Evolution of Mating Behavior in the Genus *Apis* and an Estimate of Mating Frequency in *Apis cerana* (Hymenoptera: Apidae)

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ABSTRACT Four colonies of *Apis cerana* F. were analyzed with DNA markers to determine the degree of polyandry in this species. The average observed paternity frequency was 18.0 (range, 14–27), the average worker relatedness 0.29, and the average effective paternity frequency 12.0. *A. cerana* is therefore similar to *A. mellifera* and other species of *Apis* in that the level of polyandry is extreme. A phylogeny of the genus was used as the basis for a comparative analysis of mating behavior. This analysis suggests that the enlarged penile bulb, mucus glands, and excess sperm production found in the cavity nesting species (*A. cerana*, *A. koschevnikovi*, and *A. mellifera*) are likely to be derived characters, whereas the lack of mucus glands and reduced penile bulb observed in the open-nesting species is likely to be basal.

KEY WORDS *Apis cerana*, polyandry, multiple mating, microsatellites, phylogeny

ONE OF THE more interesting problems for students of social insects is an evolutionary explanation of the widespread incidence of polyandry (Page 1980; Cole 1983; Crozier and Page 1985; Ratnieks 1990; Keller and Reeve 1994; Schmid-Hemple 1994; Moritz et al. 1995; Oldroyd et al. 1995, 1996, 1997; Crozier and Pamilo 1996). High levels of polyandry are not expected in social insects because multiple mating is assumed to have a fitness cost (Moritz 1985; Moritz et al. 1995; Oldroyd et al. 1996, 1997), and because it creates such low levels of intracolonial genetic relatedness that selective forces against eusociality may occur (Boomsma and Ratnieks 1996). Recent authors have emphasised the hypothesis that polyandry has evolved in many species and genera because the higher levels of intracolonial genetic variance it engenders increases colony fitness (Keller and Reeve 1994; Oldroyd et al. 1994, 1995, 1996, 1997; Crozier and Pamilo 1996; Reichardt and Wheeler 1996). Colonies comprising mixed genotypes may be more fit than colonies of only 1 family because (1) genetically variable colonies may have increased capacity to buffer environmental stress (Page et al. 1995) or to efficiently utilize the range of forage available (Oldroyd et al. 1991; 1992a, b; 1993; 1994), (2) multiple mating reduces the variance of reproductive success caused by genetic load from homozygosity for sex-determining alleles (Shaskolsky 1976, Page 1980, Page and Metcalf 1982, Crozier and Page 1985, Ratnieks 1990, Crozier and Pamilo 1996), (3) polyandry reduces the conflict between workers and queens over optimal sex ratios (Moritz 1985, Ratnieks and Boomsma 1995), and (4) polyandry leads to increased resistance to parasites and pathogens (Sherman et al. 1988; Shykoff and Schmid-Hempel 1991a, b; Schmid-Hemple 1994).

Understanding the evolution of extreme polyandry within a genus may be approached by comparative studies of species which have different life histories and different ecological ranges. If the number of matings is well correlated with a particular life history trait, such as migration, across a variety of species, then the evolutionary antecedents of polyandry may be suggested (Oldroyd et al. 1996, 1997). To this end, we have been attempting to estimate the degree of polyandry in all species of the genus *Apis*. These studies have demonstrated that the degree of polyandry across the genus is uniformly high (>5 matings), but extremely variable, both within and among the species so far examined (Table 1).

The Asian hive bee *Apis cerana* F. is distributed from eastern Indonesia west to Iran and north to Japan (Ruttner 1988). Three sibling species of *A. cerana* have been named, these being *A. nulensis* S. Tingek et al. (1996) from Sabah, *A. nigrocincta* F. Smith (1861) from Sulawesi, and *A. koschevnikovi* S. Tingek et al. (1988) from Borneo. Both morphological (Fuchs et al. 1996) and molecular (Arias et al. 1996) evidence suggest that *A. nuluensis* is closely related to *A. cerana*. The precise taxonomic position of *A. nigrocincta* is unknown (Hadisoesilo et al. 1995). Molecular evidence suggests that *A. koschevnikovi* diverged somewhat earlier than *A. cerana* and *A. nuluensis* (Arias et al. 1996).

