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SUSPECTED AFRICAN HONEYBEE COLONIES IN FLORIDA TESTED FOR IDENTIFYING DNA MARKERS

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ABSTRACT

Fourteen honeybee colonies, present in the vicinity of Florida ports and suspected to be of African descent, were analyzed for distinguishing mitochondrial and nuclear DNA markers. Four of the colonies, previously identified as African on the basis of morphometrics, were found to have African mitochondrial DNA. Nuclear DNA markers characteristic of African bees were found predominantly in these four colonies.

RESUMEN

Catorce colonias de abejas localizadas cerca a puertos en Florida, y las cuales se sospechaba tenían ascendencia africana, fueron analizadas por marcadores de DNA de la mitocondria y el nucleo. Se encontró que cuatro colonias, previaménte identificadas como africanas en base a morfometría, tenían DNA mitocondrial africano. Se encontró predominando en estas cuatro colonias marcadores de DNA nuclear, caracteristíco de las abejas africanas.

The American honeybee population represents a number of introduced subspecies of *Apis mellifera* L. The bees imported by early settlers were mostly of the west European race *A.m. mellifera* L. Over the past hundred years, the more docile east European types, *A.m. ligustica* Spinola, *A.m. carnica* Pollmann, and *A.m. caucasia* Gorbachev, have become preferred for beekeeping and have largely replaced managed bees from the earlier importations (Pellet 1938, Oertel 1976, Sheppard 1989). European swarms that escaped from apiaries established self-sustaining feral populations in temperate regions but not in the tropics.

African honeybees are better adapted to tropical conditions. For this reason, a subspecies from South-Central Africa, *A.m. scutellata* Lepeletier (Ruttner 1988), was introduced into Brazil in 1956 with the hope of increasing commercial honey production (Kerr 1967). Unlike the European bees, the African bees established a large feral population that has expanded through much of the neotropics over the last 35 years (Michener 1975, Taylor 1977, 1985). Within the past year, the front of the African bee population has spread into Texas. Temperate climatic and ecological conditions are expected to limit African bees to the southern tier of the United States. A hybrid zone may be

formed between them and the resident European bee population to the north (Taylor & Spivak 1984, Taylor 1985).

Although African bees are better adapted to the tropics, several characteristics make them less amenable to commercial beekeeping (Michener 1975, Taylor 1985). The most notable problem is the African bees' extremely defensive stinging which has been responsible for the deaths of animals and humans (Taylor 1986). Within a few years after African bees entered South and Central American countries, their beekeeping industries collapsed. In the United States, the value of the bee for pollination exceeds by almost a hundred fold the value for honey production (McDowell 1984, Robinson et al. 1989). If beekeepers in this country go out of business, the greatest economic damage will be from the loss of crops requiring pollination (Taylor 1985, 1988).

Due to a combination of factors, Florida will be the state most severely affected by the African bee. Because of the subtropical climate, the feral population will occupy the entire state and may reach saturating densities (Taylor & Spivak 1984). Florida has a large beekeeping industry, and many crops within the state require pollination. Annually, thousands of colonies from Florida are transported to meet pollination needs out of the state. Sensationalized publicity surrounding stinging incidents could generate an exaggerated perception of danger from African bees and seriously impact tourism.

The front of the expanding African bee population may reach the panhandle of Florida by 1995 (Taylor 1985). Over the last several years, swarms have repeatedly entered Florida ports as stowaways aboard ships arriving from South and Central American countries. The Florida Department of Agriculture and Consumer Services (FDACS) and the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA APHIS) maintain pheromone-baited hives in the proximity of the ports to intercept this type of introduction. These agencies identify the colonies suspected to be of African descent using morphometrics (reviewed by Daly 1991).

Several colonies identified as either African or European were examined here for distinguishing nuclear and mtDNA markers. Because of limitations of morphometric identification (Daly 1988, 1991), DNA testing will likely become an increasingly necessary tool for regulatory agencies (Hall 1986, 1991), especially as the African bees begin their en masse entry into Florida. The results presented here demonstrate the value and drawbacks of current DNA tests for identification of African bees. Needed improvements are discussed.

