

Levels of polyandry and intracolonial genetic relationships in *Apis koschevnikovi*

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(Received 12 February 1998, accepted subject to revision 24 April 1998, accepted for publication 15 June 1998)

SUMMARY

Adults of five *Apis koschevnikovi* colonies from Sabah, Malaysia (Borneo) were collected into alcohol and within two days, transferred to liquid nitrogen. DNA was extracted, amplified with four, or in one case six, microsatellite primer pairs or primers for a sequence tagged site, and genotypes for these loci were determined for 40 to 100 workers per colony. Four colonies were monogynous, one colony had worker daughters of two, probably highly related, queens. For the monogynous colonies, the queen genotypes and the number and genotypes of males mated by each queen was inferred from the worker genotypes. The queens mated with 10 to 32 drones. The average number of observed matings was 16.3 ± 10.5 and the average number of effective matings was 10.5 ± 8.4. The within-colony genetic relatedness was 0.31 ± 0.03. The fitness advantages of genetic variance are discussed.

Keywords: genetic variation, polyandry, microsatellite primers, DNA, multiple mating, relatedness, *Apis koschevnikovi*, polymerase chain reaction, Borneo, Malaysia

INTRODUCTION

The purpose of this study was to examine the degree of polyandry (multiple mating) and the resulting intracolonial genetic relatedness in colonies of a population of *Apis koschevnikovi*. Understanding the biological consequences of polyandry in natural populations can lead to a better understanding of the evolution of social behaviour in the genus *Apis* and provide guidance regarding functional polyandry for apicultural pursuits. Prior to the advent of *Varroa jacobsoni* in populations of *A. mellifera*, open-mated queens probably had an enriched diversity of drones as mates as a result of wild or feral colonies. Now that wild and feral colonies are rare, the question of assuring drone sources sufficient to the function of polyandry becomes important.

The evolution of eusociality in insects was probably founded on the high genetic relatedness in the offspring of single mating of females to haploid males (Hamilton, 1964; Pamilo, 1991). However, some contemporary eusocial Hymenoptera have levels of genetic relatedness only slightly higher than that of half-siblings as a result of polyandry or multiple queens (polygyny) (Page & Metcalf, 1982, Keller & Reeve, 1994). Presumably, once sociality was established, multiple mating produced substantial fitness advantages for social colonies. Five main hypotheses concerning the nature of these advantages have been offered (Crozier & Page, 1985; Keller & Reeve, 1994; Moritz, 1985):

- Increased genetic variance allows for increased caste polymorphism (Crozier & Page, 1985).
- Genetic variance increases the range of environments the colony can tolerate (Crozier & Brückner 1981; Oldroyd *et al.*, 1992) or the range of diversity of a single environment that can be effectively exploited by a colony (Oldroyd *et al.*, 1993).
- Polyandry reduces the variance in the production of diploid males among colonies making all colonies equally fit by not producing large numbers of diploid drones (Page, 1980; Ratnieks, 1990).
- Genetic variance increases colony resistance to parasites and pathogens (Sherman *et al.*, 1988; Shykoff & Schmidt-Hempel, 1991a, 1991b).
- Polyandry favours the sex ratio bias of the queen and thus decreases intracolonial conflict over the investment in reproductives (Mortiz, 1985)

Understanding the function of polyandry and evaluating these hypotheses requires good estimates of the number of matings from several species of honey bees. Comparing varied life histories and levels of polyandry will provide a way to evaluate the relative strength of the alternative hypotheses. It is especially important to include *A. koschevnikovi* in these studies, since it is a cavity-dwelling honey bee that is free of apicultural manipulations. *A. mellifera* and *A. cerana* have a long history of domestication. Also, keeping colonies in apiaries may bias the outcome of polyandry determinations.

MATERIALS AND METHODS

Samples of adult worker bees were collected from five colonies of *Apis koschevnikovi* at the Agricultural Research Station Tenom at Sabah, Malaysia in northern Borneo. These colonies were caught as wild swarms from throughout the research station. The only manipulations that they received was to be placed into hives and fed in order to prevent absconding. No queen rearing or other activity was practised that could have altered them genetically. Thus, they were representatives of the area's wild population of *A. koschevnikovi*.

