MOLECULAR GENETIC STRUCTURING AND DEMOGRAPHIC HISTORY OF THE WILLOW FLYCATCHER

(*Empidonax traillii*)

by

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ABSTRACT

Molecular Genetic Structuring And Demographic History of
The Willow Flycatcher (*Empidonax traillii*)

Eben Paxton

The willow flycatcher is a neotropical migratory bird that breeds across most of the conterminous United States, and winters from southern Mexico to northwestern South America. Based on morphological characters, the willow flycatcher has long been considered a polytypic species, with four to five subspecies usually recognized. The morphological characters used to separate the subspecies are subtle, making subspecific identification difficult. Here, I present the results of an analysis of the molecular genetics of the presumed subspecies of the willow flycatcher. Using *cytochrome-b* sequences and the Amplified Fragment Length Polymorphism (AFLP) technique, over 180 individual samples from across the willow flycatcher breeding range were analyzed. I found significant differences between all but the Pacific Northwest subspecies. The southwestern subspecies showed the greatest degree of genetic difference; overall, the *cytochrome-b* data showed greater structuring among subspecies than did the AFLP data. The present genetic patterns suggest that the willow flycatcher went through a period of low population numbers followed by a period of rapid expansion. These population size changes may have brought about the level and degree of structuring among subspecies that we see today. Molecular genetic patterns also suggest that the endangered southwestern willow flycatcher (*E. t. extimus*) may have experienced slight genetic effects from the demographic bottleneck it has passed through very recently.

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Many people helped with the collection of samples across the country. Arizona

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Chapter I: Background

NATURAL HISTORY OF THE WILLOW FLYCATCHER

The willow flycatcher (*Empidonax traillii*) is a small (10-12 g) neotropical migratory bird that breeds across most of the conterminous United States, and winters from southern Mexico south to northwest South America (Figure I). The flycatcher breeds in shrubby thickets, typically associated with stagnant or slow moving water; however, it will breed in upland habitat where wet environments promote dense vegetative growth. In arid portions of the western United States, the flycatcher is restricted to dense, mesic riparian areas. Willow flycatchers show some degree of flexibility in adapting to new habitats, using exotic species such as tamarisk (*Tamarix* spp.) and Russian olive (*Elaeagnus angustifolia*) as nest substrates in the southwest (Sogge et al. 1997), and occupying early successional stage clearcuts in the Pacific Northwest (USGS, unpub. data). Both the fragmented riparian habitat of the Southwest and the island-like habitat of the Pacific Northwest clearcuts represent discontinuous habitats. Even in relatively intact habitat the

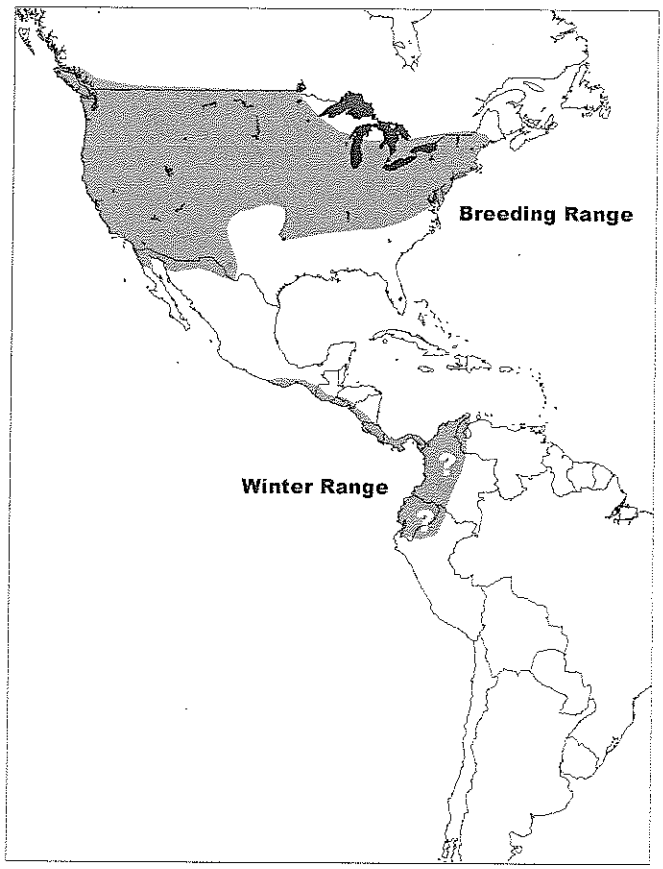


Figure I: Breeding and wintering distribution of the willow flycatcher

flycatchers tend to form breeding clusters separated by areas of unoccupied habitat (McCabe 1991). The spatially-patchy habitat and breeding clusters may decrease dispersal potential, and thus gene flow, among willow flycatcher breeding sites.

The willow flycatcher is primarily a monogamous breeder, with low levels of polygyny reported across its breeding range (Sedgwick and Knopf 1989, Whitfield and Enos 1996, Paradzick et al. 2000). Preliminary genetic analysis indicates a 30%-40% occurrence of extra-pair copulation (USGS/NAU, unpublished data). Both polygyny and extra pair copulation can have impacts on the genetic structure of a breeding population. If these breeding strategies reduce the number of males passing on their genes, then such breeding groups should have a smaller overall effective population size than groups of monogamous breeders, and will be more genetically isolated from one another than might be predicted from actual gene flow. However, extra pair copulation by non-territorial males could increase the effective population size of a breeding group, thus minimizing the inbreeding effects of polygyny.

Patterns of adult dispersal and site fidelity can also influence the genetic structure of populations. In Arizona, 38-40% of flycatchers return from their wintering grounds to the same site as the previous year (Netter et al. 1998, English et al. 1999), which is similar to the site fidelity of willow flycatchers in Oregon (45%; Sedgwick and Klus 1997) and California (36%; Whitfield and Enos 1996). Dispersal data from Arizona indicate that 13-17% of adults move to new breeding sites each year (Netter et al. 1998, English et al. 1999). Areas with multiple breeding sites that are geographically close have the highest degree of between-site movement, with longer distance dispersal fairly rare. Thus, the frequency of movement is negatively correlated with distance moved. However, willow flycatchers migrate thousands of miles between breeding and wintering sites each year, thus have the potential to move great distances from their previous

year's breeding site.

TAXONOMY OF THE WILLOW FLYCATCHER

The willow flycatcher is a member of the family Tyrannidae (Aves: Passeriformes), a diverse and widespread neotropical group of birds of which species from several genera (including *Empidonax*) breed in North America (A.O.U. 1998). The willow flycatcher is a sibling species of the alder flycatcher (*E. alnorum*), and the two were once collectively known as the Traill's flycatcher (*Empidonax traillii*; A.O.U. 1957). Although very difficult to differentiate morphologically, the two flycatchers have different song forms (Stein 1958), and studies have shown that interbreeding does not occur in areas of sympatry (Stein 1963). Subsequent genetic studies have also supported this split, with no genetic evidence of hybridization between the two species (Seutin and Simon 1988, Winker 1994).

Taxonomists have long recognized that the willow flycatcher is geographically variable and have suggested multiple subspecies (Philips 1948, Aldrich 1951, Hubbard 1987, Unitt 1987, Browning 1993). The morphological differences used to characterize the subspecies are subtle, which has led to divisions into varying numbers of subspecies, and confusion regarding exact ranges. One reason for inconsistencies in the number of proposed subspecies is that early studies included the alder flycatcher, with the result that some taxonomists included it as a distinct subspecies (Philips 1948),

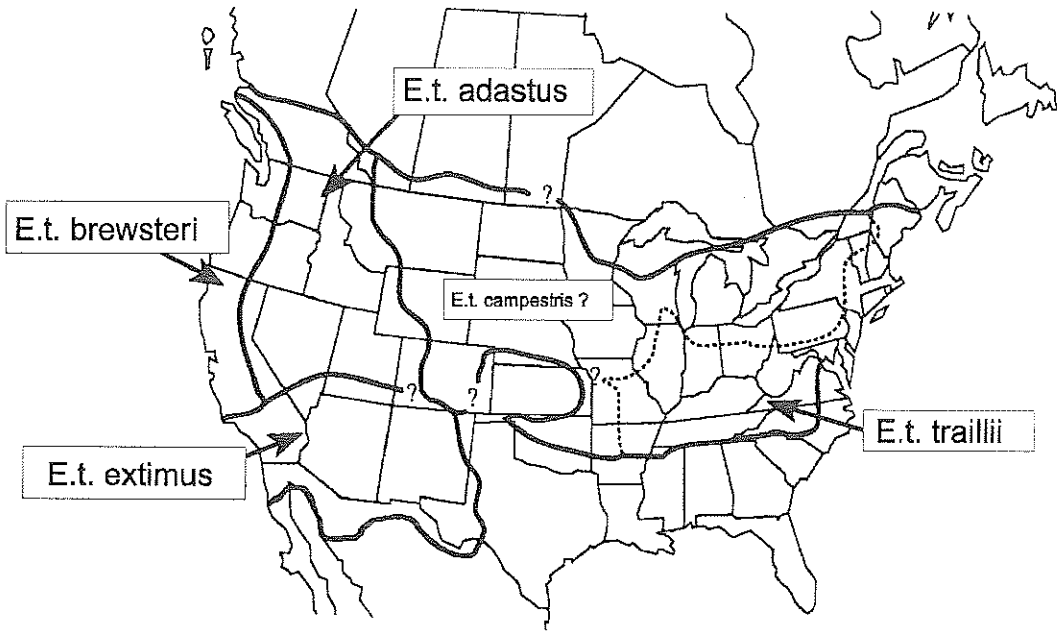


Figure II: Distribution of the breeding ranges of the four or five subspecies of the willow flycatcher. Ranges based on the work of Unitt (1987) and Browning (1993).

while others lumped it with the eastern willow flycatcher subspecies (Aldrich 1951).

Since taxonomic splitting of the Traill's flycatcher into the willow and alder flycatchers (A.O.U. 1973), three reviews of the willow flycatcher subspecies division

while others lumped it with the eastern willow flycatcher subspecies (Aldrich 1951).

Since taxonomic splitting of the Traill's flycatcher into the willow and alder flycatchers (A.O.U. 1973), three reviews of the willow flycatcher subspecies division

have been published (Hubbard 1987, Unitt 1987, Browning 1993). These studies generally agree on the distribution of the three western subspecies: a Pacific slope subspecies (*E. t. brewsteri*), a southwestern subspecies (*E. t. extimus*), and a Great Basin/northern Rocky Mountain's subspecies (*E. t. adastus*; Figure II). There is disagreement, however, about whether one or two subspecies occur in the east: Unitt (1987) and Hubbard (1987) felt that there is only one subspecies, *E. t. traillii*, while

Browning (1993) felt another subspecies (*E. t. campestris*) occurred in the Great Plains and Great Lakes region (Figure II). More study is needed to resolve this dispute, though Unitt's (pers. comm.) reevaluation of Hubbard's "*E. t. campestris*" specimens led him to conclude that they were actually alder flycatcher specimens. Therefore, the division of the flycatcher into four subspecies is the most widely accepted position at this time.

