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Complex hybridization dynamics between golden-winged and blue-winged warblers (*Vermivora chrysoptera* and *Vermivora pinus*) revealed by AFLP, microsatellite, intron and mtDNA markers

R. VALLENDER,*† R. J. ROBERTSON,† V. L. FRIESEN† and I. J. LOVETTE*
Fuller Evolutionary Biology Program, Laboratory of Ornithology, Cornell University, 159 Sapsucker Woods Road, Ithaca, NY 14850, USA, †Department of Biology, Queen's University, 4320 Biosciences, Kingston, ON, Canada K7L 3N6

Abstract

Blue-winged (*Vermivora pinus*) and golden-winged warblers (*Vermivora chrysoptera*) have an extensive mosaic hybrid zone in eastern North America. Over the past century, the general trajectory has been a rapid replacement of *chrysoptera* by *pinus* in a broad, northwardly moving area of contact. Previous mtDNA-based studies on these species' hybridization dynamics have yielded variable results: asymmetric and rapid introgression from *pinus* into *chrysoptera* in some areas and bidirectional maternal gene flow in others. To further explore the hybridization genetics of this otherwise well-studied complex, we surveyed variation in three nuclear DNA marker types — microsatellites, introns, and a panel of amplified fragment length polymorphisms (AFLPs) — with the goal of generating a multilocus assay of hybrid introgression. All markers were first tested on birds from phenotypically and mitochondrially pure parental-type populations from outside the hybrid zone. Searches for private alleles and assignment test approaches found no combination of microsatellite or intron markers that could separate the parental populations, but seven AFLP characters exhibited significant frequency differences among them. We then used the AFLP markers to examine the extent and pattern of introgression in a population where *pinus*-phenotype individuals have recently invaded a region that previously supported only a *chrysoptera*-phenotype population. Despite the low frequency of phenotypic hybrids at this location, the AFLP data suggest that almost a third of the phenotypically pure *chrysoptera* have introgressed genotypes, indicating the presence of substantial cryptic hybridization in the history of this species. The evidence for extensive cryptic introgression, combined with the lack of differentiation at other nuclear loci, cautions against hybrid assessments based on single markers or on phenotypic traits that are likely to be determined by a small number of loci. Considered in concert, these results from four classes of molecular markers indicate that *pinus* and *chrysoptera* are surprisingly weakly differentiated and that far fewer genetically 'pure' populations of *chrysoptera* may exist than previously assumed, two findings with broad implications for the conservation of this rapidly declining taxon.

Keywords: AFLP, blue-winged warbler, conservation, golden-winged warbler, hybridization, introgression

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Introduction

The study of hybridization dynamics continues to inform our understanding of evolutionary relationships and processes. In some systems, the pattern and extent of hybridization may also have profound conservation implications when it leads to the replacement of a rare species (Rhymer & Simberloff 1996; Randler 2002; Secondi *et al*. 2006; Seehausen 2006). Whereas hybridization is not often thought of as being a major contributing threat to the decline in biodiversity, there is increasing evidence that the

Correspondence: Rachel Vallender, Fax: 607-254-2486; E-mail: rf88@cornell.edu

impact of interbreeding on rare taxa is nontrivial (Rhymer & Simberloff 1996; Chan *et al*. 2006; Fredrickson & Hedrick 2006; Seehausen 2006). Extinction may result from the mixing of gene pools (Rhymer & Simberloff 1996; Chan *et al*. 2006) or via restricted mate choice resulting from the rarity of one species (Randler 2002; Chan *et al*. 2006; Secondi *et al*. 2006). Continued human-mediated introductions and habitat modification are increasing rates of hybridization worldwide. Molecular genetic studies enable us to examine hybridizing systems with greater precision, and have helped us recognize hybridization as a significant process in the extinction of species (Rhymer & Simberloff 1996; Fredrickson & Hedrick 2006; Seehausen 2006).

In eastern North America, the mosaic hybrid zone between blue-winged (*Vermivora pinus*) and golden-winged (*Vermivora chrysoptera*) warblers provides an opportunity to examine hybridization across regions with different histories of contact and potential, or known, introgression. The hybridization dynamics of these species may also have important conservation implications, as *chrysoptera* has been experiencing precipitous population declines throughout much of its breeding range in the USA since the mid-20th century (Gill 1980; Sauer *et al*. 2005). This taxon is one of the most rapidly declining songbird species in North America with regional declines as high as 15–18%/year (Sauer *et al*. 2005). *Vermivora chrysoptera* is currently being considered for protection under US Endangered Species Legislation (Confer & Tupper 2000), whereas in Canada it has recently been federally listed as Threatened (www.sararegistry.gc.ca).

Although hybrids between *pinus* and *chrysoptera* have been documented from as early as 1874 (Herrick 1874), these taxa likely occupied largely allopatric regions prior to the early 1900s, when the widespread abandonment of agricultural fields in eastern North America caused the regrowth of the early successional habitats used for breeding by both taxa (Gill 1980). *Vermivora pinus* subsequently advanced into the *chrysoptera* breeding range, and although their wide zone of contact has steadily moved northwards, hybridization remains extensive in areas where both forms occur. Typically, *chrysoptera* populations are negatively affected by hybridization and their local extirpation occurs within 50 years of the appearance of the first *pinus* individuals (Gill 1987, 1997). Two classic hybrid phenotypes known as 'Brewster's' and 'Lawrence's' warblers (Parkes 1951) are frequently depicted in popular field guides and display somewhat stereotyped plumage characteristics that enable their easy identification. However, close examination of series of hybrid individuals has suggested that there is substantial phenotypic variation in the plumage characters of hybrid individuals (Short 1963, 1969; Vallender, personal observation).

The mechanisms by which the *pinus*-phenotype predictably replaces the *chrysoptera*-phenotype after contact remain unclear. In many regions, source populations of *pinus* are likely to be substantially larger, leading to simple demographic swamping of the rarer *chrysoptera* populations. Male *pinus* may also be behaviourally dominant over male *chrysoptera* (Will 1986), and thereby obtain better quality territories in areas of sympatry, or they may have a higher rate of extra-pair copulations with females of both forms (Confer & Larkin 1998), although this has not yet been determined using molecular markers (both hybrids and *chrysoptera* show high rates of extra-pair paternity, Vallender *et al*. submitted). Other studies, however, have suggested that *chrysoptera* are more aggressive than *pinus* (Ficken & Ficken 1969). Some interspecific interactions may vary spatially: in some populations, *pinus* and *chrysoptera* do not appear to perceive each other as conspecifics and maintain overlapping territories (Confer & Knapp 1977), whereas in other locations males respond aggressively to individuals singing the other species' song (Ficken & Ficken 1969), albeit often with low frequency (Gill & Murray 1972). This regional variation in responsiveness to heterospecific song may result from differing histories of contact at those locations, or from variation in levels of genetic introgression.

