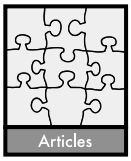

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Extensive hybridization in a contact zone between MacGillivray's warblers *Oporornis tolmiei* and mourning warblers *O. philadelphia* detected using molecular and morphological analyses

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There are many pairs of related western and eastern avian taxa in North America, and for many of these, little is known about their interactions in sympatry. One example is provided by MacGillivray's warblers *Oporornis tolmiei* and mourning warblers *Oporornis philadelphia*. There have been occasional reports of range contact and hybridization between these forms, but recent authors have doubted these reports. We show that these two species do in fact come into extensive range contact in the southern Peace Region of British Columbia, just east of the Rocky Mountains. We analyze whether patterns of variation in morphometric traits, eye-arcs, a mitochondrial DNA marker (COI), and a Z-chromosome marker (CHD1Z) are consistent with reproductive isolation or hybridization in this contact zone. Each trait shows strong differences between allopatric MacGillivray's warblers and allopatric mourning warblers, yet in the contact zone there are many birds with a combination of traits typical of both species. This is clearly seen in the molecular markers, for which 18 of 50 birds genotyped in the contact zone have both western and eastern alleles. These patterns strongly indicate the presence of an extensive hybrid zone between MacGillivray's and mourning warblers. Variation in each of the four traits is explained well by a single sigmoidal cline, with a width of roughly 150 km (or 130 km based only on the molecular markers). This is only the fourth hybrid zone known among North American wood-warblers (Parulidae).

The study of reproductive isolation has been a central component of the growing body of research on speciation (Coyne and Orr 2004, Price 2008). To test whether two groups are reproductively isolated, it is important whenever possible to study the two groups in a geographic region where they both occur. We can then determine whether they differ in various traits in a similar environment, how they interact, and whether they interbreed. These observations can help us to reconstruct the evolutionary history of the two groups, to understand their current interactions, and to more reliably predict their future. Such knowledge enables better decisions regarding taxonomic treatment and the conservation of biodiversity.

In North America, there are many examples of western and eastern avian taxa that are apparently close relatives (Newton 2003, Weir and Schluter 2004). In some cases, the two taxa have been considered subspecies of a single species (e.g. winter wrens *Troglodytes troglodytes pacificus* and *T. t. hiemalis*, Hejl et al. 2002, Drovetski et al. 2004, Toews and Irwin 2008; yellow-rumped warblers *Dendroica coronata auduboni* and *D. c. coronata*, Hunt and Flaspohler 1998, Milá et al. 2007; northern flickers *Colaptes auratus cafer* and *C. a. auratus*, Moore 1995), whereas in other cases the western and eastern taxa have been considered two species (e.g. lazuli and indigo buntings *Passerina amoena* and *P. cyanea*, Greene et al. 1996, Payne 2006, Carling and Brumfield 2008; MacGillivray's and mourning warblers *Oporornis tolmiei* and *O. philadelphia*, Pitocchelli 1993, 1995; spotted and eastern towhees *Pipilo maculatus* and *P. erythrophthalmus*, Greenlaw 1996). In some such cases, little research has occurred in areas of potential contact between the western and eastern relatives. Research from such contact areas, if they exist, could provide surprising results. For example, recent work in a contact zone between western and eastern forms of winter wrens *Troglodytes troglodytes* showed a high level of reproductive isolation, along with behavioral and genetic divergence, indicating that they are separate species that have been evolving relatively independently for millions of years (Toews and Irwin 2008; see also Drovetski et al. 2004).

Here, we examine a case of two closely related taxa that have been considered separate species since they were first described: MacGillivray's warbler *Oporornis tolmiei* (described by Townsend in 1839) in the west and mourning warbler *Oporornis philadelphia* (described by Wilson in 1810) in the east (Pitocchelli 1993, 1995, Dunn and

Garrett 1997, Weir and Schluter 2004). We resolve two key questions regarding these taxa that have been debated in the literature for some time: first, do the two taxa come into contact? Second, do they hybridize?

Almost all previous research on these two taxa took place in allopatric areas. There have been scattered reports of both range contact and hybridization between MacGillivray's and mourning warblers (e.g. Taverner 1919, Cox 1973, Patti and Meyers 1976, Hall 1979), but most of these have been doubted by more recent authors (Hall 1979, Pitocchelli 1990, 1993, Dunn and Garrett 1997). Indeed, major accounts of the species (Pitocchelli 1993, 1995, Dunn and Garrett 1997) report little if any overlap between the species. For example, Pitocchelli (1995) described "little contact between these 2 species on breeding grounds. A few MacGillivray's warblers erratically spill over into eastern foothills of Rocky Mtns. where they might contact mourning warblers, but contact is rare and irregular." Pitocchelli (1990) concluded that variation of traits within each taxon was sufficient to cause doubt that a few individuals with intermediate or apparently mismatched characteristics really are hybrids.

As Pitocchelli (1990) emphasized, determination of whether MacGillivray's and mourning warblers hybridize has been hindered by the lack of clear diagnostic characters that uniformly differ between the taxa. These two taxa are notoriously difficult to differentiate visually; the main distinguishing features are white eye-arcs in the adult MacGillivray's warbler; most mourning warblers lack these eye-arcs, but a small percentage of adult mourning warblers also have narrow white eye-arcs, across their entire geographic range (Pitocchelli 1990, 1993, Dunn and Garrett 1997). Immatures are extremely difficult to distinguish based on plumage. Morphometric data has revealed some differences between the groups, notably in the relative length of tail and wing (Lanyon and Bull 1967), but there is some overlap (Pitocchelli 1990, 1992). Song is perhaps the most reliable feature to distinguish the groups, according to Pitocchelli (1990, 1993), although the analyses that led to this conclusion used recordings made from far west and east of the possible zone of contact.

Hall (1979) concluded that "If there is to be a final solution to the problem of relationship between the two taxa, it will have to come from detailed behavioral studies as well as more collecting in the very few areas in which the ranges are contiguous." All previous work on possible contact and hybridization between MacGillivray's and mourning warblers has focused on southwestern Alberta. Here we have investigated the distributions and interactions of the two species in a different area, the southern Peace River district of northeastern British Columbia. This area is just east of a relatively low-elevation span of the Rocky Mountains.

Here, we address two questions. First, how much range overlap is there between MacGillivray's and mourning warblers in this region? Second, do the two taxa interbreed? If so, traits that differ between the two taxa in allopatry should occur in novel combinations in sympatry. To address these questions, we use variation in two molecular markers, one in the mitochondrial DNA and one on the Z-chromosome, along with morphometric and plumage variation. The use of two distinct molecular markers, one on mitochondrial DNA (inherited from the mother alone) and the other on an avian sex chromosome (the Z-chromosome, which is inherited in males from both parents), provides two relatively independent indicators of each individual's ancestry.

Methods

Sampling

During the summers (May and June) of 2006, 2007, and 2008 we captured 73 male MacGillivray's or mourning warblers for temporary study from various sites in British Columbia and Alberta (Fig. 1, App. 1). Song playbacks were used to attract birds to mist nets that were placed inside each male's territory. The playback recording was generated by combining commercially available tracks of MacGillivray's and mourning warblers recorded from allopatric areas (from the Stokes field guide to bird songs, year 1999 (western region) and 1997 (eastern region)) along with our own recordings of birds from northeastern British Columbia; we found that a combination of songs and calls generated the strongest response. This combined recording generated a strong response from virtually all males that we attempted to catch, leading to a high rate of success (roughly 80%; some birds were not captured due to difficult circumstances such as steep terrain for net placement, birds bouncing off the net, sudden rainstorms, approaching bears or moose, etc.). Morphometric measurements, a blood sample, and photographs were obtained from each bird. We focused much fieldwork on the Peace River region of northeastern British Columbia, as one of us (M. P.) had observed both MacGillivray's and mourning warblers present in the area for many years.

