

ORIGINAL ARTICLE

Asymmetric introgression of African genes in honeybee populations (*Apis mellifera* L.) in Central Mexico

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The Africanization of the honeybee (*Apis mellifera*) in South America is one of the most spectacular examples of biological invasions. In this study, we analyzed the Africanization process in Central Mexico along an altitudinal transect from 72 to 2800 m, using both mitochondrial and nuclear DNA markers. The mitochondrial analysis revealed that the two high-altitude populations had a significantly greater percentage of African mitotypes (95%) than the three lowland populations (67%), indicating successful spreading of Africanized swarms to these altitudes. All populations (highland and lowland) had a similar overall proportion of African

alleles at nuclear loci (58%). Thus, all populations showed an asymmetric introgression of African nuclear and mtDNA. Colonies with African mitotypes had, on average, significantly more African nuclear alleles (60%) than those with European mitotypes (51%). Furthermore, the three lowland populations showed clear signs of linkage disequilibrium, while the two high-altitude populations did not, indicating recent genetic introgression events into the lowland populations.

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Introduction

Increasing transport of livestock and mobility of humans has led to a sharp increase of invasive species all over the world (Vitousek *et al.*, 1997). Such biological invasions can pose a serious threat to biodiversity as well as to the public health (Mack *et al.*, 2000; McMichael and Beaglehole, 2000). One of the most spectacular, and from the point of view of the invasive organism, most successful biological invasions has been the Africanization of the honeybee (*Apis mellifera*) in the Americas (Schneider *et al.*, 2004; Moritz *et al.*, 2005).

Originally the natural distribution of *A. mellifera* was restricted to Africa, Europe and the Middle East, where about 20 subspecies, displaying distinctive morphological and behavioral features, have been described (Ruttner, 1988). The Americas were free of honeybees until the arrival of the Spaniards. Subsequently, domestic honeybees (mostly of European origin) were imported by the European settlers and successfully established domestic and feral populations (Nogueira-Neto, 1972; Spivak *et al.*, 1991). Interestingly these early honeybee populations probably already included, at a low percentage, African mitotypes (Schiff and Sheppard, 1993, 1995; Sheppard *et al.*, 1999; Clarke *et al.*, 2001); however, these did not trigger any invasive Africanization.

In the 1950s, in an attempt to improve the honey harvest and to obtain a hybrid honeybee better adapted to the tropical climate, roughly 50 *A. m. scutellata* queens were imported from South Africa to Brazil (Kerr, 1967). These 50 queens formed the nucleus for the following biological invasion in which Africanized swarms spread all over South America and finally reached the United States (Hall and Muralidharan, 1989). Their high reproductive (swarming) capacity, their pre-adaptation to tropical and subtropical climate and their unforeseen capacity to supplant honeybee populations of European origin were the key factors in this biological success story. All attempts to stop the progress of the Africanized swarms proved more or less futile and now finally only low temperature and unfavorable climatic conditions seem to have stopped their expansion towards the north in the USA (Visscher *et al.*, 1997) and towards the south of Argentina and Chile (Kerr *et al.*, 1982).

A number of studies have investigated the process of Africanization of the honeybee using various genetic markers (for example, Hall and Muralidharan, 1989; Smith *et al.*, 1989; Del Lama *et al.*, 1990; Rinderer *et al.*, 1991; Sheppard *et al.*, 1991; Lobo 1995; McMichael and Hall, 1996; Suazo *et al.*, 1998; Quezada-Euán, 2000; Clarke *et al.*, 2001, 2002; Pinto *et al.*, 2005; The Honeybee Genome Sequencing Consortium, 2006; Whitfield *et al.*, 2006). Initially, it was believed that the Africanized honeybees were a hybrid swarm of European and African honeybee subspecies, but the use of mitochondrial DNA markers soon undermined that view (Smith *et al.*, 1989). It turned out that the hybridization process is much more complex and frequently results in asymmetrical levels of

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introgression in the nuclear and mitochondrial compartments (Clarke *et al.*, 2002; Schneider *et al.*, 2004; Moritz *et al.*, 2005). This asymmetric introgression is a consequence of different behavior (migration, reproduction) of the males and females from the different subspecies. While completely Africanized populations have more than 97% African mitotypes, the introgression of African nuclear genes rarely seems to exceed 65–70% (Hall and Muralidharan, 1989; Smith *et al.*, 1989; Clarke *et al.*, 2002; Schneider *et al.*, 2004; Moritz *et al.*, 2005).

