Limited Utility of mtDNA Markers for Determining Connectivity among Breeding and Overwintering Locations in Three Neotropical Migrant Birds

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Abstract: *For the past two decades, population declines in Neotropical migrant songbirds have been both a flagship conservation issue and the subject of intensive research initiatives. Nonetheless, the design of effective conservation measures for Neotropical migrants has been hindered by a lack of information on where and how migrant populations are regulated. This problem stems in large part from the difficulty of following individual long-distance migrants throughout their annual cycles. As a result, there has been increasing interest in using genetic markers to determine patterns of connectivity between particular breeding populations and overwintering regions. In species with geographically structured genetic variation during the breeding season, genetic markers can be used to determine the origin of migrating and overwintering individuals. This information on demographic connectivity could be then used to infer the locations or seasons contributing to population trends of currently unknown origin. To date, genetic markers (primarily mitochondrial DNA) have been used to survey only a few species of migratory songbirds, with varying success. To provide examples of the geographic scale at which mtDNA markers are likely to prove most relevant to Neotropical migrant conservation, we surveyed breeding-season variation in North American populations of three long-distance migrant taxa: the Yellow-breasted Chat (*Icteria virens*), Common Yellowthroat (*Geothlypis trichas*), and Nashville Warbler (*Vermivora ruficapilla*). We then used this information to screen individuals sampled at overwintering sites in Mexico and Central America. Genetic structure was only found at the broadest continent-wide scale in all three species, which allowed us to assign overwintering individuals to eastern or western breeding lineages but did not allow us to assign overwintering individuals to breeding populations of origin on a finer scale suitable for assaying local demographic trends. Owing to mitochondrial homogeneity among widely separated breeding locations, mtDNA markers (especially when used alone) are unlikely to provide a panacea for the problem of interseasonal connectivity among migrant songbirds.*

Utilidad Limitada de los Marcadores de ADNmt en la Determinación de Conectividad entre Localidades de Reproducción y de Hibernación de Tres Aves Migratorias Neotropicales

Resumen: *En las últimas dos décadas, las declinaciones poblacionales de aves migratorias neotropicales han sido un tema insignia para la conservación así como el objeto de intensas iniciativas de investigación. No obstante, el diseño de medidas de conservación ha estado limitado por la falta de información de dónde y cómo son reguladas las poblaciones migratorias. En gran parte, este problema se deriva de la dificultad de seguir los ciclos anuales de individuos migrantes de larga distancia. En consecuencia, hay un creciente interés en utilizar marcadores genéticos para determinar patrones de conectividad entre determinadas poblaciones reproductivas y sus regiones de hibernación. Los marcadores genéticos pueden ser utilizados para determinar el origen de individuos migrantes e invernantes en especies con variación genética geográficamente estructurada*

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Paper submitted June 3, 2002; revised manuscript accepted April 21, 2003.

durante la época reproductiva. Esta información sobre conectividad demográfica podría ser utilizada para inferir las localidades o estaciones que contribuyen a las tendencias poblacionales actualmente de origen desconocido. A la fecha, los marcadores genéticos (principalmente ADN mitocondrial) se han utilizado para examinar solo unas cuantas especies de aves migratorias, con éxitos variados. Para proporcionar ejemplos de la escala geográfica en la que los marcadores de ADNmt tienen probabilidad de ser más relevantes para la conservación de migrantes neotropicales, examinamos la variación en la época reproductiva en poblaciones de Norte América de tres taxones migratorios de larga distancia: Icteria virens, Geothlypis trichas *y* Vermivora ruficapilla*. Posteriormente utilizamos esta información para investigar individuos capturados en sitios de hibernación en México y Centroamérica. En las tres especies, la estructura genética se encontró solo en la mayor escala continental, lo que nos permitió asignar a los individuos invernantes a linajes reproductivos orientales u occidentales, pero no nos permitió asignar a los individuos a poblaciones reproductivas de origen en una escala más fina adecuada para analizar tendencias demográficas locales. Debido a la homogeneidad mitocondrial entre localidades reproductivas ampliamente separadas, es poco probable que los marcadores de ADNmt (especialmente cuando se usan solos) sean la panacea para el problema de la conectividad interestacional de aves migratorias.*

Introduction

Effective conservation of highly mobile organisms like migratory birds is especially challenging because these organisms move across large distances during their annual cycle and are exposed to many spatially separated factors that could influence survival and reproduction. Recent studies of interseasonal population dynamics in long-distance Neotropical migrant passerines emphasize the importance of demographic links between breeding and overwintering populations (Sherry & Holmes 1995, 1996; Marra et al. 1998; Sillett et al. 2000). A longstanding barrier to understanding the causes of demographic trends in Neotropical migrants is the difficulty of tracking individuals during migration and thereby documenting connectivity between particular breeding and overwintering sites (Webster et al. 2002). At present, detailed information on the links between breeding populations and overwintering sites is available for only a few species of Neotropical migrant songbirds such as the Kirtland's Warbler (*D. kirtlandii*) and the Golden-cheeked Warbler (*D. chrysoparia*), both endangered species with very restricted distributions on both breeding and overwintering grounds (Haney et al. 1998; Sykes & Clench 1998; Rappole et al. 1999). Little is known about movements between breeding and overwintering locations for the majority of Neotropical migratory songbirds with larger population sizes and broader geographic distributions. Many migrants are thought to be undergoing demographic changes of uncertain extent (e.g., Robbins et al. 1989; James et al. 1992; Sauer & Droege 1992; Holmes & Sherry 2001), and identifying the processes generating these population trends is of fundamental relevance to the conservation of these species (Rubenstein et al. 2002).