All four subspecies are separated by substantial geographic and/or environmental boundaries: *E. t. adastus* and *E. t. brewsteri* by the Cascade and Sierra Nevada mountain ranges; *E. t. adastus* and *E. t. traillii* by the Rocky Mountains, and *E. t. extimus* against the others by different abiotic environments (with the exception of the northern boundary of *E. t. extimus*, which is similar to southern *E. t. adastus* habitat). These geographic boundaries are fairly well defined, but all authors agree that there are zones of intergradation between all of the subspecies. These intergradation zones are often represented by only a few museum specimens which makes determining exact boundaries between subspecies difficult. Thus, areas from which there are no representative museum specimens ("?" mark areas in Figure II), and fall between two different subspecies' ranges, have no more than a "best guess" boundary line drawn between them. Similarly, boundary lines through intergradation zones, based on only a few specimens, are approximations based on limited information. However, all of the recent studies on the flycatcher (Hubbard 1987, Unitt 1987, Browning 1993) have defined the same general areas as being occupied by morphologically distinct subspecies.

TAXONOMIC ISSUES

Beyond the difficulties associated with the subtleties of characterizing the different willow flycatcher subspecies, questions have been raised about the general

validity of assigning animal subspecies divisions based solely on morphological characters (Barrowclough 1982). The issue is whether morphological characters that vary geographically are genetically influenced as opposed to environmentally influenced. Some subspecies designations were questioned when geographic variation was attributed to environmental clines, such as American robins (*Turdus migratorius*) and red-winged blackbirds (*Agelaius phoeniceus*; James 1983, 1991). Also, several genetic studies did not support subspecies division of avian species that were considered polytypic based on morphology (e.g. downy woodpecker (*Picoides pubescens*), Ball and Avise 1992; song sparrow (*Melospiza melodia*), Zink and Dittmann 1993; red-winged blackbird, Ball et al. 1988; see also Barrowclough et al. 1985 and Zink 1997), while others found genetic patterns that differed from the morphology (dusky seaside sparrow (*Ammodramus maritimus nigrescens*); Avise and Nelson 1989). However, genetic studies have supported morphologically-based geographic patterns (e.g. loggerhead shrike (*Lanius ludovicianus*), Mundy et al. 1997; Fox sparrow (*Passerella iliaca*), Zink 1994) and it is unclear why some species show agreement between genetic and morphological patterns while others do not.

Overall, support for the use of the subspecies division as a taxonomic unit persists (Mayr 1982). It is clear that some species are truly polytypic, with numerous morphological, genetic, and ecological features supporting division. The occurrence of genetically-influenced differences indicates the presence of gene flow barriers and different evolutionary trajectories within a species that allow for divergence. Because research into evolution, ecology, and conservation often relies on subspecies designations, it is important that these designations are biologically accurate. An independent molecular genetic review is one of the best methods for testing the accuracy of a morphology-based subspecies division.

MOLECULAR GENETIC TECHNIQUES USED IN TAXONOMIC STUDIES

Molecular genetic studies of birds began in the 1950's, but analysis of genetic variation within natural populations did not occur until the late 1970's. Early genetic studies used protein electrophoresis techniques and found very few genetic differences between avian populations or subspecies (Barrowclough et al. 1985). The discovery of mitochondrial DNA as a powerful tool for the study of natural populations (Brown et al. 1979), provided a new, higher-resolution method for research that has continued to be used up to the present. Restriction fragment length polymorphism (RFLP) analysis of both mitochondrial DNA and nuclear DNA dominated avian systematic studies through the 1980's into the early 1990's (Avise 1994). Beginning in the early 1990's, following the advent of the Polymerase Chain Reaction (PCR) technique and the ability to easily sequence DNA, most studies focused on DNA sequence.

Today, there are a multitude of techniques available to researchers for studying both nuclear and mitochondrial DNA. While the rapid development of new molecular genetic techniques is exciting, one drawback is that studies conducted even a few years previous are often not directly comparable to recent studies that are based on the most current state-of-the-art techniques. Higher resolution techniques provide more information than older techniques, and genetic diversity and structuring apparent in the newer techniques may have been undetected in older techniques. However, the interpretation of the genetic patterns generated by different techniques is often similar, and many of the population genetic principles used to interpret these patterns are the same regardless of the technique.

For the study of natural populations, both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) have advantages and disadvantages; the use of both provides complimentary and powerful data sets. The properties of mtDNA within a

population make it ideal for examining intraspecific geographic patterns and it is today the choice of many avian geneticists. Mitochondrial DNA mutates at a faster rate than nDNA due to a lack of some DNA repair mechanisms associated with nuclear DNA (Brown et al. 1979, Lewin 1997), so that mutations occur more rapidly than in comparable regions within the nuclear genome. Thus, differences between groups of organisms may develop in mtDNA before they would occur in nDNA. Furthermore, mtDNA is haploid and is inherited from mother to daughter; these properties give mtDNA one fourth the effective population size of nuclear DNA. This smaller effective population size makes mtDNA much more sensitive to demographic and evolutionary processes such as changes in population size and gene flow restrictions. On the other hand, mitochondrial DNA evaluates only one locus (all DNA within the genome being inherited as one unit), and thus could theoretically provide misleading results if the mitochondrial genome has changed due to directional selective pressures, though there is no real evidence of this occurring. Analysis of nuclear DNA has the advantage of examining many different loci at once which, if there is agreement among the loci, allows for powerful conclusions about the relationships of populations.

Both mtDNA and nDNA are essentially independent of each other, and comparisons of their respective patterns can provide additional information on the history of the organism studied. Demographic changes, such as a dramatic population size change, will affect the two genomes at different rates, and could result in different genetic patterns. Mitochondrial DNA, with its smaller effective population size, will react to demographic changes at a faster rate than the nDNA, potentially creating a situation where mtDNA reflects recent demographic changes before nDNA does (Moore 1995).

In this study, I evaluated the mitochondrial DNA using sequences from the *cytochrome-b* gene, and examined the nuclear genome using DNA fingerprints

generated by the Amplified Fragment Length Polymorphism (AFLP) technique. The *cytochrome-b* gene is widely used in natural population studies, especially for interspecific comparisons (Moore and DeFilips 1997). Intraspecific studies using *cytochrome-b* have included research on avian population structure (Birt-Friesen et al. 1992, Mundy et al. 1997, Grapputo et al. 1998, Questiau et al. 1998), evolutionary change (Marshall and Baker 1998), and community level effects on intraspecific variation (Edwards and Wilson 1990). Recently, intraspecific studies have used the D-loop region because of its faster rate of mutation accumulation (Baker and Marshall 1997). I did not use the D-loop region because a preliminary analysis of willow flycatcher D-loop sequences indicated that the percentage of mutations was roughly equivalent to those found within the *cytochrome-b* gene.

AFLP is a recently described technique (Vos et al. 1995) that allows for the examination of many different loci within the nuclear genome. The advantages of AFLPs are that it requires only a small amount of DNA (ideal for endangered species work), is highly replicatable, and requires no previous knowledge of the target organism's genome. AFLP has been used to look at genetic variability in a wide variety of other organisms such as the Kanab amber snail (*Oxyloma* spp.; Miller et al. 1998) and the endangered plant *Astragalius cremnophylax* (Travis et al. 1996). AFLP is just beginning to be used in studies of wild avian populations, with one study looking at extra-pair paternity in the bluethroat (*Luscinia svecica*; Questiau et al. 1999) and another addressing genetic variability and structuring of the southwestern willow flycatcher (Busch et al., *in press*).

PURPOSE OF STUDY

The willow flycatcher is abundant in many parts of its range, particularly in the north and eastern United States, but has been a species of concern in the western U.S. due to declining populations. This trend has been particularly severe within the southwestern United States, where the willow flycatcher's breeding habitat is scarce, primarily due to riparian destruction and modification (USFWS 1995). Unitt (1987) used historical collection records, museum specimens, and literature records to show that the southwestern subspecies had been extirpated from most of the sites where it was once common. In addition, he reviewed willow flycatcher subspecies division, using morphological characteristics, and concluded that all four subspecies were valid taxa. In 1995, the USFWS listed the southwestern willow flycatcher subspecies as endangered, in part due to acceptance of this subspecies as a valid taxon.

Because willow flycatcher subspecies are difficult to separate morphologically, and morphologically-based subspecies divisions may be questionable, I undertook a review of the subspecies designations using independent molecular markers to characterize geographic structuring within this species. Furthermore, use of blood samples from live birds allowed me to obtain information from geographic areas where museum specimens had not been available for previous morphology-based analysis. This provided an opportunity for a more detailed examination of some portions of the southwestern subspecies' boundary.

Busch et al. (*in press*) examined genetic variation in the southwestern willow flycatcher and found relatively high levels of variation across the bird's range. A comparison of genetic variability with the other willow flycatcher subspecies can help determine if the southwestern subspecies has passed through a genetic bottleneck as a result of the recent decline in population size.

Chapter II: Molecular Genetic Structuring and Demographic History of the Willow Flycatcher (*Empidonax traillii*)

INTRODUCTION

Many avian species are divided into subspecies that are defined by geographically-related differences in morphology thought to result from reproductive isolation, with selection and genetic drift responsible for the observed differentiation. However, evidence that distinct morphological features may be at least partially environmentally induced (James 1983), raises questions over the genetic basis, and hence the evolutionary significance, of these differences. Analysis of the geographic patterns of independent molecular genetic markers is one technique to determine whether geographic differences in morphology are genetically derived (Avise and Ball 1990). Initial work with allozymes found few differences among avian subspecies (Barrowclough 1983). Studies using mitochondrial DNA found that less than half of the species examined showed geographic differences, and that geographic barriers (mountain ranges, deserts, isolation on islands, etc.) that could spatially isolate populations explain some, but not all, of the differences that were observed (Zink 1997). Furthermore, evidence suggests that historical events can influence present genetic patterns, such that current genetic structure within avian species cannot be predicted solely on current range and existing gene flow barriers (Avise and Walker 1998). Thus, the concordance of morphological and genetic variation must be evaluated separately for each species, with consideration of the species' demographic and evolutionary history.

The willow flycatcher (*Empidonax traillii*) is a neotropical migratory passerine that

breeds across most of the United States (Figure 1), and winters from southern Mexico through Central America into northwestern South America (DeGraaf and Rappole 1995). Most taxonomists who have examined morphological variation within the willow flycatcher recognize four (Unitt 1987, Hubbard 1987, Philip 1948) or five (Aldrich 1951, Browning 1993) subspecies. They agree on three western subspecies: *E. t. extimus* in the southwest, *E. t. brewsteri* along the Pacific slope, and *E. t. adastus* from the Great Basin east into the Northern Rockies (Figure 1). However, Aldrich (1951) and Browning (1993) felt that the eastern subspecies (*E. t. traillii*) should be split, naming the subspecies in the Great Plains and Great Lakes region *E. t. campestris* and the east-central region subspecies retaining the name *E. t. traillii*. The difference between subspecies is subtle, with the willow flycatcher considered one of the most "problematic" bird species for taxonomists interested in subspecies division (Hubbard 1987). The subtle morphological differences have led to the varying number of proposed subspecies and confusion over their exact distribution.

In 1995, the USFWS declared the southwestern subspecies (*E. t. extimus*) endangered, due to the loss of many historical breeding sites and a dramatic decrease in population size rangewide, primarily caused by loss of habitat (USFWS 1995). The listing rule accepted, and was based on, the validity of the southwestern subspecies as a distinct taxon. Given the morphological subtlety of the willow flycatcher subspecies, and the conservation and management implications of accepting the current subspecies designations, questions arose as to whether an independent molecular genetic review would support the taxonomic distinctness of *E. t. extimus*.

