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Can cuticular lipids provide sufficient information for within-colony nepotism in wasps?

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Inclusive fitness theory predicts that members of non-clonal societies will gain by directing altruistic acts towards their closest relatives. Multiple mating by queens and multiple queens creates distinct full-sister groups in many hymenopteran societies within which nepotism might occur. However, the weight of empirical data suggests that nepotism within full-sister groups is absent. It has been suggested that a lack of reliable recognition markers is responsible. In this paper, we investigated whether epicuticular lipids could provide reliable cues for intracolony kin recognition in two species of social wasps, the paper wasp *Polistes dominulus* and the hornet *Vespa crabro*. Epicuticular lipids have previously been shown to be central to kin recognition at the nest level, making them excellent candidates for within-nest discrimination. We genotyped individuals using DNA microsatellites and analysed surface chemistry by gas chromatography–mass spectrometry. We find that in both species epicuticular lipids typically could provide enough information to distinguish related nest-mates from unrelated nest-mates, a difference that occurs in colonies with multiple queens. However, in *V. crabro*, where colonies may be composed by different patrilines, information for discrimination between full sisters and half-sisters is weaker and prone to errors. Our data suggest that epicuticular lipids at best provide reliable information for intracolony nepotistic discrimination in multiple-queen colonies composed of unrelated lines.

Keywords: intracolony recognition; nepotism; cuticular lipids; social wasps; *Vespa*; *Polistes*

1. INTRODUCTION

Whether social animals are able to directly identify their closest relatives is a central question in the study of social evolution (Sherman *et al.* 1997). In social insects, the ability to discriminate between nest-mates and alien conspecifics is widespread and ensures that colonies maintain themselves as coherent units. Furthermore, workers in some species have been shown to alter sex allocation (Sundström *et al.* 1996) and worker male production (Foster & Ratnieks 2000) in response to colony kin structure, suggesting that workers can estimate overall worker–worker relatedness. Evidence that workers directly discriminate between colony members of different relatedness, however, has been much more problematic.

Many social insect colonies are composed of multiple matrilines (progeny from different mothers and, typically, fathers) and/or patrilines (progeny from different fathers). Inclusive fitness theory (Hamilton 1964), therefore, predicts that nepotism within full-sister groups would be beneficial if this does not incur excessive costs. Evidence for the existence of intracolony nepotism has been reported in some specific contexts in the honeybee *Apis mellifera* (reviewed by Breed *et al.* 1994) and the ant *Camponotus floridanus* (Carlin *et al.* 1987). However, in their 1994 review Breed and coworkers have highlighted a wealth of negative evidence for nepotism in the honeybee and indeed argue that some positive results could be due to experimental bias (Breed *et al.* 1994). A similar case has been made for the *Camponotus* data (Carlin *et al.* 1993). Furthermore, research on several other species has provided no evidence for intracolony nepotism (Queller *et al.* 1990; Balas & Adams 1996; Bernasconi & Keller 1996; Strassmann *et al.* 1997; DeHeer & Ross 1997; Solìs *et al.* 1998).

Two main arguments have been proposed for the absence of intracolony nepotism in the social insects (reviewed by Keller 1997). The first is that the benefit derived from nepotistic behaviour towards close relatives is outweighed by the costs incurred by less-related nestmates (Ratnieks & Reeve 1992). The second suggests that nepotistic behaviours are disfavoured owing to costs associated with kinship assessment errors (Reeve 1989; Sherman *et al.* 1997). Accurate kin discrimination requires both reliable genetically based cues and a self-matching mechanism, where the self provides a template for comparison (Sherman 1991). The aim of this study was to examine whether reliable genetically based cues exist in

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insect societies. Reliable genetic cues may be confounded by mixing of cues among colony members either accidentally or deliberately (Carlin *et al.* 1993; Keller 1997). Deliberate scrambling of genetic cues will be favoured by colony members that stand to lose from nepotism, such as members of minor patrilines or matrilines, and the queen (Keller 1997). Moreover, homogenization of recognition cues between colony members may be necessary for efficient nest-mate recognition.

In social insects, epicuticular lipids are considered to be central cues in nest-mate recognition, with a wealth of evidence showing that epicuticular lipids are more similar among nest-mates than non-nest-mates (reviewed in Lorenzi *et al.* 1996; Singer *et al.* 1998; Breed 1998). However, less is known about the structure of the epicuticle lipid signature used in recognition. This has been studied in honeybees (Breed 1998) and in *Polistes* (Dani *et al.* 2001), but the results obtained may not apply to other social insects. Which classes of lipids act as recognition pheromones is controversial. Some experimental work found that hydrocarbons are the only class of lipids acting as recognition cues (in the ant *Cataglyphis niger*: Lahav *et al.* 1999), while other studies have found that the oxygenated constituents are more relevant (in the honeybee: Breed 1998; Fröhlich *et al.* 2001).