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Table 1. Levels of polyandry and intracolonial genetic relationships in the genus *Apis* as determined by microsatellite analysis

Species	Paternity frequency: Observed: Mean (± SE)	Paternity frequency: Observed: Variance	Paternity frequency: Effective	Coefficient of relatedness	Author
A. mellifera	13.8 ± 2.5	30.7	12.4 ± 2.2	0.30 ± 0.009	Estoup et al. (1994)
A. florea	8.0 ± 1.6	5.5	5.6 ± 1.0	0.35 ± 0.02	Oldroyd et al. (1995)
A. dorsata	26.7 ± 6.6	117.6	20.0 ± 6.6	0.29 ± 0.007	Oldroyd et al. (1996)
A. dorsata	18.0 ± 1.6	38.4	25.6 ± 1.05	0.27 ± 0.02	Moritz et al. (1995)
A. andreniformis	13.5 ± 2.3	20.3	9.1 ± 0.83	0.30 ± 0.007	Oldroyd et al. (1997)
A. cerana	18.0 ± 3.03	36.7	12.0 ± 1.6	0.29 ± 0.005	This study

Only 2 estimates of the usual number of matings have been reported for *A. cerana*. Ruttner et al. (1973) working in Pakistan, found that the ejaculate volume of a typical drone was ≈0.2 μl, whereas the oviducts of a queen returning from her 2nd mating flight contained 2.79 μl, suggesting the queen may have mated with 14 males on that flight, and perhaps a similar number on the previous flight. Woyke (1975), working in Poona, India, again found that a typical drone produced 0.2 μl of semen, but that newly mated queens had 0–5.39 μl of semen in their oviducts. Thus the potential range of matings was 1–27.

Like *Apis mellifera* L. (Koeniger 1990, Woyciechowski et al. 1994) and *A. koschevnikovi* (Koeniger et al. 1994), *A. cerana* queens often return from mating flights with a mucus plug in their sting chamber (Woyke 1975). The plug consists of congealed mucus and corneal secretions (Koeniger 1990). The function of this plug or 'mating sign' is controversial. Koeniger (1990) argued that it assists 2nd and subsequent drones to mate with the queen by providing visual cues. Woyciechowski et al. (1994) argues that it helps the queen retain semen in the oviducts following mating. Mating signs are undoubtedly absent from the open nesting species (*A. andreniformis* F. Smith 1878, *A. florea* F. and *A. dorsata* F.) because these species lack the mucus glands necessary for their production (Koeniger and Koeniger 1990, Koeniger et al. 1991).

Dissection methods can underestimate the actual number of copulations and inseminations because queens may eject a portion of the received semen after copulation as they do in *A. mellifera* (Koeniger and Koeniger 1990), *A. andreniformis* (Oldroyd et al. 1997), *A. florea* (Oldroyd et al. 1995) *A. dorsata* (Oldroyd et al. 1996), and the ant *Acromymex versicolor* (Reichardt and Wheeler 1996). The preferred method for estimating the paternity frequency is to use highly variable genetic markers such as microsatellites (Boomsma and Ratnieks 1996). Microsatellites are sections of DNA that consist of tandem repeats of very simple motifs such as $(CT)_n$. The lengths of microsatellite sequences tend to be highly variable because of relatively high mutation rates. If the 2 DNA regions flanking a microsatellite region are sequenced, and complementary PCR primers are synthesized, then PCR reactions conducted with these primers with extracts of genomic DNA obtained from experimental animals will amplify the microsatellite region. Amplified products are usually variable in length, and these differing lengths (alleles) can be determined on sequencing gels. Microsatellite alleles are inherited in a Mendelian manner and can be used to infer pedigrees.

Here we provide estimates of paternity frequency, effective paternity frequency, and intra-colonial relatedness (Boomsma and Ratnieks 1996) in *A. cerana*, based on polymorphisms at 3 microsatellite loci (Estoup et al. 1994), and 1 sequence-tagged site (Hunt and Page 1994). We then use this estimate in a comparative analysis of mating behavior in the genus.

Materials and Methods

Four colonies of *A. cerana* were examined in this study. Colony 1 was collected by B.P.O. and T.E.R. in the town of Ban Dong, Thailand, in 1993. Colony 2 was collected from the city of Kathmandu, Nepal, in 1995 by Ratna Thapa. Colony 3 was collected by D. Sihanuntavong in Utaradit province, Thailand, in 1995. Colony 4 was collected by S.W. in Phitsanulok, Thailand, in 1996. Colony 1 was a sample of adult bees, but the colony was isolated. The other samples were brood. Therefore no drifting bees could have confounded our results. Bees were frozen in the field in liquid nitrogen or dry ice, and subsequently maintained at −70°C.

