frontiers in Ecology and Evolution

PERSPECTIVE
published: 14 August 2020
doi: 10.3389/fevo.2020.00261

Mechanisms of Change: A Population-Based Perspective on the Roles of Modularity and Pleiotropy in Diversification

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Developmental modularity has long been viewed as a hierarchical organization that facilitates evolution through modification or reuse of preexisting modules. More recently, developmental modularity has been proposed as a mechanism capable of driving rapid evolution of novel color pattern phenotypes between closely related taxa. In this scenario, recombination between modular *cis*-regulatory elements (CREs) generates novel phenotypes by shuffling genetic variation at preexisting color pattern modules into new arrangements. Recent functional evidence from *Drosophila* flies and *Heliconius* butterflies, however, provides a series of examples in which CREs function in multiple developmental contexts and are thus highly pleiotropic. The potential prevalence of pleiotropy in CRE function could be a barrier to the proposed importance of CRE modules as a mechanism for rapid evolutionary change. Here we review the concept of developmental modularity, some examples that suggest developmental modularity underlies pattern evolution, and recent evidence that indicates modular CREs may be less common than previously expected. This leads us to suggest that alternative, non-modular hypotheses should be considered alongside proposals of modular CREs. We then propose the concept of evolutionary modularity as a specific alternative to developmental modularity when discrete, seemingly modular, phenotypes occur in hybridizing taxa. We suggest that evolutionary modularity provides a potentially important pathway for exchange of phenotypic elements between hybridizing taxa independent of the underlying developmental architecture.

Keywords: modularity, pleiotropy, *cis*-regulatory element, *Heliconius*, color pattern, hybridization, evolutionary modularity

OPEN ACCESS

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Specialty section:
This article was submitted to Evolutionary Developmental Biology, a section of the journal Frontiers in Ecology and Evolution

Received: *23 December 2019*
Accepted: *24 July 2020*
Published: *14 August 2020*

Citation:
Lewis JJ and Van Belleghem SM (2020) Mechanisms of Change: A Population-Based Perspective on the Roles of Modularity and Pleiotropy in Diversification. Front. Ecol. Evol. 8:261. doi: 10.3389/fevo.2020.00261

INTRODUCTION

Diversification of animal coloration has been often used as a model for the genetics and ecology of adaptive evolution. In numerous taxa, adaptive color pattern diversity has repeatedly mapped to relatively few genomic loci. Bird plumage coloration (Toews et al., 2016; Campagna et al., 2017), cryptic hair pigmentation in mice (Steiner et al., 2007; Manceau et al., 2011), and aposematic color patterns in *Heliconius* butterflies (Reed et al., 2011; Martin et al., 2012; Nadeau et al., 2016; Westerman et al., 2018) are just a few key examples where the loci that differentiate

color morphs include only a handful of developmental genes. Consistent with the well-known trend that gene regulatory mechanisms evolve faster than coding sequence change, many of the loci driving differentiation of adaptive coloration show the strongest signal of divergence between morphs at non-coding loci presumed to capture *cis*-regulatory variants. "Combinatorial" adaptation – the restructuring of existing genetic variation in cases of rapid diversification – has become a common observation in studies of diversification and adaptive radiation (Marques et al., 2019). That is, genomic comparisons often show signals suggesting that combinations of extant alternative alleles at closely linked loci underlie the diversity seen in these color patterns. In some cases of combinatorial evolution, the architecture of *cis*-regulatory adaptation has been coined as modular and modularity of *cis*-regulatory elements (CREs) has been suggested as a potent genetic architecture to explain the rapidly evolving diversity found in these systems (Wallbank et al., 2016; Campagna et al., 2017; Van Belleghem et al., 2017).

Recent studies on the genetic basis of *Drosophila* morphology and *Heliconius* butterfly wing color pattern evolution provide the first lines of evidence that putative *cis*-regulatory modules may, instead, be pleiotropic and non-modular. Here we review the case for modular elements inferred from genomic comparisons, then consider how recent counterexamples question the ubiquity of CRE modularity. We propose that, while exchange of modular elements may indeed underlie the transfer of adaptive phenotypes, recombination of developmental modules should not be the only hypothesis considered. Instead, a genetic model combining hybrid zone homogenization of *trans* acting factors with evolutionary modules can reconcile the apparent modular exchange of alternate alleles with developmental pleiotropy and non-modular genetics.

