CellPress

Review

Gene Regulation and Speciation

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Understanding the genetic architecture of speciation is a major goal in evolutionary biology. Hybrid dysfunction is thought to arise most commonly through negative interactions between alleles at two or more loci. Divergence between interacting regulatory elements that affect gene expression (i.e., regulatory divergence) may be a common route for these negative interactions to arise. We review here how regulatory divergence between species can result in hybrid dysfunction, including recent theoretical support for this model. We then discuss the empirical evidence for regulatory divergence between species and evaluate evidence for misregulation as a source of hybrid dysfunction. Finally, we review unresolved questions in gene regulation as it pertains to speciation and point to areas that could benefit from future research.

Trends

Simulation studies suggest that hybrid incompatibilities can evolve rapidly when selection acts on regulatory pathways.

Genomic approaches have identified widespread regulatory divergence between species in *cis* and *trans*.

Cis–trans regulatory divergence increases with phylogenetic distance and has been associated with misexpression in interspecific hybrids.

Many known hybrid incompatibility genes have either a putative regulatory function or are misexpressed in hybrids.

A Role for Gene Regulation in Hybrid Sterility and Inviability

Understanding the genetic basis of speciation is a longstanding problem in evolutionary biology. The major model for the evolution of intrinsic post-zygotic isolation postulates that hybrid sterility or inviability arises from negative interactions between alleles at different loci when joined together in hybrids. The regulation of gene expression is inherently based on interactions between loci, raising the possibility that disruption of gene regulation in hybrids is a common mechanism for post-zygotic isolation. Although there is accumulating evidence that changes in gene regulation play a prominent role in adaptation (e.g., [1,2]), the role of regulatory evolution in speciation has received less attention. We evaluate here the role of regulatory evolution in speciation, and we suggest, both from recent theoretical and empirical studies, that changes in gene regulation play a major role in intrinsic post-zygotic isolation. While our focus is on post-zygotic isolation, regulatory divergence may also play an important role in establishing other reproductive barriers as a byproduct of adaptive divergence (i.e., ecological speciation).

Conceptual Framework

Single-locus models of hybrid dysfunction all suffer from the problem that mutations that lower the fitness of heterozygotes (and thus cause reproductive isolation) are unlikely to become established in a new population (e.g., [3–5]). This problem was recognized by Bateson [6], Dobzhansky [7], and Muller [8,9], who suggested instead that hybrid dysfunction could arise from negative interactions between alleles at two or more loci. In the Bateson–Dobzhansky–Muller (BDM) model, alleles that are adaptive or neutral in their own genetic background are incompatible with alleles at one or more loci on the alternative genetic background (Figure 1). Thus, diverging lineages can accumulate substitutions without any loss of fitness. There is now strong empirical support for this model of intrinsic post-zygotic isolation [10].

Gene regulation is the process by which cells control the specific amount of gene product (i.e., RNA or protein) produced. Gene regulation is a complex process involving the interaction of DNA sequences, RNA molecules, and proteins, as well as epigenetic modifications. Because the interaction of regulatory elements is required for organismal function, interacting regulatory elements are assumed to be co-adapted (e.g., [11]). When co-adapted interactions between

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 http://dx.doi.org/10.1016/j.tig.2016.11.003

