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Neo-Sex Chromosome Evolution in Treehoppers Despite Long-Term X Chromosome Conservation

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Abstract

Sex chromosomes follow distinct evolutionary trajectories compared to the rest of the genome. In many cases, sex chromosomes (X and Y or Z and W) significantly differentiate from one another resulting in heteromorphic sex chromosome systems. Such heteromorphic systems are thought to act as an evolutionary trap that prevents subsequent turnover of the sex chromosome system. For old, degenerated sex chromosome systems, chromosomal fusion with an autosome may be one way that sex chromosomes can “refresh” their sequence content. We investigated these dynamics using treehoppers (hemipteran insects of the family Membracidae), which ancestrally have XX/X0 sex chromosomes. We assembled the most complete reference assembly for treehoppers to date for *Umboonia crassicornis* and employed comparative genomic analyses of 12 additional treehopper species to analyze X chromosome variation across different evolutionary timescales. We find that the X chromosome is largely conserved, with one exception being an X-autosome fusion in *Calloconophora caliginosa*. We also compare the ancestral treehopper X with other X chromosomes in Auchenorrhyncha (the clade containing treehoppers, leafhoppers, spittlebugs, cicadas, and planthoppers), revealing X conservation across more than 300 million years. These findings shed light on chromosomal evolution dynamics in treehoppers and the role of chromosomal rearrangements in sex chromosome evolution.

Key words: sex chromosomes, neo-sex chromosomes, chromosomal fusion, chromosomal rearrangements, karyotype evolution.

Significance

The evolutionary forces underlying sex chromosome stability versus turnover have been challenging to disentangle. We present the most complete reference assembly for treehoppers to date and find evidence of long-term X chromosome conservation among treehopper species and with other hemipteran insects more generally. A key exception is the evolution of neo-XX/XY sex chromosomes via an X-autosome fusion. Sex chromosome–autosome fusions may play an important role in the evolution of otherwise “trapped” (i.e. old and degenerated) sex chromosome systems.

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Introduction

In many clades, pairs of sex chromosomes are often observed to follow distinct evolutionary trajectories in which the X (or Z) chromosome remains functional and gene rich while the Y (or W) undergoes functional degeneration and loss (Charlesworth et al. 2005; Bachtrog et al. 2011; Vicoso 2019). The emergence of such heteromorphism is predicted to act as an evolutionary trap that impedes sex chromosomes from undergoing turnover to new genomic regions (Bull 1983; Bachtrog et al. 2011). For example, evolutionary turnover from an XY to a ZW system can generate YY individuals, making such a transition unlikely if the Y is degenerated and YY individuals have low fitness. This idea has been supported by several comparative analyses (Pokorná and Kratochvíl 2009; Gamble et al. 2015; Nielsen et al. 2019), and anecdotally many clades with old sex chromosomes exhibit long-term sex chromosome conservation, such as birds, insects, mammals, and some groups of reptiles (Pokorná and Kratochvíl 2009; Rovatsos, Altmanová, et al. 2014; Rovatsos, Pokorná, et al. 2014; Fraïsse et al. 2017; Nielsen et al. 2019; Chauhan et al. 2021; Li et al. 2024; Touns and Vicoso 2023). However, there are notable exceptions (Kuroiwa et al. 2010; Vicoso and Bachtrog 2013; Pinto et al. 2023), and a comparative analysis using the Tree of Sex database did not find evidence in support of the hypothesis that heteromorphic sex chromosomes are an evolutionary trap (Pennell et al. 2018). On the other hand, some clades maintain sex chromosome homomorphism, which is thought to permit rapid and ongoing turnover in both sex chromosome identity and system (Gamble et al. 2015; Jeffries et al. 2018; Tennessen et al. 2018; Balounova et al. 2019; Darolti et al. 2019). What degree of sex chromosome divergence is necessary to create a trap and whether this affects all chromosomes equally remain unclear.

We do know that key exceptions to the evolutionary trap model of sex chromosome evolution are driven by chromosomal fusions between sex chromosomes and autosomes (Maddison and Leduc-Robert 2013; Pennell et al. 2015; Sigeman et al. 2022; Castillo et al. 2023). These fusions can generate new sex chromosome systems and “refresh” the sequence content of the sex-linked genome. Chromosomal rearrangements can have important functional genomic impacts, either directly by changing the sequences of regulatory and protein-coding regions (Stewart and Rogers 2019) or more indirectly by altering linkage and recombination among selected loci (Cicconardi et al. 2021; Näsvall et al. 2023). Fusions between an autosome and a sex chromosome present a special case with potentially more extensive impacts because of the resulting shift toward sex-linked inheritance of previously autosomal regions. These newly sex-linked regions will thus be subject to sex differences in selection, demography, and life history that can drastically alter their evolutionary trajectories (Rice 1984).

Different evolutionary forces are predicted to drive fusions between sex chromosomes and autosomes, including sexually antagonistic selection and meiotic drive; however, the relative importance of each is unclear and appears to differ across taxonomic groups (Charlesworth and Charlesworth 1980; Pennell et al. 2015; Anderson et al. 2020). Identifying the structural genomic changes involved in such events and their evolutionary drivers is thus consequential for understanding how and why sex chromosomes evolve and persist.

