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# A qPCR assay for sensitive and rapid detection of African A-lineage honey bees (*Apis mellifera*)

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**Abstract** – A real-time qPCR assay was designed to detect African-derived subspecies of western honey bees (*Apis mellifera* L.). The probes targeted the same region of the mitochondrial *cytochrome b* gene used in previous restriction enzyme assays. Using samples from 16 *A. mellifera* subspecies representing four lineages, we evaluated the efficacy of this assay to identify African- (or A-lineage) and non-A-lineage honey bees. The qPCR assay successfully differentiated A-lineage honey bees, including *A. m. scutellata* and *A. m. capensis*, from M-, C-, and O-lineage *A. mellifera* subspecies. In conclusion, the assay we developed is useful for rapid detection of A-lineage honey bees in areas where they are introduced, although it cannot be used to identify the specific *A. mellifera* subspecies within the A-lineage.

**African-derived honey bee / A-lineage / Biomarker / cytochrome b / *Apis mellifera scutellata* / BglII**

## 1. INTRODUCTION

The genus *Apis* L. (family: Apidae) consists of nine species, eight of which are native to Asia. *Apis mellifera* L., the western honey bee, is native to Africa, Europe, the Middle East, and Asia. *Apis mellifera* consists of several distinct subspecies (Ruttner 1988) that are grouped into lineages (Franck et al. 2001; Alburaki et al. 2013), with M-, C-, O-lineages represented in Europe, the Middle East, and Asia; Y-lineage in Africa; and A-lineage in Africa, Sicily, Malta, and the south-western part of the Iberian peninsula (De la Rúa et al. 2009). Historically, it was thought that *A. mellifera* split from *A. cerana* Fabricius when it expanded west, colonizing western Asia,

Europe, and Africa (Sheppard and Meixner 2003). More recent evidence suggests African or Middle Eastern origins for *A. mellifera* (Whitfield et al. 2006; Cridland et al. 2017; Tihelka et al. 2020). Based on current literature, the origin of *A. mellifera* remains unresolved, with different analyses and datasets resulting in differing origins (e.g., Han et al. 2012; Wallberg et al. 2014).

In 1956, an African subspecies of honey bee, *A. m. scutellata* Lepeletier, was introduced into Brazil where, after an accidental release in 1957, it hybridized with European-derived subspecies of honey bees managed by beekeepers there (Nogueira-Neto 1964; Kerr 1967). Since then, it spread throughout much of South America, Central America, and into parts of southern North America. These African-derived bees, often called “African,” “Africanized,” or “killer” honey bees, exhibit heightened defensive behavior, making

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them a public nuisance and safety hazard in areas they occur. Consequently, regulatory authorities in some countries monitor the spread of this bee to reduce its impact on the public and beekeeping industry.

Historically, the African honey bees of the Americas were distinguished from European-derived honey bee stocks using classical morphometrics (Rinderer et al. 1986). This led to the development of two identification methods used in the USA. The Fast Africanized Bee Identification System (FABIS) was developed by the US Department of Agriculture (USDA) to facilitate quick identification of African honey bees using wing length (Sylvester and Rinderer 1987; Rinderer 1998). However, identification using FABIS is less precise than that made using the second method developed by the USDA, USDA-ID (Rinderer et al. 1986, 1993; Meixner et al. 2013). Identification by FABIS is not always accurate and the USDA-ID system is laborious to perform. Consequently, there has been a desire for an additional tool that can be used to identify the bees quickly and accurately, hence the move toward molecular diagnostics.

Polymerase chain reaction (PCR) with species-specific primers is one of the approaches for species and subspecies identification because of its low cost, rapid turnaround, and high specificity and sensitivity (Meixner et al. 2013). Original PCR assays used to identify African bees were based on amplifying LS rRNA and *cytochrome oxidase I* and *II* (*COI*, *COII*) subunit genes, and restriction enzyme digestion with *Eco* RI, *Hinc* II, or *Xba* I (Hall and Smith 1991). Later restriction enzyme digestion of the *COI-COII* intergenic region with *Dra* I was also used to characterize *A. mellifera* mitochondrial haplotypes (Garnery et al. 1993; and see Sheppard and Smith 2000; Meixner et al. 2013 for detailed reviews of mitochondrial DNA identification methods).

Crozier et al. (1991) developed a faster test for identifying African-derived honey bees in the Americas. This improved assay amplified a region of the *cytochrome b* (*Cytb*) gene and cut the PCR product with *Bgl* II. The assay was further validated by Pinto et al. (2003) who increased the number of sample colonies. They confirmed that this *Bgl* II polymorphism can discriminate

*A. m. scutellata* (A-lineage) from *A. m. carnica* Pollmann (C-lineage), *A. m. caucasica* Pollmann (O-lineage), *A. m. cyprica* Pollmann (O-lineage), *A. m. lamarckii* Cockerell (A-lineage), *A. m. ligustica* Spinola (C-lineage), *A. m. mellifera* Linnaeus (M-lineage), *A. m. syriaca* Skorikov (O-lineage), and some geographical regions of *A. m. iberiensis* Engel (M-lineage). However, *A. m. iberiensis* from other regions, as well as *A. m. intermissa* Maa (A-lineage), could not be discriminated as they share the same mitotypes (see Chávez-Galarza et al. (2015) for a detailed overview of *A. m. iberiensis* genetic variation in the Iberian contact zone). This *Cytb/Bgl* II assay has subsequently been used as part of haplotype analyses to identify bees of the A-lineage, often in conjunction with other genes/enzymes: LS rRNA/*Eco* RI, *COI-COII/Xba* I, *COI/Hinc* II (Smith et al. 1997; Palmer et al. 2000; Pinto et al. 2004; Zamora et al. 2008; and see Ferreira, 2009). The *Cytb/Bgl* II assay has also been combined with multiplexed PCR to improve identification of African-derived bees from Arkansas, Oklahoma, and New Mexico (Szalanski and McKern 2007). Most recently, the *Cytb/Bgl* II assay has been used to investigate the distribution of African-derived bees in California (Kono and Kohn 2015; Lin et al. 2018). Therefore, the mitochondrial *Cytb* sequence is a useful target for biomarker tests that differentiate African-derived bees from European-derived honey bees in the Americas.

Building from the earlier restriction enzyme assays, real-time PCR using hydrolysis probes (e.g., Taqman probes, Holland et al. 1991) offer fast and reliable mutation detection. These probes have both a fluorescent dye and a quencher, which are close enough to one another to suppress fluorescence. When the probe sequence matches the DNA target and binds during the extension phase of PCR cycle, the DNA polymerase degrades the probe, resulting in fluorescent emission. This emission can be detected and accumulates with each cycle.

The aim of this study was to design a qPCR assay for the region targeted by the *Cytb/Bgl* II assay that enables detection and differentiation of between African- (A-lineage) and non-A-lineage honey bees. We developed an assay that can be

performed within only a few hours and is straightforward enough that it only requires basic molecular skills. Additionally, the assay is amenable for high-throughput screening, and numerous individuals from an apiary can be screened simultaneously, allowing for more informative results.

