RESEARCH ARTICLE

Disparity in Population Structuring of Southwestern Willow Flycatchers Based on Geographic Distance, Movement Patterns, and Genetic Analyses

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ABSTRACT

Estimates of population connectivity often are based on demographic analysis of movements among subpopulations, but this approach may fail to detect rare migrants or overestimate the contribution of movements into populations when migrants fail to successfully reproduce. We compared movement data of endangered Southwestern Willow Flycatchers among isolated populations in Nevada and Arizona from 1997 to 2008 to genetic analyses of samples collected between 2004 and 2008 to determine the degree to which these two methods were concordant in their estimates of population structuring. Given that documented movements of 13 color-banded adults and 23 juveniles over 10 years indicated low rates of long-distance movements, we predicted that genetic analyses would show significant population structuring between a northern (Nevada) deme and a southern (Arizona) deme. We genotyped 93 adult individuals at seven microsatellite loci and used two Bayesian clustering programs, STRUCTURE and GENELAND, to predict population structure. Both clustering algorithms produced the same structuring pattern; a cluster containing birds breeding in Pahranagat National Wildlife Refuge, the northern-most Nevada site, and a cluster comprised of all other populations. These results highlight that estimates of subpopulation connectivity based on demographic analyses may differ from those based on genetics, suggesting either temporal changes in the pattern of movements, the importance of undetected movements, or differential contribution of migrants to the subpopulations they enter. *J. Exp. Zool. 00A:1–9, 2014.*

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Population subdivision results from the interplay between forces that maintain gene flow versus those that restrict it. Populations of highly mobile or migratory species, such as birds, often see regular exchange of individuals through migration and natal dispersal that increases gene flow among populations and may increase the adaptive potential of that species (Mills and Allendorf, '96). However, high philopatry may offset the effects of these movements and lead to isolated populations more susceptible to stochastic events. This effect may be exacerbated in populations of

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threatened and endangered species that have undergone recent reductions in size and distribution (Haig, '98). In these small populations with limited immigration, random genetic drift may reduce genetic variation and the species' ability to adapt to environmental change (Lande, '88). As a result, understanding both current and historical patterns of movements among populations is important because the prevalence and distance of dispersal may directly affect fitness (Keller, '98) and population viability (Frankham, '95; Haig, '98).

Population structure is typically determined through either detailed field observations of movement patterns or genetic analysis of subpopulations. Demographic data on movement patterns determined via telemetry, mark-recapture, and behavioral observations are time consuming and often difficult to obtain with a high degree of reliability (Broquet et al., 2006; Cushman et al., 2006) and may either overestimate (Riley et al., 2006) or underestimate (Schweizer et al., 2007) gene flow among populations. For example, movement data alone provide no information on breeding status or nest success and thus may not reflect effective dispersal (Prugnolle and De Meeus, 2002). Molecular analyses may reveal levels of gene flow above that predicted by field studies due to rare, unobserved long-distance movements (Coulon et al., 2008), especially in species with low detection or recapture probabilities (Schweizer et al., 2007). Genetic patterns are influenced by both historical and recent patterns of movement, however, and therefore may not reflect recent changes in movement patterns among subpopulations. Combining movement and molecular studies can provide complementary information that studies using only one approach lack and may be especially important for studies of threatened and endangered populations that have undergone recent habitat fragmentation or population isolation (Schwartz et al., 2007).

In little more than a century, the lower Colorado River (LCR; defined here as the area downstream of Glen Canyon Dam, Arizona) has changed from a largely continuous riparian corridor to one highly fragmented into small remnant patches. The federally endangered Southwestern Willow Flycatcher (*Empidonax trailii extimus*; hereafter flycatcher) currently breeds in a subset of these remnant patches, and included among the potential threats to this subspecies is reduced gene flow due to population fragmentation (USFWS, '95; Paradzick and Woodward, 2003). High philopatry and relatively short dispersal distances (Paxton et al., 2007; McLeod et al., 2008) combined with increasing patchiness and isolation of breeding habitat due to continued habitat loss suggest that flycatcher populations along the LCR may show high levels of genetic structuring. Given the current emphasis on restoration of riparian habitat along the LCR, understanding patterns of movements and population structuring is critical for management and conservation efforts for this species. Our objective was to test whether predictions about population structure based on observed movement patterns were concordant with genetic analyses by comparing long-term demographic data and movement patterns to genetic structure of flycatcher populations along the lower Colorado River based on microsatellite analyses of breeding adults. Although Busch et al. (2000) did not detect any population structuring in this subspecies at sites in central Arizona using amplified fragment length polymorphisms (AFLPs), we believe our approach is unique in several ways. First, we use microsatellites, which are highly variable, codominant nuclear markers and can be used to detect recent changes in gene flow (Bossart and Prowell, '98). Second, we incorporate spatial data for individuals in our analysis using more advanced Bayesian clustering programs. Specifically, we tested the hypothesis that genetic structuring of flycatchers would follow the patterns expected based on geographical distance and observed movements.

