Supplemental methods

Microarray hybridization and normalization

Microarrays were constructed from 21,504 exon-specific PCR products amplified from the Oregon-R strain of *D. melanogaster* (Hild et al. 2003). PCR products were spotted onto poly-L-lysine coated slides (Thermo Scientific, Portsmouth, NH) following standard protocols (www.microarray.org). Competitive hybridizations followed a modified loop design (Supplementary Figure 7) that minimizes the distance between any two genotypes (Townsend 2003). Each genotype was assayed on four replicate arrays and separate loops were used for males and females.

Sequence mismatches resulting from divergence between species influences hybridization efficiency on microarrays and can confound inferences of differential gene expression (Gilad et al. 2005). However, in all the genotypes used here except for *mau12*, genes located outside the introgressed segment (>90% of the genome) are derived from the *sim*B strain. Interspecific divergence therefore will not influence estimates of changes in expression at genes located outside the introgressed segments (*i.e. trans*-regulated genes). *D. simulans* and *D. mauritiana* are equally divergent from *D. melanogaster* (Hey and Kliman 1993; Kliman and Hey 1993; Garrigan et al. 2012), and so *D. simulans* and *D. mauritiana* messages transcribed from genes within the introgressed segments will be equally divergent, on average, from the *D. melanogaster* sequences spotted on the microarray. We rule out spurious inference of *cis*-regulatory effects resulting from gene-specific and lineage-specific sequence divergence by means of pyrosequencing assays of allele-specific expression in introgression heterozygotes.

RNA was extracted from frozen flies using TRIzol (Invitrogen, Carlsbad, CA), chloroform and phase-lock gel tubes (5 Prime, Gaithersburg, MD) to separate aqueous and organic layers. RNA was independently extracted from multiple pools of flies from all three cohorts and pooled for cDNA synthesis. At least 20μg total RNA was used as a template for cDNA synthesis (SuperScript II, Invitrogen, Carlsbad, CA), and incorporation of fluorescent dyes and hybridization to microarrays were done with the 3DNA Array50 kit (Genisphere, Hatfield, PA) following the manufacturer's protocols. Slides were scanned in an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA) and microarray images were processed with GenePix v5.0 (Molecular Devices, Sunnyvale, CA).

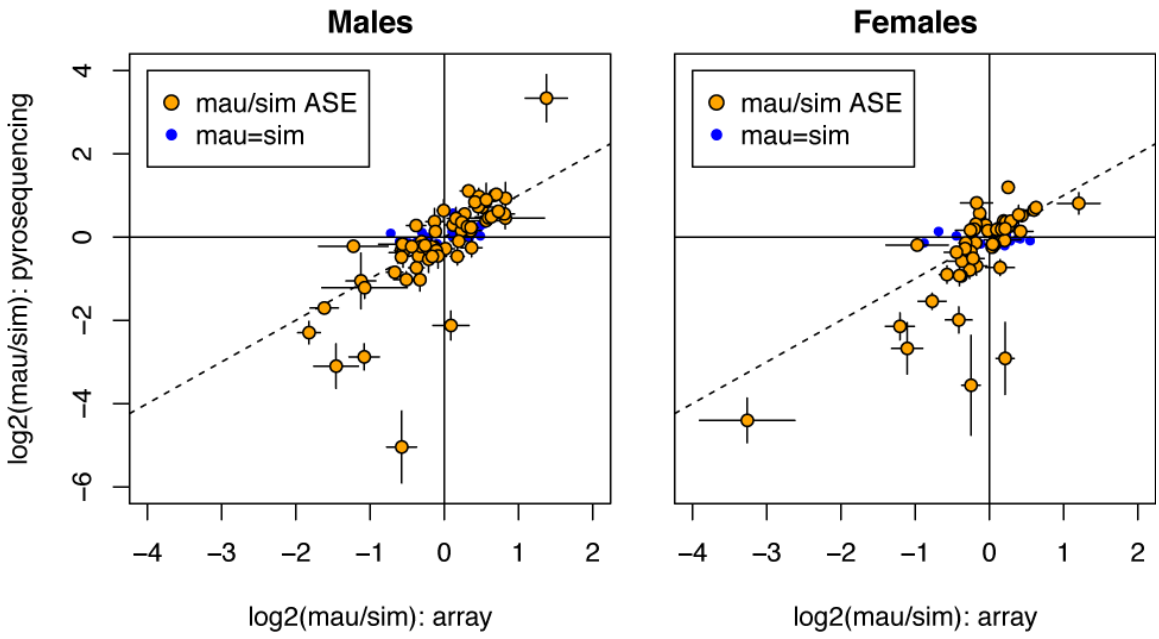
Microarray spots were filtered using the following quality control criteria: 70% of the foreground pixels within a spot were required to have a signal intensity higher than two standard deviations above the median background signal intensity in at least one of the two channels (Cy3 or Cy5); the median foreground signal intensity was required to be at least three times as great as the median background signal intensity in at least one of the two channels; a spot must have more than 30 foreground pixels. Spot-wise log2 ratios were normalized over overall spot signal intensity by loess smoothing (Smyth 2004) using a span of 0.3. To account for spatial variation in signal across arrays, spot-wise

