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Cryptic speciation in a Holarctic passerine revealed by genetic and bioacoustic analyses

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Abstract

There has been much controversy regarding the timing of speciation events in birds, and regarding the relative roles of natural and sexual selection in promoting speciation. Here, we investigate these issues using winter wrens (*Troglodytes troglodytes*), an unusual example of a passerine with a Holarctic distribution. Geographical variation has led to speculation that the western North American form *Troglodytes troglodytes pacificus* might be a distinct biological species compared to those in eastern North America (e.g. *Troglodytes troglodytes hiemalis*) and Eurasia. We located the first known area in which both forms can be found, often inhabiting neighbouring territories. Each male wren in this area sings either western or eastern song, and the differences in song are as distinct in the contact zone as they are in allopatry. The two singing types differ distinctly in mitochondrial DNA sequences and amplified fragment length polymorpism profiles. These results indicate that the two forms are reproductively isolated to a high degree where they co-occur and are therefore separate species. DNA variation suggests that the initial split between the two species occurred before the Pleistocene, quite long ago for sister species in the boreal forest. Surprisingly, the two forms are similar in morphometric traits and habitat characteristics of territories. These findings suggest that sexual selection played a larger role than habitat divergence in generating reproductive isolation, and raise the possibility that there are other such morphologically cryptic species pairs in North America.

Keywords: AFLP, cryptic species, mitochondrial DNA, pacific wren, *Troglodytes troglodytes*, winter wren

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Introduction

The relative importance of various factors in speciation is an ongoing debate (Price 1998, 2008; Schluter 2000; Coyne & Orr 2004). Ecological speciation, in which reproductive isolation evolves as a result of divergent natural selection (Schluter 2000; Rundle & Nosil 2005), is the model that is currently most discussed in the literature. An alternative is that continuous stochastic change within populations, driven by processes such as social and sexual selection, can cause reproductive isolation between geographically isolated populations that are not under divergent natural selection (West-Eberhard 1983; Price 1998, 2008). To understand patterns of biodiversity, we need to understand the relative importance of divergent natural selection and these other forces in driving speciation. One way to address this issue is to examine closely related forms that diverged in allopatry but have now come into secondary contact. We can then ask (i) how do the forms differ, that is, do they differ most in traits associated with ecology or with traits associated with social and sexual selection, and (ii) whether the forms are reproductively isolated.

It is generally considered that overlap areas between divergent avian taxa in North America represent areas of secondary contact between groups that were previously geographically isolated for extended periods of time. It has long been noted that there are large differences between the avifaunas of western and eastern North America, both in species composition and between western and eastern forms of the same species (reviewed by Newton 2003). More recently, there has been vigorous debate over the causes of these differences, with the importance of Pleistocene

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glaciations in causing population subdivision and subsequent speciation being one contentious issue (Klicka & Zink 1997; Weir & Schluter 2004; Lovette 2005). A potential challenge in resolving this debate is that many cases of cryptic speciation may not have been discovered. In some taxa, differences have been noted between West Coast and East Coast populations, but populations in intermediate locations have not been investigated to determine whether the two coastal forms represent extremes of a continuum or whether they are in fact members of distinct biological species, with a contact zone where the two co-occur without interbreeding. Here, we investigate one such group that is currently classified as a single species but exhibits notable differences between West Coast and East Coast populations.

Winter wrens (*Troglodytes troglodytes*) are an excellent system for addressing questions of biogeography, speciation, and divergence in ecologically and socially selected traits. They are noteworthy among songbirds both because of their amazingly long and complex songs (Kroodsma 1980, 2005; Kroodsma & Momose 1991; Van Horne 1995) and because they are one of the few passerine species that has a distribution spanning both North America and Eurasia (Brewer 2001; Hejl *et al*. 2002). Winter wrens display subtle geographical variation in plumage, which has led taxonomists to name more than 44 subspecies worldwide (Hejl *et al*. 2002; Kroodsma & Brewer 2005). In addition, their vocalizations display marked regional phenotypic differences. Most notably, there are large differences in song types and repertoire size between the two subspecies *Troglodytes troglodytes pacificus* at research sites in Oregon and British Columbia and the subspecies *Troglodytes troglodytes hiemalis* at sites in New York and Maine (Kroodsma 1980, 2005; Kroodsma & Momose 1991). Songs of *T. t. hiemalis* from eastern North America are more similar to songs of Eurasian forms (e.g. *Troglodytes troglodytes fumigatus* in Japan and *Troglodytes troglodytes troglodytes* in Europe) than to those of *T. t. pacificus* in western North America (Kroodsma & Momose 1991).

Variation in mitochondrial DNA (mtDNA) parallels these patterns in song (Drovetski *et al*. 2004), with the deepest divergence in the entire winter wren complex occurring between a western North America clade (i.e. *T. t. pacificus* and other western forms; see Fig. 1) and a clade representing the rest of the range (Eurasia and eastern North America, including *T. t. hiemalis*). These patterns have led to suggestions that winter wrens, which are currently treated as the single species *T. troglodytes* (e.g. Brewer 2001; Hejl *et al*. 2002; Kroodsma & Brewer 2005), may in fact consist of multiple cryptic species, with the group in western North America being specifically distinct from those in eastern North America and Eurasia (Hejl *et al*. 2002; Drovetski *et al*. 2004; Kroodsma 2005).

When considering whether two regional forms are in fact separate species, it is crucially important to gather data from an area of overlap between the groups, if such an area exists (Irwin *et al*. 2001a, b; Kroodsma 2005). Only then can it be determined whether there is (i) gradual change between the traits of the forms, suggesting gene flow between the forms, or (ii) a region of overlap between two forms with distinct differences, suggesting reproductive isolation. The second situation would provide strong evidence that the two forms are separate species under the biological species concept. Before the present study, little if any research on winter wrens had been conducted in the extensive region (spanning more than 2000 km) between eastern North America (from Ontario and Minnesota eastward) and the Pacific Coast (e.g. coastal Oregon, Washington, British Columbia, and Alaska). This lack of knowledge has led to calls for 'a careful survey of this wren's vocal behaviour from Minnesota west, especially in north Alberta

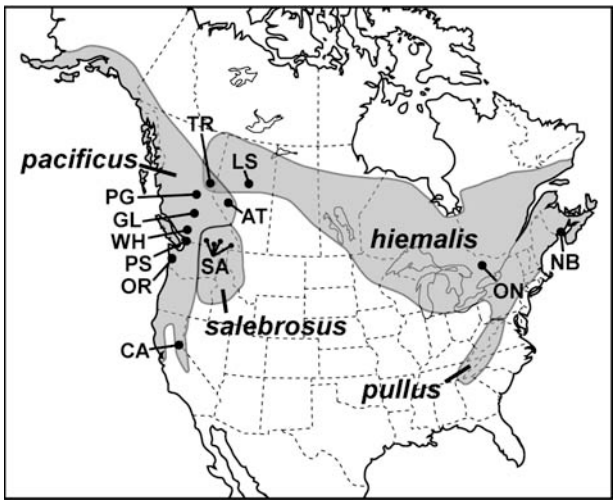

Fig. 1 Breeding distributions of winter wrens (*Troglodytes troglodytes*) in North America, along with locations of research sites. Distributions of subspecies are indicated with italicized names according to Brewer (2001) and Hejl *et al*. (2002). The western group consists of *pacificus*, *salebrosus*, and a variety of subspecies on islands off of Alaska (not shown; see Brewer 2001 and Hejl *et al*. 2002 for details; but see Kroodsma & Brewer 2005 for a differing treatment of western subspecies). The eastern group consists of *hiemalis* and *pullus*. Research sites are indicated by two-letter codes (PS, Pacific Spirit Park, Vancouver, British Columbia; WH, Whistler Interpretive Forest, Whistler, British Columbia; GL, Gavin Lake Forestry Centre, in the University of British Columbia/Alex Fraser Research Forest, north of 150 Mile House, British Columbia; PG, Prince George, British Columbia; SA, a group of four sites in southeastern British Columbia (near Penticton, Christina Lake, Nelson, and Cranbrook) that are within the area said to be occupied by *salebrosus*; AT, the Athabasca River valley, northeast of Hinton, Alberta; TR, Tumbler Ridge, British Columbia; LS, Lesser Slave Lake (Alberta). Recordings obtained from the Macauley Library came from four additional sites (OR, Corvallis, Oregon; CA, Sattley, California; ON, Algonquin Park, Ontario; and NB, Grand Manan Island, New Brunswick).

