

# Genetic structure and reproductive strategy of the ant *Cardiocondyla elegans*: strictly monogynous nests invaded by unrelated sexuals

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## Abstract

*Cardiocondyla elegans* is a Mediterranean ant that nests on river banks. It rears only wingless (ergatoid) males that live peacefully in the same nest as opposed to other species of the same genus, which have both peaceful, winged and mutually aggressive 'ergatoid' males. Using microsatellite analysis, we investigated the genetic structure of 21 colonies from three different locations as well as the parentage of sexuals of two colonies of *C. elegans*. We show that *C. elegans* is strictly monogynous, and that its nests can contain foreign sexuals. The presence of alien sexuals inside ant nests is described for the first time and probably counteracts inbreeding resulting from matings between siblings. In the laboratory, aggression tests showed that workers only allow alien males to enter their nests, while all winged female sexuals attempting to enter were attacked. Nevertheless, the presence of alien female sexuals in nests in the field seems to result from active carrying behaviour by workers during the reproductive period.

**Keywords:** *Cardiocondyla*, multiple mating, colony genetic structure, ergatoid males, inbreeding, intranidal mating

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## Introduction

Sexual selection is one of the most powerful forces in evolution and has led to an extreme diversity in mating strategies. Males compete with other males over access to females and therefore typically have evolved effective weapons, costly ornaments, or elaborate courtship displays (Andersson 1994). Social insects are a striking exception to this rule, because here mating is usually an inconspicuous event restricted to a brief period of frantic activity. Thus, male adaptations to harm or kill rival males appear to be extremely rare and occur only where males can obtain and defend a harem of queens, i.e. when mating occurs in the nest (Boomsma *et al.* 2005). In insects in general, local mating in such limited and confined areas has repeatedly led to the evolution of male polymorphisms with winged disperser males and territorial, wingless or wing-reduced fighter males (Cook *et al.* 1997), and several cases are also known from Hymenoptera (e.g. Kukuk & Schwarz 1988;

Danforth 1991; Danforth & Neff 1992; Danforth & Desjardins 1999). In ants, wingless fighter males (ergatoid males) are known only from two genera, *Hypoponera* (Hamilton 1979; Yamauchi *et al.* 1996) and *Cardiocondyla* (Kinomura & Yamauchi 1987; Stuart *et al.* 1987; Heinze & Hölldobler 1993; Cremer *et al.* 2002a; Cremer & Heinze 2003).

The genus *Cardiocondyla* is of particular interest, as male tactics and colony structure vary considerably between species. Ergatoid, wingless males as well as 'normal', winged males mate within the colony with young, virgin queens. *C. obscurior*, *C. minutior*, *C. mauritanica*, and *C. emeryi* are polygynous (several queens per colony) and ergatoid males engage in lethal fighting for access to virgin queens (Kinomura & Yamauchi 1987; Stuart *et al.* 1987; Heinze & Hölldobler 1993; Heinze *et al.* 1998). The winged males of these species are usually tolerated by the ergatoid males, probably because they chemically resemble virgin queens (Cremer *et al.* 2002b). After a short period of mating in the nest, winged males emigrate to mate with virgin queens from other colonies. *C. mauritanica* and *C. kagutsuchi* from Okinawa are polygynous, but in contrast to the other polygynous species have lost winged males (Heinze &

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Hölldobler 1993; Heinze *et al.* 2005). Finally, *C. elegans*, *C. batesii*, *C. ulianini* and several other Eurasian species are monogynous (a single queen per nest) and also lack winged males. In contrast to the polygynous species, ergatoid males of monogynous species appear to be mutually tolerant (Boomsma *et al.* 2005; Schrempf *et al.* 2005).

While several previous studies have focused on polygynous *Cardiocondyla*, comparatively little is known on monogynous species. Here we study *Cardiocondyla elegans* (Emery, 1869), a species lacking both winged males and seemingly also precluding dispersal of the female sex (ergatoid males are supposed to stay in their maternal nest during all their life and consequently mate with sisters, like in other *Cardiocondyla* species), which is supposed to lead to inbreeding. As in other *Cardiocondyla* (Heinze & Hölldobler 1993), ergatoid males have a continuous spermatogenesis and are able to mate with numerous winged female sexuals in the nest during the whole mating period in late summer and fall. Thereafter, males die and the inseminated, but still winged, female sexuals remain inside the nests for hibernation. They leave their nests and disperse on the wing or on foot in spring. We investigated how this peculiar life history affects the colony and population structure and how inbreeding depression is avoided.

