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INCIPIENT SPECIATION DESPITE LITTLE ASSORTATIVE MATING: THE YELLOW-RUMPED WARBLER HYBRID ZONE

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Hybrid zones between recently diverged taxa are natural laboratories for speciation research, allowing us to determine whether there is reproductive isolation between divergent forms and the causes of that isolation. We present a study of a classic avian hybrid zone in North America between two subspecies of the yellow-rumped warbler (*Dendroica coronata*). Although previous work has shown very little differentiation in mitochondrial DNA across this hybrid zone, we identified two nuclear loci (one sex-linked and one autosomal) that show fixed differences across the hybrid zone, in a close concordance with patterns of plumage variation. Temporal stability and limited width of the hybrid zone, along with substantial linkage disequilibrium between these two diagnostic markers in the center of the zone, indicate that there is moderate reproductive isolation between these populations, with an estimated strength of selection maintaining the zone of 18%. Pairing data indicate that assortative mating is either very weak or absent, suggesting that this reproductive isolation is largely due to postmating barriers. Thus, despite extensive hybridization the two forms are distinct evolutionary groups carrying genes for divergent adaptive peaks, and this situation appears relatively stable.

KEY WORDS: **Assortative mating, cline, linkage disequilibrium, tension zone.**

Hybrid zones are formed when genetically divergent populations come into contact and interbreed (Barton and Hewitt 1989; Harrison 1993). They represent an intermediate stage in the process of speciation, when genetic differences have evolved but are insufficient to completely prevent interbreeding (Hewitt 1988; Barton and Hewitt 1989; Harrison 1993; Jiggins and Mallet 2000). Hybrid zones may be ephemeral, resulting from the recent meeting of two divergent forms that are now blending together (Endler 1977), or may be maintained indefinitely by a balance between selection and dispersal, resulting in a stable cline (Key 1968; Bazykin 1969; Slatkin 1973; May et al. 1975; Endler 1977; Barton 1979; Mallet 1986). By studying variation in a sample of traits across a hybrid zone, we can infer (1) whether two forms are likely to have distinct evolutionary trajectories due to partial reproductive isolation, and (2) the factors that contribute to reproductive isolation. This approach can help resolve ongoing debates regarding the relative importance of premating and postmating reproductive isolation in the early stages of speciation (Coyne and Orr 2004; Price 2008), and the role of hybridization in preventing speciation (e.g., Mayr 1942) or enhancing it through the process of reinforcement (Dobzhansky 1940).

A measure of dispersal is essential to distinguishing between ephemeral and stable hybrid zones. In the case of neutral expansion, dispersal can be used to estimate the time since secondary contact necessary to explain the width of the cline (Endler 1977). If that estimated time is unreasonably small, then a model of neutral expansion can be rejected. In the alternative model of a tension zone (i.e., a hybrid zone maintained by a balance between dispersal and selection against hybrids, Key 1968), cline width and dispersal can be used to estimate the strength of selection

maintaining the hybrid zone (Barton 1979). A given cline width can be explained by high dispersal and strong selection, or low dispersal and weak selection, but these two scenarios differ in the distributions and associations of traits in the center of the zone. In the former case, many individuals in the center of the zone resemble the pure forms, leading to high trait variance and high linkage disequilibrium (LD) between distinct traits. In the latter case, the center of the zone consists of individuals that are the product of many generations of hybridization, leading to lower trait variance and lower LD. Thus, the measurement of LD allows a simultaneous estimation of both dispersal and selection, given a known hybrid zone width (Barton 1982; Szymura and Barton 1986; Barton and Gale 1993). Dispersal distance is notoriously difficult to measure directly in many organisms (Mallet et al. 1990; Sotka and Palumbi 2006), making this approach of using tension zones to estimate dispersal quite useful. Here, we apply both the neutral expansion and tension zone models to a well-known hybrid zone within the North American avifauna, with the goal of understanding the evolutionary dynamics occurring in the zone.

A hybrid zone that has been much debated in the literature (Hubbard 1969; Barrowclough 1980; Zink and McKitrick 1995; Rohwer and Wood 1998; Johnson et al. 1999; Milá et al. 2007) is found within the yellow-rumped warbler (*Dendroica coronata*) complex. Two of the four well-marked subspecies differ noticeably in plumage patterns, Audubon's warbler (*D. c. auduboni*) in the west and the myrtle warbler (*D. c. coronata*) in the east, such that they were originally considered separate species (Hubbard 1969). Discovery of the hybrid zone where their ranges meet in western Canada (Alexander 1945; Hubbard 1969) led taxonomists to lump the two taxa into a single species (AOU 1973). Recently, Milá et al. (2007) showed that ancestral common mitochondrial DNA haplotypes are shared between the forms, and used a coalescent model to infer a divergence date between the forms of approximately 16,000 years ago (but see Arbogast et al. 2002 for caveats on estimation of divergence times). Milá et al. (2007) suggested that male breeding-season plumage evolved rapidly in the myrtle warbler, likely driven by strong sexual selection, and that partial reproductive isolation may exist between the two forms.

Two previous studies on the hybrid zone reached conflicting conclusions about the degree of reproductive isolation between the subspecies. Hubbard (1969) mapped plumage and morphometric variation along two transects across the hybrid zone, and found that nearly all individuals at the center of the zone showed some evidence of admixture in plumage pattern. He speculated that the hybrid zone was narrow enough that some selection against hybrids was necessary to maintain it. In contrast, Barrowclough's (1980) analysis of Hubbard's data, pooled with additional samples he collected, concluded that the cline could be explained by a neutral expansion since secondary contact 5–10 thousand years ago. However, Barrowclough's analysis was based on an assumed dispersal distance (1 km per generation) that subsequent research (e.g., Moore and Dolbeer 1989; Paradis et al. 1998; Ruegg 2008) suggests may have been too low by as much as two orders of magnitude.

