

CHAPTER FOUR

Genetic Insights into Hybridization between Golden-winged and Blue-winged Warblers*

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Abstract. Hybridization between some pairs of closely related taxa is increasing due to anthropogenic factors such as habitat destruction and fragmentation, as well as an introduction of exotics. Outcomes of hybridization vary according to the species involved. With the advancement in molecular techniques in recent years, it is now possible to examine hybridization with far greater precision and to more fully understand hybridization systems and relationships between species. A better understanding of patterns and process can be used to inform appropriate conservation actions. Hybridization between Golden-winged Warblers (*Vermivora chrysoptera*) and Blue-winged Warblers (*V. cyanoptera*) has been ongoing for at least the past 140 years and is thought to be a primary cause of recent declines of Golden-winged Warblers in populations throughout their breeding

distribution. Here, we summarize the current knowledge of the genetics of this hybridization system. Specifically, recent analyses demonstrated extensive introgression in populations of Golden-winged Warblers throughout their breeding distribution, and bidirectional gene flow in almost all surveyed populations. To date, the use of mitochondrial genetic markers has provided the best resolution for understanding the warbler hybridization system, and a need for informative nuclear markers remains. We conclude with suggested avenues for future genetic research to increase understanding of hybridization, and to guide conservation management for Golden-winged Warblers.

Key Words: conservation, introgression, species replacement, *Vermivora chrysoptera*, *Vermivora cyanoptera*.

Hybrid zones, the physical areas where two taxa meet and interbreed, represent natural laboratories where evolutionary processes can be studied in situ (Barton and Hewitt 1985, Hewitt 1988, Rohwer and Wood 1998). Hybridization in plants is common and frequently results in the formation of new species (Ehrlich and Wilson 1991). Although less common in

animal taxa, hybridization events occur with an approximate 10% frequency in nonmarine avian species (Grant and Grant 1992, Allendorf et al. 2001, McCarthy 2006). In many cases, hybridization events occur as isolated incidences (Parkes 1978, McCarthy 2006, MacDonald et al. 2012), whereas in others they occur with great frequency (Gill 1980, Morrison and Hardy 1983). Because

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many hybrid zones contain a variety of phenotypes it is possible to address questions of sexual selection (Møller and Birkhead 1994, Higashi et al. 1999, Sheldon and Ellegren 1999), genetic control of phenotypic characters (Gill and Murray 1972, Hewitt 1988), fitness of parental species (Rohwer and Wood 1998), and forces that separate the hybridizing species (Barton and Hewitt 1985, Brumfield et al. 2001). As hybridization increases in frequency due to human-caused habitat change and other factors (Gill 1997), studies of these systems become important for conservation (Haig 1998, Allendorf et al. 2001, Randler 2002), and maintenance of species (Barton and Hewitt 1985, Avise 1994, Brumfield et al. 2001, Yuri et al. 2009).

Worldwide decreases in biodiversity tend to be attributed primarily to habitat modification or destruction, unsustainable harvest, competition with introduced species, and chains of extinction in which the extinction of one species contributes to the extinction of one-or-more ecologically dependent taxa (Rhymer and Simberloff 1996). Less attention has been paid to the effects of hybridization, even though mixing of gene pools can threaten the existence of rare species and is problematic for undertaking some conservation initiatives (Barton and Hewitt 1985, Rhymer and Simberloff 1996, Randler 2002). Understanding the evolutionary consequences of hybridization is key to prevention of further extinctions for species engaging in such genetic admixture.

Over the last 20 years, molecular techniques have become important tools for studying evolutionary dynamics of hybrid zones. Gill (1997) found that asymmetries in gene flow due to a bias in mating behavior can have a pronounced influence on the genetic architecture of a hybrid population: an effect that can go undetected by morphological analyses alone (Avise 1994, Rhymer and Simberloff 1996, Curry 2005, Vallender et al. 2009). More recently, molecular studies documented that extreme cases of hybridization can lead to reductions in genetic differentiation between species and an eventual breakdown of species integrity (Mank et al. 2004).

Whereas molecular analyses can be uniquely informative, it is important to ensure careful selection of genetic markers and techniques when examining hybridization systems (van Dongen et al. 2012). For example, several studies documented discordance between plumage characters

and genetic analyses as an indication of the location of a hybrid zone (Brumfield et al. 2001; Vallender et al. 2007b, 2009), and variation in selection pressures throughout the genome may lead to underestimates of introgression (Brumfield et al. 2001, Yuri et al. 2009). Despite these challenges, genetic analysis of hybridization dynamics has the potential to add greater understanding of evolutionary relationships among groups and individuals.

