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Combining genetic markers and stable isotopes to reveal population connectivity and migration patterns in a Neotropical migrant, Wilson's warbler (*Wilsonia pusilla*)

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Abstract

We used results from the analysis of microsatellite DNA variation and hydrogen stable-isotope ratios to characterize the population structure of a neotropical migrant passerine, the Wilson's warbler (*Wilsonia pusilla*). The resulting information was then used to infer migration patterns and population connectivity between breeding grounds in North America and overwintering areas in Mexico and Central America. The microsatellite data revealed genetic structure across the North American continent; populations in the west were found to significantly differ from the east. Minimal genetic structure was observed among western sites. The lack of isolation by distance and low variance in F_{ST} values suggests that gene flow could play an ongoing role in limiting genetic differentiation among sites in the western part of the distribution. However, additional information including estimates of effective population size and the proximity of the population to equilibrium is required before the role of gene flow can be assessed fully. Analysis of isotope data showed a negative relationship between latitude and hydrogen isotope ratios in breeding ground individuals. There was a positive relationship between wintering ground latitude and hydrogen isotope ratios for individuals that were genetically western in origin. This is consistent with a leapfrog pattern of migration, in which genetically western birds from the northernmost breeding areas overwinter at the most southerly locations in Central America. Additionally, isotopic ratios of western birds suggest that coastal breeders overwinter in western Mexico, while western birds from further inland and at high elevations overwinter in eastern Mexico. Using information from both genetic an isotopic approaches will probably be useful for identifying patterns of migration and population connectivity between breeding and overwintering areas, both important issues for conservation efforts, and may also contribute to investigation of the evolution of migration.

Keywords: leapfrog migration, microsatellites, Neotropical migrants, stable isotopes

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Introduction

Recent population declines of many neotropical migrants have ignited debate on whether populations are limited more on the breeding or on the overwintering grounds, and how certain disturbances such as habitat alteration affect demography (Robbins *et al*. 1989; Rappole & McDonald 1994; Latta & Baltz 1997). These conservation

concerns have highlighted the need for broad-scale studies of migrant systems across the annual cycle (Robbins *et al*. 1989; Berthold & Terrill 1991; Peterjohn *et al*. 1995). Demographic events during any one stage of the annual cycle may have repercussions for subsequent stages (e.g. Marra *et al*. 1998; Sillett *et al*. 2000). In such a dynamic system, information on levels of population connectivity between particular breeding and overwintering locations and knowledge of migration patterns is crucial to understand fully the limitations and threats to migrant bird populations (Webster *et al*. 2002).

To assess connectivity and establish migration patterns, individuals or populations need to be tracked as they move from breeding to wintering grounds and back; a process that often occurs across very large geographical scales. Tracking individuals across their annual cycle has met with varying success. The traditional method of bird banding has been informative for some avian groups such as shorebirds and waterfowl, but levels of band returns for migrant songbirds are often miniscule (Berthold 1993). Similarly, radio and satellite tracking are valuable for determining movements of large-bodied migrants capable of carrying heavy transmitters (Ristow *et al*. 2000; Ueta *et al*. 2000), but cannot be applied currently to small passerines. The difficulties of applying these techniques to small songbirds has led to the use of genetic and isotopic markers to track populations.

If genetic variation is structured geographically on the breeding grounds, then the breeding origin of individuals sampled at migration and overwintering locations can be identified. Genetic methods have been successful in tracking migration of shorebirds (Wenink & Baker 1996; Haig *et al*. 1997; Wennerberg 2001). However, to date, molecular studies of North American migrant songbirds based on mitochondrial DNA (mtDNA) variation have revealed low levels of population structure detectable at only broad geographical scales (Ball & Avise 1992; Milot *et al*. 2000; Kimura *et al*. 2002; Ruegg & Smith 2002). This limits the geographical resolution of connectivity to large scales. The application of molecular markers with higher mutation rates, such as highly variable microsatellites, may improve the level of population resolution that is currently attainable from mtDNA analysis. The level of geographical structure expected from microsatellite variation is yet to be determined for most North American migrant songbirds, although in a study of yellow warblers across northern North America, Gibbs *et al*. (2000) reported weak geographical structure over a continental scale.

Information on migration patterns and population connectivity can also be obtained via isotopic analysis of animal tissues, such as feathers. Isotopic signatures from tissues have been shown to be a powerful tool to infer movements of animals across different landscapes (e.g. Marra *et al*. 1998; Hobson 1999; Chamberlain *et al*. 2000; Hobson *et al*. 2001; Meehan *et al*. 2001; Rubenstein *et al*. 2002). Hydrogen isotope ratios (δD: deuterium) in the natural environment vary systematically across North America, displaying an approximate latitudinal gradient (Hobson & Wassenaar 1997). In addition, they are also affected by elevation and distance from coastlines. Generally, precipitation contains relatively more deuterium at low latitudes and elevations and near the coast (leading to heavier isotope ratios, i.e. less negative values of δD), and less deuterium at higher latitudes and elevations and towards continental interiors (lighter isotope ratios, i.e. more negative values of δD) (Rosanski *et al*. 1993). The incorporation of δD into bird feathers during growth reflects the hydrogen isotope ratios present in local environments (Chamberlain *et al*. 1997; Hobson & Wassenaar 1997; Kelly & Finch 1998). Individuals that grow new feathers on the breeding grounds thus carry the particular isotopic signature for that region. An isotopic value characteristic of where the individual bred and molted can be detected from individuals caught during migration and on overwintering grounds. The level of resolution provided by isotopic analyses is limited by the resolution of the environmental isotope map. For hydrogen isotope ratios in North America, this resolution is distributed approximately across 6° wide latitudinal bands (Kelly *et al*. 2001).

