Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4874–4877, July 1986
Genetics

DNA differences found between Africanized and European honeybees

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Communicated by Melvin Calvin, March 21, 1986

ABSTRACT **The harmful *en masse* introduction of Africanized honeybees into the United States will occur within 5 years. Possible means of control are dependent on a reliable way to distinguish the Africanized bees from the extant European bees. Current means of identification are inadequate. Reported here are the encouraging initial results to distinguish the bees by their nuclear DNA. With 9 restriction enzymes and 16 probes, six genetic differences have been found among three samples of European bees from California. Twelve additional differences were detected between the European samples and a sample of Africanized bees from Costa Rica.**

Honeybees of African descent were accidentally released from experimental hives in Brazil 28 years ago (1). African bees thrive in tropical climates and tend to swarm readily. Consequently, they have populated most of South and Central America, now as far north as Honduras, and have largely displaced the honeybees of European descent (2). African characteristics, including ferocious stinging, have predominated even after extensive migration and presumed hybridization with European bees, although the actual degree of hybridization is not known (3). African and European honeybees are classified as the same species, *Apis mellifera*, but represent different subspecies.

Isolated introductions of the Africanized bees have already occurred in this country, with a notable case this past summer in California (4), which have been largely contained. However, based on the bees' migration rate through Central America (5), they are expected to arrive in the United States *en masse* within 5 years. In 1972, the National Academy of Sciences committee on the African honeybee correctly concluded that the African bees' entry into the United States was inevitable and would be an environmental danger to the population and catastrophic to the commercial beekeeping and pollination industries (6). By U.S. Department of Agriculture estimates, the beekeeping industry will lose from $26 to $58 million annually (7). The same report calculates that $19 billion worth of agricultural products are dependent on honeybee pollination. Thus, the total loss as a result of the African bee introduction could be immense. Possible methods of control include quarantine and extermination, stock certification, and selective breeding for a gentle hybrid. All of these approaches are dependent on a reliable identification method to distinguish the Africanized bee from the European bee. Establishing a precise means of identification was a major recommendation by the National Academy of Sciences committee.

This study explores the use of nuclear DNA for identification and certification of bees. The analysis is based on fragments of DNA generated by restriction endonucleases, a method that has been effectively used with a number of organisms to determine genetic relatedness (8, 9).

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METHODS

Honeybee nuclear DNA was isolated from 4- and 5-day-old larvae with minor modifications of standard procedures. The larvae were homogenized in 0.32 M sucrose/50 mM Tris·HCl, pH 7.3/10 mM $MgCl_2$, ≈4 grams per 40 ml, with a Teflon or Dounce homogenizer. The nuclei were pelleted (Sorvall SS-34 rotor, 3000 rpm, 5 min) and resuspended in 10 ml of 75 mM NaCl/10 mM Tris·HCl, pH 7.8/10 mM EDTA. The suspension was brought to 1% $NaDodSO_4$/0.2 mg of protease K per ml, and incubated (45 min at 60°C). The solution was centrifuged again (15,000 rpm, 10 min) and the pellet was discarded. Standard phenol and chloroform/isoamyl alcohol extractions of the supernatant and EtOH precipitation of the DNA were followed. The DNA was dissolved in 4 ml of 25 mM NaCl/10 mM Tris·HCl, pH 8.0/1 mM EDTA and treated with 50 μg of α-amylase per ml and 50 μg of RNase per ml (30 min at 37°C). The DNA solution was again extracted, and the DNA was precipitated and redissolved.

Random fragments of honeybee DNA were cloned by using standard procedures (10). Total honeybee nuclear DNA was digested with the restriction enzyme *Pst* I. Plasmid pBR322 (11) was digested with *Pst* I, treated with bacterial alkaline phosphatase, extracted three times with phenol, once with chloroform, and precipitated with EtOH. The plasmid and honeybee DNA were incubated together and ligated. *Escherichia coli*, strain MM294 made competent by $CaCl_2$ treatment, were transformed by the plasmids. To obtain clones of plasmids with inserts, colonies that had gained tetracycline resistance but not ampicillin resistance were selected.

