



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

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Funding information

Deutsche Forschungsgemeinschaft, Grant/Award Number: SCHN 653/7-1 and WE 3081/25-1

Handling Editor: Daniel Stouffer

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 specialised 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 2 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 emphasise 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, indirect effects, metabolomics, population genetics, species interactions, within-species variation

1 | 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 (Rowntree, Shuker, & Preziosi, 2011; Tétard-Jones, Kertesz, Gallois, & Preziosi, 2007; Zytynska, Fleming, Tétard-Jones, Kertesz, & Preziosi, 2010). 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 Crutsinger, 2016; Rowntree et al., 2011; Whitham et al., 2012). 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, for example, 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, for example, 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 (Beyaert & Hilker, 2014; Iason et al., 2012; Kessler, 2015). Current evidence suggests that insects use ratio-odour chemical recognition rather than species-specific volatile organic compounds (VOCs) for host-plant recognition (Beyaert & Hilker, 2014; Bruce, Wadhams, & Woodcock, 2005). 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 their mutualistic ants or antagonistic natural enemies (reviewed in Zytynska & Weisser, 2016). Reduced visitation of aphids by ants on plants with high levels of a toxic defensive chemical led to reduced aphid numbers and in some cases changed the relationship between aphids and ants from mutualistic to antagonistic (Züst & Agrawal, 2017). The emission of plant VOCs can also attract natural enemies to plants—which can occur through emission of constitutive compounds in the plant (Senft, Clancy, Weisser, Schnitzler, & Zytynska, 2019) or via compounds synthesised and immediately released in response to herbivore feeding (Paré & Tumlinson, 1999). All these different interactions can influence the dynamics of herbivore populations colonising individual host plants. It is the overall sum of these direct and indirect interactions, experienced by all members of an interacting community, that lead to the structuring of ecological communities that we see in nature.

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

Controlled experiments using different aphid genotypes and plant variants (genotypes, varieties, chemotypes) have consistently shown that plant-aphid (genotype-by-genotype, or genotype-by-chemotype) interactions among these influence aphid performance and host preference (Caillaud et al., 1995; Kanvil, Powell, & Turnbull, 2014; Service, 1984; Zytynska et al., 2014; Zytynska & Preziosi, 2011). Such interactions suggest that the distribution of aphids across host plants could differ due to variation in the plant (e.g., plant chemotype or genotype) 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 co-evolution 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 (*T. 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., 2018; Clancy, Zytynska, Senft, Weisser, & Schnitzler, 2016), and this can influence the associated invertebrate community structure (Balint et al., 2016; Kleine & Müller, 2011). Recently, we have shown that plant-to-plant variation in the profile of VOCs (terpenes), identified as being putatively emitted from specialised storage structures on the leaves, affected the field colonisation of tansy plants by specialised 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 metabolotypes (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 metabolotype, 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, for example, 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., 2018, 2016; 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 whether any aphid genotype-by-plant chemotype associations were influenced by the varying abundances of ants we observed across different plant individuals (Senft et al., 2017).

2 | MATERIALS AND METHODS

2.1 | Study system and field site

Tansy (*T. 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 \pm SE)). The specialised aphid *M. fuscoviride* is obligatorily ant-tended (Flatt & Weisser, 2000), often by the black garden ant, *Lasius niger* L., or the common red ant *Myrmica rubra* L., and has a myriad of natural enemies including parasitoid wasps and generalist predators (Senft et al., 2017). The field site we used is located near Freising, Germany (Altenhausen: N 48°25'1.51"; E 11°46'1.19"), and contains around 200 individually identifiable tansy plants (Figure S1) of which 172 were visited each week in 2014 and four times in 2015; importantly, only 87 of them were colonised by aphids across both seasons leading to a heterogeneous distribution of aphids (Senft et al., 2017).

2.2 | Field survey data and aphid sample collection

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

2.3 | Plant chemical and clustering analysis

We used the plant volatile chemical information on 22 compounds, emitted from specialised storage structures on the plant (identified using GC-MS), from Clancy et al. (2016), and secondary metabolite information of 1,020 mass features as identified using LC-MS by Clancy et al. (2018) (for more details, see Appendix S1). Our focus was only on those plants that were colonised by aphids, so we performed new cluster analyses on these 87 plants to obtain chemotype and metabolotype plant groupings, using the package “pv-clust” (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, Hoeting, Volinsky, Painter, & Yeung, 2015). This analysis was not possible for the 1,020 mass features from the untargeted metabolomic 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 S2 for details). We tested the effect of compound concentration and variation (standard deviation) across the plant samples on the resulting PEP values to determine whether 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.