## 2. MATERIAL AND METHODS

### 2.1. Assay design

The mitochondrial *Cytb* sequences from eight *A. mellifera* subspecies were downloaded from GenBank (Pinto et al. 2007, Table I) and aligned using MUSCLE (Edgar 2004) as implemented in MEGA version 7 (Kumar et al. 2016). The section of *Cytb* that encompasses the *Bgl*II restriction cut site used in earlier studies was targeted (Crozier et al. 1991; Pinto et al. 2003). Two primer-probe sets were designed—one set specific for African-derived honey bees (A-lineage), and another for non-A-lineage honey bees (Table II). Both primer sets were designed to work under the same thermocycling conditions, and we used PrimeTime® qPCR 5' nuclease probes with 6-FAM fluorescent dye, ZEN as internal quencher and Iowa Black FQ as the 3' quencher (Integrated DNA Technologies Inc., Coralville, IA, USA). The A-lineage primer/probe set was designed to be specific to *A. m. scutellata*, *A. m. intermissa*, and some *A. m. iberiensis* (see introduction), while the non-A-lineage primer/probe set was designed to be specific to non-African-derived honey bees.

Both primer sets targeted the same region of *Cytb*. Using the position numbers that correspond to Crozier and Crozier (1993), and also used in Pinto et al. (2007), the A-lineage primers amplified a 169 bp product from position 219 to 387. The A-lineage probe aligned with position 285 to 319 and incorporated the T/C polymorphism targeted by *Bgl*II cutting methods (position 289, see Crozier et al. 1991). The non-A-lineage primers amplified the region from 220 to 387 resulting in a 168 bp product. The non-A-lineage probe aligned to the same positions as the A-lineage probe but targeted a T/C polymorphism at position 307 (see Pinto et al. 2007).

### 2.2. Sample preparation

Worker honey bee samples from 16 different *A. mellifera* subspecies were used. Fourteen samples, representing four lineages, were obtained from the Ruttner Bee Collection at the Bee Research Institute at Oberursel, Germany (Table III). The identity of these bees was confirmed using morphometric methods by Bee Research Institute staff. In addition, *A. m. capensis* Eschscholtz and *A. m. scutellata* samples from South Africa were included. Three honey bees were sampled from each of three locations per subspecies: Cape Town, Mooresburg, and Riversdale (*A. m. capensis*), and Springbok, Upington, and Vryburg (*A. m. scutellata*) (total  $n = 9$  for each subspecies). These samples come from apiaries that were confirmed as either *A. m. capensis* or *A. m. scutellata* based on morphometrics, microsatellites, SNPs, and mitochondrial genomes (Eimanifar et al. 2018a, b, 2020; Bustamante et al. 2020). Lastly, three immature honey bees from Florida (USA) were used to obtain fresh, high-quality DNA for assay validation and sensitivity analyses. These samples were only classified as European-derived *A. mellifera* and were not identified to subspecies.

Although sometimes contradictory to morphological lineages, we used mitochondrial lineages to classify the subspecies (Meixner et al. 2013) following recent nomenclature in Nawrocka et al. (2018). From the A-lineage, we had samples of *A. m. adansonii* Latreille, *A. m. capensis*, *A. m. lamarckii*, *A. m. litorea* Smith, *A. m. monticola* Smith, *A. m. ruttneri* Sheppard et al., *A. m. sahariensis* Baldensperger, and *A. m. scutellata*. In M-lineage, we had *A. m. iberiensis* and *A. m. mellifera*. The C-lineage samples were *A. m. carnica* and *A. m. ligustica*; and O-lineage samples were *A. m. anatoliaca* Maa, *A. m. meda* Skorikov, and *A. m. syriaca* (Table III). We also have a sample of *A. m. simensis* from the Y-lineage (Franck et al. 2001; Meixner et al. 2011).

Ethanol preserved bee tissue was equilibrated in 750  $\mu$ L  $1 \times$  PBS before being used for DNA extraction. The bee tissue was then homogenized in a Mini-Beadbeater-16 (Biospec Products; cat. #607), and extraction was performed using DNeasy Blood and Tissue Kits (Qiagen; cat.

**Table I.** List of the accession numbers used for developing primers and probes. All sequences are from Pinto et al. (2007)

Accession no.	Subspecies	Common name
EF184025-9	<i>Apis mellifera scutellata</i>	African savannah honey bee
EF184055-9	<i>Apis mellifera mellifera</i>	Dark European honey bee
EF184032-7	<i>Apis mellifera iberiensis</i>	Iberian honey bee
EF184020-4	<i>Apis mellifera intermissa</i>	Algerian honey bee
EF184041-4	<i>Apis mellifera ligustica</i>	Italian honey bee
EF184046-8	<i>Apis mellifera carnica</i>	Carniolan honey bee
EF184053-4	<i>Apis mellifera cypria</i>	Cyprian honey bee
EF184062-4	<i>Apis mellifera lamarckii</i>	Egyptian honey bee

#69506) per the manufacturer's recommended protocol for insects. DNA extractions were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific; cat. #ND2000).

### 2.3. Quantitative PCR

Quantified DNA was used in the qPCR assay, and each sample was assayed using both primer sets. Samples were assayed blind, with the molecular results later matched to the subspecies identity determined by Institute staff.

The qPCR reactions contained 5 µL Premix EX Taq PCR Master mix (Fisher Scientific, Waltham, MA), 1 µL (at least 100 ng) of DNA template, 0.5 µL (100 µM) of each primer, and 0.1 µL (100 µM) of probe. A negative control (Hyclone, Hypure Molecular Biology Grade Water) was also included to detect the presence of contaminating DNA. The real-time PCR assay was performed in a Smart Cycler (Cepheid, Sunnyvale, CA) as a tube-based reaction. The reaction was

conducted for up to 40 cycles using the following conditions: initial melting for 120 s at 95 °C followed by cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The default analysis setting of the Cepheid Smart Cycler Software was used to calculate cycle threshold (Ct) values of the assays.