Previous studies of the hybridization dynamics of these species leave many questions unanswered, but suggest that the situation is more complex than a simple survey of plumage phenotypes would indicate. Gill (1987) used morphological and allozyme variability to differentiate individuals from allopatric and phenotypically 'pure' populations of the two taxa. Whereas plumage characteristics are markedly different between *pinus* and *chrysoptera*, he found no fixed allozyme alleles that could serve as diagnostic autosomal markers for these parental forms. In a subsequent study, Gill (1997) used mitochondrial DNA (mtDNA) restriction fragment length polymorphisms (RFLP) to examine several populations with different histories of contact and hybridization. The two parental mitochondrial lineages differed by 3% nucleotide divergence, with all individuals from allopatric populations possessing their species' typical mtDNA lineage. In a region of very recent contact and known hybridization, however, *pinu*s mtDNA was present in high frequency in phenotypic *chrysoptera* as well as in almost all individuals with hybrid plumage phenotypes. This unexpectedly pervasive and directional mtDNA introgression led Gill (1997) to conclude that female *pinus* lead the northward advance into the hybrid zone, and that F_1 hybrid females preferentially backcross with *chrysoptera* males.

Two recent studies have further explored the pattern of mitochondrial introgression at five additional sites with various histories of contact and levels of hybridization (Shapiro *et al*. 2004; Dabrowski *et al*. 2005). None of these locations had a history of highly asymmetric introgression, suggesting that Gill (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 population from West Virginia. Smaller samples from Michigan and Ohio also failed to show evidence of asymmetric introgression. Likewise, similar analyses by Dabrowski *et al*. (2005) determined bidirectional gene flow and long-term persistence of *chrysoptera* mtDNA haplotypes in a sympatric population in New York with an extensive history of contact between the species. Finally, in an Ontario population in the earliest stages of hybridization (the same focal population considered here), *chrysoptera* mtDNA haplotypes were found in both *chrysoptera* and hybrid individuals (Dabrowski *et al*. 2005).

Here, we expand on these previous mtDNA-based surveys by employing three classes of nuclear DNA (nDNA) markers — microsatellites, intron sequences and amplified fragment length polymorphisms (AFLPs) — to more comprehensively explore the extent and pattern of hybridization between *pinus* and *chrysoptera* (Secondi *et al*. 2006). Our underlying motivation was to develop a panel of markers that could be used to quantify the ancestry of potentially hybrid individuals and thereby allow us to test questions about the relative fitness of pure vs. hybrid individuals, and to determine whether social and/or extra-pair mate choice is biased with respect to ancestry.

Methods

Population samples

We assayed a total of 127 individuals using a variety of molecular markers. Nineteen of these individuals came from an allopatric population of *chrysoptera* from Manitoba (MB), Canada (50°46′N, 99°30′W) and 35 came from an allopatric population of *pinus* from Kentucky (KY) and Tennessee, USA (36°32′N, 87°22′W). In both cases, the samples were collected from regions with both contemporary and historical isolation from the other taxon, and which are currently separated from populations of the other species by approximately 675 and 300 miles, respectively. These populations served as our 'control' samples of pure parental genotypes. An important assumption in our subsequent analyses is that these samples from phenotypically pure, allopatric populations are not genetically introgressed. Likewise, an additional assumption is that any differences uncovered are species-specific and not population-specific differences. This can readily be verified in future work when samples are obtained from other allopatric, phenotypically pure populations.

The remaining 73 samples (N = 21 female, N = 52 male) were collected between 2001 and 2004 from a population of *chrysoptera* at Queen's University Biological Station near Elgin, Ontario (ON), Canada (44°34′N, 76°19′W). This population has been the target of intensive monitoring efforts since 1997 (R. J. Robertson and T. Demmons, unpublished data), and has only recently been invaded by hybrid- and *pinus*-phenotype individuals. During the 2001–2004 period, the composition of the ON population was approximately 82% phenotypic *chrysoptera*, 17% hybrids, and 1% *pinus*. We classified the phenotype of each adult (male and female) sampled as pure *chysoptera*, pure *pinus*, classic 'Brewster's' warbler, classic 'Lawrence's' warbler (Parkes 1951) or phenotypically introgressed (Parkes 1951; Short 1963). An individual was characterized as introgressed if plumage characteristics did not match any of the stereotypical parental or hybrid phenotypes described by Parkes (1951). For example, an individual with *chrysoptera* facial and body patterns but a band of bright yellow feathers under the throat patch would be considered introgressed as it displays neither a classic *chrysoptera* pattern nor a 'Lawrence's' warbler pattern, but appears to be a variant of the two.

Mitochondrial DNA analyses

Several previous studies have shown that the parental plumage phenotypes of *chrysoptera* and *pinus* are associated with substantially divergent mtDNA lineages (Gill 1997; Shapiro *et al*. 2004; Dabrowski *et al*. 2005). These ancestral species lineages are separated by 3% using RFLPs and by 4.2–4.9% nucleotide divergence at the NDII gene (Shapiro *et al*. 2004; Dabrowski *et al*. 2005). By amplifying and sequencing this locus using protocols described in Dabrowski *et al*. (2005), we determined the ancestral mtDNA lineage for 97 samples: 12 phenotypically pure *chrysoptera* from MB, 12 phenotypically pure *pinus* from KY, and 73 birds of various phenotypes from our population of active hybridization in ON. Based on these NDII sequences we assigned each individual to either a *chrysoptera* or *pinus* haplotype group. Eleven of these samples were previously analysed and reported (Dabrowski *et al*. 2005).

Microsatellite analyses

We screened 54 samples from our two allopatric populations using a panel of 13 microsatellite markers (Table 1). These markers included 11 loci developed from a *chrysoptera* microsatellite library (Stenzler *et al*. 2004; Table 1) and two loci from a library developed from the Swainson's Warbler (*Limnothlypis swainsonii*; Winker *et al*. 1999; Table 1). We looked for two types of variation that could allow us to use microsatellite data to explore patterns of hybridization. First, we looked for alleles that were present in one species but not the other (i.e. private alleles). Second, we tested whether allele frequencies differed between parental populations in ways amenable to the use of assignment test methodologies.

