

*Hinf*I Variation in Mitochondrial DNA of Old World Honey Bee Subspecies

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Intraspecific taxonomy of the honey bee, *Apis mellifera*, delineates about two dozen subspecies or geographic races occurring throughout the original range in Europe, Africa, and the Middle East. Variation in behavior, morphology, and genetic markers has been reported among the subspecies and has been used to assess the racial origins of introduced populations in the New World and Australia. We examined mitochondrial DNA variation among endemic honey bee subspecies using the restriction enzyme *Hinf*I. Twenty different mtDNA haplotypes were identified and their distribution among and within the subspecies improved resolution of relationships among some racial groups and provided unambiguous identification of some taxa.

The endemic range of the honey bee, *Apis mellifera*, traverses northern Europe to southern Africa. Variation in behavior, physiology, and morphology reflects past and present barriers to panmixia and forms the basis for classification of the species into 25 subspecies or geographic races (Ruttner 1992). Morphological characters are used for honey bee intraspecific taxonomy, and evidence of biochemical and DNA differences among some of the races reinforces the hypothesis that Pleistocene glaciation led to the accumulation of genetic differences between isolated populations in Europe (Ruttner 1992). In Africa, it is likely that isolation of honey bee populations resulted from the dramatic oscillations in climate and vegetative belts that took place on the African continent during the late Quaternary (Potts and Behrensmeier 1992). Analysis of isozyme and mitochondrial DNA variation in Old World honey bees collectively permits the identification of major racial lineages similar to those based on morphology (Badino et al. 1988a; Cornuet 1982; Garnery et al. 1992; Moritz et al. 1986; Sheppard and Huettel 1988; Smith and Brown 1990). However, there has been limited success in discriminating among specific races with molecular markers. Several reasons exist for this difficulty: (1) allozyme variation in the honey bee is not extensive and the most common alleles are shared among races (Badino et al. 1988b; Comparini and Biasiolo 1991; Meixner et al. 1994; Sheppard and McPheron 1986); (2) mtDNA RFLP variation detected by 6-base

recognizing restriction enzymes differentiates among few individual races (Meixner et al. 1993; Moritz et al. 1986; Smith et al. 1991); and (3) reported sequence analyses of races represent only a small view of the variation present in the populations, because of restricted sample sizes (Garnery et al. 1992).

Recently the restriction enzyme *Hinf*I, which has a 4-base recognition site, was reported to discriminate mtDNA typical of *A. m. intermissa* from that of *A. m. scutellata*, subspecies from supra- and sub-Saharan Africa, respectively (Smith et al. 1991). Based on mitochondrial DNA restriction analysis and partial sequence comparisons, Smith et al. (1991) and Garnery et al. (1992) suggested that the honey bee populations of the Iberian peninsula represent an "intraspecific hybrid zone" between *A. m. mellifera* (the subspecies north of the Pyrenees) and *A. m. intermissa* from northern Africa. This interpretation contrasts with the view from morphological studies that these bees can be considered a distinct subspecies (Ruttner 1987; Santiago et al. 1986). Garnery et al. (1993) used the restriction enzyme *Dra*I to examine variation in the mitochondrial DNA COI-COII intergenic region of a number of honey bee populations. Subspecific identification was made by association of collecting sites with presumptive subspecific ranges. The authors reported variation among seven subspecies belonging to African (A) and western European (M) racial branches (sensu Ruttner 1987), while

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five subspecies of the eastern European branch (C) showed no variability.

In this article we report a survey for *Hinfl* variation among nine European and African honey bee races sampled from within areas of endemism. Twenty different mtDNA haplotypes were identified and their distribution among and within the subspecies improved resolution of relationships among some racial groups and provided unambiguous identification of some taxa.

