Effect of plant chemical variation and mutualistic ants on the local population genetic structure of an aphid herbivore

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Running title: Plant chemodiversity and aphid genetics

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Abstract

1. Plants exhibit impressive genetic and chemical diversity, not just between species but also within species, and the importance of plant intraspecific variation for structuring ecological communities is well known. When there is variation at the local population level, this can create a spatially-heterogeneous habitat for specialized herbivores potentially leading to non-random distribution of individuals across host-plants.
2. Plant variation can affect herbivores directly and indirectly via a third species, resulting in variable herbivore growth rates across different host plants. Herbivores also exhibit within-species variation, with some genotypes better adapted to some plant variants than others.
3. We genotyped aphids collected across two years from a field site containing ~200 patchily-distributed host plants that exhibit high chemical diversity. The distribution of aphid genotypes, their ant mutualists, and other predators was assessed across the plants.
4. We present evidence that the local distribution of aphid (*Metopeurum fuscoviride*) genotypes across host-plant individuals is associated with variation in the plant volatiles (chemotypes) and non-volatile metabolites (metabotypes) of their host plant tansy (*Tanacetum vulgare*). Furthermore, these interactions in the field were influenced by plant-host preferences of aphid-mutualist ants.
5. Our results emphasize that plant intraspecific variation can structure ecological communities not only at the species level but also at the genetic level within species, and that this effect can be enhanced through indirect interactions with a third species.

***Keywords**:* ant, aphid, chemical ecology, population genetics, species interactions, within-species variation, metabolomics, indirect effects

Introduction

Individuals within a species can differ from one another, and this leads to variation in the outcome of interactions with other species in a community context (Tétard-Jones *et al.* 2007; Zytynska *et al.* 2010; Rowntree, Shuker & Preziosi 2011). The ecological importance of intraspecific variation for community structure has been well studied in the area of community genetics, in particular for the effects of plant genetic variation on communities as diverse as invertebrates, vertebrates, plants, and microbes (reviewed in Rowntree, Shuker & Preziosi 2011; Whitham *et al.* 2012; Crutsinger 2016). Often, this is studied by comparing sets of individuals that are defined as genetically different via the use of molecular markers, or by comparing plants that vary in a genetically-based trait of interest, e.g. plant architecture or nutrient value (reviewed in Whitham *et al.* 2012). Plants are also highly chemically diverse, even among individuals within a species in a single population (Fiehn 2001); certain compounds are well known to have strong effects on multitrophic plant-insect interactions, e.g. glucosinolates in Brassicaceae (Hopkins, van Dam & van Loon 2009). Much work is focused on the role of compounds induced in plants due to herbivore feeding (Dicke & Hilker 2003), yet constitutive (non-induced, always present) compounds that can be more stable across variable environments have been shown to have strong effects on the structure of associated communities (Iason, Dicke & Hartley 2012; Beyaert & Hilker 2014; Kessler 2015). Current evidence suggests that insects use ratio-odour chemical recognition rather than species-specific volatile organic compounds (VOCs) for host-plant recognition (Bruce, Wadhams & Woodcock 2005; Beyaert & Hilker 2014). Thus, plant variation should not be considered just as the abundance of a single chemical (or genetically-based trait) but rather the whole complex mixture of compounds (or associated traits).

Plant within-species variation (genetic or chemical) can have direct and indirect influences on species in multitrophic systems. For example, for aphid herbivores that feed on plant phloem sap, plant variation can directly influence their population growth rate (performance) or host-plant preferences, and indirectly affect aphid survival via altering interactions with

78 their mutualistic ants or antagonistic natural enemies (reviewed in Zytynska & Weisser
79 2016). Reduced visitation of aphids by ants on plants with high levels of a toxic defensive
80 chemical led to reduced aphid numbers, and in some cases changed the relationship
81 between aphids and ants from mutualistic to antagonistic (Züst & Agrawal 2017). The
82 emission of plant VOCs can attract natural enemies to plants – which can occur through
83 emission of constitutive compounds in the plant (Senft *et al.* 2019) or via compounds
84 synthesised and immediately released in response to herbivore feeding (Paré & Tumlinson
85 1999). All these different interactions can influence the dynamics of herbivore populations
86 colonising individual host plants. It is the overall sum of these direct and indirect interactions,
87 experienced by all members of an interacting community, that leads to the structuring of
88 ecological communities that we see in nature.

89 Aphid-based systems are ideal to study the role of plant variation in plant-associated
90 communities. Aphids feed on the phloem sap of a restricted number of plant hosts, are
91 highly responsive to changes in host-plant quality, and interact with multiple other species in
92 the environment. In addition, they reproduce asexually during the summer months (fast
93 clonal colony growth), and often only produce winged dispersal morphs for a few weeks per
94 year after which dispersal is limited to walking between host-plants (a high risk activity).
95 When an aphid is choosing a new host, its decision is based on a combination of cues,
96 including cues from various chemicals emitted by plants (Powell, Tosh & Hardie 2006;
97 Döring 2014). The effect of plant chemical variation on aphid populations in the field has
98 been studied in a few systems, predominantly assessing the impact of dominant chemical
99 compounds on aphid numbers. More aphids were found on goldenrod plants (*Solidago*
100 *altissima L.*) containing higher levels of β-pinene (Williams & Avakian 2015), thyme plants
101 (*Thymus vulgaris L.*) with higher linalool levels (Linhart *et al.* 2005), and tansy plants
102 (*Tanacetum vulgare L.*) with lower camphor levels (Kleine & Müller 2011).

