Restriction Endonuclease Cleavage Site and Length Polymorphisms in Mitochondrial DNA of *Apis mellifera mellifera* and *A. m. carnica* (Hymenoptera: Apidae)

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ABSTRACT Restriction endonuclease cleavage maps of mitochondrial DNAs of Scandinavian *Apis mellifera mellifera* L., of German, Austrian, and Yugoslavian *A. m. carnica* Pollman, and of Austrian "Nigra" honey bees are compared with previously published maps of mitochondrial DNA from North American bees of European ancestry and Brazilian Africanized bees. *A. m. mellifera* mitochondrial DNA is characterized by a pattern of cleavage sites unique among the honey bee populations thus far investigated. Variation in size of the mitochondrial DNA molecule is common among families (hives) of *A. m. mellifera* and appears to involve several distinct regions that span a region at least 5.1 kilobase pairs in length. Some elements of size variation seem to be confined to the *A. m. mellifera* population, whereas others are shared with Africanized bees. *A. m. carnica* mitochondrial DNA is characterized by a pattern of cleavage sites, which differs from that of *A. m. mellifera* and the Africanized bees but is similar to that of the domestic North American bees of European ancestry.

KEY WORDS Insecta, *Apis mellifera*, Africanized honey bee, mitochondrial DNA

Apis mellifera L. is subdivided into approximately 24 named geographic races or subspecies (Ruttner 1988). These subspecies are believed to be recent in origin; for example, the principal subspecies in Europe may have differentiated from one another during the Pleistocene glacial periods, when European honey bee populations were probably confined to refugia around the Mediterranean (Ruttner 1988).

Apis mellifera subspecies are defined and identified largely by morphometric criteria (e.g., Alpatov 1929, Cornuet et al. 1975, Daly & Balling 1978, Ruttner et al. 1978, Ruttner 1986), but they also differ in behavior, physiology, and ecology (Adam 1951, 1954, 1961, 1964, 1977; Ruttner 1988). Differences in allele frequencies have been found among subspecies at several polymorphic loci (e.g., Mestriner & Contel 1972; Contel et al. 1977; Martins et al. 1977; Sylvester 1982; Del Lama et al. 1985, 1988; Spivak et al. 1988). These polymorphisms are useful in the study of honey bee biogeography and population biology (e.g., Cornuet 1979; Sheppard & Berlocher 1984, 1985; Cornuet et al. 1986; Sheppard & McPheron 1986) and in the study of Africanized bees (Nunamaker & Wilson 1981, Sylvester 1982, Nunamaker et al. 1984).

Polymorphisms in mitochondrial DNA (mtDNA) complement and extend studies of allozyme variation in a number of ways. Because animal mtDNA is typically maternally inherited without recombination (e.g., Dawid & Blackler 1972, Reilly & Thomas 1980; Lansman et al. 1983; Brown 1985), genetic markers on mtDNA can be used to determine the maternal ancestry of hybrid individuals and, thus, the directionality of gene flow in hybrid zones or hybrid populations (Wright et al. 1983, Avise & Saunders 1984, Gyllensten et al. 1985). In addition, because animal mtDNA does not undergo recombination during sexual reproduction, it is passed intact along maternal lineages. This allows identification of the populations that have contributed to a hybrid population even after many generations of hybridization or backcrossing, or both (Ferris et al. 1982, Wright et al. 1983, Tegelström 1987).

Several practical considerations make the use of mtDNA particularly attractive in the study of honey bee population biology. First, although honey bees appear to have relatively low levels of allozyme variability (e.g., Sheppard & Berlocher 1984, 1985), the level of variation in their mtDNA is well within the range found in other species (Avise & Lansman 1983). Second, although no fixed differences in allozymes have been found among honey bee subspecies, preliminary studies of the mtDNAs of European and African subspecies (Smith 1988; D.R.S., unpublished data) indicate that at least some have unique cleavage site patterns. Finally, because all the offspring of a queen inherit the same

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mtDNA, large quantities of mtDNA from a single source can be prepared by pooling tissue from hive mates.