Given the contradictory conclusions of Hubbard (1969) and Barrowclough (1980) regarding stability of the hybrid zone, here we test whether partial reproductive isolation has evolved between myrtle and Audubon's warblers. We show that the observed cline width and realistic ranges of dispersal and time since contact are incompatible with a neutral model of cline expansion. We test for premating isolation by comparing observed and expected differences (both genetic and phenotypic) between social mates. Because we are able to reject strong or moderate levels of premating isolation, we use cline width and LD between physically unlinked diagnostic markers to estimate dispersal and the strength of selection maintaining the hybrid zone.

Materials and Methods

FIELD SAMPLING

We sampled breeding warblers along five transects across the hybrid zone in British Columbia and Alberta (Fig. 1). Birds were captured using song playback and mist nets between late April and early July of 2005–2007, for a sample size of 661 individuals at 43 locations (Table 1). We used nonlethal sampling methods to enable observation of behavior of marked birds subsequent to capture, and because large series of specimens from this hybrid zone already exist (Hubbard 1969; Barrowclough 1980). To ensure that we sampled locally breeding warblers, individuals that were not observed singing (males only) or defending a territory (both sexes) were excluded from the analysis. For each captured bird, we scored five plumage color traits that differ between *coronata* and *auduboni*, following Hubbard's (1969) hybrid index. The scored traits were throat color (yellow in *auduboni*, white in *coronata*), auricular color (gray in *auduboni*, black in *coronata*), white supraloral spot (absent in *auduboni*, present in *coronata*), white postocular line (absent in *auduboni*, present in *coronata*), and wing pattern (single broad white patch in *auduboni*, two distinct white bars in *coronata*). A sixth trait used by Hubbard (1969), tail pattern, was excluded from analysis due to concerns over its repeatability. Auricular color was excluded from analysis in females, because in both subspecies female auriculars were brown rather than black or gray. Digital photographs were taken to verify consistency of plumage scores across observers, and a blood sample was obtained by brachial venipuncture and stored in Queens lysis buffer (Seutin et al. 1991). We attempted to identify the social mate of each female warbler captured by observing mate-guarding behavior; if a female was consistently followed or guarded by a single male, we designated that male her social mate.

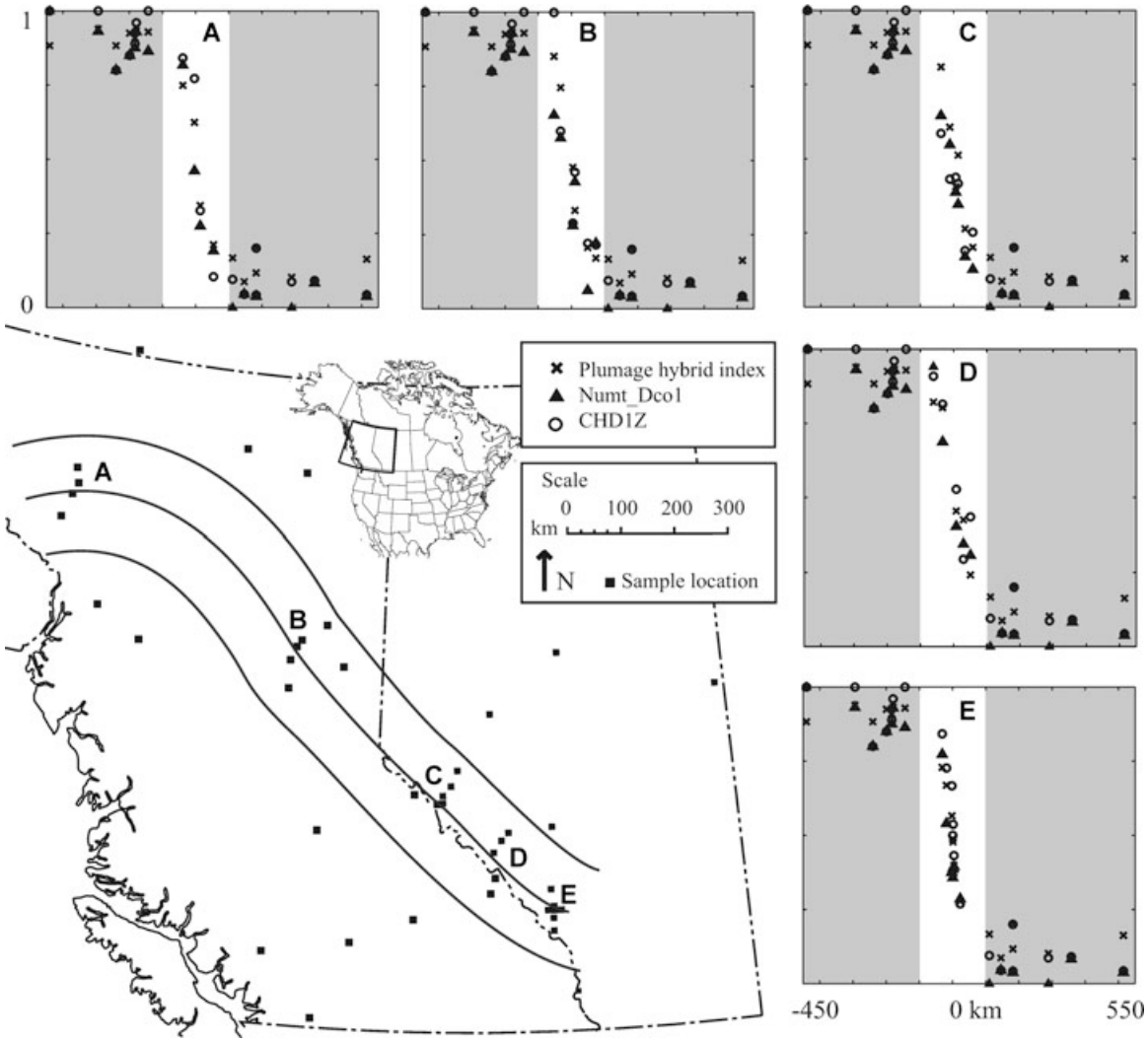

Figure 1. **Location of five hybrid zone transects and allopatric reference sample sites in British Columbia and Alberta. Parallel curves on map denote hybrid zone center and 100-km buffer. Plots of plumage pattern and two genetic markers along five transects show the consistent width of the hybrid zone between Myrtle and Audubon's warblers. Shaded areas of plots contain samples outside the 100-km hybrid zone buffer; these same samples are plotted on graphs for all five transects and were used in the cline analysis of each transect.**

