

Microsatellite Variation in Honey Bee (*Apis Mellifera* L.) Populations: Hierarchical Genetic Structure and Test of the Infinite Allele and Stepwise Mutation Models

Arnaud Estoup,* Lionel Garnery,[†] Michel Solignac* and Jean-Marie Cornuet^{†,‡}

*Laboratoire de Populations, Génétique et Evolution, CNRS, 91198 Gif-sur-Yvette cedex, France, [†]Laboratoire de Neurobiologie Comparée des Invertébrés, INRA-CNRS, URA 1190, La Guyonnerie 91440 Bures-sur-Yvette, France and [‡]School of Genetics and Human Variation, La Trobe University, Bundoora 3083 Victoria, Australia

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ABSTRACT

Samples from nine populations belonging to three African (*intermissa*, *scutellata* and *capensis*) and four European (*mellifera*, *ligustica*, *carnica* and *cecropia*) *Apis mellifera* subspecies were scored for seven microsatellite loci. A large amount of genetic variation (between seven and 30 alleles per locus) was detected. Average heterozygosity and average number of alleles were significantly higher in African than in European subspecies, in agreement with larger effective population sizes in Africa. Microsatellite analyses confirmed that *A. mellifera* evolved in three distinct and deeply differentiated lineages previously detected by morphological and mitochondrial DNA studies. Dendrogram analysis of workers from a given population indicated that super-sisters cluster together when using a sufficient number of microsatellite data whereas half-sisters do not. An index of classification was derived to summarize the clustering of different taxonomic levels in large phylogenetic trees based on individual genotypes. Finally, individual population \times loci data were used to test the adequacy of the two alternative mutation models, the infinite allele model (IAM) and the stepwise mutation models. The better fit overall of the IAM probably results from the majority of the microsatellites used including repeats of two or three different length motifs (compound microsatellites).

MORPHOMETRY has long been the only method used to study variation among honey bee populations. RUTTNER (1988) published a thorough report on the biogeography/taxonomy of this species, based almost exclusively on the analysis of morphological characters. Thanks to multivariate analysis, morphometry has shown that *Apis mellifera*, which covers a wide distribution area (Africa, Europe and Western Asia), has differentiated into numerous subspecies (24 according to RUTTNER) associated with specific behavioral and ecological characteristics and that these subspecies could be grouped in three (RUTTNER *et al.* 1978) or four (RUTTNER 1988) "evolutionary branches." Furthermore, some subspecies, such as *Apis mellifera mellifera*, are themselves composed of differentiated local populations, as evidenced by ecological observations (LOUVEAUX 1966) and confirmed by morphometry (CORNUET *et al.* 1975, 1978). However, although morphometrical analysis is very powerful in discriminating populations, morphological characters have several drawbacks such as their polygenic determinism, which hinders their use in population genetic studies.

Allozymes, the genetic interpretation of which is generally straightforward, have proved to be very useful in

many other species but have brought little additional information to our understanding of honey bees. The main reason is the low level of allozyme polymorphism, as in many other Hymenoptera (PACKER and OWEN 1992), which is generally considered as a consequence of haplodiploidy (PAMILO *et al.* 1978; PAMILO and GROZIER 1981).

In recent years, various DNA markers have been developed. One has been mitochondrial DNA (mtDNA), which is particularly suited for inferring phylogenetic relationships among the components of a species. In honey bees, the first studies confirmed the existence of three evolutionary branches, although slightly modifying their subspecies composition and their estimated time of divergence as inferred from morphometry (SMITH 1991; GARNERY *et al.* 1992). More detailed studies are now exploring the evolutionary relationships among subspecies within the same branch (GARNERY and CORNUET 1994). In parallel, nuclear probes have been isolated to discriminate between American honey bees of European and African origin. However, in the latter case, the purpose was to determine the respective influence of both origins in the genome of imported bees and the usefulness of these probes has not yet been tested on populations of the original distribution area. Random amplified polymorphic DNA (RAPD) is another category of DNA markers evidenced in honey bees. These markers have been successfully used to

Corresponding author: Jean-Marie Cornuet, School of Genetics and Human Variation, LaTrobe University, Bundoora, Victoria 3083, Australia. E-mail: genjmc@genome.latrobe.edu.au

build a genetic map of >300 loci (HUNT and PAGE 1994) and to determine the patriline structure of a colony (FONDRK *et al.* 1993). DNA fingerprints with M13 (BLANCHETOT 1991; CORLEY *et al.* 1993) or oligonucleotides (MORITZ *et al.* 1991) have also been used for the latter purpose. However, these DNA markers (RAPD and fingerprinting probes) have not been used for population genetics although TARES *et al.* (1993) found that phylogenetic relationships among four *A. mellifera* subspecies based on fingerprints with an *AhlI* family probe were in good agreement with mtDNA results.

Microsatellites are a class of DNA markers that involve a variable number (up to 100) of tandem repeats of 1–5 bp. The different alleles are characterized by the exact length in base pairs of a DNA fragment obtained by the polymerase chain reaction (PCR) performed between two fixed sequence motifs flanking the tandem repeat region. Because of their generally high mutation rate, these markers may have a large number of alleles, which make them particularly suited for genome mapping or paternity analysis. However, as codominant markers, they can be also very helpful for population genetics, especially in species where allozyme variability is low, provided that they can be found in these species. In *A. mellifera*, we have shown that microsatellites are abundant (ESTOUP *et al.* 1993) and characterized by a wide range of gene diversity (ESTOUP *et al.* 1994). However, because of their mutational modalities, microsatellites may be subject to size homoplasy (VALDES *et al.* 1993; WEBER and WRONG 1993) and some may have null alleles (CALLEN *et al.* 1993). Classically, two extreme models of mutation are considered in variable number of tandem repeats (VNTR): the infinite allele model (IAM) and the stepwise mutation model (SMM). As stated for VNTR loci, the SMM implies that an allele mutates only by losing or gaining a single tandem repeat and hence possibly towards an allele already present in the population. In contrast, under the IAM, a mutation, which can involve any number of tandem repeats, always results in an allele not encountered in the population. Because estimates of effective population size and mutation rates depend on the mutation model, we performed computations to test the adequacy of each model with the observed data.

Recent population genetic surveys in human (BOWCOCK *et al.* 1994; DI RIENZO *et al.* 1994) and other mammals (GOTTELLI *et al.* 1994; TAYLOR *et al.* 1994) and in bumble bees (A. ESTOUP, M. SOLIGNAC, J. M. CORNUET and A. SCHOLL, unpublished results) have shown that microsatellites are highly efficient at differentiating populations or groups of populations. In this paper, we investigate their usefulness for honey bee population studies by addressing the following specific questions: Are the three evolutionary branches inferred from morphometry and mtDNA confirmed by microsatellite data? Are microsatellites useful for differentiating subspecies and populations within subspecies? How does

the pattern of variability of these markers vary between branches/subspecies/populations and which factors may be invoked to explain it? Is it possible to assign a given colony to its population of origin from microsatellite data and how do the individuals from this colony cluster within the population?

MATERIALS AND METHODS

Biological material: Honey bee workers were collected from nine populations belonging to seven different subspecies from the three evolutionary branches (M, C and A). Lineage M (*A. m. mellifera*) was represented by three populations from Avignon (Southeastern France), Valenciennes (Northern France) and Umeå (Sweden), lineage C by populations from Forli (Italy, subspecies *ligustica*), Berlin (Germany, *carnica*) and Chalkidiki (Greece, *cecropia*) and lineage A by populations from Johannesburg (South-Africa, *scutellata*), Cape Town (South-Africa, *capensis*) and Tiznit (Morocco, *intermissa*), respectively. The samples of *mellifera*, *ligustica*, *cecropia*, *scutellata* and *intermissa* were composed of unrelated workers (one worker per colony from several beeyards in a circular area of a maximum of 25 km radius). The *capensis* sample included workers from different colonies collected in two beeyards 150 km from one another. The *carnica* sample was composed of workers from three different colonies, each worker being representative of a different patriline. For this population, allele frequencies were determined from the genotypes of the parents (queens and males) as deduced from the worker genotypes. The number of sampled chromosomes per population ranged from 20 to 60. In addition, a second *mellifera* sample from Avignon was composed of workers representing five different patrilines (five workers per patriline) taken at random from a single colony. The patrilines of this colony were determined elsewhere (ESTOUP *et al.* 1994).

DNA isolation and microsatellite analysis: Individual DNA extractions were performed as described by GARNERY *et al.* (1990). Among the 75 *A. mellifera* and 26 *Bombus terrestris* microsatellites available (ESTOUP *et al.* 1993), 12 were chosen to perform the required analyses. Most of the population studies were carried out with seven microsatellite loci (A7, A28, A113, B124, A43, A24 and A88). The population and patriline samples from Avignon were analyzed with five additional loci (A14, A76, A107, A29 and A35). Previous linkage analysis on the drone progeny of an artificially inseminated queen has shown that at least 11 of the 12 microsatellite loci were genetically independent (the queen was homozygous for locus A7). Radioactive PCR amplifications were carried out in 10 μ l of a mixture containing 5–10 ng of DNA template, 400 nM of each primer, 75 μ M each dGTP, 2'-deoxycytidine 5'-triphosphate (dCTP) and 2'-deoxythymidine 5'-triphosphate (dTTP), 6 μ M dATP, 0.7 μ Ci 35 S-dATP, 1.2–1.7 mM MgCl₂, 20 μ g/ml bovine serum albumin (BSA), 1 \times Promega reaction buffer and 0.4 unit of *Taq* polymerase. After a denaturing step of 3 min at 94 $^{\circ}$, samples were processed through 30 cycles consisting of 30 sec at 94 $^{\circ}$, 30 sec at an optimal annealing temperature and 30 sec at 72 $^{\circ}$. The last elongation step was lengthened to 10 min. Aliquots of 7 μ l of amplified DNA were mixed with 5 μ l of formamide solution. Two microliters of the mixture were heated for 5 min at 85 $^{\circ}$ and electrophoresed on 6% denaturing polyacrylamide sequencing gels. Multiplex PCR for pairs of microsatellites A88-A24, B124-A43 and A76-A107 were processed in a similar mixture containing the primers of both microsatellite loci. The sequences of primers and the optimal PCR conditions are given for each locus in Table 1.