Materials and Methods

Honeybee colony samples were collected in the vicinity of Florida ports by agents of the FDACS and the USDA APHIS. The samples used for this study were randomly provided from a larger number collected. Samples came from swarms caught in wooden or fiber-pot bait hives, from swarm clusters on ships or containers, and from colonies in apiaries. Brood and some adult samples were transported on ice and then frozen. Other adult samples were killed, left at ambient temperatures for an unknown number of hours, put into 95% ethanol, and stored refrigerated thereafter.

The FDACS initially screened the samples using forewing length, a component of a shortened morphometric method (Rinderer et al. 1987). Samples that scored a 90% or higher probability of being African were then tested by the USDA Bee Research Laboratories in either Baton Rouge, LA, or Beltsville, MD, using more thorough morphometric analyses. The fourteen colonies tested in this study are listed in Table 1 which gives the dates and locations of the collections. Where a ship was involved, the previous port of call is given. The probability that the sample was African, based on forewing length, is also provided. These scores, obtained by Dr. Lionel Stange and provided by Mr. Laurence Cutts, Chief Apiary Inspector, FDACS, are part of the public record.

As of September 1991, seven colonies in Florida had been identified as African. Four of these were included in this study.

The DNA isolation protocols for adults and brood were as previously described (Hall 1990). DNA was isolated from a mixture of workers from each colony. Samples 1 through 9 were from larvae or pupae; samples 10 through 14 were from adults. Twenty individuals were used in each isolation. Restriction endonuclease digestions, electrophoretic separation, blotting, probe preparation, probe labelling, and hybridizations were as previously described (Hall 1986, 1990). The polymerase chain reaction (PCR) and digestion of the amplified segments were performed as previously described (Hall & Smith 1991) except that the PCR reaction profile was modified as follows: 95°C for 1 min, 35 cycles of 93°C for 30 sec, 62°C for 90 sec, 72°C for 1 min, and a final 72°C for 15 min.

RESULTS

The fourteen honeybee colonies listed in Table 1 were tested for mitochondrial and nuclear DNA polymorphisms that either completely or partially distinguish African from European honeybees. Four of the colonies, 3, 12, 13 and 14, had been previously identified as African by morphometrics.

Mitochondrial DNA

To identify the honeybee mtDNA type, a rapid, accurate procedure, employing the polymerase chain reaction (PCR) was utilized (Hall & Smith 1991). Two regions containing informative polymorphisms were amplified. One region includes a segment between

TABLE 1. SUSPECTED AFRICAN HONEYBEE COLONIES IN THE VICINITY OF FLORIDA PORTS.

	Date Collected	Port	Location Previous ports	Score[2]
—				
1.	Apr 30, 1987	Panama City	Swarm in tree	0.96
2.	Apr 30, 1987	Panama City	Swarm in tree	0.92
3.	[1]Apr 24, 1987	Panama City	On ship Marco trader from Guatemala	1.00
4.	Jun 28, 1988	Fernandina Beach	Bait hive	0.70
5.	May 31, 1989	Tampa	Bait hive	N.A.
6.	Sep 21, 1989	Palmetto	Apiary colony	0.98
7.	Sep 21, 1989	Palmetto	Apiary colony	0.70
8.	Oct 06, 1989	Tampa	Bait Hive	0.86
9.	Oct 06, 1989	Tampa	Bait Hive	0.86
10.	Jul 01, 1987	Tampa	In pipe at terminal	0.21
11.	Jan 03, 1988	Tampa	Apiary colony	0.11
12.	[1]May 06, 1988	Ft. Lauderdale	On ship Senator, from Guatemala and Honduras	1.00
13.	[1]Apr 24, 1989	Miami	On container - ship Werner from Suriname	1.00
14.	[1]Feb 07, 1990	Ft. Lauderdale	On ship Water Stoker, from Guatemala	1.00

[1]Colonies determined to be African based on more extensive morphometric analysis by the USDA.
[2]Score is the probability of African descent based on forewing length, determined by the Florida Department of Agriculture and Consumer Services.

