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DIFFERENTIAL MIGRATORY TIMING OF WESTERN POPULATIONS OF WILSON'S WARBLER (*CARDELLINA PUSILLA*) REVEALED BY MITOCHONDRIAL DNA AND STABLE ISOTOPES

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Abstract.—Molecular markers and stable isotopes have provided important insights into the migratory connectivity of small landbirds. Research integrating these two methods has primarily focused on linking breeding and wintering sites, rather than focusing on timing of migratory movement of different breeding populations. We used mitochondrial DNA and isotopic markers to infer the timing of various breeding populations of migrating Wilson's Warblers (*Cardellina pusilla*) moving through a migratory stopover site, demonstrating the value of multiple sources of information in estimating the origin of migrants. Using mixed-stock analysis, we found that early spring migrants sampled in southwestern Arizona were dominated by warblers migrating to the West Coast of the contiguous United States, whereas later migrants included a large pulse of birds migrating to Alaska and western Canadian provinces. Stable hydrogen isotope data from individual birds showed the same timing pattern as genetic data. Had we used stable isotopes alone, we would not have been able to infer whether birds later in the migration season were most likely migrating to Alaska or the Interior West, given the large overlap in isotope values between those regions. The lack of mitochondrial group 2, common in the Interior West, in late-season migrants strongly suggests that these birds were migrating to breeding areas in Alaska or other northern regions. Studies that reveal the timing of migration of different breeding populations through stopover sites lay the foundation for more in-depth examination of seasonal interactions between migration and the stationary phases of the annual cycle. *Received 18 June 2013, accepted 13 September 2013.*

Key words: *Cardellina pusilla*, migration timing, migratory connectivity, mitochondrial DNA, mtDNA, stable isotopes, stopover ecology, Wilson's Warbler.

Sincronización Migratoria Diferencial en las Poblaciones Occidentales de *Cardellina pusilla* Revelada por ADN Mitocondrial e Isótopos Estables

Resumen.—Los marcadores moleculares y los isótopos estables han revelado aspectos importantes de la conectividad migratoria en aves terrestres pequeñas. Las investigaciones que integran estos dos métodos se han enfocado principalmente en la conexión entre los sitios de invernada y de reproducción, en vez de enfocarse en la sincronización de los movimientos migratorios en diferentes poblaciones reproductivas. Usamos ADN mitocondrial y marcadores isotópicos para inferir el tiempo de migración de varias poblaciones reproductivas de *Cardellina pusilla* que pasan por un sitio de parada migratoria, demostrando el valor de múltiples fuentes de información para estimar el origen de los migrantes. Usando modelos de acervos mixtos, encontramos que los migrantes del principio de la primavera en el sureste de Arizona eran principalmente aves que se dirigían a la costa oeste de los Estados Unidos, mientras que los migrantes tardíos incluyeron una gran cantidad de aves migrando hacia Alaska y las provincias canadienses occidentales. Los datos de isótopos estables de hidrógeno de aves individuales mostraron el mismo patrón de sincronización migratoria que los datos genéticos. Si hubiésemos usado únicamente los datos de isótopos estables, no habríamos podido determinar si las aves de finales de la temporada migratoria migraban más probablemente hacia Alaska o al occidente interior, dada la alta superposición en los valores de isótopos de esas regiones. La falta del grupo mitocondrial 2 en los migrantes al final de la temporada, el cual es común en el occidente interior, sugiere fuertemente que estas aves migraban hacia áreas reproductivas en Alaska o en otras regiones del norte. Los estudios que revelan la sincronización migratoria de diferentes poblaciones reproductivas a través de los sitios de parada migratoria sientan las bases para examinar más profundamente las interacciones estacionales entre la migración y las fases estacionarias del ciclo anual.

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Knowledge of migration routes and timing of movement between breeding and nonbreeding regions is essential for a full understanding of the ecology, evolution, and conservation of migratory species (Moore et al. 1995, Webster et al. 2002, Smith et al. 2005, Irwin et al. 2011, Ryder et al. 2011, Wilson et al. 2011), yet relatively little is known about patterns of migratory connectivity within most species of birds. Analyses of morphological variation (e.g., subspecies differences) and band recoveries have revealed some patterns of migratory connectivity over the past century, but only in species with strong geographic variation in morphology or a high recovery rate of banded birds (Ramos and Warner 1980, Bell 1997, Bairlein 2001). Moreover, the large geographic area over which migrants move during migration has limited the use of these methods in identifying migration routes and timing of migration for specific populations. Yet population trajectories for many migratory species vary across the breeding range (North American Bird Conservation Initiative Canada 2012), underscoring the need for knowledge of connectivity between migratory and stationary phases of the annual cycle.

During the past two decades, our ability to discern patterns of within-species migratory connectivity has dramatically increased with the use of intrinsic markers such as isotopes and genetic markers (Webster et al. 2002, Veen 2013). Genetic and isotopic analyses to date have revealed different components of geographic variation in Nearctic–Neotropic migrants. Molecular markers have revealed strong genetic differences primarily between eastern and western populations, with less structure within groups (Smith et al. 2005). By contrast, the latitudinal gradient in hydrogen isotope ratios across North America (Bowen et al. 2005) has allowed for inferences to be made regarding movement of breeding populations along a north–south gradient (Hobson and Wassenaar 2008). However, the level of resolution is limited for each individual marker, which results in large uncertainty of migratory connectivity for most species. The integration of genetic and isotope markers allows finer-scale geographic resolution (Clegg et al. 2003, Boulet et al. 2006, Rundel et al. 2013).

