RUTTNER, F. 1986. Geographical variability and classification. pp. 23-52. In: Bee Genetics and Breeding, T. E. Rinderer, ed. Academic Press. Orlando, Florida.
SNEATH, P., AND R. SOKAL. 1973. Numerical Taxonomy. Freeman Co., San Francisco, California.

# RIBOSOMAL DNA PROBES FOR IDENTIFICATION OF MEMBER SPECIES OF THE *ANOPHELES GAMBIAE* COMPLEX

VICTORIA FINNERTY
Emory University
Atlanta, Georgia 30322

FRANK H. COLLINS
Centers for Disease Control, Malaria Branch
Chamblee, Georgia 30333

ABSTRACT

Many insects belong to species complexes wherein member species are morphologically indistinguishable. Yet, in numerous cases, both economic and medical exigencies require that the species of such individuals be reliably determined. The specific problem addressed here concerns the *Anopheles gambiae* mosquito complex which includes *An. gambiae* and *An. arabiensis*, currently the two major African malaria vectors. The present work uses a ribosomal DNA gene probe to differentiate member species of this complex. The method is shown to be extremely useful and sensitive because it can easily test just portions of a single dried adult. The rationale for using ribosomal DNA clones to provide such diagnostic probes to distinguish among other morphologically identical insect species is also discussed.

RESUMEN

Muchos insectos pertenecen a un complejo de especies donde los miembros son morfológicamente indistinguibles. Sin embargo, en muchos casos, exigencias económicas y médicas requieren que la especie sea identificada con seguridad. El problema específico tratado aquí le concierne al complejo del mosquito *Anopheles gambiae*, que incluye a *An. gambiae* y *An. arabiensis*, que son actualmente los dos vectores principales de la malaria Africana. El presente trabajo usa un gene ribosomal de DNA como sonda para diferenciar especies de este complejo. Se demostró que el método es extremadamente útil y sensitivo porque facilmente puede probar solo porciones de un solo adulto secado. Se discute tamabién la racional del uso de clones ribosomal de DNA para proveer sondas diagnósticas para distinguir entre otras especies de insectos morfológicamente idénticas.

Malaria is the most debilitating disease in the world today; among the 92 million new cases each year, there are close to 1 million deaths, nearly all of which are young children (Service 1985). Although malaria is found world-wide, the problem is most acute in subsaharan Africa where the disease presents an enormous obstacle to social and economic development. Although malaria was eradicated from most of its original

temperate range in the 1960s, its incidence in the tropics continues to increase, due in part to insecticide resistance in the mosquito vector and chemoprophylactic drug resistance in the malaria parasite (Service 1985).

Two of the principal African malaria vectors, *An. gambiae* and *An. arabiensis*, belong to the *Anopheles gambiae* species complex, which contains six member species. All of the species are morphologically indistinguishable, and two (and in some areas as many as four) of the species are often sympatric in many parts of Africa (White 1974). The species' two principal vectors, *An. gambiae* and *An. arabiensis*, differ in behavior and preferred habitat. Moreover, there is abundant evidence suggesting that the two major vector species may not be equally involved in malaria transmission, depending upon the season and location (Coluzzi et al. 1977). Therefore, one of the problems for epidemiological studies of these insect vectors is to determine whether an individual female mosquito is infected with the malaria parasite, and also, to what species does she belong. The latter consideration is most pressing for ecological studies of habitat and reproductive behavior, which then provide information essential for the design of various control strategies.

We have focused our work upon two areas: One involves development of a rapid, accurate and easy means of distinguishing the species of single mosquitoes. The other involves what will ultimately become a fairly detailed analysis of the ribosomal RNA genes from the perspective of population structure and evolution of the species complex.

