

Recognition of host-specific chemical stimulants in two sympatric host races of the pea aphid *Acyrtosiphon pisum*

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Abstract. 1. In *ecological speciation*, adaptation to variation in the external environment provides the crucial push that starts the process of genetic divergence and eventually leads to speciation. This emphasis on the role of ecological specialisation in speciation events has brought with it a renewed interest in its proximate mechanisms in recently diverged groups such as host races. Here, the proximate mechanisms of feeding specialisation are investigated in two host races of the pea aphid *Acyrtosiphon pisum*.

2. Using alfalfa and clover extracts, enclosed in diet chambers or applied on whole plants, it is shown that feeding specialisation depends on recognition of stimulants specific to the host plant, not on deterrents or toxins specific to the non-host plants.

3. Because pea aphids mate on their host plant, feeding specialisation leads to *de facto* assortative mating. This study suggests that behavioural recognition of host-specific chemicals, rather than avoidance of deterrents or/and plant toxins, contributes to gene flow restriction between the alfalfa and clover host races.

Key words. Ecological speciation, feeding behaviour, plant chemistry, plant–herbivore interactions, stimulant.

Introduction

Adaptation to different environments is recognised increasingly as a force that can cause reproductive isolation and speciation by natural selection (Schluter, 1998). This emphasis on the role of environment in speciation events has brought with it a renewed interest in the proximate mechanisms of ecological specialisation, in particular in genetically divergent lineages close to the species boundary. The study of mechanisms in such taxa permits a focus on the characters that actually play a role in the evolution of reproductive isolation rather than on interspecies differences that may have accumulated after speciation is complete (Templeton, 1981).

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Knowledge of the precise nature of the factors affecting reproductive isolation may allow significant progress to be made in unravelling the genetics of speciation. For instance, using the fact that mating between *Drosophila melanogaster* and *D. simulans* species was unsuccessful owing to differences in their cuticular hydrocarbons (Cobb & Jallon, 1990), Coyne (1996) used these cuticular hydrocarbons as signal traits in genetic analyses to map mating preferences onto chromosome III. Elucidation of the underlying genetic architecture of traits that produce assortative mating is pivotal for understanding the link between ecological specialisation and speciation (Hawthorne & Via, 2001; Via & Hawthorne, in press). In the work reported here, the proximate mechanisms of ecological specialisation and probable assortative mating were investigated in two host races of a plant-feeding insect, the pea aphid *Acyrtosiphon pisum*.

Ecological specialisation within a herbivorous insect was demonstrated first by Edmunds and Alstad (1978). Populations appeared to perform better on *home* (natal)

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than on *away* (foreign) plants, suggesting that they were adapted to the plant habitat with which they interacted over evolutionary time. Since then, considerable evidence has accumulated about intraspecific host plant specialisation (Mopper & Strauss, 1997). The particular link between such intraspecific variation in resource use and speciation has also been discussed at length: in organisms that mate on the host plant, adaptation to alternative host plants may lead to the evolution of reproductive isolation among populations because it produces assortative mating (Bush, 1969, 1994; Mopper & Strauss, 1997; Feder, 1998). Populations exhibiting fine-scale genetic differentiation in association with plant species (host races) are currently being used as model systems to investigate the process of host race formation (Itami *et al.*, 1997; Feder, 1998; Via, 1999). The physiological and behavioural determinants of variation in plant specificity among these host races has received less attention (but see Feder *et al.*, 1997; Caillaud & Via, 2000); however knowledge of the mechanisms that lead to plant specificity and assortative mating is crucial for a detailed understanding of how population divergence could occur under selection by different resources or habitats (Schluter, 1998; Hawthorne & Via, 2001).

There is little doubt that plant chemistry plays a major role in shaping insect–plant interactions and in determining the variety of plants that can be exploited by an insect species (Berenbaum, 1991; Bernays & Chapman, 1994; Schoonhoven *et al.*, 1998). Chemical cues are important in host finding and recognition by the insect, and can alter rates of food intake. Plant compounds can also interfere with food-utilisation efficiency, reduce fecundity, or even cause mortality. As a consequence, differential adaptation to host-plant allelochemicals is thought to be largely responsible for patterns of host specialisation among different insect species (e.g. Bierbaum & Bush, 1990; Frey & Bush, 1990), however the role of variation in response to plant chemicals in host race formation is largely unknown (Berenbaum & Zangerl, 1997).

