Opinion

The importance of alternative splicing in adaptive evolution

Pooja Singh[1,2,3] and Ehsan Pashay Ahi[4]

[1]Department of Biological Sciences, University of Calgary, Calgary, Canada

[2]Institute of Ecology and Evolution, University of Bern, Bern, Switzerland

[3]Swiss Federal Institute of Aquatic Science and Technology (EAWAG), Kastanienbaum, Switzerland

[4]Organismal and Evolutionary Biology Research Programme, University of Helsinki, Helsinki, Finland

Correspondence to pooja.singh09@gmail.com

Twitter: @pooja_singh09 and @ehsanpashayahi

Abstract

Although alternative splicing is a ubiquitous gene regulatory mechanism in plants, animals and fungi, its contribution to evolutionary transitions is understudied. Alternative splicing enables different mRNA isoforms to be generated from the same gene, expanding transcriptomic and thus proteomic diversity. While the role of gene expression variation in adaptive evolution is widely accepted, biologists still debate the functional impact of alternative isoforms on phenotype. In light of recent empirical research linking splice variation to ecological adaptations, we propose that alternative splicing is an important substrate for adaptive evolution and speciation, particularly at short timescales. We synthesise what is known about the role of alternative splicing in adaptive evolution. We discuss the contribution of standing splice variation to phenotypic plasticity and how hybridisation can produce novel

splice forms. Going forwards, we propose that alternative splicing be included as a standard analysis alongside gene expression analysis so we can better understand of how alternative splicing contributes to adaptive divergence at the micro- and macroevolutionary levels.

Keywords: alternative splicing, evolution, adaptation, Iso-seq, RNA-seq, gene regulation

What is alternative splicing and why does it matter?

Alternative splicing is a form of transcriptional regulation that enables the production of different mRNA isoforms from a single gene, which may then be translated to produce different proteins (Kim, Magen, & Ast, 2007; Kornblihtt et al., 2013). These different mRNA isoforms are generated largely through the differential incorporation and/or excision of exons and introns in the final mRNA molecule that is transcribed (Figure 1). The different forms of alternative splicing are exon skipping, intron retention, exon shuffling, and use of alternative 5’ or 3’ sites or transcription initiation sites. Discovered over 40 years ago, alternative splicing formed a large part of the puzzle explaining how proteomic complexity can be achieved with a limited set of genes (Alt et al., 1980; Nilsen & Graveley, 2010). Alternative splicing was considered an anomaly until high-throughput sequencing data revealed that almost all multiexonic genes in model vertebrates and up to 70% of multiexonic genes in plants are spliced (Chaudhary et al., 2019; Merkin, Russell, Chen, & Burge, 2012). In fungi, the extent of alternative splicing can range from 0.2% to 18.2% in fungi (Fang et al., 2020). Traditionally, alternative splicing was thought to be a form of post-transcriptional regulation, however a large body of evidence has found that splicing occurs co-transcriptionally due to the influence of chromatin structure on the splicing process (Jabre et al., 2019; Luco, Allo, Schor, Kornblihtt, & Misteli, 2011). The coupling of transcription and splicing suggests that

epigenetic regulation shapes not just how genes are expressed but also how they are spliced.

There exists a strong positive relationship between organismal complexity (measured as the unique number of cell types) and percentage of alternatively spliced genes, even after accounting for covariables such as gene content and protein interactivity (L. Chen, Bush, Tovar-Corona, Castillo-Morales, & Urrutia, 2014). Even though alternative splicing is major source of transcriptomic and potentially proteomic and phenotypic variation, little is known about its role in adaptation and ecological speciation but see (Jacobs & Elmer, 2021; Mallarino, Linden, Linnen, & Hoekstra, 2016; Singh, Börger, More, & Sturmbauer, 2017; Tovar-Corona et al., 2015). This is because transcriptomic studies in ecology and evolutionary biology in the last decade have focused mostly on gene expression variation, which was easier to study with older sequencing technologies and bioinformatic tools (Brawand et al., 2011; El Taher et al., 2021; Hill, Vande Zande, & Wittkopp, 2021; Wray, 2007). The scepticism associated with alternative splicing chiefly stems from how difficult it is to functionally characterise the impact of alternatively spliced isoforms on phenotype (Blencowe, 2017; Tress, Abascal, & Valencia, 2017a). Here we discuss research that investigates the contribution of alternative splicing to major evolutionary transitions and morphological innovation, particularly at short evolutionary timescales. We highlight technological advances that will make the study of alternative splicing more accessible, and we propose testable hypotheses about alternative splicing and adaptive diversity as avenues for future research. This discussion is timely and important to emphasise that alternative splicing may be an important substrate for organismal diversification. We hope this article will provide impetus for evolutionary biologists to attain a deeper understanding of how alternative splicing evolves, how splice variation is generated and maintained, and how it contributes to phenotypic novelty.

How does alternative splicing evolve?

Novel isoforms arise when the splicing machinery called the spliceosome interacts with splice-sites found at intron-exon boundaries. There are several different alternative splicing events such as exon skipping, intron retention (exonisation), alternative 5' and 3' splice sites, alternative transcription initiation, and any one of N possibilities of exon shuffling (Figure 1). Intron retention is thought to be the predominant type of alternative splicing in plants, while exon skipping is most common in animals (Chaudhary et al., 2019).

Alternative splicing can evolve via either *cis*- or *trans*- mutations. *Cis*-mutations can generate new isoforms via (1) exon skipping, mutations which cause an exon to be transformed into an intron (2) intron retention, mutations that convert an intron to an exon (3) exon shuffling, mutations that lead to differential inclusion of exons (4) alternative 5' or 3' sites (Figure 1) (Ast, 2004; Keren, Lev-Maor, & Ast, 2010). Intron retention can rapidly generate coding variation and may be important for adaptation at short evolutionary time scales (Singh et al., 2017). That being said, normally, the introduction of a new exon in a gene would cause frameshift mutations and be subject to negative selection. However, if a new alternative isoform is expressed at low levels via exon creation, negative selection pressure against it is relaxed because the ancestral isoform continues to be expressed at normal levels (Figure 4A). In this way, alternative splicing can provide neutral or nearly neutral paths for accelerated paths of evolution (Xing & Lee, 2006). In a novel ecological environment, this minor isoform may have adaptive value and thus rise in frequency across the population. Evidence supporting this was reported during mammalian evolution where alternative spliced genes has 7-fold lower selection pressure, thus creating evolutionary hotspots of biologically functional variation (Xing & Lee, 2005). More studies looking at signatures of selection versus neutral evolution in alternative spliced genes and spliceosome genes are needed as it

is still not well understood how the alternative splicing process evolves and is regulated. It would especially be interesting to see how this differs between genes underlying adaptive phenotypes versus the genomic background. Furthermore, it is not well understood how splice variation is maintained at the species level, in locally adapted populations, or between sexes.

Differences in alternative splicing can arise from *cis*-regulatory mutations or *trans*-regulatory mutations (Ule & Blencowe, 2019), Both *cis*- and *trans*-regulatory mutations can contribute to divergent splicing patterns, but their respective contribution to adaptive evolution are largely unknown. While *cis*-mutations are more likely to affect single gene by altering splice-sites of RNA-elements, trans-regulatory splice mutations alter splicing factors and can result in wide-scale effects on many genes (Kornblihtt et al., 2013). *Trans*-regulatory mutations in spliceosome proteins that affect splice-site recognition can also generate novel splice forms (Ast, 2004). Empirical findings have pivoted towards the importance of *cis*-effects in directing novel splice variation (Kondrashov & Koonin, 2003; Merkin et al., 2012). However, recent evidence suggests that *trans*-regulatory variation in spliceosome genes contributes to the evolution of splice variation and has large scale pleiotropic effects on phenotype (Smith et al., 2018). The relative contribution of *cis* versus *trans* regulatory mutations to the evolution of splicing and the timescales at which operate, is still an open-ended question. Similar to investigations on gene expression variation (Wittkopp et al., 2004), it is yet to be determined if many small effect *cis*-regulatory mutations or few large effect trans-regulatory mutations are more important for adaptive evolution.