and throughout British Columbia, ... to determine how eastern and western wrens behave if they meet.' (Hejl *et al*. 2002, p. 9). Evidence from this region is essential in determining whether the two forms of winter wren are separate species (Hejl *et al*. 2002; Kroodsma 2005). We studied wrens in this region and found an area where *pacificus* and *hiemalis* come into contact, near the town of Tumbler Ridge, British Columbia, in the eastern foothills of the Rocky Mountains. We then determined (i) whether they are reproductively isolated where they meet, by analysing song characteristics, mtDNA sequences, and nuclear DNA profiles (amplified fragment length polymorphisms; Vos *et al*. 1995; Bensch & Åkesson 2005), and (ii) whether naturally selected traits (e.g. habitat use and morphometrics) or sexually selected traits (e.g. song) differ most in sympatry.

Materials and methods

Study organism

Winter wrens, alternatively referred to as northern wrens (e.g. Kroodsma & Brewer 2005) or simply wrens (in Eurasia, where they are the only species of wren, e.g. Knightley *et al*. 1998), have traditionally been classified within the genus *Troglodytes*, which contains 13 species according to Kroodsma & Brewer (2005). Recently, two studies (Rice *et al*. 1999; Gómez *et al*. 2005) have presented molecular data indicating that the *Troglodytes* genus as currently defined is not a monophyletic group. These studies have shown that winter wrens, traditionally referred to as *Troglodytes troglodytes*, are the most distantly related of all species within *Troglodytes*, and have suggested that two other groups, the timberline wren (*Thryorchilus browni*) and the four species within the genus *Cistothorus*, are within the clade defined by all of the *Troglodytes*. Thus, to make *Troglodytes* a monophyletic clade, there are two possible solutions. Winter wrens could be placed in their own genus, *Nannus*, as suggested by Rice *et al*. (1999) and Gómez *et al*. (2005). Alternatively, as suggested by Gómez *et al*. (2005), *Troglodytes* could be made more inclusive by assigning the *Troglodytes* genus to the current *Thryorchilus* and *Cistothorus* genera. We view these studies as important in that they show the current taxonomy likely needs to be changed. However, we think that more work needs to be carried out to clarify relationships and that it would be premature to change genus names now. Hence, we refer to winter wrens as *Troglodytes troglodytes* (consistent with the current American Ornithologists' Union list), while acknowledging that the genus name might eventually be changed.

Field research

We first studied allopatric populations of *pacificus* (at Gavin Lake, British Columbia; see Fig. 1 for locations) and *hiemalis* (at Lesser Slave Lake, Alberta) and then travelled between these localities with the goal of determining whether there is a gradient between these subspecies across this region or whether two distinct types meet in a contact zone. Searching techniques included playback of both *pacificus* recordings (from Gavin Lake) and *hiemalis* recordings (from Lesser Slave Lake) and listening for responses along forest roads and trails in the potential region of contact.

Although wren density in northwestern Alberta and northeastern British Columbia was low, in May 2005, we located an area where both subspecies could be found, near the town of Tumbler Ridge in northeastern British Columbia. Initially, the main characters that we used to recognize the two subspecies were distinct and easily identifiable macrogeographical differences in songs, which we call 'singing types'. These qualitative differences primarily include the dominant frequencies of notes (*pacificus* singers with more higher frequency notes as compared to *hiemalis* singers) and the rhythm of note delivery (*pacificus* singers with a more staccato note delivery including more trills as compared to *hiemalis* singers). The term 'singing type' should not be confused with the term 'song type', which refers to a distinct type of individual song that has a unique series of notes and has been used by previous researchers to evaluate individual repertoire size and song complexity (Kroodsma 1980; Kroodsma & Momose 1991; Van Horne 1995). Each bird can have multiple song types, but each bird belongs to only one singing type (see Results). These singing types can be relatively easily distinguished by ear, and our quantitative analysis confirms their distinctiveness (see Results).

Over three field seasons (2005–2007), we found approximately 48 male wrens in the Tumbler Ridge area, of which 22 were caught for temporary study in mist nets using song playbacks (Fig. 2). Each was marked with a unique colour band combination so that recordings or other observations taken at different times could be matched to a given bird with certainty. Morphometric measurements and a blood sample (for later genetic analysis) were also taken from captured birds. Eighteen of the captured wrens were *pacificus* singers, while four were *hiemalis* singers. The maximum pairwise distance between wrens studied in this area was 44 km, and the minimum distance between wrens present in the same season was less than 0.2 km (i.e. neighbouring territories). Three of the *hiemalis* singers had a neighbouring *pacificus* singer observed less than 0.4 km away in the same season (for the fourth *hiemalis* singer, the closest known neighbour was a *pacificus* singer 1.4 km away).

To compare patterns in sympatry (i.e. in the Tumbler Ridge area) with those in allopatry, we studied wrens at several allopatric sites (Fig. 1). We included samples from the region of southeastern British Columbia that is described by some authors as being inhabited by the subspecies *salebrosus* (Brewer 2001; Hejl *et al*. 2002), allowing us to test

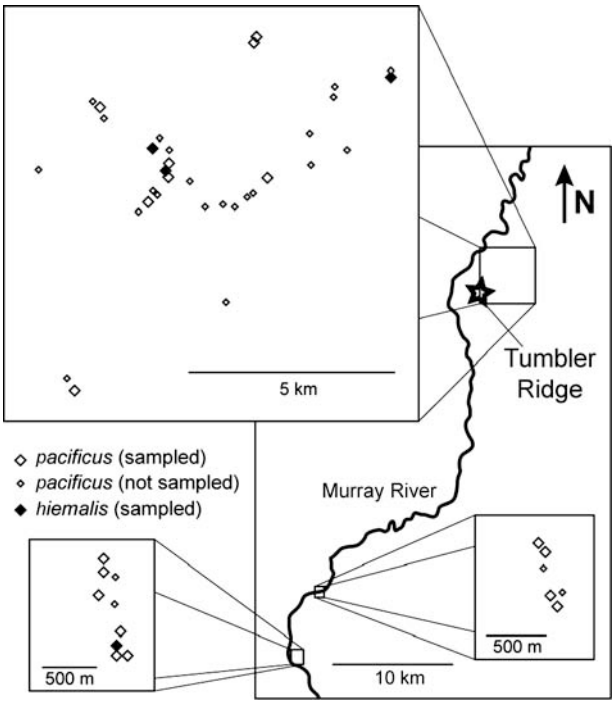

Fig. 2 Map showing the study region near Tumbler Ridge, British Columbia, Canada. Many areas were searched, and wrens were found primarily in the three areas shown in greater detail. These areas correspond to pristine old-growth forest, a habitat type that is becoming quite rare on the landscape. Each diamond symbol indicates a single male wren that was observed holding a territory, with large diamonds indicating wrens that were actually caught temporarily for genetic sampling and/or morphometric analysis. Open symbols indicate *pacificus*, and closed indicate *hiemalis*, determined according to song and/or genetic data. Data from 2005, 2006, and 2007 are shown. The patterns suggest that the two taxa have similar habitat requirements; every time we found a *hiemalis* in this area ($n = 4$), the same territory was inhabited by a *pacificus* the following year.

whether wrens in that area are genetically differentiated from those elsewhere.

