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Rare hybrid solves "genetic problem" of linked plumage traits

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We study hybrids, in part, because they are broken. Not in the sense that they are literally broken—although in many cases they do have low fitness compared to parental species—but because the recombination that occurs with multiple generations of hybridization breaks up the genomes of two species and puts it back together in unpredictable ways. This genetic mixing allows researchers to associate regions of the genome with phenotypes that differ between the parental species. Research in two hybridizing songbird species, the golden-winged warbler (*Vermivora chrysoptera*) and blue-winged warbler (*V. cyanoptera*), have allowed us to do just that for plumage pigmentation traits (Toews et al. 2016; Baiz et al. 2020). Natural recombinant *Vermivora* hybrids also allow researchers to tease apart how different parental plumage traits affect processes of hybridization and diversification.

The inheritance of plumage pigmentation in *Vermivora* warblers has had a long history. Early in the twentieth century, John Treadwell Nichols (1908) hypothesized that throat coloration follows Mendelian inheritance in hybrid populations. Natural variation among parental species and their hybrids (Parkes 1951) and recent genomic sequencing (Toews et al. 2016, Baiz et al. 2020) has corroborated this prediction. Specifically, the presence/absence of the black throat patch, and the usually co-occurring black face mask (Figure 1a), is linked to a small ~10-15 kb region upstream—in the presumed promoter region—of the Agouti signaling protein (*ASIP*) gene on chromosome 20, a key player in the melanin (i.e. black) pigmentation pathway.

In *Vermivora* warblers, throat patch color and face mask color are nearly perfectly correlated (n = 222 wild-caught individuals for which we examined full plumage traits), meaning that it was impossible for previous genomic analyses to tease apart the connection between *ASIP* variation and throat versus mask coloration. In golden-winged warblers, and in certain hybrids, individuals that have a black throat patch almost always have a broad black face mask (Figure

1a). In blue-winged warblers, and in other hybrids, individuals have a plain throat and nearly universally lack the black face mask (Figure 1a). Consequently, previous studies have assumed the two phenotypes—mask and throat melanation—are controlled by exactly the same locus (e.g., Short 1963), or have assumed perfect linkage, disregarding one plumage trait or the other in scoring hybrid admixture (e.g., Gill 1980).

However, there exists at least one specimen exhibiting a rare, mismatched throat/mask phenotype. In 1951, Kenneth Parkes reported a *Vermivora* individual with a black face mask, but lacking a black throat patch that was collected in 1934 from an area of hybridization in southern Michigan. He speculated that due to the close correlation in throat and mask melanation, if each trait is controlled by a separate locus, they would need to be tightly linked such that recombination rarely breaks up their association. Lacking the genetic tools to test his hypothesis, Parkes (1951) proposed the linkage of the phenotypes as a "genetic problem for future study".

Here, we document our own recent observation of a hybrid *Vermivora* individual in central Pennsylvania that also carries this rare mismatched phenotype, exhibiting a black face mask but lacking a black throat patch (Figure 1b). Unlike Parkes, however, we now employ whole genome sequencing of this mismatched hybrid and compare it to other matched hybrids and parental individuals to address this "genetic problem" using modern tools.

Despite striking differences in their plumage coloration, blue-winged warblers and golden-winged warblers exhibit exceptionally high levels of genetic similarity (Toews et al. 2016). This is likely due to a long history of hybridization, and hybrids can be found everywhere the breeding ranges of the parental species overlap, along the Great Lakes of the Eastern USA and across the Appalachian Mountains. Their hybrids exhibit a range of plumage phenotypes mixing various characteristics of the parental species (Toews et al. 2016) (Figure 1). Further, in these areas, the parental species can be found in very similar habitats (Will 1986), likely facilitating hybridization. Recent declines in populations of golden-winged warblers, and in some areas their gradual replacement by blue-winged warblers (Bennett et al. 2017), suggest the shifting hybrid zones may be important in shaping their evolutionary trajectories.

We opportunistically observed this mismatched hybrid on June 8, 2020 in Huntingdon County, Pennsylvania (40.651635, -77.942584), within a region that has both parental phenotypes and "typical" (matched) hybrids. We used a recorded golden-winged warbler song playback to lure the individual into a mist net. Upon capture, we assessed its plumage using the scoring criteria of Gill (1980) with the addition of the following traits: mask, eyeline, and mustache color. We also affixed a USGS aluminum band to the bird (band no. 283030117) and attained a blood sample from the brachial vein for genetic analysis.