the cytochrome c oxidase subunit I and II genes, that has an insert of several sizes in west European and African honeybees. An insert of approximately 70 base pairs (bp) is most common among neotropical African bees. An insert of about 270 bp is present at a lower frequency among neotropical African bees but is the most common class among South African and west European bees (Hall & Smith 1991). Figure 1 shows an electrophoretic agarose gel of this amplified fragment from the different samples. Samples 3, 12 and 13 each carried mtDNA with the 70 bp insert, and sample 14 carried mtDNA with both the 70 and 270 bp inserts. The second amplified region was within the cytochrome c oxidase subunit I gene that has a *Hinc*II restriction site in west European but not in east European or African bees. From all the samples tested here, this amplified fragment was not cleaved with *Hinc*II (not shown). Thus, colonies 3, 12, 13, and 14 had African mtDNA, and the other colonies had east European mtDNA.

MtDNA is maternally inherited, and, with a few exceptions, the mtDNA molecules of an individual animal are identical (reviewed in Wilson et al. 1985). Since workers comprising a honeybee colony are progeny of a single queen or, occasionally, also of a coexisting daughter queen, the mtDNA within a colony is identical. However, as seen in Figure 1, sample 14 had two African mtDNA size classes. When individual bees were tested, each carried one size class or the other but not both. Thus, the presence of two mtDNA classes was not due to heteroplasmy. Rather, the swarm apparently represented an amalgamation of bees from more than one colony, a common occurrence among African bees (Kigatiira 1988). This swarm was described as being unusually large (L. Cutts, personal communication).

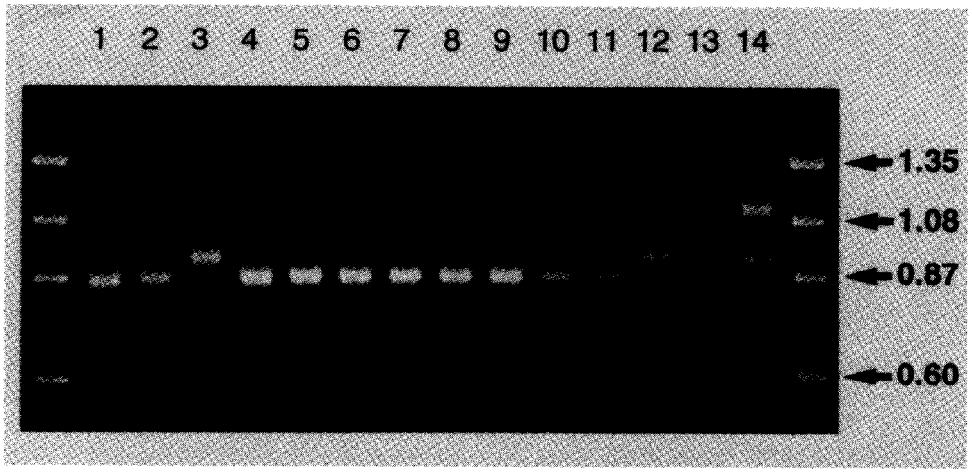

Fig. 1. Samples of Florida honeybee colonies, suspected to be of African descent, tested for a distinguishing mtDNA length polymorphism.

A region of the mitochondrial genome that includes part of the cytochrome c oxidase subunit I and II genes and the intervening segment was amplified by the PCR. The amplified region from each sample was separated by electrophoresis and stained with ethidium bromide. From samples 3, 12, 13 and 14, an amplified fragment of about a 918 bp (base pairs) in length was obtained, about 70 bp longer than the 848 bp amplified fragment from the other samples. From sample 14, an additional amplified fragment of about 1118 bp in length was obtained, about 270 bp longer. The inserts are characteristic of both west European and African bees. The mtDNA of these samples were determined not to be west European, because it lacked a *Hinc*II restriction site in another amplified region within the cytochrome c oxidase subunit I gene. Molecular size standard in outer lanes is bacteriophage ΦX174 disgusted with *HAE*III. Sizes given in Kilobases.

Nuclear DNA

Using standard RFLP (restriction fragment length polymorphism) analyses, the samples were tested with four cloned nuclear DNA probes. The clones carried random inserts of honeybee genomic DNA and, thus, likely represented four separate loci (Hall 1986). In the following paragraphs, a brief description is given of the alleles detected by each probe and their population distributions. More detail can be found in the reports cited or in preparation.