Table 1 Details of PCR conditions and microsatellite loci used on the total sample of individuals from the two allopatric populations included in the analyses

	Manitoba GWWA				Kentucky BWWA				Both sites			
Locus	No. of alleles	H_O	H_E	N_A freq.	No. of alleles	H_O	H_E	N_A freq.	No. of alleles	H_O	H_E	N_A freq.
VeCr 01	5	0.211	0.579	0.442	4	0.314	0.351	0.104	6	0.278	0.440	0.235
VeCr 02	8	0.611	0.754	0.095	11	0.829	0.795	0.025	12	0.755	0.781	0.017
VeCr 04	12	0.421	0.872	0.334	12	0.743	0.836	0.050	14	0.630	0.847	0.139
VeCr 05	4	0.444	0.449	0.031	5	0.314	0.481	0.221	5	0.358	0.468	0.136
VeCr 06	3	0.056	0.110	0.453	2	0.086	0.083	0.013	4	0.075	0.092	0.172
VeCr 07	5	0.421	0.710	0.243	7	0.543	0.628	0.072	8	0.500	0.669	0.147
VeCr 08	20	0.737	0.930	0.106	24	0.794	0.950	0.080	32	0.774	0.948	0.096
VeCr 10	3	0.158	0.152	0.032	3	0.371	0.323	0.094	3	0.296	0.265	0.073
VeCr 11	1	0.000	0.000	0.000	2	0.029	0.029	0.002	2	0.019	0.019	0.000
VeCr 14	11	0.895	0.808	0.070	11	0.697	0.662	0.031	13	0.769	0.692	0.039
VeCr 16	2	0.368	0.422	0.055	3	0.629	0.528	0.099	3	0.537	0.498	0.046
Lswμ 12	20	0.895	0.957	0.021	17	0.929	0.950	0.009	23	0.909	0.953	0.017
Lsw μ5B	9	0.526	0.801	0.200	13	0.559	0.750	0.128	15	0.547	0.772	0.159
Average	7.923	0.442	0.580		8.769	0.526	0.567		10.769	0.496	0.573	

GWWA, golden-winged warbler, *chrysoptera* ($N = 19$); BWWA, blue-winged warbler, *pinus* ($N = 35$); Both sites, samples from both populations combined ($N = 54$). H_O, observed heterozygosity; H_E, expected heterozygosity; N_A freq., null allele frequency.

Laboratory protocols for amplifying and screening these loci followed Vallender *et al*. (submitted) and Stenzler *et al*. (2004). The microsatellite data set comprised 673 genotypes of a possible 702 (i.e. 54 individuals × 13 loci). CERVUS version 2.0 (Marshall *et al*. 1998; available at www.fieldgenetics.com/pages/home.jsp) was used to calculate allele frequencies, the expected frequency of heterozygotes (H_E), the observed frequency of heterozygotes (H_O) and the null allele frequency at all loci (Table 1).

We used the assignment test methods implemented in STRUCTURE 2.1 (Pritchard *et al*. 2000) to test whether the microsatellite data grouped individuals from the allopatric populations into clusters that were consistent with their respective sampling locations. These analyses were conducted without providing any information regarding sampling origin, under the no-admixture model to consider allele frequencies independent among populations; these parameters assume that the populations have not had recent mixed ancestry (Pritchard *et al*. 2000). An initial burn-in of 50 000 generations was followed by 100 000 iterations of the Markov chain.

Intron sequence analyses

To explore whether intron sequences provide a ready source of alleles diagnostic of the parental taxa, we conducted preliminary DNA sequence surveys of four intron loci that we have employed in broader phylogenetic surveys of wood-warblers and which vary substantially among species in this family (I. J. Lovette, unpublished data; Table 2). The intron tests included 13 *pinus* samples and 10 *chrysoptera* from the allopatric, phenotypically pure parental populations. Alleles associated with only one parental population would be candidates for further screening to determine whether they were diagnostic of that taxon.

All amplifications (10 μL) contained 10–100 ng of genomic DNA, 0.25 U of Jumpstart *Taq* Polymerase (Sigma), 10 mM Tris-HCL (pH 8.3), 50 mM KCl, $MgCl_2$ specific to each locus (Table 2), 200 μM of dNTPs (Invitrogen), and 2.0 pmol each of forward and reverse primers. Cycling profiles included 1 cycle at 95 °C for three min, 30–35 cycles of 1 min at 95 °C, 1 min at the locus-specific annealing temperature (T_a; Table 2), and 1 min at 72 °C, followed by a final extension cycle of 5 min at 72 °C. Annealing temperatures followed by an 's' in Table 2 employed a step-down profile in which the initial T_a was 5 °C higher than indicated, with the T_a lowered successively by 0.5 °C on each of the next nine cycles. Cycle sequencing employed primers listed in Table 2 and followed protocols described in Dabrowski *et al*. (2005). The resulting chromatograms were assembled and compared using SEQUENCHER 4.5 (Gene Codes Corp. 1998).

Allelic variation was low at all four intron loci, making it straightforward to infer the sequences of both alleles in heterozygotes as well as the numerous homozygous individuals. We used the gene genealogy approached implemented in TCS 1.21 to reconstruct the historical relationship among alleles. This approach assumes a lack of recombination at each locus; although recombination

Table 2 Details of nuclear intron primers and PCR conditions used in analyses

Locus	Primer†	Sequence (5′–3′)	T_a‡	mM $MgCl_2$	Source
aconitase 1 intron 9 (z-linked)	ACO-I10F	CTGTGGGAATGCTGAGAGATTT	55 s	2.0	F.K. Barker, unpub.
	ACO-I10R2	CAACTTTGTCCTGGGGTCTTT			F.K. Barker, unpub.
	ACO-I10intF*	CCTCTGTGGTAAMCACAAAGCA			F.K. Barker, unpub.
	ACO-I10intR*	GCAGACCCAAACACAAGTTACAA			F.K. Barker, unpub.
beta-fibrinogen intron 5	Fib5	CGCCATACAGAGTATACTGTGGACA	52 s	3.0	F.K. Barker, unpub.
	Fib6	GCCATCCTGGCGATTCTGAA			F.K. Barker, unpub.
muscle-specific tyrosine kinase	MUSK-I4F2	AAATAACCCGACCACCTGTAAA	55 s	2.0	F.K. Barker, unpub.
intron 4 (z-linked)	MUSK-I4R	CTCTGAACATTGTGGATCCTCAA			F.K. Barker, unpub.
transforming growth factor beta-2 intron 5	TGFB2–15F	GAAGCGTGATGTAGATGCTG	52 s	2.0	Primmer *et al*. 2002
	TGFB2–15R	AGGCAGCAATTATCCTGCAC			Primmer *et al*. 2002

†asterisks indicate internal primer used only for cycle sequencing; ‡PCR annealing temperatures; 's' indicates step-down cycling profile (see Methods).

might influence the allele network structure, for our purposes here, violations of this assumption would not affect the relevant allele–source population associations.