Alternative splicing as a substrate for adaptive evolution at long and short timescales

The recent years have played host to several studies documenting the emerging role of alternative splicing during evolution using whole-transcriptome mRNA sequencing (RNA-seq). A convincing argument for the importance of alternative splicing in organismal evolution was made by Chen et al (2014). They demonstrated that alternative splicing complexity (i.e. number of alternatively spliced genes) has gradually increased over the last 1,400 million years of eukaryotic evolution and is highly correlated with organismal complexity (i.e. number of unique cells types). Two of the most influential studies on the evolutionary significance of alternative splicing were by Merkin et al. (2012) and Barbosa-Morais, Irimia, Pan, Xiong, & Gueroussov, (2012) who explored the role of gene and isoform expression over ~350 million years ago (MYA) of evolution in vertebrates and found significant differences in alternative splicing complexity among vertebrate species, with primates harbouring the highest complexity. Both these studies also found that gene expression variation was conserved at the tissue-specific level and alternative splicing was conserved at the species-specific level. This pattern was prominent in lineages that diverged < 6 MYA suggesting that alternative splicing was diverging faster than gene expression variation at shorter timescales. Remarkably, almost a decade prior to this, alternative splicing complexity of a gene had been correlated with explosive speciation in the cichlid fishes of the East African Great lakes (Terai, Morikawa, Kawakami, & Okada, 2003). This was the hagoromo gene that is involved in body pigmentation (Kawakami et al., 2000). Cichlid lineages that had undergone adaptive radiation in < 3.8 MYA (Irisarri et al., 2018) and evolved hundreds of species with extensive body colour variation had a greater variety of isoforms in their skin than species that had not radiated (Terai et al., 2003). As body colour is involved in mate choice and assortative mating (Seehausen et al., 2008) the findings from this study highlighted a link between alternative splicing and ecological speciation.

Since then, research across the tree of life has empirically demonstrated a relationship between splice variation and ecological adaptations that are under selection. For example, in

deer mice (genus *Peromyscus*), alternative isoforms of the Agouti gene has repeatedly given rise to locally adapted light-coloured populations from dark-coloured ancestor in two sub-populations of two Peromyscus sub-species found in USA in the last 10,000 years (Mallarino et al., 2016). The coat colour in *Peromyscus* is an important camouflage to avoid predation and thus is under strong selection pressure (Dice, 1940). In two subspecies of lice, alternative splicing was found to be associated with divergent adaptation to distinct ecological trophic niches (the human head and body) approximately 170,000 years ago (Tovar-Corona et al., 2015). The evidence is not just limited to animals. Plant domestication has produced some of the most exciting examples of rapid adaptation via alternative splicing. For instance, a novel isoform of the circadian clock gene (EAM8) is responsible for early flowering in a barley landrace from the Tibetan plateau, which is a short-season adaptation to colder climates (Xia et al., 2017). In sunflowers, human-mediated domestication that occurred < 5000 years ago in North America led to large frequency shifts of alternative isoforms in seedlings between wild and domesticated populations (Smith et al., 2018). In this case, standing ancestral splice forms were alternatively fixed (or increased in frequency) in the wild versus domesticated groups; though some novel isoforms were also documented. Evidence from the abovementioned studies clearly highlights how alternative splicing can rapidly give rise to ecological adaptations that lead to population and species divergence. This makes alternative splicing especially relevant for understanding evolutionary processes such as adaptive radiation and domestication. The similarities and differences in patterns of alternative splicing underlying adaptive change at short and long timescales is not well understood and more studies, especially at short timescales, are needed to achieve insights into this contrast.

Not only is alternative splicing species-specific, it has been shown to be sex-specific too (Blekhman, Marioni, Zumbo, Stephens, & Gilad, 2010). In humans, genes on the X chromosome have the highest rate of alternative splicing, such that splicing may allow X

chromosome genes to escape X inactivation (Karlebach et al., 2020). Extensive sex-specific splicing was identified in a meta-analysis of male and females birds, and these differences were associated with phenotypic differences in sexes (Rogers, Palmer, & Wright, 2020). It was also found that sexual selection was driving the rapid evolution of sex-specific alternative splicing (Rogers et al., 2020); suggesting that not only differential gene expression but also alternative splicing differences may contribute to resolving conflict between sexes during the speciation process. Little is known about the broad patterns sex-specific selection on alternative splicing beyond model organisms (see (Gómez-Redondo, Planells, Navarrete, & Gutiérrez-Adán, 2021) for a review on the role of alternative splicing in model vertebrates). Given the importance of sexual selection in evolution and the role of sex-determination in speciation, it would be really important to investigate the role of that alternative splicing plays in sex-determination and sex-associated phenotypic adaptations.

Both variation in gene expression and alternative splicing have the potential to impact phenotypic variation (Gueroussov et al., 2015; Josephs, 2021) (Figure 2). While gene promoter activity quantitatively regulates the number of transcripts, alternative splicing changes the structure of transcripts and the encoded proteins (Tellier, Maudlin, & Murphy, 2020). However, the regulatory relationship of these two transcriptional mechanisms is not well understood. Empirical evidence from a few studies suggests that these two mechanisms may be independent (Grantham & Brisson, 2018; Healy & Schulte, 2019; Jacobs & Elmer, 2021; Singh et al., 2017). For instance, we found that differences in alternative splicing far exceeded differences in gene expression in the jaws of cichlid fishes adapted to divergent trophic niches (Singh et al., 2017). While alternatively spliced genes were associated with jaw remodelling, differentially expressed genes were mostly associated with fundamental cellular processes. This pointed to non-overlapping regulatory roles of alternative splicing and differential gene expression. Little to no overlap between differentially spliced genes and differentially expressed genes was also reported in *Drosophila* (Jakšić & Schlötterer, 2016),

aphids (Grantham & Brisson, 2018), salmonids (Jacobs & Elmer, 2021). In contrast, in zebrafish, killifish and stickleback it was found that many of the genes that were differentially expressed in response to cold temperatures were also alternatively spliced (Healy & Schulte, 2019). So, more research is needed to understand the regulatory interplay between splicing and gene expression variation, especially across different tissues and developmental stages of different organisms. Such research would also be crucial to understand how regulation of gene expression and alternative splicing plays out at long and short timescales; in adaptive and plastic responses.

Phenotypic plasticity and alternative splicing

Phenotypic plasticity is the ability of a genotype to display phenotypic variation in heterogeneous environments (West-Eberhard, 1989). It is recognised now that plasticity followed by genetic assimilation can result in population divergence, adaptation and speciation (Ehrenreich & Pfennig, 2015). The contribution of gene expression to plasticity is well studied (Schlichting & Pigliucci, 1998) but few studies have investigated the contribution of alternative splicing to this process, especially in animals (Somero, 2018). Alternative splicing has the potential to generate phenotypic diversity extremely rapidly (Pleiss, Whitworth, Bergkessel, & Guthrie, 2007) by drawing upon standing and cryptic genetic variation present in populations. To this end, alternative splicing has been linked to response to several instances of phenotypic plastic responses. For example, in plants alternative splicing is considered a possible ‘molecular thermometer’ that responds to environmental stressors (Mastrangelo, Marone, Laidò, De Leonardis, & De Vita, 2012). Splice variation has also been associated with cold stress acclimation in fishes. Interestingly, a complex interaction of genotype and phenotypic plasticity was revealed in alternative splicing patterns

at different temperatures in *Drosophila melanogaster* (Jakšić & Schlötterer, 2016). (Healy & Schulte, 2019).