In studies on epicuticular lipids, multivariate statistics are most commonly used to demonstrate differences among groups of individuals. Whether differences in lipid profiles discerned by these methods can also be detected by the insects can be determined only through acceptance experiments. Studies based on bioassays showed that manipulation of cuticular lipid composition significantly affects nest-mate acceptance (Breed 1998; Dani *et al.* 2001; Ruther *et al.* 2002). Lures applied with cuticle extracts of nest-mates were accepted whereas lures applied with extracts of non-nest-mates were rejected (Dani *et al.* 1996; Lorenzi *et al.* 1997). Moreover, the capacity to discriminate between complex mixtures of cuticular lipids was demonstrated for honeybees by using a conditioning assay that takes advantage of the proboscis extension reflex (Getzt *et al.* 1988; Fröhlich *et al.* 2001).

Cuticular lipids also differ at the intracolony level among castes and physiological states, suggesting that they may play a role in caste recognition or as a signal of reproductive status (Lorenzi *et al.* 1996; Peeters *et al.* 1999; Liebig *et al.* 2000; Sledge *et al.* 2001). Epicuticular lipids, therefore, seem to be a likely candidate for intracolony kin recognition.

Differences in the lipid composition have been shown between two worker patrilines in honeybee colonies headed by artificially doubly mated queens (Page *et al.* 1991). Workers in a honeybee colony with a single queen mated 16 times could be correctly assigned to their patriline on the basis of their cuticular lipid composition when the workers were isolated and when they were allowed to remain in their colony (Arnold *et al.* 1996). Honeybee queens normally mate 7–20 times (Estoup *et al.* 1994) so a 16-patriline colony is not unusual. In a following paper, Arnold *et al.* (2000) reanalysed the same data with mixed results showing that within-hive homogenization of cuticular hydrocarbons occurs among some patrilines but not in others.

In this study, we investigated the link between genetic relatedness within colonies and cuticular lipid composition in the hornet *Vespa crabro* and the paper wasp *Polistes dominulus*, two species with colony kin structures differing from each other and from honeybees. Evidence of intracolony kin recognition has not been reported for these two species. *Vespa crabro* colonies typically consist of one to three patrilines and a single matriline, although occasionally two matrilines occur, owing to nest take-overs by usurping queens (Foster *et al.* 2000). Conversely, *P. dominulus* queens are always singly mated (Queller *et al.* 2000), but colonies are often founded by several queens, which may often be unrelated (Queller *et al.* 2000).

Genetic relatedness and chemical analysis, therefore, allowed us to analyse differences in the cuticular lipids within colonies of the following genetic structures: (i) single patriline (*V. crabro* and *Polistes*); (ii) multiple patriline (*V. crabro*); and (iii) multiple matriline (*V. crabro* and *Polistes*).

2. MATERIAL AND METHODS

Polistes dominulus colonies were collected in July 1996 in a 2 ha area of countryside *ca.* 100 km southeast of Florence, when worker number in the colonies varies from 1 to 40 (R. Cervo, personal communication). *Vespa crabro* colonies were collected in the New Forest, Hampshire, UK, in summer 1998, and contained at least 50 workers. In both cases the entire colonies were immediately killed by freezing after collection and kept at -20 C until genetic and chemical analysis. We did not isolate individuals to eliminate any effects of sharing hydrocarbons because we were interested in what cues are present in natural colonies. Age of the individuals was unknown.

Worker thoraces were used for the genetic analysis, and worker abdomens for chemical analyses. For both species, up to 10 workers, randomly chosen, from each colony were analysed. For *V. crabro*, up to 10 additional workers were analysed in colonies with more than one matriline or patriline, except for one mixed-matriline colony. Six DNA microsatellite loci (Paco3434AAT, Pan80AAT, Pbe102TAG, Pbe128TAG, Pbe269AAG, Pbe430AAG; see Queller *et al.* (2000)) were analysed for *P. dominulus* and four (Foster *et al.* 1999) for *V. crabro*. Workers cohabiting the same colony were identified as full sisters when:

(i) all shared one allele at each locus (from the haploid father);
(ii) when all collectively had no more than two other alleles at each locus (from the mother, who could be a heterozygote); and
(iii) when all the likelihood ratios between the members of the group, estimated through the program KINSHIP v. 1.3 (Queller & Goodnight 1989), were higher than the ratio needed to reject the null hypothesis.