Song analysis

Songs were recorded using an Audio-Technica 815a microphone and a Sony TCD-D100 DAT recorder (in 2005) or a Marantz PMD660 solid state recorder (in 2006). Recordings ranged in length from three to more than 50 songs. Occasionally, song playbacks were used to induce birds to sing before recording. While such playbacks undoubtedly affected singing behaviour (e.g. song rate and loudness), we are confident as a result of much informal experimentation that they did not noticeably affect the content of song (e.g. the shape of syllables, or *pacificus* vs. *hiemalis* type) and thus did not affect the conclusions reported herein.

To analyse songs quantitatively, we first examined recordings of wrens from allopatric sites. In addition to our own recordings (*pacificus*: two from Gavin Lake, two from Whistler Interpretive Forest, two from Pacific Spirit Park in Vancouver, and one from Prince George; *hiemalis*: five individuals from Lesser Slave Lake; see Fig. 1 for locations), we obtained recordings from the Macaulay Library at the Cornell Laboratory of Ornithology (*pacificus*: two from Oregon and two from California; *hiemalis*: two from New Brunswick and two from Ontario; Table S1, Supplementary material). We used only these allopatric songs to select the following variables that could be consistently and reliably measured in all songs. These variables included:

1 *Length*, the duration of a song (in seconds), as measured visually using on-screen spectrograms in the program RAVEN (Cornell Laboratory of Ornithology, version 1.2.1).
2 *Min freq*, the minimum frequency (in kilohertz) of a song, measured visually using RAVEN.
3 *Max freq*, the maximum frequency (in kilohertz) of a song, measured visually using RAVEN.
4 *Mean freq*, the mean frequency (in kilohertz) of a song, measured by starting at the first whole quarter second after the start of a song, then determining the frequency of sound with the largest amplitude at points in time distributed every 0.25 s throughout the song. These measurements were semi-automated in RAVEN, using 'frequency at maximum decibel' measurements at a series of time points imported into the program. We manually removed from the analysis points in time when the individual was not singing (i.e. silence between notes). This analysis enabled an accurate, objective and relatively quick method to determine the mean frequency of a bird's vocalization.
5 *SD freq*, the standard deviation (in kilohertz) of the frequencies at time points throughout the song, obtained as described above for *mean freq*. This variable quantifies the amount of variation in frequency of a bird's song.
6 *Percent blank*, to calculate the percentage of time points, as measured above for *mean freq*, where the bird was not singing, we divided the number of 'blank' points by the total number of points in the song.
7 *Trans/sec*, to quantify the temporal pacing of a bird's song in terms of the rate at which it switches between low and high frequency sounds, we used onscreen spectrograms (in RAVEN) to manually count the number of times in a song that the fundamental frequency of sound changes from below 5.5 kHz to above 5.5 kHz (this value was chosen because it is roughly the mid-point in the range of frequencies in typical winter wren song). The number of such transitions was then divided by the length of the song to give number of transitions per second.

To ensure consistency, the first author (D.P.L.T.) obtained all of the song measurements. Five songs were chosen for measurement at random from the total songs recorded for each individual. Songs that contained disruptive background noise or other bird species were excluded from the analysis. One extremely short song was also excluded.

We measured songs from 11 *pacificus* and nine *hiemalis* allopatric birds (described above) and from eight *pacificus* singers and four *hiemalis* singers that were captured in the contact zone at Tumbler Ridge. We then conducted principal components analysis (PCA) in R (R Development Core Team 2006) using individual means of each bird's songs (five songs per bird, in all cases but one in which four songs were used). Analysis included all seven variables described above. All variables except *percent blank* were log-transformed before PCA. Factor loadings from the PCA on individual means were then used to calculate principal component scores for each song that was measured. This method of analysis preserved statistical independence by using each bird once in the PCA that generated factor loadings, but then for graphing purposes allowed us to apply these factor loadings to all of the songs measured. The individual means were also used to test, using Welch two-sample *t*-tests, whether there were significant differences between *pacificus* and *hiemalis* in each basic variable as well as PC1.

Molecular analysis

To determine whether singing type was predictive of genotype, we generated sequences of the ND2 mtDNA gene and amplified fragment length polymorphism (AFLP) profiles from 12 *pacificus* singers and four *hiemalis* singers in the sympatric area (those samples that were obtained in 2005 and 2006; Tables S2–S3, Supplementary material). We compared them to *pacificus* and *hiemalis* samples from allopatric areas.

Blood samples (roughly 5–40 L) were taken in the field from the brachial vein and stored immediately in 500 L of 'Queen's lysis buffer' (0.01 M Tris, 0.01 M NaCl, 0.01 M EDTA, and 1% *n*-lauroylsarcosine, pH 7.5; Seutin *et al*. 1991) and left at ambient temperature until returned to the laboratory and frozen. Total genomic DNA was extracted using a standard phenol–chloroform extraction protocol. Following extraction, the DNA pellet was resuspended in 100 L of 1× TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at 4 C.

The complete mitochondrial ND2 gene (1041 bp) was amplified using Invitrogen polymerase chain reaction (PCR) reagents and *Taq* polymerase, supplied by New England Biolabs. The fragment was amplified with primers L5215 (Hackett 1996) and H1064 (Drovetski *et al*. 2004) with the following thermal cycling temperature profile: 3 min at 95 C, 35 cycles of 95 C (30 s), 55 C (30 s), and 72 C (30 s), followed by a final extension of 72 C for 10 min. PCR fragments were sequenced by Macrogen Genomics in Seoul, Korea. Sequences contained no indels and were aligned manually using BIOEDIT sequence editor (Hall 2005). We compared sequences from the sympatric area with allopatric sequences from 22 *pacificus* and 8 *hiemalis* that we sampled, in addition to six sequences from GenBank (Drovetski *et al*. 2004; *pacificus* from Washington: AY460323, AY460330, AY460332; *hiemalis* from Ontario: AY460291, AY460292, AY460294).

To create a haplotype network, we imported the sequence alignment into MEGA 3.1 (Kumar *et al*. 2004), which calculated the number of nucleotide differences between each of the samples. Because of the small number of differences within and the large differences between the subspecies, we were able to create haplotype groupings manually.

We generated AFLP profiles for a total of 72 samples (Table S3), including the 16 from the sympatric area (described above), 44 from the allopatric *pacificus* area, and 12 from the allopatric *hiemalis* area. Our AFLP analysis followed the protocol of Vos *et al*. (1995) with only minor modifications. DNA was digested with the endonucleases *Eco*RI and *Mse*I followed by ligation of the E- and M-adaptors (100 M). These fragments were pre-amplified using complementary E- and M-primers. The products from this reaction were diluted 40× and stored as a stock solution for the selective amplification. Combinations of the E- and M-primers (with three additional bases at the 3' end; see Table 1) were used for selective amplification during touchdown PCR in a volume of 10 L. The E-primers were fluorescently labelled with either IR-700 or IR-800 dyes so that reactions could be duplexed with two E-primers and one M-primer. Bands were separated on a LI-COR 4300 in a 6.5% polyacrylamide gel and the presence or absence of fragments was binary coded (1 or 0) in SAGA version 2.0. All data analyses were performed using only unambiguous AFLP loci in which both presence and absence occurred in two or more individuals.

Table 1 AFLP primer combinations resulting in informative, unambiguous polymorphic fragments

Primer combination	*Eco*RI primer (NNN-3')	*Mse*I primer (NNN-3')	Number of polymorphic fragments
1	AGC	CAT	18
2	ACC	CAT	17
3	AGG	CAC	15
4	AAG	CAC	10
5	AGC	CAC	17
6	AAC	CAC	13

Patterns of variation in the AFLP data were summarized using PCA, which is useful in summarizing patterns of covariation in multilocus data (e.g. Irwin *et al*. 2005; Van Treuren *et al*. 2005). STRUCTURE 2.2 (Pritchard *et al*. 2000; Falush *et al*. 2007) was used to calculate assignment probabilities of individuals to a number of clusters (K) ranging from 1 to 5. Each run consisted of a 50 000 step burn-in with 50 000 additional cycles, and for each value of K, the parameter set was run for 10 iterations. To deal with a statistical artefact produced by STRUCTURE that results in higher likelihoods and variance with larger K, which can make determining the true number of clusters in a data set problematic, we identified the K value with the largest ΔK according to Evanno *et al*. (2005).

