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Chemical and genetic evidences that multiple hornet colonies attack honey bee colonies.

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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 betweencolony 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.

1 Introduction

Biological invasions are one of the main factors behind the global decline in biodiversity, as 2 natural communities are already being threatened by habitat modifications or climate change 3 (Walker and Steffen 1997). The yellow-legged hornet, Vespa velutina nigrithorax 4 (Hymenoptera, Vespidae), was first reported in France in 2005, in the administrative department 5 of Lot-et-Garonne (Villemant et al. 2006a). The hornet responsible for this introduction event 6 7 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 8 et al. 2015). Vespa velutina spread very rapidly across France, at a rate of around 100 km per 9 year (Muller et al. 2013; Robinet et al. 2017), and then moved into other European countries 10 (Budge et al. 2017; Robinet et al. 2018; Husemann et al. 2020; see Rome and Villemant 2019 11 for an updated map on the MNHN website). There have been two main consequences of this 12 introduction. First, V. velutina preys on honey bees (Apis mellifera), causing serious problems 13 for apiaries (Villemant et al. 2011, Monceau et al. 2014; Darrouzet 2019; Requier et al, 2018, 14 2019). It may also have a strong ecological impact on wild pollinating insects (Potts et al. 2010) 15 16 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. 17 2010; Laborde-Castérot et al. 2021): its sting can result in severe symptoms and even death. 18

At present, there are no effective methods for controlling this hornet species. The traps used to 19 capture V. velutina are often not selective and affect entomofauna (Rome et al. 2013; Darrouzet 20 2019). Research is underway to develop new effective trapping strategies; notably, pheromones 21 are being employed to increase trap selectivity (Cappa et al. 2019). Nest elimination is an 22 extreme method that exclusively targets V. velutina, but it is expensive and time consuming. 23 24 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 25 know the number of hornet colonies that prey upon a given apiary. Moreover, this type of 26 information could reveal useful knowledge about hornet predation dynamics. Indeed, the 27 28 effects of predation could be intensified if hornet workers from different colonies are preying 29 upon bees from the same apiary. Mechanistically, if intraspecific competition among hornet 30 colonies is absent, hornets could focus fully on their bee prey, without dedicating any energy 31 to competitive interactions. Fleshing out our understanding of this system will add to what we 32 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 33

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

The insect cuticle is covered by cuticular hydrocarbons (CHCs) that act as contact pheromones 36 for inter- and intraspecific recognition among insects (Blomquist and Bagnères 2010). CHCs 37 are mainly involved in the recognition of nestmates (van Zweden and D'Ettorre 2010), castes 38 (Bagnères et al. 1998; Kaib et al. 2000), and worker tasks (Rahman et al. 2016). CHC profiles 39 are modified not only by the social or biotic environment but are also under genetic control in 40 41 both social and solitary insects (Dronnet et al. 2006; Etges et al. 2009). In eusocial insects, 42 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 43 profiling has yielded useful results in numerous chemotaxonomy studies in insects (Blomquist 44 & Bagnères 2010). In a previous study on V. velutina, individuals could be distinguished based 45 46 on their CHC profiles, which varied according to caste, sex, and colony origin (Gévar et al. 2017). 47

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. 57 *velutina* collected in front of apiaries to determine whether multiple hornet colonies could prey 58 59 on individual behives. First, we explored hornet genetic diversity at three different levels: (1) within the population by analyzing microsatellite loci using one worker per colony (population 60 level); (2) within colonies (colony level); and (3) among hornets captured in front of beehives 61 within different apiaries (apiary level). Second, we characterized the CHC profiles of hornets 62 sampled from their home colonies and in front of beehives within different apiaries. We 63 expected that hornets from different colonies would differ both chemically and genetically. 64

66 Materials and Methods

67 <u>Sampling</u>

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 74 V. velutina nests, which were then placed at -20°C for at least 48 hours. One individual worker 75 was taken from each of these colonies and placed in an individual glass tube. These 27 76 individuals were then used for the population-level analysis of microsatellite loci and to explore 77 genetic diversity. We also froze 5 entire colonies, from which 20 workers were sampled and 78 79 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 80 81 colony-level microsatellite and chemical data.

