Pre-hibernation mating by a solitary bee, *Ceratina flavipes* (Hymenoptera: Apidae: Xylocopinae)

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Abstract

At 17 localities in Japan, 71–81% of overwintering adult females of the solitary bee *Ceratina flavipes* mated before hibernation. The occurrence of males with inseminated females in their natal nests suggested the possible occurrence of inbreeding. Therefore, we examined the seasonal trends of inbreeding coefficients, calculated by comparing the band patterns of microsatellite DNA between a female and her mating partners (indicated by sperm in her spermatheca). The mean coefficient of inbreeding was 0.80 on 25 August and 0.60 on 1 November versus 0.31 on 5 June and 0.38 on 28 June. These results demonstrate a tendency towards inbreeding before winter and towards outbreeding during dispersal after hibernation.

Keywords: Coefficient of inbreeding, hibernaculum, life cycle, microsatellite, sperm

Introduction

Whether solitary or social, all non-parasitoid aculeate wasps that have a hibernation season in their life cycle mate before hibernation (Michener 2000; Wilson 2000). Before the beginning of winter, males die off and the inseminated females hibernate to await the coming of spring (Wilson 2000). In the family Apidae, social bees such as honeybees and bumblebees also mate before hibernation (Wilson 2000). However, most species of solitary bees are assumed to mate after emergence in spring (Eickwort and Ginsberg 1980). For example, solitary bees of the genus *Osmia* overwinter as larva or pupa and mate after eclosion in spring (Medler 1967; Danforth 1991a, 1991b; Rust 1991; Stone 1995; Bosch and Vicens 2002). Even species that overwinter in the adult stage, such as *Xylocopa appendiculata* (Sugiura 1995), *Andrena agilissima* (Paxton et al. 1999), and *A. jacobi* (Paxton et al. 1996), are also believed to mate after hibernation. In these species, males overwinter and survive until the following spring, and females of *A. agilissima* have been observed to mate after hibernation (Paxton et al. 1996).
Previous studies by Sakagami and Maeta described the life cycles of some *Ceratina* spp. of solitary bee that overwinter as adults (e.g. Maeta and Sakagami 1955; Sakagami and Maeta 1987a, 1987b; Maeta et al. 1997). Because *C. japonica* mate after hibernation in Iwate prefecture in northern Japan, Sakagami and Maeta (1984) concluded that most, if not all, *Ceratina* species mate after hibernation (Sakagami and Maeta 1987b; Maeta et al. 1992; Sakagami et al. 1995). However, 48–100% of *C. megastigmata* females were inseminated before hibernation (Maeta and Katayama 1978; Katayama and Maeta 1979), and nearly all the overwintering females of *C. flavipes* were already inseminated in a coastal habitat near Sapporo, in northernmost Japan (Kidokoro et al. 2003), indicating that mating can occur before hibernation. Our objective was to examine the generality of mating by *C. flavipes* prior to overwintering.

**Materials and methods**

**Sampling of hibernating individuals**

In November and December 2002, we sampled overwintering *C. flavipes* at 17 Japanese localities (identified in Figure 1). At each locality, we searched dead grass shoots for *C. flavipes* hibernacula. We collected at least 30 females at each locality except Naraha and

![Figure 1. Percentage of pre-hibernation insemination of females at 17 localities in Japan. Values are 100% unless otherwise indicated. Cool-temperate zone: 1, Sapporo; 2, Yakumo; 3, Toi; 4, Esashi; 5, Matsumae. Temperate zone: 6, Tsugaru; 7, Shimokita; 8, Oga; 9, Miyako; 10, Morioka; 11, Sakata; 12, Matsushima; 13, Niigata; 14, Naraha; 15, Kaga; 16, Matsue; 17, Mt Sanbe.](image-url)
Matsue, where the nest density was low. We dissected the females under a microscope to confirm whether or not they had sperm in their spermathecae.

Survey of the life cycle in Sapporo

To examine the life cycle of *C. flavipes* in Sapporo, where the bees mate before hibernation (Kidokoro et al. 2003), we collected 10 wild nests at each sampling, 340 nests in total. We conducted sampling at intervals of 3 days before the hibernation season and before the nesting season, and at intervals of 6 days in the nesting season.

We counted the immatures, males, and females in each nest, and reared immatures on pollen masses to adulthood in a plastic case at room temperature (about 24°C). Females were dissected under a microscope to look for sperm in their spermathecae. The nesting period of *C. flavipes* is short, a few days, and the border with the following breeding season is vague, so that we included the nesting with the breeding season. Overwintered and newly emerged females were distinguished by the numbers of nicks on the wing margin (Packer and Knerer 1986).

