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Temporal variation in the genetic structure of a drone congregation area: an insight into the population dynamics of wild African honeybees (*Apis mellifera scutellata*)

R. JAFFÉ,* V. DIETEMANN,*†‡ R. M. CREWE† and R. F. A. MORITZ*†
Molecular Ecology Research Group, Institute of Biology, Martin Luther University, Halle-Wittenberg, Hoher Weg 4, 06120 Halle (Saale), Germany, †Social Insect Research Group, Department of Zoology and Entomology, University of Pretoria, Pretoria 0002, South Africa, ‡Swiss Bee Research Center, Agroscope Liebefeld-Posieux Research Station ALP, Schwarzenburgstrasse 161, CH-3003 Bern, Switzerland

Abstract

The mating system of the honeybee (*Apis mellifera*) has been regarded as one of the most panmictic in the animal kingdom, with thousands of males aggregating in drone congregation areas (DCAs) that virgin queens visit to mate with tens of partners. Although males from many colonies gather at such congregations, the temporal changes in the colonies contributing drones remain unknown. Yet, changes in the DCAs' genetic structure will ultimately determine population gene flow and effective population size. By repeatedly sampling drones from an African DCA over a period of 3 years, we studied the temporal changes in the genetic structure of a wild honeybee population. Using three sets of tightly linked microsatellite markers, we were able to reconstruct individual queen genotypes with a high accuracy, follow them through time and estimate their rate of replacement. The number of queens contributing drones to the DCA varied from 12 to 72 and was correlated with temperature and rainfall. We found that more than 80% of these queens were replaced by mostly unrelated ones in successive eight months sampling intervals, which resulted in a clear temporal genetic differentiation of the DCA. Our results suggest that the frequent long-range migration of colonies without nest-site fidelity is the main driver of this high queen turnover. DCAs of African honeybees should thus be regarded as extremely dynamic systems which together with migration boost the effective population size and maintain a high genetic diversity in the population.

Keywords: African honeybee, *Apis mellifera scutellata*, drone congregation area, linked microsatellite markers, seasonal migrations, sibship reconstruction analyses

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Introduction

In eusocial Hymenoptera (ants, bees and wasps), the combination of eusociality, haplodiploidy and complementary sex determination, constrains the genetically effective population size (N_e) to a greater extent than in other insect taxa (Hedrick & Parker 1997; Chapman & Bourke 2001; Packer & Owen 2001). Eusociality is characterized by the occurrence of one or few reproductive females per colony (the queens). Since N_e is mostly limited by the abundance of the rare sex, eusocial Hymenoptera have smaller effective population sizes than solitary species with a larger number of reproductive individuals per unit area (Wilson 1963; Chapman & Bourke 2001). In addition, eusocial hymenopterans are haplodiploid (males originating parthenogenetically from unfertilized eggs), and thus the haploid condition of males reduces the number of copies of a given allele in the population. As a consequence, under equal sex ratios the effective population size of haplodiploids is ¾ the effective population size of diploid organisms (Hedrick & Parker 1997). Finally, the common sex determination system based on the complementary action of specific sex alleles at an autosomal locus, inevitably results in a fraction of inviable or effectively sterile diploid males in the population. The production of diploid males

Correspondence: R. Jaffé, Fax: +49-345-5527264, E-mail: rodolfo.jaffe@zoologie.uni-halle.de

not only limits colony growth (Van Wilgenburg *et al*. 2006), but also biases the effective breeding sex ratio in favour of haploid males, further reducing N_e (Zayed 2004). The combination of these factors makes populations of eusocial Hymenoptera more susceptible to losing genetic variability due to genetic drift. Hence, reproductive and dispersal strategies are expected to maximize gene flow in order to counterbalance drift and avoid the deleterious effects associated with inbreeding (Keller & Passera 1993; Nunney 1993; Crozier & Pamilo 1996; Haag-Liautard *et al*. 2008).

Regarded as one of the most panmictic in the animal kingdom, the honeybee (*Apis mellifera*) mating system involves the aggregation of several thousands males at specific drone congregation areas (DCAs), which virgin queens visit in order to mate repeatedly with tens of partners (Estoup *et al*. 1994; Moritz & Southwick 1995). Sexually mature drones leave their maternal hives to join the nearest congregation area (Koeniger *et al*. 2005), where they fly 15–60 m above the ground, following elliptical trajectories with a 60–200 m diameter (Loper *et al*. 1988). Whenever a virgin queen appears, several drones pursue her forming a 'mating comet'. After mating in the air, drones die and fall to the ground, leaving the queen free to mate again with another partner. DCAs are formed irrespective of the presence of a queen, at open sites delimited by conspicuous geographical landmarks, such as valleys, river shores or forest openings (Ruttner & Ruttner 1966). Moreover, they are stable through time, with some locations known to have served as DCAs for decades (Ruttner & Ruttner 1968).

Baudry *et al*. (1998) found more than 200 colonies contributing drones to a single DCA at a given moment in time, suggesting that these congregations gather drones from most colonies within the recruitment range, thus promoting panmixis. At the population level, however, gene flow will not only be affected by the total number of drone-contributing colonies at any given time but also by the turnover of these colonies over time. Colony turnover is mediated through queen replacement, which can result from three events: reproductive swarming, absconding and queen supersedure. During reproductive swarming, old queens leave the nest along with a large proportion of their workers to ground a new nest at a different location (Seeley 1985). Pupae containing daughter queens, who will take over reproduction, are left behind in the old nest along with the remaining workers. A colony may produce one prime swarm, headed by the old queen, and one or more 'afterswarms', headed by new queens (Winston 1987; Schneider & McNally 1992). By leaving the nest site and re-establishing at a new location (absconding), a whole colony can escape from adverse circumstances, such as nest destruction, predation and infection by parasites or pathogens (Hepburn & Radloff 1998). Finally, queen supersedure is the mechanism by which an old queen, with a reduced egg-laying performance and pheromone production, is replaced by a daughter queen reared by the workers (Winston 1987).