MOLECULAR METHODS

We extracted DNA from blood samples using standard phenol–chloroform methods. We then screened loci from both autosomes and sex chromosome Z (in birds, males are ZZ and females ZW) for a diagnostic variation between *coronata* and *auduboni* by sequencing each locus in birds sampled far from the hybrid zone. Initial screening used two birds from each subspecies; diagnostic status (i.e., frequency difference >0.9 between allopatric populations) of potentially informative markers was confirmed by sequencing or genotyping additional individuals (see below). We tested primers for 18 introns chosen from two studies on *Ficedula* flycatchers (Borge et al. 2005; Backström et al. 2006), of which nine primer pairs reliably produced single PCR products. Additionally, we screened an intron of *CHD1Z* that has been used for molecular sexing of birds (Fridolfsson and Ellegren 1999), and a nuclear sequence of mitochondrial origin ("numt") that we name *Numt-Dco1*, which was initially sequenced inadvertently in an effort to sequence a fragment of the mitochondrial control region. *Numt-Dco1* almost certainly originated from a mitochondrial sequence based on its 83–90% sequence identity to other warbler control region sequences, and is clearly located on an autosome, because many individuals of both sexes carry two different copies of this sequence, both of which are substantially (15–17%) divergent from the yellow-rumped warbler mitochondrial version. In total, we screened six autosomal and five Z-linked loci; details of sequenced loci are found in Table S1. A typical PCR reaction for sequencing included 1x PCR buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen, Carlsbad, CA), 0.2 mM dNTP mix (New England Biolabs, Beverly, MA), 0.5 μM forward and reverse primer, 1.2 units Taq DNA polymerase (New England Biolabs), and 75 ng

Table 1. Location, sample size, and marker frequencies for sampling localities used in this study. Negative distances from the hybrid zone center are on Audubon's (southwest) side of the zone, and positive distances are on the Myrtle (northeast) side.

Site name	Lat.	Long.	Distance from center (km)	*n*	Plumage	*CHD1Z*	*Numt-Dco1*
Hope	49.21	−121.36	−440	12	0.88	1.00	1.00
Whistler	50.16	−122.94	−435	10	0.92	1.00	1.00
Lac Le Jeune	50.50	−120.51	−294	30	0.94	1.00	0.93
Hazelton	55.25	−127.40	−240	10	0.88	0.80	0.80
Nass R.	55.70	−128.79	−199	10	0.93	0.85	0.85
Gavin Lk.	52.49	−121.71	−183	20	0.91	0.89	0.88
Yard Ck.	50.90	−118.82	−179	14	0.93	0.96	0.93
Crowsnest Pass	49.70	−114.56	−143	11	0.93	1.00	0.86
Golden	51.40	−116.99	−61	17	0.82	0.91	0.94
Whiskers Point	54.94	−122.99	−53	13	0.85	1.00	0.65
Bob Quinn Lk.	57.06	−130.27	−40	11	0.75	0.84	0.82
Mt. Robson	53.02	−119.28	−37	15	0.81	0.58	0.64
Blaeberry	51.61	−116.77	−33	16	0.80	0.81	0.68
Kennedy Lk.	55.12	−122.81	−33	13	0.75	0.60	0.58
Lougheed	50.78	−115.15	−31	11	0.73	0.84	0.77
Evan Thomas	50.89	−115.13	−19	25	0.67	0.73	0.54
Willow Ck.	57.44	−130.24	−5	13	0.62	0.77	0.46
Sibbald Ck. W.	51.03	−115.02	−2	28	0.57	0.67	0.38
Rafter 6	51.07	−115.02	1	7	0.49	0.50	0.36
Yamnuska	51.12	−115.08	1	23	0.48	0.54	0.39
Pine Pass W.	55.49	−122.78	4	17	0.48	0.29	0.28
Sibbald Ck. E.	51.04	−114.91	4	20	0.40	0.43	0.39
Pyramid Lk.	52.90	−118.09	7	13	0.40	0.43	0.38
Saskatch. R. X-ing	52.00	−116.66	8	31	0.45	0.53	0.40
Pine Pass E.	55.52	−122.67	10	8	0.33	0.46	0.43
Yellowhead Pass	52.89	−118.39	10	11	0.60	0.43	0.55
Todagin	57.62	−130.08	12	29	0.34	0.33	0.28
Palisades	53.02	−118.09	15	16	0.51	0.41	0.34
Waiparous	51.38	−115.01	23	14	0.28	0.27	0.29
Cline R.	52.16	−116.46	30	26	0.43	0.30	0.35
Pocahontas	53.20	−117.92	36	9	0.26	0.19	0.17
Tumbler Ridge	55.10	−121.11	49	13	0.21	0.22	0.06
Abraham Lk.	52.34	−116.34	51	13	0.24	0.43	0.31
Morchuea Lk.	57.98	−130.07	52	13	0.21	0.11	0.19
Hinton	53.39	−117.74	59	12	0.20	0.25	0.13
Moberly Lk.	55.77	−121.60	74	12	0.17	0.22	0.22
Rocky Mtn. House	52.43	−115.00	111	11	0.17	0.10	0.00
Prophet R.	57.97	−122.78	146	12	0.09	0.05	0.05
Fox Ck.	54.37	−116.89	182	13	0.20	0.20	0.04
Toad R.	58.87	−125.38	182	13	0.12	0.04	0.04
Watson Lk.	60.08	−128.86	289	12	0.10	0.09	0.00
Slave Lk.	55.48	−114.85	358	12	0.09	0.09	0.08
Cold Lk.	54.72	−110.09	515	13	0.16	0.04	0.04

genomic template DNA, in a total volume of 30 μl. The thermal cycling profile was 3 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at an annealing temperature that varied by locus (Table S1), and 45 sec at 72°C, ending with 5 min at 72°C.

