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Letter

Phylogenomics resolves key relationships in *Rumex* and uncovers a dynamic history of independently evolving sex chromosomes

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Abstract

Sex chromosomes have evolved independently many times across eukaryotes. Despite a considerable body of literature on sex chromosome evolution, the causes and consequences of variation in their formation, degeneration, and turnover remain poorly understood. Chromosomal rearrangements are thought to play an important role in these processes by promoting or extending the suppression of recombination on sex chromosomes. Sex chromosome variation may also contribute to barriers to gene flow, limiting introgression among species. Comparative approaches in groups with sexual system variation can be valuable for understanding these questions. *Rumex* is a diverse genus of flowering plants harboring significant sexual system and karyotypic variation, including hermaphroditic and dioecious clades with XY (and XYY) sex chromosomes. Previous disagreement in the phylogenetic relationships among key species has rendered the history of sex chromosome evolution uncertain. Resolving this history is important for investigating the interplay of chromosomal rearrangements, introgression, and sex chromosome evolution in the genus. Here, we use new transcriptome assemblies from 11 species representing major clades in the genus, along with a whole-genome assembly generated for a key hermaphroditic species. Using phylogenomic approaches, we find evidence for the independent evolution of sex chromosomes across two major clades, and introgression from unsampled lineages likely predating the formation of sex chromosomes in the genus. Comparative genomic approaches revealed high rates of chromosomal rearrangement, especially in dioecious species, with evidence for a complex origin of the sex chromosomes through multiple chromosomal fusions. However, we found no evidence of elevated rates of fusion on the sex chromosomes in comparison with autosomes, providing no support for an adaptive hypothesis of sex chromosome expansion due to sexually antagonistic selection. Overall, our results highlight a complex history of karyotypic evolution in *Rumex*, raising questions about the role that chromosomal rearrangements might play in the evolution of large heteromorphic sex chromosomes.

Keywords: phylogenetics, evolutionary genomics, sex chromosome, plants

Lay Summary

Sex chromosomes have arisen independently in different groups of organisms. The reasons why some groups of organisms evolve sex chromosomes while others do not, as well as the consequences of this variation, are uncertain. Chromosomal rearrangements (changes in the number and configuration of chromosomes) are thought to be an important factor in promoting sex chromosome formation by linking favorable combinations of genes. Once arisen, sex chromosomes may contribute to species formation by harboring genetic incompatibilities between populations. We investigated these questions using genetic data in *Rumex*, a group of flowering plants with diverse sexual systems and sex chromosome configurations. Analyses of evolutionary relationships suggested two independent origins of XY sex chromosomes, arising from hermaphroditic (male and female reproductive organs in the same individual) ancestors. We also found evidence for hybridization predating the formation of these sex chromosomes and among contemporary hermaphroditic species, consistent with the role of sex chromosomes in maintaining species barriers. Finally, rates of chromosomal rearrangement were generally high in species with sex chromosomes but were not elevated in the sex chromosomes specifically compared to other chromosome pairs. Overall, our results highlight the evolutionary consequences of repeated sex chromosome formation and raise questions about the role of chromosomal rearrangement in this process.

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Introduction

Despite the near universality of sexual reproduction in eukaryotes, the mechanisms that determine an organism's sex vary widely (Bachtrog et al., 2014; Bull, 1983). In those with separate male and female individuals, sex is often genetically determined by the inheritance of sex chromosomes. Classic models propose that sex chromosomes arise from a pair of ancestral autosomes as a resolution to sexually antagonistic selection (Charlesworth & Charlesworth, 1980; Rice, 1987). Suppressed recombination linking antagonistic alleles to the sex in which they are beneficial results in differentiation of the sex chromosomes and often degeneration of the Y or W chromosome over time (Bachtrog, 2013; Charlesworth, 1991; Charlesworth & Charlesworth, 2000). However, these models in isolation are unable to explain why there is so much variation in the age, degree of degeneration, and rate of turnover of sex chromosomes across eukaryotes. Some groups—such as mammals (Graves & Watson, 1991; Hughes & Page, 2015) and birds (Fridolfsson et al., 1998; Handley et al., 2004)—have mostly ancient, highly conserved, and highly differentiated sex chromosomes. Others, such as amphibians, fishes, and flowering plants, have much younger sex chromosomes and exhibit rapid sex chromosome turnover, with polymorphisms among closely related species or even within species (Jeffries et al., 2018; Ming et al., 2011, El Taher et al., 2021). The macroevolutionary consequences of this variation are also of interest, as sex chromosomes are known to have significant impacts on factors such as the evolution of gene expression and reproductive incompatibilities.

Recent work has begun to address the potential causes and consequences of sex chromosome variation among species. One important factor is the role of chromosomal rearrangements. The distinct genetic architecture of sex chromosomes may favor the evolution of large-scale chromosomal rearrangements, contributing significantly to the evolution of karyotypic differences among species (Charlesworth et al., 1987; Connallon et al., 2018; White, 1940). Certain kinds of rearrangements, such as inversions and sex chromosome-autosome fusions, are thought to enable rapid linkage of sexually antagonistic variation to the sex-determining region by extending the region of recombination suppression (Charlesworth & Charlesworth, 1980; Charlesworth et al., 2005; Rice, 1987). Alternatively, inversions that capture beneficial haplotypes on the Y or W chromosome may promote recombination suppression and degeneration in the absence of sexual antagonism (Lenormand & Roze, 2022; Lenormand et al., 2020). Sex chromosome-autosome fusions may also be favored to reduce aneuploidy in heterogametic sex chromosomes (Blackmon & Brandvain, 2017). While the importance of structural rearrangements for the formation of new evolutionary strata (Bergero et al., 2007; Handley et al., 2004; Lahn & Page, 1999) and neo-sex chromosomes (Bracewell et al., 2017; Castillo et al., 2014; Kitano et al., 2009; Pala et al., 2012) is increasingly well understood, their contributions to macroevolutionary patterns of sex chromosome variation remain understudied. Comparative approaches can be used to test whether sex chromosome-autosome fusions occur at a higher rate than autosomal fusions, as expected if fusions with sex chromosomes are favored as recombination modifiers due to sexually antagonistic selection (Anderson et al., 2020; Charlesworth & Charlesworth, 1980). Estimating the evolutionary timescale of recombination suppression relative to the origins of chromosomal inversions can also be informative for theories of sex chromosome degeneration. Thus, comparative genomic approaches in a phylogenetic context can be valuable for understanding such processes of karyotype evolution.

A long-standing body of literature predicts a disproportionate contribution of sex chromosomes to reproductive isolation among species. This may occur due to the unmasking of recessive incompatibilities in the heterogametic sex (i.e., Haldane's rule; Haldane, 1922; Laurie, 1997; Schilthuizen et al., 2011), faster rates of molecular evolution on the sex chromosomes (i.e., the faster X-effect; Charlesworth et al., 1987; Meisel & Connallon, 2013), and/or enrichment of mutations with impacts on hybrid fitness (i.e., the large X-effect; Coyne, 1992; Presgraves, 2018). In addition, reduced recombination on the sex chromosomes may result in stronger linked selection against introgressed haplotypes containing deleterious variants (Brandvain et al., 2014; Geraldes et al., 2011; Martin et al., 2019). Chromosomal rearrangements can play an important role in these processes on both sex chromosomes and autosomes; for instance, co-adapted "supergene" complexes can be maintained in the face of gene flow by large chromosomal inversions (Kirkpatrick & Barton, 2006; Lowry & Willis, 2010; Tuttle et al., 2016). Empirical investigations of these ideas have focused primarily on barriers to contemporary hybridization among extant species, and comparatively few studies have investigated the impacts of sex chromosome variation on long-term patterns of introgression and the maintenance of species barriers (but see Dufresnes et al., 2020; Xue et al., 2024). Investigating such patterns will aid in building an understanding of the role that sex chromosomes play in the process of speciation and diversification.

The docks and sorrels (*Rumex*) offer exciting possibilities for investigating the evolutionary dynamics of sex chromosomes and chromosomal rearrangements. A globally distributed plant genus with approximately 200 described species (Grant et al., 2022), *Rumex* harbors significant sexual system diversity, ranging from hermaphroditism to dioecy (Löve & Kapoor, 1967, Navajas-Perez et al., 2005). Notably, karyotypes across the genus are highly variable, including successive reductions in chromosome number (Navajas-Perez et al., 2005), the evolution of XY sex determination systems in dioecious species, and within-species sex chromosome polymorphism (e.g., *R. hastatulus*—Beaudry et al., 2020, 2022; Hough et al., 2014; Ming et al., 2011; Rifkin et al., 2021; Smith, 1964). Populations of *R. hastatulus* from eastern N. America, in addition to multiple species in a separate clade of *Rumex*, have evolved an XYY sex chromosome system from an ancestral XY configuration. This process can arise via an X-autosome fusion, as is thought to have occurred in *R. hastatulus* (Grabowska-Joachimiak et al., 2015; Kasjaniuk et al., 2019; Sacchi & Humphries et al., 2024; Smith, 1964), or potentially via fission of the Y chromosome. Comparatively close hermaphroditic relatives of dioecious species allow for the identification of ancestral autosomal homologs of the sex chromosomes, providing a powerful genomic framework for studying the forces promoting sex chromosome evolution. Previous work on *R. hastatulus* uncovered genome-wide recombination suppression, including in the autosomal homolog of the neo-X chromosome found in eastern populations (Rifkin et al., 2021, 2022), Y chromosome degeneration (Sacchi & Humphries et al., 2024), and a role for sex chromosome differences in shaping barriers to contemporary hybridization (Beaudry et al., 2022). However, sex chromosome evolution across the rest of the genus remains poorly understood.