One method of solving this problem is to track individual birds between breeding and overwintering sites, but this is a daunting prospect at present because many species of Neotropical migrants are too small to carry long-lived radio transmitters. An alternative approach is to use population-specific markers that vary with geography to examine connectivity between breeding and overwintering sites. Population-level genetic markers are a potentially powerful source of information on the level of connectivity between breeding and overwintering sites (Webster et al. 2002), and they have been applied with varying success to studies of shorebird migrants (Wenink & Baker 1996; Haig et al. 1997; Wennerberg 2001), Palearctic migrant passerines (Bensch & Hasselquist 1999), and a few Neotropical migrant passerines such as Wilson's Warbler (*Wilsonia pusilla*; Kimura et al. 2002) and Swainson's Thrush (*Catharus ustulatus*; Ruegg & Smith 2002). Two important prerequisites for using molecular markers to link breeding and overwintering populations are (1) that breeding ground populations are not panmictic and (2) that the molecular marker is sensitive enough to resolve any genetic structure that exists. Effective genetic panmixis could result from even rare episodes of long-distance gene flow among breeding populations or from rapid demographic expansions from small, genetically bottlenecked populations. Marker sensitivity is important in that the mutation rate of the marker needs to be high enough to produce intraspecific variation that differs among populations. Although mitochondrial markers have been highly informative in studies of intraspecific avian phylogeography (reviewed in Zink 1996; Avise & Walker 1998), it is not presently known how variable these markers are at the local population or regional level in most Neotropical migrant passerines.

Here, we survey the broad-scale pattern of mtDNA variation in three Neotropical migrants with transcontinental North American breeding distributions: Yellow-breasted chat (*Icteria virens*), Common Yellowthroat (*Geothlypis trichas*), and Nashville Warbler (*Vermivora ruficapilla*). These species span a gradient of breeding-range disjunction: the Common Yellowthroat is common across its

contiguous transcontinental range, the Yellow-breasted Chat has a transcontinental distribution but exhibits a fragmented distribution in the West, and the Nashville Warbler shows a large geographic disjunction between eastern and western populations. These taxa are each represented by two or more subspecies in North America, and the presence of this morphological variation suggests that historical biogeographic processes have played a role in their diversification. These species therefore represent the category of migratory taxa in which geographically structured mtDNA variation might be most likely. Our goals here are to describe the general magnitude of phylogeographic variation across each species' breeding range and to use that information to determine the scale at which mitochondrial markers can be applied when assessing breeding and overwintering connectivity.

Methods

Study Species

The Yellow-breasted Chat has a transcontinental breeding distribution that extends from southwestern Canada south into northern and central Mexico. The two currently recognized subspecies differ subtly in size, plumage coloration, and song structure (Eckerle & Thompson 2001). *I. v. virens* breeds in eastern North America west to the Great Plains and occupies a nearly continuous distribution within its breeding range. Based on the plumage phenotypes of specimens collected during winter and the few recoveries of banded individuals, this eastern subspecies is thought to winter in eastern Mexico and Central America south to Panama (Eckerle & Thomson 2001). *I. v. auricollis* has a more fragmented and localized distribution throughout western North America (Fig. 1), where it breeds predominantly in riparian habitats. This western subspecies is thought to winter in western Mexico and Central America south to Guatemala (Eckerle & Thomson 2001). Although the Yellow-breasted Chat has long been classified as a wood-warbler (Aves: Parulidae), recent molecular phylogenetic evidence has confirmed previous suspicions that this monotypic genus falls outside the Parulidae clade (Lovette & Bermingham 2002).

The Common Yellowthroat has one of the broadest distributions of any North American wood-warbler, with breeding populations spanning much of the United States, Canada, and northern Mexico (Fig. 1). Northerly breeding populations are migratory, whereas some populations in the southern United States and Mexico are nonmigratory (reviewed in Guzy & Ritchison 1999). The species shows marked morphological variation across its broad breeding range, with 13 subspecies currently recognized (Lowery & Monroe 1968). Our sample of mtDNA haplotypes was drawn from a broad geographic region spanning North America, but we did not sample many described

