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## Geographic variation in polyandry of the Eastern Honey Bee, *Apis cerana*, in Thailand

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### Abstract

The repeated evolution of extreme polyandry in advanced social insects is exceptional and its explanation has attracted significant attention. However, most reported estimates of the number of matings are derived from limited sampling. Temporal and geographic variation in mating behavior of social insects has not been sufficiently studied. Worker offspring of 18 Eastern Honey Bee (*Apis cerana* Fabr.) queens from three populations across Thailand were genotyped at five microsatellite markers to test for population differences of mating behavior across three different ecosystems. The number of matings decreased from a northern, more seasonal environment to a southern tropical population and was lowest in a tropical island population. Our study confirms earlier findings that social insect mating behavior shows biogeographic variation and highlights that data from several populations are needed for reliable species-specific estimates of the number of matings. Detailed studies of populations that show significant differentiation in the number of matings may be able to discriminate effectively among the different hypotheses that have been proposed to explain the evolution of polyandry in honey bees and other advanced social insects.

### Keywords

Polyandry; Social evolution; Behavioral ecology; Division of labor; Honeybees; *Apis cerana*; Biogeographic variation

### Introduction

Social insects owe their ecological and evolutionary success in part to an efficient division of labor, most notably between numerous non-reproductive workers and one or a few reproductives in each colony. The mating behavior of reproductive individuals is central to

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understanding social evolution because it determines the sociogenetic structure of the resulting societies. Although monogamy was presumably ancestral and significant for the evolution of eusociality in the Hymenoptera, multiple mating of reproductive females (queens) has evolved repeatedly in different social lineages of this order (Hughes et al., 2008). High levels of polyandry have evolved in several clades with advanced social organization, most notably in ants (Fjerdingstad and Boomsma, 2000; Kronauer et al., 2007), wasps (Goodisman et al., 2002), and bees (Tarpy et al., 2004).

The highest levels of polyandry have been reported in honey bees, with some records exceeding 100 matings of individual queens (Wattanachaiyingcharoen et al., 2003) and the overall estimates for honey bee species vary between 11.6 and 54.9 (Tarpy et al., 2004). These species-specific estimates often derive from limited sampling of one population. However, most of the few existing comparative studies (Corley and Fjerdingstad, 2011; El-Niweiri and Moritz, 2011; Franck et al., 2000) report significant variation of social insect mating behavior (but see Rattanawanee et al., 2012).

To explain the evolution of polyandrous mating systems in social insects, multiple hypotheses have been proposed. Polyandry may have evolved to improve colony division of labor (Crozier and Page, 1985) or disease resistance (Sherman et al., 1988). Multiple mating may also decrease variability of brood viability due to inbreeding effects (Page, 1980) or kin conflict (Ratnieks, 1988). Additionally, multiple mating may have evolved to ensure adequate sperm supply in advanced social insects (Cole, 1983). Most of these hypotheses are not mutually exclusive (Brown and Schmid-Hempel, 2003; Crozier and Fjerdingstad, 2001; Crozier and Page, 1985; Rueppell et al., 2008; Strassmann, 2001). Targeted studies have provided experimental support for almost all hypotheses, which is illustrated in the following for the Western Honey Bee, *Apis mellifera* (L.): Polyandry increases the genetic diversity of the colony members and has been shown to improve disease resistance (Seeley and Tarpy, 2007) and division of labor among workers (Mattila and Seeley, 2007). The number of matings has also been found to correspond to male sperm quantities, lending tentative support for the mating-for-sperm hypothesis (Kraus et al., 2004). In addition, multiple mating decreases the potential for intra-colonial kin conflict over reproduction, which has also been empirically demonstrated (Mattila et al., 2012). Furthermore, polyandry in honey bees reduces the chance that a substantial portion of the queen's offspring develops into infertile, diploid males due to inbreeding effects (Page, 1980). Most of these hypotheses predict micro-evolutionary differentiation among populations living in different environments but the extent and details of such differentiation differ. Thus, comparative studies of behavior across different populations of species present a powerful approach to understanding behavioral evolution (Foster, 1999). More empirical data demonstrating and quantifying relevant geographic variation in polyandry of honey bees is needed.

