolve dramatic differences in chromosome structure, gene content, and expression. Given these dynamics, it seems inevitable that genomic specialization and reorganization following a sex chromosome-autosome fusion would proceed along unique trajectories in allopatriotic populations and upon secondary contact, hybrid incompatibilities would emerge. However, few studies have examined the dynamics of neo-sex chromosome specialization and degeneration within and between natural populations. Our results indicate that neo-XY gene specialization and neo-Y structural divergence can rapidly evolve along independent trajectories between populations. This process of rapid neo-sex chromosome evolution should be enriched within reproductive pathways (e.g., spermatogenesis), which could further facilitate the persistence of reproductive barriers in the face of gene flow. Here we have focused primarily on male fertility, but the observation of delayed development in some crosses raises the intriguing possibility that rapid neo-sex chromosome evolution impacts a broader range of developmental processes.

Although we observed the most striking patterns of population genetic and functional differentiation on the neo-sex chromosomes, our results do not rule out important contributions of the ancestral X chromosome to reproductive isolation in mountain pine beetles. Indeed, neo-sex chromosome systems derived from recent sex-autosomal fusions do not appear to be subject to the same range of processes (specialization, degeneration, meiotic drive, faster-X, dominance theory) that could contribute to the evolution of hybrid male incompatibilities (Fig. 1). Given this, functional divergence during this crucial non-equilibrium stage of sex chromosome evolution is likely to be much more rapid when compared to the evolution of established heteromorphic sex chromosomes (Fig. 1). However, these major transitions are likely to span millions of years, and therefore could be a recurrent driver of population divergence and speciation.

That a rapid neo-sex chromosome speciation event appears to be unfolding in a phytophagous beetle irrespective of typical ecological drivers of divergence is all the more intriguing. Specialization in plant feeding insects is thought to be largely driven by ecological drivers of divergence is all the more intriguing. Specialization, degeneration, meiotic drive, faster-X, dominance theory that could contribute to the evolution of hybrid male incompatibilities (Fig. 1). However, these major transitions are likely to span millions of years, and therefore could be a recurrent driver of population divergence and speciation.

Phenotypic analyses of hybrid male sterility. To assess hybrid male reproduction in more detail we first quantified sperm transfer to females. On their collection day, each female had her spermathecal pump and sac removed and transferred to a drop of distilled water on a microscope slide. Each structure was immediately scored under ×400 magnification on a scale of 0 (no sperm) to 4 (sperm immobilized and structure universally opaque). Differences in the sperm quantities were determined using Wilcoxon rank sum tests of all pairwise comparisons with a Bonferroni correction (Supplementary Fig. 5). We examined sperm production in MT×nCA hybrid males (22 crosses) collected during the peak of emergence from rearing containers. Hybrid males (n = 5–7 per pairing) and nCA males (n = 20) were dissected in 60 µl of phosphate buffered saline (PBS) and each testis, attached seminal vesicle, and accessory glands were isolated and transferred to 20 µl of PBS. Spermatozoa were released from the seminal vesicle into solution using fine dissection tools and 5 µl of homogenized solution was then transferred to a Makler Counting Chamber (Seif-Medical Instruments, Ltd.). Sperm lying ≥50% within the counting grid were counted, averaging counts across the two seminal vesicles for each male. Differences between hybrid males and intrapopulation males were determined using a Wilcoxon rank sum test (Supplementary Fig. 5).

Pheromone component production. Pheromone samples were obtained from adult beetles collected near OR and ID (44° 16′ N, 115° 23′ W). Brood adults newly-emerged from logs cut from naturally-infested lodgepole pine were induced to mine into a freshly-cut lodgepole pine log in the laboratory. Solitary females (the gallery-initiating sex) that had been mating <1 d and that had been allowed to pair 1 d earlier in the entomological laboratory from the same site were excised from the bark and held in chemical adsorbent-containing microvials for 1 d at 22 °C58. To sample volatiles released by the live beetles, these steps were repeated twice. Compounds previously reported as having behavioural activity with Dendroctonus59 were identified in the hexane extracts of the adsorbent by gas chromatography-mass spectrometry by using matches of retention times and mass spectra with identified standards; compounds were quantified against a dilution curve of standards. Quantities were normalized as the percentage of the sum of all compounds found in either sex, transformed by arcsin square root, and subjected to a principal component analysis (PCA) utilizing a covariance matrix. Peaks detected in no more than trace amounts in a sex were excluded from the PCA for that sex.