In this study, we analyzed five honeybee populations in Veracruz (Central Mexico) along an altitudinal transect from 72 to 2800 m above sea level (Figure 1) using both mtDNA and nuclear microsatellite markers. Africanized bees arrived in this region in 1987 (Moffett *et al.*, 1987), 12 years before the sampling, presumably allowing the Africanization process enough time to be completed.

The sample design allowed for the assessment of the impact of environmental conditions on the spread of African genes in the populations with different kinds of markers, either inherited maternally (mtDNA) or by both sexes (microsatellites). Since Africanized colonies are supposed to be adapted to tropical conditions, we would expect them to have become established at lower altitudes. In contrast European colonies, which are more adapted to colder climate, were expected to have persisted at higher altitudes.

Materials and methods

Sampling and DNA isolation

The first population along the vertical transect was situated at Soledad de Doblado (further abbreviated *V1*; 30 colonies sampled) at an altitude of 72 m, the second at Córdoba (*V2*; 30 colonies) at 700 m, the third at Ixcuatlan del Café (*V3*; 30 colonies) at 1300 m, the fourth at Calchualco (*V4*; 14 colonies) at 1900 m and the fifth at Cuiyachapa (*V5*; 16 colonies) between 2500 and 2800 m (Figure 1). The samples in the lower altitude populations (*V1*, *V2* and *V3*) were collected directly from colonies in a 3 km radius, exclusively from beekeepers who were keeping colonies established from locally caught swarms which had no imported European queens. In the two highest locations (*V4* and *V5*) the bees had to be collected from feral colonies because there were no beekeepers in these localities.

To be able to perform a comparison, two additional populations were collected in Mexico (Figure 1) (i) a population located in the far south of Mexico in Tapachula, Chiapas State, close to the Guatemalan border at 100 m altitude, was collected in 1999 as reference for earlier Africanized honeybee populations (*Mex99*; 30 colonies) and (ii) a population located in Tamaulipas State at 250 m altitude, was collected in 1988 as a reference for pre-Africanization honeybee populations (*Mex88*; 30 colonies). Finally, since our analysis also required an African population as a reference, we used previously published mitochondrial (Franck *et al.*, 2001) and microsatellite data (Franck *et al.*, 2000b) for an *A. m. scutellata* population from South Africa (*Afric*) located near Pretoria. In this particular population the allele frequencies at microsatellite loci were calculated based on the genotypes of the parental generation derived by genotyping the worker offspring of six colonies. A

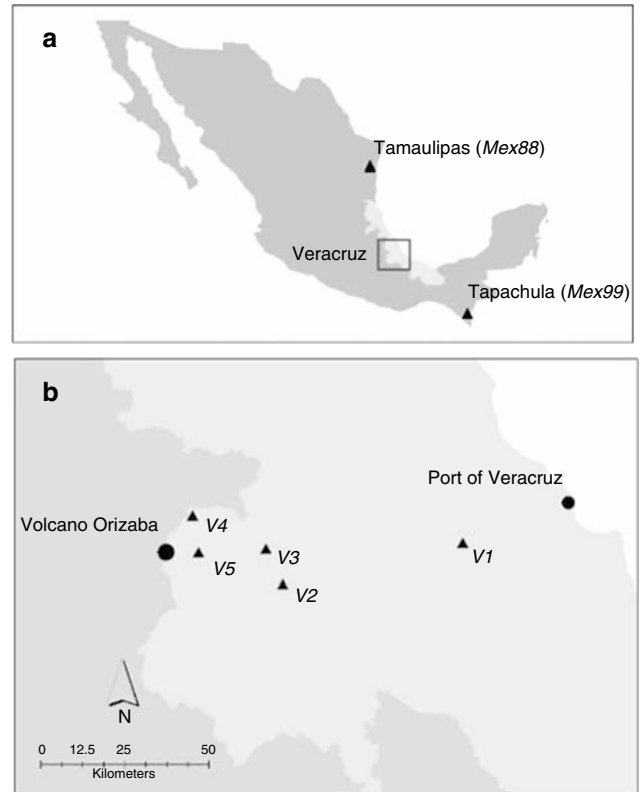


Figure 1 Map of Mexico and Veracruz; (a) a map of Mexico with the three Mexican sampling locations, Tamaulipas (*Mex88*, 250 m), Tapachula (*Mex99*, 100 m) and the Veracruz populations (*V1*, 72 m; *V2*, 700 m; *V3*, 1300 m, *V4*, 1900 m, *V5*, >2500 m). (b) The detailed view of the State of Veracruz and the location of the Veracruz populations there are shown.

summary of the names of all populations analyzed, the sampling locations, their abbreviations, the altitude and the number of colonies sampled are given in Table 1.