Morphometric and eye-arc analyses

We measured six morphometric variables from each captured bird (all according to Pyle 1997): wing chord, tail length, tarsus length, bill length (from nares to tip), and bill depth and width (both measured at the anterior end of the nostrils). To estimate the difference in each trait between allopatric MacGillivray's and mourning warblers, we calculated means and standard deviations for each trait first from birds sampled from west of the Rocky Mountains, which were assumed to represent allopatric MacGillivray's warblers, and second from those sampled from central Alberta, which were assumed to represent allopatric mourning warblers. Welch two-sample *t*-tests were used to test whether trait means differed between these groups, revealing which traits might be most useful in distinguishing birds in the contact zone.

Previous studies of the morphometrics of the two species (e.g., Lanyon and Bull 1967, Pitocchelli 1990, 1992) have focused on using wing length minus tail length as a distinguishing feature. We also focused our analysis on this composite variable, using it to represent morphometric variation in the cline analysis (see below). Note that we used wing chord (unflattened) to represent wing length, whereas most previous studies (e.g. Pitocchelli 1990, 1992) used flattened wing length; wing chord is likely shorter than flattened wing length, hence our measurement of wing

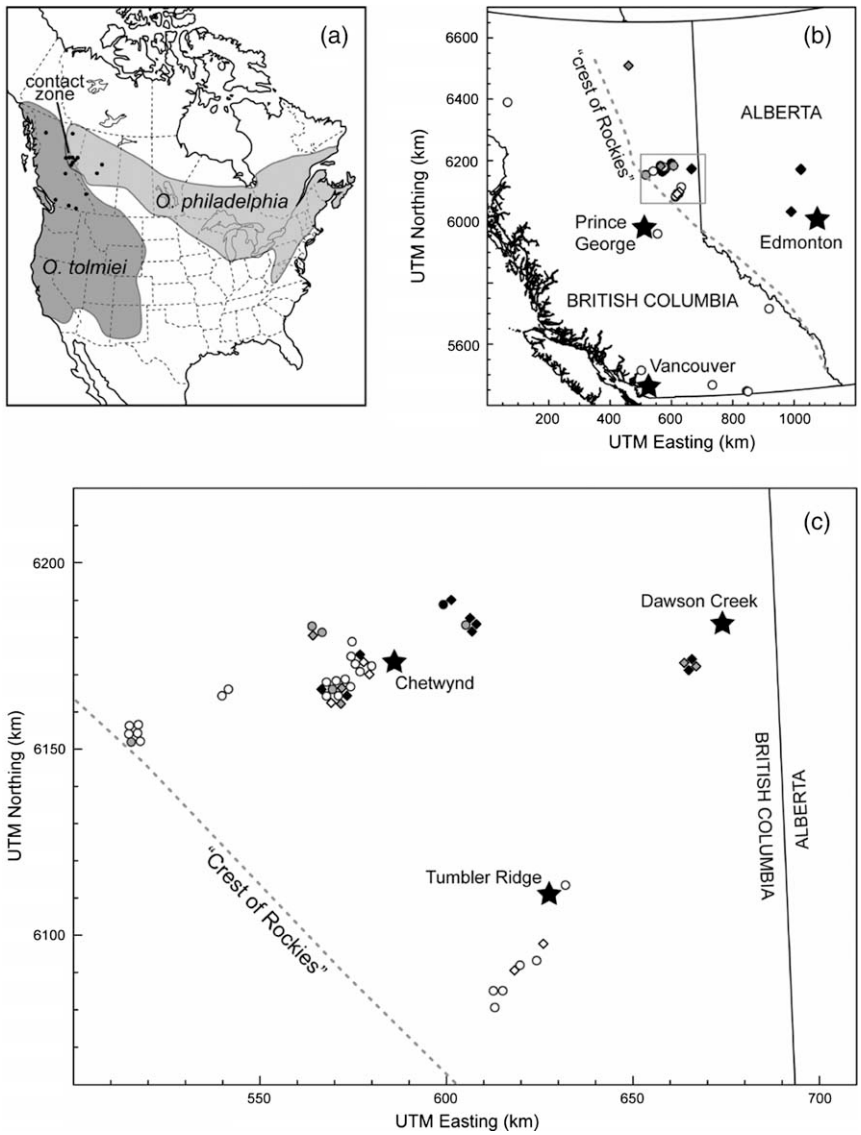

Figure 1. Maps showing: (a) the breeding ranges of MacGillivray's warblers *Oporornis tolmiei* and mourning warblers *O. philadelphia* across North America, (b) sampling sites within British Columbia and Alberta, and (c) locations of each bird sampled within the contact zone in the southern Peace Region of British Columbia, near the towns of Chetwynd, Tumbler Ridge, and Dawson Creek. Sampling sites are indicated by dots (a), or symbols indicating genotype of samples at two molecular markers (b, c). Circles indicate western mitochondria, whereas diamonds indicate eastern mitochondria. Darkness of the symbol indicates CHD1Z genotype, with white corresponding to homozygous western, black corresponding to homozygous eastern, and grey corresponding to heterozygotes. In (c), some symbols have been moved slightly to avoid overlap. The line through the "crest of the Rockies" is shown in (b) and (c); this line was used to measure location of sampling sites (measured as the closest distance to this line) for purposes of the cline analyses (Fig. 3 and 4, Table 3). In (b) and (c), the maps are plotted according the the Universal Transverse Mercator (UTM) coordinate system, based on Zone 10.

minus tail is likely to be slightly (e.g. 1 or 2 mm) shorter than that of Pitocchelli (1990, 1992). Nonetheless, the relative difference between the species should be similar with the two methods of measuring wing length.

Using photographs of each bird, we scored all birds for the presence of eye-arcs (0 = strong eye-arcs; 0.5 = faint or incomplete eye-arcs; 1 = no eye-arcs; Fig. 2).

DNA analysis

Blood samples for genetic analysis were stored in Queen's Lysis buffer (Seutin et al. 1991). We extracted DNA from blood samples using a standard phenol-chloroform procedure. We genotyped 69 out 73 samples (in the remaining four, extraction quality was insufficient) at both a mitochondrial marker (the COI gene) and a nuclear marker (the CHD1Z gene), as described below.

Mitochondrial DNA

We downloaded partial mitochondrial sequences of both species from Genbank (accession numbers AY650204.1 and AY650206.1; Lovette and Hochachka 2006), and used NEBcutter (available online at http://tools.neb.com) to design a PCR-RFLP assay (Avise 2004) in a region of the