I examined patterns of nuclear and mitochondrial molecular variation to review the subspecies divisions in the willow flycatcher. Nuclear DNA (nDNA) was evaluated using the Amplified Fragment Length Polymorphism (AFLP) technique (Vos et al.

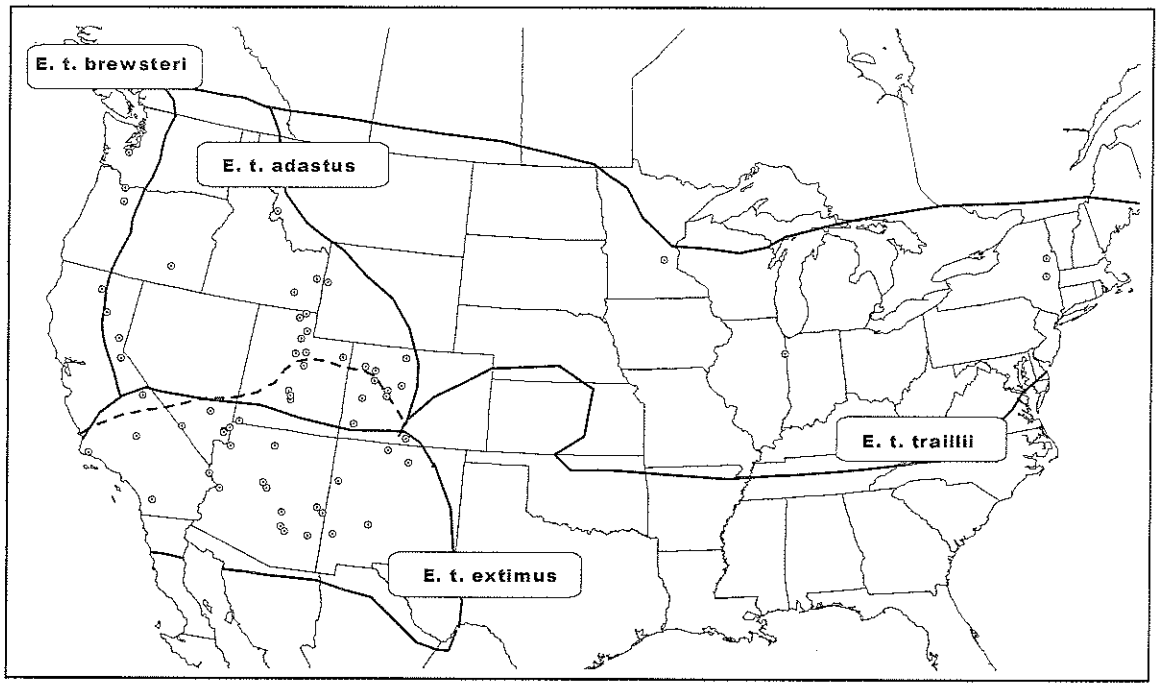


Figure 1: Map showing the breeding distribution of the willow flycatcher (*Empidonax traillii*), range of its subspecies, and sample locations. Solid lines indicate subspecies boundaries based on published taxonomic studies (Unitt 1987, Browning 1993); dashed lines show the current USFWS administrative boundary for the northern portion of the endangered southwestern subspecies, *E. t. extimus*. Circles with central dots are sample locations (see Table 1 for detailed sample site information).

1995). This DNA fingerprinting technique generates data from large numbers of loci throughout the nuclear genome. AFLPs are ideal for population level studies (Mueller and Wolfenbarger 1999), and are just beginning to be used in studies of wild avian populations (Questiau et al. 1999, Busch et al., *in press*). In addition, I determined *cytochrome-b* gene sequences to evaluate mitochondrial DNA (mtDNA) differences between subspecies. This gene is commonly used to characterize intra-specific differences within animals (Moore and DeFilipps 1997). The use of molecular markers from both genomes provides two independent perspectives on subspecies questions, and greater confidence that research results reflect the actual evolutionary history of the

species (Moritz 1994, Moore 1995).

This paper presents the results from complimentary analysis of genetic variation among willow flycatcher subspecies. It explores the demographic history of the willow flycatcher and attempts to provide a better understanding of the origin of the genetic differences observed. Lastly, this paper evaluates whether the endangered southwestern subspecies (*E. t. extimus*) demonstrates a loss of genetic variation due to its recent population decline.

METHODS

Field collections-- I collected genetic samples from 232 adult willow flycatchers at 49 sites in 14 states from 1996-1998 (Figure 1 and Table 1). Birds were captured using mist nets (Ralph et al. 1993) to obtain a non-lethal blood sample, banded, and immediately released. To assure that no migrants were used in this study, I included only adults that were territorial at a site during the non-migrant period (June 15 - July 20; Unitt 1987), or were known by field observation to be resident breeders. Blood samples were taken by clipping a toenail to the vascularized "quick", with a drop of blood rinsed into a 1.5 ml tube with approximately 40 ul of collection buffer (1xSSC, 50 mM EDTA). Samples were stored on ice until they can be frozen, with DNA isolated from the blood following the procedure described by Mullenbach et al. (1989). Blood was digested overnight at 55° C in lysis buffer (10 nM Tris, 1 nM EDTA, 1% SDS, 100 nM NaCl, pH 8) with 200 ug/ml proteinase K and 2 nM DTT (final concentration of both). This lysate was extracted with chloroform and followed by an isopropanol precipitation. An aliquot of each DNA extraction was then electrophoresed on 0.7% agarose gels to assess template quality and quantity.

AFLP-- AFLP markers were generated using the procedure of Vos et al. (1995), with modifications as discussed by Bush et al. (*in press*). Adenine was used as the

Table 1. Willow flycatcher blood sample collection sites, the site's subspecies designation (Based on Unitt 1987, Browning 1993), and number of individuals used for AFLP and *cyt-b* sequencing.

Site Name	Site Code	State	Subspecies	Sample numbers AFLPs	*cyt-b*
Alpine, Apache Co.	ALPI	AZ	*E. t. extimus*	0	2
Camp Verde/Tuzigoot, Yavapai Co.	CAVE	.	.	0	3
Greer, Apache Co.	GREE	.	.	0	2
Roosevelt Lake, Gila Co.	ROOS	.	.	8	12
Safford, Graham Co.	GILA	.	.	0	2
San Pedro River, Pinal Co.	SAPE	.	.	8	6
Topock Marsh, Mohave Co.	TOPO	.	.	7	3
Kern River Preserve, Kern Co.	KERN	CA	.	6	8
Owen's River at Bishop, Inyo Co.	OWEN	.	.	3	3
San Luis Rey, San Diego Co.	SLRE	.	.	5	9
Santa Ynez River, Santa Barbara Co.	SAYE	.	.	6	6
Alamosa NWR/Mcintire Springs, Alamosa Co., Conejos Co.	MCSP	CO	.	6	4
Ash Meadows/Parhanagat Lake, Nye Co., Lincoln Co.	ASME	NV	.	3	5
Virgin River/Lake Mead, Clark Co.	MEAD	.	.	9	6
Gila/Cliff, Grant Co.	GICL	NM	.	6	5
San Marcial, Soccoro Co.	SAMA	.	.	3	0
Tierra Azul, Taos Co.	AZUL	.	.	0	3
St. George, Washington Co.	SEEG	UT	.	4	3
Perazzo Meadow, Sierra Co.	PERA	CA	*E. t. adastus*	4	4
Red Lake, Alpine Co.	REDL	.	.	4	4
Arapahoe National Wildlife Refuge, Jackson Co.	ARAP	CO	.	5	3
Beaver Creek/Clear Creek, Dolores Co.	BBCO	.	.	6	5
Rio Blanco Lake, Rio Blanco Co.	RICO	.	.	6	3
Fall Creek, Bonneville Co.	JACK	ID	.	0	6
Hamon Memorial, Ravilli Co.	HANN	MT	.	6	9
Malheur NWR, Harney Co.	MALH	OR	.	6	8
East Canyon Reservoir, Morgan Co.	EACA	UT	.	6	3
Fish Creek, Caribou Co.	FICR	.	.	6	3
Fish Lake, Sevier Co.	FILA	.	.	6	3
Logan Canyon, Cache Co.	LOCA	.	.	6	3
Logan River, Cache Co.	LORI	.	.	5	2
Lost Creek, Morgan Co.	LOCR	.	.	6	2
Provo River, Utah Co.	PRPA	.	.	4	1
Stewart Lake, Unitah Co.	STLA	.	.	5	3
Strawberry River, Wasatch Co.	STRI	.	.	5	3
Bigelow Meadows, Siskiyou Co.	BIGE	CA	*E. t. brewsteri*	5	7
Warner Valley, Plumas Co.	WARN	.	.	5	7
Fish Creek/Salmon River, Clackamas Co.	FISH	OR	.	4	5
Lacey, Thurston Co.	LAWA	WA	.	2	2
Willow Slew, Newton Co.	WILL	IN	*E. t. traillii*	4	4
Elm Creek, Hennepin Co.	ELMC	MN	.	6	6
Black Creek SWMA, Albanny Co.	BCMA	NY	.	6	4

selective nucleotide in the first amplification; while ACG/AGG, ACG/AGC, ACG/ACC, ACG/ACA, ACG/ACG, ACG/AAG (*Eco*RI/*Mse*I primers, respectively) were used for the second selective amplification. I manually scored the polymorphic AFLP markers, using only distinct and unambiguous polymorphic markers that conformed to the 95% polymorphic rule for this study (Hartl and Clark 1997).

Cytochrome-b-- This study uses 1063 nucleotides of the *cytochrome-b* gene that is 80 nucleotides downstream from the start codon of the gene. All sequences for this region have double strand confirmation. DNA extracts were directly sequenced using Polymerase Chain Reaction (PCR) with primers obtained from Helm-Bychowski and Cracraft (1993; L14827: 5' CCACACTCCACACAGGCCTAATTAA 3', H16065: 5' GGAGTCTTCAGTCTCTGGTTTACAAGAC 3'). PCR reactions consist of 50 ng of DNA, 1x PCR buffer, 3 mM $MgCl_2$, 200 μM of dNTPs, and 1 μM each of primer, and 1 U of *Taq* DNA polymerase, with 35 cycles of 30 seconds at 94° C, 30 seconds at 55° C, and 2 minutes at 72° C. PCR products were concentrated using a Qiagen QIAquick PCR purification kit, then sequenced using the dye-nucleotide termination method with an ABI 377 DNA sequencer. I aligned the sequences obtained manually, and edited them using Sequence Navigator version 1.0.1 (Applied Biosystems).

Data analysis-- I classified samples by subspecies using published subspecies boundaries (Figure 1) based on Unitt (1987) and Browning (1993). My sampling in the eastern United States was not sufficient to address the question of one versus two eastern subspecies; therefore, all eastern samples were pooled into one subspecies (the "majority opinion" in the literature) and a four subspecies division was used. Each individual was analyzed by sequencing the *cytochrome-b*, using AFLPs, or both. Out of the 232 flycatchers analyzed, roughly equal numbers (190 for AFLP, 182 for *cytochrome-b*) were analyzed from most of the same sites. I calculated expected heterozygosity (termed diversity [D] hereafter) and percent polymorphic loci using Tools

for Population Genetic Analyzes software package (TFPGA; Miller 1997a). Nucleotide diversity was calculated using DNAsp (Rozas and Rozas 1999), as were the tests of population growth. A graphical representation of average between-site similarities was obtained using the UPGMA cluster analysis feature of TFPGA, and Nei's (1972) algorithm (Miller 1997a).

