The Interplay of Temperature and Genotype on Patterns of Alternative Splicing in *Drosophila melanogaster*

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ABSTRACT Alternative splicing is the highly regulated process of variation in the removal of introns from premessenger-RNA transcripts. The consequences of alternative splicing on the phenotype are well documented, but the impact of the environment on alternative splicing is not yet clear. We studied variation in alternative splicing among four different temperatures, 13, 18, 23, and 29°, in two *Drosophila melanogaster* genotypes. We show plasticity of alternative splicing with up to 10% of the expressed genes being differentially spliced between the most extreme temperatures for a given genotype. Comparing the two genotypes at different temperatures, we found <1% of the genes being differentially spliced at 18°. At extreme temperatures, however, we detected substantial differences in alternative splicing—with almost 10% of the genes having differential splicing between the genotypes: a magnitude similar to between species differences. Genes with differential alternative splicing between genotypes frequently exhibit dominant inheritance. Remarkably, the pattern of surplus of differences in alternative splicing at extreme temperatures resembled the pattern seen for gene expression intensity. Since different sets of genes were involved for the two phenotypes, we propose that purifying selection results in the reduction of differences at benign temperatures. Relaxed purifying selection at temperature extremes, on the other hand, may cause the divergence in gene expression and alternative splicing between the two strains in rarely encountered environments.

KEYWORDS alternative splicing; temperature; plasticity; dominance

SPLICING, the removal of introns from precursor messenger RNAs (mRNAs) together with the subsequent ligation of exons, is an integral part of gene expression regulation. Alternative splicing is the combination of different exons from the same precursor mRNA and provides the basis for the impressive diversity of gene products originating from a substantially smaller set of genes (Pan *et al.* 2008; Nilsen and Graveley 2010; Brown *et al.* 2014). There are several types of alternative splicing; such as the exclusion of exons, sometimes mutually exclusive; or the retention of intronic sequence in the mature transcript. Furthermore, the alternative selection of 5′ or 3′ splice sites, a special form of exon skipping (Koren *et al.* 2007), has been shown to make an important contribution to transcript diversification.

Splicing, in particular alternative splicing, is a highly regulated process that depends on *cis*-regulatory sequences (splicing enhancers and suppressors) and *trans*-regulatory splicing factors, such as heterogeneous nuclear ribonucleoproteins and SR and SR-related proteins (Nilsen and Graveley 2010). The repertoire of isoforms, different mature mRNAs originating from a single gene, differs widely among tissues, developmental stages, and environmental conditions (Barberan-Soler and Zahler 2008; Gan *et al.* 2010; Barbosa-Morais *et al.* 2012; Bartok *et al.* 2013; Leviatan *et al.* 2013; Long *et al.* 2013; Reyes *et al.* 2013; Telonis-Scott *et al.* 2013; Brown *et al.* 2014; Chang *et al.* 2014; Vitulo *et al.* 2014). It could, therefore, be considered as a prototype for phenotypic plasticity on the molecular level (Mastrangelo *et al.* 2012; Chen *et al.* 2015b).

Phenotypic plasticity describes the ability of a given genotype to display a range of phenotypes as a response to environmental heterogeneity. On the organismal level, phenotypic plasticity has been of key interest to evolutionary biologists as it provides the opportunity to respond quickly

doi: 10.1534/genetics.116.192310
Manuscript received June 3, 2016; accepted for publication July 8, 2016; published Early Online July 19, 2016.
Available freely online through the author-supported open access option.
Supplemental material is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.192310/-/DC1.
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to environmental changes. On the cellular level, phenotypic plasticity is the impressive manifestation of cellular differentiation of multicellular organisms; a property favored by natural selection.

While the selective advantage of both the presence or absence of phenotypic plasticity is conceptually appealing, their relative importance is not yet clear. Traditionally, plasticity has been studied using high-order phenotypes, such as morphology and life history traits, which integrate the effects of many genes. Nevertheless, the advances in molecular biology have opened the possibility to expand these studies to lower-level phenotypes such as gene expression and alternative splicing. Over the past years, an impressive amount of data has been collected demonstrating plasticity of gene expression and alternative splicing in different tissues and developmental stages (Jin *et al.* 2001; Wang *et al.* 2008; Graveley *et al.* 2011; Zhou *et al.* 2012; Smith *et al.* 2013; Brown *et al.* 2014; Etges *et al.* 2015). Much less is known about the influence of environmental conditions on this plasticity (Levine *et al.* 2011; Yampolsky *et al.* 2012; Telonis-Scott *et al.* 2013; Brown *et al.* 2014; Chang *et al.* 2014; Sikkink *et al.* 2014; Vitulo *et al.* 2014; Yampolsky *et al.* 2014; Chen *et al.* 2015a; Zhao *et al.* 2015), and the conservation of these patterns across genetically diverged organisms (Barberan-Soler and Zahler 2008; Etges *et al.* 2015; Chen *et al.* 2015a; Zhao *et al.* 2015).

Temperature is one of the key environmental parameters, in particular for ectotherms such as *Drosophila*. A broad range of morphological, behavioral, and physiological responses to temperature has been described, but few studies attempted to compare the patterns of gene expression plasticity across temperatures. Most of these studies compared the pattern of gene expression at two temperatures (Sikkink *et al.* 2014; Zhao *et al.* 2015) and found a large number of genes significantly affected by temperature. Recently, Chen *et al.* (2015a) attempted a more refined characterization of the temperature effect on gene expression by describing the reaction norm of gene expression across a broad temperature range (13–29°). Remarkably, they found that the reaction norm did not only cluster genes according to function, but also explained some of the underlying regulatory architecture (Chen *et al.* 2015b).

Extending the plasticity analysis to diverged genotypes often found significant differences in the reaction norm between genotypes. In studies that compared gene expression between differentially evolved genotypes, differences in gene expression plasticity were good indicators for direct or indirect selection targets (Telonis-Scott *et al.* 2009; Yampolsky *et al.* 2012). An interesting pattern was found when Chen *et al.* (2015a) contrasted the pattern of gene expression between two genotypes at different temperatures. At 18° the authors observed almost no differences in gene expression intensity between two inbred *Drosophila* laboratory strains, but at more extreme temperatures the expression divergence increased. This pattern was interpreted as evidence for canalized gene expression at 18°, which becomes lost when flies are exposed to more extreme environments (decanalization) (Chen *et al.* 2015a).

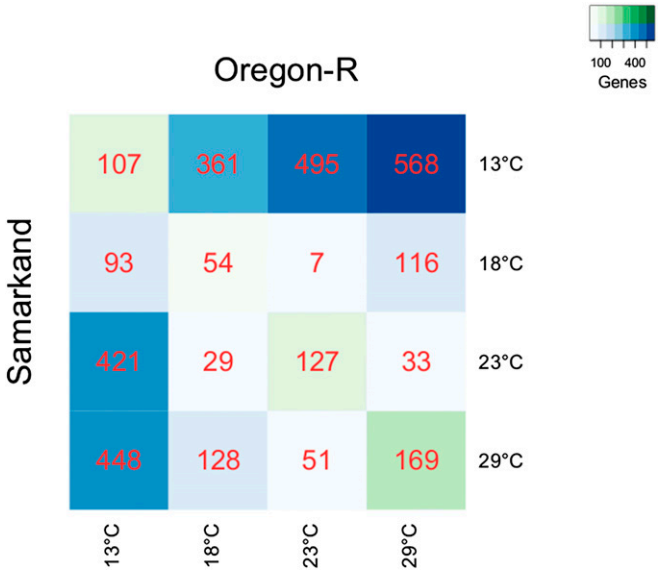

Figure 1 Temperature-dependent differential splicing between temperatures and between genotypes. Differentially spliced genes for pairwise temperature comparisons (blue) are shown for each of the genotypes, above the diagonal for Oregon-R and below the diagonal for Samarkand. Differential splicing between genotypes at a given temperature is shown on the diagonal (green). The heat map of pairwise temperature comparisons of differential splicing shows strong plasticity in both strains when the most extreme temperatures (13 and 29°) are compared. Alternative splicing between the more benign temperatures, 18 and 23°, however, exhibited only a weak plastic response to temperature in both genotypes. The data are based on reads mapping to the 3′ side of the transcript (see File S1 for more details).

Despite its well-characterized influence on the phenotype, alternative splicing plasticity has been studied only in the context of exposure to acute stress conditions (Mastrangelo *et al.* 2012; Long *et al.* 2013; Telonis-Scott *et al.* 2013; Vitulo *et al.* 2014). Very little is known, however, about how long-term exposure to typically encountered environments modulates alternative splicing.

Here, we have used the data from the gene expression study by Chen *et al.* (2015a), studied the influence of temperature on the pattern of alternative splicing in *Drosophila melanogaster*, and compared this response between two genotypes. We contrasted the patterns of alternative splicing to those of gene expression intensities from a study by Chen *et al.* (2015a). Like for gene expression intensities, we found that temperature has a strong effect on alternative splicing, resulting in up to 568 (10.4%) genes being differentially spliced between the two most extreme temperatures for a given genotype. Even more surprising was the consistency of the pattern of increasing differences between the genotypes on both levels of the phenotype at extreme temperatures: at 18° only very few genes were differentially spliced between the two genotypes, whereas at extreme temperatures we detected the largest number of genes with differential splicing. Despite the similarity of this pattern, the involved genes did not overlap more than expected by chance.

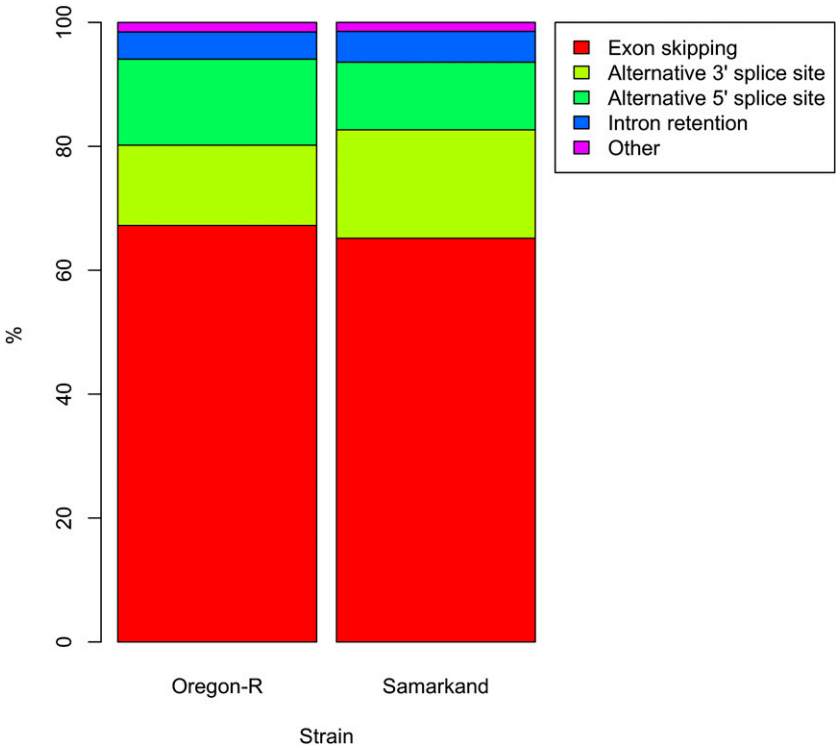

Figure 2 Distribution of splice types for genes with differential splicing between 13 and 29° for each of the two genotypes. The most prevalent splice type is exon skipping (red), followed by alternative 3′ splice site usage (yellow), 5′ splice site usage (green), and intron retention (blue). Overall, our statistically inferred splicing differences are also reflected by Sashimi plots based on reads covering exon junctions (Figure 3).