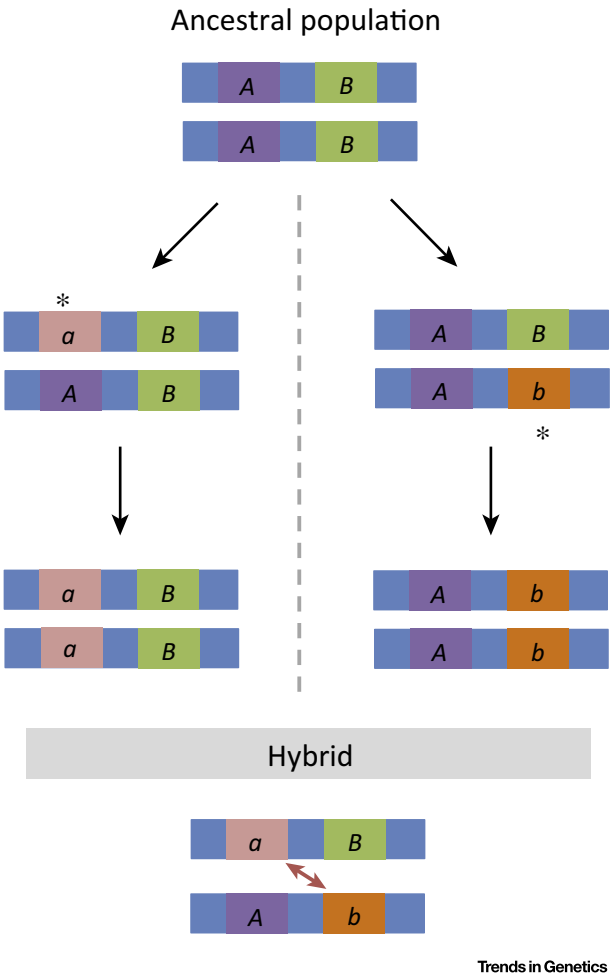

Figure 1. The Bateson–Dobzhansky–Muller Model of Hybrid Incompatibility. In the ancestral population, the genotype is *AABB*. After the two populations are isolated, new mutations arise independently on each lineage as indicated by the asterisks. In one population, *A* evolves into *a*, in the other population *B* evolves into *b*. In hybrids, negative interactions between the *a* and *b* alleles can result in sterility or inviability. The *a* and *b* alleles are found together for the first time in hybrids, explaining how this incompatibility could evolve without either lineage experiencing an intermediate state of reduced fitness.

regulatory elements are disrupted, downstream targets of these elements may be misregulated. While disrupted interactions between any of pair of regulatory elements or sequences could result in hybrid incompatibilities, the process of transcription initiation has received the most attention. While we focus mainly on transcriptional control, divergence between regulatory elements affecting other levels of gene regulation (e.g., translation) may also play a role in speciation.

Transcription is regulated by the interaction of *cis*-regulatory elements and *trans*-acting factors. *Cis*-regulatory elements are stretches of non-coding DNA (e.g., promoters, enhancers) that act as binding sites for *trans*-acting factors to regulate mRNA abundance. In the simplest case, the *trans*-acting factors are transcription factor proteins, although other proteins have also been known to act in *trans* to regulate gene expression [12]. Mutations in *cis*-regulatory regions or in transcription factors can affect mRNA abundance. Transcription factors frequently interact with multiple downstream target sequences and thus may be pleiotropic. By contrast, a single gene may have multiple *cis*-regulatory regions that regulate it

in a tissue-and context-specific manner. As a consequence, changes in *cis*-regulatory regions are thought to be less pleiotropic than changes to the transcription factors they bind. The modularity of *cis*-regulatory regions has given rise to the idea that changes to these regions may play a large role in phenotypic evolution, an idea that is now well supported by empirical research [13,14]. However, while transcription factors are assumed to evolve more slowly than *cis*-regulatory regions, they can evolve quickly compared to other gene classes [15]. Changes to transcription factor proteins have also been implicated in the evolution of novel phenotypes (e.g., [16]).

Despite the role of transcriptional variation in phenotypic evolution, mRNA levels are often constrained on long timescales [17]. Genome-wide comparisons of mRNA levels between species show widespread reductions in divergence compared to neutral expectations [18–20], suggesting that changes in transcript levels are frequently deleterious. Despite the existing constraint on transcript levels, gene regulatory networks themselves are not necessarily well conserved between species [21]. Interestingly, data on mRNA abundance from yeast, worms, and flies suggest that expression evolution best fits a 'house of cards' model of stabilizing selection [22] in which mutations generally have large effects that exceed the standing genetic variation [23,24]. As a consequence, mutations that affect mRNA abundance can bring down the evolutionary house of cards and cause a cascade of changes between co-evolved *cis* and *trans* factors within a gene regulatory network.

Given these theoretical and empirical considerations, the epistatic interactions that underlie gene regulatory networks may lead to dysfunction in hybrids. In the simplest case, regulatory incompatibilities may arise either as a result of (i) the independent divergence of interacting elements between lineages (Figure 2A) or (ii) lineage specific co-evolution between elements (Figure 2B). In the first model, populations respond differently to drift or parallel or opposing directional selection. One population fixes a *cis*-regulatory change, the other fixes a *trans* change. In the second model, a *cis* change that affects expression is compensated for by changes to an interacting *trans*-acting factor, or vice versa. In either model, negative interactions between divergent regulatory elements in hybrids may result in the misregulation of downstream targets. More complicated models are possible, including *cis* and *trans* changes in both lineages or interactions between more than two loci.

Recent simulations and mathematical models indicate that these types of regulatory incompatibilities can evolve quickly if selection is acting [25–29]. In particular, regulatory incompatibilities will evolve most quickly as a byproduct of adaptation when *cis* and *trans* regulatory elements diverge under positive selection [25,28]. Incompatibilities will evolve more slowly under a model of stabilizing selection, where compensatory changes follow genetic drift [28]. Because transcription factors often regulate the expression of many genes, opposing selective pressures may constrain functional divergence and slow the evolution of regulatory incompatibilities. However, it was recently shown that it is possible for substantial hybrid misregulation to arise even when transcription factors are under moderate pleiotropic constraint [30].

