Midsouth Entomologist 9:67-76

ISSN: 1936-6019
www.midsouthentomologist.org.msstate.edu

Research article

Genetic diversity, pathogens and parasites of feral honey bees and swarms from eight Mississippi counties

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Received: 28-VI-2016 Accepted: 31-X-2016

Abstract: A study was conducted on the genetic diversity of honey bees, *Apis mellifera* L., from feral colonies and swarms collected from Mississippi by sequencing a portion of the mitochondrial DNA COI – COII region. From the 38 samples analyzed, we observed 11 haplotypes collected from eight counties. These haplotypes represented three of the four *A. mellifera* lineages known to have been imported into the United States (C, M and O). Of the seven C lineage haplotypes, three were not found in previous queen breeder studies in the United States. The O lineage, with two haplotypes observed, accounted for 18% of unmanaged colonies. This lineage has not been observed in queen breeder studies in the United States. The M lineage accounted for 16% of samples with two haplotypes observed. Samples were also screened for the honey bee pathogens *Nosema ceranae*, *N. apis* and the honey bee parasite *Varroa destructor*. Four samples were positive for *Nosema* (3 *N. apis* and 1 *N. ceranae*) and four were positive for *V. destructor*, with two samples positive for both *Nosema* and *V. destructor*. The detection of genetically distinct maternal lineages of feral honey bees in Mississippi relative to managed honey bee colonies suggests that these haplotypes may have existed outside of the managed honey bee population for a long period of time.

Keywords*: Apis mellifera,* genetic variation, mitochondrial DNA, COI-COII

Introduction

Honey bees, *Apis mellifera* L. (Hymenoptera: Formicidae), have more than 26 subspecies which have been placed into five evolutionary lineages based primarily on morphometrics and their historical geographic distribution (Ruttner 1988, Sheppard et al. 1997, Sheppard and Meixner 2003, Ferreira et al. 2008). These lineages include the A (African group), M (North and Western Europe), C (Southeastern Europe), O (Near East and Middle East) (Ruttner et al. 1978, Ruttner 1988, Franck et al. 2001, Kandemir et al. 2006, El Niweiri and Moritz 2008, Ferreira et al. 2008, Shaibi 2009) and Y (Ethiopia) (Franck et al. 2001). Eight subspecies from four lineages (A, C, M and O) were introduced to the United States (Pellett 1938, Sheppard 1989a, 1989b) before the enactment of the 1922 Honey Bee Act which ended all importation of adult honey bees due to the tracheal mite, *Acarapis woodi* (Rennie) (Trombiformes: Tarsonemidae), the causative agent of the Isle of Wight disease (Phillips 1923).

DNA sequence analysis of a portion of the mitochondrial DNA (mtDNA) COI-COII genome can provide information on the mitochondrial lineage of the queen honey bee in a colony. Mitochondrial DNA markers, unlike nuclear DNA markers, are maternally inherited, allowing mtDNA analysis to focus on the genealogies of individual lineages (Lansman et al. 1981). This also has the advantage of using a single individual honey bee worker to genetically characterize an entire honey bee colony (Sheppard and Smith, 2000). Studies on the genetic variation of the mtDNA COI-COII region of queen breeder populations in

the United States have revealed that the vast majority of queen breeders have only the C lineage (Delaney et al. 2009, Magnus et al. 2011). However, several studies on mtDNA diversity of feral honey bee colonies and swarms from continental United States and Hawaii (Magnus and Szalanski 2010, Magnus et al. 2014, Szalanski et al. 2016) have revealed a number of M and O lineage haplotypes that have not been observed in previous studies of queen breeders (Delaney et al. 2009, Magnus et al. 2011).

It is believed that feral colonies of European honey bees in the United States are rare in natural areas due to parasites and pathogens, especially after the arrival of *Varroa destructor* Anderson and Trueman (Parisitiformies: Varroidae) (Seeley et al. 2015). On the other hand, several studies have shown that feral colonies of honey bees have persisted for at least 10 years with infections of *V. destructor* in New York State (Seeley 2007) and in Europe (Fries et al. 2006, Le Conte et al. 2007). Queen breeders seeking to increase resilience against pests, parasites and pathogens have become interested in the possibility that feral populations may be 'survivor stock' with desirable qualities that could be incorporated into managed populations (McNeil 2009a, 2009b, Jacobson 2010). These feral colonies may not have been manipulated by beekeepers for a long period of time and may have adapted to the various stresses that threaten their survival (Loper and Sammataro 2006, Seeley 2007, Villa et al. 2008).