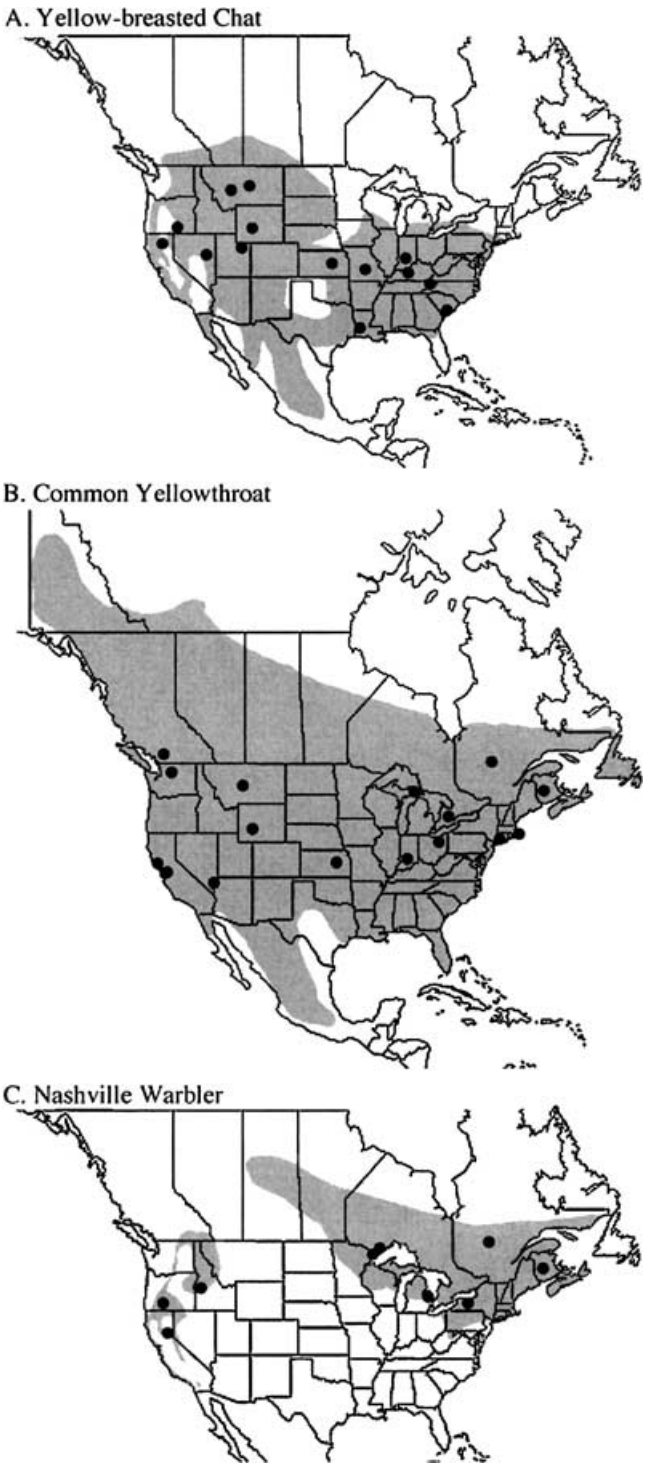

*Figure 1. Breeding distributions of the (a) Yellow-breasted Chat (*Icteria virens*), (b) Common Yellowthroat (*Geothlypis trichas*), and (c) Nashville Warbler (*Vermivora ruficapilla*). Localities indicated by black circles correspond to sampling sites described in Table 1.*

subspecies with restricted geographic ranges. We therefore provide a summary of genetic variation on a continent-wide scale encompassing the populations that give rise to the majority of migratory individuals but insufficient to reconstruct the historical pattern of relationship among all morphologically defined races.

The Nashville Warbler occupies two highly disjunct breeding regions in eastern and western North America (Fig. 1). These disjunct populations differ subtly in plumage coloration and are recognized as separate subspecies, the northeastern population as *V. r. ruficapilla* and the western population as *V. r. ridgwayi*. These two Nashville Warbler races are in turn closely related to several other members of the *Vermivora* complex that breed in the southwestern United States and northern Mexico, particularly *V. virginiae*, *V. crissalis*, and *V. luciae*, within which the two *V. ruficapilla* forms may not be sister taxa (Zink et al. 2000). Nonetheless, because these eastern and western forms are difficult to separate from one another in the hand but are more readily distinguished from the other taxonomic species in this complex, DNA-based techniques for discriminating overwintering individuals are likely to be useful in the context of migratory connectivity. All populations of *V. ruficapilla* are long-distance migrants and overwinter from southern Texas through Mexico to northern Central America.

Sampling and Laboratory Techniques

We obtained blood and feather samples from birds trapped in mist-nets at breeding sites in Canada and the United States and at overwintering areas in Mexico and Central America between 1996 and 2000. The majority of the feather samples from breeding sites were generously provided by participants in the Monitoring Avian Productivity and Survivorship Program coordinated by the Institute for Bird Populations. From breeding sites, we assayed only adult birds (for ageing of birds, see Pyle 1997) that were sampled between the end of May and August, when most individuals would have been on their breeding territories (e.g., Williams 1996; Guzy & Ritchison 1999; Eckerle & Thompson 2001), and that assay showed evidence of breeding (cloacal protuberance in males and a brood patch in females). The dates of sampling of overwintering individuals were within the period when the majority of individuals occupy their overwintering territories (Ecklerle & Thompson 2001) (Table 1). Yellow-breasted Chats were sampled between 12 December and 21 February; Common Yellowthroats between 30 November and 22 February, and Nashville Warblers between 14 November and 27 February. We obtained blood samples via subbrachial venipuncture and collected 20–40 μL of blood in heparinized capillary tubes. We obtained feather samples by plucking the outermost two retrices.

We extracted genomic DNA from most blood samples with standard techniques of digestion with proteinase K, phenol-chloroform separation, and DNA precipitation in cold ethanol (Sambrook et al. 1989). Genomic DNAs were extracted from some blood samples and all feather samples via single-use DNeasy extraction columns (Qiagen N.V., Venlo), following the manufacturer's protocols for tissue or nucleated blood. The feather material used for DNA extraction included the proximal 3–4 mm of feather shaft, which was sliced directly into the extraction tube with a sterile scalpel.

All PCR and sequencing reactions targeted a 1018-nucleotide region of the mtDNA genome that included the complete tRNA-lysine, ATPase 8, and ATPase 6 genes and short flanking regions of the cytochrome oxidase II and III genes. This region has been used in studies of phylogeographic variation in a number of other avian taxa, and the laboratory techniques, primers, reaction conditions, and automated sequencing methods have been reported previously (Hunt et al. 2001). For convenience we refer to these gene sequences as the "ATPase region" or as "ATPase haplotypes," even though they included flanking sequences surrounding the ATPase genes.

We chose a protein-coding mtDNA marker instead of potentially more variable sequences from the mtDNA control region because we were concerned about amplifying nuclear-encoded copies ("numts") of the mtDNA genome. Numts typically have a much slower rate of nucleotide change than their paralogues in the mtDNA genome, and the inclusion of unrecognized numt sequences can introduce large biases into phylogeographic reconstructions. Numts are a particular problem when avian blood samples are used as a DNA source. Avian erythrocytes are nucleated but lack mitochondria, so there is a preponderance of nuclear-encoded template in genomic DNA samples extracted from avian blood (Sorenson & Quinn 1998). We previously encountered and dealt with problematic numt contamination in control-region sequences of other wood-warbler species (Kimura et al. 2002). Furthermore, because the pattern of control-region molecular variation is less predictable than variation at protein-coding loci, it is easier to recognize numt contamination in a set of protein-coding sequences.

