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Geographic variability in mitochondrial introgression among hybridizing populations of Golden-winged (*Vermivora chrysoptera*) and Blue-winged (*V. pinus*) Warblers

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Abstract

The rapidly declining Golden-winged Warbler (*Vermivora chrysoptera*) is of conservation concern owing in part to hybridization with the closely related Blue-winged Warbler (*V. pinus*). These species hybridize extensively in eastern North America and over the past century the Blue-winged Warbler has displaced the Golden-winged Warbler from substantial regions of its historic breeding range. A previous study suggested that these genetic interactions result in rapid and asymmetric introgression of Blue-winged Warbler mitochondrial DNA (mtDNA) into Golden-winged phenotype populations within the zones of contact, but more recent and extensive surveys have documented a more complex pattern of genetic interchange between these taxa. We surveyed mtDNA/phenotype associations in 104 individuals of known phenotype drawn from two locations with different histories of contact and found substantial variation between sites in the extent of introgression. Where both species have co-existed for more than a century, we found evidence of bi-directional introgression and the long-term persistence of Golden-winged mtDNA haplotypes. At the leading edge of the northward expansion of Blue-winged Warblers, we found predominantly Golden-winged Warbler mtDNA haplotypes in both Golden-winged and hybrid-phenotype individuals. Across both sites, genetic swamping does not appear to be occurring via the early immigration of Blue-winged Warbler females into populations dominated by Golden-winged Warbler phenotypes. Instead, the differing patterns of mitochondrial introgression may be driven by the relative local population sizes of the parental species coupled with subtle between-species differences in mate choice and habitat preferences.

Introduction

Natural hybridization can result in conservation problems when a declining species is genetically swamped by introgression from closely related taxa. Such genetic mixing has resulted in the extinction of some species and is likely to become increasingly prevalent as species' distributions are modified by anthropogenic changes that bring formerly allopatric taxa into contact (Rhymer and Simberloff 1996). Hybridization can also pose legal dilemmas related to the definition of species or other taxonomic units of conservation (Allendorf et al. 2001; Avise 2004). Understanding the frequency and

pattern of hybrid introgression is therefore important for the effective conservation of taxa threatened by interspecific genetic interactions.

The Golden-winged Warbler (*Vermivora chrysoptera*) has declined throughout much of its breeding range in northeastern North America, and the species is currently being considered for listing within the USA Endangered Species Act. Causes of this decline include the reduction of shrub habitat as abandoned farmlands enter later stages of succession (Confer and Pascoe 2003) as well as behavioral (Confer et al. 2003) and genetic (Gill 1997) interactions with the closely related Blue-winged Warbler (*V. pinus*) (Gill 2004). The geographic range displacement of the Golden-winged Warbler has been paralleled by range expansions by the Blue-winged Warbler, and the two species hybridize frequently within their shifting zone of breeding overlap (e.g. Short 1963), with some sympatric populations exhibiting 7–15% hybrid phenotypes in surveys of singing males (Confer 1992).

Two hybrid phenotypes are particularly common and were named by early workers who initially described them as new species (reviewed in Parkes 1951). The F_1 phenotype is termed ''Brewster's Warbler'' and generally resembles the Golden-winged Warbler in plumage, but is lacking that species' black eye and throat patch, and exhibits the black eye-stripe of the Blue-winged Warbler. The far less common ''Lawrence's Warbler'' phenotype has a combination of the Golden-winged face pattern with the yellow plumage characteristic of the Blue-winged Warbler and probably results from a backcross (Parkes 1951).

Two previous studies have used mitochondrial markers to explore the pattern of female-mediated introgression between Blue- and Golden-winged warblers. The first was conducted by Gill (1997), who documented the rapid and asymmetric introgression of mitochondrial DNA (mtDNA) from Blue-winged Warblers into phenotypic Golden-winged Warblers. Gill surveyed mtDNA/phenotype associations in two contexts: a late-stage, lowland site (in the Delaware River valley of northeastern Pennsylvania and nearby northwestern Pennsylvania) where the two species had been hybridizing for a minimum of several decades, and an early-stage site (in northeastern Pennsylvania) where observations suggested that the two species had more recently come into contact. In the late-stage population, the RFLP haplotype diagnostic of Blue-winged mtDNA was almost fixed (40 of 41 individuals) regardless of phenotype: all six of the Golden-winged phenotype individuals had Blue-winged mtDNA, as did six of the seven Brewster's hybrid individuals and all 28 Blue-winged phenotype individuals. Blue-winged phenotype individuals were rare at the early stage site, but nonetheless the frequency of Blue-winged mtDNA in Golden-winged phenotype individuals increased from 27% to 70% over the 1988–1992 sampling interval. In the pooled sample from both years at this site, nearly half (10 of 21) of the Golden-winged phenotype individuals had Blue-winged mtDNA, as did the single Brewster's hybrid individual; no Blue-winged phenotype individuals were sampled. These patterns suggested that introgression of Blue-winged Warbler mtDNA into Golden-winged Warbler populations proceeds rapidly and that there is little reciprocal introgression of Golden-winged mtDNA into Blue-winged lineages. As mtDNA is inherited matrilineally, Gill (1997) suggested that pioneering female Blue-winged Warblers lead the advance into Golden-winged dominated populations. This is plausible as female dispersal into new regions is common in avian species (Greenwood and Harvey 1980), although the more inconspicuous behavior of females may cause them to be overlooked (Gill 1997), leading researchers to attribute initial arrival to conspicuous, singing males.