AFLP analyses

AFLP analyses were carried out following Berres *et al*. (in press) with the modifications outlined here. DNA was extracted from blood stored in a lysis buffer (White & Densmore 1992) using Eppendorf Perfect gDNA Blood Mini Isolation Kits (Brinkmann Instruments, Inc.) with a final elution volume of 200 μL 10 mM Tris-Cl, pH 9.0. DNA quality was confirmed by running 4 μL of extraction product through 0.8% agarose-TBE gels. This step is important because high quality DNA is needed for accurate AFLP results (Ritland & Ritland 2000) as degradation can lead to excessive background and erroneous peaks (Kingston & Rosel 2004).

Approximately 800–1000 ng of genomic DNA was then digested overnight at 37 °C with 2.0 U EcoR 1 (20 000 U/mL, New England Biolabs, Ipswich, Massachusetts) and 0.5 U *Bfa*I (5000 U/mL, New England Biolabs) and 1× NEBuffer 4 (New England Biolabs). After a maximum of 12 h, we ran 4 μL of the restricted DNA through 1.5% agarose gels along with a control sample of uncut DNA as well as samples that had been cut with only one enzyme. These controls confirmed that both restriction endonucleases were working effectively, and simultaneously allowed us to check for complete digestion, as partial digestion can severely bias AFLP methodologies (Vos *et al*. 1995).

We then added 20 μL of a ligation mixture [including T4 buffer; 4 U T4 DNA ligase (400 000 U/mL, New England Biolabs); 0.38 μM E-adaptor (5′-CTCGTCGACTGCGTACC-3′); 0.38 μM B-adaptor (5′-AATTGGTACGCAGTCTAC-3′)] to each sample. The samples were held at 16 °C overnight (maximum of 12 h). The digested DNA with ligated adaptors was then diluted with 80 μL 10 mM Tris (pH 8.5), and 10 μL of this mixture was used in a preselective polymerase chain reaction (PCR) which included 10× reaction buffer (Sigma-Aldrich), 1.5 mM $MgCl_2$ (Sigma), 0.75 μM each of Bfa 1 + T preselective primer (5′-GAT-GAGTCCTGAGTAGT-3′) and EcoR 1 + G preselective primer (5′-GACTGCGTACCAATTCG-3′), 0.08 μM dNTP (each), 1.25 U JumpStart *Taq* polymerase (Sigma) and DNA grade ddH20 (Fisher Scientific) to a final volume of 50 μL. Fragments were amplified using the PCR conditions of 72 °C for 15 min followed by 20 cycles of 94 °C for 50 s, 56 °C for 60 s and 72 °C for 120 s.

Following this preselective PCR, we further diluted each sample with 80 μL 10 mM Tris (pH 8.5) and used 5 μL of the dilute mixture in a selective PCR to amplify a subset of restriction sites (Vos *et al*. 1995). We added 10× reaction buffer (Sigma), 1.5 mM $MgCl_2$ (Sigma), 0.25 μM EcoR 1 selective primer (EcoR 1 + G preselective primer plus three additional bases, Table 3) labelled with a 5′-flourescent tag, 1.25 μM Bfa 1 primer (Bfa 1 + T preselective primer plus three additional bases, Table 3), 0.05 μM dNTP (each), 1.25 U JumpStart *Taq* polymerase (Sigma) and DNA grade ddH20 (Fisher Scientific) to a final volume of 25 μL. Our touchdown temperature profile involved 94 °C for 50 s, 65–57 °C for 60 s with the temperature lowered 1 °C per cycle, 72 °C for 120 s. This was followed by 20 cycles of 94 °C for 50 s, 56 °C for 1 min, 72 °C for 2 min. The program ended with a cycle of 72 °C for 10 min.

Successful selective amplification was confirmed via electrophoresis of 4 μL of product through a 1.25% agarose gel. Samples were then prepared for electrophoresis in an ABI 3100 automated sequencer (Applied Biosystems) by diluting 1 μL of PCR product with 27 μL DNA grade ddH20 (Fisher Scientific). This was further diluted by adding 1 μL of this mixture to 10 μL Hi-Di Formamide (Applied Biosystems) along with 1 μL GeneFlo 625 DNA ladder (CHIMERx; 1:5 dilution with Hi-Di formamide).

GENESCAN (Applied Biosystems) was used to convert files into a format that enabled their visualization in GENOGRAPHER version 1.6.0 (Montana State University;

Table 3 Details of selective primer combinations and fragments resulting from AFLP analyses

EcoRI primer* (NNN-3′)	BfaI primer† (NNN-3′)	Number of amplified fragments‡	Number of monomorphic fragments	Number of species indicative fragments
GCG	TGG	65	19	2
GCG	TCG	104	22	2
GA	TGA	32	11	1
GA	TAA	42	15	1
GA	TAG	38	8	1

*5′-GAT GAG TCC TGA GTA GT-3′; †5′-GAC TGC GTA CCA ATT CG-3′; ‡in the range from 50 to 400 bp.

available at http://hordeum.oscs.montana.edu/genographer). Genographer assembles the data from separate electrophoresis capillaries into a virtual gel, which fosters the comparison of AFLP alleles among individuals. Fragments were scored as present (1) or absent (0) for each individual. For fragments between 50 and 250 bp in size we used a cut-off of 150 fluorescent units; for fragments of size 251–400 bp, we used a cut-off of 50 U. This approach filters out background and helps prevent problems associated with uneven amplification among samples. As such, any fragments that fell below these cut-offs were scored as being absent (Kingston & Rosel 2004).

We used STRUCTURE to examine AFLP differences among the allopatric and actively hybridizing populations, and to assign individuals to one of two ancestral populations. We first tested whether STRUCTURE could group individuals from the two allopatric populations into clusters that corresponded with sampling sites. We considered these two populations to be species reference groups (and not just population reference groups) and used them to assign the ancestry of the samples from the area of active hybridization using a Bayesian clustering approach (Pritchard *et al*. 2000). We used an admixture model for our individuals of unknown ancestry, set the rate of migration between the populations as 0.001 and set allele frequencies as being independent among populations. Our burn-in length remained at 50 000 followed by 100 000 iterations of the Markov chain.

In order to verify results obtained using STRUCTURE, we also employed AFLPOP (Duchesne & Bernatchez 2002; available at www2.bio.ulaval.ca/louisbernatchez/) to assign individuals from the ON population to the KY or MB populations. We used the default settings for all analysis parameters (Duchesne & Bernatchez 2002) and considered that populations may contain F_1, F_2 and backcross hybrids. This means that ON individuals could be assigned to either of the parental populations, designated as an F_1 hybrid, an $F_1 \times$ GWWA hybrid, an $F_1 \times$ BWWA hybrid or an F_2 hybrid (Duchesne & Bernatchez 2002).