2.4 | 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 S3). Briefly, the library was prepared using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (New England BioLabs GmbH), 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 100 bp according to the manufacturer's instructions (Illumina Inc.). 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). The final PCR multiplex conditions were as follows: 1 µl DNA diluted 1:4, 5 × MyTaq™ Reaction Buffer (Bioline), 2 Units MyTaq™, specific primer mix, up to 20 µl with molecular grade water, run at 95°C for 2 min, 30 cycles of 95°C for 15 s, 60°C for 15 s, 72°C for 15 s, and then a final step at 72°C for 2 min. Fragment data were analysed using the software GeneMarker (version 1.75) (SoftGenetics LLC).

2.5 | 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 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.

2.6 | 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-square analysis, with individual combinations deemed

significant when above the critical value for 1 df at $\alpha = 0.05$, that is, 3.84.

To identify individual chemicals of interest within the chemotypes associated with aphid genetic structuring, we used the BMA method to identify which of the 22 volatile compounds explained variation in the aphid genotype clustering. For all compounds retained in >5% of the models, we ran post-hoc linear models to determine any associations between the compound and the plant chemotype class or aphid genotype cluster, and compared the contingency analysis results. Due to statistical limitations, we were not able to perform this analysis on the 1,020 mass features from the metabolomic data.

2.7 | Aphid genotype—plant chemotype associations mediated by interacting species

To explore potential effects of interacting species on the aphid genotype—plant chemotype interactions, we used only the 2014 data that included information on interacting ants and parasitoid wasps. We used the presence of each ant species (*L. niger* and *M. rubra*) before aphid colonisation as a measure of ant preference because ants were almost always present after aphid colonisation. For the parasitoid wasp analysis, we used the presence of parasitised aphids on a plant. Following methods for analysing contingency tables using loglinear models (Everitt, 1992), we first created separate contingency tables for each dataset that counted the number of aphids within each aphid genotype on each plant chemotype (or metabotype). For example, for the *L. niger* dataset, one contingency table was created for plants with *L. niger* present before aphid colonisation and second for those plants without *L. niger* before aphid colonisation. Separate models were run for plant chemotype and metabotype (no association between the volatile and metabolomic profile of the plants [Mantel test: $r = 0.034$, $p = 0.004$; (Clancy et al., 2018)]). In R, these tables were converted to a data frame, and generalised linear models (GLM) with Poisson error distribution were used to analyse the effect of ant (or parasitoid) presence, aphid genotype, and plant chemotype (or metabotype). For such three-way contingency tables (i.e., aphid genotype—by plant chemotype—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 whether each factor (e.g., ant presence, plant chemotype, and aphid genotype) can be considered independent or there are associations by considering all two-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.

3 | RESULTS

3.1 | Plant chemo/metabotypes

Across the 2 years of data collection, aphids colonised 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 colonised 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 subclass (identified in the current analyses) to which the plants belong. Overall, plants with similar chemotype profiles were not spatially clustered based on chemical distance, that is, there was no spatial autocorrelation and therefore neighbouring plants were not more similar to each other (Mantel test, $r = 0.050$, $p = 0.112$; Figure 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 (Figure 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, $r = 0.038$, $p = 0.019$; Figure S2b).

3.2 | 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 Figure S4 for a visualisation of the multiplex mixes).

3.3 | 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 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 $r = -0.002$, $p = 0.481$; Figure 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; Figure S5) than the three other clusters, which showed stronger differentiation from all others (clusters 3, 4, and 6; Figure S5).

3.4 | Association between aphid genotypes and plant chemo/metabotypes

There was no association between plant volatile chemotype and metabotype, that is, 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 (Fisher's exact test: $p = 1.0 \times 10^{-7}$; Figure 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; Figure 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.

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	15 July	61	145	108	19.4 (1.21)	5.4×10^{-6}	0.003
2015	11 June	12	21	19	19.0 (0.00)	0.026	0.479
	9 July	42	106	72	17.9 (1.57)	2.2×10^{-6}	0.006
	23 July	21	56	37	16.9 (1.54)	0.0002	0.011
	6 August	10	21	15	15.0 (0.00)	0.018	0.027
Pooled data		87	349	228	19.2 (1.35)	1.0×10^{-7}	2.0×10^{-7}

Note.: Expected number of MLGs (multilocus 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 are from 271 aphids. Abbreviation: SE, standard error.