103 Controlled experiments using different aphid genotypes and plant variants (genotypes,
104 varieties, chemotypes) have consistently shown that plant-aphid (genotype-by-genotype, or

genotype-by-chemotype) interactions among these influence aphid performance and host-preference (Service 1984; Caillaud *et al.* 1995; Zytynska & Preziosi 2011; Kanvil, Powell & Turnbull 2014; Zytynska *et al.* 2014). Such interactions suggest that the distribution of aphids across host plants could differ due to variation in the plant (plant chemotype) and variation in the aphid (aphid genotype). Genetic variation is the raw material for evolution of a species and therefore interactions that alter the distribution of genotypes, or lead to reduced mixing of genotypes within a population, can influence the evolutionary trajectory of a species (Stireman, Nason & Heard 2005). In extreme cases, such associations can lead to coevolution between plant variants and their herbivores, and potentially drive speciation events.

We investigated the effect of tansy plant chemical variation in a natural field site on the distribution of aphid genotypes across different host plants and asked how these associations could be mediated by the larger interacting community. Tansy plants (*Tanacetum vulgare* L.) are characterised by high chemical variability in terpenoids, which has a genetic basis (Keskitalo, Linden & Valkonen 1998). These plants exhibit high variation in their volatile and non-volatile chemical compounds even within a single population (Clancy *et al.* 2016; Clancy *et al.* 2018), and this can influence the associated invertebrate community structure (Kleine & Müller 2011; Balint *et al.* 2016). Recently, we have shown that plant-to-plant variation in the profile of VOCs (terpenes), identified as being putatively-emitted from specialized storage structures on the leaves, affected the field colonization of tansy plants by specialized aphids (*Metopeurum fuscoviride* Stroyan (Aphididae)) in the early part of the season (Clancy *et al.* 2016). In addition to variation in the VOCs, we showed–through untargeted metabolomic profiling of the leaves–that all plants of certain metabotypes (clusters of plants with similar metabolomic profiles) were colonised by aphids at the peak of the season (even on ‘less preferred’ volatile chemotypes) (Clancy *et al.* 2018). Importantly, these effects were not a result of chemicals induced by aphid feeding, but rather resulted from differences in plant constitutive chemicals. Interestingly, there was no association

between plant volatile chemotype and metabotype, leading to a unique system where we can disentangle effects of these two aspects of chemical diversity (Clancy *et al.* 2018). The two common mutualistic ant species in this system also responded to plant chemical variation (Clancy *et al.* 2016), and the presence of ants increased colonisation success and benefited the population growth of *M. fuscoviride* aphids (Flatt & Weisser 2000; Senft, Weisser & Zytynska 2017). The role of plant volatile chemotypes on aphid population growth and survival, mediated via interactions with ants and predators, was recently confirmed in a controlled manipulation experiment (Senft *et al.* 2019). This work indicates that plant chemical variation can have strong direct and indirect effects on the aphid specialists in this system.

Here we explore how plant chemical variation, both in volatile and non-volatile metabolites, can influence the distribution of aphid genotypes across host plants, at a very small scale, i.e. across neighbouring plants within a population. Based on the strong effects of both volatile and non-volatile chemical compounds in the plants on aphid-ant interactions in this system (Clancy *et al.* 2016; Clancy *et al.* 2018; Senft *et al.* 2019), we asked whether plant chemical variation could also lead to fine-scale structuring of the aphid population at the genetic level. We further wanted to determine if any aphid genotype by plant chemotype associations were influenced by the varying abundances of ants we observed across different plant individuals (Senft, Weisser & Zytynska 2017).

Material and Methods

Study system and field site. Tansy (*Tanacetum vulgare* L.) is a chemically diverse, perennial herbaceous plant that is native to Eurasia, and is regionally rare but locally common (over 100 plants within a single site), growing on well-drained and less-managed sites. Tansy plants grow in patches of genetically identical shoots (in our field there were 18±8.7 shoots per plant (mean±SE)). The specialized aphid *Metopeurum fuscoviride* is

158 obligatorily ant-tended (Flatt & Weisser 2000), often by the black garden ant, *Lasius niger* L.,
159 or the common red ant *Myrmica rubra* L., and has a myriad of natural enemies including
160 parasitoid wasps and generalist predators (Senft, Weisser & Zytynska 2017). The field site
161 we used is located near Freising, Germany (Altenhausen: N 48°25'1.51"; E 11°46'1.19"), and
162 contains around 200 individually identifiable tansy plants (Fig. S1) of which 172 were visited
163 each week in 2014 and four times in 2015; importantly, only 87 of them were colonized by
164 aphids across both seasons leading to a heterogeneous distribution of aphids (Senft,
165 Weisser & Zytynska 2017).

166 **Field survey data and aphid sample collection.** We conducted an intensive weekly survey
167 in this field site throughout the 2014 growing season (May to October) (Senft, Weisser &
168 Zytynska 2017). For the current analysis, we used data from this survey on ant presence (*L.*
169 *niger* and *M. rubra*) in the weeks before aphid arrival (for ant preference), and specialist
170 natural enemy abundance (parasitoid mummies). One aphid per colony (a close group of
171 aphids, likely produced from the same mother aphid and therefore the same clone, as
172 aphids reproduce asexually during the summer months) was collected from every plant that
173 hosted aphids (up to five colonies per plant) once in 2014 (15th July). Due to the nature of
174 the plant, as it regrows in the same location each year, plants could be followed across
175 years. In 2015, we revisited the plants and collected aphids four times across the season in
176 2015 (11th June, 9th July, 23rd July, and 6th August); plant size and aphid number data were
177 collected once in early July. All aphids were stored in 100% ethanol at -20 °C until DNA
178 extraction. Aphid DNA was extracted using the salting-out procedure (Sunnucks & Hales
179 1996).

