Honey Bees (Hymenoptera: Apidae) of African Origin Exist in Non-Africanized Areas of the Southern United States: Evidence from Mitochondrial DNA

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ABSTRACT Descendents of *Apis mellifera scutellata* Lepeletier (Hymenoptera: Apidae) (the Africanized honey bee) arrived in the United States in 1990. Whether this was the first introduction is uncertain. A survey of feral honey bees from non-Africanized areas of the southern United States revealed three colonies (from Georgia, Texas, and New Mexico) with a diagnostic African mitochondrial DNA cytochrome *b*/*Bgl*II fragment pattern. To assess maternal origin of these colonies, we developed a primer pair for amplification of a cytochrome *b* fragment and sequenced using internal sequencing primers. Samples of the three reported honey bee colonies plus another 42 representing the 10 subspecies known to have been introduced in the United States were sequenced. Of the three colonies, the colonies from Texas and New Mexico matched subspecies of European maternal ancestry, whereas the colony from Georgia was of African ancestry. Contrary to expectations, the mitotype of the latter colony was more similar to that exhibited by sub-Saharan *A. m. scutellata* than to the mitotypes common in north African *A. m. intermissa* Maa or Portuguese and Spanish *A. m. iberiensis* Engel. This finding was consistent with anecdotal evidence that *A. m. scutellata* has been sporadically introduced into the United States before the arrival of the Africanized honey bee from South America.

KEY WORDS Africanized honey bee, *Apis mellifera*, mitochondrial DNA, cytochrome *b*, mitotype

The western honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), is naturally distributed in central and western Asia, Africa, and Europe. In its wide endemic range, *A. mellifera* has diverged into >26 subspecies (Ruttner 1988, Engel 1999, Sheppard and Meixner 2003). Based on analysis of mitochondrial DNA (mtDNA), the subspecies have been further grouped into at least four main mitochondrial lineages: A (African), C (eastern Mediterranean), M (western European), and O (Middle Eastern) (Cornuet and Garnery 1991, Garnery et al. 1992, Arias and Sheppard 1996, Franck et al. 2000a, Palmer et al. 2000). Over the past 400 yr, several subspecies were introduced into the United States, with some exemplars from each mitochondrial lineage. In the early to mid-1600s, *Apis mellifera mellifera* L. *and* probably *Apis mellifera iberiensis* Engel were brought from western Europe by English and Spanish settlers (Sheppard 1989a,b). Between 1859 and 1922, beekeepers imported seven more subspecies from varying geographic origins: *Apis mellifera caucasia* Pollmann, *Apis mellifera ligustica* Spinola, and *Apis mellifera carnica* Pollmann from Europe; *Apis mellifera intermissa* Maa and *Apis mellifera lamarckii* Cockerell from north Africa; and *Apis mellifera cypria* Pollmann and *Apis mellifera syriaca* Skorikov from the Middle East (Sheppard 1989a,b). In the 1990s, in what is the most publicized introduction, the decendents of the sub-Saharan African subspecies, *Apis mellifera scutellata* Lepeletier, arrived and expanded into the southwestern United States. Before the Africanization process in this area, honey bees of the region primarily exhibited genes of European origin (Loper et al. 1999; Pinto et al. 2004, 2005; Schiff et al. 1994; Schiff and Sheppard 1995, 1996).

Before the arrival of descendents of *A. m. scutellata* (referred to as Africanized honey bees) in the United States, scientists developed various molecular and nonmolecular methods for their detection and identification. Among the molecular methods, polymerase chain reaction (PCR) amplification of specific regions

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of mtDNA followed by digestion of those fragments with restriction enzymes has been widely used in colony screening for research and regulatory purposes (Sheppard and Smith 2000). Most PCR-based assays involve amplification of more than one gene fragment followed by digestion with more than one restriction enzyme for maternal identification of Africanized honey bees (Hall and Smith 1991, Nielsen et al. 2000).

