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Keywords

Invasive species, microsatellite DNA, cuticular hydrocarbons, clustering, *Vespa velutina*

Abstract

The yellow-legged hornet, *Vespa velutina nigrithorax*, is an invasive species that is causing numerous ecological and economic problems, particularly for beekeepers, whose apiaries are seriously affected by hornet predation. The effects may be intensified if hornet workers from different colonies are preying upon bees from the same apiary. Therefore, to determine whether such could occur, we sampled hornets found within identifiable colonies versus in front of bee hives. We employed two complementary methodological approaches: the analysis of chemical markers (i.e., cuticular hydrocarbons) and genetic markers (i.e., microsatellites). Although there was chemical variation among hornets, we determined that at least two chemically homogeneous hornet groups could be found within each apiary studied (using K-means clustering). Furthermore, when hornet chemical dissimilarity was quantified at three different levels (within apiaries, within hornet colonies, and between hornet colonies), we discovered that the within-apiary dissimilarity was intermediate relative to within-colony and between-colony dissimilarity, suggesting that the hornets within a given apiary represented a mixture of individuals from more than one colony. Based on the genetic markers, hornet genetic diversity was low at the population level, as expected for this introduced species. That said, the genetic results mirrored the chemical results: genetic dissimilarity was larger between colonies and smaller within colonies. However, the hornets within a given apiary displayed an intermediate dissimilarity value. Consequently, both our chemical and genetic findings suggested that apiaries could be attacked by hornets from more than one colony.

Introduction

Biological invasions are one of the main factors behind the global decline in biodiversity, as natural communities are already being threatened by habitat modifications or climate change (Walker and Steffen 1997). The yellow-legged hornet, *Vespa velutina nigrithorax* (Hymenoptera, Vespidae), was first reported in France in 2005, in the administrative department of Lot-et-Garonne (Villemant *et al.* 2006a). The hornet responsible for this introduction event was likely a single female (fertilized by three males in her native range) that had come from China, notably from an area somewhere between the provinces of Zhejiang and Jiangsu (Arca *et al*. 2015). *Vespa velutina* spread very rapidly across France, at a rate of around 100 km per year (Muller *et al.* 2013; Robinet *et al.* 2017), and then moved into other European countries (Budge et al. 2017; Robinet *et al.* 2018; Husemann et al. 2020; see Rome and Villemant 2019 for an updated map on the MNHN website). There have been two main consequences of this introduction. First, *V. velutina* preys on honey bees (*Apis mellifera*), causing serious problems for apiaries (Villemant *et al.* 2011, Monceau *et al.* 2014; Darrouzet 2019; Requier et al, 2018, 2019). It may also have a strong ecological impact on wild pollinating insects (Potts *et al.* 2010) and compete with endemic predators such as the European hornet, *Vespa crabro* (Cini et al. 2018, Kwon and Choi 2020). Second, *V. velutina* can directly harm humans (de Haro *et al.* 2010; Laborde-Castérot *et al.* 2021): its sting can result in severe symptoms and even death.

At present, there are no effective methods for controlling this hornet species. The traps used to capture *V. velutina* are often not selective and affect entomofauna (Rome *et al.* 2013; Darrouzet 2019). Research is underway to develop new effective trapping strategies; notably, pheromones are being employed to increase trap selectivity (Cappa *et al.* 2019). Nest elimination is an extreme method that exclusively targets *V. velutina*, but it is expensive and time consuming. Implementation is also challenging because the species' nests are difficult to locate, given that they occur high up in the vegetation. To improve nest elimination efforts, it could be helpful to know the number of hornet colonies that prey upon a given apiary. Moreover, this type of information could reveal useful knowledge about hornet predation dynamics. Indeed, the effects of predation could be intensified if hornet workers from different colonies are preying upon bees from the same apiary. Mechanistically, if intraspecific competition among hornet colonies is absent, hornets could focus fully on their bee prey, without dedicating any energy to competitive interactions. Fleshing out our understanding of this system will add to what we already know about the hornet's role as a predator (Monceau *et al.* 2013a; Tan *et al.* 2012; Requier *et al*. 2018). To address these questions, it is necessary to use tools that allow hornets

from different colonies to be identified: chemical and genetic analyses are classically used for this purpose .

The insect cuticle is covered by cuticular hydrocarbons (CHCs) that act as contact pheromones for inter- and intraspecific recognition among insects (Blomquist and Bagnères 2010). CHCs are mainly involved in the recognition of nestmates (van Zweden and D'Ettorre 2010), castes (Bagnères et al. 1998; Kaib et al. 2000), and worker tasks (Rahman et al. 2016). CHC profiles are modified not only by the social or biotic environment but are also under genetic control in both social and solitary insects (Dronnet et al. 2006; Etges et al. 2009). In eusocial insects, variation in CHC profiles is commonly used to identify species (Bagnères and Wicker-Thomas 2010) and to differentiate among populations and colonies (Bonelli et al. 2015). Indeed, CHC profiling has yielded useful results in numerous chemotaxonomy studies in insects (Blomquist & Bagnères 2010). In a previous study on *V. velutina*, individuals could be distinguished based on their CHC profiles, which varied according to caste, sex, and colony origin (Gévar *et al.* 2017).

Arca *et al.* (2015) showed that molecular markers (i.e., microsatellites) can be used when analyzing the dynamics of *V. velutina* populations. However, the researchers were working over a large area at a phylogeographical scale with the objective of measuring hornet genetic diversity within the species' introduced range. Theoretically, though, their microsatellite markers should also be useful at smaller scales, such as within local populations.

Finally, Dronnet et al. (2006) and Wilgenburg *et al.* (2010) showed that, in social insects, CHC variation can be correlated with microsatellite variation. It thus seems possible that combining analyses of chemical and genetic markers could serve as a complementary approach for characterizing patterns in *V. velutina* occurrence within apiaries.