Amplified PCR products were sequenced along both strands by Macrogen, Inc. (Seoul, South Korea), using an ABI 3730 XL sequencer (Applied Biosystems, Foster City, CA). Forward and reverse sequences were aligned in BioEdit (Hall 1999) and

checked by eye against electropherograms. Haplotypes were inferred using PHASE (Stephens and Donnelly 2003), and within-taxon polymorphism, F_{ST}, and net sequence divergence were calculated for each locus using DnaSP (Rozas et al. 2003). We designed restriction fragment length polymorphism (RFLP) assays for diagnostic single-nucleotide polymorphisms (SNPs) using NEBcutter (Vincze et al. 2003), and used these assays to genotype all sampled warblers. PCR conditions for RFLP genotyping were identical to those for sequencing, but reactions were run at 10 μl volumes. Restriction digests were carried out on 2 μl of PCR product, using two units of restriction enzyme (*Hin*dIII for *CHD1Z*, *Mnl*I for *Numt-Dco1*) and 1× concentration of the appropriate buffer (New England Biolabs) in a total volume of 6 μl. Products were digested for 2 h at 37°C, and restriction enzymes inactivated by 15 min at 70°C. Digested DNA was visualized by electrophoresis on 2% agarose gel stained with SYBRSafe (Invitrogen).

CLINE WIDTH ANALYSIS

To enable comparisons between transects, we converted our two-dimensional location data to a set of five linear transects by calculating the distance from the midpoint of the hybrid zone (Fig. 1) for each sampled individual. The location of the cline midpoint was determined as follows: First, we calculated the average plumage-based hybrid index for each sampling site, and for each transect we found the two sites that bracket the midpoint value of 0.5. We then plotted a line between the centers of these two sampling sites, and interpolated the location along this line at which the expected phenotypic hybrid index would equal 0.5. For example, if the two bracketing sample sites had average hybrid index values of 0.4 and 0.6, the center of the hybrid zone would be at the midpoint of the line connecting the two sample sites. We then drew a curve (representing the hybrid zone center; Fig. 1) connecting the five transect centers; over most of the distance, this curve paralleled the local orientation of the Rocky Mountains, because the hybrid zone is broadly coincident with the Rockies over long distances. Finally, we measured minimum straight-line distances from each sampled bird to this curve, giving a single spatial dimension that is comparable among multiple transects. All spatial measurements and plotting were conducted using Google Earth.

We estimated cline width using the program C-fit (Gay et al. 2008). C-fit uses maximum likelihood to fit sigmoid curves with exponential tails of introgression on each side of the cline, using methods described by Szymura and Barton (1986). Concordance of clines among markers, among transects, and between plumage data from 1965 (taken from Hubbard 1969) and 2005–2007 (this study) was evaluated using likelihood-ratio tests, following the approach of Fel-Clair et al. (1996). Cline width was calculated as the inverse of the maximum slope (Endler 1977). Samples that were taken more than 100 km from the center of the zone were used in the cline analysis for every transect, whereas those taken within 100 km from the center of the zone were used only in a single transect.

LINKAGE DISEQUILIBRIUM ANALYSIS

We estimated composite digenic LD between two diagnostic markers (Weir 1996, ch. 3) among birds sampled within 20 km of the center of each of the five transects. Lumping samples collected a range of distances from the cline center might spuriously inflate estimates of LD by the Wahlund effect (Sinnock 1975). To account for this, we recalculated LD for samples ranging from within 1 km to 75 km of the hybrid zone center. Confidence intervals were estimated by bootstrapping with 10,000 replicate samples using Matlab (The MathWorks, Inc., Natick, MA).

When the origin of dispersing organisms differs in allele frequencies from the destination, dispersers carry gametes that retain their original associations between alleles at different loci, causing LD (Barton 1982). In a tension zone of a given width, Barton (1982) showed that LD is related to the square of dispersal: $D = \frac{\sigma^2}{rw^2}$, where D is LD, σ is the standard deviation of parent–offspring distance (hereafter, "dispersal"), w is cline width, and r is recombination rate between loci. Barton and Gale (1993) modified the equation for samples taken after dispersal and before breeding $D = \frac{\sigma^2(1+r)}{rw^2}$. We used this second equation (for samples taken after dispersal) along with our measurements of LD and cline width to estimate dispersal σ (Barton and Gale 1993). This model assumes that mating is random, the hybrid zone is maintained by selection against intermediate genotypes, and that there are no epistatic interactions among the loci used to measure LD (Barton 1982; Szymura and Barton 1986; Barton and Gale 1993). The model was also developed using an assumption of weak selection, but subsequent simulations incorporating stronger selection gave results consistent with the model (Barton and Gale 1993; Kruuk et al. 1999).

