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Genetic Distance and Age Affect the Cuticular Chemical Profiles of the Clonal Ant *Cerapachys biroi*

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Abstract Although cuticular hydrocarbons (CHCs) have received much attention from biologists because of their important role in insect communication, few studies have addressed the chemical ecology of clonal species of eusocial insects. In this study we investigated whether and how differences in CHCs relate to the genetics and reproductive dynamics of the parthenogenetic ant *Cerapachys biroi*. We collected individuals of different ages and subcastes from several colonies belonging to four clonal lineages, and analyzed their cuticular chemical signature. CHCs varied according to colonies and clonal lineages in two independent data sets, and correlations were found between genetic and chemical distances between colonies. This supports the results of previous research showing that *C. biroi* workers discriminate between nestmates and non-nestmates, especially when they belong to different clonal lineages. In *C. biroi*, the production of individuals of a morphological subcaste specialized in reproduction is inversely proportional to colony-level fertility. As chemical signatures usually correlate with fertility and reproductive activity in social Hymenoptera, we asked whether CHCs could function as fertility-signaling primer pheromones determining larval subcaste fate in *C. biroi*. Interestingly, and contrary to findings for several other ant species, fertility and reproductive activity showed no correlation with chemical signatures, suggesting the absence of fertility related CHCs. This implies that other cues are responsible for subcaste differentiation in this species.

Keywords Social insects · Ants · *Cerapachys biroi* · Cuticular hydrocarbons · Pheromones · Fertility signaling · Biological invasions · Clonality

Introduction

In social Hymenoptera, the hydrocarbons present on the surface of the cuticle (cuticular hydrocarbons or CHCs) play an essential role in social communication (Howard and Blomquist 2005), while their primary function is to limit desiccation and infiltration of microorganisms (Gibbs 2002; Gibbs and Crockett 1998; Martin et al. 2009). Much research on social evolution and behavior, where insect societies are

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extensively used as model systems, has focused on the evolution and the functional role of CHC variability. A significant amount of work has been conducted to understand how chemical signatures can both enable colony-level coherence and bear information about caste, age, and reproductive status of individuals within colonies (Denis et al. 2006, Ichinose and Lenoir 2009; Le Conte and Hefetz 2008; Liebig et al. 2000; Monnin 2006; Smith et al. 2008). From this perspective, clonal hymenopteran species have received little attention, despite their potential importance in the understanding of these issues. Genetic homogeneity within colonies allows experimental control over the genotype of individuals, and clonal species can, therefore, be used to tease apart the effects of genetics versus environment or caste (either behavioral or morphological) on chemical signatures. In the cerapachyine ant *Cerapachys biroi*, females lack a spermatheca, i.e., they cannot be inseminated, and reproduce via thelytokous parthenogenesis (Tsuji and Yamauchi 1995). Male production occurs exceedingly rarely in laboratory colonies (Kronauer et al. 2012). Even though several clonal lineages have been found in *C. biroi* (Kronauer et al. 2012), as far as we know, natural colonies are always monoclonal, i.e., all the individuals in a colony belong to the same clonal lineage with intra-colonial relatedness=0.99 (Kronauer et al. 2013; Oxley et al. 2014). A recent study has shown that individuals are able to discriminate between nestmates and non-nestmates, especially when they are of different clonal origin (Kronauer et al. 2013). As social recognition in ants is based on the divergence of CHC profiles, we explored the inter-colonial variability of chemical signatures in *C. biroi* and the relationship between genetic and chemical distances between colonies from the same or different clonal lineages.