Ultimately, hybridization is a question of mate choice. Why mate with a heterospecific when a conspecific is available? Genetic techniques can be especially informative in answering this question. Several well-studied avian hybridization systems include four species pairs: Collared (*Ficedula albicollis*) and Pied Flycatchers (*F. hypoleuca*), Black-capped (*Poecile atricapillus*) and Carolina Chickadees (*P. carolinensis*), Townsend's (*Setophaga townsendi*) and Hermit Warblers (*S. occidentalis*), and between Mallards (*Anas platyrhynchos*) and American Black Ducks (*A. rubripes*). In all four of these systems, analysis of genetic markers revealed clear patterns of mate choice. Collared and Pied Flycatchers paired with heterospecifics sought extra-pair copulations with conspecifics, presumably to minimize the fitness costs of heterospecific pairing (Veen et al. 2001). Furthermore, the sex ratio of young was biased in accordance with "Haldane's Rule" when offspring were produced through a heterospecific pairing (Veen et al. 2001). "Haldane's Rule" suggests that hybrids of the heterogametic sex are more likely to be infertile (Haldane 1922).

Female Black-capped and Carolina Chickadees both appeared to prefer Carolina Chickadee males for their extra-pair partners in a zone of sympatry (Reudink et al. 2006), perhaps due to the dominance of Carolina Chickadee males over Black-capped Chickadee males (Bronson et al. 2003; Reudink et al. 2006, 2007). Interestingly, in a hybridization system between Black-capped and Mountain Chickadees (*P. gambeli*), extra-pair copulations between Mountain Chickadee females and Black-capped Chickadee males appear to result from Black-capped Chickadee males being behaviorally dominant over Mountain Chickadee males (Grava et al. 2012).

A similar phenomenon is thought to occur in the Mallard and American Black Duck system where females of both species preferentially select more dominant Mallard males, perhaps because these males are more able to protect their mates

from harassment by intruding males (including forced copulations), through predator alerting mechanisms, or by acquisition of superior feeding sites (Brodsky et al. 1988). Likewise, in the Townsend's Warbler and Hermit Warbler hybridization system, females of both species socially prefer the more aggressive, behaviorally dominant Townsend's Warbler males (Rohwer and Wood 1998, Pearson 2000), although this has not yet been quantified using genetic parentage analysis.

GOLDEN-WINGED × BLUE-WINGED WARBLERS

In eastern North America, there is a widespread mosaic hybrid zone between Blue-winged Warblers (*Vermivora cyanoptera*) and Golden-winged Warblers (*V. chrysoptera*). Hybrids of these two species were documented as early as 1870 (Herrick 1874) and hybridization is common (Gill 1980). The taxa likely occupied largely allopatric distributions prior to the early 1900s, after which the widespread abandonment of agricultural fields in eastern North America led to the growth of early-successional vegetation favored by both species, thereby putting them into secondary contact (Gill 1980). The breeding distribution of Blue-winged Warblers subsequently advanced northward into the Golden-winged Warbler range and, although their wide contact zone has steadily moved northwestward, hybridization remains extensive in areas where both species occur. Typically, Golden-winged Warbler breeding populations are negatively affected by hybridization and their local extirpation occurs within 50 years of the appearance of the first Blue-winged Warblers (Gill 1987, 1997). Two classic hybrid phenotypes known as "Brewster's" and "Lawrence's" warblers depicted in most popular field guides, were mistakenly ascribed new species names following their initial discovery (Parkes 1951). Subsequent examination of hybrid phenotypes revealed much more variation in plumage characters than was originally assumed (Short 1963, 1969; Vallender et al. 2009). Unlike other well-studied avian hybrid systems (Veen et al. 2001; Bronson et al. 2003, 2005; Grava et al. 2012), work examining mate-choice patterns is still largely lacking for the Blue-winged × Golden-winged Warbler hybridization system. Only one analysis of genetic mate choice has been carried out in this system (Vallender et al. 2007a), even though heterospecific extra-pair

fertilizations, which override assortative mating preferences, can play a significant role in hybridization and genetic introgression (Hartman et al. 2012). Vallender et al. (2007a) concluded that female Golden-winged Warblers do not appear to avoid hybrid males as either social partners or extra-pair sires, but their work was conducted in a region with a general paucity of hybrids and Blue-winged Warblers and they were unable to fully elucidate any pattern in mate-choice decisions by females of either species.

Mate-choice preferences are not fully understood, but a general pattern of the progression of hybridization has been recognized for this species pair: advancement of Blue-winged Warblers into the Golden-winged Warbler breeding distribution is followed by a period of hybridization and subsequent replacement of the Golden-winged Warbler phenotype with the Blue-winged Warbler phenotype. The predictable temporal sequence provides a context to examine hybridization in areas with different histories of contact and introgression.