## Materials and methods

### Study site and species

*Cardiocondyla elegans* colonies are monogynous (only one wingless queen was found in each excavated nest) and have only mutually tolerant ergatoid males. One nest can contain several hundred workers (mean  $\pm$  SD = 204.43  $\pm$  182.09; range: [15–802],  $N = 42$ ), about 5 males (5.27  $\pm$  4.31; range: [1–17],  $N = 37$ ) and dozens of winged females (76.64  $\pm$  78.15; range: [2–367],  $N = 42$ ) (J-C Lenoir & J-L Mercier unpublished data).

We investigated 226 workers from 21 colonies collected during the summer (June till August) 2004 at three different sites separated by at least 200 km: Montlouis sur Loire (Indre et Loire, France, colonies 'M'), Carennac and Pinsac (Lot, France, colonies 'C' and 'P'), and Chemilly (Allier, France, colonies 'A'). All studied populations were located on the sand banks of the rivers Loire, Dordogne, and Allier, respectively.

For one colony each from Montlouis sur Loire and Chemilly, we also genotyped all males found in the nest ( $N = 8$  and 3), 10 winged females, and the sperm of their spermathecae.

### Microsatellite analysis of individuals and sperm

Five specific polymorphic microsatellites of *Cardiocondyla elegans* (CE2–3A, CE2–4A, CE2–5D, CE2–12D and CE2–4E)

(Lenoir *et al.* 2005) were used to determine the genotype of workers, males, winged females and the sperm. DNA was extracted from individual ants using a Puregene DNA Isolation Kit (Gentra Systems), according to Foitzik & Herbers (2001). For the analysis of sperm, winged females were kept in 96% ethanol for approximately 10 days to dehydrate the tissue. Thereafter, they were dissected in distilled water, the spermatheca was isolated and its membrane was crushed to release the dried pellet of sperm. Thus, only male material was used for sperm analysis. Sperm DNA was extracted using the protocol by Chapuisat (1998): sperm was incubated for 2 h at 37 °C in a final concentration of 0.05 mg/mL proteinase K, 1.75  $\mu$ M SDS and 20 mM DTT. Proteinase K was then inactivated by heating the lysate at 95 °C for 10 min and this solution was used in polymerase chain reactions (PCR).

PCR (Biometra T1 thermocycler, Whatman) was carried out in a final 20  $\mu$ L reaction volume containing 1–50 ng DNA, 10 $\times$  polymerase buffer (50 mM KCl, 0.1% Triton X-100, 10 mM Tris-HCl), 2 mM MgCl<sub>2</sub>, 200  $\mu$ M per dNTP (MBI Fermentas), 0.6  $\mu$ M unlabelled reverse primer, 0.6  $\mu$ M labelled (TET, FAM and HEX dyes) forward primer, 0.5 U *Taq* DNA polymerase (Q BIOgene). PCR was performed using the following program: initial denaturation step at 94 °C (3 min), followed by 40 cycles at 94 °C (45 s), 60 °C (45 s) and 72 °C (45 s), with a final extension step at 72 °C (7 min). PCR products were visualized on an ABI PRISM 310 Genetic Analyser (PE Biosystems), and allele size was analysed by using GENESCAN 500 size standard (Tamra) and GENESCAN 3.1 software (PE Biosystems) (Lenoir *et al.* 2005).

### Colony structure and population genetic analysis

We used MATESOFT software (Moilanen *et al.* 2004) (<http://www.zi.ku.dk/staff/jspedersen/matesoft/>) to determine the genotypes of the queen and her mate(s) from worker genotypes. We also calculated the effective paternity for each colony ( $m_{e(s)}$ ) and the population effective mating frequency ( $m_{e(s)pop}$ ) following Starr (1984):

$$m_{e(s)} = \frac{1}{\sum_i p_i^2} \text{ and } m_{e(s)pop} = \frac{n_{colo}}{\sum_j \sum_i p_{ij}^2}$$

where  $p_i$  is the proportional contribution of the  $i$ th male that mated with the queen of the colony and  $p_{ij}$  the proportional contribution of the  $i$ th male in the  $j$ th of  $n_{colo}$  colonies.

These values were compared with unbiased sample statistic of  $m_e$  corrected for nonsampling error as suggested by Pamilo (1993):

$$m_{e(p)} = \frac{n_{ind} - 1}{n_{ind} \sum_i p_i^2 - 1}$$

where  $n_{ind}$  is the number of workers genotyped in the respective colony. Moreover, we adapted this correction to the population effective mating frequency  $m_{e(p)pop}$  using the mean number of genotyped workers per colony:

$$m_{e(p)pop} = \frac{n_{colo}(n_{ind} - 1)}{\sum_j n_{ind} \sum_i p_{ij}^2 - 1}$$

We estimated 95% confidence intervals of  $m_{e(p)}$  and  $m_{e(p)pop}$  using equation 9 of Tarpy & Nielsen (2002):

$$\ln[95\% \text{ CI of } m_{e(p)}] = 0.643 - 1.101 \cdot \ln(n_{ind}) + 1.637 \cdot \ln(N)$$

where  $N$  is the minimum number of patrines obtained by MATESOFT.