Most research on migratory connectivity has focused on linking wintering and breeding populations (e.g., Clegg et al. 2003, Irwin et al. 2011, Rundel et al. 2013), with comparatively little attention given to inferring the routes and migration timing of birds actually sampled during migration itself (but see Kelly et al. 2002, Kelly 2006, Paxton et al. 2007). Here, we utilize both genetic markers and stable isotopes to better understand which breeding populations of Wilson's Warbler (*Cardellina pusilla*) are moving through a stopover site during their northward migration, as well as the relative timing of movement of those populations. The extensive background on patterns of variation in genetic markers and stable hydrogen isotopes for this species (Kelly et al. 2002, Kimura et al. 2002, Clegg et al. 2003, Smith et al. 2005, Paxton et al. 2007, Irwin et al. 2011, Rundel et al. 2013) allows us to build upon results from past studies to enhance our understanding of migratory connectivity. Previous studies of molecular variation have shown strong differentiation between western and eastern breeding regions of Wilson's Warblers, specifically in terms of mitochondrial DNA (mtDNA; Kimura et al. 2002), up to nine microsatellites (Clegg et al. 2003, Rundel et al. 2013), and 257 nuclear DNA markers (AFLPs; Irwin et al. 2011), with less structure within regions than between. Here, we show that by focusing on inferring the breeding regions of groups of birds (rather than specific individuals), mtDNA variation is much more informative than previously thought (Kimura et al. 2002, Clegg et al. 2003) in inferring patterns of migratory connectivity within the western breeding group.

We first reexamined genetic structure within western breeding populations, using mtDNA data presented by Kimura et al. (2002), and designed a method for assignment of individuals to a variety of mitochondrial haplotype groups. We then determined mitochondrial group membership and feather hydrogen isotope values of individuals sampled during spring migration at a stopover site in southwestern Arizona, with the goal of inferring breeding regions of groups of individuals moving through at different times of the season. Specifically, we examined how mitochondrial haplotype groups changed across the migratory season and corresponded to changes in stable hydrogen isotope values collected from the same individuals. Previous studies with stable isotopes have indicated that Wilson's Warblers have a "leap-frog migration" in which the more northern breeders migrate southward earlier in the fall, winter farther south, and migrate northward later in the spring (Kelly et al. 2002, Clegg et al. 2003, Paxton et al. 2007). However, precise assignment of birds to particular breeding regions in western North America on the basis of stable hydrogen isotopes alone has been confounded by the influence of elevation and other geographic factors on stable hydrogen isotope values (Ingraham 1998). This study illustrates that genetic and isotopic analysis can be useful in understanding the movements of various breeding groups through migratory stopover sites, enhancing our ability to understand movements during the full annual cycle of migratory birds.

Methods

Sampling at stopover site.—Wilson's Warblers were sampled during spring migration at Cibola National Wildlife Refuge (NWR; 33°18′N, 114°41′W; elevation 60 m) in La Paz County, Arizona. Located on the main branch of the lower Colorado River, Cibola NWR is composed of narrow fragmented strips of riparian vegetation adjacent to the river corridor, surrounded by desert upland and agricultural fields (Anderson et al. 2004, Paxton et al. 2008). We passively sampled warblers with mist nets daily between 17 March and 16 May in 2008 and 2009. We banded all warblers captured with a federal aluminum bird band and recorded standard morphological measurements. For stable isotope analysis we pulled two rectrices (R4 on each side) and stored feathers in labeled, sealed envelopes until analysis. Wilson's Warblers molt their feathers on their breeding grounds prior to fall migration (Kelly et al. 2002, Paxton et al. 2007); thus, isotope characteristics of a bird's feather collected during spring migration should reflect that bird's previous breeding region. For genetic analysis, we took a blood sample via the brachial vein and mixed a drop of whole blood in Queen's lysis buffer (0.01 M Tris, 0.01 M NaCl, 0.01 M EDTA, 1% *n*-lauroylsarcosine, pH 7.5; Seutin et al. 1991). Samples were stored on ice and then frozen within 6 h of collection. Later, in the laboratory, DNA was extracted using standard phenol-chloroform extraction and diluted to a working concentration of 25 ng μL^{-1}.

Mitochondrial typing.—We closely examined Wilson's Warbler mitochondrial control-region sequences (343 base pairs [bp]) that were originally generated and summarized by Kimura et al. (2002). Sequences were sent to us directly by Mari Kimura,

who has attested to their validity. We identified three restriction enzymes (*BsaI*: zero or one cut site; *MspI*: zero or one cut site; and *StyD4I*: 2 or 3 cut sites) that could be used in combination to distinguish major groups of haplotypes (Fig. 1 and Table 1). The relative frequencies of these haplotype digestion groups differ strongly between different sampling regions (Table 2).

To amplify the control region, we used primers MT_H417 (5′-CGG TTC TCG TGA GAA GCG C- 3′), designed by us, and LGL2 (5′- GGC CAC ATC AGA CAG TCC AT- 3′) (Milá et al. 2007). Each 20-μL reaction consisted of 1.5 μL of template DNA (at 25 ng/μL), 2 μL of 10X polymerase chain reaction (PCR) Buffer (Invitrogen, Carlsbad, California), 2.5 mM $MgCl_2$ (Invitrogen), 0.25 mM dNTPs (New England Biolabs [NEB], Ipswich, Massachusetts), 1.76 μL of each primer (at 10 μM), and 0.5 *U* of Taq polymerase (NEB). The thermal cycling protocol was 95°C for 3 min, 42°C for 60 s, 72°C for 60 s, followed by 10 cycles of 94°C for 60 s, 42°C for 60 s, 72°C for 60 s, followed by 32 cycles of 92°C for 60 s, 40°C for 45 s, 72°C for 45 s, followed by 72°C for 5 min. To ensure proper amplification, 5 μL of each product was run for 12 min on a 2% agarose gel in 0.5X TAE, along with 1 μL of 100 bp DNA size standard in a lane for comparison. The amplified product (376 bp, including primer sequences) appeared as a band next to the 400-bp size standard.