In developing a rapid means of distinguishing species we chose to use some feature of DNA because of its stability in dried tissue. Dessication is the easiest way todeal with field specimens, and further, a rapid, highly sensitive assay was already in use for detecting the presence of the malaria sporozoite antigen in single dry mosquitoes (Collins et al. 1984). We chose therefore to focus on the ribosomal DNA gene family because they are most likely to be present in hundreds of copies per genome, thus increasing the sensitivity of a probe derived from this region. More important, the ribosomal genes contain a highly conserved coding region as well as a spacer region, and although the latter is transcribed, its sequences are processed out, and it is apparently subject to fairly rapid divergence (Beckingham 1982). Thus, when used as probes, sequences from portions of the coding region flanked by spacer sequence are expected to reveal species-specific restriction fragment length polymorphisms (RFLP). Such characteristic RFLP have been used in numerous instances to distinguish among closely related species (Jeffreys 1981, Avise et al. 1979, Langley et al. 1982).

## Methods

A genomic library from *An. gambiae* was constructed in EMBL-3 as described in Collins et al. (1987). A variety of clones containing ribosomal coding sequences were isolated from this library as previously described (Collins et al. 1987), then restricted with EcoRI and SalI or whith EcoRI and BamHI and subjected to Southern analysis. The blots were probed with a ribosomal DNA clone isolated from *Sciara coprophila* (Renkawitz et al. 1979). Since the coding regions tend to be highly conserved they were expected to hybridize with the *Sciara* clone. However, the non-vector non-hybridizing fragments were expected to derive from spacer or intron regions, and we therefore chose these fragments for further study.

Southern analysis and probe preparation were carried out as described in Collins et al. (1987). An rDNA (pBC2) clone from *S. coprophila* was provided by S. Gerbi. The 18S and 28S coding region clones, derived from *Calliphora erythrocephala*, were provided by K. Beckingham (Beckingham 1980). Mosquitoes were supplied by the Malaria Branch, Centers for Disease Control, Atlanta. DNA extraction is a modification of a method reported by Livak (1984). All of the techniques used for Southern analysis are

fully described by Maniatis (1982); certain modifications are described in Collins et al. (1987).

## RESULTS

Preliminary characterization of clone λAGr12. In order to be certain of the nature of the fragments we would ultimately use as probes to reveal a species-characteristic RFLP, we chose one representative clone for further study. A preliminary restriction map of this clone is shown in Figure 1. The coding regions were roughly delineated by Southern analysis, again using separate *Calliphora* 18S and 28S clones as probes. The arrangement left to right of 28S-18S-non-coding-28S is as expected for insect ribosomal genes, and this clone evidently contains one entire repeat unit. The fragments of interest were those which include both coding and adjacent spacer sequence.

Identification of species-diagnostic RFLP. The interesting fragments (identified according to the procedure outlined in Methods) were those which did not appear to hybridize to the heterologous rDNA probe, pBC2. Figure 1 shows the position of two fragments, pAGr12A and pAGr12B, within the rDNA colone. Such fragments were either subcloned or eluted from a gel and then used individually to probe blots containing genomic DNA from different members of the *An. gambiae* ccomplex. One such blot is shown in Figure 2A. This fragment (pAgr12A), when used as a probe, reveals a characteristic RFLP which, as demonstrated in the figure, can distinguish all five of the member species. Another fragment (pAGr12B) was sued to probe a similar blot, revealing a different RFLP which is shown in Figure 2B. The RFLP revealed in Figure 2A most likely reflects the changed position of the underlined EcoRI site (Figure 1). The RFLP shown in Figure 2B may reflect the changed position of the underlined HindIII site shown in Figure 1, although variation in the HindIII site to its left is also possible.

Ribosomal DNA probes are particularly useful because of their great sensitivity. Since the rDNA genes of many insects are present in 200-500 copies per nucleus, they are readily visualized with an overnight exposure of Southern blots hybridized with a moderately hot ($10^7$ cpm/μg) probe. The blot shown in figure 2 contains an amount of DNA equivalent to one fourth of a mosquito per lane.

