Genomic identification of intergeneric hybrids in New World wood-warblers (Aves: Parulidae)

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The documentation of hybrids between distantly related taxa can illustrate an initial step to explain how genes might move between species that do not exhibit complete reproductive isolation. In birds, some of the most phylogenetically distant hybrid combinations occur between genera. Traditionally, morphological and plumage characters have been used to assign the identity of the parental species of a putative hybrid, although recently, nuclear introns also have been used. Here, we demonstrate how high-throughput short-read DNA sequence data can be used to identify the parentage of a putative intergeneric hybrid, in this case between a blue-winged warbler (Vermivora cyanoptera) and a cerulean warbler (Setophaga cerulea). This hybrid had mitochondrial DNA of a cerulean warbler, indicating the maternal parent. For hundreds of single nucleotide polymorphisms within six regions of the nuclear genome that differentiate blue-winged warblers and golden-winged warblers (Vermivora chrysoptera), the hybrid had roughly equal ancestry assignment to blue-winged and cerulean warblers, suggesting a blue-winged warbler as the paternal parent species and demonstrating that this was a first generation (F1) hybrid between these species. Unlike other recently characterized intergeneric warbler hybrids, this individual hybrid learned to song match its maternal parent species, suggesting that it might have been the result of an extra-pair mating and raised in a cerulean warbler nest.

ADDITIONAL KEYWORDS: birds – genomics – hybridization.

INTRODUCTION

In his paper, ‘Still another Parulid intergeneric hybrid ...’, Kenneth Parkes (1978) described the morphological evidence for a putative hybrid between two species of wood-warbler (Aves: Parulidae). In this case, the hybrid was between a black-and-white warbler [Mniotilta varia (Linnaeus, 1766)] and a cerulean warbler [Setophaga cerulea (Wilson, 1810)]. Parkes (1978) interpreted this finding from both taxonomic and evolutionary perspectives, with a viable hybrid between such deeply diverged taxa having implications for understanding the reproductive barriers among members of the wood-warbler family. Since that time, the theoretical understanding of avian speciation has advanced (Price, 2008), as has knowledge of phylogenetics based on molecular markers (Lovette et al., 2010). The documentation of intergeneric hybrids, although still of interest, now provides less taxonomic insight, depending on the prevalence of hybrids, owing to the widespread recognition of
the modern biological species concept that allows for low levels of hybridization between distinct taxonomic species. However, hybrids between divergent taxa can still reveal how reproductive boundaries, or lack thereof, among species could facilitate the movement of genes among distinct taxa (Ottenburghs, 2019; Grant & Grant, 2020) and how different characters, such as coloration, vocalization and habitat preference, are inherited (Toews et al., 2016; Brelsford et al., 2017).

Vallender et al. (2009) was one of the first to document a putative intergeneric hybrid between bird species (and its probable parental species) using molecular markers. In that case, Vallender et al. (2009) used a DNA sequence from a nuclear intron and a mitochondrial DNA (mtDNA) gene to compare the sequence of the hybrid with these same sequences in other parulid warblers and identified the parental species as a black-and-white warbler and a myrtle warbler [Setophaga coronata coronata (Linnaeus, 1766)]. Incorporation of molecular data is an objective approach to parental species identification, which contrasts with some previous studies that relied solely on interpretations of morphometric, plumage or bioacoustic characters to assign parentage (Parkes, 1961, 1978). More recent documentation of hybrid warblers has also used mtDNA and nuclear DNA in addition to plumage characters to confirm hybrid identity (Ralston et al., 2015; Toews et al., 2018b; Trimbath et al., 2019).

High-throughput sequencing provides an even more robust means of assigning parental taxa compared with matching sequences from a small number of nuclear introns. Short-read data from across the genome of a putative hybrid can be compared against the same genetic sequences of putative parental taxa. Although such high-throughput sequencing generates more data than necessary for simply ascertaining species parentage, there are several advantages to this approach. First, because of the random nature of sequencing library preparation, high-throughput sequencing does not require the optimization of polymerase chain reaction primers, which can be difficult to develop when comparing divergent taxa that might have diverged in their priming sites. Second, nuclear introns evolve slowly; therefore, even if optimization and amplification are successful, informative DNA sites might not be present within the intron. Finally, nuclear introns can be difficult to sequence in samples that are old or contain degraded DNA; this issue is not as problematic with short-read sequencing technologies (Billerman & Walsh, 2019). Here we demonstrate the efficacy of using short-read sequencing to identify the parentage of a putative intergeneric avian hybrid.