The pattern of advancement and replacement by Blue-winged Warblers has clear conservation implications for Golden-winged Warblers, which have experienced precipitous population declines throughout much of their breeding distribution in the U.S. and Canada over the past 50 years (Gill 1980; Sauer et al. 2012; Chapter 1, this volume). Currently, Golden-winged Warblers are one of the most rapidly declining passerines in North America, with regional declines as high as 25% per year (2001–2011) and an average distribution-wide decline of 2.6% per year (Sauer et al. 2012). Since 2007, Golden-winged Warblers have been listed as a Threatened species under the Canadian Species At Risk Act (SARA 2015) and the species is currently under consideration for federal listing in the U.S. under the Endangered Species Act (U.S. Endangered Species Act 2015). Note that Blue-winged Warbler populations have also declined (as high as 6% per year) in some portions of their breeding distribution (2001–2011; Sauer et al. 2012). However, currently, Blue-winged Warblers are not protected, or under consideration for protection, by federal species-at-risk legislation in either Canada or the U.S.

The mechanisms by which the Blue-winged Warbler phenotype predictably replaces the Golden-winged Warbler phenotype following contact remain unclear. Male Blue-winged

Warblers may be behaviorally dominant over male Golden-winged Warblers (Will 1986), thereby obtaining better quality territories in areas of sympatry, or they may have a higher rate of extra-pair fertilizations with females of both species (Confer and Larkin 1998). In contrast, Ficken and Ficken (1968) suggested that Golden-winged Warblers are generally more aggressive than Blue-winged Warblers. That said, there appears to be no clear pattern of behavioral dominance, suggesting possible genetic dominance of Blue-winged Warblers over Golden-winged Warblers. Moreover, interspecific interactions may vary spatially: in some populations Blue-winged and Golden-winged Warblers do not appear to perceive each other as conspecifics and maintain overlapping territories (Confer and Knapp 1977), whereas in other locations males respond aggressively to individuals singing the other species' song (Ficken and Ficken 1968), albeit often with low frequency (Gill and Murray 1972). Reasons for regional variation in responsiveness to heterospecific song may relate to the history of contact between the species or may be due to variation in levels of genetic introgression.

EXAMINING HYBRIDIZATION USING MITOCHONDRIAL DNA

Molecular tools have been used since the 1990s to better understand the Blue-winged Warbler × Golden-winged Warbler hybridization system. In his pioneering mitochondrial DNA (mtDNA) work, Gill (1997) used restriction fragment length polymorphisms (RFLP) to differentiate Golden-winged Warblers from Blue-winged Warblers. He examined several populations with different histories of contact and hybridization and found that the two parental mitochondrial lineages differed by 3% sequence divergence, a level of separation that is on par with many other pairs of taxa that comprise clear biological species (reviewed by Johnson and Cicero 2004), with all individuals from allopatric populations possessing their species' typical mtDNA lineage. In a sympatric region of recent contact and known hybridization, however, Blue-winged Warbler mtDNA was present in high frequency in phenotypic Golden-winged Warblers and in almost all individuals with hybrid plumage phenotypes. Based on an unexpectedly pervasive and directional mtDNA introgression, Gill (1997) concluded that female Blue-winged

Warblers led the northward advance into the Golden-winged Warbler breeding distribution, and that F₁ hybrid females preferentially backcross with male Golden-winged Warblers.

Two studies further explored the pattern of mtDNA introgression at five sites with various histories of contact and documented levels of hybridization (Shapiro et al. 2004, Dabrowski et al. 2005). At none of these sites was there evidence of asymmetric mtDNA introgression, suggesting that Gill's (1997) findings may not be broadly applicable to other populations. Instead, Shapiro et al. (2004) found approximately equal frequencies of mismatches between phenotype and mtDNA haplotypes in a primarily Golden-winged Warbler population from West Virginia. Small samples from mixed populations in Michigan and Ohio also failed to show evidence of asymmetric introgression. Likewise, using similar analyses, Dabrowski et al. (2005) found bidirectional gene flow and long-term persistence of Golden-winged Warbler mtDNA haplotypes in a sympatric population in New York with more than a century of co-existence of these two species (Eaton 1914). Last, in an Ontario population thought to be in the earliest stages of hybridization, Golden-winged Warbler mtDNA haplotypes were found in both Golden-winged Warbler and phenotypic hybrid individuals (Dabrowski et al. 2005). The results indicate that genetic swamping by Blue-winged Warblers does not appear to be occurring in any of these populations.

Most recently, Vallender et al. (2009) surveyed 753 Golden-winged and Blue-winged Warbler samples from nine U.S. states and three Canadian provinces using a PCR-restriction assay modified from earlier mtDNA-sequencing methodologies. The only population in which all phenotypic Golden-winged Warblers had the Golden-winged Warbler mtDNA type was in Manitoba, near the northwestern edge of the Golden-winged Warbler breeding distribution. All other tested populations had some proportion of phenotype-mtDNA haplotype mismatch indicating presence of cryptic hybrids among phenotypically pure individuals of both species. Of 608 Golden-winged Warblers sampled, 49 (8.0%) showed mtDNA-phenotype mismatch, whereas 14 of the 145 (9.6%) Blue-winged Warblers sampled had a mismatch between their phenotype and mtDNA haplotype. Vallender et al. (2009) concluded that there are far fewer

genetically pure populations of Golden-winged Warblers than previously believed.