The Carniolan honey bee, *Apis mellifera carnica* Pollmann, is native to Yugoslavia, Austria south of the Alps, and parts of Hungary, Rumania, and Bulgaria (Ruttner 1975, 1988). Because of its gentle disposition, good overwintering abilities, and good honey production, this subspecies is popular among commercial and amateur beekeepers and has been imported to other parts of Europe and to the New World. It is now one of the most widely distributed of the honey bee subspecies (Ruttner 1975). *Apis mellifera mellifera* L. originally occupied Britain nd north and central Europe (Ruttner 1988). Toay its range, at least in domesticated populations, as been dramatically altered by human prefernces. *A. m. ligustica* Spinola and *A. m. carnica* ıave been introduced into many parts of *A. m. mellifera*'s range, and in some domestic populations (e.g., Germany, Switzerland) they have largely replaced native *A. m. mellifera* (Ruttner 1975). Thus care was exercized in the selection of the *A. m. mellifera* populations we sampled. The Swiss and Austrian "Nigra" honey bees are hybrids of *A. m. mellifera*, originally native to these areas, and imported *A. m. carnica*.

Our study presents cleavage site maps and an analysis of length variation in *A. m. mellifera*, *A. m. carnica*, and "Nigra" mtDNA as well as comparisons between these mtDNAs and those of North American bees of European ancestry and Brazilian Africanized bees. This is part of a continuing survey of mtDNA in *A. mellifera* subspecies.

Materials and Methods

Collections. Fifty to 200 adult worker bees were collected from each hive. Heads and thoraces were frozen in liquid nitrogen and transported to the laboratory, where they were stored at −80°C until they were used in the preparation of mtDNA.

Samples of *A. m. carnica* were collected from Graz, Villach, and the Institut für Bienenkunde, Lunz-am-See, Austria. The research apiary at Lunz-am-See maintains imported colonies as well as local honey bees. Samples collected at the Institut für Bienenkunde included hives from Medvode (Slovenia) and Split (Dalmatia), Yugoslavia; Hamburg, West Germany; and Vienna and Lunz-am-See, Austria. Graz, Villach, Vienna, Medvode, and Split lie within the original range of *A. m. carnica*. Lunz-am-See lies outside the original range of *A. m. carnica*, but *A. m. carnica* is well established in the area now and most domestic hives are this subspecies (Ruttner 1988). The Hamburg sample is an example of the *A. m. carnica* that have been imported extensively into northern and western Europe and that have largely replaced domesticated *A. m. mellifera* over much of that subspecies' original range.

Because *A. m. mellifera* has been replaced or hybridized with other subspecies throughout much of its range, bees for this study were collected from apiaries whose hives were known to consist mainly of *A. m. mellifera* and for which the history of importation and breeding was relatively well known. Samples of *A. m. mellifera* were collected from Laesø, Denmark (the island of Laesø is now a preserve for *A. m. mellifera*, and importation of other subspecies is forbidden; S. Toft, personal communication); Billingstad, near Asker, Norway; and Uppsala, Sweden. Samples of the honey bee strain "Nigra" were collected from Ötztalbanhof Austria. Subspecies identifications have been corroborated by morphological measurements and allozyme analysis (D.R.S. & B. Crespi, unpublished data).

MtDNA was prepared from three hives each from Graz (Graz1–3), Villach (Vill1–3), and Split (Dalm1–3); two hives each from Vienna (Wien1–2) and Medvode (Slov1–2); one hive each from Lunz-am-See (Lunz1) and Hamburg (Hamb1); seven hives from Laesø (Laesø1–7); three hives each from Asker (Asker1–3) and Uppsala (Upps1–3); and from nine hives from Ötztalbanhof (Nigra1–9).

