TITLE

Within-species floral odor variation is maintained by spatial and temporal heterogeneity in pollinator communities

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1   **ABSTRACT**

2   Floral odor is a complex trait that mediates many biotic interactions, including pollination.

3   While high intraspecific floral odor variation appears to be common, the ecological and

4   evolutionary drivers of this variation are often unclear. Here, we investigated the influence of

5   spatially and temporally heterogeneous pollinator communities on floral odor variation in

6   *Arum maculatum* (Araceae). Through Europe-wide field surveys, we identified high floral odor

7   diversity and shifts in the dominant pollinator species within several *A. maculatum* populations

8   compared to pollinator data from the same sites ten years ago. Using common-garden

9   experiments, we further confirmed that inflorescences from native and foreign pollinator

10   backgrounds were equally efficient at attracting local pollinators. The substantial within-

11   population floral odor variation we observed may therefore be advantageous when facing

12   temporally heterogeneous pollinator communities. We propose spatio-temporal heterogeneity

13   in pollinators as one potential mechanism maintaining diverse floral odor bouquets in

14   angiosperms.

15 **INTRODUCTION**

16 The rapid diversification of angiosperms relative to other land plants has long fascinated

17 evolutionary biologists, dating back to Darwin (1903). Today, we can attribute much of the

18 variation in angiosperm diversification rates to plant-pollinator interactions (Hernández-

19 Hernández and Wiens 2020). As the dominant pollinators across all terrestrial ecosystems,

20 insects play a key role in floral trait evolution (Sprengel 1793, Henslow 1888; Grant 1949;

21 Raven 1977; Kevan and Baker 1983; Crepet and Niklas 2009; van der Niet and Johnson 2012;

22 Schiestl and Johnson 2013). These plant-insect interactions are mediated by multimodal floral

23 signals (reviewed in Junker and Parachnowitsch 2015), which include odor (Holopainen 2004;

24 Raguso 2008ab; Whitehead and Peakall 2009), color (Goyret et al. 2007; du Plessis et al. 2018),

25 morphology (Ayasse et al. 2003; Ibanez et al. 2010), nectar (Parachnowitsch et al. 2019), and

26 pollen (Dobson and Bergström 2000). Nowadays, we are able to identify many plant volatile

27 organic compounds (VOCs) with relative ease (Knudsen and Gershenzon 2020). However, the

28 ecological and evolutionary drivers of floral odor variation often remain unclear, due to

29 difficulties in collecting and integrating data on floral odor variation and spatio-temporal

30 variation in pollinators.

31

32 In a recent review, Delle-Vedove et al. (2017) found that intraspecific floral odor variation is

33 frequently identified within diverse angiosperm lineages. Since heterogenous pollinator

34 communities are known to influence variation in many plant traits (Schemske and Horvitz

35 1988; Fishbein and Venable 1996; Price et al. 2005; Weber et al. 2019), it can be argued that

36 floral odor variation should also be influenced by spatio-temporal variation in pollinators

37 (Delle-Vedove et al. 2017, Haveramp et al. 2018, Dormont et al. 2019, Friberg et al. 2019,

38 Burkle et al. 2020, Farré-Armengol et al. 2020). However, only a small fraction of studies to

39 date have been able to explicitly link floral odor variation and variation in pollinator

3

40   community structure (e.g. Chess et al. 2008; Klahre et al. 2011; Breitkopf et al. 2013; Peter

41   and Johnson 2014; Sun et al. 2013; Gross et al. 2016). Several factors may explain this

42   discrepancy, including phylogenetic constraints in the biosynthetic pathways for the production

43   of VOCs (Raguso et al. 2006), tradeoffs between pollinator attraction and chemical defense

44   against herbivory (Schiestl et al. 2014), biochemical and energetic limitations (Delle-Vedove

45   et al. 2011), genetic drift (Suinyuy et al. 2012), or gene flow (Svensson et al. 2005). Moreover,

46   most of the aforementioned surveys of floral odor and pollinator community variation were

47   carried out at a single timepoint. As a result, the impact of temporal heterogeneity in pollinator

48   communities on floral odor variation remains unknown; here, we aim to address this gap in our

49   knowledge.

50

51   If pollinator communities vary temporally within populations, heterogeneous and/or

52   frequency-dependent selective pressures may favor the maintenance of floral odor variation

53   through balancing selection (Delph and Kelly 2013; Wittmann et al. 2017; Bertram and Masel

54   2019). Alternatively, if pollinators only vary spatially among populations, we might expect

55   divergent selective pressures to occur, leading to local adaptation (Leimu and Fischer 2008,

56   Gómez et al. 2009; Gross et al. 2016; Gervasi and Schiestl 2017; Suinyuy and Johnson 2018;

57   Sayers et al. 2020). Variation may also result from phenotypic plasticity under variable abiotic

58   conditions or biotic interactions (Majetic et al. 2009; Campbell et al. 2019), if several floral

59   odor phenotypes can result from a single genotype. Here, we investigated how each of these

60   three processes influence floral odor variation, using wild populations of *Arum maculatum*

61   (Araceae).

62

63   Several aspects of *A. maculatum*'s ecology make it a suitable model system for studying the

64   relationship between pollinators and floral odor. *Arum maculatum* inflorescences attract and

4

65 temporarily trap their pollinators, mainly the moth flies *Psychoda phalaenoides* and *Psycha*

66 *grisescens* (Diptera: Psychodidae), using complex blends of VOCs which appear to vary across

67 the species distribution range (Kite 1995; Kite et al. 1998; Diaz and Kite 2002; Chartier et al.

68 2011; Chartier et al. 2013). These odor blends are thought to mimic the odor of dung or rotting

69 organic matter, the natural brood sites of these Psychodidae (Lack and Diaz 1991). Pollinators

70 are trapped in a highly specialized floral chamber (Bröderbauer et al. 2013) until the day after

71 VOC emissions (Gibernau et al. 2004). As a result, we are able to accurately quantify the

72 complete pollinator community attracted by each inflorescence, and unambiguously relate

73 pollinator abundances to VOC emissions. Since *A. maculatum* inflorescences do not offer any

74 rewards to their pollinators (Lack and Diaz 1991), and Psychodidae appear to be attracted by

75 VOCs alone (Dormer 1960; Kite 1995; Urru et al. 2011), the reproductive success of an

76 individual inflorescence (i.e. its ability to attract efficient pollinators) should be closely tied to

77 its unique floral odor. Selfing is also avoided through protogyny; female flowers are no longer

78 receptive to pollen when the male flowers mature (Lack and Diaz 1991).

79

80 This unique pollination ecology provides an opportunity to address key questions on how and

81 why floral odor variation is maintained in widely distributed species. Namely, is variation

82 maintained across species ranges, due to spatial variation in pollinators, or within populations,

83 due to temporally heterogeneous pollinators? Spatial and temporal variation in pollinators may

84 drive floral odor variation through one or more of the aforementioned evolutionary processes

85 (i.e. balancing selection, local adaptation, or plasticity). Below, we summarize the patterns in

86 floral odor diversity we expected to observe under each scenario:

87

88 1) Balancing selection is expected to maintain greater variation in deceptively pollinated

89 species compared to rewarding species (Ackerman et al. 2011), through temporal variation in

90 pollinator communities (Schemske and Horvitz 1989), frequency-dependent selection driven

91 by pollinator learning (Ayasse et al. 2000), or relaxed selection (Salzmann et al. 2007). The

92 tetraploid genome of *A. maculatum* (Turco et al. 2014) may allow balancing selection to

93 maintain variation within individual genomes as well (Mable et al. 2018). If *A. maculatum*

94 floral odor is a balanced polymorphic trait, we would expect to observe high variation in floral

95 odor both within and among populations. This variation may be a consequence of either: a)

96 temporally heterogeneous pollinator communities within populations, whose varying

97 preferences maintain floral odor diversity, or b) pollinator learning, if pollinator communities

98 are stable within populations through time.

99

100 2) Populations of *A. maculatum* are known to predominantly attract either *P. phalaenoides* or

101 *P. grisescens* in different regions across their distribution (Espíndola et al. 2010). Following

102 the 'local vs. foreign' definition of local adaptation (Kawecki and Ebert 2004) we might

103 alternatively predict that *A. maculatum* populations harbor distinct floral odor variations, which

104 maximize the attraction of their dominant local pollinator species. If these variations are true

105 local adaptations, we would expect to observe decreased pollinator attraction efficiency when

106 *A. maculatum* inflorescences are transplanted to non-native pollinator backgrounds.

107

108 3) As organisms rooted in place, plants benefit from adaptive plasticity in response to variable

109 environmental conditions or biotic interactions (Majetic et al. 2009; Metlen et al. 2009). If *A.*

110 *maculatum* floral odor is a plastic trait, we would expect to observe a correlation between

111 climatic variables and floral odor variation across the wide geographic range of *A. maculatum*,

112 and/or a shift in VOC emissions when inflorescences are transplanted to non-native

113 environments.

114

6

115   Here, we tested these three sets of predictions by surveying natural variation in *A. maculatum*

116   floral odor and pollinator attraction across Europe, and by transplanting individuals from all

117   sampled populations to two common garden sites dominated by different pollinator species

118   (either *Psychoda phalaenoides* or *Psycha grisescens*). Importantly, we selected sampling sites

119   with pollinator data collected approximately ten years ago by Espíndola et al. (2010), allowing

120   us to investigate temporal heterogeneity in pollinator communities within our study. Together,

121   these data allowed us to understand the extent of within- and between-population variation in

122   *A. maculatum* floral scent, how this variation is organized across a wide geographic range, and

123   how both spatial and temporal pollinator community dynamics influence floral odor variation.

124

125   **MATERIALS AND METHODS**

126   <u>Field sampling sites</u>

127   We sampled eleven populations of *A. maculatum*, including three in France (Forêt du Gâvre,

128   Conteville, and Chaumont), two in Switzerland (Neuchâtel and Cortaillod), two in Italy

129   (Montese and Rifreddo), one in Croatia (Visuć), two in Serbia (Gostilje and Sokobanja), and

130   one in Bulgaria (Chiflik), as shown in Appendix S1, Figure S1. Full sampling information,

131   including location data and sample sizes are given in Appendix S1, Table S1. We selected Forêt

132   du Gâvre and Neuchâtel, which are respectively dominated by *Psycha grisescens* and *Psychoda*

133   *phalaenoides*, as our two common garden sites. Five to ten *A. maculatum* individuals from each

134   population were potted and transplanted to both sites; inflorescences from Forêt du Gâvre and

135   Neuchâtel were also reciprocally transplanted as part of this experiment.

136

137   <u>Floral odor and pollinator sampling</u>

138   During the typical flowering period of *A. maculatum* (April – May), we conducted: 1) field

139   sampling between 2017 and 2019, and 2) common garden experiments in Neuchâtel in 2018

140   and 2019, and in Forêt du Gâvre in 2019. In both field surveys and common garden

141   experiments, we collected dynamic headspace VOCs and identified pollinators using identical

142   methods, as detailed in Appendix S2: Supplementary Methods. Briefly, we collected VOCs

143   from *A. maculatum* inflorescences undergoing anthesis in the early evening on

144   polydimethylsiloxane (PDMS) coated Twister® stir bars (Gerstel: Mülheim an der Ruhr,

145   Germany), at a rate of 200 mL min⁻¹ for 30 minutes. Twisters® were kept on ice in sealed glass

146   containers until gas chromatography–mass spectrometry (GC-MS) analysis, where volatiles

147   were thermally desorbed and separated on a HP-5MS column. During the morning following

148   VOC sampling, we collected all insects trapped within inflorescences and preserved them in

149   70% ethanol until identification.

150

151   <u>Testing for balancing selection and temporal heterogeneity in pollinators</u>

152   First, we investigated Europe-wide patterns in floral odor variation, by calculating Bray-Curtis

153   similarities between all individuals in our *in situ* field surveys, and visualizing the result using

154   nonmetric multidimensional scaling (NMDS). We then assessed the effect of population (fixed

155   effect factor) on the entire VOCs matrix using permutational multivariate analysis of variance

156   (PERMANOVA, Bray-Curtis distance, n = 999 permutations) with the *adonis* function in the

157   R v.3.6.1 (R Core Team 2019) package *vegan* (Oksanen et al. 2019). Then, we compared the

158   extent of both within- and between-population variation in floral odor using Bray-Curtis

159   similarity matrices, visualizing the resulting similarity scores using boxplots.

160

161   Second, we investigated shifts in the dominant pollinators trapped by *A. maculatum* over the

162   past decade, both in terms of 1) Psychodidae species and 2) other insect families, comparing

163   our observations with data (mean abundances) from the same sites in Espíndola et al. (2010).

164   We began by calculating the mean quantities of each pollinator species trapped per

8

165   inflorescence within each population. Then, to investigate shifts in pollinators, we calculated

166   Bray-Curtis distances between pollinator communities now (2017-2019) and approximately a

167   decade ago (2006-2008) for all sites with data from both timepoints, and visualized these

168   results using NMDS ordinations.

169

170   <u>Testing for local adaptation to pollinators using common garden experiments</u>

171   First, we visualized geographic patterns in mean pollinator attraction for each population *in*

172   *situ*, and following transplants to both common garden sites. Then, following the 'local vs.

