

Genetic variation and migration in the Mexican free-tailed bat (*Tadarida brasiliensis mexicana*)

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Abstract

Incomplete lineage sorting can genetically link populations long after they have diverged, and will exert a more powerful influence on larger populations. The effects of this stochastic process can easily be confounded with those of gene flow, potentially leading to inaccurate estimates of dispersal capabilities or erroneous designation of evolutionarily significant units (ESUs). We have used phylogenetic, population genetic, and coalescent methods to examine genetic structuring in large populations of a widely dispersing bat species and to test hypotheses concerning the influences of coalescent stochasticity vs. gene flow. The Mexican free-tailed bat, *Tadarida brasiliensis mexicana*, exhibits variation in both migratory tendency and route over its range. Observations of the species' migratory behaviour have led to the description of behaviourally and geographically defined migratory groups, with the prediction that these groups compose structured gene pools. Here, we used mtDNA sequence analyses coupled with existing information from allozyme, banding, and natural history studies to evaluate hypotheses regarding the relationship between migration and genetic structure. Analyses of molecular variance revealed no significant genetic structuring of behaviourally distinct migratory groups. Demographic analyses were consistent with population growth, although the timing of population expansion events differs between migratory and nonmigratory populations. Hypotheses concerning the role of gene flow vs. incomplete lineage sorting on these data are explored using coalescent simulations. Our study demonstrates the importance of accounting for coalescent stochasticity in formulating phylogeographical hypotheses, and indicates that analyses that do not take such processes into account can lead to false conclusions regarding a species' phylogeographical history.

Keywords: behaviour, coalescent simulation, genetic structure, migration, phylogeography, *Tadarida brasiliensis*

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Introduction

Separate populations of numerous wide-ranging species are known to exhibit differences in migratory behaviours such as their tendency to migrate, and the magnitude, direction, and timing of their movements. Differences in migratory behaviour can potentially separate migratory from nonmigratory populations during the mating season, and be an effective force promoting reproductive isolation. Phylogeographical studies of many bird species have

revealed that differences in migratory behaviour are typically correlated with genetic structure among populations (Helbig 2003). It has even been possible to identify genetic regions that control different aspects of migratory behaviour, supporting the idea that these behaviours in some birds are innate and not learned (Pulido & Berthold 2003). The same pattern has been found for aquatic animals that return to their natal areas to breed, such as loggerhead turtles (*Caretta caretta*; Hatase *et al.* 2002) and Chinook salmon (*Oncorhynchus tshawytscha*; Gall *et al.* 1992). Recent analyses of the migratory European eel (*Anguilla anguilla*) indicate significant genetic structuring among regional populations, which may be due to differences in the timing of their use of a common breeding area (Wirth & Bernatchez

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2001). A consistent pattern in these examples is that genetically distinct migratory populations show geographical or temporal allopatry during the breeding season.

Migration patterns in bats are often poorly understood and such patterns of allopatry are difficult to determine (Koopman 1983). Among bat studies, the tendency to migrate is often invoked as an explanation for observed patterns of genetic structuring (reviewed in Burland & Worthington Wilmer 2001), but the link between pattern and process has rarely been tested (Newton *et al.* 2003). Typically, population genetic structuring in bats conforms to the expected model of considerable genetic structure within nonmigratory species (Worthington Wilmer *et al.* 1994; Burland *et al.* 1999; Rossiter *et al.* 2000) and little differentiation among populations of migratory species (McCracken *et al.* 1994; Webb & Tidemann 1996; Wilkinson & Fleming 1996), but there are exceptions. In some migratory species, long distances (e.g. *Nyctalus noctula*; Petit *et al.* 1999) or nontraversable geographical features (e.g. *Myotis myotis*; Castella *et al.* 2000) may have led to the isolation of gene pools. Philopatry among both males and females apparently has resulted in genetic differentiation of geographically contiguous populations in the migratory bat *Miniopterus schreibersii natalensis* (Miller-Butterworth *et al.* 2003). The relationship between migration and population genetic structure might be profitably investigated by studying a species that demonstrates intraspecific variation in migratory behaviour, such as *Tadarida brasiliensis*.

The Brazilian free-tailed bat, *T. brasiliensis* (Chiroptera: Molossidae), is one of the most abundant bat species in the Western Hemisphere, occurring in colonies numbering up to tens of millions of individuals (McCracken *et al.* 1994). It is found throughout Central and South America and in North America from the Atlantic to the Pacific coast, north to 40°N latitude (Hall 1981). Within North America, the subspecies *Tadarida brasiliensis mexicana* occurs in Mexico and much of the southern and western portions of the United States (Fig. 1). Colonies in the southwestern United States and northern Mexico are migratory, with bats overwintering in central Mexico (Villa-R. 1956; Villa-R. & Cockrum 1962) and dispersing in February and March. Migration appears to be female biased with many males moving shorter distances and remaining in central Mexico. Mating occurs during February and March in transient roosts located in northern Mexico and in Texas (Villa-R. & Cockrum 1962; McCracken & Wilkinson 2000; Keeley & Keeley 2004). In June and July pregnant females congregate in maternity colonies in caves located as far north as southern Kansas (Glass 1958; Davis *et al.* 1962; Kunz *et al.* 1980). After giving birth, the females and young remain in maternity colonies until the young are weaned. The bats then disperse and form smaller colonies from August to early October in buildings, caves, and mines. Beginning as early as September, they return to central Mexico.



Fig. 1 Range of *Tadarida brasiliensis mexicana* with ranges of the putative migratory groups given as shaded areas (from Cockrum 1969). Sampled populations are indicated.

During the 1950s and 1960s extensive banding studies were conducted in which over 430 000 bats were banded throughout much of the southwestern United States and Mexico. Approximately 1.5% of the banded bats were recovered (reviewed in Cockrum 1969; Glass 1982; McCracken *et al.* 1994; Russell & McCracken in press). From band recapture records, Cockrum (1969) described four geographically and behaviourally distinct migratory groups (Fig. 1), characterized as follows: group A consists of resident populations in California and southern Oregon that do not migrate, engage only in local seasonal movements, and hibernate in winter; group B, located in western Arizona, southern Nevada, and southeastern California, migrates relatively short distances, perhaps into Baja California or into the low interior valleys of southern California; group C, located in central and eastern Arizona and western New Mexico, migrates long distances through Sonora and Sinaloa and utilizes a flyway on the western side of the Sierra Madre Mountains; group D, located in eastern New Mexico, Kansas, Oklahoma, and Texas, migrates long distances along a flyway on the eastern side of the Sierra Madre. Thus, this single subspecies demonstrates variation in migratory tendency (migratory vs. nonmigratory) and the use of separate migratory corridors (eastern vs. western).

Cockrum (1969) hypothesized that the four described migratory groups may be genetically distinct, but subsequent allozyme analyses of groups A, C, and D have not supported this hypothesis (McCracken *et al.* 1994; McCracken & Gassel 1997; but see Svoboda *et al.* 1985). Here, we examine mtDNA sequence variation, coupled with more complete geographical sampling than in the allozyme studies, to further test the hypothesis that migratory behaviour as described in the literature genetically structures populations of *T. b. mexicana* into separate gene pools. We also evaluate the roles of lineage sorting and gene flow, and consider the hypotheses that (i) rapidly evolving mitochondrial DNA (mtDNA) sequences may show more evidence of lineage sorting correlating with behavioural data than is revealed at more slowly evolving allozyme loci, and (ii) differing patterns of structure may be revealed by allozyme data and maternally inherited mtDNA haplotypes because of the gender-specific migratory behaviour of this species. This study is one of a few that explicitly incorporate stochastic lineage sorting in phylogeographical hypothesis testing (Knowles 2001; Carstens *et al.* 2005). We emphasize the power of flexible coalescent simulations, in combination with traditional statistical analyses of genetic diversity and population structuring, to detect the influence of gene flow vs. coalescent stochasticity on the data.