Insects are a promising system for investigating the relative role of fusions in sex chromosome evolution. Karyotypic data indicate that many species exhibit frequent genome rearrangements, with significant changes in chromosome number (The Tree of Sex Consortium 2014; Blackmon et al. 2017), and so we might expect sex chromosome–autosome fusions to be common if there was a selective advantage (Pennell et al. 2015). While many insect species do exhibit neo-sex chromosomes (Yoshido et al. 2011; Zhou and Bachtrog 2012; Zhou et al. 2012; Maddison and Leduc-Robert 2013; Nguyen et al. 2013; Vicoso and Bachtrog 2015; Blackmon et al. 2017; Mongue et al. 2017; Palacios-Gimenez et al. 2018; Bracewell et al. 2024; Decroly et al. 2024), it remains unclear whether the frequency of fusions involving sex chromosomes is consistent with genome-wide rates of rearrangement. There is growing evidence that the insect X chromosome has been conserved for long time periods (Chauhan et al. 2021; Li et al. 2024; Touns and Vicoso 2023), but the occurrence of neo-sex chromosomes appears to vary substantially across taxonomic groups (Mathers et al. 2021; Bracewell et al. 2024).

To investigate patterns of stability versus turnover of sex chromosomes, we employ whole-genome sequencing across 13 treehopper species spanning ~45 million years of evolution. Treehoppers (Membracidae) are a group of hemipteran insects best known for their morphologically diverse pronota or “helmets” (Prud’homme et al. 2011; Fisher et al. 2020). Knowledge of treehopper genome evolution has been relatively limited in comparison to other well-studied hemipteran groups such as aphids (Aphididae) (Jaquiéry et al. 2012, 2013; Li et al. 2020; Mathers et al. 2021) and planthoppers (Delphacidae) (Ma et al. 2021; Ye et al. 2021; Hu et al. 2022), whose genomes show differing levels of synteny but X chromosome conservation within each clade. In contrast to aphids, treehoppers are obligately sexually reproducing and exhibit a variety of sex-related phenotypes like vibrational courtship signals and parental care. The most prevalent sex chromosome configuration in treehoppers based on karyotype information is XX/X0 (Kornhauser 1919; Halkka 1959, 1962, 1964; Halkka and Heinonen 1964), in which females carry two X chromosomes, and males carry a single X. However, multiple species carrying XX/XY systems have been identified by cytological studies (Kornhauser 1914; Tian and Yuan

1997; Anjos et al. 2019), indicating the repeated emergence of new Y chromosomes. Cytological studies indicate that total chromosome number can range from 5 to 11 pairs of chromosomes, with the mode being 11 pairs. These data suggest that treehopper chromosomes undergo frequent chromosomal rearrangements.

Here, we build the most complete reference assembly for treehoppers to date for *Umbonia crassicornis*, generate genome assemblies using data from male and female individuals of 12 additional treehopper species spanning short, medium, and long-term evolutionary distances, and combine our data with published hemipteran genomes. We interrogate the relationship between chromosomal fusions and sex chromosome evolution and test the extent to which long-term conservation of the X chromosome previously observed across broad insect groups (Chauhan et al. 2021; Li et al. 2024; Toups and Vicoso 2023) is a feature of treehopper evolution.

Results and Discussion

Umbonia crassicornis Genome Assembly

We assembled the most complete reference assembly for treehoppers to date using 10x linked reads and Hi-C data for *U. crassicornis* (supplementary table S1, Supplementary Material online). The length of the final genome assembly was 1.2 Gb, the scaffold N50 was 75 Mb, and the average scaffold length was 10,566 bp. The genome assembly included ten large scaffolds (whose sizes range from 54.3 to 164 Mb, accounting for 70% of the genome) and an additional 113,655 small scaffolds (1,201,059,541 bases in total). These ten large scaffolds are coincident with the number of chromosomes observed by cytological analysis, which indicated that the species has nine pairs of autosomal chromosomes plus an X element (Escudero and Virkki 1976). Importantly, our Hi-C map corroborates this finding and shows the longest ten scaffolds clustering as distinct units (supplementary fig. S1, Supplementary Material online). The ancestral diploid number for Membracidae is ten pairs of autosomes + X0 (Emeljanov and Kirillova 1992; Kuznetsova and Aguin-Pombo 2015), implying that *U. crassicornis* underwent a reduction in chromosome number caused by a fusion between two autosomes. Consistent with this cytological evidence, we observed that the largest scaffold (164 Mb) in our assembly is roughly twice as large as the other nine major scaffolds (average 77.5 Mb). We propose that the largest scaffold represents the product of an autosome–autosome fusion. The genome assembly is available under BioProject PRJNA1122077 in the National Center for Biotechnology Information (NCBI).

Identifying and Comparing X-Linked Sequences across Treehoppers

We next leveraged the *U. crassicornis* genome assembly to identify the X chromosome in this species. We mapped

paired-end reads from two male and two female samples to the genome assembly. We then calculated the \log_2 male-to-female coverage ratio for each of the ten major pseudochromosomes (hereafter referred to as chromosomes). For a species with an XX/X0 system, this approach is expected to yield a value of 0 (equal coverage among males and females) for autosomal sequences and -1 (half the coverage in males compared to females) for X-linked sequences. Here, we report results based on read mapping to the ten chromosomes. We repeated the analyses using the full genome assembly, and results were qualitatively the same. Our results showed average \log_2 male-to-female coverage close to 0 for chromosomes 1 through 9, in contrast to chromosome 10, which showed an average \log_2 male-to-female coverage ratio of -0.978 (Fig. 1). We further investigated sex differences in coverage in windows across each of the chromosomes to confirm the expected signatures for an XX/X0 system and observed a reduction of male coverage across the entire length of chromosome 10 (Fig. 1a). Based on this evidence, we conclude that chromosome 10 is the X chromosome in *U. crassicornis*.

