Lack of Population Genetic Structure in the Bat Fly (*Trichobius major*) in Kansas, Oklahoma, and Texas based on DNA Sequence Data for the Cytochrome Oxidase I (COI) and NADH Dehydrogenase 4 (ND4) Genes

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The bat fly, *Trichobius major*, is an ectoparasite which resides for most of its life on the cave myotis, *Myotis velifer*. We used portions of the cytochrome oxidase I (COI) and NADH dehydrogenase 4 (ND4) genes of the mitochondrial genome (mtDNA) to infer population genetic structure and gene flow of *T. major* in Kansas, Oklahoma, and Texas. DNA sequence data for 48 (COI) and 41 (ND4) *T. major* collected from widely separated caves did not reveal mtDNA sequence variation. This finding suggests that the COI and ND4 might not be suitable genetic markers to recover intraspecific variation in *T. major*. Alternatively, the lack of DNA sequence variation together with the morphological similarity observed among bat flies from different cave localities might suggest a population bottle neck in the recent evolutionary history of *T. major*.

INTRODUCTION

The Streblidae, or bat flies (*Trichobius major*), [Diptera (Kessel 1925; Zeve 1958; Zeve and Howell 1962)] are obligate, blood feeding ectoparasites on the cave myotis (*Myotis velifer*), which serves as the primary host species (Caire et al., 1981; Caire and Hornuff 1982, 1986; Overal 1974; Reisen et al., 1976; Ross 1961). *Trichobius major* breed in the roosts of cave-dwelling bats and produce one larva at a time, retaining and nourishing it until maturity (Ross 1960, 1961; Zeve 1958; Caire et al., 1981; Caire and Hornuff 1982). Once the new adults emerge, several stimuli, such as carbon dioxide concentrations and heat, are used to locate a host in a cave environment (Caire et al., 1985; Overal 1980). Aside from being found on the host, *T. major* can also be observed in crevices or on surfaces of walls at the entrance or inside of the cave environment (Zeve 1958).

Although prior studies of *T. major* have emphasized ecology, external morphology, taxonomy, and distribution (Caire et al., 1981; Caire and Hornuff 1982, 1986; Caire et al., 1985; Kessel 1925; Ross 1961; Overal 1974, 1980; Reisen et al., 1976; Zeve 1958; Zeve and Howell 1962), none have used molecular techniques to address dispersal and gene flow of bat flies. Previous morphological studies have shown no significant difference in wing morphology and femur lengths in *T. major* among cave localities in western Oklahoma (Caire et al., 1981). This finding led Caire et al. (1981) to conclude that adequate gene flow in *T. major* was occurring among cave localities to overcome the effects of genetic drift.

However, Caire and Hornuff (1982) noted that *T. major* are not good fliers and postulated that movement of bat flies is probably occurring via the host species. In a laboratory setting, Caire and Hornuff (1982:356) reported that *T. major* exhibited an "upward expanding spiraling flight." They suggested that the spiraling flight patterns exhibited by *T. major* might be an adaptive

advantage from the standpoint of providing an efficient search pattern to locate bats clustered or clumped on the ceiling of a single cave (Caire and Hornuff, 1982). However, this flight behavior might not be a selective advantage for independent dispersal from one cave locality to another.

Because gene flow in *T. major* is difficult to measure directly using traditional methods (e.g., mark-recapture techniques), it might be possible to use genetic markers (Avise 2000; Hedin 1997; Moritz and Hillis 1996) to evaluate bat fly dispersal. Genetic markers have been used in other studies examining population genetic structure and gene flow for a number of cave-dwelling arthropods (Caccone 1985; Hedin 1997; Slatkin 1985). The goal of our study is to investigate genetic structuring of *T. major* that inhabit caves throughout Kansas, Oklahoma, and Texas based on two mtDNA markers. We hypothesize that gene flow between populations of *T. major* has been reduced as a result of their inability to independently disperse from one roost site to another.

MATERIALS and METHODS

Bat flies ($n = 316$) were collected from 4 caves in south-central Kansas, 8 caves in western Oklahoma, and from a single cave in south-central Texas (Fig. 1). From December 2002, December 2003 through March 2004, and January 2005, specimens were collected with forceps from the external auditory meatus and ears of hibernating bats in caves in Kansas and Oklahoma. Bat flies were collected in Texas during July 2004 from the surface of rocks near the entrance of a cave and by netting bats and searching their entire bodies for *T. major*.