Materials and Methods

Honey bee subspecies were sampled by collecting adult workers from colonies located in reported areas of endemism, and subspecific identifications were verified with discriminant analysis of morphological characters (Ruttner et al. 1978). Subspecies, collection locations, and number of colonies were as follows: *A. m. carnica*, Austria—Bruckl (5); Slovenia—Kamnik (3), Podkoren (2), Semic (2), Senicno (3), Vrhnik (1); *A. m. iberica*, Portugal—Malveira (7); Spain—Breda (1), Gualta (7), Guaro (2), Lecrin (4), Lora del Rio (2), Moura (9), Orba (4), Pelayo (2), Serra (2), Sevilla (2), Tarragoda (3), Ubrique (1); *A. m. intermissa*, Morocco—Adouar (1), Ain Oirma (2), Asilah (1), Beni Mellal (4), El Koudia (1), Inezerki (1), Moulay Idriss (3), Oualidia (1), Sidi Bennour (1), Larache (8); *A. m. lamarckii*, Egypt—Assiut (25); *A. m. ligustica*, Italy—Reggio Emilia (10), Udine (1); *A. m. mellifera*, France—Ille sur tet (7), Coll de Nargo (3); Norway—Birkeland (1), Loland (1), Skeibrok (2); *A. m. monticola*, Kenya—Kibaranyaki (1), Kimbo (8), Mt. Elgon (1); *A. m. sahariensis*, Morocco—Donar Teussikte (1), Hmame (2), Id Boukhtir (4), Ourika Ouaourmas (2), Ouarzazate (2); and *A. m. scutellata*, Kenya—Chiakari-ga (2), Gatimbi (3), Kaaga (1), Kimilili (5), Kiria (1), Ngong (2), Rwito (1), Tunyai (1).

Initially, at least 10 colonies per subspecies were characterized by restriction enzyme analysis of their mitochondrial DNA using the enzymes *EcoRI* and *Hinfl*. Within the *A. m. iberica* samples, we analyzed separately each of three distinct *EcoRI* haplotypes: the common "African" pattern (Smith 1988), the common "*A. m. mellifera*" pattern (Smith et al. 1989), and a third pattern, previously reported as "African" (Sheppard et al. 1991a). *EcoRI* restriction fragment patterns have been used to broadly differentiate mitochondrial origins of African and European races and their descendants (Hall and Muralidharan 1989; Rinderer et al. 1991; Shep-

pard et al. 1991a,b; Smith et al. 1989) and to distinguish *A. m. mellifera* from *A. m. carnica/A. m. ligustica* among European races (Sheppard et al. 1991a; Smith et al. 1991). Nucleic acid extraction, electrophoresis, hybridization conditions, and visualization of mtDNA fragments have been previously described (Sheppard and McPherson 1991; Sheppard et al. 1991a), although several modifications were made to resolve *Hinfl* restriction fragments. These included electrophoresis in 1.25%–1.50% agarose (Bio-Rad, Hercules, California) gel at 16 V for 18 h and Southern blotting to nylon or nitrocellulose filters.

Results and Discussion

Four haplotypes were observed among the 172 colonies of honey bees screened for *EcoRI* variation (Figure 1). In addition to the aforementioned three found in Iberian populations, these included a haplotype, first described by Moritz et al. (1986), present in all *A. m. carnica* and *A. m. ligustica* colonies sampled. *A. mellifera mellifera* from France and Norway exhibited a single *EcoRI* haplotype, previously reported by Smith et al. (1989). All 85 colonies sampled from the five African subspecies (*A. m. intermissa*, *A. m. lamarckii*, *A. m. monticola*, *A. m. sahariensis*, and *A. m. scutellata*) exhibited a single *EcoRI* haplotype.