173   foreign' definition of local adaptation (Kawecki and Ebert 2004), we tested the hypotheses

174   that: in the Neuchâtel common garden, transplanted *A. maculatum* inflorescences which attract

175   *P. phalaenoides* in their native population should catch 1) more *P. phalaenoides* and/or 2) more

176   insects in total than inflorescences which attract *P. grisescens* in their native population. We

177   expected the opposite pattern in the Forêt du Gâvre common garden (i.e. transplanted

178   inflorescences that attract *P. grisescens* in their native population should perform better on

179   average in Forêt du Gâvre). These predictions were tested using a two-way ANOVA on log+1

180   transformed pollinator counts, including 'native pollinator' (i.e. *P. phalaenoides*- or *P.*

181   *grisescens*-dominated) and 'common garden location' (i.e. whether the common garden site

182   was dominated by *P. phalaenoides* or *P. grisescens*) and their interaction as fixed factors. Here,

183   a significant interaction would suggest local adaptation to native pollinator communities.

184   Finally, we plotted 'deme × habitat' interactions (per Kawecki and Ebert 2004)—deme and

185   habitat referring respectively to a local population and its local environmental conditions (here

186   the pollinator community)—for mean attraction rates of *P. phalaenoides*, *P. grisescens*, and all

187   insects. Data for these plots were subset based on each inflorescence's native pollinator and

188   common garden location, as described above.

189

9

190 Testing for phenotypic plasticity and climatic correlates of floral odor variation

191 To address the correlation between climatic variation across Europe, floral odor, and pollinator

192 communities trapped within inflorescences, we used two complementary approaches. First, we

193 clustered individuals based on similarities in their proportional VOC emissions using

194 unsupervised learning algorithms (as detailed in Appendix S2: Supplementary Methods) to test

195 for VOC bouquet convergence due to spatial segregation or local adaptation to pollinators

196 (Andersson et al. 2002; Hetherington-Rauth and Ramírez 2016). We then investigated whether

197 the resulting floral odor clusters were differentially attractive to pollinator groups, including

198 Psychodidae (identified to the species level), Brachycera, Nematocera, and Staphylinidae. To

199 control for potential biases caused by differences in the dominant pollinator species among all

200 sampled populations, we split these comparisons based on whether individuals were sampled

201 in a site typically dominated by *P. phalaenoides* or *P. grisescens*. We used Kruskal-Wallis tests

202 to determine whether clusters attracted different pollinator species in each pollinator

203 background.

204 Next, we investigated the influence of large-scale climatic variation on floral odor variation,

205 by performing a mantel test (Euclidean distance, 999 replicates) between 19 bioclimatic layers

206 (BIO1-BIO19) at 30-seconds resolution from WorldClim (Hijmans et al. 2005) and population-

207 level VOC data from our field surveys. Then, we investigated whether the types or proportions

208 of odor variations within populations shifted as result of transplanting by comparing the

209 relative abundances of each VOC cluster, both within populations of origin and following

210 transplants.

211

212 Finally, we investigated whether specific VOCs were associated with the attraction of *P.*

213 *phalaenoides* or *P. grisescens*. We identified candidate compounds using the Random Forest

214 implementation in the R package *randomForest* (Liaw and Wiener 2002), with permutation

10

215   importance enabled (*ntree* = 500, *mtry* = 8; optimized using the *tuneRF* function). Then, we

216   calculated conditional feature contributions, and identified the combinations of compounds

217   which had the greatest influence on the predictive strength of the model above, using the

218   Python package *TreeInterpreter* (Saabas 2019). Finally, we performed Mann-Whitney *U*-tests

219   to identify shifts in the candidate compounds most strongly associated with *P. phalaenoides* or

220   *P. grisescens* attraction. Specifically, we tested whether populations with sufficiently large

221   sample sizes (*n* ≥8) emitted different quantities of candidate compounds, when comparing

222   between the two common gardens.

223

224   **RESULTS**

225   Balancing selection and temporal heterogeneity in pollinators

226   We observed that *A. maculatum* floral odor is highly variable across Europe. After filtering out

227   compounds present in blank samples, we retained 18 *A. maculatum* floral VOCs present in

228   relative abundances above 1% (Table 1). Many of the major compounds we identified (e.g.

229   indole, p-cresol, 2-heptanone, β-citronellene, and three unnamed sesquiterpenes) have been

230   previously reported in studies of *A. maculatum* floral odor (Diaz and Kite 2002; Chartier et al.

231   2013; Marotz-Clausen et al. 2018). Populations across Europe differed in their proportional

232   emissions of VOCs *in situ* (PERMANOVA, $R^2 = 0.38$, Pr(>F) = 0.001). However, we observed

233   substantial within- and among-population variation in floral odor (Appendix S1, Figure S2),

234   resulting in no clear regional differentiation in the NMDS ordination (Appendix S1, Figure

235   S3).

236

237   We also observed that *A. maculatum* pollinator communities are temporally variable.

238   Ultimately, we were able to re-survey pollinators trapped by *A. maculatum* inflorescences in

239   six populations (listed in Figure 1), approximately 10 years after the surveys conducted by

11

240   Espíndola et al. (2010). The total insect communities trapped by *A. maculatum* appear to have

241   shifted in most of these populations - only Forêt du Gâvre remained relatively consistent over

242   the past decade (Figure 1). When focusing on Psychodidae only, we observed shifts in the

243   dominant psychodid pollinator trapped within inflorescences in four out of six populations:

244   Chaumont FR, Conteville FR, Gostilje SRB, and Visuć, HR (Figure 1). Additionally, we

245   identified a substantial decrease in the average quantity of pollinators trapped within

246   inflorescences in Conteville FR: from 47.6 *P. phalaenoides* and 2.3 *P. grisescens* per

247   inflorescence in Espíndola et al. (2010), to 0.4 *P. phalaenoides* and 1.3 *P. grisescens* in our

248   sampling (full results in Appendix S1, Table S2).

249

250   <u>Local adaptation to pollinators</u>

251   We observed that transplanted inflorescences typically attracted the dominant local pollinator

252   species as efficiently as native inflorescences in both common gardens (Figure 2). While

253   common garden location had a significant effect on the quantity of *P. phalaenoides* and *P.*

254   *grisescens* caught by *A. maculatum* (i.e. the two transplant sites were indeed dominated by

255   different Psychodidae species), no native pollinator × common garden location interaction

256   effect was observed (2-way ANOVA, Pr(>F) > 0.05; full results in Appendix S1, Table S3).

257   Together, these results show that *A. maculatum* populations are generally not locally adapted

258   to a single pollinator species (Figure 3), with some possible exceptions in Forêt du Gâvre, and

259   the two Serbian populations Gostilje and Sokobanja, which will be discussed below.

260

261   While populations generally may not be locally adapted to specific pollinators, not all floral

262   odor blends were equally attractive to all pollinator species. Notably, one population (Forêt du

263   Gâvre) continued to exclusively attract their native pollinator *P. grisescens* when transplanted

264   to the Neuchâtel common garden, despite *P. phalaenoides* being the most abundant species in

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265 Neuchâtel when these transplanted inflorescences opened (see Appendix S1, Figure S4).

266 Additionally, transplanted inflorescences occasionally attracted 'non-dominant' Psychodidae

267 species in both common garden sites, and a third pollinator species (*Psychoda trinodulosa*) was

268 also identified within inflorescences in Croatia and Serbia during our field surveys (Figure 2).

269 This species was also observed in the Neuchâtel common garden, but not in Forêt du Gâvre.

270 Inflorescences from both Serbian populations (Gostilje and Sokobanja) continued to

271 occasionally attract *P. trinodulosa* when transplanted the Neuchâtel common garden.

272

273 <u>Phenotypic plasticity and climatic correlates of floral odor variation</u>

274 The cluster validation process identified two groups, clustered using the PAM (Partitioning

275 Around Medoids) algorithm, to be optimal for our VOC dataset (Appendix S1, Figures S5 and

276 S6). Cluster 1 was characterized by high mono- and sesquiterpene emissions, while Cluster 2

277 was characterized by high indole emissions. Five compounds (indole, skatole, Z-

278 caryophyllene, β-caryophyllene, and unnamed sesquiterpene RI 1473) varied significantly

279 between PAM clusters (Kruskal-Wallis test, $p < 0.05$, df = 2). The aforementioned unnamed

280 sesquiterpene (Kovats index apolar 1473) has been previously identified in the floral scent of

281 *Arum italicum* (M. Gibernau, unpublished data). The average VOC blends of both PAM

282 clusters are summarized in Table 1, and visualized in Appendix S1, Figure S7. Both clusters

283 were widely distributed across Europe; indole-dominated Cluster 2 was not observed in

284 Rifreddo or Visuć, though smaller sample sizes in these populations may have contributed to

285 this result (Appendix S1, Figure S8). Ultimately, whether an inflorescence belonged to Cluster

286 1 or 2 did not appear to strongly influence pollinator attraction, with considerable variation

287 observed within both Clusters (Appendix S1, Figure S9). We did not identify any significant

288 differences between Clusters 1 and 2, in terms of species-specific Psychodidae attraction

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289   (Kruskal-Wallis tests, $p > 0.05$, df = 2), after controlling for the dominant pollinator species

290   where each sample was collected.

291

292   Random Forest analyses identified one compound positively correlated with *P. phalaenoides*

293   attraction (β-humulene), and three compounds positively correlated with *P. grisescens*

294   attraction (unnamed sesquiterpenes RI 1473 and 1681, and α-selinene). After evaluating

295   combined feature contributions within the above Random Forest model (Appendix S1, Figure

296   S10), we found that the predictive strength of the model was most strongly influenced by

297   unnamed sesquiterpene (RI 1681) alone. Other strong combinations included unnamed

298   sesquiterpene RI 1681 paired with unnamed sesquiterpene RI 1473 or α-selinene, as well as β-

299   humulene alone, mirroring the results in our initial variable importance plot (full

300   *Treeinterpreter* results in Appendix S1, Table S4).

301

302   We observed that inter-population variation in VOC blends overall did not correlate with

303   bioclimatic variables (Mantel test, $p = 0.823$). Average sesquiterpene emissions remained

304   relatively consistent between *in situ* samples and following transplants to Neuchâtel, but

305   appeared to increase after inflorescences were transplanted to Forêt du Gâvre (Appendix S1,

306   Figure S11). Among the four compounds linked to species-specific Psychodidae attraction,

307   we only observed significant shifts between reciprocally transplanted inflorescences from

308   Neuchatel and Forêt du Gâvre (Mann-Whitney tests, full result in Appendix S1, Table S5). For

309   the two other populations with sufficiently large sample sizes (Montese, IT and Chaumont,

310   FR), we did not observe significant differences in the emissions of these four compounds when

311   comparing between the two common garden sites (Appendix S1, Table S5) . Together, these

312   results suggest that plasticity may contribute to the floral odor variation we observed (e.g. in

313   the case of transplanted inflorescences from Neuchatel), but in general, the effect of

14

314   transplanting on inflorescences appears to be relatively minor in comparison to natural

315   intrapopulation variation in floral odor.

316

317   **DISCUSSION**

318   In this study, we performed a range-wide survey of *Arum maculatum* floral odor and pollinator

319   attraction, which identified substantial within-population variation in floral odor (Appendix

320   S1, Figures S2 and S3), and shifts in pollinator community composition in several populations

321   over the past decade (Figure 1). Through common garden experiments, we further

322   demonstrated that *A. maculatum* populations typically are not locally adapted to attract

323   exclusively *Psychoda phalaenoides* or *Psycha grisescens* (Figures 2 and 3), though local

324   adaptation might have contributed to pollinator attraction patterns in one French and two

325   Serbian populations. Excepting host-pathogen case studies (reviewed in Delph and Kelly

326   2013), our study is among the first to demonstrate that temporally fluctuating selective

327   pressures may act to maintain high variation in a key plant trait.

328

329   Balancing selection may maintain variation within populations through several selective

330   regimes, including relaxed selection, negative frequency-dependent selection due to pollinator

331   learning, or environmental heterogeneity (Delph and Kelly 2013). While relaxed selection on

332   floral odor has been observed in some angiosperms (Salzmann et al. 2007; Ibanez et al. 2010),

333   this is unlikely the case for *A. maculatum*, given that pollinator attraction is driven by floral

334   odor alone (Dormer 1960; Lack and Diaz 1991). Furthermore, while pollinator learning has

335   been shown to maintain polymorphism in floral color (Gigord et al. 2001) and odor (Ayasse et

336   al. 2000), we would argue that this is less likely to occur in the case of *A. maculatum*. Since

337   several Psychodidae species are trapped at rates that appear to vary over short time scales, the

338   resulting selective pressure on pollinators is likely too inconsistent to lead to adaptive pollinator

15

339   learning (Renner 2006). This leaves variable conditions (i.e. pollinator communities) as the

340   mechanism most likely contributing to the maintenance of diverse floral odor bouquets in *A.*

341   *maculatum*. Further research on temporal variation in pollinators may provide greater clarity

342   in other cases where floral trait variation cannot be explained by pollinator learning (e.g.

343   Pellegrino et al. 2005; Jersáková et al. 2006). Our results may also explain why evidence for

344   greater floral odor diversity in deceptive species compared to rewarding species is weak

345   (Ackerman et al. 2011) or largely absent (Delle-Vedove et al. 2017).

346

347   Building on reciprocal transplants between two *A. maculatum* populations conducted by

348   Chartier et al. (2013), we found that local adaptation is counteracted by temporally

349   heterogeneous pollinator communities across Europe, with one possible exception. The Forêt

350   du Gâvre population in France appears to have lost the ability to attract *P. phalaenoides*, either

351   due to local adaptation resulting from the large, stable populations of *P. grisescens* we observed

352   in their native habitat (Figure 1) or genetic drift, which is known to occur at the limits of species

353   ranges (Geber 2011; Gould et al. 2013). Similarly, the fact that the two Serbian populations

354   continued to attract a third psychodid species (*P. trinodulosa*) in the Neuchâtel common

355   garden, while inflorescences from other regions did not, also argues for some level of local

356   adaptation (Figure 2). These patterns suggest that specific compounds may also be

357   differentially attractive to *P. trinodulosa*, but this species was too infrequently observed in our

358   study to make any firm conclusions. These results thus do not exclude the possibility that

359   individual VOCs are differentially attractive to certain Psychodidae species, which might be

360   expected given previously reported differences in antennal sensilla (Faucheux and Gibernau

361   2011).