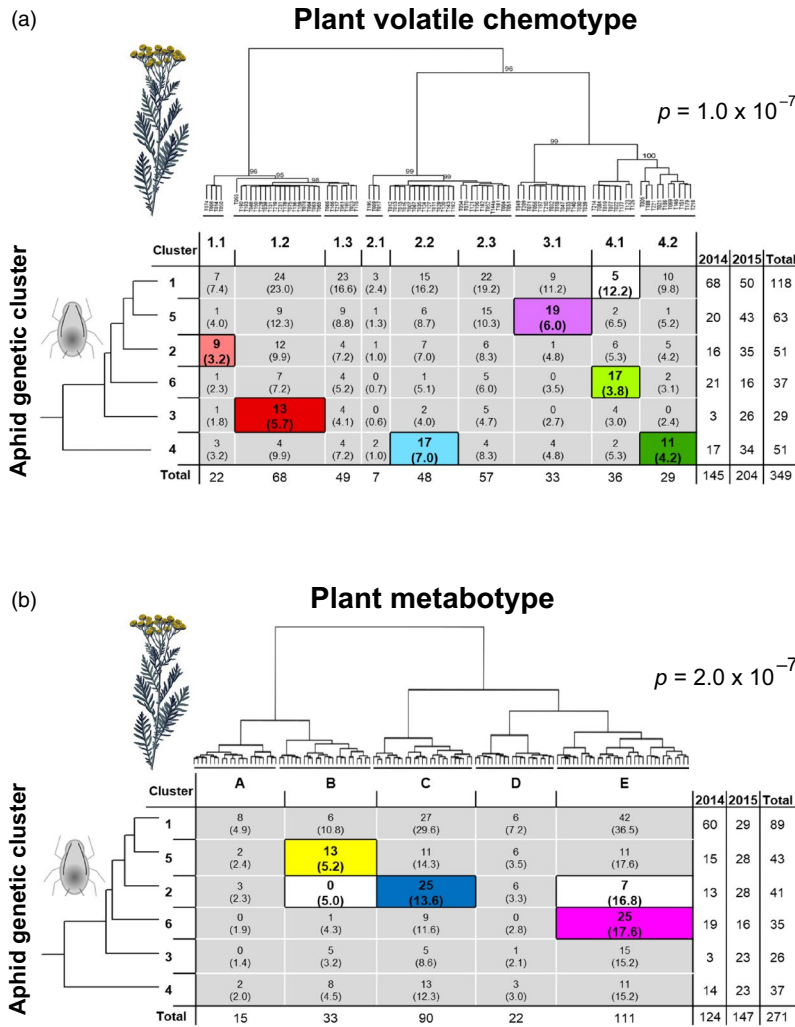


FIGURE 1 Distribution of aphid genotypes across plants as associated with (a) plant volatile chemotype and (b) plant metabotype. The plants clustered into nine chemotype classes and five metabotype groups. Aphids were structured into six different genetic groups (clusters). Aphids from different genetic 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

Using 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 -terpinol with a PEP of 52.4%; Figure S3b). Nevertheless, we identified nine compounds (eucalyptol, (Z)- β -terpineol, (E)-dihydrocarvone, α -copaene, terpinol, 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 (Figure S6). These compounds were also all found in higher concentrations in plants within the chemotype class 4.1 (Figure S7), where more aphids from this cluster than expected were also observed (Figure 1a). Other notable associations include there being more aphids from genetic cluster 3 on plants within chemotype class 1.2 than expected (Figure 1a), which could be driven by lower levels of α -pinene (Figures S6, and S7), or the association between aphids in cluster 5 and plants in 3.1 influenced by higher

eucalyptol concentrations (Figures S6, and 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 (Fisher's 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 (Figure 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).

