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MOLECULAR DETECTION OF *NOSEMA APIS* AND *N. CERANAE* FROM SOUTHWESTERN AND SOUTH CENTRAL USA FERAL AFRICANIZED AND EUROPEAN HONEY BEES, *APIS MELLIFERA* (HYMENOPTERA: APIDAE)

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ABSTRACT

A Polymerase Chain Reaction (PCR) molecular diagnostic survey for the honey bee pathogens *Nosema apis* Zander and *N. ceranae* Fries was conducted on feral Africanized honey bee (AHB) and European honey bee (EHB), *Apis mellifera* L., populations sampled from Mississippi, Arkansas, Texas, Utah, Oklahoma, and New Mexico. Polymerase Chain Reaction – Restriction Fragment Length polymorphism (PCR-RFLP) analysis of a 220 bp small subunit (SSU) marker was conducted on 517 samples, consisting of 245 AHB and 272 EHB individuals. A total of 43 samples (8.3%) were positive for *Nosema;* of these, 82.1% were *N. ceranae*, and the remainder were *N. apis*. No mixed samples were observed. For the AHB samples, *Nosema* was detected in 9.0% of the samples with 89.5% of the *Nosema* identified as *N. ceranae*, and 10.5% as *N. apis*. With the EHB samples, 7.7% had *Nosema*; 75.0% of these with *N. ceranae*, and 25.0% with *N. apis*. No significant difference was observed between AHB and EHB feral samples for occurrence of each *Nosema* species and prevalence of *Nosema* infection. Among the AHB samples, *Nosema* was more common in Utah and Texas than in New Mexico and Oklahoma. *Nosema* infection rates for feral honey bees was considerably lower than levels observed with managed honey bees in studies from New York, South Dakota, and Virginia.

Key Words: honey bee, molecular diagnostics, *Nosema*

RESUMEN

Se realizó un sondeo diagnóstico molecular de la reacción en cadena de la polimerasa (RCP) para los patógenos de las abejas de miel *Nosema apis* Zander y *N. ceranae* Fries en las abejas de miel, *Apis mellifera* L, Africanizadas (AMA) salvajes y Europeas (AME), en poblaciones muestreadas de Mississippi, Arkansas, Texas, Utah, Oklahoma y Nuevo México. Se realizó un análisis de la reacción en cadena de la polimerasa de los fragmentos de restricción de longitud polimórfica (PCR- RFLP) de una subunidad pequeña (USP) de un marcador de 220 pb en 517 muestras, que contenia 245 individuos de AMA y 272 de AME. Un total de 43 muestras (8.3%) fueron positivas para *Nosema*, de éstas 82.1% fue *N. ceranae*, y el resto fue *N. apis*. No se observaron muestras mixtas. Se detectó *Nosema* en 9.0% de las muestras de AMA, con el 89.5% de la *Nosema* identificada como *N. ceranae* y el 10.5% como *N. apis*. Con las muestras de AME, el 7.7% tenían *Nosema*; el 75.0% de éstas fue *N. ceranae*, y el 25.0% fue *N. apis*. No se observó ninguna diferencia significativa entre las muestras de las abejas salvajes de AMA y AME para la ocurrencia de cada especie de *Nosema* y la prevalencia de la infección por *Nosema*. Entre las muestras de AMA, el *Nosema* fue más común en Utah y Texas que en Nuevo México y Oklahoma. La tasa de infección por *Nosema* en las abejas salvajes fueron considerablemente menor que los niveles observados en las abejas manejadas en los estudios de Nueva York, Dakota del Sur y Virginia.

Palabras Clave: abeja de la miel, diagnóstico molecular, *Nosema*

Nosema apis Zander, is a microsporidian pathogen of honey bees, *Apis mellifera* L*.,* causing nosemosis in its host (Bailey & Ball 1991; Fries 1997). *Nosema apis* was first observed in the United States in 1910 (White 1914), and was assumed to be the only *Nosema* species infecting honey bees in the country. Another *Nosema* species, *N. ceranae* Fries, was observed in the Asian honey bee, *Apis cerana* F., by Fries et al. (1996), and then first reported in *A. mellifera* in 2004 (Higes et al. 2006). Further studies have revealed *N. ceranae* has been present in the United States since at least 1995 (Chen et al. 2008), and in Europe since 1998 (Paxton et al. 2007). *Nosema ceranae* is highly pathogenic in honey bees (Higes et al. 2008) and is associated with reduced honey production and increased winter mortality (Higes et al. 2006). Molecular diagnostics using Poly-