Regulatory Divergence Between Species Is Widespread

Recent genomic surveys have found abundant evidence for transcriptional regulatory divergence between species. Divergence in putative *cis*-regulatory regions can be inferred through comparisons of transcription factor binding sites between species. While the loss and gain of transcription factor binding sites has generally been rapid over evolutionary time [31], examination of individual *cis*-regulatory elements has demonstrated that regulatory function can be maintained despite significant sequence divergence [32–34]. This observation may be explained by the fixation of functionally compensatory mutations.

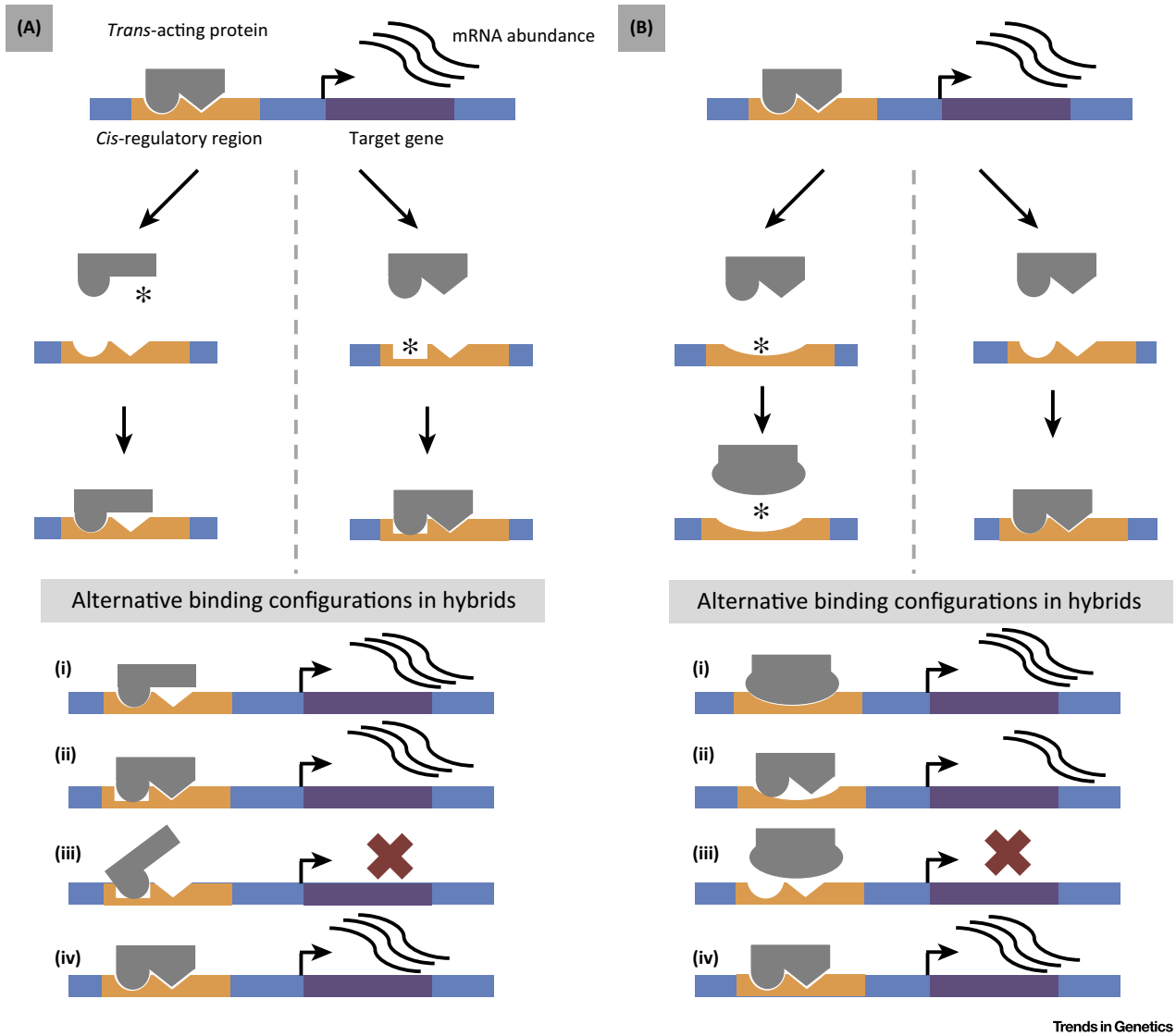

Figure 2. Regulatory Divergence as a Source of Hybrid Incompatibilities. Panels (A) and (B) are schematics of a two-locus model for hybrid incompatibilities. Each hybrid incompatibility arises as a consequence of the molecular interactions between a *cis*-regulatory region and a *trans*-acting factor. Changes in binding between interacting regulatory elements affect the expression of a downstream gene. Asterisks represent mutations that become fixed along a lineage. (A) A change to a *cis*-regulatory region in one species and the interacting *trans*-acting factor in the other result in hybrid dysfunction. Divergence in this example may be the result of drift or selection. In hybrids, the binding configuration represented by (iii) results in misregulation, while (i), (ii), and (iv) produce normal transcriptional output. (B) Lineage specific co-evolution between *cis*- and *trans*-regulatory elements result in hybrid dysfunction. In this example, a change in *cis* is followed by a compensatory change in *trans* to mask the deleterious effect of the first mutation. In hybrids, the binding configuration represented by (iii) results in misregulation. The binding configuration represented by (ii) results in reduced expression compared to the parents, while the binding configurations represented by (i) and (iv) result in the same expression as in the parents.