log2 ratios were subsequently normalized by a second loess smoothing over the physical location of the spots on the slide, using a span of 0.002, which corresponds to an effective neighborhood of ~ 250 spots. Only probes that could be computationally localized to a single position on one of the six chromosome arms in the assembled *D. melanogaster* genome (R5.48) were retained for analysis.

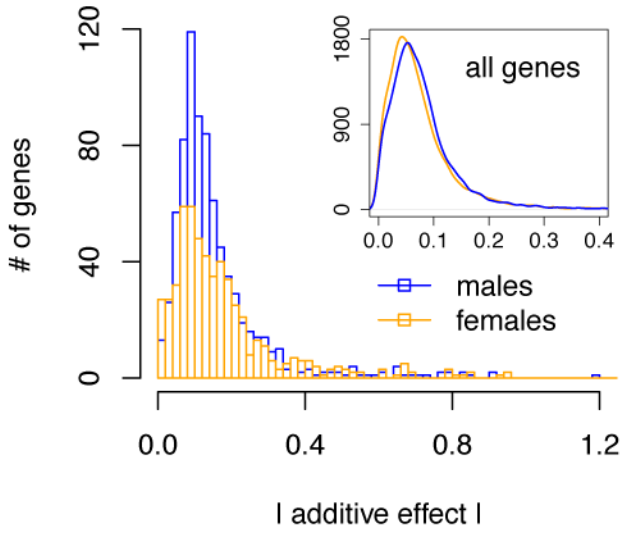
Pyrosequencing

Allele-specific expression was assayed by pyrosequencing 90 candidate genes identified from the microarrays as potentially harboring *cis*-regulatory variants (Supplementary Figure 8, Supplementary Table 8). Candidates included genes with significant evidence for eQTL in one or both sexes, as well as candidates with associations that were significant with a less stringent cutoff than was used for the final analysis. Each gene was assayed in males and females separately using two replicate RNA extractions from two different heterozygous introgression genotypes generated from both reciprocal crosses, resulting in eight biologically independent replicates in each sex. RNA was extracted using the same TRIzol/chloroform/phase-lock gel protocol as for the microarrays, and at least 2.5μg RNA was DNase treated (NEB, Ipswitch, MA) and used as a template for cDNA synthesis (SuperScript III, Invitrogen, Carlsbad, CA) using oligo-dT priming. Genomic DNA was extracted from heterozygous introgression females using phenol, chloroform, phase-lock gel tubes and an RNaseA treatment.