METHODS

We banded flycatchers with unique color bands at five sites along the LCR and its tributaries in southern Nevada and western Arizona from 1997 to 2008. Pahranagat National Wildlife Refuge (hereafter PAHR) consisted of a series of lakes and marshes in the Pahranagat Valley, Nevada, where flycatchers bred in patches of riparian vegetation dominated by native Goodding's Willow (*Salix gooddingii*), with isolated patches of Coyote Willow (*Salix exigua*), Fremont Cottonwood (*Populus fremontii*), and a few individual Tamarisk trees (*Tamarix* spp.) covering approximately 6.5 ha. Mesquite (hereafter MESQ) lies along the Virgin River, Nevada and consisted of a relatively equal mix of Tamarisk (*Tamarix* spp.) and Coyote Willow encompassing 12 ha occupied by breeding flycatchers. Mormon Mesa (hereafter MOME), also on the Virgin River, consisted mainly of Tamarisk, although flycatcher breeding areas were located in patches that contained Goodding's and Coyote Willow as well as Tamarisk. Overall, MOME covered 125 ha of land, though only 10 ha were used by breeding flycatchers. Topock Marsh (hereafter TOPO), located in the Havasu National Wildlife Refuge, Arizona was an expansive stand of nearly monotypic Tamarisk bordering a large marsh interspersed with isolated emergent Goodding's Willow trees. Our survey area was limited to historically and/or currently occupied habitat spanning 65 ha, though breeding was limited to approximately 30 ha. The Bill Williams River National Wildlife Refuge (hereafter BIWI), the southernmost known breeding location for flycatchers on the LCR, contained the largest contiguous stand of native (Goodding's Willow and Fremont Cottonwood) riparian vegetation along the Colorado River with an understory of tamarisk. It encompassed 86 ha, though breeding flycatchers were found in only 17 ha.

We collected blood samples from all flycatchers captured from 2004 to 2008. We caught individuals via passive or target mist-netting, and we fitted each with a USFWS band and a unique color band. We collected blood samples by clipping a toenail to the vascularized tissues, with blood samples stored temporarily in a commercial freezer, and ultimately maintained in an ultracold

freezer at −80°C until genetic analysis. Because we only used adult individuals that were associated with a nest in the analyses, we used nest UTMs as the sample location. These methods were approved by the Institutional Animal Care and Use Committee at Northern Arizona University.

Extensive re-sighting every year yielded high detection probabilities of adult flycatchers (77–88%, McLeod et al., 2008), so we were confident that we were able to re-sight most returning adults. We located and monitored flycatcher territories and active nests using the Breeding Bird Research and Monitoring Database (BBIRD) protocol (Martin et al., '97) from May through August 2003–2008. We determined parentage of nests based on behavior. We defined effective dispersals as a movement (adult or juvenile) followed by a successful breeding attempt. We examined adult movements and juvenile dispersals separately.

DNA Analysis

We genotyped all adults that were known to have been reproductively active between 2004 and 2008 at all sites without regard to nest outcome. We extracted DNA from blood samples using QIAGEN DNEasy blood and tissue extraction kits and pre-screened samples to evaluate DNA concentration (Morin et al., 2010). We used seven microsatellite primer sets specific to this subspecies (Pearson et al., 2006). PCR reactions were optimized at 10 μL with final concentrations of 2.5 mM MgCl2, 0.3 μM dNTPs, 0.4 μM each of forward and reverse primers, 0.4 μM *Taq* polymerase. The thermal profile consisted of one cycle at 94°C for 2 min, 35 cycles of 20 sec at 94°C, 15 sec at 60°C, and 40 sec at 72°C, followed by a final extension step for 5 min at 72°C. We sequenced the undiluted PCR products on an ABI 3730XL automated sequencer and identified alleles and genotypes manually using GeneMapper version 4.0 (Applied Biosystems, Foster City, CA, USA).