With the exception of throat and eyeline plumage, we assigned golden-winged warbler scores in all of the plumage traits assessed for the mismatched hybrid (Appendix S1: Table S1). The mismatched hybrid exhibits a plumage score of 35, where the maximum score (38) represents the golden-winged warbler plumage phenotype and the minimum score (0) represents the blue-winged warbler plumage phenotype. In *Vermivora* hybrids, plumage scores are closely correlated with genetic scores of admixture (Toews et al 2016), so the mismatched hybrid is not likely an early-generation hybrid, but likely a result of multiple generations of backcrossing into golden-winged warblers.

Because the mismatched hybrid exhibits mostly golden-winged warbler plumage traits (and likely genetic background), but shares the non-melanated throat patch of blue-winged warblers, we reasoned it may be possible to identify the genomic region underlying the face mask separate from the throat patch by quantifying transitions in ancestry from the parental species in the genome of this hybrid. We extracted the mismatched hybrid's DNA and used whole genome re-sequencing following the approach described in Toews et al. (2016) to quantify genomic variation around *ASIP*—a region where parental species exhibit fixed differences—by aligning sequences to the yellow-rumped warbler genome assembly (NCBI accession #PRJNA325157, Baiz et al. 2021). We then compared genotypes in this region for the mismatched hybrid to genotypes from matched hybrids and parental individuals previously sequenced in Toews et al. (2016) and Baiz et al. (2020). For the mismatched hybrid, we obtained 47 million paired reads, resulting in 10X coverage across chromosome 20.

Consistent with our previous work (Baiz et al. 2020), we identified a ~10 kb region upstream of *ASIP* where hybrids that exhibit a black throat share ancestry with golden-winged warblers (i.e. "matched hybrids" in Figure 2). Within this region, we identified 8 SNPs (between 4,247,547 bp – 4,252,746 bp) where black-throated individuals are homozygous for the golden-winged allele and—consistent with homozygous recessive inheritance of the trait (Parkes 1951)—plain-throated individuals are either heterozygous or homozygous for the blue-winged allele (Figure 2b). We posit that the SNPs that with this 5 kb region are likely part of the warbler *ASIP* throat-color gene promoter. Moreover, unlike all black-throated birds, the mismatched hybrid was nearly uniformly heterozygous for all SNPs in this presumed promoter region.

Importantly, we also identified a single SNP (4,245,971 bp) where the mismatched hybrid and other black-masked individuals are homozygous for the golden-winged allele, and non-masked individuals are either heterozygous or homozygous for the blue-winged allele (Figure 2b). We posit that this SNP—only 1.5 kb upstream of the SNPs linked to throat coloration—likely falls within the warbler *ASIP* mask-color gene promoter. Together with the observed high rate of linkage between the throat and mask phenotype (>99.5% of all *Vermivora*), and the previously described association between both phenotypes and *ASIP* (Toews et al. 2016, Baiz et al. 2020), these results support the hypothesis of extremely tight genetic linkage between two separate loci controlling these traits and suggest the mask promoter is adjacent to the throat promoter upstream of *ASIP*.

Discerning the genomic position underlying the mask separately from the throat phenotype in *Vermivora* raises intriguing questions about the mechanisms underlying plumage differentiation and diversification in wood warblers. Bold plumage patches like those discussed here may serve as signals of dominance, mate quality and species recognition (Santos et al. 2011, Møller 1990, Uy et al. 2009). Although reproductive isolation is generally weak in *Vermivora* and social pairs involving hybrids are common (Vallender et al. 2007), the presence/absence of the melanic plumage patches may influence male reproductive success in the hybrid zone. In a previous study of golden-winged warblers, males that were experimentally manipulated to remove their black throat patch and mask largely lost their breeding territories and failed to obtain mates (Leichty & Grier 2006). This suggests hybrid males lacking melanic patches that otherwise resemble golden-winged warblers may be outcompeted by golden-winged males, rejected by golden-winged females, or both. Consistent with this, Confer et al. (2020) recently documented that phenotypic hybrids were less likely to have social mates during the breeding season. While this study did not consider non-social mates (*i.e.* extra pair copulations), it leaves open the question of reduced hybrid fitness.

In this case, we were not able to quantify the reproductive success of the mismatched hybrid. However, we speculate that it could be greater for mismatched than for “matched” hybrids: because they exhibit one clear golden-winged trait (*i.e.* melanic mask) and one clear trait of blue-winged warblers (*i.e.* a pale throat), mismatched males may be appealing to breeding females of both species. We note we observed a female golden-winged warbler within the presumed breeding territory of the mismatched hybrid, although we do not know if they were paired. Similarly, mismatched males may not be perceived as a conspecific threat by territorial males, possibly resulting in higher rates of extra-pair copulations involving mismatched males. Thus, when linkage between these signal traits is broken, hybridization may be facilitated.