Information on population connectivity and migration patterns will be resolved at finer geographical scales if high levels of population structure are detectable on breeding grounds. Using genetic and isotopic approaches in concert has the potential to provide this finer scale geographical resolution than each separately, especially as studies often show genetic structuring in North America migrants occurs longitudinally while isotopic clines typically occur across differing latitudes (Webster *et al*. 2002). Here, we use information from both methods to investigate patterns of population subdivision and connectivity in Wilson's warbler (*Wilsonia pusilla*), a widespread songbird that breeds in the United States and Canada and overwinters in Mexico and Central America (Fig. 1). We examine first the degree of population structure using eight microsatellite loci and hydrogen isotope ratios. We then use this information to describe patterns of connectivity between breeding and overwintering areas, and migratory patterns between these areas.

Materials and methods

Sampling

Blood and feather samples were collected from individuals on the breeding and wintering areas (Fig. 1, Table 1). Precautions were taken to reduce the probability that migrating individuals were included in the breeding location samples. Only individuals in full adult plumage

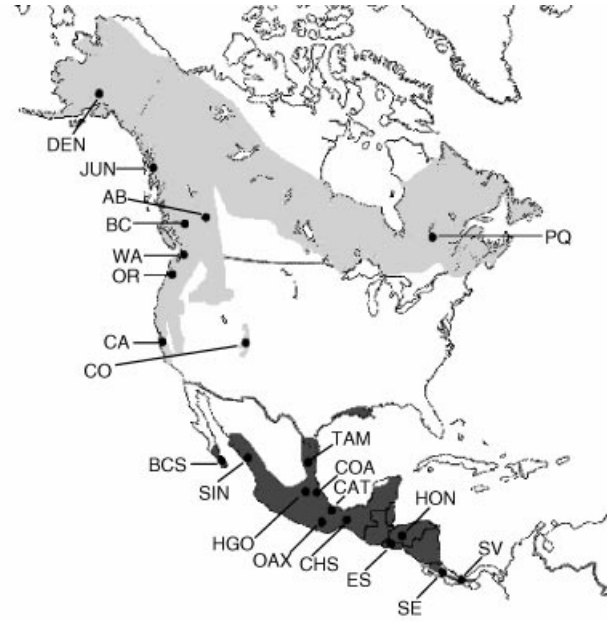

Fig. 1 Location of sampling sites on breeding and overwintering grounds. See Table 1 for location codes. Breeding grounds and overwintering grounds are indicated by light and dark shading, respectively (distribution based on Ammon & Gilbert 1999).

showing either a cloacal protuberance (males) or brood patch (females) that would indicate breeding activity were included (see Pyle 1997). To ensure further that we sampled only breeding individuals, genetic samples were taken between the end of May and August, when most individuals would have been on their breeding territories (Ammon & Gilbert 1999).

An additional requirement for the isotopic analysis in breeding-ground feathers is that the samples were collected before individuals had moulted into autumn plumage, which occurs towards the end of the breeding season (Ammon & Gilbert 1999). The premoult feathers carry the isotopic signature generated at the end of the previous breeding season, hence providing information of breeding latitude for the previous year. We used 15 July as the cut-off date for breeding ground feather samples for isotope analysis. In contrast, isotope values of feathers collected on the wintering ground indicated the breeding latitude of the current year.

Samples from overwintering individuals were collected between 20 November and 5 March. The majority of overwintering individuals arrive at their winter quarters by mid-October and leave in mid-March at the earliest (Ammon & Gilbert 1999).

Molecular genetic methods

Nuclear DNA variation was assayed using eight microsatellite loci. Four of these loci were developed from commercially made Wilson's warbler microsatellite-enriched libraries (Genetic Identification Services, CA) (Table 2). Reverse primers were designed with a GTTT 'pigtail' to reduce variability in adenylation of products and thereby improve genotyping consistency (Brownstein *et al*. 1995). The remaining four loci were developed by Dawson *et al*. (1997) from a yellow warbler (*Dendroica petechia*) library. One primer from each locus set was fluorescently labelled (Table 2). For polymerase chain reaction (PCR) amplification of Wilson's warbler-derived loci, the following reagents were combined in a 12.5-μL reaction: ~50 ng genomic DNA, 0.16 mM dNTPs, one-tenth volume of PCR buffer (10 mM Tris-HCl pH 8.3, 500 mM KCl), 1.5 mM $MgCl_2$, 50 ng/μL BSA, 4% DMSO, 0.1 μM each primer (except WpD4F where 0.01 μM was added) and 0.5 units Taq DNA polymerase (Amplitaq, Applied Biosystems). For yellow warbler-derived loci, primer concentration was increased to 0.3 μM. All loci were amplified via touch-down cycling conditions of: initial denaturation at 94 °C for 5 min; 25 cycles of 94 °C for 30 s, 60–52 °C for 40 s (where the annealing temperature was decreased by 2 °C every five cycles), 72 °C for 30 s; 25 cycles of 94 °C for 30 s, 50 °C for 40 s, 72 °C for 30 s; final extension of 72 °C for 5 min.

Two mL of PCR product was mixed with 2.5 mL formamide loading dye and 0.2 mL of a size standard [350 bp TAMRA- or ROX-labelled fragments (Applied Biosystems Inc., Foster City, CA)]. Products were electrophoresed on a 5.8% acrylamide gel on an Applied Biosystems 377 automated system at the Conservation Genetics Laboratory, San Francisco State University. Microsatellite allele sizes were scored using GENESCAN software after calibration with the internal size standard.