Preparation and Analysis of mtDNA. Mitochondrial DNA was prepared from frozen thoraces of about 25 adult worker bees from each hive by the method described by Wright et al. (1983) with the following modifications of the tissue homogenization procedure. Batches of 15–25 thoraces were ground to a powder in liquid nitrogen with a ceramic mortar and pestle. The powdered tissue (mostly flight muscle) was resuspended either in 10 ml of 10 m*M* NaCl, 10 m*M* Tris, 200 m*M* EDTA, pH 7.5, or in 10 ml of 10 m*M* Tris, 10 m*M* EDTA (TE), pH 7.5. 1.2 ml of 20% sodium dodecyl sulfate (SDS) was added, and the mixture was incubated for 10–20 min at room temperature to lyse cell and organelle membranes. 3.5 ml of CsCl-saturated TE was then added, and the mixture was incubated on ice for 15–30 min to precipitate SDS and proteins. The mixture was centrifuged at 17,000 × *g* (12,000 rpm in a Beckman JA-17 rotor) at 4°C for 10 min to pellet cellular debris; 1–2 ml of propidium iodide (2 mg/ml in TE) was added to the supernatant, and the density of the solution was adjusted to 1.56–1.57 g/ml with solid CsCl. CsCl density centrifugation and sample recovery followed the methods described in Wright et al. (1983).

Aliquots of each mtDNA sample were digested with each of 15 restriction enzymes (AccI, AvaI, BclI, BglII, EcoO109, EcoRI, EcoRV, HincII, HindIII, NdeI, PstI, PvuII, SpeI, XbaI, and XhoI), using the buffer conditions recommended by the suppliers (Bethesda Research Laboratories, Gaithersburg, Md.; International Biotechnologies, New Haven, Conn.; New England BioLabs, Beverly, Mass.; Boehringer Mannheim Biochemicals, Indianapolis). Each of these enzymes recognizes and cleaves a particular sequence or sequences of six basepairs. The resulting DNA fragments were ra-

Table 1. Percentage sequence divergence among the mitochondrial genomes of *A. m. carnica*, *A. m. mellifera*, a North American hive of European ancestry (USA1), and a Brazilian Africanized hive (Brz1)

	Car1	Car2	Car3	USA1	Brz1	Mel1	Mel2	Mel3	Mel4
Car1	—	0.0097	0.0033	0.0033	0.0269	0.0372	0.0340	0.0291	0.0324
Car2	0.0057	—	0.0063	0.0063	0.0291	0.0388	0.0356	0.0310	0.0341
Car3	0.0033	0.0045	—	0.0065	0.0304	0.0405	0.0372	0.0324	0.0356
USA1	0.0033	0.0045	0.0047	—	0.0223	0.0324	0.0291	0.0246	0.0278
Brz1	0.0109	0.0111	0.0116	0.0096	—	0.0269	0.0233	0.0269	0.0223
Mel1	0.0129	0.0129	0.0135	0.0117	0.0109	—	0.0034	0.0068	0.0033
Mel2	0.0123	0.0123	0.0129	0.0111	0.0101	0.0035	—	0.0105	0.0068
Mel3	0.0111	0.0112	0.0117	0.0099	0.0109	0.0049	0.0063	—	0.0033
Mel4	0.0117	0.0117	0.0123	0.0106	0.0096	0.0033	0.0049	0.0033	—

The method of Nei & Tajima (1983) was used to estimate the mean number of nucleotide substitutions per site (above the diagonal) and standard deviation of the mean (below the diagonal) using comparisons of the mapped cleavage sites produced by nondegenerate six base pair restriction enzymes (BclI, BglII, EcoO109, EcoRI, EcoRV, HindIII, NdeI, PstI, PvuII, SpeI, XbaI, and XhoI). *A. m. mellifera* restriction morphs (Mel1–Mel4) as in Fig. 2, *A. m. carnica* restriction morphs (Car1–Car3) as in Fig. 3.

