

# Phylogenetics and population genetics of the louse fly, *Lipoptena mazamae*, from Arkansas, U.S.A.

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**Abstract.** Louse flies, also known as deer keds (*Lipoptena mazamae* Rondani), infest cervids such as white-tailed deer, *Odocoileus virginianus* and vector pathogens such as *Anaplasma* and *Bartonella schoenbuchensis* to cattle and humans, respectively. The population genetic structure of 30 *L. mazamae* collected from white-tailed deer in four regions of Arkansas, U.S.A., designated by county boundaries, was examined using DNA sequences of a 259-bp region of the mitochondrial DNA rRNA 16S gene. Of the 259 nucleotide characters, 33 were variable and 6 haplotypes were identified. Two haplotypes occurred only once (haplotype 3 and 4), whereas two other haplotypes occurred in 43% (haplotype 1 in two regions) and 40% (haplotype 6 in three regions) of the samples. Phylogenetic relationships of the six *L. mazamae* haplotypes were constructed with other Hippoboscidae and Glossinid samples and two clades resulted. Clade 1 was located in the north and western Ozarks whereas clade 2 was found in the northern and eastern Ozarks. Results from the present study indicate that *Lipoptena* may be a polyphyletic genus; consequently, more research into genetic variation within this genus is necessary.

**Key words.** *Lipoptena mazamae*, population genetics, 16S.

## Introduction

*Lipoptena mazamae* Rondani (Diptera: Hippoboscidae) is an ectoparasite of white-tailed deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemionus*) and brocket deer (*Mazama* spp.) (Drummond, 1966) that spend a majority of their adult life within the host pelage (Mogi, 1975; Kettle, 1990). Samuel & Trainer (1972) and Forrester *et al.* (1996) reported that this species was the most prevalent ectoparasite of white-tailed deer in southern Texas and Florida. Adult flies are morphologically adapted to live on mammalian hosts possessing well-developed claws, are dorsoventrally flattened and shed their wings as adults upon finding a suitable host. These flies are larviparous; the egg and larva develop in the female uterus and are deposited at the end of the last larval stage (Curran, 1965). The extruded larva falls from the host to the ground, where it quickly develops into a puparium. The adult then emerges from the puparium and seeks a host (Maa & Peterson, 1987).

Because of their biology (adenotropic viviparity and loss of functional wings as adults), a majority of their dispersal is

dependent upon host movement. Mule deer in Colorado can travel between 7 and 15 km (Conner & Miller, 2004) assisting their ectoparasites with dispersal. The newly emerged adult has fully developed wings used for dispersal, but once attached to the host the wings break off near the base (Kettle, 1990). Active adult dispersal occurs from late March through early December, peaking in July (Samuel & Trainer, 1972; Cline & Thorne, 1993). Both males and females blood feed, but females are more voracious, requiring extra nutrition for larval development (Bequaert, 1952).

*Lipoptena* spp. are known to act as vectors for *Bartonella* spp. in deer (Halos *et al.*, 2004; Reeves *et al.*, 2006) as well as *Bartonella henselae* in felines (Kordick *et al.*, 1999), *Anaplasma* in cattle (Drummond, 1966) and *Trypanosoma cervi* in cervids (Strickland *et al.*, 1981). Additional transmission may occur when the wingless adult moves from adult deer to their nursing fawns (Samuel & Trainer, 1972). In addition, the flies may opportunistically feed on humans leading to lesions at the bite site (Rantanen *et al.*, 1982). Bites to humans may transmit *Bartonella schoenbuchensis*, causing deer ked

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dermatitis with lesions that can persist for a year (Rantanen *et al.*, 1982; Dehio *et al.*, 2004).

