Observation by microsatellite DNA analysis of sperm usage in naturally mated honeybee queens (*Apis mellifera ligustica*) over a period of two years

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ABSTRACT

The aim of this study was to observe sperm utilization in *Apis mellifera ligustica* naturally mated honeybee queens over a two year period. The study was conducted by microsatellite analysis of the progenies of 6 honeybee queens sampled during two consecutive egg-laying seasons (from June to October in the first year and from March to June in the second year). Four microsatellite markers were initially analysed. However, the most variable locus A76 alone was sufficient to distinguish the genotype of the queens and the patrilines of the six colonies. The effective number of matings based on the frequencies of the patrilines and corrected for the sample size, was computed and was 8.10, slightly lower than the number found by other authors. No significant differences were noted in the observed frequencies of the patrilines in the two reproductive seasons. Moreover, there was a similar distribution of the patrilines in the first month (June 2002) with respect to the last month (June 2003). These results indicated that there was a temporal conservation of the genetic structure of the colonies.

Key words: Sperm, Honeybee, Microsatellite, Patriline, *Apis mellifera ligustica*.

RIASSUNTO

UTILIZZO DELLO SPERMA IN API REGINE (*APIS MELLIFERA LIGUSTICA*) A FECONDAZIONE NATURALE: INDAGINE BIENNALE MEDIANTE ANALISI DI MARCATORI MICROSATELLITI

Lo scopo di questo studio è stato quello di analizzare, nel corso di un biennio, l’utilizzo dello sperma in api regine (*Apis mellifera ligustica*) feconde naturalmente. Lo studio è stato condotto tramite l’analisi del DNA microsatellite delle progenie di 6 api regine, campionate durante due stagioni riproduttive consecutive (da Giugno ad Ottobre nel primo anno e da Marzo a Giugno nel secondo). Tra i loci microsatellitari esaminati (A7, A24, A76, B124) il locus altamente variabile A76 è stato ritenuto sufficiente per discriminare
il genotipo delle regine e della maggior parte delle linee paterne delle 6 colonie. Utilizzando il numero di linee paterne le frequenze di accoppiamento effettive (numero di fuchi con cui la regina si è accoppiata), il cui valore medio è risultato essere di 8,10 fuchi, era leggermente più basso di quello osservato da altri autori. Non sono state individuate differenze significative tra le frequenze delle linee paterne osservate nelle due stagioni riproduttive, e la simile distribuzione di tali linee nel primo mese (Giugno 2002) e nell’ultimo (Giugno 2003) confermano la conservazione temporale della struttura genetica della colonia.

Parole chiave: Sperma, Api, Microsatelliti, Linee paterne, Apis mellifera ligustica.

Introduction

Understanding natural sperm storage mechanisms and sperm stratification in honeybees (Apis mellifera L.) is of applied importance because the knowledge of sperm utilization and sperm competition could help breeding programs and efforts to store and maintain genetically diverse lines (Collins, 2000). The creation of colonies with multiple paternal lines of workers or subfamilies is evolutionarily desirable as these colonies have the capability of responding readily to broad changes in the environment (Jones et al., 2004). Extreme polyandry increases the fitness of the queen by reducing the colony-level impact of her laying non-viable, diploid drone eggs (Tarpy and Page, 2001). Many authors have reported that high genetic variance within the colony results in increased colony fitness (Schmid-Hempel, 1994; Page et al., 1995; Fuchs and Moritz, 1998; Tarpy and Page, 2001; Mattila and Seeley, 2007).

More recently Mattila et al. (2008) have seen that extreme polyandry by honeybee queens enhances the production of worker-worker communication signals that facilitate the swift discovery and exploitation of food resources.