Statistical analyses: Unbiased estimates and standard devia-

TABLE 1
Microsatellite core sequences of cloned alleles and corresponding primer sequences and PCR conditions

Locus	Core sequence	Primers	PCR conditions	
			Tm (°)	MgCl ₂ (mM)
A7 ^a	(CT) ₃ (T) ₇ CCTTCG(CT) ₂₄	5'GTTAGTGCCCTCCTCTTGC3' 5'CCCTTCTCTTTCATCTTCC3'	58	1.2
A28 ^a	(CCT) ₃ GCT(CCT) ₆ (CT) ₅ TT(CT) ₄	5'GAAGAGCGTTGGTTGCAGG3' 5'GCCGTTTCATGGTTACCACG3'	54	1.7
A113	(TC) ₂ C(TC) ₂ TT(TC) ₅ TT(TC) ₈ TT(TC) ₅	5'CTCGAATCGTGGCGTCC3' 5'CCTGTATTTTGCAACCTCGC3'	60	1.2
B124 ^a	(CT) ₈ TCCTCTTC . . . (CT) ₁₄ CCTC(GC) ₃ . . . (GGCT) ₈	5'GCAACAGGCGGGTTAGAG3' 5'CAGGATAGGGTAGGTAAGAG3'	55	1.5
A43 ^a	(CT) ₁₃	5'CACCGAAACAAGATGCAAG3' 5'CCGCTCATTAAAGATATCCG3'	55	1.5
A24	(CT) ₁₁	5'CACAAGTTCCAACAATGC3' 5'CACATTGAGGATGAGCG3'	55	1.2
A88	(CT) ₁₀ TC(CCTT) ₂ (CTTT) ₃ . . . (GGA) ₇	5'CGAATTAACCGATTGTGCG3' 5'GATCGCAATTATTGAAGGAG3'	55	1.2
A76 ^a	(CT) ₃₂ CATT(CT) ₂ CA(CT) ₃	5'GCCAATACTCTCGAACAATG3' 5'GTCCAATTCACATGTCGACATC3'	58	1.2
A107 ^a	(GCTC) ₂ (GCT) ₂ (CT) ₂₃	5'CCGTGGGAGGTTTATTGTGCG3' 5'CCTTCGTAACGGATGACACC3'	58	1.2
A14 ^a	(CT) ₁₃ . . . (GGT) ₉	5'GTGTCGCAATCGACGTAACC3' 5'GTCGATTACCGATCGTGACC3'	58	1.7
A29 ^a	(GT) ₂₄	5'AAACAGTACATTTGTGACCC3' 5'CAACTTCAACTGAAATCCG3'	54	1.0
A35 ^a	(GT) ₁₄	5'GTACACGGTTGCACGGTTG3' 5'CTTCGATGGTTCGTTGTACCC	57	1.0

PCR conditions are defined by annealing temperature (Tm) and MgCl₂ concentration. All microsatellite loci were cloned from *Apis mellifera* except for B124, which was cloned from *Bombus terrestris*.

^aAlready published in ESTOUP *et al.* (1994), although PCR conditions may be sometimes slightly different.

tions of heterozygosity were calculated according to NEI (1987). Exact tests for Hardy-Weinberg equilibrium, genotypic linkage disequilibrium and genetic structure were computed using the GENEPOP package version 1.2 (RAYMOND and ROUSSET 1994). Differences in average unbiased heterozygosities between population samples were assessed by Wilcoxon's signed rank test (SNEDECOR and COCHRAN 1978).

CAVALLI-SFORZA and EDWARDS (1967) chord distances between populations were estimated with BIOSYS-1 package version 1.7 (SWOFFORD and SELANDER 1989). These distances were used to build a neighbor-joining (NJ) tree (SAITOU and NEI 1987). In addition, shared allele distances (D_{AS}) (CHAKRABORTY and JIN 1993) were computed between (diploid) individuals as a basis for a NJ tree using a program written by J.-M. CORNUET. This distance was computed by averaging the values over all available loci between two individuals. At a given locus, the distance is one if both individuals have the same genotype, 0 if they have no allele in common and 0.5 otherwise. The distances between two populations was taken as the average of the distances between one individual from one population and one individual from the other population. Bootstrap values were computed over 2000 replications (HEDGES 1992) for trees based on both distances by resampling either loci or individuals within population. For NJ trees

performed on individuals, only loci were resampled and 2000 replications were also performed.

F statistics were computed according to WEIR and COCKERAN (1984). The population from Berlin was excluded from any *F*-statistics computations because it was sampled inappropriately for this purpose. *F*_{is} values for each sample, *F*_{st} between any pair of samples and global *F*_{st} among the three *mellifera* samples were first estimated using the program DIPLOID (WEIR 1990) modified by J. GOUDET (personal communication). Departures from 0 were tested using resampling methods. A hierarchical analysis was then carried out (MICHALAKIS *et al.* 1993). We estimated *F*_{st} for pairs of subspecies within the same evolutionary lineage (*F*_{st}^{WL}) or between evolutionary lineages (*F*_{st}^{BL}). Because three population samples were available for the *mellifera* subspecies and only one in the other subspecies, three analyses were carried out by taking a different *mellifera* population each time. This resulted in three estimates of *F*_{st}^{WL} and *F*_{st}^{BL}. To obtain 95% confidence intervals (CI) on these estimates, 800 bootstrap replicates were performed, resampling loci. It should be noted that *F*_{st}^{BL} measures the differentiation between lineages M, C and A and that *F*_{st}^{WL} measures the differentiation of subspecies within the lineages C and A only. The total geographic differentiation, *F*_{st}^{TOT}, was

deduced from the equation $1 - F_{st}^{TOT} = (1 - F_{st}^{WL})(1 - F_{st}^{BL})$ (WEIR and COCKERHAM 1984).

To evaluate the adequacy of both mutation models (IAM and SMM), we computed the expected number of alleles (k_e) given the observed heterozygosity (H_o) and the expected heterozygosity (H_e) given the observed number of alleles (k_o) under each mutation model (DEKA *et al.* 1991; EDWARDS *et al.* 1992; SHRIVER *et al.* 1993), as explained below.

Expected number of alleles under the IAM: According to EWENS (1972), the expected number of alleles in a sample of n chromosomes is equal to $k_e = 1 + M/(M+1) + M/(M+2) + \dots + M/(M+n-1)$, where $M = 4N_e\mu$, N_e being the effective population size and μ , the mutation rate of the locus. At mutation-drift equilibrium, $M \approx H/(1-H)$ (CROW and KIMURA 1970), H being the heterozygosity. However, when H is based on a single locus, the latter formula is biased (ZOUROS 1979) and a bias-corrected estimator of M was obtained by solving the equation (CHAKRABORTY and WEISS 1991) $M^3 + (7 - M_o)M^2 + (8 - 5M_o)M - 6M_o = 0$ in which $M_o = H_o/(1 - H_o)$.

Expected heterozygosity under the IAM: Solving EWENS's equation above for an observed value of k leads to a corresponding value of M , from which H_e is deduced as $[M/(1+M)]$.

Expected number of alleles under the SMM: As under the IAM, a bias-corrected estimator of M was computed by solving the following equation also derived from ZOUROS (1979): $1.7M^4 + (25 - 1.7M_o)M^3 + (24.5 - 13M_o)M^2 + (9 - 22.5M_o)M - 6M_o = 0$ in which $M_o = 0.5[(1 - H_o)^{-2} - 1]$ (OHTA and KIMURA 1973).

We then followed the method given by KIMURA and OHTA (1978) to compute the equilibrium distribution of allele frequencies $\Phi(x)$. This involves several double and triple integrals, which were obtained by numerical integration. The $0,2\pi$ integration interval was divided in 40 steps (MORAN 1976). KIMURA and OHTA (1978) gave only the average number of alleles in a diploid population of size N , which is equal to $\int_{1/(2N)}^1 \Phi(x) dx$. Following EWENS's (1972) approach, the average number in a sample of n chromosomes was computed by integrating $[1 - (1-x)^n] \Phi(x) dx$ on the same interval $1/(2N), 1$. The value of N was arbitrarily fixed to 1000 (see below) in all cases. This latter integral was obtained by numerical integration with steps of 0.00001.

Expected heterozygosity under the SMM: As under the IAM, this parameter was estimated by starting with two extreme values of H_e , getting the corresponding values of the number of alleles through the method described above and iteratively reducing the gap between the observed number of alleles and the computed values until the difference between both values of H_e was < 0.001 .

In addition, we also computed the probability of observing a value as different as the theoretical value under both models. This has been done by adding the probabilities to get a number of alleles between 1 and k_o , when $k_o < k_e$, or between k_o and n , when $k_o > k_e$. Under the IAM, we used the explicit formulae given by EWENS (1972). Under the SMM, our computations were based on the rationale of EWENS (1972), but we replaced the equilibrium distribution of allele frequencies of the IAM by that of the SMM and performed computations by numerical integrations as above. The sequential Bonferroni method (RICE 1989) was applied to adjust for the number of simultaneous tests.