Phylogeographic Reconstructions and Assays of Overwintering Individuals

We aligned sequences by eye and imported them into Paup*4.0b10 (Swofford 1999) for analyses of molecular variation, genetic divergence, and preliminary phylogenetic reconstructions. We then used the program TCS 1.13 (Clement et al. 2000) to reconstruct a gene genealogy for the haplotypes of each taxon.

Based on the clusters of haplotypes identified in the haplotype networks (see Results), we identified polymorphic nucleotide sites that defined eastern or western lineages of breeding populations from sequence data for each species with the program MacClade (Maddison &

Table 1 Locations of breeding and overwintering sampling sites and numbers of genetic samples obtained from three species of migratory wood-warblers.

Site, state/providence, country[a]	*Site code*	*Latitude and longitude*	*No. of individuals*[b]		
			YBCH	COYE	NAWA
Breeding populations					
Pitt Lake, British Columbia, Canada	BC	49°22′N 122°40′W	—	2	—
Mt. Baker National Forest, Washington	WA	48°09′N 121°27′W	—	3	—
Paisley, Oregon	OR1	42°30′N 120°39′W	1	—	—
Williams, Oregon	OR2	42°09′N 123°25′W	—	—	4
Boise, Idaho	ID	43°36′N 116°03′W	—	—	3
Shasta, California	CA1	41°03′N 122°21′W	4	—	—
Bolinas, California	CA2	37°55′N 122°41jW	—	1	—
Los Banos, California	CA3	37°10′N 120°45′W	—	3	—
Foresthill, California	CA4	38°52′N 120°52′W	—	—	2
Ruby Lake, Nevada	NV1	40°12′N 115°29′W	4	—	—
Lake Mead, Nevada	NV2	35°59′N 114°50′W	—	3	—
Holter Dam, Montana	MT1	46°55′N 111°55′W	1	4	—
Denton, Montana	MT2	47°21′N 109°41′W	1	—	—
Atlantic City, Wyoming	WY	42°37′N 108°38′W	3	1	—
Manila, Utah	UT	40°58′N 109°44′W	2	—	—
Junction City, Kansas	KS	39°06′N 096°49′W	1	4	—
Fort Leonard Wood, Missouri	MO	37°46′N 092°10′W	4	—	—
Owensburg, Indiana	IN	38°53′086°44′W	3	4	—
Fort Knox, Kentucky	KY	37°48′085°49′W	3	—	—
Cuyahoga, Ohio	OH	41°25′081°39′W	—	3	—
Seney National Refuge, Michigan	MI1	46°18′N 085°10′W	—	1	—
Dearborn, Michigan	MI2	42°19′N 083°11′W	—	—	4
Bristol, Tennessee	TN	36°34′N 082°07′W	4	—	—
Charleston, South Carolina	SC	32°42′N 079°58′W	2	—	—
Finland, Minnesota	MN	47°22′N 091°11′W	—	—	2
Kakabeka, Ontario, Canada	ON1	48°09′089°49′W	—	—	4
Hilliardton, Ontario, Canada	ON2	47°30′N 079°40′W	—	2	—
Camp Myrica, Quebec, Canada	PQ	49°43′073°20′W	—	5	3
Fredericton, New Brunswick, Canada	NB	45°48′N 066°39′W	—	4	1
Rochester, New York	NY	43°15′N 077°39′W	—	—	4
South Britain, Connecticut	CT	41°28′073°15′W	—	2	—
Truro, Massacusettes	MA	42°01′070°02′W	—	4	—
Fort Polk, Lousisana	LA	31°01′093°04′W	1	—	—
Overwintering sites					
Los Cabos, Baja California Sur, Mexico	BCS	22°53′N 109°54′W	7	11	—
Chupaderos, Sinaloa, Mexico	SIN	23°50′N 102°20′W	2	—	12
Las Joyas Biosphere Reserve					
Jalisco, Mexico	JAL	19°46′N 105°22′W	1	—	30
Nevado, Colima, Mexico	COL	19°30′N 103°37′W	0	—	2
Huautla, Morelos, Mexico	MOR	21°02′N 98°17′W	1	—	7
Coatepec, Veracruz, Mexico	VER1	19°27′N 96°58′W	1	—	—
Las Tuxtlas, Veracruz, Mexico	VER2	18°25′N 95°07′W	5	—	—
Chila, Oaxaca, Mexico	OAX1	18°55′N 99°43′W	5	—	6
Animas de Trujano,					
Oaxaca, Mexico	OAX2	17°03′N 96°43′W	2	9	23
Hidalgo, Oaxaca, Mexico	OAX3	15°50′N 97°10′W	—	—	6
El Ocote Reserve, Chiapas, Mexico	CHS	16°45′N 93°07′W	7	—	—
Zacualtipán, Hidalgo, Mexico	HGO	20°39′N 98°36′W	—	—	5
San Ignacio, Belize	BZ	17°09′N 89°04′W	13	9	—
Boqueron, El Salvador	ES	13°42′N 86°23′W	2	—	2

[a] *Country is United States unless otherwise indicated.*
[b] *Abbreviations: YBCH, Yellow-breasted Chat; COYE, Common Yellowthroat; NAWA, Nashville Warbler.*

Maddison 1992). For each species, we found two restriction enzymes that cleaved different variable sites, one diagnosing the eastern lineage and the other the western lineage (Table 2). We then assayed overwintering individuals from each species with the appropriate diagnostic enzymes to determine if they were of eastern or western breeding origin. In the Yellow-breasted Chat and Common Yellowthroat, we amplified the full 1018 base-pair

Table 2 Restriction sites diagnostic of eastern or western mitochondrial DNA lineages of bird species.