AFLP repeatability

To test for repeatability of AFLP results we ran five samples from our ON population twice from DNA extraction through to selective PCR. In all cases the banding pattern was very similar and produced identical results for all seven of our diagnostic characters, even though there was some variation in the quality of the PCR (as evidenced by differences in band intensity between replicates). These results indicate that the repeatability of AFLPs is high and that it is unlikely that PCR artefacts have substantially affected our analyses.

Results

Applying the plumage-based criteria to our 73 samples from ON suggested that 49 were of the *chrysoptera* parental phenotype, 14 were clear hybrids, 9 showed introgressed plumage that differed from the classic hybrid phenotypes, and one was a phenotypic *pinus*. Introgressed individuals typically displayed plumage characteristics intermediate between the two named hybrid forms (e.g. yellow plumage on the belly of an otherwise phenotypic *chrysoptera*). Males who had parental plumage phenotypes but that sang a species-inappropriate song were not considered genetically introgressed, as song is a learned trait (Highsmith 1989; Podos *et al*. 2004), and it is likely that these individuals were raised in a sympatric population and learned their songs from a neighbour of the alternate species (Podos *et al*. 2004).

Mitochondrial DNA

All of our 12 *chrysoptera* samples from MB belonged to the typical *chrysoptera* mtDNA lineage. Likewise, all 12 samples from the KY *pinus* population had the corresponding *pinus* mtDNA lineage. As we also found in an earlier study based on mtDNA markers (Dabrowski *et al*. 2005), birds from the actively hybridizing ON population had a diverse set of mtDNA–phenotype associations

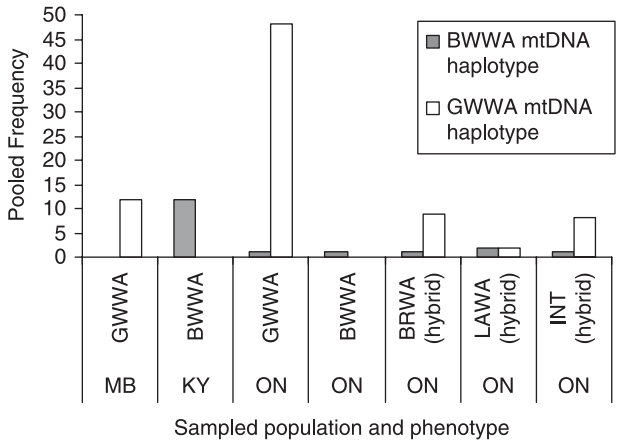


Fig. 1 Plumage characteristics by mtDNA haplotypes for each of three populations. Ontario (hybridizing population, ON); Manitoba (allopatric *chrysoptera* population, MB); Kentucky (allopatric *pinus* population, KY). GWWA, golden-winged warbler (*chrysoptera*); BWWA, blue-winged warbler (*pinus*); BRWA, 'Brewster's' warbler hybrid phenotype; LAWA, 'Lawrence's' warbler hybrid phenotype; INT., other introgressed phenotype.

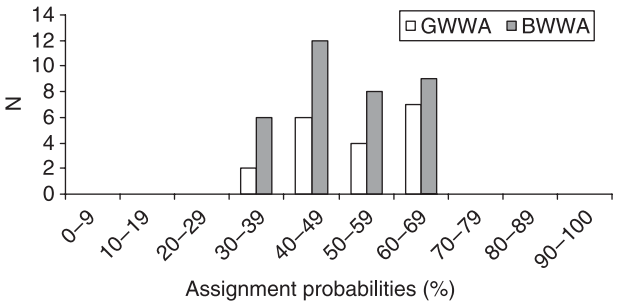


Fig. 2 Assignment probabilities to the ancestral *chrysoptera* population for allopatric *chrysoptera* ($N = 19$) and allopatric *pinus* ($N = 35$) based on 13 microsatellite loci. GWWA, golden-winged warbler (*chrysoptera*); BWWA, blue-winged warbler (*pinus*). Mean values by species are: GWWA = 0.53 ± 0.10 SD, BWWA = 0.51 ± 0.09 SD.

(Fig. 1). Specifically, whereas 48 of the 49 phenotypic *chrysoptera* samples from ON had the *chrysoptera* mtDNA lineage, one had the *pinus* mtDNA lineage. Likewise, the one *pinus* sample belonged to the *pinus* mtDNA lineage. Eight of the nine individuals with introgressed plumage had the *chrysoptera* mtDNA lineage, whereas one had the *pinus* mtDNA lineage. Of the individuals with named hybrid phenotypes, nine 'Brewster's' warblers had the *chrysoptera* group and one had the *pinus* mtDNA type. Finally, the four 'Lawrence's' warbler individuals were evenly split between the *pinus* and *chrysoptera* mtDNA lineages (Fig. 1).

Microsatellites

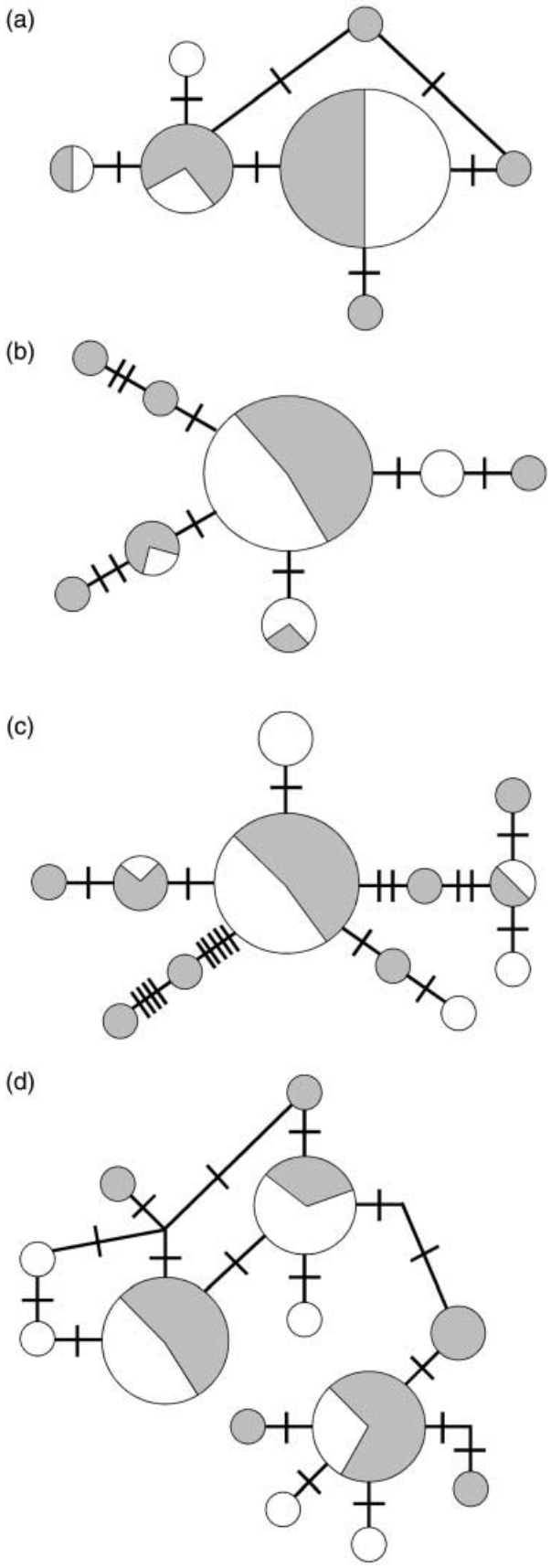
The number of microsatellite alleles ranged from two to 32 per locus, with a mean of 10 alleles per locus (Table 1). Observed and expected heterozygosity values, as well as null allele frequencies by locus (all samples combined as well as analysed separately by species) are also reported in Table 1. No common alleles were restricted to only one of the parental populations, and this absence of private alleles made it impossible to score ancestry simply on the presence/absence of diagnostic microsatellite markers.