In Apis mellifera extreme polyandry occurs as the queen can copulate with many drones in quick succession, at least 7 according to Estoup et al. (1994), and the whole mating process takes less than 5 seconds (Koeniger and Koeniger, 1991). At the end of the mating flight, the honeybee queen returns to the hive with an average of 80-90 million spermatozoa in the lateral oviducts (Koeniger, 1986). Motility of the sperm and contraction of the longitudinal muscles in the walls of the spermatheca duct cause a vacuum pump effect that results in about 5 million spermatozoa reaching the spermatheca (Page, 1986; Koeniger and Ruttner, 1989). The remainder of the semen is expelled within the following 24 hours. The sperm in the spermatheca is quiescent for the entire life of the queen and it survives at ambient temperatures for years and even decades (Taber and Blum, 1960; Boomsma et al., 2005).

The sequence in which sperm from different males is used for fertilization has been of great interest after Hamilton (1964) recognized that multiple mating of the queen reduces the average genetic relationship between colony members, introducing the concept of inclusive fitness and kin selection. Haploid males only contribute a single set of genes to their daughters (Crozier and Pamilo, 1996) that have high relatedness (r=0.75). To rescue the kin selection Taber (1955), Parker (1970), Orlove (1975), Trivers and Hare (1976), Charnov (1978), and Kerr et al. (1980), assumed sperm clumping of the spermatozoa of individual males in polyandrous species, thereby theoretically restoring the single mating situation. Several studies, however, including Alber et al. (1955), Crozier and Brückner (1981), Page and Metcalf (1982), and Laidlaw and Page (1984) demonstrated that the sperm of the
drones that mated with the queen is mixed within the spermatheca, and Moritz (1983, 1986) and Estoup et al. (1994) found no statistically significant fluctuations in sperm usage. Furthermore, Page et al. (1984) by histological sectioning studied the migration of the semen and concluded that spermatozoa readily diffuse throughout the available space of the spermatheca and found no sperm aggregation of any kind.

Haberl and Tautz (1998) demonstrated that the sperm is mixed completely inside the queen’s spermatheca and that the queen uses the spermatozoa from all her mates while Franck et al. (1999) demonstrated that the sperm mixing is incomplete at the beginning of the egg-laying period and increases progressively over time.

Schlüns et al. (2004) demonstrated non-random sperm utilization in artificially inseminated queens: the volume of semen from each drone was significant in determining the frequency of the resulting patriline, while the insemination sequence was not.

Moritz (1986) showed a weak last male advantage for multiply inseminated queens, as the last semen with which a queen was inseminated had a higher frequency in the offspring than the first semen used in the insemination, but further research by Franck et al. (2002) did not confirm this phenomenon.

The determination of the patrilines in a colony can be obtained by using highly variable DNA markers. As the workers of the same colony share the same alleles inherited from the queen it is possible, by analysing worker bees, to identify the alleles coming from the fathers. Thus, through analysis of microsatellite loci in many workers of the same colony it is possible to determine the genotype of the queen and of the different drones she mated with (Estoup et al., 1995).

The aim of this study was to observe in Apis mellifera ligustica naturally mated queens the utilization of the semen in the spermatheca over two egg-laying seasons by genotyping the progeny of 6 naturally mated honeybee queens with four variable microsatellites.

Material and methods

Honey bee samples
Pupae of worker honeybees (Apis mellifera ligustica) were collected monthly from hives headed by naturally mated queens belonging to professional queen breeders from the Central and Northern Italy, during the egg-laying seasons of the years 2002 and 2003. The queens mated in April 2002. For the present study we chose 6 hives (identified as 12, 18, 22, 27, 42 and 68) in which we were certain of the presence of the same egg laying queen throughout both years (June-October 2002 and March-June 2003). A total number of 1080 honeybee worker pupae were collected, stored in 80% ethanol and genotyped. The samples included about 20 workers per queen per month, which were collected during the egg-laying period of the queens in two consecutive years with a 4-month interruption (November-February) during the winter. Approximately 150 worker pupae from each of the six queens were analysed (minimum 143 and maximum 172).