Materials and Methods

Females (f) from Oregon-R (O) and Samarkand (S) laboratory strains were crossed with males (m) from both strains (Of × Om, Of × Sm, Sf × Sm, Sf × Om) in three replicates. After 2 days of egg laying at 23°, the eggs were transferred to one of the four assaying temperatures (13, 18, 23, and 29°). Virgin females were used for extraction and sequencing of mRNA. Further details on fly rearing can be found in Chen *et al.* (2015a) and Supplemental Material, File S1. Library preparation and sequencing are described in Chen *et al.* (2015a). Raw sequence reads (National Center for Biotechnology Information accession number SRP041398 and SRP041395) were trimmed based on sequencing quality using PoPoolation2 (Kofler *et al.* 2011) and mapped to the *D. melanogaster* reference genome (Flybase assembly 5) using the **g**enomic **s**hort-read nucleotide **a**lignment **p**rogram (GSNAP) (Wu and Nacu 2010). All mapped RNA sequencing (RNA-seq) reads were randomly downsampled to the same coverage and counted with a DEXSeq counter. Differential exon usage analysis was conducted using the DEXSeq R package (Anders *et al.* 2012). Due to 3′ gene-transcript coverage bias in some samples, we restricted some analyses by using only the reads mapping to the 3′ side of the transcript (Figure A, File S1). Inheritance assignment followed the procedures described in McManus *et al.* (2010) and Chen *et al.* (2015a) and is described in detail in File S1. Splice types were assigned based on the *D. melanogaster* annotation (see File S1). Gene ontology (GO) analysis was performed using Gowinda (Kofler and Schlötterer 2012) and accounted for different splicing opportunities (*i.e.*, intron numbers) among GO categories. Gene set overlaps were assessed using receiver operating characteristic (ROC)-like curves, which indicate if the overlap between two sets of ranked data are higher or lower than expected by chance (curve above and below the diagonal). Further details about the methods used are described in File S1.

Data availability

All raw sequence data used in this study is deposited in the National Center for Biotechnology Information Sequence Read Archive with accession numbers SRP041398 (Oregon-R and Samarkand) and SRP041395 (F1). All unfiltered read counts, custom scripts, and protocols will be available at DataDryad.org.

Results

We used 100-bp paired-end RNA-seq reads from two *D. melanogaster* genotypes, Samarkand (S) and Oregon-R (O), which were exposed to four different developmental temperatures ranging from 13 to 29° (Chen *et al.* 2015a). Each genotype–temperature combination was analyzed in three replicates. We measured alternative splicing by means of exon usage (Anders *et al.* 2012), using only those multi-exon genes with an average of at least 50 reads across all samples in the analysis (Table C, File S1).

Temperature-mediated plasticity of splicing

Pairwise comparisons of alternative splicing revealed a substantial effect of temperature, with up to 10.4% (568 out of 5463) of the multi-exon genes showing differential splicing

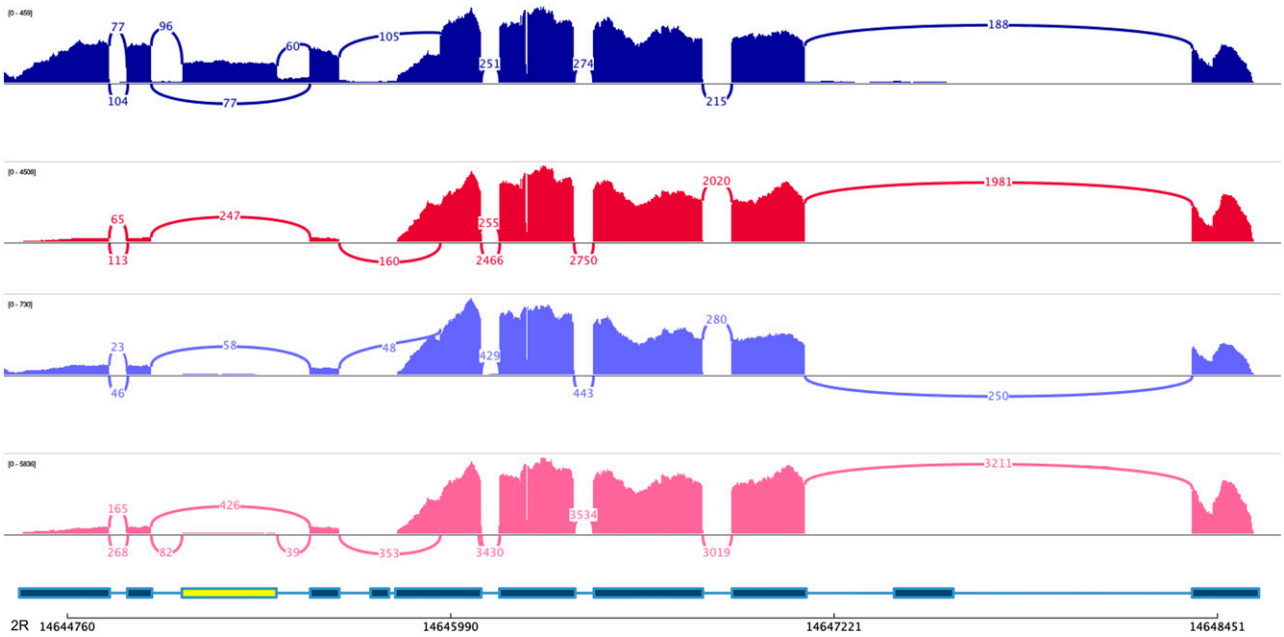

Figure 3 Genotype- and temperature-dependent alternative splicing. Sashimi plots for exon skipping of the third exon (marked in yellow) of the gene CG42351. Dark blue, Oregon-R 13°; dark red, Oregon-R 29°; light blue, Samarkand 13°; pink, Samarkand 29°.

between two temperatures for a given genotype. The highest plasticity of splicing was seen between the two extreme temperatures, but as few as seven genes differed in splicing between 18 and 23° in Oregon-R. Overall both *D. melanogaster* strains showed the same pattern of differential splicing with exons being more commonly retained at 13° and spliced out at 29° in both strains (Figure G, File S1). Oregon-R was more plastic than Samarkand (Figure 1).

The splicing differences between the two most extreme temperatures (13 and 29°) within genotypes were mostly caused by exon skipping (O = 67%, S = 65%) followed by both alternative 3′ (O = 13%, S = 17%) and 5′ (O = 14%, S = 11%) splice site selection, and with least changes caused by intron retention (O = 4%, S = 5%; Figure 2).

Temperature-dependent differences in alternative splicing between genotypes

Despite the overall similarity of the two strains in splicing patterns across temperatures, we systematically tested for differential splicing between the two genotypes (Oregon-R and Samarkand) at each of the four developmental temperatures (Figure 3 and Figure 4). The highest similarity in splicing between the two strains for a given temperature was observed at 18°, with 1.21% of all tested multi-exon genes (97 out of 8021) showing significantly different splicing patterns. However, at the other three temperatures, 13, 23 and 29°, splicing differed between the two genotypes for 1.95% (173 out of 8858), 7.99% (646 out of 8090) and 12.81% (1049 out of 8186) genes, respectively; (Figure 4) suggesting that difference in alternative splicing between strains is strongly dependent on the assaying temperature. This pattern was previously observed for gene expression intensities (Figure 4 inset). The difference in alternative splicing between the two genotypes at 13° becomes clearer after adjusting for variance in the 3′ gene-body read coverage across replicates (see File S1).

With temperature stress resulting in increasing differences in the splicing pattern between the two strains, we were interested to understand this better. Since different reaction norms of alternative splicing between the two strains may have caused the differences between genotypes at a certain temperature, we related these patterns to the intrastrain plasticity between different temperatures. Plotting the fold change in exon expression between genotypes within a temperature for each exon (corrected for overall gene expression) and fold changes of exons with splicing plasticity (differences between temperatures, within a strain) against each other, clearly indicated that the two are not congruent. Hence, we conclude that differences between strains are not a consequence of different reaction norms for alternative splicing of the two genotypes (Figure 5). Further support for this lack of congruence comes from ROC for exon expression intensities as well as a difference in GO term enrichment for genes with significant genetic differences and plasticity (Figure H, File S1).

Out of all splicing events that differed between the strains for a given temperature, exon skipping was the most frequent one (76%), followed by 3′ alternative splicing site usage (14–30%), and 5′ alternative splice site usage (3–5%). The least frequent event was intron retention (1%). This pattern was very similar across the entire temperature range, with a trend toward more exon skipping at higher temperatures (Figure 6). Importantly, a similar distribution of alternative splicing events has been described previously (McManus *et al.* 2014).