Effective population sizes (N_e) and mutation rates (μ) were estimated simultaneously, following CHAKRABORTY and NEEL's (1989) method, which assumes that populations are in mutation-drift equilibrium for the loci under study and that the loci evolve under the IAM model. Because only the product $N_e\mu$ is estimable, computations were performed with the constraint that the geometric mean of effective population sizes is set to 1000. This last figure is based on the following simplified

rationale for estimating N_e . The queen of a given colony belongs to a population defined by the number of colonies the drones of which could have mated with her. Assuming an average cumulated mating flight range of 7 km (drone 5 km + queen 2 km) and a colony density of 3 per km² (see DISCUSSION), this leads to a number of $3,14 \times 7^2 \times 3 \approx 460$ colonies. Applying KERR's (1967) formula $N_e = 15C/7$ (C , number of colonies) results in $N_e = 986$, which has been rounded to 1000.

To detect a possible bottleneck in populations of branch M , we determined the theoretical relationship between the excess, $K_o - K_e$, of the observed number of alleles compared with the expected number and the observed number K_o . Following the rationale and the equations given by MARUYAMA and FUERST (1984), K_e corresponds to the expected number of alleles in a population that is at mutation-drift equilibrium under the IAM and it is obtained through EWENS' (1972) formula (as above) in which $M = H(t)/[1 - H(t)]$. $H(t)$ is the level of heterozygosity at time t and equal to (NEI *et al.*, 1975): $H(t) = H(\infty)(1 - e^{-(1+4N_e\mu)t})$ in which $H(\infty) = 4N_e\mu/(1 + 4N_e\mu)$.

K_o was computed using equations (6–8) from MARUYAMA and FUERST (1984), in which the frequency and time intervals were set to 0.001 and 0.0000038, respectively. Different combinations of $4N_e\mu$ and time since bottleneck occurred were used to compute K_o and K_e in a sample size of 50 genes (average sample size in populations of branch M), assuming $H(0) = 0$.

Index of classification (I_c): In the classical phylogenetic approach, specialists are generally concerned with phylogenetic relationships between every single OTU (operational taxonomic unit), which generally represents taxonomic units equal to or higher than the species level. With the hypervariability of microsatellites, individuals are uniquely defined, and it is tempting to use clustering techniques directly on them (*e.g.*, BOWCOCK *et al.* 1994). This may result in trees including a large number of OTUs (> 200 in the present study) in which it may not be easy to describe how these OTUs are clustered. To measure how well individuals from a given group (branch, population, colony) cluster together in a tree, we introduce the following index of classification I_c : $I_c = (d_T - d_C)/(d_T - d_M)$ where d_T , d_C and d_M are average distances between two OTUs taken from the total sample, from the group under study and from a monophyletic group of the same size as the group under study, respectively. If the group under study is monophyletic, then $d_C = d_M$ and $I_c = 1$. If the average distance between individuals of this group, (d_C) is the same as the average distance between any OTU (d_T), then $I_c = 0$. The distance between two OTUs is defined as the number of OTUs deriving from the most external node linking the two OTUs. This distance has the property of being independent of the topology of a monophyletic group and the average distance within a monophyletic group of n OTUs is simply $2(n+1)/3$ (see APPENDIX). Because the total set of N individuals making up the tree is monophyletic, the formula of the index of classification may also be written $I_c = (N+1 - 1.5d_C)/(N-n)$. Negative values can be reached if the average distance in a group exceeds the average distance in the whole tree, the minimum being -0.5 when a group includes only OTUs branched paraphyletically to all others.

RESULTS

Allele frequencies distribution, heterozygosities, Hardy-Weinberg equilibrium and linkage disequilibrium: Allele frequencies, proportions of heterozygotes and heterozygosities (gene diversities) are given for each population in Table 2. The total number of alleles

TABLE 2

Allele frequencies, proportion of heterozygotes (H_p) and unbiased gene diversities (H_d) of honey bee populations

Locus	Allele	Avignon	Valenciennes	Umeå	Forli	Berlin	Chalkidiki	Johannesburg	Cape Town	Tiznit
B124	(bp)	(n = 60)	(n = 44)	(n = 42)	(n = 60)	(n = 46)	(n = 60)	(n = 36)	(n = 20)	(n = 56)
	212				0.017		0.100			
	214					0.043		0.028		
	216		0.045	0.048	0.583	0.413	0.133	0.056	0.150	0.089
	218				0.267	0.261	0.083	0.028		0.036
	220	0.433	0.295	0.048	0.017	0.043	0.233	0.139	0.100	0.232
	222	0.100	0.091	0.071	0.017	0.087	0.067	0.139	0.300	0.214
	224	0.067	0.091	0.190	0.100	0.065	0.167	0.278	0.050	
	226		0.023	0.024			0.050	0.111		
	228	0.033	0.114				0.017	0.056		
	230	0.050	0.068	0.167		0.043	0.033	0.056	0.050	0.018
	232	0.133	0.091	0.190		0.022	0.017	0.028	0.050	0.071
	234	0.100	0.045	0.167			0.017	0.028	0.100	0.054
	236	0.033	0.091	0.071		0.022	0.067			0.054
	238	0.017	0.023				0.017		0.150	0.107
	240	0.033	0.023					0.028		0.089
	242									0.036
	252								0.050	
	256			0.024						
	262							0.028		
H_p		0.800	0.864	0.857	0.633	ND	0.967	0.800	0.900	0.821
H_d		0.777	0.876	0.877	0.588	0.759	0.884	0.883	0.879	0.875
Locus	Allele	Avignon	Valenciennes	Umeå	Forli	Berlin	Chalkidiki	Johannesburg	Cape Town	Tiznit
A7	(bp)	(n = 60)	(n = 44)	(n = 42)	(n = 60)	(n = 42)	(n = 54)	(n = 34)	(n = 20)	(n = 46)
	098							0.029		0.109
	099							0.059		
	100							0.235	0.300	
	102							0.088		
	103							0.029		0.022
	104									0.043
	105	0.017				0.024				0.022
	106							0.176	0.200	0.087
	107	0.033	0.045		0.283			0.059		0.043
	108							0.118	0.050	0.022
	109			0.048				0.029	0.050	0.065
	110							0.029		
	111	0.783	0.909	0.929	0.083	0.262		0.088	0.200	0.391
	112							0.029	0.100	
	113	0.117				0.024				
	114					0.024	0.019	0.029		0.043
	115					0.024				0.022
	116		0.023		0.150	0.238	0.537		0.050	0.130
	118	0.017	0.023	0.024	0.267	0.333	0.167		0.050	
	120				0.067		0.019			
	122				0.050	0.048	0.037			
	123						0.019			
	126						0.056			
	128	0.017			0.100	0.024	0.019			
	130						0.056			
	132						0.019			
	136						0.019			
	142						0.019			
	140						0.019			
	148	0.017								
H_p		0.400	0.182	0.143	0.800	ND	0.593	0.706	0.800	0.696
H_d		0.377	0.174	0.138	0.816	0.777	0.686	0.898	0.853	0.816
Locus	Allele	Avignon	Valenciennes	Umeå	Forli	Berlin	Chalkidiki	Johannesburg	Cape Town	Tiznit
A24	(bp)	(n = 60)	(n = 42)	(n = 44)	(n = 60)	(n = 44)	(n = 60)	(n = 36)	(n = 20)	(n = 48)
	098	0.917	0.833	0.864	0.117	0.205		0.361	0.150	0.583
	100							0.028		
	102							0.278	0.150	0.021
	104				0.017		0.017	0.139	0.100	0.271

TABLE 2
Continued

Locus	Allele	Avignon	Valenciennes	Umeå	Forli	Berlin	Chalkidiki	Johannesburg	Cape Town	Tiznit
A24	(bp)	(n = 60)	(n = 42)	(n = 44)	(n = 60)	(n = 44)	(n = 60)	(n = 36)	(n = 20)	(n = 48)
	106	0.050	0.095	0.091	0.350	0.545	0.467	0.083	0.150	0.125
	108	0.033	0.071	0.045	0.517	0.227	0.517	0.028	0.400	
	110					0.023		0.083	0.050	
H_p		0.100	0.286	0.182	0.600	ND	0.567	0.778	0.600	0.625
H_d		0.159	0.298	0.249	0.607	0.623	0.524	0.779	0.800	0.582
Locus	Allele	Avignon	Valenciennes	Umeå	Forli	Berlin	Chalkidiki	Johannesburg	Cape Town	Tiznit
A113	(bp)	(n = 52)	(n = 46)	(n = 48)	(n = 58)	(n = 44)	(n = 60)	(n = 36)	(n = 20)	(n = 56)
	200							0.056		
	202	0.250	0.217	0.021		0.045			0.050	
	204									0.125
	206									0.089
	208	0.019						0.028	0.150	
	210							0.083	0.250	0.036
	212							0.056	0.050	0.018
	214	0.038	0.043	0.021	0.310	0.477	0.800	0.222	0.250	0.411
	216							0.111		0.018
	218							0.111		0.036
	220	0.538	0.674	0.854	0.672	0.295	0.017	0.083		0.214
	222	0.038	0.022	0.021				0.111	0.100	0.018
	224	0.019		0.042	0.017			0.083	0.150	0.036
	226	0.019	0.043	0.042		0.045	0.050	0.056		
	228	0.038				0.023	0.017			
	230					0.023	0.067			
	234	0.019					0.033			
	236	0.019				0.091				
	238						0.017			
H_p		0.692	0.565	0.292	0.483	ND	0.367	0.833	0.900	0.679
H_d		0.654	0.505	0.271	0.459	0.687	0.357	0.908	0.858	0.771
Locus	Allele	Avignon	Valenciennes	Umeå	Forli	Berlin	Chalkidiki	Johannesburg	Cape Town	Tiznit
A28	(bp)	(n = 60)	(n = 46)	(n = 44)	(n = 60)	(n = 46)	(n = 52)	(n = 34)	(n = 20)	(n = 52)
	122							0.029		
	127							0.059		0.038
	129								0.100	
	130							0.265		0.038
	131							0.029	0.050	
	132	0.783	0.957	0.977	0.017	0.109			0.050	0.115
	133							0.029	0.050	0.346
	134					0.022	0.096	0.029	0.050	0.154
	135							0.176	0.050	0.231
	136							0.147	0.250	
	138	0.067	0.043	0.023	0.967	0.870	0.885		0.300	
	139									0.019
	140						0.019	0.118	0.050	0.058
	141				0.017			0.088	0.050	
	144	0.150								
	145							0.029		
H_p		0.433	0.087	0.045	0.067	ND	0.192	0.824	0.900	0.731
H_d		0.366	0.085	0.045	0.066	0.237	0.212	0.873	0.863	0.799
Locus	Allele	Avignon	Valenciennes	Umeå	Forli	Berlin	Chalkidiki	Johannesburg	Cape Town	Tiznit
A88	(bp)	(n = 60)	(n = 46)	(n = 48)	(n = 60)	(n = 26)	(n = 60)	(n = 36)	(n = 20)	(n = 54)
	138						0.017			
	143	0.033					0.217	0.028		0.019
	144							0.194	0.050	
	146	0.950	0.978	1.00	0.033	0.152	0.033	0.111		0.259
	147									0.037
	149							0.056		
	150							0.028	0.150	0.241
	152				0.067	0.109	0.133	0.056		0.167
	153							0.083	0.150	0.111
	154	0.017	0.022		0.900	0.630	0.600	0.028		0.056