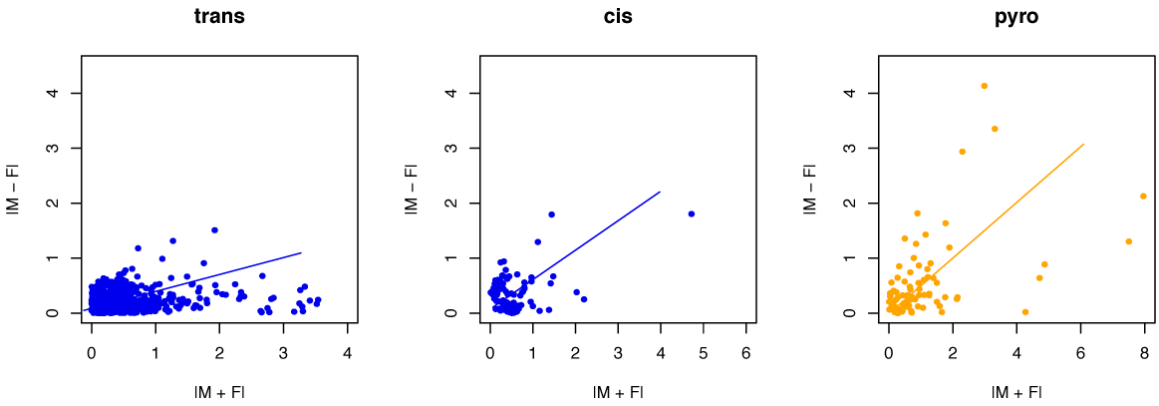
Each transcript to be assayed was PCR amplified (Promega GoTaq, Madison, WI) from cDNA and the PCR products were used in pyrosequencing reactions (Qiagen, Valencia, CA) following standard protocols (Wittkopp 2011). Two independent genomic DNA samples for each heterozygous introgression genotype were assayed by pyrosequencing and the resulting ratios were used to normalize the corresponding measurements of allele-specific expression from cDNA. Pyrosequencing ratios from gDNA at four genes differed strongly between the two heterozygous introgression genotypes, and Sanger sequencing identified SNPs resulting from *de novo* mutation or gene conversion in the sequences targeted by the pyrosequencing primers in the genotypes with unexpected ratios. These genotypes were removed from the analysis, and these four genes were analyzed using only one heterozygous introgression genotype (Supplementary Table 8).



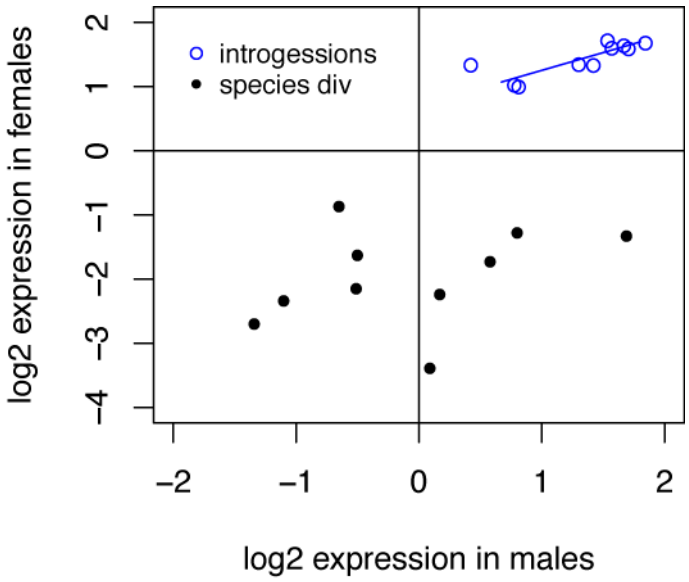
Supplemental Figure 1. Confirming candidate *cis*-regulatory variants with measures of allele-specific expression from pyrosequencing. Plotted on the ordinate is the homozygous introgression expression effect relative to the background *D. simulans* strain; on the abscissa is the log2 ratio of expression from the *D. mauritiana* and *D. simulans* alleles in introgression heterozygotes. The dashed line has a slope of 1. Error bars are only shown for genes with significant allelic differences in expression (ASE) and indicate 95% confidence limits for expression estimates. If differences in expression were due entirely to *cis*-regulatory divergence, then the ratio of allelic expression in an introgression heterozygote should equal the ratio of expression between introgression homozygotes and *sim*B. Deviations from this expectation can result from *cis*-by-*trans* genetic interactions (Wittkopp et al. 2004), as well as from comparing expression estimates using microarrays and pyrosequencing in these experiments. The allele-specific ratios obtained from pyrosequencing and the effects estimated from the microarrays are generally in good agreement ($\rho = 0.744$ and 0.719 in males and females, respectively; $P < 0.0001$), suggesting minimal *cis*-by-*trans* interaction effects. This result is expected, as > 90% of *trans*-acting factors in introgression homozygotes, introgression heterozygotes, and *sim*B are derived from the same inbred *D. simulans* strain.