A more comprehensive recent survey (Shapiro et al. 2004) examined phenotype-haplotype associations in a greatly expanded sample of individual warblers from additional study populations. In contrast to the rapid and unidirectional introgression seen at Gill's sites, Shapiro et al. (2004) found bi-directional introgression at all locations where both species were well represented, with no tendency for disproportionate introgression as indicated by Blue-winged haplotypes in Golden-winged phenotype individuals. Birds with the ''Brewster's'' and ''Lawrence's'' hybrid phenotypes similarly had *pinus* and *chrysoptera* haplotypes in approximate equal proportions. In areas where only one pure species-level phenotype was present, all individuals sampled had the corresponding mtDNA haplotype. These results led Shapiro et al. (2004) to conclude that the hybridization dynamics of these taxa are more complex than indicated by Gill's earlier study.

Here, we expand on these previous surveys of mitochondrial/phenotype associations to include additional sites with contrasting histories of contact between Blue- and Golden-winged Warblers, including an unusual site where both species have been present for more than a century and a site sampled during the first appearance of Blue-winged phenotype individuals in a large population of Golden-winged phenotypes. Our results are broadly congruent with those of Shapiro et al. (2004) in suggesting that there are substantial differences in patterns of introgression across sites and that the initial contact between these species is not invariably followed by rapid mtDNA swamping of Golden-winged Warblers.

Methods

Populations Sampled

We obtained genetic samples from two sites that have each been the focus of multi-year demographic studies. These sites differ markedly in their history of contact between Blue- and Golden-winged Warblers, as one site has a long-term history of species co-occurrence and the other site is at the edge of the current Blue-winged Warbler geographic expansion.

The long-term contact site is within Sterling Forest State Park (SFSP), a part of the Hudson Highlands of southern New York (41°10′×74°23′). At this site, both species nest in successional fields, power-line rights-of-way, and alder (*Alnus rugosa*) swamps. In addition, the Golden-winged Warbler is partially segregated from Blue-winged Warblers by its occurrence in swamp forests (Confer and Tupper 2000). Both Golden-winged and Blue-winged Warblers have coexisted at the site for a century (Eaton 1914). Genetic samples from this site were collected during two successive breeding seasons, between 21 May–3 July, 2002 (n = 16) and 15 May–25 June, 2003 (n = 29). Since mtDNA is inherited maternally, we included samples from male and female adults and one representative nestling from clutches where the mother was not captured but her phenotype was documented. Warblers sampled from SFSP had pure parental phenotypes or the distinct Brewster's phenotype without apparent plumage introgression, following the criteria of Gill (1980).

The recent contact site was located at the Queen's University Biological Station (QUBS) in southeastern Ontario, Canada (44°34′N, 76°19′W). The habitat consists of abandoned farmland, swamps, tracts of second growth forest, and rocky outcrops associated with the Canadian Shield. In this region the arrival and prevalence of the Golden-winged Warbler has been well documented. The first Golden-winged Warbler reported in the Kingston area (approximately 50 km south of QUBS) was a single male in 1954 (Quilliam 1973). From 1960 onwards the species was reported every spring, and nesting behavior was confirmed in 1961 (Quilliam 1973; Weir 1989). This population consisted solely of Golden-winged phenotype individuals until the mid-1990's, when the first hybrid individuals were recorded at the study site. The number of hybrid-phenotype adults has increased with successive breeding seasons to approximately 14% during the summer of 2003 (R. Fraser unpub. data). The first phenotypically pure Blue-winged Warblers were noted on the study sites in the late 1990's and are likely to increase in frequency in the near future. Samples from the QUBS site were collected during two periods: before the earliest sightings of pure Blue-winged phenotype individuals from 4 June–16 June, 1998 (n = 14), and during the first years of phenotypic admixture from 9 May–4 July 2001 (n = 5), 15 May–6 July 2002 (n = 28), and 8 May–30 July 2003 (n = 12). Samples from male and female adults were included in this study, and phenotype was diagnosed in the field into four plumage categories: pure Golden-winged, pure Blue-winged, Brewster's hybrid phenotype, and Lawrence's hybrid phenotype. All QUBS individuals included in this study had the 'classic' phenotypes associated with these designations (Parkes 1951).

Blood samples were obtained by brachial venipuncture and stored in Queen's lysis buffer (Seutin et al. 1991) or on filter paper. Because both study populations are the focus of intensive demographic and behavioral studies, no voucher specimens were collected from these sites. For laboratory protocol development and to confirm that DNAs extracted from nuclear-enriched blood samples and mitochondrial DNA (mtDNA) enriched pectoral muscle samples (e.g., Sorenson and Quinn 1998) gave identical sequences, we acquired blood and/or tissue samples from the genetic resources collection at the Cornell University

Museum of Vertebrates (specimens 44030 and 44172).

Laboratory techniques

We extracted genomic DNA from the buffer-preserved blood samples using either a DNeasy kit (QIAGEN Inc., Valencia, CA, USA) or a Perfect gDNA Blood Mini Isolation kit (Eppendorf Scientific, Inc., Westbury, NY, USA), in each case following the manufacturers' protocols for nucleated blood samples. Blood samples stored on filter paper were frozen at −20 °C and genomic DNA was extracted using standard phenol-chloroform protocol (Sambrook and Russell 2001), followed by ethanol precipitation. The dried DNA pellet was then reconstituted in 50 μl H_2O.

