Identification of Africanized Honey Bee (Hymenoptera: Apidae) Mitochondrial DNA: Validation of a Rapid Polymerase Chain Reaction-Based Assay

M. ALICE PINTO,[1, 2] J. SPENCER JOHNSTON,[1] WILLIAM L. RUBINK,[3] ROBERT N. COULSON,[4] JOHN C. PATTON,[5] AND WALTER S. SHEPPARD[6]

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ABSTRACT Polymerase chain reaction (PCR)-amplified mitochondrial DNA (mtDNA) assays have been used in studies of the Africanization process in neotropical feral and managed honey bee populations. The approach has been adopted, in conjunction with morphometric analysis, to identify Africanized bees for regulatory purposes in the United States such as in California. In this study, 211 Old World colonies, representing all known introduced subspecies in the United States, and 451 colonies from non-Africanized areas of the southern United States were screened to validate a rapid PCR-based assay for identification of Africanized honey bee mtDNA. This PCR-based assay requires a single enzyme digestion (*Bgl*II) of a single PCR-amplified segment of the cytochrome *b* gene. The *Bgl*II polymorphism discriminates the mitochondrial haplotype (mitotype) of *Apis mellifera scutellata* L. (ancestor of Africanized bees) from that of *A. m. mellifera*, *A. m. caucasia*, *A. m. ligustica*, *A. m. carnica*, *A. m. lamarcki*, *A. m. cypria*, *A. m. syriaca*, and some *A. m. iberiensis*, but not from that of *A. m. intermissa* and some *A. m. iberiensis*. Nonetheless, given the very low frequency (<1%) of African non-*A. m. scutellata* mitotype present before arrival of Africanized bees in the United States, cytochrome *b*/*Bgl*II assay can be used to identify maternally Africanized bees with a high degree of reliability.

KEY WORDS Africanized honey bee, *A. m. scutellata*, mitochondrial DNA, mtDNA haplotype, mitotype

THE HISTORY OF HONEY bee colonization of the United States can be divided into the period before and the period after Africanization. Historical records indicate that during the pre-Africanization period, several races were introduced from their native range in Europe, north Africa, and Middle East into the United States. In the early to mid 1600s, *Apis mellifera mellifera* L. and probably *A. m. iberiensis* were brought by English and Spanish settlers (Sheppard 1989a, 1989b). Between 1859 and 1922, beekeepers imported seven more subspecies: *A. m. caucasia*, *A. m. ligustica*, *A. m. carnica* (Europe), *A. m. intermissa*, *A. m. lamarckii* (north Africa), *A. m. cypria*, and *A. m. syriaca* (Middle East) (Sheppard 1989a, 1989b). The latter four subspecies were only tried briefly, whereas *A. m. carnica*, *A. m. caucasia*, and *A. m. ligustica* have remained available as commercial strains from United States queen producers (Sheppard 1989b). The genetic composition of pre-Africanized managed and feral United States honey bee populations largely reflects the aforementioned introductions and historical preferences of beekeepers. Indeed, mitochondrial DNA (mtDNA) analyses of the United States commercial queen-breeding population have detected a high frequency of the mitotype common to *A. m. ligustica*, *A. m. carnica*, and *A. m. caucasia* (96%), while only 4% of the colonies exhibited the mitotype common to *A. m. mellifera* (Schiff and Sheppard 1995, 1996). In the feral population, the frequency of the *A. m. mellifera* mitotype was much higher (37%), and a low frequency of maternal descendants of the Egyptian bee *A. m. lamarckii* was also found (Schiff and Sheppard 1993, Schiff et al. 1994, Nielsen et al. 2000). Nonetheless, because the pre-Africanized gene pool of the United States population has been derived predominantly from European subspecies, these bees have been traditionally referred to as being of European origin.

In 1990, with the arrival of Africanized bees in Texas (Sugden and Williams 1990), a new period of honey

[1] Department of Entomology, Texas A&M University, College Station, TX 77843–2475.