In *Alu*I digests, probe P130 detects an allele, *E*, that is characteristic of the Italian race, *A.m. ligustica*, (100% frequency in a small number of samples) and perhaps of other east European races. Allele *E* is found at about a 75% frequency in United States honeybee populations. The alternate allele, *O*, is fixed in west European and African bees (Hall 1990). Figure 2A shows that samples 3, 12, 13 and 14 lacked allele *E*. This finding was consistent with the mtDNA and morphometric results identifying these colonies as African, although the absence of allele *E* could have reflected a west European background.

In *Alu*I digests, probe 2A2 detects an allele, *B*, present at a high frequency (about 80 to 90%) in South African bees, present at a lower frequency (about 25%) in west European bees and absent in east European bees. So far, among North American colonies of European descent, allele *B* has been found at a low frequency (about 6%) only among feral colonies in northern Mexico (prior to African bee invasion) (Hall 1992). Figure 2B shows that allele *B* was present in samples 3, 12, 13 and 14. Similar to the findings with probe P130, this result was consistent with the morphometric and mtDNA results identifying an African ancestry, but allele *B* could have come from a west European lineage. Sample 9 also carried the allele. The other results did not indicate that this sample had an African background. Nevertheless, the presence of allele *B* may have resulted from hybridization with African drones.

In *Mbo*I digests, probe P271 detects an allele, *S*, found only in African-derived populations. The frequencies of this allele are approximately 25% to 45% in Old and New World African populations (McMichael & Hall, manuscript in preparation). Figure 2C shows that samples 12, 13 and 14 carried the allele, again consistent with the morphometric and mtDNA results. Due to the specificity of this marker compared to those described above, its presence indicated an African background with greater certainty. This allele was absent in sample 3 which was an African matriline. A faint indication of the African allele was seen in sample 11. As with sample 9 carrying allele 2A2-*B*, the other results did not point to an African background for sample 11. However, the results did not preclude that this colony had some progeny of African paternity. This colony was originally suspect because of its excessive defensive stinging but, on the basis of forewing length, had a low probability of being African (11%).

In *Msp*I digests, probe P178 detects several alleles specific for African bees, collectively referred to here as the *A* alleles, characterized by the presence of a 1.1kb fragment. The *A* alleles, as a group, are present at about a 15% frequency in South African bees and at a 15 to 25% frequency in neotropical populations (McMichael & Hall, manuscript in preparation). Figure 2D shows that samples 13 and 14 carried *A* alleles, reliably indicating an African background, consistent with the morphometric and mtDNA results. The *A* alleles were absent in samples 3 and 12 which had an African maternal origin.

In *Msp*I digests, probe P178 detects another allele, *M*, present in almost all bees tested of the west European race *A.m. mellifera* but absent in east European bees. It is also found at about a 5% frequency in South African bees (McMichael & Hall, manuscript in preparation). Figure 2D shows that samples 1, 3, 4, 5, 6, 8, 9, 11 and 14 carried this allele. The strong presence of the P178-*M* allele in samples 3 and 14, which had African mtDNA but not the P130-*E* nuclear allele characteristic of the east European *A.m.*