3.5 | 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 loglinear 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 *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: Ant (*L. niger*) × Plant variant, and Ant (*M. rubra*) × Plant variant). 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 (Figure 2). Further, *L. niger* also exhibited preferences across plant metabotypes, with ants observed on 78% of plants from metabotype B (Figure 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 ($\chi^2 = 5.54$, $p = 0.020$). 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 (Figure 2); while aphid preference was still found to play a role, with more aphids than expected even when no ants had been observed ($\chi^2 = 5.22$, $p = 0.022$), this effect was stronger in the presence of *L. niger* ants ($\chi^2 = 8.22$, $p = 0.004$). Ant nests were distributed throughout the field site (Senft et al., 2017), and thus, these associations are not explained by ant nest distribution. The presence of *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 (Figure 2), potentially through confounding effects of competitive exclusion by *L. niger* (Senft et al., 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 colonise, but rather that there was higher parasitism success in certain combinations of plant and aphid. For example, there were higher parasitism rates on plants from chemotype class 2.1 ($t = 4.34$, $p < 0.001$; Figure S8) and on plants colonised more frequently by aphids from genotype cluster 5 ($t = 2.95$, $p = 0.004$; Figure S8).

4 | 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

TABLE 2 Summary of 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 (<i>L. niger</i>)	1	6.4	0.012*	1	6.4	0.012*
Aphid genotype	5	80.6	<0.001***	5	80.6	<0.001***
Plant variant	8	18.4	0.019*	4	74.1	<0.001***
Ant (<i>L. niger</i>) × Aphid genotype	5	17.8	0.003**	5	17.8	0.003**
Ant (<i>L. niger</i>) × Plant variant	8	39.2	<0.001***	4	12.9	0.012*
Aphid genotype × Plant variant	40	88.4	<0.001***	20	44.8	<0.001***
Ant (<i>M. rubra</i>)	1	4.7	0.031*	1	4.7	0.031*
Aphid genotype	5	80.6	<0.001***	5	80.6	<0.001***
Plant variant	8	18.4	0.019*	4	74.1	<0.001***
Ant (<i>M. rubra</i>) × Aphid genotype	5	7.3	0.200	5	7.3	0.200
Ant (<i>M. rubra</i>) × Plant variant	8	37.1	<0.001***	4	4.0	0.405
Aphid genotype × Plant variant	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 variant	8	18.4	0.019*	4	74.1	<0.001***
Parasitoids × Aphid genotype	5	15.4	0.009**	5	15.4	0.009**
Parasitoids × Plant variant	8	23.1	0.003**	4	11.8	0.019*
Aphid genotype × Plant variant	40	81.0	<0.001***	20	42.3	0.003**

Note: 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 three associated species (for 2014 data only).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

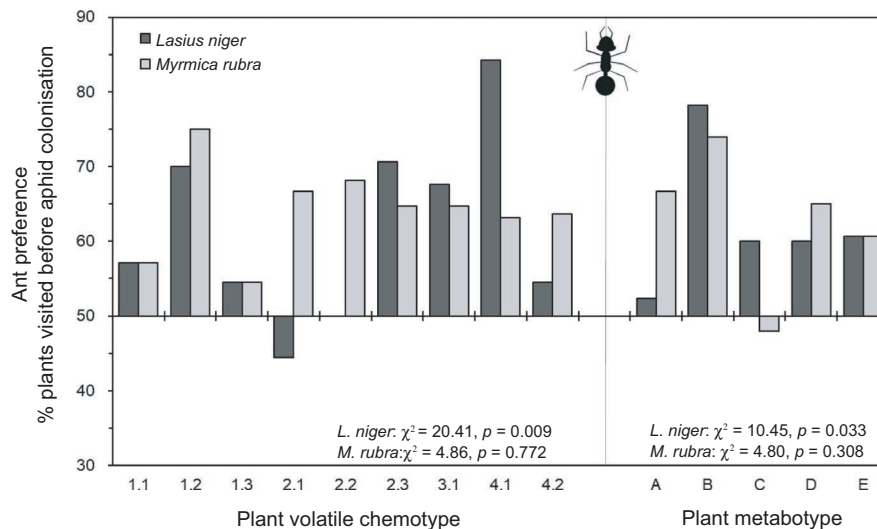


FIGURE 2 The presence of ants (*Lasius niger* and *Myrmica rubra*) before aphid colonisation across different plant volatile chemotypes and metabotypes. Data show the percentage of plants on which the ants were present before aphid colonisation (2014 data only). Analysis used binomial GLM to further 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

as having a strong ecological impact of the structure of associated communities and species interactions (Balint et al., 2016; Crutsinger, 2016; Rowntree et al., 2011; Senft et al., 2019; Whitham et al., 2012). 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) (Caillaud et al., 1995; Kanvil et al., 2014; Service, 1984; Zytynska et al., 2014; Zytynska & Preziosi, 2011), 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 et al., 2005).