Previous work constructed phylogenies of *Rumex* using nuclear and chloroplast markers (Grant et al., 2022; Koenemann et al., 2023; Navajas-Perez et al., 2005). These studies have agreed that there are two primary clades with sex chromosomes; one clade with an ancestral XY system (including *R. hastatulus*, which also has XYY populations), and another with an ancestral XYY system.

However, these phylogenies disagree on the placement of *R. bucephalophorus*, a hermaphroditic/gynomonoecious (plants with both hermaphroditic and female flowers) species (see Talavera et al., 2011) that lacks sex chromosomes, relative to these clades (Figure 1). The nuclear and chloroplast trees of Navajas-Pérez et al. (2005) placed the XY and XYY clades as sister, with *R. bucephalophorus* more distantly related (Figure 1A), whereas the other two chloroplast studies placed *R. bucephalophorus* as sister to the XY clade, and more distantly related to the XYY clade (Figure 1B and C). Significantly, these two phylogenetic hypotheses have different implications for the sequence of events in the evolution of sex chromosomes in the genus. The former suggests the possibility of a single origin of XY sex chromosomes (Figure 1A), whereas the latter requires two independent changes: either two origins of XY sex chromosomes (Figure 1B)—which also has support from preliminary transcriptome-based identification of sex-linked genes (Crowson et al., 2017)—or a single origin followed by a loss of sex chromosomes in *R. bucephalophorus* (Figure 1C). Phylogenies constructed from small numbers of genetic markers can be vulnerable to both technical errors and biological sources of uncertainty such as incomplete lineage sorting and introgression (Degnan & Rosenberg, 2009; Maddison, 1997). Resolving the history of sex chromosome evolution in *Rumex* therefore requires the analysis of genome-scale datasets with modern coalescent approaches. This resolution, combined with comparative genomics, should provide valuable insights into the role of chromosomal rearrangements in shaping the formation of sex chromosomes and species barriers.

Here, we present new transcriptome assemblies for 10 *Rumex* species representing the major clades in the genus. We also generate a new high-quality long-read genome assembly for *R. bucephalophorus* and compare genome structure and gene order with assemblies of several additional species in the genus (Sacchi & Humphries et al., 2024). Applying phylogenomic analyses, we find support for the largely independent evolution of sex chromosomes across two major dioecious clades, consistent with the scenario in Figure 1B. We also find evidence for ancestral introgression events from unsampled lineages, likely predating the formation of sex chromosomes in the genus, but no evidence for more contemporary introgression involving extant dioecious species. Lastly, using synteny-based approaches, we find evidence for extensive chromosomal rearrangements in two dioecious species compared to their hermaphroditic relatives, but no evidence for elevated rates of translocations on the sex chromosomes. Together, our results highlight the potential for pre-existing genomic architecture, in addition to ongoing karyotypic rearrangement, to play a pivotal role in the evolution of sex chromosomes.

Materials and methods

Genomic and transcriptomic data

We conducted RNA-Seq on leaf, flower bud, and pollen tissue, and assembled the transcriptomes of 10 *Rumex* species (Supplementary Table S1) using Trinity v2.11.0 (Grabherr et al., 2011) and EvidentialGene (Gilbert, 2013). In addition to RNA-Seq, we sequenced and assembled the genome of *R. bucephalophorus* using HiFi PacBio sequencing and Dovetail Omni-C sequencing. To complement our new datasets, we obtained recently published genome assemblies of *R. hastatulus* (Sacchi & Humphries et al., 2024) and Tartary buckwheat, *Fagopyrum tataricum* (Zhang et al., 2017), for a total of 12 species. See the *Supplementary materials and methods* section of Supplementary Materials for more detailed sequencing and assembly methods.

Testing for whole-genome duplications

We used the distribution of D_S values between paralogs within each of the 12 species to evaluate the presence of whole-genome duplications (WGDs). We first estimated gene trees from each orthogroup codon alignment (see *Orthogroup identification and alignment* section of Supplementary Materials) using IQ-TREE (Minh et al., 2020) with the default settings. These gene trees and their corresponding alignments were given to the *codeml* method implemented in PAML (Yang, 2007) to estimate values of D_N and D_S between each pair of sequences. A custom Python 3 script was used to extract D_S estimates between pairs of sequences belonging to the same species. We then used the R package *mclust*

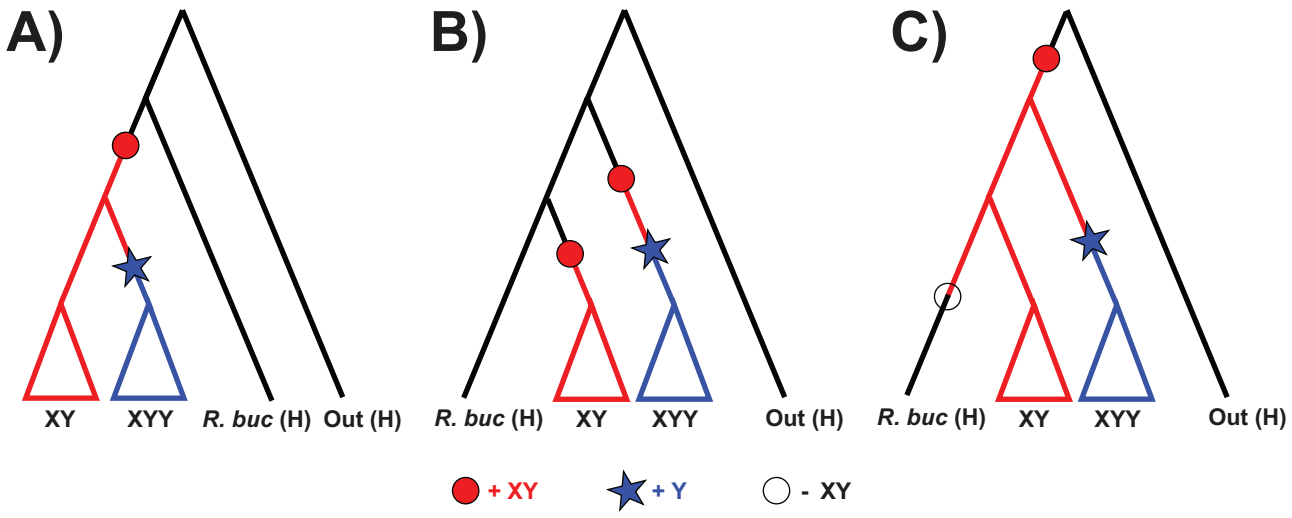

Figure 1. Hypotheses for the evolution and origins of sex chromosomes in *Rumex*. Stars indicate the gain of XY sex chromosomes; filled dots indicate the gain of an additional Y chromosome; and empty dots indicate the loss of XY sex chromosomes. (A) The XY and XYY clades are sister, consistent with the phylogeny of Navajas-Pérez et al. (2005) and implying a single origin of the sex chromosomes. (B) The XY clade is sister to *R. bucephalophorus* and more distantly related to the XYY clade, consistent with the phylogenies of Grant et al. (2022) and Koenemann et al. (2023). This scenario proposes two independent sex chromosome origins, one in each clade. (C) Same phylogeny as in scenario (B), but now proposing a single sex chromosome origin in the ancestor of the XY clade, XYY clade, and *R. bucephalophorus*, followed by a loss of sex chromosomes in *R. bucephalophorus*.

(Scrucca et al., 2016) to evaluate the presence of multimodal distributions of log(D_S) values within each species using the Bayesian information criterion (BIC). We evaluated the fit of models including 1–9 components, each with equal variance or varying variance. The best-fitting model was selected using the minimum BIC value.

Phylogenetic inference

After allowing missing data and single-species duplicates to increase ortholog sampling, we obtained a dataset of 5,263 single-copy genes across 12 species. We used IQ-TREE to estimate a maximum-likelihood phylogeny from a concatenated alignment of all orthologs. To account for the potential effects of incomplete lineage sorting, we also used ASTRAL-III to estimate a summary phylogeny from gene trees estimated for each ortholog. We then time-calibrated our IQ-TREE phylogeny based on fossil evidence and previously estimated node ages (Koenemann et al., 2023). More detailed methods can be found in the *Supplementary materials and methods* section of Supplementary Materials.

