The Auk 121(4):1019–1030, 2004

RECIPROCAL INTROGRESSION BETWEEN GOLDEN-WINGED WARBLERS (*VERMIVORA CHRYSOPTERA*) AND BLUE-WINGED WARBLERS (*V. PINUS*) IN EASTERN NORTH AMERICA

LEO H. SHAPIRO,[1,4] RONALD A. CANTERBURY,[2,3] DOLLIE M. STOVER,[3] AND ROBERT C. FLEISCHER[1]

[1]*Molecular Genetics Lab, National Zoological Park, Washington, D.C. 20008, USA;*
[2]*Department of Biology, Concord College, Athens, West Virginia 24712, USA; and*
[3]*Southern West Virginia Bird Research Center, P.O. Box 72, Harper, West Virginia 25851, USA*

ABSTRACT.—Golden-winged Warblers (*Vermivora chrysoptera*) and Blue-winged Warblers (*V. pinus*) are small, brightly colored Neotropical migrant birds that breed in eastern North America. Wherever the two species occur together, they hybridize to a limited degree, producing distinctive hybrid phenotypes. In recent decades, *chrysoptera* has experienced dramatic population declines across much of its range. Those declines have often been correlated with establishment and increase of *pinus* in the same areas, but it remains uncertain what, if any, role *pinus* has played in driving the decline of *chrysoptera*. In a first attempt at molecular genetic analysis of *chrysoptera–pinus* population dynamics, Gill (1997) reported cryptic, completely asymmetric, and possibly very rapid introgression of *pinus* mitochondrial DNA (mtDNA) into *chrysoptera*, causing what he termed "local cytonuclear extinction" of *chrysoptera*. As Gill (1997) noted, however, those results were based on relatively small samples from a single area in Pennsylvania. To begin to investigate the generality of Gill's findings and to establish a baseline for long-term genetic and ecological studies, we intensively sampled one new study area (in southern West Virginia) and also sampled more broadly across two other areas (in Michigan and Ohio) that have experienced *pinus* invasions and *chrysoptera* declines. In southern West Virginia, introgression of mtDNA appeared to be roughly symmetrical: 15% (11 of 72) of *pinus* phenotypes possessed *chrysoptera* mtDNA, and 12% (17 of 137) of *chrysoptera* phenotypes possessed *pinus* mtDNA. Results from much smaller samples from Michigan and Ohio also failed to show any evidence of asymmetric mitochondrial introgression. The results we report here, based on mtDNA and plumage phenotype information for 337 birds representing much of the range of the two species, indicate that previous genetic results and inferences from Pennsylvania may not be broadly applicable to the many areas of contact between *chrysoptera* and *pinus* in eastern North America. *Received 29 August 2003, accepted 24 June 2004.*

RESUMEN.—Las reinitas *Vermivora chrysoptera* y *V. pinus* son aves migratorias pequeñas de colores brillantes que se reproducen en el este de Norte América. En lugares donde las dos especies se encuentran juntas, éstas se hibridizan de forma limitada, produciendo fenotipos híbridos distintivos. En décadas recientes, las poblaciones de *chrysoptera* han disminuido dramáticamente a través de gran parte de su rango de distribución. Estas disminuciones a menudo han estado correlacionadas con el establecimiento e incremento de poblaciones de *pinus* en las mismas áreas, pero aún es incierto si *pinus* ha jugado algún papel causando la disminución de *chrysoptera*. En un primer análisis basado en genética molecular de la dinámica poblacional de *chrysoptera–pinus*, Gill (1997) documentó la ocurrencia de introgresión críptica, completamente asimétrica y posiblemente muy rápida, del ADN mitocondrial (ADNmt) de *pinus* a *chrysoptera*, causando lo que él llamó "extinción citonuclear local" de *chrysoptera*. Sin embargo, como lo mencionó Gill (1997), sus resultados se basaron en muestras relativamente pequeñas de una sola área ubicada en Pennsylvania. En este trabajo muestreamos intensivamente un área de estudio nueva (en el sur de West Virginia) y muestreamos más ampliamente a través de otras dos áreas (en Michigan y Ohio) en donde han ocurrido invasiones de *pinus* y disminuciones de *chrysoptera* para comenzar a investigar la generalidad de los hallazgos de Gill y para establecer una base para estudios genéticos y ecológicos de largo plazo. En el sur de West Virginia, la introgresión del ADNmt pareció ser aproximadamente simétrica: el 15% (11 de 72) de las aves con fenotipo de *pinus* presentaron ADNmt de *chrysoptera*, y el 12% (17

[4]Present address: Department of Biology, 1210 Biology–Psychology Building, University of Maryland, College Park, Maryland 20742, USA. E-mail: lshapiro@umd.edu

de 137) de las aves con fenotipo de *chrysoptera* presentaron ADNmt de *pinus*. Los resultados basados en muestras mucho más pequeñas de Michigan y Ohio tampoco mostraron evidencia alguna de introgresión mitocondrial asimétrica. Nuestros resultados, basados en información sobre ADNmt y plumaje para 337 aves de buena parte del rango de distribución de las dos especies, indican que los resultados genéticos previos y las inferencias de Pennsylvania no serían ampliamente aplicables a las múltiples zonas de contacto entre *chrysoptera* y *pinus* en el este de Norte América.

GOLDEN-WINGED WARBLERS (*Vermivora chrysoptera*) and Blue-winged Warblers (*V. pinus*) are small, brightly colored Neotropical migrants that breed in eastern North America. During the past several decades, *chrysoptera* populations have declined precipitously in many regions, disappearing entirely from some areas (Confer 1992, Canterbury et al. 1993, Gill et al. 2001, Klaus and Buehler 2001). The cause of the decline remains uncertain, but it has been associated, in time and space, with newly initiated contact with *pinus* resulting from an ongoing *pinus* range expansion (the range of *chrysoptera* has also been expanding, but that expansion has been mainly northward into regions not yet inhabited by *pinus*). Despite intensive study of ecological and behavioral interactions between sympatric *chrysoptera* and *pinus*, there is still no clear evidence of competition between the two (e.g. Confer 1992, Confer and Larkin 1998, Gill et al. 2001). Interspecific pairings are relatively uncommon even in actively hybridizing populations, but hybrids appear to be fully fertile and to backcross readily with parental phenotypes—though Confer and Tupper (2000) suggest that hybrid males may have less success obtaining mates.

