Genomics at the evolving species boundary

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Abstract

Molecular studies on hybridization date back to Dobzhansky who compared chromosomal banding patterns to determine if interspecific gene flow occurred in nature [1]. Now, the advent of high-throughput sequencing provides increasingly fine insights into genomic differentiation between incipient taxa that are changing our view of adaptation and speciation and the links between the two. Empirical data from hybridizing taxa demonstrate highly heterogeneous patterns of genomic differentiation. Although underlining reasons for this heterogeneity are complex, studies of hybridizing taxa offers some of the best insights into the regions of the genome under divergent selection and the role these regions play in species boundaries. The challenge moving forward is to develop a better theoretical framework that fully leverages these powerful natural experiments.

Hybrid zones (HZs) are regions where genetically distinct populations meet, mate and produce offspring of mixed ancestry [2]. These zones are often narrow relative to the geographic distributions of the parental populations and are associated with steep clines in phenotypic and genetic variation [2-5]. HZs are typically classified along several axes, including geographic structure (**mosaic** versus **clinal**), historical origin (**primary** versus **secondary contact**), and the degree of reproductive isolation (**unimodal** versus **bimodal**). They are ubiquitous in animals and plants and create remarkable natural experiments for research into the nature of species and population differences.

There is a long history of hybridization research and the study of insect HZs has catalyzed debate about i) **genomic architecture** of adaptation and speciation [6], ii) the importance of hybridization in adaptation [7*], and iii) the role of natural selection in speciation [8,9]. Advancing technologies allow us to characterize differences across the genome with increasingly fine detail and make research on hybridizing taxa even more relevant today. Here, we highlight some recent research on insect HZs that demonstrate how genomic data is being integrated into traditional HZ research to increase our understanding of ecological and evolutionary processes. Moreover, we use this opportunity to stress some analytical limitations that need to be solved to take full advantage of genomic data and move HZ research forward. For more general recent reviews on hybridization and HZs, see Harrison and Larson [7*] and Abbott, *et al.* [10].

Genomics across the speciation continuum: Genomic research on hybridizing taxa at various stages across the **speciation continuum** (Figure 1) extends decades of ecological studies providing rich empirical systems for addressing some of the most challenging questions in evolutionary and ecological research, including i) what is the architecture of complex adaptations [11-15]?, ii) how does natural selection and gene flow shape genome-wide patterns of divergence [16-18**-20*]?, and iii) how might species adapt to a rapidly changing world [21-24]?

In HZs where hybridization is frequent (Stage 1 in Figure 1), differentiation is expected only around regions of the genome closely associated with functionally important variation. These regions contribute to isolation during the earliest stages of divergence and pinpointing their location, their number, and their effect size is important for our understanding of the architecture of adaptive variation and how this architecture either facilitates or impedes further divergence. In these zones, reduced gene flow at loci involved in (or closely linked to) the traits underlying divergent traits will be highly differentiated relative to regions of the genome not under selection (Figure 1B). High resolution **divergence mapping** or **population association mapping** can, therefore, be extremely useful in localizing genomic regions and genes involved in phenotypic differences, particularly when traits have a relatively simple genetic basis ([25**]; Box 1). Work on the genetic basis of mimetic wing color patterns of *Heliconius* butterflies provides a particularly compelling example of how genomic technologies can be used to identify functionally important variation. Many *Heliconius* species are composed of a number of highly divergent color pattern races. Color pattern races are often, but not always [26], distributed **parapatrically** and linked together by a patchwork of narrow HZs [13]. Classical crossing experiments have shown that much of the color variation across the genus is controlled by allelic variation at a handful of major effect loci [27]. Recently, association mapping across replicate HZs among divergent color pattern races has identified candidate genes and the functional regions around them that cause the striking variation in the vivid wing patterns that characterize the group [11,13,28-30] (Box 1).