We generated estimates of genetic differentiation using two methods. First, AFLP-SURV (Vekemans 2002) was used to calculate F_{ST}, the fraction of variance in allele frequencies that is explained by differences between *pacificus* and *hiemalis*. In order to avoid biases in the estimation of allele frequencies from dominant markers (Lynch & Milligan 1994; Zhivitovsky 1999), allele frequencies were estimated using a Bayesian approach (with uniform prior distribution of allele frequencies) assuming Hardy–Weinberg equilibrium within each species. AFLP-SURV used these allele frequencies to calculate a global F_{ST} based on all 90 loci. We also calculated F_{ST} for each locus, according to

$$F_{ST} = \frac{(p_1 - \bar{p})^2 + (p_2 - \bar{p})^2}{2\bar{p}(1 - \bar{p})}$$

where p_x is the frequency of the allele for band presence in population x, and $\bar{p}$ is the mean of p_1 and p_2. Second, ARLEQUIN 3.11 (Excoffier *et al*. 2005) was used to calculate F_{ST} based on band frequencies rather than allele frequencies. Note that AFLP markers are dominant markers; hence, this second F_{ST} is not based on allele frequencies and should not be directly compared to F_{ST} values calculated from codominant markers such as microsatellites. While ARLEQUIN was not designed to be used with dominant markers such as AFLPs, it is often used for this purpose (Svensson *et al*. 2004; Bensch & Åkesson 2005; Helbig *et al*. 2005; Irwin *et al*. 2005; Parchman *et al*. 2006). Thus, we calculated this band-based F_{ST} to facilitate comparison between studies. This band-based F_{ST} was used to measure pairwise differentiation between *pacificus* and *hiemalis* as well as between main sampling sites.

Morphometric analysis

For each captured bird, we measured six morphological characters (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 eliminate possible error due to different observers, only those birds measured by one of us (D.E.I.) were included in the analysis (Table S4, Supplementary material). We conducted a PCA after log-transforming variables and standardizing to mean zero and variance one. We used ANOVA to test whether there were differences in principal components between allopatric *pacificus*, sympatric *pacificus*, sympatric *hiemalis*, and allopatric *hiemalis*, with taxonomic identity of each sample being determined by the song and/or molecular analyses. We used *t*-tests to determine whether sympatric *pacificus* and sympatric *hiemalis* differed in each basic variable. Statistical analyses were carried out using R (R Development Core Team 2006).

Estimation of coalescence time

To place the divergence of these subspecies into a historical biogeographical context, Drovetski *et al*. (2004) estimated a coalescence time of approximately 1.5 million years ago between the *pacificus* and *hiemalis* groups based on a well-supported maximum-likelihood tree from mitochondrial ND2 sequences. This date, however, was based on a molecular clock calibrated for Galapagos mockingbirds (5.5% sequence divergence per million years; Arbogast *et al*. 2006) that is at the upper end of most molecular-clock calibrations, for which 2% sequence divergence per million years in mtDNA coding regions has been suggested as a consensus estimate (Garvia-Moreno 2004; Lovette 2004; Weir & Schluter 2004; Weir 2006; Price 2008). To incorporate possible error into this calculation and to better assess the initial divergence of these two groups, we applied a range of reasonable molecular clocks (2% to 5.5% sequence divergence per million years) to the molecular distance between *pacificus* and *hiemalis* presented by Drovetski *et al*. (2004). We were especially interested if this timing fit other patterns in avian biogeography in North America that suggest glaciations during the Pleistocene epoch (which began 1.81 million years ago; Gradstein *et al*. 2004) promoted speciation in boreal forest birds (Weir & Schluter 2004).

Results

Song analysis

Songs recorded at Gavin Lake, British Columbia; Whistler Interpretive Forest, British Columbia; Prince George, British Columbia; Vancouver, British Columbia; Corvallis, Oregon; and Sattley, California (Fig. 3) were clearly similar to published songs of *Troglodytes troglodytes pacificus* (e.g. Kroodsma 1980; Van Horne 1995; Hejl *et al*. 2002), whereas songs recorded at Lesser Slave Lake, Alberta; Grand Manan Island, New Brunswick; and Algonquin, Ontario (Fig. 3) were clearly similar to published songs of *Troglodytes troglodytes hiemalis* (Kroodsma 1980; Hejl *et al*. 2002). Individual wrens at Tumbler Ridge sang either distinctly *pacificus* songs or