All samples were stored in 96% ethanol at -20°C until DNA extraction. Total DNA was isolated from the thorax of one individual bee per colony using a chloroform phenol extraction following the procedure described by Kocher *et al.* (1989). By sampling one bee per colony the mitotype of a given colony can be described, as well as 50% of the nuclear genome of a given queen and 100% of the genome of one of the drones she mated with. Thus with 14–30 colonies sampled (depending on population and availability of colonies), it is possible to obtain a representative sample of both the nuclear and mitochondrial DNA variation in a population.

mtDNA analysis

The mitochondrial analysis was carried out according to Garnery *et al.* (1992), amplifying the tRNA^{leu}-COII mitochondrial region by PCR using the primers E2 (5'-GGCAGAATAAGTGCATTG-3') and H2 (5'-CAATAT CATTGATGACC-3'), followed by a restriction of the PCR product with the enzyme *DraI*. The length of the obtained PCR products was determined on 1% agarose gels. After the digestion of the PCR product with *DraI*, the restriction fragments were electrophoretically separated on 10% polyacrylamide gels and visualized in UV light after staining with ethidium bromide.

Table 1 The names of the analyzed populations, their abbreviations, their altitude (in meters), the number of mitochondrial genes (n_1) and the number of nuclear genes (n_2) sampled per population

Population	Altitude	mtDNA									Microsatellites			
		n_1	n_2	A1	A4	A4'	C1	C2	M3	n_a	a_g	H_e	H_o	
Soledad (V1)	72	30	60	0.47	0.17	/	0.27	0.10	/	9.5±3.2	7.3±1.6	0.81±0.02	0.80±0.03	
Cordoba (V2)	700	30	60	0.43	0.17	/	0.30	0.07	0.03	11.4±3.4	8.5±1.7	0.85±0.02	0.86±0.02	
Ixhuatlan (V3)	1300	30	60	0.57	0.20	/	0.17	0.03	0.03	10.9±2.4	8.0±1.3	0.83±0.02	0.83±0.03	
Calchualco (V4)	1900	14	28	0.79	0.21	/	/	/	/	8.9±1.8	8.3±1.5	0.84±0.02	0.80±0.04	
Cuiyachapa (V5)	>2500	16	32	0.50	0.44	/	/	/	0.06	8.5±2.5	8.0±2.2	0.85±0.02	0.86±0.03	
Tapachula (Mex99)	100	30	60	0.57	0.37	/	0.03	0.03	/	10.1±2.2	7.8±1.3	0.83±0.02	0.80±0.03	
Tamaulipas (Mex88)	250	30	60	0.13	/	/	0.52	0.35	/	6.0±1.9	4.8±1.5	0.64±0.02	0.61±0.04	
Pretoria (Afric)	1300	10	58	0.20	0.70	0.10	/	/	/	12.9±2.2	8.9±1.4	0.87±0.02	0.86±0.03	

For the mtDNA the frequencies of the detected mitotypes (A1, A4, C1, C2, M3) are given. For the microsatellites the average number of alleles (n_a), the allelic richness (a_g), expected (H_e) and observed (H_o) heterozygosity are given.

Microsatellite genotyping

We used eight microsatellite loci A014, A043, A(B)024, A088, A113, Ap043, A028 and A008 (Solignac *et al.*, 2003) to genotype the individual bees at nuclear genetic level. Radioactive PCR were performed under standard conditions as reported by Solignac *et al.* (2003) except for the A014 locus that was amplified with the primers 5'-GTGTCGCAATCGACGTAACC-3' and 5'-CGATGACGCGTGGATAAGC-3' with 1.7 mM of MgCl₂ in the reaction buffer and 58°C as annealing temperature. The resulting PCR products of each of the eight loci were heated 5 min at 90°C and were electrophoretically separated on 6% denaturing polyacrylamide sequencing gels for fragment length analysis.

Statistical analysis

Unbiased estimates and s.d. of gene diversity (H_e) were calculated according to Nei (1973). Using the software *HP-Rare* (Kalinowski, 2005), we calculated the allelic richness (a_g) in the populations taking into account differences in sample size (Leberg, 2002). We further tested for Hardy–Weinberg equilibrium, linkage equilibrium, cytonuclear disequilibrium and population differentiation using Fisher exact tests (Raymond and Rousset, 1995a) implemented in *Genepop* (online version; Raymond and Rousset, 1995b). Pairwise F_{st} values were calculated and tested for their significant deviation from zero using *FSTAT ver. 2.9.3.2* (Goudet, 2001).