We employed polymerase chain reactions (PCRs) that targeted a region of the mitochondrial genome that includes the entire NADH dehydrogenase subunit II (NDII) gene using primers METb and TRPc (Hunt et al. 2001). PCR reactions were seeded with 1 μl genomic DNA in 25 μl reaction volumes consisting of 10–100 ng genomic DNA, 0.75 units recombinant *Taq* DNA polymerase (Invitrogen), 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 6.25 picomoles of each primer and 250 μM dNTPs (Invitrogen). Reactions were denatured for 30 s at 95 °C, followed by 16 thermal cycles of 95 °C denaturing for 30 s, an annealing step that started at 60 °C and decreased by 0.5 °C every cycle for 45 s, and 72 °C extension for 1 min 15 s. This was followed by 26 additional thermal cycles of 95 °C denaturing for 30 s, 54 °C annealing for 45 s, and 72 °C extension for 1 min 15 s. Reactions were terminated by a final extension at 72 °C for 4 min 30 s.

Dideoxy terminator cycle sequencing reactions were carried out following manufacturer's protocols (Applied Biosystems Division of Perkin-Elmer) using the METb amplification primer and an internal primer SS (5′- TGCAAGGGAGAGTAGGGTTAGAAGTAG–3′) that together gave complete double-stranded sequence for a 690 bp region of the NDII gene (bases 5267–5956 in the chicken mitochondrial genome (Desjardins and Morais 1990). Sequences were generated via electrophoresis on an Applied Biosystems 3100 automated sequencer.

Some of our preliminary tests of mitochondrial primers for this study targeted another mitochondrial gene, a portion of cytochrome oxidase subunit I (COI). However, sequences based on amplifications from this locus using a variety of bird- and wood-warbler-specific primer pairs had sequence artifacts typical of co-amplification of both mtDNA copies and nuclear-encoded copies (''numts''). These artifacts included: (1) the presence of multiple product bands of different lengths in PCR reactions, (2) consistent ''double peaks'' at particular nucleotide sites in otherwise clean sequencing chromatograms, and (3) a level of nucleotide variation that was atypically low for avian mtDNA. Numt contamination is a common problem (e.g., Price et al. 2000; Kimura et al. 2002) in studies that employ DNA samples derived from whole avian blood because avian erythrocytes retain their nuclei at maturation but loose their mitochondria, causing a bias towards amplification of the nuclear-encoded copies (Sorenson and Quinn 1998). Given the strong evidence for numt contamination using the COI primers, we used several lines of evidence to confirm that sequences obtained from the NDII locus were of mitochondrial origin: (1) PCRs performed using the NDII primers consistently produced strong single product bands of correct size, (2) the resulting sequences had the high transition:transversion ratio expected of mtDNA coding sequence and an absence of unexpected stop codons, (3) the sequence chromatograms had no ''double peaks,'' and (4) identical sequences were obtained from DNA originating from blood samples and mtDNA-enriched muscle samples from the same individual vouchered specimens.

Introgression analyses

Sequences were imported into Sequencher v.4.1.2 (Gene Codes Corp. 1998), double-stranded reactions were reverse-complemented and aligned, and all sequences were scanned by eye for sequencing artifacts or other errors. We found a large number of similar haplotypes, as described below. Genetic distances among haplotypes were estimated using PAUP* (Swofford 2002), primarily by counting the uncorrected number of substitutions because the low level of differentiation suggests that these distances are unlikely to be biased strongly by homoplasy. The low magnitude of divergence in these population-level samples also suggested that some haplotypes in our samples are likely to be

ancestral to other sampled haplotypes. We therefore used the program TCS 1.13 (Clement et al. 2000) to reconstruct a gene genealogy (haplotype network) that summarizes the relationships among haplotypes. This network was not rooted to sequences from other taxa because extensive mtDNA and nuclear sequence-based phylogenies indicate that the Blue-winged/Golden-winged Warbler lineage is not closely allied to the remaining extant species of *Vermivora* or to any other parulid lineage (Lovette and Bermingham 2002, Lovette, unpub. data). In previous studies (Gill 1997; Shapiro et al. 2004) of this system, patterns of mtDNA introgression have been explored through simple contingency table-based statistics on haplotype-phenotype associations. This approach assumes that individuals are sampled randomly from each study population with respect to both their phenotype and mitochondrial haplotype. We are aware of no strong biases in our field sampling, but as the genetic samples in our study were collected largely as part of other projects, such biases could be present, particularly those enhancing the likelihood of sampling of the rarer but interesting individuals with visible hybrid phenotypes. We therefore present only summary statistics for phenotype-haplotype associations.

Results

We sequenced a 690 bp region of the mitochondrial NDII gene from 104 individual warblers, including 45 from Sterling Forest State Park, New York and 59 from the Queens University Biological Station, Ontario. In the pooled sample from these two sites, 70 individuals were classified in the field as phenotypically pure Golden-winged Warblers, 15 as phenotypically pure Blue-winged Warblers, 14 as having the Brewster's Warbler hybrid phenotype, and five as having the Lawrence's Warbler hybrid phenotype.

Across all samples, the 690 bp region had a total of 52 variable nucleotide sites, which exhibited a 46:7 transition/transversion ratio. Forty-one of the total base substitutions were synonymous, whereas 12 resulted in amino acid substitutions. All but one of these 12 amino acid substitutions involved changes between amino acids with very similar structural and chemical properties (e.g., Grantham 1974). This pattern of nucleotide substitution is typical of population-level variation at the avian NDII locus, and we saw no evidence of other artifacts (such as double chromatogram peaks) indicative of co-amplification of nuclear-encoded pseudogene sequences (Sorenson and Quinn 1998). We therefore conclude that all ND2 sequences reported here are of mitochondrial origin.