Introgression analysis

We tested for introgression among both ancestral and extant lineages using two approaches: the gene tree-based test statistic Δ (Huson et al., 2005; Vanderpool et al., 2020) and a pseudolikelihood approach to estimating phylogenetic networks implemented in the software *PhyloNet* (Than et al., 2008; Yu & Nakhleh, 2015). Δ tests for an asymmetry in discordant gene tree counts for a rooted triplet, a classic signature of introgression (Durand et al., 2011; Green et al., 2010). Phylogenetic network estimation is a likelihood-based approach that constructs a network structure containing horizontal branches that denote introgression events. When significant tests involved overlapping sets of taxa, we collapsed them into more ancestral events based on parsimony. More detailed methods can be found in the *Supplementary materials and methods* section of Supplementary Materials.

Resolving the history of sex chromosome evolution

We conducted three analyses to distinguish between two independent origins of sex chromosomes vs. a single origin followed by a loss in *R. bucephalophorus*. First, we BLAST (Altschul et al., 1990) searched a previously generated list of sex-linked genes in *R. rothschildianus* (Crowson et al., 2017) against the genome of XYY *R. hastatulus* to identify shared homologous genes/regions. Second, we conducted a permutation analysis to test for a significant overlap of sex-linked genes between *R. hastatulus* and *R. rothschildianus*. Third, we estimated gene trees for orthologous genes found in *R. bucephalophorus* and the X and Y chromosomes of *R. hastatulus*, with excess affinity of *R. bucephalophorus* genes to either the X or Y suggesting a potential loss of sex chromosomes. More detailed methods can be found in the *Supplementary materials and methods* section of Supplementary Materials.

Synteny and chromosomal rearrangement analyses

Orthology and synteny between protein-coding genes in *R. bucephalophorus*, *R. salicifolius*, the XY cytotype of *R. hastatulus* (with a chimeric sex chromosome assembly), and both haplotypes of the *R. hastatulus* XYY cytotype (with phased sex chromosome assemblies) were inferred using GENESPACEv1.1.8 (Lovell et al., 2022). GENESPACE uses MCScanX (Wang et al., 2012) to infer syntenic gene blocks and implements ORTHOFINDERv2.5.4 (Emms & Kelly, 2019) and DIAMONDv2.1.4.158 (Buchfink et al., 2021) to find orthogroups within syntenic blocks. Analyses were run and riparian plots were visualized in Rv4.1.0. We used the non-default parameter "onewayBlast = TRUE," which is appropriate for species within the same genus, all other parameters were set to default. *Rumex bucephalophorus* scaffolds 9 and 10 were excluded from the GENESPACE run as they are very likely to represent separately assembled heterozygous copies of other chromosomes based on the expected chromosome number of 8, and the strong similarity of these scaffolds with fragments from other main scaffolds (Supplementary Figure S2) Scaffolds with fewer than 500 genes were excluded from the plots in all cases. In addition to visualization, we used the outputs of GENESPACE to obtain broad estimates of rates of chromosomal rearrangement in *R. bucephalophorus* and *R. hastatulus* relative to *R. salicifolius* (assuming it carries the ancestral karyotype) by counting syntenic regions. See the *Supplementary materials and methods* section of Supplementary Materials for more detailed information.

Results

Sequencing and assembly

We assembled transcriptomes from RNA-Seq data for 10 species that are representative of the major clades of *Rumex* (Supplementary Data S2). Our assemblies were broadly high-quality, with BUSCO-completeness scores ranging from 89% to 95%. The number of main transcripts varied from 23,000 in *R. bucephalophorus* to 56,000 in *R. thyrsiflorus* and was positively correlated with genome size estimates from flow cytometry (Supplementary Data S1). A total of 81% of BUSCO genes were duplicated in *R. thyrsiflorus*; this value ranged from 5% to 16% in other species. Given the lack of previous evidence for polyploidy in this species (Supplementary Table S5), one possible explanation is high heterozygosity driving spurious assembly of multiple haplotypes of the same gene.

We additionally generated a high-quality chromosome-scale assembly of *R. bucephalophorus* using high-coverage HiFi PAC Bio sequencing and Dovetail Omni-C sequencing (Supplementary Figure S1; Supplementary Table S2). After removing erroneously separately assembled chromosome haplotypes (see Supplementary Figures S2 and S4), the assembly size is 2.062 GB (compared to flow cytometry estimates of 1.96 GB; Supplementary Data S1), with 88% of the genome found in the main eight scaffolds, consistent with karyotypic evidence for eight autosomes in the species (Navajas-Perez et al., 2005).

Mixed evidence for recent whole-genome duplications

Whole-genome duplications, if left unaccounted for, can have consequences for phylogenetic inference. Previous work has identified an ancient WGD shared by buckwheat and *Rumex* (Fawcett et al., 2023; Zhang et al., 2017). In addition, *R. acetosella*, *R. scutatus*, and *R. paucifolius* are known to have natural polyploid populations (Supplementary Table S5; Löve, 1940; Löve, 1942; Smith, 1968). To further assess the presence of recent polyploidy events in our dataset, we calculated D_S values between gene paralogs for each species. In the absence of WGDs, D_S values should be exponentially distributed (normally distributed in log-space) following a birth–death model for gene gain and loss (Blanc & Wolfe, 2004; Lynch & Conery, 2000). Whole-genome duplications introduce numerous gene duplications at the same point in time, which should result in additional peaks in the distribution of D_S values

between paralogs. We found mixed evidence for WGDs in *R. acetosella*, *R. paucifolius*, and *R. thyrsiflorus* (Supplementary Figures S3 and S5). Autopolyploidy events are unlikely to substantially affect our inferences, given our approach to retaining orthologues, but allopolyploidy could still manifest as a signal of introgression (see *Discussion* section; *Supplementary materials and methods* section of Supplementary Materials for additional details).

Whole-transcriptome phylogeny supports *R. bucephalophorus* as sister to the XY clade

We used our 12-species transcriptomic/genomic dataset to infer a phylogeny of *Rumex* using both concatenated maximum-likelihood (IQ-TREE) and gene tree summary (ASTRAL-III) approaches. Our maximum-likelihood tree was estimated with strong statistical and genealogical support, with all nodes having 100% support in both SH-aLRT and ultrafast bootstrap measures (Figure 2). The topology is in general agreement with previous studies, supporting two monophyletic clades with sex chromosomes and two earlier-diverging hermaphroditic clades. We place *R. bucephalophorus* as sister to the XY clade, to the exclusion of the more distant XYY clade, in agreement with the chloroplast phylogenies of Grant et al. (2022) and Koenemann et al. (2023), but in contrast to the phylogeny of Navajas-Pérez et al. (2005). We also infer a sister relationship between *R. hastatulus* and *R. paucifolius*; this inference agrees with Koenemann et al. (2023), but contrasts with Navajas-Pérez et al. (2005) and Grant et al. (2022), who placed *R. hastatulus* as sister to *R. acetosella*. Our divergence time estimates generally agree with those of Koenemann et al. (2023), though we infer an older node age for the XY clade (5.6 MYA vs. 2.61 MYA), and a more recent node age for the root (16 MYA vs. 22.13 MYA).

Gene tree discordance varied among clades but was not prevalent enough to generate substantial phylogenetic uncertainty. Highlighting this, our ASTRAL-III phylogeny returned the same topology as maximum-likelihood (Supplementary Figure S6). As a gene tree summary approach, ASTRAL-III is more robust to high rates of incomplete lineage sorting that can mislead standard ML approaches (Mirarab et al., 2014). Gene concordance factors (gCFs), a measure of the proportion of gene trees in the dataset consistent with each branch, varied from 47.1% to 93.7% (Figure 2; Supplementary Table S3). The lowest gCF was at the node where *R. bucephalophorus* splits from the ancestor of the XY clade, at 47.1%. We observed similar patterns using site concordance factors (sCFs; Supplementary Table S4). This finding helps explain the uncertainty in its placement in previous studies, as it suggests that an individual locus such as the chloroplast or a nuclear marker has a high chance of being discordant with respect to this node. In contrast, most branches in the phylogeny exhibit modest levels of discordance, being supported by between 60% and 94% of gene trees (Figure 2). Given these values, each node in the phylogeny is supported by an excess of hundreds to thousands of gene trees, so we can be confident that our statistical support measures (SH-aLRT and ultrafast bootstrap) are returning strong support for the true species relationships, and not alternate histories generated by incomplete lineage sorting (ILS) and/or introgression.