MATERIAL AND METHODS

FIELD SITE

A putative hybrid (Fig. 1D–F) was encountered in the Sandhill Wildlife Area, Wood County, WI, USA (44.356°N, 90.180°W) in May 2017, during an ongoing study of blue-winged warblers (Vermivora cyanoptera Olson & Reveal, 2009) and golden-winged warblers (Vermivora chrysoptera (Linnaeus, 1766)) (Kramer et al., 2018). All fieldwork was conducted under protocol no. 1004A80575, approved by the University of Minnesota Institutional Animal Care and Use Committee and US Geological Survey (USGS) Bird Banding Permit #21631 (D.E.A.). The bird was first observed on 29 May 2017. On 31 May 2017, G.R.K. and B.E.C. captured the bird in a mist net using broadcasts of blue-winged and golden-winged warbler songs. The putative hybrid was photographed, measured, and had a coloured and a USGS aluminum band affixed to its legs. G.R.K. and B.E.C. also took a small blood sample from the brachial vein using a needle and capillary tube, which was stored in Queen’s lysis buffer (Seutin et al., 1991) and left at ambient temperature until it was returned to the laboratory for analysis. The putative hybrid was observed multiple times during additional visits to the site and exhibited behaviour consistent with defending a territory (singing from a prominent perch, etc.). G.R.K. also recorded singing of the putative hybrid on 3 June 2017 (Fig. 2; Xeno-Canto accession #XC554152).

GENETIC ANALYSES

Our genetic analyses included three components. First, we sequenced mitochondrial DNA to determine the maternal ancestry of the putative hybrid. Second, we used high-throughput short-read data from the putative hybrid, but aligned those reads to previously sequenced nuclear introns, which have also been sequenced in all parulid warblers, to confirm the maternal species and identify the paternal parent species. Third, we used short-read data from the presumed parental species to provide further insight into the genomic composition of the hybrid.

MITOCHONDRIAL DNA SEQUENCING

We first extracted DNA from the blood sample obtained from the putative hybrid, using Qiagen DNAeasy spin columns (Qiagen, Valencia, CA, USA). To determine the maternal ancestry of the hybrid, we amplified a portion of the mitochondrial genome using primers for the NADH dehydrogenase subunit 2 (ND2) gene and protocols from Drovetski et al. (2004). We then sequenced these products on an ABI 3730 Genetic Analyzer at the Case Western Reserve University Genomics Center (Cleveland,
OH, USA) using BigDye Terminator 1.1 and 3.1 Cycle sequencing kits (Applied Biosystems, Foster City, CA, USA). We aligned sequences against $ND2$ sequences from other Parulidae warblers available on GenBank using Geneious v.9.0 (Kearse et al., 2012).

**Short-read alignment to nuclear introns**

We then generated short-read genomic data from the hybrid (and putative parental species, see Results section below) using an Illumina TruSeq Nano library preparation kit (Illumina, San Diego, CA, USA) targeting a 350 bp insert size. In each case, samples were individually indexed, although they were included within a larger warbler sequencing project consistently running 24 individuals per lane on an Illumina NextSeq 500. We used the paired-end 150 bp sequencing chemistry.

We compared read data in the hybrid with previously published nuclear intron sequence data deposited in GenBank both to confirm the maternal parent species (ascertained from the mtDNA sequencing, above) and to identify the putative paternal parent. To do this, we first aligned the short-read data from the hybrid to a concatenated assembly of six nuclear introns that had been sequenced previously across every extant species of parulid warbler (we used Setophaga coronata sequences as a reference to align the reads from the hybrid; Lovette et al., 2010). We mapped the reads for the hybrid to the concatenated intron sequence using BOWTIE2 (Langmead & Salzberg, 2012), using the ‘very sensitive local’ set of alignment pre-sets. From this resulting read alignment, we extracted raw reads in Geneious (v.9.0). For one intron region that we found to have both sufficient coverage (i.e. more than ten reads) and heterozygous sites, we used BLAST to compare the species ancestry from previously sequenced DNA. This method allowed us provisionally to identify the putative paternal parent species and to confirm the maternal parent species (see Results).

**Genomic comparison of hybrid with putative parental species**

The analyses described above indicated that $Vermivora$ spp. × cerulean warbler ($S$. cerulea) were the most likely parents. We then conducted additional analyses that also incorporated short-read genomic data from these likely parental taxa. We obtained nuclear genomic data from the putative parental species from several sources. Initially, we used previously published whole-genome sequencing data from ten $Vermivora$ warblers ($N = 5$ golden-winged and $N = 5$ blue-winged warblers; Toews et al., 2016) derived from blood obtained from wild-caught birds and which are phenotypically representative of the ends of the $Vermivora$ plumage spectrum (Toews et al., 2016).

