

1 Discoveries

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3 Biased retention of environment-responsive genes 4 following genome fractionation

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1 **Abstract**

2 The molecular underpinnings and consequences of cycles of whole-genome duplication (WGD)
3 and subsequent gene loss through subgenome fractionation remain largely elusive. Endogenous
4 drivers, such as transposable elements, have been postulated to shape genome-wide dominance
5 and biased fractionation leading to a conserved least-fractionated (LF) and a degenerated most-
6 fractionated (MF) subgenome. In contrast, the role of exogenous factors, such as those induced
7 by environmental stresses, has been overlooked. A chromosome-scale assembly of the alpine
8 Buckler Mustard (*Biscutella laevigata*; Brassicaceae) that underwent a WGD event about 11
9 million years ago is here coupled with transcriptional responses to heat, cold, drought and
10 herbivory to assess how gene expression is associated with differential gene retention across the
11 MF and LF subgenomes. Counteracting the impact of transposable elements in reducing the
12 expression and retention of nearby genes across the MF subgenome, dosage balance is
13 highlighted as a main endogenous promoter of the retention of duplicated gene products under
14 purifying selection. Consistent with the “turn a hobby into a job” model, about one third of
15 environment-responsive duplicates exhibit novel expression patterns, with one copy typically
16 remaining conditionally-expressed, whereas the other copy has evolved constitutive expression,
17 highlighting exogenous factors as a major driver of gene retention. Showing uneven patterns of
18 fractionation, with regions remaining unbiased while others show high bias and significant
19 enrichment in environment-responsive genes, this mesopolyploid genome presents evolutionary
20 signatures consistent with an interplay of endogenous and exogenous factors having driven gene
21 content following WGD-fractionation cycles.

22

23 **Keywords:** conditionally-expressed genes; dosage balance; environmental stress; subgenome
24 dominance; transposable elements; whole-genome duplication.

25

1 **Introduction**

2 Cycles of whole-genome duplication (WGD) followed by diploidization have been pervasive during
3 the radiation of eukaryotes, especially in angiosperms (Leebens-Mack et al. 2019; Jiao et al. 2011;
4 Schranz et al. 2012; van de Peer et al. 2017). Counteracting WGD events that increase the number
5 of co-existing genomes in the nucleus and initially results in all loci being duplicated, genome
6 fractionation (i.e. gene loss) and dysploidy (i.e. reduction of chromosome number) gradually lead
7 to genome downsizing and a return to a diploid-like state (Lynch and Conery 2000; Mandáková
8 and Lysak 2018; Tank et al. 2015). Despite their contribution to the architecture of genomes,
9 neither the molecular underpinnings of such “wondrous cycles”, nor the evolutionary mechanisms
10 driving the fate of duplicated genes are fully understood (Freeling et al. 2012; Soltis et al. 2016;
11 Wendel 2015).

12 Assuming an overarching connection between gene expression levels and the strength of
13 selection acting on them, the differential expression of genes between subgenomes resulting from
14 WGD has been postulated to drive genome fractionation by promoting the adaptive retention of
15 specific duplicates against the accumulation of deleterious mutations and pseudogenization
16 (Freeling 2009; Koonin and Wolf 2010). Following WGD, constraints due to the necessary dosage
17 balance of interacting gene products are thus expected to promote the long-term retention of
18 numerous duplicated genes with conserved functions (Birchler and Veitia 2012), whereas the
19 partitioning of ancestral expression patterns between duplicates (i.e. sub-functionalization)
20 supports their retention under purifying selection. In contrast, the evolution of novel functions or
21 expression patterns is promoted by positive selection (i.e. neo-functionalization; Birchler and Yang
22 2022). Despite the null hypothesis that duplicated subgenomes undergo similar rates of sequence
23 turnover, many studies have highlighted that one subgenome (coined as dominant) commonly
24 retains more genes following WGD and is therefore “least fractionated” (LF) compared to the
25 other subgenome(s) which appear more degenerated with fewer genes and described as the
26 “most fractionated” (MF) (e.g. Chalhoub et al. 2014; Garsmeur et al. 2014). Although such biased
27 fractionation is commonly regarded as non-random, underlying processes remain elusive and rely
28 on partially overlapping hypotheses of genome-wide dominance against loci presenting lower
29 expression (Alger and Edger 2020; Woodhouse et al. 2014). In particular, interspersed copies of

1 transposable elements (TEs) are expected to reduce the expression of nearby genes (Hollister et
2 al. 2011) and have been predicted to influence subgenome-wide expression levels, determining
3 dominance and patterns of biased fractionation between genomes with unbalanced TE loads
4 (Freeling et al. 2015). Although an association between TE abundance and subgenome dominance
5 has been documented in recently formed as well as ancient polyploid genomes (e.g. Edger et al.
6 2017; Garsmeur et al. 2014), several counterexamples indicate that other factors may also be at
7 play (Douglas et al. 2015; Renny-Byfield et al. 2015; Zhao et al. 2017). Furthermore, the parental
8 legacy of TEs associated with gene expression levels was recently shown to be insufficient to
9 explain subgenome-wide dominance in experimental allotetraploids of *Brassica* (Zhang et al.
10 2023).

11 Beyond endogenous genomic features, exogenous factors such as different environmental
12 conditions may also be involved in promoting the differential expression of loci between
13 subgenomes (e.g. Shimizu-Inatsugi et al. 2017) and result in biased fractionation. The possible
14 interactions between transcriptional plasticity in response to environmental changes and genome
15 fractionation are indeed virtually unknown (Blischak et al. 2018).

16 The mustard family (Brassicaceae) that includes the model plant *Arabidopsis thaliana* contains
17 numerous examples of taxa having undergone multiple rounds of WGD and thus offers pertinent
18 model systems to investigate drivers and consequences of post-polyploid genome fractionation
19 (Hendriks et al. 2023; Kagale et al. 2014; Mandáková et al. 2017). On top of the family-specific
20 paleotetraploidy event (α -WGD) that occurred some 32 million years ago (mya; Hohmann et al.
21 2015) and left several duplicated genes in all extant diploid genomes of Brassicaceae, the genus
22 *Biscutella* comprising more than 50 species of annual herbs or perennial shrublets has rapidly
23 radiated across the Mediterranean basin following a presumably shared mesotetraploidy event
24 (Geiser et al. 2016). Although this WGD event was associated with hybridization between two
25 closely related, structurally similar genomes and resulted in a functionally redundant ancestral
26 karyotype of *Biscutella* (Guo et al. 2020), comparative chromosome painting coupled with
27 transcriptomics in Buckler Mustard (i.e. *B. laevigata*; $x=9$) has shown that chromosomal segments
28 conserved as duplicates are side-by-side with loci having undergone fractionation in this
29 mesopolyploid (Geiser et al. 2016). Although many recently active TEs were identified in this