For each of the remaining 12 species (Fig. 2a), we generated short-read sequence data for males and females (supplementary table S2, Supplementary Material online) and built de novo genome assemblies (supplementary table S3, Supplementary Material online). We then used the same coverage approach as above to identify autosomal and sex-linked sequences in each species. Based on published karyotype data and our newly generated karyotype data for *Cyphonia clavata*, *Cyphonia claviger*, and *Entylia carinata*, these species show variation in diploid chromosome number ranging from $2n = 14\text{♀}/13\text{♂}$ to $2n = 22\text{♀}/21\text{♂}$ (supplementary table S2 and fig. S3, Supplementary Material online). Comparing the repertoire of autosomal versus sex-linked sequences among species should therefore reveal chromosomal rearrangements involving sex chromosomes (Lasne et al. 2023). Since we did not have chromosome-level resolution for these other taxa, we anchored sequences back to the *U. crassicornis* assembly to ask whether the identity of sex-linked sequences is shared. For all species but two, we found that most sequences with autosomal coverage patterns corresponded to autosomes in *U. crassicornis* (Fig. 2b). We also found that sequences with X-linked coverage patterns corresponded to the X chromosome in *Umbonia*, suggesting conservation of X chromosome identity across species, spanning 45 million years of treehopper evolution. From here, we refer to this shared X chromosome as the ancestral treehopper X chromosome.

The qualitative exceptions to this trend were *Calloconophora* and *Bolbonota*. Sequences with X-linked coverage patterns in *Bolbonota* were distributed evenly across *Umbonia* autosomes, although the greatest proportion was still found on the *Umbonia* X. This noise is likely a