Two common indicators of genetic differentiation were calculated: F_{ST} (Weir 1996, Weir and Cockerham 1984) and AMOVA (Excoffier et al. 1992). F_{ST} was calculated using TFPGA (Miller 1997a). Use of this procedure, on the presumed dominant AFLP markers generated in this study, required the assumption that each marker corresponded to an independently-segregating Mendelian locus with genotype frequencies corresponding to Hardy-Weinberg equilibrium. Based on these assumptions, TFPGA estimated allele frequencies using the Taylor expansion approach of Lynch and Milligan (1994) and calculated F_{ST} under the assumption of random mating (Weir and Cockerham 1984). The significance of F_{ST} was tested by generating 95% confidence intervals around the statistic through the use of a bootstrapping procedure (5000 replicates). Confidence limits around F_{ST} that were non-overlapping with 0 were taken as evidence for significant genetic differentiation at the 95% confidence level. I used an Analysis of Molecular Variance (AMOVA) to obtain Φ_{ST} estimates (Excoffier et al. 1992), which were calculated using Arlequin (Schneider et al. 1997). Data files used in the analysis were prepared from raw data with AMOVA-PREP 1.01 (Miller 1997b) using the Euclidean distance metric of Excoffier et al. (1992).

Molecular clock estimates of 2% nucleotide change per million years are employed to estimate the age of the *cyt-b* haplotype diversity (Klicka and Zink 1997). Nucleotide diversity (π) was multiplied by 10^6 and divided by 0.02 to achieve this estimate.

RESULTS

Diversity, mitochondrial DNA-- Of the 1063 *cytochrome-b* nucleotides determined in 182 samples, 43 nucleotide positions (3.8%) were polymorphic and resulted in 40 unique haplotypes (Figure 2). Overall, nucleotide diversity (π) is low (0.00189 for all subspecies), with *E. t. extimus* and *E. t. traillii* the least diverse, and *E. t. brewsteri* the most diverse (Table 2). However, the *cytochrome-b* gene shows high haplotype diversity, with an overall diversity (D) of 0.859. Of the subspecies, *E. t. extimus* has the lowest while *E. t. adastus* has the highest levels of diversity (Table 2). Very little structuring is evident in the *cyt-b* gene-tree, with no more than two mutations separating any two sister haplotypes, and only six mutations separating the two most distant haplotypes (Figure 2). A standard molecular clock calibration of 2% change per million years (Klicka and Zink 1997) estimated the age of the gene-tree's origin at approximately 94,500 years before present.

Maximum parsimony analysis (PAUP 4.0; Swofford 1998) of the *cyt-b* haplotypes rooted with *cyt-b* gene sequence from the alder flycatcher, indicates that the D1 haplotype is the most likely ancestral haplotype from which all the others are derived (analysis not shown). In addition, D1 is no more than three mutational steps from all other haplotypes, at least one mutation less than any other of the haplotypes. The other three core haplotypes (A1, B1, C1) appear to be derived from haplotype D1, and all the *terminal node* haplotypes (except E2 and E3) appear to be directly derived from the four core haplotypes. Much of the haplotype diversity occurs in low frequency, with 24 out of the 40 haplotypes (60%) found only in a single individual, and 88% (35 out of 40) of the haplotypes occurring in no more than four individuals; the *terminal*

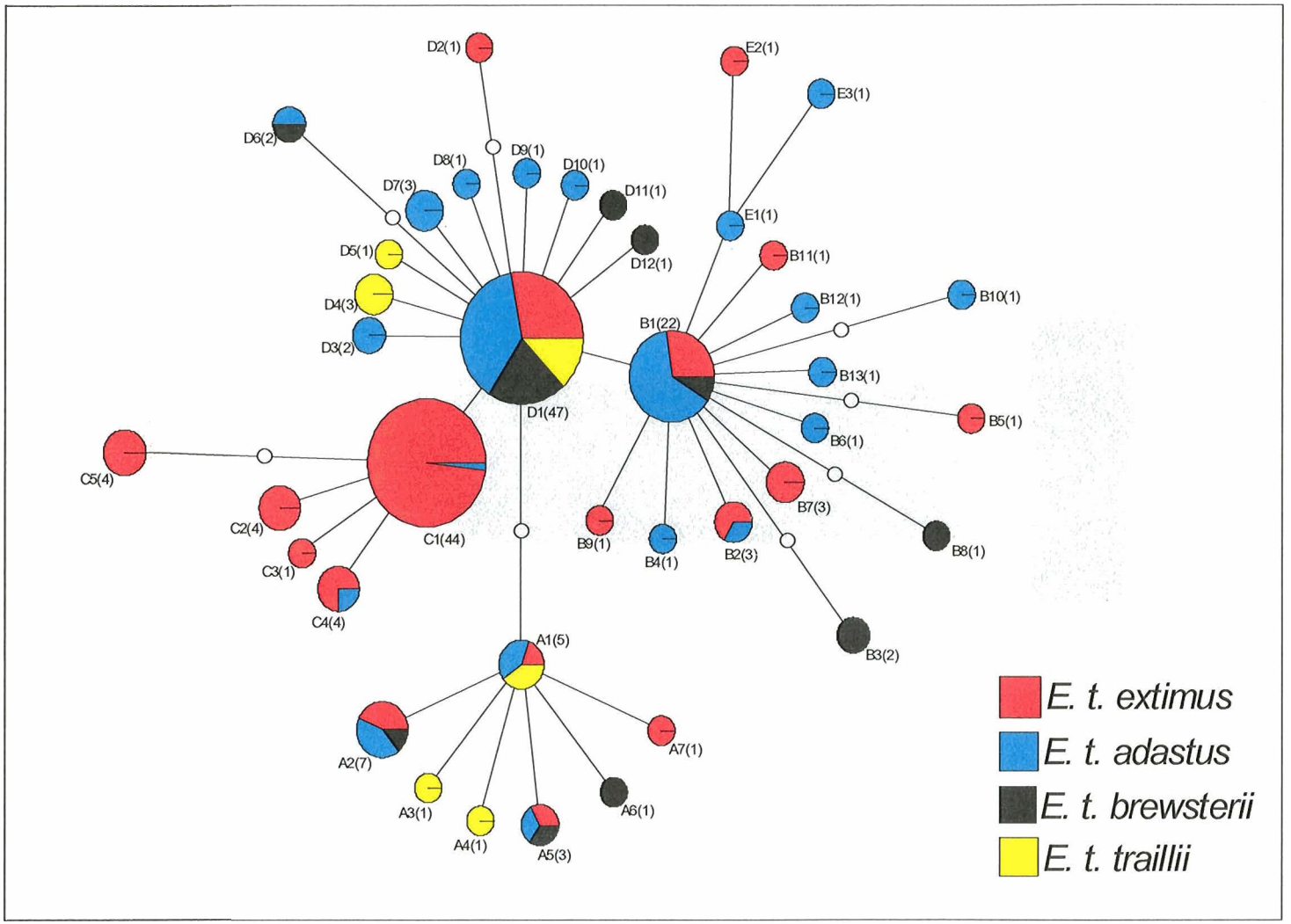
Table 2. Levels of genetic diversity within the willow flycatcher based on AFLPs and *cytochrome-b* sequences. Column headings are: n=number of individuals analyzed, D=estimated gene diversity, P=percent polymorphic loci, N_H=number of unique haplotypes detected, and π=nucleotide diversity.

	AFLPs (104 loci)			*Cytochrome-b* (1063 bp)			
Subspecies	n	D	P	n	N_H	D	π
E. t. extimus	74	0.2031	63.5	83	17	0.709	0.00155
E. t. traillii	16	0.2015	61.5	14	6	0.791	0.00157
E. t. adastus	83	0.2075	60.6	64	23	0.835	0.00167
E. t. brewsteri	16	0.1967	63.5	21	10	0.776	0.00197
All	189	0.2096	69.2	182	40	0.859	0.00189

node haplotypes are the source of the high heterozygosity found at low frequency.

Diversity, nuclear DNA-- I used 104 polymorphic markers, which were clear and unambiguous, across 189 individuals for this study. A set of 20 individuals were blindly replicated and indicated 0% error in scoring and marker consistency of those loci used in this study. All individual AFLP genotypes were unique. Diversity (D) is high for all subspecies, with an overall level of 0.2096 (Table 2); *E. t. adastus* has the highest

Figure 2 (following page): Mitochondrial Gene-tree showing the relationships between willow flycatcher cytochrome-b haplotypes. Each circle represents a unique DNA haplotype, with the lines connecting them representing their inferred evolutionary relationships with one another using parsimony theory. Each line between a haplotype represents a single mutation. Small, open circles represent an inferred ancestral haplotype not detected by this study. Each haplotype's arbitrarily assigned name is given next to the circle (e. g. A1, A2, etc.), and the frequency of occurrence is in parentheses. Additionally, the relative frequency of each haplotype is indicated by the relative size of the circle, and the proportion found within each subspecies is represented by the pie fraction and color designated to each subspecies. Haplotypes A1, B1, C1, and D1 are referred to as core haplotypes, with all other haplotypes referred to as *terminal node* haplotypes. Haplotype groups are defined as each core haplotype and their respective *terminal node* haplotypes (e. g. "C-group" would include C1, C2, C3, C4, and C5). Subspecies designations follow boundaries defined by Unitt (1987) and Browning (1993).


D2(1)
E2(1)
E3(1)
D6(2)
D8(1)
D9(1)
D10(1)
D7(3)
D11(1)
E1(1)
D5(1)
D12(1)
B11(1)
D4(3)
B12(1)
B10(1)
B1(22)
D3(2)
B13(1)
D1(47)
B5(1)
B6(1)
C5(4)
B7(3)
C2(4)
C1(44)
B9(1)
B4(1)
B2(3)
B8(1)
C3(1)
C4(4)
A1(5)
B3(2)
A2(7)
A7(1)
A3(1)
A6(1)
A4(1)
A5(3)
E. t. extimus
E. t. adastus
E. t. brewsterii
E. t. traillii

within subspecies diversity (D), followed by *E. t. extimus*, *E. t. traillii* , and *E. t. brewsteri.* Percent polymorphic loci (P) is also high overall (69.2%), with *E. t. extimus* and *E. t. brewsteri* showing the highest levels among the four subspecies.

Genetic structuring-- AMOVA and Wright's F_{ST} analysis show significant, but variable, structuring between most pairwise subspecies comparisons (Table 3). *Cyt-b* data analysis indicates greater differences among the subspecies than AFLP data, with overall F_{ST} values of 0.1540 compared to 0.0325, and overall AMOVA group values of 17.5% compared to 3.5%, *cyt-b* to AFLP respectively. No significant difference is seen between *E. t. brewsteri* and *E. t. adastus* in either mtDNA or nDNA.

