Parental Analysis of Introgressive Hybridization Between African and European Honeybees Using Nuclear DNA RFLPs

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ABSTRACT

African honeybees, introduced into Brazil 33 years ago, have spread through most of South and Central America and have largely replaced the extant European bees. Due to a paucity of genetic markers, genetic interactions between European and African bees are not well understood. Three restriction fragment length polymorphisms (RFLPs), detected with random, nuclear DNA probes, are described. The polymorphisms are specific to bees of European descent, possibly specific to certain European races. Each European marker was found present at a high frequency in U.S. colonies but absent in South African bees. Previous mitochondrial DNA studies of neotropical bees have revealed negligible maternal gene flow from managed European apiaries into feral African populations. The findings reported here with nuclear DNA show paternal gene flow between the two but suggest asymmetries in levels of introgressive hybridization. Managed colonies in southern Mexico, derived from European maternal lines, showed diminished levels of the European nuclear markers, reflecting significant hybridization with African drones. The European alleles were present only at low frequencies in feral swarms from the same area. The swarms were of African maternal descent. In Venezuelan colonies, also derived from African maternal lines, the European markers were almost totally absent. The results point to limited paternal introgression from European colonies into the African honeybee populations. These findings dispute other views regarding modes of Africanization.

HONEYBEES (*Apis mellifera* L.) of European descent have been repeatedly imported to the Americas. Early introductions included the subspecies *A. m. mellifera* (Black bee of Northern Europe) and *A. m. iberica* (Spanish), and later *A. m. ligustica* (Italian), *A. m. carnica* (Carniolan), and *A. m. caucasia* (Caucasian) (for honeybee systematics, see RUTTNER 1988). The Italian race has become predominant in commercial beekeeping throughout the Americas (PELLET 1938; OERTEL 1976).

In 1956–1957 African honeybees (*A. m. scutellata*, formerly believed to be of the race *A. m. adansonii*; RUTTNER 1988) were introduced into Brazil. The purpose was to provide a honeybee more adapted to the tropics than the European subspecies (KERR 1967; WOYKE 1969; MICHENER 1975). Since then, African bees have spread through most of South and Central America. In the tropics, European bees have not established large self-sustaining feral populations. Their presence has been limited mostly to managed apiaries. In contrast, African honeybees have not only built substantial feral populations but have also largely replaced European bees in apiaries. The migrating front of African bees will enter the United States in 1990 (TAYLOR 1985a). Due to temperate climate constraints, they are expected to be largely restricted to, but well established in, the southern tier of states (TAYLOR and SPIVAK 1984).

African honeybees are easily provoked and exhibit extremely defensive stinging. They tend to swarm and abscond frequently (MICHENER 1975; WINSTON, TAYLOR and OTIS 1983). African bees are difficult to manage and are not amenable to the large scale movement required to meet U.S. pollination needs, estimated at an annual value from $9 to $19 billion (ROBINSON, NOWOGRODSKI and MORSE 1989; MCDOWELL 1984). If African bees cannot be excluded from commercial apiaries, beekeepers will abandon their businesses, and the numbers of colonies available for pollination will become inadequate. Managed sources of European bees, that might remain competitive against feral African bee population buildup, would disappear (TAYLOR 1985a). African bees pose a real, although sensationalized, public health threat (TAYLOR 1986).

Mechanisms responsible for African bee takeover in the tropics are not well understood. A number of reports contend that the primary driving force of "Africanization" is paternal gene flow from African drones into the extant European populations (RINDERER *et al.* 1985, 1987a; ERICKSON, ERICKSON and YOUNG 1986; RINDERER 1986). Consequently, the term "Africanized bees" is used to refer to all African-derived bees in the Americas, and the neotropical African population has been called a "hybrid swarm" (RINDERER 1986). However, the overwhelming dom-

inance and persistence of African behavioral qualities have been difficult to reconcile with this view and suggest maintenance of the African genotype (MICHENER *et al.* 1972; MICHENER 1975; TAYLOR 1985a, 1988; FLETCHER 1988). Regulatory efforts have been based on the assumption that gene flow and hybridization between African and European bees are not limited (RINDERER 1986; RINDERER *et al.* 1987b; TEW, BARE and VILLA 1988). However, the actual extent of genetic exchange between African and European bees has not been well documented and remains controversial.

African and European bees are difficult to distinguish by both phenotype (morphometrics) and genotype (for discussion on identification, see HALL 1986, 1988; DALY 1988). Among protein polymorphisms reported previously, only five have significant frequency differences between African and European honeybee populations (SYLVESTER 1982, 1986; SPIVAK *et al.* 1988). None of the alleles is subspecies specific. Limited protein polymorphism is generally characteristic of Hymenopteran insects (bees, ants and wasps), probably due to the low population sizes of reproductives (GRAUR 1985) and possibly due to selection against haploid males (CROZIER 1977). The paucity of genetic markers has limited understanding of African and European honeybee interactions and the mechanisms of African honeybee spread.

Nuclear DNA restriction fragment length polymorphisms (RFLPs) are being sought that can distinguish African and European bees and identify their hybrids (HALL 1986, 1988). To determine the African or European specificity of the polymorphisms, a number of Old and New World honeybee populations have been examined. Reported here are three RFLP markers that appear to be specific to bees of European descent and, perhaps, specific to certain European races.

Recently, mitochondrial DNA analyses have revealed that neotropical African bees have spread as continuous African maternal lineages and that European maternal gene flow into the feral African population has been negligible (HALL and MURALIDHARAN 1989; SMITH, TAYLOR and BROWN 1989). During characterization of the nuclear DNA markers, patterns of paternal introgression between African and European bees have been revealed. The colony parental analyses suggest asymmetries in favor of African bee genotypes. The nuclear and mitochondrial DNA findings together do not support the "hybrid swarm" perception of neotropical African bees.