We identified 28 unique NDII haplotypes among the 104 surveyed individuals and each unique sequence has been deposited in GenBank (accession numbers AY642934-AY642961). A parsimony-based network depicting the relationship among these 28 haplotypes is shown in Figure 1. This network was concordant with simple distance comparisons in suggesting the presence of two distinct haplotype groups. Nucleotide variation within each group was modest (1–6 nucleotide substitutions), whereas a much larger degree of divergence (31–37 substitutions) separates the two haplotype clusters. Several lines of evidence suggest that these groups represent the ancestral Blue-winged and Golden-winged lineages. First, the magnitude of divergence between the two groups is similar to the findings of Gill's (1997) RFLP-based survey, and Shapiro et al.'s (2004) NDII findings from Blue-winged and Golden-winged populations. Second, each haplotype had a predominant phenotype that was geographically consistent with the respective distributions of the two species. Finally, the site that has historically been isolated from Blue-winged populations has a very high frequency of the haplotype group we have inferred to be the ancestral Golden-winged mtDNA lineage. Hereafter we refer to these two haplotype clusters as the AGW (''ancestral Golden-winged'') and ABW (''ancestral Blue-winged'') groups.

Haplotype–phenotype variation at sterling forest state park, New York

Both Blue-winged and Golden-winged phenotypes, along with Brewster's type hybrid phenotypes have been present in Sterling forest state park for the past century. Table 1 summarizes the phenotype–haplotype associations at this site. Of 15 phenotypically pure Blue-winged individuals, 13 possessed ABW mtDNA and two possessed AGW mtDNA. Of the 28 phenotypic Golden-winged Warblers at the site, 16 had AGW

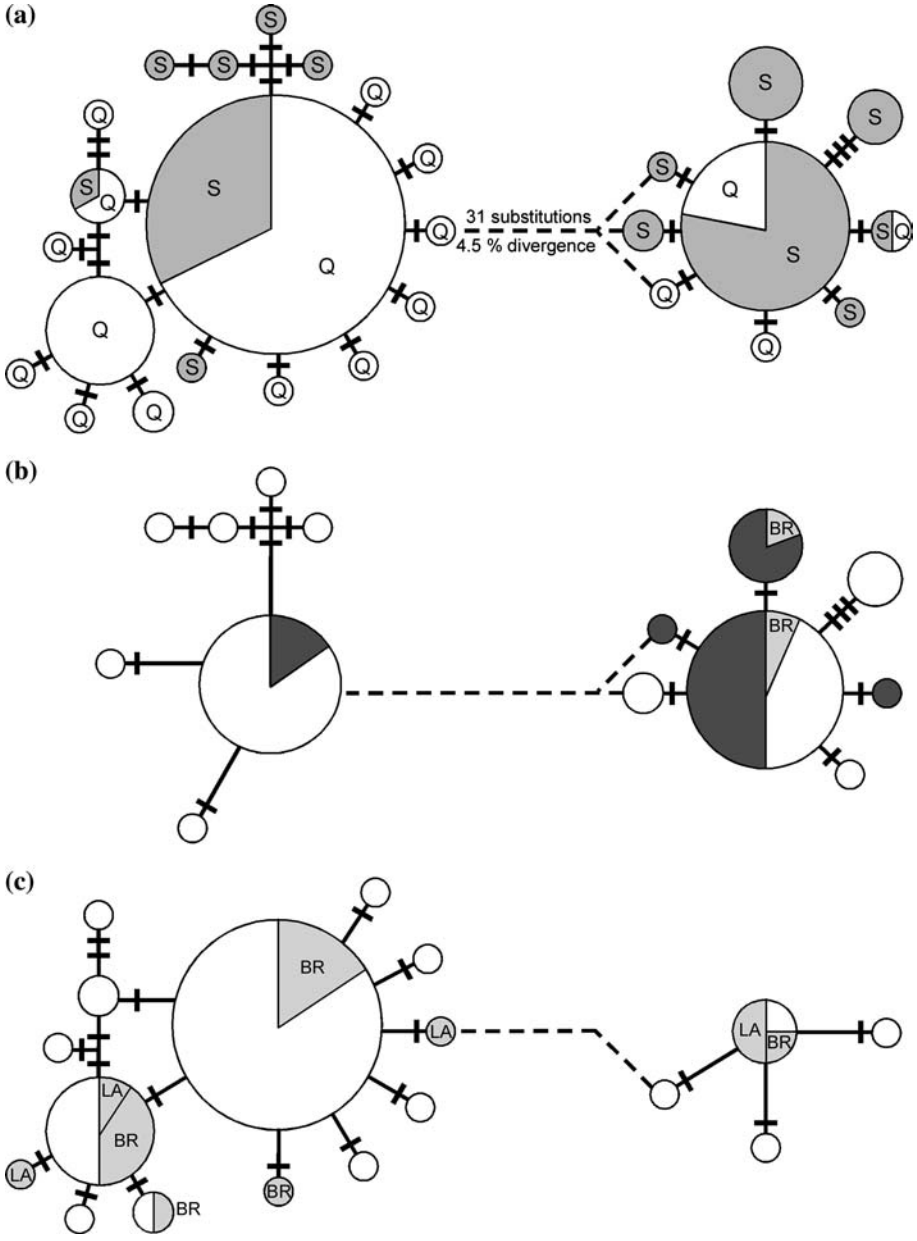

Figure 1. MtDNA haplotype networks for two mixed Blue-winged/Golden-winged Warbler populations. Each unique haplotype is indicated by a circle with an area proportional to the number of sampled individuals with that haplotype. Hatch marks along branches represent single nucleotide substitutions. The left-hand haplotype cluster represents the assumed ancestral Golden-winged group (AGW), and the right-hand cluster the assumed ancestral Blue-winged group (ABW). (a). Pooled samples from both study sites: shaded regions represent individuals from Sterling Forest State Park (SFSP) and white regions represent individuals sampled at Queen's University Biological Station (QUBS). (b). Subset of haplotypes found at SFSP. (c). Subset of haplotypes found at QUBS. In parts B and C, haplotype positions correspond to the pooled sample in A, and white areas represent phenotypic Golden-winged Warblers, black areas represent phenotypic Blue-winged Warblers, and shaded areas represent hybrid phenotypes (BR indicates Brewster's hybrid phenotype and LA Lawrence's phenotype).

