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

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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.

1 **Introduction**

2 Biological invasions are one of the main factors behind the global decline in biodiversity, as
3 natural communities are already being threatened by habitat modifications or climate change
4 (Walker and Steffen 1997). The yellow-legged hornet, *Vespa velutina nigrithorax*
5 (Hymenoptera, Vespidae), was first reported in France in 2005, in the administrative department
6 of Lot-et-Garonne (Villemant *et al.* 2006a). The hornet responsible for this introduction event
7 was likely a single female (fertilized by three males in her native range) that had come from
8 China, notably from an area somewhere between the provinces of Zhejiang and Jiangsu (Arca
9 *et al.* 2015). *Vespa velutina* spread very rapidly across France, at a rate of around 100 km per
10 year (Muller *et al.* 2013; Robinet *et al.* 2017), and then moved into other European countries
11 (Budge *et al.* 2017; Robinet *et al.* 2018; Husemann *et al.* 2020; see Rome and Villemant 2019
12 for an updated map on the MNHN website). There have been two main consequences of this
13 introduction. First, *V. velutina* preys on honey bees (*Apis mellifera*), causing serious problems
14 for apiaries (Villemant *et al.* 2011, Monceau *et al.* 2014; Darrouzet 2019; Requier *et al.* 2018,
15 2019). It may also have a strong ecological impact on wild pollinating insects (Potts *et al.* 2010)
16 and compete with endemic predators such as the European hornet, *Vespa crabro* (Cini *et al.*
17 2018, Kwon and Choi 2020). Second, *V. velutina* can directly harm humans (de Haro *et al.*
18 2010; Laborde-Castérot *et al.* 2021): its sting can result in severe symptoms and even death.
19 At present, there are no effective methods for controlling this hornet species. The traps used to
20 capture *V. velutina* are often not selective and affect entomofauna (Rome *et al.* 2013; Darrouzet
21 2019). Research is underway to develop new effective trapping strategies; notably, pheromones
22 are being employed to increase trap selectivity (Cappa *et al.* 2019). Nest elimination is an
23 extreme method that exclusively targets *V. velutina*, but it is expensive and time consuming.
24 Implementation is also challenging because the species' nests are difficult to locate, given that
25 they occur high up in the vegetation. To improve nest elimination efforts, it could be helpful to
26 know the number of hornet colonies that prey upon a given apiary. Moreover, this type of
27 information could reveal useful knowledge about hornet predation dynamics. Indeed, the
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;
33 Requier *et al.* 2018). To address these questions, it is necessary to use tools that allow hornets

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

36 The insect cuticle is covered by cuticular hydrocarbons (CHCs) that act as contact pheromones
37 for inter- and intraspecific recognition among insects (Blomquist and Bagnères 2010). CHCs
38 are mainly involved in the recognition of nestmates (van Zweden and D’Ettorre 2010), castes
39 (Bagnères et al. 1998; Kaib et al. 2000), and worker tasks (Rahman et al. 2016). CHC profiles
40 are modified not only by the social or biotic environment but are also under genetic control in
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
43 2010) and to differentiate among populations and colonies (Bonelli et al. 2015). Indeed, CHC
44 profiling has yielded useful results in numerous chemotaxonomy studies in insects (Blomquist
45 & Bagnères 2010). In a previous study on *V. velutina*, individuals could be distinguished based
46 on their CHC profiles, which varied according to caste, sex, and colony origin (Gévar *et al.*
47 2017).

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

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

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

65

66 **Materials and Methods**

67 Sampling

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

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

82 During the second sampling campaign, in September and October 2016 (a period of significant
83 predation activity), we concentrated our sampling efforts on collecting hornets while they were
84 attacking bees. Indeed, hornet nest density usually peaks in the autumn, and hornet predation
85 on honey bee beehives greatly intensifies between early August and early November (Monceau
86 *et al.* 2013b). A total of 188 hornets were collected in front of four different beehives (47 per
87 beehive). Each beehive belonged to a different apiary; the apiaries 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,
90 killed by freezing, and stored at -20°C until the chemical and genetic analyses were performed.