dioactively end-labeled with ^{32}P-deoxynucleotides using the large (Klenow) fragment of DNA polymerase I and separated by electrophoresis on 1% agarose and 4% polyacrylamide gels (Brown 1980, Ferris et al. 1981, Wright et al. 1983). The fragments were visualized by autoradiography. The lengths (in thousands of base pairs, or kilobase pairs, kb) of the fragments were estimated by comparison of their mobilities with those of known-size standards run on each gel (Brown 1980, Wright et al. 1983). A map of the six base pair (bp) cleavage sites was constructed for each mtDNA by means of double digestions (Brown & Vinograd 1974) and by comparison with previously constructed maps from North American honey bees of presumed European ancestry (probably *A. m. ligustica* or *A. m. carnica*) and Brazilian Africanized bees, presumed to be descendants of introduced *A. m. scutellata* (Smith & Brown 1988). BglII restriction sites in the mtDNA of French *A. m. mellifera* and Italian *A. m. ligustica* were first mapped by J.-M. Cornuet (personal communication). Their location was confirmed in this study. Percentages of mtDNA sequence divergences among *A. m. mellifera*, *A. m. carnica*, an American hive of European ancestry, and a Brazilian Africanized hive were estimated by the method of Nei & Tajima (1983) using only nondegenerate six base pair restriction enzyme cleavage sites (Table 1).

Results and Discussion

Both cleavage site and length polymorphisms occur in honey bee mtDNA. Fig. 1 shows examples of restriction-site polymorphisms among the mtDNAs of *A. m. carnica*, *A. m. mellifera*, and a hive of Brazilian Africanized bees. Two distinct restriction enzyme cleavage maps were found in this study, one typical of *A. m. mellifera* (Fig. 2) and a second typical of *A. m. carnica* (Fig. 3). Seven of the nine "Nigra" samples had *A. m. mellifera* mtDNA and two, Nigra 3 and 7, had *A. m. carnica* mtDNA.

The mtDNAs of *A. m. mellifera* and *A. m. carnica* show very little intrapopulation variation in cleavage sites. Only the presence of two SpeI sites and one BclI site varied among Scandinavian *A. m. mellifera* and *mellifera*-like "Nigra" hives (Fig. 2). *A. m. carnica* and *carnica*-like "Nigra" hives differed in the presence or absence of two XbaI sites and one NdeI site (Fig. 3). The similarity of mtDNA cleavage maps within each subspecies is reflected in the low estimate of sequence divergence among the *A. m. mellifera* mtDNAs (0.33–

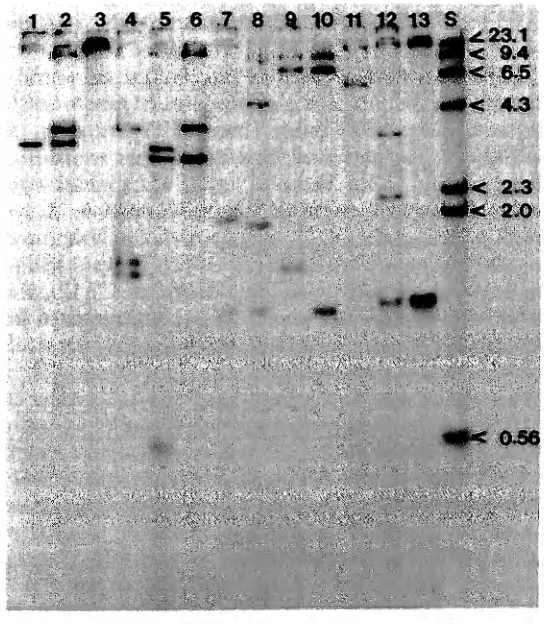

Fig. 1. Autoradiograph of 1% agarose gel showing restriction fragment length polymorphisms in mtDNA of *A. m. carnica* (lanes 1, 4, 7, 8, and 11), *A. m. mellifera* (lanes 2, 5, 9, and 12), and a Brazilian Africanized honey bee (lanes 3, 6, 10, and 13). Samples were digested with the restriction endonucleases BglII (lanes 1–3), EcoRI (lanes 4–6), XbaI (lanes 7–10), and a double digest with EcoO109 and SpeI (lanes 11–13; the band at approximately 3.3 kb in lane 11 is the result of partial digestion at the 0.5 kb SpeI site). The size standard (lane S) is a HindIII digest of wild-type lambda phage DNA; size of fragments is given in kilobase pairs.