180 **Plant chemical and clustering analysis.** We used the plant volatile chemical information
181 on 22 compounds, emitted from specialised storage structures on the plant (identified using
182 GC-MS, from (Clancy *et al.* 2016)), and secondary metabolite information of 1020 mass
183 features as identified using LC-MS by (Clancy *et al.* 2018) (for more details see Appendix 1).
184 Our focus was only on those plants that were colonized by aphids, so we performed new

cluster analyses on these 87 plants to obtain chemotype and metabotype plant groupings, using the package 'pvclust' (Suzuki & Shimodaira 2015) in R v3.3.0 in RStudio v0.99.896. ANOSIM (Analysis of Similarity, using the Community Analysis Package, Pisces Conservation) was used to show that the groupings were significantly different from one another.

To test the relative influence of the 22 individual volatile compounds on the plant chemotype clustering, we used Bayesian Model Averaging (BMA) as implemented in the R package 'BMA' (Raftery *et al.* 2015). This analysis was not possible for the 1020 mass features from the untargeted metabolome analysis (Clancy *et al.* 2018) due to model saturation from limited degrees of freedom (87 plant individuals). Essentially, BMA runs multiple linear models with each compound as an explanatory variable and calculates a posterior effect probability (PEP), which is equivalent to the proportion of models in which each variable was retained (see Appendix 2 for details). We tested the effect of compound concentration and variation (standard deviation) across the plant samples on the resulting PEP values to determine if our clustering analysis was biased towards either the more abundant or more variable compounds.

Mantel tests were used to determine the extent of geographic clustering of plant volatile and metabolomic profiles in the field, which if detected would infer confounding effects of spatial autocorrelation.

Aphid genome sequencing and microsatellite development. In order to develop new microsatellite primers for *M. fuscoviride*, we genome-sequenced one field-collected aphid (for full details see Appendix 3). Briefly, the library was prepared using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (New England BioLabs GmbH, Frankfurt am Main, Germany), with NEBNext Multiplex Oligos for Illumina adapters. Next generation sequencing using the Illumina HiSeq™ 2500 was conducted on a paired-end flow cell with a read length of 100bp according to the manufacturer's instructions (Illumina Inc., San Diego, USA). Microsatellites were identified and 18 primer pairs were chosen to develop a PCR-

multiplex leading to two multiplex combinations with nine primer pairs in each, using three fluorescent dyes: 6-FAM, HEX, and TAMRA, alongside the ROX size standard run on an ABI 3130xl Genetic Analyzer (Applied Biosystems - Life Technologies GmbH, Darmstadt, Germany). The final PCR multiplex conditions were: 1 µl DNA diluted 1:4, 5 x MyTaq™ Reaction Buffer (Bioline, UK), 2 Units MyTaq™, specific primer mix, up to 20 µl with molecular grade water, run at 95 °C for 2 mins, 30 cycles of 95 °C for 15 sec, 60 °C for 15 sec, 72 °C for 15 secs, and then a final step at 72 °C for 2 min. Fragment data was analyzed using the software GeneMarker (version 1.75) (Softgenetics LLC, State College, PA, USA).

Aphid genetic data analysis. Basic descriptive molecular statistics, such as the number of multi-locus genotypes (MLGs), were obtained using the package 'poppr' in R (Kamvar, Brooks & Grünwald 2015). To cluster the aphids into genotype clusters, we used K-means hierarchical clustering in the package 'poppr'. Since we were looking for fine-scale genetic structuring, the BIC (Bayesian Information Criterion) was calculated for different numbers of groups (K). When the difference between K=n and K=n+1 was close to zero (i.e. no further information obtained by splitting into more groups), this group number was chosen. We ran the analysis both on the pooled data across years and for each year separately, to allow comparisons. UPGMA (Unweighted Pair Group Method with Arithmetic mean) clustering using Nei's (1972) original distance was used to show the relationship among aphid genetic clusters.

Analysis of the association between aphid genotypes and plant chemo(metabo)types. We created a contingency table of the number of aphids within each genetic cluster (pooled number of individuals across all sampling times) collected on all plants within each of the different plant chemotype classes or metabotype groups. Non-random associations between 'aphid genotype' and 'plant chemotype', or 'metabotype', were analysed using a Fisher's Exact test using Monte Carlo simulated P-values, with 1.0×10^7 replicates, as the frequency table was larger than 2 by 2. Individual contributions were assessed using post-hoc Chi-

238 square analysis, with individual combinations deemed significant when above the critical
239 value for 1df at α=0.05, i.e. 3.84.

240 To identify individual chemicals of interest within the chemotypes associated with aphid
241 genetic structuring, we used the BMA method to identify which of the 22 volatile compounds
242 explained variation in the aphid genotype clustering. For all compounds retained in >5% of
243 the models, we ran posthoc linear models to determine any associations between the
244 compound and the plant chemotype class or aphid genotype cluster, and compared to the
245 contingency analysis results. Due to statistical limitations we were not able perform this
246 analysis on the 1020 mass features from the metabolomics data.