THE CASE FOR EXCHANGE OF MODULAR ELEMENTS AS A MECHANISM OF DIVERSIFICATION

Modularity has long been a mainstay of developmental biology and evo-devo. Examples of modular genetic mechanisms that underlie trait development, such as melanin patterning across *Drosophila* species (Rebeiz et al., 2009) and *Hox* gene expression domains (Kuratani, 2009), have demonstrated the importance of modular architectures in determining organismal form. More recently, comparative and evolutionary genomics have begun to propose modularity as a mechanism capable of facilitating phenotypic diversification (e.g., Hughes and Leips, 2017; Van Belleghem et al., 2017; Mason et al., 2020). Modularity as a combinatorial mechanism of diversification has been associated with exchange of modular CREs, such as enhancer, promoter, insulator, and silencer elements. In this model of diversification, transfer of autonomous CREs via hybridization and recombination of specific genomic loci from one population to another allows for swapping of discrete phenotypic elements to generate new phenotypes from ancestral genetic components (Wallbank et al., 2016; Van Belleghem et al., 2017).

The concept of a "module" or "modular element" is critical to grasping the evidence for modularity as a mechanism of evolution. Despite our focus here on modular CREs, modularity is not specific to non-coding regulatory elements, and thus we aim for a more general definition that fits both non-coding and coding loci alike. While the abstract concept "modularity" is a difficult term to define, autonomy of function (i.e., module components mostly function independently; von Dassow et al., 2000; Schlosser, 2004; Wagner et al., 2007; Monteiro and Podlaha, 2009; Espinosa-Soto and Wagner, 2010; Lacquaniti et al., 2013; Melo et al., 2016) and sufficiency (i.e., modules include all novel elements necessary to induce a phenotype; Monteiro and Podlaha, 2009; Arnoult et al., 2013; Henry et al., 2015; Koshikawa, 2015; Merrill et al., 2019) are common requirements of developmental modules and we continue this practice here. For this perspective, *we adopt the definition of a developmental module as: A genomic locus or set of loci sufficient to semi-autonomously induce a phenotype when activated in any common genetic background within a species.* Developmental modularity then refers to a developmental system consisting of one or more distinct modules [e.g., induced eyeless expression is sufficient to produce ectopic eyes in non-retinal tissues (Weasner et al., 2009)]. This can be contrasted against non-modular architectures, such as seen in many polygenic traits (e.g., Gudbjartsson et al., 2008) and additive *cis*-regulatory control of non-specific gene expression (e.g., Fulco et al., 2016).

When considered as a mechanism for producing novel adaptive phenotypes, modular loci seem most likely to underlie variation in phenotypes with discrete pattern elements, such as bird plumage patches, fish scale pigmentation, or butterfly color pattern elements. Specific examples indicative of recombination of modular elements include capuchino seedeaters in Argentina (Campagna et al., 2017) and North American warblers (Toews et al., 2016; **Figure 1A**). In both species groups, discrete plumage color pattern elements appear to be exchanged via hybridization and the strongest signal of genomic differentiation almost exclusively maps to intergenic, putatively *cis*-regulatory, loci. A modular mechanism of pattern diversification has been proposed in cichlids (Maan and Sefc, 2013), and stripe variation among clownfishes (Litsios and Salamin, 2014; Salis et al., 2018) appears modular as well (**Figure 1A**). Consistent with modular stripe evolution in fish, CRE differences at the gene *csf*1 can explain the transitions between striped and non-striped zebrafish species (Patterson et al., 2014). Direct evidence for the mechanism of phenotypic exchange in fish and birds is sparse, however, and studies have limited speculation on the role of modular loci in generating novel color pattern phenotypes. Nonetheless, closely related, hybridizing taxa that appear to exchange color pattern elements to produce novel phenotypes provide an ideal scenario for evolution via modular genomic loci.