Because of *Lipoptena*'s hematophagous behavior and potential to vector several pathogens, it is important to get a better understanding of their population structure and gene flow as it may relate to potential pathogen transmission using population genetics (Tabachnick & Black, 1995). Previous phylogenetic studies on Hippoboscoidea identified Glossinidae, Nyceterbiidae, Streblidae and Hippoboscidae as four distinct families (Nirmala *et al.*, 2001; Dittmar *et al.*, 2006; Peterson *et al.*, 2007). Although these studies included numerous species of Hippoboscoidea, study of genetic variation within any of these families is lacking. The limited genetic research on *Lipoptena* identified the genus as polyphyletic (Peterson *et al.*, 2007). Studies of genetic variation in a similar fly, *Glossina morsitans* Westwood, found a large amount of singleton haplotypes with great genetic diversity among African populations (Krafsur *et al.*, 2000, 2003). Because *Lipoptena* flies are similar to tsetse flies with low reproductive rates and habitat limitations connected to hosts, pronounced genetic variation in *Lipoptena* populations should be found.

The current study presents a phylogenetic analysis and population genetics of *L. mazamae* collected from white-tailed deer. The objectives of this study were to determine the extent of genetic variation within and among populations of *Lipoptena* flies, to compare genetic variation among deer keds collected from four regions of Arkansas, U.S.A. and to construct a phylogenetic relationship of *L. mazamae* relative to other Hippoboscidae.

## Materials and methods

### *Lipoptena* collection and preservation

Staff of the Arkansas State Game and Fish Commission collected *L. mazamae* specimens from white-tailed deer harvested during the 2007–2008 hunting season. Twenty-two deer were selected randomly from this cohort all from the northwest areas of the state and arbitrarily grouped by political boundary (four counties represented as regions) (34.8°N, 92.2°W; 137,002 km<sup>2</sup>) (Fig. 1, Table 3). Flies were individually removed from the host animal and stored in 100% ethanol. Sheep keds (*Melophagus ovinus* L.) reared on sheep (*Ovis aries*) were obtained from Montana State University. All collection information including date, Universal Transverse Mercator (UTM) coordinates and host species were recorded. Collected keds were identified to species using keys of Maa & Peterson (1987). All specimens were maintained in 100% alcohol until removed for DNA extraction. Voucher specimens are maintained in the University of Arkansas Arthropod Museum, Fayetteville, AR, U.S.A.

### DNA preparation and PCR amplification

To minimize DNA contamination, DNA extractions and PCR were conducted in different laboratories, negative controls were used, and supplies were dedicated to each procedure. Ked

DNA was isolated in the Veterinary Entomology Laboratory at the University of Arkansas after each specimen was allowed to dry on paper towels. Each ked specimen was longitudinally cut and subjected to the Qiagen Dneasy Insect Protocol (Qiagen Inc. Rohm and Haas Company, Valencia, CA, U.S.A.). Samples were stored in a freezer at –20 °C to delay DNA degradation until further analyzes.

PCR was conducted in the Social Insect Genetics Laboratory at the University of Arkansas. Each PCR set included at least one negative control and a positive control (*Apis mellifera* L.). A master mix reaction was prepared according to the protocol of Szalanski *et al.* (1997) and a Flexigene thermocycler (Techne Duxford, Cambridge, U.K.) was used for all reactions. Negative controls substituted sterile water for template DNA. Reaction products were analyzed by agarose gel electrophoresis in 1% agarose gels stained with ethidium bromide and visualized and documented with a UVP biodoc-it system (UVP Inc., Upland, CA, U.S.A.).