The Eastern Honey Bee, *A. cerana* (Fabr.), shares a number of biological features with *A. mellifera*, is commercially important, and has a wider biogeographic distribution than most other honey bee species (Ruttner, 1988). The only reliable study of polyandry in *A. cerana* has reported an average estimate of 18.8 matings per queen, based on four colonies from separate populations (Oldroyd et al., 1998; Tarpy et al., 2004). Following several studies that demonstrate a distinct population structure of *A. cerana* in Thailand (Rueppell et al.,

2011; Warrit et al., 2006), we investigated the geographic variation in queen mating behavior in this species. We selected six colonies from each of three populations. These populations came from a northern mountainous, a tropical mainland, and a tropical island environment. To infer the number of matings, we determined the number of patriline in each colony by microsatellite genotyping. We report significant differences in the number of matings per queen among these three local populations, with highest numbers in the North and lowest in the tropical island population.

## Material and methods

Semi-domesticated colonies of *Apis cerana* were sampled from three distinct populations. We collected samples in Chiang Mai province, north of Chiang Mai, in the Si Lanna mountains that were mainly covered by coffee plantations and secondary forests (18.8°N, 99.0°E). This environment represented a more seasonal climate and spatially heterogeneous environment than the other populations. In addition, we sampled colonies near Phatthalung (7.6°N, 100.1°E) in the tropical Phatthalung province mostly in rubber tree plantations. The third population was collected from Ko Samui (9.5°N, 100.0°E), a tropical island of mixed use and vegetation, which is approximately 16 km from the mainland and genetically isolated (Rueppell et al., 2011). The variance and range of monthly average minimum and maximum temperatures for Chiang Mai exceed the respective values of the other two populations by 75% or more.

From each population, six colonies were included. These colonies were provided by traditional Thai beekeepers, who do not breed or extensively maintain *A. cerana* hives but trap colonies annually until destructively harvesting honey. Between 30 and 100 random workers were collected from each colony and stored in RNAlater™ (Qiagen, Germany) until DNA extraction.

One leg was separated from individual workers, crushed in liquid nitrogen, and boiled for 10 minutes in 200 µl of 10% Chelex (Bio-Rad, California) solution. After centrifugation (13,000g for 5 minutes), 1–4 µl of the supernatant was used in the subsequent PCR reactions. Microsatellite loci were amplified and genotyped following previously published methods (Rueppell et al., 2011). Briefly, we used a tailed-primer approach to fluorescently label PCR products. All loci were amplified in a touchdown PCR with annealing temperatures decreasing from 68 to 50 °C. PCR product sizes were determined by denaturing polyacrylamide electrophoresis on a DNAnalyzer (LI-COR Biosciences, NE, USA). Fifty-five loci previously identified for *Apis mellifera* and *A. cerana* (Rueppell et al., 2011; Solignac et al., 2007; Takahashi et al., 2009) were screened in eight random individuals. The five most reliable and polymorphic loci (Table 1) were selected to genotype between 30 and 66 workers per colony. The number of alleles and the observed heterozygosity were determined directly from the worker genotypes. Expected heterozygosities were calculated from allele frequencies after estimating family structure (see below) to account for the non-independence of the multiple sister workers that were genotyped.

Maternal alleles for each colony were directly inferred from the data and used to exclude workers that were not offspring of the queen. These workers presumably drifted from other colonies into our study colonies and were not very common (0–3 per colony). Patriline numbers per colony were estimated with the computer program COLONY1.2 (Jones and Wang, 2010). The six colonies from each population were combined into one analysis file to estimate population-specific allele frequencies that were then used to infer patriline identity of each individual using a maximum likelihood approach. Allele frequencies were estimated from the data and updated every 1000 steps. Repeat analyses with random seeds for the random number generator varied slightly in their estimates. Therefore, the average of ten estimates for each colony was used to calculate the observed number of matings ( $k$ ).