DNA was extracted from the antennae (adults) or the hind leg (pupae) of individual bees by boiling the ground tissue in 1 ml 5% Chelex 100 resin (BioRad, Sydney) for 15 min (Walsh et al. 1991). DNA extractions were then amplified using the polymerase chain reaction (PCR) using primer sets specific to the loci listed in Table 2. For each primer pair, the reverse primer was radio-actively end-labeled. To do this, the γ-phosphate from ^{33}P-ATP (Bresatec, Adelaide) was transferred to the 5′-terminus of the primer, using T4 polynucleotide kinase (Amrad/Pharmacia, Sydney). PCRs were then conducted in 10 μl volumes, which were composed of 5 μl of the Chelex DNA extraction, 400 nM of each primer, 20 μM of each dNTP, 1.2–1.5 mM $MgCl_2$, 1X reaction buffer, and 0.45 units of *Taq* polymerase (Biotech International, Perth). The precise Mg^+ concentrations and cycling conditions used for each locus are given in Table 2.

Polymer Chain Reaction products were run on 6% polyacrylamide sequencing gels with M13 control DNA sequencing reactions run on the same gel as size

Table 2. Primer sequences and PCR conditions used to screen for polymorphic loci in *A. cerana*

Locus	Primers[a]	Range of annealing temps, °C	MgCL 2, mM	No. cycles	No. alleles observed
A8	5′CGAAGGTAAGGTAAATGGAAC 5′GGCGGTTAAAGTTCTGG	51–60	1.5	30	No product
A14	5′GTGTCGCAATCGACGTAACC 5′GTCGATTACCGATCGTGACG	58	1.5	30	1
A28	5′GAAGAGCGTTGGTTGCGAGG 5′GCCGTTCATGGTTACCACG	51–58	1.2	30	No product
A29	5′AAACAGTACATTTGTGACCC 5′CAACTTCAACTGAAATCCG	51–57	1.0	30	No product
A35	5′GTACACGGTTGCACGGTTG 5′CTTCGATGGTCGTTGTACCC	51–57	1.2	30	Nonspecific products
A43	5′CACCGAAACAAGATGCAAG 5′CCGCTCATTAAGATATATCCG	51–55	1.2	30	No product
A76	5′GCCAATACTCTCGAACAATCG 5′GTCCAATTCACATGTCGACATC	58	1.2	30	1
A81	5′GCCGAGTTCTTCGACTCCC 5′GGACTTTGCCAAATGGGTC	51	1.2	30	5
A88	5′GCGAATTAACCGATTTGTCG 5′GATCGCAATTATTGAAGGAG	57	1.2	35	1
A107	5′CCGTGGGAGGTTTATTGTCG 5′GGTTCGTAACGGATGATGACACC	58	1.2	30	9, but unreliable
A113	5′CTCGAATCGTGGCGTCC 5′CCTGTATTTTGCAACCTCGC	51–60	1.2	30	3, but unreliable
B116	5′GAATCAGGAGGCGCACG 5′CGCAGCCTAAGCCACG	53	1.2	30	1
B124	5′GCAACAGGTCGGGTTAGAG 5′CAGGATAGGGTAGGTAAGCAG	57	1.2	35	7
Q	5′AGTGCAGCCAGCTACTGAGAG 5′AGTGCAGCCACGTGCCTGAAT	53	1.5	35	8

[a] Microsatellite primer sequences are from Estoup et al. (1994, 1995, 1996), except for Q locus from Hunt and Page (1994), and A81, kindly provided by A. Estoup.

standards. Microsatellite (A81, A107, and B124) allele lengths were determined by reference to the M13 sequencing lanes. Because the 'Q' locus primers produce a PCR product of ≈600 bp (Hunt and Page 1994), sequence reaction products could not be used as size standards because they are too short. Therefore for the Q locus, we identified alleles by running a set of standard bees alongside the unknowns. We named Q-locus alleles 1–12 in order of ascending size. In agarose gels, Q locus products form a heterodimer (Hunt and Page 1994). Because our gels were denaturing, heterodimers were not observed.

Analyses. Queen genotype was inferred from worker genotypes, and the genotype of each fathering drone in the sample inferred by standard methods (Estoup et al. 1994; Oldroyd et al. 1995, 1996; Kryger and Moritz1997). The average coefficient of relatedness—g_{ww} weighted according to the relative proportions of each subfamily in our samples and corrected for finite sample size—was computed from

$$gww = \frac{1}{4} + \frac{1}{2}\sum_{i+1}^{k} p_i^2 \qquad [1]$$

(Boomsma and Ratnieks 1996), where

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

is calculated as

$$\left(N\sum_{i=1}^{k} y_i^2 - 1\right) \Big/ N - 1,$$

k is the number of subfamilies observed, y_i is the observed proportion of the i th subfamily, and N is the number of workers scored. The effective insemination frequency (Crozier and Pamilo 1996; Boomsma and Ratnieks 1996) (m) was computed from

$$m = \frac{1}{\sum_{i=1}^{k} p_i^2}. \qquad [2]$$

(Starr 1984).