The greatest degree of *cyt-b* difference is between *E. t. extimus* and the three northern subspecies. *E. t. extimus* versus the other subspecies results in pairwise F_{ST} values from 0.2094 to 0.2484, indicating "great" to "very great" genetic differentiation (Wright 1978). Similar differences are seen in the AMOVA results (Table 3). The three northern subspecies show less mtDNA difference, with F_{ST} values indicating "little" to "moderate" genetic differences (Wright 1978), and AMOVA results indicating genetic variation is not as well explained by grouping individuals into subspecies. The relationship among willow flycatcher breeding sites also follows this pattern, with southwestern sites tending to group with one another, but less geographic structuring among the three northern subspecies' sites (Figure 3).

AFLP results show significant differences among most subspecies, though with smaller F_{ST} and AMOVA values than seen in the *cyt-b* sequences. No single AFLP allele is unique for a particular subspecies, so subspecies differences are based on marker frequency differences. In contrast with the *cyt-b* results, *E. t. traillii* is the most genetically distinct among the subspecies, with *E. t. extimus* being the next most distinct (Table 3), which is consistent with presumably nDNA-derived morphological

Table 3. Genetic structuring within the willow flycatcher and between pairwise comparison of the four subspecies. AMOVA values measure the following: Group: amount of genetic variation explained by grouping individuals within a subspecies, Population (Pop.): amount of variation that is explained by individuals being from the same breeding site, and Individual (Indiv.): amount of variation found among the individuals from all the sites. AMOVA Group percentages and Wright's F_{ST} values that are significant (P<0.05) are marked with an asterisk, and values between p=0.10 and p=0.05 are marked with a cross.

	AMOVA - AFLPs			AMOVA - *Cytochrome-b*			F_{ST}	
Subspecies	Group	Pop.	Indiv.	Group	Pop.	Indiv.	AFLPs	*cyt-b*
E. t. extimus								
vs. E. t. traillii	9.0%*	7.7%	83.3%	22.2%*	16.5%	61.3%	0.1012*	0.2484*
E. t. extimus								
vs. E. t. adastus	3.3%*	5.9%	90.8%	21.8%*	13.1%	65.1%	0.0365*	0.2276*
E. t. extimus								
vs. E. t. brewsteri	2.2%†	9.1%	88.7%	18.3%*	14.2%	67.5%	0.0384*	0.2094*
E. t. traillii								
vs. E. t. adastus	4.2%*	2.1%	93.7%	11.8%*	8.2%	80.0%	0.0450*	0.1302*
E. t. traillii								
vs. E. t. brewsteri	4.4%*	2.0%	93.6%	3.7%	2.6%	93.7%	0.0490*	0.0454*
E. t. adastus								
vs. E. t. brewsteri	0%	3.0%	97.0%	0.7%	6.8%	92.5%	0.0024	0.0183
All Subspecies	3.5%*	5.4%	91.1%	17.5%*	11.6%	70.9%	0.0325*	0.1540*

differences (Unitt 1987). Overall, the AFLP data show less geographic grouping of sites than the *cyt-b* data (Figure 4).

Demographic history– The distribution of pairwise differences in both the *cyt-b* sequence and the AFLP profiles can be evaluated to determine if there has been stability in the population size of the willow flycatcher, or whether recent demographic changes have occurred (Rogers and Harpending 1992). I examined differences for all

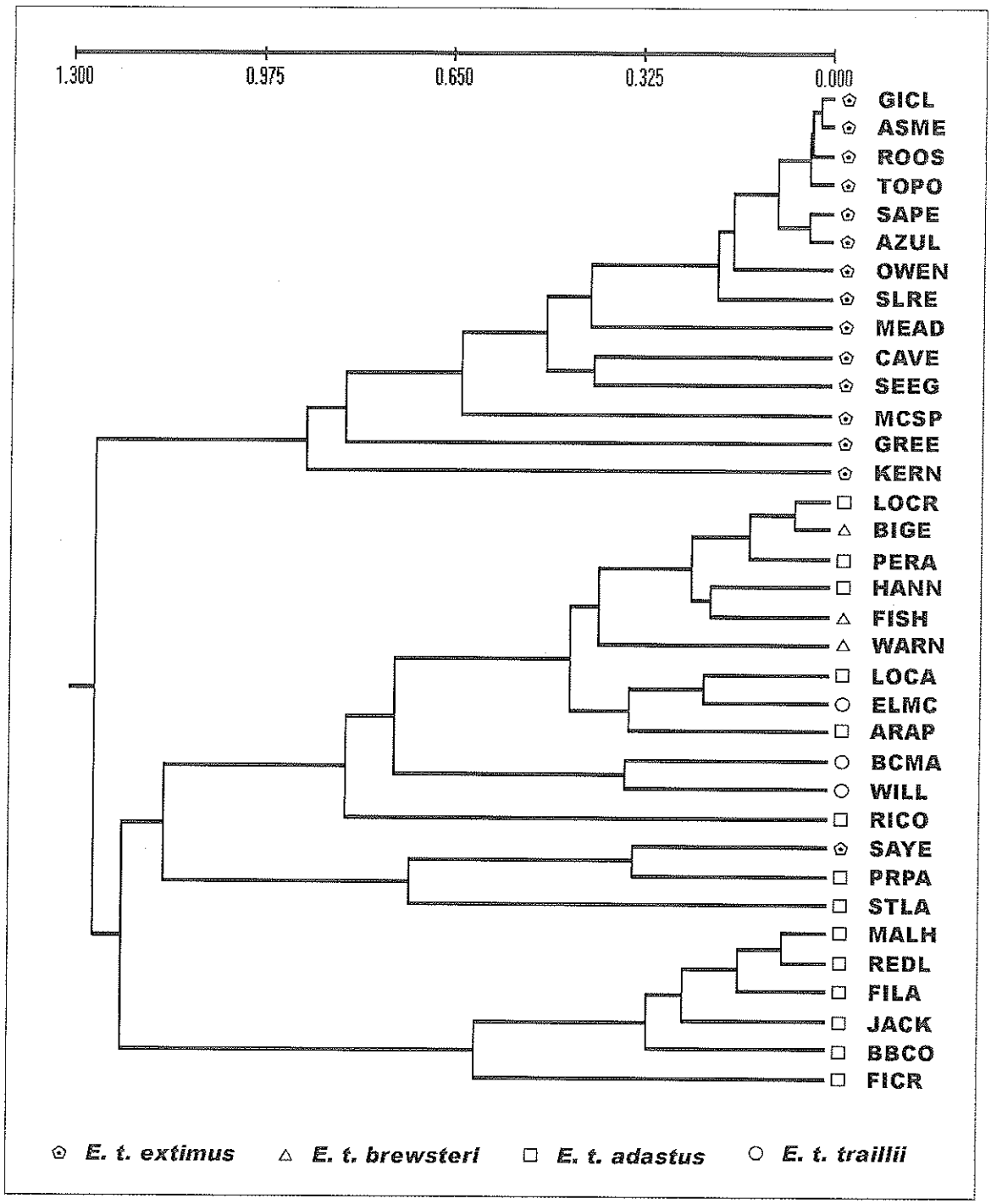


Figure 3: UPGMA dendogram showing the relationship of sites using *cytochrome-b* sequence data. Based on Nei 1972 using TFPGA (Miller 1997a). See Table 1 for explanation of site locations. Subspecies designations are based on subspecies maps of Unitt (1987), Browning (1993), and USFWS administrative boundaries. Sites with fewer than 3 samples are pooled with a geographically neighboring site.

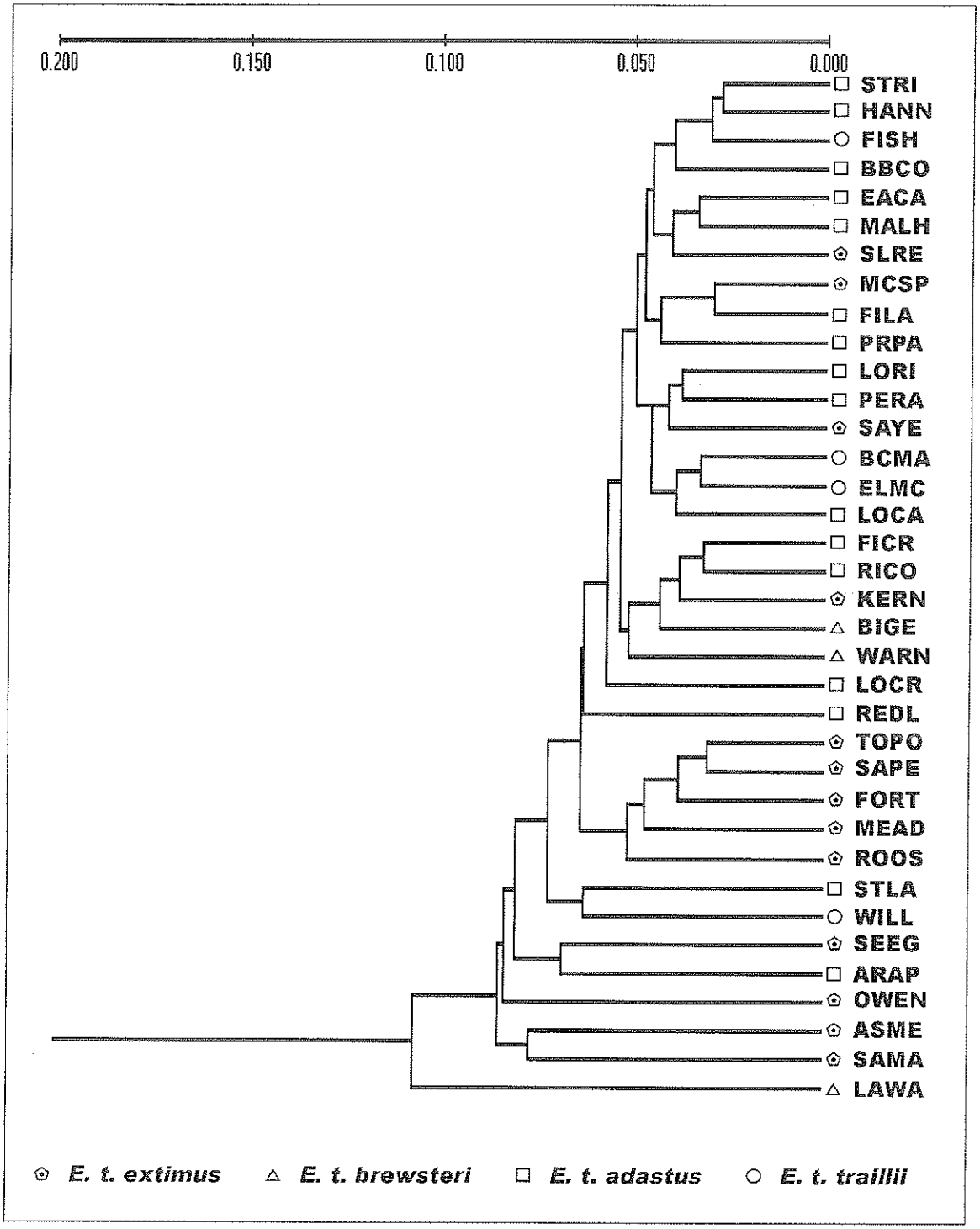


Figure 4: UPGMA dendogram showing the relationship of sites using AFLP markers. Based on Nei 1972 using TFPGA (Miller 1997a). See Table 1 for explanation of site locations. Subspecies designations are based subspecies maps of Unitt (1987), Browning (1993), and USFWS administrative boundaries.

willow flycatcher subspecies combined, and for the two subspecies with an adequate sample size (*E. t. extimus* and *E. t. adastus*). Both the mtDNA and nDNA suggest that the willow flycatcher (all subspecies combined) and *E. t. adastus* have gone through a period of rapid population growth (Figures 5A, B, D, E). Nuclear DNA patterns of *E. t. extimus* (Figure 5F) suggest a period of rapid growth, but the *cyt-b* exhibits a geometric distribution more along the lines of a population that has not rapidly increased (Figure 5C), or a population that has recently declined.