The colonies of *C. biroi* include two worker subcastes that differ in morphology, behavior and fertility levels, referred to as high and low reproductive individuals, or HRIs and LRIs (Teseo et al. 2013, 2014). These are also called intercastes and workers by Ravary and Jaisson (2004) and ergatoid queens and workers by Lecoutey et al. (2011). Low reproductive individuals (LRIs) have two ovarioles and lay eggs exclusively during their first four-five months of life, during which they also provide care to the developing brood. After this stage, they become nonreproductive foragers (Ravary and Jaisson 2004). High reproductive individuals (HRIs), which constitute approximately 5 % of the individuals in a colony, show low activity levels and produce up to eight eggs during each reproductive phase, having four to six ovarioles in total (Ravary and Jaisson 2004). These individuals probably remain fertile for a much longer time compared with LRIs. From a functional perspective, colonies therefore comprise two groups: old LRIs that behave as reproductively-inactive foragers, as well as young LRIs and HRIs of all ages that act as nurses and are fertile. In *C. biroi*, the production of HRIs is regulated via a feedback system based on the actual fertility level of the colony (Lecoutey et al. 2011). The more fertile a colony is, the greater its proportion of HRIs and young LRIs, and the less HRIs it produces, and *vice versa*. In social Hymenoptera, CHC signatures often are correlated with fertility, and are used by reproducers to signal their presence and reproductive status (reviewed by Monnin 2006). As a previous study on *C. biroi* indicated that the HRI regulation system is not based on volatile chemical signals (Lecoutey et al. 2011), we hypothesized that non-volatile, cuticular chemical cues could signal fertility and/or reproductive status. We further hypothesized that these cues act as primer pheromones that inhibit the HRI developmental trajectory. *Cerapachys biroi* colonies undergo stereotypic reproductive cycles in which they alternate between a reproductive and a foraging phase (Ravary and Jaisson 2002, 2004; Ravary et al. 2006). In the reproductive phase, all individuals aggregate while a new batch of eggs is laid by fertile individuals, whereas in the foraging phase, young individuals and HRIs remain inside the nest chamber while older LRIs forage for prey. We analyzed the CHC profiles of HRIs, the most fertile individuals, throughout the reproductive cycle, in order to assess whether the phasic ovarian activity of egg-layers (Teseo et al. 2013) correlated with changes in cuticular profiles. A reproductive activity-related change in chemical signatures could possibly play a role in the feedback regulation of HRI production. We thus compared intranidal (more fertile) workers and foragers (less fertile workers) in the foraging phase, when the two types of individuals can easily be distinguished on the basis of their behavior. This allowed us to assess whether chemical differences between both groups could be involved in this fertility-related cue.

Methods and Materials

Colonies The geographic origins, clonal lineages, and collection dates of the *C. biroi* colonies used in this study are listed in Table 1. These were kept in the laboratory under constant conditions of 27 °C, ~70 % relative humidity, and 12 h L:12 h D photoperiod. Nests were made of ~30 x 30 x 10 cm plastic boxes with Fluon®-coated edges, with a ca. 2.5 cm thick floor made of plaster of Paris. A single nest chamber was dug in the center of the nest and covered with a red glass sheet. Colonies were fed twice a week with live pupae of the ant *Aphaenogaster senilis* during foraging phases.

Genetic Analyses Prior to chemical analyses, one individual of each of ten colonies (Table 1) was genotyped at 30 microsatellite loci and sequenced for two mitochondrial gene fragments (658 bp of *cytochrome oxidase I* and 575 bp of *cytochrome oxidase II*) to determine the clonal origin of each

Table 1 Colonies used in this study

Colony	Clonal lineage	Origin	Field collection date	Used in Set
J1	A (MLL1)	Java, Indonesia	2005	1, 2
O4	A (MLL1)	Okinawa, Japan	2006	1
O5	A (MLL1)	Okinawa, Japan	2006	1,2
T5	A (MLL1)	Taiwan	2001	1,2
O6	B (MLL4)	Okinawa, Japan	2006	1
T1	B (MLL4)	Taiwan	1997	1,2
T3	B (MLL4)	Taiwan	2000	1,2
C10	C (MLL6)	Okinawa, Japan	2008	1
C9	C (MLL6)	Okinawa, Japan	2008	1
T4	D (MLL3)	Taiwan	2001	1,2

colony (data reported in Kronauer et al. 2012). Ten individuals then were randomly collected from each colony and genotyped at 17 polymorphic microsatellite loci (CKPWC, D6CNC, B3KAG, EFOHK, D3N3P, ESOCS, E27C5, B8PND, DK371, ESI77, ETWBP, EH2OX, ESA52, E324Z, ED6BM, EPCI6, D8EP1) as in Kronauer et al. 2012, in order to estimate the inter-individual relatedness within our colonies and to assure that the initially selected worker was representative of the genetic makeup of the colony. The software GenClone 2.0 (Arnaud-Haond and Belkhir 2007) was used to assign individuals to multilocus genotypes (MLGs) based on these 17 microsatellite loci. Multilocus genotypes (MLGs) were used to produce matrices containing pair-wise Euclidean genetic distances between individuals of the same clonal lineage. Based on these matrices, we calculated the average pairwise relatedness within colonies as $r=(A - D) / A$, where A is the maximum possible allele distance given the markers, and D is the average observed allele distance between individuals in a given colony (Kronauer et al. 2013). Given that *C. biroi* reproduces asexually and that the studied populations show very low clonal diversity (Kronauer et al. 2012), we did not perform standard calculations of pairwise regression relatedness (e.g., Queller and Goodnight 1989).