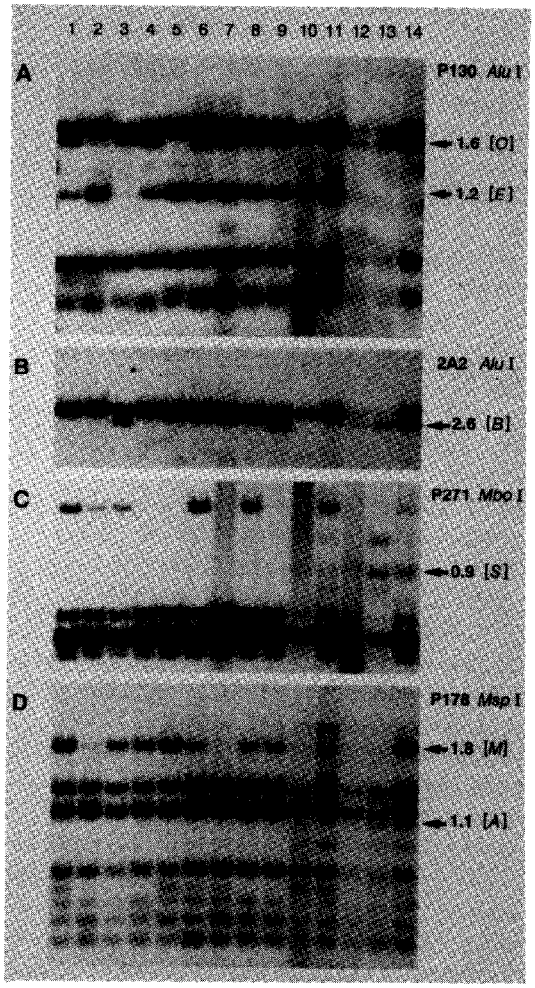

Fig. 2. The honeybee samples tested for nuclear DNA RFLPs. Digested DNA from a mixture of sibling workers from the different colonies is in the separate lanes. Different intensities of bands in the lanes are due to varying quantities and qualities of the isolated DNA loaded on the gels.

A. An RFLP generated by *Alu*I and detected with probe P130: samples 3, 12, 13 and 14, and only these samples, lacked the 1.2 kb (kilobase) fragment characteristic of the east European *A.m. ligustica* allele *E*. The 1.6 kb fragment is characteristic of the alternate allele *O*. In the original study (Hall 1990), another fragment between the arrows was also detected but not with this preparation of the probe.

B. An RFLP generated by *Alu*I and detected with probe 2A2: samples 3, 9, 12, 13 and 14 carry the 2.6 kb fragment characteristic of allele *B*. The larger fragment is 2.8 kb in length and is characteristic of allele *A*. Allele *B* is present at a high frequency in African bees but is also present in west European bees.

C. An RFLP generated by *Mbo*I and detected with probe P271: samples 11, 12, 13 and 14 carry the 0.9 kb fragment present in allele *S*. This allele is specific to African bees.

D. Two RFLPs generated by *Msp*I and detected with probe P178: samples 13 and 14 carry the 1.1 kb fragment characteristic of several alleles specific to African bees, collectively referred to here as the *A* alleles. The 1.8 kb fragment indicated by the open arrow is characteristic of allele *M* found predominantly in west European bees.

ligustica, suggested that these African matrilines had hybridized with west, but not east, European bees.

Discussion

Among the suspected African honeybee colonies found in Florida and tested in this study, there was a strong correlation among the mtDNA types, the nuclear DNA markers and the previous morphometric identifications. The same four colonies, 3, 12, 13 and 14, identified as African by morphometrics were determined here to have African mtDNA. All four colonies carried two nuclear DNA markers (alleles P130-*O* and 2A2-*B*) common to African bees, although the markers are also present in west European bees. Sample 12 also carried one African-specific marker (allele P271-*S*), and samples 13 and 14 also carried two African-specific markers (allele P271-*S* and P178-*A*). These alleles are absent in the majority of African bees, which may be the reason that sample 12 lacked one of the two alleles and sample 3 lacked both. Alternatively, the absence of these African alleles could have been due to hybridization with European bees. In addition to the four samples that showed consistent indications of an African identity, sample 11 carried a very low level of the P271-*S* allele, and sample 9 carried the 2A2-*B* allele. The presence of these markers may have been due to some African paternal introgression, although the latter may have reflected a west European lineage.

The results from the three types of identification methods strongly reinforce each other. Separately, they provide a less certain identification, reflecting inherent limitations in the different types of analyses or the individual markers.

The morphometric method provides a probability that bees are either African or European but does not distinguish hybrids. Intermediate morphometric scores were found to be more common among bees in the tropical-temperate transition zone in South America, a result taken as evidence for African-European bee hybridization (Sheppard et al. 1991). However, intermediate probabilities mean that the bees cannot be confidently identified. Previous DNA studies have revealed little hybridization of feral neotropical African colonies with European bees (Hall 1990). The limited admixture has probably helped retain the effectiveness of the morphometric method and may be responsible for the good correlation with the DNA results reported here. With increased African-European hybridization expected as African bees approach the temperate climates of the United States (Taylor and Spivak 1984, Taylor 1985, Lobo et al. 1989), the reliability of morphometric identification is likely to suffer.