1 species (Bardil et al. 2015), the lack of appropriate genomic resources to anchor genes and TEs
2 under scrutiny to specific loci and subgenomes precluded the characterization of genome
3 fractionation. Using an annotated chromosome-level assembly of the alpine Buckler Mustard
4 genome (*Biscutella laevigata* subsp. *austriaca*), this work thus addresses the role of endogenous
5 TEs and environmental factors on gene expression and long-term retention. Specifically, we (i)
6 characterized the mesopolyploid WGD event in the context of TE activity in the Buckler Mustard,
7 (ii) assessed biased fractionation by comparing syntenic genes in the LF and MF subgenomes in
8 relation to their expression, TE loads and patterns of selection, and (iii) quantified how
9 transcriptional changes in response to exogenous factors support the retention of duplicated
10 genes and shapes genomic regions with low versus high levels of biased fractionation.

11 **Results**

12 *Assembly and annotation of the Buckler Mustard genome*

13 The allogamous sample of Buckler Mustard (*Biscutella laevigata* subsp. *austriaca*) here sequenced
14 with a combination of long and short reads (total coverage of 282X; Table S1) was estimated to
15 have a haploid genome size of 904 Mb based on the flow cytometry analysis. K-mers estimated a
16 total size of 832 Mb and a moderate heterozygosity rate of 1.88-1.92%, which is in between the
17 Col-0 accession of *A. thaliana* (0.22%; Kang et al. 2023) and the T16 accession of *Brassica oleracea*
18 (up to 5.78%; Li et al. 2024), and matches estimates for typically outcrossing diploid plants such
19 as *Arabidopsis lyrata* (i.e. 1.4% to 2.1%; Ross-Ibarra et al. 2008) as well as the sampled population
20 of *B. laevigata* (i.e. 3.3%; Grünig et al. 2024). Following long-range scaffolding (Fig. S1), collapsing
21 of similar scaffolds to reduce heterozygosity while retaining duplicates arising from WGD events
22 (Fig. S1c) and gap filling, a final assembly consisting of 6,350 scaffolds with a total length of 873.75
23 Mb (<https://genomevolution.org/coge/GenomeInfo.pl?gid=67230>; N50 = 71.38 Mb; Fig. S2) was
24 produced, showing 98.2% of complete BUSCO genes (i.e. 78.1% single copy and 20.1% duplicated;
25 Table S2). The Hi-C contact map was manually curated and scaffolded into chromosomes that
26 showed a band of high contact density along their diagonal, reflecting the well-ordered underlying
27 assembly submitted as a supplementary dataset (Figure S2.b, Dataset S1). Synteny comparisons
28 with the ancestral genomic blocks of Brassicaceae (Lysak et al. 2016) and comparative
29 chromosome painting confirmed the structure of the 13 largest scaffolds and supported their

1 arrangement into the nine nearly-complete chromosomes (total length: 764.46 Mb; Fig. 1a). The
2 chromosome structure of Ba5 and Ba6, characterized by the combination of genomic blocks
3 O+P+W+R and an inactive paleocentromere (Fig. S3), align with chromosome AK6/8 of the
4 ancestral Proto-Calepineae Karyotype ($n = 8$), identified as the common ancestor of the tribe
5 Biscutelleae (Guo et al. 2020). With its largely duplicated blocks, the assembly presented
6 significant synteny with the closely related *B. laevigata* subsp. *varia* (Geiser et al. 2016), including
7 two interstitial 5S rDNA loci identified at the pericentromeric heterochromatin of chromosomes
8 Ba1 and Ba4, and two terminal 35S rDNA loci on chromosomes Ba2 and Ba3 (Fig. S3).

9 Annotated transposable elements (TEs) comprised 539.14 Mb, encompassing 68.63% of the nine
10 chromosome-scale scaffolds (Table S3), with a majority (ca. 40%) of long terminal repeat (LTR)
11 retrotransposons. Among them, a total of 2,993 full-length copies were identified (Table S4)
12 among the main lineages of Copia (e.g. 864 Ale, 301 Ivana) and Gypsy (e.g. 293 Athila, 21 Tekay)
13 which are predicted to have been active recently in Brassicaceae (Zhou et al. 2021). The
14 distribution of TEs supported the structure of the assembly, with a higher abundance of LTR-
15 retrotransposons located towards the centromeric regions (Fig. 1a). Two centromeric tandem
16 repeats were identified (213- and 468-bp; Fig. S3). *Ab initio*-predicted genes supported by RNAseq
17 data from seven tissues under mesic environments as well as from leaf tissue of clone plants
18 subjected to cold, drought, heat and herbivory resulted in the high-quality annotation of 43,632
19 gene models, and 86.3% of the complete BUSCO genes. Despite the basal split of Biscutelleae
20 from other Brassicaceae clades, here dated to be c. 20 mya based on the plastid phylogeny (Fig.
21 1b), at least 13,221 orthogroups (23,247 genes) presented a clear ortholog in all or all-but-one of
22 the representative genomes of Brassicaceae (Fig. S4; Table S5). Together with 864 such
23 orthogroups being absent from the *B. laevigata* assembly, these genes were classified as
24 conserved orthologs across Brassicaceae.

25

26 *Main molecular drivers of mesopolyploid genome evolution*

27 The mesopolyploid nature of the Buckler Mustard genome seen in the structure of the assembly
28 and the karyotype was confirmed by a peak in the distribution of synonymous substitutions (Ks)

1 among paralogs at 0.34 (\pm 0.15 standard deviation) in addition to the peak around 0.96 (\pm 0.42)
2 that is indicative of the α -WGD event shared with *A. thaliana* (Fig. 1c + Fig. S5). Consistent with
3 prior estimates, the younger meso-WGD event was dated to be younger than 11.5 mya. To assess
4 whether it promoted the concomitant activation of TEs according to the genome-shock hypothesis
5 or subsequently supported effective transposition due to relaxed selection (Parisod et al. 2010),
6 we dated the amplification of TE copies by estimating their divergence from consensus sequences
7 in the main TE lineages of the Buckler Mustard genome (Maumus and Quesneville 2014).
8 Identified peaks indicative of transposition bursts were observed to range from 10 to 6% sequence
9 divergence and indicated the ongoing transposition of several LTR retrotransposons between 10
10 and 5 mya (Fig. 1d). The lower divergence of TE copies than duplicated genes is consistent with a
11 more recent onset of TE proliferation than the mesopolyploid WGD event and hence high TE
12 dynamics during the early stages of genome fractionation in *B. laevigata*.