On the basis of their work in central New York, Confer and Knapp (1981) suggested that *chrysoptera* is more narrowly specialized on early-successional habitats, abandoning them several decades before *pinus* does, and that loss of such habitats is a major force driving the decline of *chrysoptera*. If that scenario is correct, we should expect local *pinus* declines to follow on the heels of *chrysoptera* declines, but it is not yet clear whether that is occurring. More importantly, although availability of early-successional habitat in eastern North America has decreased dramatically during the past century (Hunter et al. 2001), *chrysoptera* declines have been noted even where apparently suitable habitat remains (e.g. Confer et al. 2003), and habitat use by *chrysoptera* and *pinus* across their geographic ranges appears to vary considerably (e.g. Bent 1953; Ewert 1981; Will 1986; Frech and Confer 1987; Confer 1992; Canterbury et al. 1993, 1996; Kelly 1996; Gill et al. 2001; Hanowski 2002; Confer et al. 2003). Thus, effects of habitat succession can provide, at best, only a partial explanation for the decline of *chrysoptera*.

The possible direct role of hybridization with *pinus* in causing the decline of *chrysoptera* has been difficult to study. Nearly everywhere *chrysoptera* and *pinus* occur together, there is clear evidence of limited hybridization and introgression. Early-generation hybrid offspring exhibit typical plumage phenotypes that were initially described as distinct species and are still formally recognized today as "Brewster's Warbler" and "Lawrence's Warbler." Those two hybrid phenotypes differ in several major plumage features that can be largely explained by a simple Mendelian genetic model (Parkes 1951). Brewster's hybrid phenotypes combine the dominant black eye-line characteristic of *pinus* with the gray back and whitish underparts of *chrysoptera*; the much rarer Lawrence's hybrid phenotypes combine the recessive broad black mask and throat of *chrysoptera* with the yellow back and underparts of *pinus*. (Although the two phenotype categories are useful, careful study reveals far broader and more continuous multicharacter variation than is superficially evident [Short 1963, Shapiro et al. unpubl. data]). It is possible that hybridization with *pinus* could provide a primary explanation for eventual replacement of *chrysoptera* phenotypes by *pinus* phenotypes after several decades of hybridization. For example, if *chrysoptera* females have a higher rate of interspecific extrapair fertilizations than *pinus* females, then *chrysoptera* females will produce fewer "pure" progenies than will *pinus* females, resulting in population-level introgression of *pinus* alleles. Similarly, if backcrossing hybrids reproduce more frequently or more successfully with *chrysoptera* than with *pinus*, the result could be a "dilution" of the *chrysoptera* genome. Indeed, there is some evidence from extensive field observations of

interspecific behavioral interactions involving hybrid individuals that the latter mating bias may occur (Confer and Larkin 1998, R. A. Canterbury unpubl. data), though much work remains to be done in documenting mating patterns (using both direct behavioral observations and analysis of molecular markers) and—once those empirical data are available—in modeling projected changes in genetic structure of mixed populations through time.

Recently, Gill (1997) examined cytonuclear genotype frequencies in two *chrysoptera–pinus* populations near the Pennsylvania–New Jersey border, using plumage pattern as a rough surrogate for the multilocus nuclear genotype and assessing mitochondrial (mtDNA) haplotype using restriction enzymes. He found *pinus* mtDNA not only in *pinus* phenotypes, but also in *chrysoptera* and Brewster's phenotypes, with no reciprocal introgression of *chrysoptera* mtDNA. In an actively hybridizing mixed population in the Delaware River Valley consisting of about two-thirds *pinus* phenotypes, 98% (40 of 41) of individuals sampled had *pinus* mtDNA, including all 6 (visibly introgressed) *chrysoptera* phenotypes and 6 of 7 (86%) Brewster's phenotypes. In the second population, from upland regenerating clearcuts in the nearby Pocono Mountains, no *pinus* phenotypes were seen, but 48% (10 of 21) of birds sampled nevertheless possessed *pinus* mtDNA. On the basis of those data, Gill (1997) suggested that the pattern of plumage phenotype replacement he had previously documented in his studies of museum skins and the literature (Gill 1980) may be preceded by cytoplasmic replacement of the *chrysoptera* mitochondrial genome when introgression in a population is still barely, or not at all, evident from an inspection of plumage phenotypes.

Gill's (1997) striking results were based on relatively small samples from a single area, and he noted the importance of sampling other areas of contact for comparison. To begin to investigate the generality of Gill's (1997) findings and to establish a baseline for long-term genetic and ecological studies, we intensively sampled a new study area in southern West Virginia. That region has breeding densities of *chrysoptera* that are among the highest known anywhere (Canterbury et al. 1996, Wells and Rosenberg 1996, Canterbury and Stover 1999, R. A. Canterbury unpubl. data). It falls within the known historical distribution of *chrysoptera* and lies well east of the Ohio River drainage, a historical *pinus* stronghold. *Vermivora pinus* is a recent arrival to southern West Virginia, having moved across the Kanawha River Valley and through higher elevations in the Allegheny Plateau of West Virginia. Although *pinus* has been present in the Ohio and Kanawha River valleys since at least the late 1950s, it was not observed in southern West Virginia until 1989 (Hall 1983, Canterbury 1990, Canterbury et al. 1993). Many West Virginia sites that now harbor *pinus* had none when monitoring first began in the late 1980s (Canterbury et al. 1993, Gill et al. 2001). Thus, the region offers an excellent opportunity to carefully monitor changes in warbler populations, starting from an ecologically and genetically well-documented first contact. In addition to our intensive sampling in West Virginia, we sampled smaller numbers of birds from several other areas (in Ohio and Michigan) that have experienced *pinus* invasions and *chrysoptera* declines.

Methods

The data set presented here includes mtDNA and plumage phenotype information for 337 birds representing much of the range of *chrysoptera* and *pinus* in eastern North America (Fig. 1; geographic sampling is summarized in Table 1). For our intensive sampling, we captured 222 birds (*chrysoptera*, *pinus*, and hybrids) from numerous sites within a radius of ~40 km in the southern tip of West Virginia, where R.A.C. has been monitoring *chrysoptera* and *pinus* distributions, ecology, breeding, and behavior for 17 years (Fig. 2A and Appendix; sampling coordinates for each bird are available from the authors). Most sampled sites have been monitored since 1987 and have been the focus of an active banding program by R.A.C. and assistants since 1993. All our West Virginia sites are in the Allegheny Plateau south of the Kanawha River Drainage (Appendix). For the present study, birds were sampled each breeding season between 1997 and 2001, and six additional hybrids were added to our sample in 2002 (Table 2). Because local sampling sites are near each other (8–20 km apart) and because return rates of both young and adult birds to particular sites appear to be very low (Gill et al. 2001, R. A. Canterbury unpubl. data), we pooled data from our West Virginia sites for analysis, treating all sites as part of a larger metapopulation. There was no indication of any trends across our five field seasons, so we pooled our data across years as well.