The samples were collected into cold ethanol (80%) and stored in a freezer for two days. The alcohol was drained from them, and they were placed into liquid nitrogen for shipment to the USA where they were placed in an ultra-low freezer (–86°C).

Template DNA was extracted from individual workers in a 10% Chelex solution. Gasters were removed from bees before they were ground in 400 µl of solution. The mixture was vortexed for 10 s, incubated at 56°C for 30 min, vortexed for 10 s, boiled in a water bath for 5 min, vortexed for 10 s and centrifuged for 3 min at 12 000 rpm to remove fragments from the solution. Template DNA was then stored at –20°C until used.

Template DNA was prepared for polymerase chain reaction (PCR) using up to six primer pairs depending upon the colony. Individual reactions were conducted for each bee-primer pair combination. Five primer pairs were selected which are known to amplify microsatellite sequences in *A. mellifera* (Estoup *et al.*, 1994; Oldroyd *et al.* 1997a; Rowe *et al.*, 1997). One primer pair was selected which is known to amplify a polymorphic DNA sequence in *A. mellifera* (Hunt & Page, 1994). One primer of each pair was end-labeled with CY5 for visualization using an ALF automated sequencing machine following the manufacturer's guidelines.

PCRs were carried out in 5 µl volumes with 0.3 µl of template DNA solution, 164 µM dNTP, 0.8 µM of each of the forward and reverse primers, 1.5 mM $MgCl_2$, 100 ng BSA and 0.2 units of Taq polymerase (Promega). Initial denaturation was done at 94°C for 3 min, and followed by a varying number of cycles (table 1) of denaturation at 94°C for 45 s, annealing at the primer specific temperature for 45 s (table 1), and extension at 72°C for 45 s. A final extension of 72°C for 10 min followed the last cycle.

PCR products were run on 5% acrylamide gels for 4–6 h depending on the expected size of the fragments. Sequenced M13 DNA was used as a standard. Additionally, two internal standards were run in each lane with the PCR product. These internal standards varied from 100 bp to 300 bp depending on the expected size of the PCR product.

Results were scored using the ALF automated sequencer software. Unknown product peaks were matched to the standard M13 sequence to determine base pair size. Accuracy was confirmed by comparing

TABLE 1. Polymerase chain reaction (PCR) conditions, primer sequences and references for seven microsatellite loci used to detect paternity in *Apis koschevnikovi*.

Locus	Primers	Annealing temperature (°C)	Number of cycles
A81	5'GCCGAGTTCTTCGACTCCC 5'GGACTTTGCCAAATGGGTC	55	35
A107	5'GCCGTGGGAGGTTTATTGTCG 5'CCTTCGTAACGGATGACACC	50	35
IC1	5'GGTTTGATGCTCGTAAGGG 5'GGCACCTCTTGCCATCTG	58	30
QI	5'AGTGCAGCCAGCTACTGAGAG 5'AGTGCAGCCACGTGCCTGAAT	55	35
A14	5'GTGTCGCAATCGACGTAACC 5'GTCGATTACCGATCGTGACG	58	30
A76	5'GCCAATACTCTCGAACAATG 5'GTCCAATTCACATGTCGACATC	58	30

Primer sequences are from Estoup *et al.*, 1994 (A107, A14, A76); Hunt & Page, 1994 (QI); Rowe *et al.*, 1997 (Ic1); and A Estoup, personal communication (A81).

the internal standards to the same M13 standard. The standard sequence was counted beginning at the termination of the primer site in order to provide absolute fragment size data.