The qPCR assay was originally designed and implemented on the Cepheid SmartCycler platform. As the tubes designed for use on this platform are no longer available, the qPCR assay was also evaluated on the Applied Biosystems QuantStudio 5 platform. PCRs were set up as 20 µL reactions with the following constituents: 2 µL of genomic DNA extract, 10 µL SensiFAST Probe Lo-ROX Mix (Bioline – Meridian Bioscience), 1 µL forward primer (100 µM), 1 µL reverse primer (100 µM) of each primer, 0.2 µL of probe (100 µM), and 5.8 µL water. Thermocycle conditions on the QuantStudio 5 were as follows: (1) initial denaturing at 95 °C for 2 min, and (2) 40 cycles of 95 °C for 5 s with 62 °C extension time of 30 s. ROX was set as the

**Table II.** List of primers and probes for the qPCR assay. The bold, underlined base pairs show where the A-lineage and non-A-lineage primer/probe sequences differ

	A-lineage primer/probe set	Non-A-lineage primer/probe set
Forward (5'-3')	<u>C</u> ATCT <u>A</u> AATCCTCTTGGATCAA <u>A</u> TT	ATCC <u>A</u> AATCCTCTTGGATCAA <u>A</u> CTT
Reverse (5'-3')	GGATTTGCAATTTTAA <u>A</u> TTA <u>T</u> TCTGG	GGATTTGCAATTTTAA <u>A</u> ATT <u>G</u> TCTGG
Probe (6-FAM-ZEN-Iowa Black FQ)	AAGAC <u>C</u> TTT <u>T</u> TAGGATTTTA TAT <u>T</u> ATCTTATTTATC	AAGAT <u>T</u> CTTT <u>T</u> TAGGATTTTA TAT <u>C</u> ATCTTATTTATC

**Table III.** List of subspecies samples used to test the assay. The samples were obtained from the Ruttner Bee Collection at the Bee Research Institute in Oberursel, Germany (numerical sample ID used by Institute staff) or collected from South Africa or Florida, USA (two letter sample ID). For Ruttner Collection samples, co-ordinates were obtained from location data, not using a GPS device

Sample ID	<i>Apis mellifera</i> subspecies	Lineage <sup>a</sup>	Country	Location	Latitude	Longitude	Collector	Year
1277	<i>A. m. adansonii</i>	A	Central African Republic	Landjia (nr Bangui)	4° 23' N	18° 37' E	Kate de Bold	1984
CT	<i>A. m. capensis</i>	A	South Africa	Cape Town	33° 57' 36" S	18° 27' 00" E	T. Bustamante, M. Dykes, Dr. A. Mortensen, Dr. D. Schmehl	2014
MB	<i>A. m. capensis</i>	A	South Africa	Moorreesburg	33° 06' 00" S	18° 44' 24" E	T. Bustamante, M. Dykes, Dr. A. Mortensen, Dr. D. Schmehl	2014
RD	<i>A. m. capensis</i>	A	South Africa	Riversdale	34° 06' 00" S	21° 12' 00" E	T. Bustamante, M. Dykes, Dr. A. Mortensen, Dr. D. Schmehl	2014
2271	<i>A. m. lamarckii</i>	A	Egypt	Cairo	30° 03' N	31° 14' E	Prof. Schrickler	1997
1072	<i>A. m. litorea</i>	A	South Africa	Sodwana Bay	27° 33' S	32° 40' E	Prof. W. Haas	1983
1264	<i>A. m. monticola</i>	A	Kenya	Mt. Kenya	0° 09' S	37° 18' E	Prof. T. Rinderer	1984
2052	<i>A. m. ruthneri</i>	A	Malta	St Julian's	35° 55' N	14° 29' E	Dr. W. S. Sheppard	1995
29	<i>A. m. sahariensis</i>	A	Morocco	Sidi Yahya El Gharb	34° 18' N	6° 18' W	Brother Adam	1962
3596	<i>A. m. sahariensis</i>	A	Morocco	Erfoud area	31° 26' N	4° 14' W	Zairi Khalid	2010
SP	<i>A. m. scutellata</i>	A	South Africa	Springbok	29° 39' 00" S	17° 49' 48" E	T. Bustamante, M. Dykes, Dr. A. Mortensen, Dr. D. Schmehl	2014
UP	<i>A. m. scutellata</i>	A	South Africa	Upington	28° 31' 12" S	21° 12' 36" E	T. Bustamante, M. Dykes, Dr. A. Mortensen, Dr. D. Schmehl	2014
VR	<i>A. m. scutellata</i>	A	South Africa	Vryburg	26° 57' 36" S	24° 45' 36" E	T. Bustamante, M. Dykes, Dr. A. Mortensen, Dr. D. Schmehl	2014
2721	<i>A. m. simensis</i>	Y	Ethiopia	Gimbi	9° 11' N	35° 50' E	Messele Abebe Leta	1998
1808	<i>A. m. carnica</i>	C	Croatia	Zagreb	45° 48' N	15° 57' E	N. Kesic	1992
1715	<i>A. m. ligustica</i>	C	Italy	Reggio Emilia	44° 41' N	10° 37' E	Dr. M. Meixner	1990
1952	<i>A. m. iberiensis</i>	M	Spain	Serra	39° 41' N	0° 25' W	Dr. W. S. Sheppard	1991
1410	<i>A. m. mellifera</i>	M	Norway	Flekkenfjord	58° 17' N	6° 39' E	Prof. F. Ruttner	1987
3361	<i>A. m. anatoliaca</i>	O	Turkey	Çalioba	40° 10' N	27° 43' E	I. Çakmak	2002
3485	<i>A. m. meda</i>	O	Turkmenistan	Zakhmet	37° 36' N	62° 21' E	Mohammad Reza Pourelmi	2006



**Table III** (continued)

Sample ID	<i>Apis mellifera</i> subspecies	Lineage <sup>a</sup>	Country	Location	Latitude	Longitude	Collector	Year
1729	<i>A. m. syriaca</i>	O	Syria	Damascus	33° 31' N	36° 18' E	Dr.S.Fuchs	1990
QB	<i>A. mellifera</i> <sup>b</sup>		USA	Florida (Hillsborough Co.)	28° 09' 33" N	82° 23' 03" W	Rebecah Dineen (FDACS)	2020

<sup>a</sup> See, e.g., Nawrocka et al. (2018)

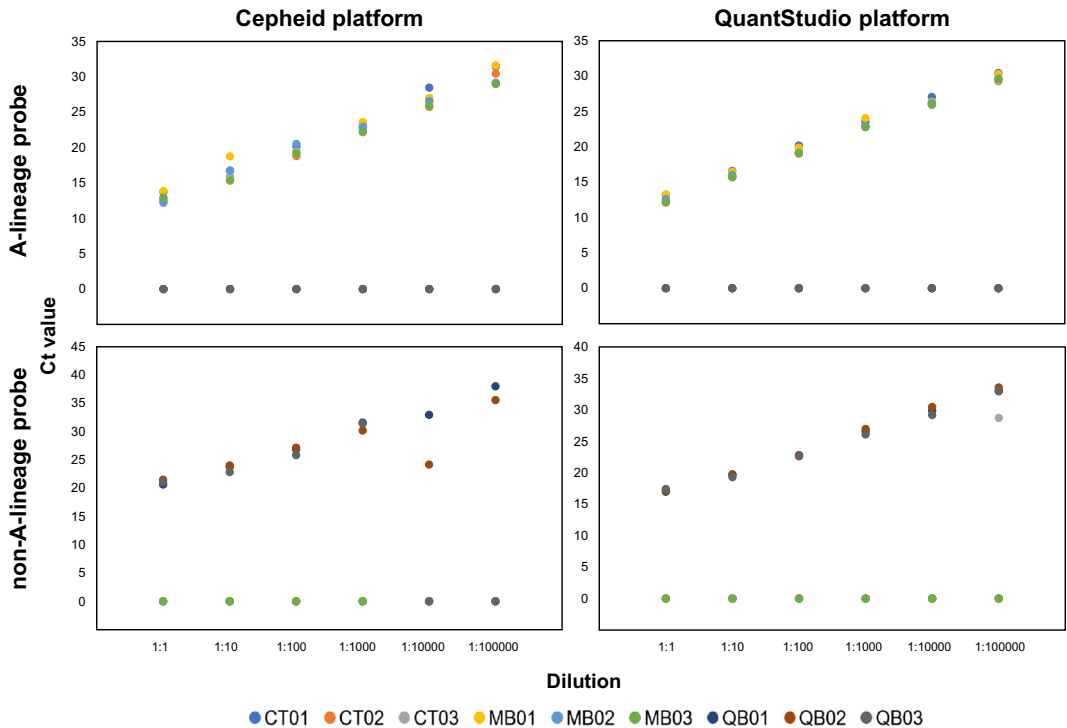
<sup>b</sup> This sample was not identified to subspecies. It was used to validate the assay and compare qPCR platforms (see Figure 1)