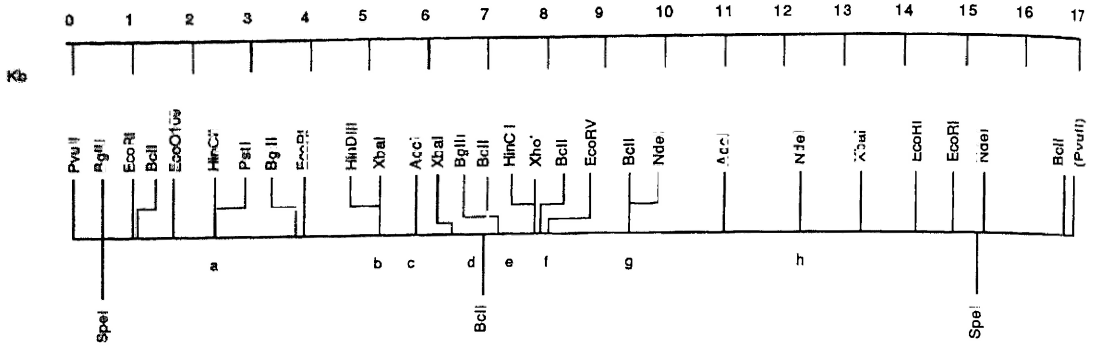

Fig. 2. Restriction enzyme cleavage site map of mtDNAs of Scandinavian *A. m. mellifera* and *mellifera*-like Austrian "Nigra" bees. The cleavage site for PvuII was arbitrarily chosen as the starting point for the map. Cleavage sites above the horizontal were common to all *A. m. mellifera* and *mellifera*-like "Nigra" mapped. The presence of sites below the horizontal (SpeI at 0.5 kb, SpeI at 15.16 kb, and BclI at 6.89 kb) varied among restriction morphs (Mel1–Mel4) as follows: Mel1, both SpeI sites present, BclI site absent, Laesø1–3, Asker1 and 3, Nigra6 and 8; Mel2, SpeI at 0.05 present, BclI and SpeI at 15.16 absent, Upps1 and 2; Mel3, SpeI at 15.16 and BclI present, SpeI at 0.05 ent, Upps3, Mel4, all 3 sites present, Laesø4–7, Asker2, Nigra2,4,5,9. Restriction-site mapping indicated that the clI and PstI sites at 2.4 kb were approximately 10 bp apart, the HincII site occurring before the PstI site bsequent sequencing of this region by Vlasak et al. (1987) shows these sites to be 9 bp apart; the region sequenced 0–3.0 kb on our map) encompasses most of the large subunit ribosomal DNA (Vlasak et al. 1987). The NdeI and II sites at approximately 9.4 kb were indistinguishable by restriction-site mapping. Subsequent sequencing of this gion (Crozier et al. 1989) shows that these two restriction sites are overlapping. The HindIII and XbaI sites at 3.2 kb are approximately 50 bp apart, the HindIII site occurring before the XbaI site. The HincII and XhoI sites at approximately 7.8 kb are approximately 15 bp apart; their relative positions have not been determined. The letters below the horizontal refer to regions of size variation presented in Table 2.

1.00%) and among the *A. m. carnica* mtDNAs (0.33–0.97%; Table 1).

However, the restriction site maps show substantial variation among subspecies. *A. m. mellifera* mtDNAs differ from those of *A. m. carnica* and the American and Brazilian bees examined (Fig. 4), and from those of other subspecies currently under investigation (*A. m. ligustica*, *A. m. scutellata* Lepeletier, *A. m. capensis* Escholtz, and *A. m. iberica* Goetze; Smith [1988] and unpublished data). In particular, the HincII site at 7.7 kb, the EcoRI site at 14.7 kb, and the SpeI site at 16.16 kb (Fig. 2) have been found so far only in *A. m. mellifera* samples. Likewise, the absence of the HindIII site at approximately 6.3 kb and the AccI site at 11.03 kb appear to be characteristic of *A. m. mellifera*. The mtDNAs of *A. m. carnica* are not substantially different from that of USA1, a domestic North American hive (0.33–0.47% sequence divergence; Table 1). This is not surprising, because domestic North American bees are primarily descendants of *A. m. ligustica* and *A. m. carnica*, and these two subspecies are themselves thought to be very closely related (Ruttner 1988). The differences between the cleavage maps of *A. m. mellifera*, *A. m. carnica*, USA1, and a Brazilian Africanized hive