Later stages in the speciation process (Stage 2 and 3, Figure 1) are characterized by a strong reduction in the frequency of hybrids because of a combination of ecological and reproductive differences. Taxa at these stages along the continuum provide a wealth of information about the relative importance of **extrinsic** and **intrinsic** forces in the earliest stages of speciation. Classic insect examples include scenarios in which ecological divergence (extrinsic) results in assortative mating and reduced hybridization as a

byproduct. For instance, divergence in host-plant use in *Timema* walking sticks [31], fruit flies in the genus *Rhagoletis* [32] and the pea aphid *Acyrtosiphon pisum* [33] has shown to be a key factor resulting in assortative mating on host plants in the early stages of speciation. In other systems, such as the field crickets of the genus *Gryllus* [34] and *Heliconius* butterflies [35,36], divergence in mating cues, not directly related to mating location, are important. Additionally, hybrids often show intrinsic postzygotic isolation that reveals the importance of other processes, including different rates of evolution on the sex chromosome, in the origins of reproductive isolation [37,38*,39]. Study of HZs with these types of dynamics has profoundly shaped our understanding of how speciation occurs, including a renewed appreciation of the role of natural selection in the origins of new species and the recognition that certain genomic architectures facilitate speciation [40,41].

The application of genomic technologies to the study of taxa where hybrids are rare permits insight into how genomes diverge during speciation and how a permeable species boundary can contribute to adaptation through a process called adaptive introgression (Box 2). Not surprisingly, patterns of genomic divergence among these taxa are far more complex than those observed between freely hybridizing taxa (Figure 1). Indeed, one of the strongest patterns emerging from genomic research is just how highly heterogeneous the patterns of genomic divergence are between hybridizing taxa. The heterogeneity reflects a complex interplay between natural and sexual selection, gene flow, demography, and recombination in divergence and admixture. Unfortunately, differing combinations of evolutionary processes and modes of speciation can generate similar patterns of genomic divergence. For example, the evolution of reproductive barriers results in a genome wide reduction of gene flow, which should permit genome-wide differentiation. Similarly, even with moderate gene flow, selection at multiple loci involved in ecological adaptation may not only reduce effective gene flow at regions physically linked to these genes, but also across the entire genome, a process called genomic hitchhiking [42,43*]. This is particularly true when

the traits under selection are influenced by a large number of interacting loci [44]. The rate at which genomes “congeal” into highly differentiated units can be rapid [16,45] and disentangling the factors driving divergence is challenging (Box 3 and below). Of course, genome data are much more informative when combined with detailed ecological studies and coupled with other types of genetic, functional genetic, and experimental research [46,47].

Despite the challenges with inferring process from pattern, genomic examination of highly differentiated taxa has demonstrated just how porous the species boundary actually is. As data accumulate, we are increasingly recognizing that species are often incompletely isolated and exchange genetic material for millions of years after they form. For example, recent studies suggest that up to 40% of the genome is exchanged between sympatric and ecologically divergent *Heliconius* species [16,18]. These data also include some of the most compelling examples of adaptive introgression (Box 2) between well-differentiated taxa, challenging the view that hybridization is always “unnatural and maladaptive” [48]. In this respect, a porous species boundary provides a source of new genetic variation and hybridization has been shown to have played an important role in the radiation of wing color patterns in *Heliconius* butterflies [49], as well as, the spread of insecticide resistance alleles and changes in ecological traits that influence the spread of human malaria in the *Anopheles* species complex [50,51**] (Box 2).

Moving forward: HZs will continue to provide exceptional experimental systems for combining genomic data with data on naturally occurring variation in form, function, and fitness. Genomic technologies will improve and, as they do, it is easy to imagine HZ studies where fine-scale descriptions of genomic variation from many individuals are layered onto one another and integrated with data on individual differences in morphology, ecology, and behavior.

To take full advantage of these incredibly rich data sets requires the continued elaboration of theory and statistical methods that more fully leverage all the information contained within genomic data (Box 3). The summary statistics often used to quantify genetic differences and to identify genomic regions where gene flow is restricted or where admixture has occurred can be misleading and inferences drawn from them can be wrong. As an example, Cruickshank and Hahn [52*] recently reanalyzed several datasets that identified areas of elevated divergence, so-called “islands” of speciation or differentiation, in the genomes of hybridizing taxa. The “island” metaphor was first coined by Turner and colleagues [53] who, in one of the first population genomic studies, identified several highly divergent regions in genomes of hybridizing but ecological divergent strains of *A. gambiae* [53]. The metaphor has since become established in the lexicon of evolutionary biology [54], because the underlying logic is so compelling. In taxa that hybridize but maintain their distinctiveness, those regions that are resistant to gene flow are likely to harbor the variation responsible for differences in ecology, morphology and behavior. However, Cruickshank and Hahn [52*] found that islands disappeared when they used a measure of divergence that was insensitive to levels of within species variation. Moreover, in all cases, previously identified islands fell in regions of the genome with reduced diversity leading Cruickshank and Hahn to hypothesize that the observed patterns of differentiation were best explained by local selective sweeps, rather than restricted gene flow. These regions may still harbor functionally important variation, but not necessarily the variation underlying the differences that distinguish incipient forms. This is a somewhat subtle but important distinction.