Recently, we validated a PCR-based assay (Pinto et al. 2003) previously proposed by Crozier et al. (1991), which requires a single enzyme digestion (*Bgl*II) of a single PCR-amplified fragment of the cytochrome *b* gene. We screened a large Old World honey bee collection representing the subspecies known to have been introduced in the United States and showed that the *Bgl*II polymorphism discriminated maternal descendents of *A. m. scutellata* (one-band pattern) from *A. m. mellifera*, *A. m. caucasia*, *A. m. ligustica*, *A. m. carnica*, *A. m. lamarckii*, *A. m. cypria*, *A. m. syriaca*, and some *A. m. iberiensis* (two-band pattern). The *Bgl*II polymorphism did not differentiate *A. m. scutellata* from *A. m. intermissa* and some *A. m. iberiensis* (Pinto et al. 2003).

Further testing of a large feral honey bee collection from non-Africanized areas of the southern United States showed a very low frequency (three of 451 colonies) of the one-band mitochondrial haplotype (mitotype) characteristic of *A. m. scutellata*, *A. m. intermissa*, and some *A. m. iberiensis.* The results suggested that the cytochrome *b*/*Bgl*II assay could be used to identify maternally Africanized honey bees in the United States with a high degree of reliability and low estimated error rate (Pinto et al. 2003).

In this study, we investigated the maternal origin of the three aforementioned colonies that exhibited the one-band ctyochrome *b*/*Bgl*II pattern by the increased resolution afforded by sequence analysis of cytochrome *b.* We hypothesized that the three colonies are maternal descendants of either *A. m. intermissa* or *A. m. iberiensis* introduced in historical times. To address that point, an mtDNA fragment of 743 bp of cytochrome *b*, which includes the diagnostic restriction site, was sequenced from samples of the three southern United States colonies and from 42 honey bees representing the 10 subspecies introduced in the United States.

Materials and Methods

Samples and DNA Extraction. A range of Old World and New World colonies were examined. The Old World samples were made up of 42 honey bee workers, each representing a different colony that had been morphometrically identified to subspecies (with the exception of *A. m. caucasia*). Subspecies, collection locations, and samples sizes are as follows: *A. m. mellifera*, France (five); *A. m. carnica*, Germany, Slovenia, and Austria (three); *A. m. ligustica*, Italy (four); *A. m. caucasia*, Turkey (three); *A. m. syriaca*, Syria (two); *A. m. cypria*, Cyprus (two); *A. m. lamarckii*, Egypt (three); *A. m. iberiensis*, Portugal and Spain (nine); *A. m. intermissa*, Morocco (five); and *A. m. scutellata*, Kenya (six). Five honey bee samples from the New World were analyzed. Three samples were the feral colonies from non-Africanized areas of Georgia, New Mexico, and Texas identified in Pinto et al. (2003) as having an African mitotype. One sample was from an Africanized honey bee colony from Brazil, chosen to represent the Africanized honey bee, and one sample was from the colony chosen for the Honey bee Nuclear Genome Sequencing Project.

DNA was extracted from the thorax of a single honey bee worker from the sample from Texas and the Africanized sample using a QIAamp DNA Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. With the exception of DNA's from *A. m. caucasia*, which were provided by D. R. Smith (University of Kansas, Lawrence, KS), all other Old and New World DNAs were made from single honey bee extractions with the phenol-chloroform method of Sheppard and McPheron (1991) in the laboratory of W.S.S.