mtDNA and 12 had ABW mtDNA. The two Brewster's-type individuals both had ABW mtDNA. Although there is a tendency towards directional introgression of ABW haplotypes into phenotypically Golden-winged individuals, this process has not been extensive enough to cause the

Table 1. Phenotype/mtDNA haplotype associations (number of individuals) at two intensively sampled sites

Phenotype	Sterling forest state park		Queen's university biological station	
	ABW*	AGW**	ABW	AGW
Blue-winged	13	2	0	0
Golden-winged	12	16	4	38
Brewster's hybrid	2	0	1	11
Lawrence's hybrid	0	0	2	3

*Ancestral Blue-winged haplotype group
**Ancestral Golden-winged haplotype group

extinction of the AGW haplotype group despite the long-term presence of the two taxa. In addition, the process of introgression has also occurred to a limited extent in the opposite direction, as AGW haplotypes were found in phenotypically pure Blue-winged individuals.

Haplotype–phenotype concordance at QUBS, Ontario

The Queen's University site was occupied exclusively by phenotypic Golden-winged Warblers from the mid-1900's until the mid to late 1990's. Of the 42 phenotypically pure Golden-winged individuals sampled at this site, 38 had AGW mtDNA and four had ABW mtDNA (Table 1). Of 12 Brewster's type individuals, 11 had AGW mtDNA and one had ABW mtDNA. Of the five Lawrence's type individuals, three had AGW mtDNA and two had ABW mtDNA. Of the 42 phenotypic Golden-winged Warblers at this site, 14 were sampled in 1998, prior to any sightings of phenotypically pure Blue-winged individuals in the area; there was, however, a single Brewster's-type individual on the study site at that time. Among these 14 pre-contact phenotypic Golden-winged individuals, two had ABW-type mtDNA.

Mitochondrial affinities of hybrid individuals

Hybrid individuals of these species are traditionally characterized as either Brewster's type individuals or the far less common Lawrence's type. In this study, phenotypically hybrid individuals had both ABW and AGW mtDNA haplotypes. Nearly all Brewster's type individuals at QUBS had AGW mtDNA, while both of the two Brewster's-type individuals at SFSP had ABW mtDNA. Lawrence's-type individuals were found only at the Queen's University site, and had nearly equal numbers of AGW and ABW mtDNA haplotypes.

Discussion

This comparison across sites indicates that populations of Golden-winged and Blue-winged Warblers have site-specific patterns of mitochondrial introgression associated with the historical context of species interactions at each location. These results parallel those found recently by Shapiro et al. (2004) who conducted analyses using samples from other populations in eastern North America. Factors likely to influence this relationship include the duration of contact and the relative local population sizes of each species.

Species-level phylogenetic distinctiveness

We found a substantial amount (~4.5%) of mtDNA sequence divergence between the haplotype groups that we assume represent the ancestral Blue-winged and Golden-winged Warbler lineages. This magnitude of divergence is very similar to that calculated by Shapiro et al. (2004) who determined that the mean nucleotide divergence ranged from 4.3 to 4.9% depending on the population sampled. These estimates are also similar to those estimated by Gill (1997) based on RFLP assays (~3.0–3.2%) of the mtDNA molecule. Some previous workers have suggested that the frequent hybridization between these morphological species indicates their particularly recent common ancestry, perhaps during the most recent glacial maxima at approximately 20,000 years before present (e.g., Short 1963). Furthermore, this extensive hybridization has led some workers to suggest that the two forms should be treated as

conspecific (e.g., Mayr and Short 1970). This inference of recent ancestry could cause the declining Golden-winged Warbler to be down-weighted in conservation ranking schemes that use phylogenetic distinctiveness as a priority criterion.

In contrast, the mitochondrial sequence evidence suggests that these lineages are substantially older and well differentiated. The few calibrations of a passerine mitochondrial clock have suggested that protein-coding mtDNA divergence accumulates at 1.4–2.5% per million years in at least some songbird lineages (Tarr and Fleischer 1995, Fleischer et al. 1998). Although dating diversification events using avian mitochondrial clocks is fraught with many potential sources of error (Lovette 2004a), the mtDNA divergence between the AGW and ABW haplotype groups is several orders of magnitude greater than expected if these lineages split near the Pleistocene-Holocene boundary. Furthermore, comparisons with other passerine genera that have radiated in North America suggests that the magnitude of nucleotide divergence between the ABW and AGW haplotype groups is equivalent to the separation of many other pairs of taxa that comprise clear biological species (reviewed in Johnson and Cicero 2004).

Site-specific patterns of mtDNA introgression

Gill's (1997) previous study of mtDNA introgression between Blue-winged and Golden-winged Warblers found rapid and asymmetric introgression of Blue-winged mtDNA into Golden-winged populations and at one site this mtDNA swamping occurred before Blue-winged phenotype individuals were present in high frequency. The pace and scope of this apparently unidirectional introgression resulted in predictions that the Golden-winged Warbler (or its mitochondrial lineage) has a high likelihood of extinction caused be genetic swamping by its more common congener (Gill 1997).