91

92 Chemical analyses

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 µl of each chemical extract was transferred to a new tube. Immediately
95 before the analyses were performed, 10 µl of n-C20 10⁻³g/ml (n-eicosane) was added to each
96 tube as an internal standard. Then, 2-µl 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 µm); helium was the carrier gas (1.7 ml/min). The

100 following temperature program was used: 1) a climb from 50 to 200°C at a ramp rate of
101 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
102 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).

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

113

114 Genetic analyses

115 We extracted genomic DNA from each of the 315 hornets (27 individuals at the population
116 level, 100 at the colony level, and 188 from within the apiaries). An individual's two antennae
117 and right mesothoracic leg were placed in a 1.5-ml tube and then ground with a pestle after the
118 tube had been immersed in liquid nitrogen. DNA extraction was performed using the Wizard®
119 Genomic Purification Kit (Promega) in accordance with the manufacturer's instructions. The
120 hornets were genotyped using 13 microsatellite markers previously described in Arca *et al.*
121 (2015). They were part of a set for which optimal multiplex polymerase chain reaction (PCR)
122 procedures have been developed by GenoScreen (Lille, France) (Table S3). We used a 3-plex
123 assay: R1-36, D2-185, R1-75, and R1-169 (multiplex 1); R4-114, R4-26, D2-142, R4100, and
124 D3-15 (multiplex 2); and R1-80, R1-77, R4-33, and R1-137 (multiplex 3). The multiplex PCR
125 mixtures contained 0.5 U of Taq DNA polymerase, 6 pmol of dNTP, 37.5 pmol of MgCl₂, 1 µl
126 of DNA (dilution: 1/5), and 1 µl of primers. The primers were labeled with fluorochromes (6-
127 FAM, NED, VIC, or PET; Table S3). The amplification conditions were as follows: an initial
128 10-min denaturation step at 95°C, 40 30-sec cycles at 95°C, a 30-sec annealing step at 50°C, an
129 initial 1-min extension step at 72°C, and a final 10-min extension step at 72°C. The PCR
130 products were analyzed by GenoScreen; an ABI 3730XL sequencing system (Applied
131 Biosystems) and a GeneScan™ 500LIZ® Size Standard (Applied Biosystems) were used. The
132 hornets were then genotyped at all 13 microsatellite loci using Geneious 9.4 (Biomatters).

133 Of these 13 loci, D2-142 and R1-77 had to be excluded because they were monomorphic; R4-
134 26 was also excluded because of unreadable traces. Consequently, all the genetic analyses were
135 performed using the 10 remaining microsatellite loci.

136

137

138 Statistical analyses for the chemical data

139 Multivariate and clustering analyses

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

150 We then performed more detailed analyses to explore grouping patterns among the individual
151 hornets collected from within the apiaries based on the results of both the chemical and genetic
152 analyses. K-means clustering analyses (Kaufman and Rousseeuw 1990) were performed to
153 define groups based on CHC similarities, using the packages *factoextra* (v. 1.0.4) and *ggplot*
154 (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
158 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 Statistical analyses for the genetic data

164 Genetic diversity and structure at both population and colony levels

165 Population-level genetic diversity was quantified based on the 27 individuals representing the
166 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_O), 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.

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

177

178 Multivariate and clustering analyses for the genetic data

179 We explored the genotypic differences among hornets using another PCA. This time, however,
180 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:

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

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

193

194 Analyses of differences among individuals from the genetic data

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

199 formula was used: $1 - [(\text{number of total alleles} - \text{number of alleles differing within the pair}) /$
200 $\text{number of total alleles}]$.

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

209

210 **Results**

211 Multivariate and clustering analyses of the chemical data

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

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

225

226 Differences in the hornet cuticular hydrocarbon profiles

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

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

233

234 Genetic diversity

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

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

248 Finally, at the apiary level, the mean number of alleles per locus was 2.5 and ranged from 2 to
249 4, a comparable level of genetic diversity to that above.