The number of workers sampled per colony varied among populations, requiring a resampling (=rarefaction) approach to compare mating behavior without sample size bias (Tarpay and Nielsen, 2002). Thirty workers were randomly sampled without replacement from each colony for each resampled data set. The sample size of 30 workers per colony was selected because it presents our smallest actual colony sample and paternity estimates require as large sample sizes as possible (Tarpay and Nielsen, 2002). One-hundred resampled data sets were generated and separately analyzed with COLONY1.2 as before to obtain sample-size controlled values of the number of matings ( $k'$ ) that could be directly compared among all 18 colonies. Additionally, we calculated the effective paternity for each colony based on the full and the resampled data sets. After comparing the different estimators (Nielsen et al., 2003) we selected to express effective paternity as  $k_e$  (sample-size controlled:  $k_e'$ ) because  $k_{e2}$   $k_{e3}$  and did not produce meaningful results: their values were in some cases much larger than the observed number of matings  $k$ . Population differentiation of observed and effective paternity based on the means of the 100 re-samplings for each colony was assessed by one-way ANOVA, followed by Tukey's and Duncan's post-hoc tests, using SPSS 21.0 (IBM, New York, USA) after it was verified that the data did not deviate significantly from normality.

## Results

Every selected microsatellite locus was polymorphic in each population but the Ko Samui population was less variable in terms of allelic diversity and heterozygosity estimates (Table 2). Across the three populations, the estimates for the observed number of matings ( $k$ ) ranged between 5.0 and 24.1 mates per queen with an average of 15.6 and effective paternity estimates ( $k_e$ ) ranged from 3.6 to 18.2 with an average of 11.5 (electronic supplement Table S1).

Relative estimates of the number of matings based on the resampled sets of 30 workers per colony were slightly lower than the estimates based on the full data. The relative estimates demonstrated significant differences among populations for observed and effective numbers of mating ( $F_{(2,15)} = 19.2$ ,  $p < 0.001$ ;  $F_{(2,15)} = 19.1$ ,  $p < 0.001$ , respectively; Fig. 1). For the observed number of matings, Duncan's post-hoc test indicated significant differences among all 3 populations. In contrast, Tukey's post-hoc test suggested that the difference between the Phatthalung and Chiang Mai populations was not significant ( $p = 0.116$ ), even though both were significantly more polyandrous than the Ko Samui population (Phatthalung:  $p =$

0.003; Chiang Mai:  $p < 0.001$ ). Likewise, both post-hoc tests indicated that the effective paternity was significantly higher in the Phatthalung and Chiang Mai populations than in Ko Samui (Tukey's  $p = 0.002$  and  $p < 0.001$ , respectively), but the difference between Phatthalung and Chiang Mai was not significant ( $p = 0.215$ ).

## Discussion

In general, our finding that mating behavior varies among populations indicates that species-level estimates of the number of matings that derive from the study of a single population need to be interpreted cautiously. Intra-specific population differences may be as high as differences that have been reported between species, particularly in highly polyandrous taxa, such as honey bees. Most species-level estimates of the number of matings in social insects have been determined without considering such population differences. However, a few population comparisons of polyandry in social insects exist. Studies on African *A. mellifera* subspecies (El-Niweiri and Moritz, 2011; Franck et al., 2000) and the ant *Lasius niger* (Corley and Fjerdingstad, 2011) agree with our conclusion that population differentiation is important, in contrast to a study of the wasp *Vespula germanica* (Goodisman et al., 2002). Our study thus provides important support for the theoretical prediction that queen mating behavior in social insects depends on the local environment (Rueppell et al., 2008).

Our results significantly add to the previous estimate of the number of matings of *A. cerana*, which was based on only four colonies (Oldroyd et al., 1998). An earlier attempt to estimate *A. cerana* mating frequencies from multiple populations in Japan remained inconclusive due to the lack of genetic marker resolution (Rozalski et al., 1996). Overall, our study is consistent with the earlier estimate. The observed average of polyandry in our study across the three populations is below the previously reported value of 18.8 (Oldroyd et al., 1998; Tarpy et al., 2004). However, our smaller sample size per colony in some cases may have led to an underestimate of the true values (Tarpy and Nielsen, 2002). The consistency between our results and the previous study (Oldroyd et al., 1998) is remarkable, given the difference in sample origins and the heterogeneity among populations discovered within our study.