Colony hybridizations (ref. 12, as described in ref. 10) were used to distinguish clones containing repetitive DNA. Replicas of the colonies were grown on nitrocellulose membranes and the plasmids were amplified with chloramphenicol. The bacteria were lysed by placing the membranes on filter paper soaked with the following for 5 min each: 10% $NaDodSO_4$, then 0.5 M NaOH/1.5 M NaCl, followed by 1.5 M NaCl/0.5 M Tris·HCl, pH 8.0. The DNA was baked onto the membranes (2 hr at 80°C, under vacuum). The membranes were washed in 1 M NaCl/50 mM Tris·HCl, pH 8.0/1 mM EDTA/0.1% $NaDodSO_4$ (2 hr at 42°C) to remove the bacterial debris. Total honeybee nuclear DNA was labeled with ^{32}P-labeled deoxycytidine (Amersham) by nick-translation (13) and separated from the free labeled nucleotide on a Sephadex G-50 column. The membranes were prehybridized in 1 M NaCl/0.1 M sodium citrate, pH 7.0/0.5% $NaDodSO_4$/0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin/0.1 mg of denatured sheared salmon sperm DNA per ml (8–10 hr at 68°C), followed by hybridization with the heat-denatured labeled probe in the same solution with 10 mM EDTA (48 hr at 68°C). The membranes were washed in 0.3 M NaCl/30 mM sodium citrate, pH 7.0/0.1% $NaDodSO_4$ (30 min at room temperature), followed by washing in 15 mM NaCl/1.5 mM sodium citrate/0.5% $NaDodSO_4$, pH 7.0 (2 hr at 68°C), and exposed to x-ray film for 2 days.

Plasmids were isolated (ref. 14, as described in ref. 10) by suspending the bacteria in 8% sucrose/0.5% Triton X-100/10

mM Tris·HCl, pH 8.0/50 mM EDTA, adding 0.75 mg of lysozyme per ml, boiling for 40 sec, and removing the bacterial debris and chromosomal DNA by Eppendorf centrifugation. The supernatant was extracted with phenol/chloroform, and the plasmid DNA was precipitated with alcohol.

Circular plasmids and plasmids cut with *Pst* I were analyzed for insert size by electrophoresis in a 0.6% agarose gel using, as the running buffer, 40 mM Tris acetate/2 mM EDTA/0.5 μg of ethidium bromide per ml. Gels were viewed and photographed under ultraviolet light.

Restriction fragment analysis was done by Southern blotting (15). Samples of honeybee nuclear DNA were digested separately by each of the following restriction enzymes: *Msp* I, *Sau*96I, *Alu* I, *Hae* III, *Hha* I, *Nci* I, *Mbo* I, *Hin*fI, and *Dde* I (Bethesda Research Laboratories). The restriction fragments were separated on 2% agarose gels (10–15 μg of DNA per lane) run for 8 hr at 4°C at 100 mA. The gels were blotted for 24 hr with 0.4 M NaOH/0.6 M NaCl, onto GeneScreen-*Plus* membranes (New England Nuclear), and the membranes were washed in 1 M NaCl/0.5 M Tris·HCl, pH 7.0, and air dried. λ phage DNA digested with *Hin*dIII and φX174 phage digested with *Hae* III were used as molecular weight standards (20 ng per well). Probe DNA, isolated as described above, was labeled with ^{32}P-labeled deoxycytidine by nick-translation. Phage marker DNA was included during the last 10 min of a 1-hr reaction. As described above for the colony hybridizations, the blots were prehybridized for 12 hr, hybridized with the denatured labeled probes for 48 hr, washed, and exposed to x-ray film for 3 days.

RESULTS

Establishment of a Library of Honeybee DNA Probes. As a source of probes, random fragments of honeybee nuclear DNA were cloned by using the *E. coli* plasmid pBR322. For the restriction fragment analysis, probes to either single-copy or low-copy-number sequences that reveal a limited number of discrete bands on Southern blots were needed. Bacterial colonies containing plasmids with inserts were hybridized to total honeybee nuclear DNA. Because of the high concentration of repetitive sequences and the low concentration of low-copy-number sequences, only colonies containing inserts of repetitive DNA exhibited significant hybridization (Fig. 1). Clones exhibiting faint hybridization were selected. To increase the number of possible restriction sites that the probes could overlap and thus detect, clones carrying inserts >4 kilobases were further selected.