[2] Departamento Florestal, Escola Superior Agrária, Instituto Politécnico de Bragança, Campus de Santa Apolónia, Apartado 172, 5301-854 Bragança, Portugal.

[3] United States Department of Agriculture/Agricultural Research Service/BIRU Honey Bee Research, 2413 East Highway 83, Building 213, Weslaco, TX 78596.

[4] Knowledge Engineering Laboratory, Department of Entomology, Texas A&M University, College Station, TX 77843–2475.

[5] Department of Wildlife and Fisheries, Texas A&M University, College Station, TX 77843–2258.

[6] Department of Entomology, Washington State University, Pullman, WA 99164–6382.

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Table 1. PCR-based assays that have been used to identify honey bee matrilineal origins

PCR-generated DNA fragment	Restriction enzyme	Mitotype cleaved fragment	Mitotype uncleaved fragment	Authors
Cytochrome *b*	*Bgl*II	Non-Africanized	Africanized	Crozier et al. (1991)
Ls rRNA	*Eco*RI	Eastern European[a] (*A. m. ligustica*, *A. m. carnica*, *A. m. caucasia*)	Western European (*A. m. mellifera* and *A. m. iberiensis* with *A. m. mellifera*-like mtDNA)	Hall and Smith (1991)
COI	*Hinc*II	Western European (*A. m. mellifera* and *A. m. iberiensis* with *A. m. mellifera*-like mtDNA)	Eastern European[a] (*A. m. ligustica*, *A. m. carnica*, *A. m. caucasia*)	Hall and Smith (1991)
COI	*Xba*I	Eastern European[a] (*A. m. ligustica*, *A. m. carnica*, *A. m. caucasia*)	Western European (*A. m. mellifera* and *A. m. iberiensis* with *A. m. mellifera*-like mtDNA)	Hall and Smith (1991)
COI	*Hinf*I	*A. m. lamarckii*	Non-*A. m. lamarckii*	Nielsen et al. (2000)
COI-COII Intergenic Region[b]	*Dra*I			Garnery et al. (1993)

[a] Also named eastern Mediterranean.

[b] This mtDNA region contains length and *Dra*I restriction site polymorphisms. More than 60 distinct mitotypes have been reported for honey bee populations (Garnery et al. 1993, 1995; Franck et al. 1998, 2000a, 2000b, 2001). Therefore, a simple allocation in two mitotype categories, as given in Table 1, is not possible.

bee colonization began. Africanized honey bees are descendants of a sub-Saharan African subspecies, *A. m. scutellata*, introduced into Brazil from South Africa in 1956 (Kerr 1967). Since their accidental release in 1957 (Nogueira-Neto 1964, Kerr 1967), they established a self-sustaining feral population that expanded throughout the neotropics with differential levels of genetic introgression from European genes (Hall and Muralidharan 1989; Lobo et al. 1989; Smith et al. 1989; Hall 1990; Hall and Smith 1991; Rinderer et al. 1991; Sheppard et al. 1991a, 1991b; Quezada-Euán et al. 1996; Clarke et al. 2001, 2002). In the ensuing 46 yr, Africanized bees colonized a broad area encompassing most of South America, Central America, Mexico, and the southwestern United States (Texas, New Mexico, Arizona, Nevada, and California).