Seasonal inbreeding as estimated by DNA analysis

Sakagami and Maeta (1984) suggested that newly emerged adults of *C. japonica* remain in their natal nests until they disperse to hibernation sites, and Kidokoro et al. (2003) found that some males and females of *C. flavipes* appeared to stay together in their natal nests and that the females were already inseminated, suggesting that inbreeding occurred before hibernation. Accordingly, we examined seasonal trends of inbreeding and outbreeding by using microsatellite DNA to calculate inbreeding coefficients.

We searched for microsatellite loci of *C. flavipes* by the magnetic bead method (Matthew et al., 1999). We collected 10 females on 25 August and 1 November 2002 and on 5 and 28 June 2003, preserved them in 100% ethanol at least for 5 days, and then placed each in a glass Petri dish filled with 100% ethanol to pick out spermathecae and thoracic muscle tissues under a binocular microscope. Each spermatheca was placed on another Petri dish and the sperm ball was extruded from the spermatheca without contamination by tissues of the female. The sperm ball was used for extracting DNA of male(s) that had copulated with the female, while the thoracic muscle tissue was used for extracting DNA of the female.

The protocol for DNA extraction from the thoracic muscle tissues was as follows (Katada’s method, unpublished): put the sperm ball in a 1.5-ml tube containing a mixture of 370 µl lysis buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% SDS), 20 µl proteinase K (10 mg ml⁻¹), and 10 µl RNase A (5 mg ml⁻¹). Incubate for 3 h in a 55°C water bath and centrifuge for 20 s at 10,000 rpm. Add 700 µl phenol–chloroform by gently rotating for 15 min. Centrifuge for 5 min at 15,000 rpm. Pipette off the aqueous phase into a new tube; add 40 µl CH₃COONa, 1 µl glycogen and 700 µl 70% ethanol. Store at −80°C for 30 min to overnight. After several replications of centrifugation for 12,000–15,000 rpm at 4°C, dispose of the supernatant by decanting and adding 180–200 µl 70% chilled ethanol, then dry the pellet in a vacuum concentrator and add 10 µl double-deionized water. Store in the refrigerator.

The protocol for DNA extraction from the thoracic muscle tissues was as follows (CTAB method): pick up the thoracic muscle tissues and dry by pressing between filter paper. Put them into a 1.5-ml tube with 10 µl CTAB buffer (2 g CTAB, 4 ml sterile 0.5 M EDTA (pH 8.0), 1 ml sterile 1 M Tris-HCl (pH 8.0), 28 ml sterile 5 M NaCl and sterile H₂O to
make up to 100 ml). Add 690 μl CTAB buffer, 45 mg polyvinylpyrrolidone and 10 μl proteinase K (20 mg ml⁻¹). Incubate at 55°C for 2 h. Add 700 μl chloroform: isoamylalcohol (24:1, v/v); centrifuge for 10 min at maximum rpm. Pipette off the aqueous phase and place into a new tube. Add 500 μl chloroform: isoamylalcohol; centrifuge for 10 min at maximum rpm. Pipette off the aqueous phase and place into a new tube. Add 400 μl isopropanol at −20°C; gently mix by tipping several times. Store for a few hours to overnight at −20°C. Centrifuge for 30 min at 12,000 rpm at 4°C. Pour off excess liquid by decanting. Add 180 μl 70% chilled ethanol to wash the pellet and mix by turning the tube. Centrifuge for 5 min at 10,000 rpm and throw away the supernatant by decanting. Dry the pellet in a vacuum concentrator and add 10 μl double-deionized water. Store in the refrigerator.

We amplified these DNA extracts by polymerase chain reaction (PCR) with primers specific to Ceratina microsatellite loci designed by Azuma et al. (2005). In a total volume of 19.7 μl containing 1.0 μl template DNA, 0.5 μM each primer, 0.2 mM dNTP mix, 2 μl reaction buffer, 1.5 mM MgCl₂, and 0.25 units Taq polymerase (Takara), all PCRs were performed as follows: initial denaturation at 93°C for 10 min; then 35 cycles of 30 s at 92°C, 30 s at 50°C, and 30 s at 72°C. The PCR products were analysed with 3100 GeneScan Software (Applied Biosystems) to automate measurement of allele length.

We estimated the allele frequencies by using the Relatedness 5.0 computer program (Goodnight and Queller 1994) with weighted equality, and calculated the coefficients of inbreeding (F) from Wright's equation (Crow 1983).