362

16

363 Through Random-Forest analyses, we identified four VOCs correlated with the attraction of *P.*

364 *phalaenoides* (β-humulene) and *P. grisescens* (unidentified sesquiterpenes RI 1473 and 1681,

365 and α-selinene). Indole, another abundant VOC in the *A. maculatum* odor bouquet, appears to

366 be generally attractive to females of both species (Appendix S1, Figure S9 and S10), consistent

367 with previous findings (Kite et al. 1998). Further research using Gas Chromatography -

368 Electroantennography (Cork et al. 1990) could confirm which VOCs elicit species-specific

369 responses, and whether these biologically active compounds are maintained at frequencies

370 expected under balancing selection.

371

372 Adaptive plasticity is the optimal solution in situations of environmental heterogeneity when

373 possible (Kawecki and Ebert 2004), and floral odor is known to vary with changing

374 environmental conditions (Burkle and Runyon 2017). Recently, a "Genomic Storage Effect"

375 has been proposed (Gulisija et al. 2016), whereby balanced polymorphism is promoted by

376 adaptive plasticity resulting from temporally varying selection. In such a scenario, a portion of

377 the population acts as a store of variation (i.e. compounds attractive to specific pollinators)

378 until conditions (i.e. dominant pollinator species) change (Chesson 2000). We found that

379 individuals from Neuchâtel and Cortaillod emitted proportionally lower quantities of indole

380 following transplants to Forêt du Gâvre (Appendix S1, Figure S8). This result is likely due to

381 a combination of high inter-individual variation in floral odor, and phenotypic plasticity driven

382 by environmental variation. Furthermore, in the case of polyploids such as *A. maculatum*, the

383 Genomic Storage Effect could even be at work at the within-individual level. Currently, it is

384 not known whether *P. phalaenoides* and *P. grisescens* phenologies are influenced by

385 environmental conditions; our data suggest that at least in Forêt du Gâvre, *P. phalaenoides* may

386 emerge slightly later than *P. grisescens* (Appendix S1, Figure S4b). If environmental variation

387 influences pollinator phenology, then plasticity in floral odor based on environmental cues

17

388 could enhance pollinator attraction. Consequently, plasticity may have contributed to the

389 variation in the four candidate compounds linked to species-specific pollinator attraction

390 (Appendix S1, Figure S11). However, in most cases, we did not observe significant shifts

391 consistent with plasticity (Appendix S1, Figure Table S5). After correction for multiple testing,

392 we only found a significant shift in the emission of unnamed sesquiterpene RI 1473 (correlated

393 with *P. grisescens* attraction) when comparing native and transplanted inflorescences from

394 Neuchâtel. This result demonstrates that the emission of some specific compounds may be

395 influenced environmental variation. While we cannot yet fully disentangle plasticity from

396 variation resulting from balancing selection, the high diversity in floral odor we observed in

397 our common garden sites is unlikely to be the result of plasticity alone.

398

399 High gene flow may also lead to the maintenance of floral odor variation. The short adult

400 lifespan (approx. one week) and limited dispersal capacity of Psychodidae (Lack and Diaz

401 1991) implies that *A. maculatum* gene flow is likely driven by seed dispersal, which is mainly

402 carried out by frugivorous birds (Snow and Snow 1988). There appears to be a strong barrier

403 to gene flow between populations from north/central Europe and from Italy and the Balkans

404 (Espíndola and Alvarez 2011), yet floral odor variation is shown to be widely maintained

405 across this barrier, suggesting that — at least regionally — balancing selection driven by

406 heterogenous pollinator community composition is at work, in association with some level of

407 local adaptation and plasticity.

408

409 <u>Conclusion</u>

410 While trait variation often appears to be more strongly influenced by spatial heterogeneity in

411 selection than temporal heterogeneity (Hedrick 1986) – as demonstrated by the extensive

412 literature on local adaptation in plants (Leimu and Fischer 2008; Anderson et al. 2011) – our

18

413   study highlights how temporal heterogeneity in pollinators may also be a contributing factor in

414   maintaining highly diverse floral odor bouquets. Although earlier models could not always

415   demonstrate the maintenance of polymorphism through temporal heterogeneity (Hedrick

416   1976), recent models (e.g. Gulisija et al. 2016) provide a mechanism by which frequent shifts

417   in pollinator communities may maintain trait variation and counteract local adaptation. To date,

418   almost all studies have sampled floral odors and pollinators at a single timepoint — possibly

419   contributing to the numerous cases where floral odor diversity appears to exceed pollinator

420   diversity (Delle-Vedove et al. 2017). Further research on the temporal dynamics of pollinator

421   communities has the potential to advance our understanding on how and why many flowering

422   plant lineages maintain high diversity in key traits such as floral odor.

19

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681 **TABLES**

682 **Table 1**. Proportional VOC blend compositions of 147 *Arum maculatum* individuals from 11

683 populations. The average blends for both K-medoids (PAM) clusters identified in this study are shown

684 below, and visualized in Appendix S1, Figure S7.

Compound	K1 (%) N=97	K2 (%) N=50
2-heptanone	1.84	1.19
β-citronellene	4.31	2.09
cis β-ocimene	0.47	0.54
p-cresol	1.68	0.94
indole ***	**24.35**	**65.39**
skatole ***	**2.52**	**1.03**
α-copaene	0.14	0.76
Z-caryophyllene *	**8.37**	**3.28**
β-caryophyllene *	**2.59**	**0.83**
α-humulene	6.68	2.92
alloaromadendrene	8.13	3.45
β-humulene	3.54	1.87
α-selinene	0.44	0.33
bicyclogermacrene	7.76	2.44
d-cadinene	2.11	0.83
Unnamed sesquiterpene (RI 1404)	4.97	2.06
Unnamed sesquiterpene (RI 1473) *	**5.76**	**2.37**
Unnamed sesquiterpene (RI 1681)	14.35	7.70

685 * denotes compounds that vary significantly between groups (Kruskal-Wallis test, $p < 0.05$, df = 1)

31

686   **FIGURES**
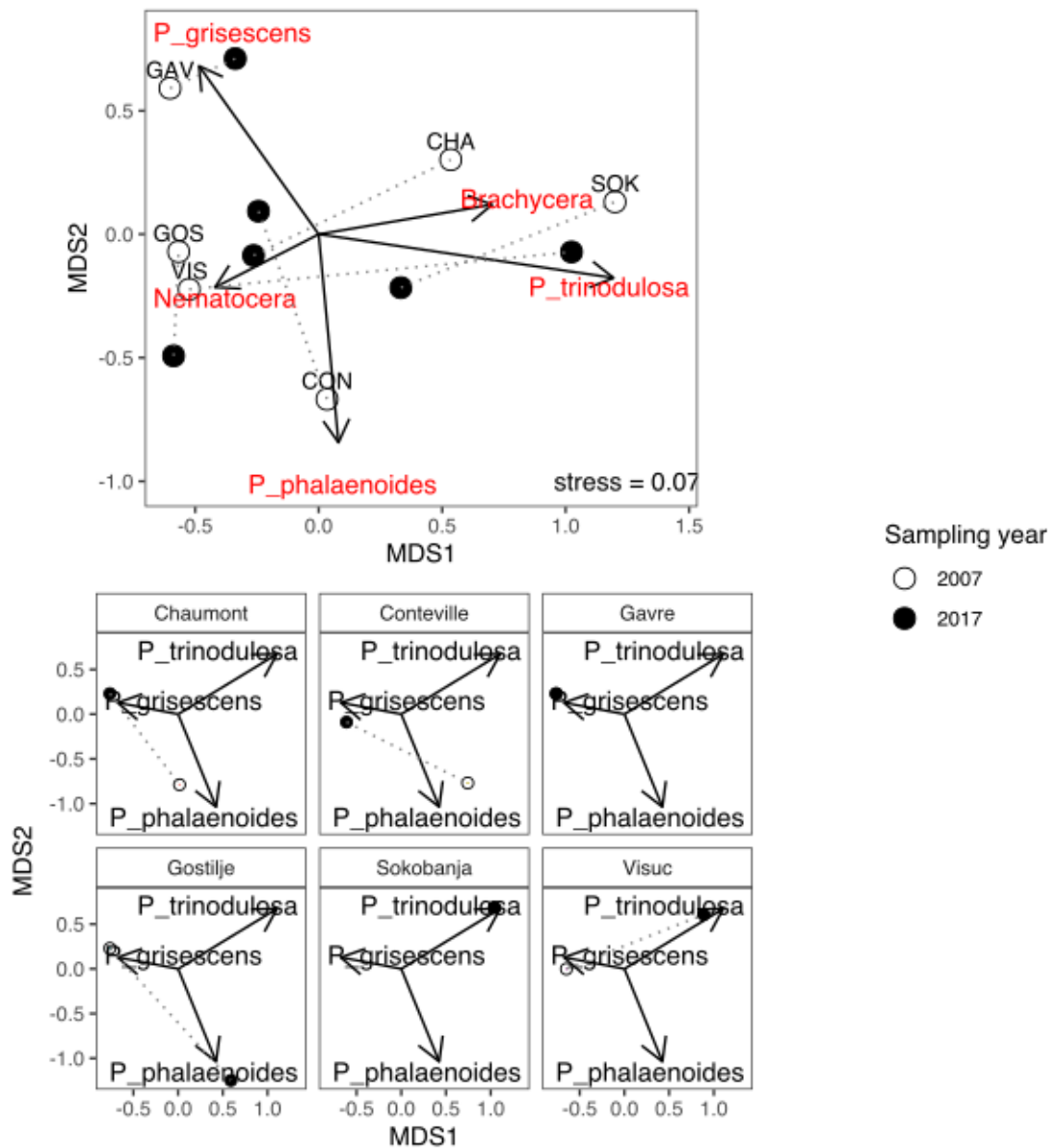
687   **Figure 1**. Shifts in the total pollinator communities (upper plot) and Psychodidae species (lower plots)

688   trapped by *Arum maculatum* inflorescences. Over the past decade, the dominant Psychodid pollinator

689   appears to have shifted in four out of six re-sampled populations.
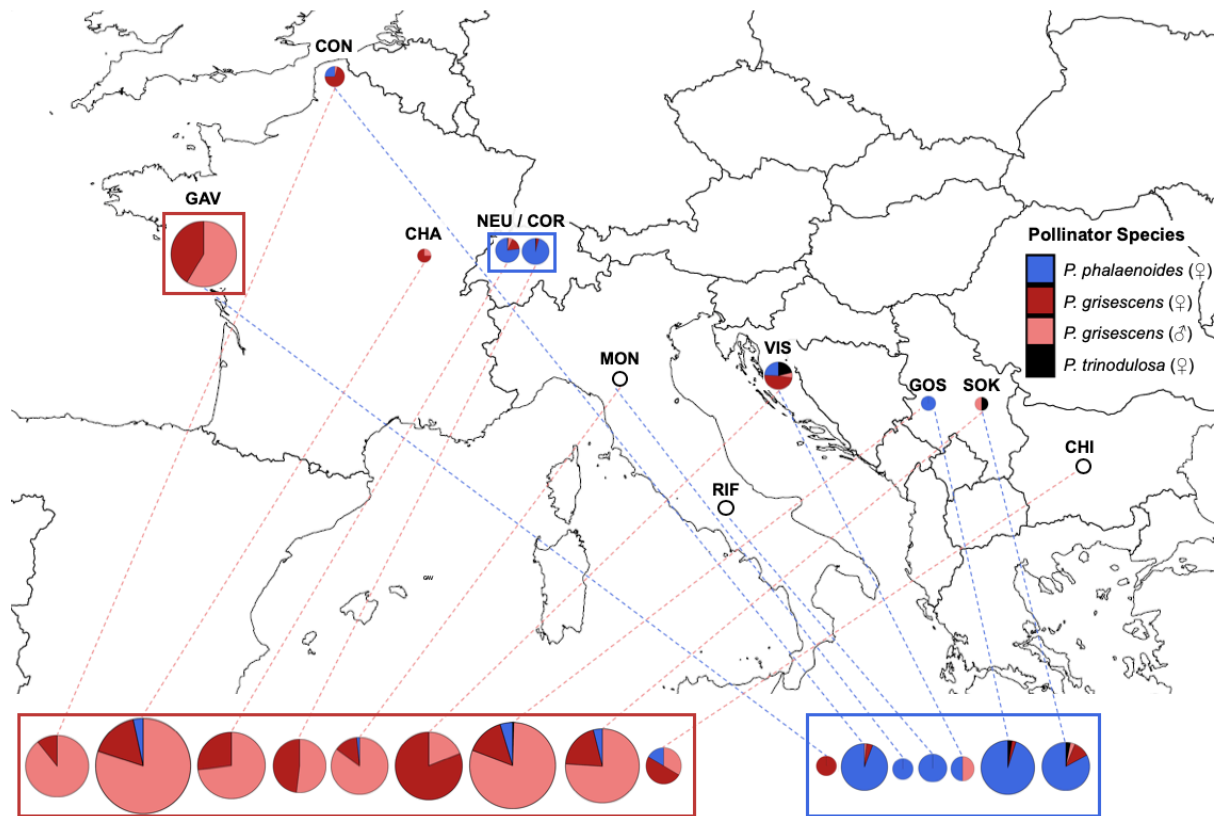
Figure 2. *Psychoda* spp. trapped by *Arum maculatum* inflorescences during field surveys, and following transplants to two common garden sites. Dotted lines link each field survey result (plots placed on the map) with the two corresponding transplant results (plots below map).