passive reference. The ramp rate was 2 °C/s. The entire thermocycle took approximately 45 min to complete. FAM Ct values were calculated by the QuantStudio Design and Analysis Software v.1.5.0 with default settings (automatic threshold set at 0.2; automatic baseline with start cycle at 3, end cycle 15).

Sensitivity testing for A-lineage and non-A-lineage probes were conducted on both the Cepheid and QuantStudio platforms (Figure 1). Samples from two *A. m. capensis* locations (CT and MB) were used as positive controls for A-lineage, and European-derived *A. mellifera* from Florida was used as positive control for the non-A-lineage probe. qPCRs were conducted with a series of sample dilutions using decreasing DNA concentrations with the same primer and probe concentration. The DNA concentrations used were in 10-fold dilutions (i.e., 1:10, 1:100, 1:1000, 1:10,000 and 1:100,000).

## 2.4. Sanger sequencing: *Cytb* gene

Sanger sequencing of a fragment of the *cytochrome b* gene was run on the same samples, to confirm the qPCR results. PCRs were set up as 25 µL reactions using Kapa HiFi HotStart ReadyMix kits, including the primers F1: 5'-CATA TTGAGGTGCAACAGT-3' and R2: 5'-ATCA TTCTGGTTTAATGTGAG-3'. Reactions were 120 s at 95 °C, followed by 32 cycles of 30 s at 98 °C, 30 s at 50 °C and 40 s at 72 °C, and 7 min at 72 °C. PCR products of ~ 420bp were stained and visualized on a 1.5% agarose gel. Positive PCRs were purified with Qiagen QIAquick PCR Purification Kits. Purified PCR products were then prepared for sequencing with the Applied Biosystems BigDye Terminator v.3.1 Cycle Sequencing Kit. Bidirectional sequencing was performed on Applied Biosystems 3130 Genetic Analyzer and SeqStudio platforms at Florida Department of Agriculture and Consumer Services, Division of Plant Industry (FDACS-DPI, Gainesville, FL). Sequences were trimmed, assembled, and aligned in Geneious Prime 2019.0.4 (Kearse et al. 2012). All newly generated sequence data from this study were deposited in GenBank (accession numbers: MT572346-MT572369, MW530560-MW530571, Table IV). Sequencing data were compared to Popset 126513039 (Pinto et al. 2007).



**Figure 1.** Sensitivity analysis for A-lineage probe (top), and non-A-lineage probe (bottom) on both the Cepheid (left) and QuantStudio (right) platforms. Two *Apis mellifera capensis* (CT, and MB) were used as positive controls for A-lineage, and European-derived *A. mellifera* (Florida, QB) was used as non-A-lineage probe positive control. Tenfold dilutions were made: 1:10, 1:100, 1:1000, 1:10,000, and 1:100,000. Not all data with a Ct value of 0 are visible.

### 3. RESULTS

The non-A-lineage probe was positive for *A. m. anatoliaca*, *A. m. carnica*, *A. m. iberiensis*, *A. m. lamarckii*, *A. m. ligustica*, *A. m. meda*, *A. m. mellifera*, and *A. m. syriaca* (Table IV). The A-lineage probe was positive for *A. m. adansonii*, *A. m. capensis*, *A. m. litorea*, *A. m. monticola*, *A. m. ruttneri*, *A. m. scutellata*, and *A. m. simensis*. One of the *A. m. sahariensis* samples was positive for A-lineage, and another was positive for non-A-lineage.

Sequencing of *Cytb* confirmed the qPCR results and facilitated a prediction of *Bgl*II mitotypes of previously unstudied subspecies (Table IV, Figure 2). The only exception to this was Y-lineage *A. m. simensis* that was positive for the A-lineage probe but has a *Bgl*II restriction site that matches that of non-A-lineage species. This

likely occurred as a result of the A-lineage probe binding to the sequence despite a 1 bp mismatch (other non-A-lineage honey bees have 2 bp mismatch, see Figure 2).

### 4. DISCUSSION

PCR-based mitochondrial assays have been used to identify African honey bee populations (Hall and Smith 1991; Lobo 1995; Quezada-Euan and Hinsull 1995; Clarke et al. 2001; Kono and Kohn 2015) and are being used along with morphometric analysis for African honey bee identification in California, USA (Nielsen et al. 1999, 2000). In developing the qPCR assay, we focused on accuracy, speed, and reliability of the identification method. Many laboratories providing African honey bee identification services rely primarily on FABIS and USDA-ID morphology-



**Table IV.** Results of the qPCR assays (run on Cepheid platform), Sanger sequences, and comparisons to known *Bgl*II mitotypes. The grey shading indicates a discrepancy between qPCR result, and prediction from *Cytb* sequence—see text for discussion. Samples are ordered to match Figure 2