Results

Hibernation of females and males

In all localities, overwintering females found in grass hibernacula were inseminated at rates of 80–100% (Figure 1). The number of individuals (males and females) per hibernaculum ranged from 1 to 33 (Table I). Variation in this value across localities was not statistically significant (ANOVA: F(1,16) = 1.46, P = 0.113). The mean sex ratio (mean ratio of males to all individuals) was 0.14–0.56 (Table I), with no significant variation across localities (ANOVA: F(1,16) = 1.31, P = 0.188). We found only four dead males and two dead females in this survey, indicating very low mortality during the winter.

Life cycle in Sapporo

Overwintered males and females dispersed in spring, and accordingly by 30 May each nest was composed of one female or one male. Eggs were observed from 8 June to 12 August, so we regarded this period as the nesting season of this solitary bee species. In nests inhabited by older, overwintered females, female and male offspring started to eclose by 12 August. The coexistence of a mother and her daughters and sons was observed until 2 September, when we found some nests composed of only one new female or male, suggesting the beginning of pre-hibernation dispersal. In late August, more than 70% of females were inseminated (Figure 2), suggesting the frequent occurrence of mating between brothers and sisters in their natal nests. Since males that occurred subsequently during the last summer nesting season from the previous winter had died off by 18 August, these males probably did not contribute to the copulations. On 9 September, we found some new hibernacula, indicating the beginning of the hibernation season.
The rearing of the immatures in the laboratory demonstrated that many early-laid eggs grew up to be females (diploid), whereas most late-laid eggs were males (haploid). However, as shown in Table II, Spearman’s rank correlation between the order (date) of oviposition and the ratio of females was not statistically significant ($P=0.13$).

**Coefficients of inbreeding**

We detected eight polymorphic loci (Table III). The success rate of PCR was low in loci $Cera391$, $Cera363$, and $Cera556$, but relatively high in the others, although $Cera558$ had only two alleles. So we used four loci, $Cera512$, $Cera308$, $Cera92$, and $Cera368$. These loci exhibited heterozygosity from 0.48 to 0.87 (Table IV).

On 25 August, the mean ($\pm SE$) coefficient of inbreeding ($F$) was high at 0.80 $\pm$ 0.061 (Table V), suggesting the frequent occurrence of inbreeding within the natal nests. $F$ remained high at 0.60 $\pm$ 0.131 in the hibernation season (1 November), not significantly different from 0.80 (Bonferroni–Dunn test: $P=0.339$). On 5 June, however, $F$ was 0.31 $\pm$ 0.082, which was significantly different from 0.60 ($P=0.024$), suggesting the frequent occurrence of outbreeding during the post-hibernation nesting period. This value was not significantly different from 0.38 $\pm$ 0.105 on 28 June ($P=0.963$), suggesting that matings are rare in the breeding season, even though a few overwintered males survived until mid-August (Figure 2).

**Discussion**

The occurrence of pre-hibernation mating is premised on pre-hibernation eclosion, which may also enable the coexistence of a mother and her daughters, one of the conditions for the evolution of eusociality (Roubik 1989; Wilson 2000). The mother–daughter coexistence has been observed in some *Ceratina* species (Maeta and Sakagami 1955;
Sakagami and Maeta 1984; Maeta et al. 1997). Collaboration and polyethism occur among adult females artificially encased in glass tubes in *C. japonica*, *C. okinawana*, and *C. flavipes* (Sakagami and Maeta 1984, 1987a, 1987b; Sakagami et al. 1995), so that the genus

![Diagram](image)

Figure 2. (A) Seasonal fluctuations in the numbers of adult females and males per nest; (B) the insemination rate of females and coefficients of inbreeding.

Table II. Relationship between order of oviposition and ratio of females reared in the laboratory.

| Order of oviposition | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | Total |
|---------------------|----|----|----|----|----|----|----|----|----|----|----|----|-------|
| N                   | 21 | 21 | 20 | 15 | 13 | 12 | 9  | 7  | 5  | 4  | 3  | 2  | 132   |
| No. of females      | 16 | 12 | 13 | 12 | 3  | 4  | 4  | 1  | 5  | 0  | 2  | 2  | 73    |
| Ratio               | 0.76| 0.57| 0.65| 0.80| 0.23| 0.33| 0.44| 0.71| 0.00| 0.50| 0.67| 0.00| 0.55  |

Spearman’s rank correlation between the sex ratio and the order of oviposition was $-0.456$, not statistically significant ($P=0.1302$).
Ceratina has been considered as almost solitary but at the initial stage of the evolutionary process for eusociality. This recognition was supported by our result, the high possibility of inbreeding of *C. flavipes*, because the occurrence of inbreeding in haplo-diploids is one of the predisposing factors for the evolutionary origin of eusociality (Linksvayer and Wade 2005). Moreover, eusociality will often arise in species where the female overwinters as an inseminated adult (Seger 1983; Linksvayer and Wade 2005).