Beyond playing a role in temperature buffering, alternative splicing has also been showing to play a role in ecologically important plastic phenotypes. For example, extensive alternative splicing was found to underlie wing and reproductive polyphenisms in female aphids with identical genotypes (Grantham & Brisson, 2018). In bumble bees, isoform switching was associated with behavioural changes that delineate the caste system (J. Price et al., 2018). Overall, there is not much known about the role of alternative splicing in producing plastic phenotypes in response to novel ecological environments. This would be key to understanding how alternative splicing can facilitate plastic phenotypes that can eventually lead to divergent phenotypes and speciation. More research is also needed to investigate the broad applicability of the role of alternative splicing as a buffer of environmental stress. This would be a pertinent avenue for future research, particularly as climate change exposes organisms to rapidly changing environments.

Dynamics of hybridisation and alternative splicing

While the role of hybridisation in generating new allelic combinations that may contribute to the evolution of phenotypic and species diversity is becoming increasingly accepted in plants as well as animals (Lewontin & Birch, 1966; Seehausen, 2004), the molecular mechanisms that generate this diversity are less well understood. It is thought that the altered regulatory environment in the hybrids is responsible for “hybrid effects” (Burke & Arnold, 2001); and considerable research has focused on studying transgressive gene expression variation patterns in conferring novel traits (Z. J. Chen, 2010). Extremely little attention has been paid to the alternative splicing, which is also an important part of the regulatory landscape of

hybrids. In poplar trees, interspecific hybrids were discovered to harbour novel gene isoforms that were absent in the parents (Scascitelli, Cognet, & Adams, 2010). Novel isoforms were also reported in sunflower intraspecific hybrids (Smith, Rieseberg, Hulke, & Kane, 2021). So it seems that hybridisation can generate novel splice variation but it is not known what the adaptive or non-adaptive value of this source of variation is. It is also not known how often such novel isoforms arises from inter and intra-specific hybridisation in animals. Although, more progress is needed to verify the functional importance of novel isoforms, hybridisation coupled with alternative splicing has the potential for generating novel genetic variation that can act as an important substrate for rapid adaptive evolution. Intriguingly, it has been suggested that aberrant splicing may contribute to reproductive isolation by reducing hybrid fitness, with spliceosome genes acting as Bateson-Dobzhansky-Muller incompatibility loci (Smith et al., 2021). This proposal presents tantalising prospects for the role of alternative splicing in speciation.

Expansion of gene regulatory networks by alternative splicing

Networks of interacting transcription factors that regulate networks of downstream elements/genes are known as gene regulatory networks (GRNs). GRNs are considered fundamental molecular mechanisms controlling developmental events in an organism (Davidson & Erwin, 2006). Components of GRNs are diverse. They evolve at different rates and in distinctive ways and hence are important for the evolution of evolvability (Crombach & Hogeweg, 2008). GRNs were initially thought to be deterministic with multiple stable states (Lauffenburger, 2000). It was later discovered that alternative splicing, along with other factors, can dynamically expand GRNs, rendering the strict deterministic modelling of GRN dynamics incomplete (Braunschweig, Gueroussov, Plocik, Graveley, & Blencowe, 2013). Also, GRNs containing factors that regulate chromatin and transcription complexes may

impact the alternative splicing process itself (Braunschweig et al., 2013). Thus, combining the functional consequences of alternative splicing with GRNs permits developmental versatility, physiological plasticity and adaptive responsiveness without the need for genome expansion (Braunschweig et al., 2013; Niklas, Bondos, Dunker, & Newman, 2015) (Figure 3A). Over the past decade, there was a rapid shift in literature regarding the transcriptional studies of adaptive evolutionary responses from reporting only changes in lists of single genes towards more comprehensive view of identifying changes in GRNs and their potential regulatory interactions. However, such a shift has not yet happened in the realm of alternative splicing studies, and thus, studies on the potential influence of AS on GRNs underlying adaptation has remained in its infancy (Ule & Blencowe, 2019). In our opinion, the time has ripe to integrate GRN-based view in future studies of AS in the context of adaptive evolution.

At detailed molecular level, alternative splicing can influence the interaction of transcription factors, and consequently their downstream *cis*-acting elements under different intra- or extracellular conditions (Talavera, Robertson, & Lovell, 2013). On the other hand, changes of *cis*-acting elements can directly affect splicing itself depending on expression of specific proteins in the same cell (Boutz et al., 2007). In Figure 3B, you can find simplified examples for potential transcriptional outcome of introducing only a single splice variant for a transcription factor that acts upstream of a GRN unit. This illustrates the tremendous possibilities arising from going beyond the strict deterministic (and reductionist) GRN view. Although the depicted examples are mainly emphasising the effects of alternative splicing on GRN dynamics, the number of genomic factors influencing alternative splicing is significantly large as well (Xiong et al., 2015). The integration of GRNs and alternative splicing emphasises the importance of developing a multidimensional understanding of alternative splicing control in the context of adaptive evolution (Wang, Weng, Li, & Xiao, 2017). Even though some progress has been made towards understanding GRNs in ecology and

evolutionary biology (Filteau, Pavey, St-Cyr, & Bernatchez, 2013; Singh, Ahi, & Sturmbauer, 2021), extremely little is known about how alternative splicing influences GRNs expansion in the context of evolution and adaptation (Schmitz et al., 2017). There are several reasons that such potentially important connections remain unexplored; the first reason comes from technical drawbacks in the past, for example difficulties in obtaining long sequencing reads to study isoforms. The second reason arises from the fact that analysis of both GRNs and alternative splicing requires different and rather complex sets of bioinformatics methods that have only been developed in the past decade. The third reason is that robust analytical tools to integrate GRN and alternative splicing are lacking. Thus, investigating alternative splicing regulatory networks underlying adaptive and speciation traits presents an open and important avenue for future research in the field of ecology and evolutionary biology.

Progress and limitations in studying alternative splicing

The complexity of the alternative splicing process has presented many challenges for empirical research. Despite mRNA-sequencing (RNA-seq) revolutionising research into genome-wide patterns of splicing; there are difficulties associated with computational methods that can accurately reconstruct isoforms from short-read sequencing data (Pertea, Kim, Pertea, Leek, & Salzberg, 2016). These methods involve either analysis of exons, transcript isoforms, alternative splicing events, or splice junctions (Alamancos, Agirre, & Eyras, 2014). However, most of these methods fail to account for biological variability across multiple conditions or consider splice events in the context of genome-wide alternative splicing variability (Trincado et al., 2018). Recent advances in long-read isoform sequencing (Iso-seq) from Pacific Biosciences has solved the problem of inaccurate isoform assembly (Gonzalez-Garay, 2016). Iso-seq generates full-length transcript sequences, providing unprecedented resolution into the alternative splicing landscape. It has allowed the

identification of thousands of novel isoforms in plants and animal (Abdel-Ghany et al., 2016; Ali, Thorgaard, & Salem, 2021; Feng, Xu, Liu, Cui, & Zhou, 2019; Nudelman et al., 2018). Iso-seq will help answer the many outstanding questions relating to the role of alternative splicing in adaptation and speciation. Oxford Nanopore Direct RNA Sequencing (ONT-DRS) is another long-read sequencing technology that has also revolutionised the study of transcriptional dynamics because it can sequence full isoforms as single reads (Clark et al., 2020). Additionally, Nanopore can provide base modification information, which allows the association of epigenetic regulation (such as methylation) and alternative splicing (A. M. Price et al., 2020). Both Iso-seq and ONT-DRS hold great promise to revolutionise the way we study the regulation of alternative splicing and shed light on its importance in evolutionary biology.