4.1 | 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., 2018, 2016) 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 et al., 2006). Our results support this, with a stronger effect of plant volatile chemotype on the distribution of aphid genotypes (indicating variation in aphid preference) during the main dispersal phase in July when winged aphids are abundant (Senft et al., 2017). The lack of association between plant metabotypes and aphid genotypes in the very first sampling period in 2015, but significant associations in all the later three periods, highlights the role of plant secondary metabolites on aphid performance, with high population growth rates leading to longer colony persistence (Senft et al., 2017), and stronger genotype–metabotype associations. We previously showed that aphids colonised “preferred” volatile chemotypes in the early part of the season (Clancy et al., 2016) and later on colonised almost all plants belonging to the “preferred” metabotypes irrespective of which volatile chemotypes they belonged (Clancy et al., 2018). Hence, while aphid genotypes might actively choose a host based on the volatile profile, the probability of successfully colonising a plant and persisting on the plant across the season is increased on certain “optimal” metabotypes where population growth rates are increased; higher population sizes were also found to reduce the chance of local extinction through predation in this system (Senft et al., 2017).

Across the plant volatile chemotypes, we found that some of these associations could be explained by specific plant compounds, including (Z)- β -terpineol, (E)-dihydrocarvone, α -copaene, β -cubebene, (Z)-sabinene hydrate, α -pinene, and eucalyptol. Many of these chemical compounds have previously been found to have contact and fumigant toxicity to invertebrates (Imdorf, Kilchenmann, Bogdanov, Bachofen, & Beretta, 1995; Isman, 2000; Tripathi, Prajapati, & Kumar, 2003). In our system, chemical diversity was high, and while any potentially toxic compound will have a strong impact in high concentrations, it is most likely the odour ration or “plume” of the plant volatiles that drive these associations (Beyaert & Hilker, 2014; Bruce et al., 2005). Indeed, we found that it is not the most dominant chemicals that drive the associations between plant chemotype and aphid genotype clusters, but rather those of intermediate abundance. This is perhaps not surprising as a dominant chemical may only provide sufficient cues for a specialist herbivore to find a patch of host plants (effective at the landscape scale), rather than allowing it to distinguish among individuals within a patch (effective at the population scale) (Beyaert & Hilker, 2014; Szendrei & Rodriguez-Saona, 2010; Webster & Card, 2017).

4.2 | Indirect effects of plant chemical variation

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

5 | CONCLUSIONS

Overall, we could show that the aphid population exhibits fine-scale genetic structuring across our field site. The distribution of aphid genotypes, across 2 years of data collection, was associated with plant within-species chemical variation in plant volatile and non-volatile chemicals. This effect was both direct between the plant and aphid, and indirect, as mediated by interactions with mutualistic ants. Studies on plant chemicals often focus on those induced by the feeding herbivores, such as volatiles that attract natural enemies (Dicke & Baldwin, 2010), or other plant secondary metabolites (Bernhardsson et al., 2013; Jansen et al., 2009; Macel, Van Dam, & Keurentjes, 2010;

Marti et al., 2013). Our work shows that community interactions can occur at the level of the individual host plant due to the response of the 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, Klecka, Melián, & Altermatt, 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.

ACKNOWLEDGEMENTS

We thank Jose Lopez for DNA extractions, Andreas Moeller for field work, and Irene Haslberger for the permission to work on the field site. This project was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) (project numbers: WE 3081/25-1 and SCHN 653/7-1). Chemical data are available through Clancy et al. (2016), Clancy et al. (2018), field data through Senft et al. (2017), and the molecular data are available through the Dryad Digital Repository (details below).

AUTHORS' CONTRIBUTIONS

This study was designed by S.E.Z., W.W.W., and J.-P.S. Field data were collected by S.E.Z., M.S., and M.V.C. Genome analysis and microsatellite development were performed by Y.G., S.S., S.D.P., M.S., S.E.Z., and C.W. All data were analysed by S.E.Z., a first draft written by S.E.Z., and all authors contributed to revisions.

DATA ACCESSIBILITY

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.mm7bj56> (Zytynska et al., 2019).

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How to cite this article: Zytynska SE, Guenay Y, Sturm S, et al. Effect of plant chemical variation and mutualistic ants on the local population genetic structure of an aphid herbivore. *J Anim Ecol*. 2019;88:1089–1099. <https://doi.org/10.1111/1365-2656.12995>