After bat flies were collected, they were placed in 1.5 ml eppendorf tubes containing 500 µl of lysis buffer (Longmire et al., 1997). The entire fly was macerated and whole genomic DNA was extracted using the protocol of Longmire et al. (1997). After extraction, DNA samples were stored in 1X TE at 4°C. Polymerase chain reaction (PCR) was used

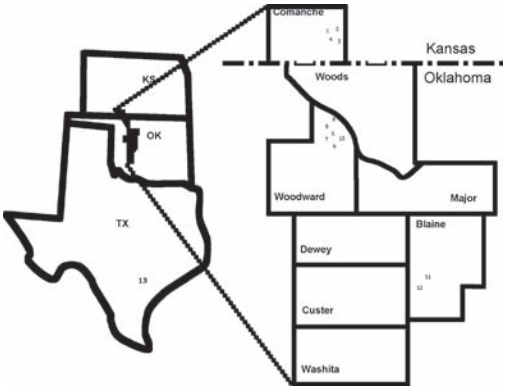

Figure 1. Locations of caves in Kansas, Oklahoma, and Texas where bat flies were collected. Numbers represent caves (followed by sample size for the cytochrome oxidase I (COI) and NADH dehydrogenase 4 (ND4) genes of the mitochondrial genome, respectively), 1) Double Entrance S Cave (n = 0, 4), 2) Sink Valley Cave (n = 0, 3), 3) Swartz Cave (n = 0, 3), 4) Parker Bat Cave (n = 0, 3), 5) Skylight (n = 5, 3), 6) Alabaster Caverns (n = 4, 3), 7) Bear Cave (n = 3, 4), 8) Skunkeater Cave (n = 3, 3), 9) Green's Cathedral Cave (n = 7, 3), 10) Cattle Cave (n = 2, 3), 11) Washita Bat Cave 1 (n = 7, 3), 12) Washita Bat Cave 2 (n = 4, 3), and 13) Eckert James River Bat Cave (n = 13, 3). Map modified from Loucks (1996). Specific cave localities are available in Loucks (1996).

to amplify a 1296 base pair (bp) fragment of the cytochrome oxidase I (COI) gene of the mitochondrial genome using the primers C1-J-1718 and TL2-N-3014 (Bernasconi et al., 2000). PCR was also used to amplify a 647 bp fragment of the NADH dehydrogenase 4 (ND4) gene of the mtDNA genome using the primers ND4ar and ND4c (Uribe Soto et al., 2001). Amplification reactions for the COI and ND4 genes were performed in 50 µl volumes following the PCR profile reported by Bernasconi et al. (2000) and Uribe Soto et al. (2001), respectively.

PCR products (i.e., 5 µl) were electrophoresed through a 0.8% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. Successful amplicons were purified using the Wizard

PCR Prep DNA Purification System (Promega, Madison, Wisconsin). DNA sequence data were obtained by cycle sequencing according to the manufacturer's instructions (BigDye™; Perkin Elmer, Foster City, California). Cycling conditions for the ND4 and COI genes were as follows: 25 cycles at 96ºC for 10 seconds, 50ºC for 5 seconds, and 60ºC for 4 minutes. Sequence product for each individual was electrophoresed on an Applied Biosystems Inc. (Foster City, California) 377 automated DNA sequencer.

After COI sequences from several *T. major* were aligned using the Clustal W option of the MacVector v6.5 computer program (Oxford Molecular 1998), we developed internal primers that amplified a 450 bp fragment at the 5' end of the COI gene (1718F—AATGGAGCTGGTACTGGATGAAC and 1718R—TGCTAGTATTGCGTAGAT). Reaction mixes consisted of 50-200 ng DNA, 5 μl of 10X buffer, 1 mM of each dNTP, 0.5 mM of each primer (1718F and 1718R), 2.5 mM $MgCl_2$, and 1.25 units of *Taq* DNA polymerase (Promega, Madison, Wisconsin). PCR cycles were as follows: 94ºC for 3 minutes, followed by 35 cycles of 94ºC for 30 seconds, 45ºC for 30 seconds, and 72ºC for 45 seconds with a final elongation period of 72ºC for 30 minutes.