TABLE 2

Continued

Locus	Allele	Avignon	Valenciennes	Umeå	Forli	Berlin	Chalkidiki	Johannesburg	Cape Town	Tiznit
A88	(bp)	(n = 60)	(n = 46)	(n = 48)	(n = 60)	(n = 26)	(n = 60)	(n = 36)	(n = 20)	(n = 54)
	155							0.278	0.500	0.074
	156					0.109		0.028		0.037
	157							0.083	0.050	
	158								0.100	
	159							0.028		
H_p		0.100	0.043	0.000	0.200	ND	0.567	0.889	0.500	0.889
H_d		0.098	0.043	0.000	0.188	0.568	0.584	0.873	0.726	0.839
Locus	Allele	Avignon	Valenciennes	Umeå	Forli	Berlin	Chalkidiki	Johannesburg	Cape Town	Tiznit
A43	(bp)	(n = 60)	(n = 44)	(n = 44)	(n = 60)	(n = 46)	(n = 60)	(n = 34)	(n = 18)	(n = 58)
	126									0.052
	127				0.100	0.130	0.083			
	128	0.733	0.841	0.682	0.267	0.196		0.029	0.111	0.259
	130	0.017								
	132							0.088	0.056	0.034
	134							0.118	0.056	0.017
	136							0.029	0.111	
	138							0.118	0.056	0.034
	139	0.017					0.033			
	140	0.150	0.114	0.295		0.043		0.059		0.138
	141	0.050	0.045	0.023	0.633	0.565	0.850			
	142							0.147	0.278	
	143					0.065	0.017			
	144							0.059	0.111	0.086
	145						0.017			
	146	0.017						0.118	0.167	0.121
	148	0.017						0.235	0.056	0.259
H_p		0.400	0.318	0.455	0.400	ND	0.267	0.941	0.889	0.793
H_d		0.444	0.284	0.458	0.527	0.522	0.273	0.891	0.895	0.834
		Avignon	Valenciennes	Umeå	Forli	Berlin	Chalkidiki	Johannesburg	Cape Town	Tiznit
No. of alleles per locus ^a		6.1 ± 1.2	4.4 ± 1.3	4.0 ± 1.2	4.1 ± 0.6	5.9 ± 0.9	7.0 ± 1.6	11.0 ± 0.8	7.9 ± 0.6	9.0 ± 1.0
Polymorphic loci (0.95 criterion) (%)		100.00	71.43	71.43	85.71	100.00	100.00	100.00	100.00	100.00
Polymorphic loci (0.99 criterion) (%)		100.00	100.00	85.71	100.00	100.00	100.00	100.00	100.00	100.00
H_p averaged ^a		0.418 ± 0.101	0.335 ± 0.110	0.282 ± 0.112	0.455 ± 0.097	ND	0.503 ± 0.098	0.829 ± 0.028	0.784 ± 0.063	0.748 ± 0.035
H_d averaged ^a		0.410 ± 0.092	0.324 ± 0.109	0.291 ± 0.114	0.464 ± 0.097	0.612 ± 0.069	0.503 ± 0.091	0.872 ± 0.016	0.839 ± 0.022	0.788 ± 0.036

n = number of chromosomes scored for each sample and for each locus. H_p could not be determined for the sample from Berlin because of haploid data (see MATERIALS AND METHODS).

^a Values are means ± SE.

detected in all samples ranges from seven (locus A24) to 30 (locus A7) and the mean number of allele per locus per sample ranges from four (Umeå) to 11 (Johannesburg). Proportions of heterozygotes and heterozygosities were not significantly different. Average heterozygosities ranged from 0.788 to 0.872, 0.464 to 0.612 and 0.291 to 0.410 for the populations of branch A, C and M, respectively. The three African populations have significantly higher average heterozygosities than the six European populations (Wilcoxon's signed rank test, $0.018 \leq P \leq 0.046$ for all 3×6 combinations).

No significant departure ($0.08 < P < 0.73$) from Hardy-Weinberg equilibrium was detected for any locus (Fisher's exact test). Global tests by population revealed a significant departure from Hardy-Weinberg equilibrium for the population of Cape Town ($P = 0.0006$). However, F_{is} statistics were not significantly different from 0 for any sample including the Cape Town sample ($F_{is} = 0.070$, $P = 0.095$).

Exact tests for linkage disequilibrium resulted in 11 significant values out of 171 comparisons (nine significant values are expected at the 5% level). No pair

of loci appeared in linkage disequilibrium in more than one population, suggesting the absence of physical linkage between loci. The populations of Avignon and Berlin included four and three of the 11 significant values, respectively. Other populations gave one or no significant value. Testing each population separately, no linkage disequilibrium was detected, except for the Avignon population, which showed a highly significant linkage disequilibrium ($P = 0.003$). The probability value for the population of Berlin was low but not significant at the 5% level ($P = 0.07$).

Testing the two mutation models: Table 3 provides the observed and expected values of the number of alleles and heterozygosities for each combination population \times locus. Monomorphic loci (*i.e.*, for which $k_0 = 1$) were excluded from these computations because they provide no information on the mutation model. Applying the sequential Bonferroni procedure with a global threshold value of 0.05, there is no significant departure from the infinite allele mutation model and only one significant departure from the stepwise mutation model (Chalkidiki \times locus A7) out of 60 possible combinations.

When comparing observed and expected numbers of alleles (under the IAM), it appears that excesses of observed numbers occur more frequently in populations of the M lineage and that the reverse seems true in lineage A. To test if there is a significant tendency here, we compared the observed numbers with the median values of the theoretical distribution (by definition, a value drawn from a given distribution has a probability equal to 0.5 of being larger than the median). When populations were taken separately, no significant departure from half could be found (global threshold of 0.05). When they were grouped by lineage (15/18 for lineage M, 13/21 for lineage C and 9/21 in lineage A), a significant bias appeared in lineage M ($\chi^2 = 8$, $0.005 > P > 0.001$). Such a bias might be explained by a bottleneck in branch M, as suggested by the low level of mtDNA variability evidenced in this branch (GARNERY 1992; GARNERY *et al.* 1992). After a bottleneck, there is a transient excess of alleles compared with the number expected if the population was at mutation-drift equilibrium with an identical average heterozygosity (MARUYAMA and FUERST 1984). To examine this hypothesis, for each locus we plotted the average difference between the observed and expected numbers of alleles as a function of the average of the observed numbers of alleles for the three populations of branch M and compared it with the theoretical expectation for samples of similar size taken from a population recovering from a severe bottleneck (Figure 1). Theoretical curves were computed for three different times after the bottleneck (0.2, 0.3 and 0.5 times $2N_e$ generations). Apart from one point corresponding to locus B124, the observed curve for the branch M is in close agreement with the first theoretical curve, suggesting that popula-

tions of branch M experienced a bottleneck $\sim 0.4N_e$ generations ago.

Effective population sizes and mutation rates: Because the infinite allele model could not be rejected, the method of CHAKRABORTY and NEEL, which relies on this model, can be validly applied to our data. Relative estimates of effective population sizes and mutation rates are indicated in Table 4. These estimates have been scaled by assuming that the geometric mean of the effective population sizes is equal to 1000, *cf.* MATERIALS AND METHODS. Although standard errors are quite large, it is clear that African populations have larger effective sizes than European. It is also worth noticing that the range of population sizes is wide, the largest one being six times the size of the smallest.

Relationships between and within populations: NJ trees of populations based on CAVALLI-SFORZA and EDWARDS' chord distance (D_{CE}) or shared allele distance (D_{AS}) provide similar topologies in which the three main evolutionary lineages are obvious (Figure 2). However, the position of the Moroccan sample (Tiznit) is ambiguous, being at the root of the African lineage with D_{CE} and at the root of lineage M with D_{AS} .