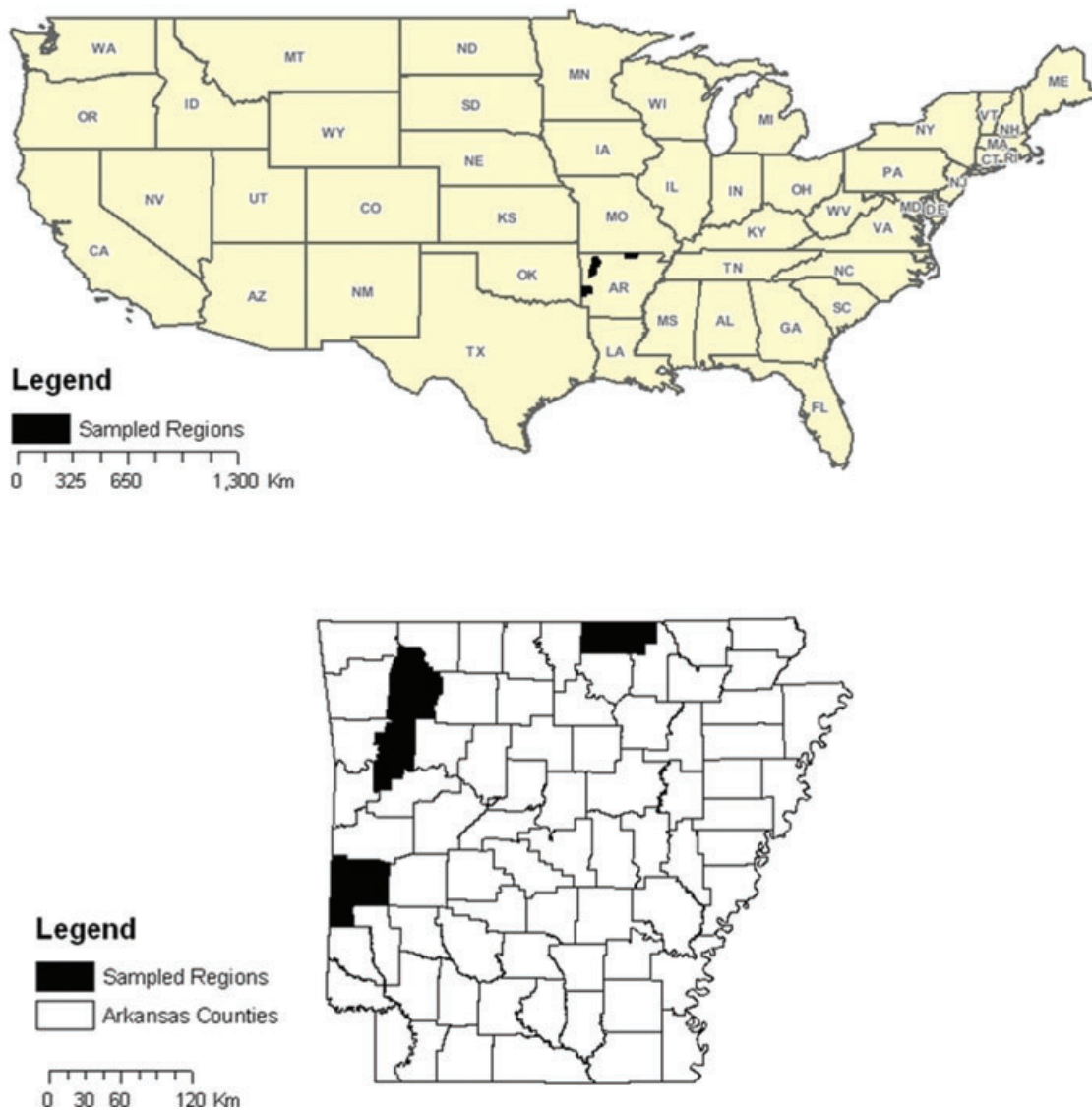
PCR was conducted with insect universal primers 16SR (5' CGC CTG TTT ATC AAA AAC AT 3') (Kambhampati & Smith, 1995) and 16SF (5' CGC CTG TTT ATC AAA AAC AT 3') (Simon *et al.*, 1994). Each reaction was initially denatured for 2 min at 94 °C. Amplification included 40 cycles of denaturing at 94 °C for 45 s, annealing at 46 °C for 60 s and extension at 72 °C for 60 s. At the end of the cycles, the samples were held for a final extension at 72 °C for 5 min (Simon *et al.*, 1994). Reaction products were analyzed with gel electrophoresis in 1% agarose gels stained with ethidium bromide and visualized with a UVP biodoc-it system (UVP Inc., Upland, CA, U.S.A.). Amplified 16S DNA from the 30 *L. mazamae* and 5 *M. ovinus* were purified and concentrated with minicolumns according to the manufacturer's instructions (Wizard PCRpreps; Promega). Samples were sent to the University of Arkansas Medical Sciences DNA Sequencing Facility (Little Rock, AR, U.S.A.) for direct sequencing in both directions using an Applied Biosystems Genetic Analyzer (Model 3100, Carlsbad, CA, U.S.A.).