13 Synteny between the *B. laevigata* assembly and sequences of *A. thaliana* identified 14,923 genes
14 retained in the high-confidence chromosomal segments of syntenic duplicates derived from the
15 meso-WGD event among a total of 122 non-overlapping windows spanning 81.2% of the nine
16 main scaffolds (i.e. chromosomes; Fig. S6). Downstream analyses were performed on these
17 syntenic windows, which were shown to be mostly shorter than 2 Mb (median length 1.58 Mb;
18 Fig. S6b), to ensure that our conclusions were largely unaffected by potential misassemblies that
19 were shown to possibly span genome segments \geq 7 Mb (Fig. S2c). These duplicated windows
20 included 6,436 duplicates in 3,218 pairs (Fig. 2a, Table S6) that were assigned to the LF and the
21 MF subgenomes based on corresponding gene trees with high node support (Fig. S7). Beyond
22 these retained duplicates representing 43.1% of the genes among syntenic windows, the LF and
23 MF subgenomes presented a total of 8,542 and 6,381 intact genes, respectively, supporting
24 differential gene retention which is consistent with biased fractionation.

25 To further assess the underpinnings of genome evolution, we turned to expression data and
26 confirmed the predicted association between expression and selection by showing that expressed
27 genes had lower ratios of non-synonymous (K_a) per synonymous substitution rates (K_s) than genes
28 considered to be unexpressed (Wilcoxon test, $p < 0.001$). The maximum level of gene expression
29 was shown to be linearly associated with K_a/K_s values (slope = -0.10, $p < 0.001$, Fig. S8), matching

1 the expectation that highly expressed genes are conserved under stronger purifying selection (i.e.
2 lower values of Ka/Ks). The majority of genes under scrutiny in the Buckler Mustard indeed
3 showed conserved coding sequences under pervasive purifying selection (i.e. 14,745 genes with
4 $Ka/Ks < 0.79$ as compared to *A. thaliana*). Despite similar signals of purifying selection across both
5 subgenomes, pairs of duplicated genes presented significantly lower Ka/Ks ratios than genes that
6 had returned to singleton state and this difference was particularly pronounced in the LF
7 subgenome (Fig. 2b; $p < 0.001$).

8 Addressing how expression and selection shaped gene retention between subgenomes, we
9 characterized the expressed genes in the LF (6,771) compared with the MF subgenome (4,920; z-
10 test, $p < 0.01$) and showed significantly higher gene expression in the LF than in the MF
11 subgenome (Wilcoxon test, $p < 0.001$; Fig. 2c and Fig. S9). As expected, expressed genes in the MF
12 subgenome accordingly showed stronger signals of purifying selection than those in the LF
13 subgenome (average Ka/Ks of 0.170 and 0.175, respectively; Wilcoxon test, $p < 0.01$), suggesting
14 that high gene expression associated with strong purifying selection has been necessary to
15 support gene retention in the MF subgenome.

16 In contrast to genes, the density of TEs was significantly lower in the LF subgenome than in the
17 MF subgenome (Wilcoxon test p -value < 0.001 ; Fig. 2d). Given that the presence of TE copies up
18 to 2,000 bp upstream or downstream of genes was significantly associated with their lower levels
19 of expression (Fig. S10 and Table S7), such differential TE load likely played a role in shaping gene
20 expression and hence selection across the LF and MF subgenomes. The presence of a TE copy
21 within 201 to 2000 bp was seen to reduce the median expression of nearby genes by 28.14% (1.83
22 \log_2 fold change), as expected by their epigenetic silencing locally affecting flanking loci. Although
23 such indirect effects of TEs may have contributed to the long-term biased fractionation under
24 pervasive purifying selection, the observed association between TE density and gene retention
25 does not exclude other drivers of fractionation or other triggers of gene expression across
26 subgenomes as drivers of fractionation.

27

28

1 *Environmental triggers of duplicate retention following WGD*

2 To what extent conditional gene expression in response to environmental cues has been shaping
3 genome fractionation was first assessed by inspecting the 977 single-copy and 760 duplicated
4 genes from 966 pairs that were differentially expressed (DE) in response to experimental cold,
5 heat, drought and herbivory treatments out of the 14,923 syntenic genes under scrutiny in the LF
6 and MF subgenomes (Fig. 3a; Fig. S10). Noticeably, the Ka/Ks ratios of DE genes were significantly
7 lower than for genes with other expression patterns (Wilcoxon tests, $p < 0.001$; see Fig. 3b),
8 indicating that coding sequences conditionally-expressed in response to environmental cues were
9 more likely to be retained under stronger purifying selection than constitutively-expressed ones.
10 It is notable that the patterns of selection in DE duplicated genes were consistent with retention
11 under even stronger purifying selection than DE single copy genes (i.e. Ka/Ks of 0.155 and 0.176,
12 respectively; Wilcoxon test, $p < 0.001$), indicating that abiotic and biotic cues promoted the long-
13 term adaptive retention of duplicates in this mesopolyploid genome. Matching the genome-wide
14 pattern of fractionation, retained DE genes were significantly more numerous in the LF than the
15 MF subgenome, as shown for conserved orthologs across Brassicaceae in Fig. 3c.

16 By cross-matching patterns of expression and signals of selection in the Buckler Mustard, we
17 assessed evolutionary underpinnings of 2,196 expressed pairs of retained duplicates across
18 syntenic windows, considering their orthologs in *A. thaliana* as “progenitor singletons” (Table S6).
19 Consistent with the advanced fractionation of the mesopolyploid genome, only eight pairs of
20 duplicated genes (0.36%) presented signals of neutral divergence ($0.79 < \text{Ka/Ks} \leq 1.21$) for either
21 both or one member of the pair and were hence possibly retained without selection. Only four
22 pairs of duplicates (0.18%) presented one member with a clear signal of positive selection (Ka/Ks
23 > 1.21) pointing to possible neofunctionalization, while the other member was retained under
24 purifying selection.