The width of a tension zone at equilibrium is given by $w = \frac{4\sigma}{\sqrt{2s}}$ where s is selection against a heterozygote (Bazykin 1969); this assumes that the cline is maintained by heterozygote disadvantage at a single locus, but authors have argued that the relationship holds approximately for more realistic multilocus clines and a wide variety of types of selection (Moore and Price 1993; Kruuk et al. 1999; Price 2008), and the equation is routinely used in analyses of hybrid zones (e.g., Alexandrino et al. 2005; Raufaste et al. 2005; Sequeira et al. 2005; Macholán et al. 2007). Using this equation, we estimated the strength of selection (s) necessary to maintain the cline.

We used a second method of estimating LD from variance in a hybrid index (Barton and Gale 1993) using plumage. Treating five plumage color traits as independent "loci," we obtained a phenotypic estimate of LD to complement our estimate based on genetic markers. (In reality, plumage traits are unlikely to

be genetically independent; see Results.) Correlation coefficients among five plumage traits and two genetic markers in populations within 20 km of the hybrid zone center were calculated using Matlab.

TEST FOR ASSORTATIVE MATING

To test whether yellow-rumped warbler subspecies mate assortatively, we used randomization tests to determine whether mated pairs ($n = 77$) were more genetically or phenotypically similar than would be expected by chance. First, we calculated a genetic hybrid index based on the two diagnostic genetic markers. This index was defined as the number of Audubon's alleles carried by an individual divided by the total number of alleles (two *CHD1Z* alleles plus two *Numt-Dco1* alleles for males; one *CHD1Z* allele plus two *Numt-Dco1* alleles for females), with possible values from zero to one in increments of 0.25 for males and 0.33 for females. We then calculated the correlation between genetic indices of females and their mates. To determine the expected level of association, we randomly assigned each female a male from the same sampling locality (generally within 10 km) and calculated the overall genetic correlation between paired individuals. This randomization was repeated 100,000 times, producing a distribution of within-pair correlations expected under random mating.

Randomization tests were also used to assess the power of our mate-choice sample to detect varying strengths of assortative mating. In these tests, we modeled mate choice as a process in which a female warbler sequentially evaluates a number n of potential mates sampled from the males caught at the same study site, choosing to pair with the first male she finds acceptable. If no acceptable males are encountered, the females pairs with the nth male. Acceptability is defined by the parameter A, the maximum difference between a female's genetic hybrid index and that of an acceptable mate. For example, a pure myrtle female (hybrid index 0) would pair with a pure Audubon's male (hybrid index 1) only if $A = 1$, or if the male is the nth potential mate she has evaluated. We simulated pairings according to this model for the 77 females with known mates, with 100,000 replicates for each parameter combination. We then compared the simulated correlation to the observed value to determine the parameter combinations compatible with the observed pattern of mate choice. This analysis was repeated using Hubbard's (1969) phenotypic hybrid index.

Results

We identified two diagnostic markers among the 11 screened. One diagnostic marker (*CHD1Z*) is Z-linked; the other (*Numt-Dco1*) is autosomal; both exhibited very nearly fixed differences between allopatric myrtle and Audubon's warblers (Table 1, localities Hope, Whistler, Cold Lk.) as well as very low levels of polymorphism within subspecies (Table 2). Sequences have been deposited in GenBank (accession numbers GQ457569–GQ457722).

Patterns of variation across the hybrid zone closely conformed to the expected sigmoidal pattern predicted by tension zone models (Barton and Gale 1993). Cline widths at diagnostic markers and plumage traits did not differ significantly among the five sampled transects (Fig. 1, Pairwise comparisons using likelihood-ratio test described by Fel-Clair et al. 1996: χ^2 ranged from 0.00 to 1.25, df = 1, P ranged from 0.26 to 1.), and overall

Table 2. Details of loci sequenced. Sample size indicates the number of chromosomes sequenced (two chromosomes per individual). Diagnostic loci (*CHD1Z* and *Numt-Dco1*) have much greater F_{ST} between Myrtle and Audubon's forms, and lower within-taxon polymorphism (π_A for Audubon's, π_M for Myrtle) than other loci. Net sequence divergence (D_A) varies among the loci. Average polymorphism is more than two times higher among autosomal than Z-linked loci.

Locus	Sample size	Chromosome	Total base pairs	Coding bp	Noncoding bp	F_{ST}	π_A	π_M	D_A
GH1	8	1	594	8	586	−0.1783	0.0077	0.0139	−0.0016
RPL30	8	2	945	0	945	0.1827	0.0198	0.0127	0.0036
LHCGR	22	3	648	6	642	0.0045	0.0126	0.0167	0.0001
Numt-Dco1	28	4	343	0	343	0.6709	0.0004	0.0020	0.0025
RPL5	8	8	573	24	549	0.2821	0.0059	0.0023	0.0016
CEPU1	8	24	567	0	567	0.0513	0.0072	0.0039	0.0003
24555	8	Z	500	56	444	0.1458	0.0060	0.0077	0.0012
ADAMTS6	8	Z	546	49	497	−0.0667	0.0037	0.0012	−0.0002
BRM	8	Z	332	35	297	0.0000	0.0015	0.0015	0.0000
CHD1Z	40	Z	613	127	486	0.6882	0.0006	0.0013	0.0021
PTCH	8	Z	554	22	532	−0.0208	0.0127	0.0021	−0.0002
Mean, Diagnostic						0.6795	0.0005	0.0017	0.0023
Mean, Other Z		Z				0.0146	0.0060	0.0031	0.0002
Mean, Other Auto.		various				0.0685	0.0106	0.0099	0.0008

Table 3. Proportion of randomization trials resulting in less-than-observed correlation between genotypes or phenotypes of social mates. Parameter *A* is the maximum difference between a female's hybrid index and that of an acceptable mate; a low *A* signifies a stringent mate preference. Parameter *n* is the maximum number of potential mates a female evaluates before mating randomly; an *n* of 1 or an *A* of 1 corresponds to random mating. Values in bold indicate parameter combinations incompatible with observed pairing data (α=0.05).