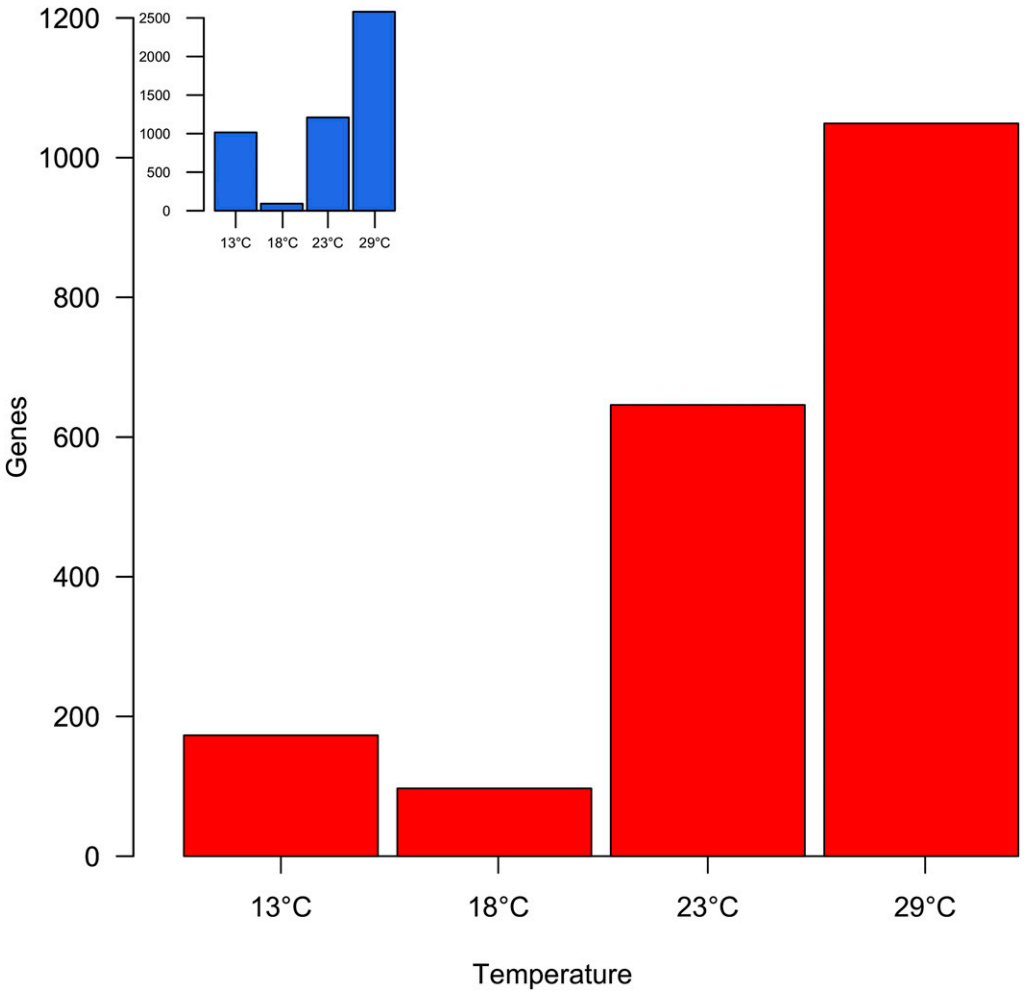

Figure 4 Genotype-specific alternative splicing. At 18° only a few genes differ in splicing between Oregon-R and Samarkand, while at more extreme temperatures the splicing patterns become increasingly different. This pattern resembles the one seen for gene expression intensity (blue inset; redrawn from Chen *et al.* 2015a).

Candidate genes for differential exon skipping

Previously, Chen *et al.* (2015a) showed enrichment in the GO categories "spliceosome" and "mRNA splicing, via spliceosome", indicating that the expression differences in the core splicing machinery could result in the differences in alternative splicing between the genotypes. Dominant inheritance of alternative splicing between the genotypes also suggested that alternative splicing regulation is guided mostly by *trans*-acting factors. To test this hypothesis further, we took advantage of *trans*-acting factors with genome-wide influence on alternative splicing. The exon junction complex serves a central role in splicing (Tange *et al.* 2004). Knockdown of two members of the exon junction complex, *mago nashi* and *eIF4AIII*, increases the rate of exon skipping (Tange *et al.* 2004; Ashton-Beaucage *et al.* 2010; Wang *et al.* 2014). The three core exon junction complex genes that can be found in the nucleus and can, therefore, have the ability to interact with the splicing process, show a consistent expression pattern across temperatures. At 13° they are more expressed in Oregon-R, while at 29° Samarkand has the higher expression level (Table 1). If the exon junction complex is involved in the splicing differences between the two strains, we expect to find more exon skipping in Oregon-R at 13°, while Samarkand would have more exon skipping at higher temperatures. In support of this hypothesis, in Samarkand flies we find on average downregulation of differentially spliced exons at 13°, and the opposite pattern at higher temperatures (Table 1). While these results strongly suggest a substantial influence of the exon junction complex on the alternative splicing, the observation of different genes being alternatively spliced across temperatures indicates that other splicing factors may also shape the plasticity of alternative splicing. Brooks *et al.* (2015) recently reported 56 splicing factors and their target genes. We used this set of splicing factors to further test our hypothesis. In our data, 49 of the factors reported by Brooks *et al.* were expressed (on average at least 20 mapped reads across all samples). A total of 31 (63%) of the splicing factors showed a similar pattern as the exon junction complex genes: they were upregulated in one strain at 13° and upregulated in the other strain at 29°. Genes with differential splicing between the two genotypes were enriched with genes regulated for 19 splicing factors (Table D, File S1). Five splicing factors, *snRNP-U1-70K* (FBgn0016978), *RpS3* (FBgn0002622), *SC35* (FBgn0265298), *RnpS1* (FBgn0037707), and *Hrb27C* (FBgn0004838) showed the same concordance of expression level and exon skipping as core exon junction complex genes (Table D, File S1). The protein components of the spliceosome, *snRNP-U1-70K* and *SC35*, are strong candidates for regulating

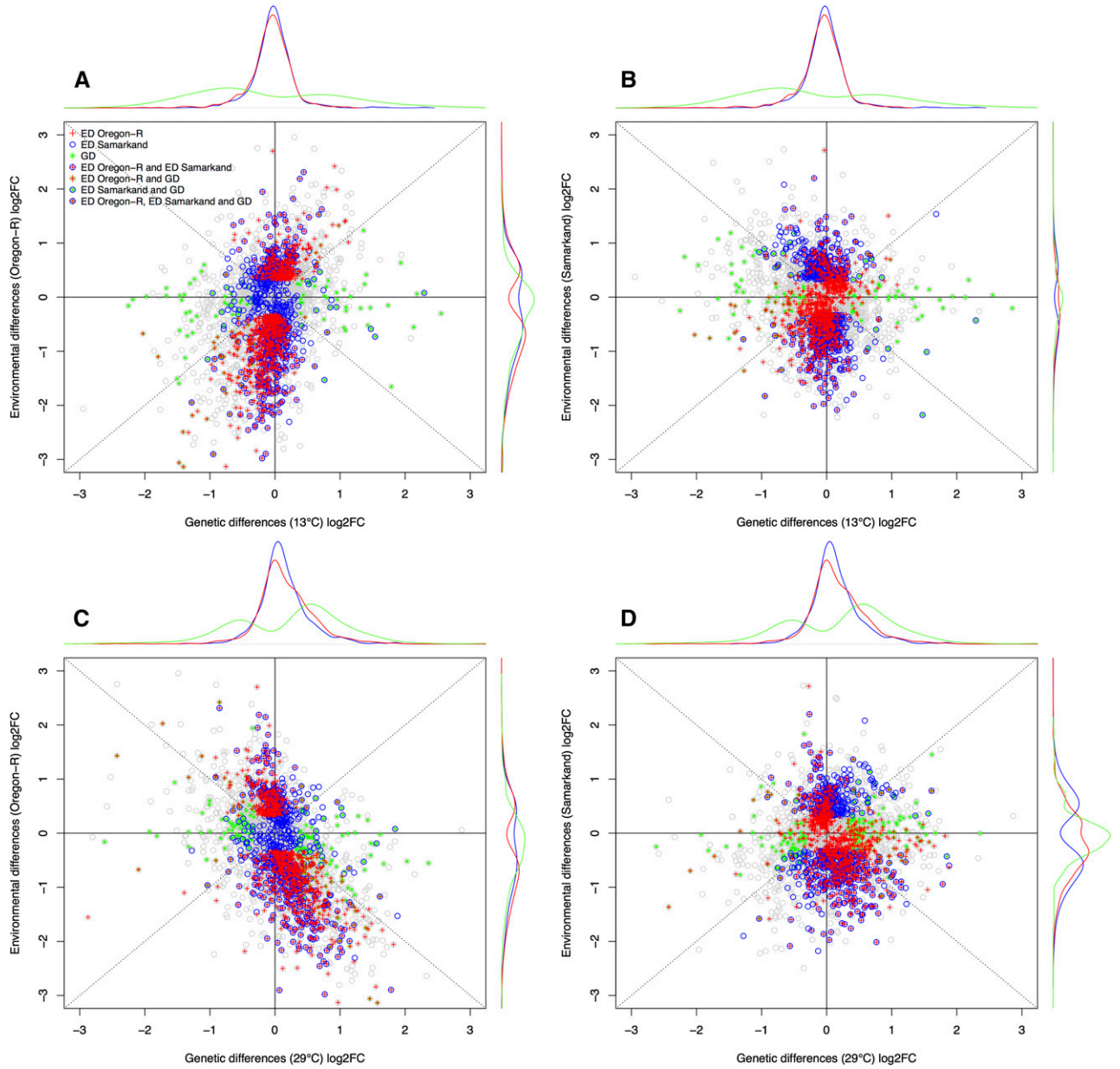

Figure 5 Genetically *vs.* environmentally induced differences in alternative splicing. Log_2-fold changes between Oregon-R and Samarkand at (A and B) 13° and (C and D) 29° are plotted against log_2-fold changes between 13 and 29° in (A and C) Oregon-R and in (B and D) Samarkand. Exons that are significantly differentially spliced between genotypes (GD, green) mostly do not overlap with the exons that are plastic (ED) in Oregon-R (red) or Samarkand (blue). Density plots on the top and on the right show the distribution of plotted points with corresponding colors. ED, environmental differences; GD, genetic differences.

differential splicing between the two genotypes. The auxiliary protein component of the exon junction complex *RnpS1* provides further support for the importance of the exon junction complex for the alternative splicing patterns seen in this study.