To obtain a geographically broader picture of

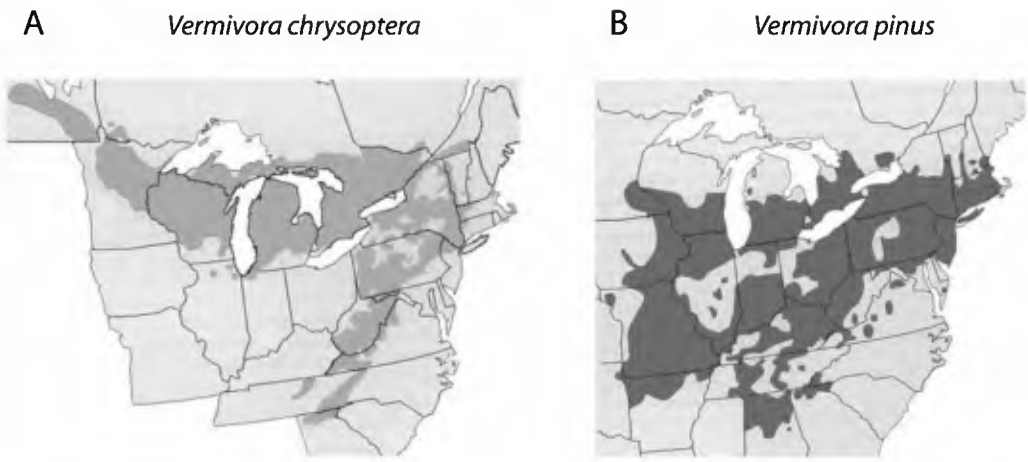

FIG. 1. Approximate geographic distributions of (A) *Vermivora chrysoptera* (light gray) and (B) *V. pinus* (dark gray) (based on Dunn and Garrett 1997).

TABLE 1. Cytonuclear composition of *Vermivora chrysoptera* and *V. pinus* populations.

	mtDNA haplotype		
Plumage phenotype	*chrysoptera*	*pinus*	Total
Mixed populations:			
West Virginia			
chrysoptera	120	17	137
pinus	11	61	72
Brewster's hybrids	5	4	9
Lawrence's hybrids	1	3	4
Total	137	85	222
Ohio			
chrysoptera	1	1	2
pinus	0	24	24
Brewster's hybrids	0	0	0
Lawrence's hybrids	0	0	0
Total	1	25	26
Michigan			
chrysoptera	2	0	2
pinus	2	27	29
Brewster's hybrids	3	0	3
Lawrence's hybrids	0	0	0
Total	7	27	34
Allopatric populations:			
Minnesota (northern)			
chrysoptera	17	0	17
Manitoba (western)			
chrysoptera	19	0	19
North Carolina (western)			
chrysoptera	1	0	1
Missouri (southern)			
pinus	0	18	18
Grand total	182	155	337

mtDNA introgression patterns, we also captured and sampled smaller numbers of birds from northeastern Ohio (n = 26) in 1997 and central and southwestern Michigan (n = 34) in 1998; in those areas, *chrysoptera* populations have been declining, and *pinus* populations increasing, for many decades (Fig. 2B and Table 1). Finally, to permit discrimination of *chrysoptera* and *pinus* mtDNA haplotypes, we sampled distinctly allopatric *chrysoptera* from northern Minnesota and allopatric *pinus* from southern Missouri and obtained samples from an isolated *chrysoptera* population in western Manitoba (courtesy of K. Hobson and H. L. Gibbs) and from a single *chrysoptera* nestling from western North Carolina (courtesy of N. Klaus). Those samples were supplemented by an additional five Missouri *pinus* tissue samples provided by The Academy of Natural Sciences of Philadelphia (originally collected by M. Robbins).

To minimize the possibility of including nonresidents in our data set, in each region we confined sampling to a period during which only resident individuals would likely be present (in West Virginia, 25 April–15 July; Ohio, 12 May–13 June; Michigan, 16 May–11 June; Missouri, 14 May–26 May; Minnesota, 4 June–13 June; Manitoba, 21 May–4 July). Most of those date ranges are consistent with general guidelines established for sampling breeding *chrysoptera* by the Cornell Laboratory of Ornithology's Golden-winged Warbler Atlas Project (GOWAP). West Virginia capture dates run significantly earlier and later than those guidelines, but territorial behavior of individual birds was closely monitored throughout the breeding season, so we are confident that sampled birds were residents. Capture dates for Manitoba *chrysoptera* extended about two weeks beyond general GOWAP guidelines, but that is the northern edge of *chrysoptera*'s distribution (Fig. 1A), so sampling of nonresident

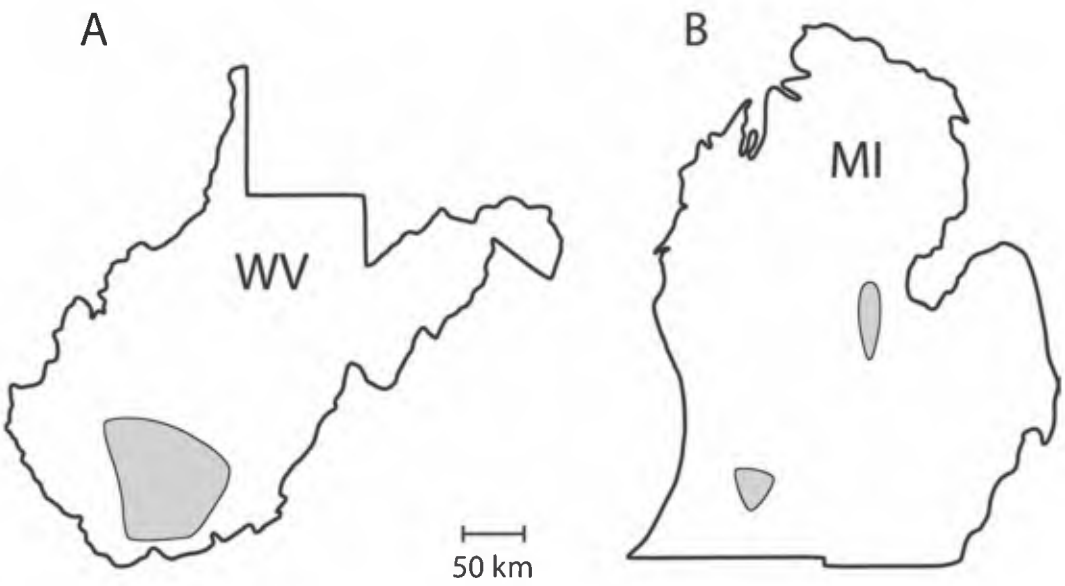

FIG. 2. (A) Sampling area in southern West Virginia. (B) Sampling areas in Michigan.

