

Research



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Evolutionary biology

A wood-warbler produced through both interspecific and intergeneric hybridization

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Hybridization between divergent taxa can provide insight into the breakdown of characters used in mate choice, as well as reproductive compatibility across deep evolutionary timescales. Hybridization can also occur more frequently in declining populations, as there is a smaller pool of conspecific mates from which to choose. Here, we report an unusual combination of factors that has resulted in a rare, three-species hybridization event among two genera of warblers, one of which is experiencing significant population declines. We use bioacoustic, morphometric and genetic data, to demonstrate that an early generation female hybrid between a golden-winged warbler (*Vermivora chrysoptera*) and a blue-winged warbler (*V. cyanoptera*) went on to mate and successfully reproduce with a chestnut-sided warbler (*Setophaga pensylvanica*). We studied the product of this event—a putative *chrysoptera* × *cyanoptera* × *pensylvanica* hybrid—and show that this male offspring sang songs like *S. pensylvanica*, but had morphometric traits similar to *Vermivora* warblers. The hybrid's maternal parent had *V. chrysoptera* mitochondrial DNA and, with six plumage-associated loci, we predicted the maternal parent's phenotype to show that it was likely an early generation *Vermivora* hybrid. That this hybridization event occurred within a population of *Vermivora* warblers in significant decline suggests that females may be making the best of a bad situation, and that wood-warblers in general have remained genetically compatible long after they evolved major phenotypic differences.

1. Introduction

Hybridization between species can result in wasted reproductive effort if resulting hybrids are less fit than the parental taxa [1]. This is particularly relevant for species of conservation concern, because wasted reproductive effort can exacerbate population declines [1,2]. These hybridization events can also speak more generally to mate choice and reproductive compatibility between divergent taxa.

Here, we report an unusual combination of factors that has resulted in a rare, three-species hybrid between two genera of parulid warblers. Golden-winged warblers (*Vermivora chrysoptera*) and blue-winged warblers (*V. cyanoptera*) commonly hybridize where they co-occur across eastern North America [3–5]. Their hybrids are fertile, and hybridization is one of several factors presumed to be contributing to the declines of golden-winged warblers [4,6,7]. Genetic data suggest they diverged over 1 Myr [3], but that hybridization has resulted in genomic similarity, except for six small regions, associated with genes for feather pigmentation [8].

A third species, chestnut-sided warblers (*Setophaga pensylvanica*), regularly interact in territorial disputes with *Vermivora* warblers [9,10]. Whereas there

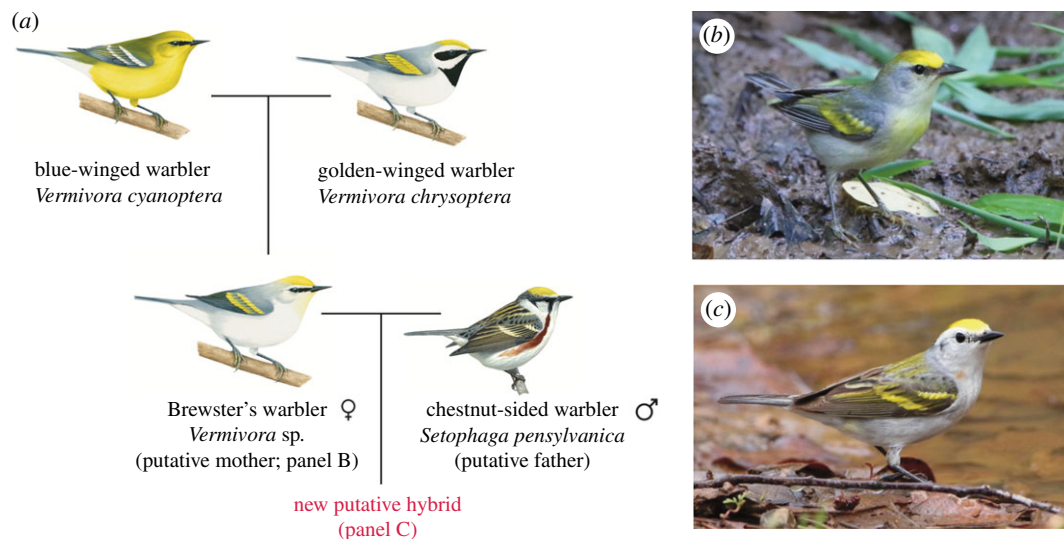


Figure 1. (a) The predicted formation of this new hybrid. (b) A female Brewster's warbler in 2017 at the site where the 2018 hybrid (c) was captured. *Vermivora* illustrations by Liz Clayton Fuller, chestnut-sided warbler from [20]. Photos in (b,c) by L.B.

are well-documented agonistic male–male interactions between chestnut-sided and *Vermivora* warblers, it is unknown how females interact, and whether they would mate intergenerically [11–14]. We suspected that hybridization would be most likely in the declining Appalachian populations, where females have a smaller pool of mates. To date, however, there have been no reports of interbreeding between *Vermivora* warblers and any of the 30+ species in the *Setophaga* genus.