Because some subfamilies were found only once, indicating that some further ones were not found at all, we used sample coverage estimators (Chao and Lee 1992, Chao et al. 1996) to estimate the actual numbers of subfamilies, including those absent from the sample. The rationale behind this approach is that if a subfamily is present at high frequency in the sample, then it is likely to be found in any other sample that might be drawn. However, a rare subfamily in a sample has a much lower probability of being found in another sample. Thus the likelihood of seeing all subfamilies in a given sample is a function of how adequately the

sample reflects the true population, and this can be estimated from the data as the sample coverage, C. Let f_n equal the number subfamilies in which the number of bees observed is n. C can then be estimated as

$$\hat{C} = 1 - f_1/N \quad [3]$$

(i.e., 1 minus the number of singleton subfamilies divided by the number of workers scored) (Chao and Lee 1992). Thus the adequacy of the sample coverage depends on the number of rare subfamilies and the size of the sample. If there are many rare subfamilies, C will decrease, and a larger sample size is needed so that all subfamilies will be observed. However, Chao and Lee (1992) show that the true paternity frequency, $\hat{k}$, can actually be estimated from $\hat{C}$ and k. When f_n is equal for all subfamilies, an unbiased estimator of the true paternity frequency $\hat{k}$ is $D/\hat{C}$ where D is the observed number of classes (Chao and Lee 1992). Where the f_ns are unequal, the value of $\hat{C}$ can be adjusted using the coefficient of variation of f_n (Chao and Lee 1992) to produce estimates of $\hat{k}$, via a method that makes no a priori assumptions about the distribution of subfamily frequencies (Chao and Lee 1992). We used the program kindly supplied by A. Chao (National Tsing Hua University Taiwan) to make these calculations. To run the program, subfamilies with < 10 individuals in the sample were classed as rare (Chao et al. 1996). We determined 95% confidence intervals for our estimates using equation 4 of Chao (1989) and the values from the Chao program; these confidence intervals are asymmetric.

Results

We attempted to amplify 13 microsatellite loci identified by Estoup et al. (1994, 1995) from *A. mellifera* and *Bombus terrestris* genomic libraries. Of these loci, 4 (A8, A28, A29, and A43, Table 3) were not sufficiently conserved across species to permit successful PCR amplification in *A. cerana*. Four loci (A14, A76, A88, and B116, Table 3) were monomorphic in a sample of 40 bees and so were discarded. A107 was polymorphic, but could not be used in colonies 3 and 4 because heterogenous allele amplification made interpretation difficult in those colonies. A35 also was discarded because of heterogenous allele amplification in all colonies.

The A81, B124, and Q loci were polymorphic and gave reliable allele amplification in all colonies. A107 was polymorphic and produced satisfactory results only in colonies 1 and 2, although a null allele was present in the queen of colony 2 (see below). Using these 4 polymorphic loci, we determined that the *A. cerana* queens heading the colonies sampled in this study mated at least 14–27 times, producing an average relatedness, g_{WW}, of workers of 0.29 ± 0.005 (mean ± SE), and an average effective number of matings of 18 ± 3.03 (Tables 1, 3, and 4). In applying the Chao program to our data, we found that the cv was small, indicating use of the low-cv estimator of Chao and Lee (1992). The observed numbers of matings are therefore likely to be very close to the actual number of drones that inseminated each queen (Table 4).

There is no evidence for polygyny in the 4 colonies examined. Because queens can transmit only 1 of 2 alleles to worker offspring at any 1 locus, the presence of more than 2 homozygous classes of workers would be evidence for polygyny provided no null alleles were carried by the queen at the loci studied (Oldroyd et al. 1996). Seven classes of homozygous workers were observed for the A107 locus in colony 2. However, because no more than 2 homozygous worker classes were detected at the other 3 loci, we concluded that the queen of colony 2 carried a null allele, rather than the presence of polygyny in this colony (Oldroyd et al. 1996).

Discussion

These results demonstrate that the paternity frequency (the number of males that father offspring) (Boomsma and Ratnieks 1996) in *A. cerana* is ≈15–30, a frequency similar to that observed in *A. mellifera* and *A. andreniformis* but higher than in *A. florea*, and considerably lower than in *A. dorsata* (Table 1). Although our sample size is small, the 4 colonies examined were from diverse localities. Our estimates may therefore reflect typical paternity frequencies for the species as a whole, but should still be regarded as a restricted data set.