Honeybee life history: In following this report, it is important to consider honeybee colony and population structure. A colony is an expanded family, usually headed by a single mother queen. Matings take place in the air, several kilometers from the hive. Typically, within her first 2 weeks as an adult, a queen mates with an average of 6–17 random drones (TABER 1954; ADAMS *et al.* 1977). Sperm stored within her at that time are used through her reproductive life. Unfertilized eggs develop into haploid males (drones) and fertilized eggs into diploid females (workers and queens). Colony workers thus represent a collection of super sisters (same patriline) and half sisters (different patrilines). Paternal gene flow occurs as drones mate with queens from distant colonies. After mating, however, queens return to their hives. Maternal gene flow occurs through swarming, whereby a queen and large contingent of workers fly to another location to establish a new colony. European swarms migrate usually no more than 1.6 km, but possibly as far as 5 km, whereas African swarms may migrate as far as 160 km (LINDAUER 1955; TAYLOR 1977).

Because of the European origin of U.S. bees, they will be referred to as "European," while recognizing that they probably represent a mixture of subspecies. Likewise, "African bees" will be used when referring to African maternal lines in the Americas, without necessarily implying that they are genetically identical to populations in Africa. The term "Africanized bees" will be limited to European maternal lines mated to African drones.

MATERIALS AND METHODS

Most honeybee samples were collected as either larvae or pupae. Worker larvae were distinguished by the cell sizes in which they were reared. All drone samples tested were pupae which had morphological features clearly distinguishable from those of workers. Shortly after collection in the field, brood samples were either frozen or equilibrated in several changes of ice cold 95% ethanol for 2–4 days. Alcohol preservation allowed shipping or transport at ambient temperatures. For storage thereafter, samples were kept at -20°. Use of brood rather than adults ensures their colony origin, since adults can drift among hives. Furthermore, soft larval and pupal tissue facilitates DNA isolation.

Samples were obtained from a total of 126 colonies from Europe, the United States, Mexico, Costa Rica, Venezuela, and South Africa. European samples came from France (4 colonies of *A. m. mellifera*), Italy (3 colonies of *A. m. ligustica*), and Russia (2 colonies of *A. m. caucasia*) (all provided by B. VAISSIERE, Texas A&M, and J.-M. CORNUET, INRA Monfavet, France). U.S. samples included a closed breeding population (PAGE, ERICKSON and LAIDLAW 1982; SEVERSON, PAGE and ERICKSON 1986), maintained in Arizona (total of 20 colonies, provided G. WALLER and E. ERICKSON, USDA-ARS, Tucson) and in Florida (5 colonies, provided by N. GARY, University of California, Davis). The population was comprised of 25 European stocks from a USDA-ARS laboratory at Madison, Wisconsin, and commercial apiaries from the following states: Alabama, California, Florida, Georgia, Hawaii, Louisiana, Mississippi, North Carolina, Tennessee, and Texas (R. PAGE, personal communication). Additional colonies from Florida (total of 15) and from California (total of 4) were also tested. South African samples, collected in late 1987, came from near Pretoria, Douglas, Durban, Port Elizabeth (*A. m. scutellata*, total of 15) and Cape Town (*A. m. capensis*, total of 2) (provided by G. PRETORIUS, Pretoria,

Republic of South Africa, and W. VAN DER PUT, Edinburg, Texas). Samples from experimental apiaries near Acarigua, Venezuela, were collected between 1986 and 1988 (provided by R. HELLMICH, J. VILLA, A. COLLINS and T. RINDERER, USDA-ARS, Baton Rouge). These included feral swarms caught just prior to sampling (total of 20 tested with probes P130 and P138, 10 tested with P170) and managed colonies established from swarms 2–24 months earlier (total of 12). In January 1988, feral swarms (total of 13) were collected near Tapachula, Mexico, from bait hives maintained by the Mexican agency SARH (Secretary of Agriculture and Hydrologic Resources). In the same region, at the same time, samples were collected from an apiary established prior to African bee arrival (total of 6 samples) and from an apiary established during their arrival (total of 2). Samples from one swarm and from two colonies previously established from swarms came from Costa Rica (provided by M. SPIVAK and O. TAYLOR, Kansas University).

Samples in alcohol were equilibrated in 0.32 M sucrose, 50 mM Tris-HCl (pH 7.3), 10 mM $MgCl_2$ (STM) overnight at 4°, with gentle agitation. Colony DNA samples came from a mixture of 10–20 sibling workers. DNA isolations proceeded according to the protocol described previously (HALL 1986). When DNA was isolated from individuals, volumes were scaled down. Each larva or pupa was homogenized with a rotating Teflon pestle (2 ml) in STM (0.5 ml per drone; 0.3 ml per worker), and the homogenate was centrifuged at 6.5K rpm for 5 min (microfuge). The pellet was resuspended in 75 mM NaCl, 10 mM Tris-HCl (pH 7.8), 10 mM EDTA (STE) (0.5 ml per drone; 0.3 ml per worker), then 20% SDS, 0.5 M EDTA, and protease K (15 mg/ml) were added (each: 30 μl per drone; 20 μl per worker). The mixture was incubated at 60° for 30 min then at 40° for 12 hr. The suspension was centrifuged at 13K for 10 min (microfuge). The supernatant was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and then with chloroform/isoamyl alcohol (24:1). After each extraction, phases were separated by centrifugation at 13K rpm for 10 min (microfuge). Centrifugation speeds were high and times were long to allow removal of translucent particles containing α-amylase-sensitive material (presumably glycogen), more abundant in larvae. DNA was precipitated with two volumes ethanol at −20° overnight and then centrifuged at 6.5K rpm for 5 min. The DNA pellet was air-dried and dissolved in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (TE) (50 μl/worker, 100μl/drone). Boiled pancreatic RNase was added (16 μg/100 μl), and the sample was incubated at 37° for 30 min. Sufficient DNA can be obtained from a single drone pupa for six to eight separate restriction endonuclease digests.