Most solitary bees, even those that overwinter as adults, are believed to mate after hibernation (Alcock 1995; Michener 2000). Post-hibernation mating has been observed in some adult-overwintering species (Sakagami and Maeta 1984; Alcock 1995, 1997). In this study, the results of DNA analysis, the variations of the coefficient of inbreeding, indicated that almost all females of *C. flavipes* mate at least twice, first with their brothers (inbreeding) before hibernation and then later with an unrelated male or males (outbreeding) after hibernation. Furthermore, on 5 June, after hibernation, the DNA bands of sperm were a single band at four loci, with a few exceptions, even though the DNA bands of sperm stored at the spermathecae of the females mated multiply should show multiple bands. It may indicate occurrence of sperm replacement.

Our results suggest that the spermathecae of all adult-overwintering solitary bees should be dissected to determine whether or not the females have been inseminated before overwintering. Since bees generally mate shortly after eclosion (Alcock 1979; Eickwort and

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**Table III. Microsatellite DNA primers developed (the top four were used in the present study).**

| Locus name | No. of alleles | Repeat pattern | Primer sequence (5’–3’)a | $T_a$ (°C) | GeneBank accession no. |
|------------|----------------|----------------|--------------------------|-----------|-----------------------|
| Cera512    | 8              | (CT)16         | F: TCTCTTTCCTAGCCCTCTGTC R: TGCTACCGACGAGAGATAG | 50        | AB182397             |
| Cera308    | 6              | (CT)7          | F: TCTCACTATACGCCGCTGTA R: AGTTGTCGTTGTCGAGGAAGA | 50        | AB182398             |
| Cera92     | 4              | (GTC)3–(GTC)9 | F: CCAAAGAAAAGAGAAAATC R: TCGTATTTCGACCCAGGA | 45        | AB182402             |
| Cera368    | 3              | (CT)10         | F: AATTCGCCAGTTCCTG | 45        | AB182404             |
| Cera391    | 7              | (CGT)9         | F: TCTTTGCTCGGTACTC R: AGAGGCAACGGGAATAGCT | 50        | AB182399             |
| Cera558    | 2              | (GCA)5         | F: ATCAGCTACGCAGCAACA R: ACCTGCTGCAACTGACT | 50        | AB182400             |
| Cera363    | 6              | (GCA)6–(GCA)5 | F: CCCACCAACACGATAG C | 54        | AB182403             |
| Cera556    | 7              | (CT)13         | F: GCCATCGTGAAAAATATCGA R: ACGAAGCGGAAAGGTTTA | 50        | AB182404             |

_aF, forward; R, reverse._

**Table IV. Allele frequency and expected heterozygosity ($H_E$) of four microsatellite loci used in the present study.**

| Locus   | a   | b   | c   | d   | e   | f   | g   | h   | $H_E$ |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-------|
| Cera512 | 0.052 | 0.278 | 0.186 | 0.134 | 0.258 | 0.021 | 0.041 | 0.031 | 0.87   |
| Cera308 | 0.806 | 0.103 | 0.057 | 0.011 | 0.011 | 0.011 |       |       | 0.48   |
| Cera92  | 0.616 | 0.110 | 0.041 | 0.233 |       |       |       |       | 0.57   |
| Cera368 | 0.462 | 0.225 | 0.312 |       |       |       |       |       | 0.57   |
Ginsberg 1980), it is likely that most, if not all, adult-overwintering bees mate before hibernation.

Although males are relatively ephemeral in many species of Hymenoptera, no matter whether social or solitary, our data demonstrated long-living males of *C. flavipes*. Two causes may be considered for the longevity of males of *C. flavipes*, one is the phylogenetic trait and the other is sperm replacement. The subfamily of genus *Ceratina* is Xylocopinae. Males of some Xylocopine bees, subfamily Xylocopinae, are relatively long-lived and occasionally exhibit territoriality (Alcock 1995). The mean life span of *C. flavipes* males is approximately 9 months, which is exceptional even among the Xylocopinae. This exceptional longevity may be partly associated with sperm replacement, whereby later-mating males have an advantage (Thornhill and Alcock 1983).

Under the condition of haplo-diploidy, which has been considered to reduce inbreeding depression (Axelrod and Hamilton 1981), pre-hibernation mating seems adaptive not only for males (Alcock 1995), but also for females, as an insurance against failure to mate after hibernation. If the female, dispersed to the new nesting location after hibernation, succeeds in mating with non-related males, the offspring may be given the advantage rather than inbred individuals.

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