Our study used both chemical and genetic approaches to distinguish among individuals of *V. velutina* collected in front of apiaries to determine whether multiple hornet colonies could prey on individual beehives. First, we explored hornet genetic diversity at three different levels: (1) within the population by analyzing microsatellite loci using one worker per colony (population level); (2) within colonies (colony level); and (3) among hornets captured in front of beehives within different apiaries (apiary level). Second, we characterized the CHC profiles of hornets sampled from their home colonies and in front of beehives within different apiaries. We expected that hornets from different colonies would differ both chemically and genetically.

Materials and Methods

Sampling

We conducted two sampling campaigns in the administrative department of Indre-et-Loire (France) to carry out this study. The first sampling campaign focused collecting *V. velutina* workers directly from identifiable colonies (after removing nests located in the field). The data gathered provided genetic and chemical references at the population and colony levels. The second sampling campaign focused capturing hornets that were preying on *A. mellifera* in front of beehives in different apiaries, without knowing hornet colony of origin.

During the first sampling campaign, between May and October 2015, we collected 27 *V. velutina* nests, which were then placed at -20°C for at least 48 hours. One individual worker was taken from each of these colonies and placed in an individual glass tube. These 27 individuals were then used for the population-level analysis of microsatellite loci and to explore genetic diversity. We also froze 5 entire colonies, from which 20 workers were sampled and placed in individual tubes. This technique prevented CHC mixing among individuals since we wanted hornet-specific chemical signatures. These 100 individuals were analyzed to obtain colony-level microsatellite and chemical data.

During the second sampling campaign, in September and October 2016 (a period of significant predation activity), we concentrated our sampling efforts on collecting hornets while they were attacking bees. Indeed, hornet nest density usually peaks in the autumn, and hornet predation on honey bee beehives greatly intensifies between early August and early November (Monceau *et al.* 2013b). A total of 188 hornets were collected in front of four different beehives (47 per beehive). Each beehive belonged to a different apiary; the aparies could comprise several beehives. The geographical distances between two given apiaries were between 1.5 km and 4 kilometres (see Table S1 for locations). Again, each individual was placed in its own glass tube, killed by freezing, and stored at -20°C until the chemical and genetic analyses were performed.

Chemical analyses

CHCs were extracted by washing each *V. velutina* worker in 1 ml of heptane for 2 x 1 min using a vortex. Then, 500 µl of each chemical extract was transferred to a new tube. Immediately before the analyses were performed, 10 µl of n-C20 10^{-3}g/ml (n-eicosane) was added to each tube as an internal standard. Then, 2-µl samples of this mixture were analyzed using gas chromatography (GC). We employed a CPG Agilent Technologies 7820A GC System equipped with a flame ionization detector (FID) and a capillary column (HP-5 Agilent Technology, Santa Clara, USA; 30 m x 0.32 mm x 0.25 µm); helium was the carrier gas (1.7 ml/min). The

following temperature program was used: 1) a climb from 50 to 200°C at a ramp rate of 8°C/min; 2) a climb from 200 to 315°C at a ramp rate of 5°C/min; and a final hold at 315°C for 5 min.

Of the 71 compounds detected (Gévar *et al.* 2017), we only focused on the 18 main compounds (displaying a relative abundance of at least 1%) in our statistical analyses. Indeed, we had to take into account the constraints of multivariate analysis, where the number of variables should be 2–3 times lower than sample size. We then calculated the relative areas of each peak (Table S2).

After preliminary chemical and genetic analyses of the raw data, the dataset for the hornets captured within apiaries was reduced from 188 hornets to 171 hornets (46 for Apiary 1, 42 for Apiary 2, 41 for Apiary 3, and 42 for Apiary 4) to ensure data consistency. Indeed, nine individuals had to be excluded because they turned out to be triploid; for the other eight individuals, certain loci were not properly amplified.

Genetic analyses

We extracted genomic DNA from each of the 315 hornets (27 individuals at the population level, 100 at the colony level, and 188 from within the apiaries). An individual's two antennae and right mesothoracic leg were placed in a 1.5-ml tube and then ground with a pestle after the tube had been immersed in liquid nitrogen. DNA extraction was performed using the Wizard® Genomic Purification Kit (Promega) in accordance with the manufacturer's instructions. The hornets were genotyped using 13 microsatellite markers previously described in Arca *et al.* (2015). They were part of a set for which optimal multiplex polymerase chain reaction (PCR) procedures have been developed by GenoScreen (Lille, France) (Table S3). We used a 3-plex assay: R1-36, D2-185, R1-75, and R1-169 (multiplex 1); R4-114, R4-26, D2-142, R4100, and D3-15 (multiplex 2); and R1-80, R1-77, R4-33, and R1-137 (multiplex 3). The multiplex PCR mixtures contained 0.5 U of Taq DNA polymerase, 6 pmol of dNTP, 37.5 pmol of $MgCl_2$, 1 µl of DNA (dilution: 1/5), and 1 µl of primers. The primers were labeled with fluorochromes (6-FAM, NED, VIC, or PET; Table S3). The amplification conditions were as follows: an initial 10-min denaturation step at 95°C, 40 30-sec cycles at 95°C, a 30-sec annealing step at 50°C, an initial 1-min extension step at 72°C, and a final 10-min extension step at 72°C. The PCR products were analyzed by GenoScreen; an ABI 3730XL sequencing system (Applied Biosystems) and a GeneScan™ 500LIZ® Size Standard (Applied Biosystems) were used. The hornets were then genotyped at all 13 microsatellite loci using Geneious 9.4 (Biomatters).