Some adult samples were also used (10 Venezuelan swarms). About 20 frozen workers were minced with a razor blade. The pieces were suspended in 5 ml of 0.025 M citric acid at 4° in a 15 ml polypropylene test tube and homogenized further with a Teckmar tissumizer (100EN shaft) (TAYLOR, YEOMAN and BUSCH 1975). Five to six 15-sec bursts were used, at number 70 setting. Samples were kept on ice during the homogenization. The homogenate was centrifuged at 3.5K rpm, the supernatant removed and the pellet suspended in STE. The procedure continued as previously described (HALL 1986).

A pBR322 plasmid library, containing *Pst*I-digested inserts of random European honeybee DNA, served as a source of cloned probes. Probe formation and selection, restriction endonuclease digestions, electrophoretic separations, alkaline blotting, probe labeling, prehybridizations, hybridizations, washes and X-ray film exposures followed standard protocols described or cited previously (HALL 1986). For exposures, blots were sealed wet in bags to allow probe removal later. Blots were stripped of radioactive probes with 0.4 N NaOH and neutralized in 0.1 × SSC, 0.1% SDS, 0.2 M Tris-HCl (pH 7.5), each step for 30 min at 45° (Amersham technical note).

RESULTS

Clones of random, honeybee, nuclear, DNA fragments were used as probes to detect RFLPs between European and African derived honeybees. The search for polymorphisms involved an initial comparison of DNA samples from a single U.S. colony and from either a Costa Rican or South African colony, each digested by nine separate restriction endonucleases. A total of 28 probes were individually tested, 19 of which showed polymorphisms with one or more enzymes, for a total of 61 RFLPs. Multiple samples from Old and New World honeybee populations were then examined for 26 of these RFLPs seen with 12 probes. Three polymorphisms, detected with three probes, were found to be present only in European-derived populations. These RFLP alleles were not present in all European bees but will be referred to as "European."

Characterization of the DNA polymorphisms: Advantage was taken of haploid males to clarify fragment patterns generated by the polymorphisms. In DNA digests from individual drones, analyzed by each probe, the relationship between the European fragments and the fragments to which they were allelic was apparent (Figure 1). In *Alu*I-digested samples, probe P130 detected a 1.2-kb fragment allelic to a 1.6-kb fragment (alleles *P130-E* and *P130-O*, respectively). Probe P138 revealed a 1.5-kb fragment, generated by *Alu*I, allelic to a 1.0-kb fragment (alleles *P138-E* and *P138-O*, respectively). Numerous faint bands were also seen with probe P138 in *Alu*I digests. Probe P170 detected a 7.2-kb fragment, generated by *Nci*I, allelic to a 3.6-kb fragment (alleles *P170-E* and *P170-O*, respectively). The presence of one fragment corresponding to the absence of only one other suggested that the polymorphisms were due to single base changes outside the probe regions. The RFLPs could have been a consequence of insertions, but no evidence for this was found from other enzyme digests.

Only one or the other of the allelic fragments was seen in each haploid drone, indicating that a single locus was recognized by each probe. The probes were hybridized to genomic DNA digested with *Pst*I, the enzyme used to produce the probe inserts. Each probe revealed a prominent single band (not shown), which further indicated that they represented single loci. The bands seen with probes P130 and P170 were equal in length to the probe inserts (6.0 kb and 13 kb, respectively). The band detected with probe P138 was larger (7.3 kb) than the insert (6.5 kb). Although

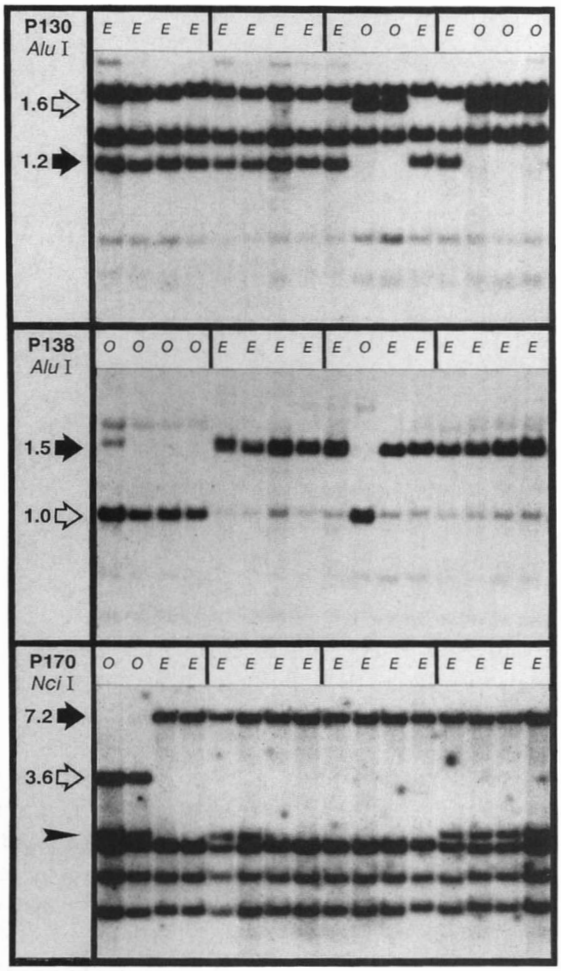

FIGURE 1.—Haplotype DNA polymorphisms in drone honeybees detected by three probes. In each lane is the digested DNA from an individual drone. Four drones from each of four different queens near Gainesville, Florida, are represented (colony members grouped together). Each individual was separately analyzed with three nuclear honeybee DNA probes (top, center and bottom panels). Fragment relationships are revealed in the haploid genotype of drone bees. The European fragments (solid arrows) and the fragments to which they are allelic (open arrows) can be seen. The allele comprising the haploid genotype is indicated at the top of each lane: *E* = the European allele, *O* = the alternate allele. Top panel: Probe P130 reveals a 1.2-kb European fragment allelic to a 1.6-kb fragment in *Alu*I digests. Center panel: Probe P138 detects a 1.5-kb European fragment allelic to the 1.0-kb fragment, also in *Alu*I digests. Many faint DNA bands are also detected with probe P138; the bands likely represent scattered repetitive sequences. In individuals with the prominent 1.5-kb fragment, a faint band is present that approximates but is slightly larger than the 1.0-kb fragment. This is probably one of the repetitive fragments, with a different identity than the 1.0-kb fragment. Bottom panel: Probe P170 detects a 7.2-kb European fragment allelic to a 3.6-kb fragment in *Nci*I-digested samples. In a few individuals, probe P170 detects an extra fragment (arrowhead).

probe P138 primarily represented a single locus, apparently another part of the probe recognized a scattered repetitive sequence seen as the additional faint bands.

