Multicoloniality in the highly polygynous ant *Crematogaster pygmaea* (Formicidae: Myrmicinae)

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Abstract. In social insects, the high variability in the number of queens per colony raises fundamental questions about the evolution of altruism. It is hypothesized, for instance, that nestmate recognition should be less efficient in polygynous than in monogynous colonies because the presence of several breeders increases the diversity of genetically determined recognition cues, leading to a less specific colonial signature. Recent studies, however, have shown that the link between the number of queens in a colony and the recognition abilities of its members is more complex than previously suggested. Here, we studied intraspecific aggression, diversity of potential recognition cues and genetic structure of colonies in the highly polygynous ant *Crematogaster pygmaea*. Our results reveal that workers of this species are clearly aggressive towards non-nestmates in field experiments but not in more artificial bioassays conducted in Petri dishes, underscoring the importance of context-dependent aspects of the assessment of nestmate recognition. Behavioural, genetic and chemical data show that *C. pygmaea* is a multicolonial species, forming spatially restricted and well-defined entities. Therefore, the postulated negative correlation between recognition ability of workers and queen number in a colony is not supported by the results of this study.

INTRODUCTION

In social insects, nestmate recognition is essential to ensure the cohesion of the colony. In monogynous and monoandrous species, nestmate recognition leads to particularly efficient kin discrimination since all members of the colony are closely related. Therefore, nestmate recognition is an efficient mechanism allowing the evolution of altruism by kin selection (Hamilton, 1964a, b; Crozier & Pamilo, 1996). However, colonies with several queens are frequently recorded in social insects, particularly ants (Bourke & Franks, 1995), and polygyny is generally associated with a lower relatedness among nestmate workers and constitutes a major evolutionary paradox (Keller, 1995).

It is hypothesized that the presence of several queens in the same colony may decrease the recognition ability of the workers and the specificity of the colony odour, leading to a reduced aggressiveness towards foreign individuals (Bourke & Franks, 1995). Several studies have confirmed that nestmate recognition is less efficient in polygynous than in monogynous colonies and/or that polygynous colonies are less aggressive than monogynous ones (e.g. *Linepithema humile*: Keller & Passera, 1989; *Solenopsis invicta*: Morel et al., 1990; *Messor barbarus*: Provost & Cerdan, 1990, *Pseudomyrmex pallidus*: Starks et al., 1998). In several highly polygynous species forming large unicolonial structures or super-colonies,

aggression between workers from relatively distant nests is completely absent (e.g. Tsutsui et al., 2000; Holway et al., 2002). Some studies, however, have shown that the negative correlation between the number of queens in a colony and its tolerance towards non-nestmates is not a rule (e.g. *Leptothorax ambiguous*: Stuart, 1991; *Camponotus yamaokai*: Satoh & Hirota, 2005; *Formica selysi*: Rosset et al., 2007; *Myrmica rubra*: Garnas et al., 2007; *Dolichoderus mariae*: Laskis & Tschinkel, 2008). Moreover, most polygynous ant species are territorial. Even in highly polgynous species, aggression is observed between workers belonging to different super-colonies (e.g. Vogel et al., 2009; Drescher et al., 2010). The relationship between social structure (monogynous vs. polygynous) and colony closure in ants is therefore more complex than previously suggested.

Living along the North-East coast of Brazil, *Crematogaster pygmaea* (Forel, 1904) is a terricolous species which forms large polydomous colonies (Quinet et al., 2009). Each colony is formed by dozens of nests (each comprising 1 to 4 chambers) dug in the ground and covering about 250 m². Each nest opens at the soil surface and all the entries of a colony are interconnected by a network of trails on the surface. A queen/worker ratio, reaching 1/100 (Quinet et al., 2009), is similar to that found in tramp species such as *L. humile*, *Monomorium pharaonis* or *Wasmannia auropunctata* (Passera, 1994).

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* This paper is dedicated to his memory.