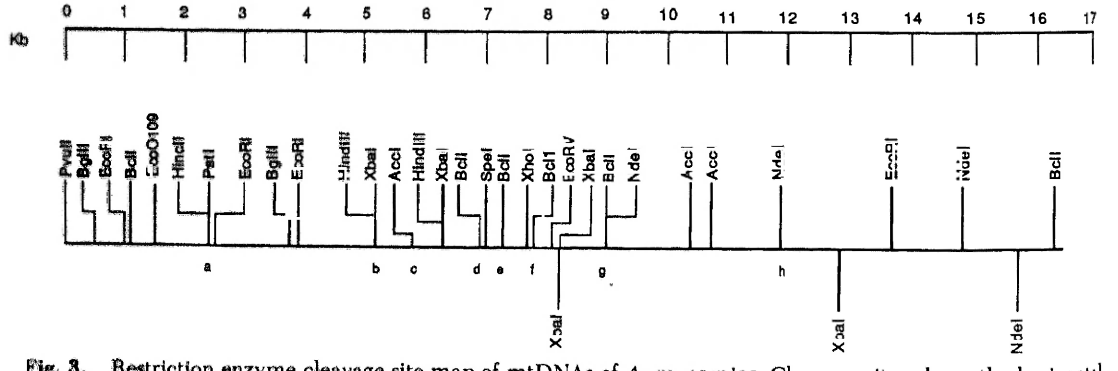

Fig. 3. Restriction enzyme cleavage site map of mtDNAs of *A. m. carnica*. Cleavage sites above the horizontal were common to all *A. m. carnica* mapped. The presence of the sites below the horizontal (XbaI at 8.23 and 12.89, NdeI at 15.74) varied among restriction morphs (Car1–Car3) as follows: Car1, both XbaI sites and NdeI site absent, Graz1 and 3, Slov1; Car2, both XbaI sites and NdeI site present, Vill3, Dalm1 and 3; Car3, both XbaI sites absent, NdeI site present, Hamb1, Wien1 and 2, Lunz1, Vill1 and 2, Graz2, Dalm2, and Slov2. The letters below the horizontal refer to regions of size variation discussed in Table 2.