DNA Amplification and Sequencing. The primers used in the cytochrome *b*/*Bgl*II assay (Crozier et al. 1991, Pinto et al. 2003) produce fragments that typically are not amenable to direct sequence analysis. To allow sequence analysis of this region, two new primers were developed by J.C.P. The first primer, 5′-ACT CCA TTT GAT TTA ATT GAA GG-3′, complements the forward primer of Crozier et al. (1991) (5′-TAT GTA CTA CCA TGA GGA CAA ATA TC-3′) to produce a fragment of ≈1,200 bp. The forward primer of Crozier et al. (1991) lies 397 bp inside the cytochrome *b* gene (Crozier and Crozier 1993), whereas the new reverse primer begins at base pair 335 of the ND1 gene. The second primer, 5′-TTG CAA ATC CAA TAA ATA CTC C-3′, was used as the forward internal sequencing primer, whereas 5′-ATT ACA CCT CCT AAT TTA TTA GGA AT-3′ from Crozier et al. (1991) was used as the internal reverse primer. These primers lie 778 bp (forward sequencing primer) and 881 bp (reverse sequencing primer), respectively, from the beginning of the cytochrome *b* gene and span the polymorphic *Bgl*II cut site. PCR amplifications were performed in 25-μl total volume containing 0.5X *Taq*DNA polymerase buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 2 pM each primer, 5–50 ng of DNA, and 1.25 U of *Taq*DNA polymerase (Promega). Alternatively, 5–50 ng DNA was used in a 50-μl reaction volume with 10 mM Tris-Cl, pH 8.7, 50 mM KCI, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 10 μM each of the two primers, and 1.25 U of AmpliTaq DNA polymerase (PerkinElmer Life and Analytical Sciences, Boston, MA). Reactions were heated to 95°C for 3 min followed by 36 cycles of amplification. Each cycle consisted of 45 s at 95°C, 30 s at 50°C, and 2 min at 70°C. Primers and nucleotides were removed from PCR reactions by using the QIAquick PCR purification kit (QIAGEN). Fragments were sequenced with Big Dye (Applied Biosystems, Foster City, CA) following protocols suggested by the supplier. Sequencing reactions were cleaned with Sephadex columns and then run in an ABI Prism 377 automated DNA sequencer (Applied Biosystems).

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3208 INTER	-	-	C	G	-	C	-	-	-	-	-	-	**C**	-	T	-	-	-	-	-	-	-	-	-	-	-	-	T	C	-	-	-	-	-	-	-	-	-
5343 INTER	-	-	C	G	-	C	-	-	-	-	-	-	**C**	-	T	-	-	-	-	-	-	-	-	-	-	-	T	T	C	-	-	-	-	-	-	-	-	-
2161 INTER	-	-	C	G	-	C	-	-	-	-	-	-	**C**	-	T	-	-	-	-	-	T	-	-	-	-	-	T	T	C	-	-	-	-	-	-	-	-	-
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8554 SCUT	-	-	C	-	-	C	-	-	-	-	-	-	**C**	-	T	-	-	-	-	-	T	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-
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99E10 LAMAR	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	C	-	-	-	T	-	-	-	-	-	-	-	-	G	-	-	-	-	-
99E13 LAMAR	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	C	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Fig. 1. Cytochrome *b* region variable sites. The numbers correspond to the *A. m. ligustica* positions as in Crozier and Crozier (1993). The dashes indicate nucleotide identity. The key to each taxon is described in Fig. 2. The nucleotides in *Bgl*II cut site and the three colonies from the southern United States are marked in bold.

Sequence Alignment and Phylogenetic Analysis. Sequences were aligned and analyzed using Sequencher software (Genecodes Corp., Ann Arbor, MI). Alignments, which resulted in sequences of 743 bp of cytochrome *b*, were transferred to PAUP 4.0b10 (Swofford 2002) for phylogenetic analysis. Using this software, neighbor joining (Saitou and Nei 1987) and parsimony analyses (Swofford 2002) were performed taking the "*A. m. scutellata*" cluster as the monophyletic sister clade (Arias and Sheppard 1996). Heuristic analysis was done using 25 randomized inputs of data from individual taxa. Data were treated as unordered, and the tree-bisection-reconnection (TBR) branch-swapping algorithm was used. Bootstrap parsimony was generated for 1,000 replicates using the criteria described for the heuristic analysis. Bremer support (Bremer 1988, 1994) was calculated using TreeRot.v2 (Sorenson 1999).

Results

The 743-bp fragment of cytochrome *b* sequenced for 47 honey bees revealed a strong A+T bias (80%). Sequence variation was observed at 38 sites of which 29 were parsimony informative. Transition mutations accounted for 86.8% (33 of 38) of the total mutations. C↔T mutations were found to account for 90.9% (30 of 33) of the transitional changes with A↔G mutations, making the remaining 9.1% (3 of 33) of the transitions. Two of the four possible classes of transversion mutations were detected. The frequency of A↔C and A↔T transversions were 7.9% (3 of 38) and 5.3% (2 of 38), respectively. Examination of the *Bgl*II cut site of the three colonies from the southern United States revealed that the one-band mitotype was generated by convergence. The colony from Georgia lost the cut site by a third position (T→C) transition mutation, whereas the colonies from New Mexico and Texas lost the cut site by a first position (C→A) transversion mutation (Fig. 1).