Queen replacement changes the genetic structure of DCAs since local drones will be produced by novel queens. A limited dispersal of swarming colonies or the return of migrant colonies to their original nesting sites would result in patches of related colonies contributing drones and virgin queens to the same DCA for a long period of time, structuring the colonies of the region into stable subpopulations. This population structuring would constrain gene flow and increase inbreeding. Migratory colonies of the Asian honeybee *Apis dorsata*, for instance, exhibit nest-site fidelity by returning to specific nesting locations after having abandoned them for many months (Neumann *et al*. 2000; Paar *et al*. 2000). Similarly, *Apis mellifera scutellata* bees have been claimed to perform annual two-way migrations in South Africa, moving to the highlands of KwaZulu Natal, in the late summer and returning to the Muden river valley the following spring (Fletcher 1991). *A. m. scutellata* populations have also been found to be highly mobile in the Okavango river delta, Botswana, with reproduction and migration being associated to seasonal fluctuations in forage availability (McNally & Schneider 1992). Although these studies report on fluctuations in population density, they neither identified the source nor the sink populations. Moreover, even though a considerable amount of work has shed light into the dynamics of feral Africanized bees (reviewed by Schneider *et al*. 2004), reliable measurements of effective dispersal or possible nest-site fidelity in wild *A. mellifera* populations in their native range have not been made.

In order to study dispersal and queen turnover in honeybee populations, it is necessary to identify colonies individually. Molecular DNA tools have been shown to be reliable means of identifying individual honeybee queens (Estoup *et al*. 1994; Baudry *et al*. 1998). In particular, the use of tightly linked microsatellite markers allows the accurate reconstruction of queen genotypes from random samples of honeybee drones (Moritz *et al*. 2007a). Shaibi *et al*. (2008) recently developed a toolkit for studying population structure in honeybees, consisting of sets of tightly linked microsatellite loci on three different chromosomes. Due to the extremely high recombination rates found in *A. mellifera* (Beye *et al*. 2006), this series of independent linkage groups can increase the detection power by more than five orders of magnitude in comparison with unlinked markers (Shaibi *et al*. 2008).

Here we studied the temporal dynamics of a wild honeybee population at a particular DCA. In many regions of Africa, apiculture is poorly developed and truly wild honeybee populations can be found (Moritz *et al*. 2005). We therefore studied the temporal dynamics of an African DCA located inside a nature reserve, without the interference of beekeeping activities. Using three sets of linked microsatellites

Table 1 Sample sizes, successfully amplified loci and genetic diversity measures for five samples of *Apis mellifera scutellata* drones collected from a single DCA over a period of 3 years. Sample sizes (*n*) are given as the number of drones collected, genotyped and assigned to a specific haplotype in at least one of the three sets of tightly linked microsatellite markers employed (LG-3, LG-13 and LG-16). Number of alleles and gene diversity (expected heterozygosity) are given as means ± SD

	Number of drones (*n*)			Amplified loci				
Sample	Collected	Genotyped	Assigned	LG-3	LG-13	LG-16	Number of alleles (*A*)	Gene diversity (H_E)
March 2004	96	96	92	3	4	5	11.00 ± 4.56	0.76 ± 0.14
November 2004	373	96	94	3	4	5	12.50 ± 5.12	0.84 ± 0.07
April 2007	88	88	86	3	4	5	9.25 ± 2.98	0.78 ± 0.08
September 2007	24	24	24	3	3	4	5.10 ± 2.12	0.81 ± 0.12
December 2007	220	220	214	3	4	4	13.00 ± 5.88	0.84 ± 0.09
Total	801	524	510				10.17 ± 3.19	0.80 ± 0.04

(Shaibi *et al*. 2008), we genotyped honeybee drones sequentially sampled from this DCA over a period of 3 years. In this way, we were able to reconstruct individual queen genotypes with a high accuracy and follow them through time to estimate their rate of replacement.

Materials and methods

Sampling

The study was carried out in the Ezemvelo Nature Reserve, located 25 km from Bronkhorstspruit, South Africa. The Reserve is situated on the 'Bankenveld', the transition ecotone between grassland and savanna. With more than 10 000 ha, it comprises well-preserved grasslands, savanna, rocky outcrops and wetlands, and is crossed by the Wilge River. A DCA was previously identified using a trap baited with synthetic queen pheromone (9-ODA) kept *c*. 20 m above ground by a weather balloon (Moritz *et al*. 2007b). The precise location of the DCA (25°42′34.03″S, 28°59′59.62″E) was established by slowly driving through the Reserve with the trap attached to a car. The closest apiaries in the vicinity of the reserve are found 25 km away in Bronkhorstpruit. Fifteen kilometres being the maximal mating distance (drone + queen flight distance) ever recorded (Jensen *et al*. 2005b), it was very unlikely that some drones from these managed hives flew to our study DCA. Drones can only fly up to 7 km (Ruttner & Ruttner 1972) and their average flight distance has been estimated at 900 m (Taylor & Rowell 1988). Thus, our drone samples most likely originated from colonies located in the vicinity of our study DCA. South African beekeepers mostly maintain small and nonmobile operations that are typically established by trapping wild swarms (Radloff *et al*. 2002). Given that both wild and managed bees comprise the same wild and unselected gene pool, we are confident that we observed the dynamics of a wild honeybee population, even in the unlikely case of drones from the managed hives being present at the DCA. Five drone samples were collected from this DCA over a period of 3 years (Table 1). Each sampling episode consisted of five to seven consecutive days of trapping, when the pheromone trap was flown between 13:00 and 18:00 hours under sunny and nonwindy conditions. All individuals collected were kept in 95% ethanol until genetic analysis.

