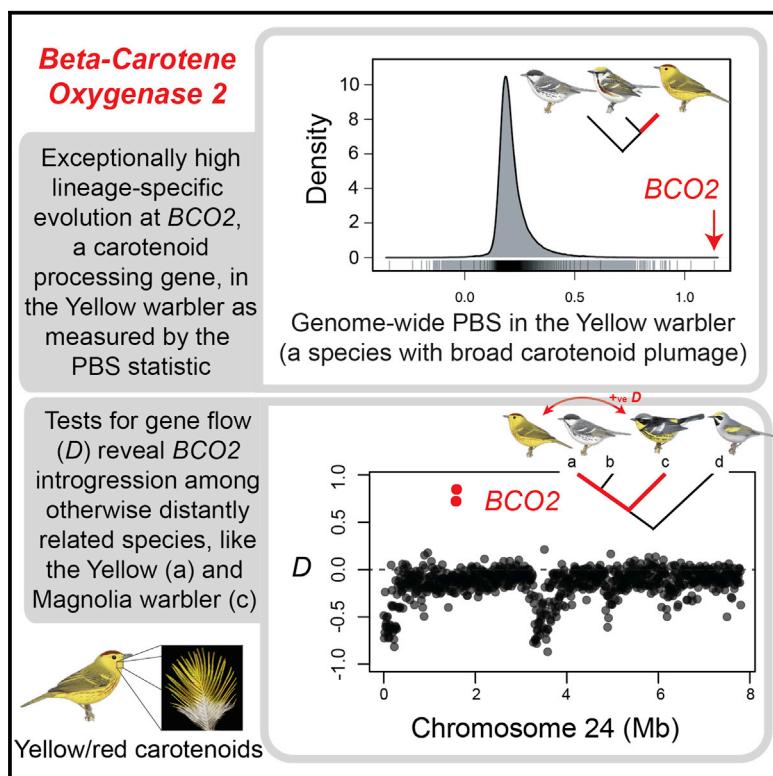


## Pigmentation Genes Show Evidence of Repeated Divergence and Multiple Bouts of Introgression in *Setophaga* Warblers

### Graphical Abstract



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### In Brief

Baiz et al. employ whole-genome sequencing across colorful *Setophaga* warblers to examine genome-wide differentiation between closely related species. Divergence in plumage coloration is mirrored by differentiation at two plumage pigmentation genes, with different evolutionary histories shaped by repeated mutation and introgression.

### Highlights

- Sister *Setophaga* species pairs share large  $F_{ST}$  peaks, especially on autosomes
- Divergence in AS/P suggests repeated mutation underlies melanistic color differences
- Introgression at *BCO2* occurred among species with carotenoid-rich plumage
- The *BCO2* introgressed region includes a putatively adaptive amino acid substitution



## Report

# Pigmentation Genes Show Evidence of Repeated Divergence and Multiple Bouts of Introgression in *Setophaga* Warblers

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## SUMMARY

Species radiations have long served as model systems in evolutionary biology.<sup>1,2</sup> However, it has only recently become possible to study the genetic bases of the traits responsible for diversification and only in a small number of model systems.<sup>3</sup> Here, we use genomes of 36 species of North, Central, and South American warblers to highlight the role of pigmentation genes—involved in melanin and carotenoid processing—in the diversification of this group. We show that *agouti signaling protein* (*ASIP*) and *beta-carotene oxygenase 2* (*BCO2*) are predictably divergent between species that differ in the distribution of melanin and carotenoid in their plumages, respectively. Among species, sequence variation at *ASIP* broadly mirrors the species' phylogenetic history, consistent with repeated, independent mutations generating melanin-based variation. In contrast, *BCO2* variation is highly discordant from the species tree, with evidence of cross-lineage introgression among species like the yellow warbler (*Setophaga petechia*) and magnolia warbler (*S. magnolia*) with extensive carotenoid-based coloration. We also detect introgression of a small part of the *BCO2* coding region (<3 kb) in *S. discolor* and *S. vitellina*, including an amino acid substitution that is unique to warblers but otherwise highly conserved across birds. Lateral transfer of carotenoid-processing genes has been documented in arthropods, but introgression of *BCO2* as demonstrated here—presumably adaptive—represents the first example of carotenoid gene transfer among vertebrates. These contrasting genomic patterns show that both independent evolution in a common set of genes and past hybridization have fueled plumage diversification in this colorful avian radiation.

## RESULTS AND DISCUSSION

Evolutionary radiations—where lineages emerge at an exceptionally high rate—have long served as essential model systems in evolutionary biology.<sup>1,2</sup> What has only been possible recently, however, is studying the genetic bases of the traits associated with exceptional diversification.<sup>3</sup> With this information, we can ask: do the genomes of rapidly emerging species exhibit unique features conducive to divergence? Have the same genes been recruited repeatedly to create divergent phenotypes? Has hybridization fueled the diversification process?