Similar patterns of temperature-dependent differences among strains for gene expression and alternative splicing

Interestingly, the striking temperature dependence of differential splicing between Oregon-R and Samarkand is mirrored for gene expression intensity (Figure 4) (Chen *et al.* 2015a). While at 18° the differences in both splicing and gene expression intensity between genotypes are very small, at extreme temperatures the differences increase. Given these parallel patterns, we were interested in whether the same genes were affected and compared the expression intensity differences of the entire gene against the expression differences in each exon (Figure 7; Figure I, File S1). Independent of the developmental temperature, genes with significant differences in gene expression intensity have only limited overlap with genes with differential splicing (Figure 7). These results suggest that despite the overall similarity in temperature dependence of

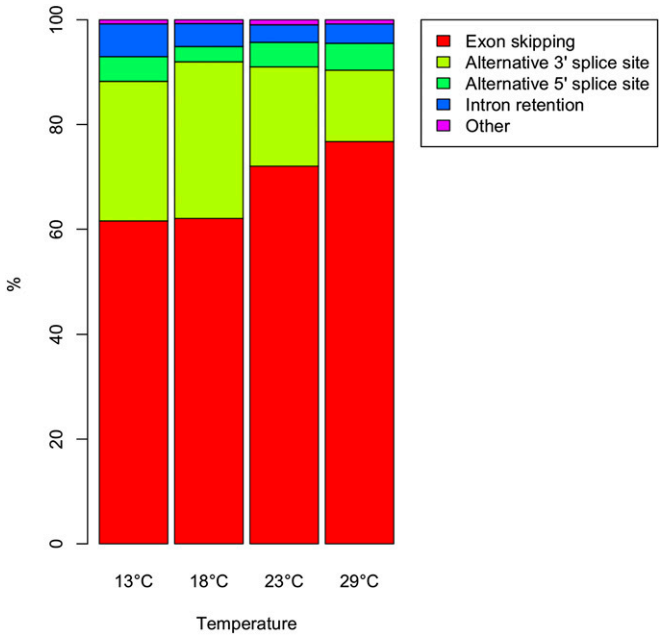

Figure 6 Contribution of splice types to differential splicing contrasting Oregon-R and Samarkand at each of the four temperatures. Exon skipping is the most frequent splice type among genes with differential splicing between genotypes and with increasing temperatures this pattern becomes even more pronounced.

differential splicing and gene expression intensities, both processes are regulated by different mechanisms. This conclusion is further substantiated in a comparison of GO categories that are enriched for genes with significant differential splicing or gene expression intensities at 23 and 29°. Despite both categories harboring a significant enrichment for some genes, there is very little similarity in the enrichment patterns (GO categories) between differential splicing and gene expression (Figure I, File S1). A similar pattern has been observed by Brooks *et al.* (2015) who found that expression levels of splicing factors that regulate alternative splicing of thousands of genes do not influence their expression intensities (Brooks *et al.* 2015).

Dominance prevails for differential splicing

The mode of inheritance of alternative splicing can be studied by contrasting two parental genotypes to offspring of a cross between them. Between 92 and 99% of the genes did not differ significantly from the splicing pattern of both parents. Splicing of most (83–96%) remaining genes matched one of parents (*i.e.*, were dominant; Table 2). Unexpectedly, this dominance was not evenly distributed between the two parental genotypes and differed strikingly among temperatures (Figure 8). This pattern was most extreme at 13 and 29°. While at 13° the splicing pattern of Samarkand was dominant for the majority of genes (58%) and made up for 70% of all genes with dominant splicing inheritance, at 29° splicing of most genes in F_1 individuals matched Oregon-R (92%) and corresponded to 96% of all dominant genes. At 18°, no such imbalance of dominance was found (44% Oregon-R dominant *vs.* 56% Samarkand dominant). To test to what extent allele-specific gene expression may have affected our inference of dominance, we evaluated if genes with dominant splicing also have imbalanced allele-specific expression favoring the allele coming from the dominant parent. On average, 21.75% of the dominant genes have imbalanced allele-specific expression favoring the dominant allele (Figure J, File S1). Nevertheless, even if only genes with no allele specific differences are considered, we still find the same temperature-dependent dominance pattern (Figure J, File S1).

Table 1 Expression of nuclear core exon junction complex negatively correlates with exon skipping at extreme temperatures

	13°		29°	
	FDR	log_2FC	FDR	log_2FC
mago nashi	0.077	0.915	0.006	−0.923
tsunagi	0.769	0.136	0.001	−1.024
eIF4AIII	0.035	0.914	0.024	−0.659
Mean log_2FC expression of exons differentially spliced between Oregon-R and Samarkand	−0.26		0.3	

FDR, false discovery rate; FC, fold change.

Similar patterns of swapping dominance for gene expression and alternative splicing

This change in the direction of dominance is not restricted to the patterns of alternative splicing but it can be also be found for gene expression intensities (Chen *et al.* 2015a). Particularly remarkable is that for gene expression intensity and alternative splicing, more genes in the F_1 resemble the Samarkand parent, while at 29° the pattern of the Oregon-R parent is dominant. Despite this overall similarity, we did not find an overlap between the genes showing swapping dominance for alternative splicing and gene expression intensity; suggesting different regulatory mechanisms.

Discussion

This study evaluates the interplay of temperature and genotype on the patterns of alternative splicing. We show that both temperature and genotype have a significant effect on the splicing patterns and that the interaction of both causes a highly complex splicing signature. We identified the exon junction complex as a strong candidate for regulation of temperature-dependent alternative splicing.

Temperature has a very strong effect on alternative splicing. Ranging from only a few genes having differential splicing at 18° to about the same fraction of genes with differentially spliced exons as found in interspecific comparisons (McManus *et al.* 2014). The same pattern has been observed for gene expression (Chen *et al.* 2015a). In comparing the differences in alternative splicing to gene expression between the two genotypes across a range of temperatures, we found that even though these two phenotypic levels behave in a similar manner, they operate on clearly distinct groups of genes. To shed light on this phenomenon it is necessary to consider different

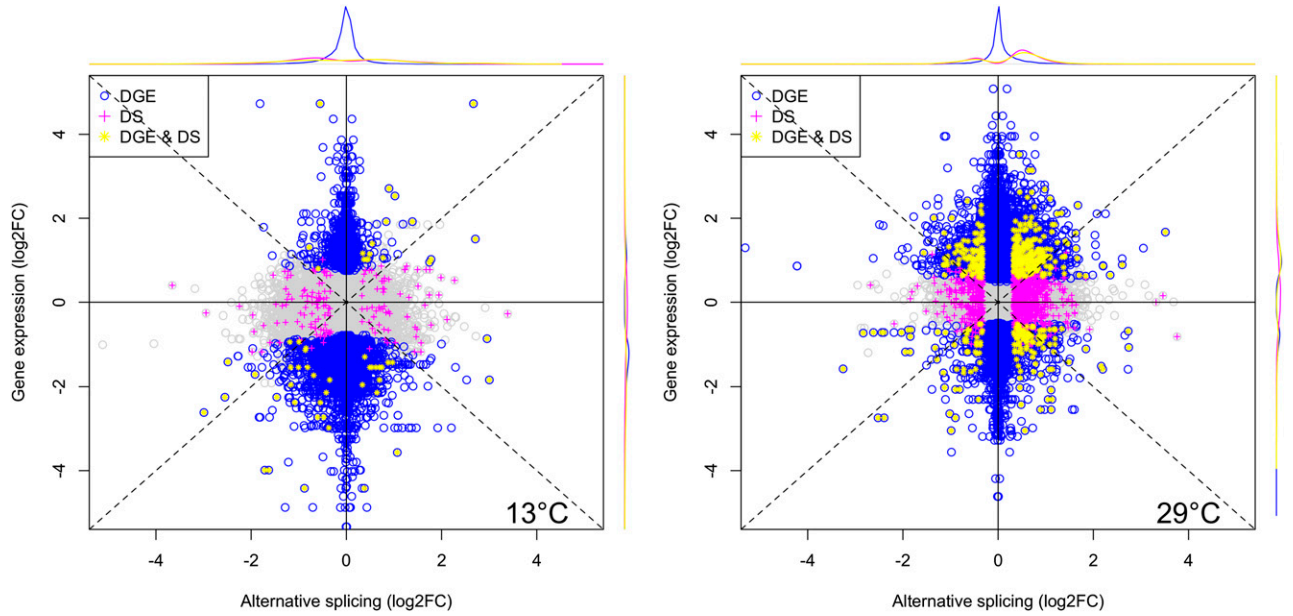

Figure 7 Limited overlap between genes with differential splicing and those that differ in expression intensity. Differential splicing between Oregon-R and Samarkand is plotted against differential gene expression between the two strains. Significant differences are indicated in color (blue, differential gene expression only; magenta, differential splicing only; yellow, significant for both categories). Density plots on top and on the right of the figure indicate nonoverlapping distribution of genes with significant differences for gene expression and alternative splicing.

factors that may influence it, such as the underlying regulation of the two phenotypic levels and selection forces that might shape the differences between the two.

Mode of inheritance

In this study we report, for the first time, an inheritance mode of alternative splicing and its temperature dependence. Our analyses of alternative splicing revealed a striking pattern of prevailing dominant inheritance. Furthermore, dominance of alternative splicing showed temperature dependence by preferably using alternative splicing patterns of Oregon-R at warm temperatures and of Samarkand at cold temperatures. This suggests that temperature-specific alternative splicing might stem from the usage of splicing factors carrying alleles or interacting with alleles that enable them to perform better at a certain temperature. Using the identical data set, Chen *et al.* (2015a) found the same pattern for gene expression intensities, despite different genes being affected. Interestingly, this prevalence of dominance was also found for other inter- and intraspecific gene expression studies in *Drosophila* and *Cirsium* (Gibson *et al.* 2004; McManus *et al.* 2010; Bell *et al.* 2013; Suvorov *et al.* 2013; Meiklejohn *et al.* 2014; Chen *et al.* 2015a). Strong departure from additivity was seen in oysters, with the highest proportion of differentially expressed genes being overdominant (Hedgecock *et al.* 2007). Nevertheless, the majority of studies in different organisms reported prevalent additive effects with only a minor portion of genes showing dominant or other types of nonadditive inheritance for gene expression (Vuylsteke *et al.* 2005; Cui *et al.* 2006; Stupar *et al.* 2008). This study supports the prevalence of dominant inheritance in *Drosophila* by extending the study of inheritance to alternative splicing, and suggests that *trans*-acting factors may be important regulators for alternative splicing as was shown for gene expression (Lemos *et al.* 2008; Suvorov *et al.* 2013; Meiklejohn *et al.* 2014; Chen *et al.* 2015a).

Selection for genotype × temperature interaction

By analyzing alternative splicing in adult *D. melanogaster* females from two different genotypes developing at four different temperatures, we observed a complex pattern of plasticity: alternative splicing showed pronounced genotype–temperature interactions. Interestingly, the genotypic differences in splicing were most pronounced at extreme temperatures, while at 18° almost no differences in alternative splicing could be recognized.

Strong differences between genotypes at extreme temperatures were previously described for gene expression intensity (Chen *et al.* 2015a). The authors argued that this pattern suggests temperature–stress-mediated decanalization of gene expression, reasoning that 18° represents the most benign temperature for *D. melanogaster*.

What evolutionary forces caused this striking pattern at both phenotypic levels? Unfortunately, the impact of gene expression and alternative splicing differences on organismal fitness is not yet understood. For the sake of argument, we will distinguish extreme scenarios and discuss their consequences.