Chemical Analyses Initially, a set of one hundred individuals was used for the identification of the CHC peaks of *C. biroi*. Fifty individuals were randomly collected in the foraging area and fifty individuals in the nest chamber of one colony (T5, clonal lineage A, Table 1). Ants were frozen and pooled, and their CHCs were extracted in 2 ml pentane during 1 h. The extract was analyzed with a VGM250Q GC/MS equipped with a DB-5 column (30 m x 0.32 mm x 0.25 μm, J & W, Agilent Technologies, Palo Alto, CA, USA). Helium was used as carrier gas, with a 28.57 cm/s flow. The column temperature was held at 150 °C during 2 min, then was increased to 300 °C at 5 °C/min, and finally held at 300 °C for 30 min. The injection port was maintained at 200 °C. The MS detector was a Fisons MD 800 (Foremost Equipment, Rochester, NY, USA) set at 70 eV. This experimental step was conducted as a first exploration of the chemical signature of *C. biroi*. We used a temperature program to maximize the definition of the part of the spectrum including CHC peaks, thus facilitating their identification.

Origin and Preparation of Samples

We used two further independent sets of individuals to explore different aspects of the chemical signature of *C. biroi*. Set 1 included foragers that show low fertility levels on average, and intranidal workers with higher fertility on average, collected at the beginning of the foraging phase from different colonies belonging to different clonal lineages. Set 2 included HRIs collected throughout the complete colony reproductive cycle from different colonies from different clonal lineages. Analyzing individuals from set 1 allowed exploration of the CHC variability within and between colonies and clonal lineages, thereby highlighting CHC-related differences on the basis of the behavioral subcastes of individuals and their clonal origin. Set 2 individuals were used to investigate whether and how chemical signatures correlate with the changes in reproductive activity related to the biphasic colony cycle of *C. biroi*.

Set 1 For each colony, 80 callow LRIs, 40 foraging LRIs and 40 HRIs were collected 5 d after the beginning of the foraging phase without previous feeding. Since ants do not forage and eat during the reproductive phase, which lasts around 18 d, the collected individuals had not eaten during at least the previous 23 d. This helped minimize any diet-dependent influences on the chemical profiles. All collected individuals were killed by freezing, except for 40 callow LRIs per colony that were placed in separate nests with larvae for one colony cycle of around 34 d. These individuals then were collected after the beginning of the following foraging phase for chemical analyses, and were considered as young fertile intranidal LRIs. Thus, four morphological/behavioral subcastes

were analyzed for each colony: 1) five-7-d-old callow LRIs with ovaries not yet functional, 2) intranidal fertile LRIs (nurses), 1–1.5 mo-old, 3) foragers (reproductively inactive LRIs older than 4 months), and 4) HRIs, fertile and at least 1 mo-old. For each of ten colonies and each of these four categories, five groups of eight individuals were pooled and extracted during 1 h in 200 μl of pentane containing 10 μl/l *n*-tetradecane and 15 μl/l *n*-tetracosane as internal standards. Cuticular hydrocarbon components were identified on the basis of their Retention Indices relative to those of *n*-alkanes and their mass spectral fragmentation patterns.

Chemical analyses (200 samples in total) were carried out using a Varian GC 3900 gas chromatograph equipped with a VF-5 ms column (30 m x 0.32 mm x 0.25 μm film thickness; Varian). The GC injection port was set to 220 °C and the flame ionization detector at 300 °C. The column temperature was held at 60 °C during 2 min, then was increased to 300 °C at 10 °C/min, and finally held at 300 °C for 10 min. Helium was used as carrier gas at 1 ml/min.

Set 2 To evaluate possible variation during the two phases of the colony cycle, five HRIs were collected every third day throughout one colony cycle from six colonies (three colonies from A, two from B, and one for D, total of 375 HRIs). We chose HRIs to be sure that the analyzed individuals were fertile, thus able to lay eggs and showing, if present, fertility-related components in their cuticular profiles. Cuticular washes were prepared by immersing single ants for 10 min in 20 μl of a pentane solution with *n*-triacontane (10 ng/ml) as internal standard. We used a lower quantity of solvent because ants were extracted individually and not in groups of eight, and in this way we increased the concentration of CHC's in the samples. Aliquots of each extract (2 μl) were injected manually onto an Agilent Technologies 7890A Gas Chromatography System connected to an Agilent Technologies 5975C mass spectrometer. The GC column was an HP- 5MS capillary column (30 m×250 μm, 0.25 μm thickness; Agilent), and the oven temperature was kept at 70 °C for 1 min, increased at 30 °C/min to 260 °C, then at 5 °C/min to 300 °C, then at 20 °C/min to 320 °C, and held for 3 min. This method was aimed at minimizing the time of each run by focusing on the part of the spectrum including the CHC peaks identified previously.

Statistical Analyses

Eighteen peaks corresponding to CHCs appeared in all samples of Set 1, while only 16 peaks appeared persistently in Set 2. We, thus, used 18 peaks for the statistical analyses of Set 1 and 16 peaks for the statistical analyses of Set 2 (details in Table 2 and Fig. 1). The relative concentrations of the compounds used for the discriminant analyses were transformed into proportions of the total, and then imported in the software PRIMER (Clarke and Gorley 2006) with the PERMANOVA^{+} add-on package. Data were square root normalized and transformed in matrices of Euclidean inter-individual distances prior to statistical analyses. We used PERMANOVA tests in order to include random factors in our statistical design. This allowed taking the inter-colony and inter-subcaste variability into account, while pooling individuals for statistical tests.