DNA extraction
DNA was extracted using the PrepMan Ultra protocol (Applied Biosystems, Foster City, CA, USA) where 50 µl of PrepMan Ultra reagent was put in a 0.2 ml tube for each leg and incubated for 24 min at 100°C. The lysate was removed and placed in a fresh tube and soon after a nucleic acid precipitation was used to remove PCR inhibitors, putting first TE buffer and then sodium acetate 3M and isopropanol. The samples stood at room temperature for at least 15 minutes and then were centrifuged at 13000 x g for
10 minutes. The supernatant was removed and the pellets of the samples were air-dried and suspended in 30 µl of water.

**PCR method**

DNA was amplified using a set of four microsatellite loci (A7, A24, A76 and B124; Estoup *et al.* 1995; Châline *et al.* 2002). The 1080 honeybee worker pupae were analysed using all four microsatellites. The reactions were carried out in 50 µl of a mixture containing 100 ng of DNA template, 10 µM of each primer, 2.5 µl of 10X AccuPrime™ PCR Buffer I, 2.5 µl of 10X AccuPrime™ PCR Buffer II (containing 15mM MgCl₂ and 2mM of each Dntp; Invitrogen, Carlsbad, CA, USA) and 0.7 µl AccuPrime™ Taq DNA polymerase (Invitrogen). The primers were labelled with fluorescent dye markers FAM.

Samples were amplified on an Applied Biosystems 9700 Thermocycler with a denaturing step of 2 min at 94°C followed by 25 cycles of 30s at 94°C, annealing for 30s at 55°C and extension of 1 min at 68°C.

The fragment lengths were analysed with the internal size marker Genescan ROX 400 HD size standard (Applied Biosystems) using an Applied Biosystems ABI 3100 DNA Sequencer and scored with Gene Mapper Software (Version 2.0; Applied Biosystems) to obtain allele size (category).

**Identification of queen genotype**

The genotypes of the queens were easily identified when the worker pupae were homozygous (daughter’s allele is the same as its mother’s), whereas when the worker pupae were not homozygous if two alleles were always present together or alternately (each in half of workers) it was deduced that they were the queen’s genotype. For example, if there are daughters each with genotype 264/293, 344/364, 293/330, 282/344 or 293/344 it is possible to deduce that the queen’s genotype is 293/344.

The paternal genotypes were deduced from the workers’ genotype by subtraction of the queen genotype. Paternal alleles were considered to be those not carried by the queen. When a worker had both queen alleles the determination of father’s allele was not possible and these workers were not considered.

**Genotyping and estimates of mating frequencies**

To correct for not detecting a drone because of the finite sample size of the worker offspring analysed per queen (non-sampling error) we computed the estimated number of matings according to Cornuet and Aries (1980):

\[ o = k - k(1 - 1/k)^n \]

where \( n \) is the sample size of the workers, \( o \) is the number of observed matings and \( k \) is the number of estimated matings.

The effective number of matings \( m_e \) based on the frequencies of the patrilines and corrected for the sample size, was computed according to Pamilo (1993) and Boomsma and Ratnieks (1996):

\[ m_e = \frac{(n-1)}{(n \Sigma p_i^2 - 1)} \]

where \( n \) is the sample size, \( l \) is the number of siring drones and \( p_i \) is the proportion of workers sired by the \( i \)-th drone.

The effective number of matings of a queen, which is usually lower than the observed number of matings, is the mating number if all drones are represented equally within her offspring.

**Statistical analysis**

To calculate the expected heterozygosity
the following formula was used:

\[ H_e = 1 - \sum_{i=1}^{k} p_i^2 \]

where: \( k \) is the number of alleles and \( p_i \) is the frequency of the i-th allele.

As a first approach Chi-square analysis was performed to verify the influence of time of sampling (month and/or year) and hives on the alleles (drones). The data were then further analysed using a log-linear test with SPSS procedure (Norusis, 1992). The variables considered were: months in the first year (MONTHS1), months in the second year (MONTHS2) and years (YEAR).