Genotyping

Three sets of tightly linked microsatellite markers were used to determine individual genotypes (Shaibi *et al*. 2008). The three linkage groups (LG) are located on different chromosomes in the following sequence: spanning 11.6 Kb of chromosome 3, loci HB-SEX-01, UN351 and HB-SEX-03 (LG-3); spanning 11.2 Kb of chromosome 13, loci HB-THE-01, HB-THE-02, HB-THE-03 and HB-THE-04 (LG-13) and spanning 16.4 Kb of chromosome 16, loci HB-16-01, AC006, HB-16-02, HB-16-03 and HB-16-05 (LG-16). Based on the average recombination rate of the honeybee genome (22.04 cM/Mb) estimated by Solignac *et al*. (2007), the recombination rates within each linkage group can be calculated in 0.26, 0.25 and 0.36 cM for LG-3, LG-13 and LG-16, respectively. DNA extractions were performed using a Chelex protocol (Walsh *et al*. 1991). The microsatellite target sequences were amplified by multiplex polymerase chain reactions (PCR) containing the same combination of fluorescent-labelled primers and the Promega PCR Master Mix (see Shaibi *et al*. 2008 for PCR conditions). Samples containing no DNA were included in all plates as negative controls. PCR products were then resolved in a MegaBACE 1000 capillary sequencer to determine allele sizes. Allele calling was performed using the Genetic Profiler software (Amersham Biosciences). Ambiguous allele callings were always left as blanks. In those cases where less than four loci from one individual showed unambiguous alleles, we re-amplified its DNA to confirm allele callings (in the much larger sample of drones collected in December 2007

seven individuals were left with less than four successfully amplified loci).

Haplotype assignment and reconstruction of queen genotypes

Given that drones are produced parthenogenetically, and thus only carry alleles from their mother, genotyping allows for their assignment to specific mother queens and the subsequent reconstruction of the queens' genotypes. For each sample set, we constructed tables with the genotypes of all drones, grouping the loci from each linkage group. We then proceeded to group all individuals sharing the same allelic combination at all loci within each linkage group. Individuals sharing a particular allelic combination were assigned to a single haplotype. Some individuals could be assigned to two or more possible haplotypes (unassigned individuals or UI), because of low polymorphism or misamplifications at some loci. In these cases, the information provided by the other two linkage groups was used to help assign UI to a single haplotype. We first pooled all samples into a single haplotype assignment analysis, and later separated each one of the five temporal drone samples in order to re-analyse the remaining UI within these smaller sample sets. Individuals that could not be assigned to a specific haplotype in at least one linkage group in these smaller sample sets were excluded from all subsequent analyses (haplotypes assignments are shown in Table S1, Supporting information).

In order to assign drones to specific mother queens and reconstruct the genotypes of drone-producing queens, we performed a sibship reconstruction analysis based on the associations of haplotypes found among the three linkage groups. Given that queens are diploid, each queen genotype consists of two haplotypes from each linkage group. The probability of not sampling the second haplotype of a heterozygous queen out of a random sample of drones, decreases with increasing sampling size (e.g. $P_{2\text{drones}} = 0.25$; $P_{3\text{drones}} = 0.125$; $P_{4\text{drones}} = 0.063$). Thus, whereas the genotype of queens represented by many drones can be reconstructed very accurately, the genotype reconstruction of queens represented by few drones is more difficult. In the first case, the queen genotype reconstruction was done by hand employing the Microsoft Excel filter functions. The genotype of low-represented queens was reconstructed employing the software Colony 1.3 (Wang 2004), which follows a maximum likelihood approach to reconstruct sibship based on the population allele (or haplotype) frequencies. Colony was run several times, and the results from these runs were then compared in order to obtain a consensus reconstruction of the sibships throughout the study (shown in Table S1). In those cases where queen genotypes could be reconstructed from drones collected in different sampling periods, we only considered as unambiguous assignments those queens showing at least two haplotypes from two different linkage groups in common. To detect any association between weather and the number of collected drones and drone-contributing queens at a given time, we performed a Spearman rank correlation using the number of drones caught and queens found at each sampling event and the associated mean monthly rainfall and temperature records.

Genetic diversity, nondetection and nonsampling errors

The mean number of alleles (A) and the gene diversity (H_E) per locus were calculated using Arlequin version 3.11 (Excoffier *et al*. 2005), based on the allelic diversity observed among all typed loci (Table S2, Supporting information). We then computed the haplotype diversity (H), following:

$$H = 1 - \sum_{i=1}^{j} p_i^2 \qquad \text{(eqn 1)}$$

where p is the frequency of the ith haplotype in each linkage group within each sample.

Two kinds of errors affected our estimated number of drone-producing queens: nondetection errors (the probability of obtaining two identical genotypes in two different individuals by chance, determined by the number of markers employed and their level of polymorphism) and nonsampling errors (the number of queens remaining undetected because of an insufficient sampling). Nondetection errors were calculated based on the haplotype frequencies following Boomsma & Ratnieks (1996). Nonsampling errors were assessed through a fitted Poisson distribution of the drones among the assigned haplotypes (Chapman *et al*. 2003). In this way, we estimated the number of undetected haplotypes in each of our five samples. Likewise, based on the distribution of the drones among the reconstructed queen genotypes, we estimated the actual number of queens unrepresented in our samples. Adding the number of detected and undetected haplotypes or queens, we finally obtained the expected number of haplotypes or queens in each sample.