A report from a bird watcher of a possible male *Vermivora* warbler singing a chestnut-sided warbler song was therefore surprising. Using genetic, morphometric and bioacoustic data, we show that this individual is indeed the rare product of a *Vermivora* warbler hybrid having itself hybridized with a chestnut-sided warbler.

2. Methods

The putative hybrid was first identified on 7 May 2018 near Roaring Spring, Pennsylvania (Macaulay Library no. ML98973521). On 26 June 2018 D.P.L.T. captured the bird using playback of chestnut-sided warbler song, taking photographs, standardized measurements, a blood sample (in Queen's buffer [15]) and affixing an aluminum band (nos. 2850–44901).

To determine how this putative hybrid compared in morphospace to the parental species, we measured specimens from the Cornell University Museum of Vertebrates (CUMV). Of the standard measurements—wing, tail, tarsus and bill length—the most distinctive between *Vermivora* and chestnut-sided warblers is the length of the bill [16–18]. Wing length also differs among these taxa, although only distinguishes golden-winged and blue-winged warblers. The same observer (D.P.L.T.) obtained all measurements.

To determine the maternal species, we sequenced a portion of the mitochondrial DNA (mtDNA) COI region using the primers COIbirdF1 and COIbirdR2 [19]. We used the PCR protocol as outlined in [8], using 30 cycles at a 49.3°C annealing temperature. We found the maternal parent had golden-winged warbler mtDNA (see below). We were then interested in whether the *Vermivora* parent was itself a hybrid (mtDNA is not predictive of phenotypes in mixed populations; [3,5]). *Vermivora* warblers were not commonly observed breeding on the property

where we captured the putative hybrid, which has been surveyed over several years. The exception is in 2017, when a female 'Brewster's' warbler was observed throughout the breeding season. The name 'Brewster's warbler' is given to early generation hybrids between golden-winged and blue-winged warblers. These birds have a white/yellow breast and face (figure 1a) and were once considered a distinct species [21].

To determine the *Vermivora* maternal phenotype, we used the set of six loci that occur in the few regions of the genome that are highly differentiated between golden-winged and blue-winged warblers [8]. To determine which variants came from the paternal parent, we also genotyped two chestnut-sided warblers (CUMV no. BT4072 and no. BT4920).

We used the PCR protocols as described in [8]. For five of the six plumage-associated loci, we used Sanger sequencing to determine genotypes. In the sequence around these SNPs (1077 bp), we also estimated how many SNPs that differed between the two chestnut-sided warblers and *Vermivora* warblers were heterozygous in the hybrid. We used whole-genome data to determine the *Vermivora* sequences [8]. For the sixth SNP—on warbler scaffold 38—we used a RFLP assay [8].

For each SNP, we identified the paternal and maternal allelic variants (see electronic supplementary material, text S1). For the maternal *Vermivora* parent, we estimated a genetic index across the SNPs. We compared this index to those values in previously sampled *Vermivora* warblers ($n = 256$).

3. Results

We observed the putative hybrid singing on seven occasions, and it sang songs characteristic of a chestnut-sided warbler (electronic supplementary material, figure S1a,b; Macaulay Library ML487980 for video). These songs differed from the 'buzzy' notes characteristic of *Vermivora* warblers (electronic supplementary material, figure S1c,d). Two small but distinct rufous patches on the throat of the putative hybrid were suggestive of chestnut-sided warbler ancestry (figure 1c). However, the olive back feathers of this hybrid contrasted with the dark feathers observed on adult male chestnut-sided warblers. The hybrid looked most like a juvenile chestnut-sided warbler—unlikely in early May. Moreover, the white feathers of the supercilium—black in adult male chestnut-sided warblers, and olive/grey in juveniles or