250

251 Multivariate and clustering analyses of the genetic data

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).
255 In the Bayesian clustering analysis, the likelihood of hornet membership in a given cluster was
256 assessed. For Apiaries 1 and 4, hornets could not be assigned to a given cluster with a high
257 degree of likelihood; instead, they had a probability of around 50% of belonging to two clusters.
258 In contrast, for Apiaries 2 and 3, hornets could be assigned to one of two or three clusters,
259 respectively, with a likelihood of more than 80%.

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

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

264

265 Differences in hornet genotypes

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

273

274 **Discussion**

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

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

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

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
307 around the apiaries, even if it would have been helpful to characterize the colonies' specific
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
310 microsatellite diversity was low, as expected given the significant loss of genetic diversity in
311 the hornet's invasive range (France, Arca et al. 2015; Korean peninsula, Choi et al. 2013).
312 However, our study nonetheless yielded sufficient baseline genetic information at the
313 population level and colony level to carry out the F_{ST} analyses and PCA on the genetic data
314 among colonies. The PCA results at the apiary level showed that, within each apiary, some
315 hornets had more similar genotypes than others. By itself, this exploratory analysis did not
316 indicate whether one or more *V. velutina* colonies were represented by the hornets sampled. We
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
320 which hornets grouped. These two methods arrived at different estimates of cluster number,
321 likely because they employ different approaches. The Bayesian method calculates the
322 likelihood that individuals belong to a given homogeneous cluster or clusters. In contrast,
323 DAPC maximizes differences between groups while minimizing variation within groups.
324 Nonetheless, both showed that there was a certain level of genetic differentiation among hornets
325 caught within the same apiary. This result mirrors the clustering that was observed with the
326 CHC data.

327 Finally, we found that, for the genetic data, within-apiary dissimilarity was significantly
328 different from within-colony and between-colony dissimilarity. More specifically, dissimilarity
329 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
331 dissimilarity between hornets collected within a given apiary, which fits with the findings of
332 the chemical analyses and suggests that the hornets could have belonged to more than one
333 colony. However, there is also the possibility that these genetic differences stemmed from
334 polyandry (i.e., queens mating with multiple males), a mechanism known to increase genetic
335 diversity within colonies. Arca et al. (2015) showed that polyandry occurs in the invasive *V.*
336 *velutina* population in France. Indeed, polyandry could be an important element in
337 understanding the hornet's invasiveness, as this mechanism could increase genetic diversity
338 within colonies. In addition, some studies have shown that combining polyandry with higher
339 heterozygosity could further enhance the success of invasive populations, since heterozygosity
340 can improve survival, longevity, and competitiveness (Taylor et al., 2014).

341 Taken together, these two sets of results lay the groundwork for further reflection. Although
342 CHC profiling and microsatellite genotyping are commonly used in studies of social insects,
343 neither approach allowed us to definitively confirm whether the hornets in a given apiary came
344 from one or multiple colonies. One challenge is that we are dealing with an invasive social
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
347 (e.g., the Argentine ant, *Linepithema humile* [Van Wilgenburg et al. 2010]). As a result of
348 founding effects (e.g., bottlenecks), chemical and genetic diversity in the introduced *V. velutina*
349 population may be too low for CHC profiling and microsatellite genotyping to yield
350 unambiguous results, like in previous studies in other invasive social insects (*Reticulitermes*
351 *flavipes*, Dronnet et al. 2006; *Linepithema humile*, Brands et al. 2009). It would therefore be
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).