When the 172 colonies were screened for *Hinfl* variation, 20 distinct haplotypes were observed (Figures 1 and 2). This restriction enzyme has 11 recognition sites within a recently published complete mtDNA sequence for the honey bee (Crozier and Crozier 1993). Therefore, some fragments quite likely were undetectable by the methods we used, either due to small sizes or duplicate migrations. However, the variation that was detectable on the Southern blots of *Hinfl* restriction digests was extensive and taxonomically useful at the subspecific level. The distribution of *Hinfl* patterns among the nine subspecies and within the three *EcoRI* haplotypes of *A. m. iberica* is shown in Table 1. One of the subspecies, *A. m. lamarckii*—a race endemic to the Nile river region of Egypt—exhibited a *Hinfl* haplotype that was unique; that is, the variant was not found in any other race. Other subspecies, such as *A. m. mellifera*, *A. m. ligustica*, and some *A. m. iberica* (iberica-me, identified as a "*mellifera*" *EcoRI* variant) exhibited patterns that were shared (E) and some that were unique (G, O, R).

Two of the subspecies that today are

considered to be "relic" or island populations surviving from previously extensive ranges yielded patterns common in the surrounding subspecies, in addition to unique variants. One, *A. m. sahariensis*, a subspecies believed to have been widespread in Pleistocene north African savanna (Ruttner 1987), but now limited to oases in the Sahara, exhibited four patterns, including three (H, I, L) that predominate in the surrounding subspecies *A. m. intermissa*. Similarly, *A. m. monticola*, previously widespread in forested east central Africa (Ruttner 1987) but now restricted to isolated mountain refugia, exhibited three *Hinfl* haplotypes, including two (A, B) found only in the surrounding subspecies *A. m. scutellata*. Recent analysis of Kenyan *A. m. monticola* populations indicates that variation in the amount of gene flow between the two races may be related to the ecological and climatic conditions of the refugia, with a deforested lower altitude site showing extensive introgression of the two subspecific types (Meixner et al. 1994).

The distribution of *Hinfl* variation alone among the Iberian honey bees does little to resolve the issue of the subspecific status of *A. m. iberica*. Iberian honey bees with *EcoRI* haplotypes identical to *A. m. mellifera* (iberica-me in Table 1) shared two *Hinfl* patterns (E, F) with that subspecies, but additional patterns (G, O) were unique to each group. Similarly, the Iberian honey bee colonies with *EcoRI* haplotypes identical to African races (iberica-af in Table 1) share two of their three *Hinfl* haplotypes with *A. m. intermissa* and *A. m. sahariensis*, although iberica-af, *A. m. intermissa*, and *A. m. sahariensis* also have unique patterns (J, M, N, respectively). Finally, Iberian honey bees exhibiting an *EcoRI* pattern previously known from Argentina (iberica-naf in Table 1) (Sheppard et al. 1991a), shared two of the three *Hinfl* patterns found in *A. m. intermissa* and iberica-af, although they also exhibited a unique variant (K). The iberica-naf *EcoRI* pattern occurs in about 10% of 500 Iberian honey bee colonies examined (Sheppard WS, unpublished data) and was not found in any of the other races in this study. Therefore, the original source for this haplotype in South American populations is quite likely in Iberia rather than sub-Saharan Africa as previously suggested (Rinderer et al. 1991; Sheppard et al. 1991a). Garnery et al. (1993) reported extensive mtDNA variation in the COI and COII intergenic region of the "African" (A) and "mellifera" (M) lineages. They reported