Before the arrival of Africanized bees in the United States, various molecular and nonmolecular methods were developed for the detection and identification of Africanized bees and used either for population genetic studies of New World bee populations or for regulatory purposes (reviewed by Sheppard and Smith 2000). Among the molecular markers, the nonrecombining and maternally inherited mtDNA has been one of the most widely used methods. Insights into the mechanisms of expansion and maternal genetic composition of the Africanized population were provided by restriction fragment analysis of the entire mtDNA molecule of honey bees from South and Central America (Smith and Brown 1988, Hall and Muralidharan 1989, Smith et al. 1989, Rinderer et al. 1991, Sheppard et al. 1991a, 1991b, 1999). A similar molecular approach was used in mtDNA surveys of feral and commercial colonies from non-Africanized areas of the southern United States (Schiff and Sheppard 1993, 1995, 1996; Schiff et al. 1994). Although restriction analysis of the entire mitochondria proved to be very informative, widespread use of the procedure in screening programs is unlikely because of the expense and time requirements. The anticipated necessity of screening large number of colonies during the post-Africanization period, either for research or regulatory purposes, led to the development of rapid polymerase chain reaction (PCR)-amplified mtDNA assays (Crozier et al. 1991, Hall and Smith 1991, Garnery et al. 1993, Nielsen et al. 2000). These methods, summarized in Table 1, consist of PCR amplification of a given fragment, followed by restriction enzyme digestion. PCR-amplified mtDNA assays have been used in studies of the Africanization process in neotropical feral and managed populations (Hall and Smith 1991, Lobo 1995, Quezada-Euán and Hinsull 1995, Clarke et al. 2001), and have been adopted, in conjunction with morphometric analysis, to identify Africanized bees for regulatory purposes in California (Nielsen et al. 1999, 2000).

The PCR-based assays that have been used for maternal identification of bees in California, originally developed by Hall and Smith (1991) and improved by Nielsen et al. (2000), require PCR amplification of two genes (Ls rRNA and COI), followed by three restriction enzyme (*Eco*RI, *Hinc*II, and *Hinf*I) digestions. Crozier et al. (1991) designed primers, within the cytochrome *b* gene, flanking a seemingly diagnostic *Bgl*II restriction site for Africanized bees (Table 1). If this assay were shown to be highly discriminatory, then the advantage of the technique is that a single enzyme digestion of a PCR-amplified segment would be sufficient for maternal identification of Africanized bees. Considering the small sample size (15 Africanized and 10 non-Africanized) and the origin (New World, country not stated) of the colonies used by Crozier et al. (1991) as a source of baseline data, further studies are needed to ascertain the robustness of the cytochrome *b*/*Bgl*II assay so it could be used for Africanized honey bee identification. In this study, we report the results of a survey for *Bgl*II variation in the cytochrome *b* gene (Crozier et al. 1991) within 211

Table 2. Description of the Old World and United States sample collection

Origin	Subspecies (mitochondrial lineage)	Number of colonies	Number of mitotypes	
			Two-band	One-band
Old World				
France	*A. m. mellifera* (western European[a])	18	17	1
Italy	*A. m. ligustica* (eastern Mediterranean[a])	22	22	
Portugal, Spain	*A. m. iberiensis* (western European and African[a,b])	28	10	18
Syria	*A. m. syriaca* (Middle Eastern[c])	20	20	
Cyprus	*A. m. cypria*[d]	20	20	
Egypt	*A. m. lamarckii* (Middle Eastern[e])	24	24	
Morocco	*A. m. intermissa* (African[a])	16		16
Kenya	*A. m. scutellata* (African[a])	33		33
Turkey	*A. m. caucasia* (eastern Mediterranean[a,f])	11	11	
Germany, Slovenia, Austria	*A. m. carnica* (eastern Mediterranean[a])	19	19	
United States				
Alabama[g]		7	7	
Arizona[g]		146	146	
Georgia[g]		31	30	1
Louisiana[g]		51	51	
Mississippi[g]		3	3	
New Mexico[g]		58	57	1
North Carolina[g]		6	6	
Oklahoma[g]		3	3	
South Carolina[g]		15	15	
Texas[g,h]		131	130	1

Location, sample size, number of mitotypes, and mitochondrial lineages of each subspecies are indicated.

[a] Garnery et al. (1992), Arias and Sheppard (1996), Franck et al. (2001).