Identification of autosomal and sex-linked scaffolds. We first eliminated mountain pine beetle scaffold that were short (<2 Kb including N’s) or were likely long.
bacterial (identified using BLASTn against the NCBI nt database), resulting in 4,877 scaffolds (195,619,274 bp excluding Ns) for further inquiry. We then took advantage of the large amount of raw sequencing data available from the male and female genome builds29 (accessions SRX180259, SRX180261, and SRX180262) to identify putative X, Y and autosomal scaffolds using relative sequencing coverage between the sexes. We used SeqyClean version 1.8.10 to quality filter, adapter trim, and screen for common Acinetobacter contaminants (accessions CP000521, ACPN00000009). Filtered reads were then mapped to the reference male genome using BWA-MEM version 0.7.962 and PCR duplicates were removed using rmdup within samtools version 1.16.3. We counted the number of male and female reads that mapped uniquely (quality score ≥ 20) to the genome build scaffolds based on depth at 20× for significant per sex, per scaffold differences in coverage by performing exact tests with a false discovery rate of 5% for each of the 4,877 scaffolds. As a proof-of-principle, we verified that six previously identified ancestral X-linked scaffold29 showed significant female-based sequencing coverage (2.06 ± 0.03 fold more female coverage; Supplementary Fig. 6). We then binned scaffolds as sex-linked based on a minimum of ~1.8-fold excess coverage in females (anc-neo-X) or males (neo-Y), autosomal (<1.4-fold excess coverage in either sex), or unclassified (Supplementary Fig. 6). This procedure resulted in 1,178 autosomal, 664 anc+neo X, 2,272 neo-Y, and 763 unclassified scaffolds. Note that residual neo-XY sequence homology is expected to occur in some palindromic or mosaic neo-X/neo-Y scaffolds in the mountain pine beetle reference genome. These complications are common to neo-sex chromosome studies but should not compromise our functional and population genomic analyses as we took additional steps to exclude potential problem regions from subsequent analyses (described below).

**Whole genome re-sequencing.** We Illumina 100 bp paired-end (PE) re-sequenced whole genomes of two adult males and one female from each of nine populations of mountain pine beetle (Fig. 2a) including both the female (Dendrocytosis Jeffreyi) (34° 13′ N, 116° 48′ W). DNA was extracted using either Omega E.Z.N.A Tissue DNA kits or Qiagen DNAeasy kits. Sequencing libraries were prepared for fourteen samples from seven populations (1 male, 1 female; sCA, mCA, nCA, OR, ID, MT and AZ) at the University of Montana using 300 ng DNA as input per sample. Paired-end DNA sequencing was performed at the Genome Barcodes by Bioo Scientific (Austin, TX). These libraries were sequenced at the Vincent J. Coates Genomics Sequencing Laboratory, Berkeley, CA. We sequenced an additional fifteen beetle taxa including one male from each population above and two males and one female from two additional populations (UT, CO). These libraries were prepared using the Nextera DNA Prep Kit for high molecular weight DNA, the New England Biolabs NEBNext Ultra DNA Library Prep Kit.