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

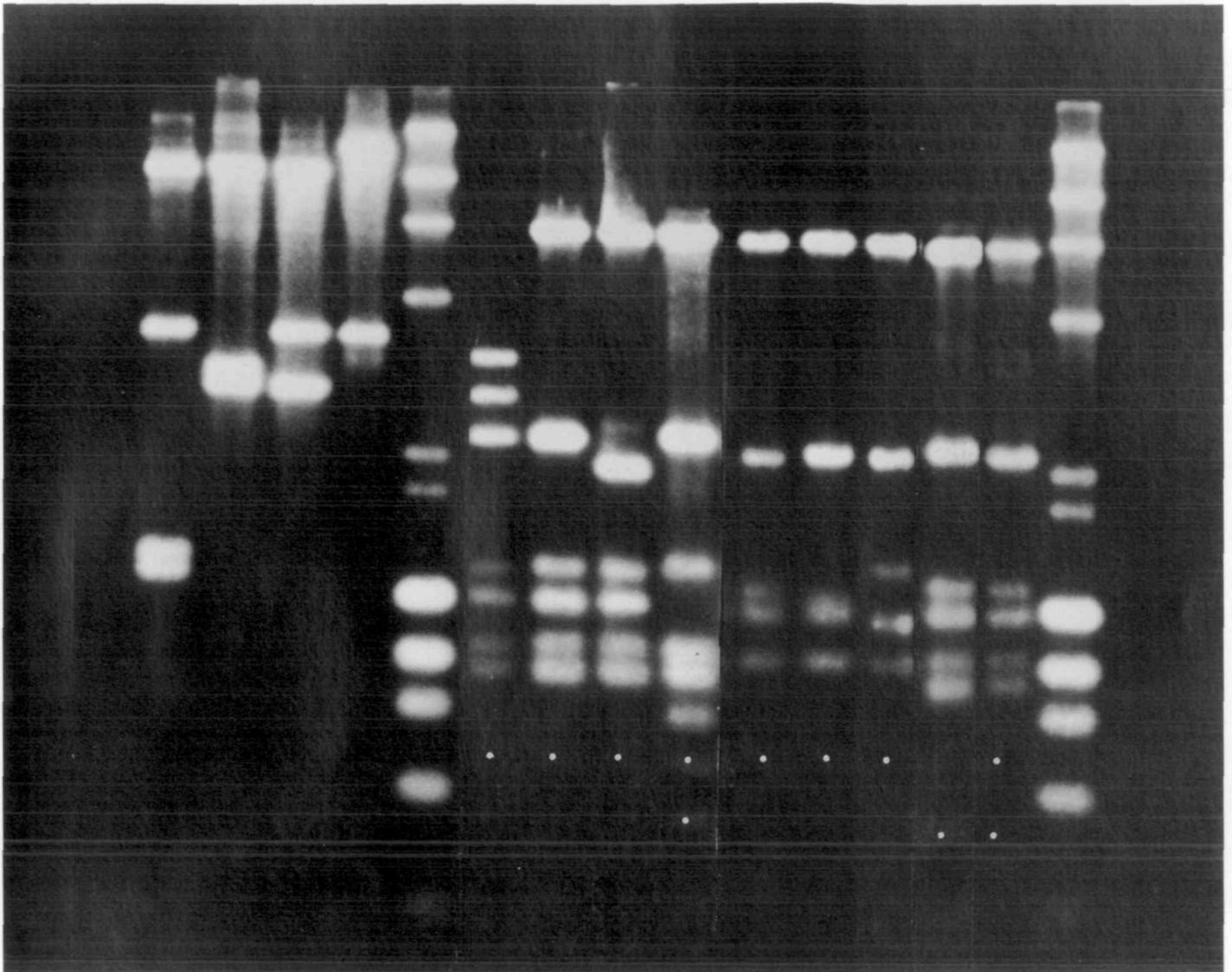


Figure 1. Autoradiograph showing *EcoRI* and *HinfI* variation in honeybee mtDNA. Lanes 1–4: *EcoRI* haplotypes common in (1) *A. m. carnica* and *A. m. ligustica*, (2) *A. m. mellifera*, (3) African subspecies (4) *A. m. iberica* (not common; see text); lanes 6–14: *HinfI* haplotypes A–I, respectively; lanes 5, 15: size standards λ /HindIII + Phi X 174/HaeIII. Presence of small fragment bands difficult to visualize in photograph are indicated by white dots.

that 3 of 75 colonies of *A. m. iberica* exhibited mtDNA haplotypes found in *A. m. intermissa*, and 33 of the 75 colonies exhibited mtDNA haplotypes found in *A. m. mellifera*. In addition to this shared variation, they also found haplotypes unique to various subspecies, similar to the present study. It is likely that the use of multiple sets of genetic markers, including those detectable in the COI–COII intergenic region and in the mtDNA using *HinfI*, can improve the resolution of genetic relationships among the subspecies.