In this study, we investigated intraspecific aggressiveness within the highly polygynous ant *C. pygmaea*, using both classic laboratory confrontations and field observations, to test for the presence of multicoloniality in this species. In order to locate colony boundaries, chemical and genetic analyses were also performed. These analyses enabled us to estimate the variability in potential recognition cues associated with different nests, the genetic structure of the colonies and the relationships between these parameters and both patterns of aggression and spatial data.

MATERIAL AND METHODS

Populations and colonies studied

Because of the occurrence of polydomy in this species (sensu Debout et al., 2007), colony boundaries were not easy to determine. In this paper, the term "nest" will be used to refer to a group of 1–4 chambers in the soil that have a single common entrance at the soil surface (Quinet et al., 2009). A colony is defined as several nests interconnected by a network of trails on which workers were regularly observed moving from one nest to another. Occasionally, brood carried by workers and queens were also observed moving along the trails. Two nests were considered as belonging to two different colonies if trails were never observed between them. Populations are defined as groups of colonies separated by a clear physical barrier (an urban zone for instance) or a distance greater than 10 km.

Twelve populations of *C. pygmaea*, designated POP-1 to POP-12, were chosen for the present study. Five of them (POP-1 to 4, and POP-10; distance between colonies: mean ± SE = 80 ± 4 m) were located on or near the campus of the State University of Ceará, in Fortaleza (north eastern Brazil). The other seven populations were situated 10 to 243 km away from the campus (Fig. 1). In each population, two colonies were selected for field experiments and laboratory analysis (distance between colonies: mean ± SE = 62.73 ± 37.17 m).

Aggression tests

Aggressiveness towards individuals from other colonies was first assessed by observing dyadic encounters between two workers of *C. pygmaea* in a neutral arena (5 cm diameter Petri dish with fluon-coated sides). For this purpose, laboratory colonies were obtained from 6 populations (POP-1, POP-3, POP-5, POP-6, POP-9 and POP-11). These 6 colonies were stored in plastic nest boxes (230 mm × 180 mm × 40 mm with fluon-coated sides) provided with several nest tubes and were maintained under natural light and temperature.

Ants were fed twice a week with dilute sugar (~0.25 M sucrose solution), water and dry cat food (PURINA© FIT32). The colonies were kept in the laboratory for a maximum of 3 days before performing the aggression tests. During each experimental encounter between two workers originating from the same colony (POP-1) (control, N = 20) or from two different colonies (POP-1, POP-3, POP-5, POP-6, POP-9 and POP-11) (N = 20 for each of the 11 pairs of colonies tested), the behaviour of the workers and the time spent in antennation were recorded for 5 min. All the workers tested were collected in the foraging area of laboratory colonies. The encounter was scored as aggressive if at least one of the following interactions was observed during the 5 min period: grips (from 1 s to several minutes), strong gaster flexions with or without application of defensive secretion, prolonged bites (see Marlier et al., 2004 and Quinet et al., 2005 for a detailed description of typical defensive behaviour of *Crematogaster* ants). After each experiment, the Petri dish was carefully cleaned with 90°C ethanol and dried to remove any chemical signals left by the workers.

A second set of experiments was carried out in the field (eleven populations studied, POP 9 was not used). In each behavioural assay, one worker (the intruder) was carefully placed on the trail of a resident colony, 10 cm from a nest entrance and the interactions between the intruder and the residents were scored for 5 min. The same behaviour as described above was used to define aggression. Workers that were not attacked after 5 min were considered as adopted by the resident colony. Generally, 10 to 30 contacts were sufficient to establish the nature of the interaction (aggression or adoption). For each pair wise test there were 20 replicates with at least 5 min between two consecutive replicates. Colonies were chosen in order to test (i) the intra-colonial aggressiveness (between nests of the same colony, N = 22), (ii) inter-colonial aggressiveness (between 2 colonies from the same population, N = 11) and (iii) inter-population aggressiveness (between colonies from different populations, N = 10, in this case POP-1 was the resident population in all the replicates) (Fig. 2). Control experiments consisted of removing a worker from a trail and reintroducing it on the same trail 4 s later (N = 22).