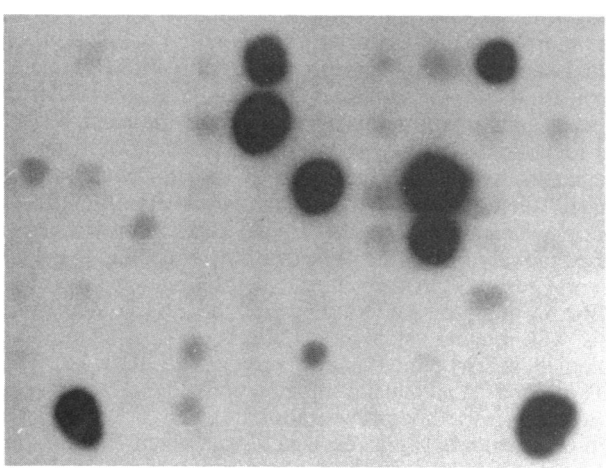

FIG. 1. Bacterial colonies, already selected for containing plasmids with honeybee DNA inserts, were hybridized to total honeybee nuclear DNA. The colonies containing inserts represented as single- or low-copy-number sequences showed little or no hybridization.

Restriction Fragment Analysis of Honeybee Samples. DNA was isolated from four samples of honeybees: two (UY and ST) designated as the Italian race (*A. m. ligustica*), one (CN) designated as Carniolan (*A. m. carnica*), and the other (AF) designated as Africanized (*A. m. scutellata*). UY and CN are both stocks maintained by controlled insemination and were obtained from the same location in Vacaville, CA (courtesy of S. Taber III). ST was obtained from an open-mated colony in Berkeley, CA. AF came from Costa Rica (through the very generous and involved efforts of O. R. Taylor and M. Spivak, Department of Entomology, University of Kansas). The samples of bee DNA were digested with nine separate restriction enzymes, and the fragments were separated by agarose gel electrophoresis, blotted onto membranes, and hybridized with the denatured radioactively labeled probes. So far, 16 probes have been tested. Among the European samples (UY, CN, and ST), fragments were found that reflected at least 6 restriction site differences. The same probes revealed fragments present in the Africanized sample but absent in all the European samples, and vice versa, that had to result from at least 12 additional restriction site differences. From estimates of nucleotide divergence (16) presented in Table 1, it is apparent that the three European samples are very closely related and the Africanized sample is more distantly related. Representative polymorphisms are shown in Fig. 2. A selection of four probes was used in this hybridization, which serves to emphasize the similarities as well as the differences. Presumably, the Africanized sample has been hybridized to some extent with European bees. However, the lack of major bands in the Africanized sample present in all the European samples (*Alu* I and *Hae* III) suggest that the loci may be homozygous for African alleles. More samples must be tested to conclude that these alleles are indeed characteristic of the African subspecies. Each DNA sample was isolated from worker larvae from a single colony, which are all progeny of a single queen mated to several drones. Thus, the restriction fragments reflect a combination of parental genotypes and not a combination of a broad population.

Probe Characteristics. The DNA isolation protocol involved nuclei isolation and lysis, followed by centrifugation to remove intact particulates such as yeast and pollen. Therefore it is unlikely that any of the probes were derived from mitochondria or gut content contamination. Furthermore, probes from the latter would likely show differences in hybridization to sources obtained under different environmental and seasonal influences. From the samples obtained at different times and locations, the same probes that re-

Table 1. Estimated divergence of DNA from the four honeybee samples

	UY	CN	ST	AF
UY	**141**	0.28%	0.10%	1.71%
CN	98.9%	**144**	0.18%	1.79%
ST	99.6%	99.3%	**142**	1.60%
AF	93.4%	93.1%	93.8%	**148**

The calculations are based on DNA digested with seven separate restriction enzymes and hybridized to eight probes. Although more enzymes and probes have been tested (see text), for these calculations, only hybridizations were used that allowed a correlation of almost all of the bands across all four samples. The numbers below the diagonal are the percentage of fragments shared by the samples paired together. Along the diagonal (boldface numbers) are the number of fragments in each sample used in the calculations. The numbers above the diagonal are the estimated percentage of nucleotide substitutions. The estimates do not consider that some fragment differences could be due to deletions or insertions. Three of the total estimated 6 site differences found among the European samples and 9 of the 12 differences between the Africanized and European samples are represented in this data.