We divided each amplified product into three 5-μL aliquots, each of which was digested with a different restriction enzyme. In the first, we added 0.11 μL *BsaI* (10,000 U mL^{-1}; NEB), 0.45 μL of NEB Buffer 4, and 3.44 μL ddH_2O. In the second, 0.11 μL *MspI*

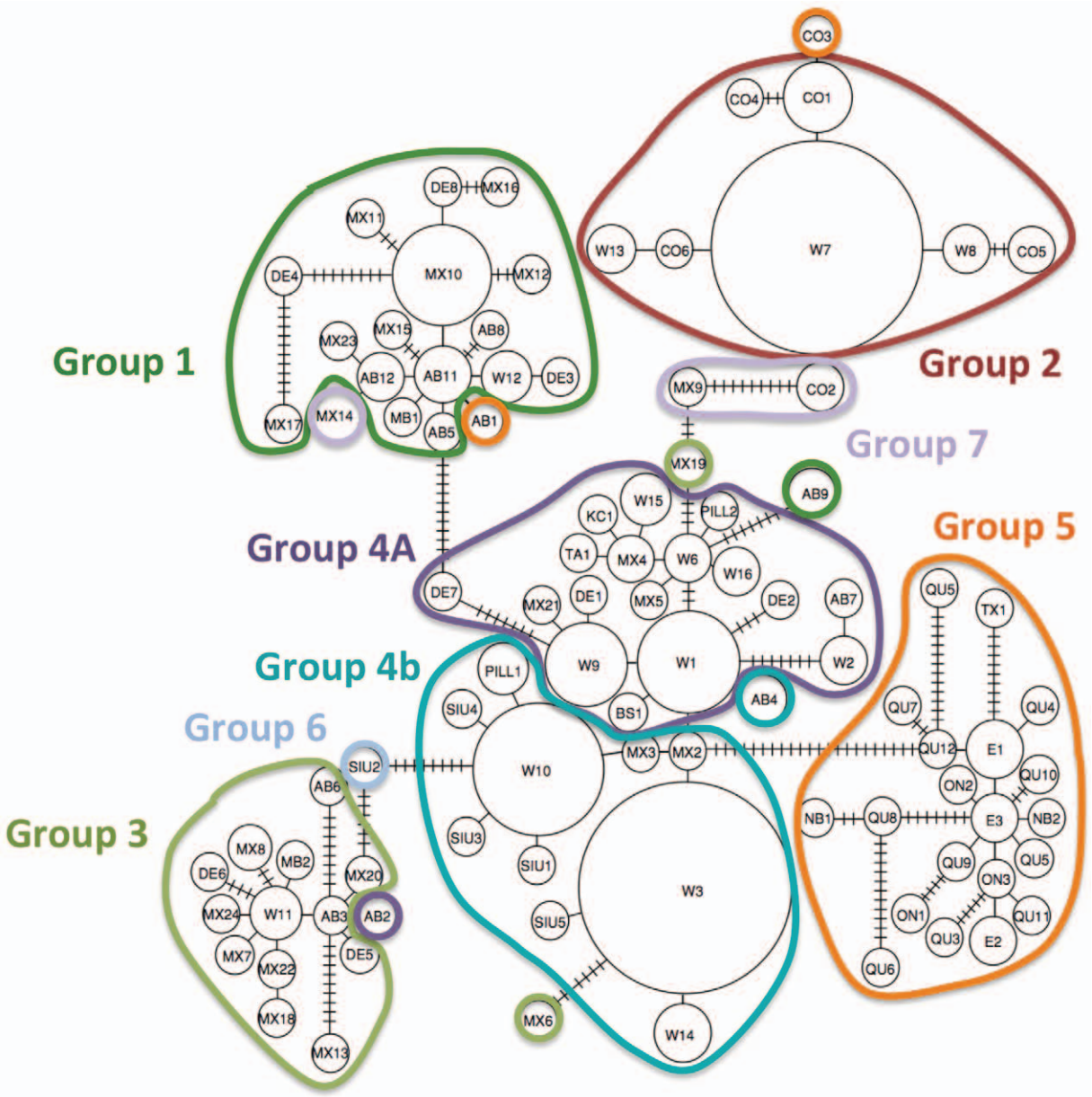

Fig. 1. Wilson's Warbler mitochondrial haplotype network presented by Kimura et al. (2002; their fig. 2), overlaid with restriction-digest group memberships (Table 1).

TABLE 1. Summary of restriction enzymes and banding patterns used to assign Wilson's Warblers to mitochondrial restriction digest groups.

	BsaI	*MspI*	*StyD4I*
Restriction enzyme	Two bands around 200 bp? (in addition to 400)	Bands at 300 and 100? (in addition to 400)	Bands just above and below 100? (in addition to 200)
Group 1	Yes	No	No
Group 2	No	No	Yes
Group 3	Yes	Yes	Yes
Group 4A	Yes	Yes	No
Group 4b	No	Yes	No
Group 5	No	No	No
Group 6	No	Yes	Yes
Group 7	Yes	No	Yes

(20,000 U mL^{-1}; NEB), 0.45 µL of NEB Buffer 4, and 3.5 µL ddH_2O were added. In the third, we added 0.11 µL *StyD4I* (10,000 U mL^{-1}; NEB), 0.45 µL of NEB Buffer 4, and 3.44 µL ddH_2O. Each digestion was then incubated for 2 h at 37°C, followed by 15 min at 70°C to terminate the reaction. All 9 µL of each digestion product was then run on a 3% agarose gel in 0.5X TBE, along with 1 µL of 100 bp DNA size standard in a neighboring lane.