Note: Pie charts are scaled to represent the square root +1 (to visualize small differences) of the average number of Psychodidae per plant. Empty charts represent populations where inflorescences did not attract any Psychodidae during field surveys.

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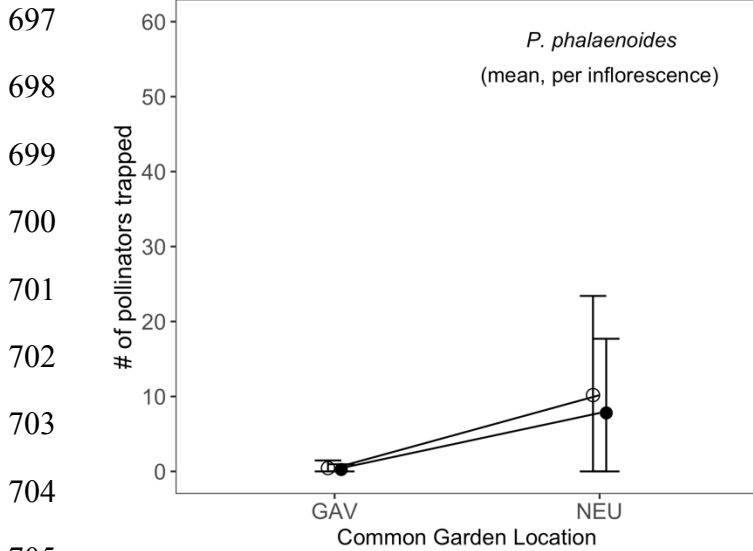
Figure 3. Mean (+/- SE) numbers of *Psychoda phalaenoides*, *Psycha grisescens*, and total insects trapped by inflorescences originating from *P. phalaenoides*-dominated sites (filled circles) and *P. grisescens*-dominated sites (hollow circles), in two common garden sites. No deme × habitat interactions (indicative of local adaptation) were identified.

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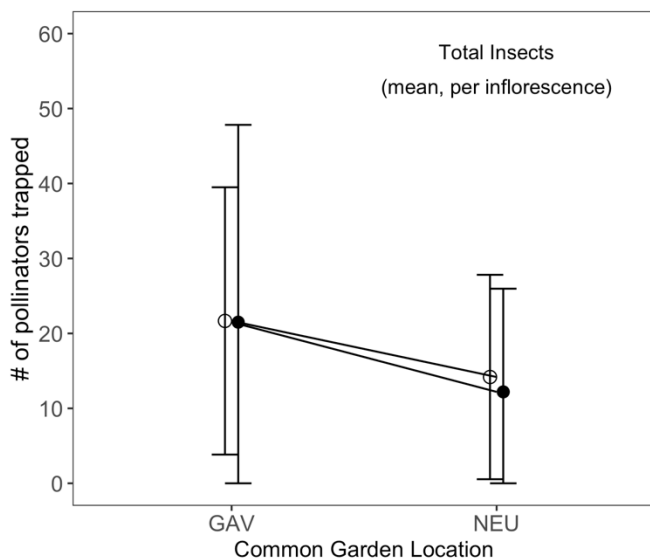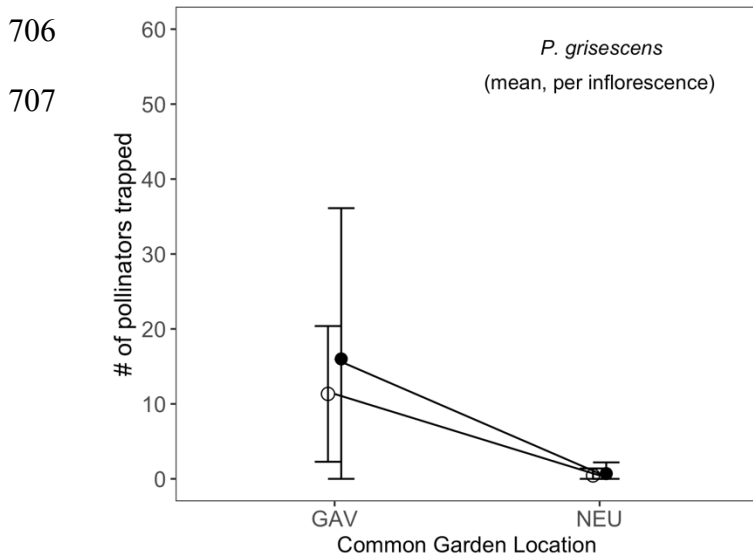
708 **APPENDIX S1 – SUPPLEMENTARY FIGURES & TABLES**
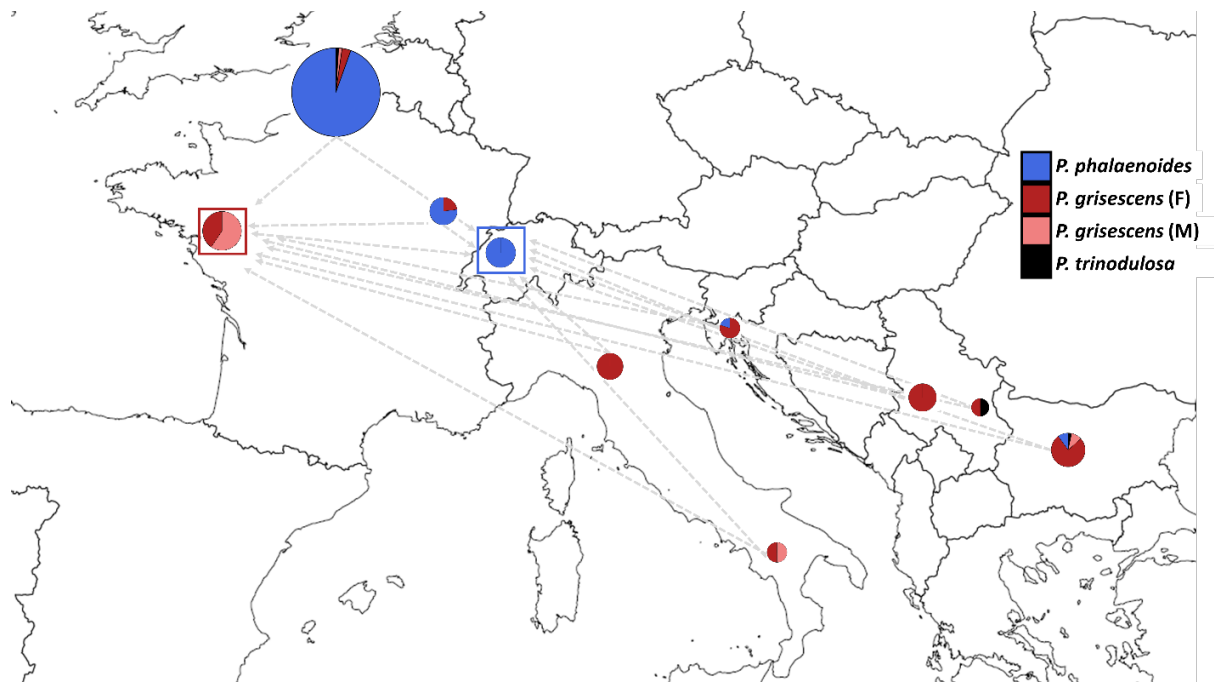
709 **Figure S1**. Outline of all *Arum maculatum* populations surveyed in this study, and the location of our

710 two common garden sites (red and blue squares). Pie charts visualize data on *Psychoda* species caught

711 by inflorescences between 2007-2009, taken from Espíndola et al. (2010). We attempted to re-survey

712 each of these sites in this study between 2017 and 2019 (result shown in Figure 2).

713 Note: Graph sizes represent the square root (plus one to visualize species differences in small pies) of

714 the mean number of psychodids per plant in each population. Gray dotted arrow indicate direction for

715 transplanting a subset of inflorescences to both common garden sites. Swiss pollinator data in this figure

716 represents Lausanne, replaced by Neuchâtel and Cortaillod populations in the present study.

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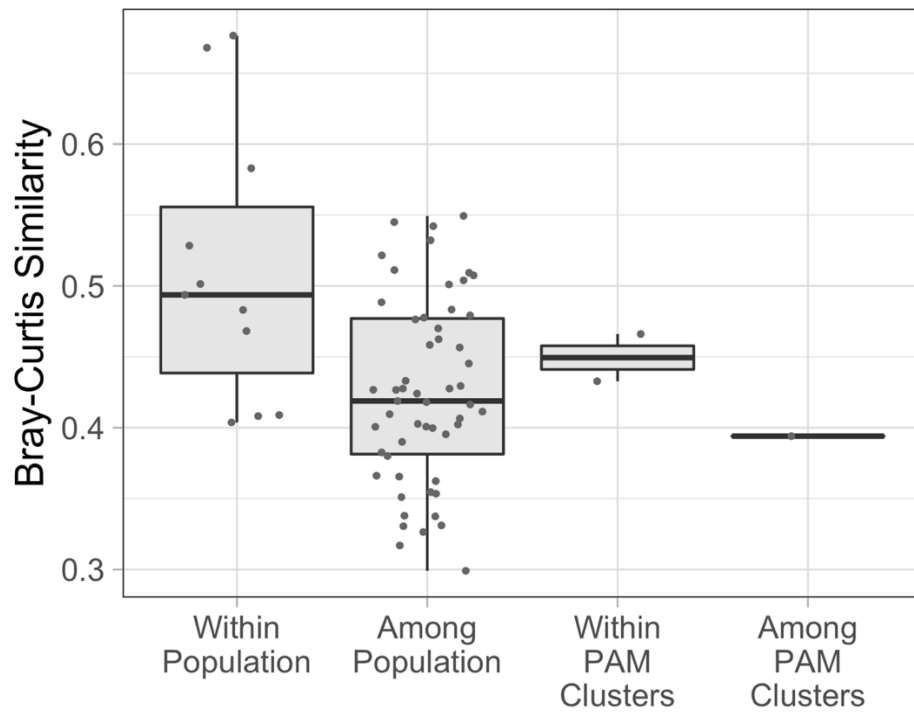
717

718 **Figure S2**. Pairwise Bray-Curtis similarity values from comparing *Arum maculatum* floral odors within

719 and among all sampled populations, and within and among the two PAM clusters identified in this

720 study.
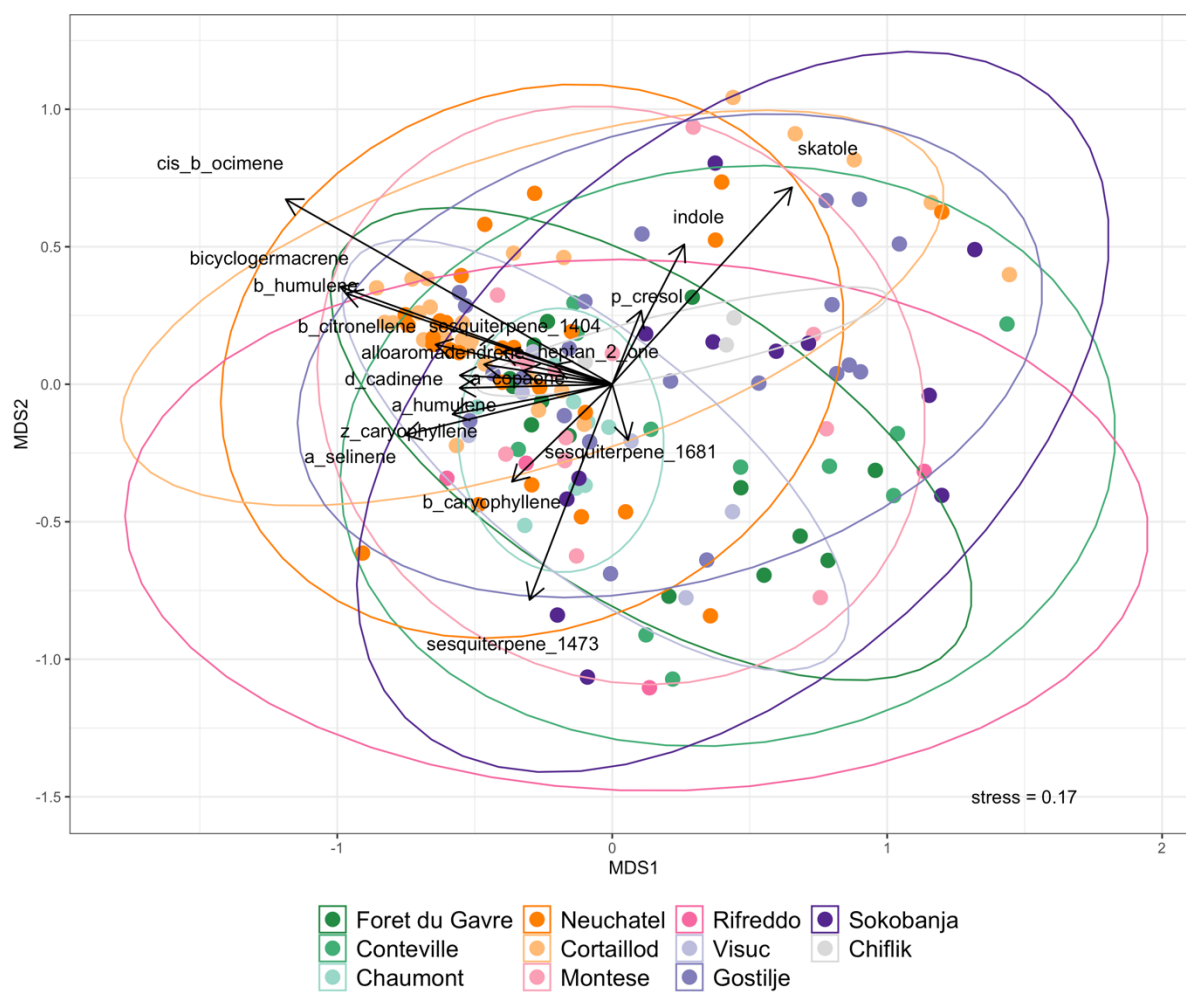
Figure S3. Multivariate representation of *Arum maculatum* volatile organic compound emissions, using nonmetric multidimensional scaling (NMDS) of Bray-Curtis distances between individuals, colored according to their population of origin.