The purpose of this study was to determine if feral colonies and swarms of European honey bees in Mississippi have genetically distinct mtDNA haplotypes relative to studies on honey bees collected from managed and queen breeder colonies in the United States using DNA sequence data. Samples were also screened for the presence of the honey bee pathogens *Nosema apis* Zander (Dissociodihaplophasida: Nosamatidae), *N. ceranae* Fries, and the parasite *V. destructor*.

Methods

Sampling. Adult European worker honey bees were collected from feral honey bee colonies during 2008 and 2009 from eight counties in Mississippi (Table 1). Following Schiff et al. (1994), feral colonies are defined as established colonies occurring in unmanaged homesites (e.g. trees, caves, buildings). Samples collected from masses of bees discovered in the open, without comb are defined as swarms. Swarms are commonly derived from unmanaged colonies, but their origin can be difficult to determine with certainty. A total of 40 samples, 35 feral and five swarms were collected in 70–100% ethanol and sent to the Insect Genetics Lab, University of Arkansas, Fayetteville, Arkansas, for genetic analysis. Each sample consisted of 25 to 40 worker honey bees.

Mitochondrial DNA Analysis. DNA was extracted from individual honey bees using a salting-out protocol with in-house reagents (Sambrook and Russell 2001). PCR Primers E2 and H2 (Garnery et al. 1993) were used to amplify extracted DNA via PCR. These primers amplify an approximately 530bp to 1230bp portion of the mtDNA COI-COII genes. A total of 2 μL of extracted DNA was used for PCR. The PCR reaction consisted of holding the samples for 5 min at 94°C, then 40 cycles of 94°C for 45s, 46°C for 1 min, and 72°C for 1 min, followed by a final extension of 72°C for 5 min. Amplicon verification was conducted by gel electrophoresis using 1% agarose gels and visualizing PCR products using a BioDoc-it™ Imaging System (UVP, Inc., Upland, CA). Samples were purified and concentrated with VWR centrifugal devices (VWR, Radnor, PA) and sent to Eurofins Genomics (Huntsville, AL) for direct sequencing in both directions. Voucher specimens are deposited at the University of Arkansas Insect Genetics Laboratory in Fayetteville, Arkansas, USA. Consensus sequences with the primer ends removed were obtained using Geneious v6.1.6. (Biomatters Ltd., Auckland, New Zealand), and unique haplotypes were identified by eye. Assignment and comparison of haplotypes was conducted by a NIH BLAST search of DNA sequences available on GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Haplotype data for 18 of the samples was previously published in Magnus et al. (2014) (Table 1).

***Nosema* and *Varroa* detection**. Molecular detection of *Nosema* was conducted using the associated honey bee worker DNA extractions, with PCR primers NosemaSSU-1F and NosemaSSU-1R (Szalanski et al. 2014). These primers amplify a 222 bp (*N. apis*) or 237 bp (*N. ceranae*) region of a SSU marker for *Nosema*. The PCR reaction conditions were: an initial denaturation of 94°C for 2 min, followed by 40 cycles of 94°C for 45s, 50°C for 60s and 72°C for 60s, and a final extension of 5 min at 72°C. PCR products were subjected to electrophoresis in 2% agarose gels, and PCR products were visualized under UV light (BioDocit Imaging System, UVP, LLC, Upland, CA). Samples which were positive for *Nosema* sp.

were subjected to PCR-Restriction Fragment Length Polymorphism (RFLP) to distinguish *N. ceranae* from *N. apis* per Szalanski et al. (2014). Occurrence of *N. ceranae* and *N. apis* for 29 of the samples was previously published in Szalanski et al. (2014) (Table 1). *Varroa destructor* mites were isolated from each sample using a mite washer modified from Oliver (2013), using 15 to 25 honey bee workers from each sample.