25 In contrast, the vast majority of expressed duplicates retained in the Buckler Mustard presented
26 both copies under purifying selection (99.4%). Considering their expression in response to cold,
27 heat, drought and/or herbivory treatments to highlight possible changes in the environmental
28 trigger(s) compared with their progenitor singletons, almost two thirds of the retained duplicates
29 in the Buckler Mustard showed either no environmental trigger (1,367 pairs) or were both

1 differentially expressed under similar conditions compared with their progenitor singletons (33
2 pairs) and were thus considered consistent with retention of conserved function under dosage
3 balance constraints (63.8% of expressed duplicates). The remaining third of duplicates retained
4 under purifying selection (796 pairs; 36.2%) showed a change in response to an environmental
5 trigger compared to the progenitor singletons. A total of 296 duplicate pairs showed both
6 members having lost their ability to respond to the environmental trigger(s), whereas 27 and 31
7 pairs presented one member with a conserved environmental trigger on the LF and MF
8 subgenome respectively, with the other member being constitutively expressed. Nevertheless,
9 the majority of the expressed duplicates (i.e. 391 pairs) presented gain(s) in environmental
10 trigger(s) in the Buckler Mustard, affecting either one or both members and indicating
11 considerable expression repatterning during fractionation.

12 Pointing to dosage balance as the chief constraint driving the retention of duplicated genes, our
13 results also highlight the importance of expression changes among environment-responding
14 duplicates following fractionation. Expression changes chiefly resulted in the constitutive
15 expression of one or both member(s) and hence increased dosage of conserved coding sequences
16 that were ancestrally stress-responding among retained duplicates in the Buckler Mustard. A
17 similar evolutionary response of constitutive expression was apparent beyond the duplicated
18 segments under scrutiny here, with most of the 2,098 progenitor singletons previously shown to
19 be environmentally responsive in *A. thaliana* being identified as singleton (1,032, 49.2%) or
20 retained as duplicate (353) presenting constitutive expression in the Buckler Mustard. Among the
21 222 progenitor singletons retained as environment-responsive duplicates in *B. laevigata*, only 57
22 had both members responding to at least one environmental treatment, while 70 presented
23 constitutive expression of one member. Such canalization of ancestrally environment-responsive
24 genes towards constitutive expression is consistent with the “turn a hobby into a job” model
25 (Conant and Wolfe 2008) and likely promoted the increased tolerance of the Buckler Mustard to
26 the stressful conditions that are typical of alpine environments where it currently thrives.

27

28

1 *Biased retention of environment-responsive genes during genome fractionation*

2 To further assess how the environment-responding genes evolved across subgenomes, we
3 partitioned the 122 windows of syntenic duplicates into (i) 36 regions comprising those with “low-
4 bias” (i.e. regions with non-significant differences in the proportion of retained genes between
5 the LF and MF subgenome), and (ii) 86 “high-bias” regions (i.e. regions characterized by a
6 significantly reduced proportion of retained genes in the MF subgenome compared with the LF
7 subgenome; Chi-square test, $p < 0.05$; Fig. 4a). “Low-bias” regions were shown to contain
8 duplicated genes characterized by significantly lower divergence than in “high-bias” regions (Fig.
9 4b) and they did not differ in TE density, unlike “high-bias” regions (Fig. 4c). These “low-bias”
10 regions are therefore considered to have undergone limited TE-driven biased fractionation and
11 possibly may have had prolonged exchanges between subgenomes that supported unbiased
12 fractionation in the absence of differential TE load. Overall, “high-bias” regions of the LF
13 subgenome were significantly enriched in constitutively-expressed duplicates (Fig. 4d).

14 The retention of genes responding to environmental triggers was otherwise consistent across
15 “high-bias” regions, showing pervasive conservation of DE and constitutively-expressed genes on
16 the LF subgenome. Biased fractionation hence supported the retention of specific environment-
17 responding genes, as seen across the genomic block U (Fig. 4e) that is duplicated on chromosomes
18 Ba1 and Ba2 and whose “high-bias” region on the LF subgenome appeared significantly enriched
19 in DE genes responding to cold (Gene ratio: 58/724, q value < 0.01 ; Table S8) and herbivory (Gene
20 ratio: 36/724, q value < 0.01). Genes related to the isopentenyl diphosphate biosynthetic process
21 (GO:0019288, comprising 15 genes; Table S9) were notably abundant across that segment of the
22 LF subgenome showing an enrichment of KEGG terms related to plant-pathogen interactions
23 (comprising eight genes; Table S10) and suggesting specialization of the locus in terpenoid
24 biosynthesis such as reported in a previous study of the Buckler Mustard (Knauer et al. 2018).
25 Genome fractionation in such regions is hence biased towards the retention of genes essential for
26 survival under harsh conditions that only polyploids can harness through an abundance of gene
27 copies being adaptively sorted (van de Peer et al. 2021).

28 Among the 14,085 orthogroups conserved in all but one of the considered Brassicaceae species,
29 93.8% were present in *B. laevigata*, supporting the necessary presence of most genes in each

1 progenitor genome. Among the syntenic windows, the LF subgenome indeed presented
2 significantly more of these conserved genes (i.e. 58.0%) than the MF subgenome (i.e. 42.0%; p-
3 value < 0.001, Fisher's exact test) and a similar enrichment was also reflected among singletons
4 (i.e. 64.1% in the LF subgenome compared to 51.7% in the MF subgenome; p-value < 0.001,
5 Fisher's exact test). This pattern held true for the specific genomic block U, in which the LF
6 subgenome harboured a significantly higher proportion of conserved genes (59.1%, p-value <
7 0.001, Fisher's exact test), among which were a higher percentage of singletons (60.3%) compared
8 to the MF subgenome (45.9%, p-value < 0.001, Fisher's exact test). Such overall and locus-specific
9 enrichments of conserved duplicates and singletons in the LF subgenome strongly support that
10 progenitor genomes have contributed similar sets of genes and, despite possible subtle
11 differences in their regulatory circuits before hybridization, had undergone post-WGD sorting that
12 chiefly shaped the two subgenomes of *B. laevigata*.

14 **Discussion**

15 The mesotetraploid genome of the Buckler Mustard originated by WGD coupled with
16 hybridization between two closely related progenitors which contributed similar gene sets before
17 the polyploid genome started to undergo diploidization over about 11.5 million years in
18 association with descending dysploidy towards 9 pairs of chromosomes (Geiser et al. 2016; Guo
19 et al. 2020). Despite bioinformatic challenges arising from aiming to reduce heterozygosity while
20 maintaining WGD-derived duplicates in a haploid assembly and those inherent to reconstructing
21 the history of polyploids and their long-extinct progenitors (Kellogg 2016), the outcomes of long-
22 term biased fractionation are still visible across the majority of 122 duplicated segments in the
23 mesotetraploid genome today. Although our results appear consistent with predictions of TE-
24 driven subgenome dominance (Alger and Edger 2020), 36 of these duplicated segments (27.0%,
25 spanning 241 Mb) actually show unbiased fractionation suggestive of locus-specific rather than
26 (sub)genome-wide drivers. Further, contrasting with the legacy of progenitor TEs determining
27 subgenome dominance, our data show that the proliferation of several types of TEs took place
28 during the early stages of genome fractionation in the Buckler Mustard and instead support the
29 prediction that relaxed selection on the initially redundant loci cumulatively fostered the biased

1 genomic divergence towards a least and a most fractionated (LF vs MF) subgenome (Bird et al.
2 2018; Woodhouse et al. 2014). Although the exact TE composition of the long-extinct progenitors
3 is unknown and their role in driving subgenome dominance immediately after WGD remains
4 elusive, the partially biased fractionation of the mesopolyploid genome of *B. laevigata* appears
5 consistent with runaway pseudogenization coupled with the loss of lowly expressed genes that
6 could only have been antagonized by strong selection resulting in the retention of highly
7 expressed genes, including duplicates mostly constrained by dosage balance (Blanc and Wolfe
8 2004).