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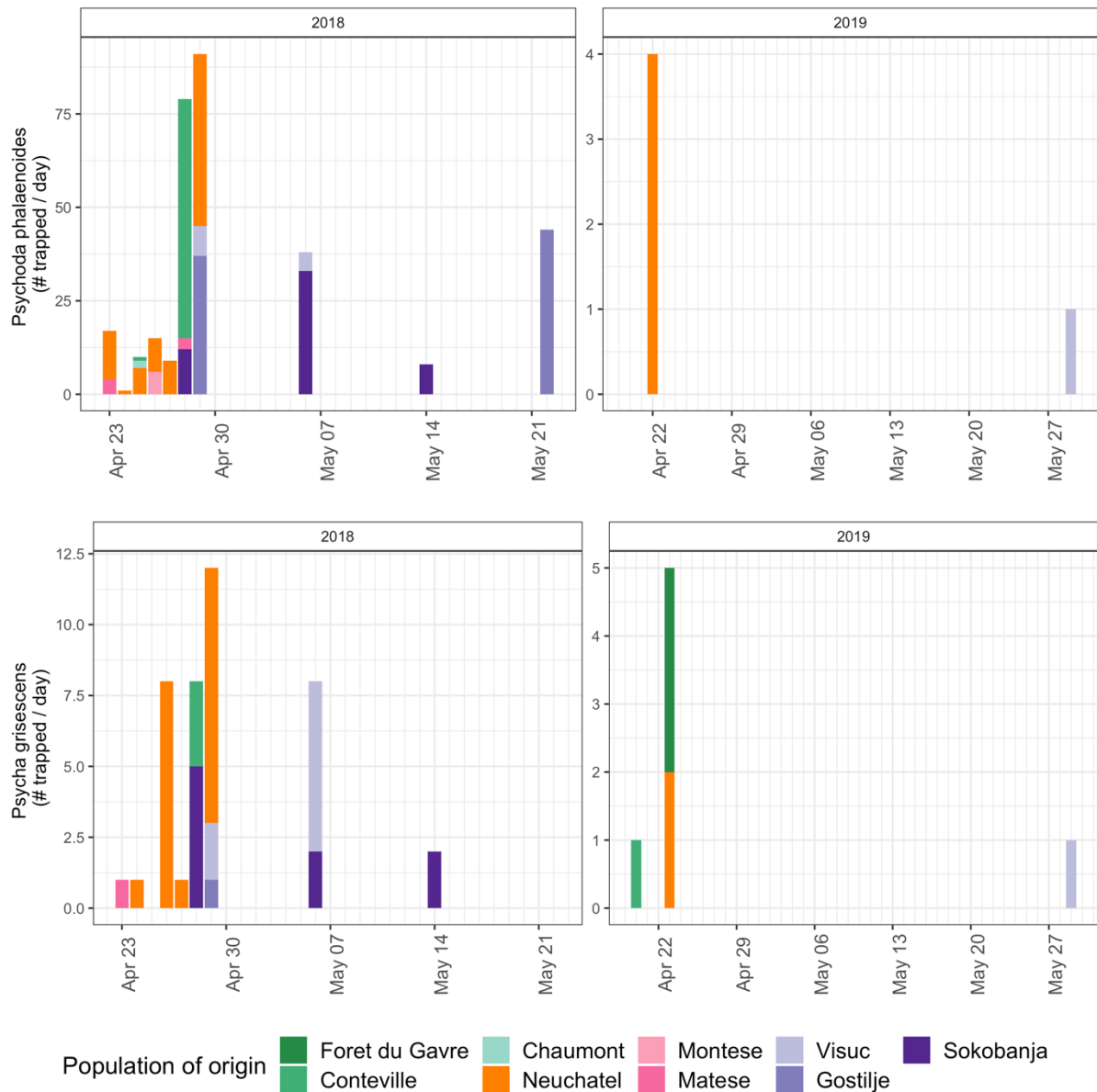
Figure S4a. Pollinators trapped by *Arum maculatum* inflorescences in the Neuchâtel common garden

(i.e. *Psychoda phalaenoides*-dominated) over two years of sampling. Both *P. phalaenoides* and *P.*

grisescens were present and trapped by inflorescences during most of the sampling season.

Note: Y-axis scale varies between plots. Bar colors represent the populations of origin for individual
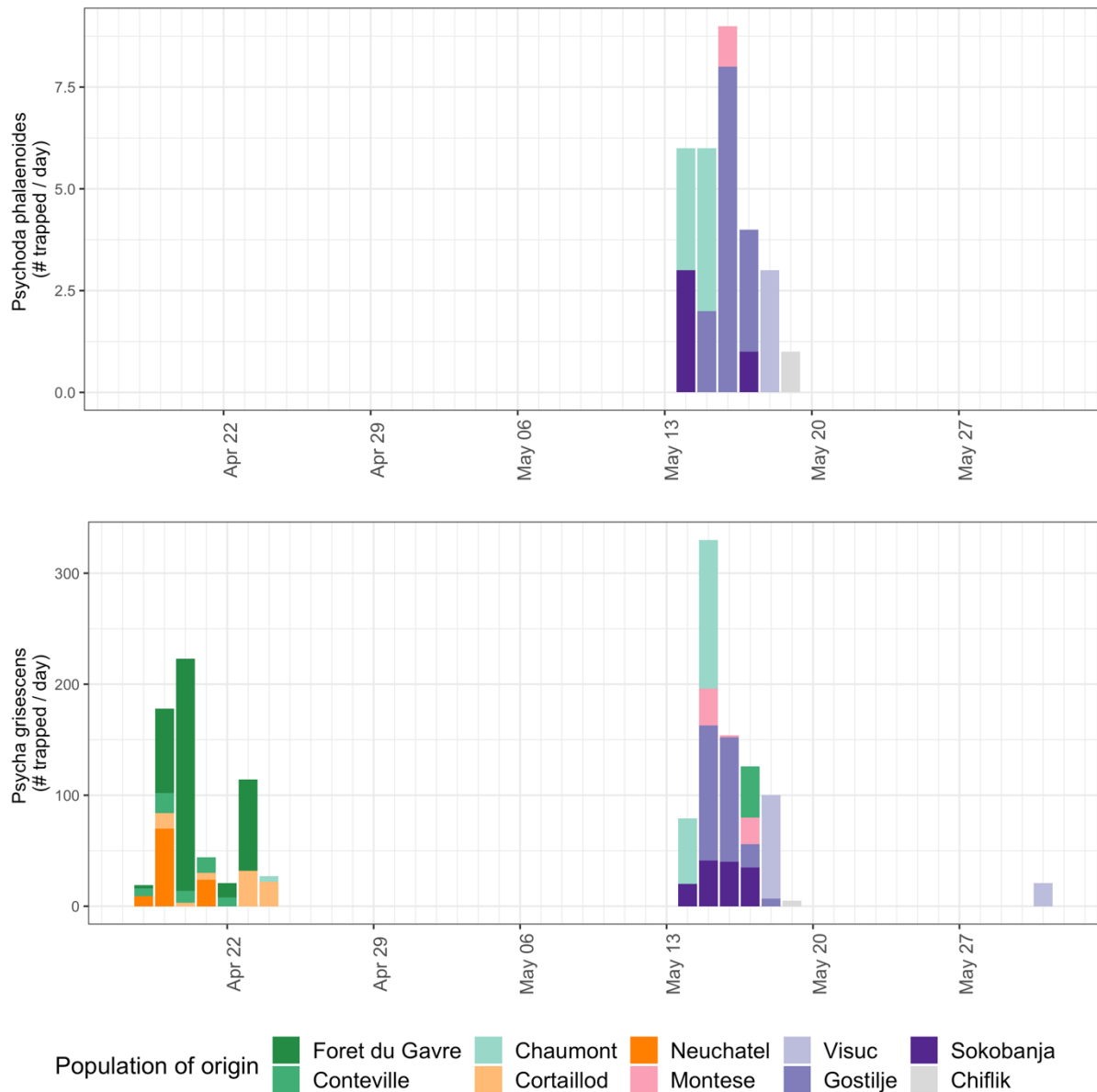
inflorescences.

Figure S4b. Pollinators trapped by *Arum maculatum* inflorescences in the Forêt du Gâvre common garden (i.e. *Psycha grisescens*-dominated) in 2019. *P. grisescens* were trapped by inflorescences over most of the sampling season, while *P. phalaenoides* were only trapped in the latter half of the season.

Note: Y-axis scale varies substantially between plots. Bar colors represent the populations of origin for individual inflorescences. No pollinator sampling was conducted between 25.Apr and 14.May.
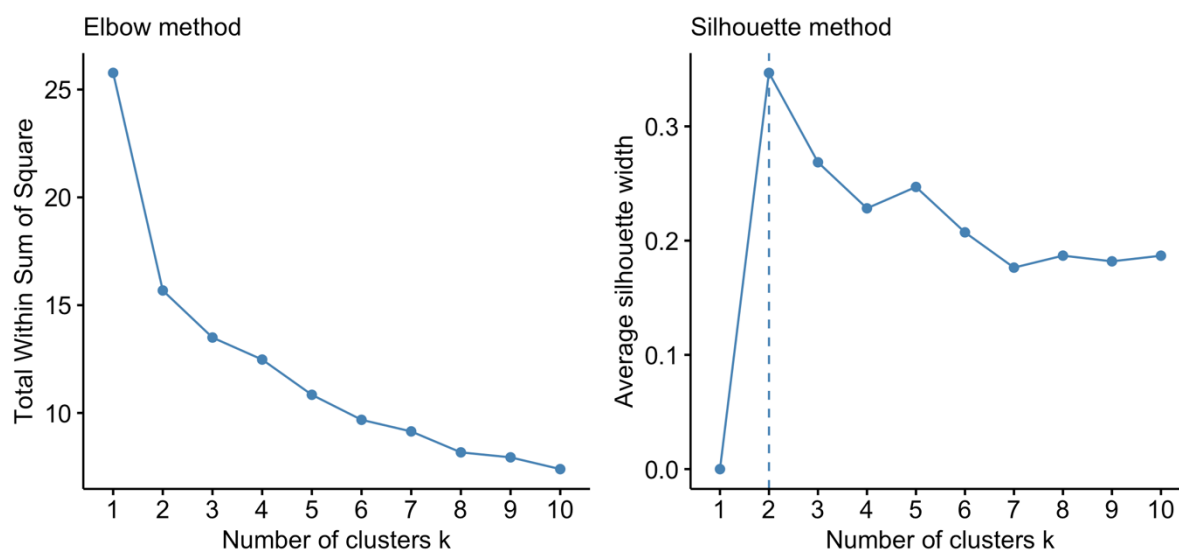
736   **Figure S5**. Cluster validation results using 'Elbow' and 'Silhouette' methods, for clustering of *Arum*

737   *maculatum* floral odor bouquets into 2-10 groups, using the PAM algorithm. Two clusters appears to