In order to categorize the genetic variation, we performed an analysis of molecular variance (AMOVA) using Arlequin, based on the haplotype frequencies found within the three linkage groups in each time sample. Genetic distances were then calculated employing all the drones collected in each sampling period, ignoring their inferred relationships. In this way, we were able to quantify the actual change in the genetic composition of the DCA, accounting for the possible variation in the drone contribution of static colonies. Nei's genetic distance (D_A) between all temporal drone samples was calculated as follows:

$$D_A = \sum_{k=1}^{L}\left(1 - \sum_{i=1}^{q_k}\sqrt{x_{ik}y_{ik}}\right)\Big/L \qquad \text{(eqn 2)}$$

where x_{ik} and y_{ik} are the haplotype frequencies at the k-th linkage group found in samples X and Y, respectively, and q_k and L are the number of haplotypes and linkage groups examined, respectively (Nei & Kumar 2000). D_A can take a maximal value of one, which indicates no haplotypes are shared between two samples. Using the web-based Clustering Calculator (Brzustowski 2002), we constructed neighbour-joining trees based on the genetic distances obtained after bootstrapping the haplotype frequencies 100 times. A consensus tree was then obtained using PHYLIP version 3.68 (Felsenstein 1989). In order to test for genetic isolation by time, we performed a Mantel test using the Isolation by Distance Web Service version 3.15 (Jensen *et al*. 2005a), based on the genetic and temporal distance matrix.

Haplotype and queen replacement rates

Given haplotypes identify specific chromosome segments, two identical haplotypes can be considered as identical by descent, that is, sharing a common ancestral queen. The rate at which haplotypes are replaced will thus be directly determined by the turnover of queens. Haplotype replacement rates therefore provide a clear and direct picture of the temporal genetic structure, without having to reconstruct individual queen genotypes. We estimated haplotype and queen replacement rates within each sampling year, based on 8 months time intervals: March–November 2004 and April–December 2007. Due to its small sample size and different timing of collection, the drone sample of September 2007 was not included in these calculations. Haplotype replacement rates (*HRR*) were calculated within each one of the three linkage groups, given that they provide information from independent, freely recombining chromosomes. *HRR* was computed based on the total number of haplotypes detected at time t ($TotalH_t$) and the number of shared haplotypes between the samples collected at time $t-1$ and t ($SharedH_{t-1,t}$):

$$HRR = \frac{TotalH_t - SharedH_{t-1,t}}{TotalH_t} \times 100 \qquad \text{(eqn 3)}$$

Queen replacement rates (*QRR*) were obtained following a similar approach as for the *HRR*, considering as shared queens those showing at least two haplotypes from two different linkage groups in common. By only including queen genotypes that could be unambiguously reconstructed from drones collected in successive sampling periods, we avoided underestimating the queen turnover. Aiming to provide a lower limit, however, we then computed a less conservative *QRR* including all the queens that could have been shared between March and November 2004 and between April and December 2007. For this estimation, we considered all possibly shared queens, even if they had less than two haplotypes from two different linkage groups in common.

Because of the tight linkage of our genetic markers, recombination events within each linkage group were rare (between two and three in 1000 individuals). Closely related queens (sisters or daughters) must therefore either share identical haplotypes in all three linkage groups or at least in two of them (given that the probability of having recombination events simultaneously in two different linkage groups is extremely low). In order to quantify the occurrence of such closely related queens, we considered all queens sharing at least two haplotypes in two different linkage groups, but clearly assigned as different queens.

Effective population size

Several methods allow for the estimation of the effective population size of natural populations using genetic markers, based on heterozygote excess, linkage disequilibrium, temporal samples or current genetic variation (reviewed in Wang 2005). Most of these methods, however, assume an isolated population without immigration, which may not be valid for most natural populations. Even a low migration can substantially alter the genetic makeup of a population and its changes over time, considerably biasing estimates of N_e (Wang 2005). We therefore did not employ any of the above-mentioned methods to estimate N_e, since large-scale seasonal migrations are well documented in *Apis mellifera scutellata* (Hepburn & Radloff 1998). Alternatively, we computed N_e following Wright (1933):

$$N_e = \frac{9N_f N_m}{(2N_f + 4N_m)} \qquad \text{(eqn 4)}$$

where N_f is the number of breeding females and N_m the number of breeding males. N_f was taken as the detected number of drone-contributing queens in a given period of time, and N_m as $N_f \times m_e$, where m_e is the effective mating frequency of *A. m. scutellata* queens (m_e = 15.29, taken as the harmonic mean of the six colonies analysed by Franck *et al*. 2000). This estimate reflects the effective number of breeding individuals in a given time window, being sensitive to the length of the chosen time window (the longer the time window the more immigrant colonies will be accounted for). We therefore first computed two N_e estimates based on all drone-contributing queens detected in 2004 and in 2007, respectively, and then computed an overall N_e based on all drone-contributing queens detected throughout the study.