In the *BsaI* digest, some individuals have a cut site (recognition sequence and cut sites: 5′-GGTCTCN/-3′ on one strand, and 3′-CCAGAGNNNNN/-5′ on the other, where "/" indicates the cut site) and others do not, due to a C-T single nucleotide polymorphism (SNP) located 189 bp from the MT_H417 end of the fragment. This results in some digests that produce two fragments of length 190/194 bp (the two numbers indicating the length of the two strands of DNA, due to the enzyme cutting at different locations on the two strands) and 186/182 bp. These two fragments tend to appear as a single band on the agarose gel, just below the 200-bp size standard, indicating the presence of the C variant at this SNP. The absence of a band near the 200-bp size standard indicated the T variant.

In the *MspI* digest, only some individuals have a cut site (5′-C/CGG-3′ on one strand, and 3′-GGC/C-5′ on the other) due to a G-A-T polymorphism located 278 bp from the MT_H417 end of the fragment. Thus, some digests have two fragments of length 275/277 bp and 101/99 bp. The presence of bands near the 300 and 100 size standards indicates the G variant at this SNP, whereas the absence of those bands indicates the A or T variants.

Finally, in the *StyD4I* digest, only some individuals have a cut site (5′-/CCNGG-3′ on one strand, 3′-GGNCC/-5′ on the other) located 259–263 bp from the MT_H417 end. Lack of that cut site is usually due to C-T-G polymorphism at 259 bp, although G-A polymorphisms were also observed at 262 bp and 263 bp (with the A variant observed in only one haplotype each). Cutting at that site results in a 118/113 bp fragment spanning from the LGL2 end, and the presence of a fragment just above the 100 bp size standard was taken to indicate the presence of this cut site.

TABLE 2. Mitochondrial digest groups names, digestion patterns (see Table 1 and Fig. 1), and frequencies among 13 sampled breeding sites grouped into four broad regions, inferred by analysis of sequences and haplotype distributions generated by Kimura et al. (2002).

Digest group:	1	2	3	4A	4b	5	6	7	
Digest pattern (*BsaI, MspI, StyD4I*):	ynn	nny	yyy	yyn	nyn	nnn	nyy	yny	Total
Boreal Forest									
Denali National Park, Alaska	3	0	3	9	0	0	0	0	15
Hinton, Alberta	6	0	2	3	1	1	0	0	13
Interior West									
Umatilla National Forest, Oregon	0	3	1	0	1	0	0	0	5
Tahoe National Forest, California	0	10	0	2	3	0	0	0	15
Kings Canyon National Park, California	0	7	0	2	3	0	0	0	12
Grand Mesa, Colorado	0	11	0	0	0	1	0	2	14
West Coast									
Mt. Baker National Forest, Washington	1	1	1	2	7	0	0	0	12
Siuslaw National Forest, Oregon	0	0	0	1	14	0	1	0	16
Pillar Point, California	0	0	0	5	12	0	0	0	17
Big Sur, California	1	0	0	5	2	0	0	0	8
East									
Camp Myrica, Quebec	0	0	0	0	0	16	0	0	16
Fredericton, New Brunswick	0	0	0	0	0	4	0	0	4
Hilliardton, Ontario	0	0	0	0	0	4	0	0	4

Most individuals also had another *StyD4I* cut site located 180–184 bp from the MT_H417 end of the fragment, resulting in a band of length 179/184 bp from the MT_H417 end, as well as bands of either 79/79 bp or 197/192 bp in the other direction (depending on whether the cut site above is present). Only the presence of the band just above 100 bp in size was diagnosed, because analysis of the sequences along with haplotype distribution data provided by Kimura et al. (2002) indicate that the cut site associated with that band was most geographically informative.

We decided to limit our analysis to the largest age and sex class, after-second-year (ASY) males, given the limits of time and budget. We focused on the samples collected in 2009, determining mitochondrial digest groups of 185 individuals. We also determined the mitochondrial groups of 62 individuals collected in 2008, so that we could compare patterns between years. Note that the 2008 samples to be genotyped were chosen with an intentional bias toward those with high or low isotopic ratios—hence, their haplotype distributions should not be viewed as a random sample.

Mixed-stock analysis. – We used a mixed-stock analysis to estimate the breeding destination of groups of migrating Wilson's Warblers on the basis of shared mtDNA haplotypes. We divided the breeding range into four regions (Boreal Forest, West Coast, Interior West, and East; Table 2) and divided the 2009 migratory season into weekly intervals. A mixed-stocked analysis estimates the frequency of mitochondrial haplotype groups for each breeding region based on samples collected across the breeding range (Table 2). Then, for each weekly group of migrants, the mixed-stock analysis determines the likelihood that observed mitochondrial haplotype groups within a group of migrants were contributed from each of the four defined breeding populations (Fournier et al. 1984). We emphasize that the mixed-stock analysis does not assign individual birds to breeding regions; rather, it provides maximum-likelihood estimates of the contribution of various breeding regions to a group of migrants, by comparing haplotype frequencies within the group with those in each breeding region. The greater the genetic differences among breeding groups, the stronger the inference, but estimates based on mixed-stock analysis are typically associated with large confidence intervals (Reynolds and Templin 2004). Mixed-stock analysis has been used widely in fisheries studies (Bolker et al. 2003, Okuyama and Bolker 2005) and occasionally in bird studies (Pearce et al. 2000, Paxton et al. 2011). We used a constrained maximum-likelihood approach with the mixed-stock package in the program R (Bolker 2012).