Methods

Sample collection

Samples representing three of Cockrum's (1969) hypothesized migratory groups, including the two long-distance migratory groups and the western nonmigratory group, were obtained with the help of participants of the Program for the Conservation of Migratory Bats (Medellín 2003) and other colleagues. Bats were captured in roosts by hand or

at roost exits using harp traps, mist nets, or hand nets. The bats were sexed, and placed in cloth bags prior to tissue sampling. Two 3-mm biopsy punches, one from each wing, were taken from each bat for DNA analysis (Worthington Wilmer & Barratt 1996). The bats were then released on the same night at the place of capture. Tissue samples were stored on ice in either NaCl-saturated 20% DMSO or silica gel desiccant in the field and at -80°C upon return to the lab. Tissue samples were obtained from a total of 94 bats from 11 locations throughout the subspecies' range, with an average sample size of 8.5 bats per location (Table 1; Fig. 1).

DNA extraction and sequencing

Total genomic DNA was isolated using a standard phenol-chloroform-isoamyl alcohol (25:24:1) method with ethanol precipitation (modified from Sambrook *et al.* 1989) or using a DNeasy® DNA isolation kit (QIAGEN), and stored in 1/10 TE. The isolates were then quantified using a Hoefer DyNA Quant® 200 fluorometer (Amersham Pharmacia), and diluted to a standard concentration of 10 ng/ μL for polymerase chain reaction (PCR). We used primers F(mt): 5'-GTTGCTGGTTTCACGGAGGTAG-3', and P(mt): 5'-TCCTACCATCAGCACCCAAAGC-3' (Wilkinson & Chapman 1991) to amplify approximately 500 bp of the mitochondrial D-loop. To obtain adequate amounts of DNA for sequencing reactions, we performed amplifications in 12 μL reaction volumes containing 1.04 mM MgCl_2 , 0.1 mM dNTPs, 1 unit *Taq* DNA polymerase (Promega), 1.2 μL Promega 10 \times buffer, 10 ng genomic DNA, and 14 pmol of each primer. The amplification involved an initial denaturation at 94°C for 2 min followed by 30 cycles at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min. The target fragment was then purified using gel band excision (MinElute® Kit, QIAGEN).

The DNA fragment was sequenced using the Big-Dye™ Terminator Cycle Sequencing Kit version 2 (Applied

Table 1 Sampling information. Colonies are listed from east to west by longitude. The sample size indicates the number of individuals that were sequenced from each colony. Samples were provided by colleagues as indicated

Colony	Location	Coordinates	Sample size	Collected by
Hidalgo	El Salitre, Mezquitlan, Hidalgo, Mexico	20°07'N, 98°44'W	9	RAM
C. Texas	James River Cave, Mason Co., TX, USA	30°44'57"N, 99°13'56"W	10	GFM
NuevoLeon	Cueva La Boca, Nuevo Leon, Mexico	25°41'N, 100°15'W	12	RAM
Michoacan	Isla de Janitzio, Michoacán, Mexico	19°42'N, 101°07'W	11	RAM
NewMexico	Carlsbad Caverns National Park, Eddy Co., NM, USA	32°10'32"N, 104°22'34"W	10	L. McWilliams, T. Best
Colorado	Grand Junction, Mesa Co., CO, USA	39°04'N, 108°33'W	4	K. Navo
Sinaloa	Ejido Juan Aldama, Sinaloa, Mexico	25°45'N, 108°57'W	12	RAM
Arizona	Eagle Creek Cave, Greenlee Co., AZ, USA	33°03'11"N, 109°19'46"W	8	GFM
C. California	Merced Co., CA, USA	37°28'11"N, 120°29'56"W	10	E. Pierson
N. California	Lava Beds National Monument, Siskiyou Co., CA, USA	41°53'18"N, 121°22'16"W	3	S. Cross
Oregon	Jackson Co., OR, USA	42°11'25"N, 122°41'58"W	5	E. Arnett

Biosystems) in a 10 μ L reaction containing 3 μ L of ready reaction mix, 7 pmol of primer, and 50–100 ng of purified PCR product. The sequencing reaction consisted of 25 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min, with temperatures changing at 1 °C per second. The sequencing reactions were cleaned of unincorporated nucleotides using Centri-Sep columns (Princeton Separations) according to the manufacturer's instructions, and analysed on an ABI 3100 automated sequencer. We sequenced 20 arbitrarily chosen individuals from both directions to quantify the average rate of sequencing error. Because this rate of error was low (c. 2.1×10^{-3} per nucleotide), we sequenced most individuals using only the P(mt) primer. All sequences were deposited in GenBank (Accession nos AY347956, AY347966, AY347976, AY347986, AY347993–AY348101).

The control region of the mitochondrial genome is characterized in many bat species by variable numbers of tandemly repeated 81-bp sequence units (Wilkinson *et al.* 1997). In *Tadarida brasiliensis*, most individuals possess three of these repeat units, although length variation representing up to six repeats was observed. In this study, we included only individuals possessing three repeat units to avoid complications in sequence alignment.

Sequence analysis

All sequences were aligned using the default settings in CLUSTAL W (Thompson *et al.* 1994). The alignment was then edited by eye and cropped to a common length of 474 bp. Sequence variation included four indels, each 1 bp in length, making alignment unambiguous. In all analyses, indels were treated as a fifth character, rather than as missing data.

We used MODELTEST version 3.06 (Posada & Crandall 1998) to determine that the D-loop of *T. b. mexicana* best fits a Tamura-Nei + I + Γ model of evolution (Tamura & Nei 1993). This model specifies unequal base frequencies and a higher rate of transition than transversion mutations, with different rates of transitions between purines than between pyrimidines. In our data, adenine constitutes nearly 50% of the nucleotides in an average sequence ($\pi_A = 0.436$, $\pi_C = 0.192$, $\pi_G = 0.101$, $\pi_T = 0.271$), and we observed nearly twice as many transitions between pyrimidines ($R_{C-T} = 76.857$) as between purines ($R_{A-G} = 44.042$). Over a third of the sites were invariant ($I = 0.359$), and the data were characterized by a gamma-distributed mutation rate with shape parameter $\alpha = 0.5402$. Subsequent phylogenetic and population genetic analyses were carried out using the assumptions of this model and parameter values specified, except where noted.

Phylogenetic analyses

Due to the large number of haplotypes, the data set was not amenable to a traditional maximum-likelihood analysis. Therefore, we used a Bayesian likelihood analysis to

look for phylogenetic signal among the migratory groups (MRBAYES version 3.0B4; Huelsenbeck & Ronquist 2001). We ran four Markov chains for 10 million generations each, with sampling every 100 generations, resulting in a sample of 100 000 trees. The chains were heated using the temperature scaling factor $T = 0.2$. The evolutionary model was approximated as GTR + I + Γ , with four rate categories. We discarded the first 10 000 trees as a burn-in, and constructed a 50% majority consensus tree from the remaining 90 000 trees in PAUP* (Swofford 1998).