During the second sampling campaign, in September and October 2016 (a period of significant 82 predation activity), we concentrated our sampling efforts on collecting hornets while they were 83 attacking bees. Indeed, hornet nest density usually peaks in the autumn, and hornet predation 84 on honey bee beehives greatly intensifies between early August and early November (Monceau 85 et al. 2013b). A total of 188 hornets were collected in front of four different beehives (47 per 86 87 beehive). Each beehive belonged to a different apiary; the aparies could comprise several 88 beehives. The geographical distances between two given apiaries were between 1.5 km and 4 89 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. 90

91

92 <u>Chemical analyses</u>

93 CHCs were extracted by washing each *V. velutina* worker in 1 ml of heptane for 2 x 1 min using 94 a vortex. Then, 500 μ l of each chemical extract was transferred to a new tube. Immediately 95 before the analyses were performed, 10 μ l of n-C20 10⁻³g/ml (n-eicosane) was added to each 96 tube as an internal standard. Then, 2- μ l samples of this mixture were analyzed using gas 97 chromatography (GC). We employed a CPG Agilent Technologies 7820A GC System equipped 98 with a flame ionization detector (FID) and a capillary column (HP-5 Agilent Technology, Santa 99 Clara, USA; 30 m x 0.32 mm x 0.25 μ m); 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.

103 Of the 71 compounds detected (Gévar *et al.* 2017), we only focused on the 18 main compounds 104 (displaying a relative abundance of at least 1%) in our statistical analyses. Indeed, we had to 105 take into account the constraints of multivariate analysis, where the number of variables should 106 be 2–3 times lower than sample size. We then calculated the relative areas of each peak (Table 107 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.

113

114 <u>Genetic analyses</u>

We extracted genomic DNA from each of the 315 hornets (27 individuals at the population 115 116 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 117 tube had been immersed in liquid nitrogen. DNA extraction was performed using the Wizard® 118 Genomic Purification Kit (Promega) in accordance with the manufacturer's instructions. The 119 120 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) 121 procedures have been developed by GenoScreen (Lille, France) (Table S3). We used a 3-plex 122 assay: R1-36, D2-185, R1-75, and R1-169 (multiplex 1); R4-114, R4-26, D2-142, R4100, and 123 124 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 MgCl₂, 1 µl 125 126 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 127 10-min denaturation step at 95°C, 40 30-sec cycles at 95°C, a 30-sec annealing step at 50°C, an 128 initial 1-min extension step at 72°C, and a final 10-min extension step at 72°C. The PCR 129 products were analyzed by GenoScreen; an ABI 3730XL sequencing system (Applied 130 Biosystems) and a GeneScanTM 500LIZ® Size Standard (Applied Biosystems) were used. The 131 hornets were then genotyped at all 13 microsatellite loci using Geneious 9.4 (Biomatters). 132

Of these 13 loci, D2-142 and R1-77 had to be excluded because they were monomorphic; R426 was also excluded because of unreadable traces. Consequently, all the genetic analyses were
performed using the 10 remaining microsatellite loci.

- 136
- 137

138 <u>Statistical analyses for the chemical data</u>

139 <u>Multivariate and clustering analyses</u>

We determined whether we could distinguish among hornets based on their CHC profiles. 140 Profiles were compared for hornets collected from the identifiable colonies and within the 141 apiaries using the (Nei distance; Nowbahari et al. 1990; Dronnet et al. 2006). We analyzed the 142 results using multivariate principal component analyses (PCAs) implemented in the R (v. 3.3.3) 143 packages FactoMineR (v. 1.35), Rcmdr (v. 2.3-2), and RcmdrPlugin.FactoMineR (v. 1.6-0). We 144 145 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 146 to conducting the clustering analyses (see below). We then looked at the broader chemical 147 variation across all the hornets by performing a PCA using the full dataset (the 171 hornets 148 149 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).