Inferring Genetic Population Structure

We used two Bayesian clustering analyses, STRUCTURE 2.3.3 (Pritchard et al., 2000) and GENELAND (Guillot et al., 2005), to describe genetic structure of populations. For the STRUCTURE analysis, we conducted six runs of 100,000 iterations after 10,000 step burn-in periods assuming one to five population clusters (K = 1–5). Runs were performed with the admixture model and correlated allele frequencies, using a priori population assignments based on breeding locations (Falush et al., 2003). We used the modal ΔK to estimate the true number of population clusters (K), which has been identified as a superior predictor of the true K than the original model's use of ln P(D) (the maximum posterior probability; Evanno et al., 2005). We confirmed that the modal ΔK was the most supported by plotting the ln P(D) values of the runs as a function of the K-values (Coulon et al., 2008).

GENELAND differs from STRUCTURE because, while both assign individual samples to inferred population clusters based on allele frequencies, GENELAND also considers spatial locations of sampling. We performed a series of runs to determine the inferred number of populations, using K = 1–5. We conducted several preliminary runs to optimize the input parameter values and to adjust them based on the behavior of the Marcov Chain Monte Carlo simulation (MCMC; Coulon et al., 2008). We then performed ten runs of 100,000 MCMC iterations with a 100 step burn-in period, varying K from one to five. Because GENELAND sometimes detects the presence of populations to which no individuals belong (ghost populations; Coulon et al., 2006), we examined output from this first set of runs and set the modal K to the number of occupied populations (i.e., after removing ghost populations; Coulon et al., 2006). We then used this modal K to perform a second set of five runs, with the above parameters, varying K from one to the modal K determined in the first step.

Population Genetics of A Priori and Inferred Populations

We used standard population genetic analyses to quantify genetic diversity within each of our five a priori populations (the breeding sites described above). We used Genalex 6.1 (Peakall and Smouse, 2006) to compute average number of alleles per locus (A), expected (H_e) and observed (H_o) heterozygosity, and the inbreeding coefficient within populations (F_{IS}). We determined allelic richness (A_R; the number of alleles adjusted for differences in the sample size per site) using F_{STAT} 2.9.3.2 (Goudet, '95). We tested for deviations from Hardy-Weinberg equilibrium and for evidence of linkage disequilibrium using the Markov chain method of Guo and Thompson ('92) using GENEPOP Version 1.2 (Raymond and Rousset, '95). We used several measures to detect the presence of population structure. First, we calculated pairwise F_{ST} values based on Nei's ('73) minimum distance algorithm; if all populations exhibit similar allele frequencies, F_{ST} will be equal to zero (Hartl and Clark, '89). Next, we performed Mantel tests (10,000 permutations) to test for isolation by distance and performed AMOVA tests to determine the genetic structuring among and within all populations (Excoffier et al., '92) using GENEPOP version 1.2 (Raymond and Rousset, '95).

RESULTS

Movement Analyses

Between 1997 and 2008, 85 banded nestlings were resighted in subsequent years; 23 of these (27%) dispersed away from their natal sites (Fig. 1A). We documented 318 adult between-year returns. The majority of adults returned to the sites where they were initially banded, with only 13 (4%) moving between sites (Fig. 1B). The majority of both adult and juvenile movements were between MESQ and MOME, the two sites closest together (26 km apart, Table 1, Fig. 1).