738   be the optimal number for our VOC dataset.
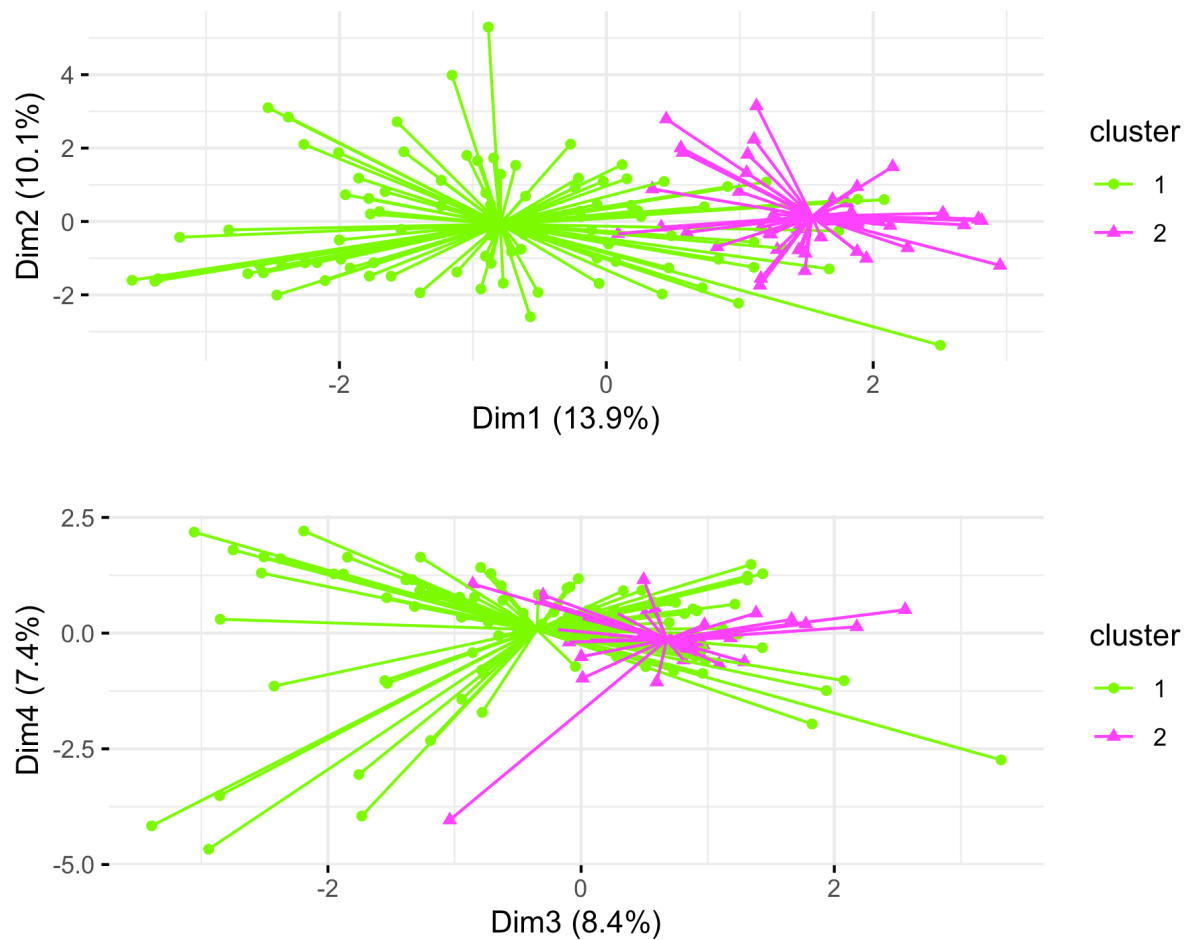
Figure S6. K-medoids (PAM) clustering plots highlighting the placement of *Arum maculatum* individuals into the two clusters we identified, along PCA axes 1 and 2 (upper plot) and 3 and 4 (lower plot).
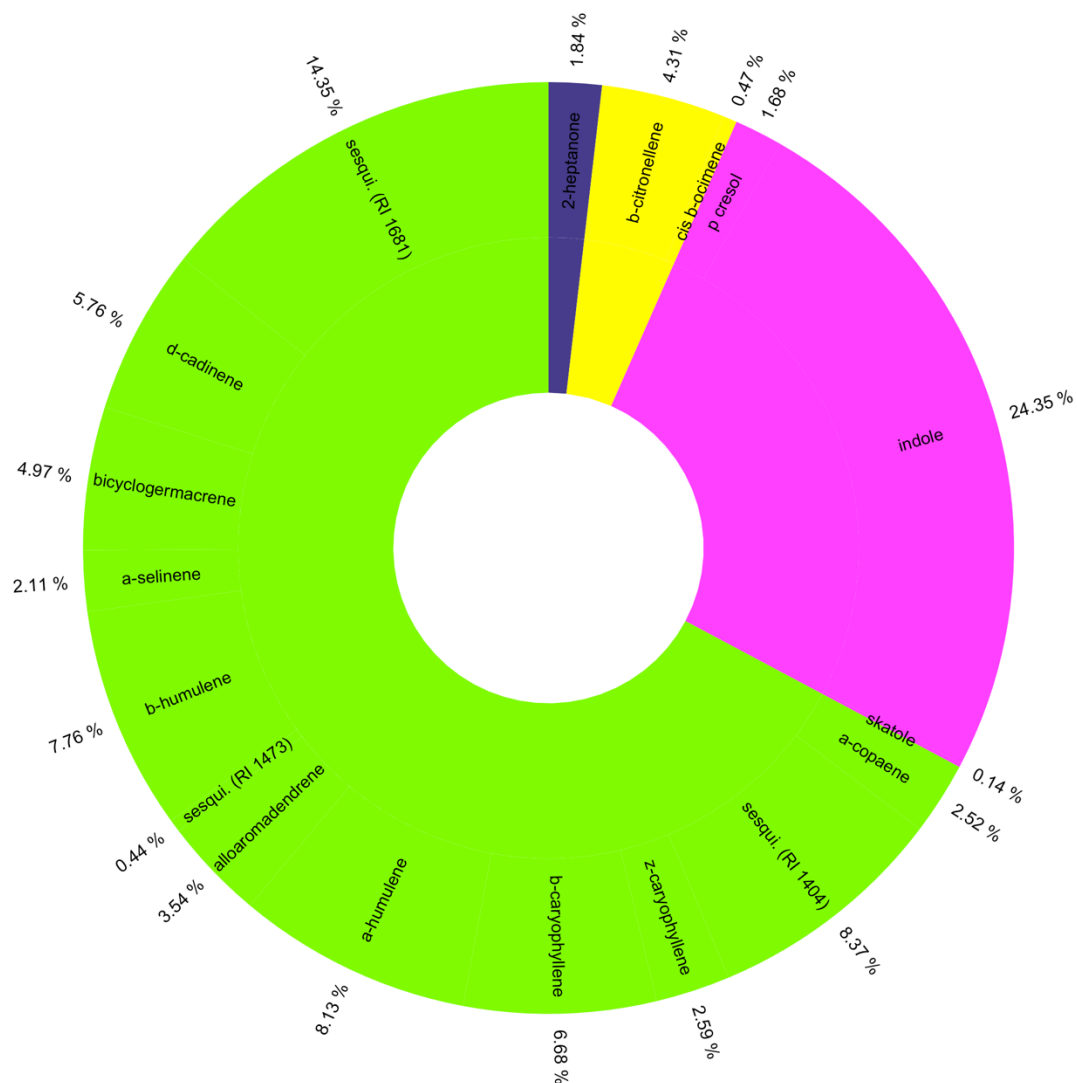
Figure S7a. The average composition of VOC blends of *Arum maculatum* inflorescences belonging to PAM Cluster 1. This cluster is mainly characterized by the proportionally large and diverse emissions of mono- and sesquiterpenes.
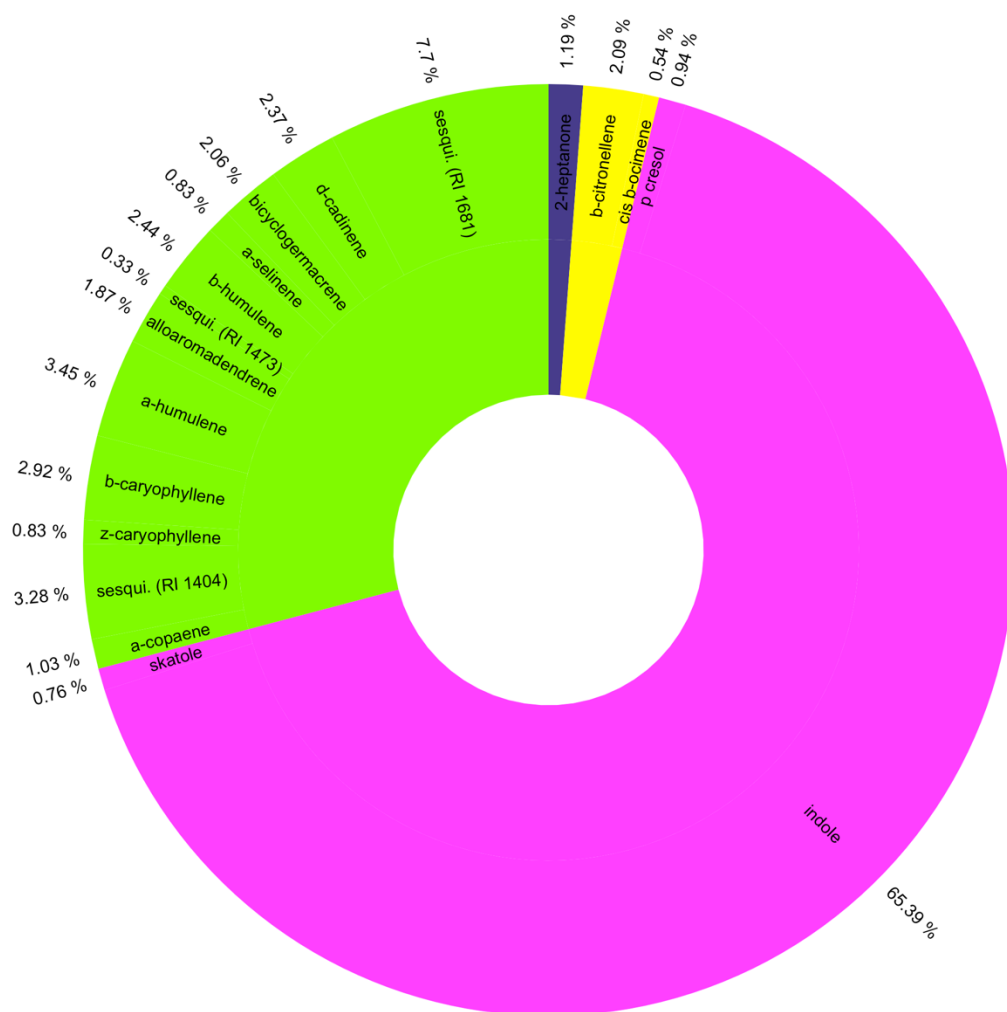
745   **Figure S7b**. The average composition of VOC blends of *Arum maculatum* inflorescences belonging to