The functional impact of most splice variants on organismal phenotype has been the source of extensive debate (Blencowe, 2017; Kelemen et al., 2013; Tress et al., 2017a; Tress, Abascal, & Valencia, 2017b) as it is largely unknown to what extent different alternative isoforms are translated into functional proteins that can alter phenotypes and hold adaptive importance. This is because large-scale mass spectrometry-based proteomics is limited in coverage and sensitivity, resulting in high false negative rates in protein quantification (Blencowe, 2017). This is major limiting factor in the characterising the functional impacts of alternative splicing on phenotypic evolution. An important future goal will be to develop high-throughput methods that can accurately quantify the function of splice variants.

Predictions and hypotheses

Differences in alternative splicing at large time scales among divergent lineages is better documented than at short timescales (Barbosa-Morais et al., 2012; Braunschweig et al.,

2014; Merkin et al., 2012). It has been shown that variation in alternative splicing correlates with organismal complexity (L. Chen et al., 2014) (Figure 4B) but see (Brett, Pospisil, Valcárcel, Reich, & Bork, 2002). One untested hypothesis is that the dramatic rate at which alternative splicing evolves may be a driving force in rapid ecological adaptation and speciation and thus demands more attention (Singh et al., 2017; Smith et al., 2018; Terai et al., 2003). We predict that alternative splicing diversity (i.e., the number of isoforms per gene) may correlate species richness and phenotypic diversity (Figure 4C). This prediction can be tested using whole transcriptome data in lineages undergoing adaptive radiation and contrasted with lineages that are not radiating, as was done by Terai et al (2003) with a single gene. If true, the results will illuminate the wider role of alternative splicing in rapid ecological adaptation and speciation. If the substrate of adaptive radiations, such as those of cichlid fishes in East Africa, was substantial standing splice variation in the ancestral lineage, another interesting prediction to test would be how splice variation is fixed through positive selection or purged via negative selection over time, as the radiation progresses (Figure 4D). Hybridisation events, which have been shown to play an important role in speciation and adaptation (Irisarri et al., 2018; Meier et al., 2017; Stankowski & Streisfeld, 2015) may replenish splice variation by providing a new suite of novel isoforms for selection to act on.

Another key test would be to investigate if different isoforms are differentially fixed in species adapted to different ecological niches (Figure 4A). And if these are isoforms of a gene underlying a key phenotype (Mallarino et al., 2016; Singh et al., 2017; Smith et al., 2018; Tovar-Corona et al., 2015). It would also be important to correlate alternative splicing variating with single nucleotide polymorphisms (SNPs) to delineate if *cis* or *trans* splicing quantitative trait loci (sQTLs) are contributing more to adaptive phenotypes. We propose that adaptive radiations and domesticated crops are the best models to test the hypotheses outlined here. To better understand how alternative splicing contributes to adaptive evolution

at short time scales, these findings must be contrasted with what is known about splicing in more divergent lineages, such as vertebrates.

Concluding remarks

Going forward, the understudied mechanism of alternative splicing needs both broadscale surveys in published and new transcriptomic datasets, as well as functional characterisation of alternatively spliced isoforms underlying adaptive phenotypes. We hope that this article will stimulate discussion and encourage evolutionary biologists to attain a deeper understanding of how alternative splicing evolves, is regulated, and how splice-mediated tinkering contributes to ecological adaptation at the population and species level, in recent and more divergent lineages.

Box 1: Outstanding questions

1. What is the contribution of alternative splicing versus gene expression variation to ecological adaptations across the tree of life? How often do these mechanisms act independently?
2. Does alternative splicing generally influence the proteome and organismal phenotype and fitness to a greater (or lesser) extent than other forms of transcriptomic variation? Does this vary among types of organisms, tissue types, and developmental stages?
3. How does splicing variation scale with species and phenotypic diversity at short time scales? How does this contrast at long time scales?
4. Is alternative splicing a predictor of evolutionary rates?
5. How do novel isoforms evolve and how frequently does selection target splice mutations?

6. What is the contribution of *cis* versus *trans* regulation of splicing during adaptive evolution?
7. How does mutation-selection-migration maintain splice variation in populations?
8. How does hybridisation generate splice variation that facilitates speciation?
9. How can splicing lead to sex-divergence and reproductive isolation?
10. What is the impact of alternative splicing on gene regulatory network evolution?
11. How can the integration of transcriptomic, proteomic, and epigenetic data shed light on regulation of alternative splicing and its impact on adaptive evolution?

Acknowledgements

I, Pooja Singh, want to dedicate this manuscript to my father, Yashwant Singh (1956 – 2022), who inspired my curiosity for nature and instilled in me the confidence to pursue my ideas. I also want to thank Sam Yeaman (University of Calgary) for making me believe that my opinion was worth sharing. We would also like to thank Anna Duenser (University of Graz) and Marija Durdevic (Medical University of Graz) for splicing related discussions; and Carlos Ramirez Rodriguez (University of Bern) for reading over a draft of this manuscript. We want to also thank Michael Koller for help making and polish the figures in this study.

Author contributions

Pooja Singh conceived the idea for this manuscript, wrote the manuscript, and made Figures 1,2,4. Ehsan Pashay Ahi contributed the section on gene regulatory network expansion and Figure 3.

Data Accessibility Statement

Not applicable. No data was used in this manuscript.

References

Abdel-Ghany, S. E., Hamilton, M., Jacobi, J. L., Ngam, P., Devitt, N., Schilkey, F., … Reddy, A. S. N. (2016). A survey of the sorghum transcriptome using single-molecule long reads. *Nature Communications*, *7*, 11706. doi: 10.1038/ncomms11706

Alamancos, G. P., Agirre, E., & Eyras, E. (2014). Methods to study splicing from high-throughput RNA sequencing data. *Methods in Molecular Biology*, *1126*, 357–397. doi: 10.1007/978-1-62703-980-2_26

Ali, A., Thorgaard, G. H., & Salem, M. (2021). PacBio Iso-Seq improves the rainbow trout genome annotation and identifies aternative splicing associated with economically important phenotypes. *Frontiers in Genetics*, *12*, 683408. doi: 10.3389/fgene.2021.683408

Alt, F. W., Bothwell, A. L. M., Knapp, M., Siden, E., Mather, E., Koshland, M., & Baltimore, D. (1980). Synthesis of secreted and membrane-bound immunoglobulin mu heavy chains is directed by mRNAs that differ at their 3′ ends. *Cell*, *20*(2), 293–301. doi: 10.1016/0092-8674(80)90615-7

Ast, G. (2004). How did alternative splicing evolve? *Nature Reviews Genetics*, *5*(10), 773–782. doi: 10.1038/nrg1451

Barbosa-Morais, N. L., Irimia, M., Pan, Q., Xiong, H. Y., & Gueroussov, S. (2012). The evolutionary landscape of alternative splicing in vertebrate species. *Science*, *338*(December), 1587–1594.