**Whole genome analyses.** All whole genome re-sequencing data was cleaned and mapped as above. We used the GATK Best Practices pipeline30 to call genotypes. All BAMs were processed using realignerTargetCreator to identify regions with potential insertion-deletion variation, locally realigned using indelrealigner, and genotyped using the GATK UnifiedGenotyper version 3.1–1. We then filtered SNPs with a MAF of >0.01 and an individual genotype quality score >30. Filtered partitions (mean MAPQ of 54.1, 59.5, 57.3 and 54.6 for autosomal, anc-neo-X, neo-Y, and neo-XY partitions, respectively) were genotyped either males or females using GATK’s UnifiedGenotyper, filtered as above, and estimated nucleotide diversity and Dxy across concatenated intervals using a custom script and generated 95% bootstrapped confidence intervals (999 replicates). Relative node depth was calculated following Feder et al.31. To characterize the sex-linked genotypic diversity relative to the autosomes, we used the same absolute coverage thresholds (≥3×) for neo-Y intervals but also eliminated regions with female coverage. We then eliminated X and Y intervals that included sites where more than one male was confidently called a heterozygote (GQ ≥20) for these are likely regions of recombinations or collapsed haplotypes. This resulted in a 40×-fold drop in the number of high-quality intervals allowing us to find regions of the degenerate neo-Y where we felt confident that we were estimating our parameters over single copy regions that were free of assembly mistakes that plague mountain pine beetle neo-Y scaffolds. Mapping qualities were similar across filtered partitions (mean MAPQ of 54.1, 59.5, 57.3 and 54.6 for autosomal, anc-neo-X, neo-Y, and neo-XY partitions, respectively). We re-analysed and re-filtered as above, and estimated nucleotide diversity and Dxy across concatenated intervals using a custom script and generated 95% bootstrapped confidence intervals (999 replicates). Relative node depth was calculated following Feder et al.31 to identify the sex-linked genotypic diversity relative to autosomes. We then tested for differences in sequence complexity and residual sequence similarity between the male and female genomes using the D-statistic35 which was implemented using a block jackknife approach in the R package evoGF. We tested the populations closest to the contact zone (OR and ID) and a Z-score value of 3 and above considered significant35. Phylogenetic NeighborNet trees were generated using SplitsTree437 (Fig. S19). Dxy values were quantified relative to the outgroup (excluding CAN) using Weir and Cockerham weighted Fst,72 estimated with VCFTools version 0.1.12b. In the next step, we evaluated the interval-based analyses to evaluate diversity and divergence between West and East beetles across all genomic partitions. To accurately identify both variant and invariant positions, we re-analysed and re-filtered our whole genome data. For the anc-X, neo-X and autosomes, we first restricted analyses to high quality intervals ≥100 bp in length where all individuals had coverage ≥3x and ≥1 SD of their total estimated mean coverage. The purpose of these thresholds was to ensure that all individuals had adequate coverage to call a variant but not extended coverage as this would alter coverage if the individual was not pure or originated from some other assembly error. We used the same absolute coverage thresholds (≥3×) for neo-Y intervals but also eliminated regions with female coverage. We then eliminated X and Y intervals that included sites where more than one male was confidently called a heterozygote (GQ ≥20) for these are likely regions of recombinations or collapsed haplotypes. These results were used in our expanded RADseq analysis. We selected 146 autosomal high quality intervals for further study. To select these intervals, we re-analysed and re-filtered the sex chromosomes using the D-statistic which was implemented using a block jackknife approach in the R package evoGF. We tested the populations closest to the contact zone (OR and ID) and a Z-score value of 3 and above considered significant35. Phylogenetic NeighborNet trees were generated using SplitsTree437 (Fig. S19). Dxy values were quantified relative to the outgroup (excluding CAN) using Weir and Cockerham weighted Fst,72 estimated with VCFTools version 0.1.12b.

**Identifying neo-Y linked genes and their neo-X gametologs.** We first identified all annotated genes that fell on neo-Y scaffolds (above, 781 genes total). We then used this list along with Ensembl and BioMart39 to identify ‘paralogous’ sequences within the draft mountain pine beetle genome. We restricted our set of genes to include only those that were single clear ‘Neo-X’ homologs that were present on a scaffold for both the ancestral (anc) and the neo-Y and where at least 60% of the neo-X gene could be aligned. Genes below this threshold were too fragmented to accurately align and were often only partial hits
of dubious homology. We then downloaded coding domain sequences and aligned the putative neo-X and neo-Y gametologs using MAFFT\textsuperscript{25} and calculated $K_s$ on 119 unique X and Y homologs (11-XY pairs as defined in Supplementary Fig. 6) using KaKs_Calculator\textsuperscript{28}.