When two males have identical genotypes, possible double mating cannot be detected, resulting in a nondetection error. The probability that two random males share the same genotype is given by  $\prod_l \sum_i p_i^2$ , where  $p_i$  is the population frequency of the  $i$ th allele at the  $l$ th locus (e.g. Boomsma & Ratnieks 1996). This probability becomes insignificant when several moderately polymorphic microsatellite markers are used (Chapuisat 1998).

Nevertheless, as inbreeding can cause a nondetection error because of identical male genotypes at all loci,  $m_e$  can be corrected for nonsampling error and nondetection error (JS Pedersen, see Schrempf *et al.* (2005)):

$$m_{e,p} = \frac{1}{\frac{n_{ind} \sum_i p_i^2 - 1}{n_{ind} - 1} \{1 - \prod_l [F_{IS} + (1 - F_{IS})(1 - H_{exp,l})]\}}$$

where  $F_{IS}$  is the inbreeding coefficient and  $H_{exp,l}$  is the expected heterozygosity at the  $l$ th locus in the absence of inbreeding (e.g. Lenoir *et al.* 2005 for *C. elegans*).

Due to limited samples for sperm-typing, queen mating frequencies were inferred from worker genotypes. Sperm-typing was used to determine whether female sexuals and their mates were brothers and sisters, i.e. shared alleles derived from the colony's queen, or were unrelated.

Allele frequencies and relatedness  $R$  among nestmates were calculated using the software RELATEDNESS 5.00 by the method of Queller & Goodnight (1989). Colonies were weighted equally and the standard errors were obtained by jackknifing over colonies. Alien and native individuals were determined by comparing their genotypes to the inferred genotype of the colony queen. According to the haplodiploid reproductive system of ants, males with at least one allele at one locus that did not match the colony queen's inferred genotype were considered as alien males. Females with at least two alleles at one locus differing from the colony queen's inferred genotype were considered as alien females. As males are haploid, the number of alleles found by sperm amplification corresponds to the minimum number of the queen's mates. GDA 1.0 software

(Lewis & Zaykin 2001) was applied to estimate  $F$  statistics with a three-level analysis according to the method of Weir & Cockerham (1984). The three levels are represented by individuals within the colony, colonies within subpopulations (corresponding to the three investigated sites) and subpopulations within the population. A comparison of pairs of genes within an individual with random genes within the subpopulation gives information on the amount of inbreeding due to nonrandom mating (in the following stated  $F_{IS}$ ), and differences of allele frequencies between subpopulations compared to random genes within the population give information on gene flow between the subpopulations (in the following referred to as  $F_{ST}$ ).

### Behavioural observations

To determine whether colonies adopt alien sexuals, we introduced sexuals (8 males and 45 winged females) from three colonies from Montlouis sur Loire one by one into the foraging arena of 15 different colonies, which came from the same subpopulation (8 colonies) or from Carennac (7 colonies). The 'adopting' colonies had been kept in the laboratory for several days to 1 year and contained a minimum of 100 workers, about 30 winged females, 30 larvae, 1 queen, and no males.

Each sexual was introduced into the foraging arena of the 'adopting' laboratory colony and the behaviour of workers in contact with the foreign sexual was recorded for the first 5 min. Within this time, males usually have entered the nests and attempted copulations with female sexuals, and winged female sexuals have been bitten and stung by several workers. As several workers can interact simultaneously with the sexual, we always noted the most aggressive behaviour towards the sexual. An aggression index was calculated following Errard & Hefetz (1997) and Errard *et al.* (2003): 0, antennal contact; 1, mandibles opening; 2, biting; and 3, gaster flexion (stinging assay). The frequency and duration of each behaviour were registered using ETHOLOG 2.25 (Otoni 2000) (<http://www.ip.usp.br/ebottoni/EthoLog/ethohome.html>). The overall aggression exhibited in each test was calculated as in Errard & Hefetz (1997) and Errard *et al.* (2003):

$$\frac{\sum_{i=1}^n AI_i \cdot t_i}{T}$$

where  $AI_i$  and  $t_i$  are the aggression index and the duration of each act, respectively, and  $T$  is the total interaction time defined as the total time during which the ants were in physical contact. The final success or failure of the adoption was also checked 3 h, 24 h and 72 h after the introduction of the sexuals.