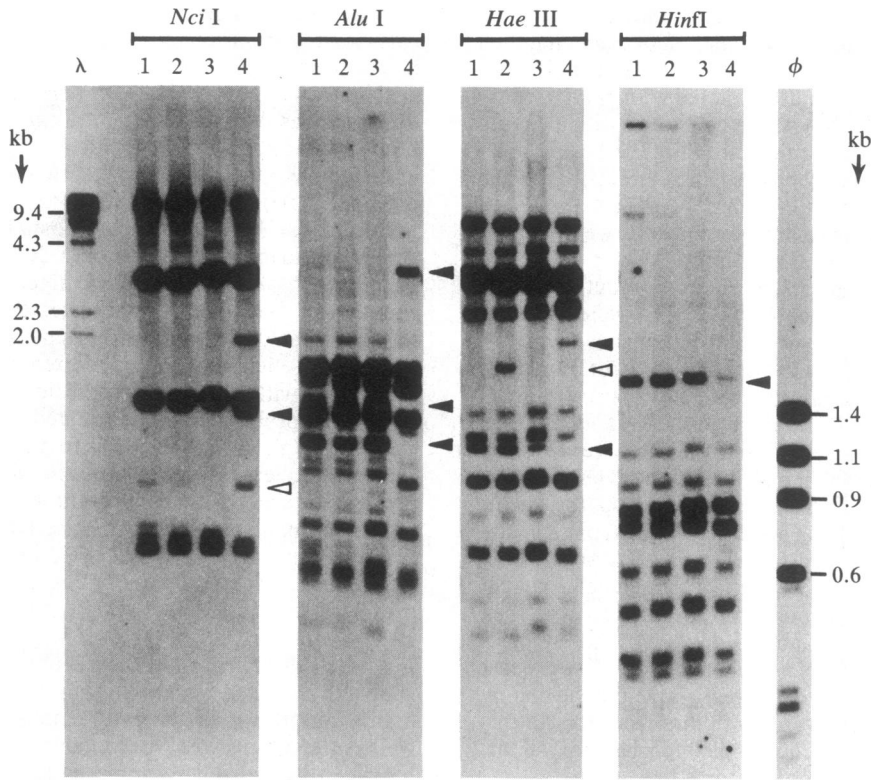

FIG. 2. Southern blot of honeybee nuclear DNA digested with four different restriction enzymes and hybridized to a mixture of four probes. The samples in each lane were the following: lanes 1, UY; lanes 2, CN; lanes 3, ST; lanes 4, AF. Fragment differences among the European samples are seen in *Nci* I, lane 3, and *Hae* III, lane 2 (open arrowheads). A number of fragments are either present or missing only in the Africanized sample (solid arrowheads). The major difference seen in the AF sample cut with *Hin*fI is a decrease in a band density. Additional differences in bands can be seen in several lanes, but they are not as straightforward as the ones indicated. λ phage DNA digested with *Hin*dIII and φX174 phage digested with *Hae* III were used as molecular weight standards. kb, Kilobase(s).

vealed polymorphisms with some enzymes showed no differences with other enzymes. Later demonstrations of Mendelian inheritance of the restriction fragments will verify the nuclear origin of the probes.

The probes used for this study reveal useful discrete bands and thus represent either single- or low-copy sequences. A number of the probes have been hybridized in Southern blots to probe-source DNA digested with *Pst* I and have, in fact, revealed single bands (not shown).

DISCUSSION

Anatomically, the African bee appears very similar to European bees of Italian descent, although somewhat smaller in size. Presently, the most effective method of identification is through morphometric statistical analysis (17). However, this method is subject to environmental influences and cannot reliably distinguish hybrids past one generation. Methods that can identify genetic alleles are required to distinguish hybrids. Honeybees sampled worldwide have few protein electrophoretic variants. With more than 40 loci examined, all but one coding for enzymes, only 5 have been found to be polymorphic. Only alleles of 3 loci are known to have significant gene frequency differences between Africanized and European bee populations, and none of the alleles is diagnostic (18, 19). Limited protein variation is characteristic of Hymenoptera (bees, ants, wasps) compared to other insects (20, 21). One likely reason is that genetic changes resulting in detrimental phenotypic or functional expression are strongly selected against in the haploid males (see introduction in ref. 21).