Morphometric analysis of 30 Iberian colonies (10 of each *EcoRI* haplotype) did not detect an association between mtDNA haplotypes and morphological variation

(Figures 3 and 4). This suggests that there is little present-day gene flow between Iberian populations and *A. m. mellifera* or *A. m. intermissa*. The independence of morphology and mtDNA haplotypes in present-day Iberia and the corresponding conclusion that gene flow with the surrounding subspecies is now low, indicates that the validity of *A. m. iberica* as a distinct subspecies should be considered in the context of overall morphological differentiation within the species. The morphological similarity among the Iberian populations sampled in this study and their distinctness from neighboring *A. m. intermissa* and *A. m. mellifera* enhances this interpretation. However, the occurrence of

mtDNA haplotypes common in *A. m. intermissa* and *A. m. mellifera* supports the suggestion by Garnery et al. (1993) and Smith et al. (1991) that populations of the Iberian peninsula may reflect a historical intrusion of European “*mellifera*” and African “*intermissa*” mtDNA. However, this intrusion would have occurred substantially before the present, perhaps during the periods of lower sea levels of the Pleistocene. In addition, the potential role of human assisted movement of African *A. m. intermissa/sahariensis* into Iberia during 800 years of Moorish occupation cannot be dismissed without careful historical investigation.