9 Here we show that, in addition to the role of endogenous factors such as TEs and genes involved
10 in dosage balance, exogenous factors, i.e. different environmental conditions driving the
11 conditional expression of genes (as shown in some previous studies; e.g. Shimizu-Inatsugi et al.
12 2017; Lee and Adams 2020) have also substantially contributed to genome fractionation. Despite
13 the many challenges inherent to distinguishing the partitioning of ancestral functions from gain(s)
14 of novel environmental triggers (Birchler and Yang 2022; Innan and Kondrashov 2010), numerous
15 ancestral environment-responsive genes with conserved coding sequences were identified as
16 having promoted increased dosage through the evolution of constitutive expression and/or the
17 retention of duplicates as a pervasive outcome of long-term fractionation. Although expression
18 changes can be expected to evolve neutrally through time (Khaitovitch et al. 2004), transcriptional
19 plasticity in response to environmental conditions was generally retained by only one member of
20 the duplicate pair, with the other showing constitutive expression that likely supported general
21 survival under stressful environmental conditions (Conant and Wolfe 2008; van de Peer et al.
22 2021). While such co-option of transcriptionally plastic genes that promoted constitutive
23 adaptation to exogenous factors may have been instrumental in shaping the current
24 mesopolyploid genome, it likely imposed costs and hence may contribute to explaining the slow
25 growth of the perennial *B. laevigata* under alpine conditions. Connections between WGD *per se*
26 and stressful conditions in the short-term remain elusive, although insights from our analysis of
27 Buckler Mustard's mesopolyploid genome point to post-WGD fractionation and particularly the
28 retention of environment-responsive duplicates coupled with expression changes as key to their
29 possible radiation across harsh environments (Dodsworth et al. 2016). Although here we have

1 unravelled plausible mechanisms linking WGD and increased stress tolerance that have operated
2 over millions of years of evolution, future work using experimentally resynthesized and recently
3 established polyploids will be needed to address how genome fractionation unfolds through time
4 and affects the fate of duplicated genes from the initial WGD event to the highly-fractionated
5 mesopolyploid genomes entering new rounds of WGD (Bird et al. 2020; Soltis et al. 2016; Parisod
6 2024).

7

8 **Materials and Methods**

9 This section gives a summary of the methodology, which is detailed in the Supplementary
10 Methods.

11 *Plant material, sequencing, assembly and annotation*

12 The same individual sample of *Biscutella laevigata* subsp. *austriaca* grown from a seed collected
13 near Schneealpe (Steiermark, Austria: 47.6968°N, 15.6100°E; 1740 m asl) was used throughout,
14 from *de novo* genome assembly to RNAseq data, using regenerated cuttings (i.e. clonal ramets).

15 The genome size was estimated by flow cytometry and high molecular weight DNA was sequenced
16 with short Illumina 10X genomics linked reads (75X) which has been shown to produce reliable
17 assembly in maize, a species that also went through WGD some 5–12 million years ago (Visendi
18 2022). Linked reads dataset was complemented with a combination of long Pacbio reads (12X) of
19 an average length of 5.3 kb, and paired-end reads (75X; Table S1). The hybrid assembler Platanus-
20 allee, which marks better performance in highly heterozygous genomes (Kajitani et al. 2019),
21 produced a draft genome that was scaffolded by using ChicagoTM (52X coverage) and Hi-C (68X
22 coverage) methods (Dovetail Genomics, Santa Cruz, CA). Hi-C maps may contain errors or
23 inaccuracies that were carefully evaluated and, combined with evidence from cytogenetic maps,
24 refined to ensure a more accurate genomic assembly. K-mers (k=21) were counted using Jellyfish,
25 and the resulting histogram was analyzed with GenomeScope2 (Ranallo-Benavidez et al. 2020) to
26 estimate genome size, heterozygosity, and repeat content. After removal of uncollapsed haplotigs
27 and gap filling, the completeness of the final assembly was assessed with the BUSCO from
28 embryophyte odb10. Merqury (Rhie et al. 2020) was then employed to compare the heterozygous

1 k-mer content before and after removal of uncollapsed haplotigs. Curation of the 13 largest
2 scaffolds into the 9 main chromosome-level scaffolds was further validated through comparative
3 chromosome painting as described in Geiser et al. (2016).

4 Repetitive elements across the assembly were first identified based on TE structural features using
5 EDTA (Ou et al. 2019). The dynamics of TEs were estimated based on the percentage of divergence
6 of each copy to the consensus according to Maumus and Quesneville (2014) and dated using 8.22
7 $\times 10^{-9}$ substitutions/synonymous site/year for Brassicaceae species (Kagale et al. 2014).

8 Genes were annotated using *ab initio* and mapped RNA-seq reads from seven tissues (i.e. roots,
9 young leaves, senescent leaves, stems, apical meristem, floral buds, and open flowers; European
10 Nucleotide Archive accession: PRJEB48599) and leaf tissues under different environmental
11 conditions (see *Gene expression in response to environmental changes*) as well as Swissprot
12 protein sequences from Viridiplantae used as homology-based support. Only annotations with an
13 edit distance below 0.5 and coding for proteins longer than 20 amino acids were considered.

14 15 *Gene expression in response to environmental changes*

16 Replicated leaf transcriptomes in response to environmental treatments (European Nucleotide
17 Archive accession: PRJEB48469) were generated from clones of the sequenced individual
18 subjected to control (22°C, 16/8h light/dark cycle), cold (24h at 4°C, 16/8h light/dark), heat (3h
19 gradual increase from 22-42°C and 6h at 42°C), drought (11.5 days without watering) and
20 herbivory condition (30h of feeding by the moth *Plutella xylostella*). Those treatments were
21 designed to mimic data available for *Arabidopsis thaliana* (Dubois et al. 2017; Klepikova et al.
22 2016; Nallu et al. 2018) as the only other plant species whose transcriptional responses to several
23 environments has been investigated.