[b] Data on mtDNA revealed the coexistence in the Iberian peninsula of mitotypes belonging to the mitochondrial African and western European lineages (Smith et al. 1991; Garnery et al. 1992, 1993, 1995; Franck et al. 1998; Clarke et al. 2001).

[c] Franck et al. (2000a).

[d] Mitochondrial lineage is not reported in the literature. Based on morphometric data, this subspecies belongs to the Middle Eastern lineage (Ruttner 1988).

[e] Arias and Sheppard (1996), Franck et al. (2000a, 2001).

[f] Smith et al. (1997).

[g] Feral colonies tested previously by restriction analysis of the entire mitochondria (Schiff and Sheppard 1993, Schiff et al. 1994).

[h] Fifty-four colonies, collected in southern Texas prior to Africanization, are newly investigated.

Old World colonies, representing all races known to have been introduced to the New World, and 451 colonies from non-Africanized areas of the southern United States. The survey allowed us to address whether cytochrome *b*/*Bgl*II is a reliable test for discriminating maternal descendants of *A. m. scutellata* from bees derived from non-*A. m. scutellata* ancestors in the United States.

Materials and Methods

Sample Collection. A collection of 662 Old World and United States colonies was screened for *Bgl*II variation in the amplified cytochrome *b* gene region (Crozier et al. 1991). The Old World collection comprises 211 colonies from the 10 subspecies known to have been introduced into the United States. These colonies include representatives from various mitochondrial lineages (Garnery et al. 1992, 1995; Arias and Sheppard 1996; Smith et al. 1997; Franck et al. 2000a, 2001), as shown in Table 2. With the exception of *A. m. caucasia*, all subspecies were morphometrically identified using standard discriminant analyses procedures (Ruttner 1988). The United States collection ($n = 451$) was obtained from feral colonies (as defined by Schiff and Sheppard 1993) throughout non-Africanized areas in the southern states (Table 2).

Analysis of mtDNA

DNA Extraction. DNA of 54 colonies from southern Texas was extracted from the thorax of a single adult worker per colony using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. DNA samples of *A. m. caucasia* were provided by D. R. Smith, University of Kansas, Lawrence, KS. All other Old and New World DNAs were from single bee extractions made with a phenol-chloroform method (Sheppard and McPheron 1991) in the laboratory of W.S.S.

Restriction Site Analysis. A 485-bp section of the cytochrome *b* gene was amplified using the primers 5′-TAT GTA CTA CCA TGA GGA CAA ATA TC-3′ and 5′-ATT ACA CCT CCT AAT TTA TTA GGA AT-3′ developed by Crozier et al. (1991). PCR amplifications were performed in 5 μl total volume containing 0.5× *Taq* DNA polymerase buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 2 pM of each primer, 0.5 μl of template DNA, and 0.25 U *Taq* DNA polymerase (Promega). The PCR temperature profile was 94°C for 3 min, followed by 30 cycles of 94°C for 15 s, 50°C for 15 s, and 68°C for 5 s. After the final cycle, an additional 10 min at 72°C was performed. After DNA amplification, samples were digested with *Bgl*II (Promega) restriction enzyme using the temperature and buffer conditions recom-

