Inbreeding, multiple mating and foreign sexuals in the ant *Cardiocondyla nigra* (Hymenoptera: Formicidae)

Alexandra SCHREMPF

**Abstract**

In the monogynous ant species *Cardiocondyla nigra* FOREL, 1905, wingless males mate with young female sexuals inside the nest. As a single mother queen produces all sexual offspring of a colony, mating is usually among siblings and a high level of inbreeding is expected. Despite of this, occasional outbreeding has been reported recently for two other monogynous species of the same monophyletic group and with the same mating system ("palearctic clade", OETTLER & al. 2010, *C. elegans* EMERY, 1869: LENOIR & al. 2007, *C. batesii* FOREL, 1894: SCHREMPF & al. 2005). Matings between unrelated individuals appear to be promoted by the active transfer of virgin queens into alien nests in one of these species (*C. elegans*: LENOIR & al. 2007). In the present study, I investigated the colony and population structure of *C. nigra* in order to examine whether this phenomenon might also contribute to outbreeding in *C. nigra*. Data revealed that more than 90% of the queens are polyandrous, and that approximately 34% of the matings are not among siblings. Moreover, in one population, "foreign" female sexuals co-occurred with workers together within one nest chamber. Thus, the exchange of sexual offspring between ant colonies might sustain genetic diversity in monogynous *Cardiocondyla* species.

**Key words:** Inbreeding, outbreeding, intranidal mating, polyandry, wingless males.

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**Introduction**

In many social insects, mating occurs in huge swarms on the wing, and it is generally assumed that this mode of mating decreases the likelihood of pairings between related individuals and thus promotes outbreeding and panmixia (HÖLLDOBLER & WILSON 1990, but see, e.g., THOMPSON & al. 2007).

In contrast, in all species of the ant genus *Cardiocondyla*, mating takes place within the nest, as wingless, "ergatoid" males occur that stay in the colony and copulate with freshly emerging virgin queens (e.g., HEINZE & HÖLLDOBLER 1993). According to that, matings among related individuals are expected.

Yet, the degree of inbreeding may vary drastically among species of the genus. Several species are still able to produce winged disperser males, and / or are highly polygynous, and thus, sexuals may easily mate with unrelated individuals and conduct – at least from time to time – outbreeding. Other species, however, have completely lost the winged male morph and produce only ergatoid males. Beyond that, several of these species with only ergatoid males are strictly monogynous ("palearctic clade" according to OETTLER & al. 2010). As sexual offspring mate within the nest and females leave the colonies only thereafter on foot, mating in the latter species is expected to occur solely between brothers and sisters, the offspring of the single reproductive queen. Yet, surprisingly, genetic data suggest a considerable amount of outbreeding in two investigated species of this monogynous group with only ergatoid males (*Cardiocondyla batesii* FOREL, 1894: SCHREMPF & al. 2005, *C. elegans* EMERY, 1869: LENOIR & al. 2007). In their study of *C. elegans*, LENOIR & al. (2007) were able to show that workers carry winged female sexuals into alien nests, where they presumably mate with unrelated males. This active transport of sexuals through workers thus strongly promotes outbreeding. Although such a carrying behavior has not been observed in the second so far investigated species *C. batesii*, the fact that queens fertilize their eggs with sperm of unrelated males (SCHREMPF & al. 2005) suggests a similar exchange of sexual offspring.

In this study, I investigated the genetic structure (relatedness between individuals, level of inbreeding and multiple mating) of colonies of *Cardiocondyla nigra* FOREL, 1905, a further species of this "palearctic clade". Aim of this study was to determine if the transfer of sexuals to promote outbreeding and maintain genetic diversity might be more widely distributed in this species group.