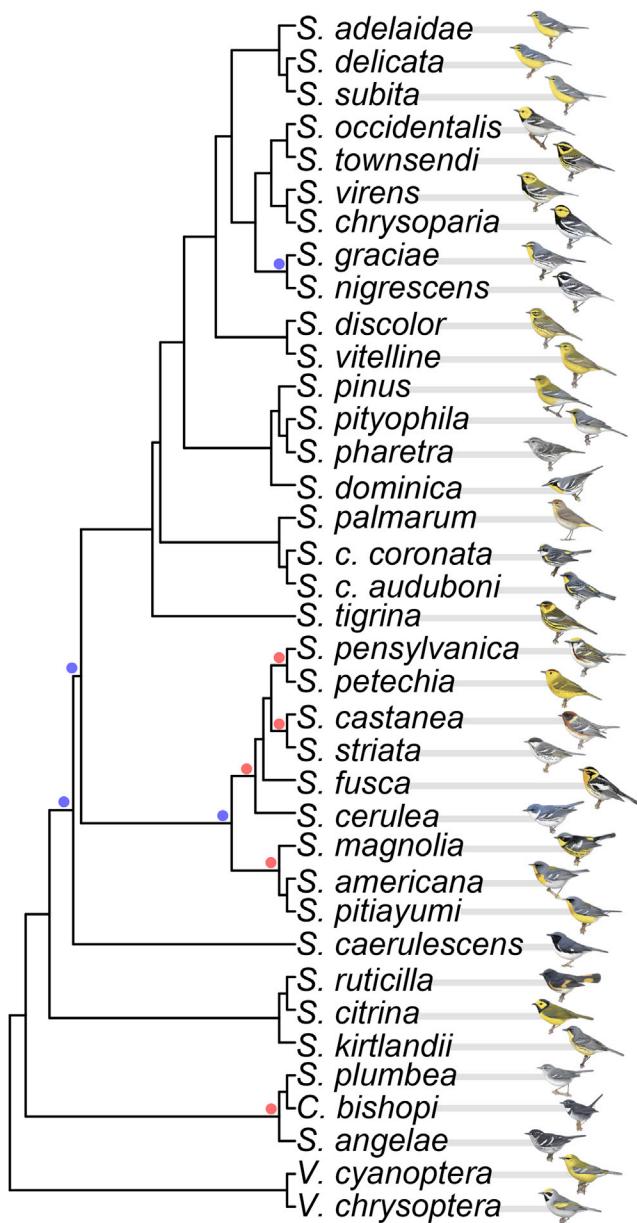
Progress in this area is exemplified by the exceptional body of work on a small number of model systems, including *Heliconius*,<sup>4</sup> *Gasterosteus*,<sup>5</sup> cichlids,<sup>6</sup> and Darwin's finches.<sup>7</sup> Addressing these questions in other radiations remains challenging because a fulsome investigation necessitates (1) high species diversity, (2) a robust phylogenetic hypothesis, (3) independent evolution of similar phenotypes, and (4) admixture—naturally or in lab crosses—to facilitate genotype-phenotype associations. This latter requirement is the most challenging in most systems where

natural hybridization is rare or absent or for which controlled crosses are intractable.<sup>8</sup> Here, we focus on an evolutionary radiation that fulfills all of these requirements.

The parulid warblers of the Americas are a well-known avian family with a rich legacy of study (Figure 1), including cornerstones of community ecology<sup>9</sup> and phylogenetic diversification.<sup>10</sup> Diverging from its sister family, Icteridae, approximately 10 mya, Parulidae radiated over the last 7 Ma.<sup>11</sup> Species accumulation in this group is exceptionally high compared to most other bird radiations; for example, among songbirds (order: Passeriformes), one of the largest shifts in diversification rate occurred in Parulidae.<sup>11</sup>

Unlike classic radiations where species show strong ecological differentiation driven by natural selection—i.e., adaptive radiations—the most conspicuous differences across Parulidae involve plumage.<sup>13</sup> In particular, coloration in this group derives from several distinct classes of pigments, primarily melanins (producing a range of brown, rufous, or black colors) and carotenoids (producing bright yellows, reds, and oranges).<sup>14</sup> The evolution of dramatic plumage color differences





**Figure 1. Phylogeny of Setophaga, Using Vermivora as an Outgroup**  
 n = 34 Species, including the monotypic *Catharopeza bishopi*<sup>12</sup>. The tree is based on bioinformatically extracted UCE loci, with 1,000 bootstraps. All branches have 100% support, except those indicated by the colored dots (red, 50%–75% support; blue, 75%–98%). Warbler illustrations © Lynx Editions.

in this group likely results from both natural and sexual selection: research on color-habitat associations<sup>15</sup> as well as on opsin gene (i.e., visual pigment) evolution<sup>16</sup> has provided a nuanced view of the joint role of both forces in driving adaptive plumage evolution.

Understanding the evolutionary history of genes involved in diversification first requires some knowledge of potential candidate genes. This is nearly universally unknown for non-model biological systems. In parulid warblers, however, detailed

genomic studies of multiple, independent hybrid zones have generated a robust set of candidate genomic regions associated with species coloration differences.<sup>17–20</sup> The present study leverages these linkages for pigmentation-associated genes to ask whether these same genes have also been recruited in the evolution of similar phenotypes across the largest genus<sup>12</sup> in the family (Setophaga) and to test explicitly for evidence for historical introgression.