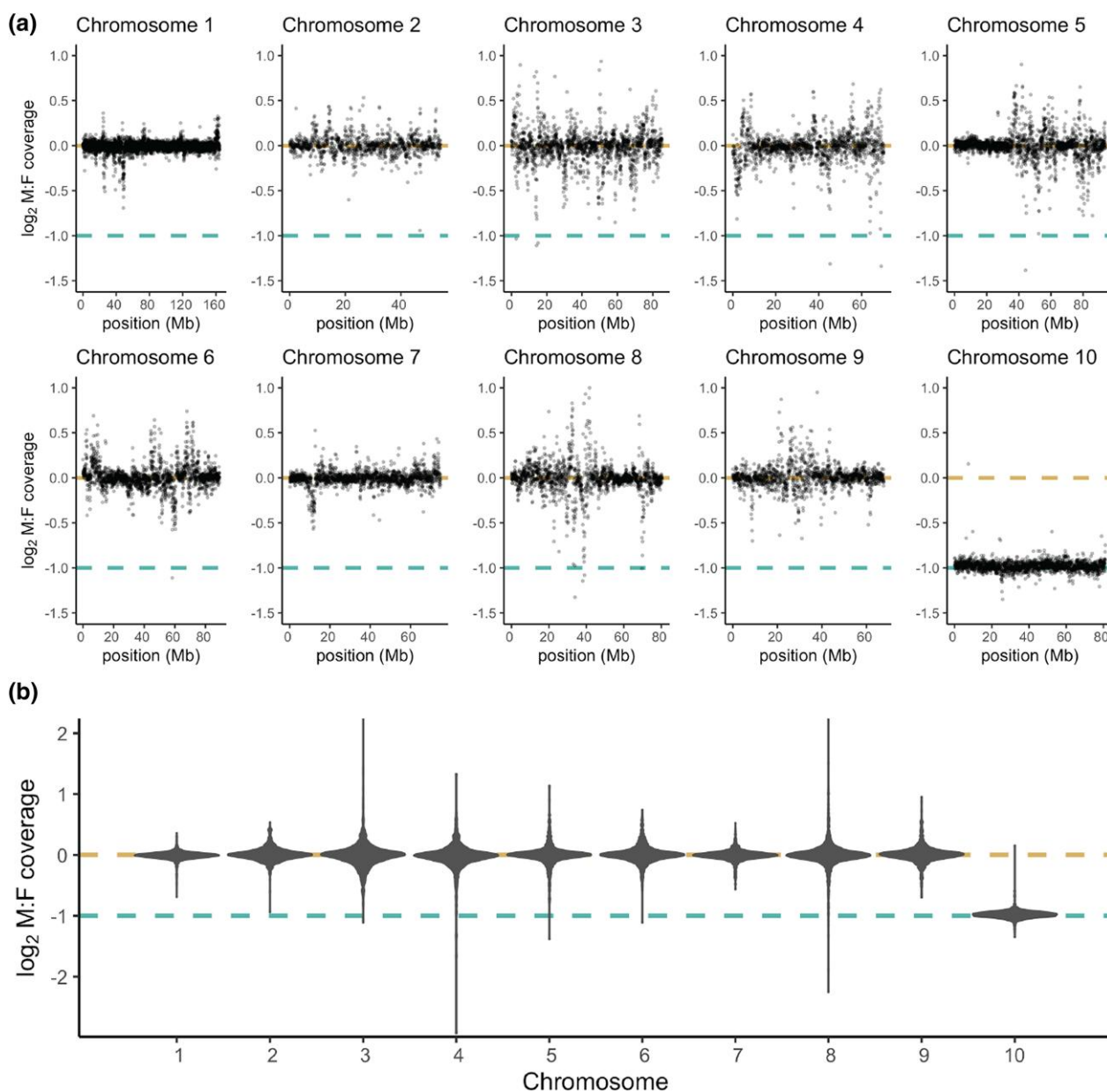


Fig. 1 Male/female (M/F) coverage ratio across *U. crassicornis* chromosomes shows reduced male coverage for chromosome 10. a) Points show M/F coverage ratio in 50-kb windows across each of the ten major scaffolds corresponding to the ten *U. crassicornis* chromosomes. b) Violins show the distribution of M/F coverage values for each chromosome. Dashed lines at $y = 0$ and $y = 1$ show the expected coverage values for autosomal and X-linked regions, respectively. Chromosome 10 was classified as the X chromosome based on the halving of M/F coverage compared to chromosomes 1 through 9 (autosomes).

product of lower sequencing coverage for individuals of this species (Supplementary table S2, Supplementary Material online). In contrast, *Calloconophora* showed clear X-linked coverage patterns for sequences corresponding to both the *Umbonia* X chromosome and chromosome 9 (which is autosomal in *Umbonia*) (Fig. 2b). Given the frequent changes in chromosome number that are known to occur in treehoppers, we hypothesized that a chromosomal fusion occurred in *Calloconophora* between the ancestral sex chromosomes and the homolog(s) of *Umbonia* chromosome 9.

Confirming a Chromosomal Fusion and Neo-X in *Calloconophora*

To further investigate the presence of a sex chromosome–autosome fusion in *Calloconophora*, we used fluorescent in situ hybridization (FISH) to visualize the chromosomes and the location of telomeric sequences. This confirmed the presence of an X and a Y chromosome and revealed the X to be ~25% larger than the Y (Fig. 3a; supplementary fig. S2, Supplementary Material online). Notably, we observed signal corresponding to telomeric

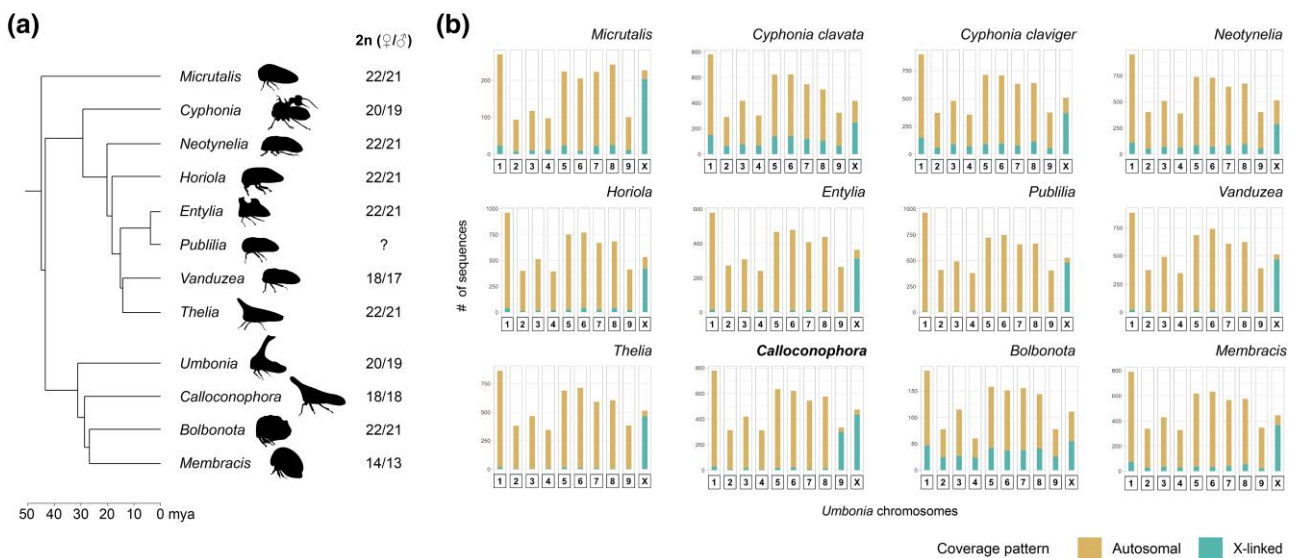


Fig. 2 X chromosome identity is largely conserved across treehopper genera. a) Phylogenetic tree of treehopper genera sampled for this study (adapted from Fletcher 2023) and diploid chromosome number (female/male) for species in this study. b) Pairwise comparisons of X- versus autosomal linkage between each focal taxon and *U. crassicornis*. Height of the bar indicates the number of focal taxon sequences that correspond to each *U. crassicornis* chromosome (along the x axis), and color of the bar shows the number of sequences that are inferred to be autosomal versus X-linked in the focal taxon.

sequence toward the middle of the X chromosome, indicating the site of a chromosomal fusion between the ancestral X and an autosome (Fig. 3a; supplementary fig. S2, Supplementary Material online).