DNA restriction fragment polymorphisms are not limited to sequences expressed as proteins, and their presence or change in noncoding sequences may not be as subject to evolutionary pressures as those on coding sequences. Since differences in restriction enzyme sites do not necessarily result in, nor does their detection depend on, functional changes, this analysis can potentially provide allele distinction at many loci within natural populations. This is of particular value with the Hymenopteran insects, where the lack of protein polymorphism limits genetic analyses. The potential to distinguish many loci is demonstrated by the initial results reported here. The number of DNA differences found in this single study already exceeds all the protein differences in honeybees reported so far.

Extensive probe testing against many more samples of European, African, and Africanized bees will establish which polymorphisms represent variability within populations and which show significant gene frequency differences among the populations. Restriction sites found only in bees of African descent, and therefore diagnostic, will be the most valuable for identification. The codominant expression of restriction fragments will enable detection of the subspecies-characteristic alleles in hybrids. With restriction site differences at many widespread loci, the alleles found in hybrids will quantitatively reflect the degree of crossbreeding, even after several generations and multiple recombinational events.

During quarantine operations (22), the morphometric analysis will remain valuable for the rapid testing of many colonies, as was done this past summer in California. When this method cannot make unambiguous identifications, the more reliable method of restriction fragment polymorphisms will be essential. This is especially true when major expensive decisions to quarantine or exterminate large numbers of colonies are to be made. Quarantine should delay the spread of Africanized bees in this country but will become increasingly difficult during the large scale immigration of the bees. Another means of control would entail certification of breed-

er stocks as non-African or as acceptable hybrids. Genotype identification by restriction fragments will be very applicable for this purpose. Regular requeening with certified lines of bees would limit the influx of harmful African traits into commercial apiaries, and certified stocks would be useful for selective breeding. As the Africanized bees become established in this country and mix with the extant European populations, diagnostic alleles will be necessary both for reliable identification and for certification (23).

Most of the bees in the United States were derived from a limited number of founding populations, among which there has probably been considerable crossbreeding. Stocks of bees in the United States, although designated as different European races, may have a similar hybrid genotype and be limited in variability. Comparison of restriction fragment polymorphisms within populations of bees in this country to more pure European samples would help establish their relationship and relative degrees of variability. This is also true of the Africanized bees. The African bee introduced to South America, originally designated *A. m. adansonii*, is now considered to be that of a distinct race from southern Africa named *A. m. scutellata*.* Restriction fragment analysis should help verify such distinctions.

Mating advantages by Africanized bees may have contributed to the retention of African qualities through their migration (24). It is suspected that other mechanisms may limit hybridization with European bees and may help preserve the African genotype, such as reproductive isolation (25) or kin recognition in queen rearing (26, 27). Restriction fragment polymorphisms would enable following the African gene flow along the bees' routes of migration and the extent of hybridization and maintenance of the African genotype through such mechanisms. Mitochondrial DNA restriction sites have been used to determine genetic relatedness in a number of other organisms (9). Since mitochondria are largely maternally inherited, mitochondrial DNA differences that may be found would reflect the spread of the African bee through swarming but not through the flight of mating drones. If the bees' distance migration has been due largely to swarming, both African mitochondrial and nuclear restriction sites should be common among the bees along their advancing front. Through this analysis, better understanding of the relative contributions of mating and swarming to the spread of the African bee would be valuable in considering control strategies.

*Ruttner, F., *Proceedings of the 25th International Apicultural Congress of Apimondia*, 1975, Bucharest, Romania, pp. 325–344.

I am very grateful to Mina Bissell and Howell Daly for their tremendous steadfast support and encouragement, to both of them and Allan Wilson for valuable reviews of the manuscript, and to Wen Hua Lee for his early help. I thank Stephen Taber, Orley Taylor, and Marla Spivak for their help in securing the bee samples, and Gary Hartshorn for transporting the AF samples. I appreciate the excellent cooperation of the officials of the Animal and Plant Health Inspection Service, U.S. Department of Agriculture, especially Phil Lima and the customs agents in Miami, and of the California Department of Food and Agriculture in the importation of frozen AF larvae. This work was accomplished while receiving support from a National Institutes of Health New Investigator Research Award and from Office of Health and Environmental Research, U.S. Department of Energy, Contract DE-AC03-76SF00098.

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