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merase Chain Reaction (PCR) primers specific for *Nosema ceranae* and *N. apis* are well established and have been conducted on managed honey bee populations from Turkey (Whitaker et al. 2011), Argentina (Medici et al. 2012), Crotia (Gajger et al. 2010) the United States (Chen et al. 2008; Chapon et al. 2009; Traver & Fell 2011; Szalanski et al. in press), and Canada (Williams et al. 2008). Despite these studies on the occurrence of *Nosema apis* and *N. ceranae* in managed honey bees in the United States, it is unknown if there is a different rate of infection with feral populations of European honey bees (EHB) or with Africanized honey bees (AHB) in the United States. Feral honey bees can be defined as those sourced from established, unmanaged colonies in a tree, building, or other cavities (Schiff et al. 1994). Only one study has been conducted on *Nosema* from feral honey bees in the United States. Gilliam & Tabler (1991) found *N. apis* from one out of seven feral honey bee colonies sampled in Arizona. A recent study by Teixeira et al. (2013) has documented that *N. ceranae* has been present in AHB in Brazil since 1979, confirming its presence in *A. mellifera* considerably earlier than was previously assumed. The objectives of this study were to determine how widespread *Nosema ceranae* and *N. apis* are in feral honey bee populations in south central and southwestern United States, and if there are any differences in *Nosema* infection rate between feral AHB and EHB populations.

Materials and Methods

Honey bee specimens were collected from Arkansas, Mississippi, Oklahoma, New Mexico, Texas, and Utah as part of other studies on genetic variation of feral EHB and AHB populations (Szalanski & McKern 2007; Szalanski & Magnus 2010) and for this study (Table 1, Fig. 1). Worker honey bees were collected from established colonies in trees, buildings, or other cavities from 1991 to 2010 and were preserved in 70-90% ethanol. The sample unit in this study was a single colony, with the number of worker honey bees collected for each sample ranging from 10 to 50 individuals. DNA was extracted separately from individual worker honey bee thoraces (AHB/EHB testing) and abdomens (*Nosema* testing) using a salting-out procedure with in-house reagents (Sambrook & Russell 2001). Molecular diagnostics using multiplex Polymerase Chain Reaction (PCR) of a mitochondrial DNA cytochrome *b* marker (Szalanski & McKern 2007) was used to determine if the sample was AHB or EHB. Two individuals were tested from each sample.

Molecular detection of *Nosema* was conducted using the associated abdominal DNA extractions, with PCR primers that were designed by finding conserved regions in aligned *Nosema* small subunit (SSU) sequences obtained from GenBank (Geneious v6.1.6, Biomatters, Auckland, New Zealand). The PCR primers that were developed were NosemaSSU-1F (5'-ACAATATGTATTAGATCTGATATA-3') and NosemaSSU-1R (5'-TAATGATATGCTTAAGTTCAAAG-3'). These primers amplify a 222 bp (*N. apis*) or 237bp (*N. ceranae*) region of a SSU marker for *Nosema*. Thermocycler 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 60 s, 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, California).

Geneious software was then used to evaluate restriction enzymes which would distinguish *N. ceranae* from *N. apis* using PCR-Restriction Fragment Length Polymorphism (RFLP). Samples which exhibited an amplicon approximately 230 bp in length were subjected to PCR-RFLP using the restriction enzymes *Dra I*, which exclusively cuts *N. ceranae* at 79 bp, and *Rsa I*, which only cuts *N. apis* at 130 bp. For each digestion, 2 µl of PCR product, 0.4 µL restriction enzyme, 1 µL enzyme buffer, and 6.2 µl ultrapure water were incubated at 37 °C overnight. An enzyme-negative control was included by substituting ultrapure water for the enzyme. Resulting products were visualized as above. Estimates of amplicon size were made by visual comparison to a 100 bp

Table 1. Number of samples from six states and year of sample for this study on the molecular detection of Nosema from feral *Apis mellifera*.

State	1991	2004	2005	2006	2007	2008	2009	2010	n
Arkansas	0	0	0	1	0	10	24	4	45
Mississippi	0	0	0	0	0	10	24	0	29*
New Mexico	0	0	13	19	16	5	0	0	53
Oklahoma	0	9	45	31	27	18	0	0	130
Texas	12	0	83	8	23	13	1	0	140
Utah	0	0	0	0	0	4	105	11	119
n	12	9	141	59	66	60	154	15	517

*Five samples from Mississippi had no associated date.