#### Whole-Genome Divergence across the Genus

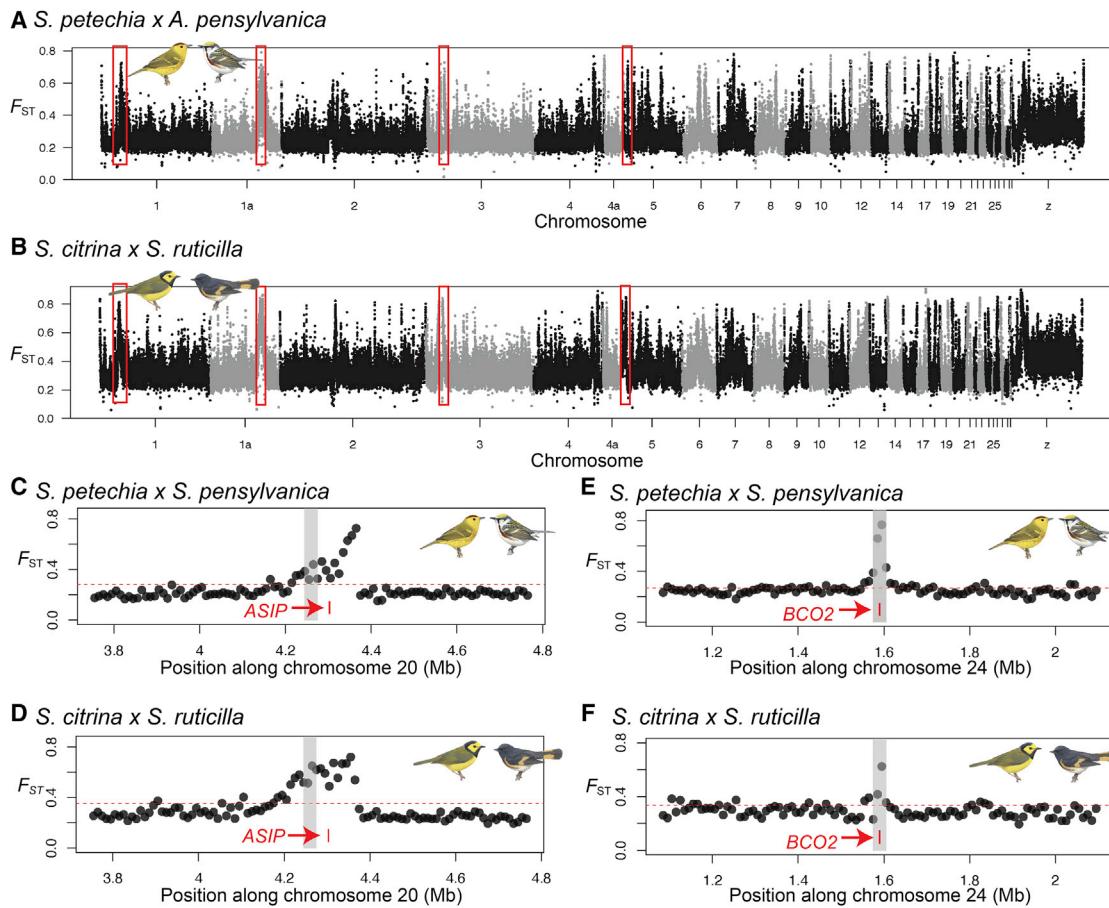
We first produced a new chromosome-level reference genome for *Setophaga coronata*. We then generated whole-genome data at 4 $\times$  to 5 $\times$  coverage for multiple individuals—usually 5 per species—for all 34 species in the *Setophaga* genus (Figure 1). The only species omitted is the most recently described, *S. flavescentis*.<sup>21</sup> We also included data from two of the *S. coronata* subspecies (*S. c. coronata*/*S. c. auduboni*)—where there is debate over species status—as well as published data from the two extant *Vermivora* species, which serve as outgroups in our phylogenetic analyses (n = 166 genomes).<sup>22</sup>

We first compared broad-scale patterns of differentiation across the genomes of nine sister species pairs (Figure S1). We identified a high level of sharing among large (>1-Mb) autosomal peaks of divergence between multiple pairs (Figure S2). This shared divergence is illustrated by two pairs: *S. graciae*/*S. nigrescens* and *S. petechia*/*S. pensylvanica* (Figures 2A and 2B). Both pairs have a highly heterogeneous pattern of divergence: overall low background  $F_{ST}$  punctuated by large, distinct peaks. Moreover, 43% of the 1-Mb peaks are shared between two or more of all the sister species pairs, with one—on chromosome 1a—shared across all but one of the nine pairs (Figure S2). We interpret this shared differentiation across independent sister species as a function of a common genomic architecture, linked selection, and reduced recombination.<sup>23,24</sup>

Much interest has been focused on the sex chromosomes and their role in speciation and divergence.<sup>25</sup> Our analyses show that (1) the Z chromosome is a hotspot of differentiation in *Setophaga* (33 large  $F_{ST}$  differentiation peaks among all pairs), but (2) patterns of differentiation across the Z appear more variable among taxa than across autosomes (only 18% of the large  $F_{ST}$  differentiation peaks are shared between 2 or more of the 9 pairs, compared to 53% of the autosomal differentiation peaks; Figures 3A–3D). For example, between *V. chrysoptera* and *V. cyanoptera* (Figure 3D), two of the six peaks in the genome occur on the Z, but these are not divergent in any of the other species pairs. Similarly, between *S. americana* and *S. pitiayumi* (Figure 3C), the only divergence peak across their genomes is restricted to a single, large, 20-Mb portion of the Z, which is possible evidence of a chromosomal inversion.