The presence of an X-autosome fusion in *Calloconophora* would imply a transition from an ancestrally XX/X0 sex chromosome system to a neo-XX/XY system, in which the fused ancestral X + chromosome 9 comprise the neo-X and the unfused homolog of chromosome 9 forms the neo-Y (Fig. 3b). Recombination among sex chromosomes becomes suppressed over time in a stepwise fashion along genomic segments known as evolutionary strata (Charlesworth et al. 2005; Wright et al. 2016). Therefore, we examined sex differences in coverage across the neo-X region to ask if strata are present in *Calloconophora* and to gain insight into the relative age of this neo-XX/XY system. This revealed similar levels of sex differences in coverage among the neo-X and ancestral X (which is entirely hemizygous), suggesting relatively advanced levels of differentiation among neo-X and neo-Y sequences (Fig. 3c). Given that we might expect some Y degeneration, this finding may initially appear inconsistent with our cytogenetic data indicating that these chromosomes are of similar physical size. However, chromosomal size as estimated by cytological data is not a reliable measure of sequence degeneration (Nanda et al. 1990; Wright et al. 2017; Moraga et al. 2023). This is because degenerating chromosomes often accumulate repetitive elements, which either compensate for reductions in size caused by deletions or increase the physical length of the Y relative to the X (Bachtrog 2013; Wright et al. 2016; Bachtrog et al. 2019).

The emergence of a neo-XX/XY system in *Calloconophora* is a marked departure from the long-term X conservation we observe in treehoppers and facilitates an “escape” from the evolutionary trap. Following the autosome-X fusion in *Calloconophora*, the neo-X would have significantly increased its gene content, and a neo-Y would have emerged where there was not one before. The functional impacts of this fusion remain unknown as well as if and how the evolution of these neo-X and neo-Y chromosomes contributes to sex-specific adaptation.

Testing for X Conservation in Auchenorrhyncha

Given that all species in the data set showed conservation of the ancestral treehopper X chromosome, we compared this ancestral X with the X chromosomes of other members of Auchenorrhyncha (the clade containing treehoppers, leafhoppers, spittlebugs, cicadas, and planthoppers) to investigate sex chromosome evolution across a deeper timescale. We took a similar approach as with the analyses among treehoppers, but this time, we compared *U. crassicornis* to publicly available chromosome-level assemblies for a leafhopper (Family Cicadellidae; *Homalodisca vitripennis*) and a planthopper (Family Delphacidae; *Nilaparvata lugens*). The divergence time between treehoppers and each group dates to ~192 and 310 million years ago, respectively (Johnson et al. 2018) (Fig. 4). In each of the pairwise comparisons, we identified homologous sequences using BLAST (Altschul et al. 1990) and assessed for concordance in the genomic location of X-linked versus autosomal sequences. In the treehopper-to-leafhopper comparison, we observed