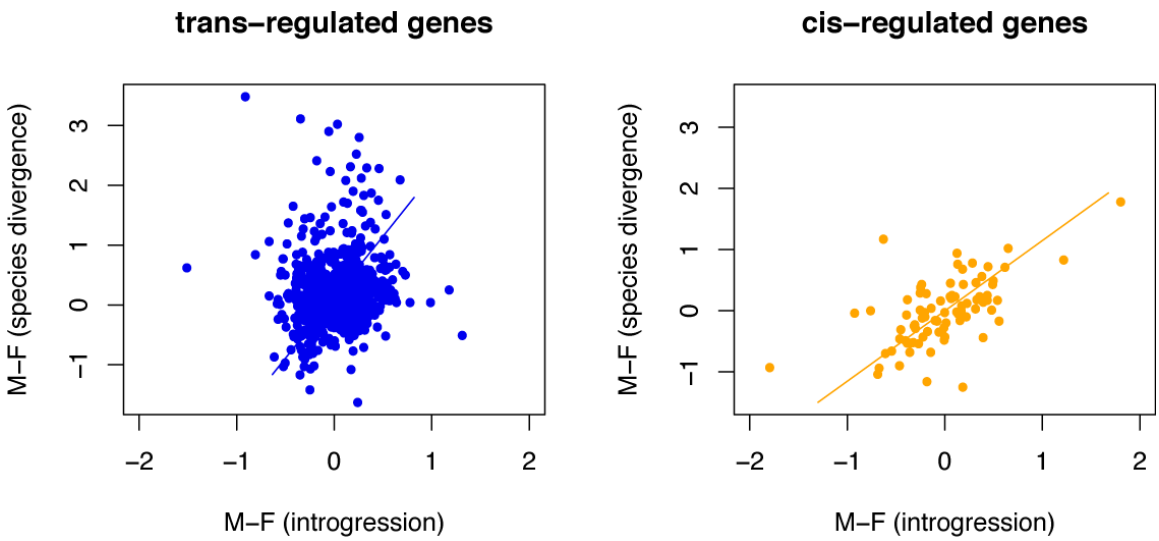
Supplemental Figure 2. Distributions of the absolute magnitude of additive effects of introgressed factors on gene expression in males and females. Divergently regulated genes are shown in the main panel; inset shows distributions for additive effects associated with the best fit marker for all genes considered in the eQTL analysis, regardless of the statistical significance of association with a genetic marker.



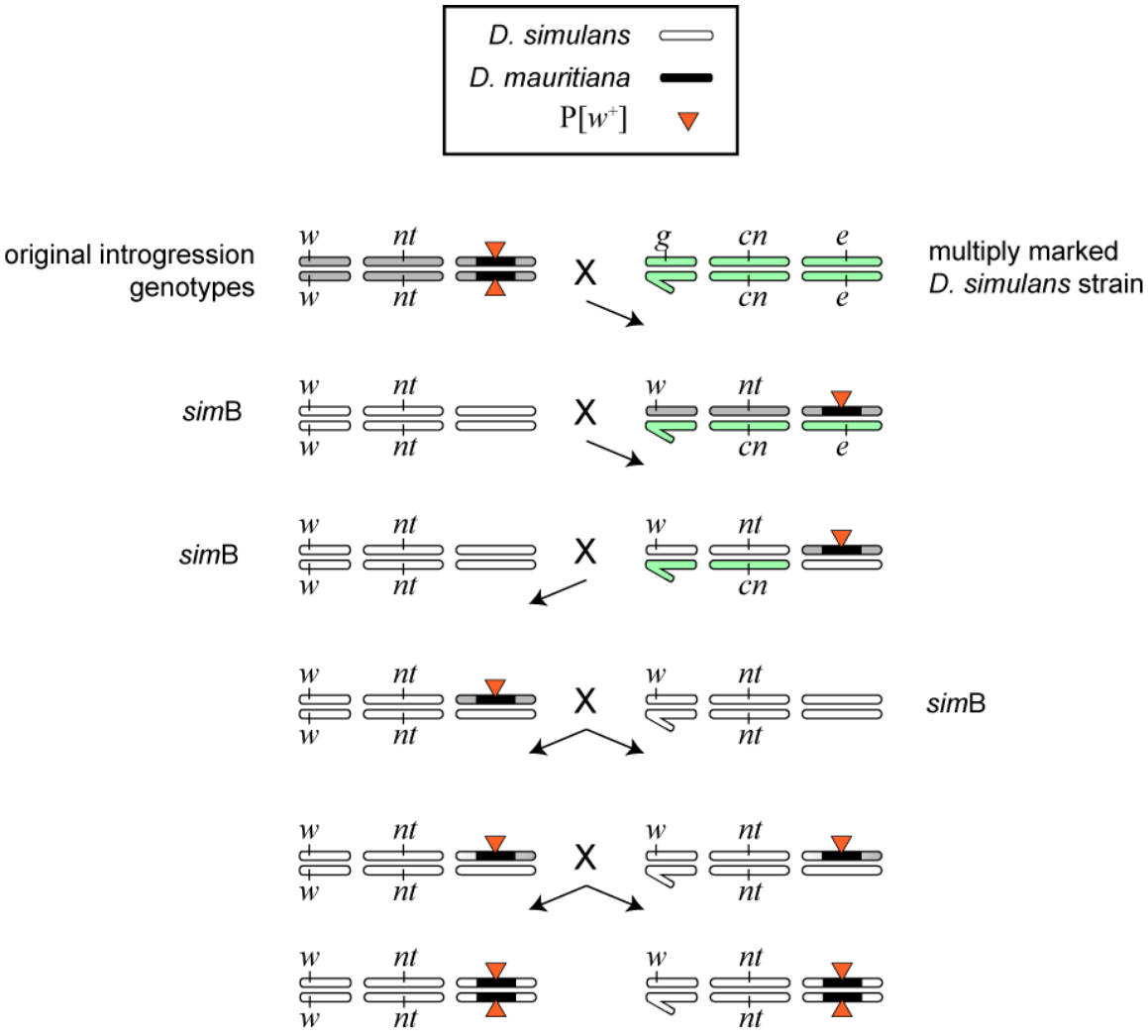
Supplemental Figure 3. Greater sexual dimorphism in expression is associated with larger average effects among introgressed *trans*-regulatory factors (slope = 0.31, $P < 0.0001$) as well as introgressed *cis*-regulatory factors assayed both by microarray (slope = 0.53, $P < 0.0001$) and pyrosequencing (slope = 0.51, $P < 0.0001$).