birds moving south from farther north should not be a problem. We captured birds in mist nets, using song playbacks and dummy birds (repainted garden ornaments), and marked them with federal bands before releasing them. We sampled feathers from all birds captured—and, in some cases, blood—for analysis. We recorded plumage data to calculate plumage scores for all adult males, using a color character index used in previous studies by Gill (e.g. table 1 in Gill 1980, 1987, 1997); the index is based on wing bar color and width, general body color, and color of chin and throat. Most birds were also photographed before release. Tail or contour feathers, or both, were either stored in vials filled with 100% ethanol and refrigerated, or placed in sealed envelopes and frozen for long-term storage. We sampled blood by pricking the brachial vein with a sterile needle, wicking a drop of blood into a 300-μL microvette, capping the open bottom, and adding a roughly equal volume of lysis buffer (0.1M Tris pH 7.8, 0.1M EDTA, 2% SDS) before refrigerating or freezing. Collecting only feathers and blood to obtain DNA permits extensive, nondestructive sampling—an especially important consideration, given the status of *chrysoptera* as a seriously declining species.

We used polymerase chain reaction (PCR) and direct sequencing to analyze mtDNA haplotype distributions. DNA was extracted from feather, blood, or tissue samples using DNeasy extraction kits (Qiagen, Valencia, California) or a standard phenol-chloroform extraction protocol. We amplified and sequenced a portion of the mitochondrial gene NADH dehydrogenase subunit 2 (ND2) using primers L5215 (5′TATCGG GCCCATACCCCGAAAAT3′; designed by J. Cracraft and K. Helm-Bychowski; see Hackett 1996) and H6113 (5′CAGTATGCAAGTCGGAGGTAGAAG3′; A. Baker pers. comm. to R. C. Fleischer lab). Our PCR reaction mix (25 μL) contained magnesium chloride (2.0 mM), primers (0.4 uM each), dNTPs (0.2 mM each), and 1 U Perkin Elmer AmpliTaq Gold DNA polymerase with the supplied buffer. Amplifications were carried out in a PTC-200 thermal cycler (MJ Research, Waltham, Massachusetts), using the following profile: 94°C (10 min)-38 cycles of 92°C (45 s), 50°C (1 min), 72°C (1 min)-72°C (5 min). The PCR products were cleaned using QIAquick PCR purification kits (Qiagen) and cycle sequenced with an ABI PRISM cycle sequencing kit (Applied Biosystems, Foster City, California). For sequencing, we used either the amplification primers or a specially designed sequencing primer, ND2H-seqC (5′CACCCTCCTAGGGCTGTT3′). Sequenced products were cleaned using CentriSep columns (Princeton Separations, Adelphia, New Jersey) and run out on an ABI 373 automated sequencer.

To identify species-diagnostic base positions, we aligned a 476-base pair (bp) region of ND2 using several *chrysoptera* and several *pinus* sequences from each sampled geographic region where they occurred and identified 18 sites (18 of 476 = 3.8%) that showed fixed nucleotide differences between *chrysoptera* and *pinus* (representative sequences have been deposited in GenBank; accession numbers AY675942–AY675964). Using those fixed differences, we scored each of the 337 birds in our data set as having either *chrysoptera* or *pinus* mtDNA, according to which of the two haplotype types it matched. To examine genetic relationships among our representative ND2 sequences,

TABLE 2. West Virginia birds captured and genotyped for mtDNA haplotype.

Year	Number of birds genotyped[a]	"Pure" plumage phenotypes			Hybrid plumage phenotypes			
		Cytonuclear mismatches (number)[b]		Cytonuclear mismatches (percentage)	Number of Brewster's genotyped		Number of Lawrence's genotyped	
		chrysoptera plumage with *pinus* mtDNA	*pinus* plumage with *chrysoptera* mtDNA		*chrysoptera* mtDNA	*pinus* mtDNA	*chrysoptera* mtDNA	*pinus* mtDNA
1997	38	5 of 24	3 of 14	21	0	1	0	1
1998	77	7 of 54	4 of 23	14	0	0	0[c]	1
1999	48	2 of 34	2 of 14	8	1	1	1	0
2000	33	2 of 16	2 of 17	12	1	0	0	0
2001	13	1 of 9	0 of 4	8	0	0	0	0
2002	–	–	–	–	3	2	0	1
Total	209	17 of 137 (12%)	11 of 72 (15%)	13	5	4	1	3

[a]Excluding Brewster's and Lawrence's phenotypes.
[b]"Cytonuclear mismatches" are individuals with either *chrysoptera* or *pinus* plumage phenotype and heterospecific mtDNA haplotype.
[c]A Lawrence's Warbler, a female with *chrysoptera* mtDNA, was sampled in 1998 too late in the season to meet our criteria for defining resident birds to be included in our analysis.

we used PAUP* 4.0b10 (Swofford 2002) to estimate genetic distances within and between regions and species and to use those data to construct a neighbor-joining tree for the sequences.

RESULTS

Mitochondrial haplotype frequencies for all populations are shown in Table 1. Judging from 476 bp from the first half of ND2, mean intraspecific genetic distances are quite low for both *chrysoptera* and *pinus* across our broad geographic sampling, ranging (depending on the populations compared) from 0.000 to 0.004 for *chrysoptera* and 0.000 to 0.001 for *pinus* (Table 3). Mean nucleotide divergence between *chrysoptera* and *pinus* is 4.3–4.9%, depending on the populations compared (Table 3). This is somewhat higher than the 3.0–3.2% estimated by Gill (1997) on the basis of restriction digests of the whole mitochondrial genome. That discrepancy may be attributed, at least in part, to the fact that in passerine birds, ND2 appears to evolve faster than most other mitochondrial genes (e.g. Hackett 1996, Omland et al. 1999). A neighbor-joining tree (using as outgroups Tennessee [*V. peregrina*], Orange-crowned [*V. celata*], and Nashville [*V. ruficapilla*] warbler sequences from GenBank; accession numbers numbers AF256494, AY030149, and AF256501, respectively) showed complete separation of *chrysoptera* versus *pinus* mtDNA, with 100% bootstrap support, but there was no indication of geographic structure within the two clades (Fig. 3).