Queen genotypes were inferred from worker genotypes. When an allele occurred in every worker, the queen was inferred to be homozygous for that allele. When every worker carried one of two alleles, the queen was inferred to be heterozygous for those two alleles (Estoup *et al.*, 1994). When all workers commonly carried one of three alleles in combination with other alleles, the colony was considered to have the progeny of more than one queen. Paternal alleles of each worker were determined by comparing the worker genotype to the inferred queen genotype. When a worker genotype was homozygous for a locus, the paternal allele was unambiguously that homozygous type. When a worker genotype was heterozygous and carried one allele not found in the queen, the paternal allele was unambiguously the allele not found in the queen. When a worker genotype was heterozygous with the same alleles as the queen, the paternal allele was uncertain, but was one of the two alleles in the heterozygous combination. Most workers were unambiguously assigned to a paternity group by their alleles at one or more loci. For some workers however, their paternity group was partially ambiguous and proportionally assigned to specific groups according to the method of Oldroyd *et al.* (1996).

The average coefficient of relatedness, *G*, is a weighted estimate which accommodates the relative frequency of each subfamily. *G* was calculated as:

$$\sum_{i=1}^{k} \{([0.75p_i] + [0.25(1-p_i)])p_i\}$$

(Laidlaw & Page, 1984) where p_i = the relative frequency of the i^{th} subfamily and k is the number of subfamilies.

The effective number of matings (m) accounts for the number and proportion of patrilines found in worker offspring (Chevalet & Cornuet, 1982; Oldroyd & Moran, 1983). It was calculated according to the method of Starr (1984) as:

$$1/\sum_{i=1}^{k} p_i^2$$

RESULTS

Four colonies were monogynous. For these monogynous colonies, the queen genotypes and the number and genotypes of males mated by each queen were inferred from the worker genotypes. The queens mated with at least 10 to 32 drones (table 2). The average number of observed matings was 16.3 ± 10.5; the average number of effective matings was 10.5 ± 8.4; the within-colony genetic relatedness was 0.31 ± 0.03 (table 3).

One colony had worker daughters of two, probably highly related, queens. This colony had groups of workers which were homozygous for three alleles of a single locus. The array of homozygous workers for various loci supported the interpretation that this colony

TABLE 2. Genotypes (PCR product length in base pairs) of queens and paternal drones in four colonies of *Apis koschevnikovi*.

Colony 1	**Microsatellite locus** A81	A107	IC1	QI		**Observed number of worker bees**
Queen allele 1	144	182	155	650		
Queen allele 2	146	184	159	660		
Drone 1	144	182	155	660		5
Drone 2	146	182	155/159	660		8
Drone 3	146	182	155/159	652		2
Drone 4	146	182	155/159	664		2
Drone 5	146	182/184	155/159	650		7
Drone 6	146	184	159	660		8
Drone 7	146	184	155/159	652		1
Drone 8	148	184	155/159	650/660		2
Drone 9	150	182	155/159	652		1
Drone 10	150	184	159	650/660		1
Drone 11	152	184	159	660		3
Total						40
Colony 2	**Microsatellite locus** A81	A107	IC1	QI		**Observed number of worker bees**
Queen allele 1	144	182	155	650		
Queen allele 2	146	184	159	660		
Drone 1	142	180	159	650/660		1
Drone 2	142	182	155/159	650/660		1
Drone 3	142	184	159	650		3
Drone 4	144	180	155/159	650		5
Drone 5	144	180	155/159	660		1
Drone 6	144	182	155/159	650		4
Drone 7	144	182	155/159	660		1
Drone 8	144/146	182	155/159	650		3
Drone 9	144/146	184	155	660		2
Drone 10	146	180	155/159	660		7
Drone 11	146	182	155	660		4
Drone 12	146	184	155/159	650		9
Total						41
Colony 3	**Microsatellite locus** A81	A107	IC1	QI	B7	**Observed number of worker bees**
Queen allele 1	144	182	155	650	163	
Queen allele 2	146	184	159	660	165	
Drone 1	144	180	155/159	650/660	157	5
Drone 2	144	182/184	155/159	652	157	1
Drone 3	144/146	180	155/159	650/660	161	1
Drone 4	144/146	180	155/159	652	163/165	1
Drone 5	144/146	182/184	155/159	652	161	1
Drone 6	144/146	184	159	660	157	1
Drone 7	146	182/184	155/159	652	163	5
Drone 8	146	184	155	650/660	157	6
Drone 9	146	184	155	660	161	9