DNA Sampling

We amplified and scored DNA at seven microsatellite loci from 93 adults of known breeding status from five sites between 2004 and

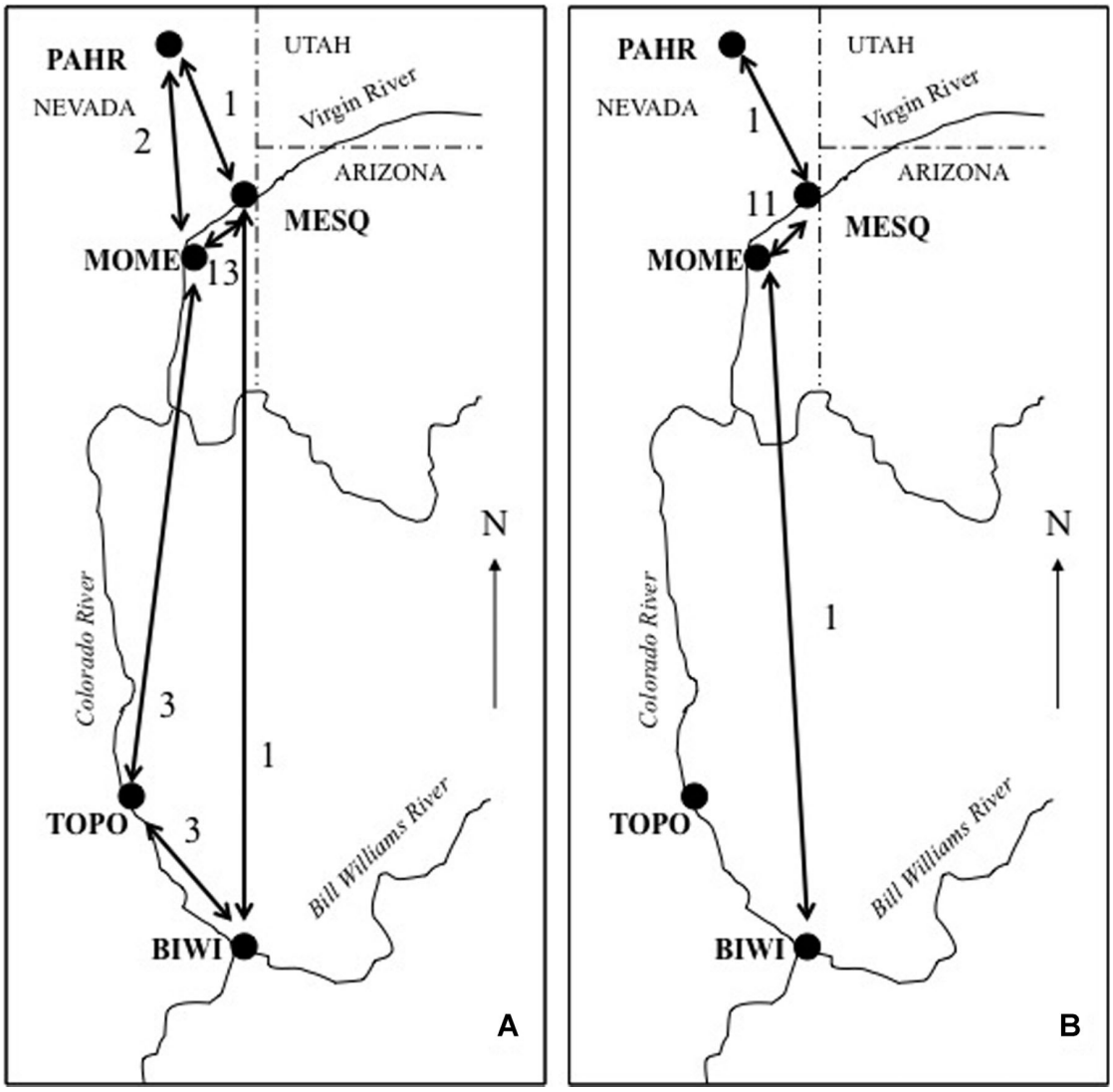

Figure 1. Map of juvenile (A) and adult (B) Southwestern Willow Flycatcher movements among the five study sites from 1997 to 2008. Numbers indicate the number of individuals that migrated between the two sites connected by arrows and total movements are shown at the bottom of each map.

Table 1. Distance (km) between study sites in Nevada and Arizona.

	PAHR	MESQ	MOME	TOPO	BIWI
PAHR		103	105	290	340
MESQ			48	240	280
MOME				201	243
TOPO					56
BIWI					

PAHR, Pahranagat National Wildlife Refuge, NV; MESQ, Mesquite, NV; MOME, Mormon Mesa, NV; TOPO, Havasu National Wildlife Refuge, AZ; BIWI, Bill Williams River National Wildlife Refuge, AZ.