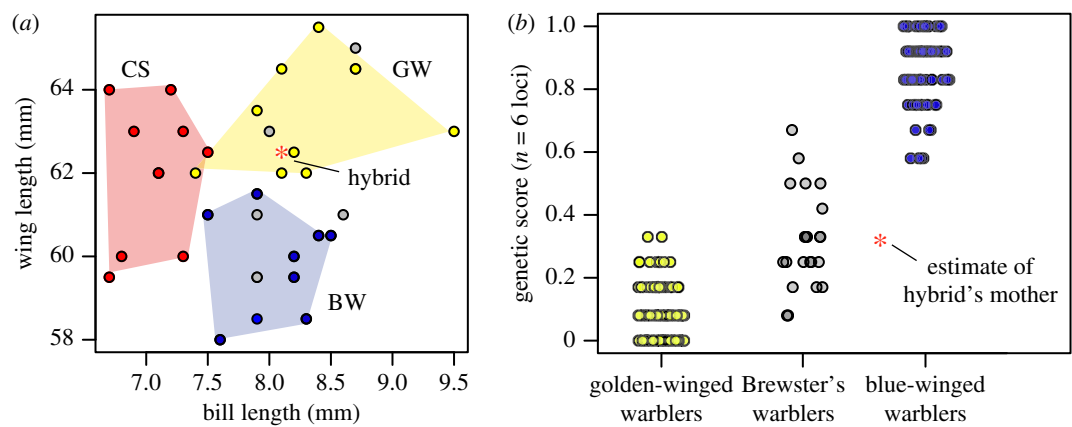


Figure 2. (a) Culmen and wing length for *Vermivora* warblers (yellow, golden-winged; blue, blue-winged; grey, Brewster's) and chestnut-sided warblers (red). The hybrid is shown with an asterisk. (b) The genetic index across six loci that distinguish *Vermivora* warblers. From the hybrid, we estimated the genetic index of its maternal parent, falling within the range of hybrids between golden-winged and blue-winged warblers (i.e. 'Brewster's warblers').

Table 1. Genotypes across SNPs that differentiate *Vermivora* warblers. Bolding illustrates which variant the hybrid received from its *Vermivora* maternal parent.

individual	scaffold-24	scaffold-38	scaffold-120	scaffold-299	scaffold-563	scaffold-653
golden-winged	G/G	C/C	G/G	C/C	A/A	T/T
blue-winged	T/T	G/G	A/A	A/A	C/C	C/C
chestnut-sided (no. 4072)	T/T	C/C	G/G	A/A	A/A	C/C
chestnut-sided (no. 4920)	T/T	C/C	G/G	A/A	A/A	C/C
putative hybrid	G/T	C/C	G/G	C/A	C/A	C/C

females—and a faint grey cheek patch, were both more characteristic of *Vermivora* warblers.

The bill length of the hybrid (8.1 mm) was 1 mm longer than chestnut-sided warblers (mean 7.1 mm $\pm$ 0.28 s.d.), but within the range of *Vermivora* warblers (figure 2a): golden-winged mean = 8.3 mm ($\pm$ 0.56 s.d.), blue-winged mean = 8.04 mm ($\pm$ 0.33 s.d.) and Brewster's mean 8.2 mm ($\pm$ 0.4 s.d.). The hybrid's wing (62.5 mm) was longer than blue-winged warblers (mean 59.8 mm $\pm$ 1.2 s.d.), but similar to chestnut-sided warblers (mean 61.9 mm $\pm$ 2.1 s.d.), golden-winged warblers (mean 63.4 mm $\pm$ 1.3 s.d.) and Brewster's warblers (mean 61.9 mm $\pm$ 2.1 s.d.).

mtDNA of the putative hybrid was 99% identical to golden-winged warblers (GenBank accession no. MH708872), and 97% identical to blue-winged warblers (these species differ by 3–4%; *Vermivora* and *Setophaga* sequences differ by greater than 8%; [3]). Across the SNPs where chestnut-sided warblers differed unambiguously from *Vermivora* warblers (n = 19 sites), the putative hybrid was heterozygous at all but one position. This site is not polymorphic in our sample of *Vermivora* warblers, but is likely variable in chestnut-sided warblers, and we have low power to detect this variation.

For the SNPs that distinguish golden-winged and blue-winged warblers, two of the six in the hybrid had alleles that originated from blue-winged warbler ancestry, and four that originated from golden-winged warbler ancestry (i.e. the maternal parent likely had one-third blue-winged alleles across these SNPs; table 1). We compared this genetic index (for the hybrid's maternal parent) to other *Vermivora* warblers (figure 2b). No phenotypic blue-winged warbler had a genetic index as low as 0.33, and only two of 149

phenotypic golden-winged warblers had indices that high. Brewster's warblers, by comparison, have a mean genetic index value of 0.32, and 23% of Brewster's warblers have exactly one-third blue-winged ancestry.