Blekhman, R., Marioni, J. C., Zumbo, P., Stephens, M., & Gilad, Y. (2010). Sex-specific and lineage-specific alternative splicing in primates. *Genome Research*, *20*, 180–189. doi: 10.1101/gr.099226.109.

Blencowe, B. J. (2017). The relationship between alternative splicing and proteomic complexity. *Trends in Biochemical Sciences*, *42*(6), 407–408. doi: 10.1016/j.tibs.2017.04.001

Boutz, P. L., Stoilov, P., Li, Q., Lin, C.-H., Chawla, G., Ostrow, K., … Black, D. L. (2007). A post-transcriptional regulatory switch in polypyrimidine tract-binding proteins reprograms alternative splicing in developing neurons. *Genes & Development* , *21*(13), 1636–1652. Retrieved from http://genesdev.cshlp.org/content/21/13/1636.abstract

Braunschweig, U., Barbosa-Morais, N. L., Pan, Q., Nachman, E. N., Alipanahi, B., Gonatopoulos-Pournatzis, T., … Blencowe, B. J. (2014). Widespread intron retention in mammals functionally tunes transcriptomes. *Genome Research*, *24*(11), 1774–1786. doi: 10.1101/gr.177790.114

Braunschweig, U., Gueroussov, S., Plocik, A. M., Graveley, B. R., & Blencowe, B. J. (2013). Dynamic integration of splicing within gene regulatory pathways. *Cell*, *152*(6), 1252–1269. doi: 10.1016/j.cell.2013.02.034

Brawand, D., Soumillon, M., Necsulea, A., Julien, P., Csárdi, G., Harrigan, P., … Kaessmann, H. (2011). The evolution of gene expression levels in mammalian organs. *Nature*, *478*(7369), 343–348. doi: 10.1038/nature10532

Brett, D., Pospisil, H., Valcárcel, J., Reich, J., & Bork, P. (2002). Alternative splicing and genome complexity. *Nature Genetics*, *30*(1), 29–30. doi: 10.1038/ng803

Burke, J. M., & Arnold, M. L. (2001). Genetics and the fitness of hybrids. *Annual Review of Genetics*, *35*, 31–52.

Chaudhary, S., Khokhar, W., Jabre, I., Reddy, A. S. N., Byrne, L. J., Wilson, C. M., & Syed, N. H. (2019). Alternative splicing and protein diversity: Plants versus animals. *Frontiers in Plant Science*, *10*, 1–14. doi: 10.3389/fpls.2019.00708

Chen, L., Bush, S. J., Tovar-Corona, J. M., Castillo-Morales, A., & Urrutia, A. O. (2014). Correcting

for Differential Transcript Coverage Reveals a Strong Relationship between Alternative Splicing and Organism Complexity. *Molecular Biology and Evolution*, *31*(6), 1402–1413. doi: 10.1093/molbev/msu083

Chen, Z. J. (2010). Molecular mechanisms of polyploidy and hybrid vigor. *Trends in Plant Science*, *15*(2), 57–71. doi: 10.1016/j.tplants.2009.12.003

Clark, M. B., Wrzesinski, T., Garcia, A. B., Hall, N. A. L., Kleinman, J. E., Hyde, T., … Tunbridge, E. M. (2020). Long-read sequencing reveals the complex splicing profile of the psychiatric risk gene CACNA1C in human brain. *Molecular Psychiatry*, *25*(1), 37–47. doi: 10.1038/s41380-019-0583-1

Crombach, A., & Hogeweg, P. (2008). Evolution of evolvability in gene regulatory networks. *PLOS Computational Biology*, *4*(7), e1000112. Retrieved from https://doi.org/10.1371/journal.pcbi.1000112

Davidson, E. H., & Erwin, D. H. (2006). Gene regulatory networks and the evolution of animal body plans. *Science*, *311*(February), 796–801. doi: 10.1126/science.1113832

Dice, L. R. (1940). Ecologic and genetic variability within species of Peromyscus. *The American Naturalist*, *74*(752), 212–221. Retrieved from http://www.jstor.org/stable/2457573

Ehrenreich, I. M., & Pfennig, D. W. (2015). Genetic assimilation: a review of its potential proximate causes and evolutionary consequences. *Annals of Botany*, *117*(5), 769–779. doi: 10.1093/aob/mcv130

El Taher, A., Böhne, A., Boileau, N., Ronco, F., Indermaur, A., Widmer, L., & Salzburger, W. (2021). Gene expression dynamics during rapid organismal diversification in African cichlid fishes. *Nature Ecology & Evolution*, *5*(2), 243–250. doi: 10.1038/s41559-020-01354-3

Fang, S., Hou, X., Qiu, K., He, R., Feng, X., & Liang, X. (2020). The occurrence and function of alternative splicing in fungi. *Fungal Biology Reviews*, *34*(4), 178–188. doi: https://doi.org/10.1016/j.fbr.2020.10.001

Feng, S., Xu, M., Liu, F., Cui, C., & Zhou, B. (2019). Reconstruction of the full-length transcriptome atlas using PacBio Iso-Seq provides insight into the alternative splicing in Gossypium australe. *BMC Plant Biology*, *19*(1), 365. doi: 10.1186/s12870-019-1968-7

Filteau, M., Pavey, S. A., St-Cyr, J., & Bernatchez, L. (2013). Gene coexpression networks reveal key drivers of phenotypic divergence in lake whitefish. *Molecular Biology and Evolution*, *30*(6), 1384–1396. doi: 10.1093/molbev/mst053

Gómez-Redondo, I., Planells, B., Navarrete, P., & Gutiérrez-Adán, A. (2021). Role of Alternative Splicing in Sex Determination in Vertebrates. *Sexual Development*, *15*(5–6), 381–391. doi: 10.1159/000519218

Gonzalez-Garay, M. L. (2016). Introduction to Isoform Sequencing Using Pacific Biosciences Technology (Iso-Seq). In J. Wu (Ed.), *Transcriptomics and gene regulation* (pp. 141–160). doi: 10.1007/978-94-017-7450-5_6

Grantham, M. E., & Brisson, J. A. (2018). Extensive differential splicing underlies phenotypically plastic aphid morphs. *Molecular Biology and Evolution*, *35*(8), 1934–1946. doi: 10.1093/molbev/msy095

Gueroussov, S., Gonatopoulos-Pournatzis, T., Irimia, M., Raj, B., Lin, Z.-Y., Gingras, A.-C., & Blencowe, B. J. (2015). An alternative splicing event amplifies evolutionary differences between vertebrates. *Science*, *349*(6250), 868–873. doi: 10.1126/science.aaa8381

Healy, T. M., & Schulte, P. M. (2019). Patterns of alternative splicing in response to cold acclimation in fish. *Journal of Experimental Biology*, *222*(5). doi: 10.1242/jeb.193516

Hill, M. S., Vande Zande, P., & Wittkopp, P. J. (2021). Molecular and evolutionary processes generating variation in gene expression. *Nature Reviews Genetics*, *22*(4), 203–215. doi: 10.1038/s41576-020-00304-w

Irisarri, I., Singh, P., Koblmüller, S., Torres-Dowdall, J., Henning, F., Franchini, P., … Meyer, A. (2018). Phylogenomics uncovers early hybridization and adaptive loci shaping the radiation of Lake Tanganyika cichlid fishes. *Nature Communications*, *9*(1). doi: 10.1038/s41467-018-05479-9

Jabre, I., Reddy, A. S. N., Kalyna, M., Chaudhary, S., Khokhar, W., Byrne, L. J., … Syed, N. H.