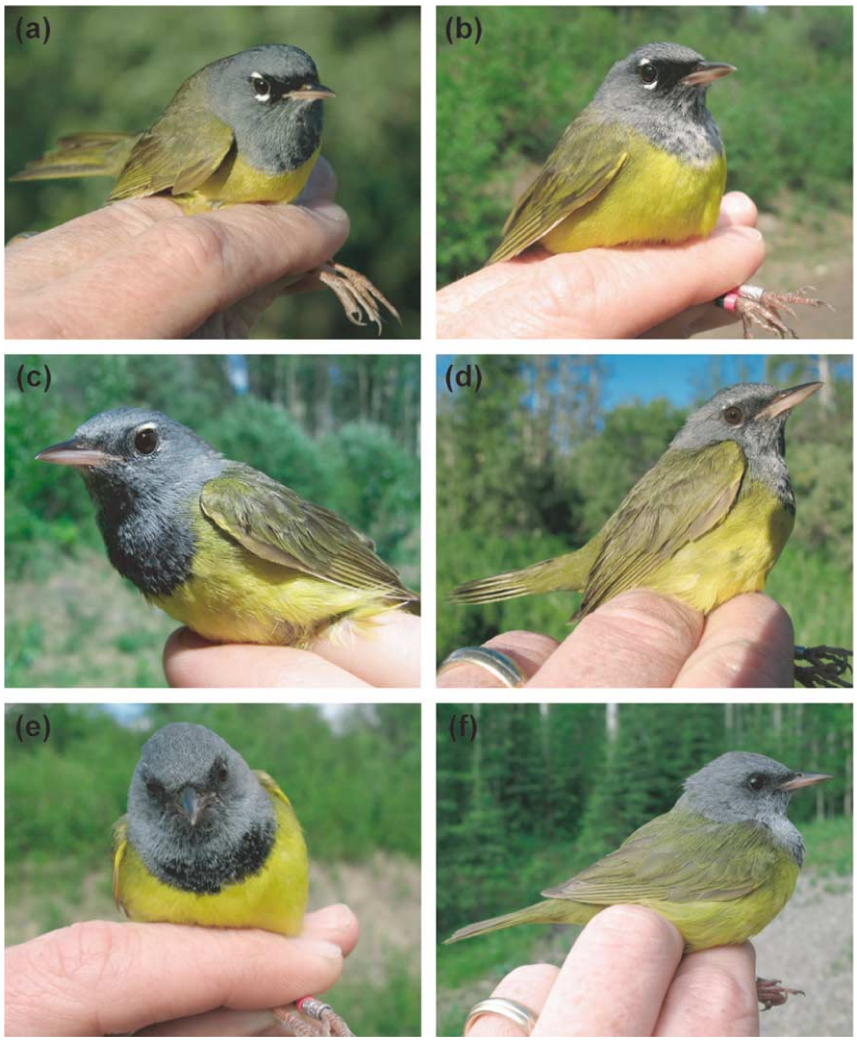

Figure 2. Six male MacGillivray's/mourning warblers from the contact zone between the two taxa, chosen to illustrate variation in eye-arcs. Complete eye-arcs (eye-arc score = 0) are displayed by (a) and (b), partial eye-arcs (score = 0.5) are displayed by (c) and (d), and lack of eye-arcs (score = 1) are displayed by (e) and (f). Each of these individuals had a combination of western and eastern molecular markers. Identity and genotypes (COI/CHD1Z) of these individuals (see Appendix 1) is as follows: (a) FF07D01 (E/W); (b) GF06D04 (E/W); (c) GF13T02 (W/E); (d) HF25D01 (E/H); (e) GF09D01 (E/H); and (f) GF13D04 (E/H).

mitochondrial genome for which primers and appropriate restriction enzymes were readily available. For all sampled warblers, we amplified a 681 base pair region of the mitochondrial COI gene, using the primers COIa-3′ (AGT ATAAGCGTCTGGGTAGTC) and COIf-5′ (CCTGC AGGAGGAGGAGAYCC) (Kessing et al. 1989). The Genbank sequences of the two species differ by 12 substitutions in this region, or 1.8% sequence divergence. Our PCR-RFLP assay genotypes one single-nucleotide polymorphism (SNP) 204 bp from the 5′ end of the fragment. This C-to-T substitution in the mourning warbler lineage generates a cut site for the restriction enzyme *Sac*I. Directionality of this substitution was inferred by comparison to sequences of other warbler species. A second *Sac*I cut site is present in both species at base pair 360. PCR reactions included 1 × PCR buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 0.2 mM dNTP mix (New England Biolabs), 0.5 μM forward and reverse primer, 0.04 units/μl Taq DNA polymerase (New England Biolabs), and 0.025 ng/μl genomic template DNA, in a total volume of 10 μl. The thermal cycling profile was 3 min at 94° C followed by 35 cycles of 30 s at 94° C, 30 s at 58° C, and 45 s at 72° C, ending with 5 min at 72° C. We then digested 2 μl of the PCR product with 2 units of the restriction enzyme *Sac*I in its appropriate buffer (New England Biolab.s), in a total volume of 6 μl. Products were digested for 2 h at 37° C, and digested DNA was visualized by electrophoresis on 2% agarose gel stained with SYBRSafe (Invitrogen). This digestion cuts the MacGillivray's PCR product into two fragments (321 and 360 bp) and the mourning product into three (156, 204, and 321 bp), thus the RFLP profile of each individual could be easily scored by eye.

Z-linked nuclear DNA

We screened an intron in the CHD1Z gene (a gene that is also used for molecular sexing of birds; Fridolfsson and Ellegren 1999) for species-informative variation by sequencing twelve males (i.e. 24 alleles, since there are two copies

in each male), six from each taxon sampled in allopatry (see Appendix 1 for identity of the samples that were sequenced). PCR reactions for sequencing used the recipe described above for COI, substituting the primer pair 2550F (GTTACTGATTCGTCTACGAGA) and 2718R (ATT GAAATGATCCAGTGCTTG, Fridolfsson and Ellegren 1999) for the COI primers, and with a reaction volume of 30 μl. We used the thermal cycling program of Fridolfsson and Ellegren (1999). Amplified fragments were sequenced bidirectionally by Macrogen, Inc., using an ABI 3730XL sequencer.

Sequences from the 12 males revealed two single nucleotide polymorphisms (SNPs) out of the 654 bp sequence of the intron and its flanking region (including the primer binding sites). The first SNP was a T/G polymorphism located 301 bp from the 5′ end of the fragment; 11 of 12 individuals sequenced were homozygous for the G variant, with one individual (a mourning warbler, GF02D02) being heterozygous for the T/G polymorphism. This SNP would obviously not be useful in distinguishing the taxa.

The second SNP was much more informative. This G/C polymorphism, located 481 bp from the 5′ end of the fragment, was nearly diagnostic of species within the 12 individuals sequenced: all six MacGillivray's warbler samples were homozygous for C in this position, and five of six mourning warblers were homozygous for G in this position, with the remaining mourning warbler (sample GF01D03) being heterozygous (C/G). For the remainder of the paper, we refer to these variants as ''MacGillivray's CHD1Z'' and ''mourning CHD1Z,'' while recognizing that each variant may occur at low frequency in the other taxon. The MacGillivray's CHD1Z (with C at this SNP) contains a cut site for the restriction enzyme *Tsp*45I, whereas the mourning CHD1Z (with G at this SNP) does not; hence we were able to use this enzyme for PCR-RFLP genotyping. PCR reactions for genotyping were identical to those for sequencing, but run at 10 μl volume. Restriction digests and visualization were carried out using the methods described above for mitochondrial DNA, with the following changes: the restriction enzyme used for CHD1Z was *Tsp*45I (New England Biolab.s), 0.66 units of enzyme were used in each reaction, and the incubation temperature was 65° C. *Tsp*45I cuts the MacGillivray's PCR product into two fragments (178 and 476 bp), but leaves the mourning product intact. An individual carrying two copies of the MacGillivray's CHD1Z sequence shows two bands on the gel, while an individual carrying two copies of the mourning sequence shows one band. Heterozygous individuals produce both cut and intact PCR products, generating a three-band (178, 476, and 654 bp) RFLP profile.

Sequences of the three types of CHD1Z intron (612 bp, without primer sequences of 21 b.p. on each end) have been deposited in Genbank (accession numbers GQ368702-GQ368704).