Based on analysis of a collection of four

1 2 3 4 5 6 7 8 9 10 11 12 13

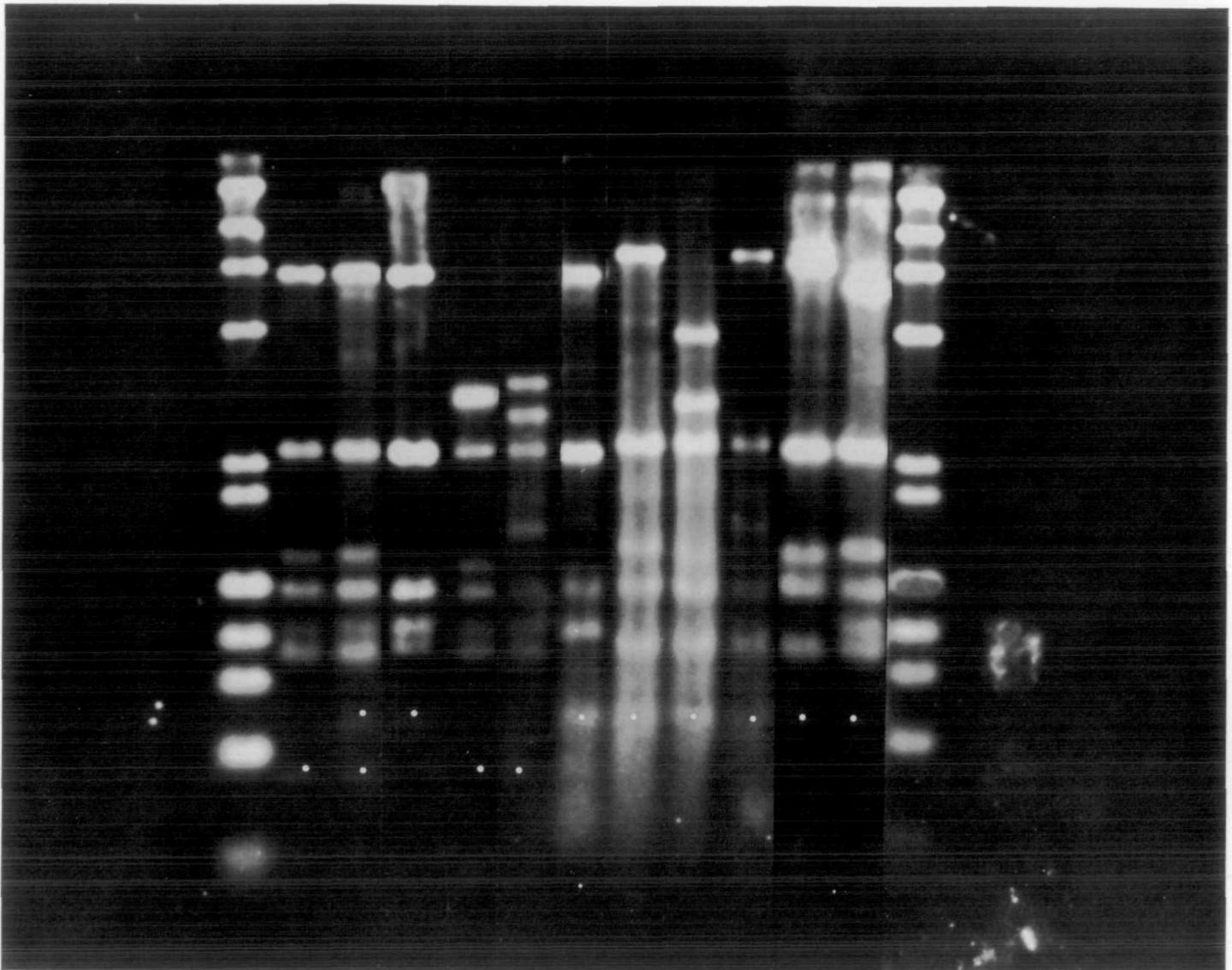


Figure 2. Autoradiograph showing *Hinfl* variation in honeybee mtDNA. Lanes 2–12: *Hinfl* haplotypes J–T, respectively; lanes 1, 13: size standards λ /HindIII + Phi X 174/HaeIII. Presence of small fragment bands difficult to visualize in photograph are indicated by white dots.

Table 1. Distribution of *Hinfl* haplotypes among the subspecies^a

| Subspecies | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S | T |
|--------------------|----|---|---|----|---|---|---|----|---|---|---|---|---|---|---|---|---|---|---|---|
| <i>camica</i> | | | | | | | | | | | | | | | | 8 | 1 | | 4 | 3 |
| <i>intermissa</i> | | | | | | | | 19 | 1 | | | 1 | 2 | | | | | | | |
| <i>lamarckii</i> | | | | 25 | | | | | | | | | | | | | | | | |
| <i>ligustica</i> | | | | | 3 | | | | | | | | | | | 7 | | 1 | | |
| <i>mellifera</i> | | | | | 6 | 7 | | | | | | | | | 1 | | | | | |
| <i>monticola</i> | 1 | 8 | 1 | | | | | | | | | | | | | | | | | |
| <i>sahariensis</i> | | | | | | | | 5 | 2 | | | 3 | | 1 | | | | | | |
| <i>scutellata</i> | 10 | 6 | | | | | | | | | | | | | | | | | | |
| <i>iberica</i> | | | | | | | | | | | | | | | | | | | | |
| -af ^b | | | | | | | | 13 | 1 | 4 | | | 1 | | | | | | | |
| -me ^b | | | | | 7 | 7 | 1 | | | | | | | | | | | | | |
| -na ^b | | | | | | | | 5 | 5 | | 2 | | | | | | | | | |

^a Numerals refer to the number of sampled colonies exhibiting a particular haplotype.