The allele frequencies in different hives were tested using the following log linear model:

\[ \ln(F^\^ {ij}) = \mu + \lambda_i^A + \lambda_j^B + \lambda_{ij}^{AB} \]

where:
- \( \ln(F^\^ {ij}) \) = log of expected cell frequency in i-th row and j-th column;
- \( \mu \) = overall mean of the natural log of the expected frequency;
- \( \lambda_i^A \) = effect of i-th ALLELE category (i=1-4);
- \( \lambda_j^B \) = effect of j-th MONTHS1/MONTHS2/YEAR category (j=1-5 for MONTHS1; j=1-4 for MONTHS2; j=1-2 for YEAR);
- \( \lambda_{ij}^{AB} \) = interaction effect.

The hypothesis that the \( k \)th-order effect was null was tested using Hierarchical Log-linear Analysis approach by likelihood ratio Chi-square (\( L^2 \)) statistics

\[ L^2 = 2 \Sigma_i \Sigma_j F_{ij} \ln \frac{F_{ij}}{F^\^ {ij}} \]

A log-linear SPSS procedure was used.

The \( \lambda \) coefficients, with \( P \) value computed using the Z-value=\( \lambda / SE(\lambda) \), were used to explain the behaviour of variables.

**Results and discussion**

The patriline information obtained from each locus (A7, A24, A76, B124) was used to calculate marker heterozygosity, which was 0.87, 0.66, 0.92 and 0.75, respectively (Table 1).

For microsatellites A24 and B124 the number of utilizable data was considerably reduced (368/1080, 335/1080) because many workers had the same allele combination as the queen and the polymorphism was low.

Using only the highly polymorphic microsatellite A76 (where the heterozygosity changed from a minimum of 0.49 to a maximum of 0.87) we could assign 944/1080 subjects and we could have a monthly view of the stratification of the semen in the spermatheca. So we used only one locus, supported by the fact that also Franck et al. (1999) investigated the patriline frequencies using only one microsatellite.

The locus A76 is already known to be highly variable. Indeed, in a study by Estoup et al. (1994) aimed at assessing the precise number of patrilines, it was observed that among the 10 microsatellite loci which were considered, A76 alone was sufficient to distinguish 12 patrilines.

The results of genotyping using microsatellite A76 only are summarized in Table 2 which shows the minimum number of drones identified in each hive.

In Table 3, we reported the observed mating frequency of each queen obtained by the number of detected patrilines and we included information about the estimated and the effective matings. Almost the same values were obtained for the observed and estimated matings thereby supporting the assumption that all drones contributed equally. The effective matings (8.10) were lower than the observed number (11). This result is lower than effective matings reported by Cornuet et al. (1986), Estoup et al. (1994), Neumann and Moritz (2000), Palmer and Oldroyd (2000), Jensen et al. (2005) and Schlüns et al. (2005) who obtained values of 12.4, 10.15, 23.95, 12.4, 10.2, and 11.8, respectively. This could possibly be due to the fact that the queens used in
this experiment belonged to professional ligustica queen breeders, who over the years have selected only a few individuals as breeding stock, thereby reducing genetic variation (Dall’Olio et al., 2007). In this study it was possible to have a complete overview for each colony of the distribution over time (2 years) of the patrilines, as shown in Tables 4 and 5. The Chi-square test revealed that there is no effect of month or year (P>0.05) on patriline frequency (ALLELE) and that in all six families drones differed significantly in their proportion of offspring (P<0.001).

The data analysis by Log-linear model also found no significant $L^2$ values in colony patrilines (ALLELE) neither in the years nor in the months; therefore, it can be concluded that the allele frequency is independent from both MONTH and YEAR. This means that no significant differences in subfamily proportions were detected between months or between years (Table 6).