Statistical analyses of genetic data

Assumptions of independence of loci and Hardy–Weinberg equilibrium of loci and populations were assessed using the linkage disequilibrium and Hardy–Weinberg options in GENEPOP 3.2a (Raymond & Rousset 1995). The probability of linkage disequilibria between each locus pair in each population was calculated where possible (in some populations, no individuals shared the same combination of genotypes for the loci being compared, thereby contrasts could not be made). A combined *P*-value was calculated for each locus pair across all populations (Fisher 1954). To determine if the loci were affected by null alleles, a specific test for heterozygote deficit was conducted for each locus and each population.

Nei's (1978) unbiased heterozygosity was calculated for each population using the software TFPGA version 3.1 (Miller 1997). Allelic diversity was calculated in GENEPOP 3.2a (Raymond & Rousset 1995) and corrected for sample

Table 1 Sampling site information. n_{ms} and n_{iso} refer to samples sizes for microsatellite and isotope analyses, respectively

Site, State/Prov, Country	Abbrev.	Latitude–longitude	n_{ms}	n_{iso}	H_O	H_E	A	δD (± SD)
Denali National Park, Alaska, USA	DEN	63°25′ N 150°26′ W	—	20	—	—	—	–107.4 (14.4)
Juneau, Alaska, USA	JUN	58°18′ N 134°25′ W	10	—	0.74	0.77	8.9	—
Hinton, Alberta, CA	AB	52°40′ N 111°18′ W	19	18	0.62	0.73	6.9	–120.9 (17.4)
100 MileHouse, British Columbia, CA	BC	51°39′ N 121°17′ W	12	—	0.47	0.73	8.2	—
Mt Baker National Forest, Washington, USA	WA	48°09′ N 121°27′ W	20	—	0.62	0.79	9.8	—
Siuslaw National Forest, Oregon, USA	OR	44°16′ N 123°51′ W	32	24	0.48	0.78	7.8	–82.1 (8.1)
Pillar Point, California, USA	CA	37°30′ N 122°29′ W	17	15	0.54	0.77	7.4	–67.9 (7.2)
Grand Mesa, Colorado, USA	CO	39°02′ N 107°57′ W	32	27	0.60	0.76	8.7	–107.4 (11.1)
Camp Myrica, Quebec, CA	PQ	49°43′ N 73°20′ W	17	13	0.58	0.70	9.2	–97.2 (16.6)
Chupaderos, Sinaloa, MX	SIN	23°50′ N 102°20′ W	—	6	—	—	—	–82.6 (8.0)
El Cielo Biosphere Reserve Tamaulipas, MX	TAM	23°00′ N 99°08′ W	—	21	—	—	—	–121.1 (13.9)
Los Cabos, Baja California Sur, MX	BCS	22°53′ N 109°54′ W	—	6	—	—	—	–77.8 (7.1)
Zacualtipán, Hidalgo, MX	HGO	20°39′ N 98°36′ W	—	5	—	—	—	–134.1 (9.8)
Coatepec, Veracruz, MX	COA	19°27′ N 96°58′ W	—	19	—	—	—	–120.5 (14.6)
Catemaco, Veracruz, MX	CAT	18°25′ N 95°07′ W	—	7	—	—	—	–118.4 (16.7)
Animas de Trujano, Oaxaca, MX	OAX	17°03′ N 96°43′ W	—	13	—	—	—	–141.6 (18.0)
El Ocote Reserve, Chiapas, MX	CHS	16°45′ N 93°07′ W	—	3	—	—	—	–138.5 (13.6)
Tegucigalpa, Francisco Morazán, HON	HON	14°06′ N 87°13′ W	—	21	—	—	—	–141.0 (16.7)
San Salvador, San Salvador, ES	ES	13°42′ N 89°12′ W	—	19	—	—	—	–143.9 (16.9)
Santa Elena, Guanacaste, CR	SE	10°56′ N 85°41′ W	—	9	—	—	—	–122.9 (20.4)
San Vito, Puntarenas, CR	SV	08°50′ N 82°58′ W	—	12	—	—	—	–125.0 (8.7)

Diversity measures for each population are calculated across five loci — H_O: observed heterozygosity, H_E: expected heterozygostiy, A: allelic diversity. Average δD is given for each site. Country abbreviations: CA: Canada; MX: Mexico; HON: Honduras; ES: El Salvador; CR: Costa Rica.

size according to Ewens (1972). Observed and expected heterozygosities for each locus across all populations were calculated in FSTAT (Goudet 1995).

To test for homogeneity of allele frequencies between each pair of populations, the significance of allele frequency differences were assessed using a Monte Carlo approximation of Fisher's exact test (Raymond & Rousset 1995) using the software TFPGA, version 3.1 (Miller 1997). Significance values were combined across loci (Fisher 1954).

F_{ST} measures were calculated to summarize between vs. among population variation (Weir & Cockerham 1984). F_{ST} is a widely used, low variance measure that performs better than other estimates of population structure when the number of loci and the number of samples are low (Gaggiotti *et al*. 1999), as is the case in this study. Global F_{ST} with 95% confidence limit was calculated using FSTAT (Goudet 1995). Pairwise F_{ST} values were calculated using the software ARLEQUIN version 1.1 (Schneider *et al*. 1997). The significance of F_{ST} from zero was assessed using 1000 bootstrap replicates. Relative measures of differentiation can be difficult to compare directly (Charlesworth 1998; Hedrick 1999), therefore alternative distance measures were also calculated. Nei's (1972) standard genetic distance (D_S) and Paetkau *et al*.'s (1997) genotype likelihood distance (D_{LR}) were calculated using programs by J. Brzustowski (University of Alberta) and available at the website http://biodb.biology.ualberta.ca/jbrzusto/.