24 Gene expression was quantified using RSEM (Li and Dewey 2011), with only genes expressed at >
25 1 transcript per million considered as “expressed”. Differentially expressed genes (DEGs)
26 presenting a log₂-fold change > 1 were identified using edgeR (Robinson et al. 2010).

27

1 *Analysis of duplicated chromosome segments*

2 The “SynMap” algorithm within CoGe (<https://genomeevolution.org/CoGe/GEvo.pl>) identified
3 duplicated genes from the mesopolyploid WGD event through collinearity within the Buckler
4 Mustard genome and with ancestral genomic blocks of Brassicaceae extracted from *A. thaliana*.
5 Following Woodhouse et al. (2011), windows of syntenic duplicates were seeded with ten
6 collinear genes, comprised 100 *A. thaliana* genes and corresponding duplicates of the Buckler
7 Mustard genome, with a maximum of 20 non-syntenic genes to be considered.

8 Windows of syntenic duplicates were assigned to the two sub-genomes according to RAxML
9 phylogenetic trees of orthologous coding sequences from *A. thaliana*, *Megadenia pygmaea* and
10 the sister genus *Heldreichia bupleurifolia* and accordingly classified as “Least Fractionated” (LF)
11 and “Most Fractionated” (MF) following Guo et al. (2020).

12 SynMap further determined synonymous substitution rates (Ks) and non-synonymous
13 substitution rates (Ka) as compared to *A. thaliana* orthologs. Approximate Gaussian distributions
14 of Ks between duplicates marking WGD events were detected by mixture models using mixtools
15 (<https://github.com/dsy109/mixtools>). The α -WGD event (mean Ks = 0.96) dated at 32.42 mya
16 (Hohmann et al. 2015) was used as a calibration point to estimate the minimum age of the
17 mesopolyploid WGD event.

18 A signal of selection was assessed using Ka/Ks values as compared to orthologous loci in *A.*
19 *thaliana*, considering genes to be under purifying selection when $Ka/Ks \leq 1-SD$ (i.e. ≤ 0.787),
20 neutral when $1-SD < Ka/Ks \leq 1+SD$, and under positive selection when $Ka/Ks > 1+SD$ (i.e. > 1.213).

21 Duplicated windows from each subgenome were further partitioned into “low-bias” regions
22 presenting quasi-unbiased fractionation (i.e. with similar number of retained syntenic genes in
23 the MF and LF subgenomes) and “high-bias” regions undergoing heavily biased fractionation (i.e.
24 significantly different number of retained syntenic genes between the MF and LF subgenomes)
25 based on chi-squared tests (non-significant difference in proportion of retained genes in MF and
26 LF (p-value > 0.05) classified as “low-bias”, and significant difference classified as “high-bias”).

27

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8
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10 genomic data; MB collected and analyzed transcriptomic data; IL collected and analyzed flow
11 cytometry data; TM and MAL collected and analyzed molecular cytogenetics data; SG collected
12 and analyzed phylogenetic data; MP analyzed HiC data; MB, RRC and CP integrated and
13 interpreted data and drafted the manuscript.

14
15 **Competing Interest Statement:** The authors declare no competing interests.

16
17 **Data and Resource Availability:** Raw sequence data are available on the European Nucleotide
18 Archive repository (<https://www.ebi.ac.uk/ena/browser/home>), as follows:

19 Genomic DNA sequence data: PacBio long-reads (SRR26423064), Paired-end Illumina short reads
20 (SRX8787129), 10X genomics linked reads (SRX8815186), Chicago Illumina short reads
21 (SRR26396391), Hi-C Illumina short reads (SRR26404274).

22 Transcriptomic data: RNAseq among tissues (ERP132985), RNAseq among environmental
23 treatments (ERP132838).

24 The genome assembly is available at:
25 <https://genomevolution.org/coge/GenomeInfo.pl?gid=67230>

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 8

9 Legends of figures

10 **Figure 1. Assembly and annotation of the Buckler Mustard mesopolyploid genome. A)** Circos
 11 plot showing (a) the nine main scaffolds (chromosomes in Mb) of *Biscutella laevigata* subsp.
 12 *austriaca* with syntenic genes shown as lines coloured and labelled by capital letters according to
 13 the ancestral genomic blocks in Brassicaceae; (b) differentially expressed duplicated genes across
 14 the least fractionated (LF) subgenome, shown as dots coloured based on expression in response
 15 to environmental treatments (blue = cold, red = heat, yellow = drought and green = herbivory); (c)
 16 differentially expressed genes across the most fractionated (MF) subgenome as in (b); (d) gene
 17 density per Mb; (e) LTR-retrotransposon density per Mb. **B)** Phylogenetic placement of Buckler
 18 Mustard among Brassicaceae and within the tribe Biscutellae based on the analysis of whole
 19 plastid genome sequences. **C)** Synonymous substitutions (Ks) among paralogs, with significant Ks
 20 peaks corresponding to the α -WGD event shown in blue and to the meso-WGD event in red. The
 21 green line indicates the Ks-based divergence between *B. laevigata* and *A. thaliana* (see Fig. S5).
 22 **D)** Dynamics of main types of transposable elements (TEs) with dated peaks indicative of
 23 transposition bursts in relation to the mean Ks values of the α -WGD and the meso-WGD events
 24 as in (C). LTR = class I long-terminal repeat retrotransposons; DNA = class II DNA transposons; DTM
 25 = mutator; DTC = CACTA; DTH = PIF-Harbinger; DTA = hAT; DTT = Tc1-Mariner; LINE = class I long
 26 interspersed nuclear element.

27
 28 **Figure 2. Patterns of fractionation in the least fractionated (LF) and the most fractionated (MF)**
 29 **subgenomes of the mesopolyploid Buckler Mustard. A)** Number of retained duplicates and genes
 30 that have returned to singleton state following WGD among the 122 hi-confidence duplicated

1 segments, showing biased fractionation with a higher number of intact genes in the LF than on
2 the MF subgenome. **B)** Non-synonymous (Ka) per synonymous (Ks) substitutions showing stronger
3 signals of purifying selection among retained duplicates than singletons in each subgenome. **C)**
4 Maximum expression levels of genes in leaf transcriptomes under cold, heat, drought and
5 herbivory treatments showing significantly higher levels of expression in the LF than in the MF
6 subgenome. **D)** Density of transposable elements (TE) in basepairs per Mb showing a significantly
7 lower TE load in the LF compared to the MF subgenome. Significance of Wilcoxon test represented
8 by adjusted p-value < 0.001 (***) and non significant (ns).