Isotopes.—Feathers were cleaned with a dilute detergent and then a 2:1 chloroform:methanol solution, following the method of Paritte and Kelly (2009). Feather material from the distal end (140 to 160 μg) of each sample was removed and wrapped in a silver capsule. All stable isotope analyses were conducted at the University of Oklahoma, using a comparative equilibrium approach with calibrated keratin standards to correct for uncontrolled isotope exchange between non-carbon-bound hydrogen in feathers and ambient water vapor (Wassenaar and Hobson 2003). As a result, values presented here are non-exchangeable feather hydrogen only. Stable hydrogen isotope ratios for both feathers and keratin standards were determined on H_2 gases, produced by high-temperature flash pyrolysis of feathers using a high temperature (1,450°C) pyrolysis elemental analyzer (TC/EA; ThermoFinnigan, Bremen, Germany) interfaced through an open split valve (Conflo III; ThermoFinnigan) with a ThermoFinnigan Delta V isotope ratio mass spectrometer. Repeat analysis of hydrogen isotope standards during the study yielded an external repeatability of ±2.1‰. Intra- and inter-assay error (± SD) were 2.2‰ and 3.6‰, respectively, based on repeat analysis of the same feather (n = 13). Stable hydrogen isotope ratios ($^2H/^1H$) are reported in delta (δ) notation, in per-mil units (‰), where δDsample = $[(R_{sample}/R_{standard}) - 1] \times 1{,}000$, in relation to a standard (Vienna standard mean ocean water [VSMOW]).

To examine how feather isotope values corresponded to latitudinal gradients in precipitation across western North America, we adjusted stable feather isotope values (δD_f) to reflect stable hydrogen isotope values of precipitation (δD_p) using the equation $\delta D_f = 1.06\ (\delta D_p) - 24.24$. The equation was generated using feather isotope values of breeding Wilson's Warblers from Paxton et al. (2007: Table 1) and stable hydrogen isotope values of precipitation from Bowen et al. (2005). Although statistical approaches have been designed to reduce uncertainty when mapping the origin of animals using stable hydrogen isotopes (Wunder and Norris 2008), our goal was not to directly map the origins of migratory birds, but to examine how broad-scale patterns of stable isotopes complement genetic data.

Results

We successfully designed a relatively efficient and inexpensive method, using PCR amplification followed by restriction digests, to assign Wilson's Warblers to major mitochondrial groups (Fig. 1). The geographic distributions (Fig. 2) of these mitochondrial groups documented by Kimura et al. (2002) could then be used to estimate the breeding destination of weekly samples of birds on migration.

Mitochondrial restriction-group membership of ASY males migrating through southwestern Arizona differed dramatically across the 2009 migration season, and in a similar way across the 2008 migration season. The mixed-stock analysis estimated that warblers migrating early in spring 2009 were dominated by breeding populations along the California, Oregon, and Washington coast (Figs. 2 and 3). In these early migrants, mitochondrial group 4b was most common, with 4A also at fairly high frequency (Fig. 2), consistent with the frequency distributions of mitochondrial groups 4b and 4A within the West Coast breeding group (66% and 25%, respectively; Table 2). The mitochondrial-group distribution of migrants shifted dramatically at about the halfway point of the migration season, on about 19 April, with group 4b decreasing in frequency and groups 1, 3, and 4A increasing in frequency (Fig. 2). The shift in the distribution of mitochondrial groups was reflected in the mixed-stock analysis, with the frequency of coastal breeding populations inferred to be migrating through the stopover site dramatically decreasing and populations breeding in the boreal forest in Alaska and Alberta increasing (Fig. 3). Altogether, the results suggest a midseason shift between birds migrating primarily to coastal California, Oregon, and Washington and those migrating farther north to western Canada and Alaska.