We tested for an isolation-by-distance effect between geographical and genetic distances among sampling sites in western United States and Canada by comparing two matrices: ln (geographical distance) and $F_{ST}/(1 - F_{ST})$ as suggested by Rousset (1997) for populations in two-dimensional space. The significance of the relationship was assessed using a Mantel test with 9999 randomizations in the R PACKAGE version 3.02 (Casgrain *et al*. 2001).

Results for the level of population genetic structure based on nuclear microsatellite loci were compared to those from mtDNA (Kimura *et al*. 2002) using samples from the same geographical regions and, where possible, the same sampling sites. MtDNA has a maternal mode of inheritance, and therefore has an effective population size four times smaller than that of biparentally inherited nuclear DNA (Birky *et al*. 1989). To account for the effect that this has on F_{ST} values estimated using the different markers, we applied a correction to mtDNA F_{ST} values according to the formula of Crochet (2000), where $F_{ST(mitochondrial)} = 4F_{ST(nuclear)}/[1 + 3F_{ST(nuclear)}]$. We compared the corrected mtDNA values to those obtained from microsatellites.

Table 2 Microsatellite primer information. Fluorescent labels were attached to the 5′ end of the forward primer

Locus	Repeat motif	Primer sequence (5′–3′)	Fluor label	Allele sizes	No. alleles	H_O	H_E
*Wp*C6	$(CCAT)_{15}$	F-CCCTGTCTGTCCATCCTTGTG R-GTTTGATGCTGATGGGGTGGATATG	HEX	84–240	17	0.56	0.79
*Wp*D23	$(GATA)_{13}$	F-GCCTGATGAATCTGTGATCTC R-GTTTGCCTAATCTCTAATGATCC	ROX	96–212	16	0.52	0.76
*Wp*D30	$(CTAC)_{15}$	F-TACATAAACTCTGCCACACG R-GTTTGAGTATGCTTGTACCTAACC	6-FAM	154–200	15	0.78	0.84
*Wp*D4	$(GATA)_{14}$	F-GACAGCAAATTACTACGACAC R-GTTTGAGGGTGATGTGGGCATTAAC	6-FAM	174–278	14	0.42	0.75
*Dpμ*01 see Dawson *et al*. (1997)			6-FAM	142–180	19	0.46	0.92
*Dpμ*03 see Dawson *et al*. (1997)			NED	129–239	31	0.42	0.87
*Dpμ*05 see Dawson *et al*. (1997)			HEX	104–305	74	0.63	0.98
*Dpμ*16 see Dawson *et al*. (1997)			6-FAM	148–194	13	0.63	0.79

Isotope analysis

Feather sample preparation followed the methods of Kelly *et al*. (2001). Flight feathers were washed in detergent and rinsed thoroughly to remove oil, dirt and residual detergent, and were then oven-dried at 100 °C to remove water. A small piece (0.1–0.2 mg) of the distal end of a feather was removed and wrapped in a silver capsule. This capsule was dropped into a high-temperature reduction furnace (Finnigan TC/EA) interfaced through an open split (Finnigan MAT Conflo II) with a mass spectrometer (Finnigan MAT Delta plus XL). The reduction furnace was used to pyrolize feather samples at 1450 °C.

We express the ratio of stable hydrogen isotopes (H2/H1) in a sample as the parts per thousand (‰) deviation from standard mean ocean water (vSMOW = 0‰). We report this deviation in delta notation (δD), which was calculated as $\delta D = ((\text{hydrogen isotope ratio}_{sample}/\text{hydrogen isotope ratio}_{standard}) - 1) \times 1000$. Higher values of δD correspond with heavier isotope composition. Values of males vs. females were compared at sites where sample sizes were at least five for both sexes. Birds captured on the breeding grounds were sexed based on plumage differences and having either a cloacal protuberance (males) or a brood patch (females). Birds captured on the wintering grounds were sexed by plumage differences. The relationship between δD and latitude (°N) was assessed by least-squares regression.

Results

Linkage disequilibrium, Hardy–Weinberg equilibrium and genetic variation

Linkage disequilibrium was not detected for any locus pair (for all pairwise comparisons $P > 0.2$) with the exception of *Wp*D23 and *Wp*D30, where $P = 0.002$. This significant value was due to results from a single population, Quebec, where $P = 0.0002$, and therefore these two loci may be considered independent.

Three loci (*Dpμ*01, *Dpμ*03 and *Dpμ*05) had a significant deficit of heterozygotes across all or most populations (critical value following Bonferroni's correction for multiple comparisons, $P < 0.006$) (Table 2), suggesting the presence of null alleles at these loci. These loci were excluded subsequently from analyses estimating distance measures, but were included where indicated to increase power of exact tests of population differentiation. Some of the five remaining loci were out of Hardy–Weinberg equilibrium at some sampling sites; however, no sampling site had this effect across all five loci, therefore samples were not taken from substructured populations.

Estimates of expected heterozygosity (H_E) and allelic diversity (Table 1) were not significantly different among breeding populations following Bonferroni corrections for multiple comparisons (for $\alpha = 0.05$ and 28 pairwise *t*-tests, adjusted significance level is 0.0018. For H_E: $P > 0.04$ in all comparisons, and for allelic diversity: $P > 0.01$).

Population genetic structure

Population genetic structure was detected across the breeding range of Wilson's warbler, with the eastern population in Quebec having highly significant allele frequency differences across eight loci compared to all other populations ($P < 0.0001$ for all comparisons). These results were the same when the analysis excluded the three loci with potential null alleles (Table 3). California, Oregon, Washington and Colorado populations were significantly different in all comparisons using eight loci. However, when the analysis was restricted to five loci, most comparisons within western North America were no longer significant, except for some pairwise comparisons involving Oregon and Colorado.