#### Pigmentation Gene Evolution Shows Repeated, Independent Evolution

Much smaller regions of divergence (i.e., <100 kb) included pigmentation genes. We focus on two with a clear connection to avian coloration: *agouti signaling protein* (*ASIP*) and *beta-carotene oxygenase 2* (*BCO2*). The *ASIP* gene product has a



**Figure 2. Differentiation between Two *Setophaga* Sister Species Pairs**

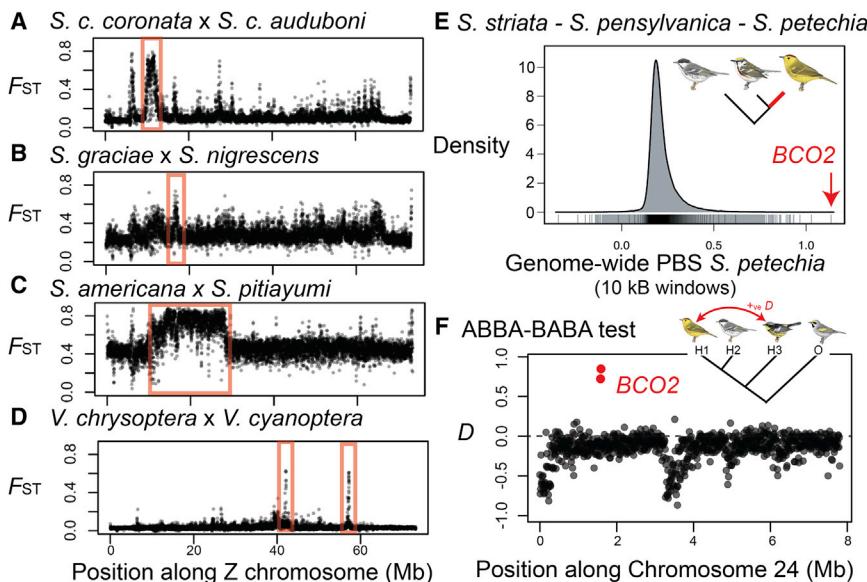
(A and B) Genome-wide distribution of  $F_{ST}$  in 10-kb windows. The red boxes indicate a subset of divergent windows that are common between the pairs (see also Figure S1). (C–F) Regions with ASIP (C and D) and BCO2 (E and F). The gray boxes indicate locations that are divergent among *Vermivora*.<sup>17</sup> The vertical red line indicates the coding regions, and the horizontal dashed red line is the chromosome average  $F_{ST}$  (see also Figures S2 and S3). Warbler illustrations © Lynx Edicions.

well-characterized role in melanogenesis, and variation in its presumed promoter is associated with throat/mask plumage in *Vermivora*<sup>17,20</sup> and hybridizing *S. townsendi*-*S. occidentalis*.<sup>19</sup> *BCO2* is involved with carotenoid processing and plumage coloration in canaries<sup>26,27</sup> and is also associated with divergent plumage characteristics in *Vermivora*.<sup>17</sup>

We found that both *ASIP* and *BCO2* gene regions were consistently and predictably divergent between multiple *Setophaga* pairs that differ in melanic (*ASIP*) or carotenoid (*BCO2*) pigmentation (Figures 2C–F and S3). For example, *S. petechia*, aptly known by its English common name of “yellow warbler,” has the most extensive carotenoid coloration of any species in *Setophaga*,<sup>28</sup> whereas its sister species, *S. pensylvanica*, has much less carotenoid-based coloration. Here, we found *BCO2* is highly divergent compared to the background level of differentiation between these two species (Figure 2E). Moreover, *S. pensylvanica* and *S. petechia* share a close common ancestor with a third species, *S. striata*, which also has little carotenoid-based plumage. We used these taxa in a three-population comparison to calculate population

branch statistics (PBS) and estimate unique evolution associated with each. Notably, the region within the *S. petechia* genome that is the strongest outlier for PBS includes *BCO2* (Figure 3E).

We next compared gene trees specific to *ASIP* and *BCO2* to the inferred species tree to test for evidence of parallel divergence and/or introgression. We generated the species tree by bioinformatically extracting sequences from each of 4,008 ultra-conserved element (UCE) loci, identified in the *S. coronata* genome, across all the species (Figure 4A). For *ASIP*, we found that only two tips showed discordance with the presumed species branching pattern (Figure S4). Given that *ASIP* is divergent between many pairs and has been directly associated with melanic coloration in two warbler hybrid zones, our interpretation of this finding is that *ASIP* has been the target of repeated mutations that have contributed to melanic plumage differences across warblers. This is consistent with other avian pigmentation studies that have identified independent mutations in the *ASIP* region across a wide phylogenetic spectrum.<sup>29–31</sup>



**Figure 3. Divergence and Introgression among Setophaga Warblers**

(A–D)  $Z$  chromosome differentiation among pairs of *Setophaga* (A–C) and (D) *Vermivora*.

(E) PBS for the three-species comparison. The 10-kb window with highest lineage-specific evolution in *S. petechia* includes the *BCO2* region (red arrow).

(F)  $D$  statistic from the ABBA-BABA test between *S. petechia* (H1), *S. striata* (H2), *S. magnolia* (H3), and *V. chrysoptera* (O) along chromosome 24. High  $D$  is consistent with introgression between *S. petechia* and *S. magnolia*. The autosomal windows with the highest  $D$  include the two 10-kb windows at *BCO2* (red points; see also Figure S2 for the whole genome comparison). Warbler illustrations © Lynx Edicions.

### Repeated Instances of Historical Introgression at *BCO2*

*BCO2* presents a strikingly different pattern from the inferred species tree, with clear evidence of introgression between otherwise highly divergent taxa (Figure 4A). As mentioned above, *S. petechia* forms a well-supported clade in the species tree with *S. pensylvanica*, *S. striata*, as well as *S. castanea*. However, at *BCO2*, *S. petechia* clusters with *S. fusca*, *S. magnolia*, and *S. ruticilla* (which we abbreviate the “PFMR group,” using the first letter of their Latin species names; Figure 4A). None of these species are closely related to one another in the species-level topology, but at *BCO2*, there is strong support for their close relationship across many SNPs (bootstrap support is >94% for all internal nodes of this clade at *BCO2*). Importantly, all four PFMR species are well known for having extensive carotenoid-based coloration. Using  $D$ -statistics—an explicit test of gene flow—we found that the 10-kb *BCO2* region has the highest excess of shared mutations across all the autosomal windows between *S. magnolia* and *S. petechia* (Figures 3F and S2).