Extraction of cuticular lipids and chemical analyses

Immediately after field confrontations, samples of 40 workers from each nest or colony tested were immersed in 300 μl of hexane for 15 min and the liquid stored until required for analysis. Cuticular lipids were analyzed by GC/MS using a Finnigan Polaris Q™ ion trap mass spectrometer interfaced with a Trace GC Ultra™ gas chromatograph equipped with a DB-5MS fused silica capillary column (30 m × 0.25 mm diameter × 0.25 μm film thickness) from J&W Scientific. The injection port and transfer line were set at 240°C and 310°C, respectively. Following splitless injection of 1 μl of the sample, oven temperature was maintained at 80°C for two min, increased to 270°C at 20°C/min and then to 310°C at 3°C/min, using helium as the carrier gas (1.2 ml/min). Qualitative and quantitative data were acquired by running the Thermo Finnigan Xcalibur™ 1.4. SR1 data system. Cuticular lipids were identified by analysing their mass spectra produced either by electron impact (ion source operating at 150°C with an ionization energy of 70eV, scan range m/z 40–700) or chemical ionization (ion source operating at 200°C, methane as reagent gas [0.3 ml/min], scan range m/z 40–700) and by comparing their GC retention times with those of reference compounds (Alkanes-mix 10: C10 to C35, Dr. Ehrenstorfer Gmbh). The proportions of the different compounds were obtained from peak areas. The Nei index was used to estimate the degree of similarity between cuticular profiles:

$$I = \frac{\sum_{i}^{n} X_i * Y_i}{\sqrt{\sum_{i}^{n} X_i^2 * \sum_{i}^{n} Y_i^2}}$$

where *n* is the number of peaks, X_i the area (%) of peak *i* for sample *x*, and Y_i the area (%) of peak *i* for sample *y*. For two strictly identical profiles we have $I = 1$, and $I = 0$ for two totally different profiles. To calculate the Nei index (using *R* software, http://cran.r-project.org/), we excluded the peaks with a relative peak area of less than 3% in all cuticular profiles.

A Mantel test was used to estimate the association between chemical differentiation and geographical distance between all pairs of samples. The matrix correlation and the correlation coefficient test were obtained using, respectively, GeneAlex 6.2 (Peakall & Smouse, 2006) and GraphPad Instat 3.06 software (GraphPad Software, San Diego California USA).

Isolation of polymorphic microsatellite loci

A genomic library was constructed and then enriched for microsatellite loci following a protocol similar to the one described by Glenn & Schable (2005). Briefly, genomic DNA was extracted from *C. pygmaea* workers using a standard phenol/chloroform extraction protocol (Sambrook et al., 1989). Genomic DNA was restricted with Sau3AI. Restricted fragments were ligated at both ends to a double-stranded linker that was used later as primer-binding site for PCR amplification. The ligation product was run on a 1% agarose gel and fragments of size 800–1,200 bp were extracted and purified with the Qiaquick Gel Extraction Kit (QIAGEN). Hybridization of fragmented and denatured genomic DNA (gDNA) with different microsatellite biotinylated oligonucleotide probes was conducted at different temperatures in the presence of streptavidin-coated magnetic beads. The hybrid gDNA/oligonucleotide probes were isolated using a magnet and the hybridized gDNA was recovered through a series of washing steps. The product of this procedure was PCR amplified (using primers binding to the previously added linkers) with the Long Expand Template PCR System Kit (Roche), and PCR products were ligated to the *p*STC1.3 no-background vector (*StabyCloning*^{TM} kit, Delphi Genetics) and transformed into competent cells (Delphi Genetics). Recombinant molecules were isolated from clones and sequences of inserted genomic DNA fragments were obtained by cycle sequencing followed by electrophoresis on an ABI 3730 sequencer (Applied Biosystems). Of the loci isolated, 45 were selected on the basis of their length (8–55 repetitions) and tested for polymorphism by genotyping several individuals from different populations. Finally, 4 loci that were sufficiently variable and had unambiguous amplification patterns were selected for genotyping workers from each population.