On the basis of the results of the mtDNA analysis, we calculated the degree of Africanization in the maternal mitochondrial genomes as the percentage of African mitotypes present in a given population sample. To infer the degree of introgression of African alleles into the Mexican populations, we used a Bayesian inference method implemented in *BAPS v. 3.1* (Bayesian Analysis of Population Structure; Corander *et al.*, 2003, 2004). This software uses allele frequencies at codominant markers (such as microsatellites loci) to infer geographically distinct groups, to estimate posterior mode of population sub-structure (combination of the geographical groups) using Markov Chain Monte Carlo (MCMC) exploration of parameter space with the assumption of Hardy–Weinberg equilibrium and linkage equilibrium. We used the admixture option based on pre-defined clustering, setting the *Afric* and *Mex88* populations as the parental population of each of the other Mexican populations.

BAPS v. 3.1 then estimated the proportion of genes from the *Afric* and *Mex88* population in each individual.

Results

mtDNA analysis

Six different mitotypes (A1, A4, A4', C1, C2 and M3), previously described (for example, Franck *et al.*, 2001) could be identified among the samples (Table 1). As expected the South African population (*Afric*) harbored exclusively the mitotypes A1, A4 and A4', characteristic for the subspecies *A. m. scutellata*. The other reference population, collected in Mexico in 1988 (*Mex88*) before the arrival of Africanized swarms, showed a predominantly European pattern (87% C1 and C2) but interestingly also had a small percentage (13%) of the A1 mitotype. The populations sampled in 1999 (*V1*, *V2*, *V3*, *V4*, *V5* and *Mex 99*) had 78% of mitotypes of the A lineage overall (53% A1 and 25% A4), 20% of C lineage (15% C1 and 5% C2; an eastern European lineages) and 2% of M lineage (M3 a western European lineage). The frequencies of the different mitotypes in the populations are given in Table 1, the joint frequency of African mitotypes is additionally shown in Figure 2.

Whereas the three populations at low altitudes (*V1*, *V2* and *V3*) had only 60–77% of African mitotypes, the two higher altitude populations (*V4* and *V5*), had 94 and 100% of African mitotypes (Table 1). Those differences in mtDNA haplotype frequencies between the low- and high-altitude populations were significant (*t*-test; $t = 4.26$; $P = 0.023$).

Microsatellite genotyping

A total of 226 individuals were analyzed with the eight microsatellites. The average number of alleles (n_a) per locus was 9.8 ± 2.1 and the allelic richness (a_g) 7.7 ± 1.2 alleles per locus over all analyzed populations (Table 1). The lowest allelic richness (a_g) was found in the *Mex88* population (4.8 ± 1.5 alleles per locus) and the highest in the *Afric* population (8.9 ± 1.4 alleles per locus). The Mexican populations from 1999 had an allelic richness between those two extremes, ranging from 7.3 ± 1.6 alleles per locus in the *V1* population to 8.5 ± 1.7 alleles per locus in the *V2* population. The average number of alleles (n_a), the allelic richness (a_g), the observed heterozygosity (H_o) and the unbiased estimates of gene

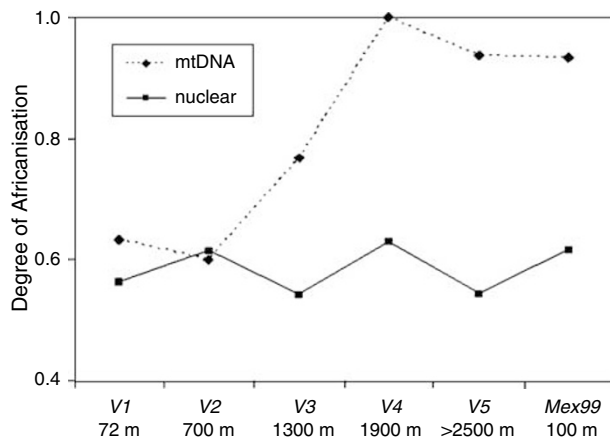


Figure 2 Degree of introgression of African genes into the populations sampled in Mexico in 1999 (V1, V2, V3, V4, V5 and Mex99). For the mtDNA the frequency of African mtDNA mitotypes is shown, while for the microsatellite markers the degree of Africanization is given as percentage of African nuclear genes estimated with *BAPS*.

diversity (H_e) are given in detail for each of the eight populations in Table 1, the microsatellite allele frequencies of the populations are given in Table 2. One population (population V3 from Mexico 1999) showed a significant deviation from Hardy–Weinberg equilibrium (at the 5% significance level).