Field observations were conducted during the reproductive period of *C. elegans* (from the end of July to the end of September) of 2003 and 2004 in Montlouis sur Loire in a 318-m<sup>2</sup> area containing 330 nests, and more anecdotally in Carennac.

**Table 1**  $F$  statistics for a total of 21 colonies computed with GDA (based on a three-level analysis; Weir & Cockerham 1984). Confidence intervals (95% CI) are based on 5000 bootstrapping repetitions. Values for  $f$  (comparison of pairs of genes within an individual with random genes within the colonies; zero or negative in case individuals of the colony are related),  $F_{IS}$  (comparison of pairs of genes within an individual with random genes within the subpopulation; positive in case of inbreeding due to nonrandom mating) and  $F_{ST}$  (subpopulation compared to whole population; positive in case of limited gene flow) are given.  $F_{ST}$  within subpopulations is taking the population substructure into account (colony within subpopulation; positive in case individuals are related)

Locus	$f$	$F_{IS}$	$F_{ST}$	
			Within subpopulations	Between subpopulations
CE2-3A	-0.1459	0.2921	0.3822	0.1537
CE2-4A	-0.2919	0.2022	0.3825	0.1561
CE2-5D	0.0345	0.6580	0.6457	0.5721
CE2-12D	-0.2197	0.5587	0.6382	0.3056
CE2-4E	-0.3015	0.2013	0.3863	0.1812
Overall loci	-0.2114	0.3732	0.4826	0.2650
Upper 95% CI	-0.1077	0.5413	0.5896	0.4226
Lower 95% CI	-0.2872	0.2184	0.3832	0.1607

## Results

### Colony and population structure

The three populations differed in allele frequencies. For example, alleles '123' and '125' of locus CE2-3A were found only in Indre et Loire, alleles '115' and '129' only at Dordogne, and alleles '105' and '113' only at Allier (Appendix). Consequently, the value of  $F_{ST}$  (allele frequency differences between subpopulations), is positive [0.265; 95% CI (0.1607–0.4226)], indicating limited gene flow between populations (Table 1).

In all investigated colonies, the genotype of the workers could be explained by a single mother queen. Only in a few colonies (C02, P03, A01, A02, A07), one or two genotyped workers appeared to be totally unrelated with the other workers of the colony and were removed from the analysis (sample sizes of less than 10 indicate that foreign workers were excluded). These workers most likely represent individual foragers that accidentally entered the wrong nest. The mean relatedness between workers of the colonies, not corrected for inbreeding effects, was  $0.43 \pm 0.06$ ,  $0.58 \pm 0.09$ , and  $0.36 \pm 0.1$ , respectively, for Montlouis sur Loire (M), Carennac plus Pinsac (C and P), and Chemilly (A). Over all subpopulations and over all loci, relatedness  $R$  is  $0.49 \pm 0.06$  (Table 2). The fact that workers of colonies are related is also expressed by negative values of  $f$  obtained by GDA, which are based on a comparison of individual genotypes with random genes within the colonies (Table 1).

The inbreeding coefficient is positive over all loci and its confidence interval does not overlap zero, indicating significant inbreeding (Table 1). The estimation of sib-mating frequency  $\alpha$  from Pamilo (1985) or Suzuki & Iwasa (1980):  $F_{IS} = \alpha / (4 - 3\alpha)$  indicates that winged females mate in 70.4% of their copulations with a brother.

Mother queens were always multiple-mated, with at least 2–9 different mates (mean number of patriline  $\pm$  SD =  $4.52 \pm 1.6$ ; Table 2). The chance that the genotypes of two random males that mate with the same female are identical (nondetection error) is 0.0004; thus, the probability to miss double mating is negligible. The mean population effective mating frequency (after Pamilo 1993) corrected for non-sampling error is  $4.37 \pm 1.65$ . Calculations, which do not take the nonsampling error into account, result in a lower number of patrilines (3.25), which is probably an underestimation. In contrast, corrections for inbreeding result in a high estimation of  $m_e$  ( $8.78 \pm 10.71$ ), which seems to be an overestimation, mainly due to a few colonies, in which a very high number of patrilines was found in a limited number of workers (see Table 2 for all results).

The correlation between the minimum number of patrilines ( $N$ ) and effective paternity ( $m_{e(p)}$ ) is positive and significant (Spearman rank correlation,  $N = 21$ ,  $r_s = 0.9194$ ,  $P = 0.001$ ). The mean number of males found in the colonies is not significantly different from the mean number of males that mate with a female (Mann–Whitney  $U$  test,  $N = 58$ ,  $U = 354$ ,  $P = 0.5733$ ).