Our SFSP site is unusual in that both Golden-winged and Blue-winged Warblers have coexisted there for over a century. If the pattern of rapid asymmetric introgression found by Gill (1997) is general, then nearly all individuals at this site should have ABW haplotypes. Instead, we found that slightly more than half of the Golden-winged phenotype individuals at SFSP had the corresponding AGW-group mtDNA. As 43% of Golden-winged phenotype individuals had haplotypes in the ABW group and 13% of Blue-winged phenotype individuals had AGW haplotypes, introgression has clearly occurred in the ancestral matrilines of a substantial proportion of SFSP individuals. The long-term local persistence of AGW haplotypes at this site could therefore have two causes. First, mtDNA introgression may have occurred at the site during the long period of sympatry but might not have been substantial enough to swamp the local Golden-winged population. Second, the SFSP Golden-winged Warbler population may have been continually augmented by immigration from a source in which the AGW haplotype group occurs at high frequency. In either case, the mixed haplotype-phenotype associations at this site suggest that introgression is pervasive where these species come into contact, but that short-term extinction of the AGW haplotype group is not a universal pattern in the hybrid zone.

The QUBS study site was sampled very early in the period of local contact between Golden-winged and Blue-winged Warblers. Consistent with the rarity of hybrid or Blue-winged phenotype individuals at this site, only 10% of the Golden-winged phenotype individuals had ABW haplotypes. Two of these ABW individuals were sampled in 1998, prior to the documented arrival of Blue-winged phenotype individuals at or near the study population. Therefore, at QUBS introgression of ABW haplotypes did precede the known immigration of Blue-winged phenotype individuals, but ABW haplotypes remain relatively uncommon and do not follow the pattern of complete genetic swamping in advance of phenotypic hybridization seen in the Pennsylvania population (Gill 1997).

Evidence against unidirectional introgression

Gill (1997) found that Blue-winged mtDNA rapidly introgresses into Golden-winged phenotype populations but that the reverse introgression of Golden-winged mtDNA into Blue-winged populations was rare or lacking in his study populations. If this is a general phenomenon, this pattern would have important implications for the conservation of these species because it suggests a mechanism for the observed displacement of Golden-winged Warblers. The highly asymmetric pattern of introgression in Gill's populations could have resulted from several non-exclusive processes, including

stochastic fixation of Blue-winged mtDNA in Golden-winged phenotype populations, particularly in small populations of Golden-winged Warblers connected via hybridization to much larger source populations of Blue-winged Warblers; sex-biased invasion of Blue-winged females into Golden-winged populations and their subsequent pairing with Golden-winged males, possibly enhanced by the behavioral dominance or competitive superiority of Blue-winged females over Golden-winged females; or a selective disadvantage in hybrids with Golden-winged mtDNA. The highly ABW-biased introgression found by Gill (1997) predicts that phenotypic hybrids will usually have ABW mtDNA, that Golden-winged phenotype individuals may have ABW mtDNA, and that Blue-winged phenotype individuals will rarely have AGW mtDNA.

In parallel with the survey of Shapiro et al. (2004), we found instead that the pattern of mtDNA introgression varies with site and history, and that there has been extensive bi-directional mtDNA introgression at some locations. At our QUBS site, 14 of 17 phenotypic hybrids had AGW mtDNA. This predominance of AGW hybrids suggests that male Blue-winged Warblers are leading the advance into Golden-winged habitat in this region, or that these hybrids are dispersing into the area from other locations. The latter is more consistent with field observations at QUBS where many hybrids have been observed, but where Blue-winged phenotypes remain rare. Only two hybrid individuals were sampled at the SFSP and both had ABW haplotypes; however, two of the 15 phenotypic Blue-winged Warblers at this site had AGW haplotypes, demonstrating past introgression of AGW mtDNA. Across both of our study sites, two of 15 phenotypic pure Blue-winged individuals had AGW mtDNA haplotypes and 16 of 70 phenotypic pure Golden-winged individuals had ABW haplotypes. This occurrence of reciprocal mtDNA introgression via presumably backcrossed individuals demonstrates that the F_1 females of both crosses between the parental species are viable and fertile. As female hybrids are most likely to suffer fitness consequences in female-heterogametic taxa (Haldane 1922), the extent of this reciprocal introgression suggests that hybrids may not be at a strong fitness disadvantage in this system.

Although phenotypic and mtDNA clines are roughly concordant in many geographically linear avian hybrid zones (Sattler and Braun 2000, Bensch et al. 2002), broad-scale displacement of mtDNA versus plumage clines has been well documented in other hybrid zones where males of one taxon either have a competitive advantage in male–male interactions (e.g., Rohwer et al. 2001) or in which males of one species exhibit traits that attract females of both species (e.g., Brodsky and Weatherhead 1984; Parsons et al. 1993; Kulikova et al. 2004). In the latter situation, introgression of plumage characters via male-mediated gene flow may advance far beyond the female-mediated mtDNA cline (Brumfield et al. 2001; Lovette 2004b). In the *Vermivora* warblers considered here, there is no evidence of strong asymmetries across species in male competitive ability or in interspecific mate attraction (e.g. Confer and Larkin 1998). As noted by Gill (2004), the mosaic hybrid zone of the Blue-winged/Golden-winged warbler system is geographically complex, with phenotypically pure and mixed populations intercalated across the middle latitudes of much of eastern North America. This complex interdigitation is driven in part by the partly overlapping habitat preferences of these two taxa (Ficken and Ficken 1968; Confer and Knapp 1981). The mosaic pattern of the Blue-winged/Golden-winged system has several implications. First, it is not surprising that patterns of introgression will differ across sites that themselves differ in their habitat landscape and in the relative population sizes of parental and hybrid individuals, as similarly diverse hybridization dynamics have been documented across a number of other mosaic hybrid zones (e.g., Ross and Harrison 2002; Vines et al. 2003). Second, genetic swamping may proceed more rapidly in mosaic zones than in tension situations where selection maintains a narrow hybrid zones separating large, geographically separated parental populations. This is especially true when small pockets of the rarer species are interspersed within a matrix of the much more common species, as is likely the case for many small populations of Golden-winged Warblers in their broad region of overlap with Blue-winged Warblers.