(2019). Does co-transcriptional regulation of alternative splicing mediate plant stress responses? *Nucleic Acids Research*, *47*(6), 2716–2726. doi: 10.1093/nar/gkz121

Jacobs, A., & Elmer, K. R. (2021). Alternative splicing and gene expression play contrasting roles in the parallel phenotypic evolution of a salmonid fish. *Molecular Ecology*, *n/a*(n/a). doi: https://doi.org/10.1111/mec.15817

Jakšić, A. M., & Schlötterer, C. (2016). The interplay of temperature and genotype on patterns of alternative splicing in Drosophila melanogaster. *Genetics*, *204*(1), 315–325. doi: 10.1534/genetics.116.192310

Josephs, E. B. (2021). Gene expression links genotype and phenotype during rapid adaptation. *Molecular Ecology*, *30*(1), 30–32. doi: https://doi.org/10.1111/mec.15729

Karlebach, G., Veiga, D. F. T., Mays, A. D., Chatzipantsiou, C., Barja, P. P., Chatzou, M., … Robinson, P. N. (2020). The impact of biological sex on alternative splicing. *BioRxiv*, 490904. doi: 10.1101/490904

Kawakami, K., Amsterdam, A., Shimoda, N., Becker, T., Mugg, J., Shima, A., & Hopkins, N. (2000). Proviral insertions in the zebrafish hagoromo gene, encoding an F-box/WD40-repeat protein, cause stripe pattern anomalies. *Current Biology*, *10*(8), 463—466. doi: 10.1016/s0960-9822(00)00444-9

Kelemen, O., Convertini, P., Zhang, Z., Wen, Y., Shen, M., Falaleeva, M., & Stamm, S. (2013). Function of alternative splicing. *Gene*, *514*(1), 1–30. doi: https://doi.org/10.1016/j.gene.2012.07.083

Keren, H., Lev-Maor, G., & Ast, G. (2010). Alternative splicing and evolution: diversification, exon definition and function. *Nature Reviews. Genetics*, *11*(5), 345–355. doi: 10.1038/nrg2776

Kim, E., Magen, A., & Ast, G. (2007). Different levels of alternative splicing among eukaryotes. *Nucleic Acids Research*, *35*(1), 125–131. doi: 10.1093/nar/gkl924

Kondrashov, F. A., & Koonin, E. V. (2003). Evolution of alternative splicing: deletions, insertions and origin of functional parts of proteins from intron sequences. *Trends in Genetics*, *19*(3), 115–119. doi: https://doi.org/10.1016/S0168-9525(02)00029-X

Kornblihtt, A. R., Schor, I. E., Alló, M., Dujardin, G., Petrillo, E., & Muñoz, M. J. (2013). Alternative splicing: a pivotal step between eukaryotic transcription and translation. *Nature Reviews. Molecular Cell Biology*, *14*(3), 153–165. doi: 10.1038/nrm3525

Lauffenburger, D. A. (2000). Cell signaling pathways as control modules: Complexity for simplicity? *Proceedings of the National Academy of Sciences*, *97*(10), 5031 LP – 5033. doi: 10.1073/pnas.97.10.5031

Lewontin, R. C., & Birch, L. C. (1966). Hybridization as a source of variation for adaptation to new environments. *Evolution*, *20*(3), 315–336. doi: 10.2307/2406633

Luco, R. F., Allo, M., Schor, I. E., Kornblihtt, A. R., & Misteli, T. (2011). Epigenetics in alternative pre-mRNA splicing. *Cell*, *144*(1), 16–26. doi: 10.1016/j.cell.2010.11.056

Mallarino, R., Linden, T. A., Linnen, C. R., & Hoekstra, H. E. (2016). The role of isoforms in the evolution of cryptic coloration in Peromyscus mice. *Molecular Ecology*, *26*, 1–14. doi: 10.1101/041087

Mastrangelo, A. M., Marone, D., Laidò, G., De Leonardis, A. M., & De Vita, P. (2012). Alternative splicing: enhancing ability to cope with stress via transcriptome plasticity. *Plant Science*, *185–186*, 40–49. doi: https://doi.org/10.1016/j.plantsci.2011.09.006

Meier, J. I., Marques, D. A., Mwaiko, S., Wagner, C. E., Excoffier, L., & Seehausen, O. (2017). Ancient hybridization fuels rapid cichlid fish adaptive radiations. *Nature Communications*, *8*(May 2016), 14363. doi: 10.1038/ncomms14363

Merkin, J., Russell, C., Chen, P., & Burge, C. B. (2012). Evolutionary dynamics of gene and isoform regulation in mammalian tissues. *Science*, *338*(December), 1593–1599. doi: 10.1126/science.1228186

Niklas, K. J., Bondos, S. E., Dunker, A. K., & Newman, S. A. (2015). Rethinking gene regulatory networks in light of alternative splicing, intrinsically disordered protein domains, and post-translational modifications. *Frontiers in Cell and Developmental Biology*, *3*(February), 1–13. doi: 10.3389/fcell.2015.00008

Nilsen, T. W., & Graveley, B. R. (2010). Expansion of the eukaryotic proteome by alternative splicing. *Nature*, *463*(7280), 457–463. doi: 10.1038/nature08909

Nudelman, G., Frasca, A., Kent, B., Sadler, K. C., Sealfon, S. C., Walsh, M. J., & Zaslavsky, E. (2018). High resolution annotation of zebrafish transcriptome using long-read sequencing. *Genome Research*, *28*(9), 1415–1425. doi: 10.1101/gr.223586.117

Pertea, M., Kim, D., Pertea, G. M., Leek, J. T., & Salzberg, S. L. (2016). Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nature Protocols*, *11*(9), 1650–1667. doi: 10.1038/nprot.2016.095

Pleiss, J. A., Whitworth, G. B., Bergkessel, M., & Guthrie, C. (2007). Rapid, transcript-specific changes in splicing in response to environmental stress. *Molecular Cell*, *27*(6), 928–937. doi: 10.1016/j.molcel.2007.07.018

Price, A. M., Hayer, K. E., McIntyre, A. B. R., Gokhale, N. S., Abebe, J. S., Della Fera, A. N., … Weitzman, M. D. (2020). Direct RNA sequencing reveals m6A modifications on adenovirus RNA are necessary for efficient splicing. *Nature Communications*, *11*(1), 6016. doi: 10.1038/s41467-020-19787-6

Price, J., Harrison, M. C., Hammond, R. L., Adams, S., Gutierrez-Marcos, J. F., & Mallon, E. B. (2018). Alternative splicing associated with phenotypic plasticity in the bumble bee Bombus terrestris. *Molecular Ecology*, *27*(4), 1036–1043. doi: 10.1111/mec.14495

Rogers, T. F., Palmer, D. H., & Wright, A. E. (2020). Sex-specific selection drives the evolution of alternative splicing in birds. *Molecular Biology and Evolution*, 1–29.

Scascitelli, M., Cognet, M., & Adams, K. L. (2010). An Interspecific plant hybrid shows novel changes in parental splice forms of genes for splicing factors. *Genetics*, *184*(4), 975 LP – 983. doi: 10.1534/genetics.109.112557

Schlichting, C. D., & Pigliucci, M. (1998). *Phenotypic evolution: a reaction norm perspective*. Sinauer Associates, Sunderland, MA.