We also identified a more fine-scale pattern of introgression at *BCO2*. Across their genomes, *S. discolor* and *S. vitellina* are sister species that are in turn sister to the crown group of nine species, including *S. virens* and *S. graciae* (Figure 1). However, for a 3-kb region of *BCO2*, *S. discolor* and *S. vitellina*—species that also have extensive carotenoid-based body coloration—share several mutations with the PFMR group (Figures 4B and S2). Notably, this 3-kb region spans the 3<sup>rd</sup> and 4<sup>th</sup> exons of *BCO2* and includes an amino acid (aa) substitution in the 3<sup>rd</sup> exon, which is unique to these warblers, with the ancestral variant being highly conserved across other birds (Figure 4B). This substitution changes *BCO2* residue 39 (relative to *Taeniopygia guttata*)—an arginine (R) across all other *Setophaga* and *Vermivora* species—to a serine (S) in the PFMR group (plus *S. discolor* and *S. vitellina*). Nearly all avian genomes (66 of 68<sup>26</sup> have either lysine (K) or R at this site, and no other S mutations have been characterized.

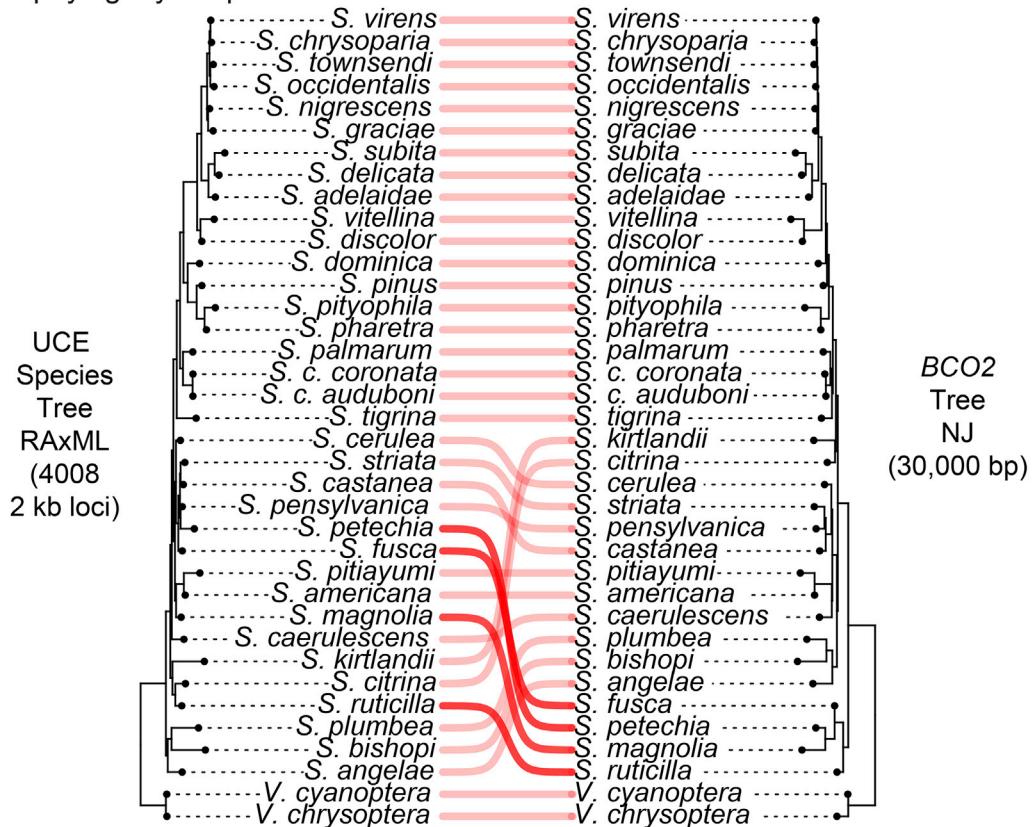
We interpret these findings at *BCO2* at three levels. First, it is consistent with at least two bouts of introgression, where a

larger fragment containing *BCO2* moved among the species in the PFMR group after their ancestral lineages diverged. The high *BCO2* PBS in *S. petechia* (Figure 3E) is likely a signal of this introgression. Second, introgression also occurred into the common ancestor of *S. discolor* and *S. vitellina*. The resulting 3-kb fragment presumably represents only a small segment of the original introgressed region yet includes the putative functional mutation that underlies carotenoid coloration. Its much smaller size compared to the block that introgressed among the PFMR group suggests introgression with the ancestor of *S. discolor* and *S. vitellina* occurred earlier.<sup>32</sup> Finally, the PFMR group clade appears basal to the main *Setophaga* radiation in the *BCO2* tree (Figures 4 and S2). This could indicate this haplotype was originally from a warbler outside the genus, although additional data will be needed to fully test this scenario.