Continued screening of samples from Canada since 2009 has further highlighted a pattern of apparent widespread introgression. Few Golden-winged or Blue-winged Warblers breed within Quebec (45.083°N, 74.217°W) where both species are considered rare (Gauthier and Aubry 1996, Sauer et al. 2012). Application of the PCR-restriction assay developed by Vallender et al. (2009) showed that rates of phenotype-mtDNA mismatch in Quebec are ~1.3% (one of eight Golden-winged Warblers samples screened had Blue-winged Warbler mtDNA). A slightly lower mismatch rate was observed in Golden-winged Warblers sampled from a site in eastern Ontario (44.617°N, 79.483°W; two of 27 Golden-winged Warblers had Blue-winged Warbler mtDNA), and in the westernmost portions of the province (48.833°N, 94.317°W; two of 28 Golden-winged Warblers had Blue-winged Warbler mtDNA; R. Vallender, unpubl. data).

Analyses of samples recently collected in Manitoba are especially informative. The first documented breeding occurrence of Golden-winged Warblers in Manitoba was in 1932 (Manitoba Avian Research Committee, pers. comm.). Consistent with the findings of Vallender et al. (2009), all 191 phenotypically-pure Golden-winged Warblers

sampled (since 2009) from Riding Mountain National Park (50.767°N, 99.500°W) in the mid-western region of the province had Golden-winged Warbler mtDNA (R. Vallender et al., unpubl. data). However, a male “Brewster’s Warbler” was found at one of the study sites in 2010 (S. Van Wilgenburg, unpubl. data) and was determined to have Blue-winged Warbler mtDNA (R. Vallender, unpubl. data). Either the male immigrated from elsewhere or there were undetected female Blue-winged Warblers or cryptic hybrid female Golden-winged Warblers breeding in the region.

Given a lack of mtDNA-phenotype mismatch in the western regions of Manitoba, and within Minnesota, it is surprising that the highest rates of mtDNA introgression uncovered in Canada in recent years were in the southeastern most region of Manitoba (49.783°N, 96.467°W). A total of 126 adult Golden-winged Warblers were sampled from this region between 2010 and 2012 and 6.3% (8 of 128) had a phenotype-mtDNA haplotype mismatch (Figure 4.1). Of eight birds with Blue-winged Warbler mtDNA, seven were sampled in 2012 (out of a total sample of 64 collected that year; L. Moulton, unpubl. data). This latest finding suggests that advancement of Blue-winged Warbler-mtDNA is occurring rapidly in this region of the province. However, the number

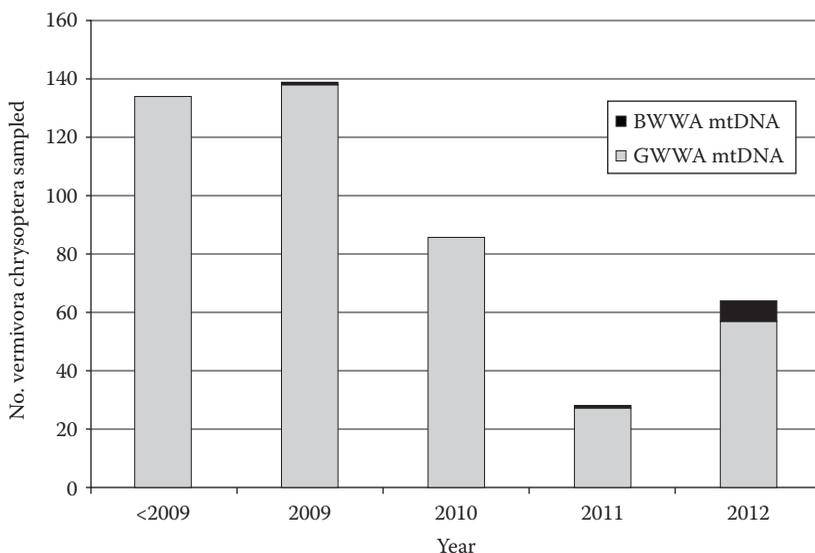


Figure 4.1. Results of mitochondrial DNA analysis of samples collected in Manitoba as of 2013. The light section of each bar represents *Vermivora chrysoptera* with *V. chrysoptera* mtDNA. The dark section of each bar represents *V. chrysoptera* with *V. cyanoptera* mtDNA (i.e., cryptic hybrids). BW mtDNA is *V. cyanoptera* mtDNA type; GW mtDNA is *V. chrysoptera* mtDNA-type. (Based on data from Vallender et al. 2009; R. Vallender, unpubl. data; L. Moulton, unpubl. data.)

of detected hybrids encountered remains low at ~3% of males on territories, and no Blue-winged Warblers were encountered through 2013 (L. Moulton, unpubl. data).