TABLE 2. Continued

Colony 3	Microsatellite locus A81	A107	IC1	QI	B7	Observed number of worker bees
Drone 10	146	184	159	660	165	10
Total						40

Colony 4	Microsatellite locus A81	A107	IC1	QI	A14	A76	Observed number of worker bees
Queen allele 1	144	182	155	650	147	95	
Queen allele 2	146	184	159	660	147	103	
Drone 1	136	184	159	660	147	95/103	1
Drone 2	142	180	155	650	145	95/103	2
Drone 3	142	180	155	660	145	95	4
Drone 4	142	180	155	660	147	95/103	1
Drone 5	142	180	159	650	147	95/103	3
Drone 6	142	182	155/159	650/660	145	95	2
Drone 7	142	182	155/159	652	145	95/103	1
Drone 8	142	182/184	155	650	147	95/103	4
Drone 9	142	184	159	660	147	95/103	5
Drone 10	142	184	155	660	147	95/103	5
Drone 11	142	184	155/159	660	145	95/103	5
Drone 12	142	184	159	650	147	95/103	4
Drone 13	142	184	159	652	147	95/103	1
Drone 14	144	180	155/159	660	145	95/103	1
Drone 15	144	182	155/159	660	145	95/103	1
Drone 16	144	182	155/159	660	147	95/103	2
Drone 17	144	184	159	650	147	95/103	7
Drone 18	144	184	159	660	147	95/103	6
Drone 19	144	184	155/159	664	147	95/103	2
Drone 20	146	180	155/159	650	145	103	3
Drone 21	146	180	159	650	147	95/103	2
Drone 22	146	182	159	650	147	95/103	4
Drone 23	146	182	159	650/660	147	103	3
Drone 24	146	184	155	650	147	95/103	6
Drone 25	146	182/184	159	652	145	95/103	1
Drone 26	146	184	155	660	145	95/103	3
Drone 27	146	184	155/159	652	147	95/103	2
Drone 28	146	184	155/159	664	147	95/103	2
Drone 29	146	184	159	650	147	95/103	5
Drone 30	146	184	159	650	145	95/103	2
Drone 31	146	184	159	660	147	95/103	9
Drone 32	150	184	155/159	652	147	95/103	1
Total							100

TABLE 3. Observed (*k*) and effective (*m*) number of matings and average coefficient of relatedness (R_c) for four colonies of *Apis koschevnikovi*.

Colony	Number of matings observed	effective	Average relatedness
1	11	7.06	0.32
2	12	7.86	0.31
3	10	4.29	0.34
4	32	22.83	0.27
Average	16.3 ± 10.5	10.5 ± 8.4	0.31 ± 0.03

had worker offspring of two queens rather than a null allele carried by one queen because the multiple homozygosity occurred at more than one locus. Also, the various classes of homozygous workers for each locus were consistent with the interpretation that progeny of a mother queen and a daughter queen were present in the colony. Such colonies would arise from swarming or other forms of queen replacement. Since this colony was not suitable to provide clear information on the level of polyandry associated with one queen, no further information was collected from this colony.

DISCUSSION

These results clearly demonstrate that *A. koschevnikovi* mate with at least 10, and often with more drones. Several circumstances support the view that these estimates, with the possible exception of colony four, are underestimates. Queens in this wild population probably have fewer colonies producing drones within their mating area than queens of species that are found or are placed in groups of nests. This would restrict their mating to the drone progeny of a smaller number of queens. Queens that are heterozygous for all four loci, such as in colony 1 and colony 2 (table 2) only produce 16 classes of drones that are distinguishable using information from those loci. Coupled with this, the loci used in this investigation had small numbers of alleles in the *A. koschevnikovi* population we studied. The highest number of alleles found at any one locus was six (A81). This increases the chances that different queens will produce at least some drones that are indistinguishable at the studied loci. For example, not only do the queens of colony 1 and colony 2 each produce only 16 genotypes of drones, these arrays are identical. Such groupings of drone genotypes may restrict the ability to detect each drone in a mating, especially when the number of colonies producing drones as mates is low. The addition of more loci, loci with more detectable alleles, more colonies in the mating range of the colonies studied and larger sample sizes would improve the ability of identify more matings (table 4). The presence of workers from other colonies as a result of drifting or robbing may have resulted in overestimates of polyandry. The determination of a patriline from the genotype of only one worker is most susceptable to this sort of error. However, this is not a very likely concern with colonies in widely scattered locations.