Of these 13 loci, D2-142 and R1-77 had to be excluded because they were monomorphic; R4-26 was also excluded because of unreadable traces. Consequently, all the genetic analyses were performed using the 10 remaining microsatellite loci.

Statistical analyses for the chemical data

Multivariate and clustering analyses

We determined whether we could distinguish among hornets based on their CHC profiles. Profiles were compared for hornets collected from the identifiable colonies and within the apiaries using the (Nei distance; Nowbahari et al. 1990; Dronnet et al. 2006). We analyzed the results using multivariate principal component analyses (PCAs) implemented in the R (v. 3.3.3) packages *FactoMineR* (v. 1.35), *Rcmdr* (v. 2.3-2), and *RcmdrPlugin.FactoMineR* (v. 1.6-0). We began by examining the results for each apiary to better understand the extent of local chemical variation among hornets. This exploratory method improved our understanding of the data prior to conducting the clustering analyses (see below). We then looked at the broader chemical variation across all the hornets by performing a PCA using the full dataset (the 171 hornets from the four apiaries).

We then performed more detailed analyses to explore grouping patterns among the individual hornets collected from within the apiaries based on the results of both the chemical and genetic analyses. K-means clustering analyses (Kaufman and Rousseeuw 1990) were performed to define groups based on CHC similarities, using the packages *factoextra* (v. 1.0.4) and *ggplot* (v. 2.2.1).

Analyses of differences among individuals from the chemical data

Differences in CHC profiles among hornets were quantified by modifying the standard genetic distance of Nei (1987) as per Dronnet *et al*. 2006. The following formula was used: $D = - \ln \sum_n x_i y_i / \sqrt{(\sum_n x_i^2 \sum_n y_i^2)}$, where n is CHC number, x_i is the relative area of hydrocarbon I for individual x, and y_i is the relative area of hydrocarbon I for individual y. Distances were calculated between all possible pairs of individuals.

Statistical analyses for the genetic data

Genetic diversity and structure at both population and colony levels

Population-level genetic diversity was quantified based on the 27 individuals representing the different colonies sampled from within the Indre-et-Loire population. We used the package *adegenet* (v. 2.0.1) implemented in R (v. 3.3.3) and the following variables: number of alleles per locus, observed heterozygosity (H_O), expected heterozygosity (H_E), and F (Wright fixation index (H_E-H_O)/H_E). H_O and H_E were compared using a paired t-test. The package *pegas* tested for deviation from overall Hardy-Weinberg equilibrium in the microsatellite loci.

We also quantified colony-level genetic diversity utilising the 20 workers from each of the 5 frozen colonies. The genetic differentiation among colonies was further characterized by calculating pairwise F_{ST} between colonies using FSTAT and by determining the significance of the differences via multiple comparisons.

We performed similar analyses at the apiary level using the 47 hornets collected from within each of the four apiaries.

Multivariate and clustering analyses for the genetic data

We explored the genotypic differences among hornets using another PCA. This time, however, the PCA was implemented using the packages *adegenet* (v. 2.0.1) and *ade4* (v. 1.7-5) at two levels: the colony level (the 20 hornets from each of the 5 frozen colonies) and the apiary level (the 47 hornets from each of the four apiaries).

To define groups based on genotypic similarities, two complementary methods were used:

(a) A Bayesian clustering analysis was performed using STRUCTURE v. 2.3.4 to infer population structure. This method estimates the number of clusters present (K) and probabilistically assigns individuals to clusters based on multilocus genotype data (Pritchard *et al.* 2000). Each run consisted of a burn-in period (length = 10,000) that was followed by 100,000 MCMC simulations using the admixture model. The optimal value of K was calculated using the Evanno method (Evanno *et al.* 2005) in Structure Harvester (v. 0.6.94).

(b) A discriminant analysis of principal components (DAPC) was carried out using the package *adegenet* (v. 2.0.1); DAPC is a multivariate method for identifying and describing clusters of genetically related individuals (Jombart *et al.* 2010).

Analyses of differences among individuals from the genetic data

Differences in genotypes among hornets were quantified. The mean pairwise distance between individuals was calculated across all the microsatellite loci using the distance measure of Kosman and Leonard (2005). All possible pairs of individuals were included in the analysis. The calculations were carried out using the package *PopGenReport* (v. 3.0.0). The following

formula was used: 1 – [(number of total alleles – number of alleles differing within the pair)/ number of total alleles].

To further interpret the chemical and genetic differences among hornets, it was important to try to identify whether hornets preying on bees from a given apiary were coming from the same colony or different colonies. We did so by comparing the dissimilarity between the hornets sampled within each apiary with the dissimilarity between the hornets sampled from the five frozen colonies. We constructed dissimilarity matrices using all the possible pairs of apiaries and colonies. Kruskal-Wallis tests were performed to determine whether there were significant differences between different categories of dissimilarity values (within apiaries, within colonies, and between colonies).

Results

Multivariate and clustering analyses of the chemical data

At the apiary level, the PCAs of the CHC data revealed that the first two axes together accounted for 63–70% of the total variation (Apiary 1: 63.05% [axis 1 = 41.55% and axis 2 = 21.50%]; Apiary 2: 70.35% [axis 1 = 47.78% and axis 2 = 22.57%]; Apiary 3: 69.12% [axis 1 = 37.12% and axis 2 = 32.00%]; Apiary 4 : 64.94% [axis 1 = 45.93% and axis 2 = 19.01%]). Within the apiaries, there were no clearly differentiated groups of hornets. The PCA analysis of all the hornets showed that there were no differences in CHC profiles among hornets captured in the different apiaries.