The Fisher's exact test for population differentiation revealed that all Mexican populations from 1999 differed significantly from the *Mex88* population and from the *Afric* population, even after Bonferroni adjustment for multiple tests (Table 3; $F_{st} > 0.086$ and $F_{st} > 0.032$, respectively). Within the Mexico 1999 populations (V1, V2, V3, V4, V5 and Mex99) 7 out of 15 pairs of populations showed significant differentiation (Table 3). The *Mex99* population from the far south of Mexico differed from three out of the five populations from Veracruz (V1, V2 and V4). Interestingly, four pairwise differences were detected between the three low- (V1, V2 and V3) and two high-altitude populations (V4 and V5), while there were no significant pairwise population differentiations within the highland or lowland populations. Overall the F_{st} values among the Mexico 1999 populations were small and often not significantly different from zero (Table 3).

Even though there was no clear-cut spatial differentiation among the Veracruz populations, we found significant differentiation if we sorted all Veracruz individuals according to their African or European mitotypes. These two resulting groups displayed significant population differentiation ($P < 0.01$) at nuclear loci and apparently form distinct genetic units within the 1999 Veracruz populations.

The tests for linkage- and cyto-nuclear disequilibrium showed a significant deviation from equilibrium in 5 out of 36 pairwise comparisons when applied over the five Veracruz 1999 populations. This is clearly more than the two significant deviations, which would be expected under the null hypothesis (at the 5% level). The three lowland populations showed five significant deviations for linkage and cyto-nuclear disequilibrium, while the two highland populations only displayed two significant deviations. Thus, only the lowland populations

displayed more deviations from linkage disequilibrium than expected by chance.

On the basis of the level of admixture calculated by *BAPS v. 3.1*, the average individual degree of Africanization over all individuals in a given population sample ranged from 54% ($\pm 17\%$ s.d.) in the V3 and V5 population to 63% ($\pm 15\%$ s.d.) in the V4 population, while V1 ($56 \pm 20\%$ s.d.) and V2 ($61 \pm 18\%$ s.d.) displayed intermediate values (Figure 2). The average introgression of African nuclear genes over all populations was 58% ($\pm 18\%$ s.d.). Furthermore, no significant differences in the average level of admixture between the three lowland populations and the two highland populations could be detected (*t*-test; $t = -0.28$; $P = 0.41$). Also pairwise testing between all five Veracruz populations for differences in the average level of introgression of African nuclear genes yielded no significant results. However, when comparing the degree of nuclear admixture in individuals with African mitotypes *vs* individuals with European mitotypes in the Veracruz populations, we found a significant difference in the degree of Africanization (*t*-test; $t = 2.36$; $P = 0.02$). Thus individuals having African mitotypes had an average higher degree of Africanization ($n = 89$; $60 \pm 18\%$ s.d.), than individuals with European mitotypes ($n = 31$; $51 \pm 18\%$ s.d.).

Discussion

The honeybee samples collected in this study were located along an altitudinal cline in Veracruz (Central Mexico) from 72 to 2800 m with the two extreme points being separated vertically by less than 100 km. Besides the differences in altitude and climate these populations were also influenced in varying degrees by human activity in form of beekeeping, and only the two highest sampling sites are free of beekeeping. We found the highest frequency of African mitotypes (96% African mitotypes) in the two high-altitude populations (V4, V5; 1800–2800 m), while in the three lower altitude populations (V1, V2, V3; 72–1800 m) the frequency of African mitotypes was significantly lower (60–76% African mitotypes). First, this implies that colonies with African mitotypes have no difficulties in coping with the harsher climatic conditions in higher altitudes, since they had nearly completely replaced all European-mitotype colonies there. We would in fact expect an adaptive advantage of the Africanized swarms over the European ones at lower altitudes, and so this result seems unexpected at first glance. The most probable explanation is human influence in form of beekeeping. Even though we took great care to take samples from beekeepers who work with captured feral swarms, there are other beekeepers in the region who regularly import European queens. The more detailed analysis of our mtDNA data is congruent with this hypothesis, since we found almost exclusively eastern European (C1 and C2) mitotypes, and these have been reported in commercially exported *A. m. ligustica* and *A. m. carnica* honeybee strains that are favored by beekeepers (Franck *et al.*, 2000a). In contrast, the only European mitotype found in the high-altitude populations was of western European origin (M3). Honeybees of this particular origin (M lineage) are normally not used in commercial beekeeping

Table 2 Allele frequency data of the microsatellites used in the genetic analysis of the eight analyzed populations