355 To conclude, our results suggest that more than one *V. velutina* colony might prey on bees
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
358 suggested that intraspecific competition may be limited. Future experiments could be performed
359 to assign hornets to their colonies of origin based on their chemical and/or genetic profiles, a
360 task that would require in-depth knowledge of the reference population. Sampling would need
361 to be carried out on a large number of nests around target apiaries. However, such research is
362 often challenging because detecting nests is difficult: they are generally hidden high up in the
363 vegetation. Nonetheless, combining chemical and molecular data could be a promising avenue

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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3

4 **Figure 1.** Clusters of *V. velutina* workers defined using the K-means clustering method based
5 on the hornets' cuticular hydrocarbon (CHC) profiles; the hornets were collected from in front
6 of beehives (one beehive per apiary). A: 46 hornets from Apiary 1; B: 42 hornets from Apiary
7 2; C: 41 hornets from Apiary 3; D: 42 hornets from Apiary 4. Circles are related to K-means.

8

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

14

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

20

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

25

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

31

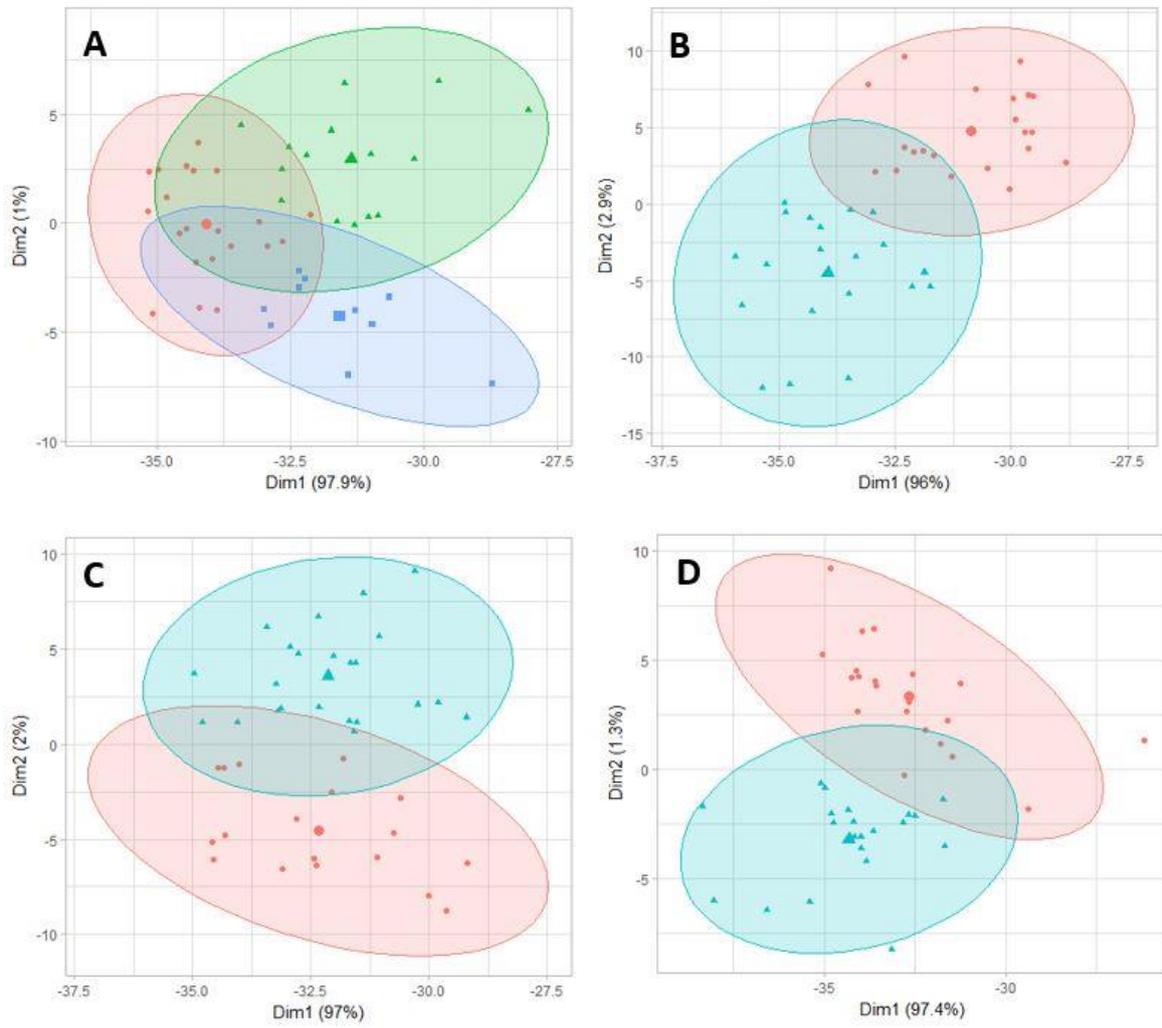
32 **Table 1.** Summary of the ten microsatellite loci used to study the genetic diversity among 27
33 *Vespa velutina nigrithorax* workers from an invasive population in Indre-et-Loire (France).
34 Data include the allele size range (in base pairs), number of alleles per locus (N_a), observed
35 (H_o)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