## DISCUSSION

Thus far, species identification in the *An. gambiae* complex relied almost exclusively on cytogenetic criteria (Coluzzi et al. 1979). This method requires a great deal of tech-

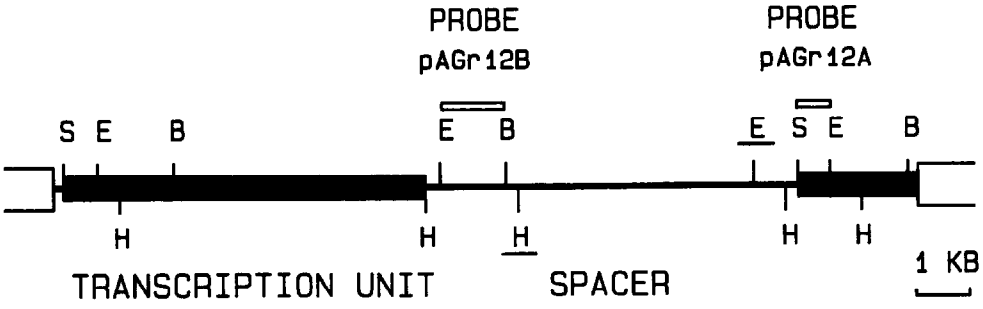



Fig. 1. Simplified restriction map of the *An. gambiae* rDNA clone, λAGr12. The dark bars represent transcribed regions, not all of which is coding region. The colone contains one complete intronless repeat unit. S = SalI, E = EcoRI, B = BamHI, H = HindIII.