Table 2. Sample size (n), average number of alleles (A), allelic richness (A_R), expected (H_e) and observed (H_o) heterozygosity, and fixation index (F_{IS}) of five populations of Southwestern Willow Flycatchers in Nevada and Arizona from 2004 to 2008.

Population	n	A	A_R	H_e	H_O	F_{IS}
BIWI	7	5	4.8	0.65	0.55	0.19
MESQ	19	6.57	5.2	0.75	0.66	0.015
MOME	8	5.43	5.2	0.66	0.64	0.002
PAHR	38	8.43	5.7	0.75	0.69	0.06
TOPO	21	7	5.0	0.68	0.66	−0.002

2008 (Table 2). All loci were highly polymorphic; average alleles per locus were between five and nine for all sites (Table 2).

Inferring Genetic Population Structure

STRUCTURE analyses revealed that the most likely K was two; one population consisting of Pahranagat and the second consisting of all other sites combined (Fig. 2). Our plot of ln P(D) did not show a distinct peak for K = 2; rather, the likelihood increased slightly until K = 2, and then dropped off significantly for values greater than two, results consistent with Evanno et al. (2005). Individuals were assigned to these two populations with a relatively high degree of probability, suggesting Pahranagat was a distinct population from all others (Fig. 2).

Our initial set of runs in GENELAND revealed either two (five of ten runs) or three populations (three of ten runs), or an equal probability of two or three (two of ten runs) populations. Maps of these runs where three populations were predicted revealed ghost populations that occurred in areas where no known flycatchers existed and no samples were taken (Fig. 3A). After removing ghost populations (Guillot et al., 2005), two populations were supported in seven out of ten runs; in each of these seven runs, Pahranagat was separate from all other populations (results not shown). We therefore set our modal K to vary from one to two for the second set of confirmatory runs. In all five of these runs, K = 2 was supported, with Pahranagat again separate from all other populations (Fig. 3B).

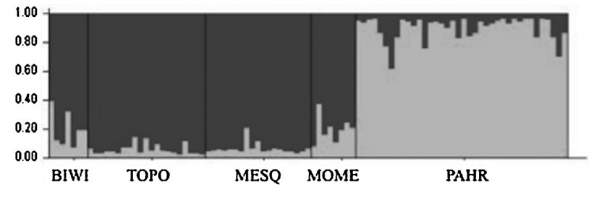

Figure 2. Genetic assignment results from the program STUCTURE for Southwestern Willow Flycatcher populations. Columns represent individuals, which are grouped according to breeding locations. Putative populations are coded with different colors and the fraction of the color is the probability of assignment to that cluster. Two clusters were supported by STRUCTURE; one for the Pahranagat population (gray) and another for all the other populations combined (dark gray).