DNA isolation and microsatellite genotyping

Immediately after field confrontations, samples of workers from each nest tested (or colonies) were stored in absolute ethanol for subsequent genetic analyses. DNA was extracted from 16 workers per colony (mean ± SE per nest = 7.11 ± 2.85), using a standard phenol/chloroform protocol. Genotype for each individual was determined for four polymorphic microsatellites.

Departures from Hardy-Weinberg equilibrium could be indicative of null alleles. The presence of alleles is readily detected in haploid males because they are not masked by the presence of another allele (Schrempf et al., 2005; Sandrock et al., 2007). Sixteen males (8 from POP-1 and 8 from POP-3) were genotyped in order to exclude the possibility of null alleles.

Amplifications were carried out in a 11.05 µl volume containing 1 µl of template DNA (~1 µg per reaction), 0.2 µl dNTPs (10 mM), 0.40 µl of each Cpyg primer (25 µM), 6 µl H_2O Mili-Q, 2 µl Q-Solution (QIAGEN), 1 µl Taq buffer (including 6 mM MgCl^2) and 0.05 µl Top Taq^{TM} DNA Polymerase (QIAGEN). Reactions were performed on a PTC-200 thermal cycler (MJ Research). After an initial denaturing step of 3 min at 94°C, the PCR consisted of 35 cycles of 30 s at 94°C, 30 s at 60°C (annealing temperature) and 1 min at 72°C. The length of the PCR products was determined using an ABI 3100 automated sequencer (Applied Biosystems) and Genemapper software (Applied Biosystems), in order to construct a multilocus genotype for each individual.

Microsatellite data analyses

Loci (from all our dataset) were examined for linkage disequilibrium using GENEPOP 3.4 on the Web (Raymond & Rousset, 1995).
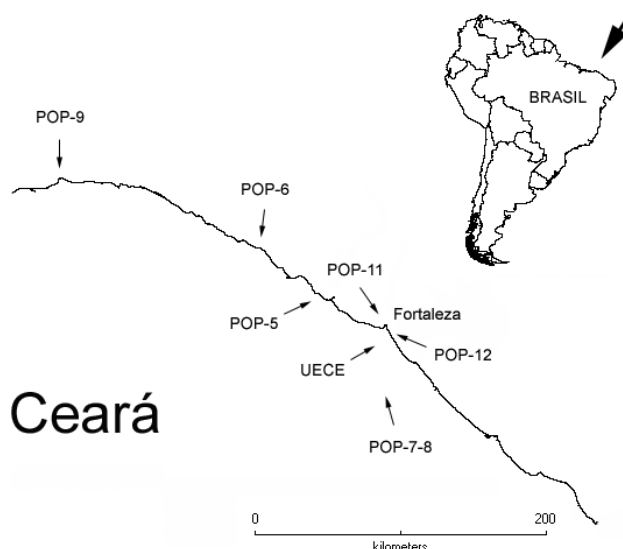

Fig. 1. Location of *C. pygmaea* populations along the coast of Ceará. "UECE" refers to populations on or near the campus of the University (POP-1, POP-2, POP-3; POP-4, POP-10).

Analyses of genetic diversity (number of alleles and private alleles, observed and expected heterozygosities, frequency of the most common allele) were calculated per locus for the eight populations using GeneAlex 6.2 software (Peakall & Smouse, 2006).

To assess the genetic boundaries of the colonies, we used pair wise *F*st values between nests. We assess these values between nests of the same colony, between colonies belonging to the same population and between colonies belonging to different populations.

All pair wise *F*st values were calculated with SPAGeDi 1.2g software (Hardy & Vekemans, 2002). In addition, the index of relatedness (Queller & Goodnight, 1989) was calculated within and between colonies sampled using SPAGeDi 1.2g software (Hardy & Vekemans, 2002) and using all 16 genotyped colonies as reference populations.

Isolation by distance was estimated using GeneAlex 6.2 (Peakall & Smouse, 2006) and GraphPad Instat 3.06 software (GraphPad Software, San Diego California USA) to determine whether there is a correlation between genetic and geographical distances.

RESULTS

High polygyny was observed in the colonies excavated during the present study (average number of queens per nest: mean ± SE, 4.27 ± 7.22, range = 0–36, N = 36; at least 15 nests per colony), confirming the results reported previously by Quinet et al. (2009).