Other alleles, not characteristic of African or European bees, were also revealed. Probe P170 detected the presence of an additional fragment in some samples (Figure 1; seen also in colony samples in Figure 5), perhaps the result of a duplication (for example: GOODBOURN *et al.* 1983; CHAPMAN, VINCENT and WILSON 1986). The extra fragment was not related to the the 7.2 kb-3.6 kb polymorphism. Between the two polymorphisms, all four possible haplotypes were seen (only three were among the samples in Figure 1). However, no more than two haplotypes came from a single queen (from the small number of individuals tested). This observation suggested that the 7.2 kb-3.6 kb and extra fragment polymorphisms came from within the same locus (probe region) or had a tight linkage. Hereafter, alleles *P170-E* (7.2 kb) and *P170-O* (3.6 kb) will be discussed without regard to the presence or absence of this other polymorphism. Probe P170 recognized a third polymorphism (Figure 2, bottom panel) altering fragments in the *P170-O* allele independent of the 3.6-kb fragment (allele *P170-X*). With probe P130, the density of another fragment band (between the 1.2-kb and 1.6-kb fragments, Figure 2, top panel) was reduced in two drones. This may be a complex polymorphism. Perhaps, the fragment carried tandem repeats, only some of which had additional restriction sites. As with the additional polymorphisms seen with probe P170, this appeared to be independent of the fragments distinguishing the *P130-E* and *P130-O* alleles.

Inheritance of the polymorphisms: By analysis of individual drone and worker progeny from a colony, segregation of the alleles can be followed. Figure 2 shows six drones and six workers from each of two colonies (A and B) from Tapachula, Mexico. Each individual was tested with all three probes. Drones carried either the European or the alternate allele fragments. Workers carried one or both allelic fragments. In some heterozygous workers, lower densities of the allelic bands, relative to the common bands, reflected the haploid amount of DNA.

Haplotypes obtained from drones provided the genotypes of the queens. Queen A (Figure 2) was apparently homozygous for *P130-O* and *P170-O* (probability determined from six progeny = 0.97), but she was heterozygous for the alleles seen with probe P138. Queen B was heterozygous for all three markers. By comparing the drone and worker genotypes, the drones with which the queen mated can often be deduced. All workers shown that carried the European markers were heterozygous. One worker (number 3) from queen A appeared to have been sired by a drone carrying the *P130-E* allele (Figure 2, top panel). The drone may have contributed the worker's *P138-E* allele as well (Figure 2, center panel), although it may have come from the heterozygous queen. It cannot be determined if the workers from queen B received any of the three European markers from her

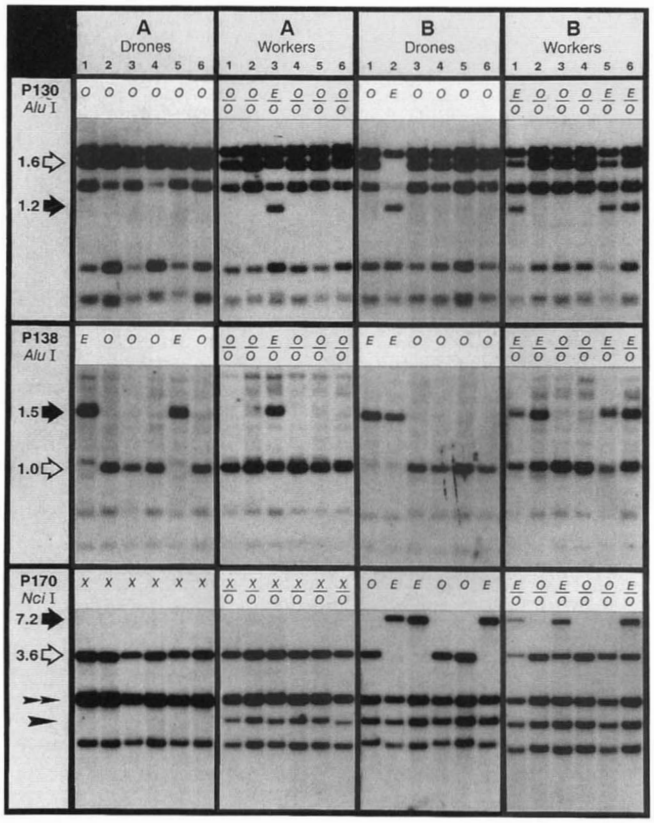

FIGURE 2.—Inheritance of the nuclear DNA markers. Six individual drones (haploid males) and six individual workers (diploid females) were tested from each of two colonies (A and B) from Tapachula, Mexico. Each individual was analyzed with the three probes separately. Top panel: probe P130 with *Alu*I digests. Center panel: probe P138 with *Alu*I digests. Bottom panel: probe P170 with *Nci*I digests. The haploid drones carried either the European fragments (solid arrows) or allelic fragments (open arrows) reflecting segregation of the mother queen's alleles. For both probes P130 and P170, queen A appeared to have been homozygous for the alleles lacking the European fragments, but she was heterozygous for the alleles seen with probe P138. Queen B was heterozygous for all three markers. All workers that carried the European alleles were heterozygous. The genotypes of these individuals are indicated at the top of each lane. Colony A carried African mtDNA and colony B carried European mtDNA. Additional alleles can be seen. Upper panels: with probe P130, the fragment band between the 1.6-kb and 1.2-kb fragments is significantly reduced in two drones (A, number 4; B, number 2). Bottom panel: probe P170 detects another allele (allele *P170-X*) from queen A, where a fragment is lost (large arrowhead) with a corresponding increase in density of the next largest fragment band (two small arrowheads). The worker progeny reveal that none of the drones with which this queen mated carried this allele.

or from their fathers. One of the other polymorphisms detected with probe P170 (Figure 2, bottom panel; allele *P170-X*) was found from queen A. None of the drones with which this queen mated, as indicated by the worker progeny, carried this allele.