In many intraspecific analyses, a hierarchical tree format may be inappropriate for representing relationships among haplotypes because the period of time over which the data have evolved is so short that ancestral and descendant haplotypes exist concurrently or because sexual reproduction or recombination can lead to reticulate relationships among haplotypes (Posada & Crandall 2001; see Kravtsov *et al.* 2004 for a discussion of mitochondrial recombination). In such cases, a haplotype network can better depict relationships among the sampled haplotypes by using multiple pathways to indicate possible recombination, homoplasy, or reverse mutations. We constructed a minimum-spanning network using ARLEQUIN version 2.001 (Schneider *et al.* 2001).

Population genetic analyses

Because barriers to gene flow that have developed recently may not be detectable using phylogenetic analyses, population genetic analyses combining information on haplotype frequency, genetic distance, and geographical distribution can be more instructive with regard to shorter-term processes (Neigel 2002). We used ARLEQUIN 2.001 to calculate descriptive parameters of molecular diversity such as haplotype diversity (h) and nucleotide diversity (π) for each colony, and identified haplotypes shared among colonies. We calculated pairwise F_{ST} values between colonies to identify pairs of colonies that were genetically distinct using the method of Slatkin (1991). We also conducted a Mantel test to look for a correlation between the standardized genetic distance [$F_{ST}/(1 - F_{ST})$] and the natural log of the geographical distance (Rousset 1997). Geographic coordinates for each colony were determined using the Astro-dienst website (<http://www.astro.com/cgi/aq.cgi?lang=e>), and the geographical distance between each pair of colonies was calculated as the direct aerial distance between geographical coordinates using the How Far Is It? website (<http://www.indo.com/distance/>).

Colonies belonging to the same migratory group were pooled for analyses of molecular variance (AMOVA; Excoffier *et al.* 1992) to examine genetic structuring among migratory groups. We also used AMOVA to test for significant structuring between nonmigratory and pooled migratory groups. The resulting measure of ϕ_{ST} is the correlation of

random haplotypes within populations relative to random pairs drawn from the whole subspecies. Significance was determined by comparing the observed variance measures with null distributions obtained from 16 000 random permutations of the data.

Demographic analyses

The historical demography of the migratory groups was inferred through a variety of methods. First, haplotype diversity (h) and nucleotide diversity (π) were calculated for the three migratory groups and for the pooled groups C and D using ARLEQUIN 2.001. Demographic processes can be inferred from characteristic patterns in these measures. Namely, high values of both h and π indicate sustained large population sizes, while a high value of h and a low value of π are consistent with recent population expansion. We also calculated the expansion coefficient (S/d), or the ratio of the number of variable sites (S) to the average number of pairwise nucleotide differences (d) (Peck & Congdon 2004). Recent analyses have shown that population growth is indicated by larger expansion coefficients, while population stationarity is indicated by smaller expansion coefficients (Von Haeseler *et al.* 1996).

We also analysed the population history of the migratory groups using mismatch distribution analyses, which compare observed frequencies of pairwise differences with those expected under various demographic models. Under a model of exponential population growth, a unimodal distribution is expected. Alternatively, a multimodal or 'ragged' distribution is expected for a population approaching mutation–drift equilibrium. We performed mismatch distribution analyses for the three migratory groups and for the pooled groups C and D with DNASP version 4.0 (Rozas *et al.* 2003), using 1000 coalescent simulations to assess the statistical significance of the raggedness (rg) statistic (Harpending *et al.* 1993).

Tests based on pairwise comparisons, such as the mismatch distribution, lose much of the historical information available in the data (Felsenstein 1992). Neutrality tests, especially Fu's (1997) F_S , has been shown to be much more powerful in detecting signals of population growth (Ramos-Onsins & Rozas 2002). Furthermore, Fu (1997) demonstrated that comparisons of neutrality tests can distinguish between genetic hitch-hiking and population expansion, two processes that can leave similar imprints on the data. If Fu & Li's (1993) F^* and D^* are significant but Fu's (1997) F_S is not, then background selection is indicated. If the reverse is true (F_S is significant, but F^* and D^* are not), then population expansion is indicated. We used DNASP 4.0 (Rozas *et al.* 2003) to calculate all neutrality test values for the three migratory groups and for the pooled groups C and D, using 1000 coalescent simulations to assess the significance of Fu's (1997) F_S .

Where a history of population expansion was indicated, we used the mismatch distribution to estimate the time since expansion, τ , scaled in mutational units. The relationship of $\tau = 2ut$, where t is the time in generations and $u = \mu k$, where μ is the mutation rate per million years (Myr) and k is the sequence length, was used to rescale the estimate to absolute time (Rogers 1995).

Coalescent-based hypothesis testing

The coalescent encompasses a body of theory that is the foundation for an increasing number of statistical analyses (Kuhner *et al.* 1995; Beerli & Felsenstein 2001; Nielsen & Wakeley 2001). However, few of these analyses are able to incorporate specific information about a species or geographical area into a coalescent-based analytical framework. We used MESQUITE (Maddison & Maddison 2004), one of the few programs that have the required flexibility for such analyses, to examine the influence of retained ancestral polymorphisms on the genetic similarity observed in the data. Because lineage sorting is a stochastic process that proceeds as a function of time and population size, it is highly probable that large populations with recent shared histories will be genetically similar, even in the complete absence of gene flow. Disregarding the potential effects of gene flow, we used coalescent simulations to model the time required for complete lineage sorting. We compared the observed results to those expected under the no gene flow model to examine the influence of retained ancestral polymorphism and ongoing gene flow on the data. Using MESQUITE version 1.04 (Maddison & Maddison 2004), we constructed population trees containing three taxa (migratory groups A, C, and D) with differing branch lengths expressed as a function of population size (N_e , $0.1N_e$, $0.01N_e$, $0.001N_e$). We simulated 1000 gene trees constrained within each population tree using a coalescent process with a conservative estimate of $N_e = 10^6$ (Russell 2003). We calculated Slatkin & Maddison's (1989) gene flow statistic (s) for each simulation, and graphed the distribution of s values for each population tree. We then compared s from the observed tree to these distributions to estimate the number of generations of lineage sorting with which the observed data is consistent under the no gene flow model. The expected time to complete lineage sorting in the absence of gene flow is given by the distribution that is consistent with an s of 2.

Results

Molecular diversity

The D-loop of *Tadarida brasiliensis mexicana* is characterized by very high levels of diversity (Table 2). From a total of 94 individuals, we identified 86 different haplotypes. Along

Table 2 Molecular diversity indices for all colonies

Colony	Migratory group	<i>n</i>	<i>n_h</i>	<i>h</i>	π
C. Texas	D	10	10	1.000	0.035 ± 0.019
NewMexico	D	10	10	1.000	0.051 ± 0.028
Hidalgo	D	9	9	1.000	0.046 ± 0.025
NuevoLeon	D	12	11	0.985	0.051 ± 0.027
Colorado	C	4	4	1.000	0.047 ± 0.032
Arizona	C	8	8	1.000	0.051 ± 0.029
Sinaloa	C	12	12	1.000	0.040 ± 0.021
Michoacan	C	11	11	1.000	0.056 ± 0.030
C. California	A	10	10	1.000	0.039 ± 0.021
N. California	A	3	3	1.000	0.056 ± 0.043
Oregon	A	5	4	0.900	0.033 ± 0.021
Overall		94	86	0.998	0.045 ± 0.022

n, number of individuals sequenced; *n_h*, number of different haplotypes; *h*, haplotype diversity; π , nucleotide diversity per site. The overall number of haplotypes is less than the sum of *n_h* for all individual colonies due to the presence of haplotypes shared between colonies.

with this high overall haplotype diversity ($h = 0.998$), this region had relatively low sequence diversity ($\pi = 0.045$). Seventy-eight of the 86 different haplotypes occurred as singletons, and the remaining eight were each found in only two individuals. Of the eight haplotypes that were shared by two individuals, five (62.5%) were in individuals in the same migratory group, of which two were in the same colony. The three haplotypes present in multiple migratory groups were shared between the two long-distance migratory groups C and D. No haplotypes were shared between individuals in migratory vs. nonmigratory groups. Sixteen individuals representing seven locations possessed more than three 81-bp repeat units, and were removed from this analysis. These individuals were found in all of the sampled migratory groups, and there was no detectable geographical bias to their distribution (data not shown).