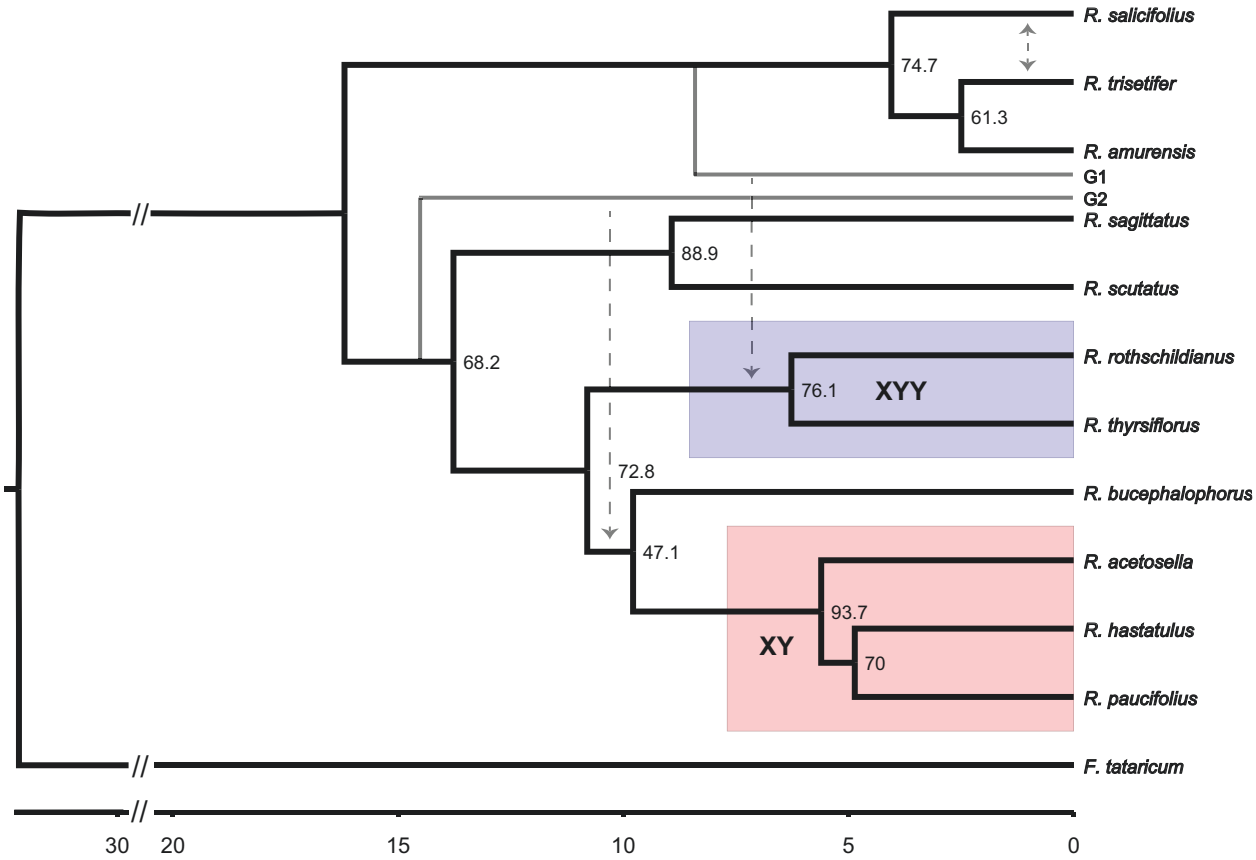

Figure 2. Whole-transcriptome maximum-likelihood phylogeny of 11 *Rumex* species, with branch length units in millions of years (x-axis scale). Branch length distance to the root is truncated for visual clarity. The XY and XYY clades are highlighted and labelled with separate boxes. Nodes are labeled with gene concordance factors. Grey dashed arrows indicate inferred introgression events. Unsampled lineages involved in introgression events are denoted with the grey branches labeled "G1" and "G2."

Signatures of ghost introgression in the *Rumex* phylogeny

We investigated signatures of introgression among our sampled species using a test statistic, Δ, based on gene tree counts, in addition to inferring phylogenetic networks with *PhyloNet*. Our Δ tests returned a multitude of highly significant results, often implying introgression between lineages that were not contemporaneous (according to the phylogeny of Figure 2) (Supplementary Data S3). On further examination of our results, we observed that many species, when included in one of the sister species positions in a test, often implied the other two species in the test as introgressing with each other, regardless of their identity. For instance, in the triplet [(A,X),Y], where A is a particular species and X and Y could be any two species with the specified relationship, introgression would always be implied between X and Y. This indicates that X is more distantly related to A than expected based on phylogenetic relationships, a classic signature of ghost introgression from an earlier-diverging donor lineage (Supplementary Figure S7) (Ottenburghs, 2020; Tricou et al., 2022a, 2022b). Such ghost introgression events might be expected in our study because we have sampled only 11 of the 200 described species in the genus. These unexpectedly distant species include *R. thyrsiflorus*, *R. rothschildianus*, *R. acetosella*, *R. hastatulus*, *R. paucifolius*, and *R. bucephalophorus*.

Our best-fitting phylogenetic network supports the existence of two ghost introgression events but disagrees with our inferred species tree topology in several places (Supplementary Figure S8). As our phylogeny has strong statistical and genealogical support, we chose to reconcile the phylogeny with our best-fitting phylogenetic network and set of significant Δ statistics to propose two ghost introgression events (Figure 2). The first event was in the common ancestor of the clade containing *R. bucephalophorus* and the XY species. The donor lineage for this event is likely a relatively early-diverging member of the clade containing the two sex chromosome subclades (branch "G2" in Figure 2), possibly *Rumex induratus* or a close relative based on the phylogeny of Grant et al. (2022). The other event may have involved the ancestor of *R. thyrsiflorus* and *R. rothschildianus*, with the donor likely a member of the comparatively early-diverging hermaphroditic clade containing *R. salicifolius* and its relatives (branch "G1" in Figure 2). Finally, we see additional evidence of introgression between *R. salicifolius* and *R. trisetifer* (Figure 2), two closely related hermaphroditic species.

Independent evolution of XY sex chromosomes

Our updated phylogeny of *Rumex* is inconsistent with a simple single-origin scenario (Figure 1A) for the evolution of sex chromosomes (although more complex single-origin scenarios are still possible; see *Discussion* section). We conducted additional analyses to distinguish between the two remaining possibilities: two independent origins of sex chromosomes (Figure 1B) vs. a single origin followed by loss of sex chromosomes in *R. bucephalophorus* (Figure 1C). First, we evaluated the chromosome of origin of sex-linked genes in the two major sex chromosome clades utilizing previous transcriptome-identified sex-linked genes from *R. rothchildianus* (XYY clade) and a recently published genome assembly of *R. hastatulus* (XY clade) (Sacchi & Humphries et al., 2024). In the simplest scenario, where a fully formed sex chromosome evolves once and is inherited by both groups, we would expect sex-linked genes in *R. rothschildianus* to map primarily to the X chromosome of *R. hastatulus* and to find significant overlap between sex-linked genes identified in the two species. Alternatively, if sex chromosomes originated or evolved independently in the two groups, sex-linked genes in *R. rothschildianus* should map to some combination of autosomes and/or the X chromosome and sex-linked genes would not overlap significantly. We found that sex-linked genes in *R. rothschildianus* mapped to all chromosomes of *R. hastatulus*, with most mapping to segments at the end of autosome 1 and the highest enrichment at the beginning of autosome 2, followed a smaller number of hits across the X and autosome 4 (Figure 3).

We observed several hits on the *R. hastatulus* X chromosome, raising the possibility that the two sex chromosome clades share a very small "core" sex-determining region with a single origin, with chromosomal rearrangements within the two clades resulting in independent origins for other regions of the X. To evaluate this hypothesis further, we separately examined chromosome of origin for hemizygous genes and genes with a Y copy present in *R. rothschildianus*. Hemizygous genes are generally expected to be older and would therefore more likely be found in an older shared sex-determining region, whereas genes with a Y copy are expected to be younger and therefore more common in younger, independently evolving regions of the sex chromosomes. In this case, most hemizygous genes mapped to the region containing the first 50 MB of autosome 2 of *R. hastatulus*, the region most enriched for XY-copy genes, with much smaller numbers of genes distributed across the remaining chromosomes (Figure 3, right column). In addition, neither XY-copy genes nor hemizygous genes in *R. rothschildianus* overlapped significantly ($p = 0.628$ and 0.15, respectively) with hemizygous genes identified from the genome of *R. hastatulus*. Overall, these results are most consistent with the independent evolution of the sex chromosomes, though we cannot rule out the single origin of a very small sex-determining region comprised of a handful of genes shared by the two groups, followed by independent expansion of sex chromosomes.

No ancestral sex chromosome system in *R. bucephalophorus*

To further resolve the history of sex chromosome evolution, we next examined the relationship of *R. bucephalophorus* genes to orthologous X/Y gametologs present in *R. hastatulus*. Evolutionary loss of sex chromosomes is generally expected to proceed via an inactivating mutation on one of the sex chromosomes that restores sex-specific functions (e.g., Vicoso & Bachtrog, 2013) and in this case, produces hermaphroditic individuals. The mutated sex chromosome then becomes an autosome, whereas the other is lost from the population. Therefore, if *R. bucephalophorus* lost an XY system that is shared by the two extant XY clades, formerly sex-linked genes should coalesce primarily with either the X chromosome (in the case of loss of the Y) or Y chromosome (in case of loss of the X) of *R. hastatulus* (Figure 4A). Alternatively, if the sex chromosomes arose independently in the XY and XYY clades, and *R. bucephalophorus* has simply retained the ancestral hermaphroditic state, then XY gametologs in *R. hastatulus* should coalesce with each other before their ortholog in *R. bucephalophorus* (Figure 4A), consistent with the phylogeny. This sets up a symmetric expectation, where a loss of sex chromosomes in *R. bucephalophorus* should lead to most trees having one of the two possible discordant histories (Figure 4A).