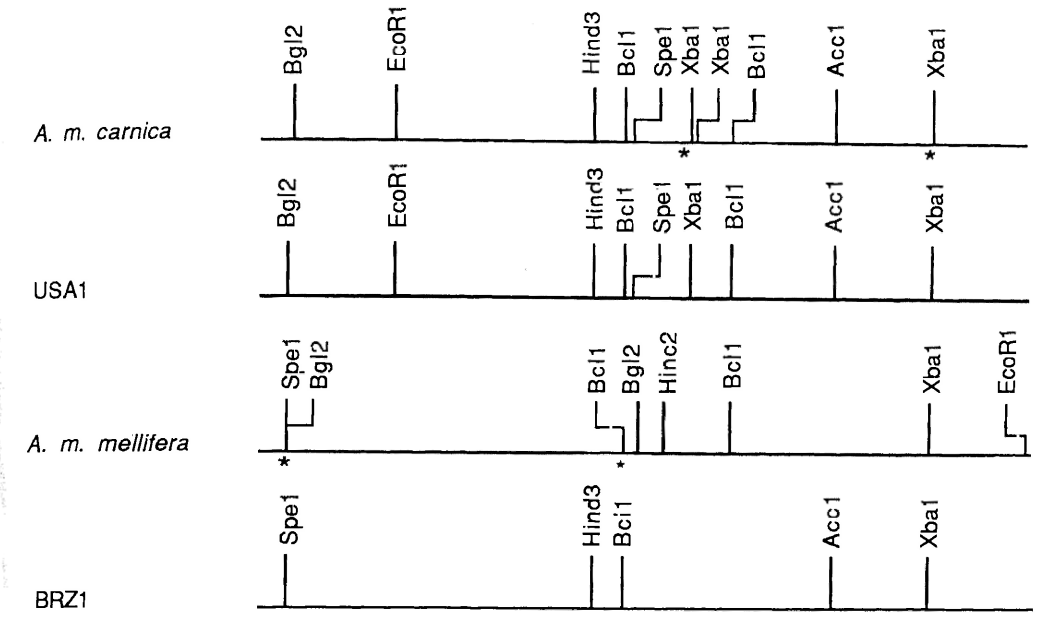

Fig. 4. Comparison of restriction enzyme cleavage site maps of *A. m. mellifera*, *A. m. carnica*, a Nor hive of European ancestry (USA1), and a Brazilian Africanized hive (BRZ1). The cleavage sites commo omitted. Asterisks indicate sites variable within populations.

(BRZ1) are reflected in their higher estimated sequence divergences (2.23–4.05%; Table 1).

There is substantial variation among families (hives) in the size of the mtDNA molecule as well as size variation in mtDNA among subspecies (Table 2). Two regions of size variation are of particular interest. The region between the XbaI site at 5.23 kb (Fig. 2, b) and the AccI site at approximately 5.8 kb (Fig. 2, c) is longer in *A. m. mellifera* and the *mellifera*-like "Nigra" than the corresponding region in the American and Brazilian mtDNAs; it also is variable among *A. m. mellifera* families (Fig. 5). This variation is most easily explained by postulating a sequence 80–100 bp in length which is present once (in Laesøl–7, Askerl–3, and Upps3), twice (in Uppsl–2), or three times (Nigral–2,4–6,8–9) in *A. m. mellifera* and *mellifera*-like "Nigra" mtDNA, and which is absent from the mtDNAs of *A. m. carnica* and the North American and Brazilian bees sampled.

The region of *A. m. mellifera* mtDN the BclI sites at 7.9 kb (Fig. 2 and 3, kb (Fig. 2 and 3, g) is 270 bp large corresponding region in the mtDNA o this respect *A. m. mellifera* and the Brazilian mtDNAs are similar. The cor region in the mtDNA of hive Upps3 mately 540 bp larger than in USA1; explained by assuming that the 270-b is present twice in the mtDNA of Upp

The region between the NdeI sites (Fig. 2, g) and 12.26 kb (Fig. 2, h) is app 80 bp larger in *A. m. mellifera* and th Africanized hive than in *A. m. carnica*

Size variation in mtDNA has been rep in individuals, among conspecific indiv among closely related species (e.g., Faur stenholme 1976, 1980a,b; Densmore e Harrison et al. 1985; Moritz & Brown 1 Solignac et al. 1986; Moritz et al. 1987)

Table 2. Comparison of size variation in kilobase pairs (kb) in the mitochondrial genomes of *A. m. ca mellifera*, and a Brazilian Africanized bee (Brzl)

Location of fragment	*A. m. carnica*	*A. m. mellifera*
1. PstI "a" to XbaI "b"	2.78	2.8–2.87
2. XbaI "b" to AccI "c"	0.53	0.62 in Laesøl–7, Askerl–3, Upps3 0.70 in Uppsl and 2 0.79 in Nigra 1–2, 4–6, 8–9
3. BclI "d" to BclI "e"	0.43	Site "d" absent
4. BclI "e" to BclI "f"	0.54	0.54
5. BclI "f" to BclI "g"	1.19	1.73 in Upps3, 1.46 in all others
6. NdeI "g" to NdeI "h"	2.87 USA1 = 2.85	2.95

A. m carnica fragment sizes are like those of a North American honey bee of European ancestry (USA1) except wher lowercase letters in quotes refer to restriction sites in Fig. 2 and 3.