Set 1 CHC profiles were analyzed using a PERMANOVA design including three factors: Subcaste (four levels (HRIs, callow, young and old LRIs), fixed), Clone (four levels (A, B, C, D), fixed), and Colony (ten levels (Table 1), random, nested within Clone). This test was aimed at investigating the subcaste-related variability in chemical signatures within colonies, and testing whether differences could be found in relation to the clonal origin of the 10 colonies we included in the study. *P* values were obtained using 999 permutations of residuals. In order to test for differences in individual CHC compounds among morphological and behavioral subcastes, we conducted separate Linear Mixed Model analyses on each CHC peak in the software STATISTICA. We implemented the subcaste as fixed factor and the colony nested within the clone as random factor.

Set 2 CHC profiles were analyzed with a PERMANOVA design using the factors Phase (two levels (reproductive or foraging phase), fixed), Clone (fixed) and Colony (six levels (Table 1), random, nested within Clone). The test was designed to investigate the influence of reproductive state of fertile individuals (HRIs) on chemical signatures and to understand whether and what type of inter-clonal differences could be found in the chemical signatures of the analyzed individuals. *P* values were obtained using 999 permutations of residuals.

Chemical and Genetic Distances In order to assess any potential associations between CHC profiles and genetic relatedness, we performed Mantel correlation tests (Mantel 1967) based on 9999 random permutations using the software GenoDive (Meirmans and Tienderen 2004). We correlated matrices containing chemical Euclidean pairwise distances between colony centroids, obtained in the software PRIMER (Clarke and Gorley 2006) from the square-root transformed areas of CHC peaks, and genetic distances between colonies. Euclidean genetic distances were obtained with the software GenoDive based on 30 nuclear microsatellite loci analyzed for 10 individuals (one for each colony).

Table 2 The 19 cuticular hydrocarbon peaks used in the present study and their identifications using Retention Indices and mass spectral fragmentation patterns

Peak #	Retention time (min)	Retention Index	Identification	MS Diagnostic ions
1	22.42	2300	C23	324
2	25.26	2500	C25	352
3	25.77	2534	11+13-me-C25	168, 224, 196, 351
4	26.22	2564	2-me-C25	323, 351
5	26.38	2575	3-me-C25	57, 337, 309, 351
6	26.76	2600	C26	366
7	27.27	2632	11+12+13-me-C26	168, 238, 182, 224, 196, 210, 365
8	27.73	2662	2-me-C26	337, 365
9	28.34	2700	C27	380
10	28.85	2734	11+13-me-C27	168, 252, 196, 224, 379
11	29.38	2769	11,15-dime-C27	168/9, 196/7, 239, 267, 393
12	29.51	2778	4,15-dime-C27	71, 365, 239, 196, 365, 393
13	29.85	2800	C28	394
14	29.94	2806	3,11-dime-C27	182, 252, 379
15	30.34	2832	13+14-me-C28	196, 238, 210, 224, 393
16	30.76	2859	11,15-dime-C28	168, 211, 239, 281, 407
17	31.38	2900	C29	408
18	31.85	2931	13+15-me-C29	196, 252, 224, 407
19	32.27	2958	13,17-dime-C29	196, 267, 421

Results

Genetic Analyses The 10 individuals (one per colony) sequenced for two mitochondrial DNA fragments and 30 nuclear microsatellite loci belonged to four previously described asexual lineages from Okinawa and Taiwan (MLL1, MLL4, MLL6, and MLL3 in Kronauer et al. 2012, referred to as A, B, C, and D, respectively, in this study (see Table 1)). Four individuals had A genotypes, three had B genotypes, two had C genotypes, and one had the D genotype. Based on the 17 polymorphic microsatellite loci analyzed for ten individuals per colony, we detected a single clonal lineage in each colony. On average, we detected 1.75 multilocus genotypes (MLGs; usually differing by a single allele across all 17 loci) per colony for the eight colonies belonging to clonal lineages A, B, and D. As predicted, average pairwise relatedness within colonies was extremely high (r=0.985 on average). Similar results have been reported previously for our two colonies from clonal lineage C (Kronauer et al. 2013). This confirms that colonies in our