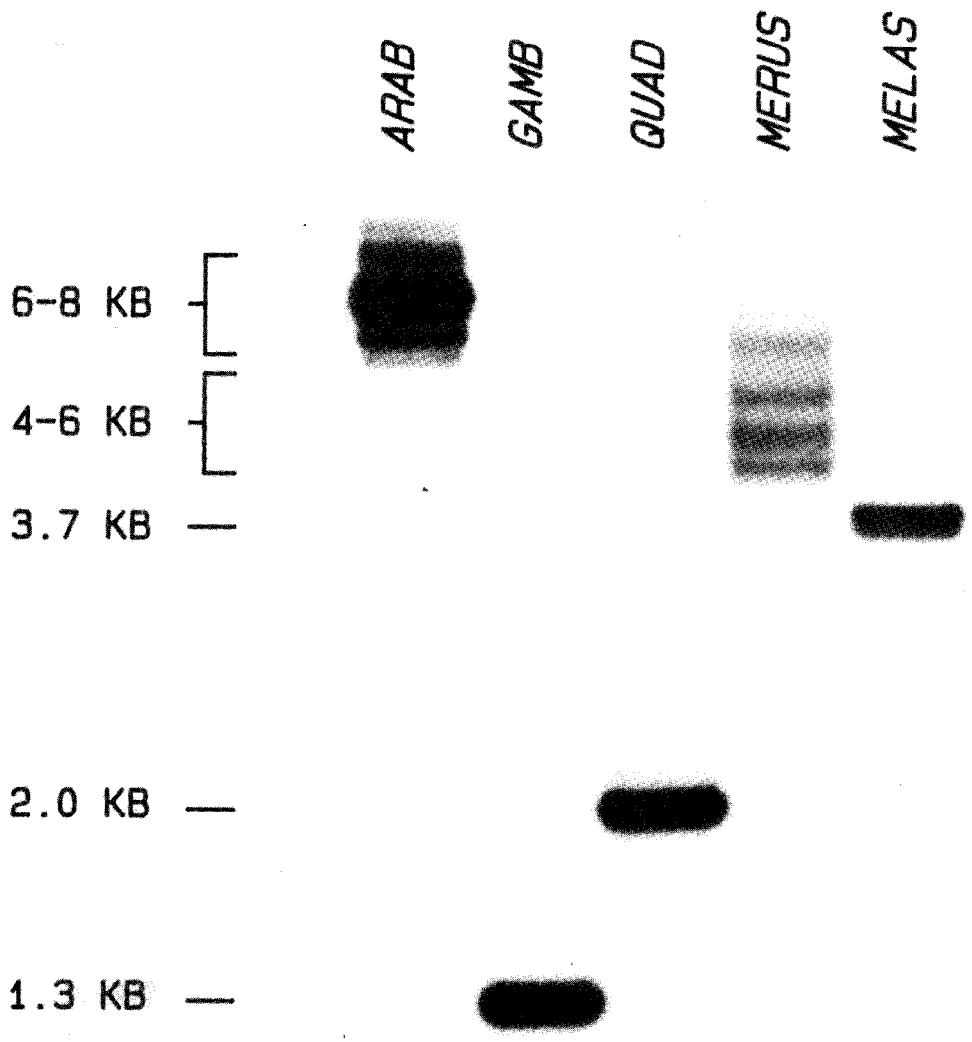



Fig. 2A. Autoradiograph of a Southern blot from an overnite exposure containing an EcoRI digest of the indicated mosquito DNAs probed with pAGr12A ($10^7$ cpm/μg). Shown from left to right are *An. arabiensis*, *An. gambiae*, *An. guadriannulatus*, *An. merus*, and *An. melas*. One quarter of a single mosquito DNA extract was used per lane.

nical expertise, as well as freshly blood fed mosquitoes. Alternative methods based upon differences in several dehydrogenase isozyme patterns have other disadvantages, among which is the requirement for fresh or frozen specimens (Miles 1978, Marchand & Mnzava 1985). Cuticular hydrocarbon profiles can be used to reliably distinguish dried specimens, but the method is not practical for large numbers of specimens or for field use (Carlson & Service 1980, Hamilton & Service 1983). Recently, we demonstrated that the RFLP revealed by rDNA probes were as reliable as either cytogenetic or isozyme methods by direct comparison of results from the same individuals or siblings from an isofemale line (Collins et al. 1988, Collins et al., unpublished). The DNA method has several clear advantages, one being that dried individuals of virtually all life stages can be used. Further, only part of the specimen is required for DNA analysis, so that