Regulatory divergence affecting the expression of individual genes can also be inferred through interspecific crosses. In F1 hybrids, differences in transcript abundance between two alleles indicates that differences between the parents at this locus are due to changes in *cis* because the two alleles in the F1 are in a common *trans*-acting environment [35] (Figure 3A). By contrast, if the two alleles in the F1 show the same level of transcript abundance, this indicates that differences between the parents are due to changes in *trans* [36] (Figure 3B), although interpretation can be complicated by dominance in regulatory pathways [37]. This approach has now been used to study genome-wide regulatory divergence between species of mice,

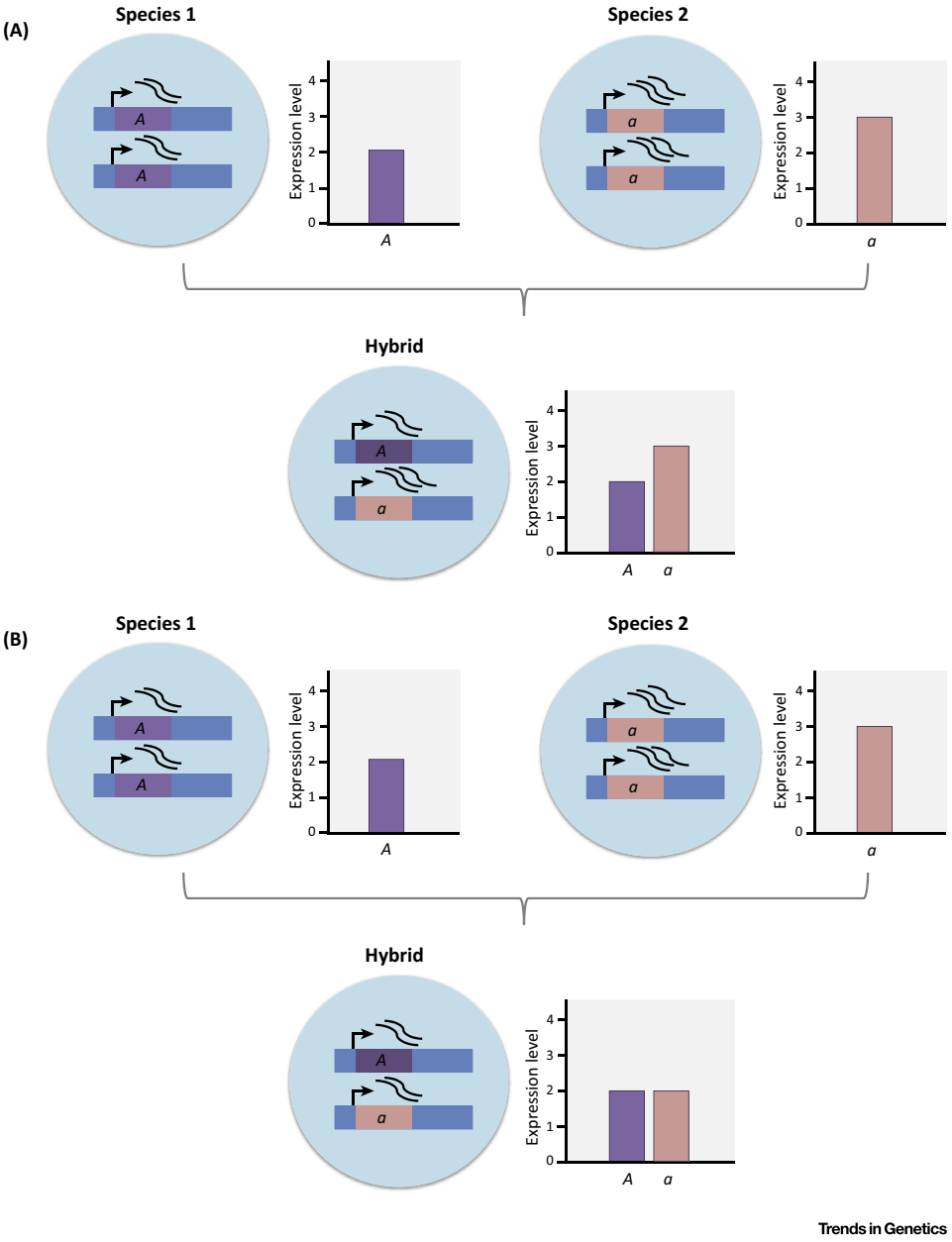

Figure 3. Using Allele-Specific Expression To Infer Regulatory Divergence between Species. Differences in the expression of alleles in an F1 can be used to determine whether expression divergence between the parents is due to changes in *cis* or to changes in *trans*. (A) Species 1 carries the *A* allele while species 2 carries the *a* allele. In the parental species, the transcript abundance of *A* is 2 and the transcript abundance of *a* is 3. Differences in the expression of the *A* and *a* alleles in the F1 hybrid suggest *cis*-regulatory divergence between species 1 and 2 because these two alleles are in the same *trans*-acting environment in the F1. (B) *A* and *a* have equal transcript abundances in the F1 hybrid despite the difference in expression seen between the parents. This suggests that differences between the parents are due to changes in *trans*.

birds, flies, yeast, and plants (e.g., [38–42]). Interspecific divergence in *cis* and *trans* is common, with *cis*-regulatory variants generally contributing more to divergence between species than variation within species [41,43,44]. However, a significant proportion of regulatory divergence can be attributed to a combination of *cis*- and *trans*-acting variants.

When *cis* and *trans* changes are found together, interactions between them can increase or decrease gene expression divergence between species. When *cis* and *trans* variants act in opposition, their effects may buffer one another in a compensatory fashion. Consistent with stabilizing selection, such *cis–trans* compensation appears to play a prominent role in regulatory evolution [38,41,42,45,46].

The proportion of genes with *cis–trans* divergence has also been shown to accumulate with phylogenetic distance. Transgenic assays called 'enhancer swaps', where orthologous regulatory regions are tested in the same *trans*-acting environment, have found that lineage-specific *cis–trans* evolution is more common in comparisons between distant than closely related taxa [47]. Similarly, pairwise comparisons between species of *Drosophila* found that, although the number of genes with *cis*-regulatory divergence increased linearly with divergence time, the number of genes with total expression divergence does not [44]. This suggests that *cis* changes are often compensated for by changes in *trans* variants, or by other *trans*-regulatory feedback mechanisms [48–50].