More rigorous assignment test analyses of allele frequency patterns further suggested that these parental populations were weakly (if at all) differentiated at these microsatellite loci. The highest probability with which an individual could be assigned to one of two ancestral populations was 65%, and the average assignment probability was 53% for *chrysoptera* individuals and 50% for *pinus* individuals (Fig. 2). This nearly random assignment of phenotypically pure individuals to their population of origin indicated that these microsatellite markers are not useful for typing the ancestry of hybrid individuals, despite the reasonably large number of loci examined and their substantial allelic variability.

Introns

We likewise found no evidence of diagnostic allelic variation at any of the four intron loci. Sequences from the different loci ranged in length between 483 and 992 nucleotides, and in total we obtained 2551 nucleotides of intron sequence from each individual (GenBank Accession nos DQ310716–DQ310720). In comparison among the four individuals, we found one to nine single nucleotide polymorphisms (SNP) at each locus (39 variable nucleotide sites in total) and two short insertions/deletions. Most variable character states found in more than one individual (i.e. potential synapomorphies) were found in alleles drawn from both parental populations.

Owing to the low total variation and the presence of numerous homozygous individuals, the sequences of the individual alleles could be determined in all cases; one locus had seven alleles, one had eight and two had 12. In each case, the most common allele was present in nearly equal frequency in individuals from each allopatric parental population (Fig. 3). Although further sampling might indicate that the parental populations differ in allele frequencies at these loci or that some rare or nonsampled private alleles occur in only one parental taxon, our initial screening demonstrates unambiguously that the populations are not reciprocally fixed at any of the four loci. This lack of fixed differences suggests that the intron sequences do not provide a simple index of the hybrid ancestry of individuals, and hence we did not further explore these or other intron loci.



AFLP screening

Initial screening of 23 individuals ($N = 10$ *chrysoptera* and 13 *pinus* from the phenotypically pure populations) with 54 selective primer combinations generated over 3600 amplified fragments (average number of fragments per primer pair = 68). Despite the large number of fragments that were variable among individuals we found no characters that were fixed within species, as has similarly been found in other closely related hybridizing taxa (e.g. Helbig *et al*. 2005). However, seven characters derived from five different primer pairs showed significant frequency differences between the two taxa (Table 4). The number of amplified fragments for these five primer pairs ranged from 32 to 104, with 16–36% of fragments being shared between the two species (Table 3). All individuals from the two allopatric populations were assigned to different populations (i.e. their ancestral ones) with > 99% confidence.

Assigning hybrid identity

The differences in allele frequencies at a small subset of variable AFLP loci suggest that individuals from our allopatric populations are weakly but significantly differentiated (Wiens & Servedio 2000). In conjunction with the known differentiation between these populations at plumage and mtDNA markers, we assumed that these AFLP loci are representative of their corresponding ancestral species. We recognize that size homoplasy can result in false interpretations of genetic similarity (O'Hanlon & Peakall 2000) but we assumed that co-migrating fragments were homologous. Furthermore, we assumed that the genetic similarity between members of these species was due to shared ancestry and not to contemporary gene flow (Allendorf *et al*. 2001). This is supported by current breeding ranges of the two species that do not overlap in the sample collection areas, by field observations that have not documented the occurrence of the alternate species, as well as mtDNA data (i.e. complete concordance between phenotype and mtDNA haplotype in all samples tested from the two allopatric populations). Accordingly, we used these allopatric populations as our reference for assignment of birds within the hybrid zone.

Fig. 3 Intron allele networks based on four loci: (a) aconitase 1 intron 9, (b) transforming growth factor beta-2 intron 5, (c) beta-fibrinogen intron 5, and (d) muscle-specific tyrosine kinase intron 4. Each network includes both alleles from 13 *pinus* individuals and 10 *chrysoptera* individuals from allopatric parental populations. Each unique allele is represented by a circle proportional in size to the number of individuals with that allele. Shaded areas indicate alleles from *pinus* individuals, whereas white areas represent alleles from *chrysoptera* individuals.

Table 4 AFLP allele frequencies for different populations, plumage phenotypes and mtDNA haplotypes. All frequencies between the Manitoba and Kentucky populations are significantly different at $P < 0.05$. MB, Manitoba; KY, Kentucky; ON, Ontario; LAWA, 'Lawrence's' warbler; BRWA, 'Brewster's' warbler; INT, phenotypically introgressed individuals

				AFLP marker loci (frequency of character)						
Pop.	Phenotype	mtDNA	N	3	5B	26	28	29	3A	5F
MB	*chrysoptera*	*chrysoptera*	10	0.00	0.00	1.00	0.00	0.75	0.00	0.10
KY	*pinus*	*pinus*	13	0.78	0.69	0.08	0.64	0.11	0.82	0.92
ON	*pinus*	*pinus*	1	0.00	0.00	1.00	0.00	1.00	0.00	1.00
ON	*chrysoptera*	*pinus*	1	—	0.00	1.00	0.00	1.00	—	1.00
ON	*chrysoptera*	*chrysoptera*	48	0.22	0.17	0.82	0.02	0.90	0.08	0.38
ON	BRWA	*pinus*	1	0.00	1.00	1.00	0.00	0.00	0.00	1.00
ON	BRWA	*chrysoptera*	9	0.11	0.22	0.88	0.00	0.63	0.00	0.44
ON	LAWA	*pinus*	2	0.00	0.00	0.50	0.50	0.50	0.00	0.00
ON	LAWA	*chrysoptera*	2	0.00	1.00	0.50	0.00	0.50	0.50	1.00
ON	INT	*pinus*	1	0.00	0.00	1.00	0.00	1.00	0.00	1.00
ON	INT	*chrysoptera*	8	0.00	0.13	0.71	0.13	0.75	0.17	0.38