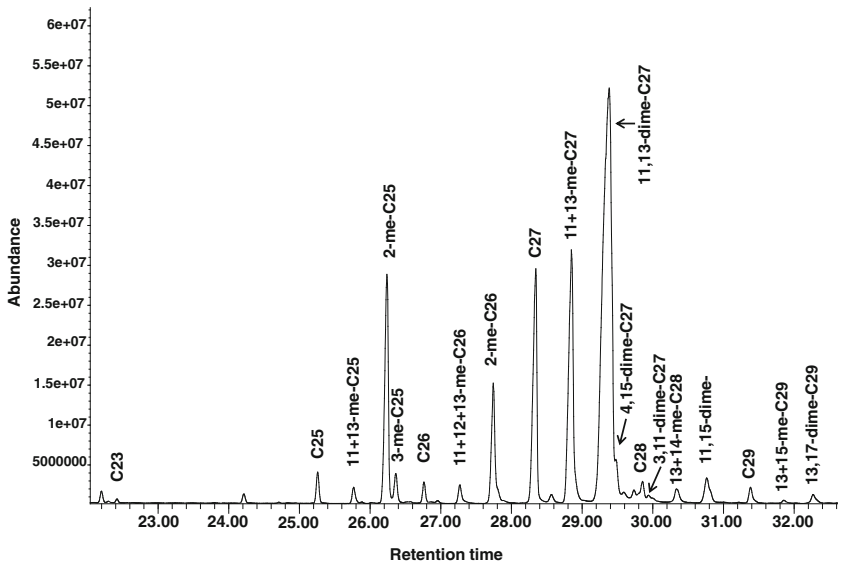

Fig. 1 The 19 cuticular hydrocarbon peaks used in the present study. We analyzed 18 peaks for Set 1, i.e. all the peaks shown except for 3-methyl pentacosane, and 16 peaks for Set 2, i.e. all the peaks shown except for tricosane, 13+15-methyl nonacosane and 13,17-dimethyl nonacosane

study were genetically homogeneous, and shows that the genotype of a single individual per colony reliably represents the genetic makeup of that colony.

Chemical Analyses

Identification of CHC's Nineteen peaks were detected in GC/MS analyses of CHC's as shown in Table **2** and Fig. 1. Several peaks contained more than one compound but were considered as one for the analyses. We analyzed 18 peaks for Set 1, i.e., all the peaks shown except for 3-methyl pentacosane. We analyzed 16 peaks for Set 2, i.e., all the peaks shown except for tricosane, 13+15-methyl nonacosane, and 13,17-dimethyl nonacosane.

Set 1 We found significant differences between experimental groups for all the factors included in our PERMANOVA analysis, and for their interactions (Table 3a). Interestingly, we found significant differences among *C. biroi* subcastes (PERMANOVA, *Pseudo F*=4.574, *df*=3, *P*=0.001, Fig. 2a). A subsequent pairwise PERMANOVA revealed that significant differences were present exclusively between callow LRIs and HRIs, between callow LRIs and young LRIs, and between callow LRIs and old LRIs (*P*=0.001, *P*=0.004 and *P*=0.015, respectively). This suggests that callow LRIs bear chemical signatures distinct from all the other groups, while the remaining three groups are identical in their CHC profiles. These results are supported by an additional Linear Mixed Model analysis in which we compared the four morphological and behavioral subcastes for each individual CHC peak (Table 4).

We also found significant differences between colonies of different clonal lineages (PERMANOVA, *Pseudo F*=5.1442, *df*=3, *P*=0.009, Fig. 2b, Table 3a). While clones A, B, and D were not significantly different from one another (pairwise PERMANOVA, all *P*>0.05), clone C was significantly different from clones A, B, and D (pairwise PERMANOVA, all *P*<0.001). A positive correlation was found between chemical and genetic distances between colonies (Mantel test, *r*=0.58, *P*<0.001). Within clonal lineages, the analyses including the factor subcaste and the interaction between the factors subcaste and colony revealed significant differences between experimental groups for all clones (Table 3b).

Set 2 We found significant differences between experimental groups for some of the factors included in the PERMANOVA analysis, and for some of their interactions (Table 3c). No significant differences were found between the chemical signatures of fertile individuals in different phases of the colony cycle, i.e., there was no influence of reproductive status (activity or inactivity) on chemical signatures (PERMANOVA, *Pseudo F*=0.1.9648, *df*=1, *P*=0.234, Fig. 2c, Table 3c). No differences among groups were found for the interaction between the clonal lineage and the phase of the colony cycle (PERMANOVA, *Pseudo F*=1.9648, *df*=1, *P*=0.198,

