Opinion

The importance of alternative splicing in adaptive evolution

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Abstract

Although alternative splicing is a ubiquitous gene regulatory mechanism in plants and animals, its contribution to evolutionary transitions is understudied. Splicing enables different mRNA isoforms to be generated from the same gene, expanding transcriptomic and proteomic diversity. While the role of gene expression 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 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 splicing be included as a standard analysis alongside gene expression

analysis so we can better understand of how splicing contributes to adaptive divergence at the micro- and macroevolutionary levels.

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

What is splicing and why does it matter?

Alternative splicing is a form of post-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 of exons and introns in the final mRNA molecule that is transcribed (Figure 1). Discovered over 40 years ago, 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). 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).

There exists a strong relationship between organismal complexity and alternative splicing, even after accounting for covariables such as gene content and protein interactivity (L. Chen, Bush, Tovar-Corona, Castillo-Morales, & Urrutia, 2014). Even though splicing is major source of transcriptomic and potentially proteomic and phenotypic variation, little is known about its role in adaptation and ecological speciation. This is because transcriptomic studies in ecology and evolutionary biology in the last decade have focused mostly on gene expression due to its established role in adaptive evolution (Brawand et al., 2011; El Taher et al., 2021; King & Wilson, 1975; Wray, 2007). The scepticism associated with 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 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 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 splicing evolves, how splice variation is generated and maintained, and how it contributes to phenotypic novelty.

Alternative splicing as a substrate for rapid adaptive evolution

The last 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 splicing complexity has gradually increased over the last 1,400 MYA of eukaryotic evolution and is highly correlated with organismal complexity. Two of the most influential studies on the evolutionary significance of 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 MYA of evolution in vertebrates and found significant differences in splicing complexity among vertebrate species, with primates harbouring the highest complexity. Both these studies also found that gene expression was conserved at the tissue-specific level and splicing was conserved at the species-specific level. This pattern was prominent in lineages that diverged < 6 MYA suggesting that splicing was diverging faster than gene expression at shorter timescales. Remarkably, almost a decade prior to this, 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 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 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, Linden, Linnen, & Hoekstra, 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 splicing can rapidly give rise to ecological adaptations that lead to population and species divergence. This makes splicing especially relevant for understanding evolutionary processes such as adaptive radiation and domestication.

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 splicing (Rogers et al., 2020); suggesting that not only differential gene expression but also 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.

Both variation in gene expression and alternative splicing have the potential to impact phenotypic variation (Figure 2). While gene promoter activity quantitatively regulates the number of transcripts, alternative splicing changes the structure of transcripts and the encoded proteins. 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. 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, Börger, More, & Sturmbauer, 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 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 and how it plays out at long and short timescales; in adaptive and plastic responses.

Phenotypic plasticity and 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 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 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, 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 (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. More research is also needed to investigate the broad applicability of the role of 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 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 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, 2020). So it seems that hybridisation can generate novel splice variation but it is not known what the adaptive value of this 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., 2020). This proposal presents tantalising prospects for the role of splicing in speciation.

Expansion of gene regulatory networks by alternative splicing

Networks of interacting transcription factors that regulate 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 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).

At detailed molecular level, 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 splicing on GRN dynamics, the number of genomic factors influencing alternative splicing is significantly large as well (Xiong et al., 2015). The crosstalk between GRNs and alternative splicing emphasises the importance of developing a multidimensional understanding of 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). Thus, it presents an open and important avenue for future research.

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 are needed as it is still not well understood how the splicing process evolves. Furthermore, it is not well understood how splice variation is maintained at the species level, in locally adapted populations, or between sexes.

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.

Predictions and hypotheses

Differences in alternative splicing at large time scales among divergent lineages is better documented than at short timescales. It is quite well established that variation in alternative splicing correlates with organismal complexity (Figure 4B). One untested hypothesis is that the dramatic rate at which splicing evolves may be a driving force in rapid ecological adaptation and speciation and thus demands more attention. We predict that splicing diversity should correlate species richness and phenotypic diversity (Figure 4C). This prediction can be tested in lineages undergoing adaptive radiation and contrasted with lineages that are not radiating. If true, the results will illuminate the wider role of alternative splicing in rapid ecological adaptation. Given substantial standing splice variation in the seeding ancestor, 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 may replenish splice variation, 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. 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.

Progress and limitations in studying splicing

The complexity of the 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 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.

The functional role of splicing 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 alternative isoforms are translated into functional proteins that can alter phenotypes. 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 splicing on phenotypic evolution. An important future goal will be to develop high-throughput methods that can accurately quantify the function of splice variants.