A few clear cases of such *cis–trans* compensatory evolution have now been reported [51,52]. In the nematodes *Caenorhabditis elegans* and *C. briggsae*, the expression of the gene *unc-47* is conserved between species even as its regulation has changed. Reciprocal swaps of *C. briggsae* and *C. elegans* regulatory elements identified lineage-specific changes consistent with compensatory *cis–trans* evolution. Regions in the *C. briggsae unc-47* promoter have co-evolved with specific changes in the *C. briggsae trans*-regulatory environment. Compensatory modifications in regulatory elements associated with *unc-47* represent an example of how gene expression can be maintained despite underlying regulatory divergence [52].

Misregulation as a Mechanism for Hybrid dysfunction

Misregulation of genes in hybrids can lead to misexpression, defined as gene expression that falls outside the range of the parental species. Novel interactions between divergent *cis* and *trans* variants are one way misexpression can arise in hybrids. Consistent with this prediction, a number of studies have associated misexpression with *cis–trans* compensatory evolution ([40,41,46,53,54], but see also [44,55]). Misexpression is commonly seen in sterile interspecific hybrids [56–61] and has been shown to accumulate with phylogenetic distance in *Drosophila* [44].

In some interspecific hybrids, abnormal expression is disproportionately observed in male-biased genes [56,57] and genes involved in spermatogenesis [62,63], suggesting that regulatory divergence might underlie some cases of hybrid male sterility. Comparisons between sterile and fertile hybrids of *Drosophila* species [64] and of house mouse subspecies [46,61] have found that a greater number of genes are misexpressed in sterile hybrids than in fertile hybrids. Moreover, in house mice, some expression quantitative trait loci (QTL) colocalize with sterility QTL in hybrids, suggesting a causal role for regulatory changes in hybrid male sterility [65]. Also in mice, misexpression in sterile hybrids is associated with compensatory *cis–trans* changes, consistent with a model where disrupted interactions between these types of loci contribute to hybrid sterility [46].

The X chromosome often plays a central role in post-zygotic isolation [10,66]. If regulatory divergence underlies hybrid dysfunction, evolutionarily diverged regulation of sex-linked genes may be expected [67]. Several recent studies have found that expression diverges faster for some genes on the X (in XY taxa) and Z (in ZW taxa) chromosomes than on the autosomes [68–73]. Faster divergence of sex-linked gene expression is especially strong for genes with sex-biased effects (male-biased effects in XY taxa and female-biased effects in ZW taxa) [69–71,74].