Cline analyses

A rich body of theory has been developed regarding contact zones that may involve hybridization (Barton and Hewitt 1989, Barton and Gale 1993, Gay et al. 2008). Many types of contact zones can be described well by a sigmoid curve depicting the expected frequency of binary traits or the expected mean value of quantitative traits across the contact zone. The parameters of the best-fitting sigmoid curve can then be used to estimate the location (i.e. the center) and width (defined as the inverse of the maximum slope) of the zone. Estimates of cline location and width can then be compared for different types of traits (e.g., Gay et al. 2008).

To generate a single geographic dimension to appropriately represent location across the contact zone, we measured the geographic distance from each bird's sampling location to a line representing the crest of the Rocky Mountains, which run northwest to southeast through western Canada (see Fig. 1 for the exact line used to represent the ''crest of the Rockies;'' this line was intended to capture the path of the mountains over a broad scale, ignoring small-scale topography). Measuring location in this way seemed appropriate given that the eastern range limit of MacGillivray's warbler and the western range limit of mourning warbler are each oriented roughly parallel to the Rocky Mountains (Fig. 1a). Note that use of this method does not assume that the contact zone is centered on the crest of the Rocky Mountains (in fact, it is offset to the east; see Results), but rather just that it is oriented roughly in parallel with the mountains.

To obtain a rough estimate of the location and width of the contact zone between MacGillivray's and mourning warblers, we used the program CFit-7 (Gay et al. 2008, Lenormand and Gay 2008) to fit sigmoid curves to each of the four traits under examination (COI, CHD1Z, eye-arcs, and wing minus tail), using a maximum likelihood approach. COI and CHD1Z were treated as simple two-allele systems, with each individual carrying a single COI allele (i.e. haploid) and two CHD1Z alleles (i.e. diploid, since all individuals were male). To convert eye-arc to a two-allele system, we recoded the eye-arc score as a diploid genotype, with eye-arc score 0 becoming the diploid genotype 0/0, eye-arc score 0.5 becoming 0/1, and eye-arc score 1 becoming 1/1. We note that this scoring system for eye-arc may be a poor approximation of reality, since we do not know the inheritance patterns for this trait. We use it here with caution, only to provide an approximate estimate of cline shape in this phenotypic trait. Wing minus tail was treated as a quantitative trait; for such traits, CFit allows trait variance to vary across the zone, in accordance with the expectation that trait variance is higher in contact zones than outside of contact zones. For wing minus tail, we ran CFit using a unimodal model (appropriate given the phenotypic distribution in the zone; see Results) and seven parameters to be fitted: cline center, slope, minimum trait value, overall trait difference, and variances of three modes (Lenormand and Gay 2008).

To determine whether cline location or slope differed significantly between the four traits, we used CFit to determine the single best-fitting curve for all traits together and used a likelihood ratio test (Gay et al. 2008; Whitlock and Schluter 2009) to compare the likelihood of the model in which all clines vary independently with the model in which a single cline is fit to all traits. We also calculated AIC (Akaike information criterion; Akaike 1974) values for each of these models; the lowest AIC value indicates the best fit to the data without having too many parameters. Runs

were repeated using different random seeds to ensure that there was convergence on similar values.

While the above analysis included each of the traits, we suggest that the most accurate estimate of the location and width of the contact zone may be provided by the two molecular markers (COI and CHD1Z), since they are close to fixation for different alleles in allopatric areas and their inheritance pattern is known. Hence, we used CFit to determine whether the two markers differed significantly in their clines and to produce a single best-fitting cline for the two markers.

We note that our estimated widths of the contact zone are likely overestimates, as our analyses included samples from a broad area across the hybrid zone (Fig. 1) and assumed that the zone is oriented in parallel with the crest of the Rockies; the width of the hybrid zone across a single transect might be substantially narrower. Our sample sizes and distribution are presently insufficient to test this possibility.

All statistical analyses aside from those provided by CFit were performed using *R* (R Development Core Team 2006).

Results

Range contact

Because eye-arc is the most obvious plumage trait that tends to differ between MacGillivray's and mourning warblers, we start by describing the geographic distribution of eye-arcs in our study area (Fig. 1 and 2). Strong eye-arcs occurred with a frequency of 100% in all samples west of the Rocky Mountains (n = 12). Far east of the Rockies, in central Alberta, eye-arcs were observed rarely (n = 7; 5 birds had no eye-arcs, and 2 had partial eye-arcs). We located a relatively narrow region in the eastern foothills of the Rocky Mountains, in the Peace Region of British Columbia, where birds with strong eye-arcs (n = 34), with partial or weak eye-arcs (n = 11), and without eye-arcs (n = 9) were each common.

Morphometric variation (Table 1, Fig. 3) shows a similar pattern as eye-arc, with three traits (tail length, beak depth, and beak width) differing significantly between birds from west of the Rockies (i.e. allopatric MacGillivray's warblers) and samples from central Alberta (i.e. allopatric mourning warblers). Sample differences in two other traits (wing length and beak length) approach statistical significance (Table 1). The sixth trait, tarsus length, is similar between the two taxa. Perhaps the most noticeable difference is in tail length, which is on average 3.4 mm longer in MacGillivray's than in mourning warblers. Most other traits (all except tarsus length) showed the opposite pattern, being slightly larger in mourning warblers. For example, wing length is on average 1.4 mm longer in our sample of mourning warblers compared to our sample of MacGillivray's warblers. Given these opposing patterns in different traits, we decided to focus on a composite variable, wing minus tail (Lanyon and Bull 1967, Pitocchelli 1990, 1992), for estimating the morphometric cline.

Both eye-arcs and wing minus tail are strongly associated with geographic location, as shown by Fig. 4. Generally, wing minus tail is low where eye-arcs are common, west of the Rockies, and high where eye-arcs are rare, in central Alberta. In the eastern foothills of the Rockies, near the towns of Chetwynd and Tumbler Ridge (Fig. 1), there is much variation in eye-arc and in wing minus tail (as well as other morphometric traits; Fig. 3).

Both the mitochondrial DNA (i.e. the COI gene) and the Z-chromosome marker (the CHD1Z gene) show a pattern similar to that of morphological variation (Fig. 4). For COI, the western type occurs in all of our genotyped samples west of the Rockies (n = 12), an eastern type occurs in all samples in central Alberta (n = 7), and a mixture of haplotypes is found within the contact zone in northeastern British Columbia (n = 50, of which 30 have the western COI and 20 have the eastern COI; Fig. 4, Table 2). For CHD1Z, all genotyped samples from west of Rockies were homozygous for the western allele (n = 24 alleles, or 12 individuals), samples from central Alberta were predominately homozygous for the eastern allele (n = 14 alleles, of which 13 were the eastern allele and 1 was the western). In the contact zone, 28 individuals were homozygous for the western allele, 11 were homozygous for the eastern allele, and 11 were heterozygous (for a total of 67 western CHD1Z alleles and 33 eastern alleles in the contact zone; Fig. 4, Table 2).

Thus all examined traits show a concordant pattern of a general difference between allopatric western and eastern birds, with an area of overlap near the towns of Chetwynd and Tumbler Ridge in northeastern British Columbia. Given this parallel overlap, we can ask whether the patterns seen in the overlap zone are consistent with: (1) complete reproductive isolation, or (2) some degree of hybridization.

Hybridization

Many birds in the overlap zone have sets of traits that were not observed west or east of the contact zone. This can be seen most clearly in the molecular traits. West of the Rockies, all genotyped samples (n = 12) had the western

Table 1. Morphometric trait means (± standard deviations) of allopatric MacGillivray's warblers (those from west of the Rocky Mountains; n = 12) and allopatric mourning warblers (those from central Alberta; n = 7), along with statistical tests (Welch two-sample t-tests) of whether their means differ. All traits are measured in units of millimeters.