Table 3 Results of the PERMANOVA analyses on the cuticular signatures of individuals. (*: *P*<0.05; **: *P*<0.01; ***: *P*<0.001; *NS*: not significant). Random factors are given in italics (***a).*** Set 1, comparisons among subcastes and clones; (***b).*** Set 1, comparisons among colonies within the same clone; (***c).*** Set 2, comparisons of the HRI signatures between the reproductive and foraging phase; (***d).*** Set 2, comparisons among groups of HRIs collected every third day throughout a colony cycle; (***e).*** Set 2, comparisons among clones)

	df	Pseudo-*F*	Significance
a.			
subcaste	3	4.574	***
clone	3	5.1442	**
colony(clone)	6	47.357	***
subcaste*clone	9	2.3327	**
colony(clone)*subcaste	18	11.498	***
b. *Clone A*			
colony	3	46.202	***
subcaste	3	74.685	***
colony*subcaste	9	15.581	***
Clone B			
colony	2	63.55	***
subcaste	3	28.954	***
colony*subcaste	6	10.554	***
Clone C			
colony	1	3.4732	**
subcaste	3	27.464	***
colony*subcaste	3	5.3728	***
c.			
clone	2	13.655	*
phase	1	1.9648	NS
colony(clone)	3	20.694	***
*clone**phase	2	2.1743	NS
phase**colony*(clone)	3	3.7517	**
d.			
clone	2	12.963	*
collection day	11	0.85237	NS
colony(clone)	3	26.968	***
*clone**collection day	22	0.95215	NS
Collection day**colony*(clone)	33	3.177	***
e.			
clone	2	13.655	*
phase	1	1.9648	NS
colony(clone)	3	20.694	***
clone*phase	2	2.1743	NS
phase**colony*(clone)	3	3.7517	**

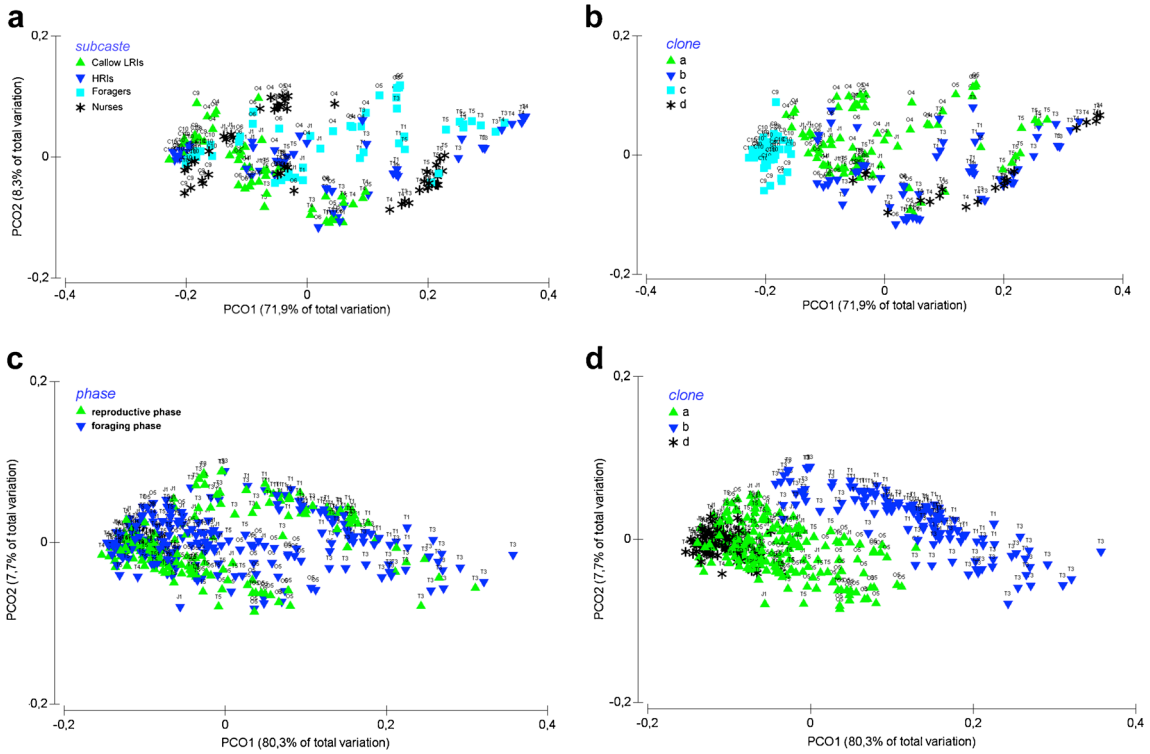

Fig. 2 Principal Coordinates Analyses on CHC data Sets 1 and 2 (**a**) and (**b**) samples of Set 1 labeled by subcaste and by clone, respectively; (**c**) and (**d**) samples of Set 2 labeled by clone and phase of colony cycle, respectively

Table 3c). In addition, no significant differences were found between cuticular signatures among any of the groups of HRIs collected every third day during a complete colony cycle (PERMANOVA, random factor: colony nested in clones; fixed factors: clones and group of HRIs collected the same day; *Pseudo F*=0.8524, *df*=11, *P*=0.6; complete results are shown in Table 3d; pairwise PERMANOVA, all *P*>0.089). The different clones showed significant differences in their chemical signature (PERMANOVA, *Pseudo F*=13.655, *df*=1, *P*=0.023, Fig. 2d; complete results in Table 3e). However, the correlation between genetic and chemical distances was marginally non-significant (Mantel test, 9999 permutations, *r*=0.7, *P*=0.064).