Colonies of *Cardiocondyla nigra* are usually small, with one to several dozens of workers and one reproductive queen per colony. In autumn, colonies produce several short-winged female sexuals and a few mutually tolerant ergatoid males. Sexuals mate within the nest, and young, inseminated queens hibernate inside the colony without re-
producing. Probably as an adaptation to intranidal mating, female sexuals do not use their wings any more and have replaced their wing muscles by fat as they disperse on foot. In spring, they shed their wings (likely correlated with the imminent egg laying) and leave the colony to find appropriate places for colony foundation in a semi-claustral way (A. Schrempf, unpubl.; as in *C. batesii*, HEINZE & al. 2002). The high number of queens produced in the colonies in autumn probably reflects the low success in colony foundation of the queens (less than 25% in the laboratory, expected to be much lower in the field; SCHREMPF & HEINZE 2007). Although several mated, young queens can be found in the colonies from autumn to early spring, colonies are strictly monogynous, and the experimental removal of the reproducing queen in spring results in fighting until only a single young queen stays alive inside the colony and begins to reproduce.

**Material and methods**

For this study, a total of 33 colonies of *Cardiocondyla nigra* were collected in March 2003 in the southern part of Cyprus at three different collecting sites (in the vicinity of Limassol, Alassa and Kalavasos, Table 1; distances (km) approximately: L. – A.: 15 km; L. – K.: 36 km; A. – K.: 49 km). Worker numbers were moderate (mean ± standard deviation, SD: 28.5 ± 35.3, min. 1, max. 160) and colonies contained a single reproductive queen. In 24 of the 33 colonies, several winged queens (mean ± SD: 32.1 ± 30.4, min. 4, max. 122; Tab. 1) could be found close together in the uppermost nest chamber, approximately 5 to 10 centimetres below the soil surface. In some colonies, queen numbers were even higher than worker numbers. Dissections of all queens from four colonies (before genetic analysis) confirmed the occurrence of only a single reproductive queen with yellow bodies, mature eggs and eggs in development. All young queens (one already wingless) had a full spermatheca but lacked eggs and developed ovaries.

To investigate colony relatedness, the amount of inbreeding and the occurrence of polyandry, I genotyped several workers of 33 colonies (for sample size see Tab. 1) from the three different collecting sites at the five available variable microsatellite loci (Card 8, Card 21; SCHREMPF & al. 2005, developed for *Cardiocondyla batessii* and CE-3A, CE-4E, CE 12-D, LENOIR & al. 2007, developed for *C. elegans*). In addition, I analysed also several winged female sexuals (Tab. 1) to determine whether they all were (full or half) sisters of the workers or whether some were “alien”, i.e., unrelated female sexuals (e.g., "imported" by workers in autumn, as described in *C. elegans*, see introduction). After the occurrence of such unrelated winged female sexuals in colonies from the Alassa population, I increased the sample size in these colonies to corroborate the results. However, as a high number of queens had been already used for other experiments, it was not always possible to increase the number of genotyped queens, but slightly more queens could be analysed from this population (Tab. 1). DNA for microsatellite analysis was isolated with an isolation kit (Puregene DNA Isolation Kit, Gentra Systems, Minneapolis), before standard PCR reactions were carried out and analysed with an ABI Prism 310 Genetic Analyzer as described in SCHREMPF & al. (2005).

Coefficients of inbreeding and population subdivision were estimated from worker and female genotypes in a three-level analysis with the program Genetic Data Analysis (GDA) 1.1 (LEWIS & ZAYKIN 2001), based on the algorithms by WEIR & COCKERHAM (1984). Confidence intervals were obtained by bootstrapping 5000 times over loci. In addition, I estimated the maximum frequency of potential null alleles as \( r = D / (2 – D) \), where \( D = (H_{\text{exp}} – H_{\text{obs}}) / H_{\text{exp}} \) (CHAKRABORTY & al. 1992, BROOKFIELD 1996), because null alleles can also cause heterozygote deficiency. Allelic richness was determined with FSTAT 2.9.3.3.2 (GOUDET 2002). Regression relatedness (QUELLER & GOODNIGHT 1989) among colony members was calculated using the program Relatedness 4.2 (GOODNIGHT & QUELLER 1994). Groups were weighted equally and standard errors were estimated by jackknifing over loci. Finally, mating frequencies of queens were calculated with the program MateSoft 1.0 (MOILANEN & al. 2004) by deducing the queen genotype from their female offspring and subsequent comparison of the alleles of the mother and their offspring using the broad deduction option (as multiple mating seems to be frequent).