Aggression tests

During dyadic encounters in neutral arenas (N = 220) aggressive interactions were never observed, whatever the origin of the two workers tested. However, the mean time spent in antennation was positively correlated with the geographical distance separating the colonies of the workers used in the bioassay (Pearson's *r* = 0.5987, *p* = 0.0397).

In field experiments, no aggressive behaviour was observed both in the control and during intra-colonial confrontations. In contrast, aggressive encounters were
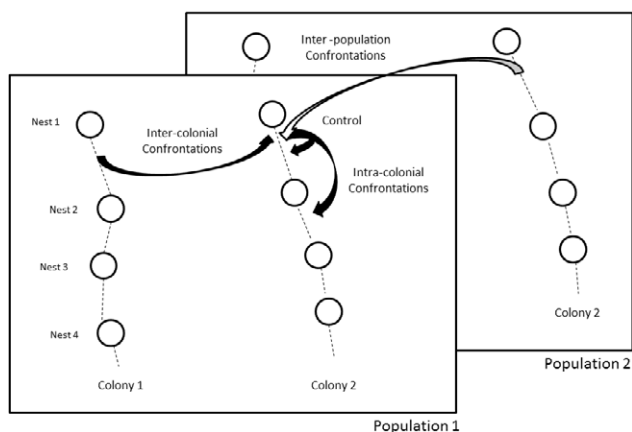
Fig. 2. Schematic representation of combinations of aggression tests carried out in the field between nests, between colonies and between populations.
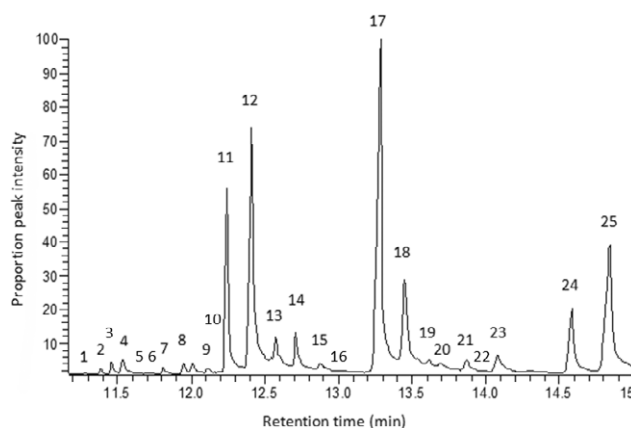

Fig. 3. GC chromatogram of cuticular hydrocarbons of *C. pygmaea* workers. 1: xC23:1; 2: n-C23; 3: unidentified hydrocarbon; 4: 9+11-MeC23; 5 and 6: unidentified hydrocarbons; 7: n-C24; 8: 10+12-MeC24; 9: xC25:1; 10: unidentified hydrocarbon; 11: n-C25; 12: 11+13-MeC25; 13: 3-MeC25; 14: n-C26; 15: 10+12-MeC26; 16: unidentified hydrocarbon; 17: n-C27; 18: 11+13-MeC27; 19: 5-MeC27; 20: 3-MeC27; 21: n-C28; 22: unidentified hydrocarbon; 23: 10+12-MeC28; 24: n-C29; 25: 11+13-MeC29.

frequent when a foreign worker was placed on a trail of a colony, with inter-population aggressiveness (percentage of aggressive encounters: mean ± SE = 90.50 ± 11.41, N = 10) being significantly higher than inter-colonial (but intra-population) aggressiveness (percentage of aggressive encounters: mean ± SE = 58.98 ± 27.17, N = 11) (Mann-Whitney test, $p < 0.0001$), at least with the reference colony used for estimating the inter-population aggressiveness.

Chemical analyses

GC/MS analyses of cuticular compounds revealed a mixture of linear and methyl-branched alkanes ranging from C23 to C30, as well as a few alkenes (Fig. 3).