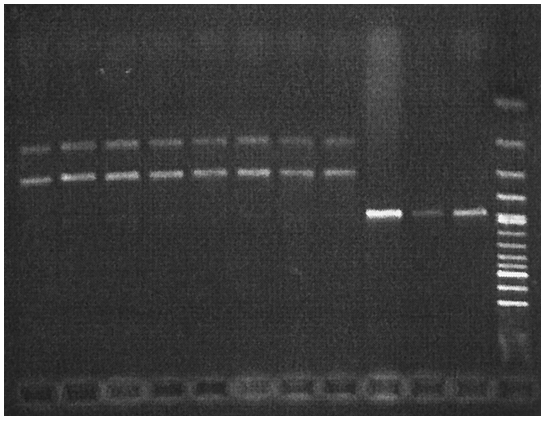

Fig. 1. Samples of mtDNA from Old World honey bee subspecies PCR amplified, using cytochrome *b* primers developed by Crozier et al. (1991), and digested with restriction enzyme *Bgl*II. (1–8) The 291- and 194-bp fragments (PCR product cut) obtained for *A. m. mellifera*, *A. m. ligustica*, *A. m. syriaca*, *A. m. cypria*, *A. m. lamarckii*, *A. m. caucasia*, *A. m. carnica*, and *A. m. iberiensis*. (9–11) The 485-bp fragment (PCR product not cut) obtained for *A. m. iberiensis*, *A. m. intermissa*, and *A. m. scutellata*. (12) Size standard 100-bp ladder (New England Biolabs, Beverly, MA).

mended by the supplier. The total digestion volume was then electrophoresed on a 2% agarose/Tris borate EDTA gel, stained with ethidium bromide, and visualized under UV light. The restriction site was scored as present (PCR product cut) or absent (PCR product not cut), based on the visualization of a two-band pattern or one-band pattern on the gel, respectively (Fig. 1).

Results

Two mitotypes (Fig. 1), as described by Crozier et al. (1991), were observed among the 211 Old World and the 451 United States colonies screened for *Bgl*II variation in the amplified cytochrome *b* gene region (Table 2). All 52 colonies sampled from the eastern Mediterranean mitochondrial lineage (*A. m. caucasia*, *A. m. ligustica*, *A. m. carnica*), and all 64 from Middle Eastern lineage (*A. m. lamarckii*, *A. m. cypria*, *A. m. syriaca*) exhibited a two-band mitotype. All 33 *A. m. scutellata* and 16 *A. m. intermissa* colonies examined from the African mitochondrial lineage shared the one-band mitotype. Colonies of morphologically identified *A. m. mellifera* and *A. m. iberiensis* displayed both mitotypes. Of the 18 sampled colonies of *A. m. mellifera*, the two-band mitotype was observed in 17 colonies and one-band mitotype in 1 colony from Ille-sur-Tet (southern France, close to the border with Spain). Finally, among *A. m. iberiensis* colonies, both mitotypes were relatively common, with 10 two-band and 18 one-band patterns observed. Sequence data from the cytochrome *b* gene (M.A.P., unpublished data) of the *A. m. mellifera* colony from Ille-sur-Tet revealed the typical African mtDNA type common in *A. m. iberiensis*, suggesting that the colony most likely represents introgression between both western European subspecies (Smith et al. 1991).

The restriction analysis of the colonies from the southern tier of the United States revealed a highly asymmetric frequency of the two mitotypes. The non-African (two-band) mitotype was present in a frequency higher than 99% (448 of the 451 colonies examined), whereas only three colonies (one from New Mexico, one from Texas, and one from Georgia) exhibited the African (one-band) mitotype. Since *A. m. intermissa* and probably *A. m. iberiensis* were introduced in the United States, the presence of African mitotypes before the arrival of Africanized bees was not unexpected.

Discussion

The baseline information developed in this study from the Old World colonies indicates that overall, the cytochrome *b*/*Bgl*II assay discriminates the mitochondrial African lineage (one-band mitotype) from the mitochondrial western European, eastern Mediterranean, and Middle Eastern lineages (two-band mitotype). Either one-band or two-band mitotypes were detected for all geographical regions but southern Europe. In the Iberian peninsula, we detected the coexistence of both African and non-African mitotypes. This result is concordant with previous studies conducted in Spain and Portugal, which have reported a mixture of western European and African mitotypes (Smith et al. 1991; Garnery et al. 1992, 1993, 1995; Franck et al. 1998; Clarke et al. 2001).