Taxon	*Restriction enzyme*	*Diagnostic site**	*Lineage cleaved*
Yellow-breasted Chat	*Rsa*I	9638	east
	*Dpn*II	9388	west
Common Yellowthroat	*Tsp*45	9399	east
	*Bst*NI	9425	west
Nashville Warbler	*Hinc*II	9390	east
	*Stu*I	9590	west

**Location of variable site in chicken mtDNA genome reference sequence (Desjardins & Morais 1990).*

fragment of mtDNA as described above. In Nashville Warblers, however, digestions of this large fragment of DNA originating from blood samples with the appropriate enzymes did not produce the restriction profiles predicted from sequences of feathers from breeding sites. Further sequencing of blood and feather samples from both breeding and overwintering sites confirmed that the restriction sites were not present in the amplifications from blood samples and that a numt copy was being preferentially amplified from the DNA samples extracted from blood. We therefore amplified a shorter fragment by using an internal primer PWL (Hunt et al. 2001) that was a perfect match for the mtDNA template but not for the numt sequence. Additional sequencing confirmed that this alternative primer combination amplified the mtDNA copy from DNA extracts of both blood and feather-tip origin. The shorter fragment contained the diagnostic sites, and we used it to assay all overwintering Nashville Warblers. Each restriction digest reaction consisted of 5 μL of PCR product and two units of enzyme. Fragments were electrophoresed on 6% polyacrylamide gels and stained with ethidium bromide. We scored individuals as belonging to the eastern or western clade based on the observed restriction profile.

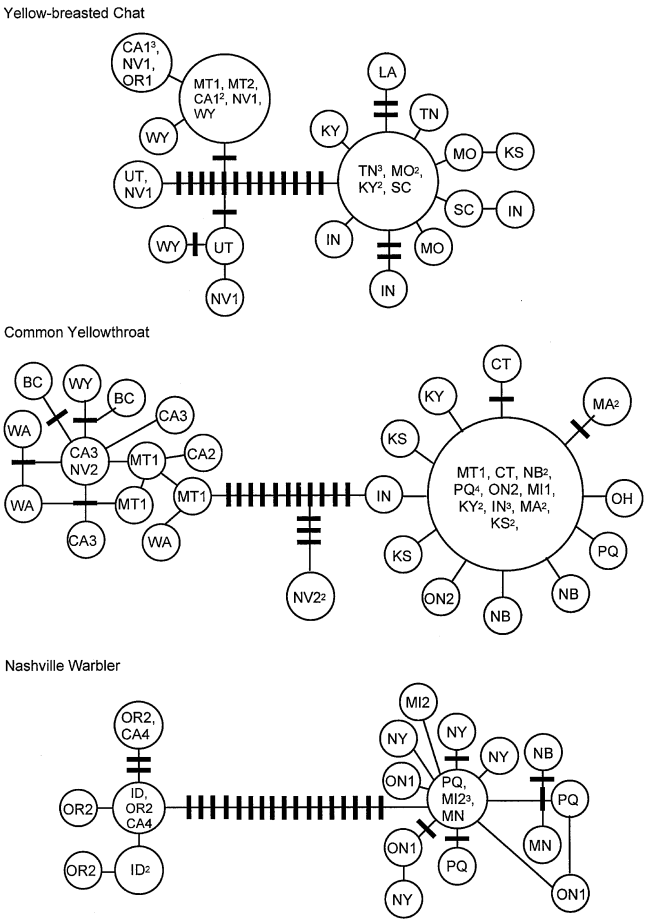

*Figure 2. The mtDNA haplotype genealogies for breeding populations of the Yellow-breasted Chat (*Icteria virens*), Common Yellowthroat (*Geothlypis trichas*), and Nashville Warbler (*Vermivora ruficapilla*). Each unique haplotype is indicated by a circle with an area proportional to the number of sampled individuals with that haplotype. Hatch marks along branches indicate inferred haplotypes differing by single nucleotide substitutions that were not sampled. Abbreviations within circles indicate the sampling site(s) from which a haplotype was recovered and are defined in Table 1.*

Results

Yellow-Breasted Chat

We obtained mtDNA sequences from a total of 34 Yellow-breasted Chats from 11 locations within the contiguous range of the eastern subspecies *I. v. virens* and from 7 locations within the more fragmented range of the western subspecies *I. v. auricollis*. We identified 18 unique ATPase haplotypes within the pooled sample of 34 individuals; the maximum differentiation among these haplotypes was 17 nucleotide substitutions (1.8% sequence divergence). The gene genealogy (Fig. 2) revealed two distinct haplotype groups, with between-group haplotypes separated by 11–17 nucleotide substitutions, that were geographically concordant with the breeding distributions of the eastern and western morphological subspecies. Within the eastern haplotype group, the pattern of haplotype relationships was star-shaped, with one central common haplotype surrounded by a number of rarer but closely allied haplotypes (one or two nucleotide changes). Haplotypes in the western group were somewhat more differentiated; the western haplotype network included two closely related, high-frequency haplotypes and six rarer haplotypes separated by one to nine substitutions from other western haplotypes. Despite the higher level of haplotype differentiation in the sample of western haplotypes, we found little indication of geographic

structuring within the western group. For example, in our sample of four birds from one site in northeastern Nevada, we found representatives of each of the two common western haplotypes and two of the most divergent rare haplotypes.

The identification of eastern and western haplotypes allowed overwintering individuals to be classified as eastern or western in breeding origin. Although sample sizes were small at the majority of overwintering sites, each site contained exclusively eastern or exclusively western mtDNA haplotypes. Western haplotypes were found only in western Mexico in Baja California Sur and from Sinaloa to Oaxaca. Eastern haplotypes were found in eastern Mexico from Veracruz to Chiapas and further south in Central America (Fig. 3).