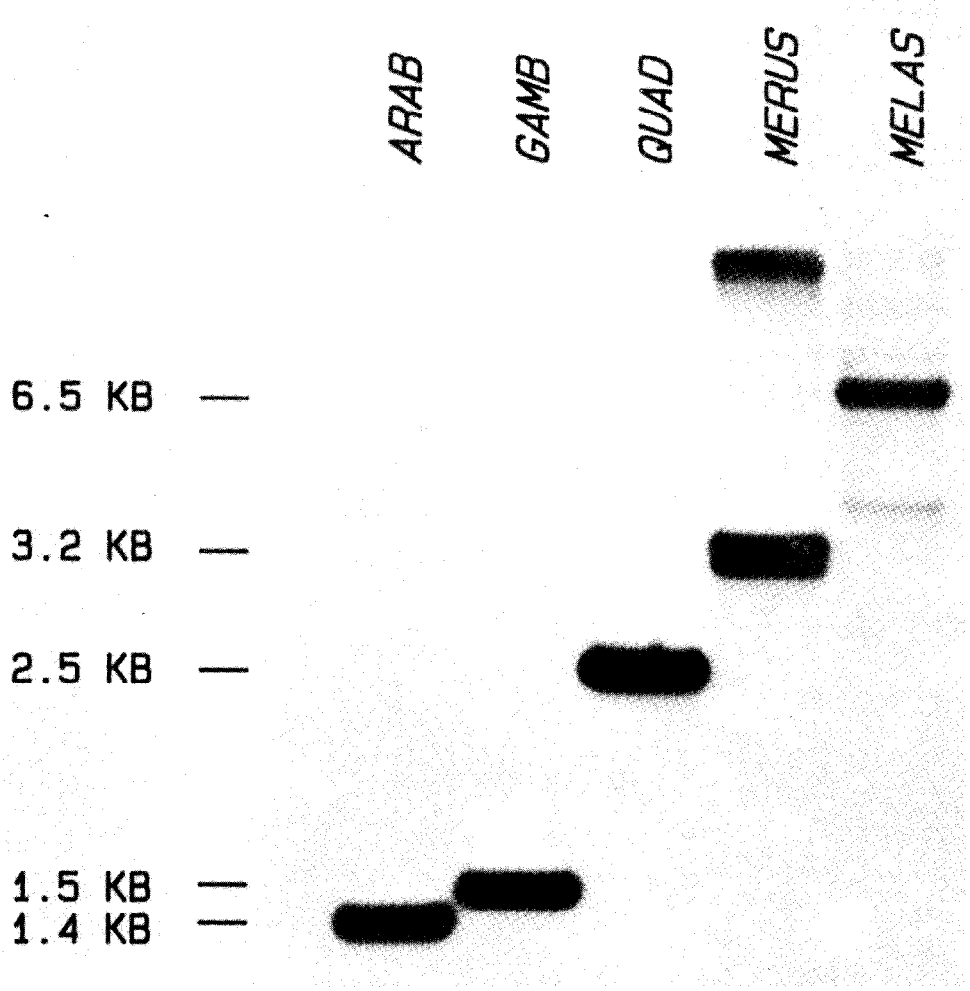



Fig. 2B. Autoradiograph of a Southern blot containing a HindIII digest of the indicated mosquito DNAs probed with pAGr12B. All other conditions are as described for figure 2A.

assessment of the blood meal source as well as the presence of sporozoite antigen can readily be done on the remainder of the specimen. However, it must be emphasized that the DNA method currently requires DNA extraction and Southern blotting. A more useful version of the test would emerge if the necessity of running and blotting a gel could be eliminated in favor of a "dot blot." Such a dot blot assay would require a series of species-specific probes. We have therefore begun to determine whether there are sequences other than the spacer within the ribosomal DNA genes which may have diverged sufficiently between species to the extent that they no longer behave as if they are homologous under the stringent conditions we use for Southern analysis. One obvious candidate is an intron region, and many Diptera contain a mixture of 28S gene types, some with a single intron, and others not having the intron, such as λAGr12, shown in Figure 1. Since mosquito rDNA genes may or may not bear such introns, the intron itself might be even more likely (than the transcribed spacer) to contain sequences which differ significantly between species (Gerbi 1985). Therefore we have examined our collection of *An. gambiae* rDNA clones in order to focus upon those which

might contain an intron in the 28S region. One such "species-specific" sequence has been subcloned from an *An. gambiae* intron. Preliminary analysis of this fragment indicates that it is perfectly useful as an *An. gambiae*-specific probe. Currently we are searching for analogous fragments in rDNA clones from the other members of the complex which could prove to be useful as species-specific probes. In contrast to the highly conserved 18S and 28S coding regions, insect rDNA is generally found to contain both spacer and intron sequences which appear to diverge more rapidly between even closely related species. The rDNA gene family is therefore an excellent candidate for providing (as it has in the present case) a number of fragments, some of which may be used to reveal a species-specific RFLP, as well as other fragments which could behave as species-specific probes.

## Acknowledgments

We thank J. M. Clark for preparing the manuscript. Research was supported by U.S. Army contract DAMD 17-85-C-5184 to VF and by U.S. Agency for International Development, PASA BST-0453-P-HC-2086-02 to FHC.

## References Cited

Avise, J., R. Lansman, and R. O. Shade. 1979. Use of restriction endonucleases to measure mtDNA sequence relatedness in natural populations. I. Peromyscus. Genetics. 92: 279-295.

Beckingham, K. 1980. The ribosomal DNA of *Calliphora erythrocephala:* An analysis of hybrid plasmids containing ribosomal DNA. J. Mol. Biol. 136: 349-373.

Beckingham, K. 1982. In: The Cell Nucleus, X. H. Busch and L. Rothblum, eds. Academic Press, New York, pp. 205-263.

Carlson, D. A., and M. W. Service. 1980. Identification of mosquitoes of *Anopheles gambiae* species complex A and B by analysis of cuticular components. Science. 207: 1089-1091.