RESULTS AND DISCUSSION

DNA sequences generated in this study for the cytochrome oxidase I (COI) and NADH dehydrogenase 4 (ND4) genes have been deposited in GenBank (accession numbers EU136047-EU136094 and EU136095-EU136135, respectively). DNA sequence data for the COI gene was obtained by the alignment of 450 bp at the 5' end for 48 *T. major* from localities in Oklahoma and Texas (Fig. 1). These DNA sequences resulted in no variable sites. A 647 bp fragment of the ND4 gene was obtained for 41 *T. major* from localities in Kansas, Oklahoma, and Texas (Fig. 1). Like the DNA sequence data for the COI gene, the ND4 sequences also resulted in no variable sites in *T. major*.

When addressing questions at the intraspecific level, it is essential to include a molecular marker that evolves at a rate appropriate to identify population structure (Avise 2000). DNA sequence data for the COI gene has been used in previous population genetic studies for tsetse fly (Wohlford et al., 1999), net-winged midges (Wishart and Hughes 2002) and dove lice (Johnson et al., 2002). The COI gene for these species exhibited a mutation rate appropriate to infer population genetic structure. Likewise, DNA sequence data for the ND4 gene was used to infer population genetic structure for sand flies (*Lutzomyia longipalpis*) from widely separated populations in Central and South America (Uribe Soto et al., 2001). Our results suggest that, in *T. major*, the 450 bp fragment of the COI gene and 647 bp fragment of the ND4 gene might not be evolving at a rate appropriate to infer population genetic structure.

The lack of genetic variation reported in our study supports findings of Caire et al. (1981 and 1982) based on morphological attributes in *T. major*. Although Caire et al. (1981 and 1982) detected significant morphological difference in wing areas related to gender, they did not detect variation within male or female *T. major* for wing morphology or femur length. The lack of morphological and DNA sequence variation might suggest the occurrence of a population bottle neck in *T. major* that occurred during their recent evolutionary history.

From a geographic standpoint, the northern-most cave in Kansas is approximately 740 km distant from the southern-most cave in Texas. It is possible that our geographic sampling did not encompass a large enough area. The host species, *M. velifer*, is distributed from southwestern and south-central United States through most of Mexico to Honduras (Fitch et al., 1981). However, populations of *M. velifer* in Kansas, Oklahoma, and northern Texas are a distinct subspecies (*M. v. grandis*) from populations in southern Texas (*M. v. incautus*; Fitch et al., 1981; Shump 1999).

Additional study is needed to infer the degree of gene flow among populations of *M. velifer*. In the Selman Cave system in northwestern Oklahoma, Caire and Hornuff (1986) documented that *M. velifer* began to increase in number from October through December, at which time population densities reached a high. However, after December, densities of bats began to decrease throughout the remainder of the winter due to relocation of individuals to other cave localities (Caire and Hornuff, 1986). Caire and Hornuff (1986) noted that the number of *T. major* were directly proportional to the number of *M. velifer*, which agreed with findings reported by Overal (1980). Understanding gene flow of the host may well provide meaningful insight into the mode and frequency of gene flow for *T. major*. For example, *T. major* might not be dispersing independently but rather moving from cave to cave via their volant, highly mobile host.

Because the COI and ND4 genes were unable to reveal population genetic structure in *T. major* from widely separated cave localities, we attempted to identify a region of the mtDNA genome that exhibits a mutation rate appropriate to infer population genetic structure in *T. major*. We tried several primers that revealed genetic structure in other invertebrates, but were repeatedly unsuccessful in our attempts to amplify the ND5 gene (Lehmann et al., 1997) and control region (Lehmann et al., 1997; Schultheis et al., 2002). In addition, we screened samples of *T. major* with several microsatellite primers (i.e., bi-parentally inherited nuclear DNA markers) which have been published for the tsetse fly (Krafsur and Endsley 2002; Solano et al., 1997; Ouma et al., 2003) blow fly (Florin and Gyllenstarand 2002), mosquito (Ravel et al., 2001), fruit fly (Machado et al., 2003), and house fly (Charkrabarti et al., 2004, Endsley et al., 2004). The microsatellite primers were unable to amplify DNA extracted from *T. major*. We suspect that the lack of amplification is due to mutations or sequence variation at the annealing site of the primers in *T. major* DNA.

In order to better understand frequency and magnitude of gene flow in *T. major*, it is essential to identify PCR primers that will amplify bi-parentally inherited nuclear markers (i.e., AFLPs and microsatellites) or additional mtDNA markers that evolve at a rate appropriate to infer population genetic structure. Examination of genetic diversity within and among populations of bat flies within caves throughout Kansas, Oklahoma, and Texas, can contribute critical information for developing and implementing prudent conservation and management plans to insure the health and biodiversity of cave ecosystems across the region.

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