We also included new sequence data from cerulean warblers ($N = 5$). Four of these samples were from...
Figure 2. Song spectrograms of the putative hybrid (A; deposited at Zeno-Canto #XC554152), a cerulean warbler (B), a blue-winged warbler (C), and a golden-winged warbler (D). Songs of the golden-winged warbler and the putative hybrid were recorded at the same time and location, in west-central Wisconsin, USA. Songs in B and C are from representative recordings obtained from the Macaulay Library.
tissues loaned from the Museum of Natural Science at Louisiana State University (LSUMZ B-3352, LSUMZ B-3353, LSUMZ B-35040 and LSUMZ B-31203). One cerulean warbler sample also came from blood collected by D.P.L.T. in Monongahela National Forest, West Virginia in 2017 and stored in 0.5% sodium dodecyl sulphate lysis buffer. In each case, we also used the Qiagen DNAeasy spin columns for DNA extraction. We note that the *Vermivora* sequence data published by Toews et al. (2016) were generated using the Illumina TruSeq PCRFree library preparation kit, targeting a 350 bp insert size.

We compared the genomic variation of the hybrid with new genome data from these putative parental taxa. We initially trimmed and collapsed overlapping read pairs across all 16 samples using ADAPTERREMOVAL (Lindgreen, 2012). We then mapped individual reads to a subset of the assembly of the myrtle warbler (*S. c. coronata*) genome assembly (Toews et al., 2016). This subset included seven warbler scaffolds, six of which were previously shown to be divergent between golden-winged warblers and blue-winged warblers (warbler scaffolds 24, 38, 120, 299, 563 and 653). We also included an additional large scaffold that does not include single nucleotide polymorphisms (SNPs) divergent between *Vermivora* species (warbler scaffold 30). This set of scaffolds covers 36 Mbp of DNA, or ~3% of the warbler genome.

To map reads to the myrtle warbler reference genome, we again used BOWTIE2 (Langmead & Salzberg, 2012). These analyses included only multivariate statistics [e.g. principal components analysis (PCA) and admixture analysis] and therefore did not require individual genotype calls, which can be more difficult to estimate accurately with low-to-moderate coverage data such as those used here. Therefore, we used genotype probabilities from the alignment files as implemented in the ANGSD bioinformatics pipeline (Korneliussen et al., 2014). We estimated allele frequencies from genotype likelihoods with the ‘-doMajorMinor 1’ function. We then used this as input for the program PCANGSD (Fumagalli et al., 2013).

To determine the sex of the hybrid, we used the bioinformatic sex determination method discussed by Toews et al. (2018a). For this, for each individual we compared the ratio of the average read depth from an autosomal scaffold (warbler scaffold 30) with a known Z chromosome scaffold (warbler scaffold 24). Given that they have two copies, we expect male birds to have roughly similar read depths between autosomes and a Z chromosome, whereas females (hemizygous) will have lower read depth.

To confirm the parental species, we first analysed all of the sequence data across the seven scaffolds from the full set of 16 individuals [blue-winged warblers (*N* = 5), golden-winged warblers (*N* = 5), cerulean warblers (*N* = 5) and the putative hybrid (*N* = 1)] using a PCA. Next, to determine the likely *Vermivora* ancestry of the putative hybrid, we restricted the analysis to only those regions along the six scaffolds that have been shown previously to be divergent between golden-winged warblers and blue-winged warblers (Toews et al., 2016). We used the ‘-admix’ function of PCANGSD to assign admixture proportions for each of the individuals, drawn from three putative ancestral populations (i.e. ‘-admix_K’ = 3, considering blue-winged warblers, golden-winged warblers and cerulean warblers). All computational analyses were run on the Institute for Computational and Data Sciences’ Advanced Cyberinfrastructure System at Pennsylvania State University.