Currently, more Golden-winged Warblers have been sampled in Manitoba than anywhere else in North America (451 Golden-winged Warblers sampled through 2013). Continued sampling in the southeastern and midwestern portions of Manitoba over the next 10–15 years is necessary to monitor introgression. Likewise, additional sampling north to Duck Mountain (Manitoba) and Porcupine Hills (Saskatchewan; Figure 4.2) will be important to determine the genetic status of Golden-winged Warblers at the northwestern extent of their breeding distribution.

Compiling all available mtDNA data reveals a clear picture of widespread introgression in Golden-winged Warblers (Figure 4.2). Of the 12 states and provinces from which Golden-winged Warbler samples have been assayed, all but those from Michigan show some proportion of genetic or cryptic hybrids. In this analysis, Michigan is

represented by only two samples. Given a known 30-year history of Golden-winged Warbler × Blue-winged Warbler hybridization (Will 1986), additional sampling is expected to reveal cryptic hybrid Golden-winged Warblers breeding in Michigan.

From a conservation perspective, the mtDNA analysis indicates Golden-winged Warblers in Manitoba appear only minimally affected by hybridization is encouraging. However, putting low levels of mtDNA introgression in Golden-winged Warbler populations breeding in Manitoba into the appropriate context is important. First, despite low rates of mtDNA introgression, there could be substantial introgression in the nuclear (nDNA) genome undetected by the studies completed to date. Second, only 1% of the global Golden-winged Warbler breeding population occurs in Manitoba (Buehler et al. 2007). Surveys provide evidence of increasing numbers of breeding Golden-winged Warblers in Manitoba (Manitoba Avian Research Committee 2003; 22.7%/year increase, $n = 2$ routes, 1988–2007, $P = 0.42$; Sauer et al. 2012) and

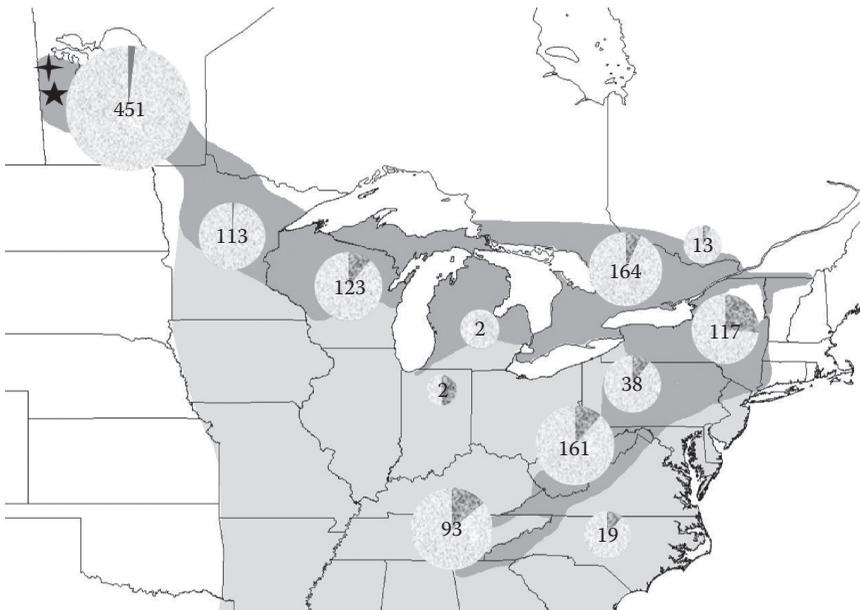


Figure 4.2. Proportion of *Vermivora chrysoptera* individuals assigned to the ancestral *V. chrysoptera* haplotype group across the breeding distribution, shown in dark gray shading on the map (approximate migration areas shown in light gray) based on a compilation of all available mitochondrial DNA results, 1998–2013. Light portions of pie charts indicate the proportion of *V. chrysoptera* samples assigned to the ancestral *V. chrysoptera* haplotype group; dark gray portions represent the proportion of *V. chrysoptera* samples assigned to the *V. cyanoptera* haplotype group as cryptic hybrids. Numbers within pie charts represent the number of *V. chrysoptera* sampled per state or province. Stars indicate sampling sites as the edge of the range at the Duck Mountains (Manitoba) and Porcupine Hills (Saskatchewan). (Based on data from Shapiro et al. 2004; Dabrowski et al. 2005; Vallender et al. 2009; L. Moulton, unpubl. data; R. Vallender, unpubl. data.)

recent, extensive field surveys suggest that earlier estimates of the number of Golden-winged Warblers breeding in Manitoba were underestimated (C. Artuso, unpubl. data). However, the density of Golden-winged Warblers in Manitoba is still low compared to breeding population densities in Minnesota, Wisconsin, or Ontario (which, support ~82% of the estimated global breeding population; Buehler et al. 2007).