Sample ID	<i>Apis mellifera</i> subspecies	Accession number	Lineage <sup>a</sup>	<i>Bgl</i> II mitotypes (literature) <sup>b</sup>	[DNA] (ng/ul)	A-lineage probe (Ct value)	Non-A-lineage probe (Ct value)	<i>Bgl</i> II recognition site from <i>Cytb</i> sequence	<i>Bgl</i> II mitotypes (predicted)
2271	<i>A. m. lamarckii</i>	MT572347	A	Two bands	3	-	26.35	AGATCT	Two bands
1729	<i>A. m. syriaca</i>	MT572346	O	Two bands	3.1	-	21.24	AGATCT	Two bands
1952	<i>A. m. iberiensis</i>	MT572349	M	One or two bands <sup>c</sup>	6.6	-	28.87	AGATCT	Two bands
1410	<i>A. m. mellifera</i>	MT572348	M	Two bands	2.7	-	21.45	AGATCT	Two bands
1808	<i>A. m. carnica</i>	MT572351	C	Two bands	2.4	-	29.47	AGATCT	Two bands
QB.01	<i>A. mellifera</i> (Florida)	MW530569			224.5	-	20.64	AGATCT	Two bands
QB.02	<i>A. mellifera</i> (Florida)	MW530570			290.9	-	21.48	AGATCT	Two bands
QB.03	<i>A. mellifera</i> (Florida)	MW530571			351.7	-	21.07	AGATCT	Two bands
3485	<i>A. m. meda</i>	MT572350	O		3.7	-	26.61	AGATCT	Two bands
3361	<i>A. m. anatoliaca</i>	MT572352	O		4.2	-	24.61	AGATCT	Two bands
29	<i>A. m. sahariensis</i>	MT572353	A		2.5	-	36.67	AGATCT	Two bands
1715	<i>A. m. ligustica</i>	MT572354	C	Two bands	2.7	-	27.86	AGATCT	Two bands
2721	<i>A. m. simensis</i>	MT572355	Y		3.2	26.84	-	AGATCT	Two bands
1072	<i>A. m. litorea</i>	MT572356	A		2	32.09	-	AGACCT	One band
2052	<i>A. m. rutneri</i>	MT572357	A		9	20.84	-	AGACCT	One band
CT.01	<i>A. m. capensis</i>	MW530560	A		135.1	13.74	-	AGACCT	One band
CT.02	<i>A. m. capensis</i>	MW530561	A		248.1	12.68	-	AGACCT	One band
CT.03	<i>A. m. capensis</i>	MW530562	A		176.6	12.14	-	AGACCT	One band
RD.01	<i>A. m. capensis</i>	MW530566	A		170.0	12.49	-	AGACCT	One band
RD.02	<i>A. m. capensis</i>	MW530567	A		132.1	12.42	-	AGACCT	One band
RD.03	<i>A. m. capensis</i>	MW530568	A		142.7	12.32	-	AGACCT	One band
3596	<i>A. m. sahariensis</i>	MT572363	A		6.3	17.97	-	AGACCT	One band
1264	<i>A. m. monticola</i>	MT572359	A		3.5	18.16	-	AGACCT	One band
1277	<i>A. m. adansonii</i>	MT572360	A		1.9	21.36	-	AGACCT	One band
VR.01	<i>A. m. scutellata</i>	MT572362	A	One band	6.1	15.59	-	AGACCT	One band
VR.02	<i>A. m. scutellata</i>	MT572361	A	One band	12.0	14.83	-	AGACCT	One band
VR.03	<i>A. m. scutellata</i>	MT572358	A	One band	8.1	15.05	-	AGACCT	One band
SP.01	<i>A. m. scutellata</i>	MT572364	A	One band	9.7	15.44	-	AGACCT	One band
SP.02	<i>A. m. scutellata</i>	MT572368	A	One band	12.6	15.89	-	AGACCT	One band
SP.03	<i>A. m. scutellata</i>	MT572367	A	One band	15.3	15.39	-	AGACCT	One band
UP.01	<i>A. m. scutellata</i>	MT572366	A	One band	11.2	16.41	-	AGACCT	One band
UP.02	<i>A. m. scutellata</i>	MT572365	A	One band	13.5	15.35	-	AGACCT	One band
UP.03	<i>A. m. scutellata</i>	MT572369	A	One band	3.3	16.53	-	AGACCT	One band
MB.01	<i>A. m. capensis</i>	MW530563	A		112.6	13.86	-	AGACCT	One band
MB.02	<i>A. m. capensis</i>	MW530564	A		166.3	12.35	-	AGACCT	One band
MB.03	<i>A. m. capensis</i>	MW530565	A		230.8	12.93	-	AGACCT	One band

<sup>a</sup> E.g., Nawrocka et al. (2018)<sup>b</sup> Crozier et al. (1991) and Pinto et al. (2003). No number indicates the *Bgl*II mitotype has not been determined<sup>c</sup> See text on A- and M-lineage *A. m. iberiensis* samples

based assays (Rinderer et al. 1986, 1993; Meixner et al. 2013). To our knowledge, no protocols using qPCR probes for the detection and differentiation of African A-lineage honey bees exist. While our assay was developed using worker bees, the possibility of extending it for use on larval, drone, and queen samples exists—something which current morphometric techniques cannot do. However, users would need to remember that *Cytb* is found on the mitochondrial genome, and as such, only

reveals maternal genetics, except in the case of embryo samples where a small proportion of mitochondrial DNA may be of paternal origin, creating temporary heteroplasmy during development (Meusel and Moritz 1993).

Our assay performed well on both platforms, even at low concentrations of DNA (Figure 1). As expected, the Ct value increased with a decrease in DNA concentration, indicating that lower concentrations of DNA took more cycles to show a