^b Refers to mtDNA haplotype based on *EcoRI* fragment pattern (see text).

colonies, *A. m. lamarckii* was reported to differ in polymorphic restriction sites (detected by 6-base recognizing restriction enzymes) from other African subspecies, and the subspecies was assigned to an eastern Mediterranean lineage of mitochondrial haplotypes (Smith 1991). According to Kamel (1991), *A. m. carnica* was extensively imported into Egypt and, by 1934, was maintained in isolated mating areas. In a study of mtDNA from 15 Egyptian colonies (Kamel 1991), the *EcoRI* haplotype common in *A. m. carnica* (Moritz et al. 1986; Smith and Brown 1990) was found in 7 colonies, while 8 others (all morphologically identified as *A. m. lamarckii*) exhibited haplotypes classified as African. In the present study, the 25 *A. m. lamarckii*

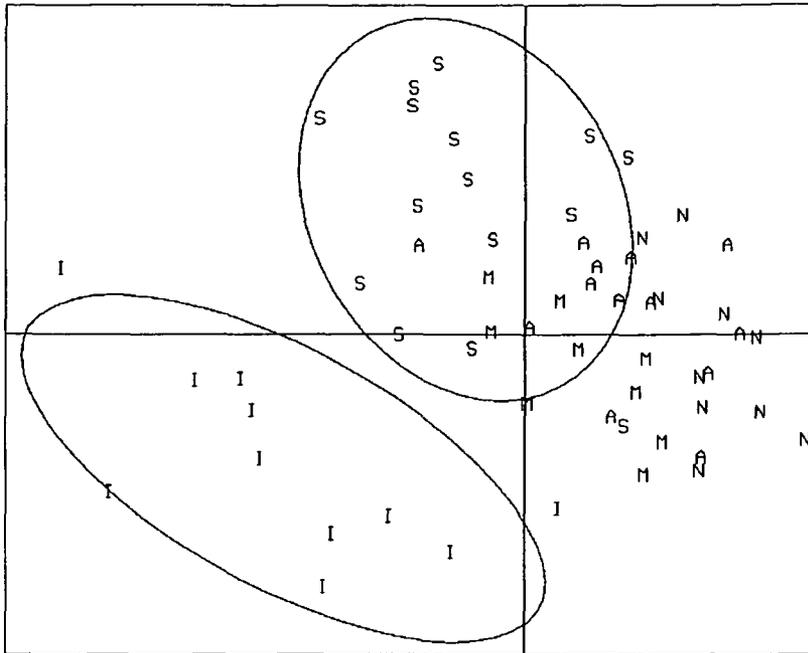


Figure 3. Factor analysis with 30 Iberian samples and *A. m. iberica* and *A. m. intermissa* from the Oberursel collection. Across: Factor 1; down: Factor 3. The ellipses of confidence (75%) are shown for the two subspecies. S: *A. m. iberica* (Oberursel collection); I: *A. m. intermissa* (Oberursel collection); A: Iberian samples with "African" *EcoRI* mtDNA haplotype; M: Iberian samples with "*A. m. mellifera*" *EcoRI* mtDNA haplotype; N: Iberian samples with the "New African" *EcoRI* mtDNA haplotype (see text).

samples were collected from isolated apiaries of traditional mud hives and were morphologically identified to subspecies. The typical "African" *EcoRI* haplotype present in these colonies suggests that the

previous assignment of *A. m. lamarckii* to the eastern Mediterranean mtDNA lineage that includes *A. m. carnica* and *A. m. ligustica* was probably the result of sampling imported European haplotypes. Notwith-