Schmitz, U., Pinello, N., Jia, F., Alasmari, S., Ritchie, W., Keightley, M.-C., … Rasko, J. E. J. (2017). Intron retention enhances gene regulatory complexity in vertebrates. *Genome Biology*, *18*(1), 216. doi: 10.1186/s13059-017-1339-3

Seehausen, O. (2004). Hybridization and adaptive radiation. *Trends in Ecology and Evolution*. doi: 10.1016/j.tree.2004.01.003

Seehausen, O., Terai, Y., Magalhaes, I. S., Carleton, K. L., Mrosso, H. D. J., Miyagi, R., … Okada, N. (2008). Speciation through sensory drive in cichlid fish. *Nature*, *455*(7213), 620–626. doi: 10.1038/nature07285

Singh, P., Ahi, E. P., & Sturmbauer, C. (2021). Gene coexpression networks reveal molecular interactions underlying cichlid jaw modularity. *BMC Ecology and Evolution*, *21*(62), 1–17. doi: 10.1186/s12862-021-01787-9

Singh, P., Börger, C., More, H., & Sturmbauer, C. (2017). The role of alternative splicing and differential gene expression in cichlid adaptive radiation. *Genome Biology and Evolution*, *9*(10), 2764–2781. doi: 10.1093/gbe/evx204

Smith, C. C. R., Rieseberg, L. H., Hulke, B. S., & Kane, N. C. (2021). Aberrant RNA splicing due to genetic incompatibilities in sunflower hybrids. *Evolution*, 1–12. doi: 10.1111/evo.14360

Smith, C. C. R., Tittes, S., Mendieta, J. P., Collier-zans, E., Rowe, H. C., Rieseberg, L. H., & Kane, N. C. (2018). Genetics of alternative splicing evolution during sunflower domestication. *PNAS*, *115*(26), 6768–6773. doi: 10.1073/pnas.1803361115

Somero, G. N. (2018). RNA thermosensors: how might animals exploit their regulatory potential? *Journal of Experimental Biology*, *221*(4). doi: 10.1242/jeb.162842

Stankowski, S., & Streisfeld, M. A. (2015). Introgressive hybridization facilitates adaptive divergence in a recent radiation of monkeyflowers. *Proceedings of the Royal Society of London B*, *282*, 20151666. doi: 10.1098/rspb.2015.1666

Talavera, D., Robertson, D. L., & Lovell, S. C. (2013). Alternative splicing and protein interaction data sets. *Nature Biotechnology*, *31*(4), 292–293. doi: 10.1038/nbt.2540

Tellier, M., Maudlin, I., & Murphy, S. (2020). Transcription and splicing: A two-way street. *Wiley Interdisciplinary Reviews: RNA*, *11*. doi: 10.1002/wrna.1593

Terai, Y., Morikawa, N., Kawakami, K., & Okada, N. (2003). The complexity of alternative splicing of hagoromo mRNAs is increased in an explosively speciated lineage in East African cichlids. *PNAS*, *100*(22), 12798–12803. doi: 10.1073/pnas.2132833100

Tovar-Corona, J. M., Castillo-Morales, A., Chen, L., Olds, B. P., Clark, J. M., Reynolds, S. E., … Urrutia, A. O. (2015). Alternative splice in alternative lice. *Molecular Biology and Evolution*, *32*(10), 2749–2759. doi: 10.1093/molbev/msv151

Tress, M. L., Abascal, F., & Valencia, A. (2017a). Alternative splicing may not be the key to proteome complexity. *Trends in Biochemical Sciences*, *42*(2), 98–110. doi: 10.1016/j.tibs.2016.08.008

Tress, M. L., Abascal, F., & Valencia, A. (2017b). Most alternative isoforms are not functionally important. *Trends in Biochemical Sciences*, *42*(6), 408–410. doi: 10.1016/j.tibs.2017.04.002

Trincado, J. L., Entizne, J. C., Hysenaj, G., Singh, B., Skalic, M., Elliott, D. J., & Eyras, E. (2018). SUPPA2: Fast, accurate, and uncertainty-aware differential splicing analysis across multiple conditions. *Genome Biology*, *19*(1), 1–11. doi: 10.1186/s13059-018-1417-1

Ule, J., & Blencowe, B. J. (2019). Alternative splicing regulatory networks: functions, mechanisms, and evolution. *Molecular Cell*, *76*(2), 329–345. doi: 10.1016/j.molcel.2019.09.017

Wang, G., Weng, L., Li, M., & Xiao, H. (2017). Response of gene expression and alternative splicing to distinct growth environments in tomato. *International Journal of Molecular Sciences*, *18*(3). doi: 10.3390/ijms18030475

West-Eberhard, M. J. (1989). Phenotypic plasticity and the origins of diversity. *Annual Review of Ecology and Systematics*, *20*(1), 249–278. doi: 10.1146/annurev.es.20.110189.001341

Wittkopp, P. J., Wittkopp, P. J., Haerum, B. K., Haerum, B. K., Clark, A. G., & Clark, A. G. (2004). Evolutionary changes in cis and trans gene regulation. *Nature*, *430*(6995), 85–88. doi: 10.1038/nature02698

Wray, G. A. (2007). The evolutionary significance of cis-regulatory mutations. *Nature Reviews Genetics*, *8*(3), 206–216. doi: 10.1038/nrg2063

Xia, T., Zhang, L., Xu, J., Wang, L., Liu, B., Hao, M., … Shen, Y. (2017). The alternative splicing of EAM8 contributes to early flowering and short-season adaptation in a landrace barley from the Qinghai-Tibetan Plateau. *Theoretical and Applied Genetics*, *130*(4), 757–766. doi: 10.1007/s00122-016-2848-2

Xing, Y., & Lee, C. (2005). Evidence of functional selection pressure for alternative splicing events that accelerate evolution of protein subsequences. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(38), 13526–13531. doi: 10.1073/pnas.0501213102

Xing, Y., & Lee, C. (2006). Alternative splicing and RNA selection pressure - evolutionary consequences for eukaryotic genomes. *Nature Reviews Genetics*, *7*(July), 499–509. doi: 10.1038/nrg1896

Xiong, H. Y., Alipanahi, B., Lee, L. J., Bretschneider, H., Merico, D., Yuen, R. K. C., … Frey, B. J. (2015). The human splicing code reveals new insights into the genetic determinants of disease. *Science*, *347*(6218), 1254806. doi: 10.1126/science.1254806

Figures

Figure 1 Different types of alternative splicing mechanisms. Coloured boxes represent alternative exons and grey boxes represent constitutively exons. Adapted from Xing and Lee (2006).

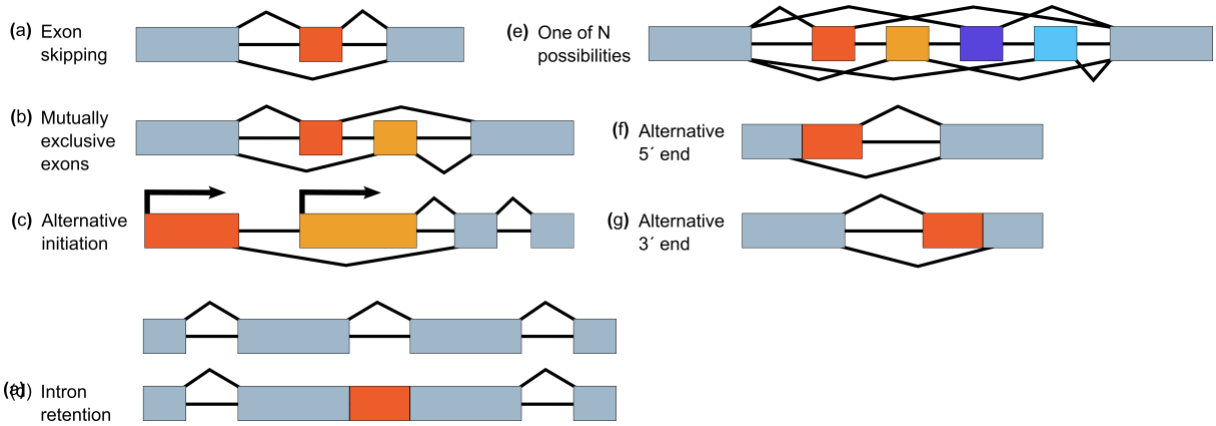

Figure 2 Schematic of how differences in (A) gene expression and (B) alternative splicing can theoretically give rise to divergent adaptive phenotypes. This schematic is based on Singh et al (2017) where the role of gene expression variation and alternative splicing in the evolutionary divergence of trophic adaptations in cichlid fishes was investigated.