Table 3 Exact tests of pairwise population differentiation. Calculations are based on five loci (above diagonal) and eight loci (below diagonal)

	JUN	BC	AB	WA	OR	CA	CO	PQ
JUN	—	0.173	0.066	0.590	0.129	0.187	0.019	< 0.001
BC	0.049	—	0.175	0.226	0.028	0.172	0.015	< 0.001
AB	0.049	0.097	—	0.375	0.010	0.723	0.021	< 0.001
WA	0.005	< 0.001	< 0.001	—	0.046	0.807	0.032	< 0.001
OR	< 0.001	0.007	< 0.001	< 0.001	—	0.230	0.018	< 0.001
CA	< 0.001	< 0.001	0.043	< 0.001	< 0.001	—	0.018	< 0.001
CO	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	—	< 0.001
PQ	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	—

An estimate of global F_{ST} (across five loci) indicates significant but low population structure across all populations (F_{ST} = 0.035, 95% confidence limits 0.007–0.091). All estimates of population structure showed the Quebec population to be the most differentiated, with all pairwise F_{ST} values being significantly positive and above 0.12 (Tables 4 and 5). When considered separately, there was little population structure among the western populations (F_{ST} = 0.005, 95% confidence limits 0.001–0.009), and the degree of genetic structure as indicated by pairwise F_{ST} estimates were mostly low, and generally not significantly different from zero (Table 4). Only two other pairwise comparisons had significantly positive pairwise F_{ST} values (Grand Mesa, Colorado vs. Juneau, Alaska and Siuslaw, Oregon vs. 100 Mile House, British Columbia) (Table 4). The additional distance measures also indicate a generally low degree of differentiation among western sites (Table 5), although higher pairwise D_{LR} values associated with Colorado population suggested some differentiation (Table 5). There was no significant isolation-by-distance effect detected among western populations (Mantel test: r = 0.17, P > 0.2) (Fig. 2). We attempted to use the program STRUCTURE (Pritchard *et al*. 2000) as a method to infer population structure. The first step in this method is to determine if there are genetically similar groups without using population information. This method failed to reveal any clear genetically defined clusters that agreed with geographical labels (results not shown). This provides a further indication of the lack of geographical population structure in the western portion of the species, or lack of power to detect structure with the number of samples and loci.

Comparisons of genetic structure calculated using corrected and uncorrected mtDNA F_{ST} values and microsatellite F_{ST} values are shown in Table 6. When the Quebec population in eastern North America is included in the calculations, the corrected mtDNA F_{ST} value is comparable to the nuclear microsatellite F_{ST} value. However, when western North American sampling sites are analysed separately the corrected mitochondrial estimate of F_{ST} is seven times higher than that estimated from nuclear microsatellite data (Table 6).

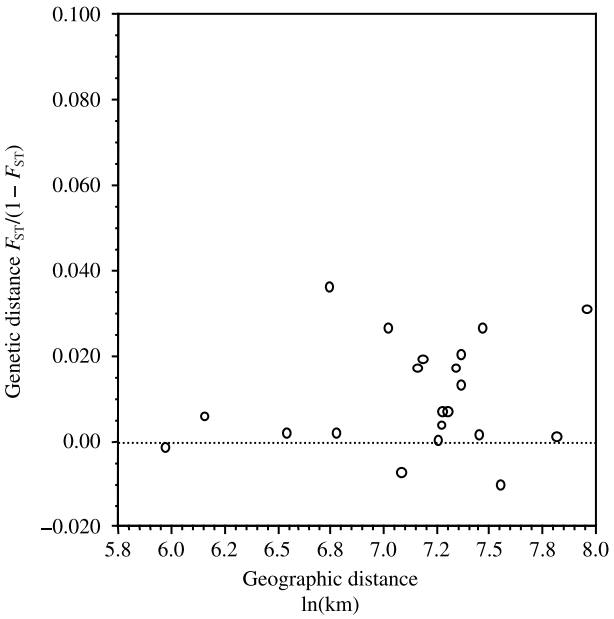

Fig. 2 Graph of geographical distance vs. genetic distance among western sampling sites. No isolation-by-distance relationship is evident.

Table 4 Pairwise F_{ST} between sampled populations

	JUN	BC	AB	WA	OR	CA	CO
BC	0.026						
AB	0.020	0.002					
WA	0.005	0.014	0.002				
OR	0.018	0.035*	0.019	0.006			
CA	0.012	0.013	–0.010	–0.007	0.004		
CO	0.030*	0.026	0.017	0.007	0.007	0.017	
PQ	0.134*	0.156*	0.145*	0.125*	0.130*	0.129*	0.138*

*Values significantly different from zero following tablewide corrections for multiple comparisons. Calculations are based on five loci and exclude the three loci that show evidence of null alleles.

Isotopic structure

There was a significant negative relationship between latitude and hydrogen isotope ratio, δD, for breeding

Table 5 Pairwise genetic distances. Nei's standard genetic distance (DS, Nei 1972) (above diagonal) and likelihood ratio genetic distance (D_{LR}, Paetkau *et al*. 1997) (lower diagonal). Calculations are based on five loci and exclude the three loci that show evidence of null alleles

	AK	BC	AB	WA	OR	CA	CO	PQ
AK		0.20	0.17	0.14	0.17	0.16	0.20	0.66
BC	−0.38		0.09	0.14	0.19	0.14	0.15	0.71
AB	0.18	−0.14		0.08	0.12	0.05	0.11	0.64
WA	−0.17	−0.33	−0.12		0.09	0.07	0.10	0.59
OR	0.02	−0.09	0.40	0.01		0.09	0.08	0.62
CA	0.14	−0.03	−0.23	−0.21	0.02		0.13	0.60
CO	0.49	0.39	0.47	0.19	0.18	0.44		0.63
PQ	3.22	3.85	4.29	3.64	4.74	3.14	4.29	