These results may underestimate the actual paternity frequency. First, finite sample sizes mean that certain rare subfamilies may not have been present in the bees analyzed. However, our analyses suggest that this bias is likely to be small. First, a sample of size 60 is adequate to detect a subfamily of $p_i = 0.1$ 95% of the time (Boomsma and Ratnieks 1996), so only rare subfamilies would be undetected with our sample sizes. Second, using the estimator of Chao and Lee (1992) to determine the number of subfamilies absent from the samples shows that the estimated number of subfamilies is very close to the actual number observed. Finally, applying Pamilo's (1993) correction for finite sample size for the estimates of effective paternity frequency makes almost no difference in the estimate.

The 2nd means by which a subfamily may remain undetected is if ≥2 fathering drones have the same genotype (particularly a null allele) at the loci investigated. It is unlikely that many subfamilies were undetected for this reason. The addition of the highly polymorphic B124 locus in colony 1 distinguished a further 3 subfamilies, but did not reveal any additional subfamilies in colony 2. However, it is almost certain that we failed to detect all subfamilies because of limited sample size or genotypically identical drones.

Another source of potential bias arises from our use of the Q locus as a genetic marker. Any drone that carries the same sex allele as the queen will have 50% of offspring homozygous at the sex locus. These homozygous individuals are diploid males, but are destroyed by the workers (Woyke 1963). This means that p_i is underestimated for these drones by 50%, resulting in an upward bias in estimates of relatedness,

Table 3. Inferred genotypes of queens and their inseminating males at 4 polymorphic loci in *A. cerana*

	Locus[a]				
	A81	A107	Q	B124	No. worker bees
Colony 1					
Queen allele 1	134	156	3	219	—
Queen allele 2	142	167	10	224	—
Drone 1	132	156/157	10	219/224	3
Drone 2	132	156/157	2	219	2
Drone 3	132	156/157	9	221	5
Drone 4	132	156/157	3/10	221	6
Drone 5	132	156/157	2	221	5
Drone 6	132	165	6	219/224	3
Drone 7	132	156	3/10	219/224	1
Drone 8	132	159	3/10	219/224	3
Drone 9	132	156/157	3/10	219/224	7
Drone 10	134	156/157	2	219	1
Drone 11	134	156/157	3/10	219/224	3
Drone 12	134/142	156/157	3/10	221	5
Drone 13	134/142	156	9	219/224	2
Drone 14	134/142	156/157	3/10	219/224	12
Drone 15	134/142	159	3/10	219/224	3
Drone 16	144	156/157	10	219	2
Drone 17	144	156/157	3/10	221	5
				Total	68
Colony 2					
Queen allele 1	132	171	3	218	—
Queen allele 2	136	Null	3	220	—
Drone 1	132	165	3	—	2
Drone 2	132	167	3	220	2
Drone 3	132	170	3	222	2
Drone 4	132	171	3	222	2
Drone 5	132	172	3	220	9
Drone 6	132	156	9	218	5
Drone 7	132	170	9	222	2
Drone 8	132	171	9	222	2
Drone 9	132	172	9	218	12
Drone 10	132	156	10	218	2
Drone 11	132	161	10	218	4
Drone 12	132	167	10	222	5
Drone 13	132	170	10	224	1
Drone 14	132	171	10	224	1
Drone 15	134	167	3	218/220	1
Drone 16	134	170	3	218/220	1
Drone 17	134	170	9	220	4
Drone 18	134	171	9	218/220	2
Drone 19	134	164	10	218/220	1
Drone 20	134	167	10	218	6
Drone 21	142	167	3	218/220	11
Drone 22	142	171	3	222	1
Drone 23	142	167	6	218	6
Drone 24	142	164	10	220	4
Drone 25	142	167	10	218/220	3
Drone 26	142	170	10	220	4
Drone 27	142	167	3	222	6
				Total	101
Colony 3					
Queen allele 1	132	—	1	219	—
Queen allele 2	142	—	3	221	—
Drone 1	132	—	9	218	3
Drone 2	132	—	10	218	8
Drone 3	132	—	9	219	4
Drone 4	132	—	12	219	9
Drone 5	132	—	10	220	3
Drone 6	132	—	10	221	3
Drone 7	134	—	9	218	6
Drone 8	134	—	10	218	2
Drone 9	134	—	10	219	16
Drone 10	134	—	9	220	2