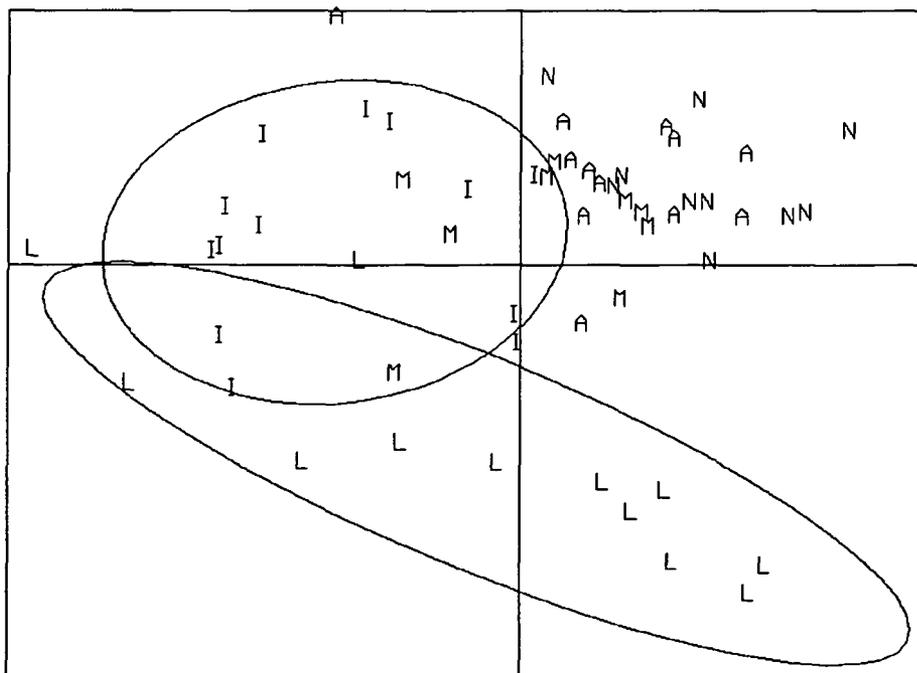


Figure 4. Factor analysis with 30 Iberian samples and *A. m. iberica* and *A. m. mellifera* from the Oberursel collection. Across: Factor 1; down: Factor 2. The ellipses of confidence (75%) are shown for the two subspecies. I: *A. m. iberica* (Oberursel collection); L: *A. m. mellifera* (Oberursel collection); A: Iberian samples with "African" *EcoRI* mtDNA haplotype; M: Iberian samples with "*A. m. mellifera*" *EcoRI* mtDNA haplotype; N: Iberian samples with the "New African" *EcoRI* mtDNA haplotype (see text).

standing, a full understanding of *A. m. lamarckii*'s genetic relationship among *A. mellifera* subspecies may require a systematic comparison with neighboring subspecies, including *A. m. intermissa*, *A. m. sahariensis*, *A. m. syriaca*, and *A. m. yemetic*.

The resolution of mtDNA haplotype associations with particular subspecies or racial lineages is obviously a complex issue. The fact that *A. m. carnica* and *A. m. ligustica*, two subspecies clearly separable by morphology, share *EcoRI* haplotypes (lane 1 in Figure 1), but are partially differentiated by *HinfI* haplotype profiles, could be explained by development of some of the *HinfI* recognition sites after isolation of the subspecies. Conversely, the *EcoRI* haplotype obviously would have arisen before the isolation of the Ligurian and Balkan refugia believed to be responsible for development of *A. m. ligustica* and *A. m. carnica*, respectively (Ruttner 1992). Recently, Ruttner's hypothesis of four major lineages among *A. mellifera* subspecies has been questioned, based on similarity of mitochondrial DNA relationships between *A. m. caucasica* and *A. m. carnica/ligustica* (Garnery et al. 1992). Only a single colony of *A. m. caucasica* was included in a sequencing analysis and three colonies in a restriction site comparison, with subspecific assignment "postulated according to geographic origin." The existence of biological variation within subspecies, well documented by morphometrics and certainly evident in our study of variation based on *HinfI* restriction sites, indicates that molecular analyses of additional representative samples of the subspecies must be conducted before support can be given to the alternative hypothesis of these authors. Future investigation of honey bee subspecific relationships will undoubtedly include molecular methodologies and will require consideration of common aspects of both population genetics and systematics regarding sampling and within taxon variation.

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