**Results**

A total of 399 individuals (237 workers, 162 winged queens, Tab. 1) of three populations were genotyped. The alleles
after PAMILO 1985, see Tab. 3).

For the number of alleles, allelic richness and expected and observed heterozygosity, see Tab. 2 of all workers and the queen offspring were consistent with a monogynous queen mating frequency me,p (with average number of matings k and the range of the number of mates) in colonies from three populations of Cardiocondyla nigra.

<table>
<thead>
<tr>
<th>Locus</th>
<th>H exp</th>
<th>H obs</th>
<th>f</th>
<th>N alleles</th>
<th>Allelic richness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Card 8</td>
<td>0.60</td>
<td>0.48</td>
<td>0.19</td>
<td>9</td>
<td>2.62</td>
</tr>
<tr>
<td>Card 21</td>
<td>0.77</td>
<td>0.52</td>
<td>0.33</td>
<td>18</td>
<td>3.70</td>
</tr>
<tr>
<td>CE-3A</td>
<td>0.57</td>
<td>0.42</td>
<td>0.27</td>
<td>6</td>
<td>2.50</td>
</tr>
<tr>
<td>CE-4E</td>
<td>0.40</td>
<td>0.28</td>
<td>0.30</td>
<td>3</td>
<td>1.87</td>
</tr>
<tr>
<td>CE-12D</td>
<td>0.85</td>
<td>0.56</td>
<td>0.34</td>
<td>9</td>
<td>4.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Population</th>
<th>H exp</th>
<th>H obs</th>
<th>f</th>
<th>N alleles (Loci 1 - 5)</th>
<th>Allelic richness (Loci 1 - 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kalavasos</td>
<td>0.57</td>
<td>0.41</td>
<td>0.28</td>
<td>6 / 4 / 5 / 3 / 7</td>
<td>3.51 / 3.00 / 2.00 / 2.05 / 2.06</td>
</tr>
<tr>
<td>Limassol</td>
<td>0.49</td>
<td>0.41</td>
<td>0.16</td>
<td>8 / 5 / 2 / 2 / 6</td>
<td>3.70 / 2.53 / 1.40 / 2.00 / 1.76</td>
</tr>
<tr>
<td>Alassa</td>
<td>0.62</td>
<td>0.49</td>
<td>0.21</td>
<td>6 / 15 / 6 / 3 / 8</td>
<td>2.30 / 3.90 / 1.60 / 1.80 / 2.50</td>
</tr>
</tbody>
</table>

Tab. 3: Regression relatedness R (± standard deviation), relatedness values corrected for inbreeding R* (after PAMILO 1985), inbreeding coefficient F (with upper and lower 95% CI) and estimated queen mating frequency me,p (with average number of matings k and the range of the number of mates) in colonies from three populations of Cardiocondyla nigra.