9
10 **Figure 3. Expression and selection of environmentally-responsive genes in the mesopolyploid**
11 **Buckler Mustard. A)** Distribution of differentially-expressed (DE) genes in response to cold, heat,
12 drought and herbivory treatments. **B)** Patterns of selection based on Ka/Ks values between
13 duplicated and single-copy genes according to their type of expression, showing stronger
14 conservation of coding sequences among environmentally-responsive (DE) genes and
15 constitutively-expressed genes compared to unresponsive genes (i.e. genes that were either
16 unexpressed or were expressed but were not DE). Significance of Wilcoxon tests represented as
17 adjusted p-value < 0.001 (***), < 0.01 (**), and non-significant (ns). **C)** Proportions of DE genes
18 conserved across Brassicaceae showing biased distribution across the least-fractionated as
19 compared to the most-fractionated subgenome. Significance of Fisher's exact tests represented
20 as adjusted p-value < 0.001 (***) and < 0.01 (**).

21
22 **Figure 4. Biased retention of environment-responsive genes across regions of the least-**
23 **fractionated (LF) vs the most-fractionated (MF) subgenomes. A)** Analysis of the 122 windows
24 partitioned into 36 "low-bias" and 86 "high-bias" regions showing similar versus significantly
25 different numbers of retained duplicate genes between the LF (open circles) and MF (filled grey
26 circles) subgenomes, respectively. **B)** Divergence based on synonymous substitutions (Ks)
27 between duplicated genes in the "low-bias" vs "high-bias" fractionation regions. **C)** Density of
28 transposable elements (TE) in basepairs per Mb in the LF and MF subgenomes is not significantly

1 different in “low-bias” regions, whereas the “high-bias” regions of the MF subgenome have
 2 significantly higher TE density than the LF subgenome. **D)** Enrichment of gene set as compared to
 3 the whole genome showing constitutively-expressed genes are significantly over-represented in
 4 the “high-bias” LF regions. **E)** Genomic segment (genomic block U) showing a pattern of biased
 5 retention of genes responding to cold (depicted in blue) and herbivory (depicted in green) within
 6 the "high-bias" LF subgenome. Constitutively-expressed genes are represented in black, while lost
 7 or unexpressed genes are depicted in grey. The first panel illustrates differentially-expressed genes
 8 related to cold and herbivory in *Arabidopsis thaliana* (labelled A. tha ortho), whereas the second
 9 and third panels show the LF and MF subgenomes in *Biscutella laevigata*, respectively. The dashed
 10 red line delineates the segment into its "low-bias" and its "high-bias" region. Significance of
 11 Wilcoxon tests represented as adjusted p-value < 0.001 (***) , < 0.01 (**) and non-significant (ns).

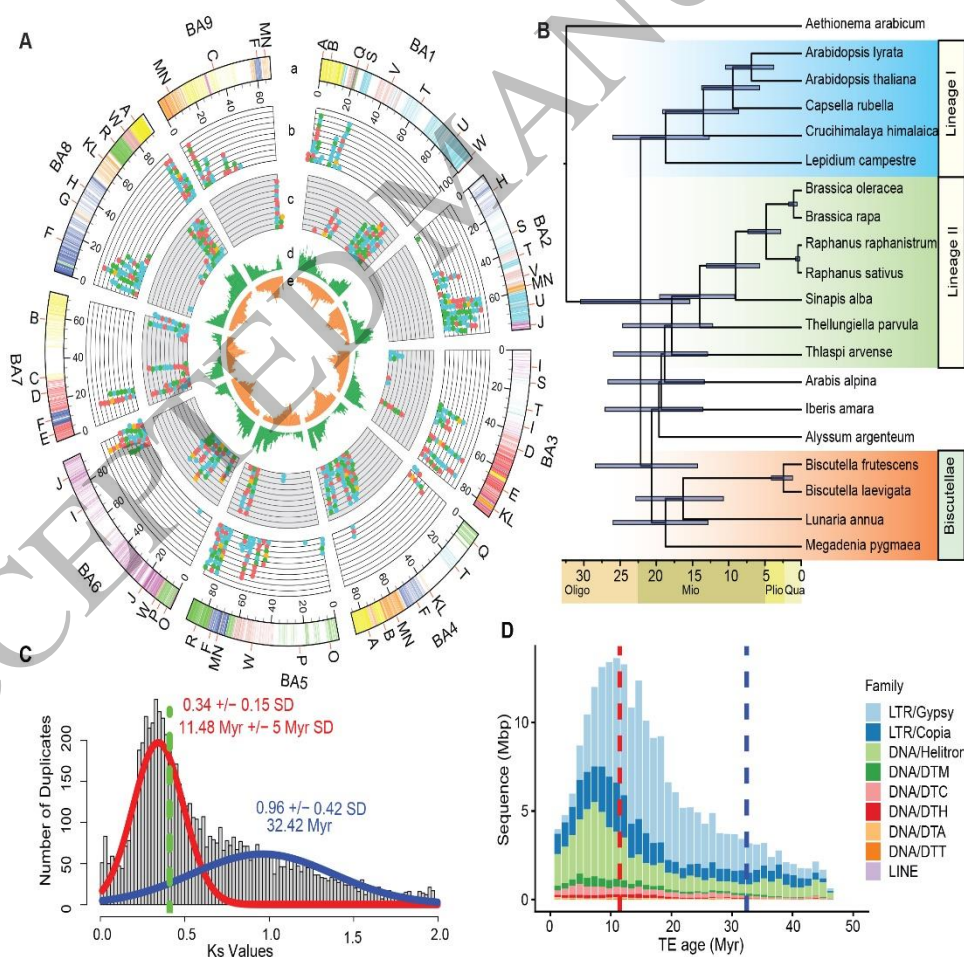


Figure 1
 220x180 mm (x DPI)