Hypothesis 1: Gene expression and alternative-splicing differences between strains are adaptive: Contrasting expression patterns of strains/populations from different habitats in a common garden setting is common practice to identify

Table 2 Inheritance modes of alternative splicing

	13°	18°	23°	29°
Testable genes Oregon-R:F_1	8982	8320	8236	8182
Testable genes Samarkand:F_1	8789	8352	8372	8340
Conserved Oregon-R	8768	8267	7864	7573
Conserved Samarkand	8672	8307	8201	8243
Conserved both	8551	8204	7792	7505
Additive	20	6	22	13
Dominant Oregon-R	36	18	249	567
Dominant Samarkand	83	23	100	26
Underdominant	0	1	3	3
Overdominant	0	0	1	1

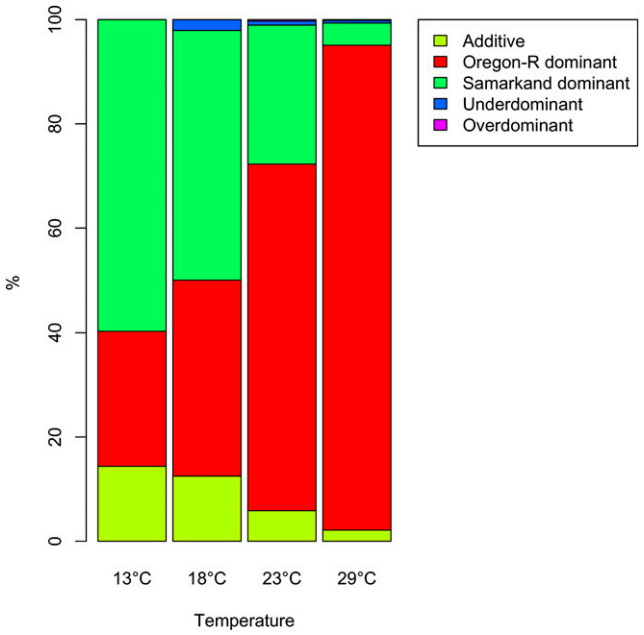

Figure 8 Temperature-dependent dominance. At 13° most of the differences in alternative splicing showed Samarkand dominance (green), while at 29° the pattern was reversed with Oregon-R dominance (red) for most genes.

differentially expressed genes, which serve as candidates for local adaptation (Telonis-Scott *et al.* 2009; Yampolsky *et al.* 2012). Along these lines, the differences at extreme temperatures seen in our experiment may be viewed as the signature of adaptation of the two strains used in this study to different environments. On the other hand, if the environments of the two strains are not as different, differences in their expression and splicing patterns may stem from convergent adaptation.

Hypothesis 2: Gene expression and alternative splicing differences between strains are maladaptive: If selection favors similar gene expression between different genotypes, mechanisms will evolve that result in a pattern of genetic canalization in a specific environment. One classic example for canalization is the *Hsp90* gene, which has been shown to suppress phenotypic differences between diverged genotypes. Once the function of *Hsp90* is compromised, through mutations or environmental stress, the genetic differences usually manifest in deleterious phenotypic differences (Rutherford and Lindquist 1998). Since these differences are typically sheltered by the action of a buffering system consisting of putative canalization factors such as *Hsp90*, they will independently accumulate between strains. Thus, once the buffering system is broken, these independently accumulated variants result in differences in gene expression and alternative splicing.

Alternatively, instead of buffering systems, purifying selection could cause the pattern of low phenotypic divergence in a given environment by removing variants causing differences in gene expression and alternative splicing. Reasoning that purifying selection is most effective in the environment in which an organism spends most time, at 18° (the favorite temperature of *D. melanogaster* larvae) (Kwon *et al.* 2008; Shen *et al.* 2011) the fewest deleterious variants are expected. Extreme temperatures, such as 13 and 29°, are avoided by *D. melanogaster.* Genes that are expressed at these temperatures can accumulate mutations that are inaccessible to purifying selection if they do not affect the gene expression patterns at 18°. In this way, variation resulting in differences in gene expression and alternative splicing could accumulate and will only be detected in environments that are rarely encountered.

Some support for this hypothesis is provided by a recent study (Richardson *et al.* 2013). Comparing the phenotypic variation in mutation-accumulation lines with and without HTZ1, a gene implicated in mutational robustness, they found no difference. Since this observation contrasted results comparing phenotypic variation with and without a gene conferring robustness, they concluded that natural selection must have purged those variants that cannot be buffered (Richardson *et al.* 2013). We follow this reasoning to explain the phenomenon seen in our data.

Several lines of evidence support the accumulation of deleterious alleles in rarely encountered environments as an explanation for the differences observed between the two strains. First, the differences in alternative splicing and gene expression are affecting different gene sets, suggesting that the two processes are independent from each other. Second, assuming that the recessive allele is the deleterious variant, the concordance of the dominance patterns for alternative splicing and gene expression intensity suggest that Oregon-R has acquired deleterious mutations in warm environments that are expressed at low-assaying temperatures. Samarkand, on the other hand, did the same in cold environments and accumulated deleterious mutations that are uncovered at high temperatures.

This leads to a clear prediction for flies from different temperature environments. While flies originating from hot environments are more likely to accumulate mutations that are deleterious in cold environments, the opposite is true for flies originating in warm environments. This phenomenon can

eventually lead to an increase in the gene expression and alternative-splicing variance between populations, which creates a pattern of high-phenotypic differentiation in extreme environments. Future work using flies evolved in extreme environments may yield new evidence in support of this hypothesis.

Acknowledgments

We thank Jun Chen, Alexander Kalinka, François Mallard, and Ray Tobler for helpful discussions. This study was supported by the Austrian Science Fund (FWF P22834 and FWF W1225) and the European Research Council (ArchAdapt).

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Communicating editor: M. W. Hahn

GENETICS

Supporting Information
www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.192310/-/DC1

The Interplay of Temperature and Genotype on Patterns of Alternative Splicing in *Drosophila melanogaster*

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DOI: 10.1534/genetics.116.192310

SUPPORTING METHODS

Fly rearing

Flies were kept in standard cornmeal-molasses-yeast agar medium with alternating 12h light and 12h dark photoperiods. Oregon-R and Samarkand strains were inbred for 7 generations by sibling pair mating at 25°C prior to the experiment. The following crosses were used in this experiment: Oregon-R female × Oregon-R male (Oregon-R), Samarkand female × Samarkand male (Samarkand), Oregon-R female × Samarkand male ($F1_A$), Samarkand female × Oregon-R male ($F1_B$). Around 80 single male and virgin female crosses were set up in three replicates for each cross combination in parallel for the RNA-seq analyses. The females laid eggs at 23°C for 2 days. After removal of the adults 20 vials of each cross combination were incubated at one of the four assaying temperatures (13°C, 18°C, 23°C and 29°C). Virgin female flies were collected upon eclosure for sequencing. Further details can be found in Chen et al. (2015a).

Library preparation

Library preparation and sequencing are described in Chen et al. (2015a).

RNA-seq read mapping and counting

Prior to mapping, RNA-seq reads were split by barcodes and their 5' ends were trimmed using Mott algorithm implemented in PoPoolation2 (Kofler, Pandey, & Schlötterer, 2011) based on a minimum sequencing quality threshold of 18 (parameters: --quality-threshold 18 --min-length 60 --no-5p-trim). We aligned the trimmed reads to the *D. melanogaster* genome (r5.49 assembly) which has been updated with strain specific SNPs using the polymorphism aware mapper GSNAP (Wu & Nacu, 2010) with default parameters (parameters: --quality-protocol illumina -N 1 -t 20 -A sam --split-output). The alignment of reads around splice sites was improved using the information of known splice sites retrieved from the *D. melanogaster* genome annotation (r5.49) and allowing for novel splice site detection (parameter: -s). Only uniquely mapped and properly paired reads (Table A) were used for further analysis. To account for sampling bias coming from the higher probability of detecting more lowly expressed

transcripts in libraries with higher read coverage, we downsampled all libraries to the same size (Table A) using samtools (Li et al., 2009). This procedure allowed us to compare differential expression between the strains across different temperatures.

Table A. Library sizes (mapped reads) before and after downsampling to the same coverage.

Library	Library size	Standard size	Downsampling factor	Downsampled library size
F1i13r1	72059984	60000000	0.83263965	60001360
F1i 13r2	59517342	53000000	0.89049676	53000146
F1i 13r3	50722344	20000000	0.39430354	19999000
F1i 18r1	63383240	53000000	0.83618319	53004970
F1i 18r2	67017068	60000000	0.89529432	59992430
F1i 18r3	52907004	20000000	0.37802178	20003484
F1i 23r1	76146878	60000000	0.78795089	60002646
F1i 23r2	55962000	20000000	0.35738537	19997390
F1i 23r3	62973096	53000000	0.84162926	53001476
F1i 29r1	72029902	53000000	0.73580553	53003510
F1i 29r2	56348786	20000000	0.35493223	19998254
F1i 29r3	98116020	60000000	0.61152093	60009958
F1ir13r1	58680110	20000000	0.34083099	19993954
F1ir 13r2	64527952	60000000	0.9298296	60000670
F1ir 13r3	58967264	53000000	0.89880378	53004986
F1ir 18r1	59605120	53000000	0.88918536	52999214
F1ir 18r2	78026730	60000000	0.76896725	60004220
F1ir 18r3	53996866	20000000	0.37039187	20000350
F1ir 23r1	67676698	53000000	0.78313513	53002896
F1ir 23r2	71735762	60000000	0.83640291	60007116
F1ir 23r3	63150190	20000000	0.3167053	19993802
F1ir 29r1	66349516	53000000	0.7988001	53000556
F1ir 29r2	55906404	20000000	0.35774077	19995934
F1ir 29r3	96667724	60000000	0.62068287	60011712
O13r1	63942400	60000000	0.93834451	59994894
O13r2	53517400	53000000	0.99033212	53517400
O13r3	37485274	20000000	0.53354285	19999868
O18r1	60632594	53000000	0.87411731	52989110
O18r2	66277860	60000000	0.90527968	59994544
O18r3	57840480	20000000	0.3457786	19998260
O23r1	54300888	20000000	0.3683181	19999284
O23r2	66632572	60000000	0.90046051	59995458
O23r3	61553042	53000000	0.86104599	52997668
O29r1	66518620	53000000	0.79676939	52994706
O29r2	66370380	20000000	0.30133924	20004220
O29r3	92474012	60000000	0.64883094	59996956
S13r1	65316152	60000000	0.91860892	60002866
S13r2	57221034	53000000	0.92623283	53002270
S13r3	20788594	20000000	0.96206603	20000144
S18r1	57098872	53000000	0.92821448	53005896
S18r2	53551020	20000000	0.37347561	19994684
S18r3	59717404	60000000	1.00473222	59717404
S23r1	62542744	20000000	0.3197813	20002222
S23r2	64233972	53000000	0.82510856	53002868
S23r3	64787418	60000000	0.92610574	59999650
S29r1	84601608	53000000	0.62646563	53001194
S29r2	78398978	20000000	0.25510537	20006018
S29r3	93306304	60000000	0.64304337	59992100

Differential splicing analysis

We characterized alternative splicing from RNA-seq data using a differential exon usage approach (Anders et al. 2012). To account for the complexity of splicing events, annotated exons are sub-divided into sub-exons ("counting bins") if used differentially by the corresponding isoforms (Anders et al. 2012). The mapped reads are counted for each sub-exon. Reads overlapping two sub-exons are counted once for both of the sub-exons. For clarity we distinguish exons from sub-exons in the Materials and Methods section, but for simplicity, we refer to sub-exon as "exons" throughout the rest manuscript and in figures and tables.