Several hypotheses have been proposed to explain how the benefits of a genetically diverse work force could outweigh the
Table 2. Genotypes of the queens and deduced drones obtained from their progeny at the microsatellite locus A76 used for the analysis (microsatellite alleles are given in bp).

|   | Hive 12                  |   | Hive 18                  |   | Hive 22                  |
|---|--------------------------|---|--------------------------|---|--------------------------|
|   | Subjects | Alleles    |   | Subjects | Alleles    |   | Subjects | Alleles    |
| Queen | 256, 320 |   |   | Queen  | 308, 332 |   | Queen | 274, 354 |
| Drone 1 | 250 |   | Drone 1 | 250 |   | Drone 1 | 250 |
| Drone 2 | 256 |   | Drone 2 | 256 |   | Drone 2 | 266 |
| Drone 3 | 266 |   | Drone 3 | 278 |   | Drone 3 | 274 |
| Drone 4 | 274 |   | Drone 4 | 296 |   | Drone 4 | 278 |
| Drone 5 | 278 |   | Drone 5 | 304 |   | Drone 5 | 296 |
| Drone 6 | 296 |   | Drone 6 | 308 |   | Drone 6 | 304 |
| Drone 7 | 308 |   | Drone 7 | 314 |   | Drone 7 | 308 |
| Drone 8 | 314 |   | Drone 8 | 320 |   | Drone 8 | 320 |
| Drone 9 | 320 |   | Drone 9 | 332 |   | Drone 9 | 332 |
| Drone 10 | 332 |   | Drone 10 | 336 |   | Drone 10 | 354 |
| Drone 11 | 364 |   | Drone 11 | 354 |   | Drone 11 | 370 |
|   |   |   | Drone 12 | 364 |   |   |   |

|   | Hive 27                  |   | Hive 42                  |   | Hive 68                  |
|---|--------------------------|---|--------------------------|---|--------------------------|
|   | Subjects | Alleles    |   | Subjects | Alleles    |   | Subjects | Alleles    |
| Queen | 278, 292 |   |   | Queen  | 292, 344 |   | Queen | 256, 320 |
| Drone 1 | 256 |   | Drone 1 | 250 |   | Drone 1 | 250 |
| Drone 2 | 274 |   | Drone 2 | 266 |   | Drone 2 | 256 |
| Drone 3 | 278 |   | Drone 3 | 274 |   | Drone 3 | 274 |
| Drone 4 | 284 |   | Drone 4 | 284 |   | Drone 4 | 278 |
| Drone 5 | 292 |   | Drone 5 | 308 |   | Drone 5 | 284 |
| Drone 6 | 296 |   | Drone 6 | 320 |   | Drone 6 | 296 |
| Drone 7 | 304 |   | Drone 7 | 332 |   | Drone 7 | 304 |
| Drone 8 | 314 |   | Drone 8 | 364 |   | Drone 8 | 308 |
| Drone 9 | 332 |   | Drone 9 | 314 |   | Drone 9 | 314 |
| Drone 10 | 354 |   | Drone 10 | 320 |   | Drone 10 | 320 |
| Drone 11 | 364 |   | Drone 11 | 332 |   | Drone 11 | 332 |
| Drone 12 | 380 |   | Drone 12 | 354 |   |   |   |
It has in fact been shown that by increasing the number of loci the precision of the analysis improves, although the size of the sample is more important than the number of loci (Kraus et al., 2005). However, the main objective of this study was to monitor the *Apis mellifera ligustica* allele frequency variation among subfamily over a long time period, in order to i) evaluate if there was a dominance effect of the genotype of some drones in the queen’s offspring and ii) if there was any kind of stratification of the spermatozoa belonging to the different drones inside the spermatheca.

Other authors have investigated the same subject: Haberl and Tautz (1998), analysing a single naturally mated queen, found that semen is completely mixed in the spermatheca and that sperm usage is random in the short term, although they don’t exclude variations in subfamily frequencies in the long term; Franck et al. (1999) investigated sperm usage in a single instrumentally inseminated queen and observed that variance of subfamily proportions, and therefore sperm admixture in the spermatheca, increased during the first few months after insemination; this result was confirmed by a later study on naturally mated queens (Franck et al., 2002); Schlüns et al. (2004) found, by analysing instrumentally inseminated queens, that the amount of semen of each drone in the spermatheca has a significant effect on subfamily frequency. Our study is the first one to address sperm usage in *Apis mellifera ligustica* queens, and one of the few that considered naturally mated rather than instrumentally inseminated queens. Only Franck et al. (2002) had previously carried out observations on naturally mated queens covering two egg-laying seasons, but the considered subspecies was *A. m. carnica*.