Using these criteria, five mixed-matriline colonies and one one-matriline (1M) colony were identified in *P. dominulus*. In *V. crabro* we chose two two-matriline (2M) colonies, three two-patriline (2P) colonies and four one-patriline (1P) colonies.

(a) *Gas chromatography–mass spectrometry analysis*

For *V. crabro*, cuticular lipids were analysed for a total of 129 workers. Lipid sampling was performed with the protocol of

Turillazzi *et al.* (1998), i.e. by gently rubbing the hornet wasp abdomen for 1 min using a cotton wool bud and then extracting the cotton wool in 1 ml of pentane for 15 min in an ultrasonic bath. This method did not give a satisfactory result for *P. dominulus*, since the signal obtained by gas chromatography–mass spectrometry was too weak. Instead, the 86 *Polistes* workers were analysed by directly extracting the abdomen in 1 ml of pentane for 10 min in the ultrasonic bath. Before cuticle extract preparation, the ovaries and the Dufour's gland were removed by carefully pulling out the sting. In this species, the Dufour's gland is known to produce the same hydrocarbons as those found on the cuticle (Dani *et al*. 1996). We also removed the ovaries because in other insects they have been reported as one of the body organs where a large proportion of internal hydrocarbons of the same kind as those found on the cuticle are accumulated (Schal *et al.* 1998).

For both species, pentane extracts were evaporated under a gentle nitrogen stream, and then resuspended in 100 l of heptane. Chemical analysis was performed on a HP5890 II series gas chromatograph coupled to a HP 5971A quadrupole mass spectrometer (using 70 eV electron impact ionization), controlled by an HP G1034C chemstation. The gas chromatograph was installed with a Restek Rtx-5MS column (30 m × 0.25 mm, 95% polydimethylsiloxane and 5% phenyl, phase thickness 0.5 m) with helium as a carrier gas. The injector port was set at 280 C. Resuspended extracts (2 l) were injected in splitless mode. The oven temperature was raised from 70 C to 150 C at a rate of 30 C min^{-1}, and after 5 min at 150 C, it was raised to the final temperature of 320 C at 5 C min^{-1}. In *V. crabro*, the alkene double bond position was determined through chemical ionization on an ion-trap mass spectrometer using acetonitrile as reagent gas (Moneti *et al.* 1997). Determination of the branching position of the methyl alkanes was based on the fragmentation pattern reported in literature (Nelson 1978) and on the comparison of their retention times with those of linear alkanes (Carlson *et al*. 1998). Citronellyl citronellate and citronellyl geranate, found in the *V. crabro* samples, have already been found for the tergal glands of this species by Wheeler *et al*. (1982). The mass spectra in our samples matched those reported by these authors. The spectra of hexadecan-1-ol and octadecan-1-ol found in the hornet samples matched with those reported in the electronic libraries and their identification was confirmed through injection of standards. In *V. crabro*, two compounds eluting late in the chromatogram were identified as octacosan-1-ol and triacontan-1-ol on the basis of

(i) their mass spectra corresponding to a primary alcohol and showing an ion corresponding to M-18 (respectively 392 for octacosan-1-ol and 420 for triacontan-1-ol); and
(ii) of their retention times close to that of the linear alkane bearing three more carbon atoms.

(b) *Data processing and statistics*

For *V. crabro*, 20 compounds producing peaks whose area was reliably integrated were considered in the data analysis. For *Polistes*, the concentration of 44 compounds was considered in the calculation of chemical distances (see § 2b(i)). However, many of these compounds were only present in traces in some of the specimens and absent from most. As the distribution of the concentration values for these compounds was markedly abnormal, these compounds were excluded from the multivariate statistic analyses (table 1) and only 24 compounds were considered.

We used three methods to analyse the data: chemical distances, principal component analysis (PCA) and discriminant analysis. The chemical distance data allow us to look for a general correlation between relatedness and chemical similarity, and examine whether chemical distance changes between groups of related and unrelated individuals. PCA whittles the chemical data down to a few important variables allowing us to test if differences existed for those variables between nest-mate lines. Finally, discriminant analysis (DA) adds to PCA and asks whether it is possible to assign an individual to its genetic group based upon its chemical profile.