However, comparisons of expression patterns in whole tissues may obscure differences in individual cell types. For example, it was recently shown that expression evolution for X-linked genes depends on the developmental stage of spermatogenesis, with genes expressed late in spermatogenesis showing slower divergence on the X [75]. Disproportionate misexpression of X-linked genes has also been reported for sterile hybrids [61,65,74,76].

There are several caveats to bear in mind when considering whether misexpression is causing hybrid sterility or inviability. First, the widespread misexpression seen in many interspecific crosses can be the result of one or a few upstream changes that cause a cascading effect on genes downstream in a regulatory network [77]. This has been seen in hybrids between *Saccharomyces cerevisiae* and *S. paradoxu*, where misexpression is primarily due to a shift in the timing of meiosis [78]. Second, while misexpression in interspecific hybrids has been the subject of intense scrutiny, misexpression has also been observed in intraspecific hybrids where dysfunction is absent [44,79]. Third, changes in cellular composition can also conflate associations between hybrid dysfunction and misexpression. Sterile and inviable animals often have gonads of differing cellular composition or suffer from atrophied tissue relative to their fertile counterparts. Because many studies isolate mRNA from whole animals or whole tissues, differences in tissue or cellular composition between sterile or inviable hybrids and parental species can produce misexpression. As a result, hybrid misexpression that is a direct result of regulatory divergence is likely to be overestimated [80]. In the future, studies that make use of sorted cell populations may mitigate this problem somewhat by comparing gene expression only in equivalent cell types [75,81,82].

Evidence from Speciation Genes

Misexpression identified in sterile hybrids provides only indirect evidence of the role of misregulation in hybrid dysfunction. 'Speciation genes' – defined here as genes that contribute to reproductive isolation – provide the best direct evidence for the role of regulatory divergence in reproductive isolation. Unfortunately, relatively few speciation genes have been identified and molecularly characterized [83,84]. Despite this limitation, some broad-scale patterns have started to emerge. Of the speciation genes identified so far, many have either a putative role in transcriptional or translational regulation, or are themselves misexpressed in hybrids (Table 1). While this pattern is intriguing, it is necessary to characterize the molecular and physiological basis of hybrid dysfunction in each case to determine whether regulatory divergence is causal. We discuss a few speciation genes that have been particularly well characterized in *Drosophila* and house mice, highlighting some of the challenges in linking specific mutations to misregulation.

Hybrid male rescue (*Hmr*) and *Lethal hybrid rescue* (*Lhr*)

Hybrid male lethality in crosses between *D. melanogaster* and *D. simulans* can be explained in part by the genes *Hmr* and *Lhr*. The protein products of *Hmr* and *Lhr* form a complex that localizes to heterochromatic regions of the genome [85,86] where they transcriptionally repress transposable elements and repetitive sequences [86,87] and play a crucial role in mitotic chromosome segregation [86].

Loss-of-function mutations at *Lhr* in *D. simulans* or at *Hmr* in D*. melanogaster* restore hybrid male viability [85,88–90]. The *D. simulans* and *D. melanogaster* orthologs of both genes have diverged extensively under positive selection [85]. These observations led to the prediction that adaptive functional divergence between *Hmr*, *Lhr*, and species-specific heterochromatin sequences causes hybrid dysfunction. However, orthologs of *Lhr* appear to be functionally equivalent: sequence divergence between *Lhr* orthologs does not affect the localization of the *Lhr* protein, and overexpression of either the *D. simulans* or *D. melanogaster* ortholog has hybrid lethal effects [91].

