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SHORT NOTE

# Assessing the Utility of a PCR Diagnostics Marker for the Identification of Africanized Honey Bee, *Apis mellifera* L., (Hymenoptera: Apidae) in the United States

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**Abstract**
An assessment of a molecular diagnostic technique for distinguishing Africanized honey bees from European honey bees in the United States was conducted. Results from multiplex PCR diagnostics of a mitochondrial DNA cyt-b marker corresponded with results based on COI-COII sequencing analysis, but differed from morphometric analysis results. We suggest utilizing both multiplex PCR and morphometric methods for Africanized honey bee diagnostics in the United States, when possible.

The Africanized honey bee was first detected in Texas in 1990 (Sugden & Williams, 1990), and USDA reports it has been established in ten states: Arizona, Arkansas, California Florida, Louisiana, Nevada, New Mexico, Oklahoma, Texas and Utah (Anonymous, 2011) Following a tragic death in 2010, Africanized honey bees were confirmed in Georgia (Berry, 2011). Hybrids between Africanized and European honey bees are morphologically similar and difficult to distinguish from one another. Two kinds of laboratory-based techniques, morphometric analysis (Rinderer et al., 1993) and molecular diagnostics (Sheppard & Smith, 2000), are commonly employed to determine Africanized status, but to our knowledge, these have not been compared.

The typical morphometric approach to Africanized honey bee detection is the Fast Africanized Bee Identification System (FABIS) method (Rinderer et al., 1987), although more precise methods such as Automatic Bee Identification System (ABIS) (Steinhage et al. 2001) are now available. Morphometric diagnostic techniques require measurements from 10 or more freshly collected specimens (Meixner et al., 2013) to assign a colony to one of four categories: 1) Africanized (AHB), 2) AHB with European (EHB) traits, 3) EHB with AHB traits and 4) EHB (Sylvester & Rinderer 1987). Molecular diagnostics rely upon mitochondrial DNA (mtDNA), typically a region of the cytochrome b gene (Pinto et al. 2003). Identification of Africanized bees with mtDNA is relatively easy, because a single worker in any condition can represent a colony's mtDNA lineage (Sheppard & Smith, 2000). Yet, since mtDNA is maternally inherited, mtDNA markers cannot determine if a European queen has mated with Africanized drones, and such colonies will remain undetected with mtDNA-based techniques. Molecular identification of Africanized bees is also conducted with an mtDNA cytochrome b marker (cyt-b) as it has a low level of intraspecific variation in honey bees (Crozier et al., 1991; Szalanski & McKern, 2007). Techniques typically used are either PCR-RFLP (Pinto et al., 2003) or multiplex PCR using a primer specific for Africanized honey bees (Szalanski & McKern, 2007). Both of these methods yield identical results. Despite the utility of molecular markers, it is unknown how well they correlate with morphometric identification and how the cyt-b marker can distinguish the A (African) lineage of honey bees relative to the O (Middle Eastern), C (Eastern Europe) and M (Western Europe) lineages (Ruttner, 1987), which are often determined through COI-COII sequencing. The objective of this study was to determine how a cyt-b multiplex technique

for distinguishing Africanized from European bees compares with COI-COII DNA sequence and FABIS morphometric techniques.

A total of 968 samples from swarms, feral colonies and managed honey bee colonies collected from Arizona, Arkansas, California, Florida, Georgia, Hawaii, Kansas, Louisiana, Missouri, Mississippi, Nebraska, New Mexico, Oklahoma, Texas and Utah during 1991-2013 were analyzed. Samples were collected by various agencies and preserved in 70-100% ethanol. Samples collected from Texas from 1991 to 2008 were identified as Africanized (AHB, n = 54), AHB with evidence of introgression of European genes (AHB.E, n = 23), EHB with evidence of Africanized genes (EHB.A, n = 22) or EHB (EHB, n = 39) using FABIS following Rinderer et al. (1993).

For the multiplex PCR diagnostics, DNA was extracted from two workers from each sampled colony, and PCR of a portion of the cyt-b region was conducted per Szalanski and McKern (2007). This multiplex results in a control amplicon of 485 bp for both Africanized and European bees and a 385 bp amplicon unique to Africanized bees. Samples exhibiting a single electrophoretic band are diagnosed as European (multiplex EHB) and those with two are diagnosed as Africanized (multiplex AHB). DNA sequencing analysis of a COI-COII marker was conducted following Szalanski and Magnus (2010), and samples from that study were additionally subjected to our multiplex procedure for comparison (n = 360). DNA sequences were identified to haplotype and assigned to lineage using GenBank BLAST searches and our own database. Africanization diagnoses from cyt-b multiplex diagnostics, COI-COII DNA sequencing and FABIS morphometric analysis were then compared with one another.