(c) *Chemical distances*

Squared Euclidean distances of the chemical concentrations (percentage data of 44 compounds for *Polistes* and 20 compounds for *V. crabro*) were calculated for all possible pairs of individuals in both samples. This measure of chemical distance is the sum of the squared differences between the concentration of each compound in the two specimens. Statistical analyses were performed with SPSS v. 7.5.

The relationship between chemical distance and relatedness was tested in two ways. First, a Spearman correlation was calculated between chemical distance and relatedness, estimated by the program RELATEDNESS v. 4.1.2 (Goodnight & Queller 1999). Second, the chemical distance for non-nest-mate pairs and for nest-mate pairs with different degrees of relatedness (unrelated nest-mates, half-sisters, full sisters) were compared using the Kruskal–Wallis test, considering the significance level based on an estimate of the exact distribution based on 10 000 sampling from the dataset (Monte Carlo Estimate, SPSS v. 7.5). Differences between each pair of groups were tested using the Mann–Whitney *U*-test, and the exact probability was compared with $\alpha_1 = 1 - (1 - \alpha)^{1/K}$, K being the number of comparisons (five for *V. crabros* and three for *Polistes*) and α being 0.05, 0.01 or 0.001 (Dunn–Šidàk correction for multiple comparisons; Sokal & Rohlf 1995).

(d) *Multivariate statistics*

PCA was performed on both the *V. crabro* and *Polistes* datasets to reduce the number of variables and to obtain orthogonal components (by the Anderson Rubin method in SPSS). Variables showing low communalities (Norušis 1992) were removed from the PCA. The Kaiser–Meyer–Olkin (KMO) value was considered to evaluate the correlation matrix adequacy (values between 0.6 and 0.8 are considered indices of acceptable matrices; Focardi 1993). For each mixed-line colony, the values of the variables excluded from the PCA and the scores obtained from each principal component (PC) were compared using the Mann–Whitney *U*-test between the individuals of the two lines (and the exact probability was considered).

Stepwise discriminant analysis was performed for each colony formed by two lines. The analysis was performed on the scores derived from the PCA for the *Polistes* colonies and for one of the two-matriline *V. crabro* colonies, which had a small number of specimens (table 2). For the other *V. crabro* colonies, a stepwise discriminant analysis was performed on the original data. Canonical correlations, Wilks' λ significance and the percentage of correct assignments were considered to evaluate the validity of the discriminant function. A problem with discriminant analysis is that with small datasets it may be able to find differences between any groups that it is given. To test whether this was the case for our dataset, we randomly assigned individuals in a colony to one of two arbitrary groups of the same size as the

real groups and tested to see if they could be separated through a discriminant analysis performed with the same procedure as on the real data. This was repeated for 50–100 permutations for the data of each colony. We then calculated the percentage of permutations for which the stepwise discriminant analysis gave a better discrimination than that on the real data. If the percentage is high then it casts doubt on the role of relatedness in obtaining the separation.

3. RESULTS

The compounds identified in the epicuticular lipids of *V. crabro* and *P. dominulus* are given in table 1. Compounds do not substantially differ from those already reported by previous authors (Butts *et al.* (1991, 1995) and Ruther *et al.* (1998) for *V. crabro*; Bonavita-Cougourdan *et al.* (1991) and Dani *et al.* (1996) for *Polistes*). However, in *V. crabro* we found some alcohols not reported in other studies, and hydrocarbons containing up to 32 carbon atoms, which is heavier than the 3-methyl heptacosane and *n*-nonacosane previously reported (Butts *et al.* 1995 and Ruther *et al.* 1998, respectively).

(a) *Chemical distances*

A significant decrease of the chemical distance when relatedness increased among nest-mates was found in *P. dominulus* ($n = 307$, $r_s = -0.329$, $p = 0.001$), but not in *V. crabro* ($n = 975$, $r_s = 0.032$, n.s.).

The median of the chemical distances (figure 1*b*) decreased from non-nest-mates through the unrelated nest-mates to full sisters for *P. dominulus* (Kruskal–Wallis, $H = 150.07$, d.f. = 2, $p = 0.000$). Significant differences in chemical distance were found between non-nest-mate pairs and different matriline pairs, and between the latter and full-sister pairs. For *V. crabro* chemical distance also differed among groups (Kruskal–Wallis, $H = 92.88$, $d.f. = 3$, $p < 0.0001$; figure 1*a*). The main cause of this was the difference between unrelated and related individuals, with non-nest-mates and unrelated nest-mates having close median values, higher than those found for half-sisters and full sisters. An unexpected result was that full sisters were significantly more chemically different than half-sisters ($p = 0.05$, considering the Dunn–Šidàk correction). However, this effect was slight, with only a marginal difference between the medians of the two classes. Overall the chemical distance data suggest that significant chemical differences occur among matrilines but not among patrilines.