Conservation implications

During the last century, the Golden-winged Warbler has been extirpated from substantial portions of its prior breeding range (Sauer et al. 2003).

Extirpation has occurred even where much suitable habitat remains (Confer et al. 2003) and where Blue-winged Warblers are now common. Habitat requirements for these species are similar (Confer et al. 2003) and this pattern of species replacement suggests that competitive interactions with Blue-winged Warblers have contributed to Golden-winged Warbler population declines.

Gill (1997) raised the specter of extremely rapid genetic swamping of Golden-winged Warblers based on mtDNA assays at two sites of recent contact, whereas the recent study of Shapiro et al. (2004) found a less predictable pattern of bi-directional introgression. Our further sampling shows that patterns of mitochondrial introgression vary with population status: in the declining Golden-winged Warbler population in the Delaware River valley, introgression of Blue-winged Warbler mtDNA into phenotypically pure Golden-winged populations was nearly complete (Gill 1997). For the coexisting populations in SFSP, introgression was bi-directional and affected less than 50% of the Golden-winged Warblers. For the Canadian population in the initial stage of contact, introgression was rare and the most common route of introgression was mating by advancing male Blue-winged Warblers with females of the established Golden-winged population.

Although many aspects of the mating behavior between Blue-winged and Golden-winged Warbler remain unknown, it is clear that continued advancement of Blue-winged and hybrid individuals into the current Golden-winged breeding range will perpetuate population declines in Golden-winged populations. Coupled with declines due to habitat loss, the future of Golden-winged Warblers in some areas of North America is grim. However, the site-specific differences in patterns of introgression exhibited in this and other (Shapiro et al. 2004) studies suggest that larger-scale demographic processes influencing the source-sink dynamics of these species are likely to mediate their long-term genetic interactions, and that complete genetic swamping of the Golden-winged Warbler is not a foregone conclusion. Furthermore, we now have the tools to assess other breeding populations to determine where populations free of maternal introgression still occur.

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References

Allendorf FW, Leary RF, Spruell P, Wenburg JK (2001) The problems with hybrids: setting conservation guidelines. *Trends Ecol. Evol.*, **16**, 613–622.

Avise JC (2004) *Molecular Markers, Natural History, and Evolution*, Sinauer Associates, Inc., Sunderland, Massachusetts.

Bensch S, Helbig AJ, Salomon M, Seibold I (2002) Amplified fragment length polymorphism analysis identifies hybrids between two subspecies of warblers. *Mol. Ecol.*, **11**, 473–481.

Brodsky LM, Weatherhead PJ (1984) Behavioral and ecological factors contributing to American Black Duck-Mallard hybridization. *J. Wildl. Manage.*, **48**, 846–851.

Brumfield RT, Jernigan RW, McDonald DB, Braun MJ (2001) Evolutionary implications of divergent clines in an avian (Manacus:Aves) hybrid zone. *Evolution*, **55**, 2070–2087.

Clement M, Posada D, Crandall K (2000) TCS: a computer program to estimate gene genealogies. *Mol. Ecol.*, **9**, 1657–1660.

Confer JL (1992) Golden-winged Warbler (*Vermivora chrysoptera*) In: The Birds of North America, no. 20. (Eds. Poole A, Stettenheim P, Gill F), The American Ornithologists' Union, Washington, DC.

Confer JL, Knapp K (1981) Golden-winged Warblers and Blue-winged Warblers: The relative success of a habitat specialist and a generalist. *Auk*, **98**, 108–114.

Confer JL, Larkin JL (1998) Behavioral interactions between Golden-winged and Blue-winged Warblers. *Auk*, **115**, 209–214.

Confer JL, Pascoe SM (2003) Avian communities on utility rights-of-ways and other managed shrublands in the northeastern United States. *Forest Ecol. Man.*, **185**, 193–205.

Confer JL, Tupper SK (2000) A reassessment of the status of Golden-winged and Blue-winged warblers in the Hudson Highlands of southern New York. *Wilson Bull.*, **112**, 544–546.

Confer JL, Allen PE, Larkin JL (2003) Effects of vegetation, interspecific competition, and brood parasitism on Golden-winged Warbler nesting success. *Auk*, **121**, 138–144.

Desjardins P, Morais R (1990) Sequence and gene organization of the chicken mitochondrial genome – a novel order in higher vertebrates. *J. Mol. Biol.*, **212**, 599–634.

Eaton EH (1914) *Birds of New York*, 2 Vol University of the State of New York, Albany.

Ficken MS, Ficken RW (1968) Ecology of Blue-winged Warblers, Golden-winged Warblers and some other Vermivora. *Am. Midl. Nat.*, **79**, 311–319.

Fleischer RC, McIntosh CE, Tarr CL (1998) Evolution on a volcanic conveyor belt: using phylogeographic reconstructions and K-Ar-based ages of the Hawaiian Islands to estimate molecular evolutionary rates. *Mol. Ecol.*, **7**, 533–545.

Gill FB (1980) Historical aspects of hybridization between Blue-winged and Golden-winged warblers. *Auk* **97**, 1–18.

Gill FB (1997) Local cytonuclear extinction of the Golden-winged Warbler. *Evolution*, **51**, 519–525.