The debate surrounding what forces, or combination of forces, generate the highly heterogeneous patterns of genomic differentiation observed among closely related taxa underscores the need to develop a more realistic theoretical framework of how genomes diverge under different evolutionary scenarios [55]. There is progress on this front [56**] (Box 3); however, most current methods do not incorporate recombination

nor **linkage disequilibrium (LD)** into their models of divergence. The extent, distribution, and patterns of LD, in particular, potentially provide much finer understanding of the history of selection, admixture and population size changes. Fortunately, new theoretical frameworks are emerging that use recombination and LD and more fully leverage the information contained in genomic datasets. These new approaches allow the recovery of the history of admixture in natural populations (see [57,58]) and offer exciting new research possibilities. In some systems, it should be possible to contrast the recombination landscape, obtained from recombination maps of controlled crosses, with that observed in natural hybridizing populations to determine the relative importance of the recombination landscape and selection in admixture [59]. Indeed, as theory catches up with technology, we will be much better positioned to disentangle “the complex interplay between selection, gene flow, and recombination” [56] that occurs when genomes collide in HZs. This is absolutely essential if we are to understand the genomic architectures of the traits under divergent selection and their relative contribution in the speciation process. In this light, comparative genomic data from insect taxa from across the speciation continuum will almost certainly be at the forefront of our continued efforts to understand the buildup of genomic differences and to link them to the origins of novel variation, the evolution of reproductive isolation and the formation of new species.

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Figure 1: Hybrid zones provide windows into the genomic pattern of differentiation at different stages of the speciation process. (A.) The speciation process is generally argued to be a continuous process with the frequency of hybridization decreasing with the buildup of reproductive isolation (RI) and genetic distance. However, at the genomic level this process is not expected to be linear [43]. Rather, as genetic differentiation increases and RI evolves, gene flow strongly decreases on a genome wide scale resulting in rapid transitions along the speciation continuum. Identifying and analyzing these transitions helps elucidate the relative importance of the various intrinsic and extrinsic factors in speciation. As indicated in the figure, the correlation between the reduction in gene flow (represented by the dashed line) by the evolution of RI and the increase in genomic differentiation (represented by the solid line) may not be perfect due to genome wide reduction in gene flow resulting from selection on multiple loci. (B.) The pattern of differentiation (100 kb *Fst* windows) along the genome (chromosomes 13 to 19) of increasingly divergent *Heliconius* populations. The left panel shows differentiation between parapatric wing color pattern races which form HZs composed primarily of admixed individuals. The two peaks in the panel are associated with yellow (chromosome 15) and red (chromosome 18) color pattern variation. When genomic differences accumulate as the hybridization frequency decreases, it becomes increasingly difficult to infer associations between loci and traits (Stage 2 and 3). (C.)

Phylogeny between the *Heliconius* species compared in the *Fst* plots. Data adapted from [16,18].

Box 1: Population mapping in contact zones can help identify functionally important variation. Population association mapping refers to experiments conducted on natural populations in which relatedness among individuals is largely unknown [60]. In this respect, HZs provide powerful comparative opportunities to explore the genetic basis of adaptation and reproductive isolation and the links between them. The underlying logic is simple- these zones often represent many generations of hybridization and admixture between divergent taxa. This mixing creates recombinant genomes and different areas of the genome are expected to mix differently depending on a combination of **intrinsic**, **extrinsic** and random factors.