The comparative analysis of the genotypes of 11 males, 20 winged female sexuals and the sperm in their spermathecae indicates that colonies contain both, alien and native sexuals. Consequently, we found that female sexuals are inseminated by their brothers as well as by alien males (Table 3). The proportion of native and alien sexual individuals in Table 3A are for the two studied colonies and are not representative for the whole population. Table 3B provides data on native and alien sexuals as well as the genotype of sperm found in the spermatheca of young queens.

### Aggression index and adoptions

Workers were significantly more aggressive towards introduced female sexuals than towards males (Mann–Whitney  $U$  test,  $N = 53$ ,  $Z = -2.8324$ ,  $P = 0.0046$ ) (Fig. 1). Whereas ergatoid males can enter alien nests (75% of the tested males were inside the nest of the adopting colony a few hours after introduction and tried to copulate with female sexuals), none of the female sexuals succeeded in entering the new nest. Moreover, 64% of them were killed within 24 h after introduction and 96% did not survive for more than 3 days. This aggressiveness of workers against female sexuals can be observed in queen-right colonies without own winged females, as well (J.-L. Mercier, unpublished data).

**Table 2** Mating frequency and relatedness in colonies of *Cardiocondyla elegans*: colony name, number of colonies ( $n_{\text{colo}}$ ), number of genotyped individuals ( $n_{\text{ind}}$ ) per colony. Mating frequency was estimated based on the minimum number of patriline (N). Effective paternity ( $m_{e(s)}$  and  $m_{e(s)pop}$ ) following Starr (1984),  $m_{e(p)}$  and  $m_{e(p)pop}$  corrected for nonsampling error following Pamilo (1993) with 95% confidence intervals, and  $m_{e,p}$  corrected for nonsampling error and nondetection error due to inbreeding following Schrempf *et al.* (2005) are given, as well as the relatedness  $R$  with standard error from jackknifing and 95% confidence intervals (CI)

Colony	$n_{\text{ind}}$	N: minimum # of patriline	Starr 1984	Pamilo 1993; Tarpy & Nielsen 2002	Schrempf <i>et al.</i> 2005	Relatedness $R$	Jackknife	95% CI
			$m_{e(s)}$	$m_{e(p)} \pm 95\% \text{ CI}$	$m_{e,p}$			
M01	10	5	4.37	6.98 ± 2.10	7.81	0.4634	0.1611	0.4471
M02	10	3	2.95	3.77 ± 0.91	4.22	0.4120	0.1403	0.3894
M03	10	3	1.80	1.98 ± 0.91	2.21	0.4872	0.2278	0.6323
M04	10	4	3.37	4.57 ± 1.46	5.11	0.6755	0.1683	0.4673
M05	10	5	3.94	5.85 ± 2.10	6.55	0.4897	0.1351	0.3751
M07	10	3	1.61	1.73 ± 0.91	1.93	0.2428	0.1298	0.3603
M34	10	3	2.33	2.74 ± 0.91	3.06	0.5431	0.2569	0.7132
M37	10	4	3.90	5.75 ± 1.46	6.43	0.0852	0.1917	0.5320
M38	26	7	4.34	4.87 ± 1.01	5.45	0.1071	0.1093	0.3033
C01	10	5	4.37	6.98 ± 2.10	7.81	0.6047	0.1436	0.3988
C02	9	3	2.26	2.68 ± 1.02	3.00	0.6349	0.2017	0.5599
C03	10	4	3.39	4.62 ± 1.46	5.16	0.7829	0.1593	0.4423
C04	10	4	3.70	5.29 ± 1.46	5.92	0.4698	0.2331	0.6469
C29	10	5	3.65	5.17 ± 2.10	5.79	0.6831	0.0455	0.1263
P01	10	6	6.97	20.74 ± 2.83	23.20	0.4773	0.1271	0.3529
P02	10	9	8.33	45.00 ± 5.50	50.35	0.2983	0.2112	0.5862
P03	9	5	4.66	8.58 ± 2.36	9.60	0.5365	0.2840	0.7885
A01	9	6	5.79	14.42 ± 3.18	16.14	0.2097	0.2514	0.6980
A02	9	4	3.12	4.24 ± 1.64	4.75	0.3824	0.1473	0.4089
A05	10	2	1.98	2.22 ± 0.47	2.48	0.6485	0.2663	0.7394
A07	8	5	3.90	6.65 ± 2.69	7.44	0.1030	0.1261	0.3501
$n_{\text{colo}} = 21$	10.76 ± 4.90	4.52 ± 1.6	$m_{e(s)pop} = 3.25$	$m_{e(p)pop} = 4.37 \pm 1.65$	8.78 ± 10.71	0.49 ± 0.06		