155

156 Analyses of differences among individuals from the chemical data

157 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

159 = $-\ln \sum n x_i y_i / \sqrt{(\sum n x_i^2 \sum n y_i^2)}$, where n is CHC number, x_i is the relative area of hydrocarbon

160 I for individual x, and y_i is the relative area of hydrocarbon I for individual y. Distances were

- 161 calculated between all possible pairs of individuals.
- 162
- 163 <u>Statistical analyses for the genetic data</u>
- 164 *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
- 167 *adegenet* (v. 2.0.1) implemented in R (v. 3.3.3) and the following variables: number of alleles
- 168 per locus, observed heterozygosity (H_0), expected heterozygosity (H_E), and F (Wright fixation
- 169 index $(H_E-H_O)/H_E$). H_O and H_E were compared using a paired t-test. The package *pegas* tested
- 170 for deviation from overall Hardy-Weinberg equilibrium in the microsatellite loci.
- 171 We also quantified colony-level genetic diversity utilising the 20 workers from each of the 5
- 172 frozen colonies. The genetic differentiation among colonies was further characterized by
- 173 calculating pairwise F_{ST} between colonies using FSTAT and by determining the significance
- 174 of the differences via multiple comparisons.
- We performed similar analyses at the apiary level using the 47 hornets collected from withineach of the four apiaries.
- 177
- 178 <u>Multivariate and clustering analyses for the genetic data</u>
- 179 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
- 181 levels: the colony level (the 20 hornets from each of the 5 frozen colonies) and the apiary level
- 182 (the 47 hornets from each of the four apiaries).
- 183 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
- 191 *und gener (v. 2.0.1), Drif e is a manifultation method for identifying and describing elasters of*
- 192 genetically related individuals (Jombart *et al.* 2010).
- 193

194 <u>Analyses of differences among individuals from the genetic data</u>

195 Differences in genotypes among hornets were quantified. The mean pairwise distance between 196 individuals was calculated across all the microsatellite loci using the distance measure of

- 197 Kosman and Leonard (2005). All possible pairs of individuals were included in the analysis.
- 198 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 201 to identify whether hornets preying on bees from a given apiary were coming from the same 202 colony or different colonies. We did so by comparing the dissimilarity between the hornets 203 sampled within each apiary with the dissimilarity between the hornets sampled from the five 204 frozen colonies. We constructed dissimilarity matrices using all the possible pairs of apiaries 205 and colonies. Kruskal-Wallis tests were performed to determine whether there were significant 206 differences between different categories of dissimilarity values (within apiaries, within 207 colonies, and between colonies). 208

209

210 **Results**

211 <u>Multivariate and clustering analyses of the chemical data</u>

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).

225

226 <u>Differences in the hornet cuticular hydrocarbon profiles</u>

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).
- 233

234 <u>Genetic diversity</u>

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 to4, a comparable level of genetic diversity to that above.

250

251 <u>Multivariate and clustering analyses of the genetic data</u>

252 At the colony level, in the PCAs of the genetic data, three distinct groupes could be observed 253 (Fig. 3). However, at the apiary level, no distinct groups of hornets emerged in the PCA results, 254 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 255 assessed. For Apiaries 1 and 4, hornets could not be assigned to a given cluster with a high 256 degree of likelihood; instead, they had a probability of around 50% of belonging to two clusters. 257 In contrast, for Apiaries 2 and 3, hornets could be assigned to one of two or three clusters, 258 259 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.

264

265 <u>Differences in hornet genotypes</u>

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).

273

274 Discussion

The objective of this study was to determine whether V. velutina hornets preying on honey bees 275 within a given apiary came from more than one colony, which would clarify whether several 276 277 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, 278 we can gather information about how many nests must be located and destroyed, an important 279 part of controlling the invasive hornet population and protecting honey bee colonies. To this 280 end, hornets were characterized based on their CHC profiles and their microsatellite genotypes. 281 With regards to the hornets' CHC profiles, the PCA results underscored the existence of 282 extensive chemical variation among individuals. More specifically, hornets collected in front 283 of a given beehive within one apiary did not have more similar CHC profiles with each other 284 285 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 286 represented because Gévar et al. (2017) found that individual hornets displayed colony-specific 287 CHC profiles. We then used the K-means clustering method on the CHC data to identify the 288 289 potential hornet clusters present within the apiaries. We discovered that each of the four apiaries 290 studied contained at least two chemically homogeneous groups of hornets. However, while this 291 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. 292

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. 296 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, 297 within-apiary dissimilarity for Apiary 3 was not significantly different from within-colony 298 dissimilarity or from within-apiary dissimilarity for the other apiaries. These findings suggest 299 that several colonies could have been preying on bees in Apiaries 2, 3, and 4. However, it is 300 important to note that the within-apiary dissimilarity was intermediate relative to within-colony 301 and between-colony dissimilarity. This result could be explained by the presence of a mixture 302 303 of individuals from the same colony and from different colonies preying on bees within individual apiaries. 304

305 The chemical analyses demonstrated that different groups of hornets may be present in the same 306 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 307 308 chemical profiles and thus ascertain whether or not the hornets had multiple origins. To confirm 309 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 310 the hornet's invasive range (France, Arca et al. 2015; Korean peninsula, Choi et al. 2013). 311 312 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 313 among colonies. The PCA results at the apiary level showed that, within each apiary, some 314 315 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 316 317 thus took the analysis further by using clustering methods.