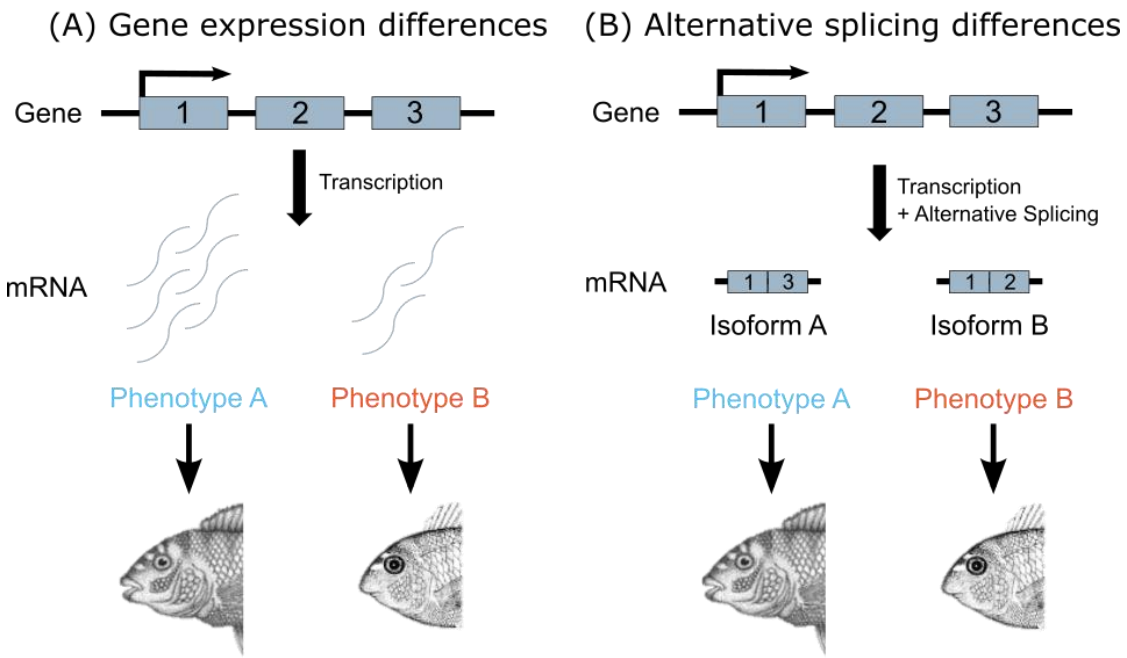

Figure 3 Gene regulatory networks and alternative splicing. A) Advantages of merging gene regulatory network and alternative splicing in understanding of processes driving evolutionary adaptation. B) An example of deterministic view of a single gene regulatory network (GRN) unit. In this GRN example, there is an upstream transcription factor inducing the transcription of a set of downstream target genes which form a module of positively co-expressed genes. C) Examples of a plethora of potential regulatory effects that can occur by alternative splicing of just the upstream transcription factor that regulates a single GRN unit. In these examples, a variety of changes are predicted to happen to the downstream co-expressed target genes, which can be translated to variations in developmental and physiological processes as well as adaptive responses. TF; transcription factor, and alphabets indicate downstream genes.

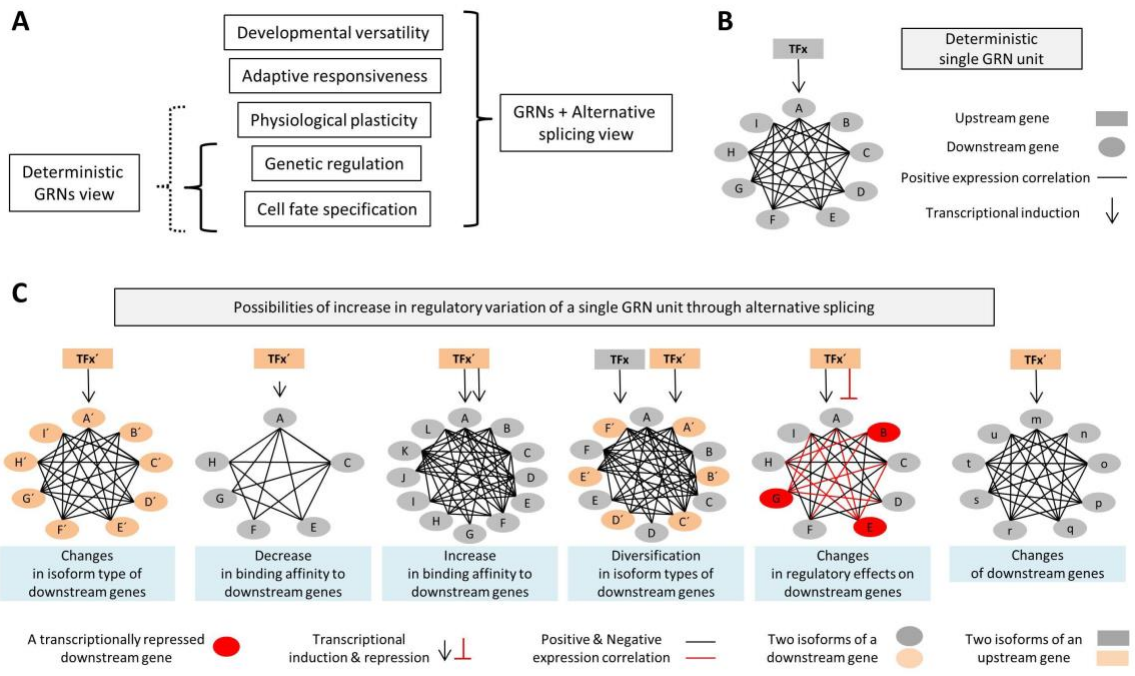

Figure 4 Predictions on the relationship of alternative splicing and adaptive evolution. (A) Alternative splicing can provide neutral or nearly neutral paths for accelerated paths of phenotypic evolution as there is low negative selection pressure against lowly expressed novel isoforms if the ancestral isoform continues to be expressed at high levels. Blue, yellow, red lines represent distinct isoforms of a gene. (B) Evidence suggests that alternative splicing complexity correlates with organismal complexity (Chen et al 2014). (C) We predict that alternative splicing diversity may correlate species richness and phenotypic diversity if splice variation is a substrate for adaptive evolution. (D) If the substrate of adaptive evolution was substantial standing splice variation in the ancestral lineage, splice variation could be fixed through positive selection or purged via negative selection over time. Hybridisation could replenish this splice variation. Blue line represents splicing variation in the absence of hybridisation and red line represents splice variation with a hybridisation event.