### Field observation

In the field, the worker behaviour is totally different. Aggression of workers against alien female sexuals has never been observed. Instead, workers were observed carrying winged female sexuals from one nest to another. Female sexuals were carried above the worker, with folded legs and antennae, liberated on the nest entrance, and entered the nest. This carrying behaviour is very frequent and was observed simultaneously during summer 2004 in the two locations of Montlouis sur Loire and Carennac. During a whole day of observation of nests in Montlouis sur Loire, workers were observed transporting females in a straight line from one colony to another 80-cm distant colony. A single winged female was carried from the first colony to the other each 4 min, from 10:00 AM (soil  $T^\circ = 31.5^\circ\text{C}$ ) to 6.30 pm (soil  $T^\circ = 32^\circ\text{C}$ ; max  $T^\circ$  during the day =  $45^\circ\text{C}$ ). Thus, during this day, dozens of females had switched from the first nest to the second one. At the same time, the first nest received some females from other locations. This high activity of exchange of female sexuals seems to differ between days and nests.

### Discussion

The genetic structure of the colonies of *Cardiocondyla elegans*, in accordance with our field data, confirms that this species is strictly monogynous. With the exception of one or two alien workers in five colonies, nestmate workers are usually offspring of a single queen and several males ( $4.52 \pm 1.6$  patriline). The real number of males that mated with the mother queen varied from 2 to 9 and was strongly correlated with effective paternity ( $m_{e(p)pop} = 4.37 \pm 1.65$ ). This means that the males that inseminated the queen contribute almost equally to the offspring. We like to point out that more than six patriline were found in three colonies by genotyping only nine or 10 workers (colonies P01, P02 and A01; Table 2), resulting in unrealistically high estimates for  $m_{e(p)}$  and  $m_{e,p}$  (values given in italics in Table 2). Although queens might mate with a large number of males (some *C. elegans* nests contain up to 17 ergatoid males), the genotyped workers might also have been offspring of multiple related queens. Dead queens may be replaced by one of the winged females of the colony (A. Schrempf & J. Heinze, unpublished data; J.-C. Lenoir & J.-L. Mercier,

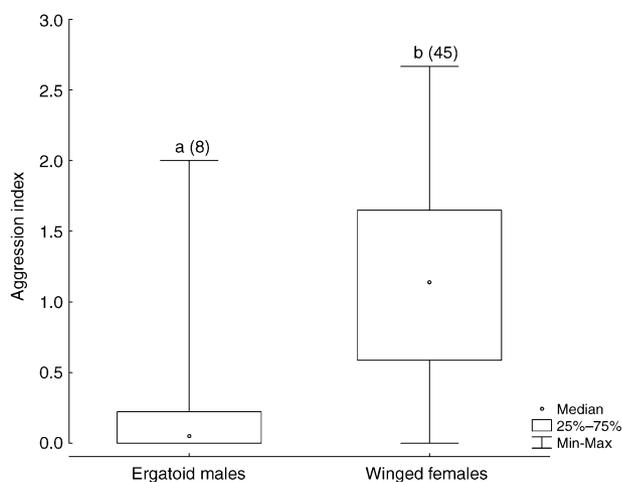
**Table 3** (A) Proportion (and full number) of native and alien sexuals found in two *Cardiocondyla elegans* nests (M38 – Indre et Loire, A01 – Allier). The sperm in the spermatheca of winged female sexuals was genotyped to determine if they had mated with males from the nest where they were found (natives) or alien males. The sperm of seven female sexuals from colony A01 could not be genotyped because of technical problems. Of the remaining three, all had mated with native males and the spermatheca of one female in addition contained sperm from at least one alien male. (B) Genotypes of some female sexuals (native/alien) found in colony M38 and their mates. Alleles, which are incompatible with the resident queen's genotype, are given in bold

A

		Males	Winged females	Spermatozooids (native/alien)
M38	Native	100% (3)	60% (6)	50%/50% (3/3)
	Alien		40% (4)	25%/75% (1/3)
A01	Native	75% (6)	0%	
	Alien	25% (2)	100% (10)	100%/33% (3/1)