(b) *Principal component analysis and discriminant analysis*

In *V. crabro*, PCA was performed on all the original variables except for tetracosene, 3-methyl heptacosane and 3-methyl pentacosane, which had the lowest communalities. The KMO test gave a value of 0.68. The analysis produced four PCs whose eigenvalues were higher than 1, which altogether accounted for 85.50% of the original variance. Significant differences were found between the two lines of the two two-matriline (2M) colonies for the scores derived from some PCs and for some variables excluded from the PCA (colony 10: $n = 6$, $n_2 = 3$; Mann–Whitney U-test = 1.000, p exact = 0.048 for PC4 scores; Mann–Whitney U-test = 0.000, p exact = 0.024 for 3-methyl heptacosane; colony 11: $n_1 = 12$, $n_2 = 8$; Mann–Whitney U-test = 22.000, p exact = 0.047 for PC1 scores; Mann–Whitney U-test = 12.000, p exact = 0.004 for PC4 scores; Mann–Whitney U-test = 17.000, p exact = 0.016 for 3-methyl pentacosane). No differences were found between the lines of the 2P colonies, again suggesting that chemical differences were limited to between-matriline comparisons.

The stepwise discriminant analysis on the 2M nest named 10, performed on PC scores only, gave a percentage of correct classifications equal to 77.8 (canonical correlation = 0.678, Wilks' $\lambda = 0.541$, $\chi^2 = 3.996$, d.f. = 1, $p = 0.046$). For the other *V. crabro* colonies, a discriminant analysis (on the original data) was calculated for the 2M colony 11 and for the two 2P colonies named 20 and 26, but not for the 2P colony 14 (table 2*a*). The percentage of correct classification was 95% for colony 11 (canonical correlation = 0.820, Wilks' $\lambda = 0.327$, $\chi^2 = 18.980$, d.f. = 2, $p = 0.000$), 85% for colony 20 (canonical correlation = 0.675, Wilks' $\lambda = 0.544$, $\chi^2 = 10.336$, d.f. = 2, $p = 0.006$) and 70% for colony 26 (canonical correlation = 0.445, Wilks' $\lambda = 0.802$, $\chi^2 = 3.869$, d.f. = 1,$p = 0.049$).

Table 2*a* summarizes for each colony the comparison between the results of the discriminant analysis performed on the real data and on the permuted data. For the 2M colony 10, a better discriminant analysis than on the real data was obtained for 14.28% of the permutations. A better discrimination was obtained than on the real data for only one of the permutations on the 2M colony 11. For the colonies 2P, 20 and 26, a better discriminant analysis was found respectively for 9% and 38% of the permutations. Therefore for only one of the 2M colonies (colony 11) we found a low percentage (1.9%) of permutations where discriminant analysis worked better than on the original data.

In *P. dominulus*, the dimethyl heptacosanes, 3-methyl heptacosane, 3-methyl nonacosane and the dimethyl tritriacontanes were excluded from the PCA. The PCA produced six components whose eigenvalues were higher than 1, which altogether accounted for 86.05% of the original variance. The KMO test gave a value of 0.624.

For each colony, except one (14-31), significant differences were found between the two lines for the scores derived from some PCs and for the values of some of the variables excluded from the PCA (colony 32-1: $n_1 = 6$, $n_2 = 4$; Mann–Whitney U-test = 0.000, p exact = 0.010 for PC4 scores; colony 29-8: $n_1 = 7$, $n_2 = 4$; Mann–Whitney U-test = 0.000, p exact = 0.006 for PC2 scores, Mann–Whitney U-test = 0.000, p exact = 0.006 for PC3 scores, Mann–Whitney U-test = 2.000, p exact = 0.021 for PC5 scores; colony 14-22: $n_1 = 7$, $n_2 = 4$; Mann–Whitney U-test = 3.000, p exact = 0.042 for PC5 scores, Mann–Whitney U-test = 3.000, p exact = 0.042 for PC5 scores, Mann–Whitney U-test = 2.000, p exact = 0.024 for 3-methyl heptacosane; colony 14-31: $n_1 = 5$, $n_2 = 3$; Mann–Whitney U-test = 0.000, p exact = 0.036 for dimethyl heptacosane).