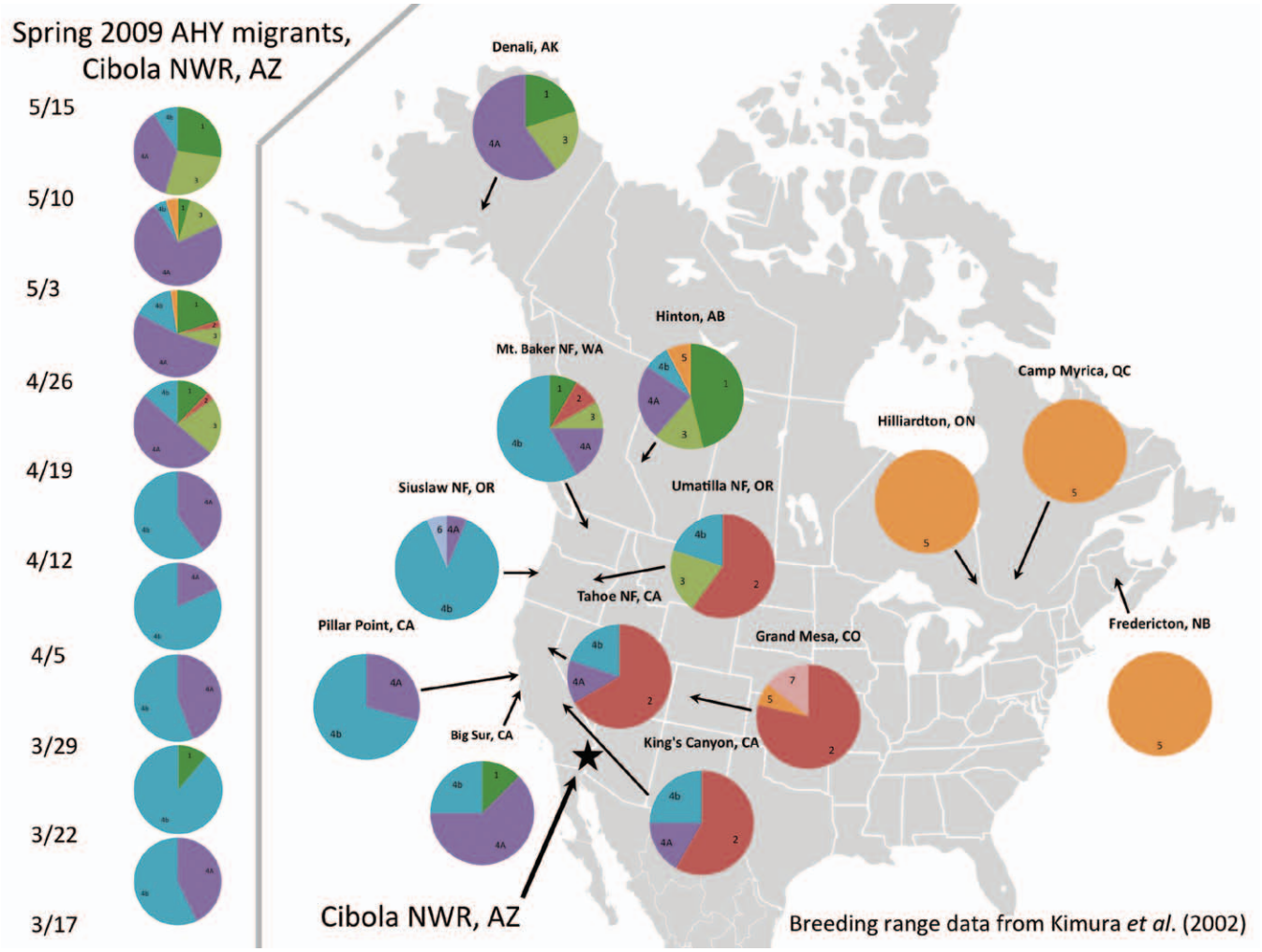

Fig. 2. Map of North America showing mitochondrial digest-group distribution at sampling sites (Table 1; based on data from Kimura et al. 2002) along with (left) weekly digest group distributions of spring 2009 after-hatch-year (AHY) migrants at Cibola National Wildlife Refuge, Arizona.

The dominant mitochondrial group of warblers breeding in the Interior West group (e.g., Colorado, eastern California, and eastern Oregon) was mitochondrial group 2 (Table 2). In our 2009 migrants, this mitochondrial group was found in only three individuals, suggesting that Interior West breeding populations are surprisingly rare migrants through southwestern Arizona (Figs. 2 and 3). Mitochondrial group 5, which is at very high frequency in eastern Canadian populations, was also very rare in our migratory samples, indicating that very few (if any) eastern birds migrate through the stopover site (Figs. 2 and 3). It should be noted that there are two rare haplotypes in Kimura et al.'s (2002) breeding-season data that have the same group-5 digestion pattern as the eastern mitochondrial group but are, in fact, related to western haplotypes in group 1 or 2 (Fig. 1); hence, the three group-5 birds in our study may not be eastern individuals.

Consideration of stable hydrogen isotope data supports and further clarifies these patterns (Fig. 4). Consistent with the mixed-stock analysis and mitochondrial group composition (4A and 4b), isotopic ratios early in the migration season tended to be more enriched (less negative isotope values), most consistent with a breeding destination along coastal California. Isotopic ratios declined gradually during the first half of the migration season, consistent with gradually more northern coastal breeding populations (e.g., Oregon) migrating through the stopover site. A fairly sudden shift in isotopic signatures, with a large pulse of birds with more depleted isotopic ratios, coincided with large increases in the frequencies of mitochondrial groups 1, 3, and 4A and a large decrease in the proportion of group 4b. Moreover, birds of mitochondrial group 3 had remarkably depleted isotopic ratios, lower than all of the isotopic ratios from the first half of the season.

Data from this study are available from Dryad (doi:10.5061/dryad.6t85v).

Discussion

The use of genetic markers to draw inferences about migratory connectivity of small Nearctic–Neotropic landbirds has been most useful in differentiating large-scale patterns between

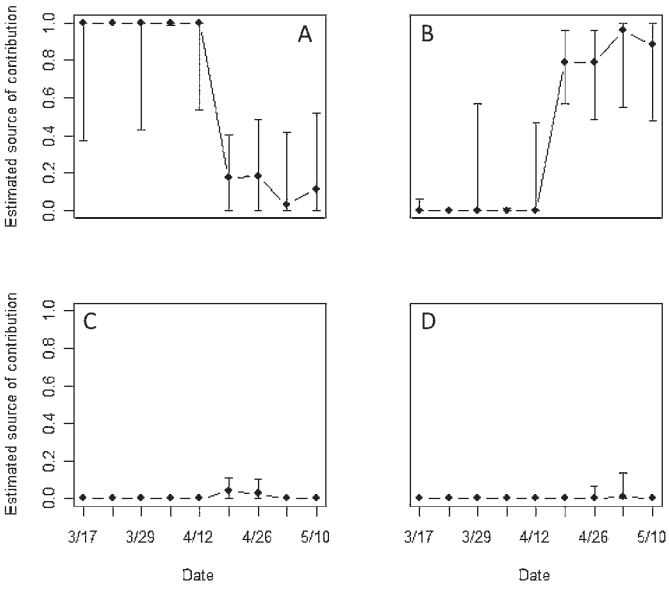

Fig. 3. Mixed-stock analysis showing the estimated contributions of the four Wilson's Warbler breeding areas, (A) West Coast, (B) Boreal Forest, (C) Interior West, and (D) East, to weekly groups of Wilson's Warblers captured during spring migration in southwestern Arizona. Bars represent 95% confidence intervals.

eastern and western populations (Kimura et al. 2002, Clegg et al. 2003, Smith et al. 2005, Irwin et al. 2011, Rundel et al. 2013). Here, we have shown that variation in mtDNA can be used to infer the likely breeding regions of groups of migrating Wilson's Warblers within western regions of North America. Early spring migrants sampled in southwestern Arizona are primarily migrating to southern West Coast regions, whereas migrants later in the season include a large pulse of birds migrating much farther north, to western Canada and Alaska. We can also infer with high confidence that very few, if any, of the warblers captured at our site are migrating to breeding regions in the Interior West of the United States (e.g., eastern California, eastern Oregon, and Colorado) or the northeastern part of the continent (e.g., Ontario, Quebec, and New Brunswick).