Discussion

Within-Colony CHC Variability Our chemical analyses revealed a high degree of homogeneity in cuticular signatures within *C. biroi* colonies, with only callow LRIs being different from all the other groups we considered. However, this finding is probably due to the fact that often in social insects the cuticular profile of recently eclosed individuals is not completely matured, and as a result they are chemically different from older nestmates (Breed et al. 2004; Ichinose and Lenoir 2009; Teseo et al. 2013).

In many species of social Hymenoptera, CHC profiles signal fertility and/or reproductive activity (see Monnin 2006), which allows non-reproductive individuals to perceive the presence of reproductives and refrain from egg-laying (Holman et al. 2010; Le Conte and Hefetz 2008; Slessor et al. 2005;) ensuring a balance between reproductive and ergonomic colony function. In some ant species where all nestmates are able to mate and produce female offspring, fertility-related cuticular hydrocarbons serve to maintain colony-level reproductive dominance hierarchies (Cuvillier-Hot et al. 2004; Heinze et al. 2002; Monnin 2006; Monnin et al. 1998; Monnin and Peeters 1999; Peeters et al. 1999). In the ponerine ant *Platythyrea punctata*, where individuals are able to produce female brood asexually, fertility and dominance signaling via cuticular signatures maintains a single reproductive individual per colony (Hartmann et al. 2003, 2005). *Cerapachys biroi* is different in its colony-level reproductive dynamics and social structure, in that all individuals can reproduce (Ravary and Jaisson 2002), and there are no reproductive dominance hierarchies. Cuticular signatures, thus, are not expected to bear fertility signals related to dominance or inducing nestmates to refrain from laying eggs. It seems improbable that the colony-level regulation of reproduction relies on cuticular signals related to individual fertility or reproductive activity. However, HRI production in *C. biroi* varies depending on the average colony-level fertility, implying the existence of some

Table 4 Set 1. *Post hoc* comparisons among subcastes for each individual CHC peak (Linear Mixed Model, Fisher's LSD test; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$). The peak of 13+15-methyl nonacosane was excluded because it did not show significant changes in the overall comparison. LRI=Low reproductive individuals; HRI=high reproductive individuals

	Callow LRIs	HRIs	Foragers	Nurses
1. Tricosane				
Callow LRIs	-	***	*	***
HRIs	-	-	0.060	0.833
Foragers	-	-	-	0.095
Nurses	-	-	-	-
2. Pentacosane				
Callow LRIs	-	***	***	*
HRIs	-	-	0.933	***
Foragers	-	-	-	***
Nurses	-	-	-	-
3. 11+13-Methyl pentacosane				
Callow LRIs	-	***	***	***
HRIs	-	-	***	***
Foragers	-	-	-	0.193
Nurses	-	-	-	
4. 2-Methyl pentacosane				
Callow LRIs	-	**	***	*
HRIs	-	-	0.054	0.464
Foragers	-	-	-	**
Nurses	-	-	-	-
5. Hexacosane				
Callow LRIs	-	***	***	***
HRIs	-	-	0.684	0.229
Foragers	-	-	-	0.421
Nurses	-	-	-	-
6. 10+12-Methyl hexacosane				
Callow LRIs	-	***	***	***
HRIs	-	-	0.298	0.276
Foragers	-	-	-	*
Nurses	-	-	-	-
7. 2-Methyl hexacosane				
Callow LRIs	-	***	***	***
HRIs	-	-	**	0.780
Foragers	-	-	-	***
Nurses	-	-	-	-
8.Heptacosane				
Callow LRIs	-	***	***	***
HRIs	-	-	0.208	*
Foragers	-	-	-	***
Nurses	-	-	-	-
9. 11+13-Methylheptacosane				
Callow LRIs	-	0.645	**	*
HRIs	-	-	*	0.098
Foragers	-	-	-	0.359
Nurses	-	-	-	-
10. 11+15 Methylheptacosane				
Callow LRIs	-	***	***	***
HRIs	-	-	0.599	*
Foragers	-	-	-	**
Nurses	-	-	-	-
11. 5, 11-Dimethylheptacosane				
Callow LRIs	-	***	0.051	*
HRIs	-	-	*	*
Foragers	-	-	-	0.559
Nurses	-	-	-	-
12. Octacosane				
Callow LRIs	-	**	***	*
HRIs	-	-	0.085	0.615
Foragers	-	-	-	*
Nurses	-	-	-	-
13. 10-Methyloctacosane				
Callow LRIs	-	0.483	0.842	*
HRIs	-	-	0.615	0.066
Foragers	-	-	-	*
Nurses	-	-	-	-
14. 12-Methyloctacosane				
Callow LRIs	-	***	***	***
HRIs	-	-	0.065	0.572
Foragers	-	-	-	0.200
Nurses	-	-	-	-
15. 14+16-Methyl octacosane				
Callow LRIs	-	***	***	***
HRIs	-	-	0.521	0.436
Foragers	-	-	-	0.155
Nurses	-	-	-	-
16. Nonacosane				
Callow LRIs	-	**	***	***
HRIs	-	-	0.070	0.160
Foragers	-	-	-	0.688
Nurses	-	-	-	-
17. 13,17-Dimethyl nonacosane				
Callow LRIs	-	***	***	***
HRIs	-	-	*	0.103
Foragers	-	-	-	0.437
Nurses	-	-	-	-