Out of 397 single-copy genes present in the old sex-linked region of the *R. hastatulus* X and Y chromosomes and *R. bucephalophorus*, 229 (57.7%) support the species phylogeny, 80 (20.1%) support the loss of Y scenario, and 88 (22.2%) support the loss of X scenario (Figure 4B). This suggests a historical absence of sex

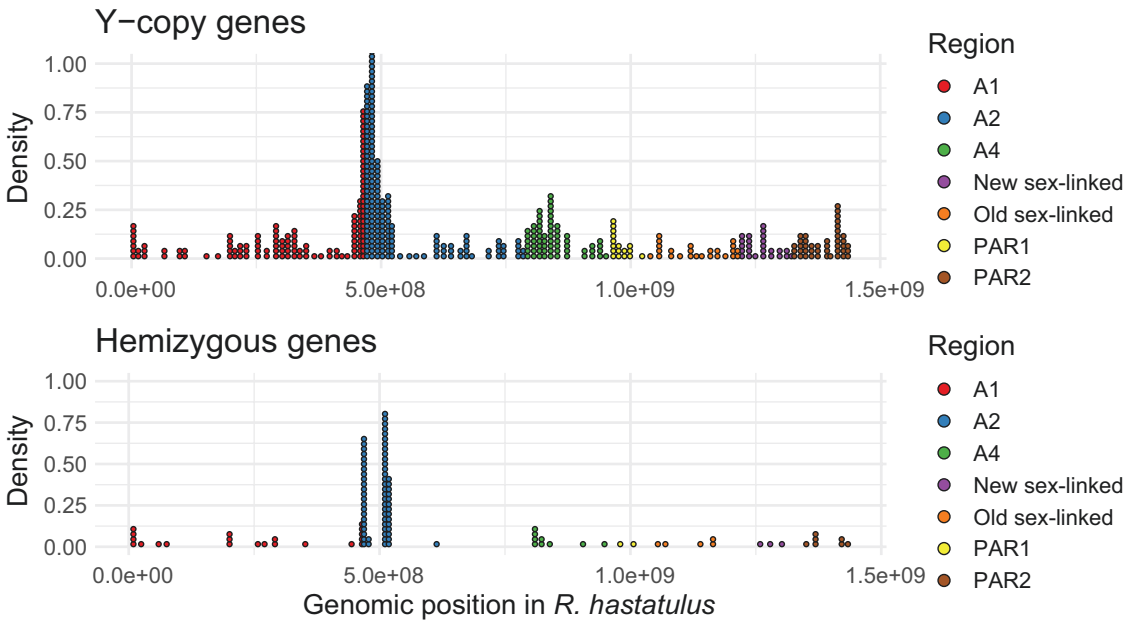

Figure 3. Distribution of BLAST hits of sex-linked genes in *Rumex rothschildianus* against the genome of *R. hastatulus*, divided into 10 Mb windows. In each plot, the x-axis is the genomic position at the start of the window, and the y-axis is the density of BLAST hits found within that window. The top row is X-linked genes in *R. rothschildianus* where a Y copy is still present; the bottom row is hemizygous X-linked genes. Chromosomal regions in *R. hastatulus* are color-coded. Note that we searched against the XYY cytotype of *R. hastatulus*, which does not contain an autosome 3. To aid in the comparison of adjacent regions, each dot represents a discrete portion of the total density within a window.

chromosomes in *R. bucephalophorus*; therefore, our results support two independent gains of sex chromosomes. Interestingly, the proportion of discordant topologies (42.3%) is much larger than the genome-wide average, where 94% of gene trees support the monophyly of the XY clade (Figure 2). One possible explanation for this pattern is that the sex chromosomes evolved relatively quickly in the common ancestor of the XY clade after its split from *R. bucephalophorus* (Figure 4C). The short amount of time separating this split from the divergence of X/Y gametologs would result in higher rates of incomplete lineage sorting (Figure 4C), producing the two discordant histories with equal frequency. Treating the X and Y as individual tips in a multispecies coalescent framework, and accounting for reduced effective population size of the sex chromosomes, our results are consistent with recombination suppression between X and Y arising approximately 500,000 years after the speciation of *R. bucephalophorus* with the XY clade, or ~9.5 MYA based on our molecular clock estimates (see *Supplementary materials and methods* section of Supplementary Materials for derivation of this result).

Broadly high rates of chromosomal rearrangement in *Rumex*

To further investigate the origin and evolution of sex chromosomes in *R. hastatulus*, we sought to identify the ancestral autosomal homologs of the sex chromosomes in the XY clade and investigate the history of rearrangements among these chromosomes relative to the autosomes. We conducted synteny analysis on genome assemblies of *R. salicifolius* (Sacchi & Humphries et al., 2024), both cytotypes of *R. hastatulus* (Sacchi & Humphries et al., 2024), and our de novo assembly of *R. bucephalophorus* using the software GENESPACE (Lovell et al., 2022) (Figure 5), and quantified chromosomal rearrangements by counting inferred syntenic regions (see *Methods* section; *Supplementary materials and methods* section of Supplementary Materials). We found that rates of chromosomal rearrangement are generally very high, with an apparent elevation in the genome of *R. hastatulus*. For interchromosomal rearrangements estimated from mapping of syntenic regions, we estimate 0.74 rearrangements per million years in *R. bucephalophorus*, and 1.2 per million years in *R. hastatulus*. For the rate of synteny evolution, we estimate 9.2 synteny breakpoints per million years in *R. bucephalophorus*, and 14 per million years in *R. hastatulus*. In contrast, Damas et al. (2022) estimated an average rate of 2 synteny breakpoints per million years in mammals, highlighting that chromosomal evolution is rapid across the board in *Rumex*. Nevertheless, these results suggest an elevated rate of chromosomal rearrangement across the genome of *R. hastatulus*, or possibly the XY clade at large (though additional genome sequencing of *R. acetosella/paucifolius* would be needed to confirm this possibility). *Rumex acetosella* and *R. paucifolius* both have reduced chromosome number ($n = 7$) relative to *R. bucephalophorus* ($n = 8$) (Navajas-Perez et al., 2005), so it is likely that at least some of this rearrangement is ancestral to the XY clade.

The sex chromosomes of *R. hastatulus* have complex chromosomal origins, with key regions sharing syntenic blocks with different chromosomes in hermaphroditic relatives (Figure 6). The old sex-linked region shared by both cytotypes (dark blue in Figure 6) is orthologous primarily with chromosome 3 of *R. bucephalophorus*, with smaller syntenic blocks on chromosomes 1 and 6; the PAR for this region (light blue, Figure 6) is also orthologous to chromosome 3. The neo-sex-linked region (yellow, Figure 6) contains syntenic blocks from chromosomes 1 and 7 of *R. bucephalophorus*, while the neo-PAR (light red, Figure 6) is orthologous with chromosome 8. These three syntenic blocks are all present on autosome 3 of XY *R. hastatulus*, suggesting their fusion predates the sex chromosome-autosome fusion that formed the XYY cytotype. A relatively small central region of chromosome 1 in *R. bucephalophorus* has independently contributed syntenic blocks to both the old and neo-X chromosomes of *R. hastatulus*, identifying this as an interesting region for future study. Finally, a reciprocal translocation of the two Y chromosomes in the XYY cytotype has