DISCUSSION

Genetic variation.-- Genetic variation in the willow flycatcher is relatively high. Heterozygosity in willow flycatcher's *cyt-b* is higher than in other widespread North American birds (e. g. song sparrow, brown-headed cowbird, etc.; Ball and Avise 1992, Fry and Zink 1998). There are no comparable AFLP studies on wild birds for direct comparison; hopefully, future AFLP-based research will provide comparative context for what appears to be high polymorphism. *Empidonax t. extimus* has significantly lower levels of *cyt-b* heterozygosity than *E. t. adastus* and lower (but not significant) levels of heterozygosity compared to the other two subspecies. This may indicate effects from the demographic bottleneck *E. t. extimus* has experienced during the last 100-150 years (USFWS 1995).

The low nucleotide diversity in the *cyt-b* suggests a recent origin of the current haplotype diversity, as no haplotype is more than three mutations removed from the root haplotype (D1). Taking the frequently used value of 2% nucleotide change per million years (Klicka and Zink 1997), I estimated the time of coalescence at 94,500 years before present. This makes the willow flycatcher's mtDNA genome one of the youngest yet described for a single bird species (Avise and Walker 1998), though the

Mitochondrial DNA

Nuclear DNA

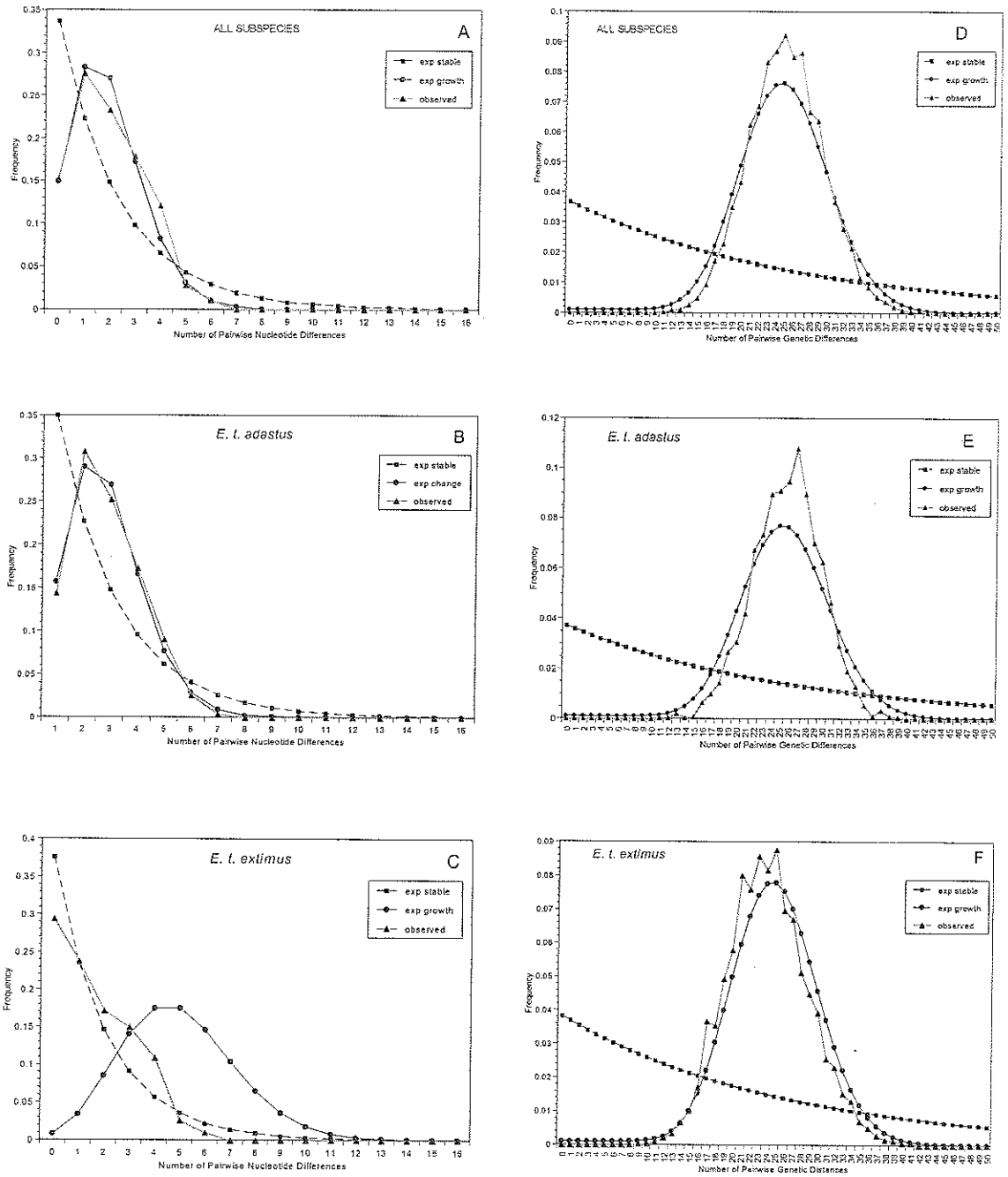


Figure 5: Distribution of pairwise genetic differences within the *cyt-b* and AFLP data. Figures A-C are from pairwise nucleotide differences in the *cytochrome-b* sequences, and figures D-F are from the pairwise genetic differences in the AFLP data. Expected and observed frequencies were calculated for all subspecies combined, and for the two subspecies with large samples sizes (*E. t. adastus* and *E. t. extimus*), using DNAsp v 3.0 (Rozas and Rozas 1999).

use of a molecular clock to determine the time of origin is fraught with difficulty (Avise 1994).

Genetic structuring-- Mitochondrial and nuclear DNA data indicate significant genetic structuring within the willow flycatcher. The mtDNA structuring fell into two categories: a highly significant degree of separation between *E. t. extimus* and the three northern subspecies, and lower levels of genetic differences among the three northern subspecies; nDNA lacked any strong levels of structuring between subspecies. The distinctness of *E. t. extimus* suggests greater isolation from the other subspecies, a different demographic history, or a combination of both scenarios.

The lack of any significant differences between *E. t. adastus* and *E. t. brewsteri* may be due to small sample size for *E. t. brewsteri*, as well as biased sampling towards *E. t. brewsteri*'s range along an intergradation zone with *E. t. adastus*. Genetic differences between the three northern subspecies results from low frequency, unshared haplotypes, and a large sample size might be necessary to detect differences.

Geographic distribution of cytochrome-b haplotypes-- The geographic patterns of *cyt-b* haplotype distribution suggest lower levels of gene flow between subspecies than are suggested by the F_{ST} values. All haplotype groups show limited geographic distribution (Figure 6), and only 10 of the 40 haplotypes (25%) were detected in more than one subspecies (Figure 2). Only the presumed ancestral haplotype D1 is found across the range of all four subspecies (Figure 6E), suggesting retained ancestral lineage. The core haplotypes have the next greatest geographic distribution, with all occurring in at least two subspecies, while the terminal node haplotypes (presumed to be more recently derived) show the smallest geographic distribution (only 17% occurring in more than one subspecies). While some of the haplotypes shared among

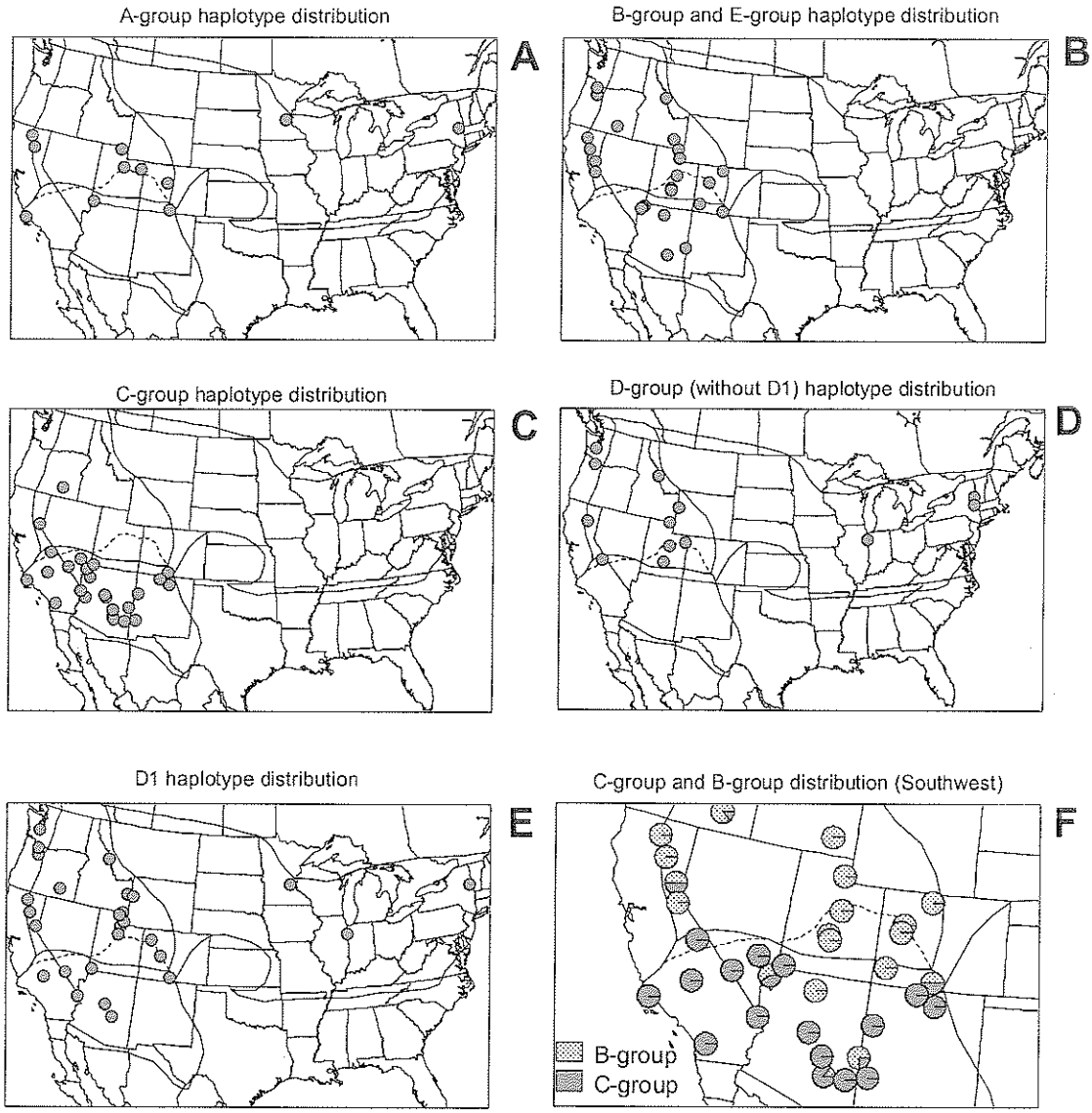


Figure 6: Geographic distribution of cytochrome-b haplotype groups (see Figure 2). Grey circles in figures A-E indicate a site with the occurrence of the particular haplotype, regardless of frequency. Circles in figure F indicate occurrence and frequency of haplotype groups C and B; frequencies are for these two haplotype groups, and not absolute frequency of all haplotypes. A, distribution of A-group; B, distribution of B-group and E-group; C, distribution of C-group; D, distribution of D-group, without the D1 haplotype that has wide distribution; E, distribution of the D1 haplotype; and F, distribution of C-group and B-group haplotypes in the southwestern United States. Subspecies boundaries are based on Unitt (1987) and Browning (1993), and the dotted line indicates the current USFWS administrative boundary for *E. t. extimus.*

subspecies probably indicate gene flow, the widespread distribution of the core haplotypes is probably due to incomplete lineage sorting, where haplotypes shared by an ancestral population are still retained by separate populations. It is difficult to distinguish between gene flow and lineage sorting, especially within the *terminal node* haplotypes, but clearly the limited distribution of *terminal node* haplotypes indicates a low level of gene flow across large geographic areas.