The K-means clustering analyses estimated the optimal number of clusters (groups of hornets) within each apiary. There were three clusters in Apiary 1 and two clusters in Apiaries 2, 3, and 4. For Apiary 1, the ellipse associated with cluster 1 overlapped with a large portion of the ellipses of the two other clusters, but individuals in cluster 1 were located quite far from individuals in clusters 2 and 3 (Fig 1. A). For Apiaries 2, 3, and 4, there was little overlap among the cluster ellipses, and the ellipse centroids were distinct (Fig 1. B, C, and D).

Differences in the hornet cuticular hydrocarbon profiles

CHC dissimilarity between the hornets collected within the apiaries (apiary level) was compared with CHC dissimilarity between hornets collected from the identifiable colonies and between hornets from the five frozen colonies (colony level). For Apiaries 1, 2, and 4, significant differences were found among within-apiary dissimilarity, within-colony

dissimilarity, and between-colony dissimilarity (Kruskal-Wallis, $P < 0.05$; Fig 2). In contrast, for Apiary 3, no significant differences were seen (Kruskal-Wallis, $P > 0.05$; Fig 2).

Genetic diversity

At the population level (i.e., the 27 hornets from each colony), the observed number of alleles at each of the ten microsatellite loci ranged from two to three, showing a moderately low level of genetic polymorphism. This result is consistent with past research findings for introduced *V. velutina* populations in France (Arca et al. 2012, 2015) and on the Korean Peninsula (Choi et al. 2013) but contrasts with those for native populations in China (Arca et al. 2012, 2015). No significant departure from HWE was detected across any of the loci (Table 1; F = -0.007, p-value = 0.5001), which suggests the absence of substructuring within the population.

At the colony level (i.e., the 20 hornets from the 5 frozen colonies), genetic diversity was also low, varying from 2 to 4 alleles. A fourth allele was detected at the R1-137 locus in one colony but not at the population level, likely because we used more individuals in the colony-level analyses than in the population-level analyses. However, we did find strong evidence for genetic differentiation between each pair of colonies. All the F_{ST} values differed significantly from zero (Table S4).

Finally, at the apiary level, the mean number of alleles per locus was 2.5 and ranged from 2 to 4, a comparable level of genetic diversity to that above.

Multivariate and clustering analyses of the genetic data

At the colony level, in the PCAs of the genetic data, three distinct groupes could be observed (Fig. 3). However, at the apiary level, no distinct groups of hornets emerged in the PCA results, even if there seemed to be genetic similarities among certain individuals in Apiary 3 (Fig. 4). In the Bayesian clustering analysis, the likelihood of hornet membership in a given cluster was assessed. For Apiaries 1 and 4, hornets could not be assigned to a given cluster with a high degree of likelihood; instead, they had a probability of around 50% of belonging to two clusters. In contrast, for Apiaries 2 and 3, hornets could be assigned to one of two or three clusters, respectively, with a likelihood of more than 80%.

Finally, the apiary-specific DAPCs estimated the optimal number of clusters into which hornets could be grouped. The optimal number of clusters is the minimum number of clusters remaining

after optimizing the Bayesian Information Criterion (BIC) (Fig. S1). The optimal number of clusters (K) was 8 for Apiary 1, 4 for Apiary 2, 6 for Apiary 3, and 6 for Apiary 4.

Differences in hornet genotypes

Genetic dissimilarity was greatest between colonies and smallest within colonies (Fig. 5); it was intermediate within apiaries. The dissimilarity between colonies was significantly different from the dissimilarity within colonies and within apiaries (Kruskal-Wallis, $P < 0.05$). The dissimilarity within colonies was also significantly different from the dissimilarity within apiaries for all four apiaries (Kruskal-Wallis, $P < 0.05$). When within-apiary dissimilarity was examined, only the dissimilarity values for Apiary 1 and Apiary 2 were not significantly different from each other (Kruskal-Wallis, $P > 0.05$).

Discussion

The objective of this study was to determine whether *V. velutina* hornets preying on honey bees within a given apiary came from more than one colony, which would clarify whether several colonies could be engaging in competition for resources in the same zone. Moreover, by determining the number of hornet colonies responsible for predation within individual apiaries, we can gather information about how many nests must be located and destroyed, an important part of controlling the invasive hornet population and protecting honey bee colonies. To this end, hornets were characterized based on their CHC profiles and their microsatellite genotypes. With regards to the hornets' CHC profiles, the PCA results underscored the existence of extensive chemical variation among individuals. More specifically, hornets collected in front of a given beehive within one apiary did not have more similar CHC profiles with each other than they did with hornets collected in front of another beehive in a different apiary. The existence of such variability provides a basis for gauging the number of hornet colonies represented because Gévar *et al.* (2017) found that individual hornets displayed colony-specific CHC profiles. We then used the K-means clustering method on the CHC data to identify the potential hornet clusters present within the apiaries. We discovered that each of the four apiaries studied contained at least two chemically homogeneous groups of hornets. However, while this result suggests that more than one hornet colony was hunting in the same area, this information alone could not tell us whether these groups truly represented different colonies.

We therefore quantified chemical dissimilarity between hornets at three different levels: within apiaries, within colonies, and between colonies. The analysis revealed that, for each apiary, within-apiary dissimilarity was significantly smaller than between-colony dissimilarity.

Furthermore, there was a significant difference between within-colony dissimilarity and within apiary dissimilarity for Apiary 1 (i.e., smaller) and Apiaries 2 and 4 (i.e., larger). In contrast, within-apiary dissimilarity for Apiary 3 was not significantly different from within-colony dissimilarity or from within-apiary dissimilarity for the other apiaries. These findings suggest that several colonies could have been preying on bees in Apiaries 2, 3, and 4. However, it is important to note that the within-apiary dissimilarity was intermediate relative to within-colony and between-colony dissimilarity. This result could be explained by the presence of a mixture of individuals from the same colony and from different colonies preying on bees within individual apiaries.