Consensus	GenBank	67	81	97	106	109	127	139	146	187	202	223	241	289	290	307	319	353
		T	C	C	A	C	C	T	T	T	C	T	T	C	C	T	C	T
<i>A. m. lamarcii</i> (Egypt, 4267 LAMAR)	EF184062.1	C	-	T	-	-	A	-	-	-	-	-	-	T	-	C	T	-
<i>A. m. lamarcii</i> (Egypt, 99E10 LAMAR)	EF184063.1	C	-	T	-	-	A	-	-	-	-	-	-	T	-	C	T	-
<i>A. m. lamarcii</i> (Egypt, 99E13 LAMAR)	EF184064.1	C	-	T	-	-	A	-	-	-	-	-	-	T	-	C	T	-
<i>A. m. lamarcii</i> (Egypt, 2271)	MT572347	C	-	T	-	-	A	-	-	-	-	-	-	T	-	C	T	-
<i>A. m. syriaca</i> (Syria, 1729)	MT572346	C	-	T	-	-	A	-	-	-	-	-	C	T	-	C	T	-
<i>A. m. syriaca</i> (Syria, 9946 SYR)	EF184061.1	C	T	T	-	-	A	-	-	-	-	-	C	T	-	C	T	-
<i>A. m. syriaca</i> (Syria, 9945 SYR)	EF184060.1	C	T	T	-	-	A	-	-	-	-	-	C	T	-	C	T	-
<i>A. m. iberiensis</i> (Spain, 1952)	MT572349	-	-	T	-	-	A	C	-	C	-	-	-	T	-	C	-	-
<i>A. m. iberiensis</i> (5290 IBER)	EF184039.1	-	-	T	-	-	A	C	-	C	-	-	-	T	-	C	-	-
<i>A. m. ligustica</i> (Italy, 5197 LIGU)	EF184044.1	-	-	T	-	-	A	C	-	C	-	-	-	T	-	C	-	-
<i>A. m. iberiensis</i> (1720 IBER)	EF184038.1	-	-	T	-	-	A	C	-	C	-	C	C	T	-	C	-	-
<i>A. m. mellifera</i> (Norway, 1410)	MT572348	-	-	T	-	-	A	C	-	C	-	C	C	T	-	C	-	-
<i>A. m. mellifera</i> (France, 1481 MELL)	EF184059.1	-	-	T	-	-	A	C	-	C	-	C	C	T	-	C	-	-
<i>A. m. mellifera</i> (France, 1938 MELL)	EF184058.1	-	-	T	-	-	A	C	-	C	-	C	C	T	-	C	-	-
<i>A. m. mellifera</i> (France, 5422 MELL)	EF184057.1	-	-	T	-	-	A	C	-	C	-	C	C	T	-	C	-	-
<i>A. m. mellifera</i> (France, 1445 MELL)	EF184055.1	-	-	T	-	-	A	C	-	C	-	C	C	T	-	C	-	-
<i>A. m. mellifera</i> (France, 1627 MELL)	EF184056.1	-	-	T	-	-	A	C	-	C	-	C	C	T	-	C	-	-
<i>A. m. cypria</i> (Cyprus, 9929 CYPYR)	EF184054.1	-	-	T	-	-	T	A	-	-	-	-	-	T	-	-	-	-
<i>A. m. cypria</i> (Cyprus, 9900 CYPYR)	EF184053.1	-	-	-	-	T	A	-	-	-	T	-	-	T	-	C	-	-
<i>A. m. carnica</i> (A1 CARN)	EF184048.1	-	-	-	-	-	T	A	-	-	-	-	-	T	-	C	-	-
<i>A. m. caucasica</i> (Turkey, 2183 CAUC)	EF184050.1	-	-	T	-	-	T	A	-	-	T	-	-	T	-	C	-	-
<i>A. m. caucasica</i> (Turkey, 2193 CAUC)	EF184052.1	-	-	T	-	-	T	A	-	-	-	-	-	T	-	C	-	C
<i>A. m. caucasica</i> (Turkey, 2187 CAUC)	EF184051.1	-	-	T	-	-	T	A	-	-	-	-	-	T	-	C	-	-
<i>A. m. carnica</i> (3384 CARN)	EF184047.1	-	-	T	-	-	T	A	-	-	-	-	-	T	-	C	-	-
<i>A. m. carnica</i> (3357 CARN)	EF184046.1	-	-	T	-	-	T	A	-	-	-	-	-	T	-	C	-	-
<i>A. m. carnica</i> (Croatia, 1808)	MT572351	-	-	T	-	-	T	A	-	-	-	-	-	T	-	C	-	-
<i>A. mellifera</i> (Florida, USA, QB.01)	MW530569	-	-	T	-	-	T	A	-	-	-	-	-	T	-	C	-	-
<i>A. mellifera</i> (Florida, USA, QB.02)	MW530570	-	-	T	-	-	T	A	-	-	-	-	-	T	-	C	-	-
<i>A. mellifera</i> (Florida, USA, QB.03)	MW530571	-	-	T	-	-	T	A	-	-	-	-	-	T	-	C	-	-
<i>A. m. meda</i> (Turkmenistan, 3485)	MT572350	-	-	T	-	-	T	A	-	-	-	-	-	T	-	C	-	-
<i>A. m. anatoliaca</i> (Turkey, 3361)	MT572352	-	-	T	-	-	T	A	-	-	-	-	-	T	-	C	-	-
<i>A. m. sahariensis</i> (Morocco, 29)	MT572353	-	-	T	-	-	T	A	-	-	-	-	-	T	-	C	-	-
<i>A. m. ligustica</i> (Italy, 1715)	MT572354	-	-	T	-	-	T	A	-	-	-	-	-	T	-	C	-	-
<i>A. m. ligustica</i> (Italy, 4426 LIGU)	EF184043.1	-	-	T	-	-	T	A	-	-	-	-	-	T	-	C	-	-
<i>A. m. ligustica</i> (Italy, 4493 LIGU)	EF184041.1	-	-	T	-	-	T	A	-	-	-	-	-	T	-	C	-	-
<b><i>A. m. ligustica</i> (Italy, AMFGENOM)</b>	<b>AMFGENOM</b>	-	-	T	-	-	T	A	-	-	-	-	-	T	-	C	-	-
<i>A. m. ligustica</i> (Italy, 5200 LIGU)	EF184042.1	-	-	T	-	-	T	A	-	-	T	-	-	T	-	C	-	-
<i>A. m. scutellata</i> (Kenya, 3335 SCUT)	EF184040.1	-	-	T	-	-	T	A	-	-	-	-	-	T	-	C	-	-
<i>A. mellifera</i> (Texas, USA, 5182 STX)	EF184049.1	-	-	T	-	-	T	A	-	-	-	-	-	T	A	C	-	-
<i>A. mellifera</i> (New Mexico, USA, 1706 N MEX)	EF184045.1	-	-	T	-	-	T	A	-	-	-	-	-	T	A	C	-	-
<i>A. m. simensis</i> (Ethiopia, 2721)	MT572355	-	-	-	-	-	A	-	-	-	-	-	-	T	-	-	-	-
<i>A. m. litorea</i> (South Africa, 1072)	MT572356	-	-	-	-	T	G	T	A	-	-	-	-	-	-	Y	-	-
<i>A. m. ruttneri</i> (Malta, 2052)	MT572357	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. capensis</i> (South Africa, CT.01)	MW530560	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. capensis</i> (South Africa, CT.02)	MW530561	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. capensis</i> (South Africa, CT.03)	MW530562	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. capensis</i> (South Africa, RD.01)	MW530566	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. capensis</i> (South Africa, RD.02)	MW530567	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. capensis</i> (South Africa, RD.03)	MW530568	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. scutellata</i> (Kenya, 8554 SCUT)	EF184027.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. scutellata</i> (Kenya, 5449 SCUT)	EF184025.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. scutellata</i> (Kenya, 5471 SCUT)	EF184026.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. scutellata</i> (Kenya, 3327 SCUT)	EF184028.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. iberiensis</i> (1995 IBER)	EF184035.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. iberiensis</i> (1962 IBER)	EF184036.1	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. iberiensis</i> (5310 IBER)	EF184034.1	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. iberiensis</i> (1998 IBER)	EF184033.1	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. iberiensis</i> (1957 IBER)	EF184032.1	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. iberiensis</i> (5390 IBER)	EF184037.1	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. intermissa</i> (Morocco, 3208 INTER)	EF184020.1	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. intermissa</i> (Morocco, 3182 INTER)	EF184024.1	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. intermissa</i> (Morocco, 5343 INTER)	EF184021.1	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. intermissa</i> (Morocco, 2161 INTER)	EF184023.1	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. intermissa</i> (Morocco, 2160 INTER)	EF184022.1	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. sahariensis</i> (Morocco, 3596)	MT572363	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. monticola</i> (Kenya, 1264)	MT572359	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. adansonii</i> (Central African Republic, 1277)	MT572360	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. mellifera</i> (Georgia, USA, 3302 GEORG)	EF184031.1	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. scutellata</i> (Kenya, 5455 SCUT)	EF184029.1	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. scutellata</i> (South Africa, VR.01)	MT572362	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. scutellata</i> (South Africa, VR.02)	MT572361	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. m. scutellata</i> (South Africa, VR.03)	MT572358	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
<b>Africanized honey bee (Mexico, KJ601784)</b>	<b>KJ601784</b>	-	-	-	-	G	-	-	-	C	-	-	-	-	-	-	-	-
<i>A. mellifera</i> (Brazil)	EF184030.1	-	-	-	-	G	-	-	-	C	-	-	-	-	-	-	-	-
<i>A. m. scutellata</i> (South Africa, SP.01)	MT572364	-	-	-	-	G	-	-	-	C	-	-	-	-	-	-	-	-
<i>A. m. scutellata</i> (South Africa, SP.02)	MT572368	-	-	-	-	G	-	-	-	C	-	-	-	-	-	-	-	-
<i>A. m. scutellata</i> (South Africa, SP.03)	MT572367	-	-	-	-	G	-	-	-	C	-	-	-	-	-	-	-	-
<i>A. m. scutellata</i> (South Africa, UP.01)	MT572366	-	-	-	-	G	-	-	-	C	-	-	-	-	-	-	-	-
<i>A. m. scutellata</i> (South Africa, UP.02)	MT572365	-	-	-	-	G	-	-	-	C	-	-	-	-	-	-	-	-
<i>A. m. scutellata</i> (South Africa, UP.03)	MT572369	-	-	-	-	G	-	-	-	C	-	-	-	-	-	-	-	-
<i>A. m. capensis</i> (South Africa, MB.01)	MW530563	-	-	-	-	G	-	-	-	C	-	-	-	-	-	-	-	-
<i>A. m. capensis</i> (South Africa, MB.03)	MW530564	-	-	-	-	G	-	-	-	C	-	-	-	-	-	-	-	-
<i>A. m. capensis</i> (South Africa, MB.02)	MW530565	-	-	-	-	G	-	-	-	C	-	-	-	-	-	-	-	-