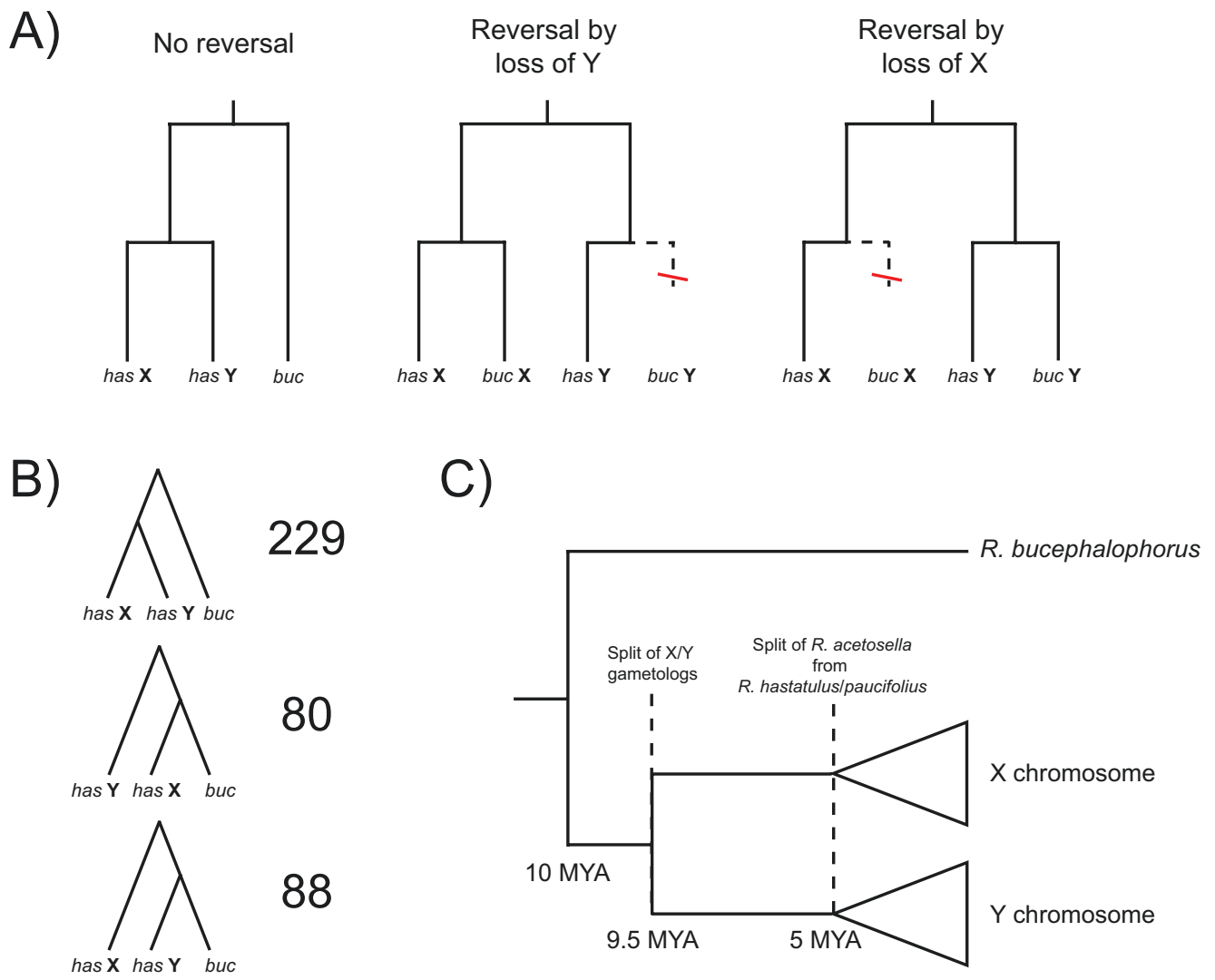

Figure 4. Relationship of *Rumex hastatulus* X/Y gametologs to *R. bucephalophorus*. (A) Three scenarios for the evolution of sex chromosomes in *R. bucephalophorus*. For each scenario, the expected majority gene tree is traced by the solid black line. (B) Counts of the three possible tree topologies in coding sequences shared by *R. bucephalophorus* and the X and Y chromosomes of *R. hastatulus*. (C) Demographic history explaining our observed gene tree counts in panel (B). Recombination suppression between the X and Y arises relatively quickly in the ancestor of the X/Y clade after its split from *R. bucephalophorus*. Subsequent speciation within the XY clade happens later on.

resulted in both old and neo-X syntenic blocks existing in both chromosomes (Figure 6, Sacchi & Humphries et al., 2024).

While the origins of the sex chromosomes of *R. hastatulus* are complex, their overall rates of rearrangement are not exceptional as compared to the autosomes. For interchromosomal rearrangements per million years, we estimate rates of 0.4375, 0.25, and 0.4375 for the X, Y_1, and Y_2 chromosomes, respectively, compared to rates of 0.5, 0.375–0.4375, and 0.1875 for autosomes 1, 2, and 4, respectively. These rates are correlated primarily with chromosome size. For synteny breakpoints per million years (either intrachromosomal or interchromosomal), we find rates of 4.93, 1.31, and 2.875 for the X, Y_1, and Y_2, compared to rates of 3.4, 3.9, and 1.9 for autosomes 1, 2, and 4. Here, the X is slightly higher than expected given its relative size, potentially suggesting an elevated rate of intrachromosomal rearrangement on the X; we do not, however, observe this pattern for the Y chromosomes. An important caveat to these estimates is that degeneration of the old sex-linked regions of the X and Y chromosomes will lead to a complete loss of synteny in some regions; as a result, we are likely under-estimating the rate of synteny breakpoint evolution on the Y in particular. Regardless, these regions of lost synteny are relatively small (Figure 6) and unlikely to change our conclusion that rates of rearrangement are unexceptional on the sex chromosomes.

Discussion

Resolving phylogenetic relationships and histories of introgression in *Rumex*

Rapid karyotypic evolution across *Rumex* provides a powerful system for studying the evolutionary interplay of sex chromosomes, chromosomal rearrangements, and post-speciation introgression. An important first step in addressing these topics is constructing a well-resolved phylogeny to enable a robust comparative framework. Previous studies estimating phylogenies of *Rumex* focused on sampling a wide range of taxa, at the cost of using a relatively small number (1–3) of genetic markers (Navajas-Perez et al., 2005, Grant et al., 2022; Koenemann et al., 2023). Whereas increased

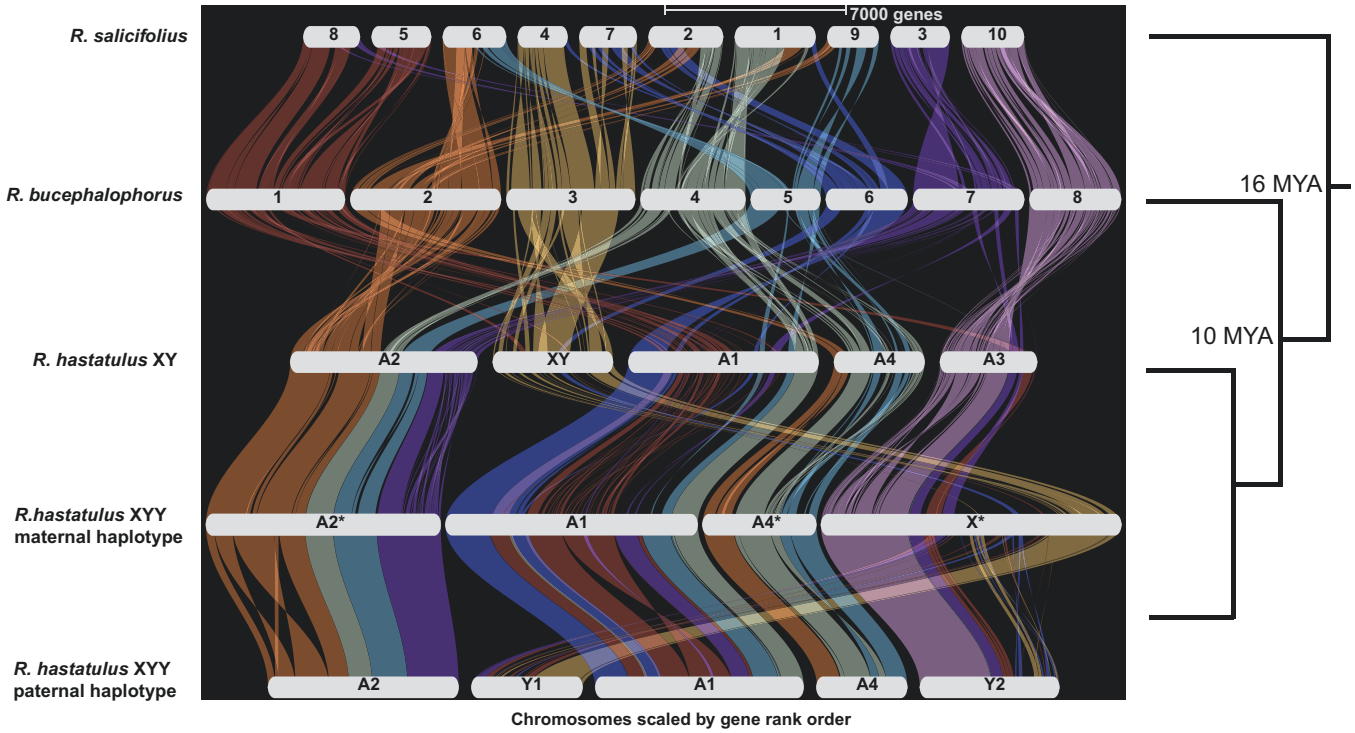

Figure 5. GENESPACE riparian plots showing synteny between *Rumex salicifolius*, *R. bucephalophorus*, and the two cytotypes of *R. hastatulus*. Syntenic blocks are ordered and colored according to the chromosome of origin in *R. bucephalophorus*. Branch lengths on the right-hand tree are not to scale. Note that chromosome size is scaled by gene rank order, rather than by physical size.