Genetic- vs. morphology-based geographic patterns-- The distribution of the haplotypes tends to follow subspecies boundaries based on the published morphologically-based taxonomy of Unitt (1987) and Browning (1993). The distribution of B-group haplotypes is widespread throughout the range of *E. t. adastus*, and occurs outside of that range primarily along its boundaries with *E. t. brewsteri* to the West, and *E. t. extimus* to the South. *E. t. traillii* shares no terminal node haplotypes with any other subspecies.

E. t. extimus is characterized by the C-group of haplotypes, which are found in high frequency throughout most of its range. Over 60% of the individuals sampled within the range of *E. t. extimus* possessed one of the C-group haplotypes, and only two occurrences were detected outside of its range. Furthermore, virtually every site within the core area of *E. t. extimus* (Southern California, Arizona, New Mexico) had a high frequency of these haplotypes. No other subspecies has this degree of frequency bias for a particular group of *cyt-b* haplotypes.

The current USFWS administered boundary of the endangered southwestern willow flycatcher (*E. t. extimus*) differs from the published taxonomic studies based on morphology (Unitt 1987, Browning 1993) along its northern boundary with *E. t. adastus* (Figure 1). The *cyt-b* pattern along this extensively sampled boundary suggests that the northern range of *E. t. extimus* is closer to the published taxonomic distributions (Unitt

1987, Browning 1993) than to the current USFWS administered boundaries. Distributions of C-group and B-group haplotypes (Figure 6F) indicate that the southern border of Utah has *E. t. extimus* stock, while sites farther north are typical of *E. t. adastus*. Subspecies status of southwestern Colorado has always been uncertain due to the small number of museum specimens (Unitt 1987). My *cyt-b* data indicate that the southern-most sites in southwestern Colorado are intergrade zones, with no *E. t. extimus* haplotypes detected farther north. The Owen's River in eastern California has a high frequency of C1 haplotypes, suggesting that willow flycatchers there should be affiliated with *E. t. extimus* (as also recommended by Unitt 1987).

The significant levels of genetic structuring within the willow flycatcher subspecies, evidence of limited gene flow across subspecies boundaries, and the general agreement of *cyt-b* haplotype distribution and subspecies boundaries, all support that the morphological characters used for the published taxonomy are primarily genetically derived.

Demographic history of the willow flycatcher species-- The patterns of the genetic data suggest the willow flycatcher's *cyt-b* diversity is relatively young. The low nucleotide diversity, corresponding lack of structuring in the *cyt-b* gene-tree, and recent molecular clock estimate (<94,500 ybp) all indicate that the haplotypes are recently derived. Furthermore, the high frequency and wide geographic distribution of the apparent ancestral haplotype D1, in contrast to all other haplotypes, is best explained by the species existing, at some point in the past, in a small, panmictic population with a relatively small number of haplotypes. Thus this study suggests the willow flycatcher existed as a single, small panmictic population less than 94,500 years ago.

The willow flycatcher shows evidence of a rapid population growth following its occurrence as a small, panmictic population. Weak structuring in the *cyt-b* gene-tree, high occurrence of low frequency polymorphisms, and a unimodal distribution of

pairwise genetic differences in both mtDNA and nDNA (Figures 5A and 5D) all indicate a rapid population growth (Lavery et al. 1996, Hey and Harris 1999). Furthermore, the Poisson-like distribution of the *cyt-b* of all subspecies combined and *E. t. adastus* indicate exponential population growth (Slatkin and Hudson 1991).

Given the current, geographically extensive breeding range of the willow flycatcher (Figure 1), such a significant population growth most likely occurred during a large range expansion that probably began from a much smaller geographic area. That would agree with the small panmictic population predicted by the *cyt-b* haplotype gene-tree structure. It is possible that willow flycatchers were forced into a small Pleistocene refuge during one of the last major ice age epochs, then underwent a rapid range expansion as the ice sheets receded. Similar scenarios have been proposed for other temperate zone birds (Zink 1997, Zink and Slowinski 1995, Avise and Walker 1998). If this occurred, the current range of the willow flycatcher may represent the maximum boundaries for the species to date, and it may still be expanding in some areas (as suggested by Browning 1993, Unitt, pers. comm.).

Implications of population changes on genetic structuring-- For genetic differences to arise among geographically separated populations, gene flow barriers must exist long enough for mutation and genetic drift to make the populations genetically distinct. The evidence for the recent occurrence of major population change, the high AMOVA and F_{ST} values among pairwise comparisons of most subspecies, and limited geographic distribution of most *cyt-b* haplotypes supports the existence of sustained gene flow barriers among the subspecies.

Without the large sample size and high resolution molecular techniques of this study, genetic differences among the willow flycatcher subspecies may not have been detected. Morphological differences are presumably driven by evolution, and are undoubtedly polygenic and able to change rapidly (Rana et al. 1999); they should reflect

demographic processes (such as genetic drift, adaptation to different habitats, etc.) before the neutral molecular markers do. The lower degree of structuring in the nuclear DNA, in contrast with the strong mitochondrial and morphological geographic patterns, suggests that the willow flycatcher's genome has not yet arrived at equilibrium. Mitochondrial DNA, with its smaller effective population size, may be more sensitive than nuclear DNA to recent demographic changes. The difference between the two genomes may be due to a delayed response of the nuclear DNA to more recent gene flow barriers and the resultant genetic structuring.

Evidence of a genetic bottleneck in the endangered southwestern subspecies--Very recent demographic changes can effect genetic patterns in species and their sub-populations. The southwestern willow flycatcher, *E. t. extimus*, passed through a severe demographic bottleneck over the last 100-150 years. Habitat destruction and modification, intensive grazing, water impoundment, and other human-caused factors throughout the southwest led to the loss of vast stretches of riparian vegetation, essential breeding habitat for *E. t. extimus* (USFWS 1995). With the reduction of detrimental land practices, restoration of some riparian corridors, and perhaps with the introduction of the exotic tamarisk which can grow in areas inhospitable to native vegetation, suitable breeding habitat for the willow flycatcher has probably increased over the last 50 years. Thus, the southwestern subspecies, while currently existing at extremely small numbers, has probably emerged from the severest point of the bottleneck. A comparison of *E. t. extimus* to the other willow flycatcher subspecies indicates that *E. t. extimus* may have lost some genetic variation during this period.

The genetic uniqueness of *E. t. extimus* may be due, in part, to the demographic bottleneck that it experienced. The dominant, high frequency distribution of C1 haplotypes throughout *E. t. extimus*' range, and lower *cyt-b* diversity, indicate a recent demographic history which differs from the other willow flycatcher subspecies.

Furthermore, the frequency of *E. t. extimus*' *cyt-b* pairwise differences suggest a population which did not experience rapid population growth or significant decline (Figure 3C; Lavery et al. 1996, Rogers and Harpending 1992). A separate demographic history for the willow flycatcher (e.g. no rapid population growth) would suggest a long term isolation from the other willow flycatcher subspecies; the lack of strong nucleotide differences between *E. t. extimus* and the other subspecies contradicts this conclusion. Therefore, it is more likely that the geometric distribution of *cyt-b* pairwise differences in *E. t. extimus*' is due to a recent population decline.

Detailed analysis of the southwestern subspecies, using AFLPs (Busch et al., *in press*), indicates high levels of nuclear genetic diversity remain across the southwestern subspecies' range; even within small isolated breeding sites. These findings are also supported by this study, which indicates high nDNA diversity when compared to other willow flycatcher subspecies. The contrast between mtDNA, which indicates a genetic bottleneck, and nDNA which does not, may indicate that the demographic bottleneck was not long enough to substantially impact the nuclear genome.

LITERATURE CITED

Aldrich, J. W. 1951. A review of the races of the Traill's Flycatcher. Wilson Bull. 63:192-197.

American Ornithologists' Union. 1957. Check-list of North American Birds. 5th ed. Am. Ornithol. Union, Baltimore.

American Ornithologists' Union. 1973. Thirty-second supplement to the American Ornithologists' Union checklist of North American birds. Auk 90:411-419.

Avise, J. C. 1994. Molecular markers, natural history and evolution. Chapman and Hall, New York.

Avise, J. C. and W. S. Nelson. 1989. Molecular genetic relationships of the extinct Dusky Seaside Sparrow. Science 243: 646-648.

Avise, J. C. and D. Walker. 1998. Pleistocene phylogeographic effects on avian populations and the speciation process. Proc. R. Soc. Lond. B 265:457-463.

Avise, J. C. and R. M. Ball. 1990. Principles of genealogical concordance in species concepts and biological taxonomy. Oxford Surveys Evol. Biol. 7:45-67.

Baker, A. J. and H. D. Marshall. 1997. Mitochondrial control region sequences as tools for understanding evolution. Pages 51-82 *in* Avian molecular evolution and systematics (D. P. Mindell, Ed.). Academic Press, San Diego.

Ball, R. M. and J. C. Avise. 1992. Mitochondrial DNA phylogeographic differentiation among avian populations and the evolutionary significance of subspecies. Auk 109:626-636.

Ball, R. M., S. Freeman, F. C. James, E. Bermingham, and J. C. Avise. 1988. Phylogeographic population structure of red-winged blackbirds assessed by mitochondrial DNA. Proc. Natl. Acad. Sci. USA 85:1558-1562.

Barrowclough, G. F. 1982. Geographic variation, predictiveness, and subspecies. Auk 99:601-603.

Barrowclough, G. F. 1983. Biochemical studies of microevolutionary processes. Pages 223-261 *in* Perspectives in Ornithology (A. H. Brush and C. A. Clark, Eds.). Cambridge Univ. Press, New York.

Barrowclough, G. F., N. K. Johnson, and R. M. Zink. 1985. On the nature of genic variation in birds. Current Ornithology 2:135-154.