Perhaps the best argument for adaptive evolution via transfer of modular CREs comes from *Heliconius* hybrid zones in two co-mimetic species. In both *Heliconius erato* and *Heliconius melpomene*, regional butterfly populations converge on the same mimicry-related phenotypes to form local morphs with discrete aposematic color pattern elements. In the *H. melpomene* clade,

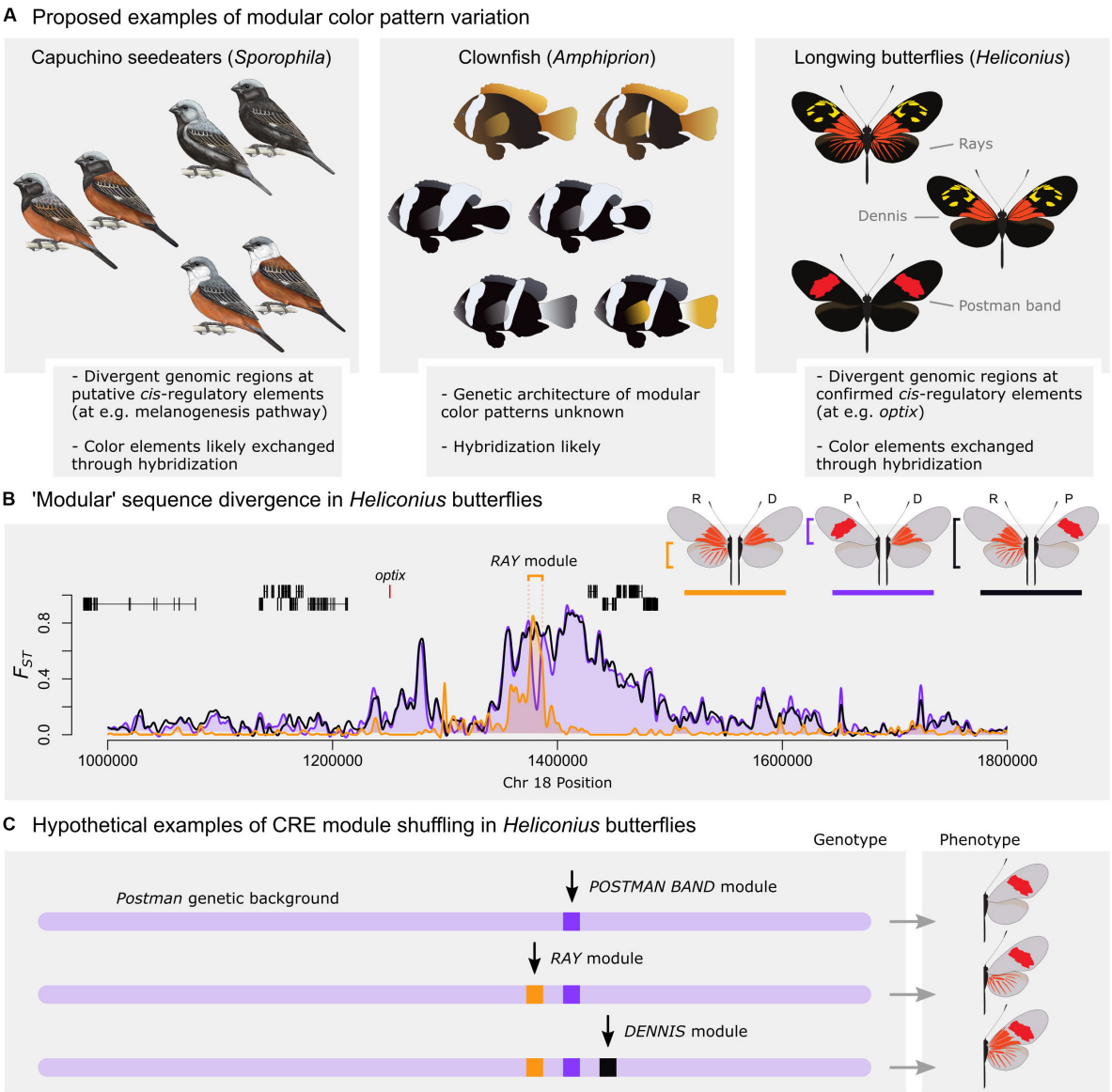

FIGURE 1 | Examples of putative modular color pattern diversity. **(A)** Examples of potential exchange of modular phenotypes via recombination include plumage patterns in capuchino seedeaters (left, reprinted from Campagna et al., 2017), white stripes in clownfish (middle, modified from Salis et al., 2018), and red wing color patterns in longwing butterflies (right). **(B)** Genomic differentiation (F_{st}) between red wing pattern morphs in *Heliconius erato* at the *optix* locus (data from hybrid zones in French Guiana; Van Belleghem et al., 2017). Orange: Differentiation between radiate (R) and dennis (D) morphs; Purple: Differentiation between postman (P) and dennis (D) morphs; and Black: Differentiation between radiate (R) and postman (P) morphs. A single locus, called Ray module, shows a putative *cis*-regulatory module controlling differentiation between the radiate and dennis morphs. **(C)** Model of CRE module shuffling assuming these divergent loci function as developmental modules. In this case, different combinations of CRE modules would generate modular alternative phenotypes. Note however that the proposed phenotypes with both a red forewing band and dennis/rays on the right have not been found in a homozygous state in nature and suggest that recombination has not occurred (F1 hybrids can have these phenotypes due to dominance effects of *optix*).

some subspecies have evolved to contain partial phenotypes completely present in neighboring subspecies. This includes, for example, the sole presence of rayed hindwing or red forewing triangle patterns in the absence of the other element (Wallbank et al., 2016). This scenario repeats in *H. erato*, where butterfly morphs again form narrow hybrid zones in which discrete red wing pattern phenotypes appear to be exchanged between morphs (Van Belleghem et al., 2017; **Figure 1A**). Importantly, in both species, the exchange of red pattern elements: (A) maps back to a *cis*-regulatory region distal to the "switch" gene *optix*, and (B) associates with recombination of specific genomic haplotypes at these loci that transfer via recombination between neighboring populations with shared wing color pattern elements (**Figure 1B**). In cases similar to *Heliconius* wing patterns, where hybridization and recombination of extant alleles can produce phenotype diversity, exchange of modular elements is an