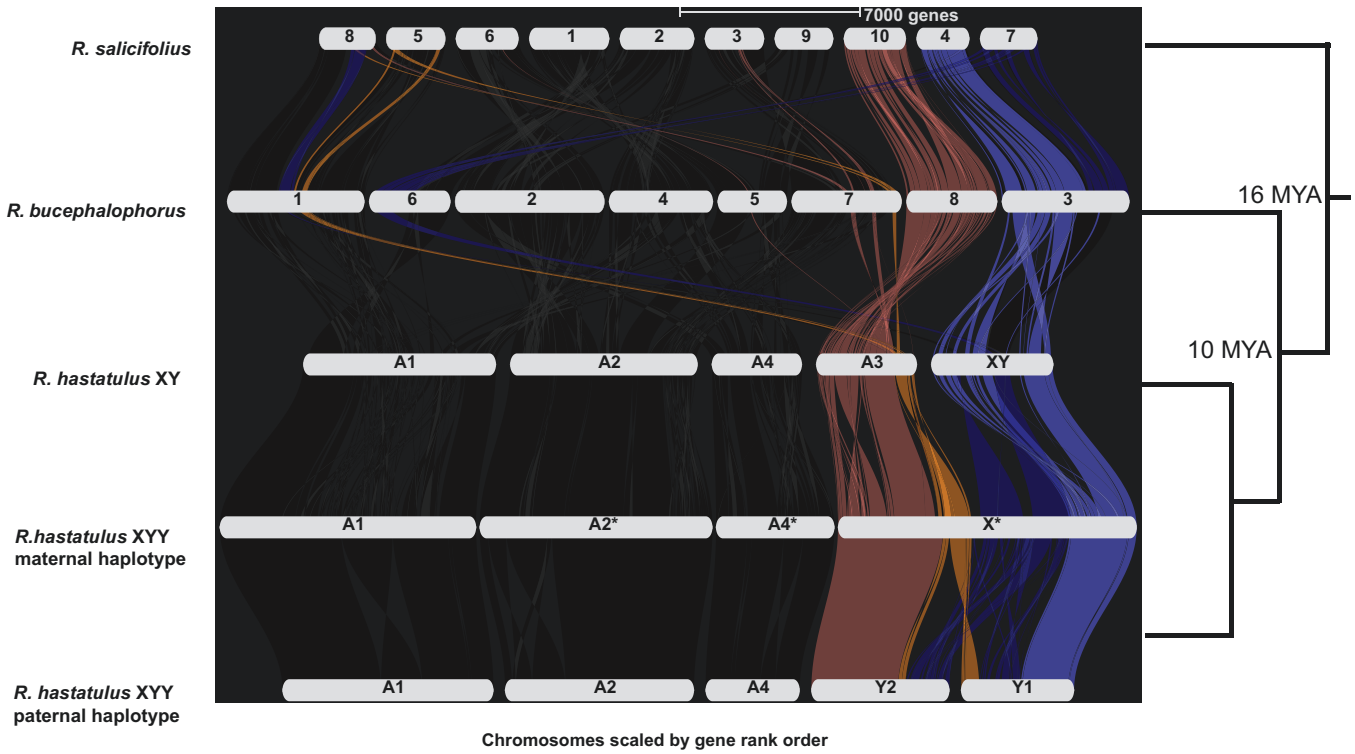

Figure 6. GENESPACE riparian plots showing synteny between *Rumex salicifolius*, *R. bucephalophorus*, and the sex chromosomes of *R. hastatulus*. Syntenic blocks are ordered and colored according to regions of the neo-X chromosome of *R. hastatulus*: neo-PAR in light red, neo-sex-linked region in yellow, old sex-linked region in dark blue, and old PAR in light blue. Branch lengths on the right-hand tree are not to scale. Note that chromosome size is scaled by gene rank order, rather than by physical size.

taxon sampling can improve statistical confidence in inferred relationships, it will not help if particular nodes are incorrectly resolved due to biological sources of gene tree discordance such as incomplete lineage sorting (Degnan & Rosenberg, 2009). Here, we applied modern coalescent-informed approaches to genome-scale data to resolve key relationships within *Rumex*, placing *R.*

bucephalophorus as a sister to the XY dioecious clade. We found that the node where *R. bucephalophorus* branches off is supported by fewer than half of our estimated gene trees (Figure 2), highlighting the need to sample many loci to accurately resolve relationships. Importantly, the phylogenetic methods we apply are still vulnerable to the effects of introgression, for which we found evidence in our data. In parts of the tree with evidence for introgression, our estimated concordance factors are sufficiently high that biologically unrealistic amounts of introgression would be required to have biased our species tree estimation. In addition, the placement of *R. bucephalophorus* has approximately equal frequencies of the two minority trees, suggesting limited evidence for introgression affecting this node. Therefore, we can be confident that our phylogenetic reconstruction is not meaningfully affected by hybridization/introgression.

Sex chromosomes have long been known to contribute to barriers to contemporary hybridization, but their role in the long-term maintenance of species barriers remains comparatively poorly understood. Introgression involving unsampled taxa ("ghost" introgression) is increasingly recognized as an issue for phylogenetic inference (Ottenburghs, 2020; Tricou et al., 2022a, 2022b), but it is still challenging to estimate from genomic data as it is easily confused with introgression among sampled taxa. Inference requires careful examination of the three species introgression results and patterns in genomic data, as we have done here. As highlighted by our results and recent simulation work (Tricou et al., 2022a, 2022b), authors should explicitly consider ghost introgression hypotheses when studying introgression. We should note, however, that such parsimony-based inferences might eliminate true instances of introgression among sampled taxa, including our study; full-likelihood methods with more explicit model selection criteria like the PhyloNet method we applied in our study can further aid in distinguishing among scenarios. Although we do not know the identity of the donor lineages, based on overlapping patterns of gene tree discordance, the timing of our proposed events likely predates the evolution of sex chromosomes in both clades (Figure 2). In addition, the one instance of more recent introgression we observe is between two closely related hermaphroditic species. While preliminary, our results are consistent with the idea that the evolution of sex chromosome and sexual system differences among *Rumex* species contributed significant barriers to more recent introgression.

Another significant source of potential bias in both our phylogenetic and introgression analyses is WGD. The potential impacts of WGD are two-fold: (1) large-scale gene duplication poses challenges for the identification of single-copy orthologs; (2) allopolyploidization events generate similar genealogical signals to introgression and can bias both phylogenetic and introgression inference. Applying mixture model analyses to the distribution of D_S values, we found mixed evidence for WGD in *R. acetosella* and *R. paucifolius*, two species with known polyploid varieties (Supplementary Table S5), in addition to *R. thyrsiflorus* (see Supplementary Materials for additional discussion of these results). Given the absence of evidence for polyploidy in *R. thyrsiflorus*, we expect the patterns in this species might be driven by the retention of highly heterozygous haplotypes in a very polymorphic species. In general, because we sampled single-gene copies and gene duplications were limited to a single species, our phylogenomic analyses should be robust to the effects of autopolyploidy across the genus, regardless of the precise history of events. In addition, our phylogenetic analyses are likely not substantially affected by the potential presence of allopolyploidy, for reasons similar to those discussed for introgression. However, it remains possible that our introgression results are influenced by the presence of allopolyploidy among our study species, since allopolyploidy is a form of hybridization; disentangling the signals of allopolyploidy and introgression remains an open methodological challenge in phylogenomics. Ultimately, whole-genome sequences will be required to fully resolve the history of WGD and introgression in *Rumex*.

Origins of XY sex chromosome systems across two clades

We found that previously identified sex-linked genes in *R. rothschildianus* are homologous to all autosomes and the X chromosome (both old X and neo-X) of *R. hastatulus*, with most hits in relatively small regions of autosomes 1 and 2 (Figure 3). Although this result adds some evidence of shared sex-linked genes between these species, we found that the proportion of these shared genes is not any greater than expected by chance. This, in addition to the large number of sex-linked genes from *R. hastatulus* autosomes, provides clear support for the significantly independent evolution of sex chromosomes in the two major clades. Because of the additional independent evolution of an XYY system (likely from an XY ancestor) in the clade with *R. rothschidianus*, we expected a priori some differences in patterns of sex linkage. Many genes mapped to the last 10 MB of autosome 1 and the first 50 MB of autosome 2. Both regions could simply be preserved syntenic blocks with ancestrally high gene content, but it is interesting that hemizygous *R. rothschildianus* genes appear to be particularly enriched at the beginning of autosome 2, suggesting the possibility that this region represents the primary source of the original sex chromosomes of this species. Despite the lack of significant overlap of sex-linked genes, we found some sex-linked genes in *R. rothschildianius* that mapped to the X of *R. hastatulus* (Figure 3), despite this region having relatively low gene density overall. This result can be explained by independent recruitment of genes with ancestrally sex-biased functions to the X chromosome, a shared sex-determining locus between the two major clades, and/or simply by random recruitment of ancestral gene orthologs due to high rates of rearrangement. Ultimately, chromosome-scale genome assemblies of *R. rothschildianus* and close relatives of *R. hastatulus* will be needed to further resolve the complex history of karyotypic evolution in the genus, but it is clear that sex chromosomes have largely arisen independently in the two clades.