<i>Ap43</i>	125	127	131	133	134	135	137	139	141	143	145	147	149	151	157	159	161	163	165	167	169	171	173	175	189
V1			0.35	0.06		0.06	0.26	0.07			0.17										0.04				
V2			0.21	0.16		0.05	0.13	0.02		0.02	0.23	0.02			0.02	0.02	0.05		0.04		0.02		0.02		0.02
V3			0.16	0.22		0.06	0.14	0.14			0.14	0.02				0.02		0.02	0.02		0.02	0.02	0.02		
V4			0.18	0.07		0.07	0.18	0.07			0.21						0.04	0.04	0.07				0.04		0.04
V5	0.04		0.19	0.15		0.04	0.08	0.12		0.04	0.12				0.04		0.04		0.08		0.04				0.04
Mex99			0.07	0.12		0.14	0.09	0.21		0.02	0.14		0.02			0.09	0.02	0.02		0.03	0.02		0.03		
Mex88			0.03	0.03		0.03	0.53			0.11	0.22	0.03													
Afric			0.02	0.13	0.21	0.07	0.32	0.08	0.02	0.04	0.02			0.04	0.02						0.03				0.02

<i>A43</i>	125	126	127	128	130	132	134	135	136	138	139	140	141	142	143	144	145	146	148	150	152	159			
V1		0.05	0.07	0.25		0.03	0.02	0.02		0.02	0.07	0.08	0.13	0.08	0.02		0.02	0.03		0.12					
V2	0.02	0.12	0.02	0.17		0.03	0.09		0.02	0.05		0.05	0.07	0.02	0.03	0.03		0.05		0.21		0.02			
V3		0.07		0.25			0.04			0.07	0.04	0.07	0.11		0.07	0.02		0.04	0.02	0.21	0.02				
V4		0.19	0.04	0.19			0.08				0.27	0.04	0.04		0.04	0.08		0.04							
V5		0.13		0.20			0.07	0.03		0.07	0.10	0.07	0.07		0.03			0.13		0.10					
Mex99		0.17		0.21		0.05			0.02	0.10	0.03	0.05	0.02	0.03		0.07		0.05		0.19					
Mex88			0.06	0.54						0.02		0.17	0.15		0.06										
Afric		0.13		0.04	0.02	0.08	0.13		0.11	0.02		0.10	0.06	0.15		0.06		0.02	0.07	0.02					

<i>A14</i>	154	156	157	158	160	161	162	164	166	168	169	170	172	178	182	184	188	190	192
V1				0.03	0.42		0.17	0.12	0.13	0.02			0.02		0.02	0.02		0.03	0.03
V2				0.02	0.35		0.08	0.12	0.18	0.03			0.12		0.02	0.07		0.02	
V3				0.02	0.43		0.10	0.12	0.13		0.03	0.02	0.03		0.02	0.03		0.07	
V4				0.07	0.29		0.11	0.11	0.11	0.04		0.04	0.14					0.04	0.07
V5				0.05	0.18		0.23	0.14	0.23			0.09	0.09						
Mex99				0.03	0.41		0.14	0.10	0.10			0.09	0.02	0.02	0.02			0.07	
Mex88					0.30		0.20	0.10	0.10	0.18		0.10				0.03			
Afric	0.02	0.02	0.02	0.19	0.20	0.04	0.19	0.20	0.09	0.02			0.02						

<i>A88</i>	138	140	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	159
V1		0.02	0.05		0.07		0.22			0.02	0.02		0.10	0.02	0.35	0.03	0.02	0.10	
V2			0.07	0.02	0.17	0.02	0.17			0.03	0.09		0.07	0.03	0.16	0.02	0.10	0.05	
V3			0.02	0.07	0.02	0.11	0.19		0.02	0.02	0.04		0.07	0.02	0.17	0.09	0.04	0.13	
V4			0.04	0.12	0.04	0.04	0.31		0.04	0.04			0.08		0.15		0.04	0.12	
V5					0.07		0.27			0.10	0.07		0.07	0.03	0.17		0.13	0.10	
Mex99			0.02		0.10		0.21		0.02	0.02	0.09		0.17	0.03	0.17		0.09	0.09	
Mex88				0.03	0.03		0.45		0.02						0.43		0.02	0.02	
Afric	0.04		0.02	0.11	0.04	0.04	0.16	0.04	0.14	0.02	0.02	0.02	0.02	0.05	0.15	0.09		0.03	0.03