continued

Table 3. continued

	Locus				No. worker bees
	A81	A107	Q	B124	
Drone 11	142	—	3	218	4
Drone 12	142	—	9	218	4
Drone 13	132/142	—	3	219	1
Drone 14	132/142	—	10	219	2
Drone 15	132/142	—	3	220	1
Drone 16	132/142	—	9	221	3
				Total	71
			Colony 4		
Queen allele 1	132	—	3	218	—
Queen allele 2	142	—	10	220	—
Drone 1	132	—	3	218	7
Drone 2	132	—	10	218	8
Drone 3	132/142	—	10	220	7
Drone 4	132	—	9	218	8
Drone 5	132/142	—	9	220	4
Drone 6	132	—	3	219	2
Drone 7	132	—	10	219	5
Drone 8	132	—	11	216	3
Drone 9	140	—	10	219	1
Drone 10	140	—	11	219	2
Drone 11	142	—	3	216	1
Drone 12	132/142	—	10	216	1
Drone 13	132/142	—	11	218	3
Drone 14	132/142	—	11	220	2
Drone 15	132/142	—	12	219	1
				Total	55

[a] Microsatellite loci (A81, A107, and B124) are given as lengths in base pairs; Q-locus allele designations are arbitrary.

and a downward bias in the estimated effective mating frequency. In *A. mellifera*, the Q locus is very tightly linked to the sex-determining locus (Hunt and Page 1994), and this linkage is likely to be similar in *A. cerana*. However, this linkage does not result in any further bias to estimates of p_i. Consider the mating between a queen and a drone carrying the same sex allele as the queen, but a different allele at an unlinked genetic marker *A*. This mating may be written as $X^1X^2A^1A^2 \times X^1A^3$ and will result in 50% $X^1X^2A^1A^3$ and 50% $X^1X^2A^2A^3$ worker offspring because all X^1X^1 individuals are killed. Thus this male's insemination frequency and effective insemination frequency (Boomsma and Ratnieks 1996) will be underestimated by 50%. Provided sample sizes are adequate, however, the estimate of paternity frequency will be unaffected. By definition (Boomsma and Ratnieks 1996), effective paternity frequency is unaffected because this measure is based on worker offspring alone. When the genetic marker is linked to the sex locus, the mating is $X^1A^1/X^2A^2 \times X^1A^3$ which, except for recombinants, results in 100% X^2A^2/X^1A^3 in the worker offspring because X^1X^1 individuals are killed. Thus although A^1A^3 individuals will be absent from this male's offspring, there is no additional affect of linkage on estimates of effective insemination frequency. We conclude that the use of the Q locus has no effect on the estimates presented here.

Finally, some subfamilies represented by only 1 bee may in fact be the result of a PCR artifact or scoring error. However, we double-checked all such bees by performing a new PCR reaction, and consider this unlikely.

The results show (Table 4) that the effective number of matings (m) is not a particularly good predictor of the observed or estimated number of matings. For example, because the semen pool of colony 3 is more skewed than that of colony 4, it has a lower effective number of matings, m, and estimated values of mating frequency, $\hat{k}$, despite having the higher observed num-

Table 4. Observed, estimated, and effective number of matings in 4 colonies of *A. cerana*

Colony	Observed no. matings	Estimator cv	Estimated no. matings[a]	95% CI	Effective insemination frequency
1	17	0.113	17.62	17.07–22.77	11.92
2	27	0.476	30.56	27.84–42.02	14.37
3	16	0.417	16.93	16.12–23.29	9.42
4	15	0.583	17.64	15.5–28.98	10.05

[a] Estimated using estimators $\hat{N}_2$ (when the coefficient of variation in $f_n \leq 0.8$) or $\hat{N}_3$ (when the coefficient of variation in $f_n \geq 0.8$) (Chao and Lee 1992).

Table 5. Estimates of number of spermatozoa found in spermathaecae of laying queens, seminal vesicles of mature drones, and median oviducts of freshly mated queens

	A. mellifera[a]	*A. cerana*[b]	*A. koschevnikovi*[c]	*A. dorsata*[d]	*A. florea*[e]	*A. andreniformis*
No. spermatozoa						
In seminal vesicles ($\times 10^6$)	5–10	1.0	1.7	2.5	0.4	0.1
In oviducts after returning from mating flight ($\times 10^6$)	170	—	18.8	—	0	—
In spermatheca ($\times 10^6$)	4.7	1.3	2.1	3.7	0.9	1.0
Copulation frequency						
Based on oviducts[f]	17	—	11.0	—	—	—
Based on spermathaecae[g]	0.5	6.4	1.3	1.5	2.0	7.9
% spermatozoa stored[h]	3.4–6.8	4.8–7.2	<11.2[i]	5.5	28.1	74.1