Phylogenetic analyses

Bayesian likelihood analysis using the South American subspecies *T. b. brasiliensis* as an outgroup revealed no evidence of long-term segregation of gene pools among migratory groups. A strict consensus summary of the 90 000 trees resulting from the analysis is completely comb shaped and reveals no structure among the data (not shown). More cladistic structure was found in a 50% majority rule consensus tree, but this structure does not correspond with any clustering of groups correlating with their migratory behaviour (Fig. 2). The highly unresolved structure in the observed genealogy may indicate a star-like phylogeny

Table 3 Pairwise ϕ_{ST} measures between migratory and nonmigratory bats and between Cockrum's (1969) migratory groups. *P* values are the significance of the ϕ_{ST} measures

	ϕ_{ST}	<i>P</i> value
Migratory vs. nonmigratory	0.021	0.054
A–C	0.018	0.128
A–D	0.024	0.065
C–D	–0.011	0.950

resulting from recent population growth (Russell 2003); in any case, it yields no clear signal for these analyses.

We constructed a minimum-spanning network to depict the relationships among haplotypes (Fig. 3). This network also shows no clear pattern of structure among behaviourally differentiated groups. Most sequential haplotypes in the network are separated by multiple mutations (mean = 6.1; range = 1–21). Although this lack of intermediate haplotypes is not unexpected in a species with very large population sizes such as *T. brasiliensis*, it does preclude a statistically rigorous analysis of genetic structuring in the network, such as a nested clade analysis (Templeton 1998).

Population genetic analyses

F_{ST} values between all pairs of colonies were low (average $F_{ST} = -0.018$; range –0.137 to 0.128) with only one pair of colonies (Nuevo Leon in group D, and Oregon in group A) showing an F_{ST} significantly greater than zero ($F_{ST} = 0.128$; $P = 0.021$). Because these analyses revealed no significant structure among colonies within the same migratory group, we pooled sequences from the same migratory group for further analyses. An analysis of molecular variance (AMOVA) among the three pooled migratory groups revealed no evidence of genetic structuring, with 99.6% of the genetic variation in the data set explained by variation among individuals within colonies ($\phi_{ST} = 0.004$, $P = 0.290$). An AMOVA between migratory and nonmigratory bats showed that only 2.1% of the total genetic variance was associated with variation in migratory tendency ($\phi_{ST} = 0.021$, $P = 0.054$; Table 3). The weak signal of genetic structuring at this level may be due to the difference in sample sizes between these two samples ($n = 76$ for all migratory colonies, $n = 18$ for all nonmigratory colonies), or it may be due in part to the genetic differentiation between the migratory colony in Nuevo Leon and the nonmigratory colony in Oregon. An AMOVA with the Nuevo Leon colony removed reveals the impact of that single colony on genetic structuring in the entire data set ($\phi_{ST} = 0.015$, $P = 0.111$). Whether the colony in Nuevo Leon is of any special significance in the life history of *T. b. mexicana* is not certain, but it is notable that this is the only colony in the sample to share a haplotype

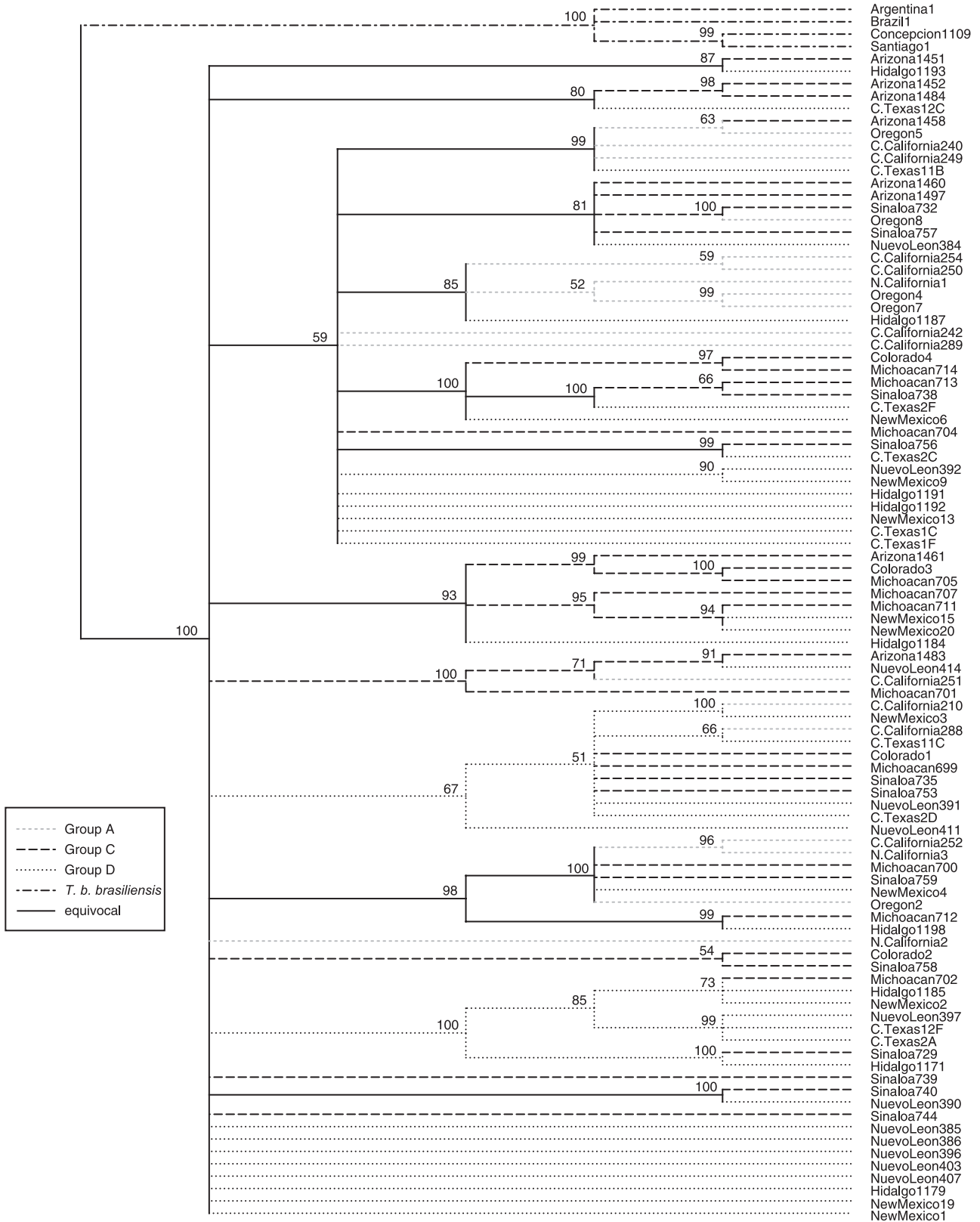


Fig. 2 A 50% consensus tree from a Bayesian phylogenetic analysis. Only trees sampled after the burn-in period were used to construct the consensus. Support indices are the percent of trees having the given partition, and are equivalent to the posterior probabilities from the Bayesian analysis. Migratory group designations are indicated by pattern and tone, following the code in the legend box. Internal branches are assigned to migratory groups following a parsimony criterion; solid black branches ('equivocal') could not be unambiguously assigned to a single group. Haplotypes from *Tadarida brasiliensis brasiliensis* were used as an outgroup.