	MacGillivray's warbler	Mourning warbler	Test of diff. in means
Wing length	59.5 (± 1.6)	60.9 (± 1.4)	$t = -1.97$, df = 13.7, $P = 0.069$
Tail length	54.0 (± 2.4)	50.6 (± 1.1)	$t = 4.31$, df = 16.4, $P < 0.001$
Tarsus length	20.7 (± 0.5)	20.7 (± 1.0)	$t = -0.17$, df = 7.6, $P = 0.867$
Beak length	7.6 (± 0.3)	7.9 (± 0.3)	$t = -1.93$, df = 14.5, $P = 0.074$
Beak depth	3.2 (± 0.1)	3.4 (± 0.1)	$t = -5.43$, df = 11.4, $P < 0.001$
Beak width	3.0 (± 0.2)	3.2 (± 0.2)	$t = -2.63$, df = 14.5, $P = 0.019$

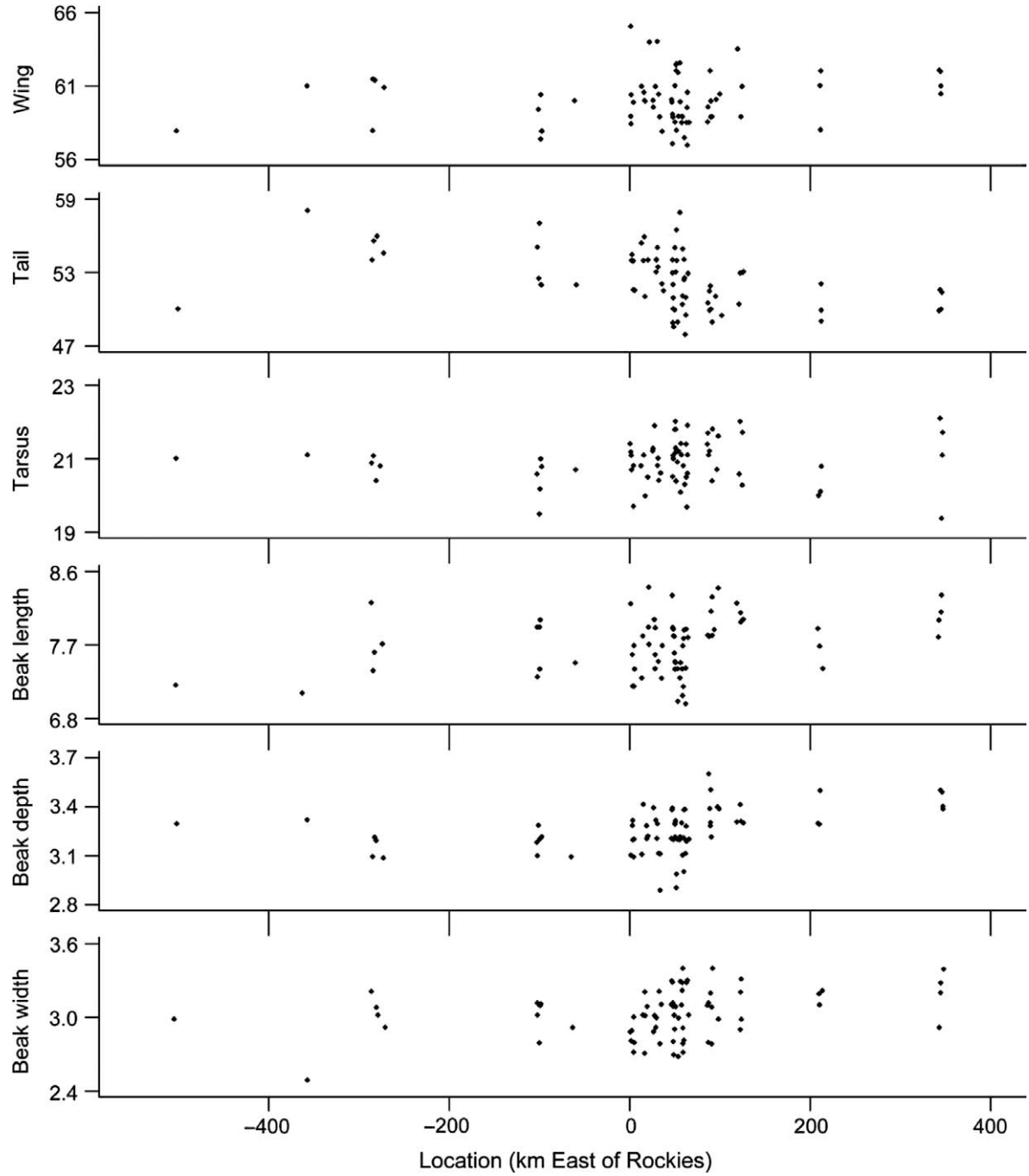

Figure 3. Variation among MacGillivray's and/or mourning warblers in six morphometric traits, in relation to location. For each trait, the location (with respect to the crest of the Rockies; Fig. 1) and trait value of each bird is indicated with a small diamond. All traits are measured in units of millimeters.

mitochondrion and were homozygous for the western CHD1Z. In central Alberta, all seven of our genotyped birds had the eastern mitochondrion; six were homozygous for the eastern CHD1Z, and one was heterozygous for CHD1Z (i.e. one western allele and one eastern). In the contact zone, defined broadly here as the Peace Region of northeastern British Columbia, apparent recombinant types (defined as a mixture of western and eastern alleles) were common, occurring in 18 out of 50 individuals (Table 2). The difference in frequency of such putative recombinants between the contact zone and outside of the contact zone (one out of 19) is statistically significant ($\chi^2_1 = 5.1$, $P = 0.024$), providing clear evidence of hybridization.

Considering other traits together with the molecular traits, further novel combinations can be seen in the contact zone (Fig. 4). For example, 10 individuals with the eastern mitochondrion have wing minus tail values lower than allopatric eastern birds. Likewise, six individuals with western mtDNA have wing minus tail values far higher than allopatric western birds. There are also six cases of birds with eastern mitochondria that have strong eye-arcs, and two cases of birds with western mitochondria that