NJ trees of individual bees based on D_{AS} show that all bees are grouped by evolutionary lineage except for the Moroccan bees (Tiznit), which are scattered across the three lineages (Figure 3). Our index of classification is equal to 1 for lineage M, 0.98 for lineage C and 0.63 for lineage A (Table 5). Within each lineage, individuals from the same subspecies or population are not so well clustered, the average index of classification being equal to 0.79, excluding the Tiznit sample, which has a value of 0.22 (Table 5). Bootstrap values have been computed but are not shown for reasons of space; they are always very low, ranging from 0 to 67%. Even the branches at the basis of lineages have very low bootstrap values (maximum 17% for lineage M). Dropping the samples of Tiznit and Berlin raises bootstrap values of these branches (41% for lineage C and 48% for lineage M, but still only 12% for lineage A). Index of classification for each sample are given in Table 5.

Figure 4 shows a NJ tree, also based on D_{AS} , including the individuals of Avignon and 25 honey bees from a single colony of the same population. These 25 bees represent five different patriline (five bees per patriline), *i.e.*, progenies of five different drones mated to the queen. The tree was rooted with a single bee from Forli. Bees belonging to the same patriline cluster together with high bootstrap values (between 80% and 97%), but the five patrilines are scattered among other bees from the same population, indicating that the variability within a colony represents a large amount of the variability of the population from which it is issued. The index of classification of the colony within the population (Figure 4) was equal to 0.25 (Table 5).

Hierarchical genetic structure: Very similar values for F_{st}^{WL} (within lineage) and F_{st}^{BL} (between lineage)

TABLE 3
Observed and expected values of the number of alleles (k_o and k_e) and heterozygosity (H_o and H_e)

Population	Locus	n	k_o	H_o	k_e (IAM)	Pr (IAM)	k_e (SMM)	Pr (SMM)	H_e (IAM)	H_e (SMM)
Avignon	B124	54	10	0.794	9.4	0.482653	6.1	0.04072	0.770	0.882
Avignon	A7	54	6	0.354	2.7	0.035537	2.4	0.00957	0.603	0.790
Avignon	A24	54	1	0.000	irrelevant					
Avignon	A113	46	10	0.668	5.8	0.037798	4.2	0.00139	0.785	0.885
Avignon	A28	54	3	0.371	2.8	0.747258	2.5	0.44828	0.336	0.475
Avignon	A88	54	1	0.000	irrelevant					
Avignon	A43	54	6	0.354	2.7	0.035537	2.4	0.00957	0.603	0.790
Valenciennes	B124	44	12	0.876	13.1	0.420445	9.3	0.16193	0.836	0.907
Valenciennes	A7	44	4	0.174	1.6	0.078291	1.7	0.02757	0.464	0.647
Valenciennes	A24	42	3	0.298	2.2	0.600520	2.1	0.30509	0.353	0.492
Valenciennes	A113	46	5	0.505	3.7	0.364041	3.1	0.14356	0.548	0.740
Valenciennes	A28	46	2	0.085	1.3	0.999998	1.5	0.38915	0.198	0.266
Valenciennes	A88	46	2	0.043	1.1	0.999997	1.4	0.34498	0.198	0.266
Valenciennes	A43	44	3	0.284	2.2	0.581754	2.1	0.28736	0.350	0.489
Umeå	B124	42	12	0.897	14.5	0.237821	10.8	0.37726	0.840	0.908
Umeå	A7	42	3	0.138	1.5	0.295508	1.6	0.11226	0.353	0.492
Umeå	A24	42	3	0.260	2.0	0.532955	2.0	0.24756	0.353	0.492
Umeå	A113	48	6	0.271	2.1	0.009028	2.0	0.00240	0.614	0.792
Umeå	A28	44	2	0.045	1.2	0.999996	1.4	0.34415	0.200	0.268
Umeå	A88	48	1	0.000	irrelevant					
Umeå	A43	44	3	0.458	3.3	0.465427	2.8	0.56931	0.350	0.489
Forli	B124	60	6	0.588	4.9	0.400689	3.7	0.11412	0.593	0.787
Forli	A7	60	7	0.816	10.7	0.097639	6.8	0.53838	0.648	0.823
Forli	A24	60	4	0.607	5.2	0.315637	3.8	0.55898	0.440	0.629
Forli	A113	58	3	0.459	3.5	0.411654	2.9	0.59898	0.332	0.470
Forli	A28	60	3	0.066	1.2	0.160196	1.5	0.07980	0.330	0.468
Forli	A88	60	3	0.188	1.8	0.431582	1.8	0.17749	0.330	0.468
Forli	A43	60	3	0.527	4.2	0.266560	3.3	0.58488	0.330	0.468
Berlin	B124	46	9	0.759	7.8	0.382411	5.3	0.04024	0.754	0.870
Berlin	A7	42	9	0.777	8.1	0.434008	5.6	0.05538	0.763	0.872
Berlin	A24	45	4	0.629	5.1	0.322027	3.9	0.57114	0.462	0.645
Berlin	A113	45	6	0.687	6.1	0.569045	4.4	0.22642	0.620	0.795
Berlin	A28	46	3	0.237	1.9	0.500314	1.9	0.22217	0.347	0.485
Berlin	A88	46	4	0.568	4.4	0.481259	3.5	0.45710	0.461	0.645
Berlin	A43	46	5	0.633	5.2	0.534285	3.9	0.32431	0.548	0.740
Chalkidiki	B124	60	13	0.884	15.5	0.258412	10.3	0.17876	0.828	0.910
Chalkidiki	A7	54	13	0.686	6.4	0.003411	4.5	0.00001**	0.837	0.912
Chalkidiki	A24	60	3	0.524	4.1	0.272045	3.3	0.58979	0.330	0.468
Chalkidiki	A113	60	7	0.357	2.7	0.009382	2.4	0.00192	0.648	0.823
Chalkidiki	A28	52	3	0.212	1.8	0.465962	1.8	0.19795	0.339	0.478
Chalkidiki	A88	60	5	0.584	4.8	0.622482	3.7	0.26822	0.525	0.729
Chalkidiki	A43	60	5	0.273	2.2	0.060720	2.1	0.01918	0.525	0.729
Johannesburg	B124	36	13	0.883	12.5	0.487081	9.4	0.08216	0.873	0.921
Johannesburg	A7	36	13	0.914	15.1	0.275070	12.2	0.43974	0.873	0.921
Johannesburg	A24	36	7	0.779	7.8	0.443398	5.5	0.27231	0.698	0.835
Johannesburg	A113	36	11	0.908	14.5	0.127607	11.5	0.50579	0.834	0.903
Johannesburg	A28	34	11	0.873	11.6	0.494631	8.7	0.19622	0.840	0.905
Johannesburg	A88	36	12	0.873	11.8	0.545077	8.8	0.10384	0.855	0.913
Johannesburg	A43	34	10	0.891	12.8	0.186120	9.9	0.56325	0.815	0.892
Cape Town	B124	20	9	0.879	9.3	0.550110	7.9	0.35842	0.851	0.900
Cape Town	A7	20	8	0.853	8.3	0.551669	6.8	0.33343	0.816	0.882
Cape Town	A24	20	6	0.800	6.8	0.442472	5.3	0.44630	0.716	0.827
Cape Town	A113	20	7	0.858	8.4	0.317833	7.0	0.59224	0.772	0.859
Cape Town	A28	20	10	0.863	8.6	0.329552	7.2	0.10210	0.879	0.916
Cape Town	A88	20	6	0.726	5.4	0.476321	4.2	0.19177	0.716	0.827
Cape Town	A43	18	9	0.895	9.4	0.512385	8.4	0.46345	0.866	0.907
Tiznit	B124	56	11	0.875	14.2	0.170672	9.5	0.32431	0.793	0.893
Tiznit	A7	44	12	0.819	9.8	0.245253	6.7	0.00905	0.836	0.907
Tiznit	A24	36	4	0.611	4.6	0.429170	3.6	0.50655	0.483	0.660
Tiznit	A113	56	10	0.771	8.7	0.360972	5.6	0.02182	0.767	0.881
Tiznit	A28	44	8	0.788	8.6	0.484488	5.8	0.17396	0.722	0.854
Tiznit	A88	44	8	0.836	10.6	0.194245	7.2	0.43410	0.722	0.854
Tiznit	A43	56	9	0.834	11.4	0.233364	7.4	0.27824	0.736	0.867

Values for alleles and heterozygosity taken under the infinite allele mutation model (IAM) and the stepwise mutation model (SMM). n , number of sampled chromosomes. Pr(IAM) and Pr(SMM) are the probabilities that the number of alleles is as or more different from the theoretical value under both mutation models.

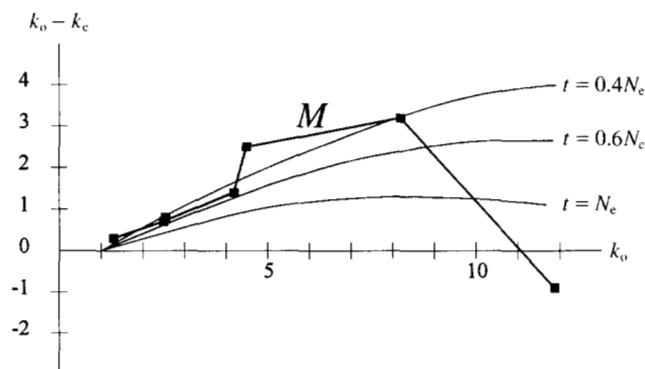


FIGURE 1.—Relationship between the excess of observed to expected number of alleles ($k_o - k_e$) and the observed number of alleles (k_o). Averages for the three populations of lineage M are plotted for each of the seven microsatellite loci and compared with theoretical curves for a sample of 50 genes taken from a population recovering from a bottleneck (the three curves correspond to three different times after the end of the bottleneck, measured in $2N_e$ generations).

were found when a different population from lineage M was included in the hierarchical analysis (Table 6). Average values were 0.34 (95%, CI: 0.24–0.45) and 0.25 (95%, CI: 0.13–0.38), respectively, for F_{st}^{wt} and F_{st}^{bl} . Thus, the differentiation within and between lineages is strong and significant. The overall geographic differentiation (F_{st}^{TOT}) was equal to 0.51 (95%, CI: 0.34–0.66).