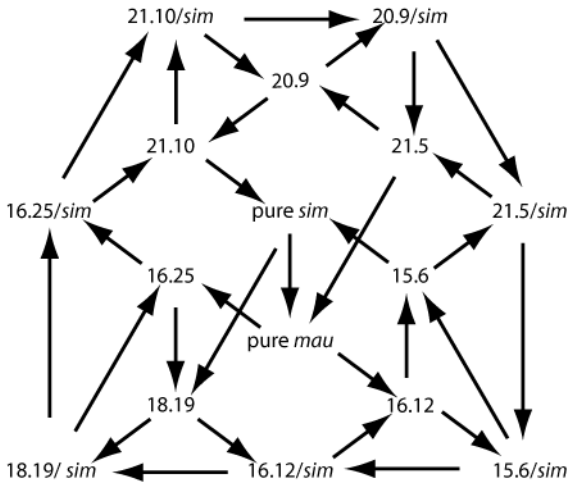
Supplemental Figure 4. Homozygous introgression effects on the expression of ten divergently regulated immunity-related genes are positively correlated between males and females ($\rho = 0.78$, $P = 0.008$), but whole-genome divergence effects on the expression of these 10 genes are uncorrelated between the sexes ($P = 0.32$).



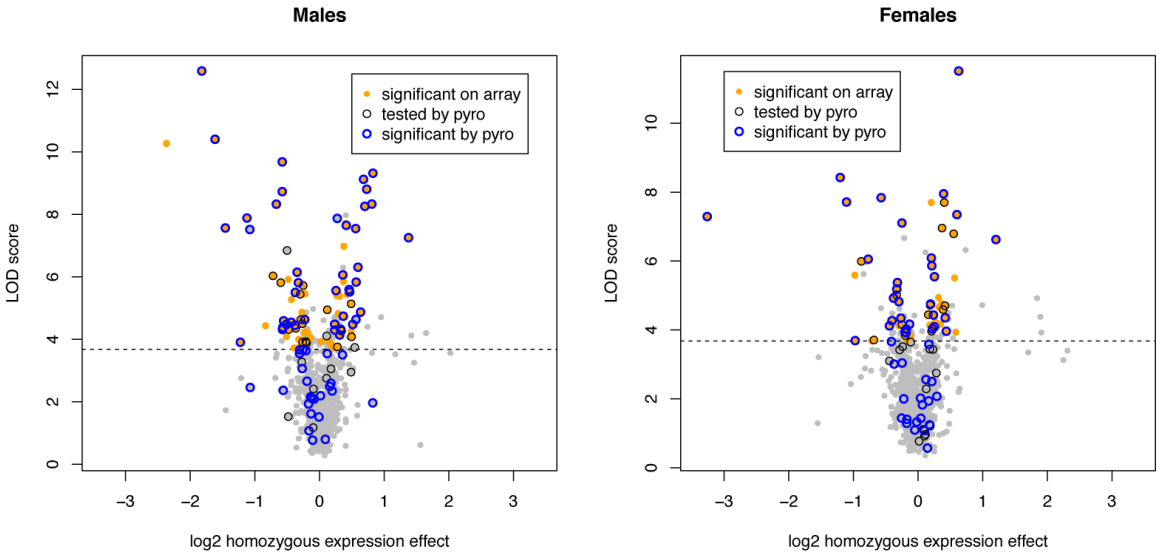
Supplemental Figure 5. Sexually dimorphic expression resulting from introgressed *trans*-regulatory factors is poorly correlated with sexually dimorphic expression at the same genes due to whole-genome divergence between *sim*B and *mau12* (slope = 2.04, ρ = 0.124, P = 0.0002) compared to sexually dimorphic expression resulting from introgressed *cis*-regulatory sequences (slope = 1.15, ρ = 0.607, P < 0.0001).