Only samples with the highest probability (1.00) of being African, on the basis of forewing length, were ultimately determined to be African using more complete morphometric tests and DNA analyses. However, most of the other samples had scores of high African probability (Table 1) but were found not to be African upon further analysis. Thus, scores less than 1.00, obtained by the abbreviated method, appear to be questionable as useful indicators of African background.

The identification of African honeybee mtDNA is precise and unambiguous, but it only determines the matrilineal origin. Colonies found with African mtDNA, as the four samples reported here, are derived as continuous maternal lineages from the bees introduced from Africa. Previous studies have demonstrated that the feral neotropical African population is comprised almost exclusively of African matrilines (Hall & Muralidharan 1989, Smith et al. 1989, Hall & Smith 1991). Therefore, in testing neotropical colonies with mtDNA alone, an African maternal ancestry can be recognized in almost all feral colonies and in most managed colonies. However, an African paternity in European matrilines, which can result in significant defensive behavior, would not be recognized. Although hybrids cannot be identified using only mtDNA, valuable insight can be ob-

tained using mitochondrial and nuclear DNA together to distinquish maternal and paternal gene flow (Hall 1990).

The presence or absence of introgression between populations can be demonstrated using frequency differences among nuclear alleles, even if the alleles are not specific. A few honeybee allozymes have been useful for this purpose (Lobo et al. 1989, Smith et al. 1989). However, to identify certain African or European paternal ancestry in individual hybrids bees, nuclear alleles specific to each group are required (Page & Erickson 1985). The most useful markers are those present at higher frequencies, thereby representing more members of the group. Alleles that are diagnostic represent virtually all members (Ayala & Powell 1972). Because segregation results in the loss of alleles in hybrids, extensive admixture reduces the effectiveness of any single marker as an identifier. Thus, it is important to have specific alleles representing a number of loci.

Most of the useful honeybee nuclear DNA polymorphisms discovered, like two employed in this study, distinguish African from east European bees. Fewer markers have been found that distinguish African from west European bees. The other two markers used here appear to have the needed African specificity but are not present at high frequencies. The multiple paternity of honeybee colonies increases the probability that even low frequency African alleles would be present among the members of an African colony. Despite the limited specificities and frequencies of these nuclear DNA markers, they are effective when used together, as shown by the high correlation obtained here among the different African identifiers. Expanding the collection of markers, particularly with specific alleles at higher frequencies, will provide greater ability to characterize African-European hybridization in the United States.

Testing of a number of individuals is necessary to include the multiple patrilines of a honeybee colony. To facilitate the screening of colonies, individuals can be mixed together as one sample, as in this study. However, to quantitate more accurately the presence of the DNA alleles and to ascertain the genotypes of the queen and of the drones with which she has mated, individuals must be tested separately (Hall 1990). For this purpose, the samples must be well-preserved. That level of accuracy was not sought here, and many of the samples that had been provided as adults were not adequately preserved to allow recovery of sufficient intact DNA from individuals for these analyses. Other useful markers are available but are only effective in testing individuals. These markers represent multiple alleles characterized by differences in several restriction fragments (Hall 1992, McMichael & Hall, unpublished data). Because the different alleles share fragments in common, allele identities can be obscured in heterozygous individuals and especially in mixed sibling samples.

A major limitation of standard restriction fragment analyses is the expense, time and labor involved, especially if a number of individuals from each colony must be tested. Furthermore, low quality DNA preparations from poorly preserved specimens can preclude analysis. This study serves to contrast a newer PCR-based method used to identify the mtDNA (Hall & Smith 1991) with the standard method used to analyze the nuclear DNA. With the new method, very low quantities of DNA are needed, and the preparations can be more crude. The entire identification procedure can be accomplished in about 12 hours and is relatively inexpensive. The standard approach involves more than a week and is much more costly. The application of the PCR to the analysis of honeybee nuclear DNA polymorphisms is forthcoming. Implementation of this technology will enhance the search for additional markers and make nuclear DNA analyses feasible for regulatory identification and breeding stock certification.