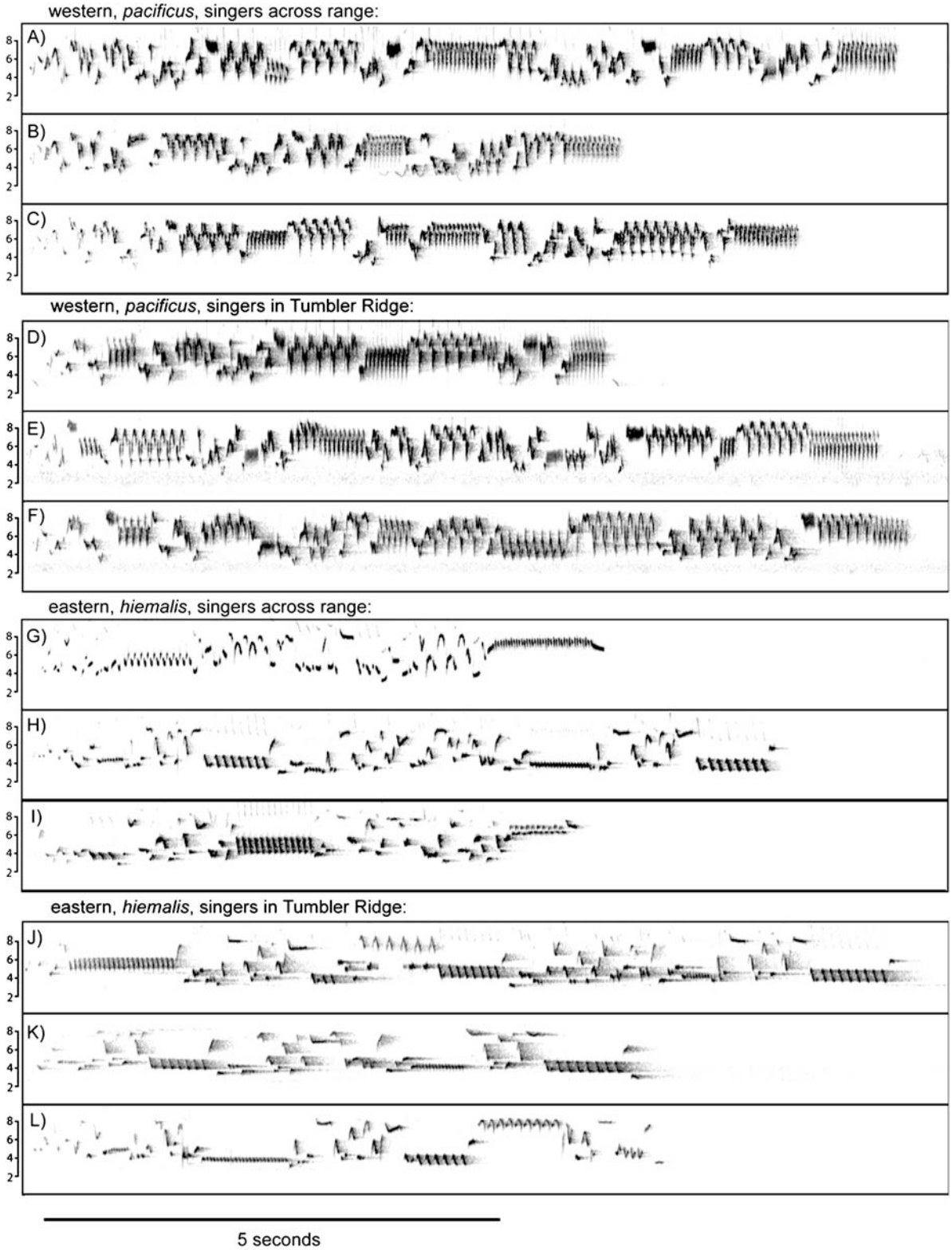

Fig. 3 Example song spectrograms from six western winter wrens [A–F, *Troglodytes* (*troglodytes*) *pacificus*] and six eastern winter wrens (G–L, *Troglodytes troglodytes hiemalis*). One song is shown for each individual from the allopatric *pacificus* populations in (A) Gavin Lake, British Columbia (B) Prince George, British Columbia, and (C) Whistler, British Columbia, and allopatric *hiemalis* populations in (G) Grand Manan Island, New Brunswick (H) Lesser Slave Lake, Alberta, and (I) Algonquin Park, Ontario. Spectrograms D–F and J–L are from *pacificus* and *hiemalis* individuals, respectively, from the overlap zone near Tumbler Ridge, British Columbia. Vertical axes show frequency in kilohertz.

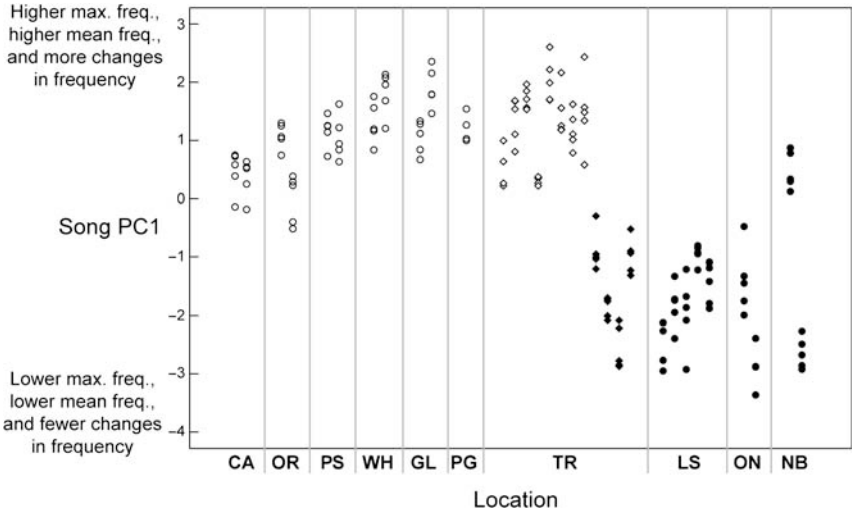

Fig. 4 Individual songs of western (*pacificus*, open symbols) and eastern (*hiemalis*, filled symbols) winter wrens are readily distinguished by PCA. The analysis summarizes variation in seven variables. The first principal component (PC1) accounts for 38.7% of variance in the entire data set. Each column represents an individual bird, ordered left to right by longitude (west to east), and each point represents a single song, of five, analysed for each individual. Songs from the sympatric population in Tumbler Ridge (diamonds) are indistinguishable from the respective allopatric songs (circles).

Table 2 Eigenvalues, variance explained, and factor loadings of the first three principal components produced in the PCA of song variables (see Fig. 4). Factor loadings are equivalent to simple correlation coefficients between a variable and a principal component

	PC1	PC2	PC3
Eigenvalue	2.71	1.22	1.16
Variance explained	38.7%	17.4%	16.6%
Factor loadings:			
Length	–0.186	0.408	–0.740
Min freq	–0.025	–0.472	–0.750
Max freq	0.912	0.127	–0.088
Mean freq	0.920	0.200	–0.166
SD freq	–0.150	0.583	–0.077
Percent blank	–0.328	0.654	0.048
Trans/sec	0.932	0.070	0.086

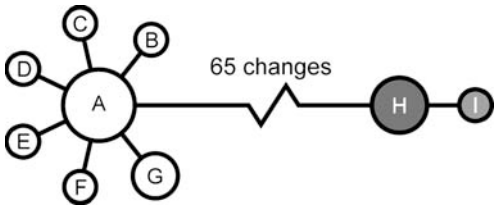

Fig. 5 Mitochondrial DNA haplotype network of 16 winter wrens occurring in sympatry in Tumbler Ridge, British Columbia, generated using 1041 bp of the ND2 gene. The frequency of haplotypes is represented by the areas of the circles (the smallest circles are each representative of one individual) and the number of nucleotide differences is represented as the number of nodes between the circles. Nodes are coloured based on the singing type of the individual (*pacificus* singers shown as white; *hiemalis* singers as shaded). The two major haplogroups, which correspond to distinct singing types, are separated by 65 mutations (6.2% sequence divergence), but haplotypes within each group differ from each other by at most one or two mutations.

distinctly *hiemalis* songs (Figs 2, 3). In no instance did we observe birds that sang both *pacificus* and *hiemalis* types (even during recording sessions on different days, ranging from 1 day to 6 weeks apart) and in no case did individual songs show intermediacy between the two types.

The distinctness of *pacificus* and *hiemalis* songs was confirmed by quantitative analysis. Of the seven basic variables that we measured, three showed highly significant differences between *pacificus* and *hiemalis*. These include *max freq* (Welch two-sample *t*-test: $t = -5.57$, d.f. = 24.5, $P < 10^{-5}$), *mean freq* ($t = -8.73$, d.f. = 14.7, $P < 10^{-6}$), and *trans/sec* ($t = -10.29$, d.f. = 14.4, $P < 10^{-7}$), each of which is higher in *pacificus*. The other song variables did not differ significantly between the groups (*length*, $t = 0.46$, d.f. = 20.4, $P = 0.65$; *min freq*, $t = 0.28$, d.f. = 28.9, $P = 0.78$; *SD freq*, $t = 1.26$, d.f. = 20.6, $P = 0.22$; *percent blank*, $t = 0.47$, d.f. = 29.5, $P = 0.65$).

Multivariate analysis further illustrates the differences between *pacificus* and *hiemalis*. The principal components analysis (Fig. 4, Table 2) shows no overlap between *pacificus* and *hiemalis* songs in sympatry. Within each taxon, songs differ little between allopatric areas and the contact zone. PC1, which is highly correlated with *max freq*, *mean freq*, and *trans/sec* (Table 2), is high in *pacificus* and low in *hiemalis* ($t = -9.43$, d.f. = 18.9, $P < 10^{-7}$).

Mitochondrial DNA analysis

Among the 16 individual winter wrens sampled in sympatry at Tumbler Ridge, we observed nine haplotypes that show a striking pattern of relationships (Fig. 5): the seven haplotypes in the 12 *pacificus* singers differ from each other by only up to 2 bp, and the two haplotypes in the four *hiemalis* singers differ from each other by only up to 1 bp; in contrast, these *pacificus* and *hiemalis* haplogroups differ

from each other by 65 or more base pairs. The two most common haplotypes (A and H, Fig. 5) correspond to sequences from allopatric individuals far to the west and east (Drovetski *et al*. 2004). These common haplotypes differ from each other at 65 of 1041 bp (6.24%). Our allopatric samples closer to the contact zone (not shown) differ by at most 5 bp from those of the corresponding group in the sympatric area. There was a perfect correspondence between singing type and haplogroup; each singer in sympatry belonged to the haplogroup of allopatric individuals of the same singing type. Thus, in every case, singing type was predictive of ND2 genotype, an association that is highly significant (Fisher's exact test using samples from the contact zone: $P = 0.00055$).

Sequences can be downloaded from GenBank (accession nos EU528850–EU528865, Table S2).