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 splicing evolves, 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 to ecological adaptations across the tree of life?
2. How does splicing variation scale with species and phenotypic diversity at short time scales? How does this contrast at long time scales?
3. Is alternative splicing a predictor of evolutionary rates?
4. How do novel isoforms evolve and how frequently does selection target splice mutations?
5. What is the contribution of *cis* versus *trans* regulation of splicing during adaptive evolution?
6. How does mutation-selection-migration maintain splice variation in populations?
7. How does hybridisation generate splice variation that facilitates speciation?
8. How can splicing lead to sex-divergence and reproductive isolation?
9. What is the impact of alternative splicing on gene regulatory network evolution?

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Figures

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

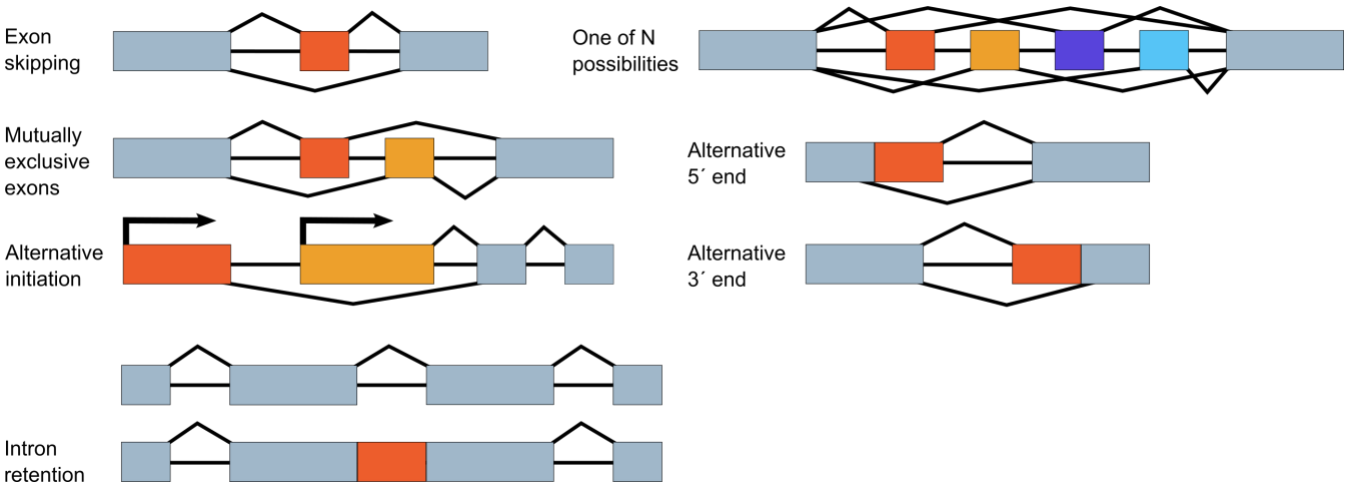

Figure 2 Schematic of how differences in (A) gene expression and (B) alternative splicing can theoretically give rise to divergent phenotypes.