Distribution of plumage scores for birds sampled in mixed-species regions (West Virginia, Ohio, and Michigan) and allopatry (Missouri and Minnesota) is shown in Figure 4. All individuals from our allopatric reference populations (*chrysoptera* from northern Minnesota, western Manitoba, and western North Carolina and *pinus* from southern Missouri) had the expected mtDNA, confirming that those populations apparently remain pure (Table 1). In Michigan, *chrysoptera* gave very weak responses to song playbacks and dummy birds, so they are under-represented in our sample as compared with their abundance in the field. Nevertheless, both of the two *chrysoptera* sampled in Michigan had *chrysoptera* mtDNA, as did all three sampled Brewster's phenotypes and two of 29 sampled *pinus* (Table 1; the two *pinus* were from southwestern Michigan, where *pinus* arrived in the

TABLE 3. Mean genetic distances (uncorrected) within and among *chrysoptera* and *pinus* populations, based on the first 476 bp of mitochondrial ND2 (each mean based on two or three individuals per region, except only one for North Carolina). Abbreviations: Manitoba (MB), Minnesota (MN), North Carolina (NC), West Virginia (WV), Michigan (MI), Ohio (OH), and Missouri (MO).

	chrysoptera						*pinus*			
	MB	MN	NC	WV	MI	OH	MO	WV	MI	OH
chrysoptera										
MB	0.000	–	–	–	–	–	–	–	–	–
MN	0.000	0.000	–	–	–	–	–	–	–	–
NC	0.000	0.000	–	–	–	–	–	–	–	–
WV	0.001	0.001	0.001	0.001	–	–	–	–	–	–
MI	0.002	0.002	0.002	0.003	0.004	–	–	–	–	–
OH	0.002	0.002	0.002	0.003	0.004	0.000	–	–	–	–
pinus										
MO	0.046	0.046	0.046	0.046	0.048	0.044	0.001	–	–	–
WV	0.046	0.046	0.046	0.045	0.048	0.043	0.001	0.001	–	–
MI	0.047	0.047	0.047	0.046	0.049	0.045	0.001	0.001	0.001	–
OH	0.046	0.046	0.046	0.046	0.048	0.044	0.001	0.001	0.001	0.000

late 19th century and had essentially replaced *chrysoptera* by the 1970s; Will 1986, Brewer et al. 1991). In Ohio, all 24 *pinus* sampled had *pinus* mtDNA, as did one of the two sampled *chrysoptera* (Table 1). Thus, our data from Michigan suggest, if anything, greater introgression by *chrysoptera* mtDNA than by *pinus* mtDNA, though our single example of plumage–mtDNA mismatch from Ohio was a *chrysoptera* individual with *pinus* mtDNA.

In West Virginia, where we have sampled intensively and where, by our estimate, *chrysoptera* phenotypes make up approximately two-thirds of the population (with frequency of hybrid phenotypes observed ranging around 1–3%, depending on year), we analyzed plumage data and mitochondrial genotypes for 222 birds, including 137 *chrysoptera* phenotypes, 72 *pinus* phenotypes, 9 Brewster's phenotypes, and 4 Lawrence's phenotypes (Table 1). Introgression of mtDNA appeared to be roughly symmetrical: 15% (11 of 72) of *pinus* phenotypes had *chrysoptera* mtDNA, and 12% (17 of 137) of *chrysoptera* phenotypes had *pinus* mtDNA (Table 2). Of the nine sampled Brewster's phenotypes, four had *pinus* mtDNA and five had *chrysoptera* mtDNA; three of four sampled Lawrence's phenotypes had *pinus* mtDNA, and one had *chrysoptera* mtDNA.

Our results from West Virginia show no indication of the pattern found by Gill in his study of *chrysoptera–pinus* in Pennsylvania (Gill 1997), in which he reported evidence of dramatic and completely asymmetric introgression of *pinus* mtDNA. For Gill's Pennsylvania populations, we find that the asymmetry of mtDNA introgression is highly significant, but our West Virginia population shows no such asymmetry (Table 4). A test of homogeneity of odds ratios (Sokal and Rohlf 1995) confirms that the apparent difference in pattern of mtDNA introgression between our West Virginia data set and Gill's Pennsylvania data set is highly significant (Table 4).

DISCUSSION

Genetic analysis of populations can be a powerful tool for inferring patterns of gene flow (e.g. see Rohwer et al. 2001) and will surely be critical in refining our understanding of interactions between *chrysoptera* and *pinus* and the possible role of *pinus* in driving the decline of *chrysoptera*. Prior to the present study, the only molecular investigations of this system were an unsuccessful attempt to identify diagnostic allozyme markers to distinguish *chrysoptera* and *pinus* (Gill 1987) and a small RFLP (restriction fragment length polymorphism) data set examining the distribution of mtDNA haplotypes in an area near the Pennsylvania–New Jersey border (Gill 1997). Our large data set from West Virginia, as well as our more limited Michigan data set, stand in sharp contrast to Gill's (1997) results from Pennsylvania. Our data show no indication of directional introgression of *pinus* mtDNA. In fact, in West Virginia, *pinus* phenotypes had a slightly higher frequency of heterospecific mtDNA than *chrysoptera*, though that difference is not significant (Table 2). Similarly,

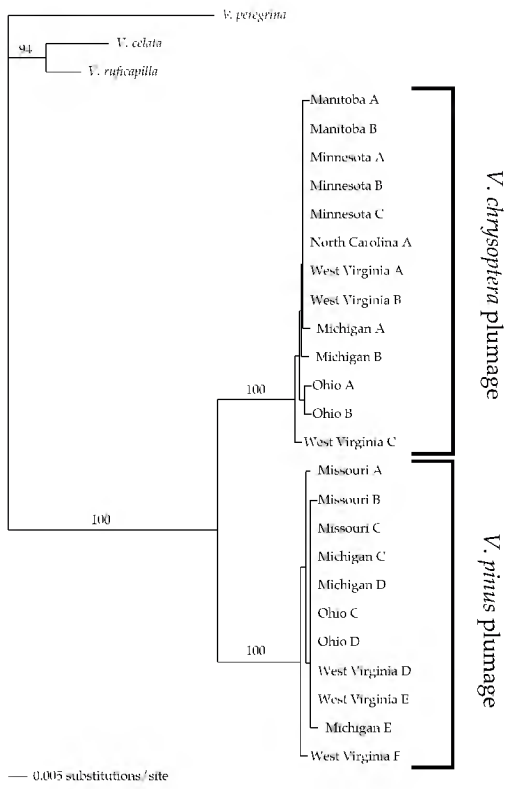

FIG. 3. Neighbor-joining tree based on 476 bp of ND2 from representative *chrysoptera* and *pinus* from all regions sampled. Sequences for outgroup taxa *Vermivora peregrina*, *V. celata*, and *V. ruficapilla* were obtained from GenBank (GenBank accession numbers AF256494, AY030149, and AF256501, respectively). Numbers above branches indicate bootstrap support for subtended clades (from 1000 pseudoreplicates; only values greater than 70% shown).