The Tiznit population appeared closer to other African populations than to West European populations, as deduced from F_{st} between pairs of populations (Tiznit-Avignon: 0.218, Tiznit-Valenciennes: 0.256, Tiznit-Umeå: 0.316, Tiznit-Cape-Town: 0.088, Tiznit-Johannesburg: 0.069). This results supports the hypothesis that *A. m. intermissa* belongs to lineage A.

In spite of a lower differentiation within lineage M compared to other lineages, F_{st} values between *A. m. mellifera* populations were significantly different from zero ($F_{st} = 0.054$, $P[F_{st} = 0] < 0.005$). F_{st} between individual pairs of populations were all significantly different from zero (Avignon-Valenciennes: 0.013, Umeå-Valenciennes: 0.042, Avignon-Umeå: 0.083).

Finally, we tested the potential of microsatellite markers to assign a given colony to the honey bee population it came from. Using the data published in ESTOUP *et al.* (1994), we considered the genotypes of the parents (the queen and the 12 fathering drones) of one colony from Avignon. Performing Fisher's exact tests, we compared the allelic frequencies of this sample with those of the three population samples of *A. m. mellifera*. This comparison was carried out with the four microsatellite loci, which were common to both studies (loci B124, A7, A28 and A43). The parental structure of the colony was not significantly different from the genetic structure of the population from Avignon ($P = 0.13$), whereas it was significantly different from those of the populations from Valenciennes ($P = 0.01$) and Umeå ($P = 0.0004$). Extending to 12 microsatellite loci for

TABLE 4

Estimates of effective population sizes and mutation rates

Population	Effective population size
Avignon	696 ± 143
Valenciennes	560 ± 128
Umeå	534 ± 124
Forli	454 ± 106
Berlin	842 ± 169
Chalkidiki	1037 ± 185
Johannesburg	2935 ± 460
Cape Town	2555 ± 508
Tiznit	1617 ± 262

Locus	Mutation rate × 10 ³
B124	1.002 ± 0.138
A7	0.686 ± 0.104
A24	0.192 ± 0.042
A113	0.523 ± 0.084
A28	0.291 ± 0.056
A88	0.256 ± 0.051
A42	0.377 ± 0.067

Estimates based on the assumption that the geometric mean of effective population sizes is equal to 1000. Values are means ± SE.

the population from Avignon alone still did not change this conclusion ($P = 0.10$).

DISCUSSION

According to the way honey bee subspecies were clustered on the first plane of a discriminant analysis based on morphometrical data, RUTTNER *et al.* (1978) proposed the hypothesis that the species evolved in three evolutionary branches which he named A, M and C, including respectively the African subspecies (branch A), the West-European *mellifera* subspecies (branch M) and Asian plus North-Mediterranean subspecies (branch C). This bold deduction was almost completely confirmed by subsequent mitochondrial DNA analyses (SMITH 1991; GARNERY *et al.* 1992).

Three evolutionary lineages: In the present study, microsatellites clearly confirm the existence and composition of the three evolutionary branches, each one represented by three different samples. This result is obtained with any of the different distances used here (CAVALLI-SFORZA and EDWARDS, D_{AS} , F -statistics). However, all these distances underestimate the real divergence between evolutionary branches because of size homoplasy, *i.e.*, independent occurrence of alleles of the same size in distant taxa due to mutational modalities and constraints on allele length variation. This last phenomenon, suggested by the comparison of allele sizes in primates species (BOWCOCK *et al.* 1994), has been analyzed in greater detail in honey bee on microsatellite A113 (ESTOUP *et al.* 1995), which has a core sequence characterized by several interruptions (Table 1). Alleles

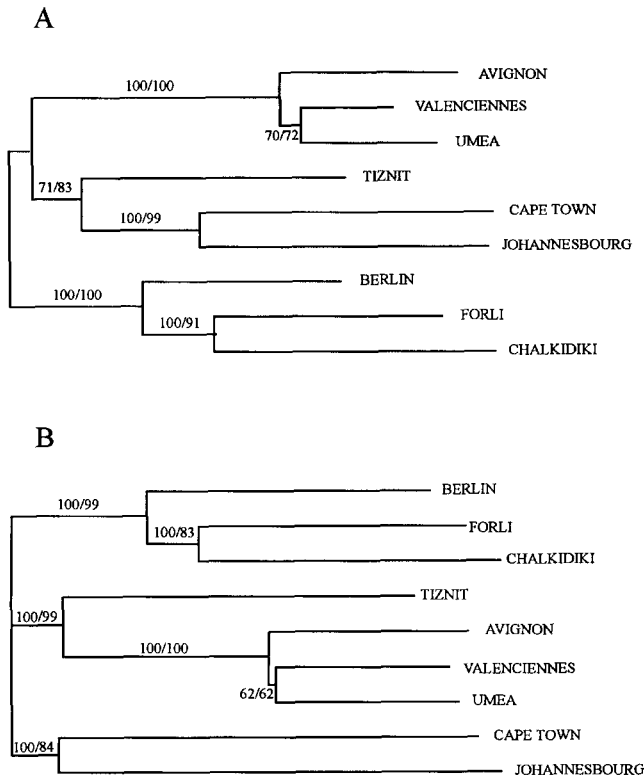


FIGURE 2.—Neighbor-joining trees of populations based on CAVALI-SFORZA and EDWARDS' distance (A) and shared allele distance (B). Bootstrap values have been computed over 2000 replications by resampling individuals within population (left values) and by resampling loci (right values) and noted as percentages.

of the same size were identical by descent within evolutionary branches, whereas they frequently had different sequences when comparing individuals from different branches (different number and/or location of interruptions). Because the estimated mutation rate for A113 is in the middle of the range in our study (Table 4), size homoplasy is likely to affect most of the microsatellites used here. This implies that the differentiation between branches is probably much deeper than suggested by our present results.

The main discrepancy between the hypothesis of RUTTNER *et al.* (1978) and the interpretation of mtDNA results was the status of the North-African subspecies (*intermissa*), which was included in branch M by RUTTNER *et al.* (1978) and in branch A by GARNERY *et al.* (1992). In a more recent survey of 192 Moroccan colonies, only African mitotypes were found (GARNERY *et al.* 1995), thus strengthening the conclusions initially based on a very small number of colonies. It is interesting to note that in our study, the sample from the North-African subspecies *intermissa* (Tiznit) swaps from one branch to the other according to the distance used. The ambiguity persists when taking other distances. With Nei's *standard* genetic distance, the Tiznit sample branches at the basis of the M lineage whereas it branches at the basis of the A lineage with Nei's *mini-*

mum distance (results not shown). Because RUTTNER *et al.*'s hypothesis was based upon morphometrical data, the genetic basis of which is nuclear as for microsatellites, including the *intermissa* subspecies within branch M was quite reasonable.

What can explain the contrast between the clear assignment of *intermissa* by mtDNA studies and the ambiguous result of microsatellites? A clue is given by the observation of the NJ tree of individual bees (Figure 3), in which the bees from Tiznit appear scattered among three clusters, each one branching at the basis of a different lineage. The majority of them (14/21) are located with other African bees, which is consistent with the *F* analysis. But the location of the other two clusters, based on a relatively high proportion of alleles shared with bees of other branches, confused the output of population level analyses. This result might suggest the hypothesis of a center of dispersion of the three branches located in North Africa. However, because it is in complete disagreement with mtDNA analyses, the above hypothesis has to be rejected. Another hypothesis is that allelic frequencies in Morocco have been modified by a natural gene flow between Spanish and Moroccan populations and/or importations of European queens or colonies, especially during the period of the French colonization. This hypothesis implies that nuclear and mitochondrial genes have experienced contrasting fates, since no European mtDNA has been found in Morocco. However, this is still quite possible because European colonies are known to be much less competitive in tropical areas than those of African subspecies, as shown by the "Africanization" episode in America (TAYLOR 1977). As has been shown in Venezuela for example (DANKA *et al.* 1992), colonies headed by European queens disappear quickly in a tropical environment filled with bees of African origin, leaving no female progeny. But the drones originating from these transient colonies may have participated in matings and thus have transmitted some nuclear genes to the local populations. A natural gene flow restricted to male sexuals from Spain to Morocco could also explain the observed pattern of variability. Finally, additional similarity between Tiznit and *mellifera* alleles may also result from size homoplasy (*cf.* above).