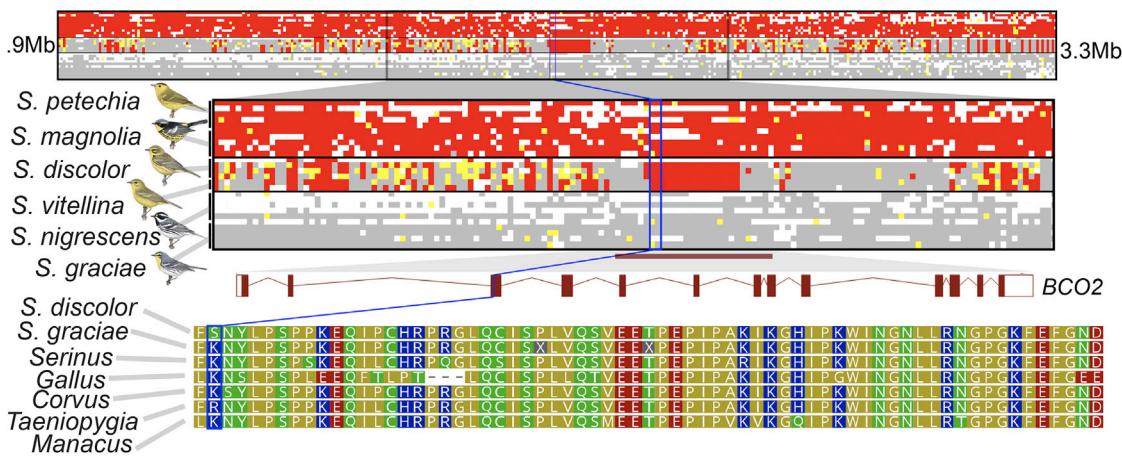
We predict that these changes in the 3<sup>rd</sup> exon of *BCO2* affect the function of the enzyme (e.g., Wu et al.<sup>33</sup>) and thus carotenoid feather pigmentation. Recent work in mosaic and urucum canaries found that downregulation<sup>26</sup> or disruption<sup>27</sup> of *BCO2* increased carotenoid deposition in feathers or bare parts, respectively, reflecting the carotenoid degradation properties of *BCO2*.<sup>27</sup> Thus, we speculate that the mutations in the 3<sup>rd</sup> exon of the PFMR group—plus *S. discolor* and *S. vitellina*—may change the function of *BCO2* and facilitate the broader deposition of carotenoids across the body feathers. We suggest future functional assays of these different *BCO2* variants in expressed cell lines, as has been done for *SCARB1*.<sup>34</sup> Movement of carotenoid genes between fungi and aphids is one of the most famous examples of horizontal gene transfer involving animals,<sup>35</sup> but introgression of *BCO2* among warblers would represent the best evidence so far for functional consequences of carotenoid gene movement among wild vertebrates.

Because parulid warblers have broadly overlapping ranges,<sup>36</sup> and different combinations of species likely came into and fell out of contact multiple times during the Pleistocene,<sup>37,38</sup> their biogeographic history has likely given them many opportunities to exchange genes compared to other groups that have been more consistently allopatric. Further, several species form hybrid zones (*Vermivora*,<sup>17</sup> *Setophaga*,<sup>19,39</sup> and *Myioborus*<sup>40</sup>), and multiple adult inter-generic hybrids have been confirmed.<sup>41,42</sup> Given

**A Co-phylogeny of species tree and BCO2 tree**



**B Genotypes at BCO2 region, exon structure, and exon 3 amino acid alignment**



**Figure 4. Multiple Bouts of Introgression at BCO2**

(A) Co-phylogeny comparing the UCE species tree with the BCO2 tree. The opaque red curves highlight discordant relationships among the PFMR group (see also Figure S4).

(B) Genotypes for high- $F_{ST}$  SNPs for the BCO2 region. Homozygous genotypes from two PFMR species are in red, gray genotypes represent homozygous genotypes for two non-PFMR species, and yellow shows heterozygous genotypes (white, missing). The middle rows include S. discolor and S. vitellina, which are homozygous for PFMR-group genotypes at this region (Figure S2). This introgressed region includes an AA substitution in exon 3, unique to the PFMR group plus S. discolor and S. vitellina. Warbler illustrations © Lynx Edicions.

this high frequency of hybridization, Parkes<sup>43</sup> speculated that many parulids have remained genetically compatible long after they evolved major phenotypic differences. The new molecular

data presented here confirm that historical hybridization, as well as independent evolution in a common set of pigmentation genes, has likely fueled their evolution and diversity.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead Contact
  - Materials availability
  - Data and code availability
- METHOD DETAILS
  - Sampling and DNA extraction
  - Reference genome
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Reference genome
  - Population genomic bioinformatic pipeline
  - Phylogenomic bioinformatic pipeline

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.cub.2020.10.094>.