Several recent studies in insects have used population mapping and these studies serve to highlight the utility (and limitation) of this approach in natural populations. Population mapping between taxa with high levels of hybridization and few reproductive barriers (Stage 1 in the speciation continuum, see Figure 1B) can help pinpoint variation responsible for phenotypic differences, especially when combined with other evidence [47]. These zones are typically composed of highly admixed individuals, the practical result of which is that any associations between DNA sequence variants and a phenotype of interest are likely due to linkage with the functional variation that underlies phenotypic differences and not other phenomena such as population structure. In *Heliconius* butterflies, for example, population genomic analyses of HZs between divergent wing color pattern races identify a small genomic interval that likely underlies differences in red pattern variation [13] (Figure B1). This approximately 65kb region shows a strong molecular signature of selection and contains numerous single nucleotide polymorphisms (SNPs) that are perfectly associated with phenotype. This divergent peak presumably contains a number of discrete regulatory regions of the transcription factor *optix* consistent with previous research that suggests that expression differences in *optix* underlies red pattern variation across the broader *Heliconius* wing color pattern radiation [11]. The striking divergence and genotype x phenotype association around *optix* stands in marked contrast to the lack of differentiation in other regions of the genome (Figure B1).

At the other extreme of the speciation continuum, population mapping of highly divergent taxa with strong ecological and reproductive differences can be informative about the genetic architecture of factors involved in adaptation and speciation. However, inferences drawn from the genomic dissection of these taxa are challenging, as reduced gene flow generates genome wide differentiation that obscures the association between genotype and phenotype [25**]. For a reasoned discussion of the power and limits of population mapping of divergent taxa see recent work by Gompert, *et al.* [61] on the genetic architecture of differences in female host plant preference and male genitalia in *Lycaeides*. As our ability to finely sample genomic variation and our theoretical understanding of the forces that shape this variation improve, population mapping of HZs promises increased understanding of the genetic basis of phenotypic variation and reproductive isolation.

Figure B1: Sliding window genomic divergence between two major *H. erato* phenotypes (“postman” and “rayed”) from three hybrid zones across a 1Mb genomic region that contains the red switch locus. There are two peaks of divergence, one centered on the transcription factor *optix* and another, much larger 65 kb peak, in a region downstream of *optix*. The 65 kb peak is in the center of a region containing no transcribed genes and is of particular interest as it also contains numerous SNPs (shown in red) perfectly associated with color phenotype (right hand axis). The two divergent peaks stand in marked contrast to the lack of differentiation at regions unlinked to color pattern, where average background genomic differences were effectively zero using a relative measure of between population differences (left hand axis). Figure modified from [13].

Box 2. Introgressive hybridization and its role in adaptation: Introgressive hybridization is the exchange of alleles between species through hybridization and subsequent gene flow. Introgressive hybridization has been recognized for over 60 years [62] but its relative importance in evolution remains contentious. Nonetheless, there is a growing recognition that hybridization is common and the advent of genomic technologies has demonstrated that species boundaries are extremely porous [7*]. As a creative force, introgressive hybridization can accelerate evolution by bringing together new adaptive combinations of alleles without the need to wait for mutations. Indeed, two recent studies of the *Anopheles gambiae* species complex emphasize an important role for introgressive hybridization in the diversification of the group. Several species in this complex are major vectors of human malaria and in the past few years there has been a rapid increase in genomic resources in the group, including the publication of 16 *Anopheles* genomes [63]. In one study, Fontaine *et al.* [51**] combined the new reference genomes with resequence data from many individuals across the range of the complex to document pervasive introgression among species (Figure B2). Their conclusions about the extent and importance of introgression rely on determining the correct historical branching order of the complex (i.e. **the species tree**). This is a nontrivial task in recently derived species because allele sharing among species could simply reflect the incomplete sorting of ancestral variation. Fontaine and colleagues went to great lengths to reconstruct the “true” relationships among species and to use this as the backdrop to identify introgressed genomic regions. In the *Anopheles* complex, phylogenetic relationships among species varied dramatically across different regions of the autosomes and between autosomes and X chromosome (Figure B2). They argued that the correct branching order of species was retained in genomic regions of the chromosomes that show the deepest divergence, which were particularly abundant on the X chromosome. Under this assumption, Fontaine *et al.* detected several major introgression events across the complex that appeared to play a role in adaptation. This included a large region that was shared among the three more distantly related anthropophilic species, *A. gambiae*, *A. coluzzii*, and *A. arabiensis*, that may have played