The identified population differences in polyandry can be used to illuminate the importance of the different hypotheses for the evolution of multiple mating in social insects. Contrasting the different hypotheses and quantifying their relative importance in population differentiation are urgently needed because many hypotheses of polyandry have some experimental support, making it difficult to decide which selective forces are actually responsible for the evolution of polyandry. Our results are incompatible with the predictions of the “diploid drone hypothesis” that multiple mating should be highest where the inbreeding risk is most severe: Ko Samui showed the lowest degree of polyandry. Instead, our data suggest that the environment is influential (Franck et al., 2000). Environmentally variable selection for increasing colony genetic diversity is more consistent with our observations than factors that are not as environmentally dependent, such as social kin-conflict, or sperm limitation. Genetic diversity may benefit disease resistance and division of labor. However, we lack sufficient knowledge on pathogen pressure and selection to

enhance division of labor in the different environments to distinguish between these two explanations of polyandry.

Given the spatial and genetic differentiation between the investigated populations (Rueppell et al., 2011), the observed population differences are presumably due to genetic differences between the populations (Foster, 1999). Yet, alternative explanations based on direct environmental influences on mating behavior cannot be excluded (Franck et al., 2000). Difficult reciprocal transplant or common garden experiments would be needed to distinguish between these two alternative explanations, phenotypic plasticity and genetic differentiation. Honey bee queens adjust their mating behavior to perceived costs (Hayworth et al., 2009), for example in response to higher rainfall (El-Niweiri and Moritz, 2011). Annual rainfall is higher in the southern part of Thailand, and particularly on Ko Samui, than in the north Chiang Mai province (<http://www.worldweatheronline.com/>). However, circumstantial evidence suggests that sexual production and mating of Thai *A. cerana* is not restricted to seasons of low precipitation.

In summary, our study confirms the notion that all species in the genus *Apis* exhibit a high level of polyandry and it confirms a previous estimate of polyandry in *A. cerana* (Oldroyd et al. 1998) with data from three distinct populations in Thailand. Furthermore, the data reveal significant intra-specific variation (El-Niweiri and Moritz, 2011; Franck et al., 2000), cautioning against the common practice of deriving species estimates of polyandry from a single population.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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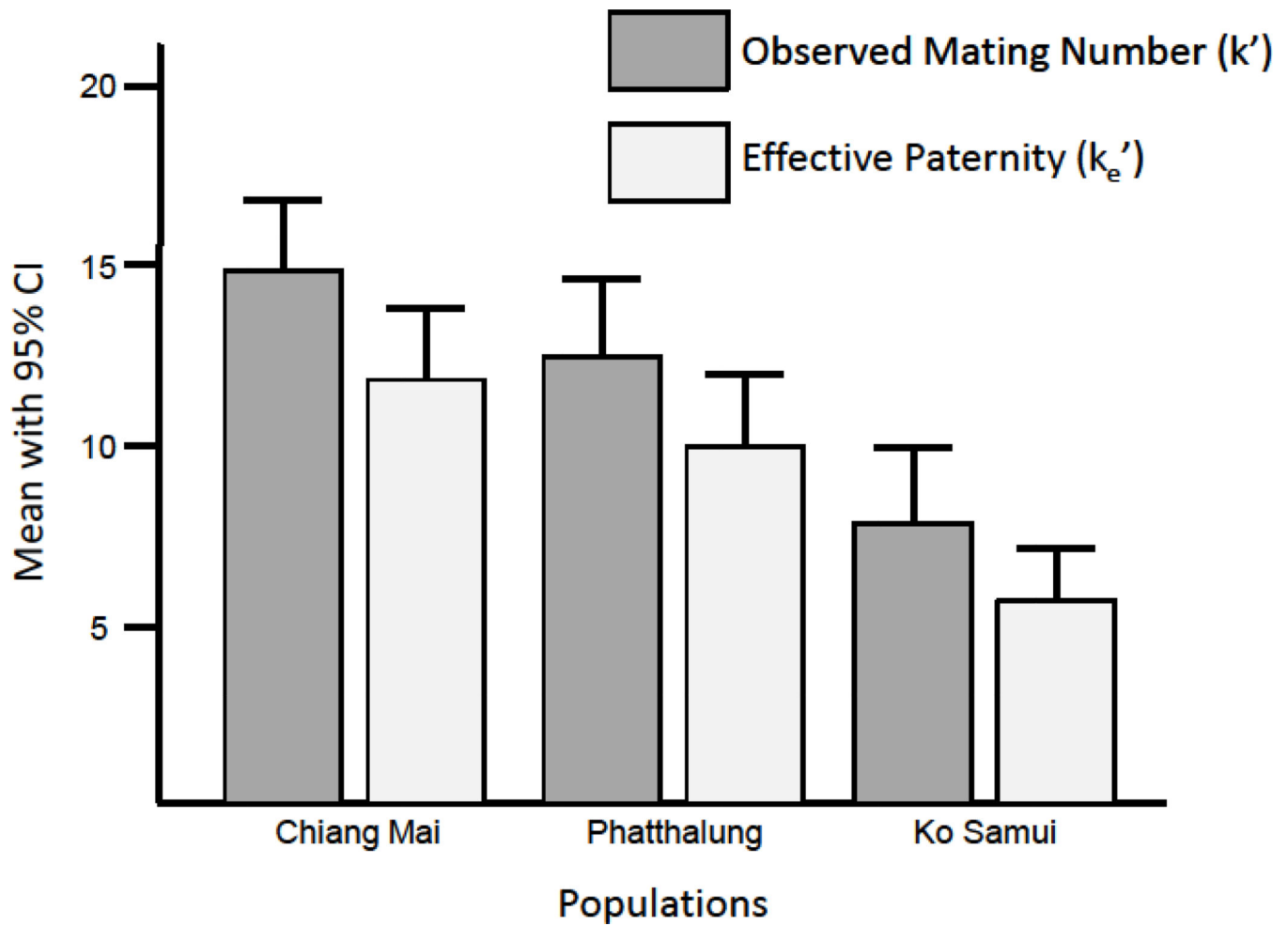
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**Figure 1.**