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Figure 1

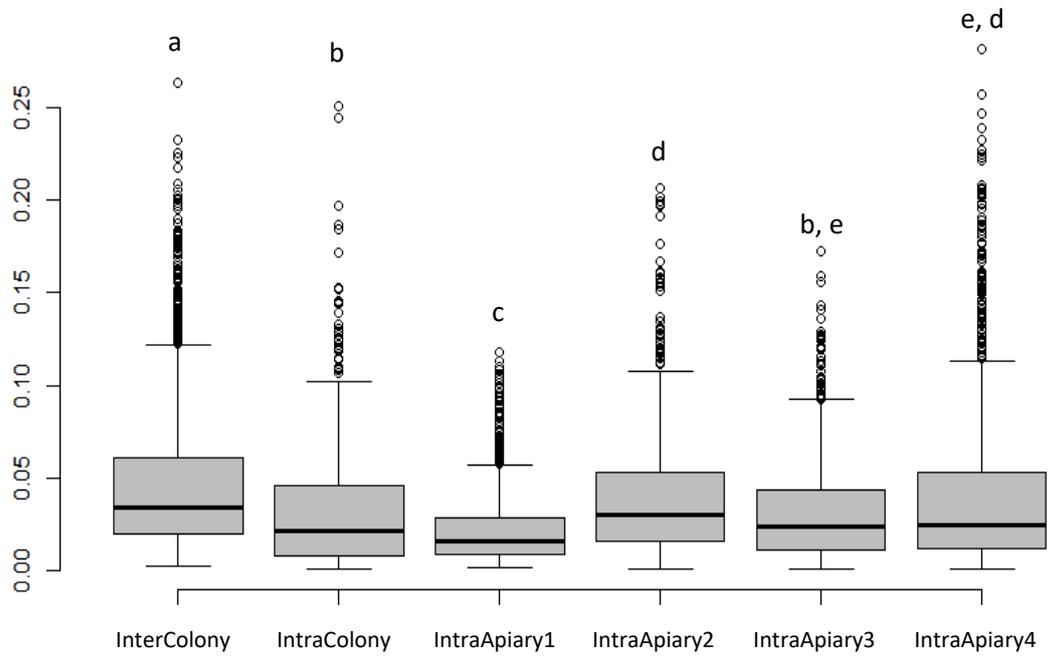