[a] Rinderer et al. 1985, Woyke 1966.
[b] Woyke 1975, Ruttner et al. 1973.
[c] Koeniger et al. 1994.
[d] Koeniger et al. 1990.
[e] Koeniger et al. 1989.
[f] Estimated as number of spermatozoa found in oviducts of queens recently returned from mating flights, divided by number found in seminal vesicles of drones.
[g] Estimated as number of spermatozoa found in spermathecae divided by number found in seminal vesicles of drones.
[h] See text for a definition of *IP*.
[i] Maximum estimate in which the insemination frequency is estimated as the copulation frequency based on spermathaecal sperm counts.

ber, k. Although m is the important parameter with such quantities as the expected proportions of diploid males, k has behavioral significance because it is correlated with mating risk (Moritz 1985; Oldroyd et al. 1996).

Our genetically determined estimates of paternity frequency in *A. cerana* are similar to copulation frequency estimates obtained from sperm counts. Tables 1 and 5 show that for *A. mellifera* and *A. cerana*, sperm counts obtained from the oviducts of freshly mated queens provide an accurate estimate of paternity frequency determined by genetic means. This is not so for *A. florea*, where virtually no spermatozoa are found in the oviducts of freshly mated queens (Koeniger et al. 1989). Data on the amount of sperm present in the oviducts of freshly mated queens are not available for other *Apis* species.

Conversely, inspection of Table 5 shows that estimates of paternity frequency based on the number of spermatozoa found in spermathaecae would be inaccurate for all species. This is because queens of all species take up only a proportion of the spermatozoa placed in their oviducts by copulating males. This proportion, *IP*, can be estimated from

$$N_S/[IF(N_D)], \qquad [4]$$

where N_S is the average number of sperm found in the spermathaecae of laying queens, N_D is the number of sperm found in the average drone of that species, and *IF* is the insemination frequency estimated by genetic or other means. We present these proportions in Table 4 and plot them on the phylogeny in Fig. 1. The calculations suggest that *A. cerana* is similar to *A. mellifera* in that >90% of semen received is excreted by the queen, even though some semen is retained from each male. We would therefore caution that estimates of paternity frequency in ants obtained via dissection of spermathaecae and seminal vesicles (reviewed by Page 1986, Keller and Reeve 1994, Boomsma and Ratnieks 1996, Crozier and Pamilo 1996) also may be gross underestimates.

Koeniger and Koeniger (1991) analyzed the evolution of mating behavior in the genus *Apis*, proposing that *A. florea* and *A. andreniformis* have a highly derived mating apparatus, including clasping organs on the penis, and in the case of *A. florea*, on the legs as well (Ruttner 1988). These adaptations, they argued, facilitate aerial mating. The cavity-nesting species, however, lack these structures. In these species, the mating pair is held together in copula by the enlarged bulb of the everted penis. Koeniger and Koeniger (1991) argued that evolution of the clasping organs facilitated the reduction in the size of the penile bulb, and the loss of the mucus glands and the mating plug which are only observed in the cavity-nesting species. Reduction of the penile bulb also permitted direct placement of sperm at the entrance to the spermathaeca instead of the median oviduct. Thus Koeniger et al. (1991) regard the mating apparatus of *A. florea* and *A. andreniformis* as the most derived, those of *A. mellifera*, *A. cerana*, and *A. koschevnikovi* the least, and *A. dorsata* as intermediate.

We accept the phylogeny for the genus given by Alexander (1991), which was based on morphology and behavior. Although Willis et al. (1992) rejected this tree on the basis of a parsimony analysis of mitochondrial COII data, Lockhart et al. (1994) showed that parsimony was inappropriate for this gene because base composition differences among the species interferes with the phylogenetic signal. However, when Lockhart et al. (1994) used the LogDet parsimony method (which compensates for base-composition differences) on the Willis et al. data, the same tree as Alexander (1991) was retrieved. More recently, Engel and Schultz (1997) again retrieved Alexander's (1991) dendogram using mitochondrial ribosomal DNA sequence data (Cameron 1991a b), the Willis et al. (1992) data and an expanded morphological analysis. We therefore accept the Alexander (1991) tree and use it as the basis for our reconstruction of ancestral traits.