Table 6 Comparisons of global F_{ST} estimates based on microsatellite and mitochondrial data. The corrected values of mtDNA F_{ST} account for the smaller effective population size of mitochondrial DNA markers (Crochet 2000)

F_{ST}	MtDNA uncorrected	MtDNA corrected	Msat DNA
All populations	0.119	0.033	0.035
Western populations only	0.127	0.035	0.005

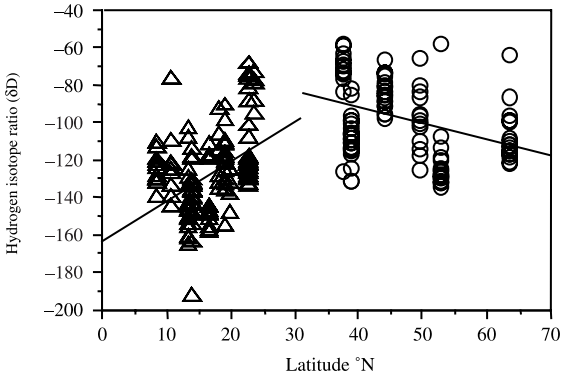

Fig. 3 Regression of δD values on latitude. Each data point represents a value for a single individual. Separate regression lines are shown for wintering ground samples (triangles) and breeding ground samples (circles).

populations of Wilson's warbler (Fig. 3). This relationship was evident when we used data from six breeding populations ($y = -0.87x - 57.19$, $n = 117$, $r^2 = 0.137$, $P < 0.0001$) and when we excluded two high-elevation/rain shadow sites (Colorado and Alberta) ($y = -1.33x - 24.1$, $n = 72$, $r^2 = 0.47$, $P < 0.0001$). These relationships are comparable qualitatively to that described by Kelly *et al*. (2001), where different samples and locations were used (breeding ground regression: $y = -1.8x + 0.1$). A statistical comparison of the regressions shows a significant difference between that from Kelly *et al*. (2001) and the regression using all six populations (ANCOVA: $F = 16.04$, 177 d.f., $P < 0.0001$), but not when the Colorado and Alberta sites are excluded ($F = 0.21$, 132 d.f., $P > 0.6$). There were no significant differences in isotope values for males vs. females in sampling sites that had sufficient sample sizes of each sex (unpaired *t*-tests, see Table 1 for location codes, AK: $t = 2.1$, $P > 0.05$; OR: $t = 0.98$, $P > 0.3$; CA: $t = 0.60$, $P > 0.5$; CO: $t = -0.57$, $P > 0.5$; COA: $t = 0.80$, $P > 0.4$, HON: $t = -1.24$, $P > 0.2$; ES: $t = -0.40$, $P > 0.6$, TAM: $t = -0.78$, $P > 0.4$).

A significant positive relationship between δD and latitude was evident in birds caught on the wintering grounds ($y = 2.13x - 162.9$, $n = 139$, $r^2 = 0.184$, $P < 0.0001$) (Fig. 3). Additional isotope patterns emerged from the overwintering samples. First, birds from Baja California and western Mexico tended to have heavier isotope ratios than those in eastern Mexico at the same latitude. For example, samples from Baja California Sur and Sinaloa (western Mexico) are considerably heavier (less negative) than those from Tamaulipas (eastern Mexico; Table 1). Second, it is apparent the values of wintering birds were generally lighter (more negative) than the birds sampled on the breeding grounds (Table 1 and Fig. 3).

Discussion

In Wilson's warbler the molecular genetic markers reveal structure in an east–west orientation, whereas isotope analysis reveals structure in a north–south orientation. The ability to distinguish genetically between eastern and western breeders on the wintering grounds, in combination with the determination of an approximate breeding latitude based on an isotopic signature, narrows down the possible breeding areas of origin. In the following sections, we discuss the details of these genetic and isotopic patterns and how this information sheds light on migratory patterns and population connectivity.

Microsatellite population genetic structure

On a continental scale there is evidence of genetic population structure in Wilson's warbler, with the eastern population differing from all populations in the west. In addition, the Grand Mesa population in Colorado was found to be significantly different in allele frequencies, as well as with some pairwise distance measures, suggesting that this population is differentiated from other western populations. A characteristic of this population that could contribute to this pattern is that it occurs both at the periphery of the main breeding range and is disjunct from other populations (Fig. 1). Otherwise, the level of microsatellite

population structure detected across the western breeding range was relatively low, as demonstrated by insignificant exact tests of population differentiation and low pairwise distance measures. These genetic results are only partially concordant with described subspecies ranges. The eastern population is genetically differentiated and is described as a separate subspecies (*W. p. pusilla*), however, the western sampling sites (which show little genetic differentiation) cover the ranges of two morphological subspecies (*W. p. chryseola* and *W. p. pileolata*) (Ammon & Gilbert 1999). The microsatellite genetic structure described is therefore only partially concordant with designated subspecies, a result that has also been found using mitochondrial DNA variation (Kimura *et al*. 2002).