in Michigan, we found evidence of introgression of *chrysoptera*, but not of *pinus*, mtDNA (Table 1). Thus, in contrast to Gill's (1997) findings in his Pennsylvania study, a genetic footprint of *chrysoptera* mtDNA may persist in some areas in Michigan long after the disappearance of *chrysoptera* phenotypes. In northeastern Ohio, where *pinus* was established by the early 1900s and had essentially replaced *chrysoptera* by the 1940s (Dawson 1903, Peterjohn and Rice 1991, Peterjohn 2001), we found only a single cytonuclear mismatch in our limited sampling: a *chrysoptera* phenotype with *pinus* mtDNA (Table 1). For both Michigan and Ohio, however, we must note that, given our limited sampling in those areas and our relative lack of long-term familiarity with those populations, it remains possible that the mixed-ancestry individuals sampled were, in fact, transients from elsewhere; we therefore hesitate to draw strong conclusions from those particular results.

With respect to mtDNA in hybrid phenotypes, it is more difficult to compare our results with those of Gill (1997), because both his and our data sets are necessarily quite small; again, however, the data from Pennsylvania are more extreme than our data from West Virginia and Michigan (no hybrid phenotypes were sampled from Ohio). Gill (1997) found that 89% (eight of nine) of Brewster's sampled in his Pennsylvania study had *pinus* mtDNA. He noted that the binomial sampling probability of that result is very low if we assume an equal probability that each of the hybrid birds' mothers carried *chrysoptera* or *pinus* mtDNA ($P = 0.017$, according to Gill [1997]; the more appropriate cumulative binomial probability is only trivially higher: $P = 0.019$). Thus, Gill interpreted the mtDNA genotypes of those Brewster's hybrids as further evidence for the preponderance of *pinus* mtDNA in Pennsylvania (though that is not necessarily a sound conclusion: for example, if pure *pinus* females are more likely to mate heterospecifically than are pure *chrysoptera* females, then frequency of *pinus* mtDNA in hybrids could be greater than that in the broader population; there is no behavioral evidence of such a mating pattern, however). Our West Virginia hybrids showed no tendency to have either *chrysoptera* or *pinus* mtDNA, despite greater abundance of *chrysoptera* mtDNA in the population. That might suggest disproportionate representation of *pinus* mtDNA in hybrids, but our hybrid sample size was very small ($n = 13$; Table 2). In southwestern Michigan, all three of the Brewster's sampled had *chrysoptera* mtDNA, despite the fact that *chrysoptera* phenotypes have been very rare in that area for several decades (e.g. Brewer et al. 1991). Overall, frequencies of *chrysoptera* and *pinus* mtDNA in our hybrid phenotypes give no clear indication of a nonrandom distribution.

The explanation for the quite different results from the present study and the Pennsylvania study (Gill 1997) is uncertain. In his multiregion historical survey of the replacement of *chrysoptera* by *pinus*, Gill (1980) roughly divided the replacement process into five phases, defined by the relative proportions of

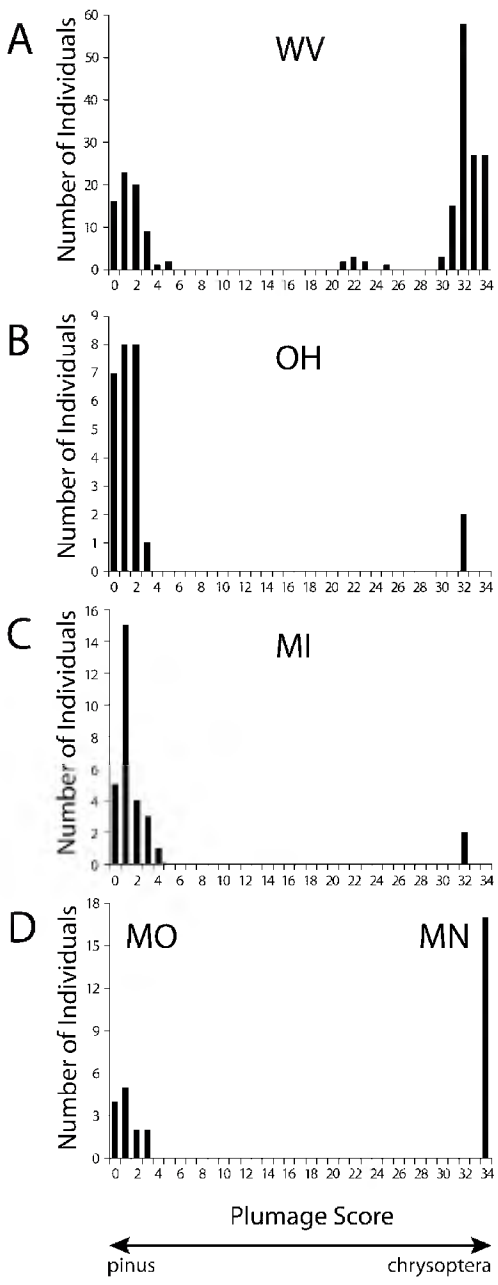

FIG. 4. Distribution of plumage scores (Gill 1980, 1987) for (A) West Virginia, (B) Ohio, (C) Michigan, and (D) allopatric *chrysoptera* from northern Minnesota and *pinus* from southern Missouri. Gill's *pinus* samples from southern Missouri ($n = 18$) were evenly divided between plumage scores of 0 and 1; Gill's northern Minnesota samples ($n = 27$) had plumage scores ranging from 27 to 32, with a median of 31 (Gill 1987, 1997). The slight difference between Gill's allopatric scores and ours (our *pinus* score range was greater, and our *chrysoptera* score range was smaller) is almost surely attributable to differences between observers in subjective scoring. Brewster's and Lawrence's hybrid phenotypes are excluded from the plot.