Results and Discussion

From the 38 honey bee samples subjected to mtDNA COI-COII DNA sequence analysis in this study, three *A. mellifera* lineages (C, M and O) were observed (Table 1, Fig. 1). The C lineage was the most commonly observed lineage and accounted for 66% of all samples sequenced in this study, followed by the O lineage (18%), and M lineage (16%). A total of 11 haplotypes were observed with seven belonging to the C lineage, two to the O lineage and two to the M lineage. Of the seven C lineage haplotypes, five (C1, C2, C11, C27 and C31) have been observed in queen breeder populations in the United States (Delaney et al. 2009, Magnus et al. 2011). The two haplotypes not observed in the queen breeder population were C26 (sample MS18 from Pontotoc County), and C34 (sample MS40 from Lamar County). Haplotype C26 has been observed from a feral honey bee sample from New Mexico (Magnus et al. 2014), while haplotype C34 has not been observed outside of this Mississippi study. For the O lineage haplotypes, O1 was the most common (n = 6), accounting for 86% of the O-lineage haplotypes. Haplotype O1 was found in unmanaged honey bee samples from Missouri, Oklahoma and Utah, and managed colonies in Hawaii (Magnus et al. 2014, Szalanski et al. 2016). Haplotype O5''b has also been observed in managed and unmanaged colonies in Arkansas (Magnus et al. 2014).

Samples were also screened for the honey bee pathogens *Nosema ceranae, N. apis* and the honey bee parasite *Varroa destructor*. Four samples were positive for *Nosema*: sample MS3 from Union county (*N. apis*); sample MS23 from Benton county (*N. ceranae*); sample MS26 from Union county (*N. apis*), and sample MS30 from Pontotoc county (*N. apis*). The percentage of Mississippi feral honey bees and swarms infected with *Nosema* (8.3%) was similar to a previous study on feral honey bee (8.8%) from south central and southwestern United States (Szalanski et al. 2014). Although the number of infected colonies was low, it is interesting that *N. apis* was more prevalent in these samples than *N. ceranae.* Most studies have shown that *N. ceranae* has become more common in recent years than *N. apis* (Szalanski et al. 2013, 2014) and may have completely displaced *N. apis* in some populations (Rangel et al. 2015). The occurrence of *Nosema* sp. in feral European honey bees is considerably lower than the levels observed in managed honey bees from New York and South Dakota (31-44%) (Szalanski et al. 2013), and by a national survey of beekeepers where of the 648 alcohol samples collected from 2013-2014, 348 (47.0%) had detectable *Nosema* spore loads (Rennich et al. 2015). This is not surprising since it has been documented that management of honey bee colonies can increase the infection rate of *Nosema* in apiaries. For example, Oertel (1967) observed that opening honey bee colonies can increase the presence of *N. apis*, and Moeller (1978) noted that honey bee equipment from colonies that died over winter can be a source of *Nosema* for new colonies established by package bees or divisions. *Nosema* contaminated honey supers or brood chambers can also inoculate uninfected colonies in the summer (Moeller 1978).

Four of the Mississippi samples (10%) were positive for *V. destructor*: samples MS24 and MS25 from Union county and two samples, MS23 from Benton county and MS26 from Union county, which were positive for both *Nosema* and *V. destructor*. The occurrence of V*. destructor* in the sampled feral and swarm Mississippi honey bees is greatly lower than its occurrence in managed honey bee colonies in the United States. A 2013-2014 national survey from 32 states, found 98.2% of their 648 samples positive for *V. destructor* (Rennich *et al*. 2015). However, this may be due to the relatively small sample size (15-25 worker honey bees per colony) used. Screening of more worker honey bees per colony (approx. 300, Dietemann et al. 2013) is needed to confirm if there is a difference in the occurrence of *V. destructor* in feral versus managed honey bees in Mississippi. It is assumed that when *V. destructor* arrived in the United States in the mid 1980's, it destroyed most of the feral European honey bee population (Wenner and Busing 1996, Sanford 2001). However, Seeley (2007) observed that feral honey bees located in the Arnot forest in New York were infested with *V. destructor* and that there appeared to be more feral colonies than a previous survey in 1978, which was before *V. destructor* was present.