The low level of population genetic structure detected among western Wilson's warbler populations may be the result of a combination of processes. Post-pleistocene population expansions from refugial, genetically bottlenecked populations have been invoked to explain the lack of mtDNA structure in a number of species (Gill *et al*. 1993; Zink 1996; Milá *et al*. 2000). Kimura *et al*. (2002) suggested that the current patterns of mtDNA variation in Wilson's warbler could be the result of expansion and mixing of populations from a number of southern refugia. However, the lack of structure detected using microsatellite variation may also be influenced by current gene flow. Hutchison & Templeton (1999) propose a method to assess the relative importance of genetic drift and gene flow in nonequilibrium populations. According to their model, there should be no relationship between geographical and genetic distances and overall low variance in genetic differentiation following a demographic expansion. This pattern is predicted to persist if ongoing gene flow remains relatively high over time. If a species distribution does not subsequently become fragmented, isolation by distance patterns are expected to appear (Hutchison & Templeton 1999). In Wilson's warbler, the lack of relationship between geographical and genetic distance and apparently low variance in genetic differentiation among populations is consistent with the hypothesis that long-distance gene flow among western populations has played an ongoing role in limiting genetic differentiation in the west. It should be noted, however, that decisions about the level of variance should include a comparison with another region. A larger sample from the eastern part of the distribution of Wilson's warbler would allow such a comparison. While ecological information on low levels of natal philopatry in Wilson's warbler (between 0% and 11%, Chase *et al*. 1997) is consistent with the idea of continuing gene flow, a full assessment of the importance of gene flow requires estimates of effective population size and the proximity of the population to equilibrium.

If a high level of contemporary gene flow is the dominant process resulting in lack of structure detected at microsatellite loci in Wilson's warbler, then the application of microsatellite markers to questions of connectivity would require large sample sizes and many more loci to increase the power to detect any slight differentiation. In any case, assaying microsatellite variation will probably not be a sufficient approach on its own to associate birds from particular breeding sites with particular overwintering sites.

Microsatellites vs. mitochondrial DNA variation

The results described above for microsatellite DNA are qualitatively similar to Kimura *et al*.'s (2002) mtDNA study in Wilson's warbler, where a divergent well-supported eastern lineage was identified in North America, although sampling between the two areas was insufficient to identify the geographical location of the east–west split. When focusing on western populations only, the quantitative estimates of differentiation appear to differ for the two types of markers. In contrast to the microsatellite results, Kimura *et al*. (2002) detected a significant amount of among-population mtDNA variance in the western USA and Canada ($F_{ST} = 0.127$, $P < 0.0001$). At microsatellite loci, pairwise distance measures indicated that the Colorado population may be slightly differentiated, but no other populations were obviously different. Some difference in estimates from the different types of markers is expected. Although microsatellites generally have higher mutation rates than mtDNA (Goldstein *et al*. 1995) the maternal mode of inheritance for mtDNA means that it has a smaller effective population size, faster geographical sorting of variation and could therefore have higher estimates of differentiation (Birky *et al*. 1989). However, even after accounting for the difference in mode of inheritance among western populations, microsatellite estimates remain seven times lower than that detected by mtDNA (Table 6). Estimates of relative measures of differentiation using microsatellites can be underestimated even when gene flow is low because within-population heterozygosity may be almost as high as the proportion in the total population (Hedrick 1999; Balloux & Lougon-Moulin 2002). However, it is unlikely that the low F_{ST} values observed among western Wilson's warblers populations are an artefact of using highly variable markers, because exact tests of population differentiation and alternative distance measures also suggested that there is a low degree of geographical population structure.

When making the comparisons discussed above, one important consideration to bear in mind is that mtDNA represents a single locus. If each microsatellite locus is considered separately, then one locus (D4), has an F_{ST} range of 0.009 ± 0.024. The upper end of the standard error (0.033) is comparable to that from the adjusted mtDNA value. Therefore, whether or not the apparent difference in magnitude of F_{ST} values is biologically meaningful is debatable in this case. In other studies, differences in the level of

structure inferred from mitochondrial vs. nuclear markers have been attributed to sex-biased dispersal, because male-biased gene flow tends to homogenize nuclear variation and female-biased dispersal tends to homogenize mitochondrial variation (e.g. Melnick & Hoelzer 1992; Paetkau *et al*. 1998; Gibbs *et al*. 2000; Haavie *et al*. 2000; Piertney *et al*. 2000; Scribner *et al*. 2001). In most of these cases, inferences from the genetic data have been consistent with behavioural observations on the dispersing sex. In birds, the general trend is for females to disperse (Weatherhead & Forbes 1994), which would tend to homogenize mtDNA variation. However, a study of genetic structure in yellow warblers (*Dendroica petechia*) reported higher mtDNA structure vs. microsatellite structure and attributed this to male-biased gene flow, despite ecological data that suggests that female yellow warblers disperse (Gibbs *et al*. 2000). Similarly, in Wilson's warbler it is possible that the level of male gene flow is higher than previously appreciated. A much larger microsatellite data set would help to determine how often single-locus nuclear estimates of differentiation approach mtDNA estimates, and therefore whether or not such a biological interpretation is warranted.

Isotopic structure

The feathers sampled from adult Wilson's warblers on the breeding grounds contain the isotopic signature of the latitude in which they were grown the previous year. The significant relationship between latitude and hydrogen isotope ratios across the breeding grounds indicates therefore that many birds were sampled at the approximate latitude where they spent the previous breeding season. This is consistent with ecological information on breeding site philopatry, with high rates of return reported for males, although not for females (Ammon & Gilbert 1999). Information on the age of adults sampled on the breeding grounds is not available for this study, however, it is possible that older adults exhibit higher site philopatry than younger adults. Isotope analysis of different age groups may provide a way of assessing differences in levels of philopatry between age groups (e.g. Graves *et al*. 1999).