746   PAM Cluster 2. This cluster can be characterized by significantly higher emissions of indole than

747   Cluster 1 ($\chi 2 = 34.92$, $p < 0.001$, $df = 1$).
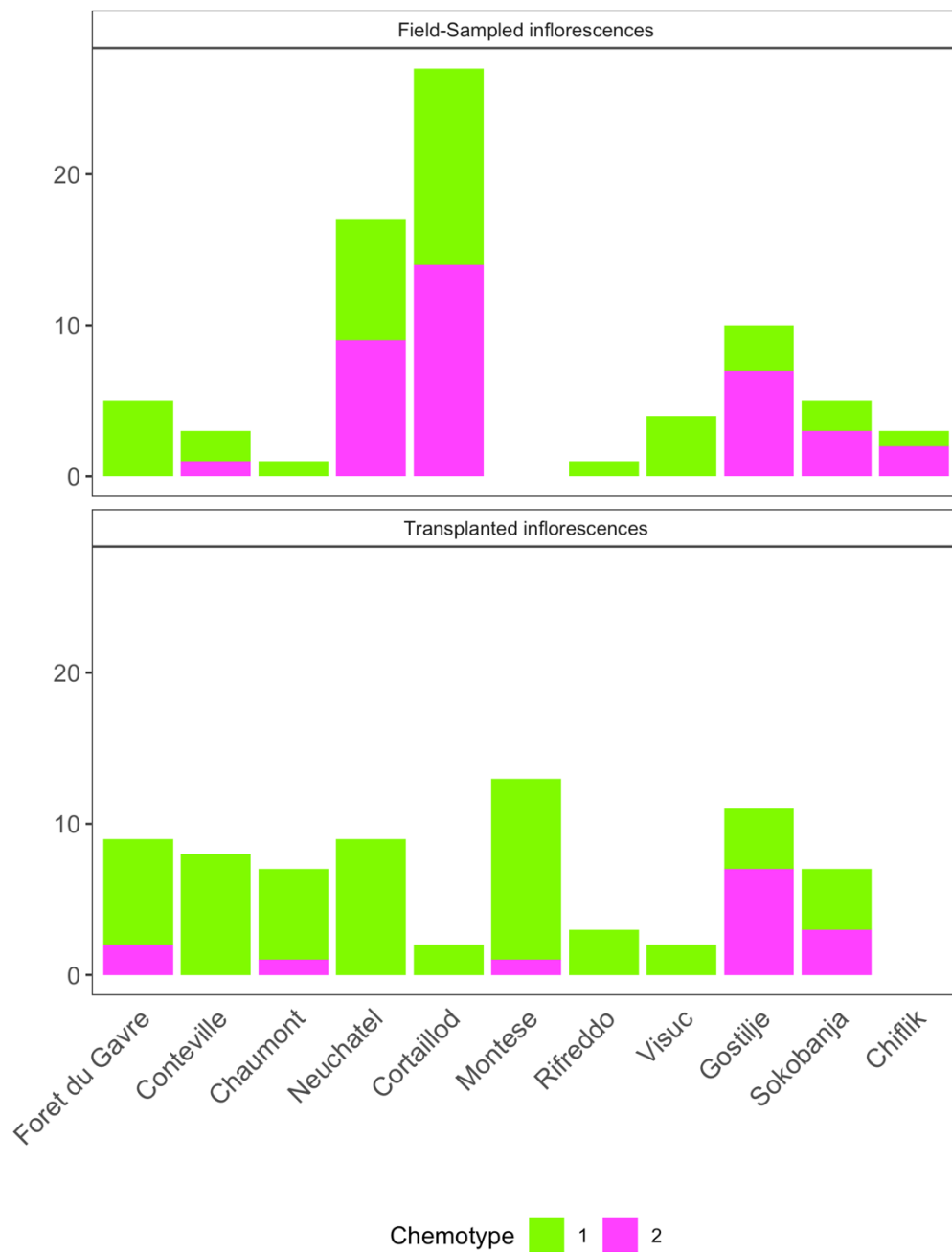
**Figure S8**. The number of *Arum maculatum* inflorescences assigned to terpenoid-dominated PAM Cluster 1, and indole-dominated PAM Cluster 2. *In situ* VOC samples are shown on the upper graph, while VOC samples from potted and transplanted individuals are shown in the lower graph. No VOCs were able to be sampled *in-situ* in Montese, and following transplants to common gardens for inflorescences from Chiflik.
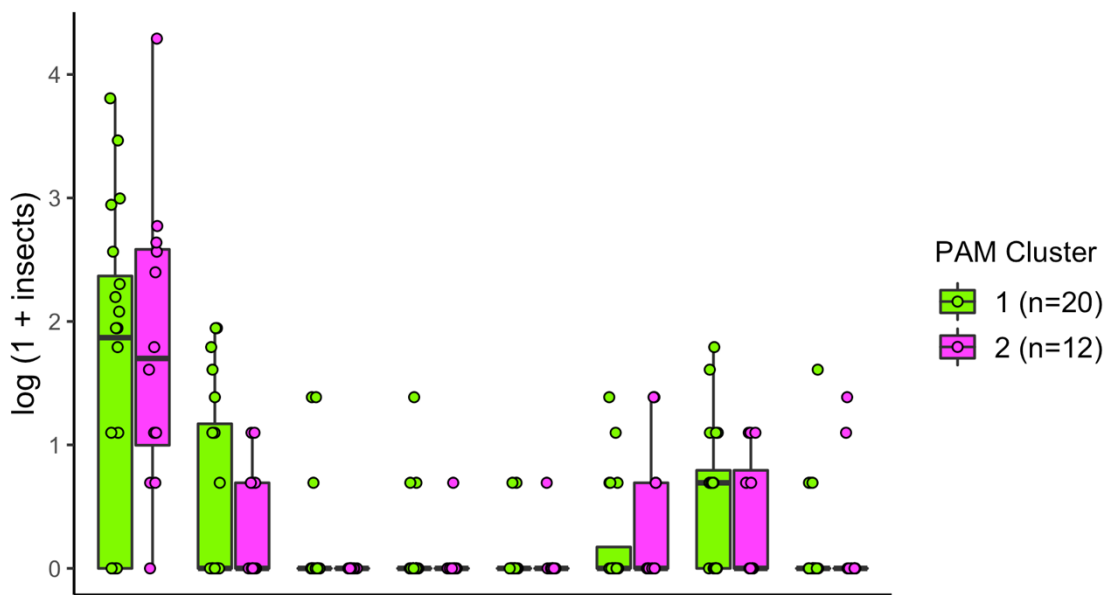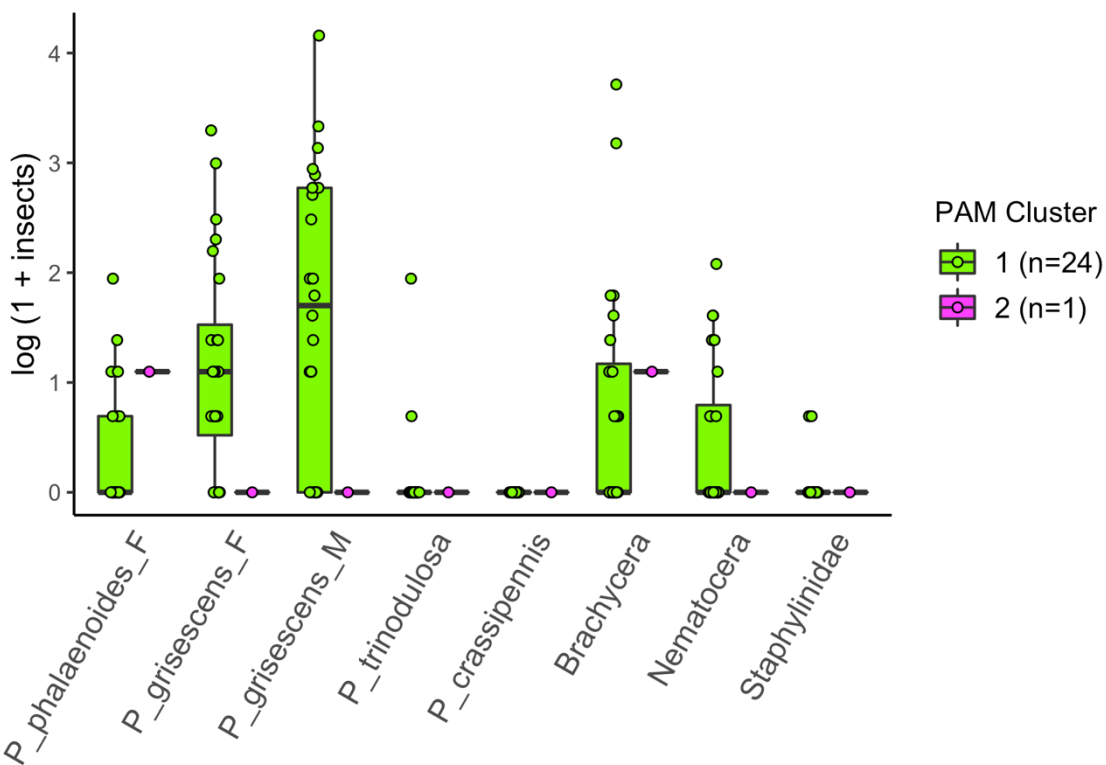
Figure S9. *log1p*-transformed abundances of pollinators trapped by *Arum maculatum* emitting high proportional quantities of terpenoids (PAM Cluster 1) and indole (PAM Cluster 2). To control for geographic variation in background pollinator communities among sampled sites, the visualization is split based on the dominant Psychodidae species where each sample was collected. Where possible, sex (M/F) is specified above; all *P. trinodulosa* and *P. crassipennis* we identified were female.
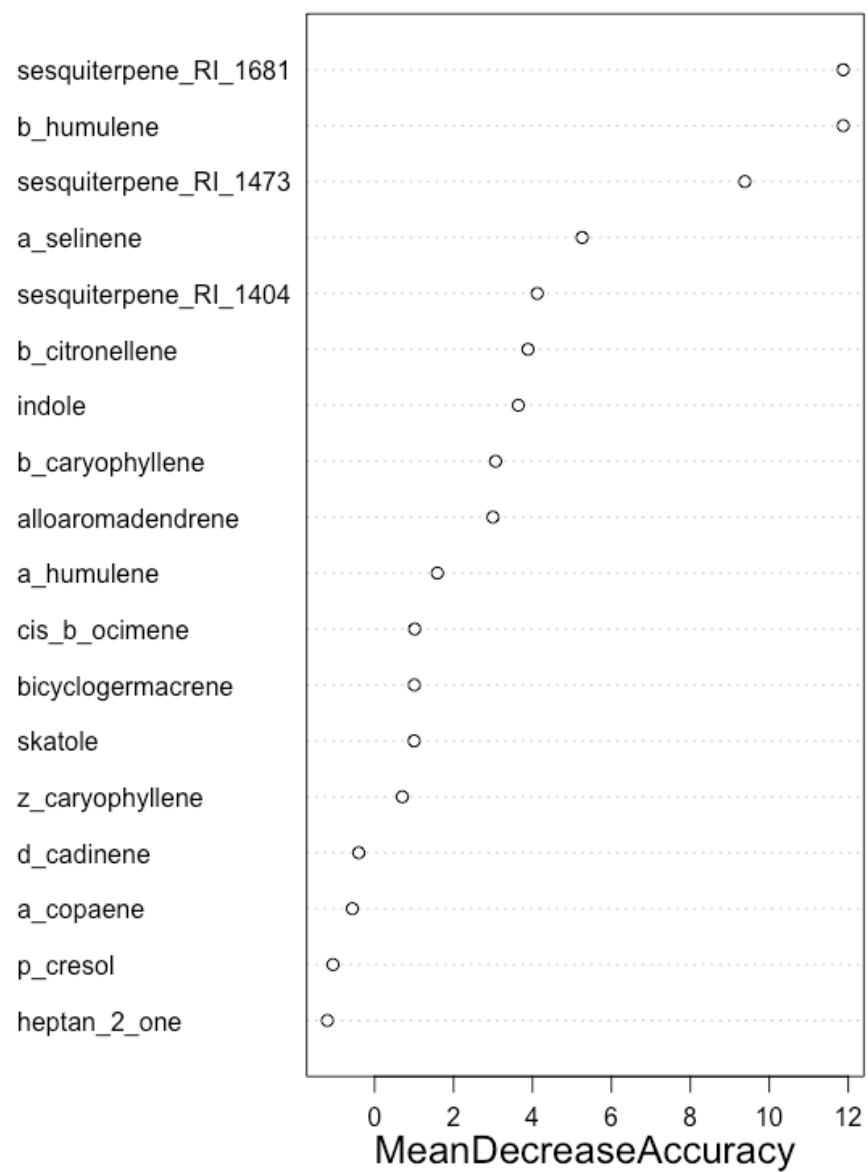
Figure S10. Variable importance plot highlighting the *Arum maculatum* VOCs with the greatest effect on the random forest model accuracy. Compounds such as β-humulene, unnamed sesquiterpenes (RI 1681 and 1473) and, α-selinene were the strongest predictors of whether an inflorescence would trap predominantly *P. phalaenoides* or *P. grisescens*.
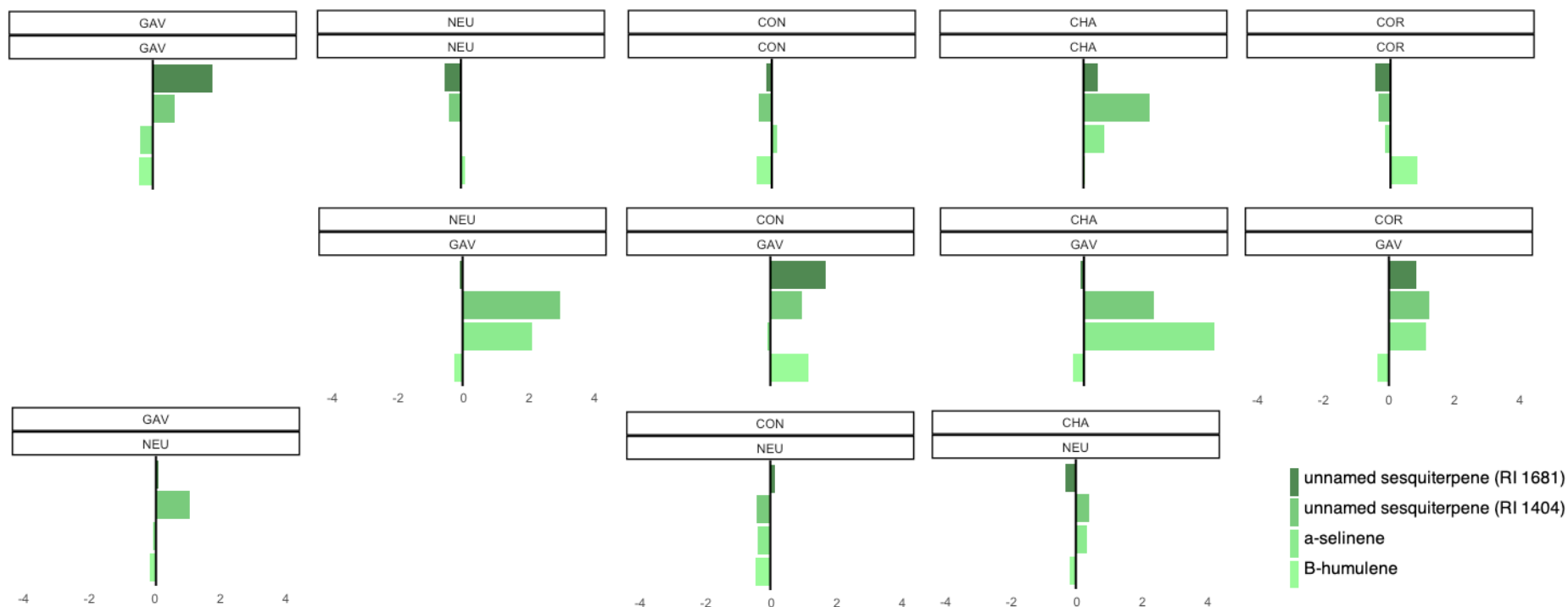
764 **Figure S11.** Shifts in the mean population standard scores (calculated as: [(raw individual VOC quantity – mean individual VOC quantity) / VOC std. dev.])

765 for the four *Arum maculatum* VOCs which were the strongest predictors of whether an inflorescence would trap predominantly *P. phalaenoides* or *P. grisescens*.