The chemical analyses demonstrated that different groups of hornets may be present in the same apiary. However, it would have been complicated to locate the hornets' colonies within the zone around the apiaries, even if it would have been helpful to characterize the colonies' specific chemical profiles and thus ascertain whether or not the hornets had multiple origins. To confirm the grouping patterns obtained in the chemical analyses, a genetic approach was utilized. Hornet microsatellite diversity was low, as expected given the significant loss of genetic diversity in the hornet's invasive range (France, Arca et al. 2015; Korean peninsula, Choi et al. 2013). However, our study nonetheless yielded sufficient baseline genetic information at the population level and colony level to carry out the F_{ST} analyses and PCA on the genetic data among colonies. The PCA results at the apiary level showed that, within each apiary, some hornets had more similar genotypes than others. By itself, this exploratory analysis did not indicate whether one or more *V. velutina* colonies were represented by the hornets sampled. We thus took the analysis further by using clustering methods.

We carried out a Bayesian analysis, which only helped define clusters for Apiaries 2 and 3. We also performed a DAPC for each apiary, which estimated the optimal number of clusters into which hornets grouped. These two methods arrived at different estimates of cluster number, likely because they employ different approaches. The Bayesian method calculates the likelihood that individuals belong to a given homogeneous cluster or clusters. In contrast, DAPC maximizes differences between groups while minimizing variation within groups. Nonetheless, both showed that there was a certain level of genetic differentiation among hornets caught within the same apiary. This result mirrors the clustering that was observed with the CHC data.

Finally, we found that, for the genetic data, within-apiary dissimilarity was significantly different from within-colony and between-colony dissimilarity. More specifically, dissimilarity was greatest between colonies and smallest within colonies, notably because individuals from

the same colony had a high degree of genotypic similarity. Interestingly, there was intermediate dissimilarity between hornets collected within a given apiary, which fits with the findings of the chemical analyses and suggests that the hornets could have belonged to more than one colony. However, there is also the possibility that these genetic differences stemmed from polyandry (i.e., queens mating with multiple males), a mechanism known to increase genetic diversity within colonies. Arca et al. (2015) showed that polyandry occurs in the invasive *V. velutina* population in France. Indeed, polyandry could be an important element in understanding the hornet's invasiveness, as this mechanism could increase genetic diversity within colonies. In addition, some studies have shown that combining polyandry with higher heterozygosity could further enhance the success of invasive populations, since heterozygosity can improve survival, longevity, and competitiveness (Taylor et al., 2014).

Taken together, these two sets of results lay the groundwork for further reflection. Although CHC profiling and microsatellite genotyping are commonly used in studies of social insects, neither approach allowed us to definitively confirm whether the hornets in a given apiary came from one or multiple colonies. One challenge is that we are dealing with an invasive social insect whose propagation dynamics and colony organization are complicated; at the same time, these complexities are useful for studying the evolutionary responses of introduced species (e.g., the Argentine ant, *Linepithema humile* [Van Wilgenburg et al. 2010]). As a result of founding effects (e.g., bottlenecks), chemical and genetic diversity in the introduced *V. velutina* population may be too low for CHC profiling and microsatellite genotyping to yield unambiguous results, like in previous studies in other invasive social insects (*Reticulitermes flavipes*, Dronnet et al. 2006; *Linepithema humile*, Brands et al. 2009). It would therefore be helpful to better characterize the hornet's population genetic structure, colony genetic structure, and use of polyandry in our study area. We could thus assess whether such factors have influenced CHC profile diversity (Crozier & Fjerdingstad 2001).

To conclude, our results suggest that more than one *V. velutina* colony might prey on bees within a given apiary. Our findings are consistent with those from a study of the local spatial distribution of *V. velutina* nests in southwestern France (Monceau & Thiery 2017), which suggested that intraspecific competition may be limited. Future experiments could be performed to assign hornets to their colonies of origin based on their chemical and/or genetic profiles, a task that would require in-depth knowledge of the reference population. Sampling would need to be carried out on a large number of nests around target apiaries. However, such research is often challenging because detecting nests is difficult: they are generally hidden high up in the vegetation. Nonetheless, combining chemical and molecular data could be a promising avenue

for advancing our understanding of *V. velutina* and should be considered in work on other invasive social insect species.

Acknowledgments

We thank C. Poirier (Abeilles Services 37) for providing some of the samples, F. Dedeine for fruitful discussions on the population genetics of invasive species, and S. Dupont for his help with the molecular genetics methodologies. We gratefully acknowledge the English-editing services of J. Pearce-Duvet. This study was supported by funding from the Centre-Val de Loire regional government for the FRELON 2 project.

Conflict of interest: The authors declare that they have no conflict of interest.

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Bouzar, Bankhead-Dronnet, Gevar and Darrouzet: Figures and Tables

Figure 1. Clusters of *V. velutina* workers defined using the K-means clustering method based on the hornets' cuticular hydrocarbon (CHC) profiles; the hornets were collected from in front of beehives (one beehive per apiary). A: 46 hornets from Apiary 1; B: 42 hornets from Apiary 2; C: 41 hornets from Apiary 3; D: 42 hornets from Apiary 4. Circles are related to K-means.

Figure 2. Dissimilarity values calculated based on the CHC distances between *V. velutina* workers: dissimilarity between the hornets captured in each of the studied apiaries (IntraApiary1, IntraApiary2, IntraApiary3, and IntraApiary4), between hornets within known colonies (IntraColon), and between hornets from different known colonies (InterColon). Differences in letters indicate statistically significant differences (Kruskal-Wallis, $P<0.05$).