The relationships between δD and latitude were qualitatively comparable to that described by Kelly *et al*. (2001), where a limited number of museum specimens of varying ages were used. When the study of Kelly *et al*. (2001) was statistically compared to this study, differences in the regression slopes were seen when the Colorado and Alberta sites were included in the comparison. The δD shifts observed in samples from these two sites is likely to be due to the elevational and continental (rainshadow) effects on the δD of feathers. The elevation effect on hydrogen isotope ratios has been well documented for precipitation, with heavy isotopes precipitating out preferentially at higher altitudes leaving behind successively lighter moisture (Dansgaard 1964; Rosanski *et al*. 1993; Graves *et al*. 1999; Poage & Chamberlain 2001). This highlights the importance of sampling from continental and high altitude areas when assessing isotope variation in widely distributed species such as Wilson's warbler.

Population connectivity and leapfrog migration

The broad-scale east–west population structure revealed here using microsatellites and previously with mtDNA provides important information when combined with isotopic data. All but a few of the samples from overwintering individuals used for isotope analysis were genetically identified as western breeders using mtDNA (Kimura *et al*. 2002), thereby reducing the geographical context to the western USA and Canada. When these data are combined with the positive relationship between latitude and hydrogen isotope ratios at overwintering sites, it suggests that birds that breed in the northernmost part of the western range leapfrog over the more southerly breeding populations to winter in the southernmost parts of the winter range. By demonstrating that individuals were of western origin using genetic markers in addition to using larger numbers of wintering samples, we are able to confirm, in a more precise fashion, a pattern of leapfrog migration suggested previously from a limited amount of isotopic data (Kelly *et al*. 2001). Isotopic information on it's own is insufficient to demonstrate leapfrog migration in a broadly distributed species such as Wilson's warbler. This is because isotope clines across North America correspond only approximately to latitude, and the difference in isotope value between an individual from sites at the same latitude in eastern and western North America can be large, because of the effects of altitude (Rocky Mountains) on δD. Thus, by combining molecular and isotopic markers to resolve both north–south and east–west population subdivision leapfrog migration can be confirmed. Leapfrog migration has been detected in some groups by identifying the distribution of species or subspecies on breeding and wintering grounds (e.g. fox sparrows: Wetmore 1926; Charadrii shorebirds: Boland 1990), as well as by using information on latitudinal clines in morphometric traits (Berthold 1993). However, combining genetic and isotopic methods may provide a better way of detecting leapfrog migration within species.

Our genetic and isotopic data suggest a further association between breeding and overwintering areas. Individuals wintering in western Mexico (Sinaloa and Baja California Sur) tended to have heavier δD values than those at comparable latitudes in eastern Mexico (Tamaulipas). This pattern could be the result of coastal breeding birds wintering in western Mexico and birds from western interior breeding regions wintering in eastern Mexico. However, due to the lack of strong genetic structure

detected in the west, it is not possible to resolve this pattern further.

While the combination of genetic and isotopic information revealed general associations between different geographical regions used by Wilson's warbler throughout the annual cycle, it remains difficult to make more precise statements about the degree of population connectivity. This may be rectified in future studies by additional sampling. In general, the samples from the breeding grounds had heavier δD values than overwintering birds, a difference possibly resulting from the summer samples being taken primarily from lower elevation coastal populations. In contrast, the overwintering samples are more likely to include birds from across the breeding range, thereby representing a greater range of δD values. In particular, it is likely that the wintering sample included individuals from breeding areas in Alaska and Canada, where densities are high (Sauer *et al*. 2001). If the regression lines are used to infer breeding origin of overwintering individuals, the predicted breeding latitude of the most southerly overwintering individuals would be above 70° N latitude. This is clearly unlikely, given that the breeding range barely extends to this latitude. It is probable that these overwintering birds breed at high latitudes (> 60° N) in the interior of Alaska and Canada, where more negative isotope values are the consequence of rain shadow effects. Additional sampling would help to resolve this question. The application of other stable isotopes such as carbon could also provide further resolution of latitudinal patterns (e.g. Rubenstein *et al*. 2002). The second limitation is that each method provided resolution only at broad geographical scales. Therefore, even the combination of genetic and isotopic information employed here does not provide the kind of fine-scale population resolution required to make precise statements about connectivity.

Evolutionary and conservation implications

The combination of genetic and isotopic information has revealed important information about population connectivity and confirmed a leapfrog migration pattern in Wilson's warbler. Due to the changes in morphology, physiology and life-history characteristics that are associated with variation in migration distance, leapfrog migration has important evolutionary implications (Berthold 1993). The physiological demands of long-distance migration may promote differences among populations at different latitudes, and result in a cascade of ecological and morphological effects, such as a shorter breeding season and faster development time in northern populations (Berthold 1999). The results from this study therefore provide a starting point to test alternative models of the evolution of leapfrog migration (Greenberg 1980; Cox 1985; Holmgren & Lundberg 1993) in addition to examining the role of such a pattern in generating and maintaining intraspecific variation in ecology and morphology in a widespread Neotropical migrant. While overall size and plumage differences among Wilson's warbler subspecies have been described, it would be interesting to look for clinal variation, especially with respect to character traits that may be important for efficient migration over long distances, such as wing shape (Lockwood *et al*. 1998).

From a conservation standpoint, the associations between breeding and wintering areas identified in this study provide a starting point to examine the effects of region on demographic processes. The southern parts of the overwintering range are probably more important for the numerically common, northern breeding individuals in western North America. Conversely, the northern parts of the overwintering range are probably more important for the more southerly breeding birds, which are less common and occupy patchier breeding habitat. Therefore, if characteristics of wintering grounds affect breeding ground productivity, then efforts to maintain southern overwintering habitats may have greatest conservation benefit in terms of maintaining population size, while a focus on northern parts of the overwintering range may contribute to maintenance of population diversity. The descriptions of connectivity are still at a relatively coarse geographical scale, however, application of the methods used for Wilson's warbler has the potential to provide basic information needed to address population declines in neotropical migrants in general.

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