The results of our molecular genetic analysis show that geographic structuring in the frequencies of mitochondrial groups enables fairly strong conclusions regarding the likely breeding destination of groups of migrants moving through a stopover site. However, these conclusions are made with respect to populations of migrants, not individual birds. As Kimura et al. (2002) noted, the sharing of mitochondrial haplotypes among western populations precludes assignment of individual birds to breeding regions with a high degree of confidence. Although we cannot assign origin to individuals, the frequencies of haplotypes in a group of migrants can be used to estimate the probability that they belong to one or more breeding populations. For example, a single individual with mitochondrial group 4A sampled at the stopover site early in the migration season could only be assigned to a very broad breeding region, including coastal and interior California, coastal Oregon and Washington, the Canadian Rockies, and Alaska, because group 4A is common in all those regions. However, a group of early-season individuals consisting of roughly half of group 4A and half of group 4b can with more confidence be assigned to breeding destinations within a much smaller area, namely the West Coast of the contiguous United States. The lack of mitochondrial groups 2, 3, 1, and 5 in the group of early-season migrants excludes the other breeding regions for the majority of the group. Although mixed-stock analysis sometimes has large uncertainty in estimates (Reynolds and Templin 2004), our results suggest a large shift in source populations over a short period, resulting in high estimated precision and highlighting the strength of the mixed-stock analysis in inferring patterns of migratory connectivity from genetic data.

The stable isotope and genetic data from this study complement each other, strengthening conclusions in areas where uncertainty from just one technique would otherwise limit inferences. Stable isotope data from individual birds showed the same timing pattern as genetic data grouped into weekly intervals, which supports the assignment of groups of birds to western breeding regions using genetic markers. Specifically, an influx of more depleted stable hydrogen isotope values coincided with a shift in mitochondrial groups at the middle of the migration season, suggesting that birds captured at the stopover site were migrating to more northern breeding areas. However, the influence of elevation and other geographic variables on stable hydrogen isotope values (Ingraham 1998) confounds the use of stable hydrogen isotopes alone to assign later migrants to particular breeding regions in western North America. For example, the range of isotope values in Alaska (–80‰ to –140‰) also occur in southwest British Columbia, Washington, and interior Oregon and California. Thus, using stable isotopes alone, we would only be able to postulate (rather than confidently determine) that later migrants were most likely migrating to Alaska. Fortunately, the rarity of mitochondrial group 2 in migrants captured at the stopover site, common in the Sierra Nevada mountains of eastern California as well as eastern Oregon, reduced the uncertainty of assignment to the regions with overlapping isotope values, strongly suggesting that birds captured at the stopover site were migrating to breeding regions in Alaska and western Canadian provinces, not interior western regions. Compared with results drawn from stable isotopes alone, the genetic marker results allow stronger inferences about the timing of migration for specific populations in our study and the leapfrog migration pattern advanced previously by Paxton et al. (2007) at this stopover site.

Likewise, stable isotope data from migrating Wilson's Warblers highlight the need for more extensive sampling of genetic markers across the breeding range, especially in northwestern Canada and Alaska. Mitochondrial group 3 was found in low frequency across the breeding range sampled by Kimura et al. (2002) but was fairly common in our migratory stopover samples in the second half of the season, which suggests that there may be a large breeding population where this mtDNA group is common. Stable isotope values from migrants captured with mitochondrial group 3 were quite depleted, consistent with a northern boreal forest location, and indicate that more sampling in western Canadian provinces and Alaska may reveal the presence of this poorly known mitochondrial group.

The rarity of mitochondrial group 2 in warblers captured at the stopover site was somewhat surprising, given the prevalence of this mitochondrial group in breeding locations northwest of