### Data analyzes

Sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) and PAUP\* (Swofford, 2001) was used for phylogenetic analyses. Consensus sequences were derived from both of the DNA sequences from an individual ked specimen using Bioedit 5.0 (Hall, 1999) to verify nucleotide polymorphisms. Outgroup taxa were obtained from GenBank and are presented in Table 1. As mentioned previously, field-collected sheep keds (*M. ovinus*) were subjected to all of the procedures to ensure the extraction, amplification and sequencing processes, and used as an outgroup taxa.

Mitochondrial DNA haplotypes were determined using MacClade v4 (Sinauer Associates, Sunderland, MA, U.S.A.). PAUP\* 4.0b10 distance matrix option (Swofford, 2001) was used to calculate genetic distances according to the Kimura two-parameter model of sequence evolution (Kimura, 1980), maximum likelihood and unweighted parsimony analysis on the alignments. Bootstrap tests were conducted to test the

## Sampled Regions



**Fig. 1.** Map of the United States (A) and Arkansas (B) highlighting regions sampled for *Lipoptena mazamae* from white-tailed deer.

reliability of the tree (Felsenstein, 1985, 1993). Parsimony bootstrap analysis and maximum likelihood analysis were conducted using the method by Tripodi *et al.* (2006) and genealogical relationships among mtDNA haplotypes were determined using TCS (Clement *et al.*, 2000) with the method described by Templeton *et al.* (1992). Briefly, PAUP\* 4.0b10 was used to conduct maximum likelihood and unweighted parsimony analysis on the alignments, the Branch and Bound algorithm of PAUP\* was used for parsimony bootstrap analysis and a heuristic search was performed using the neighbor

joining tree as the starting tree with PAUP\*. GenBank accession numbers are given in Table 2.

### Results

PCR amplification of the 16S rRNA gene resulted in a ~350-bp amplicon, an aligned 280-bp segment was used for analyzes. From the 30 DNA sequences, six unique haplotypes were observed (Table 2, Fig. 1) and summary statistics of ked genetic variation by county are presented

**Table 1.** Taxonomic information, GenBank accession number and reference for insects used in the phylogenetic tree of *Lipoptena mazamae* (Fig. 3).

Taxon	Family	GenBank	Reference
<i>Culex quinquefasciatus</i> Say	Culicidae	EU555416	Sharma <i>et al.</i> (2009)
<i>Culicoides tutti-frutti</i> (Meiswinkel)	Ceratopogonidae	AY294137	Meiswinkel & Linton (2003)
<i>Glossina mositans</i> (Westwood)	Glossinidae	EF531110	Peterson <i>et al.</i> (2007)
<i>Glossina pallidipes</i> (Austen)	Glossinidae	EF531111	Peterson <i>et al.</i> (2007)
<i>Glossina palpalis</i> (Robineau-Desvoidy)	Glossinidae	EF531112	Peterson <i>et al.</i> (2007)
<i>Glossina brevipalpis</i> (Newstead)	Glossinidae	EF531108	Peterson <i>et al.</i> (2007)
<i>Pseudolynchia</i> spp. (Macquart)	Hippoboscidae (SF: Ornithomyinae)	EF531103	Peterson <i>et al.</i> (2007)
<i>Crataerina pallida</i> (Latreille)	Hippoboscidae (SF: Ornithomyinae)	EF531107	Peterson <i>et al.</i> (2007)
<i>Ornithomya avicularia</i> (L.)	Hippoboscidae (SF: Ornithomyinae)	EF531118	Peterson <i>et al.</i> (2007)
<i>Ornithomya chloropus</i> (Bergroth)	Hippoboscidae (SF: Ornithomyinae)	EF531120	Peterson <i>et al.</i> (2007)
<i>Stenopteryx hirundinis</i> (L.)	Hippoboscidae (SF: Ornithomyinae)	EF531121	Peterson <i>et al.</i> (2007)
<i>Ortholfersia mintuta</i> (Paramonov)	Hippoboscidae (SF: Ornithomyinae)	EF531123	Peterson <i>et al.</i> (2007)
<i>Hippobosca rufipes</i> (von Olfers)	Hippoboscidae (SF: Hippoboscinae)	EF531115	Peterson <i>et al.</i> (2007)
<i>Lipoptena cervi</i> (L.)	Hippoboscidae (SF: Liopopteninae)	AF322437	Nirmala <i>et al.</i> (2001)
<i>Lipoptena cervi</i> (L.)	Hippoboscidae (SF: Liopopteninae)	EF531114	Peterson <i>et al.</i> (2007)
<i>Melophagus ovinus</i> (L.)	Hippoboscidae (SF: Liopopteninae)	EF531104	Peterson <i>et al.</i> (2007)