A discriminant function was obtained for each colony, except one (14-31), on the six PC scores, obtaining a percentage of correct classification ranging from 90.9% to 100% (colony 32-1, canonical correlation = 0.94, Wilks' $\lambda = 0.119$, $\chi^2 = 14.88$, d.f. = 2, $p = 0.001$; colony

Table 1. Constituents of *Vespa crabro* and *Polistes dominulus* cuticular lipids.
(For *V. crabro*, only the compounds in italics were considered in the computation of chemical distances—see § 2b—and in multivariate statistics. For *P. dominulus*, all the compounds were considered in the computation of chemical distances, while only those in bold were considered in multivariate statistics.)

V. crabro	*P. dominulus*
hexadecan-1-ol	***n-pentacosane***
citronellyl citronellate	*9-,11-,13-methyl pentacosane*
x-*octadecen-1-ol A*	*7-methyl pentacosane*
x-*octadencen-1-ol B*	*5-methyl pentacosane*
octadecan-1-ol	*3-methyl pentacosane*
citronellyl geranate	***n-hexacosane***
9-tricosene	***2-methyl hexacosane***
n-tricosane	***n-heptacosane***
3-methyl tricosane	***9-;11-;13-methyl heptacosane***
9-tetracosene	*7-methyl heptacosane*
n-tetracosane	***5-methyl heptacosane***
9-pentacosene	***9,17-; 9,13-; 9,15- and 11,17-dimethyl heptacosane***
n-pentacosane	***3-methyl heptacosane***
3-methyl pentacosane	*5,*x-*dimethyl heptacosane*
x-*hexacosene*	***n-octacosane***
n-hexacosane	***10-;11-;12-;13-;14-methyl octacosane***
9-heptacosene	***2-methyl octacosane***
n-heptacosane	x,y-*dimethyl octacosane*
3-methyl heptacosane	x-*nonacosene*
n-octacosane	***n-nonacosane***
n-nonacosane	***9-;11-;13-;15-methyl nonacosane***
n-hentriacontane	***7-methyl nonacosane***
octacosan-1-ol	***5-methyl nonacosane***
n-dotriacontane	***11,x-dimethylnonacosane***
triacontan-1-ol	*7,*x-*dimethyl nonacosane*
	3-methyl nonacosane
	5,15-; 5,x- and x,y-dimethyl nonacosane
	n-tricosane
	trimethylnonacosane
	11-;12-;13-;14-;15-methyl triacontane
	2-methyl triacontane
	x-*hentriacontene*
	n-hentriacontane
	11-;13-;15-methyl hentriacontane
	7-methyl hentriacontane
	13,17-;13,19-;11,17-;11,19-dimethyl hentriacontane
	*7,15- and 7,*x-*dimethyl hentriacontane*
	5,15- and 5,17-dimethyl hentriacontane
	12-,13-,14-,15-,16-methyl dotriacontane
	11-;13-;15-;17-methyl tritriacontane
	13,19- and 11,21-dimethyl tritriacontane
	*11,*x-*dimethyl tritriancontane*
	*13,21- and 13,*x-*dimethyl pentatriacontane*
	*11,*x-*; 11,17- and 11,19-dimethyl pentatricontane*

29-8, canonical correlation = 0.95, Wilks' $\lambda = 0.10$, $\chi^2 = 18.35$, d.f. = 2, $p = 0.000$; colony 34-1, canonical correlation = 0.97, Wilks' $\lambda = 0.05$, $\chi^2 = 13.49$, d.f. = 3, $p = 0.004$; colony 14-22, canonical correlation = 0.83, Wilks' $\lambda = 0.31$, $\chi^2 = 9.36$ d.f. = 2, $p = 0.009$). Table 2*b* reports for each colony the results of the discriminant analysis performed on the real data and on the permuted data. For one colony no better results could be obtained on the permuted data; for the other three colonies the percentages were 7.54%, 5.94% and 2%.

4. DISCUSSION

In both species, related nest-mates are markedly more similar than unrelated nest-mates (figure 1). Thus, variation in cuticular hydrocarbons provides information that could be used for discrimination between unrelated and related nest-mates. However, our principal question concerns the possibilities for discrimination among differently related nest-mates. The general absence of within-colony nepotism could be caused by three factors: workers may not benefit from discrimination; genetic cues may be absent or unreliable; or workers may lack a self-referent mechanism. Our study shows that there is genetic information available in hydrocarbon profiles. However, we also find that there may be limits to this information.