We used the software tool DEXSeq (Anders et al. 2012), which implements this differential exon usage analysis. We first normalized the read count data using DESeq normalization (Anders & Huber, 2010) and then we used the normalized reads to fit two generalized linear models assuming negative-binomial distribution of count data. We fit the models with count data of each sub-exon (i), gene (j) and strain or temperature (l). The reduced model is decomposed into 3 factors, overall gene expression effect (G), sub-exon effect (E) and strain (or temperature) effect (S)

$$\log M_{ijl} = G_i + E_{il} + S_{ij} \quad (1)$$

The second model that we fit, which contains additional S×E interaction term, will have a better fit, if the proportion of reads of the sub-exon in the reads of the whole gene is significantly bigger in one strain (or temperature) than in the other and will indicate differential exon usage. The significance of differential exon usage is adjusted by the Benjamini-Hochberg correction of p-values (Benjamini & Hochberg, 2009) to account for multiple testing. We analyzed only multi-exon genes (genes that contain more than one exon) and restricted our tests to sub-exons which had on average at least 50 mapped reads across all samples (Table A).

3' gene body coverage bias

Since an uneven gene body coverage of this RNA-seq data set has been previously been described (Chen, Nolte, & Schlötterer, 2015), we carefully re-evaluated the data using the RSeQC software (Wang, Wang, & Li, 2012). We confirmed an excess of read coverage at the 3' end of the gene and noted some heterogeneity in this 3' bias among the samples. Importantly, samples from the same temperatures had very similar gene body coverage (Figure A). With a similar coverage bias within temperatures, the 3' bias should not affect the between strain comparisons and no excess of false positives is expected. Indeed, this assumption is confirmed by the comparison of F1i and F1r crosses within temperatures, which did not identify any significantly differentially expressed sub-exons. We further tested the robustness of the differential exon usage to 3' coverage bias analyzing single exon genes. Reasoning that single exons are not spliced, we created a modified annotation in which single exon genes were split into 3 sub-exons of approximately the equal size. This modified annotation of single exon genes consisted of 9232 transcripts with mean transcript length 753bp. We applied the identical differential exon usage procedure to test for a signal of differential splicing, which would be the result of gene coverage bias. Consistent with our expectation of no excess of false positives due to differential gene body coverage, the proportion of significantly differentially expressed sub-exons did not exceed 5% (Table B). As a final test for robustness of our analyses, we tested if the pattern of reduction of differences between strains at 18° and increase in differences at extreme temperatures is confirmed when the analysis is restricted to first 1kb of the 3' end of transcripts where the coverage is high and homogeneous. The analysis on the 1kb transcripts yielded qualitatively same results (Chi-square test of independence $p < 2.2e\text{-}16$; SM Figure 2, A). Also an analysis of the first 500bp resulted in the same pattern (Chi-square test of independence, p= 1.125e-13; ~~SM~~ Figure ~~2,~~ B).

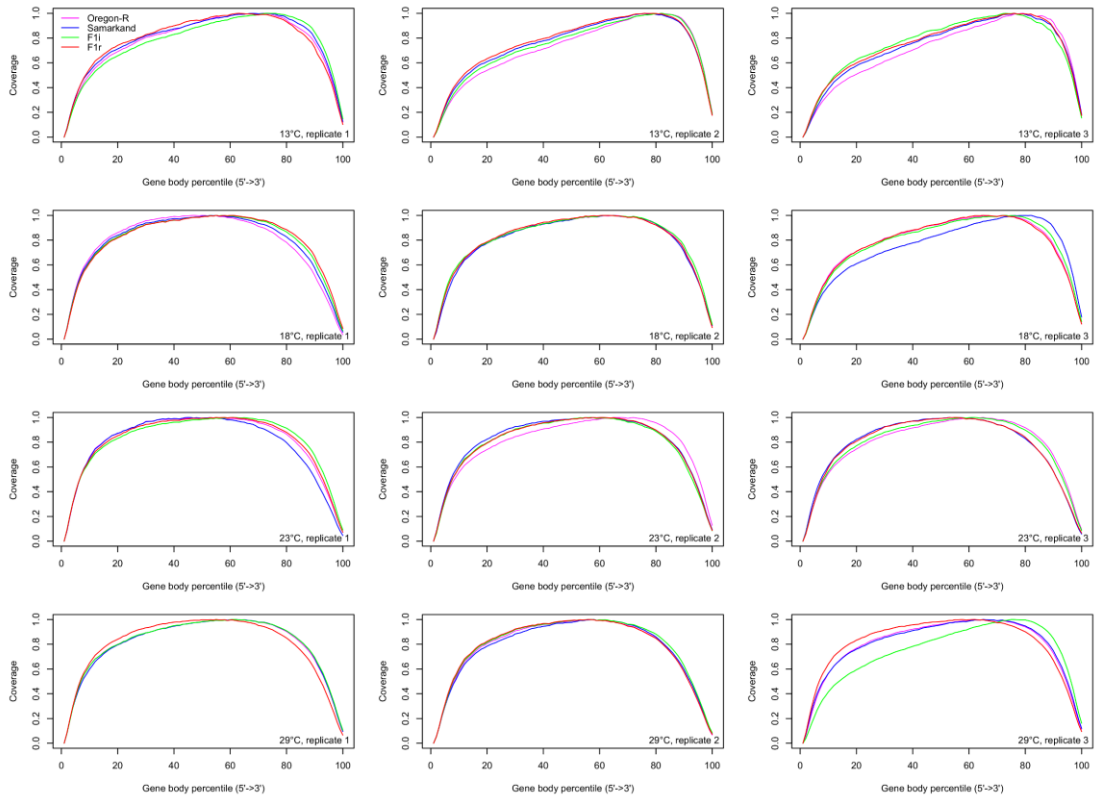

Figure A. Gene body coverage is similar across samples within replicates of the same temperature.

Table B. Differential splicing for single exon genes. Exon is divided into three spurious exons and tested for differential splicing. Less than 4% of spurious exons were significantly differentially used.

	13°C	18°C	23°C	29°C
Tested exons	2600	2359	2479	2410
Significant exons	4	5	84	65
Non-significant exons	2596	2354	2395	2345
% significant exons	0.15	0.21	3.38	2.697

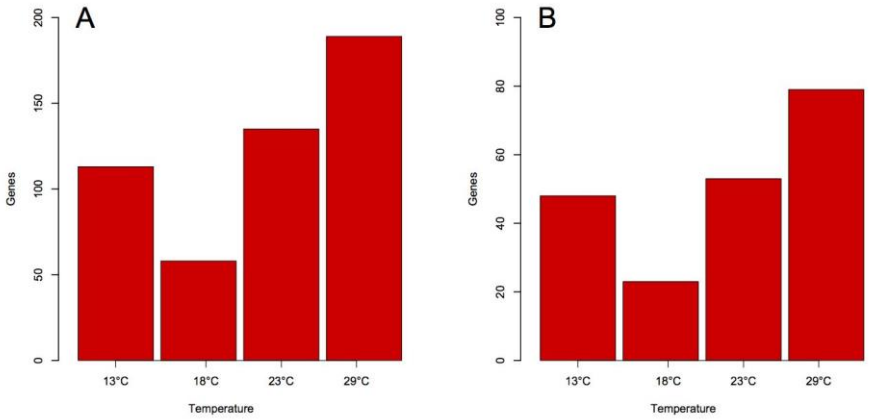

Figure B. Differential splicing of transcripts truncated to 1kb from the 3' end (A) and 500 bp from the 3'end (B). The pattern of few differences at 18°C

and many differences in splicing at extreme temperature remains when truncated transcripts are analyzed.

The comparison across temperatures is, however, complicated due to the apparent differences in gene coverage (Figure A). To account for this, we restricted all analyses between temperatures to 1kb of the 3' end of the transcript where the coverage is even (Figure C). The comparison of environmental and genetic canalization was also restricted to first 1kb of the 3'end of the transcript.

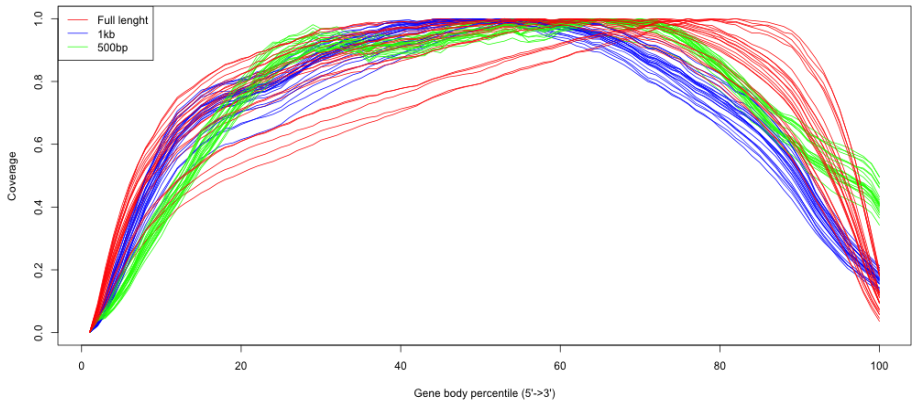

Figure C. Gene body coverage for full length transcripts (red) and transcripts truncated to 1kb (blue) and 500bp (green) in Oregon-R and Samarkand samples. Gene body coverage is biased towards the 3'end for full length transcripts, whereas truncated transcripts show even coverage.

Inference of splice types from differential exon usage patterns

Splice types were defined according to correspondence of the position of sub-exon relative to the exons in the standard annotation on the same strand.

Exon skipping: sub-exons share both 3' and 5' end position with the corresponding exon in the standard annotation file.

3' alternative splice site usage: sub-exons share their 5' end, but not the 3' end position with their corresponding exon in standard annotation.

5' alternative splice site usage: sub-exons share their 3' end, but not the 5' end position with their corresponding exon in standard annotation.

Intron retention: sub-exons share their 5' end position with the 3' end position of one and their 3' end with the 5' end position of the other exon of the standard annotation.

Splice types that could not be assigned to any of the splice types described above were grouped as “other “splice types.