Although our combined results support largely independent sex chromosome evolution in the two major dioecious clades, this does not fully rule out the possibility of a single origin of dioecy. It is possible that the two major sex chromosome clades originally shared a sex-determining region that arose in their common ancestor and has since turned over. Although we found no evidence of the loss of sex chromosomes in *R. bucephalophorus*, our analysis assumed that reversal could only proceed via the inactivation of the existing sex-determining pathways. One alternate possibility is reversal to hermaphroditism via a novel genetic pathway, as recently reported in Japanese persimmon (*Diospyros kaki*, Masuda et al., 2022), followed by restoration of recombination between incipient sex chromosome haplotypes; this would be indistinguishable from our "no reversal" scenario. Another possibility is incomplete lineage sorting of the sex-determining locus leading to its inheritance in the two major sex chromosome clades, but not *R. bucephalophorus*, following a single origin in their common ancestor (Avise & Robinson, 2008; Mendes & Hahn, 2016). This hypothesis would require the presence of ancestral

polymorphism in the genetics of sex determination, which would be consistent with observations of polymorphic sexual systems in contemporary *Rumex* species such as *R. bucephalophorus*, *R. sagittatus*, and *R. scutatus* (Navajas-Perez et al., 2005, and our own observations) in addition to a high diversity of sexual systems across the genus potentially implying polymorphic ancestral states.

Alternatively, introgression of the sex-determining locus between the XYY clade and the XY clade following its split with *R. bucephalophorus* could have led to a shared genetic basis of *SD*. In any case, subsequent rearrangements and a divergent history of recombination suppression would drive highly divergent sex linkage of many genes in the two groups, including an ancient X-autosome fusion that gave rise to the XYY karyotype. Our analyses do not provide clear support for introgression among these clades, but as previously mentioned, our results made it challenging to distinguish between ancient introgression among unsampled hermaphroditic lineages, more contemporary bouts of introgression among sampled taxa, and the effects of allopolyploidization. Identification of the causal sex-determining genes across the genus would enable a direct examination of these possibilities further. However, the evidence for divergent mechanisms of sex determination across species (X:autosome balance in the XYY clade and XY_1Y_2 *R. hastatulus* vs. Y-presence in the XY clade; Navajas-Perez et al., 2005) means that the two clades are unlikely to share a single genetic basis among extant taxa, even if there was original sharing of the mechanism of sex determination.

The role of chromosomal rearrangements in sex chromosome and genome evolution

Classic theory predicts an elevated rate of fusions involving sex chromosomes and autosomes, as well as inversions near sex-linked regions of sex chromosomes; these rearrangements are predicted to help physically link sexually antagonistic variation on the same or other chromosomes to the sex-determining region (Charlesworth & Charlesworth, 1980; Charlesworth et al., 2005; Rice, 1987). Alternatively, chromosomal inversions that capture beneficial haplotypes can become fixed on the Y and subsequent compensatory trans-regulatory evolution of the degenerating inversion haplotype can favor recombination suppression and degeneration of the Y in the absence of sexual antagonism (Lenormand & Roze, 2022; Lenormand et al., 2020). However, evidence is mixed for the importance of sexually antagonistic variation in sex chromosome evolution, and hypotheses have rarely been tested in a comparative framework, despite this body of competing models. Chromosomal rearrangements including fusions and inversions are frequent in *Rumex*, as evidenced by successive reductions in chromosome number from the ancestral $x = 10$ karyotype (Navajas-Perez et al., 2005), high rates of intraspecific rearrangement in XYY species *R. acetosa* (Parker & Wilby, 1989), high rates of inversion across the genome of *R. hastatulus* (Sacchi & Humphries et al., 2024), and our synteny analyses (Figures 5 and 6). Although we can confirm the existence of an X-autosome fusion forming a neo-X chromosome in *R. hastatulus* (Figure 6), the rate of interchromosomal rearrangements involving sex chromosomes does not appear to be elevated relative to autosomes in this species, as might be expected under a model where fusions involving the sex chromosomes are favored due to sexual antagonism. We note that karyotypes appear to be more stable in the early-diverging hermaphroditic clade containing *R. salicifolius* and its relatives (Navajas-Perez et al., 2005), so one possibility is that transitions to dioecy and XY sex determination favor broad increases in rates of chromosomal rearrangement, rather than increases targeted to specific chromosomes. Alternatively, fusions in this system may be favored across the genome as a whole to facilitate local adaptation (Guerrero & Kirkpatrick, 2014), with SA variation captured on the sex chromosomes as a consequence. Other possible explanations for high rates of fusions are meiotic drive (Blackmon et al., 2019; Sandler & Novitski, 1957) and/or a high mutational input of chromosomal rearrangements in this group.

In contrast to our results for chromosomal fissions/fusions, recent work (Sacchi & Humphries et al., 2024) found that inversions are highly enriched on the sex chromosomes relative to autosomes, at least in the genome of *R. hastatulus*, leading to an almost complete breakdown of synteny between the X and Y chromosomes, despite very short divergence times. This finding is consistent with our observation that synteny appears to be evolving faster on the X than the autosomes in *R. hastatulus*. Such an enrichment is predicted by both models of sexual antagonism and compensatory regulatory evolution. Alternatively, inversions may become more tolerated on degenerated sex chromosomes, due to reduced efficacy of selection and reduced potential deleterious fitness consequences in the absence of recombination between X and Y. Generally, our results on chromosomal rearrangements indicate a need to consider alternative hypotheses for the origins and degeneration of sex chromosomes and highlight potential directions for future research. Of particular importance is developing comparative genomic approaches to resolve the timing of chromosomal rearrangements and gene expression divergence relative to the genesis of recombination suppression. Understanding the sequence of these events could provide valuable information on the direction of cause and effect as it relates to different models of sex chromosome evolution. *Rumex* will continue to be a valuable system for developing these approaches as additional genome sequences and estimates of quantitative gene expression levels are generated across the group. Regardless of the primary cause, the continued expansion of sex-linked regions in *Rumex* will have significant long-term consequences for degeneration and genome evolution across the genus.

Sexual system variation in species lacking sex chromosomes

Finally, our study focused primarily on the sex chromosomes, but even among species without them, *Rumex* contains a variety of sexual systems. Among our studied species without differentiated sex chromosomes, *R. bucephalophorus* has been observed in our samples and described in other studies as gynomonoecious (female and bisexual flowers in the same individual) (Talavera et al., 2011), *R. sagittatus* has been described as both monoecious (male and female flowers in the same individual) and dioecious but without heteromorphic sex chromosomes (Navajas-Perez et al., 2005) (though our samples were hermaphroditic), and *R. scutatus* has been described as polygamous (male, female, and bisexual flowers in the same individual, Navajas-Perez et al., 2005). Hermaphroditic individuals have also been described for *R. bucephalophorus* and *R. scutatus*. Outside our study species, sexual system variation has also been described in the large hermaphroditic clade containing *R. salicifolius* and its relatives (Grant et al., 2022). This variation in sexual systems is important because the evolutionary transition from hermaphroditism to dioecy is expected to proceed through some of these "intermediate" sexual systems (Barrett, 2002). One important pathway is thought to be through gynodioecy (female and bisexual flowers in different individuals), via the successive fixation of male-inactivating

and female-inactivating mutations producing separate male and female individuals (Charlesworth & Charlesworth, 1978; Spigler & Ashman, 2012). Gynodioecy has not been observed among our study species, although it has been described in members of the clade that includes *R. sagittatus* and *R. scutatus* (Navajas-Perez et al., 2005). Unfortunately, given the wide variation of sexual systems in our study species and our lower taxon sampling, we have insufficient information to reconstruct the ancestral sexual system of the two sex chromosome clades. Regardless, it is clear that significant ancestral variation in sexual systems would have existed to facilitate transitions from hermaphroditism to dioecy across the genus. Integrating sexual system data across more species with transcriptomic and genomic approaches will continue to provide insights into the forces shaping sexual system transitions in flowering plants.

Supplementary material

Supplementary material is available online at *Evolution Letters*.

Data availability

Raw sequencing reads from PacBio/Omni-C and RNA-Seq, in addition to the genome assembly of *R. bucephalophorus*, are available at GenBank under BioProject PRJNA698922. Genome assembly and annotation are also available on COGE under genome ID 66598. Transcriptome assemblies are available on Dryad at doi:10.5061/dryad.w6m905qzj. Custom scripts and supplementary data files are available on GitHub at https://github.com/mhibbins/RumexComparative.

Author contributions

M.S.H., J.L.R., S.C.H.B., and S.I.W. conceptualized the project; J.L.R., B.I.C., O.V., M.Y., and S.C.H.B. conducted sample collection, processing, and sequencing; M.S.H., J.L.R., B.S., and Y.G. conducted bioinformatic analyses; M.S.H., S.C.H.B., and S.I.W. contributed to project funding and supervision; and M.S.H. and S.I.W. wrote the manuscript, with contributions and edits from all other authors.

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Conflict of interest: Editorial processing of the manuscript was conducted independently of S.I.W., an Associate Editor of *Evolution Letters*. The other authors declare no conflict of interest.

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