247 **Aphid genotype – plant chemotype associations mediated by interacting species.**
248 To explore potential effects of interacting species on the aphid genotype – plant chemotype
249 interactions, we used only the 2014 data that included information on interacting ants and
250 parasitoid wasps. We used the presence of each ant species (*L. niger* and *M. rubra*) before
251 aphid colonization as a measure of ant preference because ants were almost always present
252 after aphid colonisation. For the parasitoid wasp analysis we used the presence of
253 parasitized aphids on a plant. Following methods for analysing contingency tables using log-
254 linear models (Everitt 1992), we first created separate contingency tables for each data set
255 that counted the number of aphids within each aphid genotype on each plant chemotype (or
256 metabotype). For example, for the *L. niger* dataset, one contingency table was created for
257 plants with *L. niger* present before aphid colonisation and second for those plants without *L.*
258 *niger* before aphid colonisation. Separate models were run for plant chemotype and
259 metabotype (no association between the volatile and metabolome profile of the plants
260 [Mantel test: r=0.034, P=0.004; (Clancy *et al.* 2018)). In R, these tables were converted to a
261 data frame, and generalised linear models (glm) with poisson error distribution were used to
262 analyse the effect of ant (or parasitoid) presence, aphid genotype, and plant chemotype (or
263 metabotype). For such 3-way contingency tables (i.e. aphid genotype – by plant chemotype
264 – by ant presence/absence), deviances are calculated for each possible model of interest,

accounting for all potential interactions. These are then considered in a multinomial context to determine if each factor (e.g. ant presence, plant chemotype, aphid genotype) can be considered independent or there are associations by considering all 2-factor interactions (Everitt 1992). From this, the optimal model is chosen that best represents the data.

To explain any effect of ant presence through ant preference to different plant chemo/metabotypes we analysed ant preference using a binomial GLM on the number of times ants of each species were present on the different plants before aphids colonised, controlling for the number of weeks before aphid colonisation.

Results

Plant chemo/metabotypes. Across the two years of data collection, aphids colonized 87 of the 172 plants in the field site (61/172 in 2014 and 50/172 in 2015). In previous work, we clustered all 172 plants (aphid colonized and empty plants) into four main volatile chemotype classes (1-4) (Clancy *et al.* 2016). The 87 plants that hosted aphids exhibited finer-scale clustering, with nine distinct final chemotype classes (ANOSIM: $r=0.812$, $P<0.001$). These still fit the main classes obtained from analysing all 172 plants, and so are labelled 1.1, 1.2, 1.3, 2.1, 2.2, 2.3, 3.1, 4.1 and 4.2 to show the main class (from Clancy *et al.* 2016), followed by the sub-class (identified in the current analyses) to which the plants belong. Overall, plants with similar chemotype profiles were not spatially clustered based on chemical distance, i.e. there was no spatial autocorrelation and therefore neighbouring plants were not more similar to each other (Mantel test, $r=0.050$, $P=0.112$; Fig. S2a). There was no bias in the chemotype clustering analysis due to highly abundant compounds ($F_{1,20}=0.52$, $P=0.478$) or highly variable compounds ($F_{1,20}=0.09$, $P=0.772$) in the plants (Fig. S3a). Thus, clustering was due to the whole profile of compounds in the plants.

After clustering plants that hosted aphids by their plant non-volatile metabolomic profile, the plants grouped into the same five metabotype clusters (A-E) previously identified (Clancy *et*

al. 2018). Again, there was no evidence for spatial autocorrelation and hence no clustering of metabolically similar plants across the field site (Mantel test, r=0.038, P=0.019; Fig. S2b).

Aphid genome sequencing and microsatellite development. A total of 30,753 microsatellites [2,372 perfect (only containing pure repeats), and 28,381 imperfect (containing mutations) microsatellites] were detected. All of the final 18 microsatellites had the same optimal annealing temperature of 60 °C, leading to the successful development of two PCR-multiplexes (see Table S1 for primer details and Fig. S4 for a visualization of the multiplex mixes).

Aphid population genetic structure. We collected 145 aphids from the 61 occupied plants in 2014, and 204 aphids from the 50 occupied plants in 2015 (total 349 aphids from 87 individual plants). In total, we identified 228 MLGs (multi-locus genotypes) from 349 aphids, indicating high genetic diversity within the aphid population (Table 1). There was no association between the genetic distance of aphids and geographic distance between plants within the field site (Mantel test r = -0.002, P = 0.481; Fig. S2c), indicating no spatial clustering of aphid genotypes across the field site. The aphids clustered into six genetic clusters, pooled across all time points; both the 2014 and 2015 data showed similar structuring as the overall data. While K-means hierarchical clustering analysis showed that there was statistical evidence for six aphid genetic clusters, three of these clusters were more closely related and contained more individuals (clusters 1, 2, and 5; Fig. S5) than the three other clusters, which showed stronger differentiation from all others (clusters 3, 4, and 6; Fig S5).

Table 1. Summary of aphid samples collected in 2014 and 2015.

Year	Date of collection	Number of plants	Number of aphid colonies	MLGs	expected MLGs (SE)	Chemotype-aphid genotype (Fisher's P)	Metabotype-aphid genotype (Fisher's P)
2014	15th July	61	145	108	19.4 (1.21)	5.4×10^{-6}	0.003
2015	11th June	12	21	19	19.0 (0.00)	0.026	0.479
	9th July	42	106	72	17.9 (1.57)	2.2×10^{-6}	0.006
	23rd July	21	56	37	16.9 (1.54)	0.0002	0.011
	6th August	10	21	15	15.0 (0.00)	0.018	0.027
Pooled data		**87**	**349**	**228**	**19.2 (1.35)**	$\mathbf{1.0\times10^{-7}}$	$\mathbf{2.0\times10^{-7}}$

Expected number of MLGs (multi-locus genotypes) controls for differences in sample size by rarefaction. Chemo/metabotype-aphid genotype columns give results of Fisher's Exact tests (contingency analysis) across the different time points; metabotype data is from 271 aphids. SE, Standard Error

Association between aphid genotypes and plant chemo/metabotypes. There was no association between plant volatile chemotype and metabotype, i.e. plants of one metabotype did not belong to a particular volatile chemotype (Fisher's Exact test P=0.775).