the various types of pure and hybrid phenotypes. A population in phase I is characterized by mainly *chrysoptera* phenotypes, with a few *pinus* and hybrid individuals. In phase II, similar numbers of *chrysoptera* and *pinus* phenotypes are present, along with some hybrid (especially Brewster's) phenotypes. In phase III, *pinus* phenotypes predominate, with relatively few "pure" *chrysoptera* phenotypes but a full range of intermediate phenotypes (including Lawrence's phenotypes) present. Phase IV populations contain many *pinus* phenotypes with conspicuous evidence of introgression, but few strongly intermediate phenotypes and no *chrysoptera*. Finally, phase V populations are *pinus* populations with some variability in wing bar color and occasional Lawrence's phenotypes. According to this scheme, Gill's (1997) actively hybridizing valley population from Pennsylvania appears to be further along in the *chrysoptera*-to-*pinus* transition (in phase III) than is our West Virginia population, which appears to be in late phase I. Gill (1997) estimates that hybridization was probably initiated in his study area around 30–50 years ago, and that at the time of his study the population consisted of about two-thirds *pinus* phenotypes and about 13% Brewster's and Lawrence's phenotypes. In contrast, *pinus* has been present in southern West Virginia for only about a decade and currently constitutes only about one-third of the population overall; Brewster's and Lawrence's phenotypes together make up about 1–3% of the population in southern West Virginia. Thus, it might appear that mtDNA replacement is part of the *chrysoptera*-to-*pinus* transition, but that it is simply not yet evident in the early stage of transition. If that is the case, we should predict a rapid increase in relative frequency of *pinus* mtDNA in southern West Virginia over the next decade or two. However, different times since contact cannot fully explain the differences in introgression patterns between the Pennsylvania and West Virginia studies,

TABLE 4. Comparison of mtDNA introgression in Pennsylvania (Gill 1997) and West Virginia (present study). Introgression of mtDNA was highly asymmetric in Pennsylvania (all mismatches consisted of *pinus* mtDNA in birds with *chrysoptera* plumage; odds ratio, with continuity correction, 0.013; *G*-test of independence, $G = 28.39$, df = 1, $P < 0.0001$), but not at all in West Virginia (odds ratio, with continuity correction, 1.29; *G*-test of independence, $G = 0.33$, df = 1, $P = 0.57$). The difference in introgression patterns between the two studies is highly significant (test of homogeneity of odds ratios [Sokal and Rohlf 1995], $X^2_H = 8.96$, df = 1, $P = 0.003$).

Plumage phenotype	mtDNA haplotype		Total	Cytonuclear mismatches (%)
	Conspecific	Heterospecific		
	Pennsylvania (pooled)			
chrysoptera	11	15	26	58
pinus	28	0	28	0
Total	39	15	54	28
	West Virginia			
chrysoptera	120	17	137	12
pinus	61	11	72	15
Total	181	28	209	13

because even Gill's (1997) nearly phenotypically pure upland *chrysoptera* population in early phase I—presumably at least as early in transition as our West Virginia population—had a high frequency of *pinus* mtDNA (48%). Thus, it is possible that the dynamics of hybridization in the two regions studied are truly different. As noted above, across eastern North America there appear to be important regional differences in ecology and behavioral interactions between *chrysoptera* and *pinus*. In a few areas, in fact, there even appears to be stable long-term coexistence between the two (e.g. Frech and Confer 1987, Confer et al. 1998, Scully 1999). In some other areas, such as the mixed-species sites in central Michigan studied by Tom Will in the early 1980s (Will 1986), anecdotal evidence suggests that, in at least some habitats, *chrysoptera* may persist fairly well even in the presence of *pinus*—though overall abundance of *chrysoptera* as compared with *pinus* in central Michigan appears to have declined, perhaps substantially (Kelly 1996, L. Shapiro pers. obs.).

Our results indicate that mitochondrial introgression between *chrysoptera* and *pinus* is symmetric in southern West Virginia and, therefore, that previous intriguing genetic results and inferences from Pennsylvania (Gill 1997) may not be broadly applicable to the many areas of contact between *chrysoptera* and *pinus* in eastern North America. Although regional differences in patterns of mtDNA introgression may result from ecological and behavioral differences, it is also possible that those regional differences simply represent stochastic geographic variation. Such possibilities will need to be explored through coordinated studies of the ecology, behavior, and genetics of *chrysoptera* and *pinus* at multiple sites across eastern North America.

ACKNOWLEDGMENTS

We thank F. B. Gill for encouraging us to pursue this work. We thank N. Kotesovec, Jr., J. Meyer, A. Waldron, S. Zadar, and V. Tierce for assistance with field work. Our field work was greatly facilitated by the warm generosity of many individuals and by the cooperation of many institutions: R. Zink, University of Minnesota's Lake Itasca Forestry and Biological Station, and the Minnesota Department of Natural Resources (Minnesota); A. Dowling, K. Terry, and the Missouri Department of Conservation (Missouri); D. Powless and R. J. Adams, Jr., of the Kalamazoo Nature Center; R. Touvell, G. Kelly, and E. Kenaga of the Chippewa Nature Center; R. Keith, B. Keith, T. Will, and the Michigan Department of Natural Resources (Michigan). K. Hobson and H. L. Gibbs kindly gave us access to samples from Manitoba, N. Klaus provided samples from North Carolina, and the Academy of Natural Sciences of Philadelphia provided several samples from Missouri. L.H.S. was supported by a National Science Foundation Postdoctoral Fellowship in Biosciences Related to the Environment. Field and lab work were further supported by a Smithsonian Scholarly Studies Research Grant to R.C.F. and L.H.S., with additional support from the Smithsonian Institution's Nelson Fund. R.A.C. received funding from the Nongame Program of the West Virginia Division of Natural Resources and the U.S. Fish and Wildlife Service. We thank A. Bely, H. L. Gibbs, F. B. Gill, and three anonymous reviewers for very helpful

comments on the manuscript. Birds were captured and blood and feathers collected under appropriate federal and state permits. This work is dedicated to the memory of Dollie Stover, who devoted 18 field seasons to the study of these warblers.

Literature Cited

Bent, A. C. 1953. Life histories of North American wood warblers. Bulletin of the U.S. National Museum, no. 203.

Brewer, R., G. A. McPeek, and R. J. Adams. 1991. The Atlas of Breeding Birds of Michigan. Michigan State University Press, East Lansing.

Canterbury, R.A. 1990. Breeding ecology of the Golden-winged Warbler in Raleigh County, West Virginia. Proceedings of the West Virginia Academy of Science 62:83–93.

Canterbury, R. A., and D. M. Stover. 1999. The Golden-winged Warbler: An imperilled migrant songbird of the southern West Virginia coalfields. Greenlands 29:44–51.

Canterbury, R. A., D. M. Stover, and J. N. J. Kotesovec, Jr. 1996. Population ecology of Golden-winged Warblers in southern West Virginia. West Virginia Division of Natural Resources, Elkins.