RESULTS AND DISCUSSION

We observed and subsequently captured the putative hybrid in a willow (*Salix spp.*) and alder (*Alnus sp.*) wetland within a broader landscape of diverse mixed-age deciduous forest. The putative hybrid occupied and defended a territory with three to five territorial male golden-winged warblers in the general vicinity. Although blue-winged warblers bred in the area (i.e. at Sandhill Wildlife Area), they were never detected at the same location as the putative hybrid during any survey. *Vermivora* warblers were common across Sandhill Wildlife Area, whereas cerulean warblers were uncommon and typically absent (or undetected) from sites that were surveyed for *Vermivora* warblers, probably owing to their very different nesting habitat requirements. Cerulean warblers nest in the canopy of mature forests, whereas *Vermivora* warblers nest on the ground in, or very near to, early successional forest patches (Confer et al., 1992; Gill et al., 2001; Buehler et al., 2013). Interestingly, we only observed the putative hybrid using cover types associated with *Vermivora* warblers that were ~150 m from the nearest mature forest potentially suitable for breeding cerulean warblers.

GENETIC RESULTS

Using bioinformatic sex determination, we confirmed that the putative hybrid was male: the read depth ratio of the autosomal scaffold to the Z chromosome scaffold in the hybrid was 1.16; in known males, this ranged from 1.11 to 1.21 (*N* = 19), and in known females it ranged from 0.802 to 0.809 (*N* = 2). The mitochondrial *ND2* region of the putative hybrid aligned with 99.8% identity to a cerulean warbler sequence, confirming the ancestry of the maternal parent (the next strongest alignment was *Setophaga townsendi* at 93.94%). From the intron
alignment, we identified one region in **RHO-1** that had reasonable coverage (15 reads) and was also heterozygous in the hybrid (five polymorphic SNPs within 148 bp). Of these 15 reads, three differed from the other 12 across the same five sites. Extracting representative raw reads from these two putative haplotypes (which we denote as different ‘read-groups’ below), BLAST indicated the top hit (98.67% identity) for ‘read-group 1’ as *V. cyanoptera* (NCBI accession #GU932517). For ‘read-group 2’, the top hit (99.32%) was *S. cerulea* (NCBI accession #EU815776).

Within **RHO-1**, three of the heterozygous SNPs were highly informative. At position 287, ‘T’ is only observed in *S. cerulea*, whereas all other parulids have ‘A’. The hybrid was heterozygous for ‘T/A’. At position 290, ‘G’ is found only in *Vermivora* spp., where all other parulid warblers have ‘A’. The hybrid was also heterozygous for G/A at this site. These two SNPs provide strong evidence that the parents of the hybrid were *S. cerulea* and *Vermivora* spp. We note that there are possible sequencing errors introduced in this raw read data and that each parulid species is represented by only a single intron sequence. However, this assessment is highly consistent with our other, more detailed sequencing analysis, where we included genomic data from both the putative parental species. Importantly, this approach, wherein we only analysed short-read genomic data from the putative hybrid aligned to intron sequences from many warbler species, is a useful method for identifying the provisional species ancestry. This is true for taxa with some previous sequence data available, such as the nuclear intron data, which in this instance were originally used to generate the parulid phylogeny (*Lovette et al.*, 2010).

The comparison of the genomic data of the putative hybrid with samples of these putative parental taxa confirmed their identities and also provided additional insight into its ancestry. Sample information and accession numbers are reported in Table 1. Across the reduced genome assembly (i.e. 36 Mbp of the warbler genome), we obtained 4 844 754 sequence reads from the putative hybrid. This equated to at least one or two reads across 95% of the seven scaffolds, with a mean coverage of 14.6× across the 36 Mbp. From the PCA, including all SNPs along all seven scaffolds, two clusters separated strongly along principal component (PC) 1, distinguishing individuals by genus (i.e. *Setophaga* vs. *Vermivora*). The first PC axis explained 11.1% of the variance, whereas all others axes explained < 2%. The putative hybrid fell between these two clusters (Fig. 3).

We then restricted our analysis to only the six genomic regions that distinguish between golden-winged warblers and blue-winged warblers and estimated admixture across these regions. The putative hybrid had a roughly equal mix between *S. cerulea* and *V. cyanoptera* (Fig. 4). We did not detect any ancestry from *V. chrysoptera*, suggesting that the paternal parent of the hybrid was *V. cyanoptera*. Our use of short-read resequencing data in identifying the parental species of this hybrid meant that we were able to assign parentage with certainty that would not have been possible using a small number of nuclear introns, including confirmation that this was an F₁ hybrid rather than a later backcross, because the genetic differences between golden-winged warblers and blue-winged warblers are small and few in the genome (*Toews et al.*, 2016).