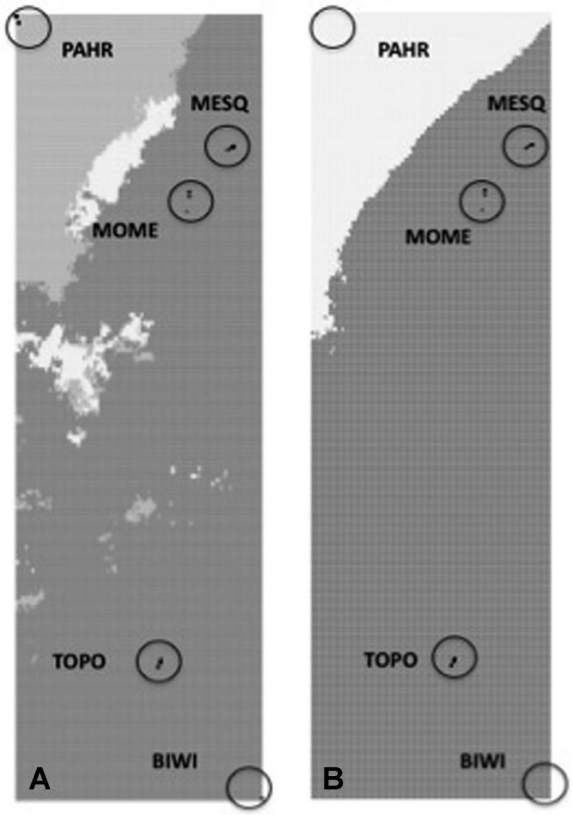

Figure 3. Genetic assignments from GENELAND for Southwestern Willow Flycatcher populations. In both maps, individual colors represent population assignments. Panel A illustrates an example of a ghost population (white), with Pahranagat (light gray) genetically distinct from all other populations (dark gray). Panel B illustrates the population assignments where two populations were supported; Paharangat (white) and all other populations (gray).

Table 3. Pairwise F_{ST} values (below diagonal) and associated *P* values (above diagonal) for five populations of Southwestern Willow Flycatchers in Arizona and Nevada from 2004 to 2008.

	BIWI	MESQ	MOME	PAHR	TOPO
BIWI		0.198	0.642	0.049	0.385
MESQ	0.032		0.388	<0.001	0.029
MOME	0.045	0.029		0.004	0.236
PAHR	0.038	0.023	0.030		<0.001
TOPO	0.033	0.026	0.041	0.034	

Population Genetics of A Priori and Inferred Populations

Allelic richness ranged from 4.8 to 5.7 (Table 2). Observed heterozygosity did not differ from expected in any of the populations. Fixation indices (F_{IS}) ranged from −0.002 at TOPO to 0.19 at BIWI (Table 2). Pairwise F_{ST} values ranged from 0.023 (MESQ, PAHR) to 0.045 (BIWI, MOME; Table 3) and did not reveal significant population structure, however several pairs were significantly different (Table 3). Mantel tests revealed significant evidence of isolation by distance ($P = 0.01$). Ninety-eight percent of the molecular variance was within and among individuals, while only 2% was among populations (AMOVA; $P = 0.001$). There was no evidence of linkage disequilibrium ($P > 0.09$ for each locus pair across all populations).

DISCUSSION

Movements of marked birds over the decade these sites were studied indicated relatively high levels of philopatry, with the majority of birds resighted returning to the site where they were originally banded, consistent with studies of Southwestern Willow Flycatchers in central Arizona (Paxton et al., 2007). Although passerines are generally regarded as having low natal philopatry due to low return rates (Weatherhead and Forbes, '94; Newton, 2008), the majority of juvenile resightings we documented were of birds returning to their natal site. The largest number of both adult and juvenile movements was between the two southern Nevada sites (MOME and MESQ) that were both located in the Virgin River drainage and separated by 48 km (Table 1). Although MESQ and MOME were nearer to the northernmost Nevada site (105 and 103 km, respectively; Table 1) than to either of the Arizona sites (201–280 km, Table 1), the number of adult and juvenile movements from these sites to PAHR and to the Arizona sites was roughly the same.

Our genetic analyses also revealed population structure patterns that were unexpected given the geographical distance among sites. Although we found significant isolation by distance based on standard genetic analyses, we found little support for subpopulation differentiation using these methods. This was not unexpected, given that many studies fail to detect population structure using traditional methods (Ball and Avise, '92; Benedict et al., 2003; Van Den Bussche et al., 2003) especially when population differentiation is low (F_{ST} around 0.03; Latch et al., 2006; Waples and Gaggiotti, 2006) as is often the case for birds (Barrowclough and Rockwell, '93). Both Bayesian approaches, however, supported differentiation of the single northernmost Nevada site (Pahranagat) from all others. This was unexpected given the greater distance (Table 1) between Nevada sites and those in Arizona and the fact that documented movements among Nevada and Arizona sites were as rare as those between the southern Nevada sites and Pahranagat (Fig. 1). We propose two non-exclusive hypotheses that may explain these results.

First, southwestern migratory birds rely on riparian habitats during migration (Yong and Finch, '97; Kelly and Hutto, 2005) and Pahranagat, which was genetically distinct from other populations, is not on the lower Colorado or Virgin River mainstems nor one of its major tributaries. If dispersing or emigrating birds tend to follow major drainages, they may be less likely to arrive at the Pahranagat site. Secondly, birds at Pahranagat may have been less likely to emigrate in response to changes in habitat quality (Mayer et al., 2009). Habitat with standing water during the breeding season is generally considered high quality flycatcher breeding habitat (Sogge and Marshall, 2000), and water levels were relatively consistent at Pahranagat from 2003 to 2007 compared to other sites, and habitat at some other sites was subjected to greater changes due to over-winter flooding (McLeod et al., 2008).