The different splice types were visually confirmed by using Sashimi plots (intron retention, Figure D; 5’ alternative splice site usage, Figure E; 3’ alternative splice site usage, Figure F).

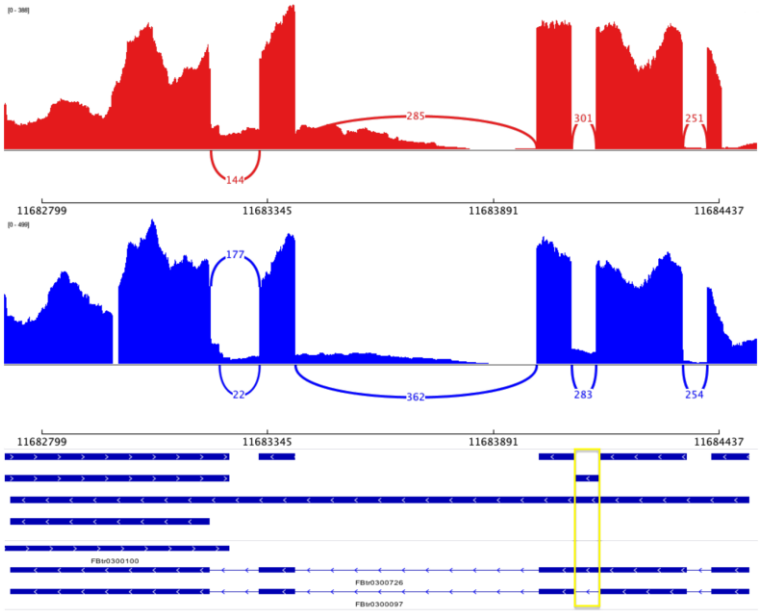

Figure D. Intron retention. Intron from transcript FBtr0300097 (indicated with yellow frame in the annotation) in gene CG14133 is spliced out in Oregon-R (red) and retained Samarkand (blue).

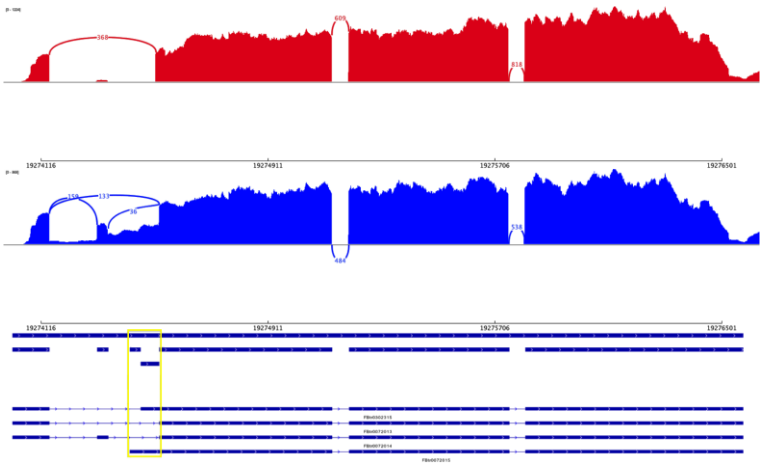

Figure E. Alternative 5’ splice site usage. Oregon-R (red) uses different 5’splice site when splicing third exon in CG10315 (indicated with yellow frame in the annotation) than Samarkand (blue)

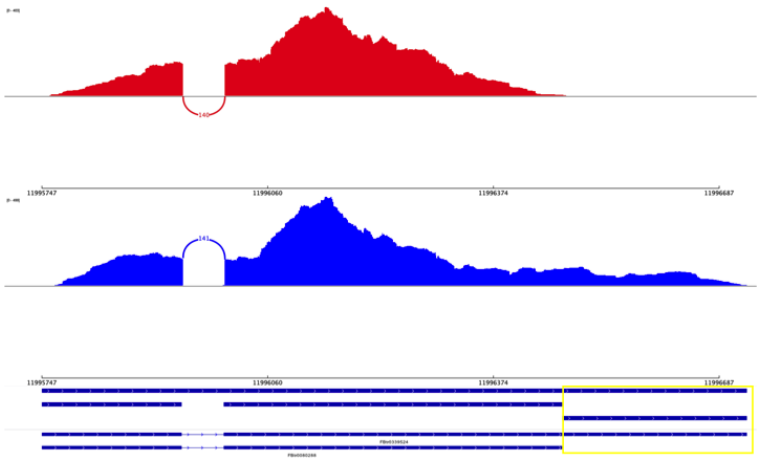

Figure F. Alternative 3' splice site usage. Oregon-R (red) uses a shorter transcript than Samarkand (blue) by splicing the second exon of CG31864 at a different 3'splice site (indicated with yellow frame in the annotation).

Mode of inheritance

We assigned the mode of inheritance of each gene by comparing the expression differences of sub-exons between F1 and parental strains.

Conserved Oregon-R inheritance: All sub-exons of a given gene in the F1 offspring do not have significant differences in expression from Oregon-R (q-value<0.05). Furthermore the log2 fold change may not exceed an absolute value of 0.32 when compared to Oregon-R.

Conserved Samarkand inheritance: All sub-exons of a given gene in the F1 offspring do not have significant differences in expression from Samarkand (q-value<0.05). Furthermore the log2 fold change may not exceed an absolute value of 0.32 when compared to Samarkand.

Conserved inheritance for both parents: The expression of all sub-exons of a given gene does not differ significantly from both parental strains Oregon-R and Samarkand.

Additive inheritance: The expression of at least one sub-exon in F1 individuals is significantly different from that in both of the parents (q-value<0.05). Furthermore the F1 expression level is intermediate to the one of the two parents.

Dominant Oregon-R inheritance: The expression of at least one sub-exon is conserved in the Oregon-R - F1 contrast, while being significantly different in the Oregon-R - Samarkand, and Samarkand - F1 contrasts.

Dominant Samarkand inheritance: The expression of at least one sub-exon is conserved in the Samarkand - F1 contrast, while being significantly different in the Oregon-R - Samarkand, and Oregon-R - F1 contrasts.

Underdominant inheritance: The expression of at least one sub-exon in F1 individuals is significantly lower than the one in Oregon-R and Samarkand.

Overdominant inheritance: The expression of at least one sub-exon in F1 individuals is significantly higher than the one in Oregon-R and Samarkand.

GO analysis

Gene expression studies typically do not account for the influence of different gene lengths. For alternative splicing, however, it is important to correct for the opportunity for alternative splicing to be somehow linked to gene length. For example, genes involved in neuronal development are among the longest genes and also contain the genes with the largest number of isoforms (Barbosa-Morais et al., 2012).

To account for the differential splicing potential among the different functional gene categories, we used the Gowinda software tool (Kofler & Schlötterer, 2012). Rather than SNPs as in the original publication, we used sub-exons as the unit of observation. We assessed the enrichment of genes by randomly sampling sub-exons from the set of all sub-exons present in our data set and compare the randomly sampled sets to the observed set of differentially expressed sub-exons. By selecting the "gene based" analysis option we avoid erroneous enrichments in GO categories containing very long genes that have higher number of sub-exons and therefore a greater chance to have at least one of their sub-exons differentially expressed. The significance of overrepresentation of spliced genes in a category is estimated from the empirical null distribution created by permutation sampling of sub-exons from the total number of sub-exons. Also an empirical false discovery rate (FDR) is calculated to correct for multiple testing. The parameter we used are --simulations 1000000 --min-significance 1 --gene-definition gene --threads 20 --mode gene --min-genes 1e used a threshold of FDR< 0.05 in this analysis.

Assessment of gene set overlap using receiver-operator curve (ROC)

To assess the overlap between two sets of sub-exons (or GO categories) we used a modified receiver-operator curve (ROC) analysis where both sets of sub-exons (or GO categories) are first ranked according to FDR corrected p-values and binned in increasingly larger bins. When equation 2 is satisfied, it indicates that the number of sub-exons (or GO categories) shared between the two data sets is expected by chance.

$$\frac{A_i \cap B_i}{n_i} = \frac{n_i}{N} \qquad (2)$$

with *A* and *B* being sub-exon sets contained in the bin *i* of size *n.* In the case that equation 2 is not met and the left side of the equation is larger (smaller) than the right side, the overlap of sub-exons (or GO categories) is higher (less) than expected by chance.

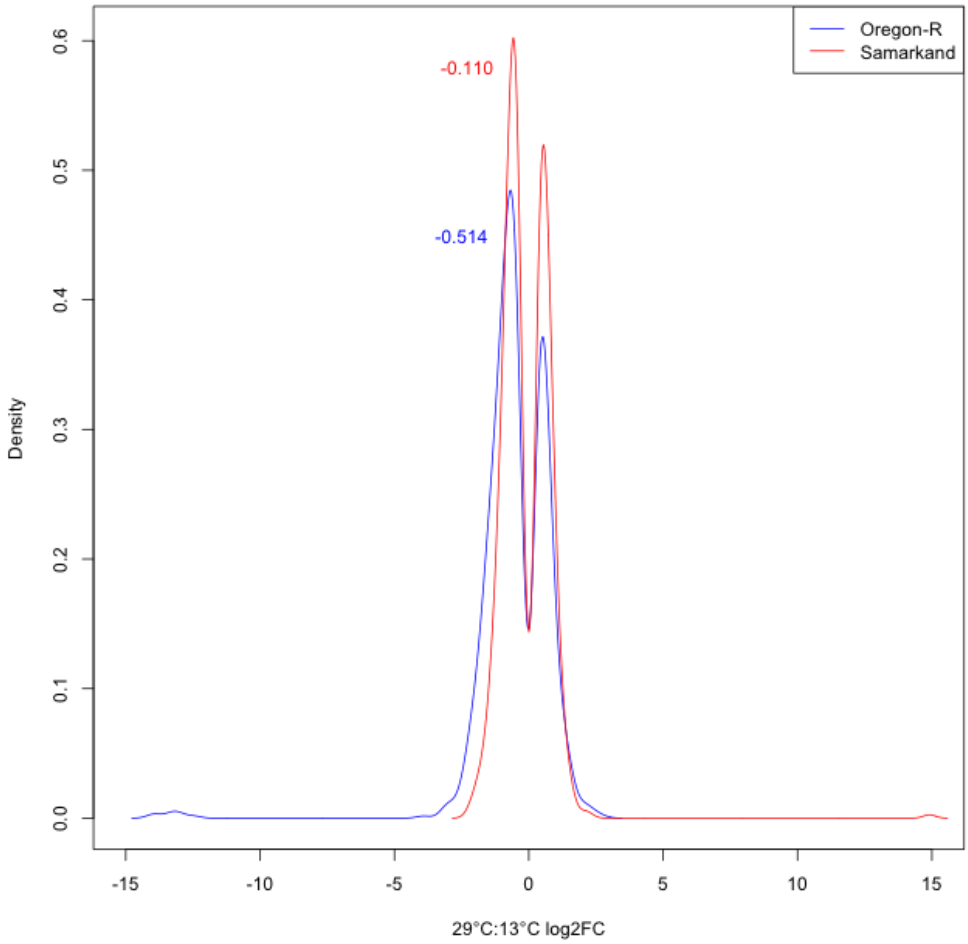

Figure G. Exon inclusion and exclusion. Density plot of log2 fold change for exons differentially expressed between 13° and 29°. Both genotypes have more transcripts with retained exons at 13° (i.e. log2 fold change < 0). Also the mean log2 fold change of all exons is negative for both strains (Oregon-R: -0.514, Samarkand (-0.110).