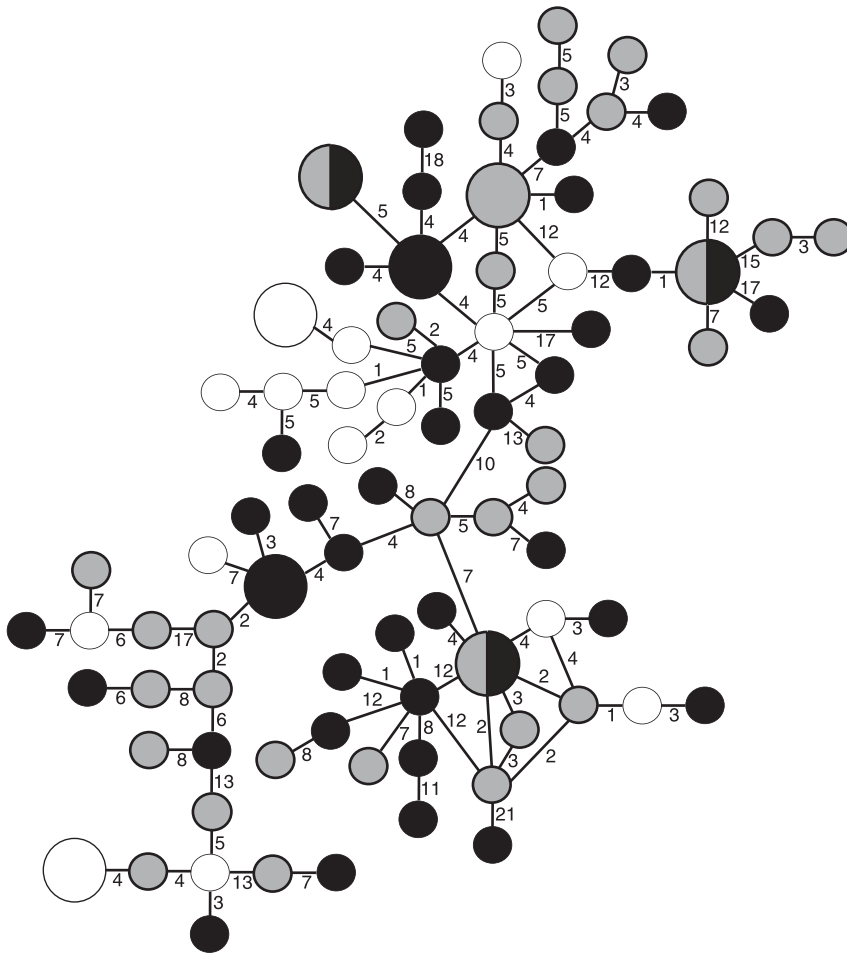


Fig. 3 Minimum-spanning network of all haplotypes. Migratory groups are indicated by tone; white is group A, grey is group C, and black is group D. Smaller circles represent unique haplotypes, and larger circles represent haplotypes found in two individuals. Haplotypes having two tones were found in two migratory groups. The number of mutations at each step of the network is indicated by the number at each connecting link.

with populations traditionally assigned to the currently recognized southeastern subspecies, *Tadarida brasiliensis cynocephala* (Russell 2003). A Mantel test revealed no correlation between genetic distance [$F_{ST}/(1 - F_{ST})$] and geographical distance ($r = -0.0013$, $P = 0.328$).

The low level of genetic structuring among Cockrum's migratory groups and between migratory and nonmigratory groups suggests high levels of gene flow between these groups (Table 3). These pairwise analyses reveal no genetic distinction between migratory colonies ($\phi_{ST} = -0.011$), and indicate that groups C and D are of the same genetic population. Pairwise measures of ϕ_{ST} were slightly higher for comparisons involving the nonmigratory group A ($\phi_{ST} = 0.018$ for A-C, and $\phi_{ST} = 0.024$ for A-D), but were not significant in either case.

Demographic analyses

We analysed the demographic history of each migratory group and for the pooled groups C and D separately (Table 4). The mismatch analysis of group A revealed a

multimodal distribution (Fig. 4A) that was as ragged as expected under a model of population stationarity ($rg_A = 0.015$, $P = 0.124$). Mismatch analyses closely fit unimodal distributions for groups C and D, both separately and together (Fig. 4B). The raggedness statistic (rg) for group C and for the pooled groups C and D was significantly low ($rg_C = 0.004$, $P = 0.011$; $rg_{CD} = 0.003$, $P = 0.035$), meaning that the observed distributions were smoother than expected under a model of population stationarity. Although the observed distribution for group D closely fit a unimodal distribution, the raggedness statistic was not significant ($rg_D = 0.006$, $P = 0.132$).

The results for the various neutrality tests are summarized in Table 4. These analyses strongly indicate population growth for groups C and D, both separately and together. Fu & Li's (1993) F^* and D^* are not significant for group C ($F^* = -1.246$, $P > 0.10$; $D^* = -0.991$, $P > 0.10$), group D ($F^* = -2.194$, $P > 0.05$; $D^* = -2.065$, $P > 0.05$), or the pooled groups C and D ($F^* = -2.080$, $P > 0.05$; $D^* = -1.900$, $P > 0.05$), while Fu's (1997) F_S was significant for these same migratory groups ($F_{SC} = -17.578$, $P < 0.01$; $F_{SD} = -21.749$, $P < 0.01$;

Table 4 Diversity statistics, results of neutrality tests, and results of mismatch distribution analyses. Results are given for each behaviourally distinct migratory group, and for the pooled Groups C and D. Expected trends in the data are given for a demographic model of exponential population growth (Hull & Girman 2005)

	Group A	Group C	Group D	Pooled Groups C & D	Expectation
Nucleotide diversity (π)	0.034	0.042	0.040	0.041	Low
Haplotype diversity (h)	0.987	0.998	0.998	0.998	High
Expansion coefficient (S/d)	3.955	5.809	6.767	8.185	High
Fu & Li's (1993) F^*	-0.704	-1.246	-2.194	-2.080	Not significant
Fu & Li's (1993) D^*	-0.586	-0.991	-2.065	-1.900	Not significant
Fu's (1997) F_S	-3.394	-17.578**	-21.749**	-55.100**	Significant
Raggedness (rg)	0.015	0.004*	0.006	0.003*	
Mismatch distribution	Multimodal	Unimodal	Unimodal	Unimodal	Unimodal
τ	8.777	14.333	12.973	13.392	—
Time since expansion (year BP)	c. 1850	c. 3000	c. 2700	c. 2800	—

Significant results are indicated by asterisks: * $P < 0.05$, ** $P < 0.01$.