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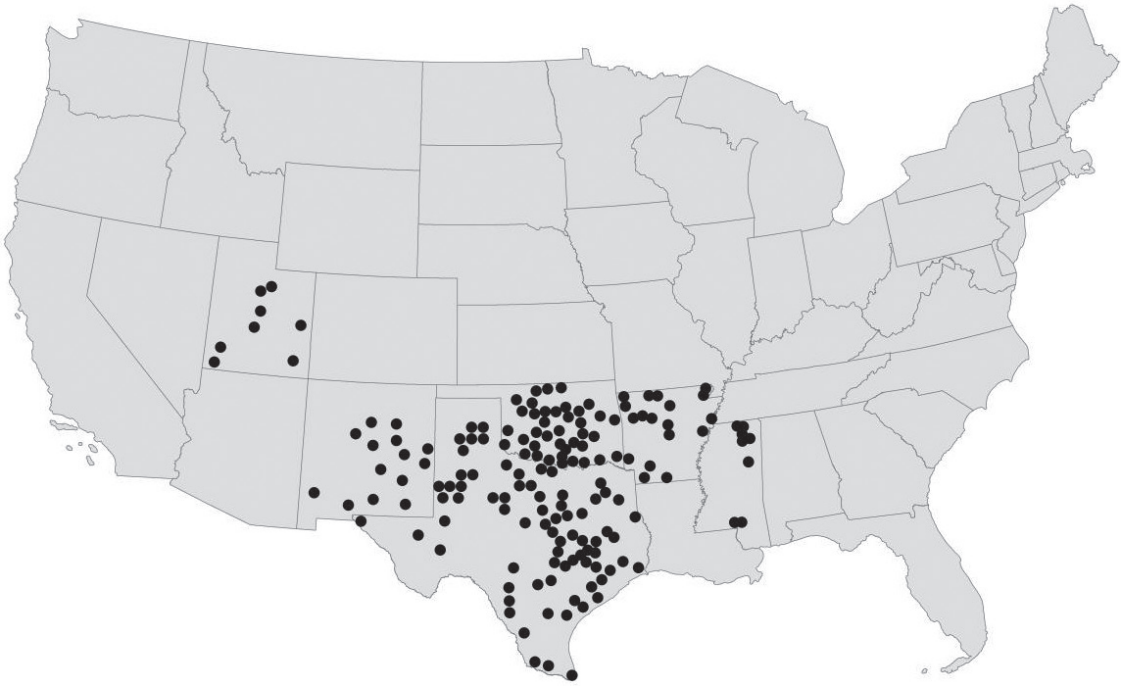


Fig. 1. Feral Africanized (AHB) and European honey bee (EHB) sample locations.

ladder. Amplified DNA from 2 *N. ceranae* and 3 *N. apis* positive PCR-RFLP samples were purified, concentrated with PES 30k filter devices (VWR, Radnor, Pennsylvania), and sent for direct sequencing in both directions (University of Arkansas Medical School, Little Rock, Arkansas). Consensus sequences were constructed using Geneious v6.1.6. Identification of samples was then confirmed using *N. ceranae* and *N. apis* SSU sequences from GenBank. Statistical analysis was conducted using pairwise comparisons via Fisher's exact tests (Sokal & Rohlf 1969).

Results and Discussion

A total of 517 feral *A. mellifera* samples (245 AHB, 272 EHB), collected from Arkansas, Mississippi, Texas, Utah, Oklahoma, and New Mexico from 1991 to 2010, were subjected to PCR-RFLP analysis for molecular identification of *Nosema apis* and *N. ceranae* (Table 1, Fig. 1). Of these, 43 samples (8.3%) were positive for *Nosema,* with 82.1% being *N. ceranae*, and the remainder were *N. apis* (Table 2). No mixed samples were observed. For the AHB samples, *Nosema* was detected in 9.0% of the samples with 89.5% of the *Nosema* identified as *N. ceranae*, and 10.5% as *N. apis*. With the EHB samples, 7.7% had *Nosema*, with 75.0% of these with *N. ceranae*, and 25.0% with *N. apis*. There was no significant difference in *Nosema* infection rate (P = 0.635), or the frequency of *N. apis* and *N. ceranae* infections (P = 0.407) between AHB and EHB populations.

Table 2. Occurrence of *Nosema ceranae* and *N. apis* in Africanized Honey Bee (AHB) and European Honey Bee (EHB) colonies from six states.

State (n)	AHB (n)	% *Nosema* positive, species (%)	EHB (n)	% *Nosema* positive, species (%)
AR (45)	1	0%	44	8.9%; *N. ceranae* (100%)
MS (29)	0		29	3.4%; *N. ceranae* (33%); *N. apis* (67%)
TX (141)	101	12%; *N. ceranae* (83%); *N. apis* (17%)	40	22%; *N. ceranae* (78%); *N. apis* (12%)
UT (119)	67	9%; *N. ceranae* (100%)	52	8%; *N. ceranae* (100%)
NM (53)	35	3%; *N. ceranae* (100%)	18	0%
OK (130)	41	7%; *Nosema* sp. (100%)	89	3%; *N. ceranae* (67%); *N. apis* (33%)
All	245	9%; *N. ceranae (89%); N. apis* (11%)	272	8%; *N. ceranae* (75%); *N. apis* (25%)

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Prevalence of *Nosema* from AHB was highest in Texas (12%) and lowest in New Mexico (3%), although there was no significant difference (P = 0.183). From the EHB, results were similar to the AHB, with *Nosema* most common in Texas (22%) and lowest in New Mexico (0% infection) (P = 0.003). There was a significant difference in overall *Nosema* infection for feral honey bees in Oklahoma compared to Texas (P = 0.007), and between Texas and New Mexico (P = 0.010), but not between Oklahoma and Utah (P = 0.302), and Texas and Utah (P =0.126).