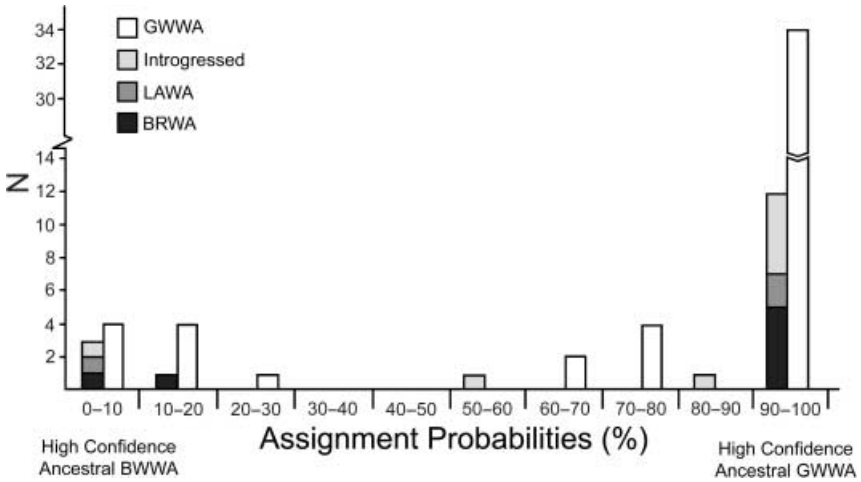


Fig. 4 Assignment probabilities to the ancestral *chrysoptera* population for *chrysoptera* and hybrids from ON, *chrysoptera* from MB and *pinus* from KY based on AFLP analyses. GWWA, golden-winged warbler (*chrysoptera*); BRWA, 'Brewster's' warbler; LAWA, 'Lawrence's' warbler.

In applying these AFLP markers to the ON population, 32 (67%) of 48 phenotypic *chrysoptera* with ancestral *chrysoptera* mtDNA haplotypes were assigned to the ancestral *chrysoptera* population with high confidence (> 95%; Fig. 4). An additional five (10%) phenotypic *chrysoptera* were assigned to this ancestral species with > 70% confidence. The remaining 11 (23%) individuals were assigned with greater probability to the ancestral *pinus* population, or were not assigned to either population (i.e. equal probability of being assigned to the *chrysoptera* or *pinus* ancestral populations). The one phenotypic *pinus* included in our analyses likewise could not be assigned to either ancestral population, suggesting this individual is a genetic hybrid.

Individuals assigned to one of the two hybrid phenotypes based on plumage characteristics showed a complex pattern of assignment (Fig. 4). Seven phenotypic hybrids were assigned to the ancestral *chrysoptera* population, whereas the remaining three hybrids were assigned to the ancestral *pinus* population. More 'Brewster's' warblers were assigned to the ancestral *chrysoptera* population than they were to the ancestral *pinus* population, whereas the 'Lawrence's' warblers were assigned approximately equally to both ancestral populations.

Birds showing only slight evidence of introgressed plumage characters as well as individuals exhibiting incongruence between phenotype and mtDNA haplotype also exhibited a complex assignment pattern. Six phenotypically introgressed individuals were assigned to the *chrysoptera* ancestral population with high confidence, while only one was assigned to the *pinus* ancestral population. One individual was not clearly assigned to either population.

Results from AFLPOP showed a similar pattern of assignment with almost 70% of ON samples being assigned

to the MB golden-winged warbler population with high confidence. No samples were assigned to the KY blue-winged warbler population. Assignments of individuals to the various hybrid categories showed no pervasive pattern with respect to plumage characteristics of individuals, and generally lacked power. Accordingly, we did not pursue this further.

Discussion

Many species that regularly hybridize are morphologically and genetically very similar (Bensch *et al*. 2002a). In these cases, molecular genetic variation may be shared between hybridizing taxa both via the retention of ancestral variation and through ongoing introgression-mediated gene flow. Indeed, even when mtDNA shows marked differentiation, nuclear markers often do not (Bensch *et al*. 1999; Bensch *et al*. 2002a, b; Kingston & Rosel 2004). This presents a challenge, because it is these autosomal markers, being biparentally inherited, that enable us to more thoroughly examine the genetic pattern and implications of hybridization. AFLP methodologies have been widely used in plant, fungal and bacterial research, but they are less frequently employed in animal studies (Bensch & Åkesson 2005). While AFLP techniques have limitations associated with their inability to detect heterozygotes (i.e. characters are scored as either present or absent), the benefits of using AFLPs include the ability to sift rapidly through the genome for loci that have atypically recent coalescences, a relatively short start-up time, and generation of data from hundreds of loci with moderate costs (Bensch & Åkesson 2005; Helbig *et al*. 2005). In cases where other marker types have revealed weak genetic structure (e.g. *Phylloscopus trochilus*, Bensch *et al*. 2002a), the power of AFLPs for future work on animals has been highlighted (Bensch & Åkesson 2005).

Whereas our initial attempts to find intron or microsatellite genotypes diagnostic of the two parental lineages failed, the AFLP approach proved somewhat more effective. Given the substantial mtDNA divergence between these ancestral lineages, we anticipated that some degree of genetic differentiation would have accumulated during a period of isolation prior to secondary contact, and that this would be evident in rapidly evolving nuclear loci such as microsatellites. The great similarity in these species' microsatellite allele distributions may be due to back mutations (i.e. homoplasy), gene flow between these populations (Balloux *et al*. 2000) or limited lineage sorting on account of the larger effective population size of the nuclear genome (Hudson & Turelli 2003; Ballard & Whitlock 2004). The low variation at intron loci is less surprising, as these loci have much lower mutation rates. Similarly low levels of interspecific variation are seen in comparisons of these same loci between other known sister species in the Parulidae (I.J. Lovette, unpublished data). Alternatively, if differences in plumage characters have evolved very recently there could be weak contemporary gene flow, but we could still see striking phenotypic differences and a lack of genetic differentiation at nuclear loci (Bensch *et al*. 1999).