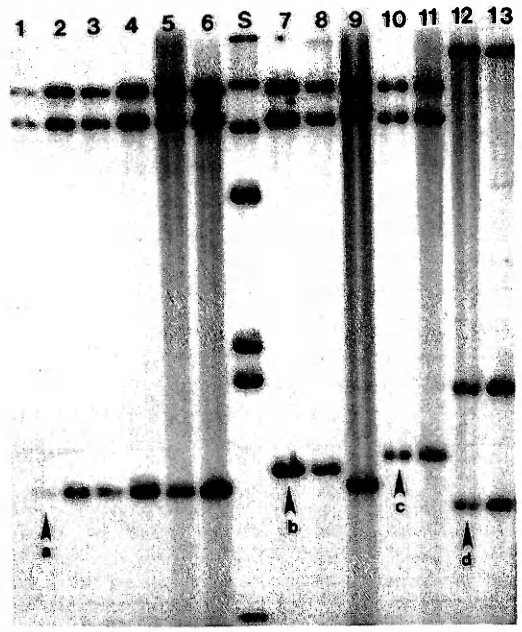

Fig. 5. Autoradiograph of 1% agarose gel showing *A. m. mellifera* and "Nigra" mtDNA digested with the enzyme XbaI. Lane 1, Laesø1; 2, Laesø2; 3, Laesø3; 4, Asker1; 5, Asker2; 6, Asker3; 7, Upps1; 8, Upps2; 9, Upps3; 10, Nigra1; 11, Nigra2; 12, *A. m. carnica* (Graz1). Size standard is HindIII-digested lambda phage DNA. Note size variation in smallest XbaI fragment.

in Brown 1983, 1985). In many cases, this variation is located in or adjacent to the noncoding control region. However, the regions to which size variation in *A. mellifera* mtDNA have been mapped span a minimum of 5.1 kb, or approximately one-third of the 16.8-kb genome, making it unlikely that all of these regions of size variation are in or adjacent to the control region.

In addition, Crozier et al. (1989) have sequenced a part of an *A. m. ligustica* mtDNA corresponding to the region from 6.5 to 9.5 kb on our maps. This region was found to include (reading from 6.5 to 9.5 kb) aspartate tRNA, cytochrome oxidase I, leucine tRNA, a small unidentified reading frame, cytochrome oxidase II, and lycine and tryptophan tRNAs. The position of the (as yet) unidentified reading frame corresponds to one of the regions of size variation, namely the site which is 270 bp larger in *A. m. mellifera* and Brazilian Africanized bees. This element of size variation clearly does not lie in the AT-rich control region.

Maternally inherited mtDNA polymorphisms can be used in conjunction with biparentally inherited characters such as allozyme or morphological phenotypes to detect the direction of gene flow in hybrid populations. This has practical application in the study of honey bee populations in the New World. For example, *A. m. mellifera* was the subspecies imported to North America by European colonists in the 17th century and was probably the most commonly imported bee until the middle of the 19th century (Oertel 1976, Pellet 1938). Today, *A. m. mellifera* has been largely replaced in North American apiaries by other subspecies, particularly *A. m. ligustica*, *A. m. carnica*, and their hybrids. It is possible that the descendants of the early *A. m. mellifera* imports are present in North American feral populations, as has been suggested by Sheppard (1988) on the basis of allozyme studies. If so, matrilineal descendants of the early *A. m. mellifera* colonists should be identifiable by their characteristic mtDNA.

mtDNA polymorphisms also are useful in analyzing the pattern of gene flow between European and Africanized bees in the New World. Earlier work has shown differences in mtDNA cleavage patterns between North American bees of European ancestry and some Brazilian Africanized bees (Smith & Brown 1988) and between European and African subspecies (Smith 1988). One critical question for the management of Africanized bees is the extent to which gene flow takes place from managed European apiary populations into feral Africanized populations. If swarms headed by European queens or hybrid queens (from European queen–African drone matings) routinely enter and survive in the feral population, the feral populations should gradually acquire more European characteristics. On the other hand, if swarms headed by European queens or European–African hybrid queens are selected against in feral populations, the Africanized population advancing north might show relatively little European influence. The success of queens of European maternal ancestry in feral Africanized populations can be assessed by the relative frequencies of European and Africanized mtDNAs in these populations.

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