Genetic introgression due to beekeeping, which might explain the ambiguous assignment of the Tiznit sample, could be detected in bees from the Avignon sample. This sample was characterized by a highly significant linkage disequilibrium ($P = 0.003$). When performing an initial NJ tree on individuals (similar to Figure 3), three bees from Avignon branched outside the cluster formed by all other bees of the M lineage. These bees (numbers 8, 20 and 28) were characterized by having alleles very rare in *mellifera* but frequent in *ligustica*. Excluding these bees from linkage disequilibrium analysis resulted in complete loss of significance ($P = 0.92$) for the Avignon sample. We concluded that

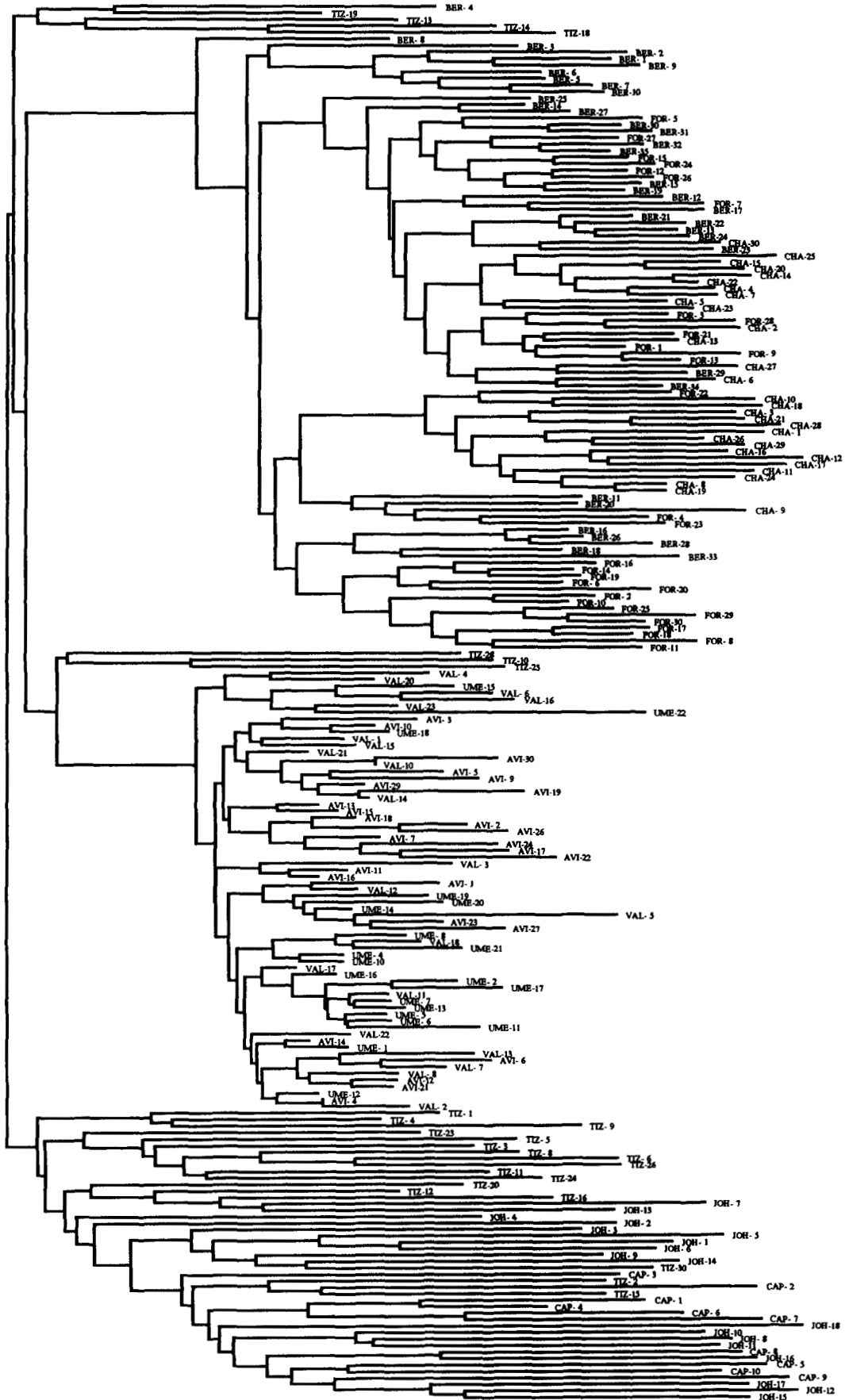


FIGURE 3.—Neighbor-joining tree of individual bees based on the shared allele distance. The geographic origin of bees is given by the first three letters of the name of the location (*e.g.*, AVI for Avignon).

TABLE 5
Index of classification (I_c) at different level
of grouping of the individual bees

Group of individuals	I_c
Branch M (Avignon + Valenciennes + Umeå)	1.00
Branch C (Forli + Berlin + Chalkidiki)	0.98
Branch A (Johannesburg + Cape Town + Tiznit)	0.63
Population Avignon	0.83
Population Valenciennes	0.73
Population Umeå	0.84
Population Forli	0.73
Population Berlin	0.58
Population Chalkidiki	0.76
Population Johannesburg	0.92
Population Cape Town	0.95
Population Tiznit	0.22
Colony (within population Avignon)	0.25
Patriline (within population Avignon)	1.00

Classifications based on the NJ tree of Figures 3 (branch and population) and 4 (colony and patriline). NJ, neighbor joining.

these bees had recent Italian ancestors and excluded them from subsequent analyses. MtDNA analysis of Avignon bees (results not shown) revealed that four bees (numbers 2, 8, 11 and 15) out of 30 had haplotypes

characteristic of the C lineage (which includes *ligustica*), indicating that genetic introgression indeed occurs in this area. This is not surprising, because many French beekeepers have repeatedly imported queens, especially from Italy. This result also implies also that withdrawing the three bees above might not be sufficient to exclude all foreign genes from this particular population.

Differentiation of subspecies within lineage and populations within subspecies: The second question addressed in the introduction also received a positive answer: microsatellites are useful to differentiate subspecies and populations within subspecies. F analysis indicates a strong differentiation between subspecies within lineages A and C (lineage M including a single subspecies). The differentiation within lineages appeared even deeper than between lineages ($F_{st}^{WL} = 0.34$, $F_{st}^{BL} = 0.25$). Although 95% confidence intervals for these parameters do overlap, this result could be due to the phenomenon of size homoplasy discussed above, which implies an underestimation of the differentiation between lineages. As a consequence, the overall geographic differentiation is most likely underestimated.

The F_{st} between *A. m. mellifera* populations were also significantly >0 , indicating that these populations are differentiated units of the same subspecies and that our markers detect this differentiation. Such a differentia-

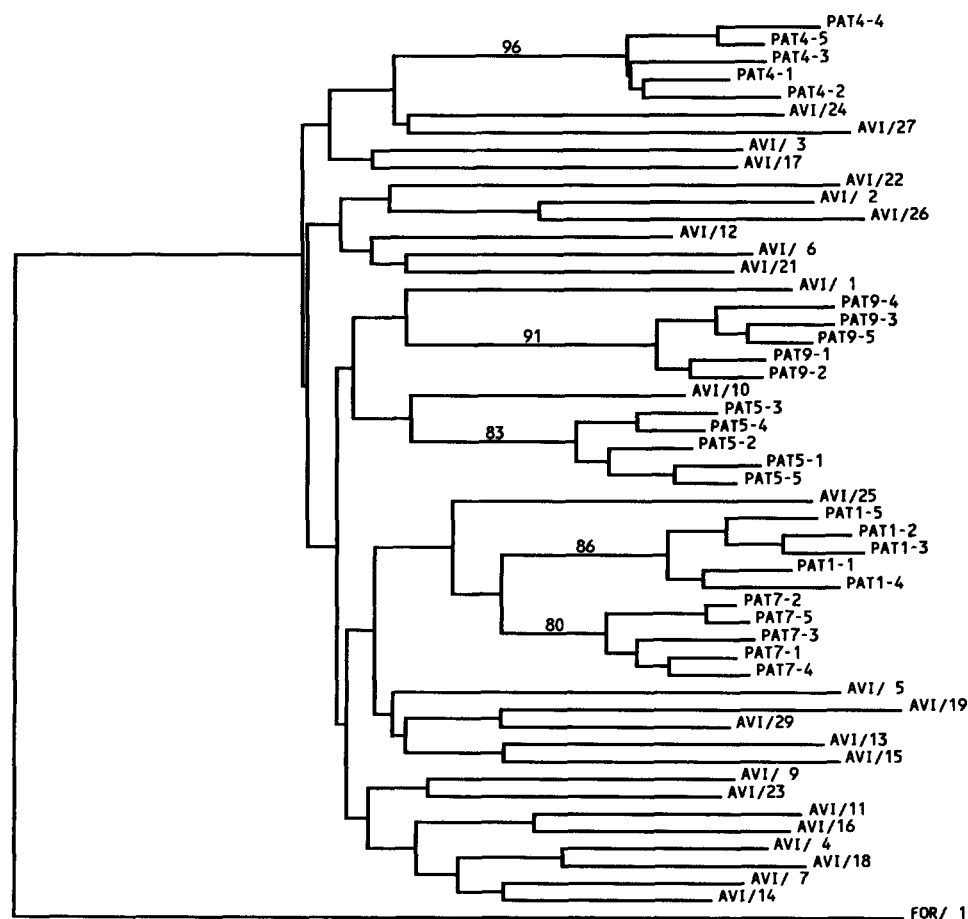


FIGURE 4.—Neighbor-joining tree of individual bees from the sample of Avignon (AVI) and from five different patriline (PAT1 to PAT5) of a colony, also from Avignon. The first bee from the sample of Forli has been added as an outgroup. Bootstrap values have been computed over 2000 replications. Only those at least equal to 80% have been noted (as percentages) on the figure. They all correspond to patriline clusters.