We found strong non-random associations between aphids from particular genetic clusters and plant chemotype classes (Fishers Exact Test: $P=1.0 \times 10^{-7}$; Fig. 1a). Within sampling times and years, we also found significant non-random associations (Table 1). The majority of associations showed that aphids were more common than expected on certain plant chemotypes, with only one cluster being observed less often than expected on a single class (aphids from genetic cluster 1 on plant chemotype class 4.1; Fig. 1a). All other aphid clusters were each found more often than expected on a single plant chemotype class, except aphid genetic cluster 4 that was found significantly more often on two chemically-distinct plant chemotype classes (2.2 and 4.2: ANOSIM between chemotype classes r = 0.896, P = 0.001). From the 2015 data, we found these aphids more often on chemotype 4.2 at the start of the season and 2.2 later in the season.

Using Bayesian Model Averaging (BMA), to assess the individual impact of the 22 volatile compounds emitted from the plants on the aphid genetic clustering, we showed that only two

compounds were retained in more than half the models (eucalyptol with a PEP of 56.3% and (*Z*)-β-terpineol with a PEP of 52.4%; Fig. S3b). Nevertheless, we identified nine compounds (eucalyptol, (*Z*)-β-terpineol, (*E*)-dihydrocarvone, α-copaene, terpineol, β-cubebene, germacrene-D, α-pinene, and (*Z*)-sabinene hydrate) that were retained in >5% of models and could explain some of the genotype-chemotype associations. The main result here showed that aphid genetic cluster 6 is most associated with changes in the concentration of different individual compounds. This aphid genetic cluster was associated with higher amounts of (*Z*)-β-terpineol, (*E*)-dihydrocarvone, α-copaene, β-cubebene, and (*Z*)-sabinene hydrate (Fig. S6). These compounds were also all found in higher concentrations in plants within the chemotype class 4.1 (Fig. S7), where more aphids from this cluster than expected were also observed (Fig. 1). Other notable associations include there being more aphids from genetic cluster 3 on plants within chemotype class 1.2 than expected (Fig. 1), which could be driven by lower levels of α-pinene (Fig. S6, S7), or the association between aphids in cluster 5 and plants in 3.1 influenced by higher eucalyptol concentrations (Fig. S6, S7). Despite these associations, other plant clusters also showed increased/decreased levels of one or more of these compounds and thus, again, any effect on the aphid structuring is unlikely a single compound effect but rather the combination of compounds.

Similarly, we also detected a strong effect of plant metabotype on the distribution of aphid genotypes among the plants (Fishers Exact Test: $P=2.0 \times 10^{-7}$). Here, aphids from genotype cluster 2 were collected more often from plants of metabotype C; aphid genotype 5 from metabotype B; and, aphid genotype 6 from metabotype E (Fig. 1b). These associations were also significant within each of the three mid- to late-season 2015 sampling points, and the single time-point in 2014 (Table 1).