AFLP analysis

Ninety polymorphic AFLP markers, from six primer pair combinations (Table 1), were scored in 72 individuals (Table S3). Principal components analysis shows no overlap between the *pacificus* group (i.e. *pacificus* and *salebrosus*) and the *hiemalis* group (assigned using song and/or mtDNA haplotype) along the first principal component axis, with a noticeable gap between the scores for the two species (Fig. 6). The first principal component (PC1) explains appreciably more variation than PC2 and PC3 (19.8%, 4.8%, and 4.0%, respectively) and clearly separates the two taxa. Individuals from the sympatric population cluster with allopatric individuals of the same taxon, and allopatric and sympatric populations within taxa are essentially indistinguishable.

There is a single individual falling between the two groups. Preliminary sequencing from mtDNA and a nuclear intron suggests this individual was likely a first generation hybrid — it had a *hiemalis* mtDNA and was heterozygous at a nuclear single nucleotide polymorphism (SNP) that shows a fixed difference between five allopatric *pacificus* and five allopatric *hiemalis* individuals (D.P.L. Toews, unpublished). Surprisingly, this individual was not captured in the sympatric population — it was captured in a western population that was thought to be exclusive to the *pacificus* singing type (site GL in Fig. 1 and 265 km from the nearest known breeding site of a pure *hiemalis*, at site TR). Unfortunately, no song recording was obtained from this only known apparent hybrid.

Calculation of ΔK using the $L(K)$ output from STRUCTURE showed a clear peak at $K = 2$ (Table 3). All individuals, with one exception (see below), were assigned to one of these populations with a high probability by STRUCTURE, with an average probability of an individual's assignment over 10 runs of 99% (± 0.01). Assignments correlate perfectly with our a priori designation of individuals as either *pacificus* or *hiemalis*. The putative hybrid individual was the one exception to this pattern as it had a roughly equal probability (45% and 55%) of being assigned to either population, consistent with expectation of a first generation hybrid.

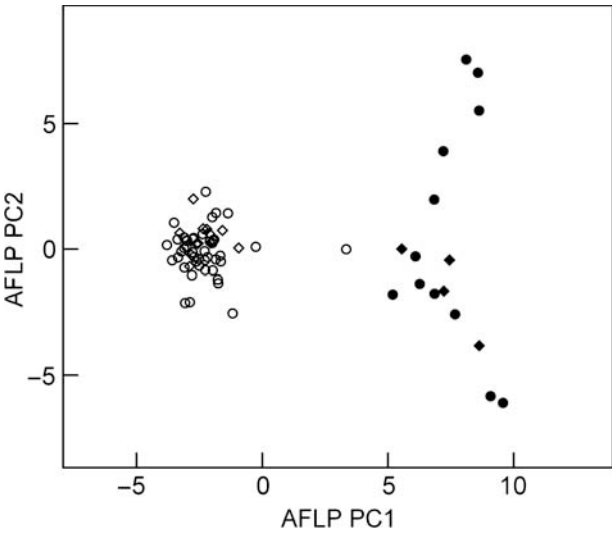

Fig. 6 Individual AFLP principle component scores of western (*pacificus*, open symbols) and eastern (*hiemalis*, closed symbols) winter wrens. The analysis summarizes variation in 90 AFLP loci. The first principal component (PC1) accounts for 19.8% of variance in the entire data set and PC2 accounts for 4.8%. Individuals from the sympatric population (diamonds, Tumbler Ridge) cluster with allopatric individuals (circles) of the same taxon. A putative first generation *pacificus-hiemalis* hybrid (from Gavin Lake, British Columbia) falls between the two clouds of points (see Results). PC1 differs significantly between *pacificus* and *hiemalis* (*t*-test with the hybrid excluded: $t_{69} = 13.57$, $P < 10^{-15}$). See Table S3 for locations and identities of samples.

Table 3 Averaged estimated log probability, variance, and *K* at different clusters (*K*) from *pacificus* and *hiemalis* AFLP profiles from 10 iterations in STRUCTURE

K	Ln	P(D)	*K*
1	–3988.2	115.8	—
2	–3325.4	305.5	440.1
3	–3449.3	646.5	2.2
4	–3346.5	590.7	13.7
5	–3405.9	784.3	—

The strong difference in AFLP profiles between *pacificus* and *hiemalis* can be further quantified using *F*-statistics. First, F_{ST} based on allele frequencies (calculated using AFLP-SURV; Vekemans 2002) is 0.308. The distribution of allele-based F_{ST} estimates among the 90 variable loci is heavily skewed, with a small number of loci showing a strong difference in frequency between the taxa while most show little difference (Figure S1, Supplementary material),

Table 4 Pairwise measures of divergence in AFLP markers between major sampling sites. Populations are indicated by their two-letter site codes (see Fig. 1; Table S3), preceded by a letter indicating western (w) or eastern (e) singing group. Above the diagonal are average AFLP distances (number of bands) between sites. On the diagonal are AFLP distances within sites. Below the diagonal are band-based F_{ST} values between sites, calculated using ARLEQUIN 3.11 (Excoffier *et al*. 2005). Italicized F_{ST} values are significant at the $P < 0.05$ level, and bold F_{ST} values are significant at the $P < 0.01$ level

	w-PS	w-SA	w-GL	w-AT	w-TR	e-TR	e-LS
w-PS	21.52	20.78	21.69	20.48	21.41	34.82	37.17
w-SA	*0.035*	18.33	20.93	19.33	19.91	34.22	35.47
w-GL	–0.021	0.024	23.00	19.30	20.47	34.30	36.85
w-AT	–0.003	0.037	–0.093	19.00	18.88	35.00	38.06
w-TR	*0.023*	0.030	–0.049	–0.045	20.24	34.71	36.92
e-TR	***0.403***	***0.457***	***0.383***	*0.457*	***0.428***	19.00	20.94
e-LS	***0.418***	***0.435***	***0.394***	***0.454***	***0.432***	0.015	21.86

a pattern generally expected for multilocus genetic data (Whitlock 2008). Second, F_{ST} based on band frequencies (calculated using ARLEQUIN 3.11; Excoffier *et al*. 2005) is 0.420 (the hybrid individual described above was excluded from these calculations). Both statistics are significantly different from zero ($P << 10^{-4}$). The average corrected pairwise difference between individuals of differing taxa is 15.1 bands (average between individuals within the *pacificus* group: 21.5; within *hiemalis*: 20.7; uncorrected difference between the taxa: 36.1). There is very little genetic differentiation between research sites within each of these groups (Table 4). Samples from the area reported to be occupied by *salebrosus* (grouped as site SA in Table 4; Brewer 2001; Hejl *et al*. 2002) were very similar genetically to samples from *pacificus* areas (Table 4). Hence, our data do not provide any genetic evidence of *salebrosus* being evolutionarily distinct from *pacificus*, although additional sampling from further south in the described range of *salebrosus* might reveal more genetic differentiation.

Morphometric analysis

In contrast to the strong differences seen between *pacificus* and *hiemalis* in song, mitochondrial DNA, and nuclear markers (AFLP), the two forms are extremely similar in morphometric traits. A PCA (Fig. 7; Table S5, Supplementary material) shows that neither major axis of variation in the morphometric data shows differences between allopatric *pacificus* ($n = 24$), sympatric *pacificus* ($n = 10$), sympatric *hiemalis* ($n = 4$), and allopatric *hiemalis* ($n = 5$). A comparison of sympatric *pacificus* and *hiemalis* shows that only wing and tail length display differences that approach statistical significance (Welch two-sample *t*-test: wing chord, $t = –2.43$, d.f. = 11.9, $P = 0.032$; tail, $t = 2.33$, d.f. = 5.0, $P = 0.067$; tarsus, $t = –0.33$, d.f. = 6.5, $P = 0.75$; beak length, $t = 0.21$, d.f. = 4.0, $P = 0.85$; beak depth, $t = 0.44$, d.f. = 5.42, $P = 0.68$; beak width, $t = 0.56$, d.f. = 4.8, $P = 0.60$), but certainly not after Bonferroni correction for multiple tests. Thus, in stark contrast to song, these morphometric traits cannot be used effectively to distinguish between *pacificus* and *hiemalis* in sympatry (Fig. 8).