Moreover, whereas the use of mtDNA markers has greatly improved understanding of hybridization between Golden-winged and Blue-winged Warblers, it provides only a partial window into patterns of hybridization given that mtDNA is maternally inherited and a non-recombining part of the avian genome. A better understanding of the patterns of introgression of nDNA in Golden-winged and Blue-winged Warblers is likely to be informative (Irwin et al. 2009, Hartman et al. 2012).

EXAMINING HYBRIDIZATION USING NUCLEAR DNA

Nuclear DNA is biparentally inherited and has the potential to improve the understanding of genetic introgression dynamics (Irwin et al. 2009, Vallender et al. 2009, Hartman et al. 2012). In the only nDNA study of Golden-winged and Blue-winged Warblers to date, Vallender et al. (2007b) used a combination of markers (intron sequences, microsatellites, and Amplified Fragment Length Polymorphisms [AFLP]) to differentiate between Golden-winged Warblers and Blue-winged Warblers from allopatric populations with no history of contact with the other species. One of the primary goals of this work was to create a panel of markers that could be applied broadly to quantify the ancestry of hybrid individuals. However, despite screening with a moderate panel of 13 microsatellite markers, analyses of allele frequency patterns suggested that the parental populations were weakly differentiated at these loci. Despite their high variability, microsatellite variation had little utility for distinguishing parental species, and did not prove useful for typing the ancestry of hybrid individuals. Likewise, no evidence of diagnostic allelic variation was found at any of the five intron loci. Sixteen variable nucleotide sites were found in >3,000 nucleotides of intron sequence, but each of the variable character states was found in alleles drawn from both parental populations.

A large panel (>1,000 separate fragments) of AFLP loci was generated using 54 different primer pairs. Despite a large number of variable fragments, Vallender et al. (2007b) found no characters that were fixed within species, although eight characters derived from seven different primer pairs showed significant frequency differences between Golden-winged and Blue-winged Warblers. Accordingly, this panel of AFLP loci was applied to 73 Golden-winged Warbler samples collected in southern Ontario where hybridization was thought to be in the initial stages with a low proportion of phenotypic hybrids and Blue-winged Warblers present. Surprisingly, almost a third of the phenotypic Golden-winged Warblers had introgressed genotypes indicating a substantial history of cryptic hybridization.

AFLP methodologies have been widely employed in plant, fungal, and bacterial research, but they have only recently been explored in animal studies (Bensch and Åkesson 2005, Toews and Irwin 2008, Rush et al. 2009, Sternkopf et al. 2010). Limitations include an inability to detect heterozygotes because characters are scored as either present or absent, but benefits of using AFLPs include the ability to rapidly sift through the genome for recent coalescences, a relatively short start-up time, and generation of data from hundreds of loci (Bensch and Åkesson 2005). In cases where other marker types have revealed weak genetic structure (such as Willow Warbler *Phylloscopus trochilus*; Bensch et al. 2002), the utility of AFLPs has been highlighted and may be useful when examining other animal hybridization systems (Bensch and Åkesson 2005).

However, generating AFLP data is time-consuming and relatively costly and not suitable for population-scale screening, which typically involves hundreds or thousands of samples. To date, attempts to circumvent these limitations by converting diagnostic AFLP fragments into a panel of single-locus, co-dominant markers have not been successful (R. Vallender, unpubl. data). It appears unlikely that AFLPs will be a useful nDNA tool to assess wide-scale patterns and levels of hybridization between Golden-winged and Blue-winged Warblers. Moreover, nDNA results to date indicate that nuclear genome differentiation between Golden-winged and Blue-winged Warblers is low and can only be detected using the most sensitive methods, consistent with the slow mutation rate and longer coalescence times

of nDNA relative to mtDNA (Moritz et al. 1987, Zink and Barrowclough 2008).

A lack of effective nDNA markers is currently the factor most limiting improved understanding of the Golden-winged Warbler × Blue-winged Warbler hybridization system. The inclusion of nDNA data from Z-linked loci that coalesce relatively quickly (Carling and Brumfield 2008, Carling et al. 2010) is essential for a better understanding of the current level of hybridization and to track the progression of introgression as Blue-winged Warblers continue to advance northward into the breeding distribution of Golden-winged Warblers.