an important role in the emergence of traits enhancing malaria transmission to humans. In addition, they identified another introgression on chromosome 2 (2La) from the ancestral *A. gambiae* species into *A. arabiensis* that has been shown to confer resistance to desiccating environments (Figure B2). In another study, Clarkson *et. al.* [50] examined the rise of an insecticide-resistance mutation in the M and S forms of *A. gambiae* using whole genome resequence data. Despite strong isolation between the forms, the new mutation has spread from the S to M form and rapidly increased in frequency consistent with strong natural selection. By sequencing the full genomes of 15 wild-caught individuals, the group showed that the introgressed region was massive (about 3Mb) and completely removed a very large peak of divergence between the two forms. Nonetheless, this introgression event did not obviously reduce the strong reproductive barriers between the two forms, recently characterized as distinct species [64]. Taken together, these two studies demonstrate that introgressive hybridization can provide the raw material for evolution without necessarily eroding the differences in ecology and mating behavior that characterize incipient species.

Figure B2: Introgression within the *Anopheles gambiae* complex. Most likely phylogenetic relationships among *A. quadriannulatus* (Q), *A. melas* (L) and *A. merus* (R) and Patterson's *D* based on sliding window calculations across 4 autosomal chromosomal arms and the X chromosome. The orange phylogeny is thought to reflect the true branching order of the three species. Comparing this expected phylogeny to the best support phylogeny generated at different gene regions indicates a large amount of phylogenetic discordance across the genome. This is particularly true when comparing gene trees generated from the X chromosome, which generally reflect the true tree, and those generated from autosome regions, which reflect the species tree less than 50% of the time. At least some of this discordance can be explained by introgressive hybridization. As an example, there is a large block in 3L where Patterson's *D* is consistently negative indicating an excess of shared derived alleles and strongly suggestive of introgression from *A. merus* (R) to *A. quadriannulatus* (Q). Figure modified from [51**].

BOX 3. Analytical tools used in genomic research. High throughput sequencing strategies will continue to improve and the current challenge is to develop analytical methods that make use of all the information in these rich, albeit large and complex, datasets. To date, most studies of hybridizing taxa have relied on inferring aspects of the history of divergence and gene flow from relatively simple summary statistics or very basic evolutionary models. For instance, many studies have identified highly divergent genomic regions using the ***F_{st}*** statistic, which measures the normalized difference in allele frequency between populations [65]. Alternatively, studies have estimated the degree of admixture between taxa using **Patterson's D** statistic [66]. This statistic and variants of it [67] measure the excess of shared derived alleles in regions across the genome as way to identify introgressed loci. Within the context of HZs, classic "cline theory" [3] can be used to identify genomic regions that contribute to species boundaries and/or local adaptation. In its simplest form, cline theory models allele frequency differences between hybridizing taxa as a function of selection and dispersal. Thus, alleles that show abrupt changes in frequency across a hybrid zone may be important for the ecological, morphological, and behavioral differences that characterize hybridizing taxa. Recently, Buerkle and colleagues have applied cline theory at the genomic level [68-70]. Their method of **genomic clines** relies on generating the expected distribution of **admixture** at neutral loci in two populations coming into secondary contact. Loci that deviate from the neutral expectations are likely experiencing selection and different forms of selection can, in theory, be distinguished.

The above strategies for analyzing genomic data enriched our understanding of genomic divergence and focused debate around what these patterns actually mean. This in turn has stimulated efforts to develop a more robust theoretical framework for analyzing genomic data. Statistical approaches are beginning to emerge that measure the likelihood of particular patterns of divergence under specific evolutionary models. These models make fuller use of genome data and concomitantly allow researchers to estimate specific parameters from the data. For instance, the observed **allele frequency spectrum** (AFS), which summarizes the joint distribution of allele frequencies across

diallelic variants in single or multiple populations, can be compared to the AFS under a specific demographic model to distinguish among competing modes of speciation [71,72]. Alternatively, the pattern of branching events in allelic genealogies from multiple **gene trees** can be examined and compared to those expected under competing evolutionary scenarios using **coalescent theory** [73,74]. These approaches assume that SNPs or gene regions used in the analysis are independently segregating. Furthermore, coalescent-based approaches assume no recombination within genomic regions being analyzed. More recent mathematical modeling approaches are beginning to make use of the patterns of **linkage disequilibrium** (LD) and haplotype information across the genome [58]. When coupled with an understanding of variation in recombination rate across the genome, these approaches will more effectively use information within genomic datasets. Presently, the broad application of these methods is restricted because they require both a good reference genome and **phased** diploid genomic data and are computationally intensive even when comparing small numbers of samples [75]. For an exceptional overview of state-of-the-science analytical methods and the challenges of making full use of genomic data see [56**].