regulation acting on larval fate (Lecoutey et al. 2011). The lack of correlation between fertility levels and cuticular signatures suggests that the regulation of HRI development is either non-chemical or does not depend on signals derived from CHCs. One possibility is that a non-CHC, non-volatile chemical signal that is present on the cuticular surface or secreted from other glandular sources is involved. Moreover, the quality of the food that may be admixed with some glandular products of adults can direct larval development toward different pathways, which underlies caste determination in many social Hymenoptera (Hölldobler and Wilson 1990; Wheeler 1986, 1991). Primer pheromones transmitted from adults to larvae via direct contact during parental care might play a complementary role in caste differentiation. Indeed, workers regularly perform a peculiar behavior during brood care that consists of licking the developing larvae ventrally under the head capsule (Lecoutey, personal observations). Further studies on the mandibular secretions of nurses are needed to investigate whether they play a role in larval differentiation in *C. biroi*.

Subcaste differentiation might also exclusively rely on the quantity of food available to larvae. Quantitative differences in food intake during pre-imaginal stages have major effects on development in insects, and give rise not only to differences in adult size, but also differential expression of adult polyphenisms (Emlen 1994; Hunt and Simmons 1997; Moczek and Emlen 2000). Adult *C. biroi* could quantitatively limit larval feeding in several ways, e.g., by actively keeping larvae away from prey items within the nest, or simply by competing with larvae for food. HRI production is inversely proportional to the proportion of fertile individuals in a colony, and fertile individuals might need a higher quantity of food in order to produce eggs. Thus, the more fertile individuals are present in a colony, the less food might be available for larvae, which possibly limits HRI production. Other types of influences on larval fate, such as mechanical stress on developing larvae due to biting from adults (Brian 1973; Pennick and Liebig 2012), also could be involved. Observations on the behavior of adult individuals towards larvae during the foraging phase will clarify the proximate factors determining subcaste differentiation in *C. biroi*.

CHC Variability Among Clones Our study shows that the colony-level chemical signatures of *C. biroi* vary according to the clonal lineage, with chemical distances between colonies growing with genetic distances. The fact that we observed only a marginally non-significant correlation between genetic and chemical distances for colonies of Set 2 was probably due to the low number of colonies included in that analysis (N=6). Colonies belonging to the same clonal lineage also show some variability in their cuticular signature, even though the chemical distances among those are lower than distances among colonies from different clones. Overall, our findings support the results of a previous study on the invasive *C. biroi* population in Okinawa, where individuals were able to discriminate between nestmates and non-nestmates, especially when non-nestmates belonged to unrelated asexual lineages (Kronauer et al. 2013). According to our results, chemical signatures might be the proximate cues indicating genetic dissimilarity between interacting individuals, which in turn might prevent fusions between unrelated colonies. This might be a reason that explains why natural *C. biroi* colonies have been found to be exclusively monoclonal (Kronauer et al. 2013). However, given that the putative native range of *C. biroi* remains still largely unexplored, it cannot be excluded that in natural populations different clones mix in chimeric colonies.

Invasive populations of ants are likely to originate via the introduction of few individuals, i.e., population bottlenecks which most of the time produce a strong founder effect (Tsutsui and Suarez 2003). As a result, invasive populations are overall genetically less diverse than native populations. This can result in individuals from different colonies displaying similar cuticular hydrocarbon profiles, potentially leading to a loss of aggression even between non-nestmates (Tsutsui and Suarez 2003). This loss of aggression is thought to promote the formation of supercolonies (Giraud et al. 2002). For example, many invasive species forming supercolonies exhibit negligible levels of between-colony aggression, even between individuals taken from nests separated by several kilometers (Blight et al. 2012; Drescher et al. 2010; Giraud et al. 2002). *Cerapachys biroi* is invasive (Kronauer et al. 2012, 2013; Wetterer et al. 2012), but to our knowledge it does not form supercolonies. This might in part be due to the maintenance of non-nestmate discrimination between colonies from different clonal lineages (Kronauer et al. 2013).

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