Among the drone samples from queen B, alleles at the three loci appeared to segregate independently (four of the possible eight combinations seen in just the six individuals, Figure 2), but sufficient numbers have not been tested to rule out linkage. The probes, derived from random pieces of DNA, are likely to represent separate loci. Because honeybees have 16 (1 N) chromosomes, the probability of random probes coming from different chromosomes is high.

Analysis of European and African colonies: Honeybees from Europe, the United States, Mexico, Costa Rica, Venezuela and South Africa were tested against the three probes revealing the European markers. For some of the populations, individual workers and drones were analyzed to obtain approximate gene frequencies (summarized in Table 1). Allele frequencies can be taken directly from haploid drone genotypes but, as discussed above, drones from the same colony reflect only the alleles carried by the queen. Workers from the same colony, derived from different patrilines, would be more representative of the local population, although the genotype of the queen would still be overrepresented. In addition to individuals, mixtures of ten to twenty worker siblings were analyzed to provide broader samplings of the populations. In autoradiographs of mixed samples, the absence or low relative density of the European bands was interpreted as an absence or minor proportion of the European alleles among colony members.

A limited number of samples from Europe, representing pure races, were available for this study. Four workers were tested from each of three *A. m. ligustica* colonies, two *A. m. caucasia* colonies and four *A. m. mellifera* colonies. All of the *A. m. ligustica* were homozygous for the European alleles detected by all three probes. All of the *A. m. caucasia* were homozygous for the European allele seen with probe P138 but homozygous for the alternate alleles seen with probes P130 and P170. All of the *A. m. mellifera* were homozygous for the alternate *P130-O* and *P170-O* alleles, and most were homozygous for the *P138-O* allele.

Among the colonies tested from the United States, 25 came from a closed breeding population comprised of stocks from many locations across the country. Additional colonies from Florida and California were analyzed. Four individual workers were tested from each of ten colonies from the closed breeding population. The allele frequencies for the European polymorphisms were: *P130-E* = 75%; *P138-E* = 83%; and *P170-E* = 70% (Table 1). From a local Florida population, allele frequencies were obtained for ten queens, each of their genotypes determined from four individual drones (some shown in Figure 1). The frequencies of the three European alleles among these queens were: *P130-E* = 75%; *P138-E* = 70%; and *P170-E* = 70% (Table 1), similar to those in the closed breeding population.

In all U.S. colonies tested as mixed sibling samples, the European fragments were seen as intense bands (Figures 3–5, group A). The ubiquitous presence of the European markers in U.S. colonies was consistent

TABLE 1

Summary of honeybee colonies analyzed, allele frequencies and mitochondrial DNA type

Population	Total No. colonies tested	Frequencies of European Alleles[a]			Basis of frequency determinations	mtDNA type[b]
		P130-E	*P138-E*	*P170-E*		
Europe						
A. m. ligustica	3	1.00	1.00	1.00	4 workers from each of 3 queens	
A. m. caucasia	2	0.00	1.00	0.00	4 workers from each of 2 queens	
A. m. mellifera	4	0.00	0.06	0.00	4 workers from each of 4 queens	
United States						
Arizona	20[c]	0.75	0.83	0.70	4 workers from each of 10 queens	E-20
Florida[d]	5	++++	++++	+++		
Florida[e]	15[f]	0.75	0.70	0.70	4 drones from each of 10 queens	E-5
California	4	++++	++++	++++		
Mexico						
Managed	8[c,g]	+/++++	+/++++	++/+++		E-6 A-2
Feral	13[c]	0.15	0.08	0.18	4 workers from each of 10 queens	A-13
Costa Rica						
Managed	2	−/++	−	−/+		A-2
Feral	1	−	−	−		A-1
Venezuela						
Managed	12[c]	−/+	−/+	−		A-10
Feral	10–20[h]	−	−	−		
South Africa						
A. m. scutellata	15[c]	−	−	−		A-8
A. m. capensis	2	−	−	−		A-2

[a] Frequencies come from tests of the individuals indicated in next column. "+" indicates density in autoradiographs of colony samples. "+/++++" indicates range of densities in autoradiographs among colony samples. "−" indicates absence in autoradiographs of colony samples.
[b] From HALL and MURALIDHARAN (1989). E = European; A = African-numbers of samples tested.
[c] Colony samples shown in Figures 3, 4 and 5.
[d] Part of a closed population related to the Arizona population.
[e] Local population.
[f] Individual samples shown in Figure 1.
[g] Six shown in Figures 3, 4, 5; two shown in Figure 2. Only mtDNA types for the six were reported in HALL and MURALIDHARAN (1989).
[h] Ten tested with P170; 20 tested with P130 and P138.

with the high frequencies estimated from individuals and indicated a widespread distribution. The absence of the European fragments in some individual U.S. bees was obscured in mixed progeny samples but betrayed by the fragments belonging to the alternate alleles. The lower density of the allelic fragment bands seen in almost all colonies reflected the higher frequencies of the European markers (Figures 3–5, group A). Samples from two colonies completely lacked the alternate allele *P130-O* (Figure 3, group A, lanes 6 and 8), and one lacked allele *P170-O* (Figure 5, group A, lane 1).

None of the three European markers was detected in samples of bees from South Africa (Figures 3–5, group E). Most of the South African samples came from the vicinity of Pretoria, the region from which African bees were imported to South America (KERR 1967).