The amplified cytochrome *b* region does not contain *Bgl*II polymorphisms that discriminate *A. m. scutellata* from *A. m. intermissa* and from some *A. m. iberiensis*. The same findings have been reported for Old and New World colony screening using other rapid PCR-based assays as Ls rRNA/*Eco*RI, COI/*Hinc*II, COI/*Xba*I (Hall and Smith 1991), and COI/*Hinf*I (Nielsen et al. 2000). The COI-COII/*Dra*I test, which has been widely used in Old World (Garnery et al. 1992, 1993, 1995; Moritz et al. 1994; Franck et al. 1998, 2000a, 2001) and New World (Clarke et al. 2001) population genetic and phylogenetic studies, has produced >60 distinct mitotypes in bees sampled from all mitochondrial lineages. Many mitotypes are subspecies specific, but some are shared, occurring at different frequencies in different subspecies. This is the case of the A1, A2, and A4 mitotypes present in *A. m. scutellata*, *A. m. intermissa*, and *A. m. iberiensis* populations (Garnery et al. 1993, Clarke et al. 2001). Therefore, the COI-COII/*Dra* assay is not *A. m. scutellata* diagnostic either. Restriction fragment-length polymorphism analysis of Africanized honey bees using the entire mtDNA and 6-base recognizing enzymes also presents limitations in making subspecies distinctions. For example, the procedure was found to overestimate the proportion of *A. m. scutellata* by ≈25% in bees from Argentina (Sheppard et al. 1999) and 5.3% in bees from the Yucatan Peninsula of Mexico (Clarke et al. 2001). These non-*A. m. scutellata* African mito-

type frequencies are much higher than that found for the United States (<1%) in this study.

The restriction data shown in this paper report, for the first time, the presence of relictual maternal descendants of probable *A. m. intermissa* and/or *A. m. iberiensis* origin in the United States. The strength of this association assumes that no other African mitotypes were introduced before the arrival of Africanized bees in 1990 and that all the variation for *Bgl*II in non-African bees was sampled. This assertion can be further confirmed by sequencing the three United States colonies carrying the African restriction pattern. However, given that the frequency of African non-*A. m. scutellata* mtDNA mitotypes in the southern United States is very low (<1%), the probability of misidentifying the matrilineal origin of African colonies is consequently quite low. Therefore, cytochrome *b*/*Bgl*II can be used to identify maternally Africanized bees with a high degree of reliability and very low estimated error rate.

Previous mtDNA surveys on feral colonies reported *A. m. lamarckii* as the only African type mitochondria present in the United States before Africanization (Schiff and Sheppard 1993, Schiff et al. 1994, Nielsen et al. 1999, 2000). Our baseline data indicate that cytochrome *b*/*Bgl*II variation can discriminate *A. m. lamarckii* and the other two Middle Eastern subspecies (*A. m. syriaca* and *A. m. cypria*) from African *A. m. scutellata*, *A. m. intermissa*, and some *A. m. iberiensis*, but not from the eastern Mediterranean *A. m. caucasia*, *A. m. ligustica*, and *A. m. carnica*, and western European *A. m. mellifera* and some *A. m. iberiensis*. Therefore, if the purpose of honey bee screening is merely identification of maternal descendants of *A. m. scutellata*, then the cytochrome *b*/*Bgl*II assay is as accurate as the two genes (Ls rRNA and COI)/three enzyme digestion (*Eco*RI, *Hinc*I, and *Hinf*I) assay used by the California Department of Food and Agriculture (Nielsen et al. 1999, 2000).

The cytochrome *b*/*Bgl*II assay, developed by Crozier et al. (1991) and further validated in this study, is a simple, rapid, and relatively inexpensive test suited for screening large number of samples. Indeed, this assay, consisting of low volume (5 μl) PCR reactions performed in less than 1 h and digested in 1 h, produces very distinctive and easily identifiable mitotypes (Fig. 1). Because mtDNA provides information about maternal ancestry, for proper identification this marker should be used in conjunction with either morphometrics, as in California (Nielsen et al. 1999), or nuclear markers. With the anticipated sequencing of the complete honey bee genome, there will be opportunities for further refinement of identification methods to include nuclear markers.

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