◀ **Figure 2.** *Cytochrome b* region variable sites for the 36 test samples (highlighted in grey), together with sequences from the reference mitogenomes for *Apis mellifera ligustica* (AMFGENOM), an Africanized bee from Mexico (KJ601784), and Popset 126513039 (Pinto et al. 2007). Where available, location data are shown in brackets, followed by the sample ID used by the authors. The three *A. m. carnica* from Pinto et al. 2007 were from Germany, Slovenia, and Austria; while *A. m. iberiensis* were from Spain and Portugal—samples could not be matched back to specific locations. The numbers correspond to the *A. m. ligustica* positions (Crozier and Crozier 1993), as used in Pinto et al. (2007). The dashes show nucleotide identity. The nucleotides within the *Bgl*II cut site are shown in red, and within the probe binding region in bold numbers. Positions 81, 202, and 353 showed no variation in our data but were included to match the previous papers.

fluorescent emission. The Ct scores for the samples tested on both platforms were strongly correlated ( $r^2 = 0.82$ ,  $y = 0.879x + 1.62$ ). Two samples that did not work at the lowest dilutions on the Cepheid platform for the non-A-lineage probe, did fluoresce on the QuantStudio platform. The qPCR results and predicted *Bgl*II mitotypes of previously unstudied subspecies (Table IV) will be useful for anyone who chooses to continue using the traditional *Cytb*/*Bgl*II assay. The only sample to give contrasting information between the qPCR and Sanger sequencing was *A. m. simensis*, belonging to the Y-lineage (see results above). This should be interpreted with caution given that we only have data for one sample presented here. The mitochondrial genome of *A. m. simensis* closely resembles that of other A-lineage honey bees (Boardman et al. 2020a); so further genetic research on this subspecies would be useful.

Based on the downloaded sequences used to design the primers (Table I, Pinto et al. 2007), *A. m. iberiensis* and *A. m. scutellata* samples should all have been positive for the A-lineage probe. While *A. m. scutellata* came back as expected, our *A. m. iberiensis* sample was negative for the A-lineage probe. This apparent anomaly may be explained in one of two ways. First, while *A. m. iberiensis* is often considered to be M-lineage, some mitochondrial studies have shown evidence of A-lineage mtDNA haplotypes

(Arias et al. 2006; reviewed in Meixner et al. 2013; Boardman et al., 2020b), and recent studies support a geographic cline between M- and A- populations (Cánovas et al. 2008, 2014; Chávez-Galarza et al. 2017; Henriques et al. 2018). Thus, samples of *A. m. iberiensis* may present either M- or A-lineage results, as found by Pinto et al. (2003). As our sample is from Spain, it is most likely representative of the European M-lineage rather than the African A-lineage. This was further supported by the similarities in *Cytb* sequences (Figure 2) between our *A. m. iberiensis* and M-lineage *A. m. mellifera* samples, and other *A. m. iberiensis* and *A. m. mellifera* samples from Pinto et al. (2007). Second, one of the constraints of using the *Bgl*II site is the inability to discriminate between *A. m. scutellata* and the related A-lineage subspecies *A. m. intermissa* and some *A. m. iberiensis* (Pinto et al. 2003). This means that samples that are positive for this test cannot definitively be identified as *A. m. scutellata* or *A. m. scutellata*-derived. This was specified at the end of the Crozier et al. (1991) and clearly shown by Pinto et al. (2007) where sequencing data revealed that only one of three suspected African-derived samples from Pinto et al. (2003) contained an African mitotype.

This is especially important for use in the United States where *A. m. iberiensis* was potentially introduced prior to 1922 (Sheppard 1989a, b; Pinto et al. 2007). In addition, six other subspecies from the A-lineage, which to our knowledge have not been tested with the *Cytb*/*Bgl*II marker, were also positive for the A-lineage probe. This suggests that *A. m. adansonii*, *A. m. capensis*, *A. m. litorea*, *A. m. monticola*, *A. m. ruttneri*, and *A. m. sahariensis* may also be indistinguishable from *A. m. scutellata* when using our qPCR assay, or the *Cytb*/*Bgl*II marker. Therefore, any interpretations as African-derived when using these methods should be interpreted with caution, especially since

published mitochondrial genomes show that seven of the other *Apis* species share this trait with A-lineage bees (i.e., no restriction cut site, AGATTT – GenBank: AP01764, GQ162109, MF565375, KF736157, KX908207, KX908208, KY799147). Only *A. florea* Fabricius (JX982136) has the *Bgl*II cut site: AGATCT.