One hypothesis for why within-nest discrimination may be less effective than between-nest discrimination is blending of recognition cues. We find mixed results on this point. In *P. dominulus*, sharing the same nest seems to

Table 2. Main results from the stepwise discriminant analysis.
(Main results from the stepwise discriminant analysis (DA) between (*a*) the two lines of each *Vespa crabro* colony and percentage of better results (see § 2d) obtained on the permuted data and (*b*) the two lines of each *Polistes* colony and percentage of better results on the permuted data. 2M, two-matriline colony; 2P, two-patriline colony.)

(*a*) *V. crabro* colonies	colony 10 (2M) PC scores only	colony 11 (2M) original variables	colony 14 (2P) original variables	colony 20 (2P) original variables	colony 26 (2P) original variables
percentage of correct classifications and p associated with Wilks' λ in the DA on the real data	77.8, 0.046	95, 0.0001	failed	85, 0.006	70, 0.049
permutations for which DA produced a better separation than that on the real data (%)	14.28	1.90		9.00	38.00
number of possible permutations	83	125 969		125 969	184 755
number of permutations executed	63	52		100	48

(*b*) *Polistes* colonies	colony 32-1 (2M) PC scores only	colony 29-8 (2M) PC scores only	colony 34-1 (2M) PC scores only	colony 14-22 (2M) PC scores only	colony 14-31 (2M) PC scores only
percentage of correct classifications and p associated with Wilks' λ in the DA on the real data	100, 0.001	100, 0.0001	100, 0.004	90.9, 0.009	failed
permutations for which DA produced a better separation than that on the real data (%)	2.00	0.00	7.54	5.94	
number of possible permutations	209	329	55	329	
number of permutations executed	49	48	53	84	

render cuticular lipid composition more similar, as demonstrated by a lower chemical distance between nest-mates belonging to different matrilines than between non-nest-mates (figure 1*b*). On the contrary, sharing the same nest does not seem to have an effect on cuticular lipid composition in *V. crabro*, where the average distance between unrelated nest-mates is similar to that between non-nest-mates (figure 1*a*). The difference observed between the two species could possibly be due a different extent of allogrooming or trophallactic interactions, favouring epicuticle lipid homogenization between colony members. It is difficult to evaluate if the different methods used for the lipid extraction, superficial sampling from the cuticle in *V. crabro* and extraction of the abdomen in solvent in *P. dominulus*, may have affected the results. Although the Dufour's gland and the ovaries (where hydrocarbons of the same kind as those found on the cuticle are present; see § 2a) had been removed before *P. dominulus* abdomens were extracted in solvent, contamination with the internal hydrocarbons may have occurred, and this may have affected the results. However, preliminary results suggest that once the Dufour's gland and the ovaries are removed, abdomen tissues release only a small amount of hydrocarbons when extracted.

The chemical distance results obtained for *P. dominulus* show a strong relationship between cuticular lipid composition and level of relatedness, as demonstrated by both the correlation between chemical distance and relatedness and by the differences in the chemical distance between nest-mates belonging to different matrilines and full sisters. The pattern for *V. crabro* is more complex. Both types of sisters are more similar than are unrelated nest-mates, as expected from closer relatedness. However, full sisters were found to be more dissimilar than half-sisters. This result is difficult to explain, and it contrasts with honeybees, where Page *et al.* (1991), using a method similar to ours, found that half-sisters were significantly more dissimilar for their epicuticular composition than full sisters.

The discriminant analyses (table 2) show more directly whether these kinds of differences could be used to effectively distinguish matrilines or patrilines within colonies. Arnold *et al.* (2000) showed that honeybee patrilines could often be distinguished in a discriminant analysis. Our results were variable from colony to colony, and generally