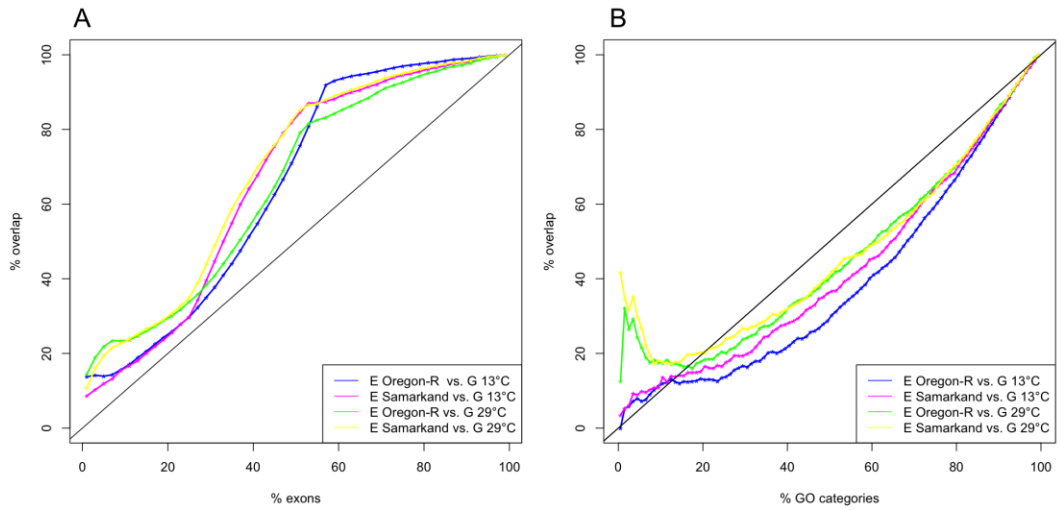

Figure H. Overlap between genetically and environmentally induced differences in splicing is only slightly higher than expected by chance. The ROC-like curve indicates that the overlap of most significantly differentially expressed exons (A) and most significantly enriched GO categories (B) is lower than 20% for both the comparisons between environmentally induced differential splicing in Oregon-R (13°C vs. 29°C) and genotypically induced differential splicing (blue at 13°C; magenta at 29°C) and for comparisons between environmentally induced differential splicing in Samarkand (13°C vs. 29°C) compared to genotypically induced differential splicing (green 13°C; yellow 29°C). Note that the GO analysis is restricted to transcripts truncated to 1kb. There was only 1 significantly enriched GO category for genotype differences at 13°C and none for 29°C.

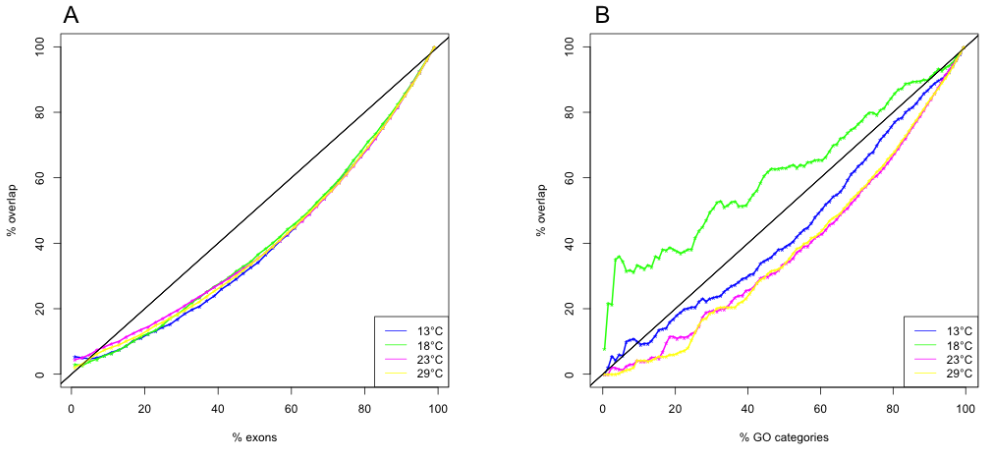

Figure I. Differential splicing and gene expression differences are not correlated. (A) ROC-like curves show limited overlap between exons that are differentially expressed and differentially spliced between Oregon-R and Samarkand. (B) ROC-like curves indicate that also on the level of GO categories no correlation can be detected between differential splicing and gene expression differences in contrasts between Samarkand and Oregon-R. Note, that at 13 and 18°C no GO category was enriched for differential splicing.

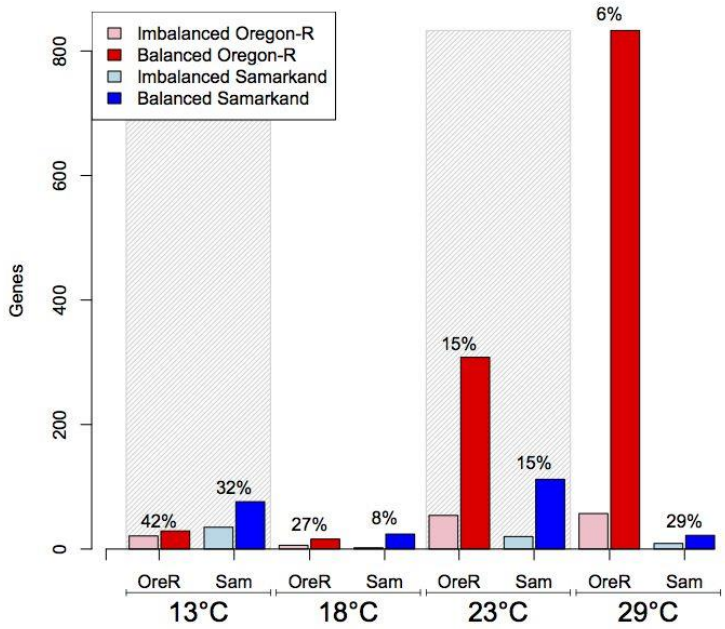

Figure J. Allelic imbalance for genes with dominant differences in alternative splicing. The widespread dominance of difference in alternative splicing cannot be attributed to allelic imbalance. Percentages indicate the proportion of genes with imbalanced allele specific gene expression. Note, that even when imbalanced genes are excluded, the pattern of temperature dependent dominance is retained.

SUPPORTING TABLES

Table C. Numbers of genes and exons used in analyses. We restricted our analyses to multi-exon genes. Only exons with an average of 50 reads across all samples were included in the analysis.

Between temperature comparisons*

	Oregon-R		Samarkand	
	Exons	Genes	Exons	Genes
13°C:18°C	18684	5398	17443	5108
13°C:23°C	18741	5411	17980	5263
13°C:29°C	18907	5463	18189	5277
18°C:23°C	16024	4738	16753	4964
18°C:29°C	16259	4818	17179	5040
23°C:29°C	16140	4788	17473	5128

Between genotype comparisons

	Exons	Genes
13°C	49675	8858
18°C	39839	8021
23°C	41415	8090
29°C	43598	8186

Parent:hybrid comparisons

	Oregon-R:F1		Samarkand:F1	
	Exons	Genes	Exons	Genes
13°C	50842	8982	47910	8789
18°C	42567	8320	42846	8352
23°C	42971	8236	43785	8372
29°C	43407	8182	45430	8340

*Analyses restricted to 1kb transcripts

Table D. Enriched splicing factors. Expression levels (fold change between genotypes) of splicing factors enriched among differentially spliced genes and mean log2FC expression of differentially spliced exons of their target genes.

Splicing factor		13° (log2FC)	29° (log2FC)	Enrichment (FDR)
FBgn0016978	Expression	0.4588179	-0.9970979	0.0003
	Target gene splicing	-0.4448631	0.2131981	
FBgn0034237	Expression	-0.3557789	1.494437	0.0008
	Target gene splicing	-0.0406573	0.2351111	
FBgn0014870	Expression	-0.04421718	0.08564842	0.0025
	Target gene splicing	-0.5431015	0.1904218	
FBgn0086895	Expression	-0.479691	-0.2391361	0.0032
	Target gene splicing	-0.1679616	0.1663099	
FBgn0052423	Expression	-0.2825182	1.046229	0.0039
	Target gene splicing	-0.3714648	0.1525109	
FBgn0043884	Expression	1.053044	0.7595915	0.0040
	Target gene splicing	0.2343859	0.2400676	
FBgn0004237	Expression	0.6325645	0.7301817	0.0054
	Target gene splicing	0.07591968	0.2010865	
FBgn0003742	Expression	0.4954141	-0.1337727	0.0054
	Target gene splicing	1.568466	0.303582	
FBgn0002622	Expression	0.4094199	-0.5025998	0.0064
	Target gene splicing	-0.1965331	0.1701375	
FBgn0003261	Expression	0.1514235	0.4385707	0.0079
	Target gene splicing	-0.1031118	0.2371531	
FBgn0265298	Expression	0.3814688	-1.101479	0.0110
	Target gene splicing	-0.2191292	0.2000744	
FBgn0015778	Expression	0.07093362	0.7919228	0.0110
	Target gene splicing	-0.4963501	0.138276	
FBgn0037707	Expression	0.5810485	-0.957719	0.0131
	Target gene splicing	-0.3245482	0.1524923	
FBgn0037081	Expression	-0.6283451	0.398747	0.0254
	Target gene splicing	-0.0378984	0.2111358	
FBgn0033378	Expression	0.1359079	-1.024293	0.0254
	Target gene splicing	0.01102423	0.152548	
FBgn0024285	Expression	-0.2871507	1.52559	0.0254
	Target gene splicing	0.2008206	0.2267776	
FBgn0004838	Expression	0.3086141	-0.04624664	0.0304
	Target gene splicing	-0.07199447	0.2308261	
FBgn0004587	Expression	-0.365944	-0.2019777	0.0304
	Target gene splicing	-0.1613062	0.1874542	
FBgn0038826	Expression	0.2015537	0.3856733	0.0362
	Target gene splicing	-0.1791964	0.1893673	

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