Results

The final sample of 510 drones collected in our study DCA over a period of 3 years showed a mean of 10.17 ± 3.19

Table 2 Haplotype diversity, number of detected haplotypes and queens, nondetection and nonsampling errors for five samples of *Apis mellifera scutellata* drones collected from a single DCA over a period of 3 years. Haplotype diversity and number of detected, undetected and expected haplotypes (detected + undetected) were estimated independently in three different sets of tightly linked microsatellite markers (LG-3, LG-13 and LG-16)

		March 2004	November 2004	April 2007	September 2007	December 2007
	Haplotype diversity	0.95	0.95	0.88	0.88	0.96
	Detected haplotypes	37	39	22	10	55
	Undetected haplotypes*	3.93	4.30	0.51	0.91	1.59
LG-3	Expected haplotypes	40.93	43.30	22.51	10.91	56.59
	Haplotype diversity	0.96	0.97	0.91	0.85	0.96
	Detected haplotypes	46	53	25	11	61
	Undetected haplotypes*	6.36	9.52	0.87	1.36	2.08
LG-13	Expected haplotypes	52.36	62.52	25.87	12.36	63.08
	Haplotype diversity	0.97	0.97	0.92	0.87	0.98
	Detected haplotypes	48	56	31	11	80
	Undetected haplotypes*	7.36	10.45	2.06	1.24	5.87
LG-16	Expected haplotypes	55.36	66.45	33.06	12.24	85.87
Detected queens		42	46	27	11	69
Undetected queens†		4.70	5.96	1.12	1.24	3.10
Expected queens		46.70	51.96	28.12	12.24	72.10
Nondetection errors‡		6.2×10^{-05}	4.4×10^{-05}	8.7×10^{-04}	2.4×10^{-03}	3.3×10^{-05}

*Based on a fitted Poisson distribution of the drones among the assigned haplotypes; †based on a fitted Poisson distribution of the drones among the reconstructed queens; ‡following Boomsma & Ratnieks (1996).

alleles per locus and an average gene diversity (expected heterozygosity) of 0.80 ± 0.04 (Table 1). This high genetic diversity translated into high haplotype diversity (ranging from 0.85 to 0.98) and low nondetection errors (ranging from 3.3×10^{-5} to 2.4×10^{-3}; Table 2). The number of undetected haplotypes due to sampling effects was lowest in the LG-3 within the April 2007 sample (0.51 haplotypes undetected) and highest in the LG-16 within the November 2004 sample (10.45 haplotypes undetected). The number of undetected queens was also lowest in the April 2007 and highest in November 2004 (1.12 and 5.96 queens undetected, respectively). The largest number of queens (i.e. colonies) contributing drones to our study DCA was found in December 2007 (72 colonies) while the September 2007 sample showed the lowest number (12 colonies, Table 2). Both the number of detected and expected drone-contributing colonies were found to be positively correlated with mean monthly rainfall and temperature (Spearman's rank correlation $r_s = 0.90$; $t_{5,2} = 3.58$; $P = 0.04$ in all cases; see Table S3, Supporting information), while the number of collected drones was found positively correlated with rainfall but not temperature ($r_s = 1.00$ and $r_s = 0.80$; $t_{5,2} = 2.31$; $P < 0.10$, respectively).

Whereas most of the drones caught in a given time window exhibited haplotypes found exclusively in this particular time window (among all haplotypes there were 74% private haplotypes in LG-3, 79% in LG-13 and 82% in LG-16; see mono-chromatic columns in Fig. S1, Supporting information), some haplotypes were shared by drones from different time samples (multichromatic columns in Fig. S1). Haplotype 8 months' replacement rates ranged from 81.13% to 91.80% (Table 3), and resulted in clearly differentiated groups of haplotypes across the five different time samples in all three linkage groups (Fig. S1). Likewise, more than 80% of all detected drone-contributing queens were found to be replaced within 8 months. Restricting the analysis to

Table 3 Haplotype replacement rates (*HRR*), queen replacement rates (*QRR*), and effective population size in a South African *Apis mellifera scutellata* population. *HRR* were calculated separately in three different sets of tightly linked microsatellite markers (LG-3, LG-13 and LG-16), over two 8-month periods in 2004 and 2007. *QRR* were computed over the same two 8-month periods in 2004 and 2007, first considering as shared queens those showing at least two haplotypes from two different linkage groups in common (QRR_{2H}), and then including all possibly shared queens (QRR_{All}). Effective population size (N_e) was calculated on a yearly basis and overall, based on all the drone-contributing queens found throughout the study

	2004	2007	Mean ± SD
HRR (LG-3)	82.05	83.64	82.84 ± 1.12
HRR (LG-13)	81.13	91.80	86.47 ± 7.55
HRR (LG-16)	85.71	87.50	86.61 ± 1.26
QRR_{2H}	91.30	97.10	94.20 ± 4.10
QRR_{All}	78.26	84.06	81.16 ± 4.10
N_e	183.02	220.05	201.54 ± 26.19
N_e (Overall)	398.71		

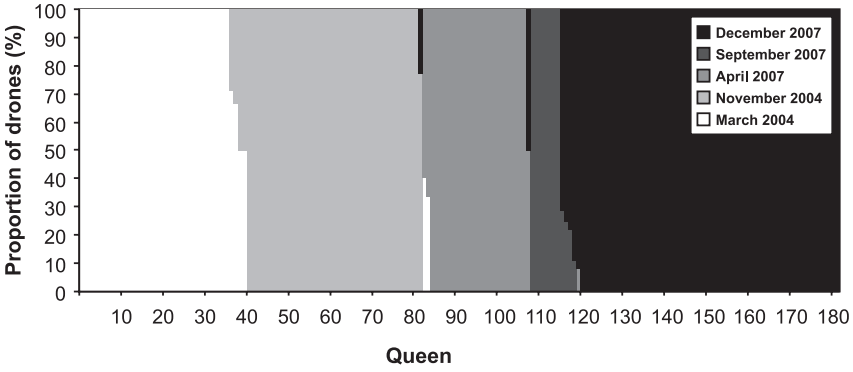

Fig. 1 Change in the composition of queens contributing drones to an *Apis mellifera scutellata* DCA over a period of 3 years. The *Y*-axis shows the proportion of drones contributed by a particular queen (on the *X*-axis) during each one of the five sampling events (shown as a grayscale). Queens showing more than one grey tone could be unambiguously reconstructed from drones collected in different sampling periods (showing at least two haplotypes from two different linkage groups in common).