The first record of honey bees in Mississippi was by Claiborne (1880) as he stated that honey bee colonies were abundant in the Natchez, Mississippi area between 1770 and 1775. By 1819 honey bees were commonly found in Mississippi (Lincecum 1905), and by 1841 feral honey bee colonies were often found (Claiborne 1906). These honey bees would have been the German dark bee, *A. m. mellifera* (M lineage), with the Italian honey bee, *A. m. ligustica* (C lineage) not being introduced to the United States until 1855 (Sheppard 1989a). Honey bees belonging to the O lineage were imported into the United States as early as the 1880's but were found to be too aggressive for most beekeepers and never became popular with beekeepers (Sheppard 1989b). By the 1920's C lineage honey bees became the predominant lineage in the United States (Oertel 1980). Our observation of a number of feral honey bee colonies in Mississippi belonging to the M and O lineages provides evidence that these colonies are not recent descendants from managed honey bee colonies and that they may have survived as feral colonies for a long period of time. These M and O mtDNA COI-COII maternal lineages observed in Mississippi could be established in managed apiaries to determine if they possess any traits that are beneficial for beekeepers. Better survival of feral colonies compared to managed honey bee colonies has been supported by a recent study by Loftus et al. (2016). They observed that honey bee colonies living in small hives similar in volume to feral colonies (42 L) had lower *Varroa* infestation rates, less disease, swarmed more often and had higher survival rates than colonies with a larger hive volume used by beekeepers (up to 168 L).

Table 1. Mitochondrial DNA haplotypes, *Nosema* species and *Varroa destructor* occurrence for feral and swarm honey bees from Mississippi**.**

Sample	County	Haplotype	Haplotype GenBank Accession No.	Feral (F) or Swarm (S)	*Nosema*	Varroa
MS1	Union	M3*	FJ743636	F	-**	-
MS2	Union	M3*	FJ743636	F	-**	-
MS3	Union	C31*	HQ287900	F	*N. apis***	-
MS4	Lee	C2*	JF934704	F	-**	-
MS5	Union	C2*	JF934704	F	-**	-
MS6	Tippah	O1*	JF934714	F	-**	-
MS8	Union	C11*	FJ037776	F	-**	-
MS9	Union	C2*	JF934704	F	-**	-
MS10	Pontotoc	O5"b*	GQ856213	F	-**	-
MS11	Tippah	C2*	JF934704	F	-**	-
MS12	Pontotoc	M4*	FJ743637	F	-**	-
MS13	Pontotoc	C11*	FJ037776	F	-**	-
MS14	Pontotoc	M4*	FJ743637	F	-**	-
MS15	Pontotoc	***		F	-**	-
MS16	Tippah	C31*	HQ287900	F	-**	-
MS17	Union	C2*	JF934704	F	-**	-
MS18	Pontotoc	C26*	JF934705	F	-**	-
MS19	Union	O1*	JF934714	F	-**	-
MS20	Tippah	C27*	HQ287899	F	-**	-
MS21	Union	M3*	FJ743636	F	-**	-
MS22	Tippah	C2*	JF934704	F	-**	-
MS23	Benton	O1	JF934714	F	*N. ceranae***	Pos
MS24	Union	C1	FJ478010	F	-**	Pos
MS25	Union	O1	JF934714	F	-**	Pos
MS26	Tippah	O1	JF934714	F	*N. apis***	Pos
MS27	Lee	C11	FJ037776	F	-**	-
MS28	Union	C1	FJ478010	S	-**	-
MS29	Union	***		F	-**	-
MS30	Pontotoc	C1	JF934714	S	*N. apis*	-
MS31	Lee	O1	JF934714	S	-	-
MS32	Lee	C1	FJ478010	F	-	-
MS33	Union	C1	FJ478010	S	-	-
MS34	Union	C1	FJ478010	F	-	-
MS35	Tippah	C11	FJ037776	F	-	-
MS36	Tippah	C1	FJ478010	F	-	-
MS37	Oktibbeha	M3	FJ743636	F	-	-
MS38	Marion	C1	FJ478010	S	-	-
MS39	Marion	C1	FJ478010	F	-	-
MS40	Lamar	C34	JF934710	F	-	-
MS41	Tippah	C31	HQ287900	F	-	-