**Whole genome insertion-deletion variation.** We counted the number of reads mapping (minimum mapping quality 20) to the 4,114 scaffolds. We then used edgeR to normalize and estimate RPKM (reads per kilobase of transcript per million reads mapped) for each individual and to test for differences in per scaffold coverage between East and West, and South versus North. We used the pheatmap package in R for clustering (hclust), restricting analysis to scaffolds with a minimum of two counts per million for at least eight individuals.

**RADseq population genomic data generation and analysis.** Approximately 400 ng total DNA from adult OR and ID beetles was used for the RADseq protocol. We developed a customized single restriction enzyme digest protocol similar to Etter and colleagues\textsuperscript{77}, with additional DNA cleaning steps and a slight modification to the A overhang. DNA was blunt-end repaired, a 5' adapter (available upon request). DNA was cleaned, digested with specific adapter was ligated to the A overhang. The indexing read barcode and Illumina program from Stacks version 1.1878 and default settings. The resulting paired end reads were processed and cleaned using the process_radtags program from Stacks version 1.18\textsuperscript{29} and default settings. The resulting paired end reads were then mapped to the male reference genome using the BWA aln and sampe command and filtered for PCR duplicates with a 0.75 minimum mapping quality. We then compared coverage of RADtags positioned within 141 putatively neo-Y deleted regions (mapping quality $\geq 20$) using samtools\textsuperscript{63} (Supplementary Fig. 11). We then genotyped RADtags from all OR and ID individuals as described above. SNP filtering differed slightly by linkage group because of differences in ploidy for the autosomes and sex chromosomes in males and the availability of male and female RAD data for different linkage groups. To call variants on the autosomes, we analysed both male and female beetle data and only kept genotypes with a minimum genotype quality of 30 and a minimum depth of 10 reads. We removed indels, excluded sites that failed quality filters, and restricted our analyses to bi-allelic sites in Hardy-Weinberg proportions where at least 20 of the 30 individuals were confidently genotyped. Neo-Y SNPs were identified from males using filters as above except we applied no minimum coverage filter and retained SNPs found in only one individual. We excluded positions with female genotypes (GG $\leq 30$) or male heterozygosity. X chromosome analyses were restricted to males and using the same filtering criterion as for the neo-Y. Population genetic analysis of RADseq population genomic data generation and analysis

The M2-ACE-like gene stood out as an interesting candidate for playing a role in hybrid male sterility. To determine if this gene might have a homolog on the neo-X, we used BLASTp to search the neo-Y protein sequence (accession ENN80010) against GENBANK and found the top two hits were to a protein on a different mountain pine beetle scaffold, Seq_1102825 (accession ENN740447) and to a single protein in the female mountain pine beetle genome (accession ERJ91207). The presence of only one other ACE-like gene in both the male and female genome builds provided some evidence that these versions might be on the neo-X. We then performed PCA of SNPs located on the putative male neo-X scaffold (Seq_1102825) and found that the first principal component split individuals by sex and explained 33.6% of the variation providing more evidence of X-linkage. Investigation of gene expression for the putative neo-X version of the gene suggested that in contrast to the neo-Y version (Fig. 5b), it is widely expressed across tissues.

**Data availability.** Sequence and crossing data that support the findings of this study have been deposited at the NCBI SRA sequence read archive (BioProject ID PRJA036777 and at Figsar [https://doi.org/10.6084/m9.figshare.5479594v1]). Any additional data that support the findings of this study are available from the corresponding author upon request.

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Author contributions
R.R.B. and J.M.G. conceived of the study. R.R.B. conducted the MT×nCA cross, and performed all crossing and genomic analyses. B.J.B. conducted the AZ×sCA cross. B.T.S. conducted pheromone analyses. R.R.B. and J.M.G. wrote the manuscript with comments from all authors.

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