All the cuticular profiles of the workers were very similar. However, similarity was significantly lower between colonies of different populations (mean Nei index ± SD = 0.938 ± 0.0456; range = 0.714–0.995; N = 1701) than between colonies of the same population (mean Nei index ± SD = 0.971 ± 0.020; range = 0.903–0.995; N = 108) (Mann-Whitney test, $p < 0.001$). Nei index between colonies of the same population were also significantly different from those between nests of the same colony (mean ± SD = 0.980 ± 0.017; range = 0.916–0.999; N = 82) (Mann-Whitney test, $p < 0.001$). However, chemical distances (Nei index calculated by comparing the mean worker's chemical profile of different colonies) were not significantly correlated with geographical distances (Matrix correlation, r = –0.015; Spearman's rank correlation coefficient, $p = 0.882$).

Microsatellite data analyses

256 workers from eight populations were examined for microsatellite variation (Table 1). Each locus was successfully amplified for all individuals. All loci were polymorphic in the eight populations sampled, with the number of alleles per locus per population ranging from 1 to 10. We thus used a total of 57 different alleles with an average of 2.37 ± 1.18 (mean ± SE) private alleles per population. All sequences were deposited in the GenBank Data Library.

The level of observed heterozygosity per locus in Ceará populations varied from 0.188 to 0.813 (mean 0.500). All loci showed a significant heterozygote deficit (Table 1; Chi2: infinity, df: 32, $p < 0.0001$) (Raymond & Rousset 1995).

These departures from HWE could be due to presence of null alleles. However, null alleles are readily detected in this system by genotyping haploid males, and in a preliminary analysis of 16 males originating from different populations, all loci were amplified successfully in all individuals (data not shown). Moreover, the four loci were successfully amplified for all workers genotyped. Thus, it is likely that null alleles are not present at a high frequency in any of the new loci. In addition, there are other biological explanations that are likely to explain the observed homozygote excess.

Workers from nests of the same colony were not genetically different (mean Fst = 0.0181 ± 0.1067, Wilcoxon rank sum test, $p = 0.7317$, $df = 26$), while workers from different colonies and/or populations were genetically differentiated (respectively, mean Fst = 0.3997 ± 0.2099, Wilcoxon rank sum test, $p < 0.0001$, $df = 38$; mean Fst = 0.4702 ± 0.1194, t test, $p < 0.0001$, $df = 562$). On average, the genetic distance between two workers from two different colonies from the same population was significantly different from the genetic distance between two workers from different populations (Mann-Whitney test, $p = 0.0202$). In addition, intra-colonial relatedness was higher (mean $r = 0.5843 ± 0.0449$) than relatedness between colonies (Wilcoxon rank sum test, $p < 0.0001$; mean $r = –0.0038 ± 0.0006$).

Neighbouring colonies tend to be more genetically similar than more distant colonies, as shown by a relatively strong and significant isolation by distance (Matrix correlation, r = 0.3162; Mantel test with Spearman's rank correlation coefficient, $p = 0.0004$). In contrast, genetic

TABLE 1. Description of the microsatellites developed for *Crematogaster pygmaea*. N_a – the number of alleles, H_o – estimates of observed, H_e – expected heterozygosities, Freq (A) – frequency of the most common allele. The calculations are based on 32 individuals per population, two colonies per population (16 individuals per colony).