Morphometric identification of honeybees is limited by the few known subtle morphological differences. MtDNA already allows for precise and rapid distinction but will always be limited to matriline identification. With the large amount of nuclear DNA, many more distinguishing polymorphisms can be found, and the application of new

technologies will greatly facilitate its use. Thus, the use of nuclear DNA for identifying bees of African ancestry has the greatest potential for continued improvement.

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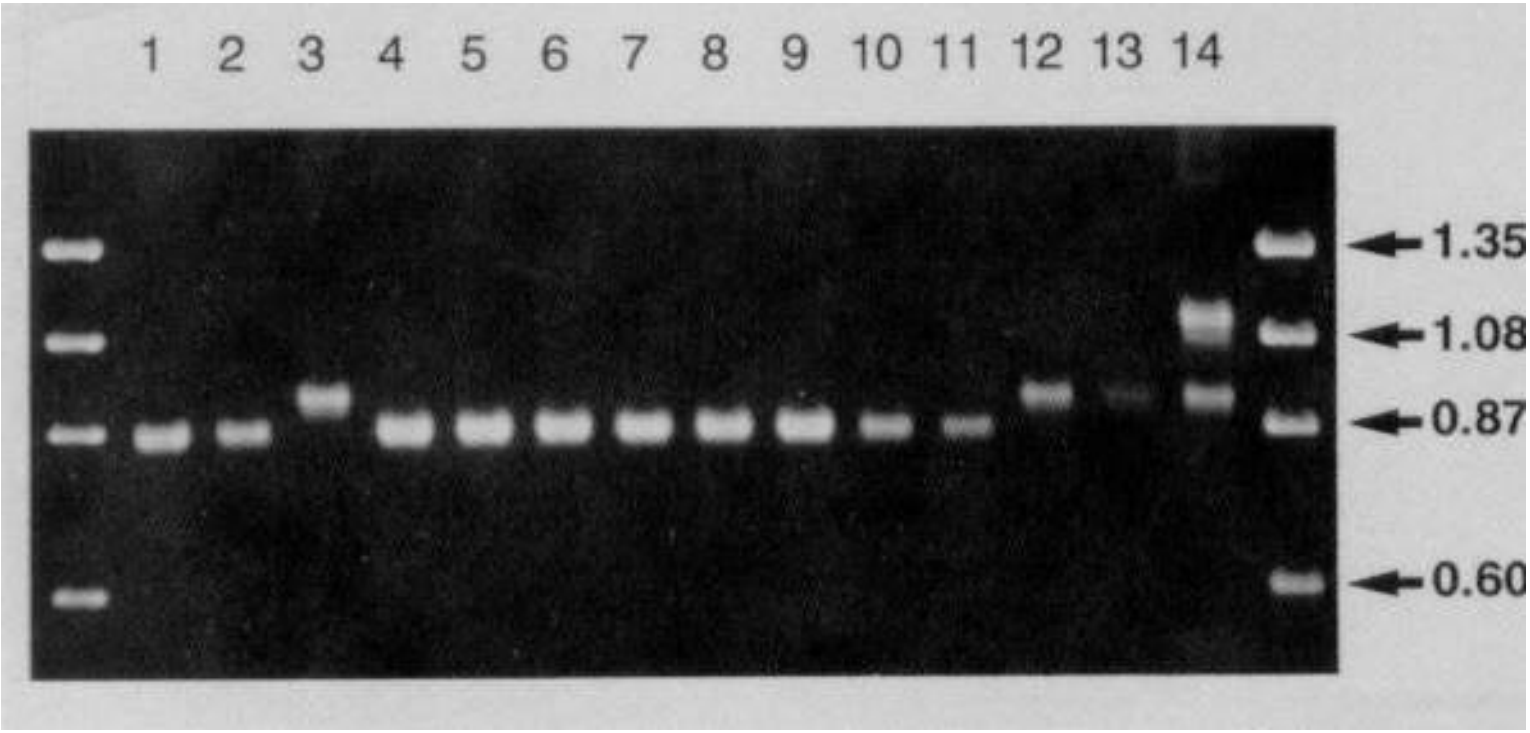
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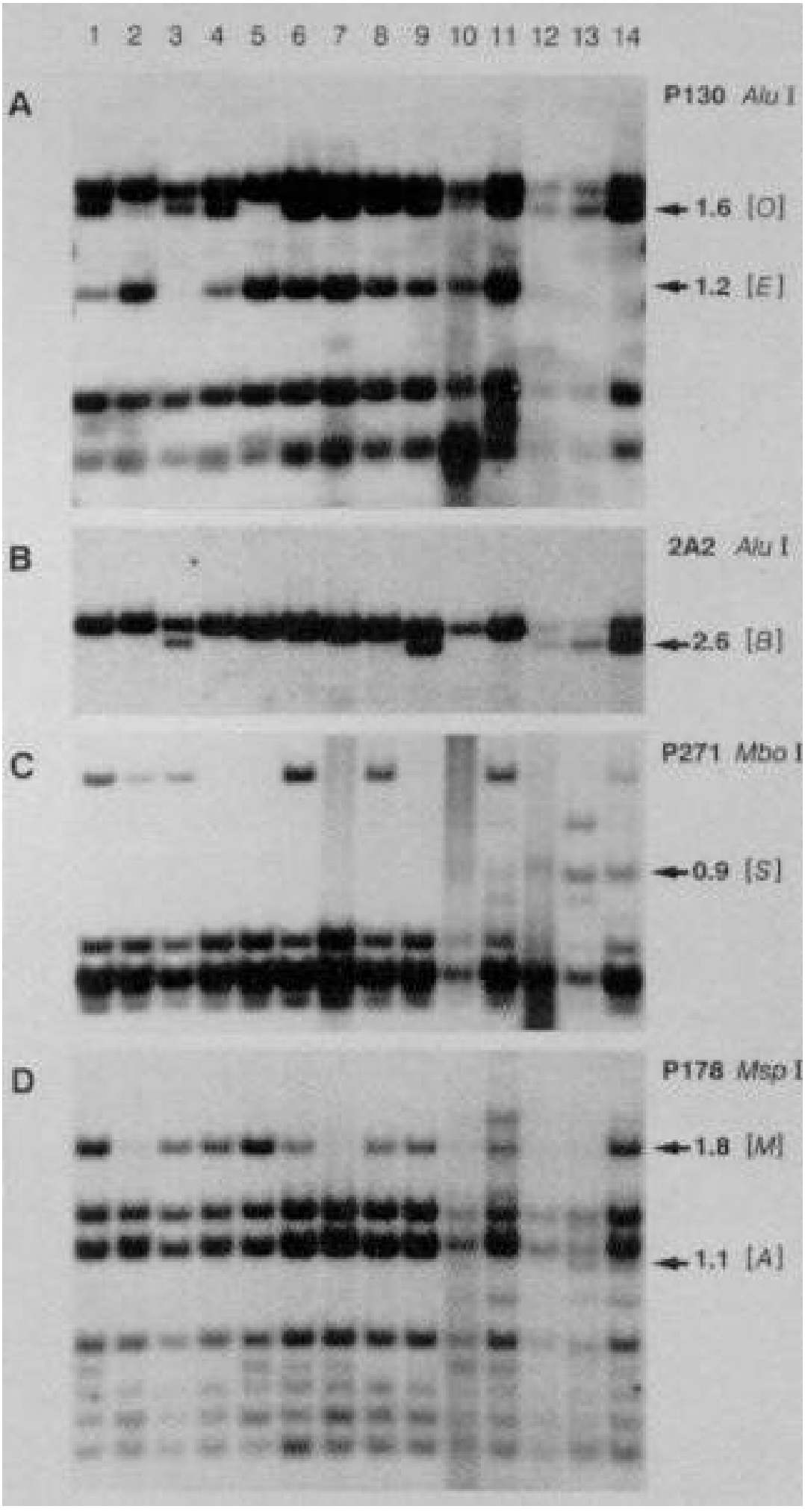
1 2 3 4 5 6 7 8 9 10 11 12 13 14
A
P130 Alu I
1.6 [O]
1.2 [E]
B
2A2 Alu I
2.6 [B]
C
P271 Mbo I
0.9 [S]
D
P178 Msp I
1.8 [M]
1.1 [A]