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DISTINGUISHING AFRICAN AND EUROPEAN HONEYBEES USING NUCLEAR DNA RESTRICTION FRAGMENT POLYMORPHISMS

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ABSTRACT

African-derived honeybees, expected to enter the United States within two years, will adversely affect the honey and pollination industries and will be an environmental hazard to the public. Control measures are dependent upon a reliable identification method, heretofore unavailable. Nuclear DNA restriction fragment polymorphisms are being found that successfully distinguish African and European bees as well as hybrids between the two. Beyond identification for regulatory purposes, DNA markers will enhance studies on African-European bee genetic interactions and population dynamics.

RESUMEN

Se espera que abejas africanizadas entren los Estados Unidos dentro de dos años y que adversamente afectarán las industria de la miel y de la polinización, y también serán un peligro al público. Las medidas de control dependen de un método de identificación de confianza, hasta ahora no disponible. El polimorfismo de la restricción de fragmentos de DNA nuclear se ha encontrado que distinguen con éxito entre abejas Africanas y abejas Europeas, así como entre híbridos de las dos. Después de la identificación por propósitos regulatorios, marcadores de DNA mejoraran los estudios sobre la interacción genética y el dinamismo de población de las abejas Africanas-Europeas.

European honeybees were imported to the American continents at the time of colonization. They are now vital to the pollination of many agricultural crops at an estimated US annual value of $19 billion (McDowell 1981). African honeybees (*Apis mellifera scutellata;* Ruttner 1976), accidently released in 1957 from experimental hives in Brazil (Kerr 1967, Woyke 1969, Michener et al. 1972, Michener 1975), have undergone a phenomenal migration with the replacement of the extant European bees throughout much of South and Central America (Taylor 1977). The bees are now in Mexico and are expected to enter Texas *en masse* within two years and the panhandle of Florida within six years (Taylor 1985). In Floridia's tropical climate, African bees could reach saturating densities (Taylor & Spivak 1984). Excessive stinging, excitability and swarming make management of African bees difficult. If they cannot be kept from apiaries, beekeepers will discontinue their business. Competitive European bee populations will be eliminated, and numbers of commercial colonies available for pollination will be reduced. Large populations of African bees will increase stinging incidents, and resulting publicity will likely impact tourism in Florida. The threat to human and animal populations has been sensationalized but is, nevertheless, real (Taylor 1986). Proposed methods of control include quarantine, extermination, and certification of breeding stock. These are dependent upon diagnostic identification of African honeybees (Page & Erickson 1985). However, current methods are inadequate. Morphometric statistical analysis, now most commonly used to identify African bees (Daly & Balling 1978, Daly et al. 1982), is subject to error due to environmental influences and overlapping distributions. The composition of cuticular hydrocarbons (Carlson & Bolten 1985, Smith 1988) has considerable promise for identification, but the inheritance of these compounds is not understood. Honeybees show limited allozyme variation which is generally characteristic of hymenopteran insects. Alleles of only four proteins are known to have significant frequency differences between African and European bee populations, but none are diagnostic. Therefore, allozyme analysis cannot make certain identifications, especially of hybrids after several generations (Rinderer & Sylvester 1981).

Honeybee subspecies are distinguished here through DNA fragments generated by restriction endonucleases. A number of studies have demonstrated the value of this approach in establishing the genetic relatedness of organisms (Brown et al. 1982, Cann et al. 1984, Ferris et al. 1982). DNA restriction fragment polymorphisms do not necessarily result in, nor their detection depend upon, functional changes subject to selection. Thus, they can provide allele distinction at many loci within natural populations. In the initial study with honeybees, greater DNA polymorphism was found than all the protein differences reported so far (Hall 1986).

Restriction enzymes recognize and cut DNA at specific short sequences, most commonly consisting of four to six nucleotides. Alterations in the sequences, due to genetic diverence, are manifested in the resulting DNA fragments, separated according to size by electrophoresis. When the total genome is digested, thousands of different-size fragments are generated. To visualize only a small portion at a time and to compare the homologous regions between individuals, use is made of DNA probes (Southern 1980, Maniatis et al. 1982). These short pieces of cloned radioactive DNA recognize homologous sequences, binding through complementary base pairing, i.e. hybridization. Thus genetic distinction is based on a small percentage of nucleotides comprising the restriction sites within the probe region (Jeffreys 1979).