B

Locus	CE2-3A	CE2-4A	CE2-5D	CE2-12D	CE2-4E
Queen genotype of the colony M38 (inferred from the genotype of workers)	119/121	184/186	208/210	136/138	139/145
Native female	103/121	184/184	208/210	138/138	145/145
Alleles found after sperm amplification: native males	119/121	186	208	136/138	145
Native female	103/121	184/186	208/210	136/138	145/145
Alleles found after sperm amplification: alien males	119/121	184/186	208/210	138	<b>143</b>
Alien female	103/119	186/186	208/210	136/138	<b>143/157</b>
Alleles found after sperm amplification: native males	119/121	184/186	208/210	138	145
Alien female	103/121	184/184	208/210	136/138	<b>143/157</b>
Alleles found after sperm amplification: alien males	119/121	186	208	138	<b>143</b>



**Fig. 1** Aggression index of workers against introduced alien sexuals (number of introduced sexuals in brackets). Significant differences are indicated by different letters.

unpublished data), which are mainly its daughters and thus share the same alleles. Thus, even if the genotypes of the workers can be explained by a single queen, in a few cases two or more queens might have contributed one after the other to the worker offspring. This phenomenon could lead to an overestimation of the number of patriline in

some colonies. Future genetic studies on *C. elegans* will therefore have to use a significantly increased number of workers. According to equation 8 in Tary & Nielsen (2002), sample size for the study of *C. elegans* should be larger than 17 individuals per colony. Nevertheless, except for the colonies mentioned above, the small difference between  $m_{e(s)}$  and  $m_{e(p)}$  indicates that the sampling error had no strong influence on the results (Tary & Nielsen 2002).

Multiple-mated mother queens produce colonies with an average worker relatedness of about 0.5 (Table 2). The positive inbreeding coefficient indicates that sexuals copulate with relatives in 70% of the copulations, and thus about one-third of all matings involve unrelated partners. This is surprising, given that ergatoid males have long been considered to stay and mate inside their natal nests because of their winglessness and yellowish colouration (e.g. Forel 1892), and female sexuals found in the nests before spring dispersal were always mated. However, our genetic data show that colonies may adopt both alien males and alien female sexuals, which guarantees a certain degree of outbreeding (Table 3A: even if no alien male was detected in colony M38, half of the native females had sperm from alien males in their spermathecae). Although these results are based only on a small sample, they nevertheless reveal the same phenomenon in two independent

populations. Studies with a larger number of genotyped sexuals are needed for a full understanding of the reproductive strategies and the frequency of the adoption of alien sexuals in this species.

The presence of foreign female sexuals in a nest is surprising because in laboratory tests they were attacked by workers and not allowed to enter (Fig. 1). In contrast, workers were frequently observed to carry winged female sexuals from one nest to another in the field. We are at present not able to determine whether workers carry related female sexuals from their own colony to another one or whether they are capturing female sexuals from an alien colony and are bringing them back to their own. Whatever the direction of female trading, it appears to be sufficient to help female sexuals to enter nests containing unrelated males and workers.

The presence of unrelated males in a colony is more easily explained because workers do not behave aggressively towards alien males (Fig. 1). Indeed, the adoption of alien males is common in ant species with mating inside the nests (Peeters 1991; Passera & Keller 1994). In the field, a few *C. elegans* males have been seen walking outside the nest (three observations), but male dispersal is probably difficult to observe due to their yellowish colouration, and not very efficient because of high soil temperature, possible desiccation (males carry less than one-third of the quantity of cuticular hydrocarbons of workers) and potential predation by the spider *Dyctina bicolor* (unpublished data). Workers carrying males have occasionally been seen at the end of the reproductive period (late September or October), but males were usually carried away from a nest and discarded, probably similar to the expulsion of supernumerary males from the hives of honey bees before winter (Morse *et al.* 1967; Free & Williams 1975; Page & Peng 2001).

In contrast, the exchange of individuals between neighbouring nests is commonly known from polydomous ant species. In such cases, monogynous colonies inhabit several queenless satellite nests surrounding a queenright nest. Contrary, in *C. elegans*, all excavated nests were headed by a single queen and all workers of a nest were offspring of a single queen. Thus, different colonies are involved in the exchange of sexuals in this species.

The presence of many sexual intruders increases the gene flow in the population, lowers nest-mate relatedness and counteracts the negative effects of inbreeding. Workers might develop a bet-hedging strategy and benefit from displacing some of their sexual sisters from their own nest, because of the high colony mortality in winter. Each year during the winter period, the temperature inside the nest decreases to less than 5 °C, nests are inundated and buried by soil, and about 40% of the nests perish (Lenoir *et al.* in preparation). Dispersing sisters to other nests could permit the survival of one's genetic lineage in case the own nest is

destroyed in winter. To determine the direction of carrying and the relations between nests that exchange females, we will in the future attempt to genotype the carrying worker and the carried winged females as well as investigate the genetic structure of the two nests that exchange females. In addition, the real proportion of alien sexuals hosted by a colony will be investigated in detail.