The non-A-lineage probe serves to confirm the results of the A-lineage probe, as subspecies with a “C” in *Bgl*II cut site, have a “T” at position 307 which is targeted by the non-A-lineage probe (Pinto et al. 2007). This probe was positive for C-, O-, and M-lineage subspecies (Table IV), including *A. m. iberiensis* (discussed above). *Apis m. lamarckii*, an A-lineage honey bee, was also positive for the non-A-lineage probe. Geographically, one may expect *A. m. lamarckii* to be from the A-lineage (which was confirmed morphometrically), but mitochondrial evidence suggests that it can be A- or O-lineage—depending on the sample collection site (Arias and Sheppard 1996; Franck et al. 2000, 2001). Similarly, our results suggest that *A. m. sahariensis* also has different mitotypes, which supports earlier links to the M-lineage (Meixner et al. 2013). It is thus likely that these three subspecies can occur in different mitochondrial lineages, and the outcome of this assay will depend on where the bees are sampled or if European-derived subspecies have been imported in the area.

The movement of honey bees confounds efforts to identify mitochondrial lineages. European honey bees are often imported into countries around the world. Furthermore, aside from the well-known introduction of African-derived honey bee in the Americas, honey bees with African mitochondrial DNA *COI-COII* haplotypes have been observed near Paris, France (Bertrand et al. 2015). Four of the African haplotypes found were characteristic of *A. m. iberiensis* populations (A1, A2, A3), or populations in central or southern Africa (A4), suggesting that European beekeepers may be importing honey bees from Africa, despite the problems with African-derived honey bees in the Americas.

It is also important to note that our data represent one sample from each of the subspecies we had available, with the exceptions noted above (*A. m. sahariensis*, *A. m. capensis*, and *A. m. scutellata*). To confirm the *Cytb* sequencing data, we compared our sequences to the matching regions from Pinto et al. (2007). Looking at this larger dataset of samples, including *A. m. cyprina*, some patterns emerge. The European-derived *A. mellifera* sample from Florida clusters with European C- and O-lineage honey bees, which show little variation for this *Cytb* region. Samples of *A. m. ligustica* and *A. m. scutellata* show the highest variation with each having four different mitotypes. This trend is discussed in detail in Pinto et al. (2007) and is likely a result of importation of honey bees and hybridization between subspecies.

The *Cytb* sequences from this study were only intended to validate the results of the qPCR assay, and help explain apparent anomalies (e.g., *A. m. simensis* result—see discussion above). This study was not designed, nor intended, to compare *Cytb* sequences or mitotypes between *A. mellifera* subspecies, mostly because this requires broader sampling distribution and more replicates per location. However, as we have multiple locations and replicates for the South African samples, we think it is interesting to discuss these results further as variation in *A. m. scutellata* mitotypes can present a problem for detection of African-derived honey bees. Here, all the *A. m. scutellata* samples were positive for the A-lineage probe and negative for the non-A-lineage probe, so the probe binding region is conserved. However, the *Cytb* sequencing showed four different mitotypes (Figure 2). In the South Africa *A. m. scutellata* samples from this study, the three samples sequenced from a single colony all had the same Sanger *Cytb* sequence. Sequences were identical for honey bees from Springbok (SP) and

Upington (UP) and these matched data for African-derived honey bees, and a sample from Brazil (Pinto et al. 2007). However, *A. m. scutellata* samples from Vryburg (VR) had a T at position 146 (Figure 2) and were the same as *A. m. adansonii*, *A. m. monticola*, and *A. m. sahariensis* from our study; and *A. m. scutellata* (Kenya), *A. mellifera* from Georgia (USA, feral/admixed sample), *A. m. intermissa* (Morocco), and *A. m. iberiensis* from Pinto et al. (2007) (Figure 2).

The *A. m. capensis* samples were all positive for the A-lineage probe and negative for the non-A-lineage probes. *Apis m. capensis* from two locations, Cape Town (CT) and Riversdale (RD), were different to South African *A. m. scutellata*, but had the same *Cytb* region as *A. m. ruttneri* from our data, and *A. m. scutellata* (Kenya) and an *A. m. iberiensis* from Pinto et al. (2007) (Figure 2). The *A. m. capensis* samples from the other South African location, Moorreesburg (MB, which is also the most northern location of our *A. m. capensis* samples), were identical to *A. m. scutellata* samples from Springbok (SP) and Upington (UP), but not Vryburg (VR). This result was unexpected, as each of these locations had *A. m. capensis* mitochondrial genomes in a previous study (Eimanifar et al. 2018b). We compared the corresponding region of this mitochondrial genome dataset to our data for the *Cytb* region and found that the T>C at position 146 was also found in *A. m. capensis* samples from Stellenbosch (ST), Lainesburg (LB), Citrusdal (CD), and a hybrid from Klaver (KL). Remembering also that this SNP was found in some *A. m. scutellata*, and African-derived honey bee samples from Brazil and Mexico (Figure 2, and see previous paragraph), this result is surprising. Geographically, these sampling locations are all found in the northwestern parts of South Africa. While confirmed as *A. m. capensis* or *A. m. scutellata* with morphometrics, microsatellites, SNPs and mitochondrial genomes (Eimanifar et al. 2018a, b, 2020; Bustamante et al. 2020), it remains possible that some level of hybridization is

ongoing, or that this region has resulted in selection for this SNP. Alternatively, these data may suggest that the differences between these subspecies are negligible (Hepburn and Radloff 2002) highlighting the need to present geographical data with all sequencing results.

Our qPCR assay has the same constraints as the *Cytb/Bgl* II restriction enzyme assay as it is based on the same polymorphism. However, the qPCR reaction has the advantage over enzyme assays of being quicker and allowing more samples to be run concurrently. Nevertheless, to distinguish fully between these subspecies and classify a honey bee as being derived from *A. m. scutellata*, additional tests and methods are needed that target additional polymorphisms or regions of genetic variation specific to that subspecies.

In conclusion, this qPCR assay provides a faster, reliable version of the *Cytb/Bgl* II restriction enzyme assay. While this assay cannot separate *A. m. scutellata* from other A-lineage honey bees that also test positive for the probe, together with morphometrics, this assay can form part of an identification pipeline. Further development of qPCR assays for subspecies identification will enable fast, cheap diagnostic services for beekeepers, and will contribute to our understanding of *A. mellifera* evolution and diversification.

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**Availability of data and material** DNA sequences are deposited in GenBank (accession numbers: MT572346-MT572369, MW530560-MW530571).



**Code availability** Not applicable.

## AUTHOR CONTRIBUTION

PS, AJ, LW, JE conceived of this research; PS, AJ, MM collected the data; LB analyzed the data and wrote the manuscript. All authors participated in revisions, and read and approved the final manuscript.

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## DECLARATIONS

**Ethics approval** Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

**Un test qPCR pour la détection sensible et rapide des abeilles africaines de la lignée A (*Apis mellifera*).**

**abeille domestique d'origine africaine / lignée A / biomarqueur / cytochrome b / *Apis mellifera scutellata* / BglII.**

**Eine qPCR-Methode für die sensitive und schnelle Detektion der afrikanischen A-Linie der Honigbiene (*Apis mellifera*).**

**Honigbiene afrikanischen Ursprungs / A-Linie / Biomarker / Cytochrome b / *Apis mellifera scutellata* / BglII.**

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