Although the number of workers in each

### Table 3. Mating frequencies of each hive.

| Hive  | Observed matings | Estimated matings | Effective matings |
|-------|------------------|-------------------|-------------------|
| 12    | 11               | 10.99             | 8.26              |
| 18    | 12               | 11.99             | 10.22             |
| 22    | 11               | 10.99             | 6.67              |
| 27    | 12               | 11.99             | 7.77              |
| 42    | 8                | 8                 | 6.94              |
| 68    | 12               | 12                | 8.78              |

The effective mating frequencies determined in this study (6.67-10.22) are surely under-estimated due to use of a single locus.
Table 4. Patriliné frequencies for each year (1, 2) and hive.

| allele | 1 (n=79) | 2 (n=64) | 1 (n=91) | 2 (n=75) | 1 (n=91) | 2 (n=56) | 1 (n=91) | 2 (n=56) | 1 (n=92) | 2 (n=75) | 1 (n=96) | 2 (n=79) |
|--------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| 250    | 0.14     | 0.16     | 0.12     | 0.20     | 0.21     | 0.15     | 0.11     | 0.07     | 0.06     | 0.09     |          |          |
| 256    | 0.05     | 0.05     | 0.04     | 0.05     |          | 0.01     | 0.08     | 0.04     | 0.08     | 0.01     |          |          |
| 266    | 0.14     | 0.09     |          | 0.11     | 0.09     |          | 0.14     | 0.17     |          |          |          |          |
| 274    | 0.05     | 0.05     |          | 0.01     | 0.04     | 0.02     |          |          | 0.02     | 0.10     |          |          |
| 278    | 0.15     | 0.13     | 0.09     | 0.04     | 0.04     | 0.04     | 0.04     | 0.07     | 0.29     | 0.18     |          |          |
| 284    |          |          |          |          |          | 0.08     | 0.18     | 0.20     | 0.16     | 0.05     |          |          |
| 292    |          |          |          |          |          | 0.01     | 0.02     |          |          |          |          |          |
| 296    | 0.14     | 0.11     | 0.10     | 0.12     | 0.03     | 0.02     | 0.13     | 0.2      | 0.09     | 0.14     |          |          |
| 304    |          | 0.05     | 0.13     | 0.19     | 0.13     | 0.09     | 0.05     |          | 0.03     | 0.04     |          |          |
| 308    | 0.08     |          | 0.01     | 0.27     | 0.22     |          | 0.07     | 0.03     | 0.05     | 0.13     |          |          |
| 314    | 0.03     | 0.26     | 0.03     |          |          | 0.08     | 0.02     |          | 0.02     | 0.04     |          |          |
| 320    | 0.18     | 0.23     | 0.09     | 0.19     | 0.03     | 0.16     |          | 0.18     | 0.13     | 0.04     |          |          |
| 332    | 0.09     | 0.06     | 0.01     | 0.01     | 0.08     | 0.13     | 0.21     | 0.25     | 0.16     | 0.27     | 0.09     | 0.12     |
| 336    |          |          |          |          | 0.11     | 0.08     |          |          |          |          |          |          |
| 354    | 0.07     | 0.07     | 0.01     | 0.02     | 0.10     | 0.11     |          |          | 0.05     | 0.09     |          |          |
| 364    | 0.04     | 0.05     | 0.05     | 0.07     |          | 0.05     | 0.11     | 0.07     | 0.09     |          |          |          |
| 370    |          |          | 0.01     | 0.02     |          |          |          |          |          |          |          |          |
| 380    |          |          |          |          |          | 0.18     | 0.14     |          |          |          |          |          |

The values are approximated by defect or by excess at two decimal numbers and $\chi^2$ is the Chi-square statistics for each hive.
Table 5. Patriline frequencies for each sampling time point (1-9), where 1=June, 2=July, 3=August, 4=September, 5=October (year 2002), 6=March, 7=April, 8=May, 9=June (year 2003).