Common Yellowthroat

We obtained ATPase sequences from 47 Common Yellowthroats from 16 sites across the northern part of this species' breeding range in the United States and Canada (Fig. 2). We identified a total of 26 unique ATPase haplotypes within the pooled sample of 47 individuals; the maximum differentiation among these haplotypes was 19 nucleotide substitutions (2.0% sequence divergence). The gene genealogy for these 26 haplotypes was composed of three distinct haplotype groups. First, samples from eastern sites from Montana east to New Brunswick and south to Kansas, Missouri, and Kentucky clustered in a star network around a single common central haplotype found in 19 individuals. Eleven peripheral haplotypes recovered from 1 or 2 individuals each clustered around this common eastern haplotype and were separated from it by 1 or 2 nucleotide substitutions. The maximum differentiation within this eastern haplotype group was four substitutions (0.4%). Second, samples from western sites in California, Washington, Montana, Nevada, and British Columbia clustered into a network of 13 closely allied haplotypes, each recovered from 1 or 2 individuals, which were separated from one another by 1–5 nucleotide substitutions. Third, we recovered a single additional differentiated haplotype from two individuals from a possibly nonmigratory population in the Colorado River valley in southern Nevada. This Nevada haplotype was separated from haplotypes in the more geographically widespread western group by 12–16 substitutions and from haplotypes in the geographically widespread eastern group by 7–9 substitutions. This Nevada population may be allied to the many semi-isolated and/or nonmigratory populations in the southern part of the breeding range that we did not sample, and it is further likely that these unsampled populations harbor additional mtDNA lineages. Nonetheless, our sparse but geographically broad sampling captured the general pattern of mitochondrial variation across much of the breeding range of this species.

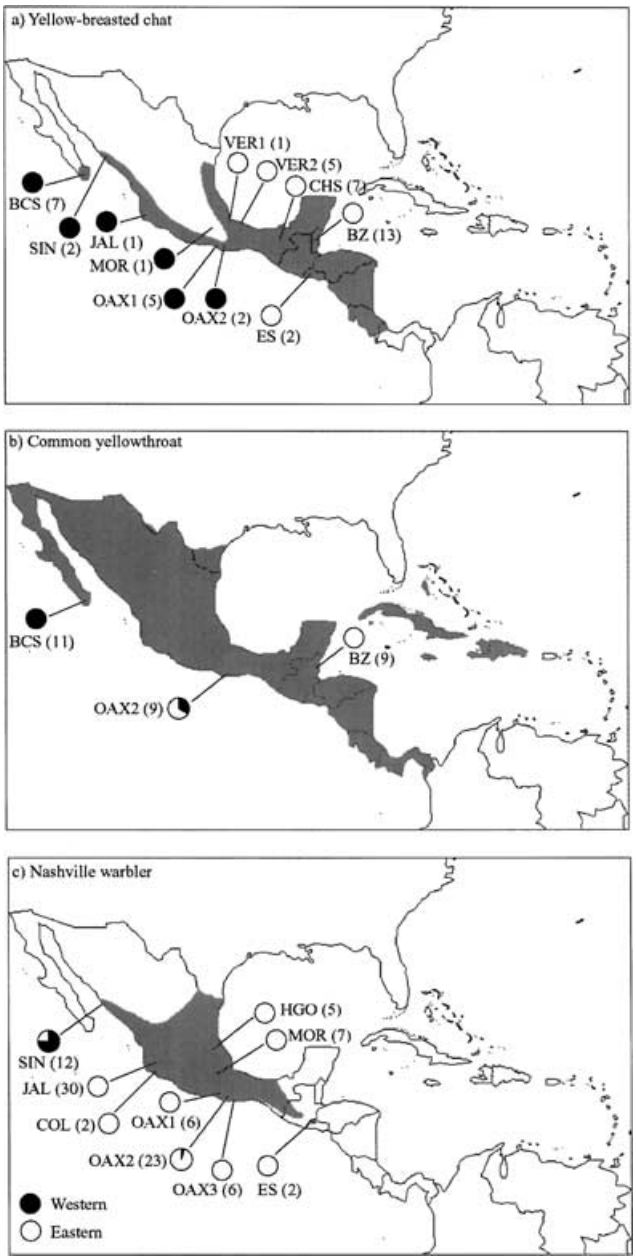

*Figure 3. Maps of Mexico and Central America showing the occurrence of eastern- and western-origin lineages as diagnosed by restriction assays for the Yellow-breasted Chat (*Icteria virens*), Common Yellowthroat (*Geothlypis trichas*), and Nashville Warbler (*Vermivora ruficapilla*). Shaded areas indicate the typical overwintering distribution of each species. Location abbreviations are defined in Table 1. Numbers in parenthesis are sample sizes for each location.*

We found two locations where individuals with differentiated haplotypes occurred in local sympatry, suggesting that these three groups—the eastern, western, and Nevada lineages—are not completely geographically isolated. First, in the sample of three birds from the Nevada

site, two individuals had the differentiated haplotype found only at that site. The third individual had a haplotype that was also recovered from an individual from a northern California breeding site and which clustered in the center of the haplotype network of the "western" group. This third individual was captured in breeding condition on 26 June during the latter half of the breeding season, making it highly unlikely that it was a passage migrant en route to a more northerly breeding location. Second, in a sample of three birds from a site in central Montana, we recovered two individuals with western-group haplotypes and a single individual with the core eastern-group haplotype.

Samples of overwintering individuals were available from only three sites, but even this limited sampling was informative. One site in Baja California Sur, Mexico, contained only individuals with western haplotypes; Oaxaca, Mexico, had representatives of both eastern and western haplotypes; and the eastern site in Belize had only eastern haplotypes.