318 We carried out a Bayesian analysis, which only helped define clusters for Apiaries 2 and 3. We 319 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, 320 321 likely because they employ different approaches. The Bayesian method calculates the likelihood that individuals belong to a given homogeneous cluster or clusters. In contrast, 322 DAPC maximizes differences between groups while minimizing variation within groups. 323 Nonetheless, both showed that there was a certain level of genetic differentiation among hornets 324 caught within the same apiary. This result mirrors the clustering that was observed with the 325 CHC data. 326

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 330 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 331 332 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 333 polyandry (i.e., queens mating with multiple males), a mechanism known to increase genetic 334 diversity within colonies. Area et al. (2015) showed that polyandry occurs in the invasive V. 335 velutina population in France. Indeed, polyandry could be an important element in 336 understanding the hornet's invasiveness, as this mechanism could increase genetic diversity 337 within colonies. In addition, some studies have shown that combining polyandry with higher 338 heterozygosity could further enhance the success of invasive populations, since heterozygosity 339 can improve survival, longevity, and competitiveness (Taylor et al., 2014). 340

Taken together, these two sets of results lay the groundwork for further reflection. Although 341 CHC profiling and microsatellite genotyping are commonly used in studies of social insects, 342 neither approach allowed us to definitively confirm whether the hornets in a given apiary came 343 from one or multiple colonies. One challenge is that we are dealing with an invasive social 344 345 insect whose propagation dynamics and colony organization are complicated; at the same time, 346 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 347 348 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 349 unambiguous results, like in previous studies in other invasive social insects (Reticulitermes 350 flavipes, Dronnet et al. 2006; Linepithema humile, Brands et al. 2009). It would therefore be 351 352 helpful to better characterize the hornet's population genetic structure, colony genetic structure, 353 and use of polyandry in our study area. We could thus assess whether such factors have 354 influenced CHC profile diversity (Crozier & Fjerdingstad 2001).

To conclude, our results suggest that more than one V. velutina colony might prey on bees 355 356 within a given apiary. Our findings are consistent with those from a study of the local spatial 357 distribution of V. velutina nests in southwestern France (Monceau & Thiery 2017), which suggested that intraspecific competition may be limited. Future experiments could be performed 358 to assign hornets to their colonies of origin based on their chemical and/or genetic profiles, a 359 360 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 361 362 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 363

364	for advancing our understanding of V. velutina and should be considered in work on other
365	invasive social insect species.
366	
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373	
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Bouzar, Bankhead-Dronnet, Gevar and Darrouzet: Figures and Tables

2 3

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.

8

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).

14

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).

20

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).

25

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_0) and expected (H_E) heterozygosities, Wright's fixation index *F* (H_E - H_0)/ H_E).

36

37







- 44 Figure 3
- 45



PC 1





PC 1

- Figure 5



Table 1.

59						
60	Locus	Alle size range	Na	H_0	$H_{ m E}$	F
61		(bp)				
62						
63	R1-36	107-116	2	0.44	0.42	-0.066
64 65	D2-185	214-220	2	0.56	0.46	-0.218
66 67	R1-75	149-161	3	0.56	0.62	0.109
68	R1-169	166-172	2	0.37	0.47	0.206
69 70	R4-114	135-142	3	0.67	0.63	-0.053
71 72	R4-100	183-187	2	0.41	0.48	0.143
73	D3-15	168-178	3	0.48	0.53	0.092
74 75	R1-80	115-117	2	0.31	0.31	0.010
76 77	R4-33	211-215	2	0.50	0.41	-0.218
78	R1-137	186-194	3	0.62	0.61	-0.015
79 – 80	Mean		2.364	0.48	0.48	-0.007