Twenty seven mitotypes were observed among the 47 honey bees sequenced. Portions of the sequences with the variable sites of the 27 mitotypes are shown in Fig. 1. The entire sequences are deposited in GeneBank under accession numbers EF184020–EF184064. The mitotypes were distributed among the same four mitochondrial lineages (Fig. 2) reported by others (Garnery et al. 1992, Arias and Sheppard 1996, Franck et al. 2000a, Palmer et al. 2000). The average ± SD of nucleotide divergence estimated using Kimura two-parameter distance is provided in Table 1. Because only moderate philopatry among honey bees relative to subspecies within lineage is suggested by the phylogeny, Table 1 involves only calculations of the level of divergence within and among lineages and does not include calculations among subspecies. Within-lineage divergence was low, ranging from 0.116 to 0.414%. This result contrasts with higher divergence values among lineages, which ranged from 1.559 to 1.920%.

The results of neighbor joining and bootstrap parsimony analyses are shown in Fig. 2A and B, respectively. The topology of the trees supports the existence of four mitochondrial lineages, which relate to the African (A), western European (M), eastern Mediterranean (C), and Middle Eastern (O) lineages. Support for the division of lineage A into two sublineages, one sublineage from northern Africa (*A. m. intermissa* and most *A. m. iberiensis*) and one sublineage from sub-Saharan Africa (*A. m. scutellata* and Africanized honey bee), is provided by both phylogenetic trees. All samples of *A. m. caucasia*, *A. m. carnica*, *A. m. cypria*, all but one sample of *A. m. ligustica*, and one sample of *A. m. scutellata* formed lineage C. All samples of *A. m. mellifera*, two samples of *A. m. iberiensis*, and the exceptional sample of *A. m. ligustica* formed lineage M, whereas the samples of *A. m lamarckii* and *A. m. syriaca* formed lineage O. Variation within the four lineages was low with most of the branching observed in the neighbor joining analysis decaying away in the bootstrap analysis.