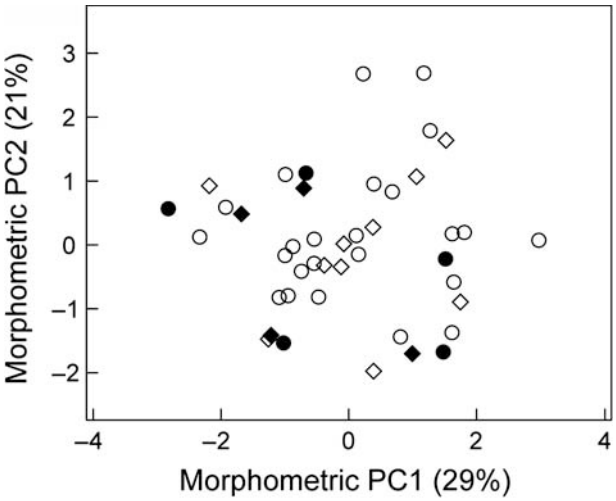

Fig. 7 Results of a PCA of morphometric variation of winter wrens, showing that morphometric traits show broad overlap between *pacificus* (circles) and *hiemalis* (diamonds), both in allopatry (open symbols) and in sympatry near Tumbler Ridge (filled symbols). Variables included in the analysis are wing chord, tail length, tarsus length, beak length, beak depth, and beak width (for factor loadings see Table S5). PC1 explains 29% of the variance in the data, while PC2 explains 21%. Neither principal component differs significantly between groups (ANOVA: PC1, $F = 0.493$, d.f. = 3 and 39, $P = 0.69$; PC2, $F = 0.615$, d.f. = 3 and 39, $P = 0.61$).

Estimation of coalescence time

Applying a reasonable range of molecular clocks (2–5.5%) to the branch lengths reported by Drovetski *et al*. (2004), we find that a large majority of the clocks in this range (83%) date the split of these two taxa before the start of the Pleistocene (1.81 million years ago; Gradstein *et al*. 2004). The generally accepted '2% rule' dates the split to 4.3 million years ago, which suggests that *pacificus* and *hiemalis* last shared a common ancestor long before the glacial cycles of the Pleistocene.

Discussion

We have described the first known area in which members of both western (e.g. *pacificus*) and eastern (e.g. *hiemalis*) groups of winter wrens in North America can be found occupying neighbouring territories, allowing a test of

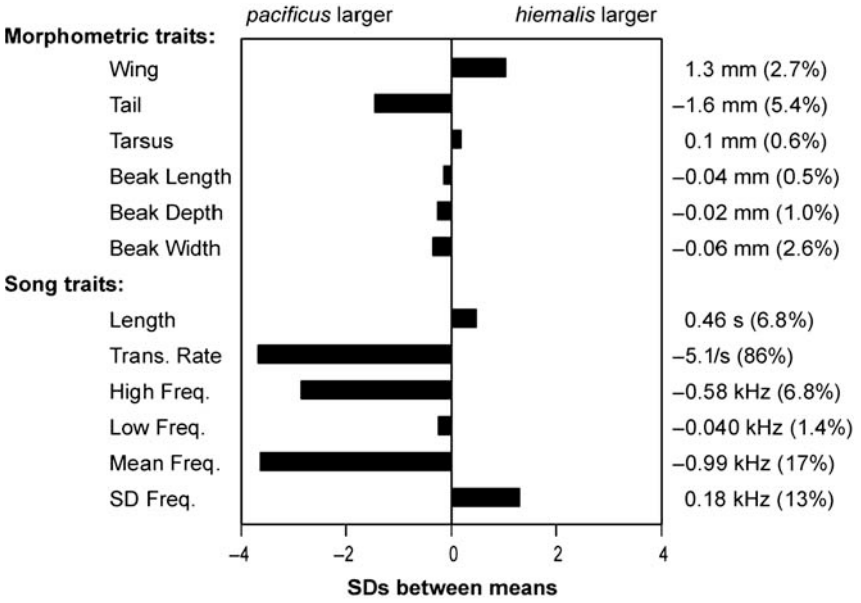

Fig. 8 The difference between sympatric *pacificus* and *hiemalis* near Tumbler Ridge in a variety of morphometric and song traits, expressed as the number of pooled standard deviations between the means of the two groups. The size of the bar can be interpreted as how distinguishable the two groups are in the corresponding trait, with bars on the left of centre indicating that *pacificus* is larger and right of centre indicating that *hiemalis* is larger. At right is shown absolute differences as well as percentage differences (i.e. absolute difference divided by the mean of the two group values, expressed as a per cent). Songs are much more powerful than morphometrics in distinguishing the two groups.

whether the two forms are reproductively isolated. Dramatic differences of singing types in sympatry are as strong as those observed between allopatric populations. The two singing types correspond to distinct and highly divergent mitochondrial clades and nuclear DNA clusters. The strong correlation between song, mtDNA, and nuclear markers in sympatry indicates there has been little if any gene flow or cultural exchange (i.e. learning of heterotypic song) between *pacificus* and *hiemalis* and suggests strong reproductive isolation.

A single hybrid individual from an otherwise allopatric population demonstrates that *pacificus* and *hiemalis* can and do occasionally interbreed; however, there are a number of reasons to think that reproductive isolation is quite strong. First, the two groups differ substantially in their nuclear genome and these differences are just as strong in sympatry as in allopatry. If hybridization were causing significant gene flow, we would expect sympatric populations to show more genetic similarity than allopatric populations. Second, the strong association between nuclear DNA, mtDNA and singing types would not be expected if isolating barriers were not strong. An alternative scenario is that the two forms might have only recently come into contact (e.g. within the last generation) and hence have not yet had a chance to interbreed or exchange singing types through cultural mixing. This possibility is unlikely, however, since the two subspecies have been recorded as occurring in northeastern British Columbia for at least the past half-century (Campbell *et al*. 1997).

We acknowledge that sample sizes in the contact zone are lower than we would prefer; both forms of winter wren prefer similar old-growth forests, a habitat type that is becoming increasingly rare in northeastern British Columbia due to logging, agriculture, and other human impacts. In addition, the eastern form is more rare in the Tumbler Ridge area than the western. Nonetheless, we believe our sample sizes are sufficient for our conclusions regarding reproductive isolation, for two reasons. First, statistical tests that take into account sample size show a highly significant relationship between song, mtDNA, and AFLP profile. Second, AFLP analysis effectively allows us to learn about the genetic ancestry of an individual; by examining a single individual, we can infer something about the genetic makeup of its many ancestors. Thus, AFLP analysis effectively integrates patterns over a much larger number of individuals than just those that were sampled. If detectable gene flow were occurring between the forms, we would expect to see evidence of it in the individuals we sampled: we would expect to see sympatric populations more similar than allopatric populations, a pattern that was not observed.

Taken together, the mtDNA and AFLP data indicate that the initial split between *pacificus* and *hiemalis* is relatively old, most likely predating the Pleistocene epoch. Our F_{ST} estimate based on AFLP band frequencies is 0.42, which is at the upper end of F_{ST} values similarly calculated from AFLP data from other avian sister species. For example, band-based F_{ST} is 0.18 between greater and lesser spotted eagles (*Aquila clanga* and *Aquila pomarina*; Helbig *et al*. 2005), 0.4 between two reproductively isolated taxa of greenish warbler (*Phylloscopus trochiloides viridanus* and *Phylloscopus trochiloides plumbeitarsus*; Irwin *et al*. 2005), and 0.38 between white-winged crossbills and Hispaniolan crossbills (*Loxia leucoptera* and *Loxia megaplaga*; Parchman *et al*. 2006). In terms of mtDNA, *pacificus* and *hiemalis* are more strongly

divergent than are all nine boreal-species pairs examined by Weir & Schluter (2004), indicating that the two taxa are quite old, even compared to species pairs with obvious plumage differences.