In January 1988, samples from 13 feral swarms were collected in the vicinity of Tapachula, Mexico, near the Guatemalan border. About 15 months earlier, African bees first crossed into Mexico (as identified by morphometrics, behavior, and allozyme frequencies). All of these swarms were previously found to carry African mtDNA (HALL and MURALIDHARAN 1989). The European nuclear DNA fragment bands, detected by the three probes, were either absent or faintly present in most of the swarms (Figures 3–5, group C). Four individual workers from each of ten swarms were analyzed. Gene frequencies for the European alleles were: *P130-E* = 15%; *P138-E* = 8%; and *P170-E* = 18% (Table 1). Eleven of the 12 *P130-E* alleles and five of the six *P138-E* alleles were found in progeny from the same three queens. One of these queens was heterozygous and another probably homozygous for allele *P130-E*. The latter queen may have also been heterozygous for the alleles seen with probe *P138*. Six of the 14 *P170-E* alleles were also found

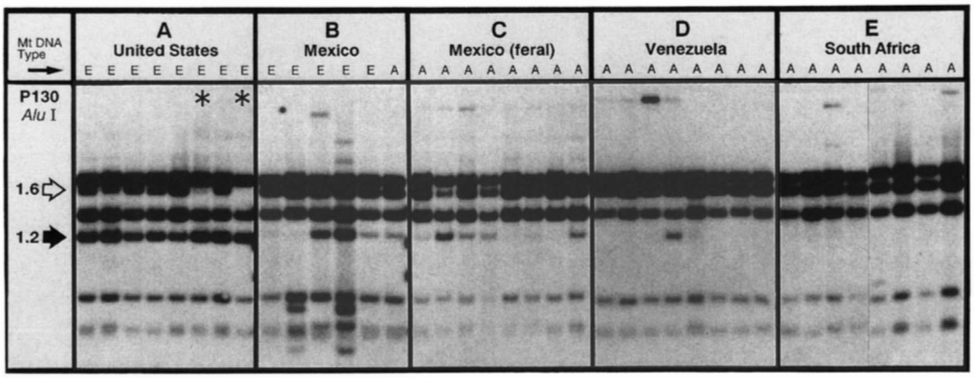

FIGURE 3.—Samples of bees from different locations analyzed for a nuclear DNA RFLP generated by *Alu*I and detected with probe P130. Each lane represents DNA isolated from a mixture of siblings from a single colony. These are representative of the total number of colonies tested. (A) Managed US (European) colonies. (B) Managed Mexican colonies established prior to and sampled 15 months after African bee arrival. (C) Feral Mexican swarms. (D) Managed Venezuelan colonies. (E) Managed South African colonies. The solid arrow points to a European 1.2-kb fragment allelic to a 1.6-kb fragment indicated by the open arrow. The 1.2-kb fragment was present in U.S. colonies (A) and absent in South African colonies (E). All individuals in two U.S. samples were homozygous for the European allele (lanes 6 and 8, asterisks). Most of the established Mexican colonies (B) showed a significant reduction in the 1.2-kb European fragment band, consistent with "Africanization" of these colonies by mating of the queens with African drones. Some feral Mexican swarms (C) showed varying faint 1.2-kb European fragment bands due to hybridization with European drones. Almost all managed Venezuelan colonies (D) lacked the 1.2-kb European fragment. The first 5 samples in group B carried European mtDNA, and the last sample carried African mtDNA. All samples in groups C, D and E carried African mtDNA (HALL and MURALIDHARAN 1989). MtDNA type from each colony is indicated above the lane (E = European; A = African).

from the same three queens, one of which was heterozygous. Seven *P170-E* alleles were found in the progeny of two other heterozygous queens.

At the same time, samples were also collected from an apiary near Tapachula, established prior to African bee arrival. The European fragment bands varied in intensity, pronounced in some samples but almost absent in others. There was an association of the markers within these samples, with the third and fourth samples in group B (Figures 3–5) exhibiting the highest band density of all three European markers. Five of these colonies were found to carry European mtDNA, and one was found with African mtDNA (HALL and MURALIDHARAN 1989). The two colonies seen in Figure 2 came from another apiary established just as African bees were moving into the area. Colony A carried African mtDNA and colony B carried European mtDNA.

Samples from Venezuela were collected between 1986 and 1988, about 10–12 years after African bees first entered the country. The samples came from recently caught feral swarms and from managed colonies established from swarms 2–24 months prior to sampling. The more recently caught swarms completely lacked the three European markers. Some of the earlier established colonies exhibited faint European bands, but most did not (Figures 3–5, group D). A few European colonies were present in the area. From the time the African colonies were established, an undetermined number of generations would have passed (one to six per year) with matings to local drones. In three samples from Costa Rica, the European polymorphisms were absent or faintly present (not shown). All of the Venezuelan and Costa Rican colonies had African mtDNA (HALL and MURALIDHARAN 1989).

DISCUSSION

The migration of the African honeybee in the Americas has been a phenomenal event, but it has not been studied in detail with genetic markers. DNA polymorphisms can overcome the lack of markers in systems such as the honeybee, where low protein polymorphism has limited understanding of their population biology. Compared to allozymes, the relative abundance of DNA RFLPs increases the likelihood of finding specific markers. With specific markers, parental analyses of individual colonies can reveal patterns of introgressive hybridization between the honeybee subspecies.

Frequency and distribution of the European DNA markers

As described here, three segments of the European honeybee nuclear genome were found to carry polymorphisms present at a high frequency and widespread distribution in U.S. bees but absent in South African bees. The frequencies were determined from small sampling sizes but were comparable in bees from both Florida and Arizona. The frequencies of the European DNA markers in U.S. bees match or exceed those of the most useful allozymes, none of which is population specific. For example, the fast allele of malate dehydrogenase can be 100% in some South African populations, but it can also be as high as 37% in European populations (SYLVESTER 1986). Consid-