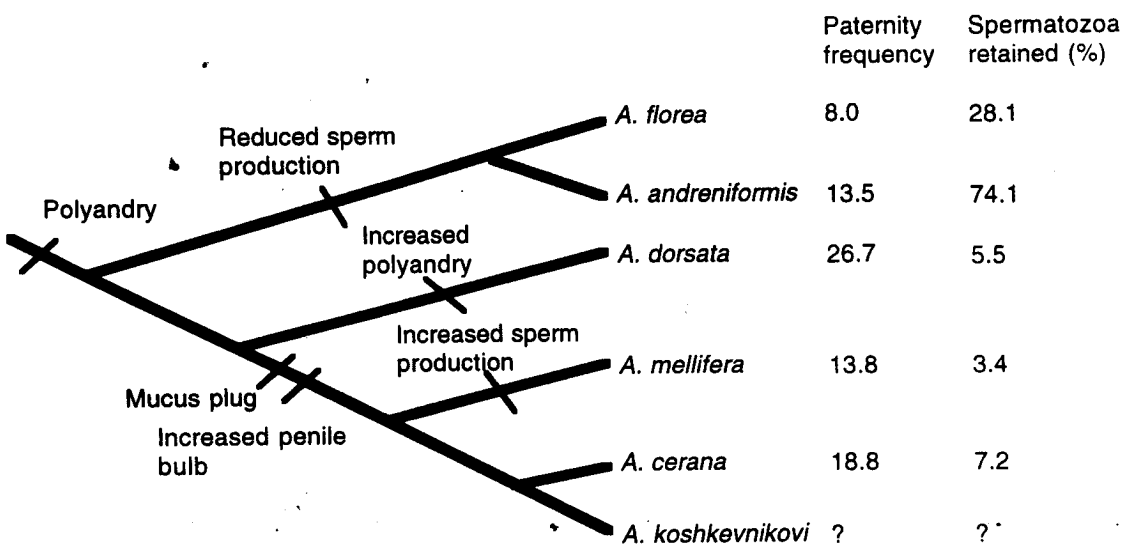

Fig. 1. Cladogram of the genus *Apis* (after Alexander 1991) with reconstructions of reproductive behavior traits. The average paternity frequency and the proportion of injected spermatozoa retained (see text) are superimposed.

A comparative analysis of the mating biology of *Apis* based on the Alexander (1991) cladogram suggests an evolutionary hypothesis slightly different from that of Koeniger and Koeniger (1991). In Fig. 1 we place the evolution of the mating sign, increased size of the penile bulb, and expulsion of semen from the oviducts between the *A. dorsata* branch and the cavity nesting clade. Under this hypothesis, the derived condition is an enlarged penile bulb as is seen in the cavity-nesting species. The alternative polarity requires loss of the mating sign twice, once in the *A. florea–A. andreniformis* branch and again in the *A. dorsata* branch. Koeniger and Koeniger (1991) argue that the presence of mating plugs in some stingless bees supports the notion that the mating sign is ancestral. We suggest that the structures are not homologous. Certainly we are not aware of the presence of mucus glands in the male genitalia of the Meliponini, which is "unequivocally" the sister group to the Apidae (Chavarria and Carpenter 1994). We concur with Koeniger and Koeniger that the clasping organs of the open-nesting species are derived.

Oldroyd et al. (1995, 1996, 1997), following Koeniger and Koeniger (1990), suggested that mating behavior in the genus has been subject to divergent sexual selection, based on the a priori notion that colony-level selection is strongly in the direction of polyandry. In the cavity-nesting species, we proposed that drones are selected to produce large numbers of spermatozoa to increase their share of paternity of potential offspring, whereas queens are selected to mate many times and expel excess semen. In the open-nesting species, direct insertion of the penile bulb into the spermathaeca appears to be an adaptation of males to increase paternity frequency while eliminating the need for massive sperm production. The current analysis supports this view. The percentage of spermatozoa retained in the spermathaeca (Table 5; Fig. 1) is higher in the open-nesting species than in the more derived cavity-nesting species.

Reference to Table 5 and Fig. 1 reveals that polyandry, excess production of semen by males, and excretion of excess semen by queens are all pleisiomorphic characters for the genus. This could be caused by phylogenetic inertia, but this seems very unlikely as high levels of polyandry are not known to exist in any other genus of social insects (Page 1986, Keller and Reeve 1994, Boomsma and Ratnieks 1996, Crozier and Pamilo 1996). This suggests that special conditions exist in the genus which selected for polyandry. These are likely to be caused by the need for genetically variable worker populations that provide increased colony fitness in the face of the varying environments generated by migration and perennial nesting, and by pressure from parasites and pathogens (Sherman et al. 1988; Shykoff and Schmid-Hempel 1991b; Oldroyd et al. 1994, 1995, 1996, 1997; Moritz et al. 1995; Crozier and Pamilo 1996). However, the explanation of why polyandry is so favored in the genus remains elusive. Perhaps the extraordinarily high number of matings in *A. dorsata* eventually will provide the key.

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