In the pea aphid, two host races utilising either alfalfa or clover have been described (Via, 1991a,b, 1999). Comparisons of estimated gene flow between the alfalfa and the clover host race with similar data on gene flow between recognised species or geographical races suggest that these aphid populations are incipient species (Via, 1999). Habitat fidelity appears to be the major barrier to gene flow between alfalfa and clover populations (Via, 1999). Gene flow is reduced further by selection against migrants and hybrids in the parental environments (Via *et al.*, 2000). Host fidelity being a crucial barrier to gene flow in this system, its proximate determinants have been examined in detail using a variety of behavioural bioassays (Caillaud & Via, 2000). Specialised aphids of each population land without discrimination on alfalfa and clover; however shortly after landing on the non-host (i.e. alfalfa for clover specialists), aphids abandon the plant. If the most specialised genotypes are prevented from leaving the non-host, they may starve to death because they are unwilling to feed on the alternate plant. Clearly, plant fidelity is *not* driven by plant toxicity

but by the willingness of aphids to accept either alfalfa or clover as food. Furthermore, pea aphids sample the plant tissues before accepting or rejecting a plant (Caillaud & Via, 2000). Thus, plant chemistry, rather than other plant cues such as leaf morphology or colour, is likely to determine plant acceptance as food.

Here, the effect of aqueous extracts of alfalfa or clover, either tested in diet chambers or applied on plant foliage, on aphid behaviour and larviposition was examined, and evidence that plant chemistry determines host-plant fidelity in the pea aphid is provided. It is also shown that the plant chemicals involved are stimulants rather than deterrents. If an individual detects the appropriate stimulant, the host plant is accepted and feeding starts. Because pea aphids mate on their host plant, host acceptance results indirectly in assortative mating within each plant habitat (Via, 1999; Caillaud & Via, 2000). Therefore, the results of this study suggest that variation in recognition of host-specific feeding stimulants plays a role in restricting gene flow between populations associated with either alfalfa or clover.

Materials and methods

Plants

Alfalfa *Medicago sativa* var. O'Neida and red clover *Trifolium pratense* var. Medium Red were grown in an environmental chamber (LD 16:8 h, 20 °C). Plants used for all procedures were 4–6 weeks old before flowering.

Plant extracts

Aqueous extracts of alfalfa and clover were prepared by boiling fresh foliage (100 g) in distilled water (500 ml) for 10 min. Hot plant extracts were then cooled in an ice-water bath and filtered through glass wool and Whatman[®] filter paper no. 1. The volume of each plant extract was reduced to 100 ml by rotary evaporation under vacuum, in a warm bath at 60 °C, so that the final concentration was equal to 1 g leaf equivalent per ml (1 GLE ml⁻¹). This concentration was selected because it is ecologically significant. Assuming that the distribution of the chemicals is homogeneous in the fresh plant tissue, the chemicals in plant extracts made from 1 g of fresh plant tissue, then dried, and diluted in 1 ml of water, represent the natural concentration of chemicals encountered in plants in the field. Fresh plant extracts were prepared weekly. Plant extracts were always allowed to warm up at room temperature prior to use in a bioassay.

Insects

The variance between host races (i.e. between alfalfa-collected and clover-collected genotypes) is considerably larger than the variance within each host race (e.g. between alfalfa-collected genotypes) (data on host performance: Via,

1991a; host preference: Via, 1999; feeding behaviour: Caillaud & Via, 2000). Thus, one genotype per host race was considered in the present study. A1 and C1 were collected respectively from alfalfa and clover in 1989, in New York state, U.S.A. (42°26'N, 76°29'W), and maintained parthenogenetically on their preferred host. These two genotypes were chosen because field experiments of demography on both host plants revealed that they typify the host specialisation of a set of field-collected clones (Caillaud & Via, 2000).