<table>
<thead>
<tr>
<th>Population</th>
<th>Relatedness R</th>
<th>Relatedness R*</th>
<th>Inbreeding F (95% CI)</th>
<th>Mating frequency me,p (k; number of mates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kalavasos</td>
<td>0.61 ± 0.06 (n = 130)</td>
<td>0.26</td>
<td>0.31 (0.45 - 0.18)</td>
<td>4.16 (2.99; 1 - 5)</td>
</tr>
<tr>
<td>Limassol</td>
<td>0.54 ± 0.08 (n = 66)</td>
<td>0.34</td>
<td>0.18 (0.38 - 0.12)</td>
<td>2.69 (2.29; 1 - 4)</td>
</tr>
<tr>
<td>Alassa all individuals included,</td>
<td>0.31 ± 0.04 (n = 203)</td>
<td>-0.07</td>
<td>0.22 (0.30 - 0.14)</td>
<td>5.41 (3.7; 2 - 4, and 7)</td>
</tr>
<tr>
<td>without alien females</td>
<td>0.57 ± 0.06 (n = 142)</td>
<td>0.31</td>
<td>0.24 (0.32 - 0.14)</td>
<td></td>
</tr>
<tr>
<td>Over all populations</td>
<td>0.45 ± 0.04 (n = 399)</td>
<td>-0.09</td>
<td>0.33 (0.36 - 0.27)</td>
<td>3.84 (2.92)</td>
</tr>
<tr>
<td>all individuals included,</td>
<td>0.58 ± 0.04 (n = 338)</td>
<td>0.11</td>
<td>0.34 (0.39 - 0.27)</td>
<td></td>
</tr>
<tr>
<td>without alien females</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The inbreeding coefficient F was positive at both loci. Heterozygote deficiency was significant both over all populations as well as in each single population (without significant differences among the populations: Alassa: 0.31, Limassol: 0.18; Kalavasos: 0.22; over all: F = 0.33; upper 95% CI: 0.36, lower 95% CI: 0.27; ANOVA over all populations: p = 1.21, df = 2, F = 1.64; Tab. 3). This indicates mating between related individuals, as expected (equates to 66% sib mating; estimated from F = a (4 - 3a); SUZUKI & IWASA 1980, PAMILO 1985). Assuming that the heterozygote deficiency is caused by null alleles or large allelic dropout alone, frequencies of 11.0% (locus 1), 18.5% (locus 2), 15.0% (locus 3), 17.6% (locus 4) and 20.0% (locus 5) of such alleles would be required. With these frequencies, a lack of amplification products in 1.2% (3 individuals, locus 1), 1.6% (3 individuals, locus 2), 2.2% (9 individuals, locus 3), 3.0% (12 individuals, locus 4) and 4.0% (16 individuals, locus 5) can be expected (r²).

In this study, the successful amplification of a PCR-product was not possible in the first two loci in six (locus 1; 1.6%) and five cases, respectively (locus 2; 1.3%). Thus, whereas null alleles could explain heterozygote deficiency at the first locus theoretically, it is unlikely that they alone account for the heterozygote deficiency at the second locus. In the remaining three primer pairs that were usually developed for Cardiocondyla elegans and conducted in addition only recently, PCR reactions were very sensitive to the DNA concentration and purity, and a successful amplification was not always possible (in 32, 30 and 38 samples, respectively), sometimes simply due to an insuffici-
ent concentration of left DNA. Hence, also in these, null alleles could explain the heterozygote deficiency theoretically. However, as values of θ were similar across all loci and results could be confirmed after adding three more loci to the analysis and are consistent across all loci, this is unlikely.

The value of \(p_0\) suggested a moderate differentiation of the three populations (\(p_0 = 0.11\); upper: 95% CI: 0.159, lower 95% CI: 0.036).

In accordance with the finding of unrelated individuals in eight colonies, MateSoft excluded those colonies from the analysis by classifying them as polygynous. For all other colonies, the power to deduce correctly the queen phenotype was high (>0.98 in 24 colonies and 0.92 in another colony). The average estimated and corrected mating frequency over all groups obtained from MateSoft was \(m_{\text{per}} = 3.84\) (average \(p_1\) over all groups = 0.51). The average number of matings detected based on the frequency distribution of the number of patrilines per group was \(k = 2.85\) (minimum average number of matings estimated from the smallest number of matings found per group \(k_{\text{min}} = 2.92\)). In three colonies (9%; Kalavasos: one colony; Limassol: two colonies), queens mated only once, whereas in the others usually two to five males contributed to worker offspring. Yet, in a single case, seven males contributed to the offspring, resulting in a higher calculated mating frequency in this population (Alassa, Tab. 3).

**Discussion**

The study indicates that queens of *Cardiocondyla nigra* are facultatively polyandrous and regularly mate with several males. Moreover, genotypes of colony members as well as the inbreeding coefficient clearly reveal that queens not only mate with their brothers but also with unrelated males.

Even though I cannot exclude that null alleles or large allelic dropout might influence heterozygote deficiency in the study, they are unlikely to account for the calculated amount of inbreeding alone, at least with regard to the second locus. Beyond that, all five loci support the same results and conclusions. Finally, taking the mating biology of the species into account, the level of inbreeding is rather extraordinary and unexpectedly low.