attractive hypothesis that posits a simple mechanistic process by which new adaptive phenotypes can be rapidly gained or lost.

CRE PLEIOTROPY AND NON-MODULAR GENE REGULATION

While indirect evidence for recombination of modular CREs between geographically close and genetically related taxa is abundant, recent studies in *Drosophila*, cell lines, and *Heliconius* butterflies suggest pervasive CRE pleiotropy and interdependence. These results present several counterexamples to key principles that support phenotype modularity derived from modular CREs. We consider evidence against the following two principles: (A) CREs are independent and highly tissue-specific and that (B) recombination of existing CREs is more important for rapid evolutionary change compared to *de novo* mutations. We discuss these findings and their consequences for CRE and phenotypic modularity in more detail below.

The first principle favoring exchange of modular CREs can be stated as: genes are frequently found to be highly pleiotropic, where a single gene often affects multiple characteristics, while *cis*-regulatory loci are often assumed to be highly tissue-specific (Prud'homme et al., 2007; Carroll, 2008). This principle provides a foundation for the exchange of phenotype-specific CREs without any corresponding alteration of fitness from undesirable pleiotropic effects. In support of this principle, many early studies of trait evolution found that evolution of one or two enhancer elements underlies variation in phenotypes. Yet recent evidence suggests that *cis*-regulatory loci may often be substantially more pleiotropic than initially expected. The overall prevalence of enhancer pleiotropy has been well covered by Sabarís et al. (2019) and the potential interpretations of "pleiotropy" in Paaby and Rockman (2013). For this perspective, we adopt the standard genetic definition of pleiotropy, where a locus is pleiotropic if it affects multiple characteristics. Some aspects of CRE biology that suggest pleiotropic elements may frequently play an important role in generating novel traits are worth considering further.