Materials and Methods

As a source of probes, random fragments of honeybee nuclear DNA were cloned in *E. coli* bacteria, by insertion into plasmid pBR322 (Hall 1986). To have probes sufficiently large to overlap a number of sites, clones carrying inserts of honeybee DNA

greater than four kilobases were also selected. Clones with low copy number sequences were distinguished from those with repetitive sequences.

DNA was isolated from uncapped honeybee larvae. Each sample came from a single colony, thus represented a combination of progeny from a single queen mated with one to a dozen drones. European bees were provided by N. Gary (Dept. of Entomology, Univ. of Calif., Davis) established by R. Page (Dept. of Entomology, Ohio State Univ.) and E. Erickson (USDA-ARS, Tucson) as members of a closed breeding population from stocks across the United States. African samples from Costa Rica and Venezuela were provided by O. Taylor, M. Spivak (Dept. of Entomology, Univ. of Kansas) R. Hellmich, A. Collins and T. Rinderer (USDA-ARS, Baton Rouge). DNA can be isolated from any stage, but the soft larval tissue facilitates DNA isolation. Also, the use of larvae ensures the colony origin of samples, since adults can drift among hives. Each DNA sample was digested separately with different restriction enzymes that recognize short nucleotide sequences (four). Short sequence enzymes generate more fragments, increasing the chances of finding differences. Each enzyme digest was placed in a separate lane in an agarose gel, and the fragments were separated by electrophoresis and blotted onto membranes of derivatized nylon. Probes were radioactively-labelled with 32P deoxycytidine by nick translation. The blots were hybridized with the denatured labelled probes, one at a time, washed and exposed to X-ray film (Southern 1980, Maniatis et al. 1982).

Results and Discussion

DNA Differences:

DNA from a few European and African samples was digested with nine separate restriction enzymes. Of sixteen probes hybridized to these samples, three fourths revealed polymorphisms. Pairwise comparison of the fragments clearly showed that European samples from the U.S. were more distantly related to an African sample from Costa Rica than they were to each other (Hall 1986). Some of the first polymorphisms have promise as diagnostic markers. Enzyme-probe combinations that revealed differences among a few samples were tested against multiple samples of European bees from different U.S. locations and African bees from Costa Rica and Venezuela. So far three of the probes reveal restriction fragments present in all members of one group and absent in all members of the other group (Hall 1988 a,b). Other fragments are totally present or absent in one group but variable in the other. Although many more samples need to be tested, these results strongly indicate that numerous diagnostic differences will be found. By a rough estimation, the probes tested so far together represent only about 1/2000th of the genome.

Since the probes were derived from random fragments of DNA, presumably they represent loci scattered throughout the genome. Although clones to low copy number sequences were selected, some polymorphisms seen in very intense bands may, nevertheless, be of repetitive sequences. These could be advantageous, since each probe would represent many loci. To form the discrete bands needed for analysis, fragments would have to be derived entirely from within the repetitive sequence. Those spanning the repetitive sequence and different flanking DNA would be of many different sizes, forming an unresolvable smear.

Restriction site variations in nuclear DNA sequences show Mendelian inheritance as codominant alleles (Cavenee et al. 1982, Gusella et al. 1984) which enables the identification of heterozygous loci. A single fragment characteristic of a subspecies will be present in the homozygotes and heterozyotes but will be absent only in homozygotes of the other subspecies. Differences detected by two thirds of the probes in the samples

from South and Central America are represented by the lack of fragments, indicating that the loci are homozygous for African alleles (or at least not heterozygous with any of the alleles in the European samples). Interestingly, among Venezuelan bees, greater homozygosity is found in feral swarms. Samples from managed apiaries show varying, although minor, amounts of European-characteristic fragments, which probably represent a minority of colony members sired by European drones.