	Genetic hybrid index					Plumage hybrid index				
n	1	2	3	5	8	1	2	3	5	8
A										
0.25	0.650	0.037	**0.001**	**0.000**	**0.000**	0.905	0.076	**0.002**	**0.000**	**0.000**
0.5	0.654	**0.021**	**0.001**	**0.000**	**0.000**	0.905	0.182	0.035	**0.008**	**0.005**
0.67	0.652	0.101	0.026	**0.008**	**0.006**	0.907	0.565	0.466	0.437	0.439
0.75	0.651	0.239	0.170	0.147	0.137	0.905	0.730	0.690	0.681	0.682
1.0	0.649	0.648	0.649	0.651	0.653	0.908	0.907	0.905	0.905	0.906

marker cline widths (i.e., when the five transects were combined) were similar to each other and to the cline in plumage pattern (Fig. 1, χ^2 ranged from 0.01 to 0.16, df = 1, P ranged from 0.69 to 0.92). Cline location (i.e., the location of the center of the clines) did not differ between *CHD1Z* and plumage ($\chi^2 = 0.18$, df = 1, $P = 0.67$), but did differ between *Numt-Dco1* and those traits (*CHD1Z*: $\chi^2 = 14.56$, df = 1, $P = 0.0001$; plumage: $\chi^2 = 10.27$, df = 1, $P = 0.0014$), with the *Numt-Dco1* cline falling approximately 14 km west of the clines in the other two markers, a small distance compared to the width of the clines. Clines in plumage hybrid index from samples taken in 1965 (Hubbard 1969) and 2005–2007 did not differ in width ($\chi^2 = 0.53$, df = 1, $P = 0.47$) or position ($\chi^2 = 0.02$, df = 1, $P = 0.66$). Like Hubbard (1969), we found that the vast majority (191 of 200) of individuals near the hybrid zone center showed some evidence of admixture. The cline width estimated from all transects using plumage and both genetic markers was 132 km.

Correlation between the hybrid indices of female warblers and their mates was 0.69 (phenotypic) and 0.54 (genetic). Observed correlations were slightly higher than the median expected under local random mating for phenotypic (0.60) and genetic (0.52) hybrid indices. However, the difference was not significant; phenotypic correlation between randomly assigned pairs was higher than the observed value in 9.5% of trials, whereas genetic correlation was higher than the observed value in 35%. Power analysis indicated that the observed pairing pattern was consistent with random or very weakly assortative mating (females may avoid mates that differ from their genotype by at least 0.75, or from their phenotype by at least 0.67), but moderate or strong assortative mating would have resulted in a significantly higher female–male correlation (Table 3). Without access to family groups, we were only able to assess social mates; extra-pair parentage is common in passerines, including some warblers (Griffith et al. 2002). Because of this constraint, we assumed that patterns of mate choice for extra-pair copulations do not differ substantially from patterns of choice of social mates.

LD between *CHD1Z* and *Numt-Dco1* was significant at two of five transects (Fig. 2), with estimates ranging from −0.007 (transect A) to 0.120 (transect C). Pooling the samples from all five transect centers gave an LD estimate of 0.067 (95% C.I. = 0.029–0.104). This level of LD is substantial; the theoretical maximum for this measure of disequilibrium is 0.25, which would be expected in the absence of hybridization. Assuming for the moment that there is no strong epistasis between the two marker loci, we can use the model of Barton and Gale (1993) to calculate a dispersal distance of 20 (13–25) km per generation, and a strength of selection of 0.18 (0.08–0.28). We note that this estimate of

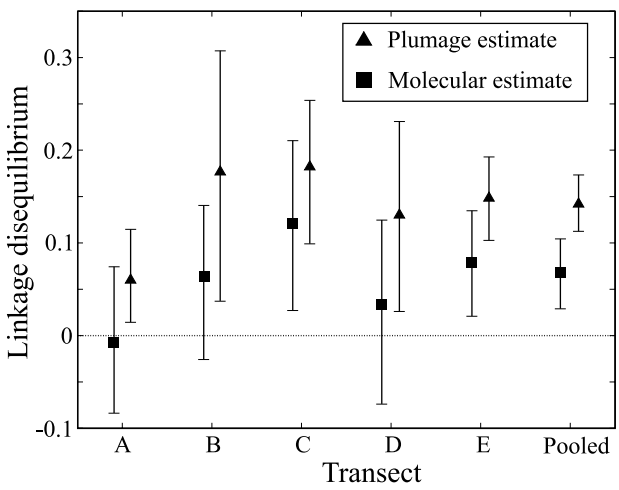

Figure 2. Linkage disequilibrium estimated from diagnostic genetic markers and from plumage traits for five transect centers (all samples within 20 km of each center) and for a pooled sample from all transect centers. Error bars represent bootstrap 95% confidence intervals. Sample sizes for each transect are 39 (A), 19 (B), 33 (C), 22 (D), 87 (E), and 200 (Pooled).

selection refers to the "effective selection pressure at a single locus that would be required to maintain a cline of the observed width" (Szymura and Barton 1986). In reality, selection is likely to act on multiple loci; hence, this estimate of selection represents the sum of selection acting on each locus multiplied by its LD with the markers we use in this analysis at the hybrid zone center. We observed evidence of the Wahlund effect only at sample threshold distances of 30 km or greater, whereas LD values calculated from samples within 30 km of the hybrid zone center were relatively insensitive to sample area (Fig. S1); we used samples within 20 km of the center to calculate LD.