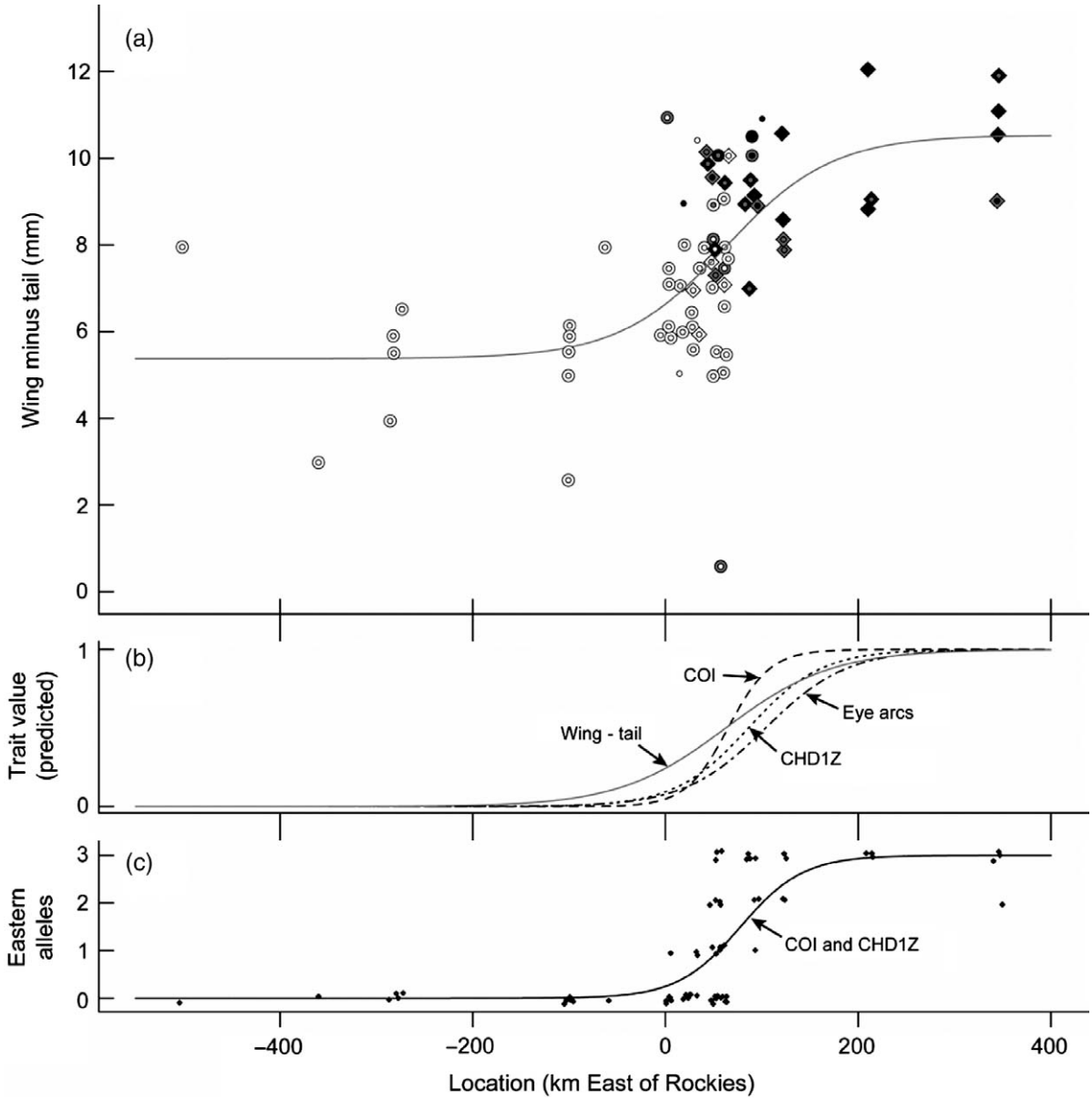

Figure 4. Relationship between breeding location and four distinct traits of MacGillivray's and mourning warblers, along with the best-fitting sigmoid clines as estimated by CFit-7 (Gay et al. 2008, Lenormand and Gay 2008). (a) Each bird is plotted according to its sampling location in relation to the crest of the Rocky Mountains (in km; see Fig. 1) and its wing length minus tail length (Fig. 3, Appendix 1). Symbols indicate eye-arc score and genotype as follows: color of small circles corresponds to complete eye-arcs (white), partial or faint eye-arcs (grey), or no eye-arcs (black). For samples that were genotyped, mitochondrial clade is indicated by large circles (western clade) or large diamonds (eastern clade), and CHD1Z genotype is indicated by color within these large symbols: white indicates homozygous western, black indicates homozygous eastern, and grey indicates heterozygous CHD1Z. The positions of some symbols were moved slightly to avoid overlap. The gray line shows the best-fitting cline for wing minus tail. (b) Best-fitting clines for each trait, standardized so that zero represents MacGillivray's warbler and one represents mourning warbler. (c) The number of eastern alleles of each bird (out of one COI allele and two CHD1Z alleles; a small amount of random jitter was added to avoid overlap of symbols), along with the best-fitting cline for the two molecular markers together. Each of the four traits is strongly associated with geographic location, with a narrow transition region just east of the crest of the Rocky Mountains.

completely lack eye-arcs. Birds with apparent hybrid genotypes can appear very much like either of the two pure forms (Fig. 2).

Morphometric variation shows a pattern of intermediacy in the contact zone that is consistent with hybridization (Fig. 3–5). For example, we observed a unimodal distribution of wing minus tail in the contact zone, with the peak centered between the two allopatric distributions (Fig. 5).

Hybrid zone location and width

Estimated centers and widths of the clines fitted separately to each type of data (COI, CHD1Z, eye-arcs, and wing minus tail) as well as to various combinations of data are provided in Table 3 and Fig. 4b. Estimates based on separate clines for each marker range from 62 to 104 km east of the crest of the Rockies for cline center and from

Table 2. Numbers of individuals with particular COI and CHD1Z genotype combinations in the contact zone. "W" refers to western (MacGillivray's warbler) alleles, and "E" to eastern (mourning warbler) alleles.

		CHD1Z genotypes:		
		WW	WE	EE
COI haplogroup:	W	23	5	2
	E	5	6	9

87 to 221 km for cline width. A model that allows each trait to have its own cline (log-likelihood = −284.3, parameters = 13, AIC = 594.5) does not provide a significantly better fit to the data than a model in which a single cline is fit to variation in all four traits (log-likelihood = −289.0, parameters = 7, AIC = 592.0; likelihood ratio test: $\chi^2 = 9.51$, df = 6, P = 0.15). Hence the different traits do not differ significantly in their estimated clines. In the cline fit to all four traits, cline center is 86 km east of the crest of the Rockies, and cline width is 150 km.

The most accurate estimate of contact zone location and width may be provided by the two molecular markers, since the morphological traits have unknown patterns of inheritance and may be subject to phenotypic plasticity. The two markers do not differ significantly in their clines (one-cline model: log-likelihood = −84.9, parameters = 2, AIC = 173.7; two-cline model: log-likelihood = −83.4, parameters = 4, AIC = 174.8; likelihood ratio test: $\chi^2 = 2.87$, df = 2, P = 0.24). The cline based solely on these two molecular markers is centered at 78 km east of the crest of the Rockies and has a width of 130 km.

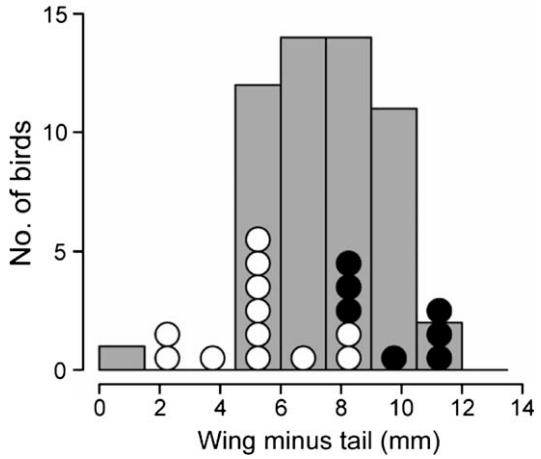

Figure 5. Histogram showing the generally unimodal distribution of wing minus tail in the contact zone (grey bars), defined here as the Peace Region of British Columbia, as well as distributions west (white circles) and east (black circles) of this region. The distribution of wing minus tail between the three regions differs significantly (ANOVA: $F_{2,70} = 15.2$, P < 0.00001), with western and eastern birds having low and high values of wing minus tail, respectively (Welch two-sample t-test: t = 6.8, df = 15.0, P < 0.001), and the contact zone being intermediate (west versus contact: t = 4.0, df = 18.1, P < 0.001; east versus contact: t = 4.5, df = 9.5, P < 0.01).