Glossary:

Adaptive introgression: Gene flow of advantageous alleles from one species into the gene pool of another by repeated backcrossing.

Admixture: The exchange of genetic material between populations or closely related species resulting in chromosomal regions of mixed ancestry.

Allele Frequency Spectrum: Matrix containing the number of diallelic genetic polymorphisms (in a whole genome or genomic region) in which the derived or minor allele was found in the corresponding number of samples from each population. The dimension of the matrix is equal to the number of populations.

Bimodal hybrid zone: HZ consisting largely of individuals resembling the parental forms with low frequencies of intermediate genotypes and/or phenotypes.

Clinal hybrid zone: HZs that show a gradual transition from one character state to another, typically associated with steep clines in phenotypic and genetic variation, and often associated with distinct environmental transitions.

Coalescent theory: The population genetic theory that describes the expected distribution of time to common ancestry of a set of alleles drawn from a population or several populations under a given set of demographic conditions.

Divergence Mapping: Using scans of genetic markers to identify ones that show allele frequency differences that are higher than expected by random chance. Similar to population association mapping, this strategy is based on the premise that the marker(s) showing elevated rates of divergence are linked to functionally important variation. Importantly, a number of approaches have been developed that incorporate underlying neutral population genetic structure.

Extrinsic factors promoting genomic differentiation: Barriers to gene flow that arise when hybrids experience lower fitness in a particular environment.

***F_{st}* (Wright's fixation index):** A measure of population differentiation. It is calculated as the fraction of the total genetic variation that is distributed among subpopulations.

Gene tree: A bifurcating tree that represent the ancestral relationships of homologous loci (more correctly haplotypes) sampled from closely related species. The topology may or may not reflect the topology of the **species tree** depending on the history of selection and gene flow.

Genomic architecture: Refers to the number, location, and effect size of loci underlying a phenotypic trait.

Genomic clines: Mathematical functions that describe the probability of locus-specific ancestry along a gradient in genome-wide admixture or hybrid index. Hybrid index is defined as the proportion of an admixed individual's genome inherited from one of two parental populations.

Intrinsic factors promoting genomic differentiation: Barriers to gene flow driven by incompatibilities in hybrids independent of the environment.

Linkage disequilibrium (LD): The nonrandom association of alleles at different loci. The level of LD is determined by a number of factors including physical linkage, population structure, and selection. LD is broken down by recombination.

Mosaic hybrid zones: HZs that show no regular transition from one parental taxon to the other. Rather parental forms associate with particular habitat mosaics, where one parental taxa is associated with one habitat type and the other with another habitat type and hybridization occurs across patch boundaries.

Patterson's *D* statistic: A statistic that uses the distribution of shared derived alleles between taxa to identify interspecific gene flow. The statistic is based on a simple premise: when considering an outgroup, alleles can be either ancestral ("A") or derived ("B"). When considering four taxa, derived alleles can be shared among taxa resulting in patterns termed "ABBA" and "BABA". An excess of ABBA or BABA patterns is indicative of gene flow between two of the taxa (see [66,67]).

Population Association Mapping: Uses the statistical association between genetic markers and particular phenotypes obtained from a collection of population samples to identify regions of the genome that may be responsible for phenotypic variation. The strategy is based on the premise that the marker(s) associated with a particular phenotype will be in close proximity to the causal locus or loci determining a phenotype.

Primary contact: When divergence takes place *in situ* between adjacent populations of a previously homogeneous species.

Secondary contact: Contact between two diverged lineages that were previously geographically isolated.

Speciation continuum: A re-envisioning of Darwin's view of speciation where there is a continuous sequence of genetically-based changes that occur as two lineages diverge from one another on the pathway to complete reproductive isolation [76].

Species tree: A bifurcating tree that reflects the true history of speciation.

Unimodal hybrid zone: HZ with high frequencies of intermediate genotypes and/or phenotypes.

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