Table 1. Hybrid Incompatibility Genes

Locus	Gene name	Species	Phenotype	Molecular function	Evidence of gene regulation[a]	Ref.
AEP2	*ATPase expression 2*	*Saccharomyces bayanus* × *S. cerevisiae*	Sterility	Mitochondrial protein	Regulates translation of *OLI1* transcripts	[118]
OLI1	*Oligomycin resistance 1*	*S. bayanus* × *S. cerevisiae*	Sterility	F0-ATP synthase subunit	Impaired translation in hybrids	[118]
Ods	*Odysseus*	*Drosophila mauritiana* × *D. simulans*	Sterility	Regulation of heterochromatic sequences	Encodes a DNA-binding protein that localizes to heterochromatic regions and regulates their decondensation	[126,127]
agt	*O-6-alkylguanine-DNA alkyltransferase*	*D. mauritiana* × *D. simulans*	Sterility	DNA binding protein	Encodes a DNA-binding protein for an alkyl-cysteine-*S*-alkyltransferase	[128]
Taf1	*TBP-associated factor 1*	*D. mauritiana* × *D. simulans*	Sterility	Transcription factor component	Encodes a DNA-binding protein for a subunit of transcription factor TFIID	[128]
Hmr	*Hybrid male rescue*	*D. melanogaster* × *D. simulans*	Inviability	Regulation of heterochromatic sequences	Overexpression of HMR/LHR complex in hybrids	[90]
Lhr	*Lethal hybrid rescue*	*D. melanogaster* × *D. simulans*	Inviability	Regulation of heterochromatic sequences	Overexpression of HMR/LHR complex in hybrids	[85]
gfzf	*Suppressor of Killer-of-prune [Su(Kpn)]*	*D. melanogaster* × *D. simulans*	Lethality	Cell-cycle regulation	Transcriptional regulator of the RAS/MAPK pathway	[129]
Nup160	*Nucleoporin 160*	*D. simulans* × *D. melanogaster*	Inviability	Nuclear pore protein	None	[111]
Nup96	*Nucleoporin 96*	*D. simulans* × *D. melanogaster*	Inviability	Nuclear pore protein	None	[110]
Ovd	*Overdrive*	*D. pseudoobscura bogatana* × *D. p. pseudoobscura*	Sterility	DNA binding	Encodes a MADF DNA-binding domain	[130]
Hhl	*Heterochromatin hybrid lethal*	*D. melanogaster* × *D. simulans, D. mauritiana, D. sechellia*	Lethality	Unknown	Unclear	[131]
Zhr	*Zygotic hybrid rescue*	*D. melanogaster* × *D. simulans*	Inviability	Unknown, repetitive DNA	Unclear	[132]
Prdm9	*PR/SET domain-containing 9*	*Mus musculus musculus* × *M. m. domesticus*	Sterility	Mediates meiotic homologous recombination	Encodes DNA-binding domains associated with transcriptional regulation	[99]
DM1/DM2	*DANGEROUS MIX 1/2*	*Arabidopsis thaliana*	Lethality	Disease resistance	None	[133]

[a]Putative evidence for regulatory function or of misexpression in hybrids for hybrid incompatibility genes.

Hybrid lethality is instead a consequence of species-specific changes in the abundance of *Hmr* and *Lhr* protein products. HMR expression is higher in *D. melanogaster*, and LHR expression is higher in *D. simulans*. Increased expression of HMR in *D. melanogaster* and LHR in *D. simulans* results in an elevated amount of the HMR–LHR complex in hybrids. The activity of the HMR–LHR complex is dosage-dependent, and overexpression leads to mislocation of the complex [86].

Because hybrid lethality is a consequence of HMR–LHR overexpression, the observed asymmetric lethal effects of *D. melanogaster Hmr* and *D. simulans Lhr* are likely the result of divergence in regulatory pathways between *D. melanogaster* and *D. simulans* rather than of functional divergence between orthologs [92]. Supporting this hypothesis, transcriptional differences between *Lhr* orthologs in hybrids has been linked to compensatory *cis*-by-*trans* divergence between species in allele-specific expression [92,93].

PR/SET domain 9 (*Prdm9*)

Crosses between *Mus musculus domesticus* and *M. m. musculus* produce sterile hybrid males [94]. A series of laboratory mapping experiments by Forejt and colleagues [95–98] led to the positional cloning and identification of *Prdm9* [99], the only known hybrid sterility gene in vertebrates. *Prdm9* is believed to interact with so far uncharacterized loci on the X chromosome and autosomes to cause spermatogenic failure in hybrids [100,101]. Sterile hybrid males show sex-specific failure to pair chromosomes during meiosis as well as misexpression of genes on the X and Y chromosomes [76]. While *Prdm9* contains conserved domains associated with transcriptional regulation [102,103], the effect of *Prdm9* on misexpression may be a secondary consequence of the role of *Prdm9* in meiotic recombination.

Prdm9 has been implicated in recombination rate variation in both humans and mice [104–106]. During meiosis in mammals, double-stranded breaks are created throughout the genome and then repaired, leading to homologous recombination. These breaks are concentrated in regions called recombination hotspots. In mice, PRDM9 appears to mediate the process of recombination at hotspots by binding to DNA sequences [104]. Intriguingly, another QTL implicated in recombination rate variation was recently found to overlap with a hybrid male sterility QTL on the X chromosome [107]. Altogether, these results suggest a genetic connection between recombination and hybrid sterility [108].

Variation in the number of PRDM9 zinc-finger tandem repeats has been implicated in house mouse sterility [99]. The PRDM9 zinc-finger array co-evolves with species-specific binding sites. Meiotic drive against recombination hotspots is thought to result in the rapid turnover of these binding sites. Species-specific erosion of PRDM9 binding sites may explain asymmetric binding of PRDM9 in F1 hybrids that is associated with hybrid sterility. Supporting this prediction, hybrid fertility can be rescued by replacing the sterility-associated zinc-finger array with an orthologous region from humans [109]. While it is clear that sterile hybrid males show misexpression of genes on the X and Y chromosomes, the direct role, if any, of *Prdm9* in this misexpression remains unclear.