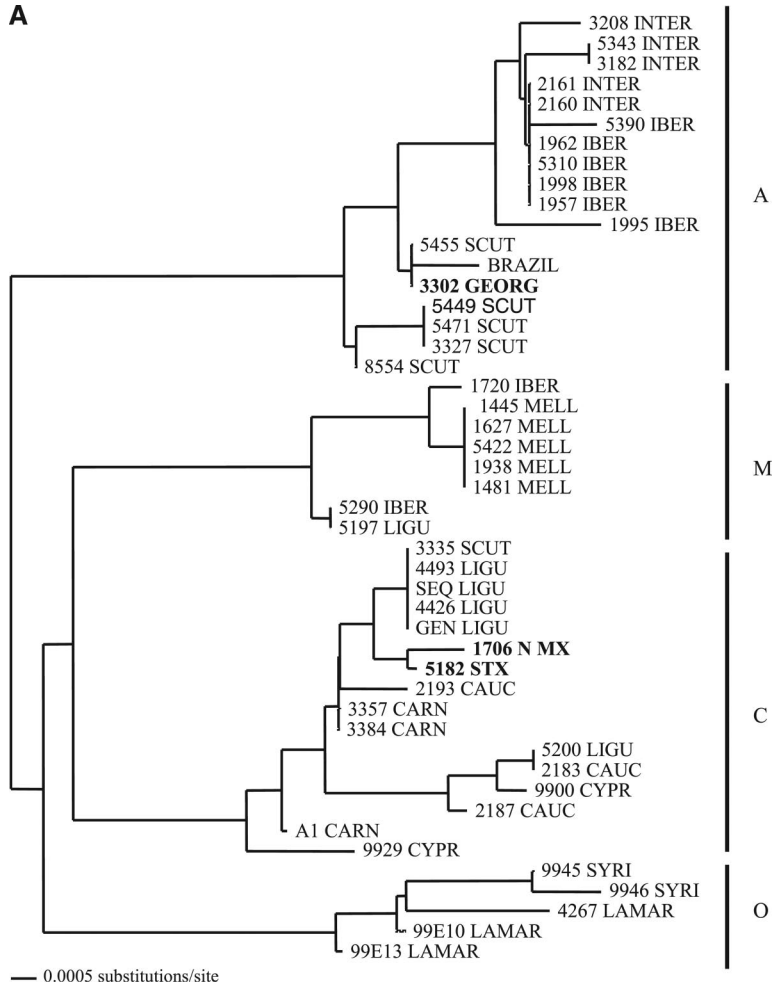

Fig. 2. Phylogenetic trees inferred from cytochrome *b* sequence data. Trees were obtained using the neighbor joining method (A) and the parsimony method (B) taking "*A. m. scutellata*" cluster as the outgroup as a monophyletic sister clade. Bootstrap (top) and Bremer support (bottom) values are indicated for each node (B). Letters A, M, C, and O refer to African, western European, eastern Mediterranean, and Middle Eastern mitochondrial lineages, respectively. The key to each taxon is as follows: INTER, *A. m. intermissa*; SCUT, *A. m. scutellata*; IBER, *A. m. iberiensis*; MELL, *A. m. mellifera*; LIGU, *A. m. ligustica*; CAUC, *A. m. caucasia*; CARN, *A. m. carnica*; CYPR, *A. m. cypria*; SYRI, *A. m. syriaca*; LAMAR, *A. m. lamarckii*; BRAZIL, Africanized honey sample from Brazil; GEN LIGU, *A. m. ligustica* from GeneBank (Crozier and Crozier 1993); SEQ LIGU, *A. m. ligustica* sample, for which nuclear genome is being sequenced; GEORG, sample from Georgia; N MX, sample from New Mexico; and STX, sample from Texas. The three latter samples, which were collected from non-Africanized honey bee areas from the southern United States, exhibited the "African" *Bgl*II mitotype as in Pinto et al. (2003).

Within this framework, the sampled colony from Georgia clustered with the A lineage (sub-Saharan group), whereas the sampled colonies from southern Texas and New Mexico were placed in the C lineage.

Discussion

In this study, we showed that the three honey bee colonies collected in non-Africanized areas of Georgia, Texas, and New Mexico that shared the one-band ctyochrome *b*/*Bgl*II pattern (Pinto et al. 2003) belong to two distinct mitochondrial lineages (Fig. 1). The colony from Georgia clustered with honey bee subspecies of the African lineage (A), whereas the colonies from New Mexico and Texas clustered with honey bee subspecies of the eastern Mediterranean lineage (C). The loss of the *Bgl*II cut site, which produced the one-band pattern, resulted from distinct mutation types. The colony from Georgia lost the cut site by a third position (T→C) transition mutation seen in *A. m. scutellata*, *A. m. intermissa*, and *A. m. iberiensis*, whereas the colonies from New Mexico and Texas lost the cut site by a first position (C→A) transversion mutation observed in no other individuals. These results led to rejection of the hypothesis that all three colonies that exhibit the one-band *Bgl*II pattern are maternal descendants of either *A. m. intermissa* or *A. m. iberiensis*, because two of them were