In any case, the data indicate that, similar to what has been found in *Cardiocondyla elegans* (see LENOIR & al. 2007), colonies may contain unrelated female sexuals. Strikingly, alien female sexuals were detected only in colonies in one of three study populations, despite of the lack of obvious ecological differences between the collecting sites. Genetic analysis revealed that the level of inbreeding is high in all three populations sampled and mating between siblings represents more than 66% of all matings. However, about one third of the matings are among unrelated individuals. The absence of alien female sexuals in the colonies from two populations may be due to low sample size, bias of genetic markers or temporal irregularities of the exchange of sexuals. Alternatively, ergatoid males rather than female sexuals might move between the colonies, as observed in *C. emeryi* FOREL, 1881 (see BOLTON 1982).

Ergatoid males are produced in autumn and do not hibernate, and therefore it would be necessary to collect colonies during autumn to detect if males also enter other colonies. LENOIR & al. (2007) found alien males in one colony of *Cardiocondyla elegans* and showed that males are easily adopted into alien nests in the laboratory. Similar adoptions were also easily possible in *C. nigra* in the laboratory (ten males in ten colonies: all accepted, A. Schrempf, unpubl.). Males might try to enter alien colonies whenever no females are available in their own nest or after insemination of all female sexuals inside their natal nest. As the testes of ergatoid males do not degenerate (in contrast to what is the case in other ant species) and they are thus not sperm-limited (HEINZE & HÖLDDOBLER 1993), they are expected to try to inseminate as many female sexuals as possible.

It might simply be that, for a female transfer, special environmental conditions are needed, and that these conditions do not necessarily occur every year in each population and not in each single colony or are even lacking in two of the three populations.

Alternatively, the active transfer of females might simply depend on nest density or available workers in the colonies. Yet, nest density was not obviously different between the populations (A. Schrempf, unpubl.), speaking against the former. Similarly, average worker numbers are even higher in the populations of Kalavasos (average number of workers: 46.0 ± 36.5) and Limassol (38.1 ± 46.3) in comparison to Alassa (12.9 ± 13.1), also making the latter explanation unlikely. However, the numbers of queens in colonies from Alasia often exceed the number of workers in the colonies, generally reflecting the high investment into reproductive offspring at the end of the season. It might be that the transfer of sexuals is only initiated after the occurrence of a huge number of queens in the colonies, and that these queens are produced at the expense of worker offspring. Under these conditions, several queens are still able to stay in the own colony to hibernate, while “surplus” queens are transferred into other colonies to increase the chance that at least some of them survive the cold season.

In any case, it seems rather unlikely that sample size was too low to detect alien sexuals in the other two populations, as I observed this phenomenon in eight of 14 colonies (with female sexuals) in the Alasia population, i.e., in more than 50% (before increasing the sample size in queen number, see Material & methods). Given the fact that I investigated six and four colonies with female sexuals in the other populations, the probability to miss this event in all of them seems to be pretty low.

In accordance with multiple mating, relatedness values in the colonies were around \(R = 0.58\), as expected for half-siblings with one mother and several fathers. The increased genetic diversity in the colonies might counteract possible negative effects of inbreeding, even though species of the genus generally seem to get along well with mating between related individuals. For example, it has been shown that sex is not determined by single locus complementary sex determination system as usual in the Hymenoptera (see, e.g., COOK 1993), and that the production of diploid males is therefore prevented (SCHREMPF & al. 2006). Yet, increased genetic diversity might still be valuable with regard to the immune system (SCHMID-HEMPEL 1998, HUGHES & BOOMSMAN 2004, 2006, ÜGELVIG & al. 2010) or to better react and/or tolerate varying environmental conditions (BOOMSMAN & RATNIK 1996). As monogyny is derived in this genus (SCHREMPF & HEINZE 2007), polyandry might have evolved in those species that have lost the genetic
diversity through polygyny. Keller & Reeve (1994) suggested an optimal level of genetic diversity, and this has been supported on an intraspecific level, e.g., in the bulldog ant (Qian & al. 2011). It remains to be investigated whether polyandry and polygyny are negatively correlated in the genus Cardiocondyla to reach an ideal degree of genetic diversity.

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