*****Magnus et al. 2014, **Szalanski et al. 2014, *** undetermined, Pos = Positive

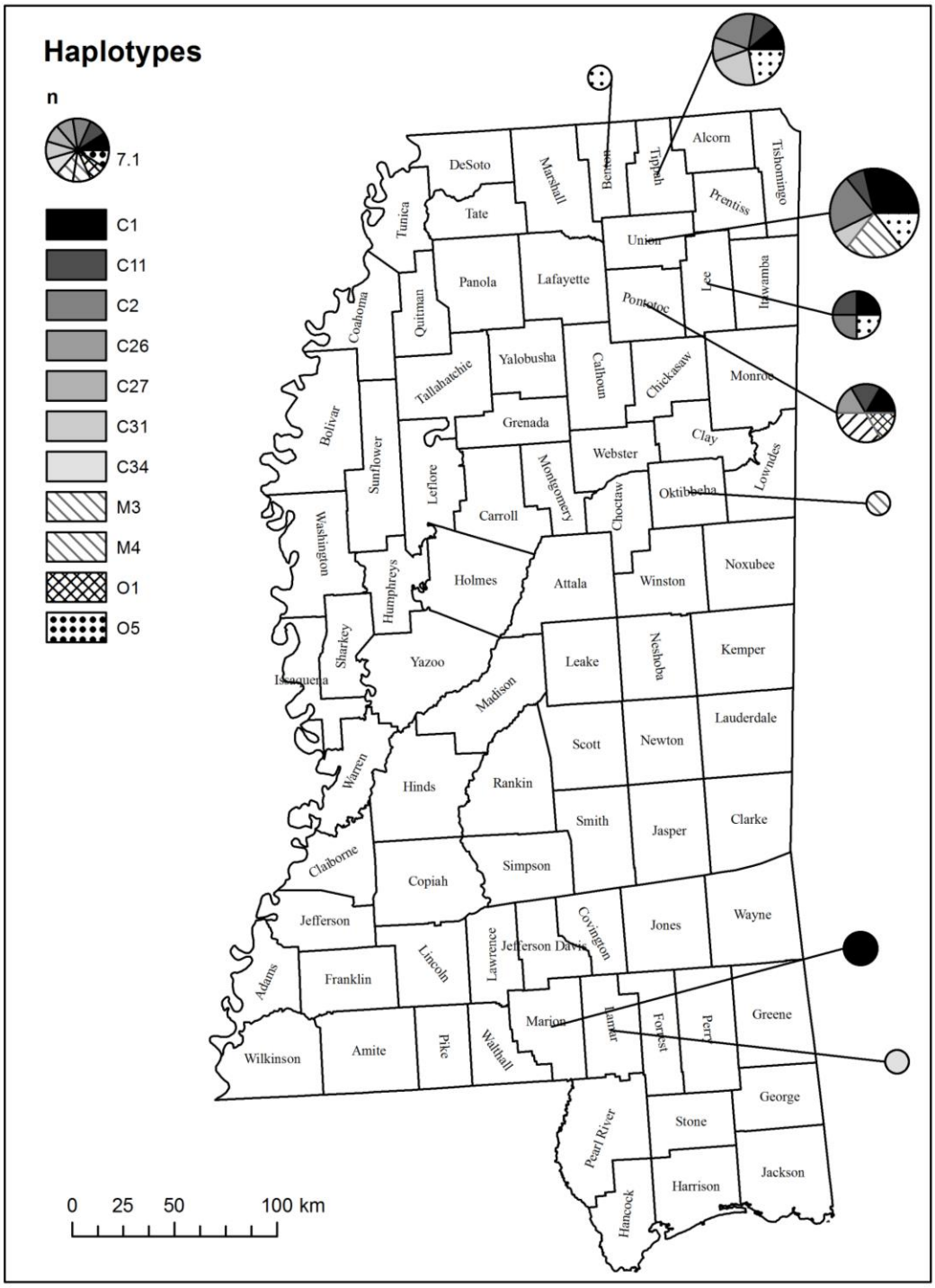

Figure 1. Distribution of COI-COII *Apis mellifera* haplotypes in Mississippi. The size of each chart is proportional to the number of samples analyzed for that county, and the slices indicate the proportion of samples of each haplotype found.

Acknowledgements

We thank Clarence Collison for providing samples. This research was supported in part by the University of Arkansas, Arkansas Agricultural Experiment Station.

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