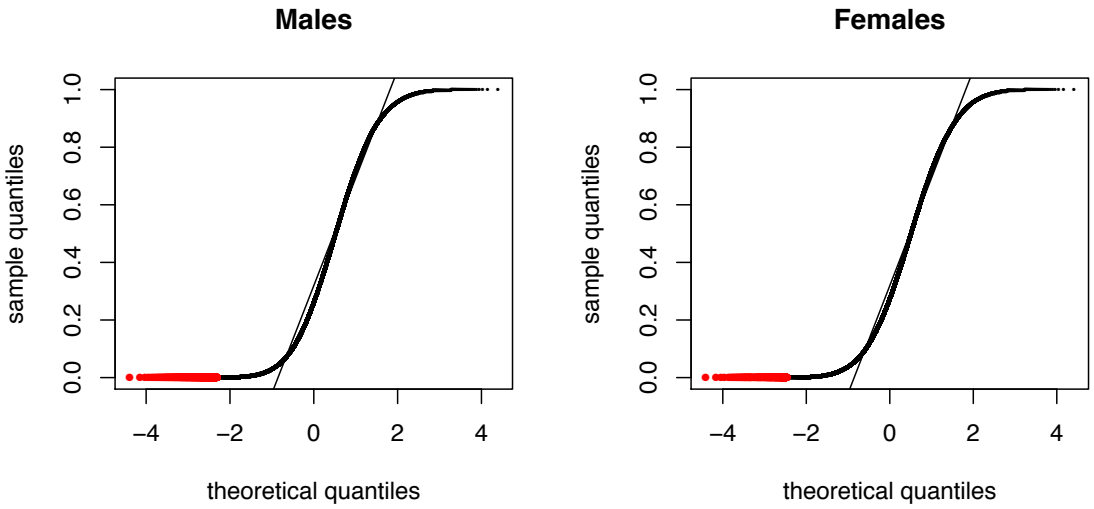
Supplemental Figure 6. Crossing scheme used to re-extract introgression 3rd chromosomes into the *sim*B genetic background (which is the same strain used for the original introgressions). Shown are the sex chromosomes and two major autosomes and the visible mutations they carry. Passage of introgression chromosomes through females in the 4th and 5th generations allows recombination and replaced some regions of chromosome arm 3L outside of the introgressed segments with genetic material from *sim*B.



Supplemental Figure 7. Loop design used for comparative hybridizations. Arrowhead indicates the sample labeled with Cy5; alternate sample was labeled with Cy3.



Supplemental Figure 8. Volcano plots indicate genes harboring candidate *cis*-regulatory divergence chosen for pyrosequencing analysis. Grey dots indicate all genes detected as expressed and located inside the introgressed region of the genome. The dashed line indicates the threshold LOD score chosen to determine significant associations between expression phenotypes and marker genotypes. Genes marked by grey dots above this threshold had expression phenotypes associated with markers distant from the gene.



Supplemental Figure 9. Q-Q plots of QTL mapping *P*-values. Lines correspond to a normal distribution with the same first and third quartiles as the data. Points in red indicate *P*-values retained after FDR correction for multiple tests.

Supplemental references

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