Using the alternate method of estimating LD from variance in a hybrid index (Barton and Gale 1993), in this case based on plumage, we obtained an LD estimate of 0.14. LD estimated from plumage traits was consistently higher than LD between genetic markers at each transect center (Fig. 2), but the two measures were strongly correlated ($R^2 = 0.79$, $P = 0.04$). These observations suggest that the five plumage traits used to calculate LD are not completely genetically independent, due to either pleiotropy or physical linkage of genes. Further support for the nonindependence of plumage traits is given by the correlation coefficients among traits, which ranged from 0.43 to 0.75, considerably higher than the correlation (0.25) between two physically unlinked genetic markers in the same sample (Table S2). Because of this nonindependence, we do not use plumage-based LD to estimate dispersal.

Discussion

Our results strongly suggest that partial reproductive isolation exists between myrtle and Audubon's warblers. Given a conservative estimate of migratory songbird dispersal of 10 km/generation (estimates based on band recoveries range from 10 to 100 km/generation, Moore and Dolbeer 1989; Paradis et al. 1998; Ruegg 2008), a neutrally diffusing cline would reach a width of 132 km in approximately 60 generations, or 108 years using a generation time of 1.8 years (Milá et al. 2007). Higher estimates of dispersal would lead to even more recent estimates of the time since initial secondary contact. A neutral cline of such recent origin would be expected to show noticeable expansion over the past four decades; thus the consistency of width of the hybrid zone between 1965 and 2005–2007 is incompatible with selectively neutral mixing of the taxa.

Assortative mating in the hybrid zone is absent or very weak, suggesting that premating isolation is unlikely to be a major factor in stabilizing the hybrid zone. The observed pairing data does not allow us to rule out assortative mating between the two pure forms, but does rules out assortative mating between hybrids and either pure form. These results are in line with Coyne and Orr's (2004, ch. 2) argument that postzygotic barriers often play a major role in the early stages of speciation. In the absence of strong assortative mating, we are able to apply tension zone models (Barton and Gale 1993) to the system to investigate the strength of the partial reproductive barriers between the two forms. Assuming that all selection maintaining the hybrid zone falls on hybrids, we find selection equivalent to a single-locus heterozygote disadvantage of 18% is necessary to account for the cline width and LD we observe. We emphasize, however, that this result is approximate. Uncertainty in LD and cline width due to sampling, assumptions inherent in the tension zone model (see Methods), and the possibility of habitat-dependent (e.g., Moore 1977) or frequency-dependent (Mallet 1986) selection on parental types indicate a low degree of confidence in the specific numerical result. In particular, we cannot rule out weakly assortative mating. To the extent that assortative mating causes sexual selection against hybrids or reduces the frequency of hybridization, this selection would be incorporated in our estimate of selection maintaining the hybrid zone. Nevertheless, our findings of a narrow and stable cline, nonzero LD at the cline center, and little to no assortative mating provide strong evidence that some form of postmating isolation maintains this hybrid zone.

The mechanism by which this selection acts remains unknown, and will require detailed behavioral, ecological, and genetic study to determine. Differences in migratory pathway (e.g., Helbig 1991; Irwin and Irwin 2005), adaptation to different environments (e.g., Price 2008, ch. 15), and intrinsic genetic incompatibilities (e.g., Bronson et al. 2005) may all play a role. Regardless of mechanism, the presence of substantial LD long after secondary contact suggests that some form of selection impedes the fusion of the Myrtle and Audubon's forms.

When two incipient species interbreed, genetic differentiation between them will erode at neutral loci but persist where selection reduces introgression (Wu 2001; Turner et al. 2005; Via and West 2008). In the context of an old and stable clinal hybrid zone, markers closely linked to selected loci will exhibit clinal variation whereas neutral, nonhitchhiking loci will not (Barton and Bengtsson 1986; Durrett et al. 2000). In the presence of LD, selection on any one locus will reduce introgression and narrow the cline for all other selected loci, such that the cline width for all selected loci will be determined by the total effective selection on all of them (Barton 1983). In this study, we did not sample enough loci to precisely determine the fraction of the genome over which selection maintains differences between the two incipient species, but our estimate of the strength of selection incorporates the selection acting on all loci that differ across this hybrid zone.

Of the 11 loci we screened, only two were reciprocally monophyletic between myrtle and Audubon's warblers. Our subsequent analyses focused on the two diagnostic markers to make use of the substantial body of hybrid zone theory developed for diagnostic markers. However, consideration of the other nine markers

can provide additional insights into the maintenance of the hybrid zone. One interpretation of the lack of reciprocal monophyly in nine of 11 screened loci is that the proportion of the genome subject to restricted introgression is roughly 2/11, or 0.18 (keeping in mind that 11 loci, six of which are on a single chromosome, is a miniscule sample relative to the total genome size). However, some of the nondiagnostic loci we screened may also be subject to restricted introgression across the hybrid zone, and the shared sequence variation could result from incomplete lineage sorting (Hudson and Coyne 2002).

The SNP frequency differences we observe in several introns suggest that gene flow between the taxa may be restricted at some nondiagnostic loci. In the future we plan to analyze changes in SNP frequency across the hybrid zone at the nine nondiagnostic introns and mitochondrial DNA; we hypothesize that several clines in SNP frequency may prove concordant with the diagnostic clines we have measured in the current study. Regardless of the genomic extent of restricted introgression across this hybrid zone, we note that any incomplete barrier to gene flow between divergent populations can be viewed as partial reproductive isolation (e.g., Mayr 2001; Rundle et al. 2001; Vines et al. 2003; Jones et al. 2006), even if such a barrier applies only to certain loci (e.g., Barton and Bengtsson 1986; Wu 2001; Navarro and Barton 2003; Gay et al. 2007).