In general, wood warblers exhibit high levels of plumage differentiation between closely related species, which often show modular variation in color across plumage patches. A genetic architecture involving multiple regulatory regions that each control deposition of pigments in different plumage patches may allow the high rates of warbler plumage differentiation to be explained by the acquisition of few mutations. Further, tight genetic linkage between these regulatory regions may ensure co-inheritance of species-specific traits, making it less likely they get broken up by hybridization and recombination. Thus, tightly-linked, modular control of plumage patch color may not only provide a mechanism for rapid plumage diversification but may also promote pre-mating reproductive isolation, as discussed above. Such a compound mechanism may help explain the extremely rapid rate of speciation in this colorful songbird family (Oliveros et al. 2019).

Further field studies are necessary to discern the role of plumage signals in *Vermivora* hybridization. For example, it may be possible to test the effects of multiple signal traits on intrasexual communication in the hybrid zone by using artificial mounts with differently colored plumage patches to challenge territorial males. Although our results suggest modular control of melanic plumage patches via regulation of *ASIP* in *Vermivora*, it will also be necessary to perform functional genetic tests of this hypothesis.

A recent study investigating the genomics of plumage traits in another wood warbler hybrid zone, between hermit (*Setophaga occidentalis*) and Townsend's (*S. townsendi*) warblers, linked the presence/absence of the black face mask to a large (200 kb) region on chromosome 20 (Wang et al. 2020). Importantly, this region also encompasses the small region we predict here is the *Vermivora ASIP* mask promoter. Further, across *Setophaga* sister-species pairs, differentiation in melanic plumage traits is mirrored by repeated differentiation near *ASIP* (Baiz et al. 2021). Thus, comparative genomic studies across wood warblers may help elucidate the relationship between the genomic architecture of regulatory variation in *ASIP*, its control of melanic plumage traits, and mechanisms of species differentiation.

Our observation highlights the value of natural hybrids to the study of plumage (and other) phenotypes. Individual hybrids, like this rare recombinant, are unique mosaics that carry different combinations of parental ancestry. Within hybrids, when breakpoints in this ancestry occur between genomic regions underlying different phenotypes, it may be possible to associate these phenotypes with precise genetic loci.

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Supporting Information

Additional supporting information may be found online at: [link to be added in production]

Open Research

Sequencing data are available from the National Center for Biotechnology Information Sequence Read Archive: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA325126/