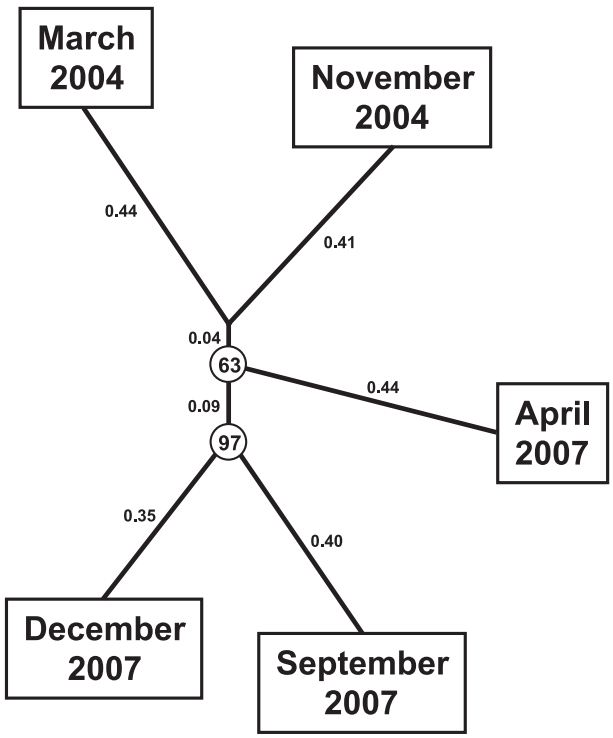

Fig. 2 Consensus neighbour-joining tree representing Nei's genetic distances (D_A) between the drone samples collected at a single *Apis mellifera scutellata* DCA over a period of 3 years. Values on the nodes represent the number of times that particular partition occurred among 100 bootstrapped trees. The actual lengths are given next to each branch.

those shared queens having at least two haplotypes from two different linkage groups in common, we found a mean 8 months queen turnover of 94.20 ± 4.10% (Table 3), with only four queens contributing drones in March as well as November 2004 and two queens contributing drones in April as well as December 2007 (Fig. 1). Only two out of the 183 queens detected throughout the study showed genotypes that could be unambiguously reconstructed from drone samples collected both in 2004 and 2007 (one queen contributed drones in November 2004 as well as December 2007, and one in March 2004 as well as April 2007, Fig. 1). In addition, we detected four pairs of related queens (sharing at least two haplotypes in two different linkage groups, but clearly assigned as different queens) sampled at different times (one pair from March 2004 and November 2004, two pairs from March 2004 and April 2007, and one pair from November 2004 and December 2007). Twenty-four pairs of queens sampled during the same period were also found to be related [one pair from March 2004, representing 5% of all queens detected in this period, one pair from November 2004 (4%), nine pairs from April 2007 (67%), and 13 pairs from December 2007 (38%)].

The AMOVA revealed highly significant variance components (among the time samples, among the linkage groups within time samples, and within the linkage groups of each time sample; $P < 0.001$ in all cases), with most of the genetic variance arising from the high polymorphism found within each linkage group (Table S4, Supporting information). Nei's genetic distances between all drone time samples ranged from 0.74 to 0.98, reflecting a large temporal-dependent differentiation (Fig. 2; Table S5, Supporting information). A significant genetic isolation by time (Mantel test with 20 000 randomizations: $Z = 227.09$, $r = 0.60$; one-sided $P = 0.032$) confirmed such differentiation. Based on the number of detected queens in 2004 and 2007 separately, we estimated an N_e approaching 200. Pooling all the queens found throughout the study doubled the N_e (Table 3).

Discussion

Using three independent sets of tightly linked microsatellite markers to genotype drone samples from a highly diverse natural population, we were able to detect most of the colonies contributing drones to a single DCA during five sampling events spaced over a period of 3 years. The number of drone-contributing colonies varied from 12 in winter to 72 in summer, being correlated with temperature and rainfall. Following these colonies through time, we found that more than 80% were replaced by mostly unrelated

colonies in successive 8 months sampling periods. This high turnover of colonies resulted in a clear temporal genetic differentiation and an effective population size estimated to range between 180 and 400, depending on the timescale chosen.

The use of three independent sets of tightly linked microsatellite markers (Shaibi *et al*. 2008) to genotype samples from a highly diverse natural honeybee population resulted in a very high detection power (Boomsma & Ratnieks 1996), even allowing us to identify closely related queens. Nondetection errors had thus little if any impact on our estimates. Sampling biases, on the other hand, represented a more important source of inaccuracy in our estimation of colony numbers. Weather factors such as wind speed and direction during the sampling could have caused an uneven scattering of the queen pheromone towards some colonies, resulting in an uneven representation of the drone-producing colonies in our samples. However, sampling over five to seven consecutive days during each collection event minimized this bias, assuring an exposure to different weather conditions (such as winds blowing in different directions). In addition, based on the distribution of the drone contributions of each colony, we estimated the number of nonsampled colonies and found that few of the drone-producing colonies located within flight distance of our study DCA remained undetected because of an insufficient sample (Table 2). Some colonies may have lacked any sexually mature drones at the time the samples were collected. These colonies could obviously not be accounted for in our estimation of colony numbers, causing an underestimation of population size. This caveat does not represent a major concern, given that all colonies belonged to the same wild population and were exposed to similar seasonal fluctuations in resource availability. Thus, by collecting drones from a DCA we obtained a robust sample of the reproductively active colonies found within flight distance during a discrete time window.