TABLE 4. The numbers of patrilines detected in four colonies using varied sizes of worker bee samples and various numbers of loci.

Colony	Number of bees	Number of loci	Number of detected patrilines
1	40	4	11
2	41	4	12
3	40	5	10
4	24	5	13
	40	5	20
	100	4	24
	100	5	32
	100	6	32

The number of matings that were detected for *A. koschevnikovi* is in excess of the six matings that add increased buffering against the genetic load resulting from sex locus homozygosity (Shaskolsky, 1976; Page, 1980; Ratneiks, 1990). Hence, although polyandry overcomes sex locus inviability, the large numbers of matings observed also supports the other hypotheses for extreme polyandry concerning fitness benefits deriving from increased intracolonial genetic variance (Crozier & Page, 1985; Keller & Reeve, 1994; Crozier & Brückner, 1981; Oldroyd *et al.*, 1993; Sherman *et al.*, 1988; Shykoff & Schmidt-Hempel, 1991a, 1991b). All of these hypotheses are complementary to the overall theme of wide intracolonial genetic variance generally supporting colony fitness in a shifting and perilous environment.

A companion hypothesis is that polyandry increases the chance that portions of the paternity array will produce workers having increased fitness through heterosis. Heterosis arises from heterozygosity at various loci in individual workers. The probability that at least some subfamilies are heterozygous at loci regulating specific traits and expressing heterosis for those traits would improve with increased polyandry, especially for loci with restricted numbers of alleles. The potential for polyandry to increase fitness though promoting heterosis also may have been an important component in the natural selection of extreme polyandry.

Aggregations of colonies might increase the chances for increased polyandry to occur and to be detected

using an analysis of microsatellite data. More drones from more colonies would be available in the immediate mating area. *A. dorsata* colonies often are found in aggregations. *A. cerana* and *A. mellifera* colonies often are artificially aggregated in apiaries. The high levels of polyandry detected in these species (Estoup *et al.*, 1994; Moritz, *et al.*, 1995; Oldroyd *et al.*, 1997a; 1996) might be an outcome of colony aggregations. However, *A. koschevnikovi* is not known to form colony aggregations nor were the queens of this study mated in an apiary setting. Yet, the level of polyandry found for *A. koschevnikovi* was high. This is also the case for *A. florea* (Oldroyd *et al.*, 1995) and *A. andreniformis* (Oldroyd *et al.*, 1997b). This suggests that intracolonial genetic variance arising from large numbers of matings is the general rule for the genus.

This rule has important implications for *A. cerana* and *A. mellifera* queen breeders and any development programmes that may attempt to propagate *A. koschevnikovi*. One breeding goal should be to produce queens that will produce colonies having fitness levels equal to that of naturally occurring colonies in regard to intracolonial genetic variance. Hence, an abundance of drones from varied sources should be raised for mating with queens. This may be especially important for *A. mellifera* queen breeders in areas where *Varroa jacobsoni* has made feral or wild colonies rare.

Acknowledgments

We thank the Sabah Ministry of Agriculture who graciously provided access to the Tenom Agricultural Research Station; Arnaud Estoup for the primer sequences for A81; and Michael Gries, Niko and Gudrun Koeniger, and Makhdzir Mardan for facilitating the collection of bees for this research. TER's and JAS's contributions are in co-operation with Louisiana Agricultural Experiment Station.

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