The finding that *pacificus* and *hiemalis* subspecies are genetically and behaviourally distinct in sympatry suggests that they are reproductively isolated and should qualify as 'good species' under the biological species concept, as well as most other major species concepts. Thus, we propose that within the currently defined *Troglodytes troglodytes*, the western subspecies, *pacificus*, along with other closely related western subspecies (e.g. *salebrosus*) should be promoted to the species level designation of *Troglodytes pacificus*. We suggest the common name 'Pacific wren' for this new species, as that name reflects its scientific name as well as its geographical distribution (although it should be noted that other subspecies of *T. troglodytes* inhabit the Pacific Coast of Asia). The eastern subspecies, *hiemalis*, and other closely related subspecies (e.g. *pullus*), as well as Old World forms, should retain the *T. troglodytes* species name for now. This includes the European form with the original 'pure' trinomial *Troglodytes troglodytes troglodytes*. We speculate that future work may determine that additional cryptic species may occur with *T. troglodytes*, as suggested by Drovetski *et al*. (2004). In particular, *Troglodytes troglodytes hiemalis* is phylogenetically distinct from Eurasian forms of *T. troglodytes* in mtDNA (Drovetski *et al*. 2004), suggesting they may be best treated as separate species. Testing their distinctiveness under the biological species concept will be difficult as it is unlikely the two encounter each other in a natural setting.

The finding that *pacificus* and *hiemalis* are reproductively isolated groups brings up the question of what is causing that isolation. Intrinsic postzygotic isolation (i.e. intrinsic genetic incompatibilities) is perhaps not a likely cause of reproductive isolation as studies have shown that hybrid sterility and inviability generally take much longer to develop than other forms of reproductive isolation in birds (Price & Bouvier 2002). The finding of a single adult hybrid also suggests that intrinsic postzygotic isolation is not very strong. Possible sources of prezygotic isolation include differing habitat preferences, differences in the timing of breeding, or female preference for homotypic plumage and/or song characteristics. Our observations do not reveal any habitat difference between *pacificus* and *hiemalis* (in fact they show that the same patch of forest can be inhabited by a *hiemalis* in 1 year and a *pacificus* in the next), and the similarity in morphometric traits suggests that the two forms have similar ecological niches. Our data are insufficient to test whether there are differences in arrival times in the overlap zone. We also have little evidence suggesting plumage differences are an important reproductive barrier as the two forms differ only subtly in plumage, most noticeably on the throat, with *pacificus* being darker than *hiemalis* (Brewer 2001; Hejl *et al*. 2002). In contrast, we suggest that the large, indeed diagnostic, differences in song may be playing a more important role.

Like any other trait, song is expected to diverge over time between species for a variety of reasons (Catchpole & Slater 1995; Irwin 2000). Females of many species are known to assess male songs when choosing a mate (Catchpole & Slater 1995; Hasselquist *et al*. 1996; Collins 2004; Price 2008); hence, it is likely that song is not only an indicator of reproductive isolation but also plays a role in generating that isolation. The very distinct differences between *pacificus* and *hiemalis* song, which can be easily recognized by a human after moderate training, are surely recognizable to a female winter wren, although this has not yet been tested. Songs have been used increasingly in recent years to recognize the existence of morphologically cryptic species of birds (e.g. Irwin *et al*. 2001a; Päckert *et al*. 2004). Cultural and social modes of speciation, often involving acoustic divergence, have also become increasingly recognized in nonavian systems (Gray & Cade 2000; Kingston *et al*. 2001; Rodriguez *et al*. 2006).

Extrinsic postzygotic isolation mechanisms could also contribute to reproductive isolation between *pacificus* and *hiemalis*. One possible source of postzygotic isolation is seasonal migratory behaviour. The overlap zone between *pacificus* and *hiemalis* may correspond to a migratory divide, a meeting place of divergent groups that migrate in different directions to their wintering grounds (Bensch *et al*. 1999; Ruegg & Smith 2002; Irwin & Irwin 2005). The two forms apparently have differing wintering areas, with *pacificus* wintering along the West Coast from California through Alaska, and *hiemalis* wintering in the southeastern USA (Hejl *et al*. 2002). Hybrids between the forms might inherit intermediate and likely less optimal migratory behaviour, resulting in selection against hybrids. Thus, differences in migratory behaviour could potentially contribute to reproductive isolation, reducing gene flow between the divergent forms and promoting speciation (Helbig 1991; Bensch *et al*. 1999; Ruegg & Smith 2002; Irwin & Irwin 2005).

The finding that *Troglodytes pacificus* and *T. troglodytes hiemalis* are distinct biological species, which have likely been evolving independently for millions of years, is especially interesting given their extreme morphological similarity in sympatry. The two forms differ only in subtle patterns of plumage colouration (Brewer 2001; Hejl *et al*. 2002) and also have broadly overlapping measurements of six morphometric traits, suggesting that they are ecologically similar. This similarity does not appear to be due to an inability to evolve in response to ecological differences: populations inhabiting unusual habitat in the Aleutian Islands of Alaska differ markedly from mainland *T. pacificus* in morphometric traits (Hejl *et al*. 2002) yet show relatively little mtDNA differentiation from mainland *T. pacificus* compared to the difference between *T. pacificus* and *T.*

troglodytes hiemalis (Pruett & Winker 2008). Hence, the morphometric similarity between *T. pacificus* and *T. troglodytes hiemalis* in northeastern British Columbia suggests that they are under similar stabilizing selection for morphometric traits. Informal playback experiments in the overlap area (D.P.L. Toews and D.E. Irwin, unpublished data) also indicate that males of each species respond aggressively to songs of the other species, suggesting the two species may be utilizing substantially similar resources. These observations suggest that social and sexual selection may have been more important than natural selection in driving reproductive isolation between *pacificus* and *hiemalis*.

These results raise the question of whether there are other morphologically similar pairs of western and eastern taxa that are currently considered conspecific but in fact are reproductively isolated. They also emphasize the importance of comparing sister species in sympatry and considering a wide variety of traits whenever possible. Research in Central Asia has revealed the presence of many such cryptic species (Alström & Olsson 1999; Irwin *et al*. 2001a), and further research in the central parts of North America, particularly in the boreal forests of western Canada, may similarly uncover more species pairs. Revealing such cryptic species may have important implications for the debate about the role of Pleistocene glaciations in speciation (reviewed by Lovette 2005). In this case, a taxon that until now was treated as a single species is in fact a species pair that is older than all of the boreal sister species splits examined so far in North America (Weir & Schluter 2004). This finding suggests that it is likely that there are other, younger pairs of cryptic species yet to be discovered.

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David P. L. Toews recently completed his M.Sc. thesis titled 'Reproductive isolation in a contact zone between divergent forms of winter wren (*Troglodytes troglodytes*).' Darren E. Irwin, an Assistant Professor of Zoology, leads the Laboratory for Molecular Biogeography at the University of British Columbia. His research focuses on the roles of geography, behaviour, genetics, morphology, and ecology in the diversification of birds in the boreal forests of North America and Eurasia.

Supplementary material

The following supplementary material is available for this article:

Fig. S1 Histogram of F_{ST} values estimated between *pacificus* and *hiemalis* for 90 variable AFLP markers. F_{ST} reported here is based on estimated allele frequencies determined by AFLP-SURV (Vekemans 2002).

Table S1 Location and identity of individuals for which sonograms were analyzed

Table S2 Identification numbers and Genbank Accession numbers for ND2 sequences obtained from individuals in Tumbler Ridge, British Columbia

Table S3 Identity and location of individuals for which AFLP profiles were generated at 90 polymorphic loci

Table S4 Identity and location of individuals that were used in the morphometric analysis. All individuals were measured by a single observer (D.E.I.)

Table S5 Eigenvalues, variance explained, and factor loadings of the first three principal components produced in the PCA on morphometric traits. Factor loadings are equivalent to simple correlation coefficients between a variable and a principal component.
This material is available as part of the online article from:
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