<i>A113</i>	200	202	204	206	208	210	212	214	216	218	220	222	224	226	228	230	234	236	
V1		0.15					0.10	0.20	0.33		0.22								
V2		0.02			0.04		0.11	0.07	0.25		0.34		0.11	0.05	0.02				
V3		0.13		0.02	0.03		0.03	0.20	0.22	0.03	0.23	0.08		0.02					
V4		0.11			0.07		0.11	0.04			0.54	0.04	0.04	0.07					
V5		0.13						0.10	0.17		0.43			0.13				0.03	
Mex99		0.12			0.02		0.05	0.13	0.17		0.40	0.05		0.03		0.03			
Mex88		0.42					0.06	0.08		0.31	0.03	0.06		0.03				0.03	
Afric	0.04	0.04	0.02		0.09	0.09	0.07	0.13	0.10	0.13	0.05	0.19	0.04	0.02		0.02			

<i>A28</i>	121	125	127	129	130	131	132	133	134	135	136	137	138	140	141	142
V1			0.18		0.07	0.12	0.18			0.22	0.05		0.12	0.02	0.03	0.02
V2		0.03	0.10	0.02	0.07	0.09	0.10	0.03	0.02	0.21	0.05	0.02	0.17	0.05	0.03	
V3		0.06	0.16			0.06	0.20			0.08	0.02		0.38	0.02	0.02	
V4			0.23			0.09	0.23			0.09	0.05		0.27	0.05		
V5			0.07		0.03	0.10	0.23			0.27	0.07		0.17	0.07		
Mex99		0.03	0.16		0.07	0.12	0.09			0.33	0.02		0.12	0.03	0.03	
Mex88							0.33			0.02			0.65			
Afric	0.02		0.09		0.05	0.09	0.03	0.03	0.05	0.24	0.07	0.05	0.09	0.09	0.10	

<i>A8</i>	148	150	152	154	156	157	158	159	160	161	162	164
V1	0.16	0.05	0.05	0.35	0.13		0.04		0.07		0.09	0.05
V2	0.25	0.10	0.08	0.17	0.19		0.06		0.08		0.02	0.06
V3	0.20	0.02	0.04	0.31	0.06		0.07	0.04	0.11		0.11	0.04
V4		0.04	0.35	0.31	0.15		0.04	0.04			0.08	
V5	0.25		0.14	0.29	0.07		0.04		0.07		0.07	0.07
Mex99	0.36		0.07	0.31	0.04		0.04		0.09		0.04	0.05

Table 2 (Continued)

A8	148	150	152	154	156	157	158	159	160	161	162	164
Mex88	0.54						0.07		0.09		0.26	0.04
Afric	0.02	0.14	0.18	0.20	0.17	0.02	0.16	0.02	0.04	0.03		0.04
B24	97	98	99	100	101	102	104	105	106	108	110	
V1	0.10	0.42				0.08	0.12		0.07	0.20	0.02	
V2	0.10	0.29	0.00			0.10	0.09		0.10	0.31		
V3	0.05	0.24				0.13	0.09		0.11	0.36	0.02	
V4	0.11	0.18				0.11	0.29		0.14	0.14	0.04	
V5	0.07	0.23				0.07	0.13		0.03	0.47		
Mex99	0.09	0.36				0.09	0.10		0.07	0.28	0.02	
Mex88		0.66		0.02					0.05	0.28		
Afric	0.04	0.21	0.04	0.03	0.02	0.25	0.13	0.02	0.10	0.18		

Allele sizes are given in base pairs.

Table 3 Pairwise F_{st} values (above diagonal) and P values for the fisher exact test for population differentiation (below diagonal)

	V1	V2	V3	V4	V5	Mex99	Mex88	Afric
V1	/	0.0109*	0.0102	0.0302*	0.0151	0.0152	0.0942*	0.0446*
V2	0.0015	/	0.0058	0.0207	-0.0014	0.0042	0.1065*	0.0338*
V3	0.0230	0.0171	/	0.0246	0.0049	0.0100	0.0868*	0.0414*
V4	0.0000 [§]	0.0011 [§]	0.0000 [§]	/	0.0077	0.0279*	0.1286*	0.0352*
V5	0.0080 [§]	0.5394	0.2626	0.1298	/	-0.0004	0.1021*	0.0322*
Mex99	0.0000 [§]	0.0158 [§]	0.0187	0.0000 [§]	0.4835	/	0.1079*	0.0436*
Mex88	0.0000 [§]	0.0000 [§]	0.0000 [§]	0.0000 [§]	0.0000 [§]	0.0000 [§]	/	0.1506*
Afric	0.0000 [§]	0.0000 [§]	0.0000 [§]	0.0000 [§]	0.0000 [§]	0.0000 [§]	0.0000 [§]	/

For the F_{st} values, *significantly different from 0; for the Fisher's exact test, [§]significant P values after Bonferroni adjustment for multiple tests.

nowadays and it is thus very unlikely that they were recently introduced by beekeepers.