Sample-size controlled number of matings and effective paternity estimates differed significantly among the three investigated populations. The estimates of mating frequency were highest in the northern Chiang Mai population, followed by the tropical mainland Phatthalung population, and the tropical island Ko Samui population. The shown relative estimates of the number of matings and effective paternity are lower than estimates based on full sample sizes (see main text) but permit an unbiased comparison among colonies and populations.

**Table 1**

Microsatellite loci used in this study

<b>Locus</b>	<b>Tailed Forward Primer</b>	<b>Reverse Primer</b>
AC35 (Takahashi et al. 2009)	CACGACGTTGTAAAACGACTTCCAGCAGGAAGTGACGGT	CGGGAAACTCGTCATTTTCGA
AC033 (Solignac et al. 2007)	CACGACGTTGTAAAACGACTACGTTTGCTCTTCCACTCGG	AATGGCGTGATAACGGGC
UN379 (Solignac et al. 2007)	CACGACGTTGTAAAACGACTGAATTGAATGGTGTTAATACTAAATGC	GCACTGCTTGTTAATCTATGCCA
K0957 (Solignac et al. 2007)	CACGACGTTGTAAAACGACTATTGCGACGATTGCGGA	GGGCATGTATTAGTGGTTCGAT
OR8_29a (Rueppell et al. 2011)	CACGACGTTGTAAAACGACCCCGGTATGCAATATGTGTG	TACCATCGGTTCAACCAAAC

Table 2

Summary of the microsatellite characteristics in the three study populations

Locus	"Chiang Mai" Population			"Patthalung" Population			"Ko Samui" Population		
	$H_{obs} / H_c$	No. alleles	No. colonies / No. individuals <sup>#</sup>	$H_{obs} / H_c$	No. alleles	No. colonies / No. individuals <sup>#</sup>	$H_{obs} / H_c$	No. alleles	No. colonies / No. individuals <sup>#</sup>
AC35(Takahashi et al. 2009)	0.80 / 0.82	11	6 / 224	0.65 / 0.68	14	6 / 280	0.48 / 0.76	9	6 / 317
AC033	0.79 / 0.87	15	6 / 208	0.67 / 0.74	12	6 / 282	0.41 / 0.78	12	6 / 311
UN379	0.77 / 0.74	23	6 / 182	0.82 / 0.82	15	6 / 270	0.72 / 0.76	9	6 / 305
K0957	0.66 / 0.81	11	6 / 162	0.61 / 0.64	8	6 / 268	0.62 / 0.53	7	6 / 304
OR8_29a	0.51 / 0.73	18	6 / 190	0.56 / 0.66	10	6 / 247	0.38 / 0.73	11	6 / 275

<sup>#</sup>The number of individuals differ among loci due to missing genotype data