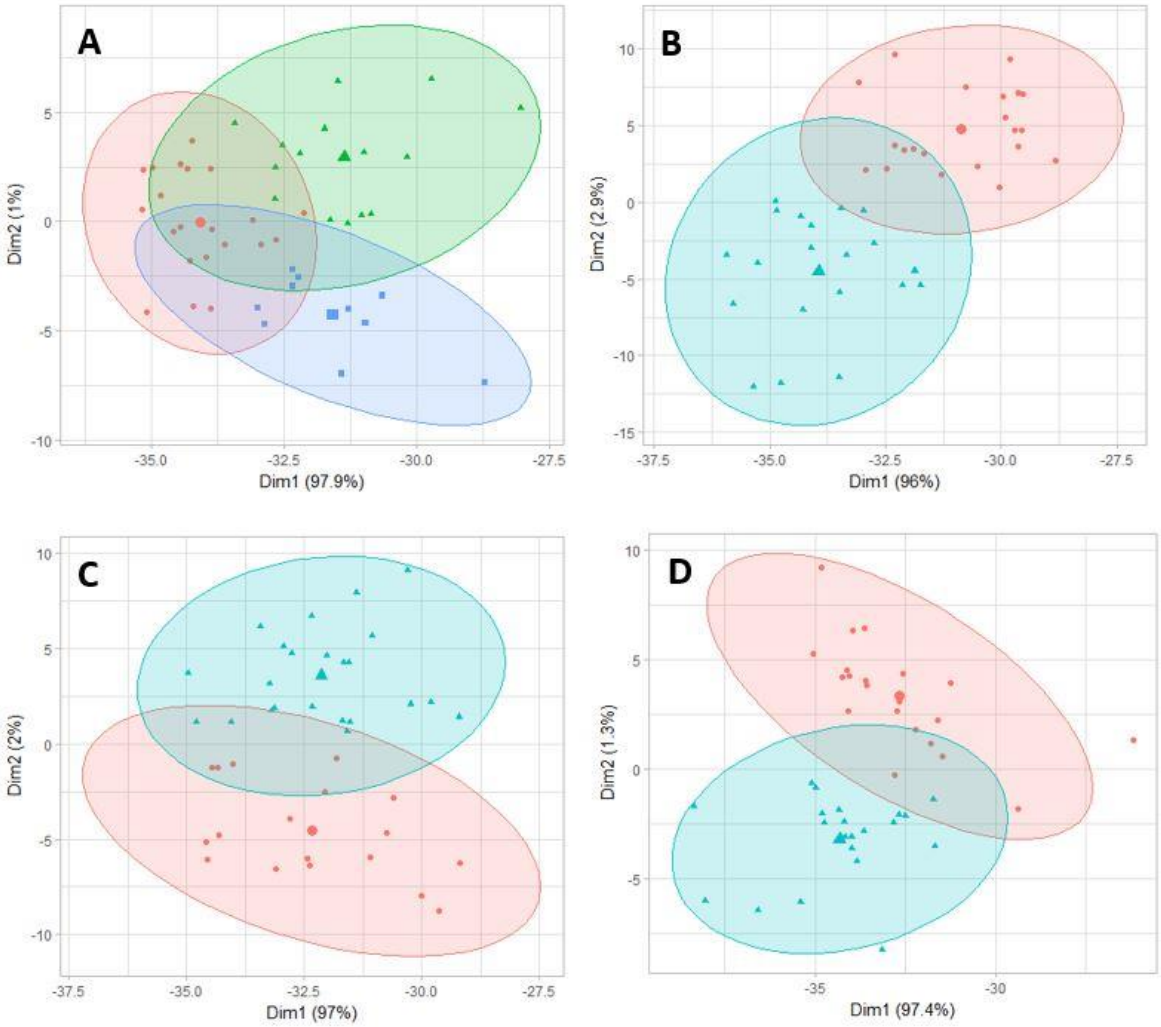
Figure 3. Results of the principal component analysis (PCA) performed on the microsatellite data for the 100 *V. velutina* workers collected from five different colonies (20 workers per colony). The genetic diversity is represented in two complementary ways: by the distances (further away = more genetically different), and by the colors (more different colors = more genetically different).

Figure 4. Results of the principal component analysis (PCA) performed on the microsatellite data for the 46 *V. velutina* workers captured in Apiary 3. The genetic diversity is represented in two complementary ways: by the distances (further away = more genetically different), and by the colors (more different colors = more genetically different).

Figure 5. Dissimilarity values calculated based on the genotypic distances between *V. velutina* workers: dissimilarity between the hornets captured in each of the study apiaries (IntraApiary1, IntraApiary2, IntraApiary3, and IntraApiary4), between hornets within known colonies (IntraColon), and between hornets from different known colonies (InterColon). Differences in letters indicate statistically significant differences (Kruskal-Wallis, $P<0.05$).

Table 1. Summary of the ten microsatellite loci used to study the genetic diversity among 27 *Vespa velutina nigrithorax* workers from an invasive population in Indre-et-Loire (France). Data include the allele size range (in base pairs), number of alleles per locus (Na), observed (H_O) and expected (H_E) heterozygosities, Wright's fixation index F (H_E-H_O)/ H_E).