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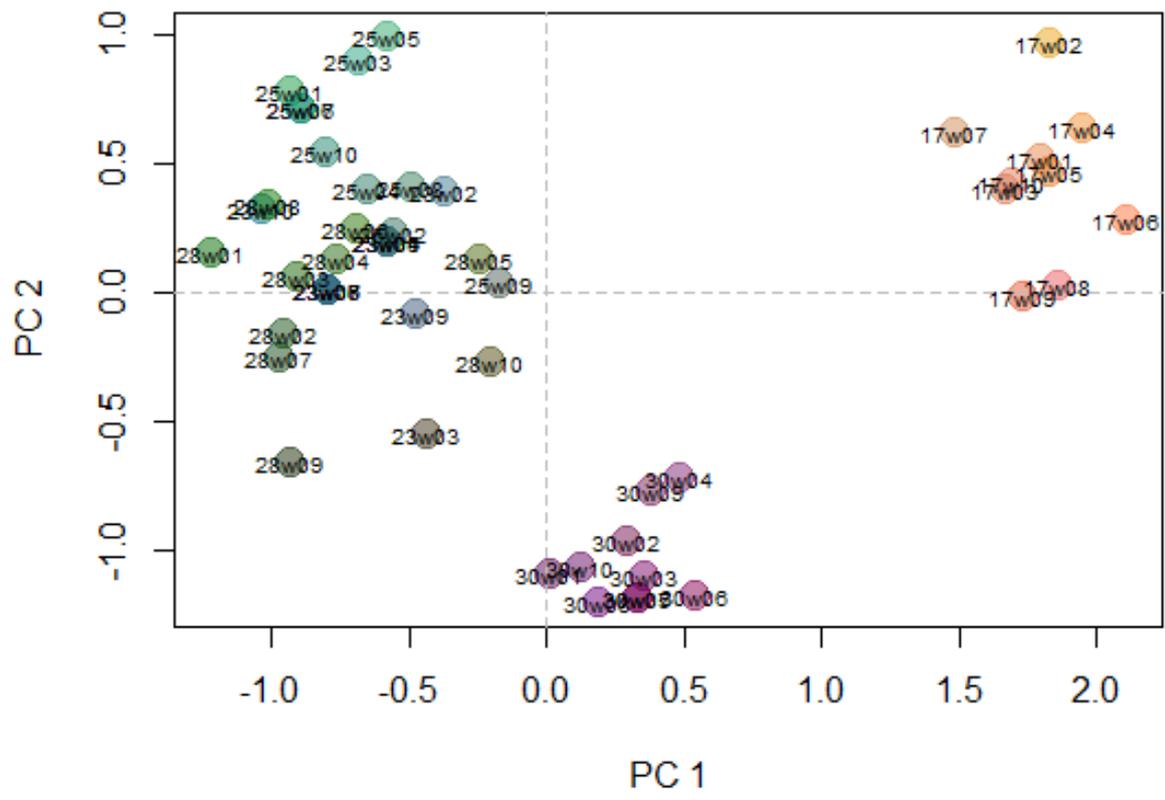
41 **Figure 2**