**Table 2.** Haplotype assignment, DNA variable sites, number of times the haplotype occurred and GenBank accession numbers.

Haplotype	DNA variable site			<i>n</i>	GenBank
	181	191	214		
1	T	T	A	12	GQ368825
2	T	C	A	3	GQ368826
3	T	C	T	1	GQ368827
4	G	T	A	1	GQ368828
5	G	C	A	2	GQ368829
6	G	C	T	11	GQ368830

in Table 3. Haplotype 1 was the most common haplotype (43% of specimens) and was found once in region 1 and 11 times in region 3. Haplotype 6 occurred in 40% of the samples and in three regions; region 1 twice, region 2 three times and was the only haplotype identified in region 4 (six times). Two haplotypes were only identified once (haplotypes 3 and 4).

Twenty-two deer were sampled for keds; eight of these deer had two keds analyzed and the remaining 14 deer had only one ked analyzed (30 keds analyzed). Because the sample number was small, genetic differences among the keds collected from host animals were not calculated, but haplotype information from each host is presented in Table 4. Half of the deer sampled for two keds were infested with two different *L. mazamae* haplotypes. Haplotype 6 was the most common haplotype found on a single deer. This haplotype was identified

from six keds samples collected from three deer and was paired with haplotypes 2 and 4 on two other deer.

Of the 259 characters used for analysis, 204 characters were constant, 22 were variable characters and 33 were parsimony-informative characters. Sequences were AT rich, with adenine consisting of 39% and thymine representing 41% of the bases (guanine 13%, cytosine 6.6%). Pairwise Tajima-Nei distances among *L. mazamae* ranged from 0.388 to 1.187% within species and 7.456 to 15.844% for the outgroup taxa (Tajima & Nei, 1984). Between the two *Lipoptena* samples (*L. mazamae* and *L. cervi*) pairwise Tajima-Nei distances ranged from 9.139 to 9.543%. This was only slightly larger than the distance from *L. mazamae* to *M. ovinus* (9.104 to 10.318%). Interestingly, the genetic distance between *L. cervi* and *M. ovinus* was 6.7 to 7.456%.

The TCS spanning tree rooted haplotype 2 as the ancestral haplotype (Fig. 2). The more common haplotypes 1 and 6 were different from haplotype 2 by one and two nucleotides, respectively. Maximum parsimony and maximum likelihood analyzes resulted in a single tree with a total length of 277 steps and a consistency index (CI) of 0.570 (Fig. 3). *Lipoptena mazamae* formed a clade separate within the Hippoboscidae but separate from the other subfamilies (Ornithomyinae, Hippoboscinae and Liopopteninae). This tree indicates *Lipoptena* is a monophyletic genus. Interestingly, *Melophagus ovinus* from Montana, U.S.A. were more similar to *L. cervi* from Europe than to *L. mazamae* from Arkansas. Variation within *L. mazamae* resulted in different clades among haplotypes 1, 2, and 3 from haplotypes 4, 5, and 6; however, support for these

**Table 3.** Summary statistics for mtDNA polymorphisms in *L. mazamae* among regions.

Region	Arkansas county	$n_s$	$n_h$	$h_n$	$H_d$	$\pi$ (k)	Haplotype (frequency)
1	Franklin	7	4	5	0.90476	0.00558 (1.42857)	1, 2, 3, 4(2), 5(2), 6(2)
2	Fulton	4	2	2	0.4	0.00313 (0.8)	4, 6(3)
3	Madison	13	12	2	0.28205	0.0011 (0.28205)	1(11), 2(2)
4	Polk	6	4	1	0	0 (0)	6(6)
All	Total	30	22	6	0.71264	0.00594 (1.52)	1(12), 2(3), 3, 4, 5(2), 6(11)

$n_s$ , number of sequences;  $n_h$ , number of hosts sampled;  $h_n$ , number of haplotypes;  $H_d$ , haplotype diversity;  $\pi$ , nucleotide diversity;  $k$ , mean number of pairwise nucleotide differences.