**Table 1.** Sampled states and results of multiplex PCR identification of samples as Africanized or European honey bees.

| State | N | Multiplex AHB | Multiplex EHB |
|---|---|---|---|
| Arizona | 1 | 0 | 1 |
| Arkansas | 143 | 1 | 142 |
| California | 3 | 3 | 0 |
| Florida | 1 | 1 | 0 |
| Georgia | 2 | 1 | 1 |
| Hawaii | 124 | 1 | 123 |
| Kansas | 18 | 0 | 18 |
| Louisiana | 24 | 0 | 24 |
| Mississippi | 20 | 0 | 20 |
| Missouri | 25 | 0 | 25 |
| Nebraska | 19 | 0 | 19 |
| New Mexico | 62 | 45 | 17 |
| Oklahoma | 178 | 58 | 120 |
| Texas | 140 | 109 | 31 |
| Utah | 208 | 99 | 109 |
| Total | 968 | 318 | 650 |

Of the 968 samples subjected to multiplex diagnostics, 318 samples were diagnosed as Africanized and 650 were diagnosed as European (Table S1). A total of 12 haplotypes from the A (African) lineage were observed, and four O (Middle Eastern), five M (Western Europe) and 11 C (Eastern Europe) haplotypes were compared with the multiplex results (Table 2). All of the multiplex PCR identified Africanized samples fell within the A lineage while the European samples were O, M or C lineages. The multiplex method reaches exactly the same diagnoses as the sequencing method but can be carried out in a single PCR step without subsequent DNA sequencing.

Africanization diagnoses in the multiplex and FABIS methods differed quite dramatically (Table 3). Of the 107 samples the exhibiting Africanized signature in the PCR-multiplex, only 84 were diagnosed as of African descent (AHB, AHB.E and EHB.A) using FABIS. This suggests that 21% of Africanized bees in our sample were cryptically Africanized and undetectable using morphology-based methods. Similarly, of the 99 bees exhibiting morphometric characteristics of Africanization (AHB, AHB.E and EHB.A), 15% were misdiagnosed as European by the multiplex. Interestingly, our results suggest that only a small proportion (15%) of colonies that exhibit Africanized morphology were founded by European queens inseminated by Africanized drones.

**Table 2.** Correlation of mitochondrial DNA COI-COII DNA sequence lineages with cyt-b multiplex PCR identification.

| Lineage (n haplotypes) | N | Multiplex AHB | Multiplex EHB |
|---|---|---|---|
| C (11) | 173 | 0 | 173 |
| M (5) | 12 | 0 | 12 |
| O (4) | 19 | 0 | 19 |
| A (12) | 156 | 156 | 0 |
| Total | 360 | 156 | 204 |

COI-COII lineage data includes haplotype determinations from Szalanski and Magnus (2010).

This study verified the utility of using a cyt-b PCR-multiplex technique for identifying honey bees of the A lineage in the United States. Identification of Africanized matrilines through cytb-b diagnosis parallels classic COI-COII matriline determination through sequencing, but in a single-step, lesser cost procedure. We also found evidence that using either FABIS or mtDNA determination alone may underestimate the occurrence of Africanized populations. For greater certainty in diagnosing Africanized populations of honey bees, we suggest utilizing both morphological and molecular methods when the number of samples makes this possible.

**Table 3** Comparison of morphometric and multiplex PCR identification of Africanized and European honey bees from Texas.

| Morphometric Identification* | N | Multiplex AHB (%) | Multiplex EHB (%) |
|---|---|---|---|
| AHB | 54 | 48 (89) | 6 (11) |
| AHB.E | 23 | 19 (83) | 4 (17) |
| EHB.A | 22 | 17 (77) | 5 (23) |
| EHB | 39 | 23 (59) | 16 (41) |
| Total | 138 | 107 | 31 |

*AHB: Africanized; AHB.E: Africanized with evidence of introgression of European genes; EHB.A: European with evidence of introgression of Africanized genes; EHB: European (Rinderer et al., 1993).

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