Birt-Friesen, V. L., W. A. Montevecchi, A. J. Gaston, and W. S. Davidson. 1992. Genetic structure of Thick-billed Murre (*Uira lomvia*) populations examined using direct sequence analysis of amplified DNA. Evolution 46:267-272.

Brown, W. M., M. George, and A. C. Wilson. 1979. Rapid evolution of animal

mitochondrial DNA. Proc. Natl. Acad. Sci. USA 76:1967-1971.

Browning, M. R. 1993. Comments on the taxonomy of *Empidonax traillii* (Willow Flycatcher). Western Birds 24:241-257.

Busch, J. D., M. P. Miller, E. H. Paxton, M. K. Sogge, and P. Keim. *in press*. Genetic variation in the endangered southwestern willow flycatcher. Auk 117.

DeGraaf, R. M. and J. H. Rappole. 1995. Neotropical migratory birds: Natural history, distribution, and population change. Comstock Publishing Associates, Ithaca, New York.

Edwards, S. V. and A. C. Wilson. 1990. Phylogenetic informative length polymorphism and sequence variability in mitochondrial DNA of Australian songbirds (*Pomatostomus*). Genetics 126:695-711.

English, H. C., E. H. Paxton, and M. K. Sogge. 1999. Survivorship and movements of southwestern willow flycatchers in Arizona - 1999. U.S. Geological Survey report to the U.S. Bureau of Reclamation, Phoenix. 47pp.

Excoffier, L., P. E. Smouse, and J. M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. Genetics 131:479-491.

Fry, A. J. and R. M. Zink. 1998. Geographic analysis of nucleotide diversity and song sparrow (Aves: Emberizidae) population history. Mol. Ecol. 7:1303-1313.

Grapputo, A., A. Pilastro., and G. Marin. 1998. Genetic variation and bill size dimorphism in a passerine bird, the Reed Bunting. Mol. Ecol. 7:1173-1182.

Hartl, D. L. and A. G. Clark. 1997. Principles of population genetics. Third edition. Sinauer Assoc., Massachusetts.

Helm-Bychowski, K. and J. Cracraft. 1993. Recovering phylogenetic signal from DNA sequences: Relationships within the Corvine assemblages (Class Aves) as inferred from complete sequences of the mitochondrial DNA *cytochrome-b* gene. Mol. Biol. Evol. 10:1196-1214.

Hey, J. and E. Harris. 1999. Population bottlenecks and patterns of human polymorphism. Mol. Biol. Evol. 16:1423-1426.

Hubbard, J. P. 1987. The status of the willow flycatcher in New Mexico. Report to the New Mexico Dept. of Game and Fish. 29 pages.

James, F. C. 1983. Environmental component of morphological differentiation in birds. Science 221:184-186.

James, F. C. 1991. Complimentary descriptive and experimental studies of clinal variation in birds. Amer. Zool. 31:694-706.

Klicka, J. and R. M. Zink. 1997. The importance of recent ice ages in speciation: a failed

paradigm. Science 277:1666-1669.

Lavery, S., C. Moritz, and D. R. Fielder. 1996. Genetic patterns suggest exponential population growth in a declining species. Mol. Biol. Evol. 13:1106-1113.

Lewin, B. 1997. Genes VI. Oxford University Press, New York. 1259 pp.

Lynch, S. M. and B. G. Milligan. 1994. Analysis of population structure with RAPD markers. Mol. Ecol. 3:91-99.

Marshall, H. D. and A. J. Baker. 1998. Rates and patterns of mitochondrial DNA sequence evolution in Friingilline Finches (*Fringilla* spp.) And the Greenfinch (*Carduelis chloris*). Mol. Biol. Evol. 15:638-646.

Mayr, E. 1982. Of what use are subspecies? Auk 99:593-595.

McCabe, R. A. 1991. The little green bird: Ecology of the willow flycatcher. Rusty Rock Press, Madison, Wisconsin. 171 pp.

Miller, M. P. 1997a. Tools for Population Genetic Analyses (TFPGA) version 1.3: A windows program for the analysis of allozyme and molecular population genetic data. Computer software distributed by author, Northern Arizona University, Flagstaff, Arizona.

Miller, M. P. 1997b. AMOVA-PREP version 1.01. A program for the preparation of AMOVA input files from dominant-marker raw data. Computer software distributed by author, Northern Arizona University, Flagstaff, Arizona.

Miller, M. P., J. Busch, J. Sorenson, L. Stevens, and P. Keim. 1998. Genetic diversity, population structure, and relationships of the Kanab Amber Snail (*Oxyloma haydeni haydeni*) in the southwest USA. Report to the National Park Service and Arizona Game and Fish Department.

Moore, W. S. 1995. Inferring phylogenies from mtDNA variation: Mitochondrial-gene trees versus nuclear-gene trees. Evolution 49:718-726.

Moore, W. S. and V. R. DeFilippis. 1997. The window of taxonomic resolution for phylogenies based on mitochondrial cytochrome b. Pages 84-119 *in* Avian molecular evolution and systematics (D. P. Mindell, Ed.). Academic Press, San Diego.

Moritz, C. 1994. Applications of mitochondrial DNA analysis in conservation: a critical review. Mol. Ecol. 3:401-411.

Mueller, U. G. and L. L. Wolfenbarger. 1999. AFLP genotyping and fingerprinting. TREE 14:389-394.

Mullenbach, R., J. P. L. Lagoda, and C. Welter. 1989. An efficient salt-chloroform extraction of DNA from blood and tissues. Trends in Genetics 5:391.

Mundy, N. I., C. S. Winchell, and D. S. Woodruff. 1997. Genetic differences in between

the endangered San Clemente Island Loggerhead Shrike *Lanius ludovicianus mearnsi* and two neighboring subspecies demonstrated by mtDNA control region and *cytochrome-b* sequence variation. Mol. Ecol. 6:29-37.

Netter, M. R., E. H. Paxton, and M. K. Sogge. 1998. Banding and Movements of the southwestern willow flycatcher at Roosevelt Lake and San Pedro/Gila River confluence, Arizona - 1998. U.S. Geological Survey report to the U.S. Bureau of Reclamation, Phoenix. 48 pp.

Paradzick, C. E., R. F. Davidson, J. W. Rourke, M. W. Sumner, A. M. Wartell, and T. D. McCarthey. 2000. Southwestern willow flycatcher 1999 survey and monitoring report. Technical report 151, Nongame and Endangered Wildlife Program, Arizona Game and Fish Department. 93 pp.

Phillips, A. R. 1948. Geographic variation in *Empidonax traillii*. Auk 65:507-514.

Questiau, S., M. C. Eybert, and P. Taberlet. 1999. Amplified fragment length polymorphism (AFLP) markers reveal extra-pair parentage in a bird species: the bluethroat (*Luscinia svecica*). Mol. Ecol. 8:1331-1339.

Questiau, S., M. C. Eybert, A. R. Gaginskaya, L. Gielly, and P. Taberlet. 1998. Recent divergence between two morphologically differentiated subspecies of bluethroat inferred from mitochondrial DNA sequence variation. Mol. Ecol. 7:239-245.

Ralph, C. J., G. R. Geupel, P. Pyle, T. E. Martin, and D. F. DeSante. 1993. Handbook of field methods for monitoring land birds. USDA Forest Service general technical report PSW-GTR-144.

Rana, B. K., D. Hewett-Emmett, L. Jin, B. H. -J. Chang, N. Sambuughin, M. Lin, S. Watkins, M. Bamshad, L. B. Jorde, M. Ramsay, T. Jenkins, and W. Li. 1999. High polymorphism at the human Melanocortin 1 receptor locus. Genetics 151:1547-1557.

Rogers, A. R. and H. Harpending. 1992. Population growth makes waves in the distribution of pairwise genetic differences. Mol. Biol. Evol. 9:552-569.

Rozas, J. and R. Rozas. 1999. DNAsp version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics 15:174-175.

Schneider, S., J. M. Kueffer, D. Rosessli, and L. Excoffier. 1997. Arlequin. Http://anthropologie.unige.ch/arlequin, University of Geneva, Geneva, Switzerland.

Sedgwick, J. A. and R. J. Klus. 1997. Injury due to leg bands in Willow Flycatchers. J. Field Ornith. 68:622-629.

Sedgwick, J. A. and F. L. Knopf. 1989. Region wide polygyny in Willow Flycatchers. Condor 91:473-475.

Seutin, G. and J. P. Simon. 1988. Genetic variation in sympatric Willow Flycatchers and Alder Flycatchers. Auk 105:235-243.

Slatkin, M. and R. R. Hudson. 1991. Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. Genetics 129:555-562.

Sogge, M. K., R. M. Marshall, S. J. Sferra, and T. J. Tibbitts. 1997. A southwestern willow flycatcher natural history summary and survey protocol. Technical report NPS/NAUCPRS/NRTR-97/12. 35 pp.

Stein, R. C. 1958. The behavioral, ecological and morphological characteristics of two populations of the Alder Flycatcher, *Empidonax traillii* (Audubon). N.Y. State Mus. Sci. Serv. Bull. 371.

Stein, R. C. 1963. Isolating mechanisms between populations of Traill's Flycatchers. Proc. Amer. Phil. Soc. 107:21-50.

Swofford, D. L. 1998. Phylogenetic analysis using parsimony (PAUP) v. 4.0 beta. Laboratory of Molecular systematics, Smithsonian Institute.

Travis, S. E., J. Maschinski, and P. Keim. 1996. An analysis of genetic variation in *Astragalus cremnophylax* var. *cremnophylax*, a critically endangered plant, using AFLP markers. Mol. Ecol. 5:735-745.

Unitt, P. 1987. *Empidonax traillii extimus*: an endangered subspecies. Western Birds 18:137-162.

U. S. Fish and Wildlife Service. 1995. Final rule determining endangered status for the southwestern willow flycatcher. Federal Register 60:10694-10715 (February 27, 1995).

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407-4414.

Weir, B. S. 1996. Genetic data analysis II: Methods for discrete population genetic data. Sinauer Associates, Inc. Sunderland, Massachusetts.

Weir, B. S., and C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution 38:1358-1370.

Whitfield, M. J. and K. M. Enos. 1996. A Brown-headed cowbird control program and monitoring for the southwestern willow flycatcher, South Fork Kern River, California, 1996. Kern River Research Center report. 20pp.

Winker, K. 1994. Divergence in the mitochondrial DNA of *Empidonax traillii* and *E. alnorum*, with notes on hybridization. Auk 111:710-713.

Wright, S. 1978. Evolution and the genetics of populations, vol. 4: Variability within and among natural populations. University of Chicago Press, Chicago.

Zink, R. M. 1994. The geography of mitochondrial DNA variation, population structure, hybridization, and species limits in the fox sparrow (*Passerella iliaca*). Evolution

48:96-111.

Zink, R. M. 1997. Phylogeographic studies of North American birds. Pages 301-324 *in* Avian molecular evolution and systematics (D. P. Mindell, Ed.). Academic Press, San Diego.

Zink, R. M. and D. L. Dittmann. 1993. Gene flow, refugia, and evolution of geographic variation in the song sparrow (*Melospiza melodia*). Evolution 47:717-729.

Zink, R. M. and J. B. Slowinski. 1995. Evidence from molecular systematics for decreased avian diversification in the Pleistocene Epoch. Proc. Natl. Acad. Sci., USA. 92:5832-5835.