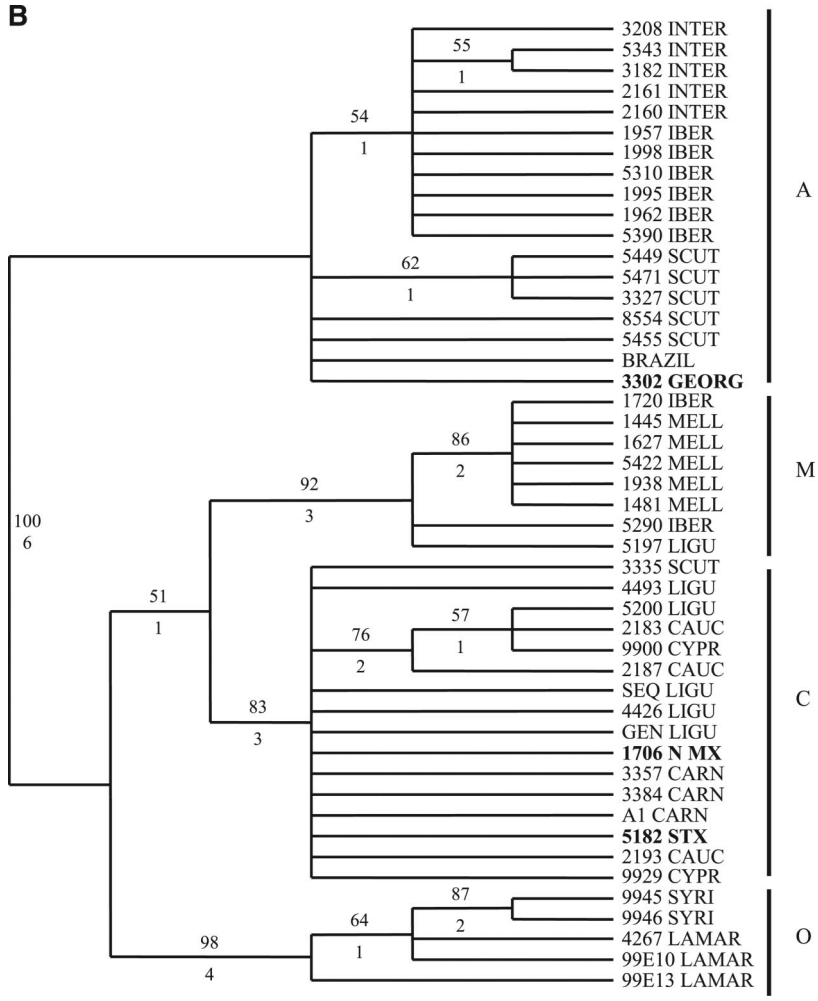

Fig. 2. *Continued.*

derived from lineage C. Therefore, the reference by Pinto et al. (2003) to the mitochondrial origins of the colonies exhibiting the one-band ctyochrome *b*/*Bgl*II pattern as African is not entirely correct as the one-band pattern may be displayed by colonies of either African or European maternal ancestry. Colonies exhibiting both maternal origins for the one-band mitotype were found in non-Africanized areas of the southern United States. Therefore, the frequency of the African mitotype reported by Pinto et al. (2003) was overestimated (1 instead of 3 of 451 colonies).

Table 1. Average (% ± SD) sequence divergence within and between African (A), eastern Mediterranean (C), western European (M), and Middle Eastern (O) lineages

	A	C	M	O
A	0.319 ± 0.206			
C	1.786 ± 0.205	0.414 ± 0.301		
M	1.855 ± 0.172	1.226 ± 0.289	0.116 ± 0.130	
O	1.920 ± 0.271	1.585 ± 0.257	1.559 ± 0.144	0.379 ± 0.178

The present data generated from a 743-bp sequence support the existence of four mitochondrial lineages in *A. mellifera*, as did data produced with different mtDNA genes and shorter sequences (Garnery et al. 1992, Arias and Sheppard 1996, Franck et al. 2000a, Palmer et al. 2000). The subspecies composition of each lineage also agrees with other mtDNA studies. Consistent with previous reports based on restriction and sequence data (Cornuet and Garnery 1991; Smith et al. 1991; Garnery et al. 1992, 1995; Arias and Sheppard 1996; Franck et al. 1998, 2000a, 2001), the African lineage is divided into a northern group, which clustered *A. m. intermissa* and most *A. m. iberiensis*, and a sub-Saharan group, which clustered *A. m. scutellata* and the Africanized honey bee colony (Fig. 2). All colonies of *A. m. mellifera* and two colonies of *A. m. iberiensis* formed a single group, matching the composition of the western European lineage reported by others (Garnery et al. 1992, Arias and Sheppard 1996, Franck et al. 1998). The presence of two divergent mitotypes in *A. m. iberiensis*, one mitotype belonging