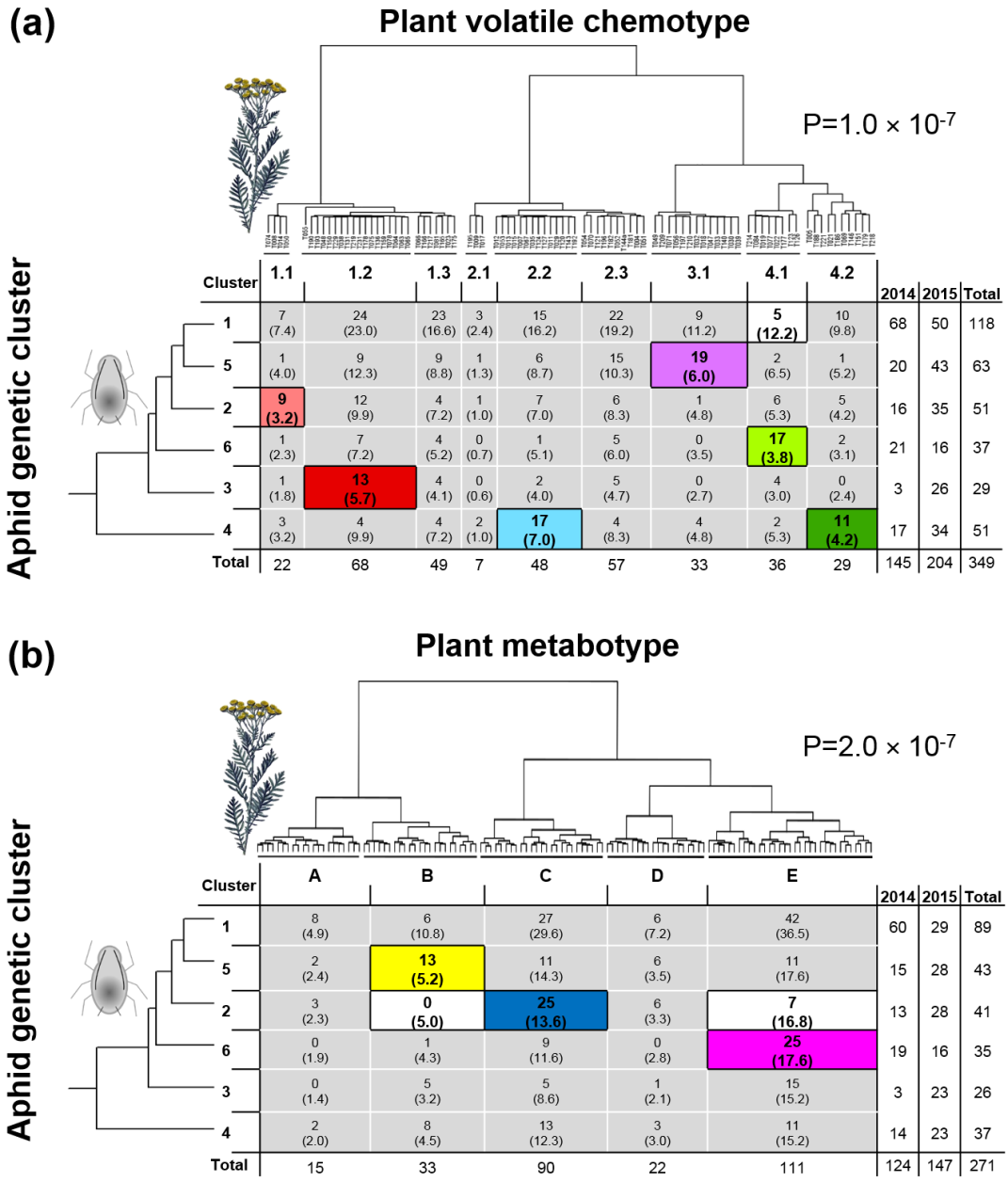

Figure 1. Distribution of aphid genotypes across plants as associated with (a) plant volatile chemotype, and (b) plant metabotype. The plants clustered into nine chemotype classes and five metabotype groups. Aphids were structured into six different genetic groups (clusters). Aphids from different genetic clusters colonised plants from different chemotype classes, and metabotypes more often than expected at random. Numbers show the observed number of aphids in each category, with expected number (from Chi-square formula) underneath in parentheses. Coloured cells (non-grey) show the combinations where aphids were observed more often than expected, and in white the single combination where aphids were observed less often than expected. Number of aphids collected in each year, and the total, are shown to the right of the tables.

Aphid genotype – plant chemo/metabotype associations mediated by interacting species.

We tested the potential impact of interacting species on aphid genotype – plant chemotype associations using log-linear models for contingency tables. We collected 76 aphids from plants on which *L. niger* ants had been observed before aphid colonisation, and 48 aphids from plants with no scouting ants, compared to 50 aphids from plants with no *M. rubra* before aphid colonisation and 74 aphids without this ant species. The number of aphids collected per genotype across the different plant chemotypes and metabotypes depended on the presence of these ants (Table 2). Before aphid colonisation, *L. niger* ants were found more often on plants from chemotype class 4.1, with ants observed on 82 % of these plants, compared to only 44 % of plants within class 2.1 (Fig. 2). Further, *L. niger* also exhibited preferences across plant metabotypes, with ants observed on 78 % of plants from metabotype B (Fig. 2). When further exploring the data, we found that the association between aphid genetic cluster 5 and plant chemotype class 3.1 depended on *L. niger* ants, with more aphids than expected from this genetic cluster on only those plants where ants had been observed patrolling before aphid arrival (X^2=5.54, P=0.020). Similarly, the association between aphids in genetic cluster 6 and plants in chemotype class 4.1, was enhanced by the increased presence of ants on these plants (Fig. 2); while aphid preference was still found to play a role, with more aphids than expected even when no ants had been observed (X^2=5.22, P=0.022), this effect was stronger in the presence of *L. niger* ants (X^2=8.22, P=0.004). Ant nests were distributed throughout the field site (Senft, Weisser & Zytynska 2017), and thus these associations are not explained by ant nest distribution. The presence *M. rubra* ants had less impact on the distribution of aphids, only altering the number of aphids across different plant chemotypes, but not across plant metabotypes (Table 2). *Myrmica rubra* ants showed some variation across chemotypes and metabotypes but this was not statistically significant (Fig. 2), potentially through confounding effects of competitive exclusion by *L. niger* (Senft, Weisser & Zytynska 2017).

The interaction between aphid genotype and parasitoid presence, or plant chemo(metabo)type (i.e. both chemotype and metabotype) and parasitoid presence (Table 2) is unlikely to mean that the parasitoid wasps can influence where aphids colonize, but rather that there was higher parasitism success in certain combinations of plant and aphid. For example, there was higher parasitism rates on plants from chemotype class 2.1 (t=4.34, P<0.001; Fig. S8), and on plants colonized more frequently by aphids from genotype cluster 5 (t=2.95, P=0.004; Fig. S8).

Table 2. Summary of using log-linear models used to analyse 3-way contingency tables to understand the effect of interacting species on the number of aphids

Response: number of aphids	Chemotype			Metabotype		
	df	**Chi-sq**	**P**	**df**	**Chi-sq**	**P**
Ant (*L. niger*)	**1**	**6.4**	**0.012***	**1**	**6.4**	**0.012***
Aphid genotype	5	80.6	<0.001***	5	80.6	<0.001***
Plant	8	18.4	0.019*	4	74.1	<0.001***
LN x Aphid	**5**	**17.8**	**0.003****	**5**	**17.8**	**0.003****
LN x Plant	**8**	**39.2**	**<0.001*****	**4**	**12.9**	**0.012***
Aphid x Plant	40	88.4	<0.001***	20	44.8	<0.001***
Ant (*M. rubra*)	**1**	**4.7**	**0.031***	**1**	**4.7**	**0.031***
Aphid genotype	5	80.6	<0.001***	5	80.6	<0.001***
Plant	8	18.4	0.019*	4	74.1	<0.001***
MR x Aphid	5	7.3	0.200	5	7.3	0.200
MR x Plant	**8**	**37.1**	**<0.001*****	4	4.0	0.405
Aphid x Plant	40	86.2	<0.001***	20	42.9	0.002**
Parasitoids	**1**	**62.3**	**<0.001*****	**1**	**62.3**	**<0.001*****
Aphid genotype	5	80.6	<0.001***	5	80.6	<0.001***
Plant	8	18.4	0.019*	4	74.1	<0.001***
Para x Aphid	**5**	**15.4**	**0.009****	**5**	**15.4**	**0.009****
Para x Plant	**8**	**23.1**	**0.003****	**4**	**11.8**	**0.019***
Aphid x Plant	40	81.0	<0.001***	20	42.3	0.003**