Canterbury, R. A., D. M. Stover, and T. C. Nelson. 1993. Golden-winged Warblers in southern West Virginia: Status and population ecology. Redstart 60:97–106.

Confer, J. L. 1992. Golden-winged Warbler. *In* The Birds of North America, no. 20 (A. Poole, P. Stettenheim, and F. Gill, Eds.). Academy of Natural Sciences, Philadelphia, and American Ornithologists' Union, Washington, D.C.

Confer, J. L., J. Gebhards, and J. Yrizzary. 1998. Golden-winged and Blue-winged warblers at Sterling Forest: A unique circumstance. Kingbird 39:50–55.

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

Confer, J. L., and J. L. Larkin. 1998. Behavioral interactions between Golden-winged and Blue-winged warblers. Auk 115:209–214.

Confer, J. L., J. L. Larkin, and P. E. Allen. 2003. Effects of vegetation, interspecific competition, and brood parasitism on Golden-winged Warbler (*Vermivora chrysoptera*) nesting success. Auk 120:138–144.

Confer, J. L., and S. K. Tupper. 2000. A reassessment of the status of Golden-winged and Blue-winged warblers in the Hudson Highlands of southern New York. Wilson Bulletin 112:544–546.

Dawson, W. L. 1903. The Birds of Ohio. Wheaton Publishing Company, Columbus, Ohio.

Dunn, J. L., and K. L. Garrett. 1997. A Field Guide to Warblers of North America. Houghton Mifflin, Boston.

Ewert, D. N. 1981. The occurrence of closely related species-pairs in central Michigan: Willow and Alder flycatchers and Golden-winged and Blue-winged warblers. Jack-Pine Warbler 59:95–98.

Frech, M. H., and J. L. Confer. 1987. The Golden-winged Warbler: Competition with the Blue-winged Warbler and habitat selection in portions of southern, central and northern New York. Kingbird 37:65–71.

Gill, F. B. 1980. Historical aspects of hybridization between Blue-winged and Golden-winged warblers. Auk 97:1–18.

Gill, F. B. 1987. Allozymes and genetic similarity of Blue-winged and Golden-winged warblers. Auk 104:444–449.

Gill, F. B. 1997. Local cytonuclear extinction of the Golden-winged Warbler. Evolution 51: 519–525.

Gill, F. B., R. A. Canterbury, and J. L. Confer. 2001. Blue-winged Warbler (*Vermivora pinus*). *In* The Birds of North America, no. 584 (A. Poole and F. Gill, Eds.). Birds of North America, Philadelphia.

Hackett, S. J. 1996. Molecular phylogenetics and biogeography of tanagers in the genus *Ramphocelus* (Aves). Molecular Phylogenetics and Evolution 5:368–382.

Hall, G. A. 1983. West Virginia Birds. Special Publication, no. 7, Carnegie Museum of Natural History, Pittsburgh, Pennsylvania.

Hanowski, J. 2002. Habitats and landscapes used by breeding Golden-winged Warblers in Great Lakes forests. Loon 74:127–133.

Hunter, W. C., D. A. Buehler, R. A. Canterbury, J. L. Confer, and P. B. Hamel. 2001. Conservation of disturbance-dependent birds in eastern North America. Wildlife Society Bulletin 29:440–455.

Kelly, G. A. 1996. A vegetational analysis of Blue-winged Warbler (*Vermivora pinus*) and Golden-winged Warbler (*Vermivora chrysoptera*) habitats in central Michigan. M.S. thesis, Central Michigan University, Mount Pleasant.

Klaus, N. A., and D. A. Buehler. 2001. Golden-winged Warbler breeding habitat characteristics and nest success in clearcuts in the southern Appalachian Mountains. Wilson Bulletin 113:297–301.

Omland, J. E., S. M. Lanyon, and S. J. Fritz. 1999. A molecular phylogeny of the New World orioles (*Icterus*): The importance of dense taxon sampling. Molecular Phylogenetics and Evolution 12:224–239.

PARKES, K. C. 1951. The genetics of the Golden-winged × Blue-winged warbler complex. Wilson Bulletin 63:5–15.

PETERJOHN, B. G. 2001. The Birds of Ohio. Wooster Book Company, Wooster, Ohio.

PETERJOHN, B. G., AND D. L. RICE. 1991. The Ohio Breeding Bird Atlas. Ohio Department of Natural Resources, Columbus.

ROHWER, S., E. BERMINGHAM, AND C. WOOD. 2001. Plumage and mitochondrial DNA haplotype variation across a moving hybrid zone. Evolution 55:405–422.

SCULLY, R. L. 1999. A field study of the Golden-winged Warbler in the Pequannock Watershed. Proceedings of the Linnean Society of New York 1977–1995:25–39.

SHORT, L. L., JR. 1963. Hybridization in the wood warblers *Vermivora pinus* and *V. chrysoptera*. Pages 147–160 *in* Proceedings XIII International Ornithological Congress (C. G. Sibley, Ed.). American Ornithologists' Union, Washington, D.C.

SOKAL, R. R., AND F. J. ROHLF. 1995. Biometry, 3rd ed. W. H. Freeman, New York.

SWOFFORD, D. L. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), version 4.0b10. Sinauer Associates, Sunderland, Massachusetts.

WELLS, J. V., AND K. V. ROSENBERG. 1996. Bird conservation in the northeast. [Online.] Birdscope vol. 10, no. 1. Available at birds.cornell.edu/Publications/birdscope/Winter1996/conserv96101.htm.

WILL, T. C. 1986. The behavioral ecology of species replacements: Blue-winged and Golden-winged warblers in Michigan. Ph.D. dissertation, University of Michigan, Ann Arbor.

Associate Editor: A. E. Burger

APPENDIX. Locations of West Virginia sampling sites.

Site	Latitude	Longitude	Elevation (m)	County
Athens	37°25′N	81°01′W	690	Mercer
Pinnacle Creek Mine	37°33′N	81°29′W	510	Wyoming
Lillybrook	37°38′N	81°13′W	750	Raleigh
Sandstone	37°45′N	80°54′W	310	Raleigh
Lilly Mountain	37°46′N	81°17′W	738	Raleigh
Peachtree Ridge	37°48′N	81°27′W	750	Raleigh
Dawson	37°51′N	80°42′W	750	Greenbrier
Highland Mountain	37°52′N	81°01′W	785	Fayette
Hobet 21 Mine	38°08′N	81°54′W	600	Boone
Cannelton Mine	38°13′N	81°16′W	628	Fayette–Kanawha