Discussion

Our results strongly indicate that: (1) MacGillivray's and mourning warblers come into substantial range contact within the Peace Region of British Columbia, and (2) the two species hybridize extensively in this contact zone. We have shown that there tend to be distinct western and eastern types of body shapes (e.g. wing minus tail), plumage (eye-arcs), mitochondrial DNA, and a Z-chromosome marker (CHD1Z), and that both types of each trait can be found in high frequency in the Peace Region. We have further found a high frequency of individual birds that display combinations of western and eastern traits in this region. Indeed, many of the birds in our sample simply cannot be assigned to either species because they have such a mixture of traits typically associated with both MacGillivray's and mourning warblers.

The presence of such an extensive hybrid zone is somewhat surprising, given the rarity of earlier reports of hybridization (Taverner 1919, Cox 1973, Hall 1979), and the doubting of those reports based on further analysis (Hall 1979, Pitocchelli 1990, 1993, Dunn and Garrett 1997). There are four reasons why such extensive hybridization was not noted earlier. First, earlier investigations of potential range contact between MacGillivray's and mourning warblers (Cox 1973, Pitocchelli 1990, 1992) have focused primarily on southwestern Alberta, where contact between these two species is apparently highly limited due to the extensive and apparently unsuitable lodgepole pine *Pinus contorta* forests separating them along the eastern foothills of the Rocky Mountains (Pitocchelli 1993). The Peace Region of British Columbia, in contrast, has received comparatively little research attention from ornithologists, and apparently has more continuous habitat for the two taxa. Second, although birds are relatively dense in the hybrid zone, the zone is quite narrow in width, making it unlikely for ornithologists to notice it. Third, the two taxa differ little in plumage traits. There is essentially only a single plumage trait (the eye-arc) that can be easily scored; other plumage differences between the taxa, such as the darkness of the lores or the presence of a dark "bib" on the breast (Pitocchelli 1993, 1995), are difficult to discern under different lighting conditions and to score repeatably. Given this single easily diagnosable plumage trait, eye-arc, and the presence of this trait in low frequency within mourning warblers across their entire range (Pitocchelli 1990), it is difficult if not impossible to confidently identify potential hybrids using plumage alone. Here, our use of two molecular markers in combination with plumage and morphometric traits gives us more confidence in our ability

Table 3. Best-fitting cline centers and widths determined for each trait individually and for combinations of traits, as determined using CFit-7 (Gay et al. 2008, Lenormand and Gay 2008).

Traits used in cline	Center (km)	Width (km)
COI only	64.9	87.5
CHD1Z only	86.1	151.7
Eye-arcs only	103.9	168.7
Wing minus tail only	62.4	220.8
All traits	86.1	150.1
COI, CHD1Z only	77.8	130.0

to identify hybrid individuals. Fourth, it is possible that the hybrid zone has formed quite recently. Mourning warblers were not known from northeastern BC during the first half of the 20th century, despite moderately extensive bird surveys (McTaggart-Cowan 1939, Munro and McTaggart-Cowan 1947, Campbell et al. 2001). In contrast, during the spring of 1974, Erskine and Davidson (1976) frequently encountered the species in northeastern BC (including the Peace Region). They noted, ''The relative status of these species (mourning and MacGillivray's warblers) in northeastern British Columbia needs further study.'' These observations suggest that mourning warblers may have spread into the Peace Region of BC relatively recently, bringing them into contact with MacGillivray's warblers. This spread may have been facilitated by forestry and other development in this region, as areas that are clearcut subsequently produce the shrubby deciduous habitat that the two taxa prefer.

Given the extensive hybridization between MacGillivray's and mourning warblers, the question arises as to whether they should continue to be treated as separate species. We advise caution regarding potential taxonomic changes based on the current evidence. On the one hand, extensive hybridization demonstrates that two groups can and do interbreed, potentially challenging their treatment as separate species. On the other hand, the zone of hybridization is reasonably narrow (roughly 130 km based on the two molecular markers) compared to the range size of the two taxa, which together span west to east across the whole of Canada. We have little evidence that the influence of hybridization extends beyond the contact zone itself. Milá et al. (2000) found very little variation in a sample of 116 mitochondria (cytochrome *b* sequence) from MacGillivray's warblers throughout the western USA and Alaska, and Pitocchelli (1990, 1992) found that plumage and morphometric variation was relatively constant across virtually the entire range of mourning warblers (neither study included samples from the southern Peace Region of British Columbia). Based on mitochondrial cytochrome *b* divergence, Weir and Schluter (2004) estimated that MacGillivray's and mourning warblers began diverging roughly 0.9–1.5 million y ago, a moderate amount of divergence compared to other pairs of boreal taxa that are considered distinct species (Weir and Schluter 2004). Narrowness of a hybrid zone can indicate that selection is maintaining the discreteness of the two taxa despite extensive hybridization, through intrinsic selection against hybrids or through a gradient in habitat and differential adaptation of the two taxa to habitat (Barton and Hewitt 1989). A full analysis of whether selection is maintaining the shape and narrowness of this hybrid zone is beyond the scope of this paper, and will require larger samples sizes and additional molecular studies. However, we note here that it appears that the amount of variation within traits is quite high in the center of the hybrid zone, a pattern that is consistent with some form of selection maintaining the narrowness of the zone (Barton and Gale 1993). The close concordance of the clines of morphometric traits (e.g. wing-tail), plumage traits (eye-arcs), mitochondrial DNA, and the Z-chromosome marker is also consistent with selection maintaining the zone. If selection against hybrids is occurring, the two taxa might maintain their distinctiveness and be free to diverge further despite extensive hybridization.

One trait that is possibly important in contributing to reproductive isolation between incipient bird species is song (Irwin et al. 2001a,b; Price 2008). Pitocchelli's (1990) analysis of song variation from allopatric MacGillivray's and mourning warblers revealed good separation of the two taxa in multivariate space, suggesting that song might be a useful distinguishing feature in an area of contact between these taxa. A full analysis of song variation is beyond the scope of this paper, but we note here that we have not noticed any differences between the taxa in the contact area. One of us (M.P.) has been observing and recording songs of the two taxa within the southern Peace Region for many years, and has come to the conclusion that song cannot be used reliably to predict the appearance of a MacGillivray's/mourning warbler in that region. This apparent similarity of songs in sympatry, in contrast to the difference in allopatric songs observed by Pitocchelli (1990), suggests that songs may have undergone a process of blending in the contact zone, due to cultural and/or genetic exchange between the taxa. Work is ongoing to investigate this possibility.

This study increases from three to four the number of known hybrid zones between well-differentiated wood-warbler (Parulidae) taxa in North America. These include hybrid zones between blue-winged and golden-winged warblers *Vermivora pinus* and *V. chrysoptera* (Parkes 1951, Vallender et al. 2007), hermit and Townsend's warblers *Dendroica occidentalis* and *D. townsendi* (Rohwer and Wood 1998, Rohwer and Martin 2007), and Audubon's and myrtle warblers *Dendroica coronata auduboni* and *D. c. coronata*, classified since 1973 as one species, the yellow-rumped warbler (Hubbard 1969, AOU 1973, Barrowclough 1980). In addition to these cases of extensive hybrid zones, there have been many other cases of rare hybridization between species of wood warblers (McCarthy 2006). It is becoming clear that hybridization between divergent forms is a common and perhaps creative phenomenon in evolution (Grant and Grant 1992, Arnold 1997, Price 2008), and may occur often during the divergence of a single species into two (Price 2008).