to the African lineage and the other mitotype belonging to the western European lineage, has been explained by secondary contact between populations from both lineages in the Iberian Peninsula (Smith et al. 1991, Garnery et al. 1995). *Apis m. cypria* formed a group with the colonies of *A. m. caucasia*, *A. m. ligustica*, and *A. m. carnica.* The mitotype of the sample used for the honey bee genome project (SEQ LIGU in Fig. 2) was found to be identical to most *A. m. ligustica* in our sample. Finally, the group formed by *A. m lamarckii* and *A. m. syriaca* supports a proposed fourth mitochondrial lineage (Arias and Sheppard 1996; Franck et al. 2000a, 2001; Palmer et al. 2000).

Two colonies were seemingly misplaced in the phylogenetic trees (Fig. 2). The sample 5197 morphometrically identified as *A. m. ligustica* exhibited a mitotype characteristic of the western European lineage. However, a more detailed study of *A. m. ligustica* from Italy showed that populations of this subspecies express relatively high frequencies of the mitotype commonly associated with *A. m. mellifera* (Franck et al. 2000b). Apparently, multiple glaciations and isolation of both mitochondrial haplotypes into Ligurian refugia established the mitochondrial polymorphism within this subspecies. Sample 3335, although morphometrically identified as *A. m. scutellata*, carried a *A. m. ligustica*-type mitotype. The Italian honey bee, *A. m. ligustica*, is highly favored for apiculture and, consequently, has been exported worldwide where it hybridized with local honey bees (De la Rúa et al. 1998, 2002). Given the history of honey bee hybridization in the Old and New Worlds, we suggest that the pattern exhibited by sample 3335 likely originated through importation of a *A. m. ligustica* queen followed by backcrossing with *A. m. scutellata* drones.

In contrast with the four well-defined mitochondrial lineages, branches within lineages were shallow and unstable (Fig. 2). Thus, the phylogenetic analysis did not provide the resolution needed to determine maternal origin of the three colonies at the subspecies level. The colonies from New Mexico and Texas could have descended from *A. m. ligustica*, *A. m. carnica*, *A. m. caucasia*, or *A. m. cypria* subspecies introduced in the 19th century. We suggest that the two colonies were likely maternally derived from *A. m. ligustica* and/or *A. m. carnica* because before Africanization most commercial and feral honey bee colonies in the southern United States were of these ancestries (Schiff et al. 1994; Schiff and Sheppard 1995, 1996). The sequence of the mitotype from Georgia was not referable to *A. m. iberiensis* or *A. m. intermissa* based on our sampling of the subspecies. Although not referable to north African mitotypes, the sequence of the Georgia sample was identical with that from one colony of *A. m. scutellata* (Fig. 2). This finding can be explained by either 1) additional variation existing in *A. m. intermissa* and *A. m. iberiensis* that includes the mitotype of the Georgia sample, or 2) that the matrilineal origin of the Georgia sample was sub-Saharan. The latter hypothesis suggests undocumented introductions of *A. m. scutellata* mitotypes into the southeastern United States in historical or recent times. Assertions of maternal origin made herein are based on the assumption that only the 10 aforementioned subspecies were introduced in the United States.

Here, we have shown how the resolution provided by sequence data can change the contours of a story. Although restriction data, associated with historical records, suggested that the three colonies from the non-Africanized areas of the southern United States were of northern African maternal ancestry (Pinto et al. 2003), sequence data revealed that the Texas and New Mexico colonies had European mitochondrial haplotypes and only the Georgia colony had an African mitotype. However, instead of being of north African origin, the maternal ancestry of the colony from Georgia was likely derived from sub-Saharan *A. m. scutellata.* The sample itself was collected in 1980 from a feral swarm hived by a beekeeper (W.S.S.). The colony exhibited no unusual defensive behavior during the time of collection. The means by which this colony was introduced in Georgia is unknown. Introductions of Africanized honey bees have occurred at major ocean ports at least in California (Berenbaum 1995) and Florida (Bronson 2005) before Africanization. The same could have happened in a port in Georgia. Other types of human-assisted dispersion, such as movement of colonies or queens, also could account for the finding.

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