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## AUTHOR CONTRIBUTIONS

Conceptualization, M.B.D., D.P.L.T., and I.J.L.; Investigation, M.B.D., A.W.W., A.B., and D.P.L.T.; Resources, I.J.L. and D.P.L.T.; Data Curation, M.D.B. and D.P.L.T.; Writing – Original Draft, M.D.B. and D.P.L.T.; Writing – Review and Editing, all authors; Visualization, M.D.B. and D.P.L.T.; Supervision and Project Administration, D.P.L.T.; Funding Acquisition, A.B., I.J.L., and D.P.L.T.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
Warbler blood and tissue samples	<sup>22</sup>	<a href="https://doi.org/10.5061/dryad.n02v6wwv">https://doi.org/10.5061/dryad.n02v6wwv</a>
Chemicals, Peptides, and Recombinant Proteins		
QIAGEN DNeasy Blood and Tissue kit	QIAGEN	Cat# 69504
UPrep Spin Columns	Genesee Scientific	Cat# 88-143
Qubit Fluorometer	ThermoFisher Scientific	Cat# Q33239
TruSeq Nano DNA	Illumina	20015964
Deposited Data		
Reference <i>Setophaga coronata</i> genome	This paper	NCBI# PRJNA325157
Whole genome re-sequencing <i>Setophaga</i> data	This paper	NCBI# PRJNA630247
Software and Algorithms		
mitoBIM 1.9.1	<sup>44</sup>	<a href="https://github.com/chrishah/MITObim">https://github.com/chrishah/MITObim</a>
AdapterRemoval 2.1.1	<sup>45</sup>	<a href="https://github.com/MikkelSchubert/adapterremoval">https://github.com/MikkelSchubert/adapterremoval</a>
BowTie2 2.3.5.1	<sup>46</sup>	<a href="http://bowtie-bio.sourceforge.net/bowtie2/">http://bowtie-bio.sourceforge.net/bowtie2/</a>
PicardTools 2.20.8	Broad Institute	<a href="https://broadinstitute.github.io/picard/">https://broadinstitute.github.io/picard/</a>
ANGSD 0.929	<sup>47</sup>	<a href="http://www.popgen.dk/angsd/index.php/ANGSD">http://www.popgen.dk/angsd/index.php/ANGSD</a>
Samtools 0.1.18	<sup>48</sup>	<a href="http://www.htslib.org/">http://www.htslib.org/</a>
Seqtk 1.3	<a href="https://github.com/lh3/seqtk">https://github.com/lh3/seqtk</a>	<a href="https://github.com/lh3/seqtk">https://github.com/lh3/seqtk</a>
Geneious 11.0.3	Geneious	<a href="https://www.geneious.com/">https://www.geneious.com/</a>
Phyluce 1.6	<sup>49</sup>	<a href="https://phyluce.readthedocs.io/en/latest/">https://phyluce.readthedocs.io/en/latest/</a>
Phytools 0.6-60	<sup>50</sup>	<a href="https://cran.r-project.org/web/packages/phytools/">https://cran.r-project.org/web/packages/phytools/</a>

## RESOURCE AVAILABILITY

## Lead Contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, David P. L. Toews ([toews@psu.edu](mailto:toews@psu.edu))

## Materials availability

This study did not generate new unique reagents.

## Data and code availability

All code used in the bioinformatic pipeline to analyzed re-sequencing data can be found in <sup>22</sup>. The “Version 2.0” *Setophaga coronata* genome can be found at NCBI #PRJNA325157. Re-sequencing data is deposited at NCBI# PRJNA630247.

## METHOD DETAILS

Information about sample origin and sample ID can be found in <sup>22</sup>; available at DataDryad <https://doi.org/10.5061/dryad.n02v6wwv>. All procedures were performed in accordance with procedures outlined in the approved IACUC at Cornell University (#015-0065) and Pennsylvania State University (#201900879). Bird banding and blood sampling was approved by the USGS (Master Banding Permit to DPLT #24043) and NYDEC (banding permit to DPLT #156).

## Sampling and DNA extraction

The majority of samples (117 of 166) were DNA extracted from tissues loaned from several museums<sup>22</sup>. For DNA extraction, we used 2 mm<sup>3</sup> of tissue digested in buffer ATL and AL and washed with AW1 and AW2 of the QIAGEN DNeasy Blood and Tissue kit—following the tissue extraction procedure—and separated DNA using UPrep spin columns (Genesee). For the remaining 49 samples, we used blood samples. Most of these were from species of significant conservation concern, or endemic to Caribbean Islands, so obtaining tissue samples is logistically challenging, and thus relied on previously collected blood in buffer.

We used 75  $\mu$ L of blood and followed the same QIAGEN DNA extraction procedure as above, except using the blood extraction protocol.

We standardized DNA concentrations after quantifying with a Qubit fluorometer, and then generated sequencing libraries with the Illumina TruSeq Nano kit, targeting 350 bp insert sizes. We individually indexed each sample and sequenced the libraries with Illumina NextSeq 500. We sequenced 24 individuals together at a time on a single lane, with paired-end 150bp chemistry, and used seven NextSeq lanes in total. Data from the 10 *Vermivora* individuals have been published previously<sup>17</sup>.

### Reference genome

We generated a new chromosome-level reference genome by combining the fragment libraries (SRA accession number SRX2019496) and mate-pair libraries (SRA accession numbers SRX2019494 and SRX2019495) from the original *Setophaga coronata* assembly, with chromosome conformation capture data, generated by Dovetail Genomics, from a newly sampled individual. This new *S. coronata* individual (USGS band #284029498) was sampled in Centre County, PA (latitude: 40.723591, longitude:  $-77.791108$ ) on June 12<sup>th</sup>, 2019.

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Reference genome

To generate the new assembly (NCBI accession #PRJNA325157), we used the Dovetail “Meraculous” pipeline. The new scaffolding data came from Hi-C and Chicago libraries using the Dovetail “Hi-Rise” pipeline. We manually adjusted the fourth largest scaffold outputted from the pipeline, as the Hi-C report and subsequent alignments suggested that various segments of it each matched a different micro-chromosome in the Zebra Finch genome. To generate the full mitochondrial genome sequence, we used the *S. coronata* tissue sample “CUMV4915” and ran mitoBIM<sup>44</sup> using the “-quick” option and the complete mitochondrial genome of a Canada warbler (*Cardellina canadensis*; NCBI accession number MK033135) as bait.

### Population genomic bioinformatic pipeline

We generated resequencing data for 156 individuals across 34 *Setophaga* species (range 1-15, mean = 4.45, individuals per species). We first used the program AdapterRemoval<sup>45</sup> to trim and collapse overlapping paired reads with the following options: “-collapse -trimns -minlength 20 -qualitybase 33.” We then used BowTie2<sup>46</sup> to align reads to the new reference genome with the “very-sensitive-local” presets and set the option “-X” (the maximum fragment length for valid paired-end alignments) to 700 bp. We used PicardTools (2.20.8; <https://broadinstitute.github.io/picard/>) to mark PCR duplicates.