Gill FB (2004) Blue-Winged Warblers (*Vermivora pinus*) versus Golden-Winged Warblers (*V.. chrysoptera*). *Auk*, **121**, 1014–1018.

Grantham R (1974) Amino acid difference formula to help explain protein evolution *Science*, **185**, 862–864.

Greenwood PJ, Harvey PH (1980) Mating systems, philopatry and dispersal in birds and mammals. *Anim. Behav.*, **28**, 1140–1162.

Haldane JBS (1922) Sex ratio and the unisexual sterility in hybrid animals *J. Genet.*, **12**, 101–109.

Hunt JS, Bermingham E, Ricklefs RE (2001) Molecular systematics and biogeography of Antillean thrashers, tremblers, and mockingbirds (Aves : Mimidae). *Auk*, **118**, 35–55.

Johnson NK, Cicero C (2004) New mitochondrial DNA data affirm the importance of Pleistocene speciation in North American birds. *Evolution*, **58**, 1122–1130.

Kimura M, Clegg SM, Lovette IJ, Holder KR, Girman DJ, Milá BJ, Wade P, Smith TB (2002) Phylogeographic approaches to assessing demographic connectivity between breeding and over-wintering sites in a Nearctic-Neotropical warbler (*Wilsonia pusilla*). *Mol. Ecol.*, **11**, 1605–1616.

Kulikova IV, Zhuravlev YN, McCracken KG (2004) Asymmetric hybridization and sex-biased gene flow between Eastern Spot-billed Ducks (*Anas zonorhyncha*) and Mallards (*A. platyrhynchos*) in the Russian Far East. *Auk*, **121**, 930–949.

Lovette IJ (2004a) Mitochondrial dating and mixed support for the "2% Rule" in birds. *Auk*, **121**, 1–6.

Lovette IJ (2004b) Molecular Phylogeny and Plumage Signal Evolution in a Trans Andean and Circum Amazonian Avian Species Complex. *Mol. Phyl. Evol.*, **32**, 512–523.

Lovette IJ, Bermingham E (2002) What is a wood-warbler? A molecular characterization of a monophyletic Parulidae. *Auk*, **119**, 695–714.

Mayr E, Short LL (1970) Species taxa of North American birds. *Publications of the Nuttall Ornithological Club, no. 9*, Nuttall Ornithological Club, Cambridge, Massachusetts.

Parkes KC (1951) The genetics of the Golden-winged × Blue-winged Warbler complex. *Wilson Bull.* **63**, 5–15.

Parsons TJ, Olson SL, Braun MJ (1993) Unidirectional spread of secondary sexual plumage traits across an avian hybrid zone. *Science*, **260**, 1643–1646.

Price T, Lovette IJ, Bermingham E, Gibbs HL, Richman AD (2000) The imprint of history on communities of North American and Asian warblers. *Am. Nat.*, **156**, 354–367.

Quilliam, HR (1973) *History of the Birds of Kingston, Ontario*, 2nd edn. Kingston Field Naturalists, Kingston, ON.

Rhymer JM, Simberloff D (1996) Extinction by hybridization and introgression. *Annu. Rev. Ecol. Syst.*, **27**, 83–109.

Rohwer S, Bermingham E, Wood C (2001) Plumage and mitochondrial DNA haplotype variation across a moving hybrid zone. *Evolution*, **55**, 405–422.

Ross CL, Harrison RG (2002) A fine-scale spatial analysis of the mosaic hybrid zone between *Gryllus firmus* and *Gryllus pennsylvanicus*. *Evolution*, **56**, 2296–2312.

Sambrook J, Russell DW (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Sattler GD, Braun MJ (2000) Morphometric variation as an indicator of genetic interactions between Black-capped and Carolina chickadees at a contact zone in the Appalachian mountains. *Auk*, **117**, 427–444.

Sauer JR, Hines JE, Fallon J (2003) *The North American Breeding Bird Survey, Results and Analysis 1966–2002*, USGS Patuxent Wildlife Research Center, Laurel, MDVersion 2003.1.

Seutin G, White BN, Boag PT (1991) Preservation of avian blood and tissue samples for DNA analyses. *Can. J. Zool.*, **69**, 82–90.

Shapiro LH, Canterbury RA, Stover DM, Fleischer RC (2004) Reciprocal introgression between Golden-Winged Warblers (*Vermivora chrysoptera*) and Blue-Winged Warblers (*V. pinus*) in eastern North America. *Auk*, **121**, 1019–1030.

Short LL (1963) Hybridization in the wood warblers *Vermivora pinus* and *V. chrysoptera*. *Proceedings of the 13th International Ornithological Congress* 147–160.

Sorenson MD, Quinn TW (1998) Numts: A Challenge for Avian Systematics and Population Biology. *Auk*, **115**, 214–221.

Swofford DL (2002) *PAUP* Phylogenetic Analysis Using Parsimony (*and other methods) v.4.0b10*, Sinauer Associates, Sunderland, Massachusetts.

Tarr CL, Fleischer RC (1995) Evolutionary relationships of the Hawaiian honeycreepers (Aves: Drepanidinae). In: Hawaiian Biogeography: Evolution on a Hot Spot Archipelago (Eds. Wagner WL, Funk VA), pp. 147–159. Smithsonian Institution Press, Washington, DC.

Vines TH, Kohler SC, Thiel M, Ghira I, Sands TR, MacCallum CJ, Barton NH, Nurnberger B (2003) The maintenance of reproductive isolation in a mosaic hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata*. *Evolution*, **57**, 1876–1888.

Weir RD (1989) The nesting season, Ontario Region. *American Birds* **43**, 1310–1313.