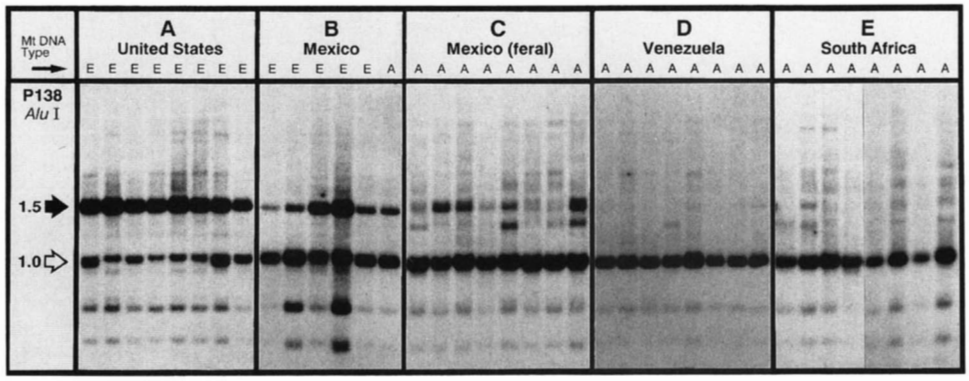

FIGURE 4.—Samples of bees from different locations analyzed for a nuclear DNA RFLP generated by *Alu*I and detected with probe P138. The samples are the same as in Figures 3 and 5. The solid arrow points to a European 1.5-kb fragment allelic to the 1.0-kb fragment indicated by the open arrow. The 1.5-kb fragment was present in U.S. colonies (A) but absent in South African colonies (E). As with the RFLP seen with probe P130, the 1.5-kb European fragment band was reduced in the established Mexican colonies (B), faintly present in feral Mexican swarms (C), but absent in Venezuelan bees (D). This probe also detects some repetitive DNA seen as many faint bands.

erable collecting of U.S. feral and managed colony samples is needed over the next few years, in advance of the African honeybee invasion. This will permit a more thorough determination of allele frequencies and distribution in the European-derived U.S. bee populations.

The DNA markers may be characteristic of specific European races. The European alleles seen with probes P130 and P170, and only these alleles, were found in only the *A. m. ligustica* (Italian) samples. Only the European allele seen with probe P138 was present in the *A. m. ligustica* and *A. m. caucasia* but was absent from almost all of the *A. m. mellifera*. Future research to establish the nuclear DNA RFLP specificity among pure European races (including *A. m. carnica* and *A. m. iberica*) should prove valuable. The frequency of the alleles in U.S. populations may correspond to the proportion of European races, and their distribution may reflect race mixing as a consequence of beekeeping.

Because of their specificity, the European markers can reveal low levels of European introgression into African colonies. Because the alternate alleles are not African-specific, they cannot reveal minor amounts of African introgression into European colonies (PAGE and ERICKSON 1985). However, DNA polymorphisms that appear to be African-specific have been found and are the subject of another report (K. MURALIDHARAN and H. G. HALL, manuscript submitted for publication).

African and European honeybee interactions

Polymorphisms in nuclear and mitochondrial DNA provide an ability to separate and determine paternal and maternal contributions to gene flow between subspecies. Both types of DNA together are providing a much more complete view of the processes involved in the establishment of African honeybee populations.

Maternal gene flow–previous findings: From two studies, a total of 54 out of 55 feral swarms (19 plus 39, minus 3 in common), randomly caught in southern Mexico, were found to carry African mtDNA (HALL and MURALIDHARAN 1989; SMITH, TAYLOR and BROWN 1989). Because mtDNA is maternally inherited, this finding demonstrates that the African bee migrating force consists of continuous African maternal lineages spreading as swarms. This conclusion contradicts an assumption that African bee spread occurs primarily through the flight of drones (RINDERER *et al.* 1985, 1987a; ERICKSON, ERICKSON and YOUNG 1986; RINDERER 1986). It is well established that apiaries increasingly exhibit African phenotypes after repeated backcrosses with African drones (MICHENER *et al.* 1972; MICHENER 1975; DALY 1988; COLLINS 1988). However, despite an African nuclear composition inherited from drones, bees descended from European maternal lines make no significant contribution to the feral "Africanizing" force. Observations of managed colonies, rather than of feral populations, has fostered the misconception that New World African populations are predominantly "Africanized" European mother lines.

African maternal migration into European apiaries would occur as beekeepers replace lost colonies with feral swarms. If not actively maintained, European maternal lines in the tropics would likely be lost through attrition. A change in mtDNA in one established Mexican colony may have been the result of a swarm invasion (HALL and MURALIDHARAN 1989), but the contribution of such direct takeovers to the Africanization process has not been adequately substantiated (MICHENER 1975; Taylor, 1985a,b).

Paternal gene flow-current findings: The observations reported here with nuclear DNA are consistent with the mtDNA results. The nuclear markers reveal paternal gene flow but point to asymmetries in levels of hybridization.

Some of the managed colonies sampled from southern Mexico expressed the defensive behavior charac-

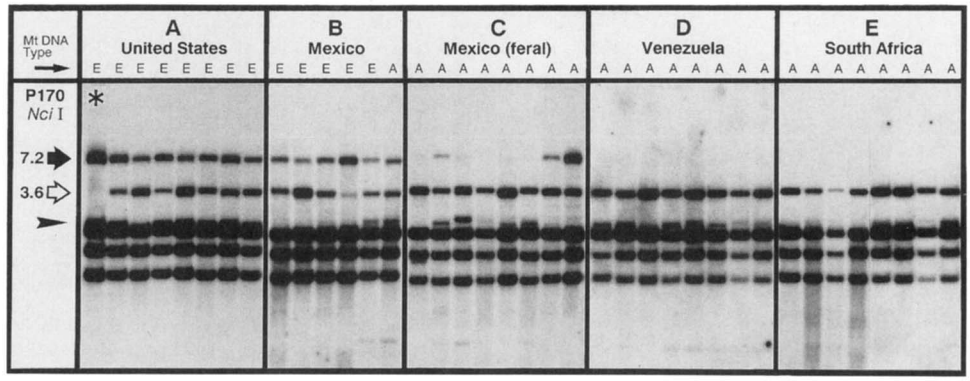

FIGURE 5.—Samples of bees from different locations analyzed for a nuclear DNA RFLP generated by *Nci*I and detected with probe P170. The samples are the same as in Figures 3 and 4. The solid arrow points to a European 7.2-kb fragment allelic to the 3.6-kb fragment indicated by the open arrow. The 7.2-kb fragment was present in U.S. colonies (A) but absent in South African colonies (E). All individuals in one U.S. sample were homozygous for the European allele (lane 1, asterisk). As with the RFLPs seen with probes P130 and P138, the 7.2-kb European fragment band was reduced in the established Mexican colonies (B), faintly present in feral Mexican swarms (C), but absent in Venezuelan bees (D). The arrowhead points to another fragment in some colonies, that is neither African nor European-specific, whose presence is not related to the 3.6-kb–7.2-kb polymorphism (seen more clearly in Figure 1, bottom panel).