Our estimated number of colonies contributing drones to the DCA at any given time (ranging from 12 to 72), is far less than that found by Baudry *et al*. (1998), who estimated 240 colonies contributing drones to a single DCA. This difference may primarily be due to the fact that Baudry *et al*. (1998) conducted their study at a German DCA surrounded by five major apiaries and located in a region of intense beekeeping (with a density of about 20 colonies/km^2). In contrast, our study was carried out in a natural setting without the influence of beekeeping. Managed hives will inevitably affect the number of drone-contributing colonies at the local scale. Furthermore, beekeepers treat hives against diseases and provide additional feeding resources, facilitating colony investment in drone production.

Without the influence of the beekeeper, brood and drone production and reproductive swarming are mainly determined by rainfall, which ultimately drives flowering and therefore availability of forage (McNally & Schneider 1992; Hepburn & Radloff 1995; Hepburn & Radloff 1998). *Apis mellifera scutellata* occupies the arid and semi-arid zones ranging from tropical East Africa to subtropical South Africa, rainfall being the main climatic feature limiting its distribution (Hepburn & Radloff 1995). In the Ezemvelo Nature Reserve, precipitation is lowest during the winter months of May to September, when it averages 7.35 mm/month. Rainfall sharply increases in October, reaching a maximum of 133.55 mm/month in January, and progressively decreases later in March and April. The number of collected drones and the number of drone-producing colonies found in the different samples closely followed this rainfall pattern, being lowest in September and highest in December, thus confirming a close relationship between reproductive investment and climate-mediated abundance of floral resources (McNally & Schneider 1992; Allsopp & Hepburn 1997).

Through genotyping drones sequentially sampled from a single DCA over a period of 3 years, we gained an insight into the spatio-temporal dynamics of a wild honeybee population. Nearly 80% of all the haplotypes found throughout the study where found exclusively within a discrete time window, representing the genotypes of different queens contributing drones at different times to our study DCA (Fig. 1 and Fig. S1). Between successive 8 months sampling periods, about 85% of all detected haplotypes were replaced by new ones (Table 3), reflecting the rapid replacement of queens by new, unrelated ones. For instance, only a small fraction of queens from different time samples (four pairs) were found to be related, representing either supersedure or short distance swarming events. Two mechanisms may explain this high queen turnover: (i) the irregular or seasonal production of drones by long-established queens that only became apparent to us occasionally, and (ii) the migration of colonies to and from our study area (long-distance reproductive swarming or absconding). From all the queens detected throughout the study, only 3% were found to produce drones during two successive samplings and even fewer (1%) contributed drones over 3 years to the DCA. This demonstrates that while some established colonies can invest in drone production throughout the year and over several years, only a minority of colonies remain stationary for extended periods of time. Moreover, large-scale seasonal migrations are well documented in *A. m. scuttelata* (McNally & Schneider 1992; Hepburn & Radloff 1995), and hence colony migrations are the most likely driver of the high queen turnover here found. Given the reproductive swarming season of *A. m. scuttelata* in the South African Highveld lies between August and December (Hepburn & Radloff 1995), the drones collected during this period were probably produced by immigrant swarms. Since absconding-driven migrations are known to occur both in late summer and during the dry

winter season (McNally & Schneider 1992; Hepburn & Radloff 1995), the drones collected in March and April might have been produced by such migrant colonies arriving shortly after the swarming season (see Fig. 1B in McNally & Schneider 1992).

Following marked queens of 30 colonies for a period of four years, Allsopp & Hepburn (1997) found that reproductive swarming in a South African *A. m. capensis* population was concentrated in spring, whereas queen supersedure occurred throughout the year and was not correlated with weather factors. In our case, most of the related queens found within a single time sample (14 out of 24 pairs) were found in late spring–summer and are thus likely to have originated from short-distance swarms (probably small afterswarms), established in the vicinity of the DCA, or from groups of related colonies immigrating together into our study location. Since the remaining related queens from a single time sample (10 out of 24 pairs) were found in late summer-fall, we cannot determine whether they originated in a similar way, or from recent supersedure, temporary polygyny or worker reproduction events. However, the latter two phenomena are rare in nature (Ratnieks 1993; Allsopp & Hepburn 1997; Dietemann *et al*. 2008) and a superseding queen is unlikely to start producing drones while those of her mother are still alive. No seasonality was observed in the proportions of related to unrelated queens, suggesting that such possible short-range swarmings are not regular events. Therefore, long-range migrations bringing colonies out of the recruitment area of their previous DCA seem to be the rule.