For example, pioneering work by the ENCODE project found that CRE availability, an accessible chromatin state important for CRE activity, tends to be maintained through cell lineages (Stergachis et al., 2013). Thus, enhancers activated in earlier cell types are often available for use through much of the remainder of development. It is likely, then, that these elements could be reutilized or co-opted by evolutionary processes to drive new expression patterns instead of generating a suite of novel CREs *de novo* (Monteiro and Podlaha, 2009). Important for evolution, CREs active in multiple tissues or during extended periods of development show increased conservation between taxa and provide an evolutionarily stable set of pre-wired regulatory loci (Lewis et al., 2016; Fish et al., 2017). The availability of accessible CREs in many tissues – a likely precursor for the evolution of enhancer pleiotropy – thus appears frequent enough to be found in meta-analyses of CREs. But does this context result in actual enhancer pleiotropy? The recent discovery of pleiotropic enhancers associated with development of leg bristles and trichome patterning explicitly demonstrates that pleiotropic CREs targeting developmental genes can and do underlie more than one important traits in *Drosophila* (Nagy et al., 2018; Preger-Ben Noon et al., 2018).

The second principle guiding the prediction of modular CRE transfer is that recombination of extant CRE modules is a logical mechanism for the rapid introduction of new alleles into a population while mitigating deleterious effects likely to occur with coding sequence variation (Prud'homme et al., 2007; Wallbank et al., 2016). While this is undoubtedly true, past studies suggest that transfer of modular elements should not necessarily be the default assumption. In many cases, such as loss of stickleback spines (Chan et al., 2010) and horizontal stripes in cichlids (Kratochwil et al., 2018), adaptive trait evolution is driven by loss of function mutations when an organism is exposed to a novel environment. Adaptive loss of phenotype requires no assumptions regarding the modularity of a trait, as a simple deletion can be sufficient to break the regulatory architecture that underlays trait development in both modular and non-modular scenarios (Prud'homme et al., 2007). In the gain of function case, numerous studies have highlighted the relatively rapid rate of *cis*-regulatory evolution (e.g., Villar et al., 2015; Lewis et al., 2016). Multiple studies have shown that mutations within enhancer elements drive variation in complex phenotypes (e.g., Gompel et al., 2005; Nagy et al., 2018). Similarly, convergent evolution can occur from independent mutations at the same loci, such as the repeated evolution of warning coloration in bumblebees (Tian et al., 2019). Thus, recombination of existing CREs is not required for rapid evolutionary change. Conditional on the number of CREs, distance between loci, and strength of selection against partial recombinants, evolution via directional selection on novel variants or some alternate process may potentially be faster than precise exchange of multiple CRE modules.

Evidence of pleiotropic enhancers and non-modular evolution of novel phenotypes does not reject the transfer of modular CREs. Instead, these counterexamples and arguments suggest we need additional studies to explicitly test for adaptation via exchange of putatively modular elements. Fortunately, recent work on the evolution of mimicry phenotypes in *Heliconius* provides the perfect case study for how evolution of non-modular genetic architectures may drive variation in apparently modular traits.

HELICONIUS WING PATTERNS AS A CASE STUDY IN NON-MODULAR PHENOTYPE EVOLUTION

Heliconius wing patterns have been proposed as a key example of how modular *cis*-regulatory alleles can generate novel color pattern adaptations (Mallet and Clarke, 1989; Wallbank et al., 2016; Van Belleghem et al., 2017). In *Heliconius erato*, red wing pattern phenotypes appear to be shuffled between hybridizing populations to produce morphs with various combinations of these pattern components. The entire list of hybrid zones described in Van Belleghem et al. (2017) is extensive, so we focus here on a particularly clear example of apparent modularity.