There has been much interest and controversy regarding the causes and timing of speciation (Klicka and Zink 1997, Weir and Schluter 2004, Lovette 2005, Price 2008), as well as the existence and location of suture zones (Remington 1968, Swenson and Howard 2004) in North American birds. These analyses rely on accurate information regarding species boundaries and patterns of hybridization. We have shown here that two taxa previously considered full species in fact hybridize extensively where they meet. An example of the inverse situation was also observed in the Peace Region: the western and eastern forms of the winter wren (*Troglodytes troglodytes pacificus* and *T. t. hiemalis*), which had been considered the same species, were recently shown to coexist in that region without interbreeding (Toews and Irwin 2008), suggesting that they are in fact separate species that have been evolving independently for several million years. Many other western and eastern pairs of related taxa

come into contact in the Peace Region, yet few have been studied closely. These findings suggest that this unique region may contain many more surprises regarding the interactions between western and eastern forms.

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Appendix 1. A list of each sampled bird's record number (record no.), date sampled location (lat., long., and distance from crest of Rocky Mountains), eye-arc score (0 for strong eye-arc, 0.5 for faint or partial eye-arc, 1 for no eye-arc), COI haplogroup (W for western, E for eastern), CHD1Z genotype (W for homozygous western, E for homozygous eastern, and H for heterozygote; asterisks indicate samples for which full sequences were obtained), and wing minus tail measurement. A few samples were not genotyped (indicated with dashes), due to lack of a blood sample or poor extraction. Samples are arranged in order of their distance from the Rockies, with negative values being on the western side and positive values on the eastern side.

Record number	Date sampled	Lat. N (°)	Long. W (°)	Dist. from Rockies (km)	Eye-arc score	COI	CHD1Z	Wing-tail (mm)
GF20D03	20/6/07	49.73	123.06	−502	0	W	W*	8.0
FF13D03	13/6/06	49.31	119.79	−360	0	W	W*	3.0
GF15A12	15/6/07	57.44	130.24	−285	0	W	W*	4.0
FF16D03	16/6/06	49.09	118.28	−282	0	W	W*	6.0
FF16D04	16/6/06	49.08	118.27	−282	0	W	W*	5.5
FF17D01	17/6/06	49.06	118.21	−274	0	W	W*	6.5
HF30D01	30/6/08	53.85	122.35	−100	0	W	W	6.0
HF30D03	30/6/08	53.85	122.35	−100	0	W	W	6.0
HF30D04	30/6/08	53.85	122.35	−100	0	W	W	5.5
HF30D05	30/6/08	53.85	122.35	−100	0	W	W	5.0
HF30D06	30/6/08	53.85	122.35	−100	0	W	W	2.5
GE21A10	21/5/07	51.44	116.98	−62	0	W	W	8.0
GF07A07	07/6/07	55.52	122.74	2	0	W	H	11.0
GF08D02	08/6/07	55.54	122.75	3	0	W	W	7.5
HF19D01	19/6/08	55.54	122.75	3	0	W	W	7.0
HF26D01	26/6/08	55.53	122.74	3	0	W	W	6.0
HF26D03	26/6/08	55.53	122.74	3	0	W	W	6.0
GF08T02	08/6/07	55.52	122.73	4	0	W	W	6.0
FE19D02	19/5/06	54.86	121.24	15	0	W	W	7.0
FE21R04	21/5/06	54.86	121.24	15	0	–	–	5.0
FE29D04	29/5/06	54.90	121.22	19	0	W	W	8.0
FF06D01	06/6/06	54.89	121.23	19	1	–	–	9.0
FF06D02	06/6/06	54.90	121.23	19	0	W	W	6.0
GE25D02	25/5/07	54.95	121.15	28	0	E	W	7.0
HF29D01	29/6/08	55.63	122.36	28	0	W	W	6.5
HF29D02	29/6/08	55.63	122.36	28	0	W	W	6.0
FF06D03	06/6/06	54.96	121.13	29	0	W	W	5.5
FF07D01	07/6/06	55.01	121.03	33	0	E	W	6.0
FF07D03	07/6/06	54.97	121.06	33	0	–	–	10.5
GE25D01	25/5/07	54.97	121.06	35	0	W	W	7.5
GF13D01	13/6/07	55.62	121.88	49	0	W	W	7.0
GF13D02	13/6/07	55.62	121.88	49	0	E	W	7.5
GF13D03	13/6/07	55.62	121.88	49	0.5	E	E	10.0
GF13D04	13/6/07	55.63	121.88	49	1	E	H	9.5
GF13D07	13/6/07	55.63	121.88	49	0	W	H	8.0
HF21D04	21/6/08	55.61	121.88	49	0.5	E	H	10.0
HF23D01	23/6/08	55.64	121.91	49	0.5	W	W	9.0
HF23D03	23/6/08	55.63	121.91	49	0	E	E	8.0
HF23D07	23/6/08	55.63	121.91	49	0	W	W	8.0
GE29D01	29/5/07	55.15	120.93	54	0	W	W	5.5
GF13T02	13/6/07	55.65	121.84	54	0.5	W	E	10.0
HF27D01	27/6/08	55.65	121.84	55	0	W	W	8.0
HF28D01	28/6/08	55.78	121.97	57	0	W	H	0.5
HF28D03	28/6/08	55.78	121.96	57	0.5	E	H	7.5
HF28D04	28/6/08	55.77	121.94	58	0	W	H	7.5
GF06D04	06/6/07	55.69	121.77	61	0	E	W	10.0
GF10D06	10/6/07	55.69	121.77	61	0	W	W	6.5
GF11D01	11/6/07	55.70	121.79	61	0	W	W	7.5
GF11D02	11/6/07	55.71	121.80	61	0	W	W	5.0
GF11D03	11/6/07	55.71	121.80	61	0.5	E	E	9.5
HF20D01	20/6/08	55.67	121.74	61	0	E	W	7.0
HF21D01	21/6/08	55.69	121.75	61	0	W	W	9.0
GF11D04	11/6/07	55.75	121.81	63	0	W	W	5.5
HF24D06	24/6/08	55.77	121.30	88	0.5	E	E	7.0
HF21D06	21/6/08	55.85	121.41	89	0.5	E	E	9.0
HF22D01	22/6/08	55.84	121.41	89	1	W	E	10.5
HF24D03	24/6/08	55.78	121.30	89	0.5	E	E	9.5
HF24D04	24/6/08	55.78	121.30	89	1	W	H	10.0
HF24D01	24/6/08	55.80	121.30	90	1	E	E	9.0
GF09D01	09/6/07	58.72	123.69	95	1	E	H	9.0
GF05D01	05/6/07	55.62	120.79	100	1	–	–	11.0
FF05D01	05/6/06	55.66	120.38	121	1	E	E*	10.5
FF05D03	05/6/06	55.68	120.36	123	1	E	E*	8.5
HF25D01	25/6/08	55.67	120.37	123	0.5	E	H	8.0
HF25D02	25/6/08	55.68	120.37	123	0.5	E	H	8.0
GF03D01	03/6/07	54.21	115.46	211	1	E	E	12.0

Appendix 1 (*Continued*)

Record number	Date sampled	Lat. N (°)	Long. W (°)	Dist. from Rockies (km)	Eye-arc score	COI	CHD1Z	Wing-tail (mm)
GF03D04	03/6/07	54.21	115.49	211	1	E	E	9.0
GF03D06	03/6/07	54.21	115.46	211	0.5	E	E	9.0
GF01D02	01/6/07	55.41	114.76	345	0.5	E	E*	12.0
GF01D03	01/6/07	55.42	114.75	345	1	E	H*	9.0
GF02D02	02/6/07	55.39	114.75	345	1	E	E*	11.0
GF02D03	02/6/07	55.42	114.74	345	1	E	E*	10.5

*Indicates samples that were sequenced for CHD1Z rather than simply genotyped by PCR-RFLP.