Figure 1



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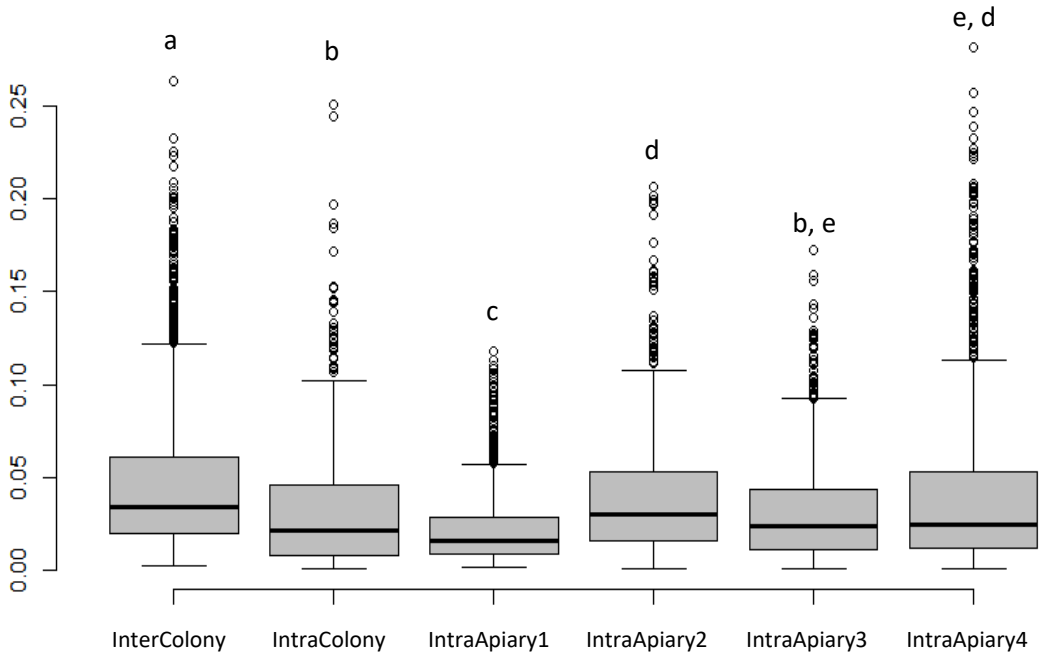
40