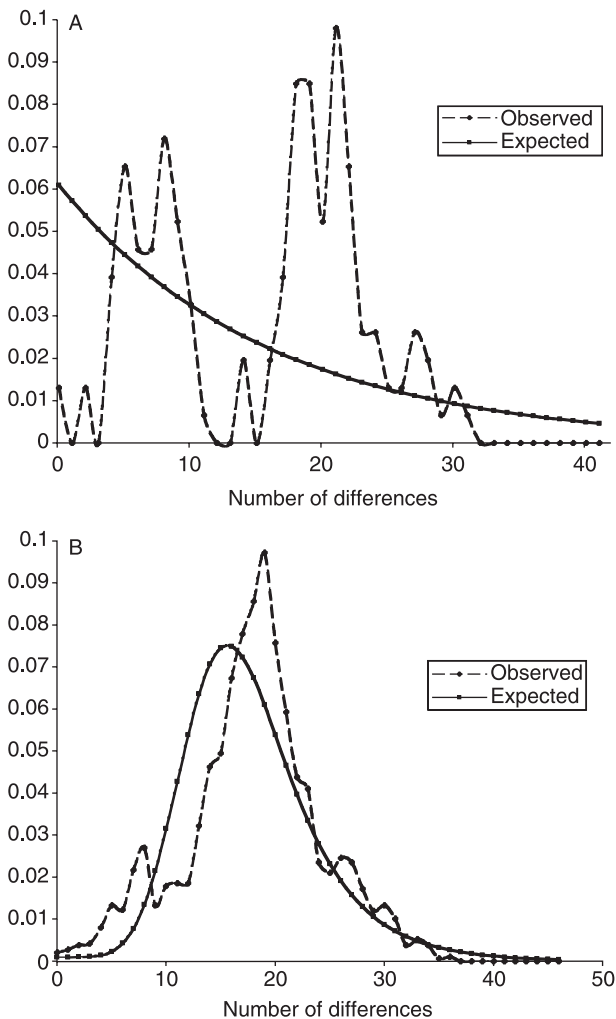


Fig. 4 Mismatch distributions for A, group A, and B, pooled groups C and D. The expected distribution for group A is based on a model describing a constant population size through time, while the expected distribution for groups C and D is based on a model of exponential population growth.

$F_{S-CD} = -55.100$, $P < 0.01$). Population growth was weakly indicated for group A; although none of the neutrality tests were significant for this group ($F^* = -0.704$, $P > 0.10$; $D^* = -0.586$, $P > 0.10$; $F_S = -3.394$, $P = 0.068$), the F_S result was strongly negative.

Estimates of the time since population expansion were taken from the mismatch distribution analyses, resulting in a τ of 8.777 for group A, 14.333 for group C, 12.973 for group D, and 13.392 for the pooled groups C and D (Table 4). Assuming a mutation rate of 10^{-5} per site and a generation time of 2 years yields a time since expansion of approximately 1850 years BP for group A, approximately 3000 years BP for group C, approximately 2700 years BP for group D, and approximately 2800 years BP for the pooled groups C and D.

Coalescent simulations

The estimated low level of genetic structure among migratory groups may result from ongoing gene flow or may be the artefact of retained ancestral polymorphism. We modelled the effect of time on lineage sorting using coalescent simulations to examine the potential impact of retained ancestral polymorphism on our data. These simulations show that, in the absence of gene flow and given an effective population size on the order of 10^6 , complete lineage sorting among migratory groups would require more than 1 million generations (Fig. 5A). Decreasing the time since population divergence by orders of magnitude reveals that the simulated gene trees are consistent with increasing levels of apparent gene flow ($\bar{s} = 14.6$, Fig. 5B; $\bar{s} = 32.9$, Fig. 5C; $\bar{s} = 37.4$, Fig. 5D). Because these simulations do not incorporate gene flow, values of s are due solely to incomplete lineage sorting. The gene flow statistic calculated from our observed tree ($s = 38$) is consistent with the divergence of migratory groups around 1000 generations ago with no subsequent gene flow (Fig. 5D).

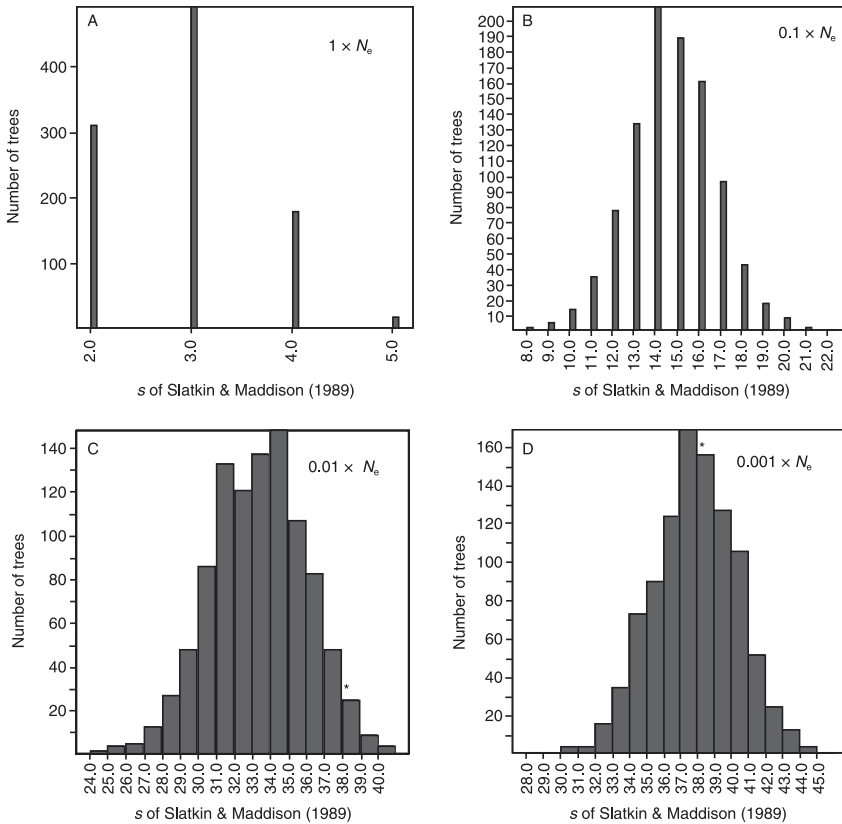


Fig. 5 Distributions of Slatkin & Maddison's (1989) gene flow statistic s for 1000 simulated gene trees within four population trees. Population trees differ in their branch lengths, expressed as a function of the effective population size (N_e). (A) Branch lengths = N_e . (B) Branch lengths = $0.1N_e$. (C) Branch lengths = $0.01N_e$. (D) Branch lengths = $0.001N_e$. Observed $s = 38$ (indicated by asterisk), indicating that the observed phylogeny is consistent with 38 gene flow events among the three migratory groups.

Discussion

Variation in migratory behaviour can spatially separate populations during mating. *Tadarida brasiliensis mexicana* consists of four groups that have been presumed to be genetically distinct due to variation in migratory behaviours; however, our mtDNA sequence data reveal no significant genetic structure either among previously described migratory groups or between migratory and nonmigratory groups of bats. A Bayesian phylogenetic analysis revealed no structuring of mitochondrial lineages corresponding to different migratory behaviour. The application of Bayesian statistics to phylogenetic analyses has been criticized as tending to overly support detected clades (Huelsenbeck *et al.* 2002). This criticism lends further support to our conclusion that the migratory groups are not genetically differentiated. Population genetic analyses also provide no evidence that migratory behaviour is associated with the genetic differentiation of migratory groups or migratory vs. nonmigratory bats, and indicate that significant gene flow occurs among these groups. These results agree with previously published allozyme data (McCracken *et al.* 1994; McCracken & Gassel 1997) that behaviourally distinct migratory groups are not genetically distinct.