**Table 4.** Descriptive results for mtDNA polymorphisms in *Lipoptena mazamae* among deer hosts.

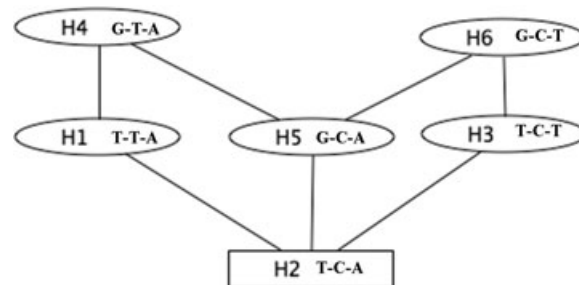
Host	Region	N samples	N haplotypes	Haplotype (frequency)
1	1	2	2	2, 6
2	1	2	1	5 (2)
3	1	1	1	3
4	1	2	2	1, 6
5	2	2	1	6 (2)
6	2	2	2	4, 6
7	3	1	1	1
8	3	1	1	2
9	3	1	1	1
10	3	1	1	1
11	3	1	1	1
12	3	1	1	1
13	3	2	2	1, 2
14	3	1	1	1
15	3	1	1	1
16	3	1	1	1
17	3	1	1	1
18	3	1	1	1
19	4	1	1	6
20	4	2	1	6 (2)
21	4	2	1	6 (2)
22	4	1	1	6

clades was relatively weak (consistency index of 0.857). When comparing the Bayesian tree to the TCS spanning tree, haplotypes 1, 2 and 3 from clade 1 are ancestral to haplotypes 4, 5, and 6 from clade 2. The maximum likelihood tree was identical in topology to the tree derived from maximum parsimony analysis. These inferred relationships were supported in 100% of the 100 bootstrap replications.

Wright's  $F_{ST}$  statistic revealed that gene flow occurred ( $F_{ST} = 0.0157$ ,  $Nm = 0.33$ ) among the populations. The number of migrants necessary to maintain the observed  $F_{ST}$  value (0.6196) was 0.153 individuals per generation and was calculated using the formula  $\{Nm \approx [(1 - F_{ST})/4F_{ST}]\}$  (Wright, 1951). The slightly negative values for Fu's  $F_s$  (-0.329), Fu's  $F_s$  (-0.329) and Tajimas D (-1.711) suggest population growth (Table 5).

**Discussion**

The current study recorded genetic variation within a regionally distributed population of *L. mazamae*. These data suggest that



**Fig. 2.** Genealogical relationships among six haplotypes of *Lipoptena mazamae* keds defined by nucleotide variability sites (181-191-214) and estimated by TCS (Clement *et al.*, 2000). A unit branch represents one mutation.

although deer ked dispersal is limited their populations are still expanding. The levels of genetic variation observed in *L. mazamae* was similar to biologically related *Glossina* flies (Krafsur *et al.*, 2000). In the study conducted by Krafsur *et al.* (2000), 26 *Glossina* haplotypes were identified with the 12S and 16S markers. Nine of their haplotypes occurred only once and seven haplotypes were identified only twice. Only six of the haplotypes (23%) were shared among boundaries (Krafsur *et al.*, 2000). Krafsur *et al.*'s (2000) results were similar to this study: identified six haplotypes, few singleton haplotypes (haplotypes 3 and 4) and one haplotype (haplotype 6) occurring in most of the regions.