teristic of African bees, and most carried low levels of European nuclear DNA polymorphisms (Figures 3–5, group B). All except one sample carried European mtDNA (HALL and MURALIDHARAN 1989). A loss of European nuclear alleles in European maternal lines would be an expected result of African paternal gene flow into apiaries through introgressive hybridization and repeated backcrossing.

However, European paternal gene flow into the African feral population does not appear to be appreciable. The Venezuelan colonies, which had been established from feral swarms, almost completely lacked the European nuclear markers (Figures 3–5, group D). The Mexican swarms carried the European alleles at a low frequency (Figures 3–5, group C) and, thus, appeared to have become "Europeanized" to some extent upon migrating into the area. It was only 15 months earlier that African swarms had moved into this region of Mexico, a region that had, up to the time of sample collection, a large population of managed European bees. The distribution of European alleles among individual workers, rather than just the frequencies, gives more insight into the hybridization of these swarms. Almost all of the European alleles that account for the frequencies came from a minority of heterozygous and possibly homozygous queens. The majority of the feral queens did not carry the European alleles and, hence, did not reflect an ancestry of hybridization with European drones. The faint European fragment bands seen in some swarms would have come from a minority of European drones mated to this most recent generation of queens.

Ideally, allele frequencies in the European population near Tapachula would have been established prior to African bee arrival, but sampling in Mexico had not started before then. If the European population had been composed largely of races lacking the markers, the African swarms could have been more "Europeanized" than that indicated by the RFLP levels. However, a prior abundance of the European alleles seems likely for several reasons: the prominent presence of the European markers in some of the established colonies from this area (group B, Figures 3–5, and both colonies in Figure 2), the high frequencies and widespread distribution of the European alleles in U.S. bees, the markers being an apparent characteristic of the Italian race predominantly used for commercial beekeeping throughout the Americas, and the high concentration of commercial apiaries in this region of Mexico.

The nuclear DNA results coincide with allozyme frequencies reported previously. Frequencies in Venezuelan bees were similar to those in Africa, while those in the Mexican swarms were somewhat more intermediate (SMITH, TAYLOR and BROWN 1989). The African bees that first entered Venezuela may have encountered fewer European colonies, or, after some initial hybridization, levels of European alleles may have become reduced as the population became established. In Panama, a transition toward a more African morphology occurred in the years following passage of the migrating front (BOREHAM and ROUBIK 1987).

Mechanisms possibly responsible for low levels of European alleles: Along the course of their migration, African bees may have encountered a million managed European colonies (O. R. TAYLOR, personal communication). Upon entering an area with European apiaries, African bees would initially be a minority. If there were no limitations to hybrid formation or survival (RINDERER 1986), significant introgression of European alleles into the migrating front and dilution of the African genotype would be expected. However, little or no attenuation of African pheno-

typic traits has been observed. The nuclear DNA findings reported here, and the mtDNA results reported previously, support the view that there has been significant maintenance of the African genotype (TAYLOR 1985a, 1988; FLETCHER 1988). A number of mechanisms could be responsible.

Offset mating periods may provide some reproductive isolation (TAYLOR, KINGSOLVER and OTIS 1990). When inter-subpecies matings occur, European alleles, expressed by worker progeny, may not necessarily enter the reproductive queens. Where African patrilines comprise the majority of a colony, one would be the likely source of the next daughter queen. European alleles would be more actively excluded if queen selection (PAGE and ERICKSON 1984; PAGE, ROBINSON and FONDRK 1989) or faster developmental times favored queens with African fathers (TAYLOR 1988).

Greater fitness of African bees in tropical climates, resulting in a feral population buildup, would cause a relative increase in African gene frequencies (MICHENER 1975, WINSTON, TAYLOR and OTIS 1983; TAYLOR 1988). The migrating front should be a selective process in itself. Introgressed European genes that reduce reproductive capability, the tendency to swarm, or the ability to achieve long distance dispersal may be strongly selected against. If such selection acted effectively on early generation hybrids, this could even reduce introgression of neutral European markers. If any genetic incompatibilities exist (more likely to be expressed in late generation hybrids), these also would tend to purge low frequency European alleles from the African population.

European apiaries, juxtaposed to a large, encroaching African feral population, would become overwhelmed by paternal introgression and, perhaps eventually, by maternal displacement. Like European bees, hybrids may survive the tropics better as managed colonies, not subject to the same selective factors as feral colonies. Successive generations of backcrossing with African bees should increase hybrid adaptability in a feral environment. Thus, swarms, produced by colonies that have become strongly "Africanized" by paternal gene flow, would be expected to have contributed significantly over the years to the migrating force of African bees. However, the failure to find "Africanized" European maternal lines in the feral population suggests that there may be persistent selection against a European maternal component, possibly reflecting some nuclear-mitochondrial interaction (HALL and MURALIDHARAN 1989).

As African bees reach temperate climates favoring European bees, the hybridization patterns observed in the tropics are likely to become reversed. A hybrid zone between African and European bees has been predicted (TAYLOR and SPIVAK 1984; TAYLOR 1985a), and a recent allozyme study indicates that a hybrid zone already exists in the temperate regions of South America (LOBO, DEL LAMA and MESTRINER 1989). The nature of selective processes should affect the characteristics of the hybrid zones and may be revealed by introgression patterns and genotype distributions. DNA markers will be valuable in the continued elucidation of African-European honeybee interactions and population dynamics.

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