Collins, F. H., F. Zavala, P. M. Graves, A. H. Cochrane, R. W. Gwadz, J. Akoh, and R. S. Nussenzweig. 1984. First field trial of an immunoradiometric assay for the detection of malaria sporozoites in mosquitoes. American J. Trop. Med. Hyg. 33: 227-230.

Collins, F. H., M. A. Mendez, M. O. Rasmussen, P. C. Mehaffey, N. J. Besansky, and V. Finnerty. 1987. A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. American J. Trop. Med. Hyg. 37: 37-41.

Collins, F. H., P. C. Mehaffey, M. O. Rasmussen, A. D. Brandling-Bennett, J. Sande Odera, and V. Finnerty. 1988. Comparison of DNA-probe and isoenzyme methods for differentiating *Anopheles gambiae* and *Anopheles arabiensis* (Diptera: Culicidae). J. Med. Entomol., in press.

Coluzzi, M., A. Sabatini, V. Petrarca, and M. A. DiDeco. 1977. Behavioral divergences between mosquitoes with different inversion karyotypes in polymorphic populations of the *Anopheles gambiae* complex. Nature. 266: 832-833.

Coluzzi, M., A. Sabatini, V. Petrarca, and M. A. DiDeco. 1979. Chromosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex. Trans. R. Soc. Trop. Med. Hyg. 73: 483-497.

Gerbi, S. 1985. Evolution of ribosomal DNA. In: Molecular Evolutionary Genetics. R. J. MacIntyre, ed. Plenum Corp., New York, pp. 419-517.

Hamilton, R. J., and M. W. Service. 1983. Value of cuticular and internal hydrocarbons for the identification of larvae of *Anopheles gambiae* Giles, *Anopheles araiensis* Patton and *Anopheles melas* Theobald. Ann. Trop. Med. parasitol. 77: 203-210.

JEFFREYS, A. J. 1981. Recent studies of gene evolution using recombinant DNA. In: Genetic Engineering, II. R. Williamson, ed. Academic Press, New York, pp. 1-48.

LANGLEY, C. H., E. MONTGOMERY, AND W. QUATTLEBAUM. 1982. Restriction map variation in the AdH region of Drosophila. Proc. Nat. Acad. Sci. USA. 79: 5631-5635.

LIVAK, K. 1984. Organization and mapping of a sequence on the *Drosophila melanogaster* X and Y chromosomes that is transcribed during spermatogenesis. Genetics. 107: 611-634.

MARCHAND, R. P., AND A. E. P. MNZAVA. 1985. A field test of a biochemical key to identify members of the *A. gambiae* group of species in northeast Tanzania. J. Trop. Med. Hyg. 88: 205-210.

MILES, S. J. 1978. Enzyme variations in the *Anopheles gambiae* Giles group of species (Diptera, Culicidae). Bull. Entomol. Res. 68: 85-96.

RENKAWITZ, R., S. A. GERBI, AND K. H. GLATZER. 1979. Ribosomal DNA of the fly *Sciara coprophila* has a very small and homogeneous repeat unit. Molec. Gen. Genet. 173: 1-13.

SERVICE, M. W. 1985. Anopheles gambiae: Africa's principal malaria vector, 1902-1984. Bull. Ent. Soc. America. Fall 1985: 8-12.

WHITE, G. B. 1974. Anopheles gambiae complex and disease and transmission in Africa. Trans. R. Soc. Trop. Med. Hyg. 68: 278-301.