Our findings indicate that the myrtle and Audubon's warblers are stable and genetically distinct forms, that parts of their genomes remain distinct despite extensive hybridization, and that selection maintains differences between the taxa. We therefore suggest that these two taxa may meet the current criterion for full species status between hybridizing North American birds, that the hybrid zone be "narrow and stable" (AOU 1998). If the recent divergence date estimated by Mila et al. (2007) from mitochondrial DNA (roughly 16,000 years) is even remotely accurate, our results would suggest a relatively rapid evolution of partial reproductive isolation between the forms. However, many authors have criticized reliance on mtDNA for dating recent species divergences (Arbogast et al. 2002; Ballard and Whitlock 2004; Edwards et al. 2005). Thus, we suggest that an alternative hypothesis merits consideration: mitochondrial introgression from one form to the other may have obscured a long history of independent evolution of each form. This phenomenon, termed "cytoplasmic capture," has been documented in plants (Rieseberg and Soltis 1991), fish (e.g., Wilson and Bernatchez 1998), birds (e.g., Weckstein et al. 2001; Irwin et al. in press), insects (e.g., Llopart et al. 2005), and mammals (e.g., Good et al. 2008). Evaluating this alternative hypothesis of ancient divergence and recent cytoplasmic capture will require analysis of multiple nuclear markers throughout the yellow-rumped warbler species complex.

The yellow-rumped warbler provides an intriguing example of bird speciation in progress. Partial postmating isolation has evolved between the forms before the onset of any measurable premating isolation, and has not yet led to reinforcement. In the face of extensive hybridization, selection maintains differences between Audubon's and myrtle forms over at least part of their genomes, including the two diagnostic markers we have found as well as the loci controlling plumage pattern differences. Given the long period of time that complete reproductive isolation usually takes to evolve (usually more than a million years; Coyne and Orr 2004; Price 2008) and the many climatic cycles that occur over such a long period, many diverging pairs of taxa likely come into secondary contact multiple times on their way to becoming full species. The yellow-rumped warbler hybrid zone, in which there is moderate postmating isolation but little if any premating isolation, may thus represent a typical early stage in the divergence of many species. If so, the study of such hybrid zones could play a central role in developing a full understanding of speciation.

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Supporting Information

The following supporting information is available for this article:

Figure S1. Linkage disequilibrium at the center of the hybrid zone increases due to Wahlund effect as samples farther than 30 km from the center are included.

Table S1. Details of loci sequenced.

Table S2. Correlation coefficients among two genetic markers (*CHD1Z, Numt-Dco1*) and five plumage traits (Throat, Auricular, Spot, Line, Wing) among samples within 20 km of the hybrid zone center.

Supporting Information may be found in the online version of this article.
(This link will take you to the article abstract).

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Table S1: Details of loci sequenced. [1]: Chromosome and nucleotide number in zebra finch genome sequence (genome.ucsc.edu, assembly July 2008) [2]: Approximate position of sequence homologous to the flanking region of *Numt-Dco1* (data not shown). [3]: The forward primer we use is the sequence reported by Milá et al. (2007) for LGL2, which because of a typo differs by one substitution from the original primer sequence (Tarr 1995). Milá et al. used Tarr's original LGL2 primer sequence.

Locus	Start pos. in zebra finch[1]	End pos. in zebra finch[1]	Forward Primer	Reverse Primer	Source	Annealing Temp. (°C)
GH1	1 112878784	1 112879348	AACCTGTTTGCCAACGCTGT	CTGGTCCTCCGGAATATAGGTG	Borge et al. 2005	55
RPL30	2 134052606	2 134051596	CCAAGTTGGTCATCCTAGCCA	GCCACTATAATGATGGACACCAGTC	Borge et al. 2005	62.5
LHCGR	3 21664487	3 21663828	TGCCTTCAATGGGACCAAG	CCGCCTGAGGTTTTTGTTGT	Borge et al. 2005	55
Numt_Dco1	4 ~9637000[2]	4 ~9637000[2]	GGCCACATCAGACAGTCCAT	AGTAGCTCGGTTCTCGTGAG	Milá et al. 2007, Tarr 1995[3]	61.5
RPL5	8 10441755	8 10441184	GTTGGCCTGACCAATTACGC	CTTCAACTTGGCCTTCATAGATCTT	Borge et al. 2005	55
CEPU1	24 6314566	24 6315079	GTGCAGTGCCTCCAACGAC	TCGCATCCGAGATGTACGG	Borge et al. 2005	55
24555	Z 6656177	Z 6656632	CCTCCAGATATTTCATTCCC	AATGGAAATGGCTGAACTTG	Backström et al. 2006	61.5
ADAMTS6	Z 50815125	Z 50815666	GGAGAGAATGGATTTCTGCC	TGATTCCAGTCTAGGAAACG	Backström et al. 2006	62.8
BRM	Z 64937586	Z 64937254	AGCACCTTTGAACAGTGGTT	TACTTTATGGAGACGACGGA	Borge et al. 2005	62.8
CHD1Z	Z 24736734	Z 24736133	GTTACTGATTCGTCTACGAGA	ATTGAAATGATCCAGTGCTTG	Fridolfsson and Ellegren 1999	58
PTCH	Z 9746535	Z 9747057	CCATTTTCTTCCAAGCAATA	TTTCTTGACAGTCCATAGCA	Borge et al. 2005	57.5

Table S2: Correlation coefficients among two genetic markers (*CHD1Z, Numt-Dco1*) and five plumage traits (Throat, Auricular, Spot, Line, Wing) among samples within 20 km of the hybrid zone center.

Trait	*CHD1Z*	*Numt-Dco1*	Throat	Auricular	Spot	Line	Wing
CHD1Z	---						
Numt-Dco1	.25	---					
Throat	.51	.35	---				
Auricular	.37	.26	.56	---			
Spot	.42	.35	.46	.43	---		
Line	.42	.35	.53	.56	.75	---	
Wing	.50	.27	.63	.44	.50	.49	---

Figure S1: Linkage disequilibrium at the center of the hybrid zone increases due to Wahlund effect as samples farther than 30 km from the center are included. We used samples within 20 km of the center to calculate LD.