Models run were GLMs, with poisson error distribution on the number of aphids. Separate models were run to determine the individual and interaction effects of plant chemotype and metabotype separately (for 2014 data only). The main effects of aphid genotype and plant chemo(metabo)type, and the aphid x plant interaction are greyed out, as this simply confirms earlier analyses that different numbers of aphids from different genotypes were collected on different plant variants. Lines in bold represent the important information on the role of interacting species on the number of aphids per genotype across plant chemo(metabo)types
•P<0.10, *P<0.05, **P<0.01, ***P<0.001

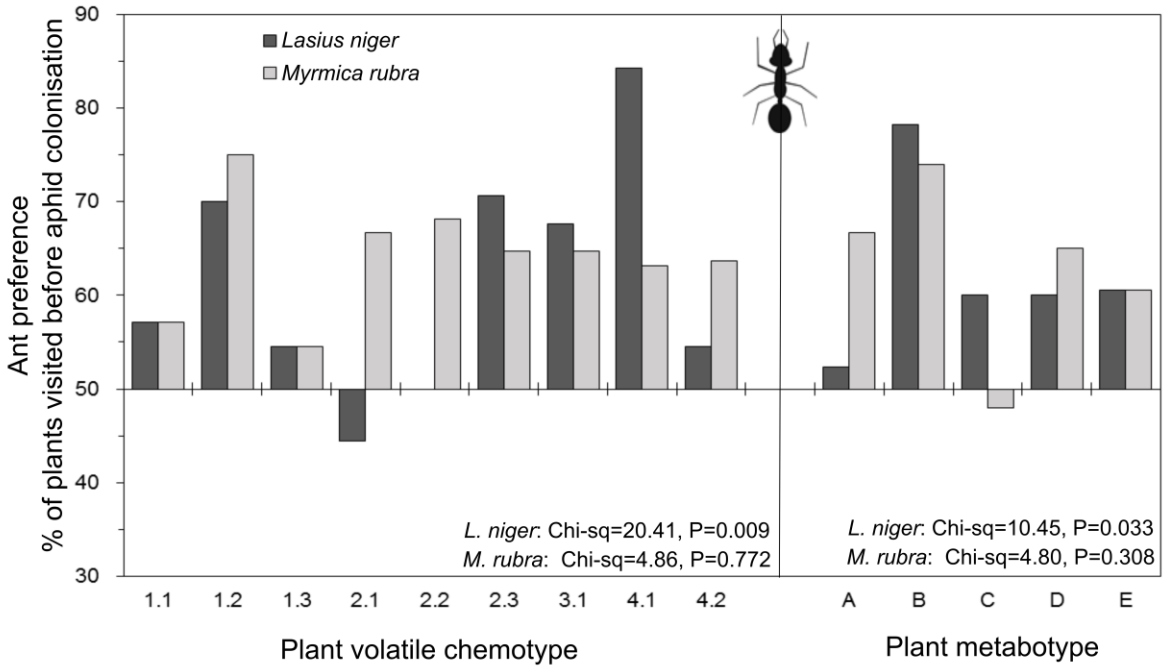

Figure 2. The presence of ants (*Lasius niger* and *Myrmica rubra*) before aphid colonisation across different plant volatile chemotypes and metabotypes. Data shows the percentage of plants on which the ants were present before aphid colonisation (2014 data only). Analysis used binomial GLM to further control for the number of weeks a plant was empty before aphid colonisation, and includes plants that were never colonised across the whole season. The intercept is set to 50 %, to highlight the groups on which ants were observed on less than half the plants.

Discussion

We found that plant within-species chemical variation of volatile and non-volatile compounds was associated with the distribution of aphid genotypes across host-plants at the small scale of a single field. These associations were mediated by interactions with aphid-tending mutualistic ants, indicating that plant chemical variation could have both direct and indirect effects on the aphid population at the genetic level. Plant within-species variation is now widely accepted as having a strong ecological impact of the structure of associated communities and species interactions (Rowntree, Shuker & Preziosi 2011; Whitham *et al.* 2012; Balint *et al.* 2016; Crutsinger 2016; Senft *et al.* 2019). Interactions between plant variants (genotypes or chemotype) have only before been documented in controlled experiments, often using highly-differentiated plants (e.g. crop varieties or morphologically distinct individuals) (Service 1984; Caillaud *et al.* 1995; Zytynska & Preziosi 2011; Kanvil,

Powell & Turnbull 2014; Zytynska *et al.* 2014), but not under natural conditions. We extend this work to show that plant chemical variation can structure herbivore populations at the genetic level in the field. This could have evolutionary consequences, for example if such interactions persist over multiple seasons co-evolutionary responses could lead towards host-associated differentiation with the potential to drive speciation (Stireman, Nason & Heard 2005).

Direct effects of plant chemical variation

We developed two successful multiplex-PCR mixes for 18 microsatellite loci, each allowing the amplification of nine microsatellite loci with which to genotype the aphids. We observed high levels of genetic diversity, confirming results from other studies on the same species (Loxdale, Kigathi & Weisser 2009; Loxdale, Massonnet & Weisser 2010). Despite this high genetic variability, the aphids clustered into six main groups. All but one of these genotype clusters was found more abundantly on a particular plant chemotype, and three observed more often on a particular plant metabotype, than would be expected with a random distribution of aphid genotypes across the plants. Since there was no pattern of spatial autocorrelation, with plants of different chemotypes and metabotypes found distributed across the whole field site, we suggest that these associations are driven by aphid genotype specific host-preferences. Host preference of aphids to different plant variants is known from various experimental studies (Zytynska & Weisser 2016). Our previous work showed that neither the plant volatile chemotypes nor the metabotypes studied here were likely induced by aphid feeding (Clancy *et al.* 2016; Clancy *et al.* 2018), and thus represent direct effect of the ‘base’ chemotype.