In French Guiana and Suriname, three red pattern morphs – named radiate, dennis, and postman (**Figure 1A**) – form a complex hybrid zone. Dennis, which has the forewing pattern of the radiate morph and the hindwing pattern of the postman, appears by eye to be a simple shuffling of these two other phenotypes via recombination of forewing and hindwing pattern-associated alleles. Consistent with this, DNA sequence analysis of these morphs has shown that a single locus downstream of the red switch gene *optix* is the only site that differentiates red wing phenotypes between the radiate and dennis morphs (Van Belleghem et al., 2017; **Figure 1B**). When adding the postman phenotype to this comparison, we see that the locus differentiating dennis from radiate morphs contains the postman allele, rather than the radiate allele. This suggests a simple evolutionary mechanism where a presumptive postman hindwing allele (lacking the Rays module) has been recombined into the radiate haplotype, resulting in the loss of the hindwing rays to produce the dennis phenotype. Thus, the evolution of the dennis morph appears to be a clear example of modular CRE transfer to create a novel phenotype. CRE modules have been similarly suggested for the other red color pattern elements, including a module for the red Dennis and Band color pattern elements (**Figure 1C**).

Recent findings, however, suggest that the origin and subsequent evolution of the radiate morph is much more complicated than expected from a few simple modular CREs (Lewis et al., 2019). These results show that at least five distinct *cis*-regulatory loci drive adaptive evolution of the radiate mimicry phenotype. Consistent with the maintenance of CRE availability through cell-lineages, wing CREs rarely differ between forewings and hindwings (Lewis and Reed, 2018; van der Burg et al., 2019). Thus, the same CRE landscape is shared near *optix* by both forewing and hindwing color patterns (Lewis et al., 2019). Inconsistent with modularity, pattern associated CREs are all interdependent and pleiotropic – CRE mutants alter both the rays and the dennis components of the radiate morph (**Figure 2A**). While hybridization seems to have spread the radiate phenotype between multiple geographic populations, recombination of a single CRE is insufficient to produce a partial or complete radiate phenotype in a postman-like morph. Perhaps most surprisingly, deletion of one CRE showed the effects of both enhancer (phenotype suppression in the mutant) and silencer (phenotype activation in the mutant) activity in different wing sections. This underscores the long-standing view that epistatic effects, and thus the genetic background, can play an important part in how traits evolve (Phillips, 2008). The dual-modality of individual CREs in *Heliconius* is consistent with evidence from *Drosophila* that activating or repressing behavior from CREs can be context dependent (Gisselbrecht et al., 2019). Similarly, epistatic control of wing-pattern CREs is supported by recent observations in various *Heliconius* species that a single gene can modulate phenotype expression associated with other unlinked color pattern loci (Concha et al., 2019).

Taken together, pleiotropic CRE activity and indication of epistasis between color pattern loci runs counter to the developmental modularity of red color pattern CREs in *Heliconius erato*. These experiments can, specifically, reject the hypothesis that a single locus is sufficient to induce wing pattern components in the absence of additional CRE alleles and the necessary genetic background. This work does, however, raise the question: Why do specific loci appear to control wing phenotypes in a modular fashion in genome sequence comparisons?

EVOLUTIONARY MODULARITY: A HYBRID ZONE MODEL

It is important to reconcile the apparent conflict between the genomic sequence comparisons and experimental data in our case study. It is our view that comparative genomic analyses indicating modular transfer of phenotype components capture an important aspect of adaptive evolution and phenotype stability in the face of gene flow. While this approach does not demonstrate developmental modularity, it can provide strong evidence for a similar concept – evolutionary modularity. *By evolutionary modules, we mean: Any locus sufficient to modulate the gain or loss of phenotype components in the local genetic context of two or more hybridizing populations.* Evolutionary modularity differs from developmental modularity by making no requirements for the autonomy or sufficiency of a module, and may be specific only to a single geographic region or pair of hybridizing taxa. That is, an evolutionary module may require a specific genetic background found only within specific geographic regions. This concept does not make any assumptions about the true developmental genetic architecture of a trait, but instead suggests that many architectures can be utilized in a modular fashion by evolutionary processes.