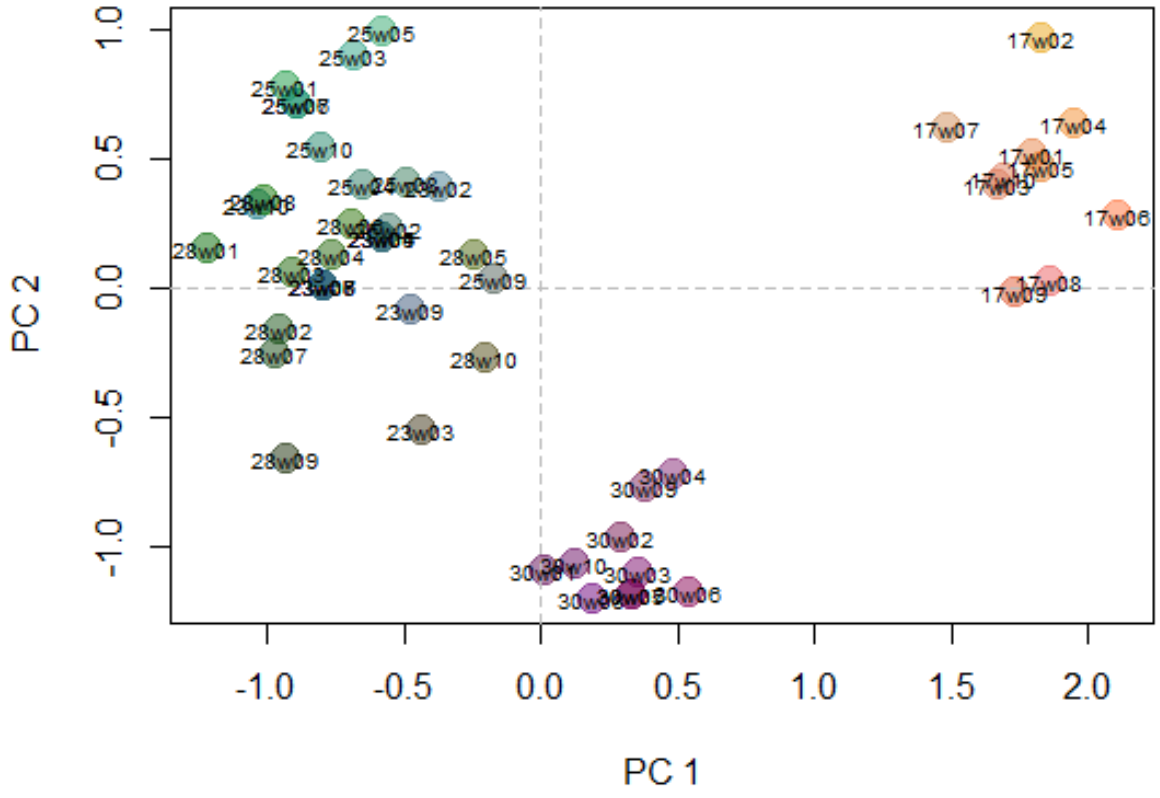
41 **Figure 2**

42



43

Figure 3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0.5
1.0
1.5
2.0
25w05
25w03
25w01
25w10
25w09
25w02
28w01
28w04
28w05
28w02
28w07
28w10
23w09
23w03
28w09
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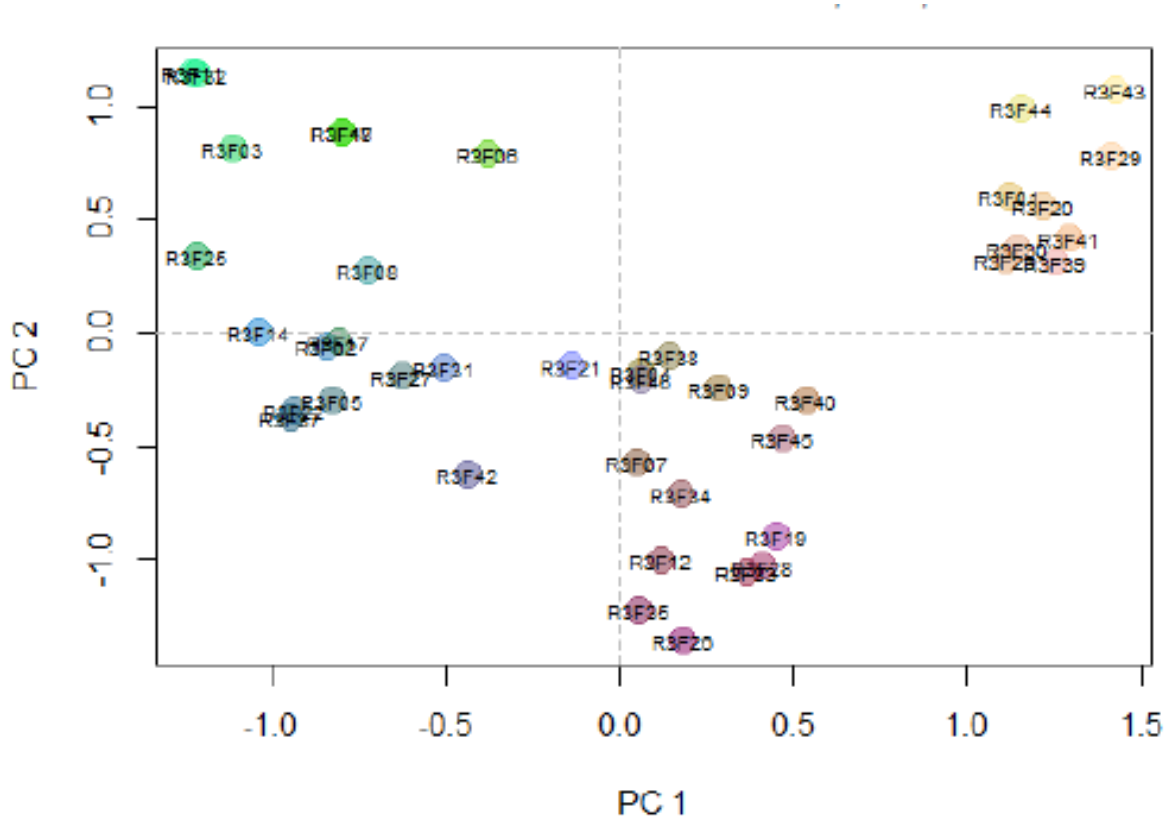
17w06
17w08

Figure 4


PC 2
PC 1
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0.0
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-1.0
-1.0
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0.0
0.5
1.0
1.5
R3F43
R3F44
R3F29
R3F03
R3F06
R3F20
R3F41
R3F26
R3F09
R3F14
R3F21
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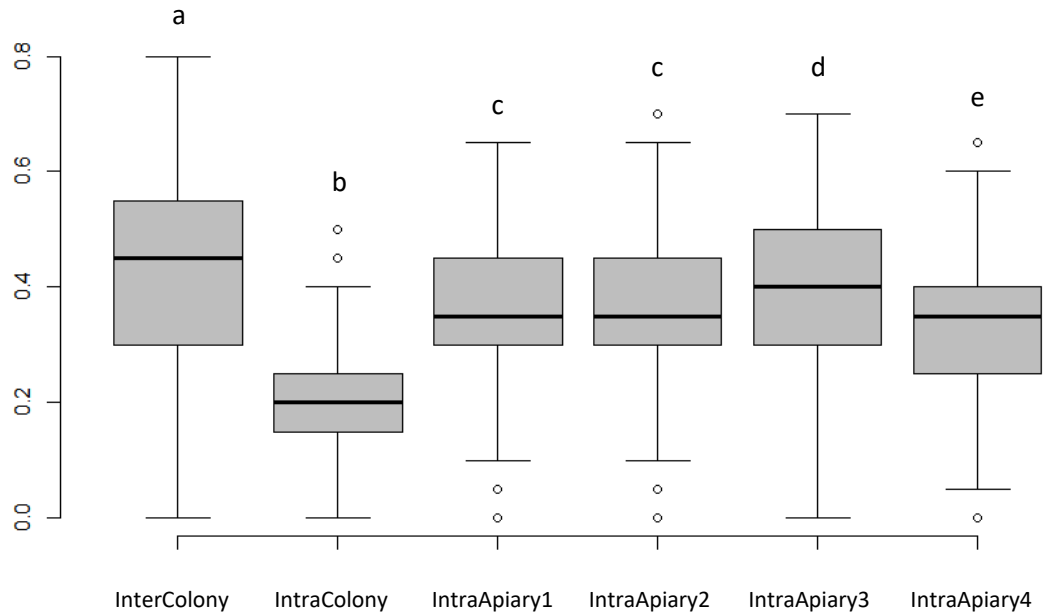
R3F12
R3F20

Figure 5


a
b
c
c
d
e
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0.2
0.4
0.6
0.8
InterColony
IntraColony
IntraApiary1
IntraApiary2
IntraApiary3
IntraApiary4

Table 1.

Locus	Alle size range (bp)	Na	H_O	H_E	F
R1-36	107-116	2	0.44	0.42	-0.066
D2-185	214-220	2	0.56	0.46	-0.218
R1-75	149-161	3	0.56	0.62	0.109
R1-169	166-172	2	0.37	0.47	0.206
R4-114	135-142	3	0.67	0.63	-0.053
R4-100	183-187	2	0.41	0.48	0.143
D3-15	168-178	3	0.48	0.53	0.092
R1-80	115-117	2	0.31	0.31	0.010
R4-33	211-215	2	0.50	0.41	-0.218
R1-137	186-194	3	0.62	0.61	-0.015
Mean		2.364	0.48	0.48	-0.007