IMPLICATIONS OF HYBRIDIZATION

Based on extensive distribution-wide molecular research and identifying phenotypic hybrids in many previously allopatric populations, it is likely that a high percentage of breeding Golden-winged Warbler populations are impacted by hybridization with Blue-winged Warblers. This impact is surprisingly far-reaching and can be detected even in Golden-winged Warbler breeding populations that are geographically distant from Blue-winged Warbler populations. Golden-winged Warbler breeding populations in the northwestern portion of their breeding distribution in Minnesota and Manitoba are exhibiting the lowest levels of mtDNA introgression as of 2013, and therefore play a significant role in conservation of Golden-winged Warblers. Continued genetic screening of Golden-winged Warblers from the northwestern portion of their breeding distribution is a conservation priority, coupled with examining related issues such as mate choice, reproductive success, habitat use and selection, parasitic infection, survival and recruitment, and linkages to nonbreeding grounds.

Cumulative genetic evidence suggests that hybrids within the Golden-winged Warbler × Blue-winged Warbler hybridization complex are not affected by post-zygotic selection. To date, post-zygotic selection has been examined in relation to the ability to attract mates of the parental species through use of song by males (Harper et al. 2010), to produce viable offspring (Vallender et al. 2007a), to produce clutches of balanced sex ratio not biased towards the homogametic sex in accordance with “Haldane’s Rule” (Neville et al. 2008), by the ability to successfully fledge young (Reed

et al. 2007, Vallender et al. 2007a), and the probability of parasitic infection (Vallender et al. 2012), although there appears to be regional variation in the vigor of hybrids (Confer and Tupper 2000, Confer 2006) and a general lack of knowledge about possible pre-zygotic selection against hybrid offspring. Moreover, the genetic data suggesting that hybrids are not at a disadvantage compared to genetically pure individuals of either parent species do not align with extensive field observations in two populations (New York and West Virginia) where selection against hybrid males by Golden-winged Warbler females has been documented (Confer 2006). Without quantifying reproductive success, together with genetic purity, it is difficult to determine if behavioral avoidance affects reproductive success for either species. However, the hypothesis that hybrid fitness may differ across the Golden-winged Warbler breeding distribution needs further examination.

Gill (1980) predicted that Golden-winged Warblers could be rare, or even extinct, by 2080. The prediction may not be accurate given the now-documented bidirectionality of gene flow between Golden-winged and Blue-winged Warblers, and persistence of the Golden-winged Warbler phenotype in at least one region where these two species co-exist, but the widespread and high frequency of hybridization suggests that it is unlikely that stable coexistence will occur over any large geographic region.

FUTURE APPLICATIONS OF GENETIC TECHNIQUES

The Golden-winged Warbler × Blue-winged Warbler hybridization system is complicated, variable, and unlikely to be halted by directed management actions. However, pursuit of the following five key research directions will increase understanding of the impact of hybridization on both Golden-winged and Blue-winged Warblers.

Identify Plumage Genes

Given their genetic similarity but strikingly dissimilar plumage characteristics, Golden-winged and Blue-winged Warblers are likely well-differentiated at ≥ 1 loci encoding plumage patterns. Plumage genes may be the most appropriate and useful regions of the genome to target

for further research examining the genetic differentiation of these species. Moreover, in other avian species the differences in the number of alleles at the *melanocortin-1 receptor* gene influence the degree of melanism, and genes associated with plumage polymorphism influence mate preference (Uy and Borgia 2000, Mundy et al. 2004, Mundy 2005).

High-Throughput Sequencing

With the continuing development of high-throughput sequencing (HTS) technologies, generating genome-scale sequence data for nonmodel organisms continues to become more attainable. Therefore, HTS is being used more frequently in ecological and evolutionary studies (Lerner and Fleischer 2010, Ekblom and Galindo 2011, Steiner et al. 2013, Toews et al. 2016) and is proving effective even when applied to historical specimens, such as museum skins, despite the challenges of DNA degradation (Bi et al. 2013, Gansauge and Meyer 2013, Guschanski et al. 2013). Therefore, with the necessary analytical resources, HTS has great potential to reveal a much more complete picture of the genetics of the Golden-winged Warbler × Blue-winged Warbler hybridization system.

Specifically, applying HTS and comparative genomic analysis to the Golden-winged Warbler × Blue-winged Warbler hybridization system may better reveal the evolutionary history of these two species as it has recently for two other hybridizing species, grizzly bears (*Ursus arctos*) and polar bears (*U. maritimus*; Miller et al. 2012). Genomic analyses may give an accurate estimate of the time of divergence for Golden-winged and Blue-winged Warblers and potential shifts since divergence between allopatry and sympatry revealed as genetic signatures of ancient admixture.