4. Discussion

The evidence strongly suggests that the warbler sampled in our study is the product of hybridization between a female *Vermivora* warbler and a male chestnut-sided warbler. Moreover, our genotyping implies that the maternal parent of this individual was itself a hybrid between a golden-winged and blue-winged warbler. The observation of a female Brewster's warbler at the same site during the previous breeding season makes it possible that this bird was the maternal parent of the 2018 hybrid. While warblers are known to have high juvenile dispersal, some *Vermivora* warblers have been observed breeding within 100 m of their territory of provenance, especially in small and isolated populations (H.M.S. 2014, personal observation). In addition, this female Brewster's warbler had the plumage characteristics consistent with at least one-third blue-winged warbler ancestry, which we estimated based on the six plumage-associated loci (figure 1b; additional details in electronic supplementary material, text S2).

The hybrid fell within a morphospace occupied by golden-winged and Brewster's warblers (figure 2a); it had a larger bill than chestnut-sided warblers, and a longer wing compared to blue-winged warblers. Both wing and bill traits are polygenic [22,23], thus F_1 hybrids would be expected to have intermediate characters [24]. Therefore, a

hybrid between an average-sized chestnut-sided warbler, and a Brewster's warbler with a larger-than-average bill, could produce an intermediate hybrid with the traits we characterized.

While this is the first published case of a *Vermivora* × *Setophaga* hybrid, there are other intergeneric hybrids that have been observed within parulids [12,13,25]. However, only one of these—a hybrid between a black-and-white warbler (*Mniotilta varia*) and a yellow-rumped warbler (*Setophaga coronata*)—had associated genetic data [11]. Also, this hybrid occurred within a highly artificial habitat, between captive birds in a free-flight aviary. To our knowledge, the present case is the first in the literature of an interspecific hybrid producing an intergeneric hybrid.

These kinds of events, in isolation, are unlikely to have significant evolutionary implications. However, it speaks to two broader aspects of warbler evolution. First, the fact that hybridization occurred within a population of *Vermivora* warblers in significant decline [6] suggests that females may be making the best of a bad situation. While plumage misidentification is unlikely driving the male–male territorial interactions between golden-winged and chestnut-sided warblers [10], hybrid females may be cuing in on other characters that promote interbreeding.

Over many generations, these rare hybridization events may also promote the movement of alleles across species. We have no knowledge of whether this hybrid successfully paired, allowing genes to move between the taxa (although we will monitor this site and potentially this bird in future years). It does, however, show how genes could move across deep phylogenetic boundaries, like *Heliconius* butterfly wing colour genes [26].

This finding of 'hybrids hybridizing' provides a novel avenue for the movement of genes between distantly related

taxa not yet observed or described in birds. It also suggests reproductive compatibility between distinct genera differing by greater than 8% in mtDNA [27]. Finally, it furthers the foundational research into hybridization within this family of birds, and reinforces Parkes [13] who also described distantly related warbler species producing viable young, and this was because warblers were a 'rapidly evolving group [whose] members have remained genetically compatible long after they evolved major morphological and behavioral differences'.

Ethics. Research was conducted under protocols approved by IACUC at Cornell University (no. 2015-0065).

Data accessibility. mtDNA sequence GenBank accession no. MH708872. Specimen measurements and nuclear sequence alignments in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.qb3sq2s> [28]. Photographs archived at Macaulay Library: ML98973521; <https://macaulaylibrary.org/asset/98973521>, ML102681801; <https://macaulaylibrary.org/asset/102681801>, ML102181871; <https://macaulaylibrary.org/asset/102181871>, ML104737301; <https://macaulaylibrary.org/asset/104737301>, ML106384041; <https://macaulaylibrary.org/asset/106384041>, ML112452071; <https://macaulaylibrary.org/asset/112452071>, video archived at the Macaulay Library, no. ML487980.

Authors' contributions. D.P.L.T., S.T. and H.S. designed the study. D.P.L.T. performed the analyses, and wrote the original draft of the manuscript. L.B. first identified the bird and obtained natural history observations. All authors contributed to the writing and editing of the manuscript and agree to be held accountable for the content therein and approved the final version of the manuscript.

Competing interests. The authors declare no competing interests.

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