To estimate  $F_{ST}$ , we used ANGSD<sup>47</sup> version 0.929, which accounts for genotyping uncertainty in low-coverage data, and estimated  $F_{ST}$  for non-overlapping 10-kb windows. For each population, we calculated the site allele frequency likelihoods using the “-dosaf 1” command. We then calculated the two-population site frequency spectrum and resulting  $F_{ST}$  estimates using “realSFS.” To calculate population branch statistics (PBS) for *S. petechia*, *S. pensylvanica*, and *S. striata*, we used a similar approach, calculating site allele frequency likelihoods for each species, and then calculating all the pairwise two population site frequency spectra. These were then used by “realSFS” with the options “-win 10000 -step 10000” to calculate the sliding-window PBS statistic for each of the three species. To calculate *D*-statistics, we used the “-doAbbababa2” function, also in ANGSD, using the options “-blockSize 10000 -doCounts 1.” For the analysis of *S. petechia*, we used it and *S. striata* and *S. magnolia* as populations 1, 2, and 3, respectively, and *Vermivora chrysoptera* as the outgroup (with the “-useLast 1” option).

To identify shared clusters of  $F_{ST}$  differentiation between independent sister species pairs, we focused our analysis on nine pairs that the UCE tree identified as sister taxa (Figure S1 shows the nine pairs included in the analysis). Given the species-status uncertainty between *S. c. coronata* and *S. c. auduboni*, we only include the nominate form (*S. c. coronata*) in this analysis and compare it to *S. palmarum*. Some possible sister taxon comparisons were excluded due to their limited sample sizes<sup>22</sup>.

We first binned the reference genome into 991, 1-Mb windows, excluding the mitochondrial genome, as well as small scaffolds not assigned to chromosomes. Then, for each pair, we used the output from 10-kb non-overlapping  $F_{ST}$  window analysis and identified the top 1% of windows (referred throughout as the ‘top 1%’ windows). This resulted in 880-1108 of the most divergent windows, depending on the species pair. We then ran a permutation sampling of 991 10-kb windows—with 10,000 replicates—to determine how many of these ‘top 1%’ windows would be expected to fall in the same larger 1-Mb window by chance. For each replicate we calculated the maximum number of 10-kb windows in each of these larger windows. Across these 10,000 replicates the maximum number of ‘top 1%’ windows occurring in a single 1-Mb window by chance was 7, and therefore used 8 ‘top 1%’ windows as our threshold to delimit the highly divergent, large  $F_{ST}$  peaks.

### Phylogenomic bioinformatic pipeline

To delineate regions analyzed for *ASIP* and *BCO2* we first identified the divergent regions near these genes between *Vermivora chrysoptera* and *V. cyanoptera* in the new *S. coronata* reference genome. *V. chrysoptera* and *V. cyanoptera* are extremely similar in their nuclear genome, except for six small regions (including *ASIP* and *BCO2*) that stand out against this background<sup>17</sup>. For *ASIP*, the divergent region spans nearly 100 kb, including a large portion in the presumed promoter region as well as the full coding region. For *BCO2*, the divergent region between *Vermivora* species spans 30 kb, which includes the upstream portion of the gene, as

well as the first four exons. The locations used for *ASIP* in the new reference assembly are on chromosome 20, between 4,215,000-4,315,000 bp; for *BCO2*, these are on chromosome 24, between 1,575,000-1,605,000 bp.

For the phylogenetic analyses, we combined data for all the individuals in each species to produce a single sequence file. We did this to generate sequence data with sufficiently high coverage to avoid large amounts of missing data. Therefore, to generate fasta files, we used the “mpileup” command in samtools version 0.1.18<sup>48</sup>, the “call -c” command in bcftools version 1.10.2, and the “vcf2fq” option to generate fastq files. Finally, we used the program seqtk (version 1.3; <https://github.com/lh3/seqtk>) to translate into fasta format. Any gaps without sequence coverage were manually closed in the alignments using Geneious (version 11.0.3). To generate trees for *ASIP* and *BCO2*, we used the Geneious Tree Builder to generate Neighbor-Joining trees using the HKY distance model.

To generate a species tree, we used ultra-conserved element (UCE) loci. To locate UCEs, we aligned the “Tetrapods-UCE-5Kv1” probe set<sup>51</sup> to the *S. coronata* reference genome using BowTie2. We removed all but the P1 probes, as well as 175 UCEs that did not align, 7 UCEs that did not align to a chromosome (i.e., they aligned to small, unplaced scaffolds), and 42 UCEs that aligned to > 1 position in the reference assembly. We then trimmed the probes such that they were at least 3,000 bp apart from any subsequent UCE locus. This resulted in 1,000 base pairs from either side (i.e., 2000 bp total) from a final set of 4,008 UCE loci.

We utilized the phyluce phylogenomics pipeline (version 1.6) to produce the species tree<sup>49</sup>. We extracted UCE loci from each species with seqtk by filtering the fasta files for the regions identified above as UCE loci. We aligned all loci using the MAFFT aligner option and retained only those alignments that included all target species (4,008 UCE loci). We then concatenated UCE loci for all species into a phylip file using the command “phyluce\_align\_format\_nexus\_files\_for\_raxml” and ran RAxML (-m GTRGAMMA) with 1000 bootstrap replicates. To compare the gene trees for *ASIP* and *BCO2* to the species tree, we used the “co.phylo” function in phytools version 0.6-60<sup>50</sup>.