Open Questions and Future Directions

While the evidence so far suggests that changes in gene regulation may contribute to the origin of new species, there are also cases where hybrid incompatibility appears to be independent of regulatory changes. For example, the speciation genes *Nup160* and *Nup96* cause hybrid inviability in crosses between *Drosophila simulans* and *D. melanogaster*. The protein products of both genes form architectural components of the nuclear pore complex and show evidence of adaptive protein evolution [110,111]. We do not wish to provoke a debate on the relative importance of coding versus regulatory mutations to speciation; both surely occur and both are likely to be important in some instances. Instead, we offer several research directions that are

Outstanding Questions

Does regulatory divergence contribute to other reproductive barriers such as mating isolation, gametic isolation, or ecological isolation?

Do disrupted interactions between post-transcriptional regulatory elements contribute to hybrid dysfunction?

Does dysregulation typically arise as a consequence of strictly adaptive evolution or as a consequence of compensatory evolution?

likely to be particularly useful in understanding the connection between regulatory divergence and speciation (see Outstanding Questions).

First, the study of speciation has benefited from studies of natural populations and from studies that utilize laboratory crosses. However, most of what is known about the role of regulatory divergence in speciation comes from laboratory studies. These studies represent a small sliver of phylogenetic diversity and they rely mainly on model systems (Table 1). If we are interested in understanding generalities of the speciation process, greater taxonomic sampling is necessary. It would also be useful to compare patterns of gene expression in naturally-occurring hybrid individuals that contain mixed genetic backgrounds to those seen in laboratory crosses.

Second, there are two aspects of many natural populations that merit further study: the presence of later-generation hybrids and the fact that alleles contributing to reproductive isolation may be polymorphic rather than fixed [112]. Studying both of these issues in the context of the role of regulatory divergence and reproductive isolation is important. For example, while great progress has been made studying F1 hybrids, using F2 or later-generation hybrids makes it possible to identify disrupted gene expression caused by recessive alleles [65].

Third, most of the focus has been on the role of regulatory divergence in intrinsic post-zygotic isolation. The role of regulatory divergence in other forms of reproductive isolation (i.e., ecological, mating, and gametic) is still largely unexplored. Regulatory divergence may commonly lead to phenotypic differences between populations that result in different types of reproductive barriers. In particular, to the extent that changes in gene regulation underlie adaptive evolution, such changes may be fairly common in ecological speciation, but this remains to be shown.

Fourth, there is a need to better integrate speciation theory with empirical evidence from gene expression studies. For example, the exposure of recessive mutations on the X (or Z) chromosome in heterogametic hybrids (i.e., XY males or ZW females) has been invoked to explain observations such as Haldane's rule and the large X effect [10,113,114]. According to this hypothesis, many of the alleles that decrease hybrid fitness are at least partially recessive. It is possible to test the dominance of expression inheritance using crosses or chromosome substitution lines [79,82,115], and this would help to link theoretical predictions with empirical observations of gene expression. Similarly, BDM incompatibilities are predicted to accumulate at a non-linear rate over evolutionary time, resulting in a 'snowball' effect [116]. Controlled gene expression studies may be able to determine whether regulatory incompatibilities conform to this prediction and increase nonlinearly with phylogenetic distance.

Fifth, the evolutionary forces that drive regulatory divergence and contribute to hybrid incompatibilities remain largely unknown. Many of the known speciation genes show a signature of positive selection [83]. While this observation is consistent with a model of adaptive divergence driving the evolution of hybrid incompatibilities, a model of compensatory evolution is equally possible. Compensatory evolution requires positive selection to fix compensatory changes to mask the deleterious effects of an earlier mutation.

Finally, while there is significant interest in the role of regulatory divergence in speciation, transcriptional control has received nearly all the attention. The regulation of gene expression is a complex process that may be modulated at many stages, including transcription, translation, and post-translation [117]. The yeast speciation genes *AEP2* and *OLI1* provide one example of how translational misregulation can result in hybrid sterility. *AEP2* encodes a mitochondrial protein that translationally regulates *OLI1*. In interspecific hybrids of *S. cerevisiae* and *S. bayanus*, the Aep2 protein is unable to bind to *OLI1* transcripts. The inability of Aep2 to mediate the translation of *OLI1* is thought to result in hybrid sterility [118]. Advances have made

the study of post-transcriptional regulation more feasible [119]. Allele-specific analyses of translational efficiency can now be used to infer *cis* and *trans* divergence acting on translation rate [120–122]. QTL mapping techniques have been employed to study intraspecific variation in translation and protein abundance [117,123–125]. Studies that combine each of these levels will provide a more complete picture of the role of regulatory divergence in speciation.

Acknowledgments

We thank Daniel Barbash, Jiri Forejt, Norman Johnson, Bret Payseur, Adam Porter, Patricia Wittkopp, and one anonymous reviewer for their thoughtful comments on this manuscript. This work was supported by National Institutes of Health (grant R01 GM074245 to M.W.N.).

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