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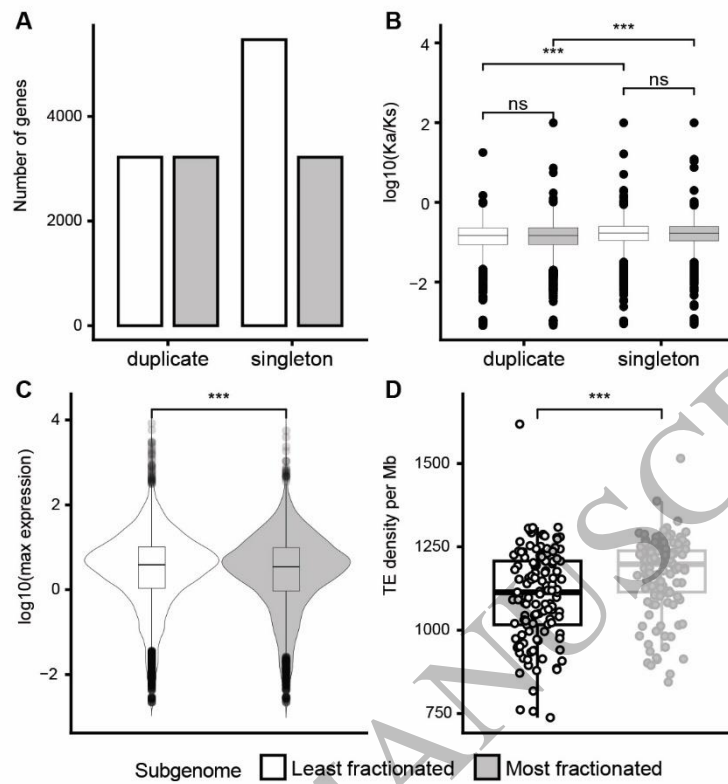


Figure 2
99x105 mm (x DPI)

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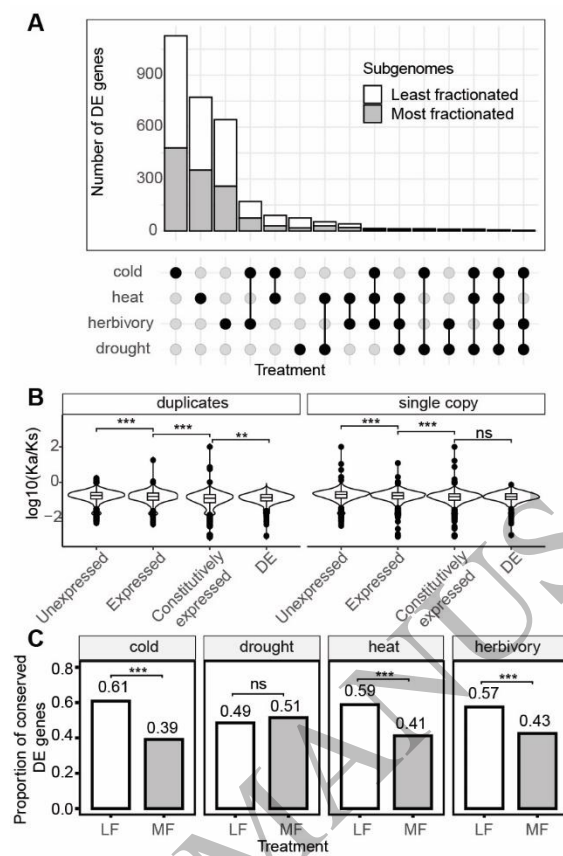


Figure 3
74x113 mm (x DPI)

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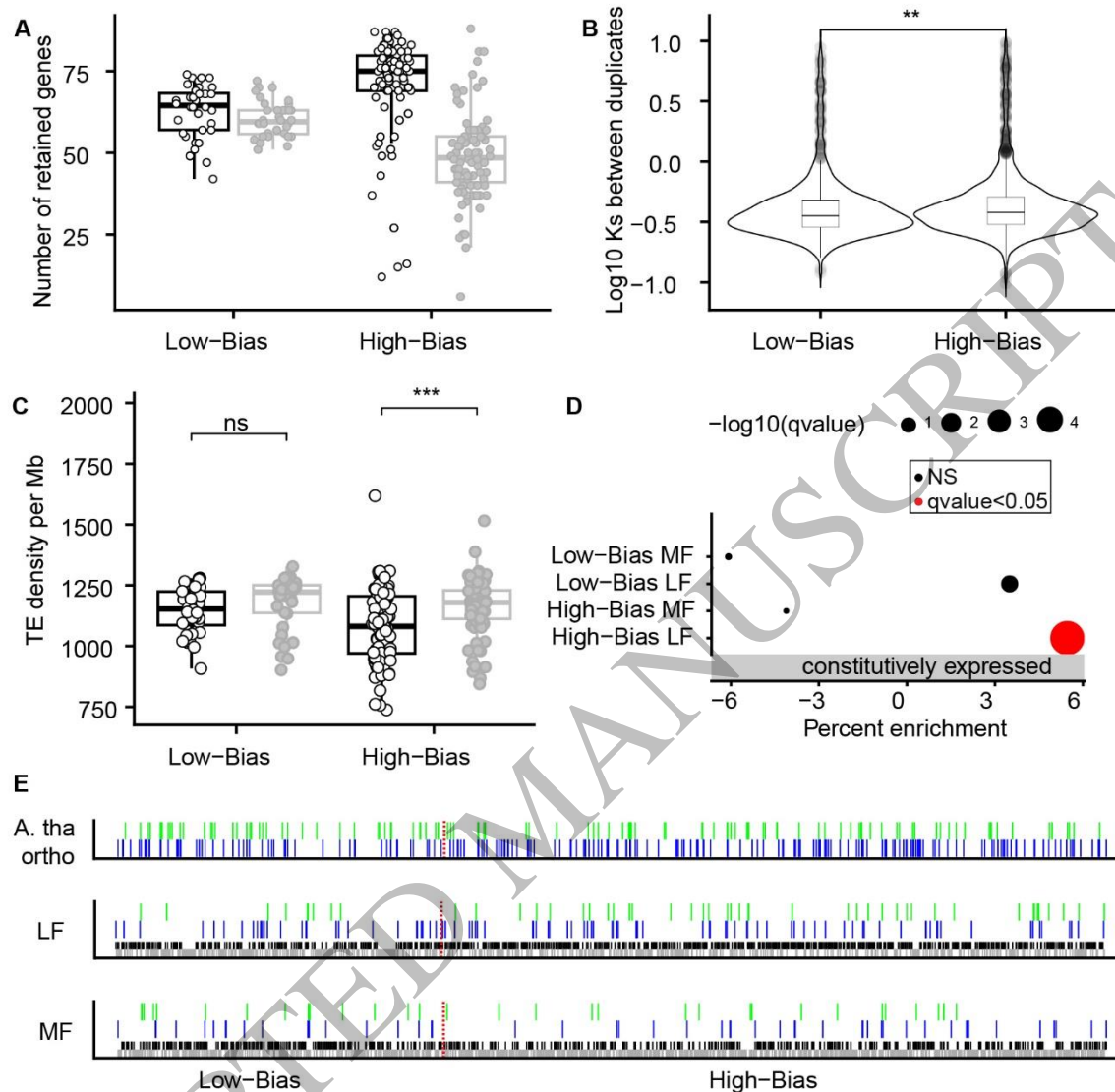


Figure 4
151x149 mm (x DPI)

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