|       | Hive 12 n=143 |       | Hive 18 n=166 |       | Hive 22 n=146 |
|-------|---------------|-------|---------------|-------|---------------|
|       | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  |
| all   | n=18 | n=19 | n=18 | n=16 | n=18 | n=17 | n=13 | n=18 | n=19 | n=18 | n=17 | n=20 | n=18 | n=17 | n=20 | n=18 | n=17 | n=20 | n=18 | n=17 | n=17 | n=0 |
| 250   | 0.22 | 0.11 | 0.31 | 0.19 | 0.24 | 0.23 | 0.21 | 0.17 | 0.16 | 0.06 | 0.1  | 0.35 | 0.11 | 0.24 | 0.16 | 0.26 | 0.18 | 0.5  | 0.15 | 0.29 |
| 256   | 0.11 | 0.05 | 0.06 | 0.06 | 0.06 | 0.06 | 0.1  | 0.05 | 0.06 | 0.1  | 0.06 | 0.11 | 0.24 | 0.05 | 0.06 | 0.06 | 0.06 | 0.1  | 0.12 |
| 266   | 0.11 | 0.16 | 0.25 | 0.17 | 0.06 | 0.06 | 0.11 | 0.12 | 0.08 | 0.11 | 0.11 | 0.24 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 |
| 274   | 0.06 | 0.05 | 0.06 | 0.06 | 0.12 | 0.08 | 0.05 | 0.24 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 |
| 278   | 0.11 | 0.05 | 0.22 | 0.31 | 0.31 | 0.11 | 0.06 | 0.17 | 0.06 | 0.24 | 0.05 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 |
| 284   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 292   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 296   | 0.22 | 0.05 | 0.13 | 0.22 | 0.06 | 0.13 | 0.17 | 0.06 | 0.08 | 0.06 | 0.05 | 0.11 | 0.29 | 0.15 | 0.1  | 0.11 | 0.12 | 0.05 | 0.05 | 0.06 | 0.06 |
| 304   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 308   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 314   | 0.05 | 0.06 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 320   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 332   | 0.16 | 0.13 | 0.11 | 0.06 | 0.06 | 0.18 | 0.05 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 336   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 354   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 364   | 0.11 | 0.06 | 0.06 | 0.06 | 0.06 | 0.17 | 0.11 | 0.1  | 0.05 | 0.12 | 0.05 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 |
| 370   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 380   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

continued >>
Table 5. >> Continuation

|       | Hive 27 n=147 |       | Hive 42 n=167 |       | Hive 68 n=172 |
|-------|---------------|-------|---------------|-------|---------------|
| all   |               |       |               |       |               |
| 1     |               |       |               |       |               |
| 2     |               |       |               |       |               |
| 3     |               |       |               |       |               |
| 4     |               |       |               |       |               |
| 5     |               |       |               |       |               |
| 6     |               |       |               |       |               |
| 7     |               |       |               |       |               |
| 8     |               |       |               |       |               |
| 9     |               |       |               |       |               |
| 1     | 0.15          | 0.1  | 0.15          | 0.06  | 0.11          | 0.06  | 0.05          | 0.05  | 0.15          | 0.05  | 0.11          | 0.20  | 0.16          |
| 2     | 0.15          | 0.07 | 0.15          | 0.06  | 0.11          | 0.06  | 0.05          | 0.05  | 0.15          | 0.05  | 0.11          | 0.22  | 0.05          |
| 3     | 0.35          | 0.1  | 0.13          | 0.1   | 0.26          | 0.18  | 0.05          | 0.21  |               |       |               |       |               |
| 4     |               |       |               |       |               |       |               |       |               |       |               |       |               |
| 5     |               |       |               |       |               |       |               |       |               |       |               |       |               |
| 6     |               |       |               |       |               |       |               |       |               |       |               |       |               |
| 7     |               |       |               |       |               |       |               |       |               |       |               |       |               |
| 8     |               |       |               |       |               |       |               |       |               |       |               |       |               |
| 9     |               |       |               |       |               |       |               |       |               |       |               |       |               |