Partial sib-mating and premating dispersal of wingless males and female sexuals probably characterizes the reproductive behaviour also of other monogynous species of *Cardiocondyla*. For example, Schrempf *et al.* (2005) indicate that female sexuals of *Cardiocondyla batesii* mate in their maternal nests before dispersing on foot (Heinze *et al.* 2002), but that one-fifth of all matings involve unrelated partners. Active displacement of female sexuals by workers and male dispersal can probably explain this fact.

The presence of foreign sexuals in a nest is puzzling: why do the workers take care of winged females that may be totally unrelated to them, both by the genotype of the female sexual as well as by the genotype of the sperm they are storing? Workers presumably benefit from adopting alien sexuals in that it leads to outbreeding of their sexual brothers and sisters, and thus helps to avoid the possible negative effects of inbreeding. These advantages might outweigh the cost of occasionally caring for an alien queen that has mated with an alien male. Furthermore, the low aggressiveness of workers against foreign sexuals inside the nest probably also comes from the fact that workers can no longer distinguish sexual sisters from foreign female sexuals after the latter have obtained the gestalt odour of the colony. The nest entrance then becomes the key point for workers to discriminate between females that are tolerated inside the nest and females to be rejected. Additional behavioural tests, such as introducing a female sexual together with native females into the nest or disturbing a nest and introducing an alien winged female when the nest reorganizes, could probably answer the question. That the 'adopting' colonies in our experiment did not contain males could also have biased worker behaviour. As males occur only in low numbers and sexuals do not develop in laboratory nests, it will be difficult to conduct similar experiments with colonies that house several males.

Our study also points out an interesting aspect of local mate competition. Whereas colonies of many other species of *Cardiocondyla* contain only a few, highly aggressive ergatoid males that fight for access to the female sexuals, nests of *C. elegans* and other monogynous species can accommodate numerous ergatoid males ( $= 5.27 \pm 4.31$ ; see also Arnoldi 1926 for *C. stambuloffii*; Marikovskiy & Yakushkin 1974 for *C. ulianini*, Schrempf *et al.* 2005 for *C. batesii*). The mean number of males present in colonies of *C. elegans* matches the mean number of males that mate with a queen ( $4.52 \pm 1.6$ ), suggesting that all males have equal access to the females sexuals. Males mating with the same female

sexual participate equally in its offspring. Fighting among males has not been observed and the match between effective paternity and the observed number of patrines suggests that sperm competition is absent or low. *C. elegans* therefore appears to have returned to the seemingly peaceful mating system of other ant genera. Nests are probably too large in terms of space and number of workers, and harbour too many female sexuals for males being able to monopolize a harem (sex ratio:  $0.91 \pm 0.07$ ,  $N = 34$ ). Furthermore, males can probably avoid competition when male numbers are too high by dispersing to other nests.

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## Appendix

Allele frequencies in the five investigated microsatellite loci from the workers of the three studied populations. Alleles are identified by their length (bp). Numbers in brackets indicate how often an allele was found in female sexuals

Alleles	Location			Alleles	Location		
	Loire	Dordogne	Allier		Loire	Dordogne	Allier
CE2-3A				CE2-12D			
103	0.135		0.167	124		0.403	
105			0.093	128			0.102
107	—	—	(1)	130	0.030		0.016
109	0.006	0.015		132		0.165	0.113
111		0.041	0.424	134	0.106	0.126	0.455
				136	0.044	0.214	0.157
113			0.017	138	0.820	0.093	
115		0.143		140			0.157
117	0.011	0.014	0.030	148	—	—	(1)
119	0.316	0.585	0.051	CE2-4E			
121	0.414	0.095	0.218	139	0.016	0.020	
123	0.090			141		0.054	
125	0.028			143	0.011		
129		0.108		145	0.398	0.074	
CE2-4A				147	—	—	(2)
178			0.382	149		0.114	0.500
182		0.201		151	—	—	(1)
184	0.429	0.155	0.175	153	0.016	0.392	
186	0.106	0.008	0.325	155	0.049	0.126	
188		0.286	0.102	157	0.170		
190		0.095	0.017	159	0.300		
192	0.464	0.255		161	0.032	0.033	0.500
CE2-5D				163	0.008		
204		0.148	0.017	165		0.033	
206	0.045	0.736	0.799	173		0.013	
208	0.906	0.116	0.184	175		0.141	
210	0.049						