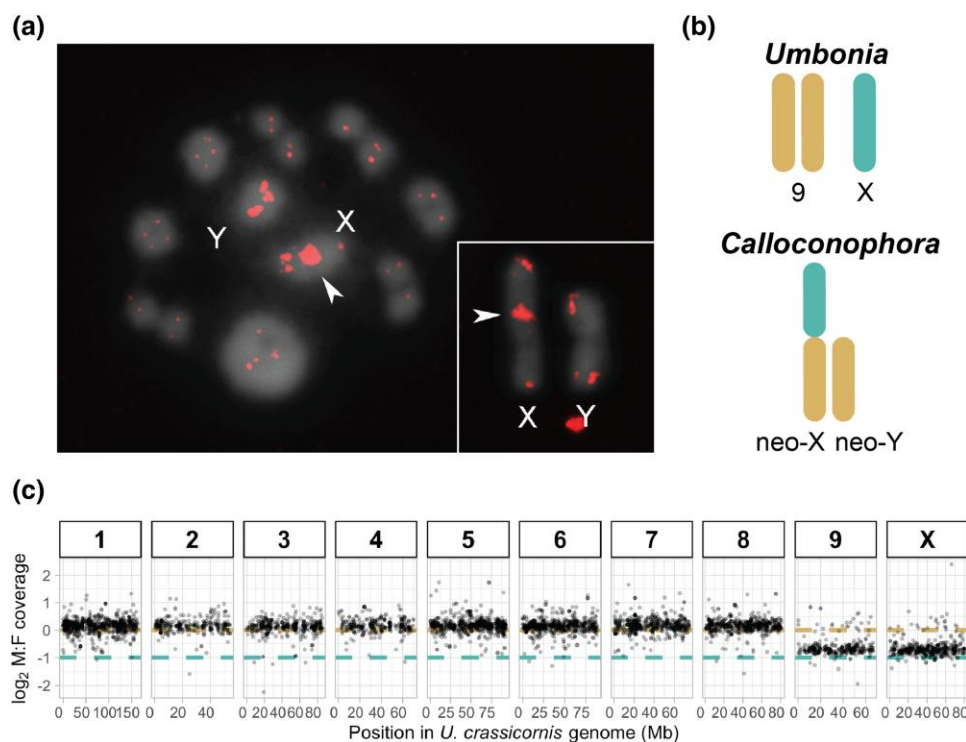


Fig. 3 An X-autosome fusion underlies the formation of neo-sex chromosomes in *Calloconophora*. a) FISH showing telomeric sequences in red at metaphase I (main panel) and mitotic metaphase (insert). Interstitial signal indicated by arrowheads shows the putative X-autosome fusion site. b) Schematic of the inferred pre- (*Umbonia*) and post-fusion (*Calloconophora*) chromosomes involved in the transition from XX/X0 to XX/XY sex chromosomes. c) Patterns of male/female sequencing coverage for *Calloconophora*. The numbered boxes and physical position along the x axis show the location of these sequences relative to the *Umbonia* reference genome. Dashed lines at $y = 0$ and $y = 1$ show the expected coverage values for autosomal and X-linked regions, respectively. *Calloconophora* sequences that correspond to the *Umbonia* X chromosome and autosome 9 show reduced coverage consistent with X-linkage.

near identical concordance in X-linked versus autosomal regions among the two genomes (Fig. 4). When comparing treehoppers with planthoppers, a similar qualitative result was seen, with most treehopper X chromosome sequences also showing X-linkage in the planthopper. Overall, these results indicate conservation of the X chromosome across extremely long time periods spanning the clade Auchenorrhyncha, consistent with recent work describing ancient origins of the insect X chromosome (Chauhan et al. 2021; Li et al. 2024; Toups and Vicoso 2023).

Based on karyotype data, treehoppers exhibit a wide array of chromosomal configurations that suggest frequent genomic rearrangements. The species in our data set represent a subset of this variation, with chromosome number ranging from $2n = 13\delta$ to $2n = 21\delta$ (Boring 1907; Kornhauser 1914; Halkka 1964; Halkka and Heinonen 1964; Tian and Yuan 1997; Anjos et al. 2019). The fact that we observed only one species with a chromosomal rearrangement involving a sex chromosome is consistent with recent work in aphids showing conservation of the X between species despite extensive autosomal rearrangements (Li et al. 2020; Mathers et al. 2021). Furthermore, it appears that the relative conservation of the X amidst dynamic

autosomal reshuffling extends beyond treehoppers, since we found signatures of shared X identity between treehoppers, leafhoppers, and planthoppers. On the other hand, comparisons within other hemipteran clades like the assassin bugs reveal high levels of synteny across autosomal regions interrupted by recurring X chromosome fission events (Panzeria et al. 1996; Mathers et al. 2021). Taken together, these results suggest differences among hemipteran clades in their propensity for and/or tolerance of genomic reorganization.

Rearrangements like chromosomal fusions and fissions are largely expected to be deleterious because of their potential to disrupt normal segregation during meiosis (Melters et al. 2012; Ruckman et al. 2020). This expectation is based on monocentric organisms (i.e. species with a single, localized centromere per chromosome), in which fusions or fissions can result in chromosome fragments with too many or too few centromeres that fail to segregate properly in meiosis. However, in holocentric species, centromeric activity is spread across the chromosome, meaning that fusions and fissions may be better tolerated. Holocentrism has evolved repeatedly in insects (and beyond) and appears to be the ancestral state for treehoppers