To parse out how evolutionary modularity would work, we return to our example of the East Amazon *Heliconius* hybrid zone. In the admixed genetic background of the hybrid zone, many combinations of pleiotropic and epistatic loci are likely to occur due to hybridization of "pure" parental phenotypes and recombination in hybrid and backcrossed offspring. When most genetic elements for a trait are homogenous among all three morphs, a single, variable locus may be sufficient to create a novel phenotype and modulate between wing pattern morphs. The apparent gain of a modular phenotype in the dennis morph can be explained as the product of this scenario: In the admixed genetic background of the hybrid zone, *trans*-acting factors are shared by both morphs and a single, *cis*-regulatory domain provides a module-like switch for swapping between phenotypes (**Figures 2B,C**). This single locus may be sufficient to maintain differentiation between the derived dennis morph and the radiate population, while more complicated differentiation patterns would separate radiate from postman. Thus, a single locus, insufficient for producing a phenotype in the absence of a specific genetic context, may act modular in localized population structures. This is different from the scenario of modular CRE shuffling presented in **Figure 1C**, as evolutionary modules require the interdependent regulatory genetic architecture to be present and may require additional *trans* factors.

The concept of evolutionary modularity points to an important feature of adaptive evolution: Evolutionary novelty arises at a specific time and place. The process of refining or separating phenotype components in derived taxa can be distinct