# DISTINGUISHING AFRICAN AND EUROPEAN HONEYBEES USING NUCLEAR DNA RESTRICTION FRAGMENT POLYMORPHISMS

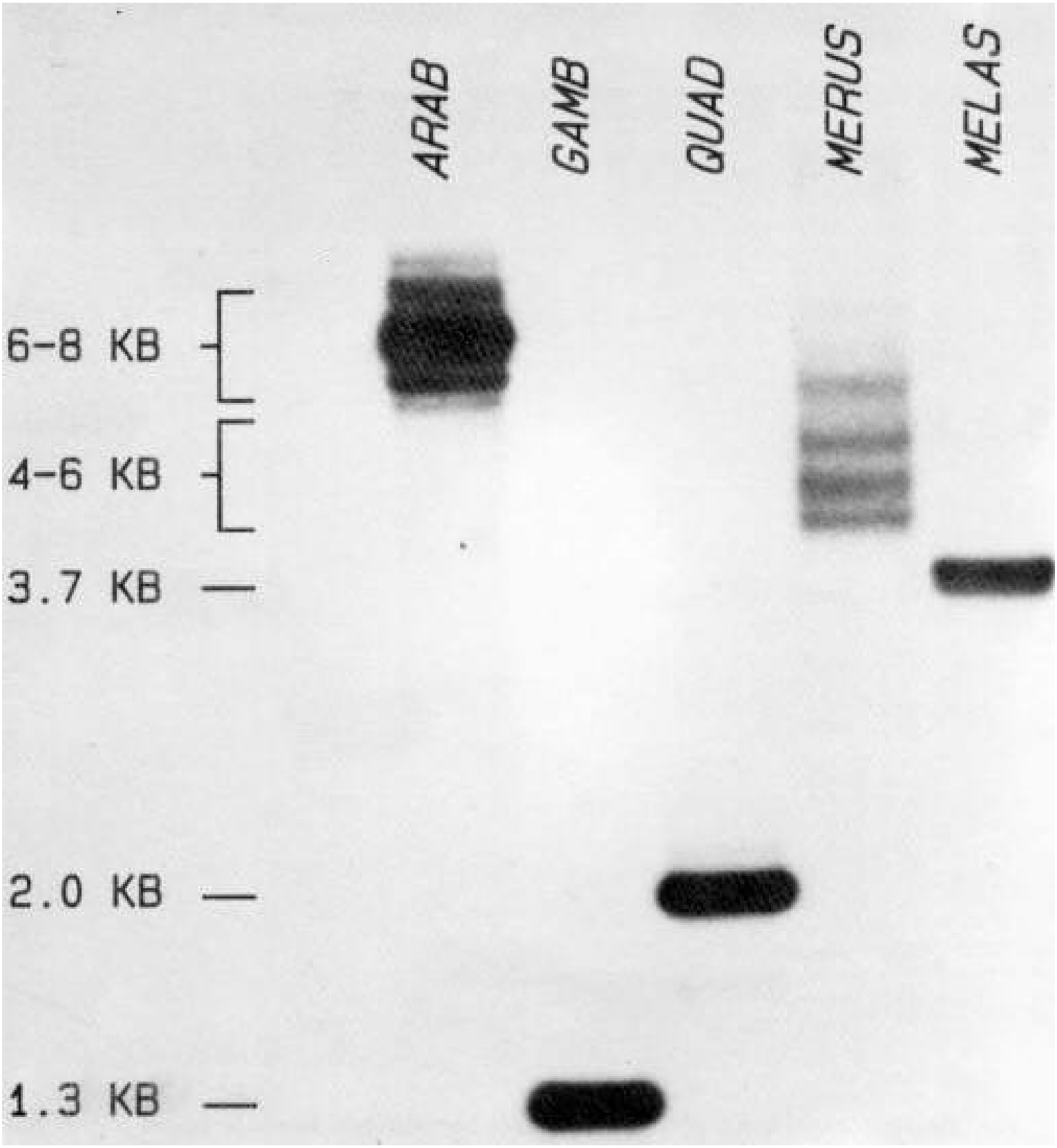
H. GLENN HALL
Department of Entomology and Nematology
University of Florida
Gainesville, Fl 32611