Disparity between movement patterns and genetic structuring like that we documented has been hypothesized in other cases to be due in part to movements by individuals that do not subsequently contribute to the gene pool of the populations they enter (Prugnolle and De Meeus, 2002). For example, immigrant male House Sparrows have been documented to contribute fewer offspring than residents (Pärn et al., 2009). We had relatively few birds whose reproductive output we could follow. One juvenile male that moved from Mesquite to Pahranagat produced 16 offspring over the subsequent 5 years, suggesting the potential for relatively large contributions the Pahranagat gene pool. Of those 16 offspring, however, only a single female successfully recruited at the site and she did not

successfully produce offspring. Four additional offspring of that immigrant male were detected at Key Pittman Wildlife Management Area, 30 km to the north and at similar elevation to Pahranagat, within the years of our study; however, intensive population monitoring there did not begin until 2010 so their breeding status is unknown (Nevada Department of Water unpubl. data). A second juvenile dispersed from Pahranagat to Mormon Mesa and subsequently fledged two offspring, one of which recruited to Mesquite. The last juvenile dispersal, from Mormon Mesa to Pahranagat, produced no fledges. Overall, these limited data do not support strong selection against successful reproduction by immigrants as a driver for the genetic differentiation we documented.

An alternate explanation for our results is that we failed to accurately assess the true rate of movements among sites because of undetected movements by adults and juveniles (Crochet, '96; Koenig et al., '96; Nathan, 2001). We banded 64–93% of nestlings at these sites (McLeod et al., 2008), yet we consistently observed unmarked breeding individuals at the beginning of each season that may have dispersed from outside of our study sites. Although this suggests we may have underestimated the total number of movements, unless the proportion of the population we did band was not representative of the population as a whole, our estimates of the relative amount of movements among sites should be robust. Likewise, we did not sample unpaired males in this study, although we did document them at our sites. Given that extra-pair paternity in Southwestern Willow Flycatchers may be higher than previously thought (Pearson et al., 2006) the contributions of these unsampled males may have altered expectations of gene flow based on movements and genetics of territorial birds. However, as there is no reason to believe that unpaired males move among populations more often than paired males, and most unpaired males were likely breeding, and therefore sampled, in prior or subsequent years, it is unlikely that their presence would increase the amount of gene flow we detected.

Alternatively, the genetic structuring we documented may reflect historical patterns of gene flow that have been altered too recently to be reflected in our genetic analyses (Crochet, '96; Koenig et al., '96; Nathan, 2001). Movement between Pahranagat and more southerly sites may have been more restricted in the past when flycatcher habitat was more widespread and flycatcher populations were larger and presumably more stable. However, although nest productivity and reproductive output was consistently higher at Pahranagat compared to any of our other study sites (McLeod et al., 2008) during the years we followed movements, that higher productivity did not result in larger numbers of movements into southern populations that would have been expected if contemporary movements were increased relative to what they had been historically. More likely, the genetic patterns we documented do reflect current patterns of gene flow, and therefore weaker demographic linkages between Pahranagat and other populations, suggesting that conservation and enhancement of those more southerly populations along the lower Colorado River will need to focus on enhancing productivity at those sites and identifying any other sites that could act as demographic sources of immigrants, potentially populations along the Bill Williams or Gila Rivers. The lack of genetic differentiation among sites in southern Nevada and Arizona suggests that these populations are likely functioning as part of a metapopulation somewhat separate from that of sites to the north like Pahranagat, results consistent with a genetic study of flycatchers in central Arizona using AFLP markers (Busch et al., 2000). Identifying how these southern Nevada and central Arizona populations may be demographically linked to other populations in Arizona, like those along the Gila River, remains an important question. Likewise, the northernmost site in Nevada, Pahranagat, should be compared to other sites at similar elevation and latitudes to the north that were not included in this study to determine whether it is representative of a more fundamental break in genetic continuity.

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