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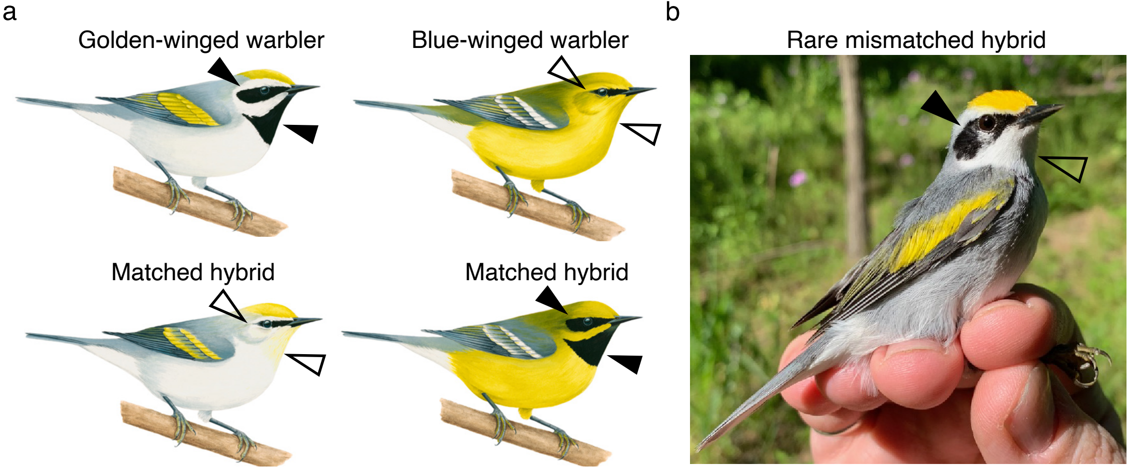
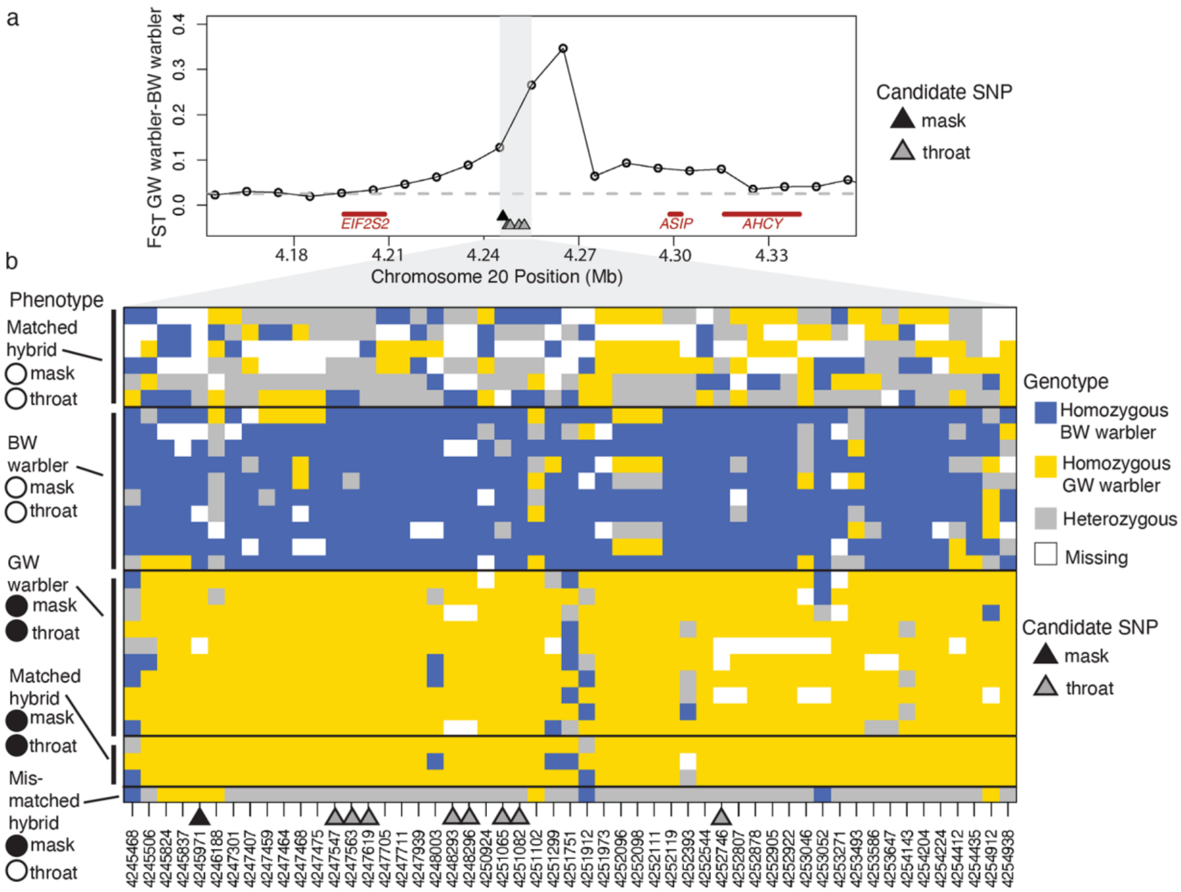
Figure 1. Plumage traits of *Vermivora* warblers and their hybrids. **a)** Typical (matched) plumage characteristics of parental species and their hybrids. Presence/absence of the melanic face mask and throat patch (referenced by triangles) are highly correlated and individuals that exhibit a broad melanic face mask almost always exhibit a melanic throat patch and vice versa. Illustrations by Liz Clayton Fuller. **b)** Rarely, hybrids exhibit a mismatched throat/mask phenotype, like this individual we observed here. We estimate this mismatched phenotype to occur at a frequency of <0.5% in hybrid populations.

Figure 2. Genomic variation near the Agouti signaling protein (*ASIP*) gene on chromosome 20 underlies mask and throat coloration in *Vermivora* warblers. **a)** Genetic differentiation estimated in 10 kb windows across the region highly differentiated between parental species (Toews et al. 2016). Mask and throat color were linked to a small ~10 kb region upstream of *ASIP* (light gray window ~4.245 Mb – 4.255 Mb, Baiz et al. 2020). Protein coding genes are labeled in red, and locations of candidate SNPs are denoted by triangles. The dashed gray line indicates mean F_{ST} across chromosome 20. **b)** Genotypes at highly differentiated SNPs ($F_{ST} > 0.7$) for parental species and hybrids within the candidate 10 kb region. Mask and throat color phenotypes are shown at left. Locations of candidate SNPs for the mask and throat phenotype were determined by comparing masked to non-masked individuals and black-throated to plain-throated individuals, respectively, as described in the text (BW=blue-winged, GW=golden-winged warblers).


a
Golden-winged warbler
Blue-winged warbler
Matched hybrid
Matched hybrid
b
Rare mismatched hybrid


a
F_{ST} GW warbler-BW warbler
0.0
0.1
0.2
0.3
0.4
EIF2S2
ASIP
AHCY
4.18
4.21
4.24
4.27
4.30
4.33
Chromosome 20 Position (Mb)
Candidate SNP
mask
throat
b
Phenotype
Matched hybrid
mask
throat
BW warbler
mask
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GW warbler
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Matched hybrid
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Homozygous BW warbler
Homozygous GW warbler
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