It is interesting to find such a high level of genetic variation within *L. mazamae* because of their distinct biology, dependence on host for dispersal and our limited sample size. Other studies of ectoparasites have included work on tsetse flies (*Glossina morsitans* Westwood) (Krafsur *et al.*, 2000), feather lice (Phthriaptera: Iscnocera) (Johnson *et al.*, 2002), stable flies (*Stomoxys calcitrans* L.) (Szalanski *et al.*, 1996) and bed bugs (*Cimex lectularius* L.) (Szalanski *et al.*, 2008). Population genetics studies of tsetse fly mitochondrial genes 12S and 16S2 yielded 26 haplotypes across the Gambian and Ethiopian regions of Africa inferred that the two tsetse fly islands (eastern and western regions of Africa) will continue to be geographically separated because of a decline in rainfall, reduction in forest and woodland cover and overall changing habitat (Krafsur *et al.*, 2000). Amongst the bird lice genera *Physconelloides* and *Columbicola* from doves there was a strong differentiation between them when using the cytochrome oxidase I gene which lead to inferences about

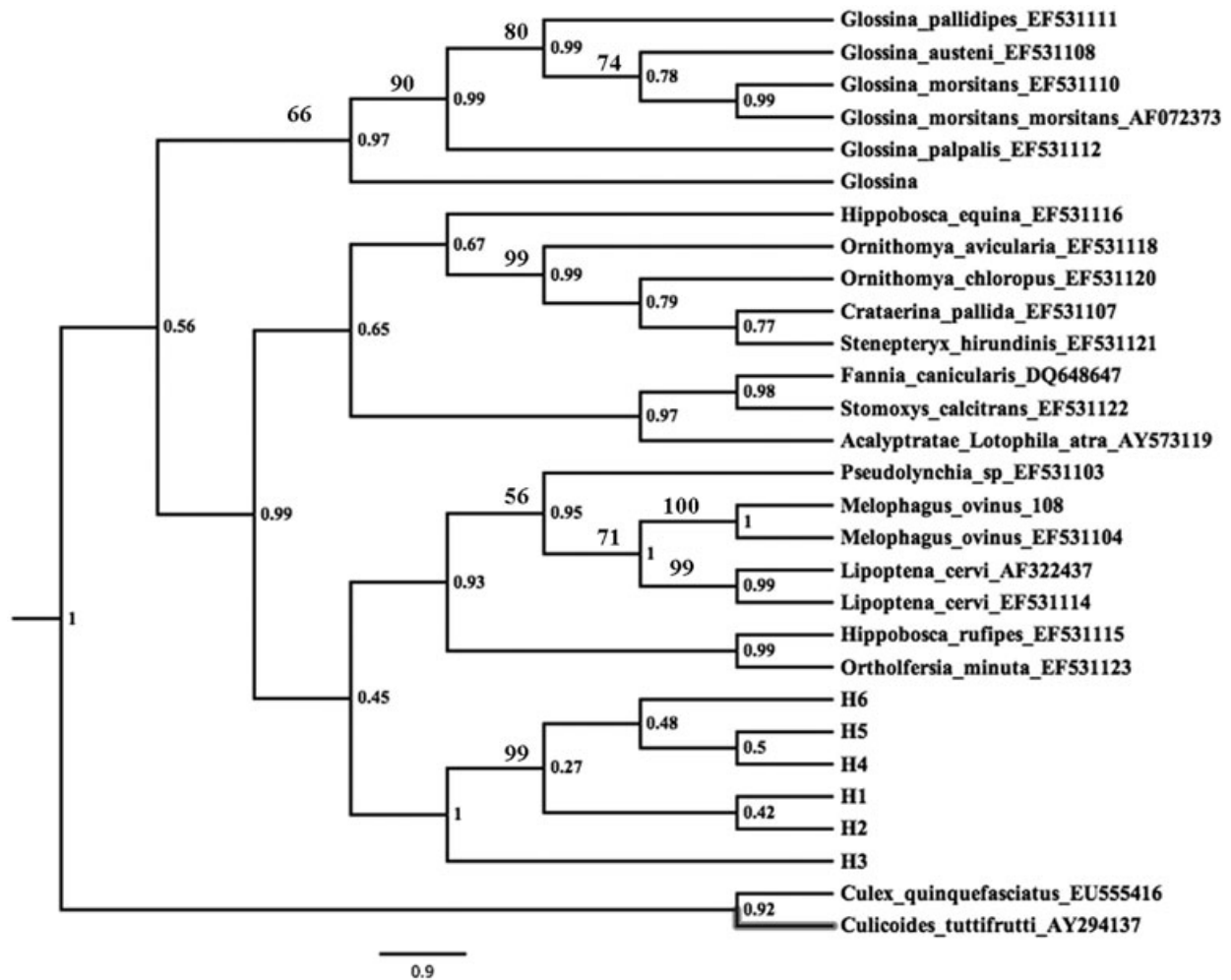


Fig. 3. Maximum likelihood and maximum parsimony tree based on 16S rRNA sequence data. Numbers in line are bootstrap support values.

Table 5. Summary statistics for mtDNA polymorphisms among all 30 sampled deer keds.

	$N$	$h$	$\pi$ ( $k$ )	$\theta_s$	$\theta_g$	D+	F+	D
All counties	30	6	0.00598 (1.52)	0.00293	0.745	0.94181	1.58257*	-1.711

\* $0.10 < P < 0.05$ .

$n$ , number of sequences;  $h$ , number of haplotypes;  $\pi$ , nucleotide diversity;  $k$ , mean number of pairwise nucleotide differences;  $\theta_s$ , theta per site;  $\theta_g$ , theta per gene; D+ and F+ statistics are detailed in the text (Fu & Li, 1993); D, Tajima & Nei (1984), detailed in the text.

host specificity resulting from their dispersal abilities (Johnson *et al.*, 2002). Genetic studies on stable flies using PCR-RFLP analyzes combined with allozyme analyzes on several mitochondrial and nuclear genes indicated high levels of genetic homogeneity among stable flies collected in several states suggesting a population bottleneck upon their original introduction with cattle (Szalanski *et al.*, 1996). Genetic work with bed bugs has identified moderate to high levels of gene flow with high levels of mitochondrial diversity, which may have occurred with recent introductions (Szalanski *et al.*, 2008).