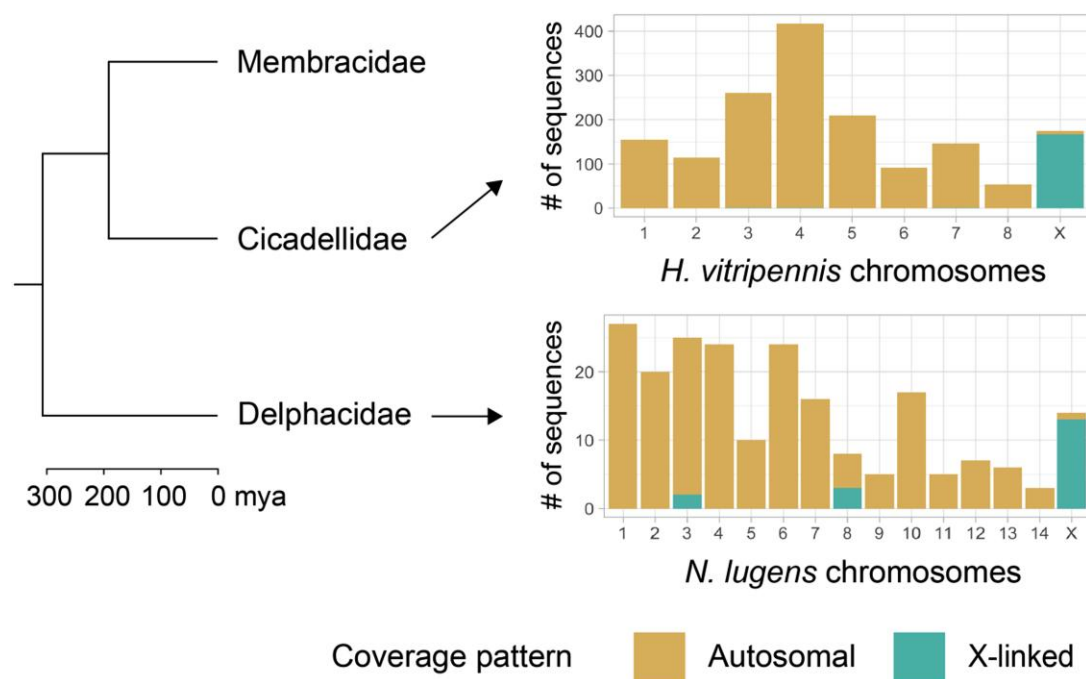


Fig. 4 X chromosome identity is conserved within Auchenorrhyncha. Pairwise comparisons between the *U. crassicornis* genome and representative genomes of Cicadellidae (leafhoppers) and Delphacidae (planthoppers). The phylogeny on the left shows the evolutionary distances based on Johnson et al. (2018). In the barplots on the right, height of the bar indicates the number of *Umbonia* sequences that correspond to the chromosomes of each species. Color of the bar shows the relative proportion of sequences that are inferred to be autosomal versus X-linked in *Umbonia*.

and the family Hemiptera (Drinnenberg et al. 2014). The presence of holocentric chromosomes in treehoppers, therefore, may be one of the factors promoting chromosomal evolution in this group, but does not explain why most rearrangements seem to be limited to the autosomes. Further investigation is also needed to understand the apparent differences in rates of chromosomal evolution among holocentric clades.

Conclusion

We find that X chromosome identity is largely conserved among treehopper species spanning 45 million years. We also find that a chromosomal fusion likely underlies the formation of a neo-XY sex chromosome system. Combining our data with published hemipteran genomes, we observe that the ancestral treehopper X chromosome is homologous to other hemipteran X chromosomes, indicating long-term conservation of the X across more than 300 million years of evolution.

Materials and Methods

Sample Collection

Adult females and males of *Micrutalis calva*, *Thelia bimaculata*, and *Vanduzeea arquata* were collected from the wild in New Jersey, USA during July 2019. Adult female and male

E. carinata, *Publilia reticulata*, and *U. crassicornis* were obtained from greenhouse populations housed at Princeton University in September/October 2019.

Adult females and males of *Bolbonota melaena*, *Calloconophora caliginosa*, *C. clavata*, *C. claviger*, *Membracis foliatafasciata*, *Horiola picta*, and *Neotynelia pubescens* were collected from the campus of UNESP-São Paulo State University, Rio Claro, Brazil between 2020 and 2021.

Umbonia crassicornis Genome Assembly

Genome Assembly

The genome of *U. crassicornis* was assembled using a single lab-reared adult female (collected in December 2016) that originated from a population in Ft. Lauderdale, FL, USA. Linked-read libraries were constructed using the 10x Genomics platform and sequenced on the Illumina HiSeq X platform, generating 150-bp paired-end reads from approximately half a lane. An additional lab-reared female from the same host plant was collected for Hi-C library preparation to aid in scaffolding the genome assembly.

Laboratory Methods

High molecular weight DNA was extracted using Qiagen Genomic Tip kits (Qiagen, USA, Catalog #10223) with slight modifications to the manufacturer's protocol.

Specimens were gently homogenized with a pestle over dry ice, followed by the addition of 350 μ L of buffer ATL and 4 μ L of RNase A. After incubation at 37°C for 30 min, 50 μ L of proteinase K and 1 mL of G2 buffer were added, and the samples were incubated overnight at 50°C. The standard genomic tip protocol was then followed, with centrifugations performed at 12,000 $\times g$ for 30 and 15 min. DNA was eluted in 50 μ L of TE buffer.

Hi-C Sequencing

To scaffold the 10 \times genome drafts, in situ Hi-C libraries were prepared following a previously described method (Jones et al. 2023). Tissue from a single individual was cross-linked, and nuclei were lysed while maintaining their integrity. DNA was restricted, and overhangs were filled in with a biotinylated base. Free ends were then ligated together in situ, followed by reversal of cross-links and shearing of DNA to 300- to 500-bp fragments. Biotinylated ligation junctions were isolated using streptavidin beads, and the recovered material was used for Illumina library construction. This involved end-repair using T4 DNA polymerase, Klenow polymerase, and T4 polynucleotide kinase, followed by A-tailing with Klenow fragment (3' to 5' exo minus) and dATP. Illumina adapters with a single "T" base overhang were ligated to the DNA fragments. The ligated DNA was PCR amplified for 8 to 12 cycles using Illumina primers, and library fragments of 400 to 600 bp were purified using solid-phase reversible immobilization beads. The purified DNA was captured on an Illumina flow cell for cluster generation and sequenced following the manufacturer's protocols.

Short-Read Genomic Sequencing of 13 Treehopper Species

For the New Jersey wild-caught samples and Princeton University greenhouse samples, DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. For the samples from Brazil, DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, WI, USA). Each sample includes DNA from a single individual (individuals were not pooled). For *U. crassicornis* and *T. bimaculata*, the head and wing tissue were used for DNA extraction. For *B. melaena*, *C. caliginosa*, *C. clavata*, *C. claviger*, *M. foliatofasciata*, *H. picta*, and *N. pubescens*, DNA was extracted from the thorax, leg, and head (excluding eye) tissue. For the remaining species, the whole body was used for DNA extraction. Homogenization was done by cutting the tissue into small pieces with fine scissors. All libraries were prepared and sequenced at the Center for Genomic Research at the University of Liverpool using standard protocols. DNA was sequenced on the Illumina NovaSeq 6000 S1 and S4, resulting in on average 259 million 150-bp paired-end reads per individual.