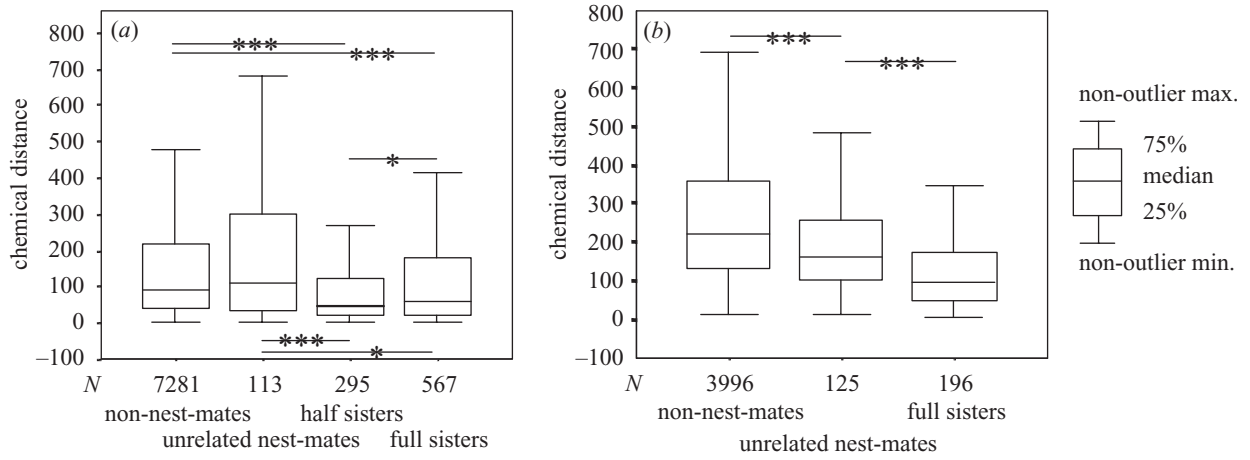

Figure 1. Chemical distances (see § 2c) between pairs of (*a*) *Vespa crabro* workers divided into non-nest-mates, unrelated nest-mates (workers belonging to different matrilines), half-sisters and full sisters, and (*b*) *Polistes dominulus* workers divided into non-nest-mates, unrelated nest-mates (workers belonging to different matrilines) and full sisters. Horizontal lines connect groups for which the exact probability p for the Mann–Whitney U-test was lower than probability applied with the Dunn–Šidàk correction (see § 2c). $^{*}p < 0.05$; $^{***}p < 0.0001$.

went in the same directions as those obtained analysing the differences in the concentration of single compounds and the PC scores. In most 2M colonies, related workers could be grouped correctly based upon their chemical profiles, and this separation was most probably due to relatedness because few random groups gave better results. For *V. crabro*, the best separation was found for one of the 2M colonies, but a mediocre discrimination was obtained for the other 2M colony. In the *P. dominulus* colonies, high separation was obtained for four out of the five 2M colonies. Alternatively, separation was obtained in two out of the three 2P *V. crabro* colonies, but it was far from being complete. This suggests that epicuticular lipid composition could be a reliable indicator of lines in most multiple matriline colonies, while only moderate information is available for discrimination between patrilines.

The weak discrimination between patrilines in *V. crabro* suggests therefore that there are limits to the level of information provided by surface chemistry and this is consistent with chemical distance being very similar among full and half-sisters. There are several possible explanations for the relatively weak patriline discrimination. It could result from their common environment blending any genetic differences, although, as noted before, the similar average chemical distance between non-nest-mates and unrelated nest-mates suggests that in *V. crabro* a common environment has little effect on epicuticular lipid composition. An alternative hypothesis is that weaker discrimination of patrilines than matrilines may derive from the smaller relatedness differences involved. Relatedness within patrilines is 0.75 and 0.25 between them. Relatedness within matrilines is also 0.75, but relatedness between unrelated matrilines is 0.

A final reason is that the genetic chemical differences between matrilines may be enhanced by environmental effects. If matrilines are produced at different times (as happens when colony usurpation occurs), other factors like age and larval diet could also contribute to the divergence of cuticle lipid composition. Recent research on *Polistes* supports the notion that cuticular lipid composition varies with age (Panek *et al.* 2001) and an effect of diet has been demonstrated in an ant species (Lian & Silverman 2000).

These results show that cuticular lipids could sometimes provide enough information to be reliable kin cues in colonies formed with unrelated lines, but are likely to lead to significant errors in discrimination among patrilines. The absence of within-patriline nepotism may, therefore, reflect the cost of using unreliable cues. Even if we assume that workers are capable of the sophisticated comparisons made by discriminant analysis, our data suggest that they would make regular mistakes in identifying their own patrilines using cuticular lipids. This conclusion is conservative because the discriminant analysis finds the best separation available using the data for each colony, and workers may be unable to do it. Of course, workers may be able to use cues other than those we have considered. However, cuticular lipid composition has been shown to be a reliable indicator of nest origin, caste and reproductive status within castes (Lorenzi *et al.* 1996; Singer *et al.* 1998; Breed 1998; Peeters *et al.* 1999; Liebig *et al.* 2000; Sledge 2001), so it is significant that it appears to be a limited indicator of within-colony relatedness in social wasps. Confirmation of this conclusion must come from additional studies examining a wide range of species and potential chemical cues.

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