766 Two Swiss populations (COR and NEU) and one Serbian population (GOS) shifted to more sesquiterpene-dominated blends following transplants to the Forêt

767 du Gâvre common garden. By contrast, most populations remained relatively consistent in their emissions of these sesquiterpenes following transplants to the

768 Neuchâtel common garden.

769 Note: Upper box represents population origin, lower box represents site where VOCs were collected.   [FIGURE CONTINUED ON NEXT PAGE]

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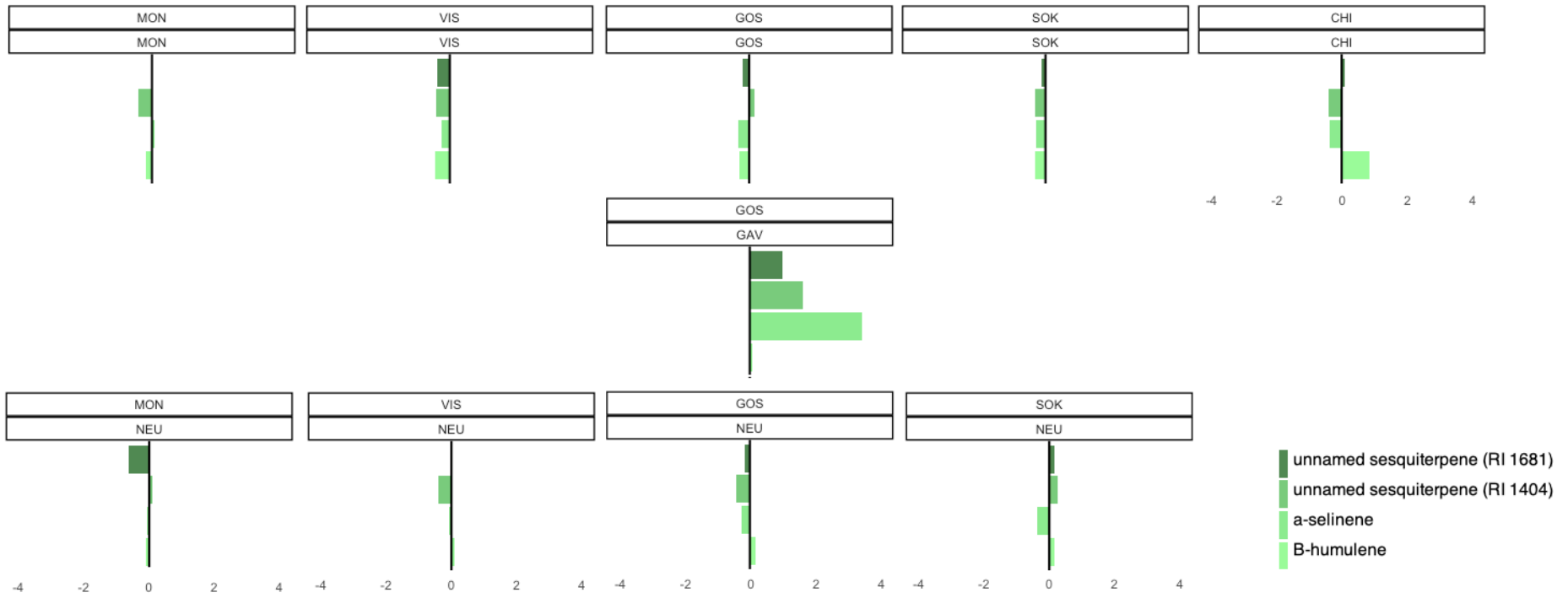
771 **Figure S11 (cont.)** – see caption on previous page

772 **Table S1**. List of study sites, GPS coordinates, number of Arum *maculatum* VOC samples passing all quality filters, and number of inflorescences with insect

773 data, during both field collections and common garden experiments.

Population	Country	Lat.	Long.	Alt. ASL (m)	# sampled VOCs (in situ)	# sampled VOCs (NEU, transplant)	# sampled VOCs (GAV, transplant)	# sampled insects (in situ)	# sampled insects (NEU, transplant)	# sampled insects (GAV, transplant)
Forêt du Gâvre	FR	47.55066	-1.86466	16	10	4	NA	5 (16)	3 (3)	NA
Conteville	FR	50.73731	1.73872	60	8	2	1	0 (5)	1 (4)	1 (6)
Chaumont	FR	48.11508	5.09475	296	1	6	2	1 (5)	2 (2)	1 (5)
Neuchatel	CH	47.00043	6.93790	556	18	NA	3	8 (30)	NA	3 (5)
Cortaillod	CH	46.93205	6.83290	430	27	NA	2	9 (14)	NA	2 (6)
Montese	IT	44.25523	10.98371	707	6	6	2	0 (0)	1 (1)	2 (4)
Rifreddo	IT	40.57235	15.82473	1172	4	0	0	0 (0)	0 (2)	0
Visuć	HRV	44.53128	15.76134	810	6	2	0	3 (4)	2 (4)	0 (2)
Gostilje	SRB	43.65561	19.83549	785	17	3	2	4 (10)	3 (4)	2 (8)
Sokobanja	SRB	43.60373	21.88755	844	10	2	0	1 (11)	2 (4)	0 (5)
Chiflik	BG	42.8130	24.52836	786	3	0	0	0 (0)	0 (0)	0 (1)

774 Note: For the insect data, we report the number of inflorescences with both VOC and insect data (i.e. data used in Figure S9); the adjacent bracketed numbers indicate number

775 of inflorescences with insect data only; this larger sampling was used for all other tests of local adaptation, as well as Figures 2 and 3.

776   **Table S2**. Mean *in-situ* quantities of *Psychoda phalaenoides* and *Psycha grisescens* caught per *Arum*

777   *maculatum* inflorescence between 2006 and 2008 (data from Espíndola et al. 2010), and in this study

778   between 2017 and 2019.

Population	*P. phalaenoides* (Espíndola 2010)	*P. grisescens* (Espíndola 2010)	*P. phalaenoides* (field surveys 2017-2019)	*P. grisescens* (field surveys 2017-2019)
Fôret du Gavre (FR)	0 (5)	6.4 (5)	0 (16)	25.7 (16)
Conteville (FR)	47.6 (8)	2.3 (8)	0.4 (14)	1.3 (14)
Chaumont (FR)	1.6 (6)	0.5 (6)	0 (7)	0.6 (7)
Lausanne/Neuchâtel (CH)	3.0 (2)	0 (2)	2.0 (44)	0.6 (44)
Montese (IT)	0 (5)	2 (5)	NA	NA
Rifreddo (IT)	0 (4)	2.5 (4)	NA	NA
Visuc (HRV)	0.1 (7)	0.6 (7)	1.3 (6)	3.0 (6)
Gostilje (SRB)	0 (5)	2.4 (5)	0.4 (18)	0 (18)
Sokobanja (SRB)	0 (5)	0.2 (5)	0 (21)	0.1 (21)
Chiflik (BUL)	0.4 (7)	4.3 (7)	NA	NA

779   Note: The number of individual inflorescences sampled *in situ* during both sampling periods are

780   indicated in parentheses. NAs indicate that no inflorescences were open yet at the time we visited

781   populations during our study.

50

782   **Table S3**. Two-way ANOVA results for pollinators trapped by transplanted *Arum maculatum* growing

783   in two common garden sites, dominated by either *Psychoda phalaenoides* or *Psycha grisescens*.

ALL POLLINATORS	df	SumSq	MeanSq	F value	Pr(>F)	
common garden loc.	1	2.34	2.3369	2.138	0.153	
native poll.	1	0.16	0.162	0.148	0.703	
common garden loc : native poll.	1	0.05	0.0538	0.049	0.826	
Residuals	34	37.16	1.0928			

P. PHALAENOIDES	df	SumSq	MeanSq	F value	Pr(>F)	
common garden loc.	1	18.38	18.379	16.2	0.000301	***
native poll.	1	0.15	0.152	0.134	0.716174	
common garden loc. : native poll.	1	0.01	0.008	0.007	0.931867	
Residuals	34	38.57	1.134			

P. GRISESCENS	df	SumSq	MeanSq	F value	Pr(>F)	
common garden loc.	1	36.28	36.28	55.84	1.14E-08	***
native poll.	1	0.19	0.19	0.287	0.596	
common garden loc. : native poll.	1	0.1	0.1	0.155	0.696	
Residuals	34	22.09	0.65			

784   <u>Factors</u>: **Native pollinator** (whether an inflorescence traps *P. phalaenoides* or *P. grisescens* in their

785   native population) and **Common Garden Location** (Neuchâtel, a site dominated *P. phalaenoides*, or

786   Forêt du Gâvre, a site dominated by *P. grisescens*). F-statistics are shown above (df = 1).

787   *** P<0.001, ** P<0.01, * P<0.05

788   **Table S4**. The top ten combinations of *Arum maculatum* VOCs with the greatest contribution to the

789   predictive strength of the random forest model (see Figure S10). Unnamed sesquiterpene (RI 1681)

790   alone was the strongest predictor of the species composition of pollinators trapped by individual *A.*

791   *maculatum* inflorescences.

Compound Blend	Feature Contribution
unnamed sesquiterpene (RI 1681)	0.1947778
unnamed sesquiterpene (RI 1473) + unnamed sesquiterpene (RI 1681)	0.0592275
α-selinene + unnamed sesquiterpene (RI 1681)	0.0502504
β-humulene	0.0436112
β-citronellene	0.0361404
α-selinene	0.0320748
β-humulene + unnamed sesquiterpene (RI 1681)	0.0180302
β-citronellene + indole	0.0138245
unnamed sesquiterpene (RI 1404) + unnamed sesquiterpene (RI 1681)	0.0119733
unnamed sesquiterpene (RI 1404)	0.0114971

792

793 **Table S5**. Mann Whitney U (Wilcoxon rank-sum) test results, comparing the emissions of four VOCs

794 associated with species-specific Psychodidae attraction. To test for shifts in VOCs related to

795 environmental variation, comparisons were made between samples collected in the Forêt du Gâvre

796 common garden and in the Neuchâtel common garden.

Pop.	α-selinene (*p*)	Z	β-humulene (*p*)	Z	Sesquiterpene RI 1473 (*p*)	Z	Sesquiterpene RI 1681 (*p*)	Z
GAV	0.0105	2.5584	0.0213	-2.3026	0.3706	-0.8954	0.1111	1.8557
NEU	0.0102	2.5700	0.7428	-0.3281	**0.0004 ***	**3.5475**	0.3128	1.0094
CHA	0.2857	1.4652	0.8591	-0.1776	0.5942	0.5327	0.8571	0.4637
MON	1.0000	0.0000	0.8591	0.1776	1.0000	0.0000	0.1798	1.3413

797 * Significant after Bonferroni correction for multiple testing

53

798   **APPENDIX S2 – SUPPLEMENTARY METHODS**

799   <u>Floral odor collection and identification</u>

800   *A. maculatum* inflorescences open for a duration of roughly 24h, with VOC emissions peaking

801   in the late afternoon / early evening of the first flowering day. We therefore carried out all

802   floral odor sampling between 18:00 at the earliest and 20:30 at the latest. We sampled dynamic

803   headspace volatile organic compounds (VOCs) using polydimethylsiloxane (PDMS) coated

804   Gerstel Twister® (Mülheim an der Ruhr, Germany) stir bar sorptive extraction. Inflorescences

805   were wrapped in inert oven bags (Tangan No34 distributed by Migros, Zurich, Switzerland)

806   cut open at least 8cm above the tip of the spathe to prevent condensation, due to the strong

807   thermogenesis of the appendix. Twisters® were inserted in a glass tube through the oven bag

808   at a height even with, but not contacting, the tip of the spadix. 6L of air was pumped over

809   Twisters® at a standard rate of 200mL per minute for 30 minutes – except for five samples

810   from Conteville, France, where sampling was carried out at the same rate over only 15 minutes.

811   At every sampling site, at least one empty oven bag was placed approximately 5 meters away

812   from any inflorescences, and ambient air was passed over Twisters® identically as with *A.*

813   *maculatum* inflorescences; these samples were used as controls to filter out ambient air VOCs.

814   All samples were transported in glass vials on ice, and stored at -21°C until analysis.

815

816   *Gas Chromatography*

817   We applied 1μL of internal standard (5μg mg/mL naphthalene in dichloromethane) directly to

818   each Twister® immediately before processing. Using a Multipurpose Sampler (Gerstel,

819   Mülheim an der Ruhr, Germany), VOCs were thermally desorbed and separated on a HP-5MS

820   column, 30 m x 0.25mm x 0.25μm at 40°C for 30 sec, increasing temperature by 5°C per min

821   to 160°C, which was held for 0.01 min before increasing 3°C per min to reach 200°C, which

822   was held for 4 min, finally increasing at 10°C per min. until reaching 250°C for 3 min.

823   *Volatile Data Processing*

824   We aligned peaks by retention time within each population. Major ions were recorded for each

825   integrated peak using Agilent Chemstation software. Putative compound identifications were

826   then derived from NIST 2.3 (library version 17) hits confirmed for the same peak in several

827   spectra; all names used in the final analysis should be considered hypotheses. Compounds

828   present in blank samples with a mean quantity anywhere near those within *A. maculatum*

829   samples were removed prior to further analyses. Quantitative values were obtained by dividing

830   compound peak areas by the internal standard, then multiplying by the internal standard

831   concentration, and finally scaling based on sampling time (for the few samples run for less than

832   30 minutes).

833

834   <u>Pollinator identification</u>

835   On the morning after floral odor collection, all trapped pollinators were collected from within

836   each inflorescence and preserved in 70% ethanol. All pollinators were identified to at least the

837   suborder level. Psychodidae were further identified to species level using taxonomic

838   information and illustrations (Ježek 1990). First, the number of antennal segments were

839   counted. 15 segments indicated specimens were likely *P. phalaenoides* or *P. crassipennis*. 16

840   segments indicated either *P. grisescens* or *P. trinodulosa* - wing venation patterns were then

841   examined for 16-segmented specimens, as *P. trinodulosa* has a characteristic disconnection in

842   one branched vein. To confirm the final species identity (particularly when intact antennae

843   were not available) and sex of all psychodids, the reproductive anatomy of specimens were

844   also examined: Psychodid abdomens were separated, flattened, cleared in a diluted solution of

845   potash, and mounted on a slide in glycerol beside their decapitated head, and wings laid out

846   flat.

847

848   <u>Clustering VOCs using Unsupervised Learning Algorithms</u>

849   The optimal clustering algorithm (k-means, PAM, or hierarchical clustering) and number of

850   clusters (k = 1 through k = 10) was selected using the clustering validation function

851   implemented in the R package *clValid* (Brock et al. 2008). Following this step, k–medoids

852   (PAM) clustering (Kaufman and Rousseeuw 1990) was used to cluster samples into the optimal

853   number of groups (validated with 'Silhouette' and 'Elbow' plots); a cluster plot was then

854   created using the R package *factoextra* (Kassambara and Mundt 2019).

855

856   We identified significant differences in VOC blend composition among the identified clusters

857   using a Kruskal-Wallis test, and then plotted the mean VOC blend of each cluster. Next, we

858   produced population-level Bray-Curtis similarity matrices, to investigate whether 1) within-

859   cluster similarity was greater than within-population similarity and 2) between-cluster

860   similarity was less than between-population similarity; the variation within each group in the

861   resulting matrices was then visualized using boxplots.

862

863   **REFERENCES**

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