Nashville Warbler

We sampled 27 Nashville Warblers, including 18 individuals from locations within the breeding range of the eastern subspecies *V. r. ruficapilla* and 9 individuals from locations within the breeding range of the western subspecies *V. r. ridgwayi*. These eastern and western populations were separated by a relatively high magnitude of divergence and were clustered as reciprocally monophyletic groups in the gene genealogy (Fig. 3). Haplotypes from the eastern population differed from those from the western population at 16–22 nucleotide sites (1.7–2.3%). Within the eastern population, the 13 haplotypes recovered differed at 1–6 nucleotide sites, whereas within the western population, the 5 haplotypes recovered differed at 1–5 nucleotide sites. In the gene genealogy networks, both the eastern and western haplotype groups clustered around a single central haplotype recovered from multiple individuals from that geographic region, and these central haplotypes were separated from other closely allied haplotypes by 1–3 nucleotide substitutions.

These analyses demonstrate that the eastern and western Nashville Warbler subspecies are each comprised of mitochondrial lineages that share a recent common ancestor, and that these eastern and western populations are reciprocally monophyletic. Inferences about the origin and relationships of these two populations are confounded, however, by the presence of additional, equally differentiated populations currently classified as different species such as *V. virginiae*, *V. crissalis*, and *V. luciae*, which previous workers (Zink et al. 2000) have shown to be closely allied to *V. ruficapilla*.

Only 10 out of 96 overwintering individuals assayed with a combination of restriction enzyme assays and DNA sequencing belonged to the western lineage (9 in Sinaloa and 1 in Oaxaca). Eastern-lineage individuals were found in both eastern and western Mexico, and in a geographically disjunct overwintering location in El Salvador (Fig. 3). We therefore found a surprisingly high frequency of birds with eastern haplotypes throughout the wintering range, particularly in western Mexico, where we expected a higher frequency of birds from western breeding sites. Given the problems with numts described above, we were concerned that our RFLP assay might be incorrectly typing birds in the western clade. We therefore sequenced 650 nucleotides of the ATPase gene with the primers used for the RFLP assay from 14 overwintering individuals. These 14 sequenced samples included 5 DNA samples extracted from blood and 9 samples extracted from feather tips, and they comprised birds with eastern RFLP types sampled from western Mexico in the states of Sinaloa, Jalisco, and Colima. All 14 sequences were free of conspicuous pseudogene artifacts, all sequences fell within the eastern haplotype group as expected on the basis of the previous RFLP assays, and none were closely allied to the numt sequences identified from previously sequenced blood samples and the non-mtDNA-specific primers. These sequences thereby confirmed the eastern breeding origin of these overwintering birds from western Mexico.

Discussion

Effective conservation of migratory birds requires information on the processes that regulate populations and on the locations where such regulation occurs. The ability to determine where individuals from particular breeding populations overwinter would help target conservation efforts toward the sites most demographically critical for these populations. Genetic markers provide one potential window on patterns of connectivity among migrant populations. Our primary goal here is to investigate the geographic scale at which mitochondrial markers (currently the most easily assayed class of genetic marker and the marker currently in broadest use) will be informative about patterns of breeding and overwintering connectivity for Neotropical migrant passerines.

The first step in this process is to document any mtDNA differentiation among breeding populations. We identified similar patterns of mitochondrial variation in the three species we surveyed, in that each has two main haplotype clusters that correspond to sampling locations in eastern or western North America. A second feature common to the gene networks of all three species (and particularly apparent in the eastern breeding populations) is the absence of genetic differentiation among distant sampling sites. The star-shaped relationship of haplotypes in each of these regional groups suggests recent demographic expansions from previously bottlenecked populations (e.g., Avise 2000). Unfortunately, this low level of genetic

variation precludes the identification of population-specific markers on a finer geographic scale.

Generally low genetic structuring was also a feature of the western haplotype groups, but the general pattern of haplotype relationships was different in that the western haplotypes tended to be more highly differentiated, with a more even distribution of haplotype frequencies. This pattern of diversity in western haplotypes probably reflects the somewhat different demographic histories of these western populations, including less severe late-Pleistocene bottlenecks (Pielou 1991; Hewitt 2000), recent demographic contractions stemming from conversion of western riparian habitats, or a higher degree of past population subdivision. Any such past subdivision has since been largely eclipsed by gene flow. As depicted in the gene networks, many geographically distant western sites share haplotypes, and relatively divergent western haplotypes frequently occur within the same local breeding population.

Our geographic sampling was designed a priori to assess the magnitude and pattern of mtDNA variation across the majority of each species' breeding distribution in the United States and Canada, with the explicit goal of investigating the general utility of mtDNA markers for examining breeding and overwintering connectivity. Our characterization of two main lineages in each species (and one additional, rarer lineage in the Common Yellowthroat) probably captures the most important features of mtDNA variation across the northern parts of the breeding ranges of these three species, but each may have differentiated lineages at unsampled locations elsewhere.

Genetic Affinities of Overwintering Individuals

We identified well-defined eastern and western haplotype groups for each species but found little evidence of mitochondrial structuring on a finer geographic scale. Nevertheless, information on migration routes and on broad-scale associations between breeding and overwintering regions can be gleaned from the identification of phylogeographic variation at a continent-wide scale, as has been shown previously for the Dunlin (*Calidris alpina*; Wenink & Baker 1996; Wennerberg 2001), Great Reed Warbler (*Acrocephalus arundinaceus*; Bensch & Hasselquist 1999), Swainson's Thrush (*Catharus ustulatus*; Ruegg & Smith 2002), and Wilson's Warbler (*Wilsonia pusilla*; Kimura et al. 2002).

For Yellow-breasted Chats, the separation of eastern- and western-origin lineages at migration and overwintering sites is consistent with what is known about the overwintering distributions of the two currently recognized subspecies. Based on the morphology of specimens from overwintering sites, previous workers (e.g., Lowery & Monroe 1968) have suggested that the eastern subspecies *virens* winters mainly from eastern Mexico south to western Panama and that the western subspecies *auricollis* winters primarily in western Mexico (Lowery & Monroe 1968; Eckerle & Thomson 2001). Our results are fully concordant with these predictions because our survey of 11 overwintering sites suggests that the two subspecies or mtDNA groups have highly segregated distributions in Mexico and Central America (Fig. 3). Overwintering Yellow-breasted Chats generally occur in lowland habitats (Howell & Webb 1995; Eckerle & Thomson 2001), suggesting that the central Mexican highlands help restrict the mixing of individuals originating from eastern and western breeding areas.