The values are approximated by defect or by excess at two decimal numbers.
monthly sample was not large enough to singularly estimate the mating frequency, the average total number of $156.8\pm12.83$ genotyped workers per queen was adequate to provide sufficient information on patriline frequency variation and on the effective mating frequency of the queens. On the whole, our data confirms previous research, the only difference being that we never observed any change in the variation of subfamily proportions, which was observed by some authors (Franck et al., 1999, 2002) to be greater in the initial stages of the queen’s reproductive life. In our case, this could be explained by the fact that sampling was started 3 months after the mating had taken place, and therefore semen admixture in the spermatheca had already taken place.

### Conclusions

The independent segregation of the allelic frequencies (patrilines) within each hive in each month shows that sperm admixture in the spermatheca occurs, thereby confirming results previously found by several authors (Alber et al., 1955; Crozier and Brückner, 1981; Page and Metcalf, 1982; Moritz, 1983, 1986; Laidlaw and Page, 1984; Page et al., 1984; Estoup et al., 1994; Haberl and Tautz, 1998; Franck et al., 2002). The lack of significant differences in the frequency of the patrilines between the two reproductive seasons, and the similar distribution of the patrilines in the first month (June 2002) with respect to the last month (June 2003) shows that there is a temporal conservation of the genetic structure of the colony.

### Table 6. Results of Log-linear model for the terms in the model.

| Hive | Month (year 1) | Month (year 2) | Year |
|------|----------------|----------------|------|
| 12   | ALLELE         | ALLELE         | ALLELE | $L^2=14.884$ | $DF=18$ | $P=0.670$ |
|      |                |                |       | $L^2=46.520$ | $DF=54$ | $P=0.755$ |
|      |                |                |       | $L^2=14.884$ | $DF=18$ | $P=0.670$ |
| 18   | ALLELE         | ALLELE         | ALLELE*YEAR | $L^2=14.884$ | $DF=18$ | $P=0.670$ |
|      |                |                |       | $L^2=37.864$ | $DF=54$ | $P=0.953$ |
| 22   | ALLELE         | ALLELE         | ALLELE*YEAR | $L^2=73.42879$ | $DF=72$ | $P=0.431$ |
|      |                |                |       | $L^2=29.392$ | $DF=36$ | $P=0.774$ |
|      |                |                |       | $L^2=11.925$ | $DF=17$ | $P=0.805$ |
|      |                |                |       | $CS=68.002$  | $DF=72$ | $P=0.953$ |
|      |                |                |       | $CS=22.847$  | $DF=36$ | $P=0.957$ |
|      |                |                |       | $CS=12.218$  | $DF=17$ | $P=0.787$ |
| 27   | ALLELE         | ALLELE         | ALLELE | $L^2=64.752$ | $DF=72$ | $P=0.715$ |
|      |                |                |       | $L^2=32.002$ | $DF=36$ | $P=0.659$ |
|      |                |                |       | $L^2=12.430$ | $DF=17$ | $P=0.773$ |
| 42   | ALLELE         | ALLELE         | ALLELE | $L^2=40.462$ | $DF=72$ | $P=0.999$ |
|      |                |                |       | $L^2=23.255$ | $DF=54$ | $P=1.000$ |
|      |                |                |       | $L^2=12.050$ | $DF=18$ | $P=0.845$ |
| 68   | ALLELE         | ALLELE         | ALLELE*YEAR | $L^2=68.991$ | $DF=72$ | $P=0.579$ |
|      |                |                |       | $L^2=49.228$ | $DF=54$ | $P=0.659$ |

The allele frequencies in different hives are tested using a log linear model considering like variability factors: first-year months, second-year months and years. The probability (P) of likelihood ratio L2 is computed by Chi-square statistics (ALLELE*YEAR is the interaction between allele and year).
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