Such frequent long-range migrations, driving the rapid replacement of the queens contributing drones to the DCA, resulted in a clear temporal genetic differentiation of the DCA (Fig. 2). Even though genetic distance was high between samples collected only a few months apart (thus the long branches in Fig. 2), we found a significant genetic isolation by time. Such a temporal genetic differentiation would not be expected under a seasonal production of drones by long-established static colonies, or the return of migrant colonies to old nesting sites. Under these scenarios, the genetic distance between the samples collected in the same season should be smaller than between those collected in different seasons, a prediction not supported by our results. Therefore, the frequent migration of colonies to and away from our study area without nest-site fidelity seems the most likely explanation for the observed temporal genetic differentiation. The reported annual two-way migrations of *A. m. scutellata* in the subtropics of South Africa (Fletcher 1991) are thus most likely undertaken by unrelated colonies that do not return to their previous nesting site. In contrast, giant honeybees (*Apis dorsata*) return to old nest sites after several months of having abandoned them (Neumann *et al*. 2000; Paar *et al*. 2000), which results in a higher relatedness within their aggregations (consisting of up to 200 colonies in a single tree) compared to the relatedness between different aggregations of colonies (Paar *et al*. 2004). *A. dorsata* colonies from different aggregations, however, have been shown to contribute drones to the same DCA in varying proportions through time (Kraus *et al*. 2005), enhancing gene flow between aggregations and counteracting inbreeding (Paar *et al*. 2004; Kraus *et al*. 2005). The maintenance of such a reproductive system suggests that nesting sites are more valuable to the giant honeybees of Asia than to African honeybees, which rather exploit the seasonal shifts in forage availability by abandoning old nesting sites depleted of forage in search for richer habitats (Hepburn & Radloff 1998). Given that the distribution of floral resources is patchy, both in time and space, being able to find the best patches seems more important than returning to a specific nesting site. For instance, migrating colonies can cover up to 100 km (Otis 1991), making their return to previous nesting sites unlikely.

Important life-history differences explain variations in population dynamics between different *Apis* species but also within *A. mellifera* (Hepburn & Radloff 1998), making it difficult to generalize our findings to other subspecies. African subspecies are known to disperse via long-distance migratory swarms, abscond more readily, and have a faster generation time and smaller colonies than European honeybees (Ratnieks 1991; Hepburn & Radloff 1998; Schneider *et al*. 2004). In consequence, gene flow and genetic diversity are higher and effective population size larger in African honeybee populations (Estoup *et al*. 1995; Moritz *et al*. 2007b). Our results suggest that a rapid queen turnover, driven by the frequent long-range migration of colonies without nest-site fidelity, is the main cause of the larger effective population size of African honeybees. Our N_e estimates provide an insight into the effective number of breeding individuals within the range of a natural DCA. However, because of the migration of colonies to and from our study area, our effective population size estimates cannot reflect the genetic properties of the larger, panmictic population, containing our detected colonies (Wang 2005). As immigrant queens arrive and add their drone contributions to the DCA, the effective number of breeding individuals within the range of the DCA increases, boosting the genetic effective size of the surrounding population. DCAs of African honeybees should thus be regarded as extremely dynamic systems, not only assuring a high genetic diversity among the queen's mates, but together with migration, boosting effective population size and guaranteeing the maintenance of the population's genetic diversity.

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This work is part of Rodolfo Jaffé's PhD at the Molecular Ecology Research Group, Institute of Biology, Martin Luther University, Halle-Wittenberg, on the evolution of mating systems in highly eusocial Hymenoptera. He is interested in sexual selection, the evolution of insect mating systems and the evolution of sociality. Vincent Dietemann is a scientific collaborator at the Center for Bee Research, Agroscope Liebefeld-Posieux ALP, Bern, Switzerland, and is interested in the evolution of the mechanisms of division of reproductive labour and how they shape sociality, in the chemical ecology of ants and bees, in the conservation of honeybees and in how they maintain homeostasis in their nest. Robin M. Crewe is the Vice-Principal of the University of Pretoria and the head of the Social Insect Research Group at the Department of Zoology and Entomology from this university. He is interested in the behavioural and chemical ecology of bees, ants and termites. Robin F. A. Moritz is the head of the Molecular Ecology Research Group at the Martin Luther University, Halle-Wittenberg. He is interested in the evolution of sociality and evolutionary processes within social systems and his main lines of research include behavioural and population ecology, population genetics and genomics.

Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1 Change in the genetic structure of an *Apis mellifera scutellata* DCA over a period of 3 years. The Y-axis shows the proportion of drones from each sampling event exhibiting a particular haplotype (on the X-axis) in three different linkage groups (LG-3, LG-13 and LG-16). Colours represent the five sampling events performed during the 3 year period. Haplotypes showing several colours were found repeatedly over time.

Table S1 Haplotype assignments and consensus reconstruction of the sibships of 510 *Apis mellifera scutellata* drones collected at a single DCA over a period of 3 years.

Table S2 Arlequin input file containing the multilocus genotype of 510 *Apis mellifera scutellata* drones collected at a single DCA over a period of 3 years. The 12 loci employed for genotyping are shown in the first line. The collection date of each sample is given as Ma, No, Ap, Se and De, referring to March 2004, November 2004, April 2007, September 2007 and December 2007, respectively.

Table S3 Mean monthly rainfall and temperature and associated number of collected drones, detected and expected queens found in five samples of *Apis mellifera scutellata* drones collected at a single DCA over a period of 3 years. Rainfall and temperature scores are given as the average values of daily records taken continuously from 2004 to 2007. Climate data from the Witbank meteorological station (30 km from the Ezemvelo Nature Reserve) was provided by the South African Weather Service.

Table S4 Analysis of molecular variance (AMOVA) showing the amount of genetic variance found among time samples, among linkage groups within time samples and within the linkage groups of each time sample. The *P* values show the significance level of each variance component after 1023 permutations.

Table S5 Genetic and temporal distance matrix. Nei's genetic distance (D_A) is shown in the lower portion of the table, while the time in months separating each sample is shown in the upper portion.