We obtained Common Yellowthroat samples from only three overwintering sites, so the genetic assignments of those samples are presented here primarily for comparative purposes. Geographically peripheral overwintering sites in the east (Belize) and the west (Baja California) contained only eastern and western haplotypes, respectively, whereas both eastern and western lineages occurred in sympatry at a geographically intermediate site in Oaxaca, Mexico.

The eastern Nashville Warbler subspecies *ruficapilla* winters primarily from northeastern Mexico south to Guatemala (Lowery & Monroe 1968), whereas the western subspecies *ridgwayi* winters mainly in western and southern Mexico (Lowery & Monroe 1968; Williams 1996). Subspecies diagnosis during migration and at overwintering sites is difficult owing to the subtle differences in plumage color that separate the two forms (Pyle 1997). Our genetic analysis of the distribution of eastern and western lineages at overwintering sites is not concordant with these previous ideas about the overwintering distributions of the two subspecies. We identified few western-origin birds in the overwintering sample, despite having reasonable numbers of samples and sampling sites in western Mexico. In contrast, eastern-origin individuals were found, often exclusively, at all sites sampled. Overwintering Nashville Warblers occur in a variety of habitats, including lowlands and highlands and a variety of vegetation types (Williams 1996). Although such habitat generalization may promote mixing of lineages at overwintering sites, it is surprising that we found such a paucity of western lineages even in western locations. It may be that birds of western origin overwinter primarily in higher-elevation habitats (most of our overwintering samples came from lowland sites).

Utility of mtDNA-Based Assessments of Population Connectivity

Genetic markers will be most useful in establishing population connectivity between breeding and overwintering populations of Neotropical migrants when two criteria are satisfied. First, the mutation rate of the marker must be high enough to generate population-specific variation in the time since the separation of the populations of interest. Second, gene flow must be low enough for genetic

differences among breeding populations to accumulate. Previous studies of intraspecific mitochondrial variation in North American songbirds have revealed a variety of patterns, including highly structured populations, isolation-by-distance relationships, and near panmixia across wide geographic ranges (reviewed in Zink 1997). Traits that appear to be associated with the degree of geographically structured variation include disjunct breeding distributions and lack of migration. In resident species, levels of genetic structuring can often be understood in terms of current range disjunctions and is often congruent with patterns of morphological variation. Long-distance migrants in particular generally have lower levels of phylogeographic variation, and this variation is frequently incongruent with subspecies limits (e.g., Ball et al. 1988; Avise & Ball 1992; Buerkle 1999; Lovette & Bermingham 1999; Gibbs et al. 2000; Milot et al. 2000; Kimura et al. 2002). The likely contrast in levels of genetic structure between long-distance migrants and more sedentary taxa is consistent with the generally high levels of natal and breeding dispersal in long-distance migrants (Paradis et al. 1998). Low natal philopatry is likely to promote gene flow between local breeding populations, which in turn would promote the homogenization of genetic diversity across broad geographic areas.

The migratory birds that are most likely to exhibit breeding-season phylogeographic structure are those with multiple morphological subspecies occupying a broad but fragmented geographic range. Our survey of three such species and previous studies of similarly diverse taxa such as the Yellow Warbler (Milot et al. 2000), Wilson's Warbler (Kimura et al. 2002), and Swainson's Thrush (Ruegg & Smith 2002), suggest that historical demography and gene flow have led to the homogenization of mtDNA diversity across substantial portions of these species' breeding ranges. As a result, mitochondrial markers can be used to establish breeding-overwintering connectivity, but they provide resolution only at the broadest of geographic scales. The low diversity and broad regional homogeneity of these mtDNA markers suggest that both approaches based on haplotype frequency and searches for diagnostic population-specific haplotypes are unlikely to provide the resolution required to assess population connectivity on a finer geographic scale. The majority of Neotropical migrant species have smaller and less fragmented geographic ranges and lower subspecific diversity than the species we surveyed here or those that have been examined in previous studies, and mtDNA markers are likely to be of limited utility for tracking breeding-overwintering connectivity in these taxa.

Acknowledgments

We thank the many people who generously contributed samples and assisted with fieldwork and logistics for this project: D. DeSante, P. Nott, K. Wilcox, and their fellow workers at the Institute for Bird Populations; the participants in the Monitoring Avian Productivity and Survivorship program, particularly J. Allen, B. Allen, M. Boyles, J. Carlisle, L. Fortin, J. Johnson, R. Lewis, G. Taylor, K. Voget and D. Vroman; G. Geupel, N. Nur, and the Point Reyes Bird Observatory; and a number of independent banders. B. Milá, O. Figueroa, M. Kimura, and K. Ruegg collected the majority of the samples from overwintering areas. We thank L. Gilbert, A. Hoffman, K. Holder, M. Kimura, B. Kuo, B. Milá, K. Ruegg, F. Sariano, R. Sehgal, and H. Slabbekoorn for discussions and their laboratory expertise and two anonymous reviewers for many helpful suggestions on the manuscript. For research permits we thank the Canadian Wildlife Service and Environment Canada, the U.S. Fish & Wildlife Service and the National Park Service, F. Villaseñor and the Instituto Nacional de Ecologia, Secretaria de Medio Ambiente y Recursos Naturales in Mexico, the Forestry Department of the Ministry of Natural Resources in Belize, and the Government of El Salvador. This work was supported by grants from the Turner Foundation, San Francisco State University, the National Institutes of Health's Office of Research on Minority Health (5P20RR11805), and the Environmental Protection Agency (R827109–01–0).

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