**PLUMAGE, MORPHOMETRICS AND SONG**

The plumage of the putative hybrid indicated that it was an adult (after second year). Adults of both parent species possess plumage characters that allow for the identification of sex: blue-winged warbler males have a black eye-line and a bright yellow breast; cerulean warbler males have a solid, blue ‘necklace’ and bright blue backs (Fig. 1A–C). Females of both species have duller plumage overall, and female cerulean warblers often lack a solid necklace. The plumage of the hybrid had none of the traits typically associated with males of either parent species. However, the putative hybrid had an enlarged cloaca, no brood patch and was actively singing and defending a territory, suggesting that it was male.

Generally, the putative hybrid possessed diluted plumage traits of both species (Fig. 1D–F). The
of characteristics of both parent species, but lacked the typical plumage markers associated with males of either species (i.e. brightly coloured plumage, highly contrasting markings), and thus would not be able to be diagnosed based on appearance alone.

The measurements of the putative hybrid suggested an intermediate morphology between *Vermivora* and *Setophaga* warblers: wing chord length of the putative hybrid was 61 mm, exposed culmen length 9 mm and tail length 45 mm. For *V. cyanoptera*, Toews et al. (2018b) reported an average of 59.8 mm for wing chord and 8.04 mm for culmen, and Gill et al. (2001) reported a tail length of 50.9 mm. For *S. cerulea*, Buehler et al. (2013) report 64.5 mm for average wing chord length, 9.6 mm for average culmen length and 42.4 mm for average tail length.

We observed and recorded the putative hybrid singing songs characteristic of a cerulean warbler (Fig. 2). These songs consisted of three parts, with each part higher in frequency than the previous one. The longest and most distinct section of the song (a component that appears to be identical between the putative hybrid and typical cerulean warbler songs) is in the second section of the song, which typically includes five two-element phrases. These phrases each begin with two short notes, followed by a clear slur, with a downward pitch profile (e.g. comparing the phrase at 20 s for the putative hybrid and 31.2 s for the cerulean example; Fig. 2). These songs differed strongly from the simpler, ‘buzzy’ notes of the songs of golden-winged warblers or blue-winged warblers (Fig. 2C, D). We did not observe or record any song switching from a cerulean warbler song to a *Vermivora* warbler song by the putative hybrid.
regardless of which song type was being broadcasted, as has been observed in *Vermivora* warblers (Kramer et al., 2020). However, the putative hybrid was responsive and aggressive toward broadcasts of both *Vermivora* warbler and cerulean warbler calls and songs. We note that the example spectrogram of the golden-winged warbler (Fig. 2D) is from a song on the same recording as the putative hybrid (i.e. a neighbouring bird).

**CONCLUSION**

Combining the genetic analyses, morphology, plumage, habitat and vocalization patterns, these findings suggest that this hybrid learned the song of its maternal parent species, a cerulean warbler, and not the song of its blue-winged warbler father. This contrasts with song inheritance patterns observed in many species of birds and in other divergent Parulidae hybrids, where hybrids either learned the song of the paternal parent (e.g. *Setophaga* pennsylvanica × *Vermivora* spp., Toews et al., 2018b; and *S. cerulea × Setophaga parula*, Trimbath et al., 2019) or sang a type of intermediate song (e.g. *M. varia × S. coronata*; Vallender et al., 2009). For the hybrid to have learned a cerulean warbler song, it would have needed to have been exposed to cerulean warbler tutors during the critical window of song learning, which is thought to be within the first 100 days of life in warblers (Kroodsma & Meservey, 1983). This suggests that the hybrid might have been the result of an extra-pair mating.

One scenario that appears plausible to us is that an extra-pair blue-winged warbler mated with a female cerulean warbler and that the offspring was raised in a cerulean warbler nest and had a cerulean warbler social father, from which this bird learned its song. Albeit highly speculative, this is consistent with a scenario where the hybrid was raised in the canopy of a mature forest, learned its song and subsequently moved to early successional forest (where cerulean warblers are rarely observed). This is consistent with a genetic component to habitat choice, in which *Vermivora* alleles are dominant; however, there are many other environmental factors that could have contributed to movement to a different type of cover.

We have used short-read DNA resequencing data to identify the parents of an intergeneric hybrid between a cerulean warbler and a blue-winged warbler. Although other intergeneric hybrids within Parulidae have been reported previously, the only other documented hybrid between *Vermivora × Setophaga* was reported only recently (Toews et al., 2018b). It is likely that ‘still more’ intergeneric hybrids will be discovered with new genomic data. Indeed, our study is the first to use short-read DNA resequencing data to identify a putative avian intergeneric hybrid, and we believe that similar approaches will become more common as genomic resources continue to develop for other taxa.

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**REFERENCES**