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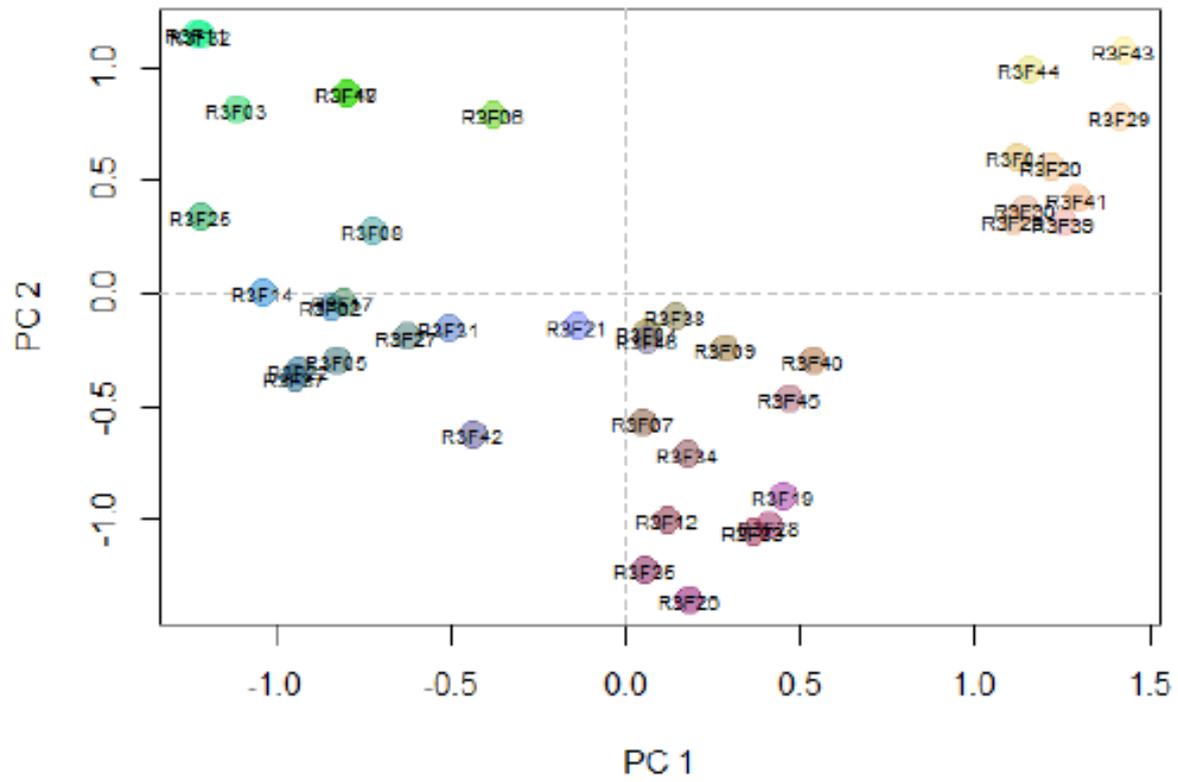


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44 **Figure 3**
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46 **Figure 4**



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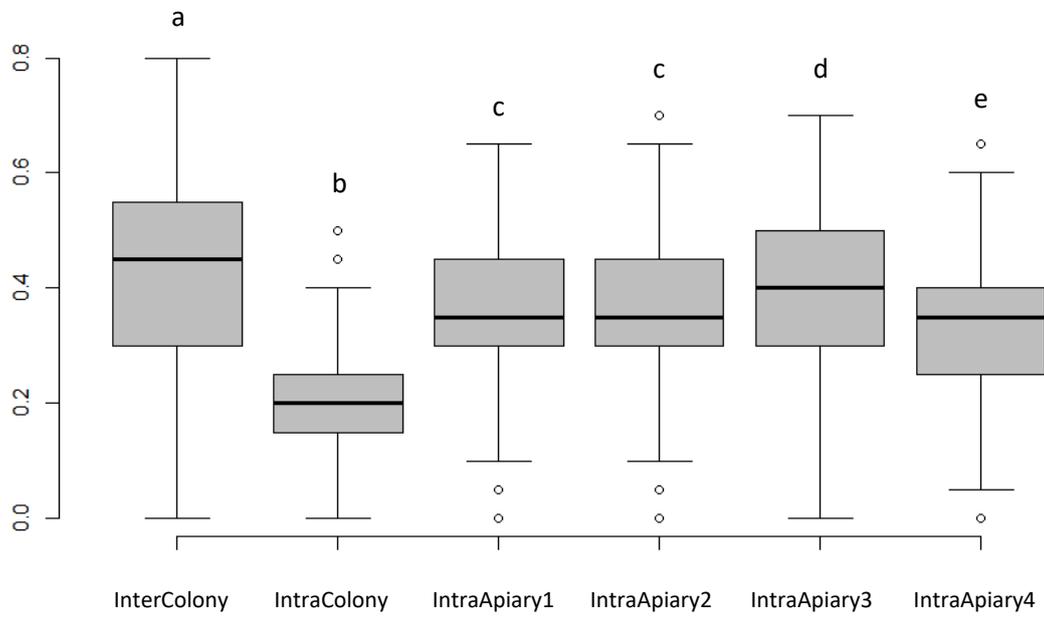
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52 **Figure 5**



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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