Locus	Primer sequences (5'-3')	Repeat motif	Size (bp)	N_a	Populations	N_a	H_o	H_e	Freq (A)	Accession no.
Cpyg1	F: GAAACAACATATAACGTTAAG R: GGTAAGACTAGTTTCAGGAA	$(AC)_{21}$	138–160	7	POP-1	3	0.500	0.562	0.578	GQ183015
					POP-2	2	0.250	0.305	0.813	
					POP-3	2	0.250	0.404	0.719	
					POP-4	4	0.469	0.410	0.750	
					POP-5	4	0.531	0.673	0.359	
					POP-6	2	0.188	0.219	0.875	
					POP-7	3	0.333	0.509	0.550	
					POP-8	6	0.563	0.723	0.344	
Cpyg2	F: CCATCCTTTGTCCCGATTTT R: ATCGAATTTTTGTCGACGTG	$(CA)_{28}$	190–230	13	POP-1	5	0.594	0.666	0.438	GQ183014
					POP-2	3	0.344	0.48	0.625	
					POP-3	5	0.563	0.697	0.422	
					POP-4	6	0.594	0.799	0.266	
					POP-5	4	0.375	0.726	0.375	
					POP-6	3	0.313	0.625	0.500	
					POP-7	5	0.531	0.645	0.531	
					POP-8	6	0.375	0.635	0.563	
Cpyg3	F: GCATTCAGGTCATTTTCAAG R: ACCTACGAAACATGTGAGTG	$(TG)_{21}$	250–288	15	POP-1	1	0.000	0.000	1.000	GQ183016
					POP-2	5	0.419	0.598	0.484	
					POP-3	3	0.313	0.521	0.625	
					POP-4	5	0.323	0.644	0.452	
					POP-5	3	0.344	0.380	0.766	
					POP-6	3	0.219	0.626	0.438	
					POP-7	5	0.281	0.630	0.547	
					POP-8	4	0.313	0.591	0.500	
Cpyg4	F: AAGTGCGTGGTGAAACCTAA R: GGGACGTAAGCATTGTACC	$(GA)_{36}$	202–298	22	POP-1	8	0.500	0.820	0.266	GQ183017
					POP-2	5	0.594	0.597	0.453	
					POP-3	6	0.188	0.665	0.500	
					POP-4	10	0.581	0.851	0.226	
					POP-5	6	0.469	0.703	0.438	
					POP-6	6	0.563	0.752	0.344	
					POP-7	7	0.594	0.782	0.328	
					POP-8	10	0.813	0.850	0.219	

distances were not significantly correlated with chemical distances (matrix correlation, r = 0.1831; Mantel test with Spearman's rank correlation coefficient, p = 0.0823).

DISCUSSION

Our results show that, although highly polygynous and polydomous, *C. pygmaea* is clearly a multicolonial species, at least in the study area. Colony boundaries, firstly estimated by observing the movements of workers between nests, were confirmed by genetic, behavioural and chemical data. Aggression was never observed between workers from different nests belonging to the same colony. By contrast, workers were very aggressive towards individuals from other colonies they encountered on their network of trails. Genetic boundaries were also very clear since we found no genetic differences between nests of the same colony whereas there were significant differences between colonies in the same population and between different populations. Profiles of cuticular hydrocarbons were very similar whatever the origin of the workers sampled, but chemical distances were significantly greater between workers of different populations or colonies than between workers of different nests of the same colony.

Due to a greater diversity in olfactory receptors and recognition cues of genetic origin, workers from polygynous colonies are expected to be less efficient in nestmate recognition (Bourke & Franks, 1995). This should be particularly true for highly polygynous species, in which non-aggression between distant colonies is reported (e.g. Tsutsui et al., 2000; Holway et al., 2002). Here, we show that workers of the highly polygynous species *C. pygmaea* can discriminate between nestmate and non-nestmate workers, and behave aggressively towards the latter, as recently described for several other highly polygynous ant species (e.g. *M. rubra* in Maine populations: Garnas et al., 2007; *D. mariae*: Laskis & Tschinkel, 2008; *Plagiolepis pygmaea*: Thurin & Aron, 2008). These results are of particular interest in *C. pygmaea*, *D. mariae* and *P. pygmaea* because high polygyny in these species is not associated with unicoloniality or supercolony structures (sensu Helanterä et al., 2009), as is the case for example in *L. humile* (Suarez et al., 1999; Tsutsui et al., 2000; Pedersen et al., 2006), *Anoplolepis gracilipes*

(Abbott et al., 2007; Drescher et al., 2007) or *L. neglectus* (Ugelvig et al., 2008). Therefore, they represent rare examples of native species forming highly polygynous and territorial colonies.