In a quite extensive study, Clarke *et al.* (2001) found that during the Africanization of honeybee populations in the Yucatan peninsula, the degree of western European mtDNA (M lineage) increased from 5 to 16%, while the eastern European mitotypes (C lineage) decreased. This effect is commonly explained by the incorporation of western European mitotypes in the advancing Africanized swarms (Schneider *et al.*, 2004) in Brazil already shortly after the first Africanization and hybridization events took place. Also a recent study by Whitfield *et al.* (2006) using single-nucleotide polymorphisms reports similar results, where Africanized honeybee populations harbor a constant proportion of M alleles, while C-lineage alleles get replaced by A-lineage alleles. Further results of Whitfield *et al.* (2006) indicate that the M lineage itself is of African origin and thus evolutionarily much closer to the A mitotypes than to the geographically neighboring C mitotypes in Europe. Even though our results show a smaller proportion of western European mitotypes in association with Africanization than reported by Clarke *et al.* (2001), they are otherwise consistent with the previous studies. In the feral high-altitude populations the only European mitotypes present are M mitotypes while the presence of the C mitotypes is exclusively confined to lower altitudes and thus most likely a result of introductions of C-lineage queens for beekeeping.

No significant differences in the level of African alleles between the low- and high-altitude populations could be detected. However, we found significant linkage

disequilibria in the three lowland populations (V1, V2 and V3), while the two highland population (V4 and V5) showed no signs of significant linkage disequilibrium. Again the most likely explanation for this phenomenon is beekeeping and importation of European queens in the lowland populations resulting in recent nuclear introgression.

Interestingly, the colonies displaying African mitotypes within the Veracruz populations had a higher proportion of African alleles (60%) than the colonies displaying European mitotypes (51%). As a result of these different proportions of African alleles there was a significant population differentiation between these two groups, indicating that in fact the colonies with European mitotypes form a genetic subunit within the Veracruz populations. A possible explanation for these differences in proportions of African and European alleles between African and European descendent colonies might be cyto-nuclear incompatibilities and negative heterosis (Smith *et al.*, 1997; Schneider *et al.*, 2003). As the presence of M mitotypes in Africanized populations and the recent findings by Whitfield *et al.* (2006) suggest, these cyto-nuclear incompatibilities are much more pronounced for the hybrids between the A and C lineage and not so much for hybrids of the A and M lineage.

In this study, we found that Africanized swarms obviously can outcompete European swarms in high altitudes. However, this phenomenon seems not to be universal. Quezada-Euán *et al.* (2003) found only around 60% African mitotypes in honeybee populations from high-altitude populations (1800–3500 m) in Peru, even though Africanization had started there 18 years before

the study. In the Mexican state of Veracruz, the climate is subtropical at low altitude. At higher altitude, that is, above 2500 m, there is an arid desert-like climate, with hot days (commonly above 30°C) and cold nights, especially during the winter, where the temperature can be as low as -5°C. Thus even though the climatic conditions in high altitudes in Veracruz are surely less suitable than at low altitudes, the Africanized bees still have a selective advantage over the European descendent populations, and are able to cope with periods of night frost.

Our study also included one 'pre-Africanization' population from Tamaulipas collected in 1988 (Mex88), which contained a small percentage (13%) of African (A1) mitotypes. This result supports the findings of previous studies which also detected African mitotypes in pre-Africanization populations in South- and Meso-America (Schiff and Sheppard, 1993, 1995; Sheppard et al., 1999; Clarke et al., 2001). This pre-Africanization occurrence of A1 mitotypes may reflect the composition of the Iberian honeybees introduced into the Americas by settlers more than 100 years ago. In accordance with this hypothesis, the A1 mitotype has been observed in samples from Spain and Portugal at frequencies reaching up to 40% (Franck et al., 2001).

In sum, these Veracruz populations showed two distinct patterns of Africanization depending on the degree of beekeeping activity present. While the three lowland populations (V1, V2 and V3), which are heavily influenced by beekeeping, displayed only a moderate asymmetry in introgression of African genes (67% African mitotypes, 57% African nuclear genes), the two highland populations (V4 and V5) did not. These two populations at high altitudes, where no beekeeping is present, were nearly exclusively constituted of African mitotype colonies (97% African mitotypes), but had a much lower degree of African nuclear alleles (59% African alleles), thus showing a strong asymmetrical introgression of African genes. The difference in the lowland populations can be explained by the constant introduction of European queens, reflected in the linkage disequilibrium in these populations. We conjecture that, without constant importation of queens, the African mitotype colonies would rapidly replace the European mitotypes leading to a strong asymmetric genetic pattern as found in the feral highland populations.

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