The estimates of $\phi_{ST} = -0.011$ – 0.024 that we found for *T. b.*

mexicana indicate a lack of genetic structure similar to that found in other widely dispersing species. Buerkle (1999) found no evidence of genetic structuring among five geographically dispersed populations of a migratory subspecies of prairie warblers, *Dendroica discolor discolor* (pairwise $\phi_{ST} = -0.014$ – 0.091). Eastern populations of the monarch butterfly, *Danaus plexippus*, overwinter in large colonies in Mexico and migrate north over several generations during the summer. Allozyme analyses revealed that summer populations are not genetically structured (average $F_{ST} = 0.009$), and the authors concluded that eastern monarch butterflies consist of a single panmictic population (Eanes & Koehn 1978). In the red-winged blackbird, *Agelaius phoeniceus*, allozyme analyses revealed that populations representing five subspecies from across the United States are not genetically structured (average $F_{ST} = 0.043$) (Gavin *et al.* 1991). While indices of genetic structuring from allozyme data are not directly comparable to those from mitochondrial data due to differences in mode of inheritance, rate of evolution, and the effective size of the population of reference, data from the different markers may be transformed as described by Crochet (2000):

$$F_{ST(\text{mitochondrial})} = \frac{4F_{ST(\text{nuclear})}}{1 + 3F_{ST(\text{nuclear})}}$$

Transforming nuclear F_{ST} estimates for monarch butterflies ($F_{ST(mtDNA)} = 0.035$) and red-winged blackbirds ($F_{ST(mtDNA)} = 0.152$) using this equation yields measures of genetic structure in these taxa that are approximately 1.5 to 6 times greater than what we observed for the most differentiated Mexican free-tailed bat migratory groups ($\phi_{ST} = 0.024$ between group A and group D), indicating that these behaviourally differentiated *T. b. mexicana* migratory groups are substantially less structured than other migratory and broadly distributed species.

Using the equation above, we transformed ϕ_{ST} from our mitochondrial data into nuclear analogues for the three sampled subgroups of *T. b. mexicana* that were examined in previous studies using allozyme data (Svoboda *et al.* 1985; McCracken *et al.* 1994; McCracken & Gassel 1997). The transformed indices of genetic structuring from our mitochondrial data are all less than one-fourth of those from nuclear allozyme data (Table 5). We suspect that this may be due to gender-biased migratory behaviour. Because females migrate longer distances than males, maternally inherited mtDNA haplotypes are more exposed to potential gene flow. Consistent with this hypothesis, separate analyses of allozyme data from males in winter colonies in Mexico ($F_{ST} = 0.016$, $P > 0.05$) and migratory females from summer colonies in the United States ($F_{ST} = 0.008$, $P > 0.05$) suggest a greater degree of genetic structure among the nonmigratory males (McCracken *et al.* 1994). It is worth emphasizing, however, that neither mitochondrial nor allozyme data show significant differentiation of populations at any spatial scale.

Table 5 Comparison of genetic structuring from nuclear allozyme and mitochondrial sequence data. Indices of genetic structure for mtDNA data are given as ϕ_{ST} values that were transformed to allow for comparison with nuclear data. McCracken *et al.* (1994) estimated genetic structure among migratory colonies in the USA from samples of females collected during the summer. Genetic structure among migratory colonies in Mexico was estimated from samples of males collected during the winter. Genetic structure among migratory and nonmigratory colonies in McCracken & Gassel (1997) was analysed using data from both males and females

Locus	F_{ST}	P value	Reference
<i>Among migratory colonies (USA)</i>			
Allozymes	0.008	ns	McCracken <i>et al.</i> (1994)
D-loop	0.002	ns	this study
<i>Among migratory colonies (Mexico)</i>			
Allozymes	0.016	ns	McCracken <i>et al.</i> (1994)
D-loop	-0.006	ns	this study
<i>Among migratory and nonmigratory colonies (USA)</i>			
Allozymes	0.014	ns	McCracken & Gassel (1997)
D-loop	-0.006	ns	this study

Historical demography

Despite the lack of genetic structure, we found that the migratory and nonmigratory groups are consistent with two different demographic scenarios. Specifically, demographic analyses strongly support a history of population growth in the central migratory groups, dating from approximately 3000–2700 years BP. The western nonmigratory group is characterized by a weaker signal of population growth that dates back to approximately 1850 years BP. These scenarios are supported by several lines of evidence, including neutrality tests, mismatch distributions, and genetic diversity statistics.

Neutrality tests show strong support for population expansion in groups C and D, both separately and together, while providing only a weak indication of population expansion in group A. Significant results for these tests can result from background selection, selective sweeps, or population expansion. These processes can be discerned by comparing different neutrality tests. Fu & Li's (1993) F^* and D^* are more strongly affected by background selection, while Fu's (1997) F_S is more strongly affected by population expansion or selective sweeps. Therefore, a significant F_S and insignificant F^* and D^* indicate an over-abundance of infrequent haplotypes due to either population expansion or selective sweeps, while the opposite indicates the influence of background selection. The results of Fu's (1997) F_S were strongly significant in groups C and D, both separately and together, and the results of Fu & Li's (1993) F^* and D^* were insignificant in those same migratory groups, indicating a history of population growth. In group A, Fu & Li's (1993) F^* and D^* were also insignificant, but Fu's (1997) F_S , although strongly negative, was not statistically significant. We interpret these tests, on their own, as providing ambivalent support for weak population growth in group A.

Additional information concerning the demographic histories of these migratory groups comes from the molecular diversity statistics. For all three groups, nucleotide diversity was relatively low, and haplotype diversity was extremely high (Table 4). The groups were also characterized by high values of the expansion coefficient (S/d). Taken together, these results are consistent with population growth in all migratory groups, although the signal of expansion in group A is much weaker than in groups C or D.

The timing of population expansion events reveals different demographic histories for the far western (nonmigratory) vs. central (migratory) populations. Environmental and archaeological evidence has shown that the period between about 7500 and 5000 years BP was characterized by a marked reduction in effective moisture throughout the greater Southwest, followed by a marked increase in effective moisture after about 5000 years BP (Aikens 1983). Archaeological remains in this region suggest that human

populations in this area expanded as effective moisture increased (Irwin-Williams 1967). Similarly, the increase in effective moisture during this period may have supported an increased diversity and abundance of flying insects, thus supporting the observed expansion of *T. b. mexicana* populations in the central migratory groups.

Impact of gene flow vs. incomplete lineage sorting

Neigel & Avise (1986) showed that the time to genetic divergence of populations is a function of population size. Large populations are expected to retain ancestral polymorphisms for a significant period of time following reproductive isolation. For historically large populations, the retention of ancestral polymorphisms can bias estimates of gene flow upwards, even for populations that are reproductively isolated (Neigel 2002). Therefore, while the presence of reciprocal monophyly is powerful evidence for the evolutionary independence of lineages, taxa may be evolving independently even in the absence of monophyly.