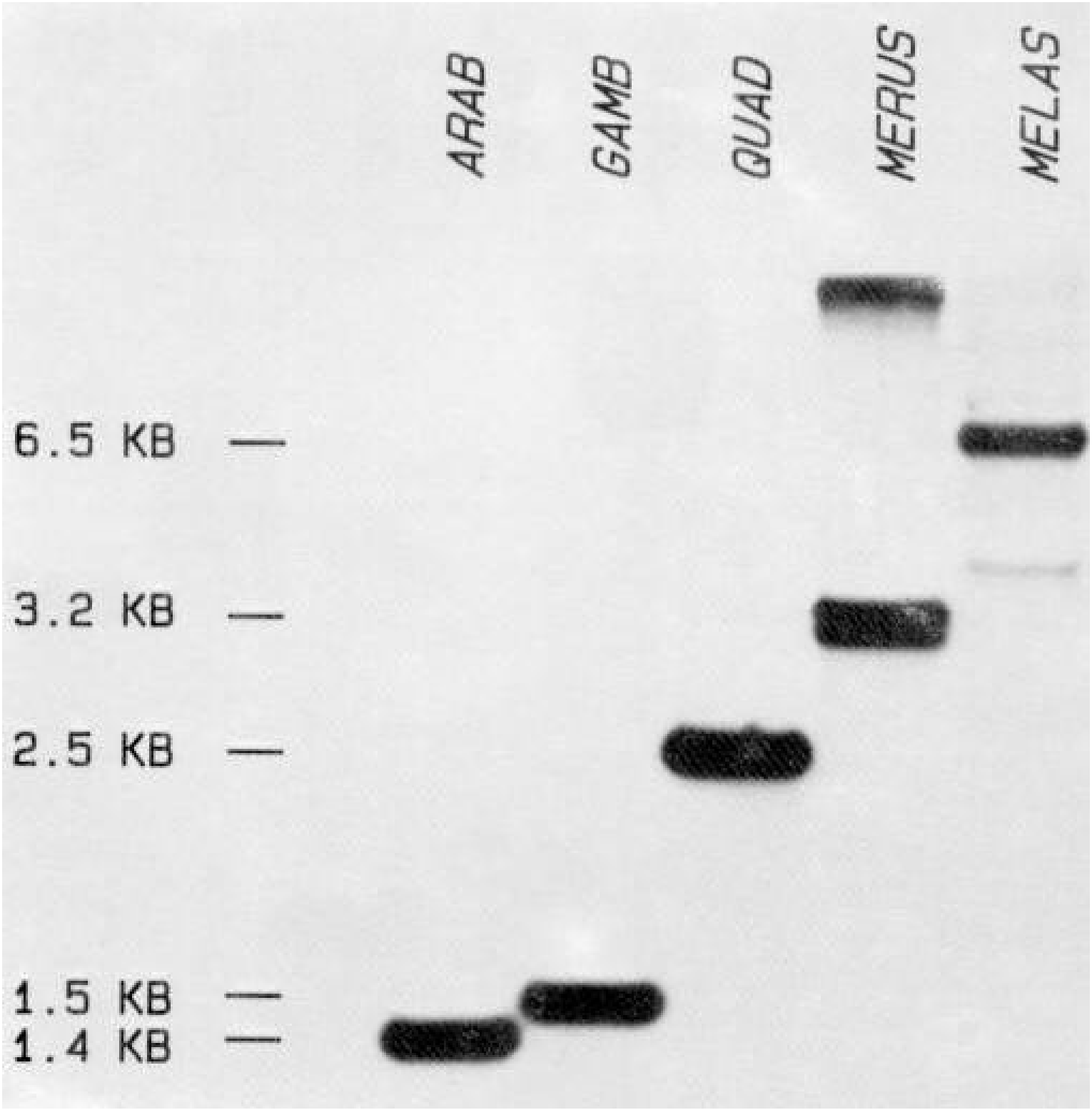
ABSTRACT

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.


ARAB
GAMB
QUAD
MERUS
MELAS
6-8 KB
4-6 KB
3.7 KB
2.0 KB
1.3 KB


ARAB
GAMB
QUAD
MERUS
MELAS
6.5 KB
3.2 KB
2.5 KB
1.5 KB
1.4 KB