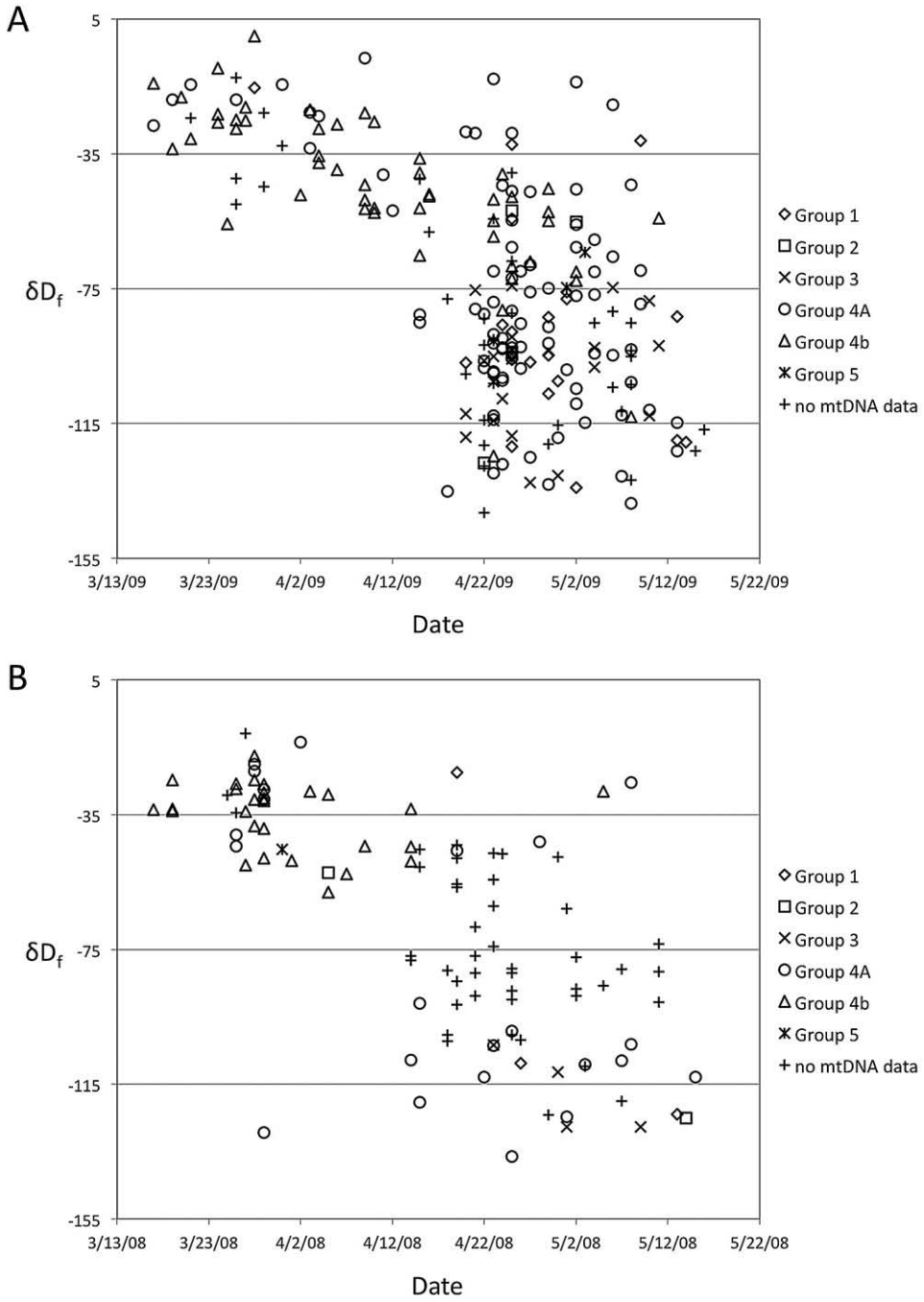

Fig. 4. Plot of capture dates and hydrogen isotope ratios of (A) spring 2009 and (B) spring 2008 after-second-year male Wilson's Warblers migrating through Cibola National Wildlife Refuge, Arizona. Each symbol represents an individual bird, with different symbols representing different mitochondrial digest groups (see Fig. 1, and Tables 1 and 2). Note that some samples in 2008 were not genotyped, and the genotyped ones were not chosen randomly.

the stopover site, including the Sierra Nevada of eastern California as well as eastern Oregon. The Colorado River is a major flyway for western migratory birds (Rosenberg et al. 1991), and we would expect birds from these breeding regions to stop over in critical riparian habitat at our stopover site on the lower Colorado River. Yet our results suggest that populations breeding in the Interior West use different migration routes than those breeding on the coast; these routes are presumably east of Cibola NWR. Using stable hydrogen isotopes, Paxton et al. (2007) found evidence for fairly strong connectivity of migration routes for Wilson's Warblers, with isotopic values differing between five stopover sites across the Southwest. Wilson's Warblers captured

at western migration stations were dominated by populations breeding within coastal regions, whereas eastern migration stations had larger proportions of individuals breeding at more northern latitudes such as the western Canadian provinces and Alaska. However, there was not a continuous gradient of change in populations migrating across the southwestern migration routes. Thus, factors such as topography and habitat features likely play a role in shaping different migratory pathways for birds breeding in coastal and interior mountainous regions.

This is an exciting time in migratory connectivity research, given the technological advances that now allow us to link the movement of small landbirds throughout their annual cycle (Veen 2013). Our results show that by focusing on groups of birds (rather than individuals), we can use mtDNA variation to infer patterns of timing of migration for Wilson's Warblers at a finer scale of resolution than was previously thought feasible (Clegg et al. 2003). The approach used in the present study has the potential to be applied to many species of birds where geographic structure has already been documented among populations using mtDNA (Newton 2003, Price 2008), potentially increasing our understanding of migratory connectivity for many species (e.g., Paxton et al. 2011). In addition, new modeling approaches offer much promise for integrating multiple markers (e.g., genetic and stable isotope markers) to infer patterns of migratory connectivity (Wunder 2010, Chabot et al. 2012, Rundel et al. 2013). For example, the Bayesian framework used by Rundel et al. (2013), which combines microsatellite and stable isotope markers for Wilson's Warblers and Hermit Thrushes (*Cathurus guttatus*), allows for more precise estimates of the breeding location of individuals captured across the wintering range. New approaches for understanding patterns of migratory connectivity of small landbirds are essential for determining the population dynamics of migratory species. Only through linking the phases of a migratory species' annual cycle and documenting seasonal interactions will we begin to develop a full understanding of the ecology of migratory species (Webster et al. 2002). The results of this study provide important insights into differences in the patterns of timing and migration routes for western breeding populations of Wilson's Warblers, laying the foundation for more in-depth examination of seasonal interactions between migratory and stationary periods of the annual cycle.

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