Use of DNA Markers to Study Honeybee Population Dynamics

DNA restriction fragment polymorphisms will enable studies that have been limited due to the lack of protein variation in natural bee populations. The term "Africanized bees" is a presumption that the honeybees in South and Central America are hybrids but further implies that they are mostly European with introgression of African genes. This was a logical assumption at the time the bees were released when, as it has been reported, there were only twenty-six African queens compared to the entire extant European population (Kerr 1967). However, the extent to which African and European bees freely interbreed and the degree of hybridization is not well documented and has become a controversial subject (Taylor 1985, Rinderer 1986).

Finding loci homozygous for African DNA alleles is consistent with the predominance of African morphological and behavioral characteristics. This may be solely a consequence of tropical environmental selection against hybrids of European bees. However, other mechanisms may exist that allow retention of the African genotype (Taylor 1985). The extent to which the South and Central American bees are hybrids can be clearly resolved with further DNA restriction fragment analysis. Possible genotype retention mechanisms can be followed in natural populations without need to introduce genetic markers that would disrupt the bees' genetic integrity.

Use of DNA Markers for Regulatory Control of African Bees

African bee identification through morphometrics is rapid, simple and can be used to test many colonies but with limited certainty. DNA and morphometric analyses together would make an effective combination for regulatory purposes. Restriction fragment analysis would be used for a smaller number of colonies when a high degree of reliability and hybrid identification are necessary. With diagnostic DNA alleles at many loci and codominant expression of restriction fragments, hybrids can be recognized after many generations and multiple segregational events. This will increase the efficacy of control measures (Stibick 1984) and will reduce the chances of misidentification leading to unnecessary regulatory steps, costly to the government and beekeepers. A quarantine operation to eliminate an isolated introduction in California (Cobey and Lawrence 1985) cost over $1 million. Regular requeening of apiary colonies with certified stocks would likely be a central control tactic. Presently, DNA analysis is the only promising approach for genetic certification. Simplification of the restriction fragment analysis, likely to be forthcoming, will make it more practical for routine use.

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USE OF DNA PROBES TO DISTINGUISH SIBLING SPECIES OF THE *ANOPHELES QUADRIMACULATUS* COMPLEX

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ABSTRACT

Isozyme electrophoresis has traditionally been the method of choice for analyzing the genetic structure of populations. It can also be useful for distinguishing morphologically identical sibling species. More recently mitochondrial DNA restriction analysis has become popular as an alternative for studying population structure; and the isolation of species-specific DNA probes has become the method of choice for distinguishing closely related species. We have evaluated the usefulness of these DNA based techniques in mosquitoes and discuss their advantages and disadvantages.

RESUMEN

La electroforesis de isoenzyma tradicionalmente ha sido el método preferido para analizar la estructura genética de poblaciones. También puede ser utilizada para distinguir entre especies hermanas morfológicamente idénticas. Más recientemente, análisis de la restricción de la mitocondria del DNA se ha hecho más popular como una alternativa para estudiar la estructura de la población; y el aislamiento de sondas de DNA específicas a especie ha sido el método preferido para distinguir especies cercamente relacionadas. Nosostros hemos evaluado la utilida de estas técnicas basadas en el DNA de mosquitos y se discuten sus ventajas y desventajas.

The *A. quadrimaculatus* species complex consists of at least four sibling species: species A, species B (Lanzaro 1986), species C and species D (unpublished data). We have used species A, B, and C of this complex to investigate the usefulness of DNA techniques in mosquito evolution research. Our first aim was an estimate of the degree of diversity between different populations (in this case the three sibling species) and the second was a rapid identification tool for wild-caught mosquitoes. These are quite different questions and have involved different approaches.

DNA restriction analysis has been widely used in vertebrate population and evolution studies (Brown 1985), and is becoming more popular in entomology (DeSalle et al. 1986, DeSalle & Giddings 1986, Hale & Beckenbach 1985, Hale & Singh 1986, Harrison et al. 1987, Hall 1986, LaTorre et al. 1986, Powell 1983, Shah & Langley 1979, Solignac