The current analysis of the phylogenetic relationships of *L. mazamae* within the Hippoboscidae was similar to Peterson *et al.* (2007) who used CAD, COI, 16S and 28S to determine phylogeny and evolution of host choice in the Hippoboscoidea. In both studies, *Lipoptena* was a polyphyletic group. This study only used the 16S rRNA gene of the mitochondrial genome instead of multiple genes because the focus of the study was population genetics. Consequently, the variation found with this single gene placed *L. mazamae* out of the Lipopteninae subfamily in the resulting tree. The use of additional genes such as those used by Peterson *et al.* (2007) may group *L. mazamae*

back with the *Lipoptena*. Using additional nuclear genes and morphological evidence to identify genetic variation within the subfamily and species will provide a better understanding of the polyphyletic findings in this study.

Understanding genetic variation for *L. mazamae* is important for pathogen transmission and epidemiology (*Bartonella* and *Anaplasma*), host economic impact (blood feeding and anemia) and control efforts (Tabachnick & Black, 1995). Predicting gene flow indirectly allows researchers to understand ked dispersal (Krafsur, 2003). The genetic analysis of *L. mazamae* in Arkansas revealed a high amount of genetic diversity among the four regions sampled. The Ozark Mountains (regions 1–3), the Arkansas River Valley (region one) and the Ouachita Mountains (region four) (Fig. 1) naturally divide the four regions and may serve as geographical barriers to dispersal. The geography of Arkansas, with the Ozark Mountains, Ouachita Mountains, and Arkansas River Valley, may limit *L. mazamae* gene flow. As *Lipoptena* flies also act as a reservoir for *Bartonella* and potentially other pathogens, the limited gene flow may identify potential areas of high risk to pathogen transmission. Continued understanding and monitoring of ked gene flow combined with pathogen detection is essential in identifying these potential high-risk areas for disease.

Phylogenetics and population genetics studies of *L. mazamae* further illustrates the impact natural geography and ecological variation has on dispersal, gene flow and pathogen transmission within this hematophagous species. Further analysis into additional genetic markers, varying geographies and multiple host animals will provide more insight into the dispersal and taxonomic status of this veterinary pest of ruminants and humans.

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