The combination of such traits as high polygyny and territoriality in *C. pygmaea* could be explained by a strong relatedness among queens within the same colony leading to a low genetic diversity among workers of the same colony, as described in *P. pygmaea* (Trontti et al., 2005). However, the absence of a simple correlation between genetic diversity and nestmate recognition is reported in two other native species, *F. paralugubris* (Chapuisat et al., 2005; Holzer et al., 2006) and *F. selysi* (Rosset et al., 2007). Although the former is unicolonial and the second is territorial, both species are polygynous and their ability to recognize nestmates is associated with low relatedness between worker nestmates (Chapuisat et al., 1997; Rosset et al., 2007). Therefore, the relationship between the number of queens, genetic diversity, recognition ability and territoriality in ants is clearly more complex than previously thought.

Although it is well known that animal behaviour is largely context dependent (Liebert & Starks, 2004; Buczkowski & Silverman, 2005; Campbell et al., 2009; Helanterä, 2009), aggressiveness in ants is generally estimated using basic bioassays. Simple dyadic encounters in neutral arenas are often used for this purpose (e.g. Dahbi et al., 1996; Tsutsui et al., 2000; Giraud et al., 2002; Fournier et al., 2009). Few studies compare results obtained in the laboratory and the field (Campbell et al., 2009; d'Ettorre & Lenoir, 2010) or using different bioassays (Roulston et al., 2003). Here, we clearly show that aggressiveness between *C. pygmaea* workers from different colonies, although unambiguously observed in the field, can not be detected by dyadic encounters in Petri dishes. Moreover, recent studies demonstrate that subtle behaviour, such as rates of trophallaxis or time spent in antennation, may reveal the presence of nestmate recognition even in the absence of clear aggressiveness (Steiner et al., 2007; Chapuisat et al., 2005; Holzer et al., 2006; Björkman-Chiswell et al., 2008). Our study therefore clearly demonstrates that tests of aggression between pairs of workers recorded in neutral arenas are insufficient to accurately assess nestmate recognition or aggressiveness between ant colonies.

Alkenes and branched alkanes are generally expected to encode stable signals used in nestmate recognition in ants (Dani et al., 2001; Howard & Blomquist, 2005; Lucas et al., 2005; Guerrieri et al., 2009; Brandt et al., 2009; Martin et al., 2009). In *C. pygmaea*, only 10 methyl alkanes and 2 alkenes were identified. However, this seems sufficient to ensure colonial identity, as suggested by both empirical (Breed, 1998; Boulay et al., 2000; Dani et al., 2001) and theoretical studies (Breed & Buchwald, 2009).

Our data show that *C. pygmaea* colonies belonging to the same population were genetically differentiated and that genetic differentiation was correlated with geographical distances. These results suggest that populations are spatially isolated and that gene flow between them is low. This could be due both to the limited dispersal of sexuals during the nuptial flights observed in this species (Quinet et al., 2009) and to frequent nest budding events (Crozier & Pamilo, 1996; Chapuisat et al., 1997). We also show that chemical and genetic distances were not correlated, a result that contrasts with that reported for the Argentine ant and two species of *Lasius* (Vogel et al., 2009; Cremer et al., 2008). Such a correlation, however, is not confirmed for *Lasius neglectus* (Ugelvig et al., 2008). A strong association between these two parameters is expected only if the chemical diversity of cuticular compounds is directly correlated with the overall genetic distance estimated using microsatellite marker loci. Although it is well known that chemical recognition cues have a genetic component, environmental conditions can also affect cuticular profiles (d'Ettorre & Lenoir, 2010), breaking the correlation between genetic and chemical distances. However, our results should be interpreted with caution given that the estimate of genetic distance was based only on 4 microsatellite loci.

In conclusion, no support was found in this study for the hypothesis that high polygyny should lead to limited recognition abilities between ant colonies. Although polydomous, *C. pygmaea* does not form super colonies (sensu Helanterä et al., 2009) since direct interactions between workers from the different nests belonging to the same colony are possible. Therefore, the most important finding of the present work is that high polygyny does not necessarily lead to large supercolonial structures extending over great distances but, on the contrary, that highly polygynous ant species can form territorially and genetically well-defined entities showing a clear multicolonial organisation at a local scale. A detailed analysis of the genetic structure of *C. pygmaea* colonies, currently under progress in our laboratory, should help us to understand the possible role of nestmate relatedness in the social closure of this species.

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