Apart from clarifying the evolutionary history of these two species, data generated by HTS could be used to improve understanding of current populations and the extent of introgression across the breeding distributions of both species. In addition to generating genome-scale datasets, HTS techniques can be used to quickly develop thousands of informative molecular markers such as single-nucleotide polymorphisms (SNPs; Twyford and Enos 2012). Alternatively, HTS can be applied directly to population samples through genotyping-by-sequencing

methodologies requiring no *a priori* knowledge of the genome of the study species (Elshire et al. 2011). These simple, low-cost methods are suitable for population-level studies and have recently been used to characterize the hybrid zone of Black-capped and Carolina Chickadees (Taylor et al. 2014). Using these approaches, Golden-winged and Blue-winged Warbler populations could be screened to assess interspecific gene flow much more effectively than is currently possible. Such an approach would likely yield a spatially-explicit representation of the extent and progression of introgressive hybridization. Additionally, estimates of the historical rate of advancement of introgression could be made by screening previously-collected blood samples and museum specimens.

Last, through comparisons with avian genome reference markers (Backström et al. 2008), genomic datasets could be used in studies of the genetic basis of adaptive phenotypic traits in Golden-winged and Blue-winged Warblers. Such an approach could lead to predictions of the potential fitness costs or benefits of introgressive gene flow for both species and such information, along with estimates of interspecific gene flow, is critical for conservation planning (Steiner et al. 2013).

Studying Mate Choice

Mate choice is a key factor driving gene flow in the Golden-winged Warbler × Blue-winged Warbler hybridization system and needs to be better understood. To this end, we recommend paternity analyses, combined with genetic-purity analyses, throughout the breeding distributions of Golden-winged Warblers and Blue-winged Warblers. Such analyses, especially in regions of extensive hybridization, where Golden-winged Warblers, Blue-winged Warblers, and their hybrids co-exist, will likely reveal mate-choice dynamics such as how often females select interspecific and hybrid mates, either as their social partners or via extra-pair copulations, and will enable the examination of why heterospecific mate choice decisions occur (Curry 2005, Vallender et al. 2007a, Hartman et al. 2012). Improving measures of realized reproductive success by including survival of fledglings might also help elucidate the adaptive significance of mate choice in Golden-winged Warblers (Streby et al. 2014).

Sampling at the Northern Boundary of Breeding Distribution

Continued sampling of Golden-winged Warblers in the northwestern portion of their breeding distribution (especially Minnesota and Manitoba) will further understanding of hybridization between Golden-winged and Blue-winged Warblers, by tracking the advancement of Blue-winged Warblers or their genes into otherwise genetically pure Golden-winged Warbler populations. Cryptic hybrids are being found with increasing frequency in the southeastern corner of Manitoba, but Golden-winged Warblers from the northwestern portion of Manitoba have not yet been sampled. If Golden-winged Warblers in northwestern Manitoba are determined to be unaffected by hybridization (based on both mtDNA and nDNA analyses), these populations may represent high-priority locations to focus management activities that benefit Golden-winged Warblers. Given the similar ecological niches of Golden-winged and Blue-winged Warblers (Confer 1992, Gill et al. 2001), it will be important to examine habitat \times genetic interactions in regions of sympatry. Such analyses have strong potential to inform on-the-ground conservation action that aims to preferentially maintain and improve habitat for Golden-winged Warblers (A. M. Roth et al., unpubl. plan). Indeed, management and conservation efforts are likely to be most effective when focused on areas where a significant proportion of Golden-winged Warblers have been determined to be genetically pure (at least based on mtDNA assays), even though some admixture may be present in all populations. Habitat loss has also been recognized as a significant threat to Golden-winged Warbler persistence and many other shrubland species (A. M. Roth et al., unpubl. plan), and mitigating this threat may be one of the most straightforward conservation actions to undertake.

Treating Golden-winged Warblers and Blue-winged Warblers as a Species Pair

Of these two species, Golden-winged Warblers have received the vast majority of funding and research attention, and considerable information exists about their ecology and conservation. In contrast, only basic ecological information on breeding, mate choice, nonbreeding-ground habitat use, and migration exists for Blue-winged

Warblers. We therefore recommend that research be conducted on life-history characteristics of Blue-winged Warblers, particularly in the phenotypically “pure” Blue-winged Warbler breeding distribution. Such work should include genetic analyses of mate choice in allopatry and sympatry, realized reproductive success, genetic purity analyses, and blood parasite load (e.g., Vallender et al. 2007a,b, 2012; Hartman et al. 2012) as these would provide for interesting comparisons with Golden-winged Warblers and would be broadly informative about the impact of hybridization on Blue-winged Warblers. Additional sampling of Blue-winged Warblers is also important, especially from regions where they have been poorly sampled to date such as both peninsulas of Michigan and within Missouri. Given their propensity to hybridize in all areas of contact with Golden-winged Warblers, unraveling individual life histories of Golden-winged and Blue-winged Warblers is unlikely and therefore studying them as a species pair is more likely to elucidate the causes and impacts of their widespread hybridization.

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