This is the first study to document the occurrence of both *N. ceranae* and *N. apis* from feral EHB and AHB in the United States. The only previous study which observed *N. apis* from feral honey bees in the United States was limited in scope and only found 1 colony out of 7 in Arizona infected with *N. apis* (Gilliam & Tabler 1991). We also found evidence that *N. ceranae* has been present in AHB colonies in Texas since 2005. A recent study by Teixeira et al. (2013) documented the existence of *N. ceranae* in a sample of AHB drones from Brazil that were collected 34 yr ago. This suggests that *N. ceranae* may have followed AHB into the United States as AHB expanded northward. We did not detect *Nosema* from the 1991 and 2004 Texas samples, but the small sample sizes (17 and 10, respectively) may be the reason for not detecting it. It may also be that, as Teixeira et al. (2013) suggest, microscopy-based detection of *Nosema* prior to the molecular age did not distinguish species as well as modern methods.

Several statewide *Nosema* surveys have been conducted on managed honey bee colonies in the United States. Traver & Fell (2011) studied 586 managed *A. mellifera* colonies from Virginia in 2009 using real time PCR to detect *Nosema*. They found 37% of the colonies to have *Nosema* with 97.3% infected with *N. ceranae* and 2.7% with *N. apis*. Szalanski et al. (2013) found 31% of 1,200 apiaries in New York and 44% of 1,009 apiaries in South Dakota to have *Nosema* based on spore counts. Of the samples subjected to molecular diagnostics they found 96% of the New York spore positive samples to have *N. ceranae*, 3% co-infected with *N. ceranae* and *N. apis*, and 1% with *N. apis*. All of their South Dakota samples subjected to molecular diagnostics were positive for *N. ceranae*. In the present study, the percentage of feral honey bees infected with *Nosema* (8.3%) was considerably lower than the levels observed in managed honey bees (31-44%). 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).

Among all 6 sampled states, *Nosema* sp. occurrence was lowest in the Spring and highest in the Winter (Table 3). Studies in Germany (Gisder et al. 2010) and in Virginia (Traver et al. 2012) have revealed that *Nosema* infection levels were highest in the Spring and lower in the Fall and Winter. It is difficult to draw conclusions from our dataset on the seasonality of *Nosema* prevalence given the broad geographical distribution of our samples, uneven sampling throughout the years, and the low incidence of *Nosema*-positive samples. Robust, regular sampling throughout the region, as conducted by Gisder et al. (2010) in Germany, would be required to determine if there are seasonal differences in *Nosema* infection rates that are driven by climate.

We found that *Nosema* was widespread throughout the southwestern and south central United States. *Nosema ceranae* was present in all sampled states, yet *N. apis* was only present in Mississippi, Texas, and Oklahoma. Despite *N. ceranae* being first detected in the United States as recently as 1995 (Chen et al. 2008), *N. ceranae* seems more common in both managed and feral populations of honey bees than *N. apis*, although increased sample sizes are needed to determine if this is truly the case. Our study is the first to report that *N. ceranae* occurs in feral AHB populations within the United States. Although our work did not show a difference in *Nosema* species occurrence between AHB and EHB populations, most (98%) of our samples were collected after the known occurrence of *N. ceranae* in the United States. If samples collected in the past become available, the molecular identification techniques employed in this study should prove useful in further investigations of the invasion history of *Nosema* and AHB into the United States.

TABLE 3. SEASONAL OCCURANCE OF *NOSEMA* IN AFRICANIZED HONEY BEE (AHB) AND EUROPEAN HONEY BEE (EHB) COLONIES FROM SIX STATES.

Month	N	*Nosema* +	*Nosema* –	% *Nosema* +
Jan	13	2	11	15.4%
Feb	15	0	15	0.0%
Mar	44	1	43	2.3%
Apr	37	3	34	8.1%
May	88	4	84	4.5%
Jun	119	4	115	3.4%
Jul	62	8	54	12.9%
Aug	77	9	68	11.7%
Sep	73	5	68	6.8%
Oct	83	10	73	12.0%
Nov	19	2	17	10.5%
Dec	21	3	15	14.3%

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