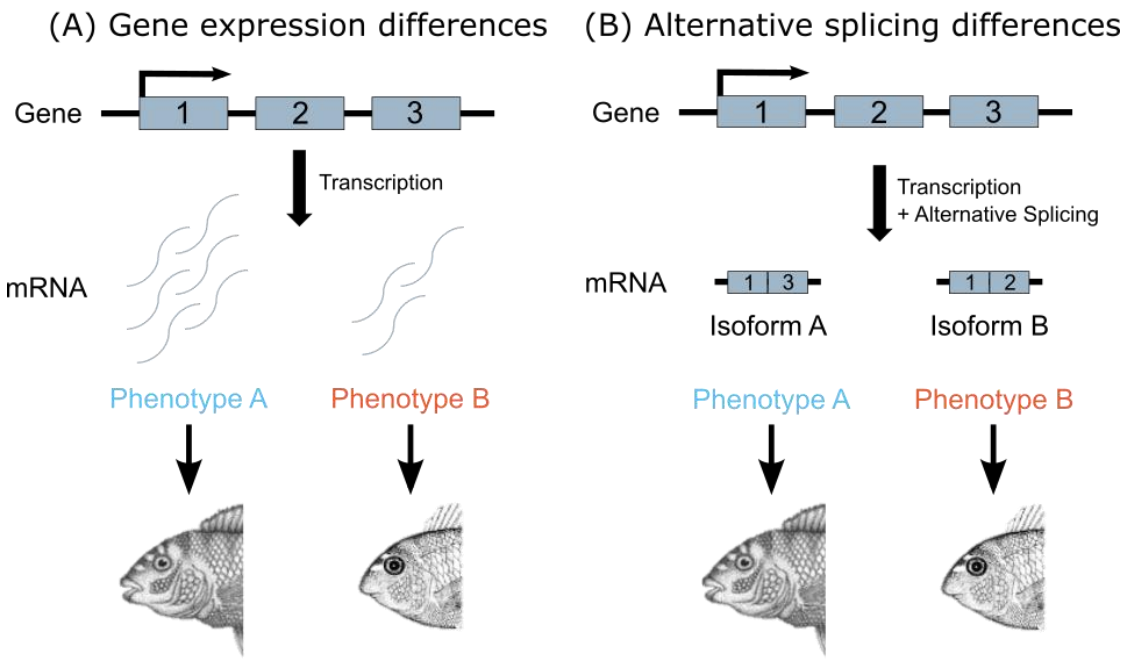

Figure 3 Gene regulatory networks and alternative splicing. A) Advantages of merging gene regulatory network and alternative splicing in understanding of processes driving evolutionary adaptation. B) Examples of plethora of potential regulatory changes by adding alternative splicing to a single gene regulatory unit. TF; transcription factor, and numbers and letters indicate isoform and downstream genes, respectively.

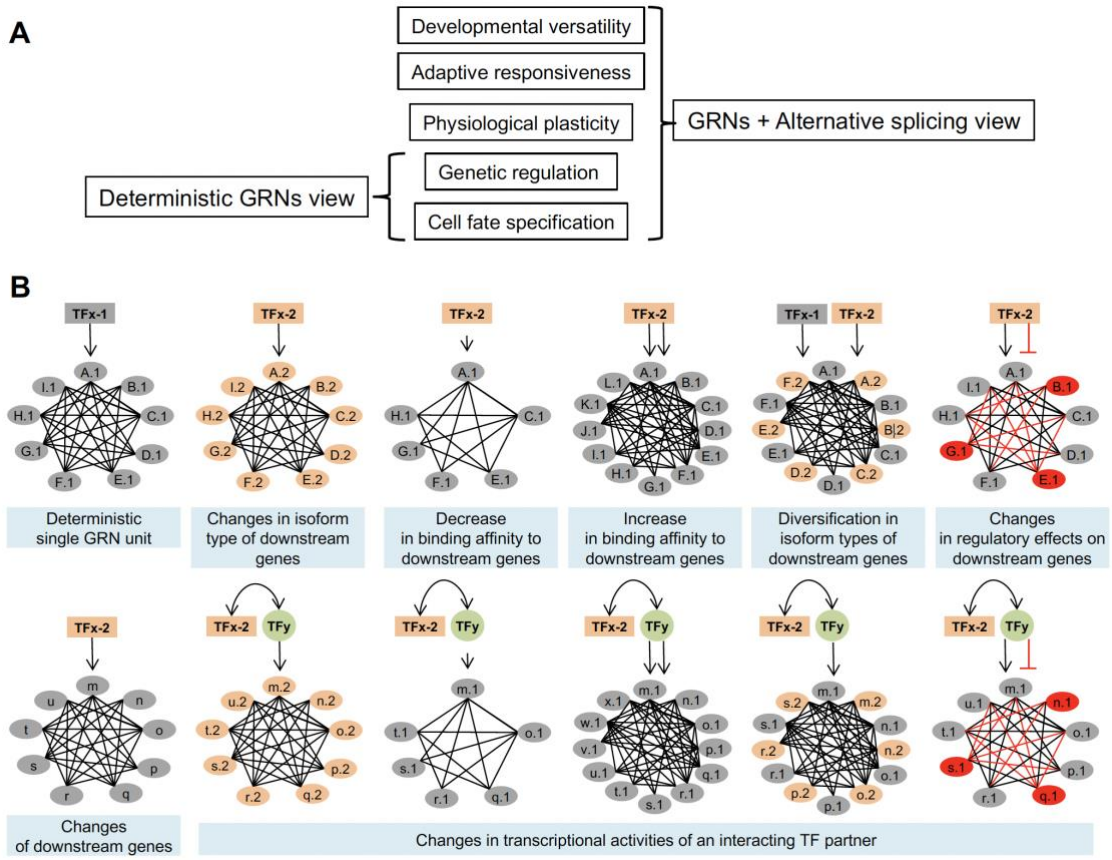

Figure 4 Predictions on the relationship of alternative splicing complexity and evolutionary complexity. (A) Blue, yellow, red lines represent distinct isoforms of a gene (D) Blue line represents splicing variation in the absence of hybridisation and red line represents splice variation with a hybridisation event.