Considered in concert, these three classes of nuclear markers provided congruent results in showing that nuclear genome differentiation between these taxa is very low, with differences between them only being detectable using the most sensitive method. Similar results were found in phenotypically distinct subspecies of hybridizing willow warblers (*Phylloscopus trochilus trochilus* and *Phylloscopus trochilus acredula*) for which both mtDNA control regions and microsatellite markers failed to differentiate the subspecies (Bensch *et al*. 1999) but a small proportion of AFLP markers were able to do so (Bensch *et al*. 2002a). Not all actively hybridizing passerines have this level of genetic similarity; for example, hybridizing black-capped chickadees (*Poecile atricapillus*) and Carolina chickadees (*Poecile carolinensis*) can be readily differentiated using microsatellite markers alone (Reudink *et al*. 2006). However, theses results are perhaps not surprising seeing as these chickadee species are more distantly related to one another (Reudink *et al*. 2006) than are the willow warblers or the subjects of this study.

Whereas *chrysoptera* and *pinus* exhibit notably differentiated plumage characters, and mtDNA nucleotide divergence (Shapiro *et al*. 2004; Dabrowski *et al*. 2005) that puts them on par with other avian biological species (reviewed in Johnson & Cicero 2004), there has long been an assumption that the widespread production of viable hybrids means the parental taxa are genetically very similar. Our actively hybridizing population provides an opportunity to test this assumption, and to further elucidate the genetic relationship between these species. Given the high preponderance of seemingly pure parental phenotypes and the strong association between phenotypic traits and mtDNA (only one of 49 individuals with pure plumage phenotypes showed a mismatch between mtDNA lineage and plumage type), we expected to find low overall rates of introgression in our actively hybridizing population in ON. Moreover, hybrids from this same area exhibited mostly ancestral *chrysoptera* mtDNA haplotypes, indicating limited bidirectional gene flow (as was also found in Dabrowski *et al*. 2005), and field observations have documented that the majority of females in the population are of the *chrysoptera* phenotype (R. Vallender, unpublished data).

In contrast, the AFLP results do not support our a priori expectations about the ancestry of this actively hybridizing population. Sporadic sightings of *pinus* individuals in the area were first reported in the 1960s (Weir 1989) but *pinus* breeding at our study sites was only confirmed in 2005. Given the continued relative rarity of *pinus* and hybrid individuals, we expected the population to have remained

primarily genetically *chrysoptera*. However, over the course of fieldwork on this population the frequency of hybrids (including those with introgressed plumage characteristics) increased from 12% in 2001 to 33% in 2003 and 27% in 2004. The AFLP evidence and the increasing number of individuals with hybrid plumage phenotypes together suggest that extensive cryptic genetic introgression occurs before it is phenotypically pervasive.

Genotypes and phenotypes

The hybrid origin of 'Brewster's' and 'Lawrence's' warblers was intermittently disputed until Parkes (1951) suggested a plausible pattern of inheritance for the plumage characters seen in the parental species. Parkes (1951) suggested that the black throat patch seen in male *chrysoptera* is the result of a single gene, with absence of the throat patch being dominant (i.e. *pinus* carry the dominant gene showing no evidence of a throat patch). As such, the hybrid phenotypes are expected to have one or the other facial pattern (Parkes 1951; Gill & Murray 1972).

The genetics of body plumage colouration is considered to be more complicated, with underpart colour initially thought to be under the control of a single gene (with white being dominant over yellow). However, the appearance of 'Brewster's' warblers (presumed to be F_1 hybrids) with yellow bellies suggests that the allele for white underparts is incompletely dominant over the allele for yellow underparts. As such a bird that is heterozygous at this locus is basically white but may show some yellow colouration (Parkes 1951).

All of the 'Brewster's' warblers in our population had white plumage on their underparts and were more likely to be classified as belonging to the ancestral *chrysoptera* population. This supports Parkes' (1951) assumption that these individuals are likely backcrosses with *chrysoptera*. Individuals of the 'Lawrence's' warbler phenotype were no more likely to be assigned to the ancestral *pinus* population than to the *chrysoptera* population, which also is consistent with Parkes' suggestion of their origin.

However, the current data suggest that Parkes (1951) analysis of plumage characters associated with F_1 and subsequent backcrosses is likely too simplistic (Allendorf *et al*. 2001). Indeed, hybrid individuals are often assumed to possess morphological characters from both parental species (Allendorf *et al*. 2001). However, our results support the idea that individuals within hybrid swarms (where a high proportion of individuals in a population are introgressed due to mating between hybrids and backcrossing with the parental species, Allendorf *et al*. 2001) may be morphologically indistinguishable from one of the parental species but contain genes from both parental taxa. It is also important to consider that even with a moderate influx of a foreign species' genes in a population, a percentage of hybrids will appear to be genetically pure based on individual diagnostic loci.

Conservation implications

As in many cases of geographically widespread hybridization, the past and present hybridization dynamics between *chrysoptera* and *pinus* are complex. Because *chrysoptera* populations are shrinking as *pinus* populations expand, the major conservation concern in this case is the preservation of pure *chrysoptera* populations. Unfortunately, two aspects of our genetic screening suggest that the prospects for maintaining viable *chrysoptera* populations are even worse than has been anticipated. First, we found no detectable differentiation among *chrysoptera* and *pinus* across a substantial number of microsatellite and nuclear intron loci, and only weak segregation at a small subset of AFLP sites. The great genetic similarity of these species helps explain why they hybridize so readily, and why hybrid individuals appear to have high fitness (Vallender *et al*. submitted). Indeed, high hybrid reproductive success suggests a lack of fitness consequences associated with hybrid pairing, and a concomitant absence of potential for reinforcement. Second, we found evidence for cryptic past hybridization in many *chrysoptera* that we scored as pure parental-type individuals using both plumage and mtDNA markers. If this finding of extensive hidden introgression is generalizable to other *chrysoptera* populations, it suggests that the number of pure populations may be much smaller than field surveys of plumage phenotypes currently suggest. Therefore, the most pressing conservation action directed at maintaining pure *chrysoptera* populations may be to preserve those populations that are most isolated from *pinus* populations.

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Rachel Vallender is a Post-doctoral Associate in the Fuller Evolutionary Biology Program at the Cornell Lab of Ornithology. Her current research is examining patterns of avian hybridization in North American wood warblers. Her main interests lie in conservation genetics and behavioural ecology. Raleigh J. Robertson holds the Baillie Family Chair in Conservation Biology at Queen's University. His research deals with the behavioural ecology and conservation biology of birds, mostly focused at the Queen's University Biological Station, where he maintains a long-term study of Tree Swallows as well as continuing studies of Golden-winged Warblers and Cerulean Warblers. Vicki Friesen is an Associate Professor of Biology at Queen's University. She uses molecular markers to study mechanisms of population differentiation in vertebrates, primarily seabirds. Much of her work has conservation implications. Irby Lovette directs the Fuller Evolutionary Biology Program at the Cornell Lab of Ornithology and has research endeavors that integrate the ecology of avian evolution, behavior, and conservation.