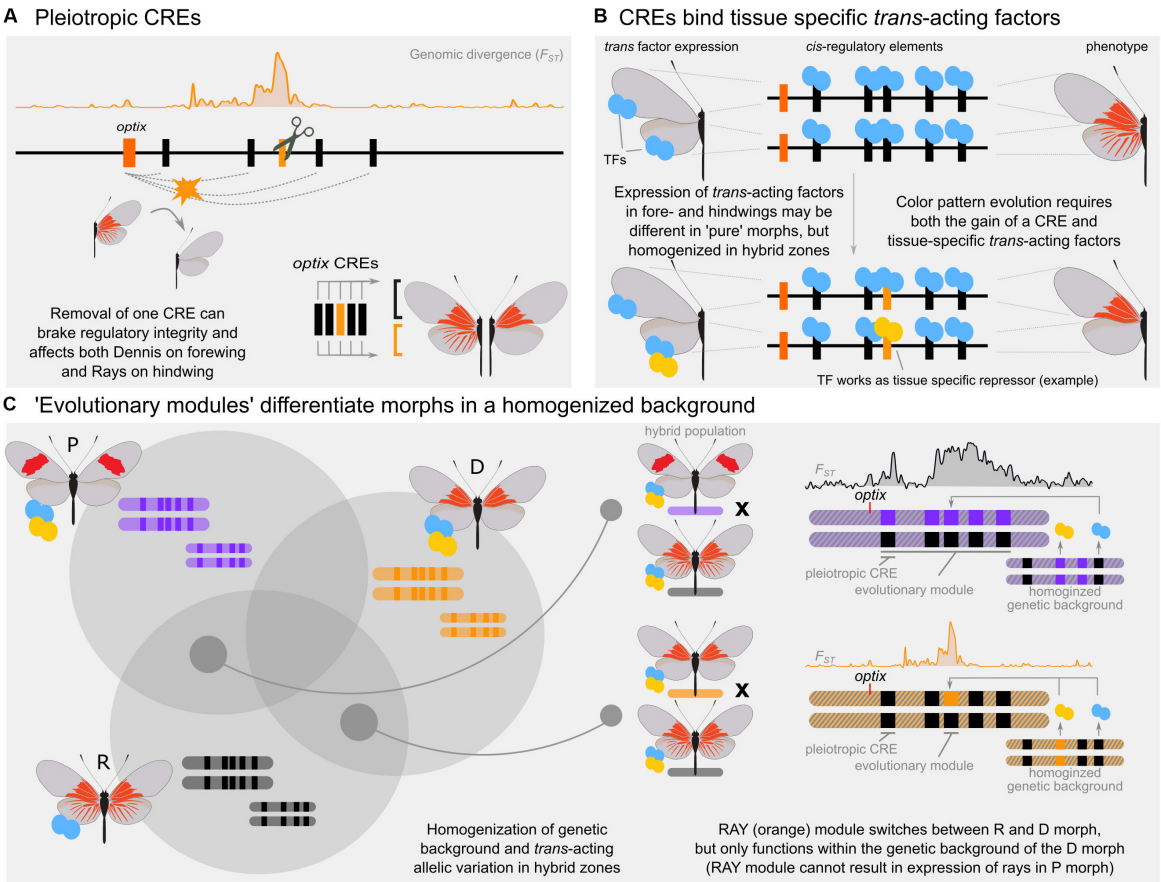

FIGURE 2 | Conceptual overview of evolutionary modularity. **(A)** CRISPR/Cas9 experiments of five putative CREs demonstrate that red wing patterns in *Heliconius erato* are controlled by an interdependent set of enhancers. Loss of any enhancer causes loss of red pattern phenotype in both fore- and hindwings and are pleiotropic (Lewis et al., 2019). **(B)** Hypothetical mechanism by which a novel, tissue specific phenotype (dennis) might evolve through the acquisition of both a CRE and a tissue-specific transcription factor. Blue and yellow symbols indicate tissue specific transcription factors (TFs) which may be unique to a "pure" population but homogenized in hybrid zones. The evolutionary module in this example includes a CRE that is accessible to TFs in both the fore- and hindwing, but may bind a TF unique to the hindwing in the dennis morph only. **(C)** Depiction of hypothesized evolutionary modularity in the East Amazon *H. erato* hybrid zone. Multiple unlinked alleles control divergence between all three morphs (Left). These loci become homogenized in narrow regions of hybridization, allowing a single locus to modulate wing pattern phenotype (Right). These homogenized loci can include *trans*-acting factors, which may not show any signal of differentiation in genomic hybrid zone comparisons.

from the processes that generate the ancestral form. This, in turn, suggests that individual hybrid zones – where modular phenotypes appear most likely – can be a breeding ground for phenotype diversity via evolutionary modularity from either modular or non-modular developmental landscapes.

PROSPECTS FOR FUTURE STUDIES OF ADAPTIVE PHENOTYPE EVOLUTION

Here we consider a single case study of whether modular CREs drive adaptation of novel phenotypes. Many more studies will be necessary before we can parse the relative significance of modular and non-modular genetic architectures for phenotypic novelty and diversification. Importantly, we are not suggesting that modular genetic elements cannot or do not underlie novel phenotypes. Our perspective simply suggests that developmental modularity should not be the default assumption, even in cases where discrete phenotypes are swapped between hybridizing populations. The larger evolutionary implications of developmental CRE modularity, or the lack thereof, are substantial: modular CREs would favor adaptation via exchange of a few large-effect loci and the potential for simple adaptive introgression of genetic elements. If, however, our concept of evolutionary modularity accurately captures a common evolutionary scenario, we might expect to see an increasing number of studies mapping traits to oligogenic and polygenic adaptive architectures. Similarly, combinatorial adaptation would thus require multiple genetically distinct recombination events.

It will be important that future cases of putative developmental modularity be demonstrated with empirical assays, rather than assumed from sequence comparisons. While consideration of genetic background is not new, and is even quite common

in many studies of developmental genetics, we predict that analysis of genomic interactions and pleiotropic effects will be increasingly important during future studies of phenotypic adaptation. We also suspect, though only time will tell, that evolutionary modularity will be an important process in the production of novel phenotypes. As a deeper understanding of the genetic basis of adaptive evolution emerges, we anticipate that complex developmental architectures will repeatedly be processed in fairly simple evolutionary scenarios via hybridization and recombination to produce ecologically significant phenotypes.

AUTHOR CONTRIBUTIONS

JL and SV contributed equally to the presented ideas, writing of the manuscript, and making the figures. Both authors contributed to the article and approved the submitted version.

FUNDING

JL was supported by the NASA 17-EXO-17-2-0112 and NSF DEB-1546049. SV was supported by the NSF EPSCoR RII Track-2 FEC (OIA 1736026) and in part by National Institutes of Health-NIGMS COBRE Phase 2 Award – Center for Neuroplasticity at the University of Puerto Rico (Grant No. 1P20GM103642). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

ACKNOWLEDGMENTS

We thank Leo Campagna and Vincent Laudet for providing us with images for capuchino seedeaters and clownfish, and Karin van der Burg, Arnaud Martin, and Joe Hanly for helpful comments.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.