TABLE 6
Hierarchical F statistics over all loci

	F_{st}^{WL}	CI	F_{st}^{BL}	CI
Computation 1 (Avignon)	0.3363	0.2360–0.4389	0.2447	0.1236–0.3664
Computation 2 (Valenciennes)	0.3442	0.2357–0.4569	0.2530	0.1227–0.3843
Computation 3 (Umeå)	0.3534	0.2464–0.4624	0.2626	0.1365–0.3929
Mean values	0.3446	0.2397–0.4527	0.2534	0.1276–0.3812

CI, 95% confidence interval. The *A. m. mellifera* samples included for the three estimations of within and between lineage M, C and A differentiation are indicated between parentheses.

tion within the *mellifera* subspecies had already been evidenced through morphometry (CORNUET *et al.* 1975, 1978). But to obtain the same level of resolution, using morphometry, much larger samples were needed (10–30 of colonies, each one with 20–25 workers) whereas our results suggest that 20–30 unrelated workers are enough with microsatellite markers. It is even possible that only a dozen bees will be sufficient if more microsatellite loci are scored as suggested by BOWCOCK *et al.*'s (1994) study on humans.

All populations had genotypic frequencies in agreement with the Hardy-Weinberg principle, except for Cape-Town. Because this sample was taken from two beeyards 150 km apart, the discrepancy from the expectation under Hardy-Weinberg equilibrium can be explained by Wahlund's principle. This result, although based on a small sample size and not confirmed by a significant F_{is} , suggests that local differentiation may occur within the *capensis* subspecies. Local differentiation within a subspecies had so far been described only in European subspecies and morphometrical studies on African subspecies such as *adansonii* (GADBIN *et al.* 1979) or *intermissa* (CORNUET *et al.* 1988; GRISSA *et al.* 1990) had failed to show differentiation between populations, at least on such a small scale. This was generally explained by the migratory behavior characterizing most African subspecies (RUTTNER 1988). Our result suggests that *capensis* colonies, living in a temperate climate, would be less mobile than other African subspecies.

Different patterns of variability: The estimates of N_e rely on the assumptions that population are at mutation-drift equilibrium and that loci evolve under the IAM model. Table 3 shows that the IAM could never be ruled out for any microsatellite under study. Furthermore, when the discrepancy between observed and expected numbers of alleles was large, it seemed consistently larger under the SMM than under the IAM. This observation disagrees with those of EDWARDS *et al.* (1992) and SHRIVER *et al.* (1993). However, as shown in Table 1, four out of our seven microsatellites have core sequences made of repeats of two or even three different length, which is likely to prevent a unique stepwise mutation process as postulated under the SMM.

To test the adequacy of the alternative mutation

model, we compared the observed values of number of alleles and heterozygosity with the expected values of both parameters under both models. SHRIVER *et al.* (1993) found that the Kimura and Ohta analytical formulation (KOAF) for the number of alleles under the SMM did not fit the results of simulation. However, there is no precise indication of how the KOAF was applied to their data. When using the computations as described here, we found a very close agreement with the results of their simulations, indicating that the KOAF for the number of alleles can be validly used for microsatellite data. In contrast, when testing our computations of the probability distribution of the number of alleles under the SMM, we found an overrepresentation of tails as compared with the results of simulations. This indicates that the SMM hypothesis might have been rejected more often if our computations were more accurate. This conservative bias may result from the approximated nature of the KOAF or from the non-Markovian behavior of the process of drawing genes when building a sample (W. J. EWENS, personal communication).

The amount of variability, expressed as the mean number of alleles or the heterozygosity, varies largely between the three evolutionary branches and is significantly higher in African than European populations. This result is consistent with large differences in effective population sizes. In Africa, effective population sizes are larger, allowing more alleles to be maintained. At least three factors affect population size: density of colonies, migratory behavior of swarms/colonies, flight distance of drones and queens to mating sites. Data on the first two factors have been collected that show higher densities and superior migratory tendencies in African subspecies compared to European ones. Actually, the densities range from 0–1 colony/km² in temperate zones to 5–100 in tropical-equatorial zones (OTIS 1991; RATNIEKS *et al.* 1991; SCHNEIDER and BLYTHER 1988). Even if differences in migratory behavior are not accounted for, such a wide range of colony densities does not fully translate in N_e . This may be partly due to smaller range mating flights in areas where the colony density is higher.

Within lineage M, although it may not be significant, the decrease of the effective population size with in-

creasing latitudes was expected because the environment is progressively less favorable to honey bees. However, in lineage C, one can note that the population of Forli has a very small effective size, which can hardly be explained by poor environmental conditions. We hypothesize that this situation results from intense queen rearing in this area (for commercial purposes) which increases the level of inbreeding of the population.

Populations of lineage M are characterized by an overall significant excess of alleles compared with the numbers expected under mutation-drift equilibrium. At least two different explanations can be given to this result. The first one is genetic introgression due to beekeeping which has been estimated to (at least) 20, 7.7 and 7.3% for Avignon, Valenciennes and Umeå populations, respectively through mtDNA characterization (L. GARNERY, unpublished results). The number of different alleles can be transiently increased by introgression of foreign genes. The second explanation is that these populations have all experienced a bottleneck in their recent past. This hypothesis, which had been initially suggested to explain the low level of mtDNA found in lineage M (GARNERY 1992; GARNERY *et al.* 1992), is supported by the agreement between observed data and theoretical computations for six out of seven loci. The curves of Figure 4 provide also a rough idea of how recent this putative bottleneck would be. Excluding locus B124, the best fit is obtained with $t = 0.4N_e$ generations, which would correspond to 500 years ago, if we take an average length of generations of 2 yr and an average N_e of 600 (Table 4). However, more loci and populations are needed to check this hypothesis and, if it is verified, to estimate when the bottleneck occurred.

Colony assignment and parental signature: The usefulness of microsatellites to classify a colony of unknown origin, has yet to be assessed, because the test has been performed on a single colony. However, some simple deductions can be made from our results. First, assigning a colony to an evolutionary branch should be errorless because individuals bees are themselves correctly classified (Figure 3), provided that they have not received too many genes from another branch (like in Tiznit). Second, a queen is naturally mated to 10–20 drones coming from the surrounding population. This implies that the parents of the colony represent a sample of 12–22 haploid genomes which can be compared in terms of allelic frequencies to reference populations. Figure 4 indicates clearly that such a colony-based sample can be quite representative of the population to which the colony belongs, confirming previous observations (ESTOUP *et al.* 1994). We conclude that in many cases, it should be possible to assign a given colony to the right population as long as the latter belongs to the set of reference. As a consequence, microsatellites should be very useful to measure the introgression of

African genes into American populations of European origin.

The study of phylogenetic relationships between workers of a given colony and those of the population it originated, indicates that honey bees from the same patriline cluster together when a sufficient number (here 12) of microsatellite data are used whereas half-sisters do not. This possibly surprising result however agrees with the observation that the average heterozygosity of a population can be estimated from a single colony with a fairly good precision (ESTOUP *et al.* 1994). As discussed above, the parents of the colony represent a significant sample of the whole population. Note that, since honey bee drones are haploid, workers of the same patriline (super-sisters) have 75% of their genes in common (by descent), *i.e.*, three times more than workers of different patrilines (half-sisters) which share only 25%. Examples given per CHAKRABORTY and JIN (1993) indicate that for an intermediate relationship (parent-offspring, 50% of gene sharing), average heterozygosities >0.7 are required to distinguish parent-offspring from random pairs with 10 loci. Because the population of Avignon has an average heterozygosity of 0.6, it is not surprising that half-sisters, which are more distantly related than parent-offspring, are scattered among the other members of the population.

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APPENDIX

The distance between two OTUs is defined as the total number of OTUs derived from the most external node linking the two OTUs.

In a monophyletic group of three OTUs, there are three pairs of OTUs. For the closest pair, the above distance is equal to two, and for the other two pairs, it is equal to three. The sum of the three distances is then equal to eight, and the average equal to $8/3$.

In a monophyletic group of four OTUs, two different topologies are possible according to whether the root is on the internal or an external branch. It is easy to check that the sum of distances between the six pairs of OTUs is in both cases equal to 20, and hence the average distance equal to $20/6$.

Suppose now that in a monophyletic group of n OTUs ($n \geq 4$), the sum of distances between pairs of OTUs, S_n , is independent of the topology and equal to:

$$S_n = n(n+1)(n-1)/3 \quad (A1)$$

Consider a monophyletic group of $(n+1)$ OTUs.

Whatever the topology of this group is, it can be split into two monophyletic subgroups linked by the most basal node. Assume that one subgroup contains i OTUs ($n \geq i \geq 1$) and the other $(n+1-i)$ OTUs. The total number of pairs of the $(n+1)$ OTUs can be split into three sets: the pairs composed of individuals of the first subgroup, the pairs composed of individuals of the second subgroup and the pairs composed of one individual taken in each subgroup. Consequently, the sum S_{n+1} is equal to

$$S_{n+1} = S_i + S_{n+1-i} + i(n+1-i)(n+1)$$

The third term corresponds to the sum of distances between any of the i OTUs of the first subgroup and any of the $(n+1-i)$ OTUs of the second subgroup, all such distances being equal to $(n+1)$.

Replacing S_i and S_{n+1-i} by their respective value taken from (1), we find after simplification:

$$\begin{aligned} S_{n+1} &= (n^3 + 3n^2 + 2n)/3 \\ &= [(n+1)^3 - (n+1)]/3 = n(n+1)(n+2)/3 \end{aligned}$$

which is identical to (1).

This shows that if (1) is valid for up to n , it is also valid for $(n+1)$. Because it is verified for $n=3$ and $n=4$, it is valid for all n .

Because there are $n(n-1)/2$ pairs of individuals in a group of size n , the average distance within a monophyletic group of n individuals, d_n , is equal to

$$d_n = 2(n-1)n(n+1)/[3n(n-1)] = 2(n+1)/3.$$