Coalescent simulations examining the effect of time on lineage sorting show that complete lineage sorting among migratory groups requires at least 1 million generations (Fig. 5). In terms of absolute time, the migratory groups would have had to exist as isolated gene pools for more than 2 Myr (= mid-Pliocene) to have achieved complete lineage sorting. Since the earliest *Tadarida brasiliensis* fossil dates to the late Pleistocene (Morgan 1985) and the earliest *Tadarida* sp. fossil in the New World dates to the late Pliocene (Morgan & Ridgway 1987), the time required for complete lineage sorting as shown in these simulations probably predates the species' origin. Our observed tree is consistent with the divergence of migratory groups around 1000 generations (= 2000 years) ago (Fig. 5d). This leads to three possible explanations for the observed pattern of genetic structure: (1) recent isolation of migratory groups with no subsequent gene flow, so the observed lack of genetic structure is due to incomplete lineage sorting alone; (2) migratory groups have existed as separate populations for a long period of time, so the observed lack of genetic structure is solely due to ongoing gene flow; or (3) relatively recent establishment of migratory groups as separate populations that are still linked by ongoing gene flow. Under this last hypothesis the observed lack of genetic structure is due to a combination of gene flow and incomplete lineage sorting. Considering the fossil record of *Tadarida* in the New World, we believe it unlikely that the migratory groups of *T. b. mexicana* have been in existence long enough that incomplete lineage sorting is not playing some role in our observed patterns of genetic structure, thus ruling out hypothesis 2. This leaves only hypotheses 1 and 3, which are distinguished by the presence or absence of gene flow among the migratory groups.

To distinguish between these hypotheses, we turned to direct estimates of intergroup migration from banding records. Over a span of 30 years, records were published of 31 bats that switched migratory groups (Mohr 1952; Villa-R. & Cockrum 1962; Mumford 1963; Constantine 1967; Barbour & Davis 1969; Glass 1982; Svoboda *et al.* 1985). In studies at Carlsbad Caverns in western New Mexico, Constantine (1967) documented recoveries of 64 banded bats in places other than where they were banded. Of these, 13 recoveries (20.3%) involved individuals that moved between migratory groups C and D. Notably, the longest documented movement was 1340 km from Carlsbad Caverns, New Mexico, in group D to Las Garrochas Cave, Jalisco, in group C (Villa-R & Cockrum 1962). In addition to the high rate of documented movements between groups C and D, demographic analyses supporting a shared demographic history between these migratory groups strongly suggest that these groups form a single, widespread population. Movement also has been documented between the other migratory groups, although much less frequently, such as one individual banded in eastern Nevada (group B) and recovered in western Kansas (group D) (Svoboda *et al.* 1985). While no movements between migratory and non-migratory populations have been documented, we suggest that, rather than being the result of restricted gene flow between these areas, the infrequency of such movements may instead reflect the fact that almost all banding studies on *T. b. mexicana* have involved only the migratory groups C and D. This observational bias has been exacerbated by the 1973 decision of the US Fish and Wildlife Service to stop issuing bands due to overwhelming evidence that banding and related activities cause bat mortality (Herreid *et al.* 1960), as well as by the near cessation of reporting the results of banding studies following that decision (Peurach 2004). The presence of substantial colonies in locations outside of the described migratory routes, such as Ojuela Cave in the Sierra Madre Occidental (McCracken *et al.* 1994) and Orient mine in the San Luis Valley of Colorado (Svoboda *et al.* 1985), both located between groups C and D, also suggests that migratory groups are not as distinct as Cockrum's (1969) hypothesis would suggest. Therefore, considering the lack of genetic structure from previous allozyme data as well as this species' capacity for long-distance dispersal, our data indicate that current gene flow, in addition to coalescent stochasticity, exerts a significant influence on the data. These data further suggest that tagging studies, using necklaces or other nonlethal identification methods (Barclay & Bell 1988), might reveal the movement of individuals between migratory and nonmigratory colonies.

Alternative explanations of mtDNA patterns

The lack of genetic differentiation among migratory groups might also be explained by the use of an inappropriate

genetic marker. If the mutation rate at the mtDNA D-loop were too high, then any phylogeographical signal would be lost due to saturation. We do not consider this to be the case for two reasons. First, use of the same sequence region to examine genetic structure among multiple subspecies of *T. brasiliensis* has shown clear patterns of genetic structure (Russell 2003; Russell & McCracken, in press). Second, our results are consistent with allozyme studies of the same migratory groups, which also did not find any genetic structuring (McCracken *et al.* 1994; McCracken & Gassel 1997). If the lack of structure in the mtDNA D-loop were due to signal saturation, the genetic structure might still be expected to manifest itself in the more slowly evolving nuclear allozymes (Crochet 2000). Therefore, we conclude that the lack of phylogeographical signal in these sequence data reflects the absence of genetic structuring among *T. b. mexicana* migratory groups and not the artefact of signal saturation in the data.

Migratory behaviour

Despite the lack of genetic differentiation, variation in migratory behaviours among colonies of *T. b. mexicana* is amply documented in the literature. Colonies of tens to thousands of bats are routinely found throughout the year in California, Oregon, Nevada, and Utah (Benson 1947; Jewett 1955; Krutzsch 1955; Ruffner *et al.* 1979; Perkins *et al.* 1990), while colonies throughout Texas, Oklahoma, New Mexico, Arizona, and Mexico undergo seasonal migrations (Villa-R. 1956; Glass 1959, 1982; Villa-R. & Cockrum 1962; Cockrum 1969). Additionally, the majority of banding data record individuals being banded and recaptured within the same migratory corridor, suggesting that the bats largely maintain distinct migratory routes. However, all tests for genetic differentiation corresponding with these behaviourally distinct groups, using a total of 68 loci from both the mitochondrial and nuclear genomes, have failed to detect such structure (Svoboda *et al.* 1985; McCracken *et al.* 1994; McCracken & Gassel 1997; this study). Therefore, it seems that differences in migratory tendency and direction have not led to significant gene pool differentiation in *T. b. mexicana*.

We postulate that migratory behaviour in *T. b. mexicana* may be a plastic behavioural response, perhaps due to differential environmental cues. Plasticity in dispersal behaviour has been demonstrated in northern goshawks (*Accipiter gentilis atricapillus*), in which juveniles provided with experimental food supplementation did not disperse, whereas those without food supplements engaged in normal dispersal behaviour (Kennedy & Ward 2003). Seasonally migrating populations of moths are a major food source for the migratory populations of *T. b. mexicana* in Texas (McCracken 1996; Lee & McCracken 2002, in press), and the availability and seasonal movements of these moths

may provide an environmental stimulus that cues the bats' migratory behaviour. Alternatively, migratory behaviours, including the tendency and route of migration, may be learned. Behaviours reported for *T. b. mexicana* circumstantially support learning. In a long-term study of the colony at Carlsbad Caverns, New Mexico, Constantine (1967) noted that in all movements the older adults preceded younger adults who 'followed [their elders'] example' (p. 25). In a review of distribution records for this species, Genoways *et al.* (2000) proposed a mechanism by which individuals dispersing from crowded maternity colonies in the late summer discover potential new roosting sites that are presumably remembered and relocated in following summers. For this long-lived species, the adaptability conferred by the ability to learn may well be advantageous.

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This work was conducted as part of Amy Russell's dissertation research at the University of Tennessee under the supervision of Gary McCracken. Amy is currently investigating the phylogeography of bats in Madagascar. Rodrigo Medellín coordinated sample gathering and contributed discussion and conservation implications, in the context of the binational program for the conservation of migratory bats. Rodrigo is involved in understanding the conservation needs and status of migratory, threatened, and endemic species of bats in Mexico. Gary, a Professor in Ecology and Evolutionary Biology at the University of Tennessee, engages in field and laboratory studies concerning bat behaviour and genetics.