Identification and Comparison of X-Linked Sequences

For *U. crassicornis*, paired-end reads from two males and two females were mapped to the reference genome using BWA (Li and Durbin 2009) version 0.7.17 with default settings. Uniquely mapped reads were extracted using the grep command "XT:A:U". SOAPcov v2.7.9 (<https://github.com/aquaskyline/SOAPcoverage>) was then used to calculate coverage depth for each scaffold and in 50-kb windows across the genome. The log ratio of male-to-female coverage was calculated for each scaffold, and for each window, using a custom script.

For each of the remaining species, female paired-end reads were used for de novo genome assembly using SOAPdenovo2 (Luo et al. 2012) r242. All reads were used during the contig and scaffold assembly steps and option -F was used during scaffolding. We used GapCloser version 1.12 (<https://anaconda.org/bioconda/soapdenovo2-gapcloser>) to close gaps generated in the scaffolding step. The optimum kmer value for each assembly was determined using kmergenie (Chikhi and Medvedev 2014) version 1.7051. We used BUSCO (Manni et al. 2021) version 5.7.1 to assess completeness for each assembly against database hemiptera_odb10. We also used BUSCO to determine the representation of bacterial and archaeal sequences. Although the assemblies show varying levels of bacterial and archaeal BUSCOs, we subsequently anchored our de novo scaffolds to the *Umbonia* assembly (see below) so that contaminating bacterial or archaeal sequences are not included in our analyses and therefore not expected to impact our results. Once each species' genome had been assembled, we mapped the male and female reads of each species to its genome assembly using BWA (Li and Durbin 2009) version 0.7.17 with default settings. We then used SOAPcov v2.7.9 (<https://github.com/aquaskyline/SOAPcoverage>) to calculate the coverage depth for each scaffold and used our custom script to obtain the log ratio of male-to-female coverage. Scaffolds with $\log_2(M:F)$ coverage less than the median coverage -0.5 were designated as X-linked. Scaffolds with $\log_2(M:F)$ coverage greater than or equal to the median coverage -0.5 were designated as autosomal.

To compare X chromosome identity among taxa, we used BLAST to assign inferred coding sequences from each taxon to a chromosomal location in the *Umbonia* genome. We first downloaded a publicly available transcriptome assembly for *E. carinata* (Fisher et al. 2020). These sequences were BLASTed against the *U. crassicornis* genome assembly using BLASTn (Altschul et al. 1990) version 2.11.0+ with parameters `-perc_identity 30 -evalue 10e-10`. In cases where there was more than one hit, the match with the higher bitscore and percent identity was chosen. We then BLASTed the *E. carinata* transcriptome sequences to each of our de novo assemblies, again using parameters `-perc_identity 30 -evalue 10e-10` and choosing

the match with the higher bitscore and percent identity in cases of multiple hits. Next, for each taxon, we intersected these BLAST results and the $\log_2(M:F)$ coverage values using a custom script to assign putative autosomal and X-linked sequences to their corresponding location in the *U. crassicornis* genome.

Comparing X-Linked Sequences among Auchenorrhyncha Families

We downloaded the coding sequences and gtf/gff annotation files for two previously published chromosome-level genome assemblies of representative Auchenorrhyncha species: *H. vitripennis* (Family Cicadellidae), and *N. lugens* (Family Delphacidae).

The *H. vitripennis* data (UT_GWSS_2.1) were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_021130785.1/), and the *N. lugens* data were downloaded from InsectBase (<http://v2.insect-genome.com/Organism/572>). For each species, coding sequences were BLASTed against the *E. carinata* transcriptome (Fisher et al. 2020) using parameters `-perc_identity 30 -evalue 10e-10` and choosing the match with the higher bitscore and percent identity in cases of multiple hits. We then used each genome's annotation file to locate X-linked and autosomal sequences and compared these to our previous BLAST results between *E. carinata* and *U. crassicornis* to determine whether they correspond to X-linked or autosomal treehopper sequences.

Cytology

Chromosomal analyses were performed by inspection of meiotic and mitotic cells obtained from male testis stained with 4',6-diamidino-2-phenylindole. The diploid number and sex chromosomes were determined here for *C. claviger*, *C. clavata*, *C. caliginosa*, and *E. carinata* while for the other species, the data are published (Boring 1907; Kornhauser 1919; Halkka and Heinonen 1964; Escudero and Virkki 1976; Anjos et al. 2019), except for *P. reticulata* in which we did not have proper material for chromosomal analysis (supplementary table S2, Supplementary Material online). For the FISH mapping of the telomeric probe in *C. caliginosa*, we followed a published protocol (Cabral-de-Mello and Marec 2021). The insect telomeric probe was synthesized by nontemplate PCR according to (Ijdo et al. 1991) using the self-complementary primers (TTAGG)₅ and (CCTAA)₅ and labeled with digoxigenin-11-dUTP by nick translation.

Supplementary Material

Supplementary material is available at *Genome Biology and Evolution* online.

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Data Availability

All raw sequence data are deposited under BioProject ID PRJNA1106845 and the *Umbonia crassicornis* genome assembly is deposited under BioProject ID PRJNA1122077 in the NCBI Sequence database.

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