Active choice of dispersing aphids to plant hosts is likely to be driven more by variation in plant volatiles (Szendrei & Rodriguez-Saona 2010) than metabolites, since the aphids can detect the volatiles even before settling on, and probing, a plant (Powell, Tosh & Hardie 2006). Our results support this, with a stronger effect of plant volatile chemotype on the

471 distribution of aphid genotypes (indicating variation in aphid preference) during the main
472 dispersal phase in July when winged aphids are abundant (Senft, Weisser & Zytynska
473 2017). The lack of association between plant metabotypes and aphid genotypes in the very
474 first sampling period in 2015, but significant associations in all the later three periods,
475 highlights the role of plant secondary metabolites on aphid performance; with high
476 population growth rates leading to longer colony persistence (Senft, Weisser & Zytynska
477 2017), and stronger genotype-metabotype associations. We previously showed that aphids
478 colonised 'preferred' volatile chemotypes in the early part of the season (Clancy *et al.* 2016)
479 and later on colonised almost all plants belonging to the 'preferred' metabotypes irrespective
480 of which volatile chemotypes they belonged (Clancy *et al.* 2018). Hence, while aphid
481 genotypes might actively choose a host based on the volatile profile, the probability of
482 successfully colonising a plant and persisting on the plant across the season is increased on
483 certain 'optimal' metabotypes where population growth rates are increased; higher
484 population sizes were also found to reduce the chance of local extinction through predation
485 in this system (Senft, Weisser & Zytynska 2017).

486 Across the plant volatile chemotypes, we found that some of these associations could be
487 explained by specific plant compounds, including (*Z*)-β-terpineol, (*E*)-dihydrocarvone, α-
488 copaene, β-cubebene, (*Z*)-sabinene hydrate, α-pinene and eucalyptol. Many of these
489 chemical compounds have previously been found to have contact and fumigant toxicity to
490 invertebrates (Imdorf *et al.* 1995; Isman 2000; Tripathi, Prajapati & Kumar 2003). In our
491 system, chemical diversity was high, and while any potentially toxic compound will have a
492 strong impact in high concentrations, it is most likely the odour-ration or 'plume' of the plant
493 volatiles that drive these associations (Bruce, Wadhams & Woodcock 2005; Beyaert & Hilker
494 2014). Indeed, we found that it is not the most dominant chemicals that drive the
495 associations between plant chemotype and aphid genotype clusters, but rather those of
496 intermediate abundance. This is perhaps not surprising as a dominant chemical may only
497 provide sufficient cues for a specialist herbivore to find a patch of host plants (effective at the

498 landscape scale), rather than allow it to distinguish among individuals within a patch
499 (effective at the population scale) (Szendrei & Rodriguez-Saona 2010; Beyaert & Hilker
500 2014; Webster & Card 2017).

501 *Indirect effects of plant chemical variation*

502 Plant chemical variation indirectly influenced aphid population genetic structure, through
503 interactions with mutualistic ants, potentially via preference for different plant volatile
504 chemotypes and metabotypes by *L. niger* (the mutualist with the strongest effect on the
505 aphids in this system (Senft, Weisser & Zytynska 2017)). Some plant chemotype-aphid
506 genotype combinations were limited to plants where these ants had been observed before
507 aphid arrival, whereas others were just enhanced by ant presence. *Lasius niger* ants have
508 previously been reported to move aphids among host-plants and stay with them until the
509 aphid settles on the plant, with speculation that host-plant suitability is assessed via aphid
510 honeydew composition (Collins & Leather 2002; Züst & Agrawal 2017), which may be
511 related to variation in plant metabotypes. In our tansy system, such interactions need to be
512 empirically tested in controlled experiments to see if ant-borne dispersal of aphid genotypes
513 across plant chemo(metabo)types occurs.

514 *Conclusions*

515 Overall, we could show that the aphid population exhibits fine-scale genetic structuring
516 across our field site. The distribution of aphid genotypes, across two years of data collection,
517 was associated with plant within-species chemical variation in plant volatile and non-volatile
518 chemicals. This effect was both direct between the plant and aphid, and indirect, as
519 mediated by interactions with mutualistic ants. Studies on plant chemicals often focus on
520 those induced by the feeding herbivores, such as volatiles that attract natural enemies
521 (Dicke & Baldwin 2010), or other plant secondary metabolites (Jansen *et al.* 2009; Macel *et*
522 *al.* 2010; Bernhardsson *et al.* 2013; Marti *et al.* 2013). Our work shows that community
523 interactions can occur at the level of the individual host-plant due to the response of the
524 interacting aphids, ants, and natural enemies to the individual plant non-induced

chemo(metabo)type; particularly for patchily-distributed host plant species such as tansy. This has implications for research in the area of metacommunity ecology where interactions across multiple trophic levels (Fronhofer *et al.* 2015; Resetarits & Silberbush 2016), as well as genetic interactions among species are often ignored. While controlled experiments are needed to empirically test aphid, ant, and natural enemy preferences, our analyses clearly show that these associations can have real ecological and evolutionary impacts in natural communities.

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Author contributions

This study was designed by SZ, WWW and JPS. Field data was collected by SZ, MS and MC. Genome analysis and microsatellite development was performed by YG, SS, SP, MS, SZ, and CW. All data were analyzed by SZ, a first draft written by SZ, and all authors contributed to revisions.

Data accessibility

Data available from the Dryad Digital Repository http://dx.doi.org/10.5061/dryad.mm7bj56 (Zytynska *et al.* 2019).

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