Bioassay 1

Plant extracts were enclosed in diet chambers. Half a millilitre of each plant extract (concentration: 1 GLE ml⁻¹) was enclosed between stretched Parafilm[®] membranes following standard procedures (reviewed by Mittler, 1988). The Parafilm[®]-enclosed solutions were then mounted in diet chambers consisting of plastic cylinders of 4 cm inner diameter (Caillaud & Rahbé, 1999). These plant extract solutions were then tested in the same trial as two control solutions (0.5 ml of distilled water and 0.5 ml of 16% sucrose) and two control plants (clover and alfalfa foliage). The control solutions were enclosed in the diet chambers described above. The foliage was placed in individual Petri dishes (10 cm diameter) with the petioles inserted in wet cotton. Two types of trial were run. In each trial, three replicates for each of the six treatments (four diets and two foliage) and each of the two aphid genotypes (A1 and C1) were tested simultaneously. Each replicate consisted of five aphids. The first type of trial involved first-instar nymphs starved for 1 h in Petri dishes containing moistened filter paper. After nymphs were placed on either diet or foliage, the number of nymphs feeding at nine time intervals was recorded: 0, 15, 30, 45 min, 1, 2, 3, 4, and 24 h. The second type of trial involved 3–4-day-old adults, starved for 3 h prior to the bioassays. After adults were placed on either diet or foliage, the total number of nymphs produced in 48 h was recorded. All trials were performed at 20 °C and a LD 16:8 h photoperiod. If the diet stimulated aphid feeding, most nymphs were expected to penetrate the Parafilm[®] in an attempt to feed from the diet. Abundant larviposition by adults was also expected. If the diet was neutral, a response on plant extract solutions comparable to that recorded for control solutions (water or sucrose) was expected. If the diet deterred aphid feeding, less probing and larviposition on plant extract solutions than on water and sucrose solutions was expected.

Bioassay 2

Plant extracts were applied on whole plants. Stems and leaves of either alfalfa or clover were immersed for 5 min in 8 ml of either alfalfa or clover extract. Treated foliage was allowed to dry at room temperature. The feeding behaviour of adult aphids (A1 or C1 individuals) was then monitored on treated alfalfa or clover plants and untreated clover or

alfalfa plants (controls). A single 3–4-day-old adult aphid was placed on top of plant foliage. Its behavioural state was recorded every minute for 30 min. Possible behavioural states were: probing, immobile, walking. Whether the aphid larviposited or left the plant was also recorded. If an extract from the non-host plant was a deterrent, few feeding attempts and low larviposition were expected on the host foliage after application of the non-host extract. If an extract from the host was a stimulant, frequent feeding attempts and high larviposition were expected on the non-host foliage after application of the host foliage extract. If an extract was neutral, no significant difference in feeding or larviposition was expected between extract-treated foliage and untreated plants.

Analyses

Feeding behaviour of nymphs in diet chambers (bioassay 1, trial type 1) was analysed for each genotype separately (A1 and C1) using repeated-measures ANOVA. This model was adequate because feeding behaviour of nymphs was sampled in a time sequence for each experimental unit (diet chambers containing five aphids) and therefore did not represent independent observations. The dependent variable was the number of aphids found probing a diet chamber at a given time. The independent variables were diet (distilled H₂O, 16% sucrose, alfalfa extract, clover extract, alfalfa foliage, clover foliage), time (sampling times: 0, 15, 30, 45 min, 1, 2, 3, 4 h), and the interaction of diet and time, to test for changes in the feeding behaviour of nymphs over time. This analysis included only the first 4 h of the bioassay because some random mortality was observed in diet chambers after 4 h, making replicates unbalanced (some with three or four nymphs per diet chamber rather than five) and thus unsuitable for repeated-measure analysis.

For adult larviposition in diet chambers (bioassay 1, trial type 2), a one-way ANOVA was used for each genotype separately. The dependent variable was the number of nymphs larviposited in a diet chamber by five adult aphids in a period of 48 h. The factor in the model was diet (distilled H₂O, 16% sucrose, alfalfa extract, clover extract, alfalfa foliage, clover foliage).

Feeding behaviour of adult aphids on extract-treated foliage (bioassay 2) was analysed using two-way ANOVA for each genotype separately. The dependent variable was the number of observations at which an aphid was seen feeding during a period of 30 min. The factors in the model were plant, extract, and the interaction of plant and extract. All statistical analyses were performed using SPSS version 10.0.

Results

Bioassay 1. feeding behaviour of nymphs in diet chambers

For genotype A1, the alfalfa specialist, there was a significant main effect of diet ($F_{\text{diet}} = 87.97$, d.f. = 5,

$P < 0.001$), with more probing by nymphs on their native host (alfalfa) and alfalfa extract than on clover, clover extract, or control solutions (water and sucrose). Furthermore, there was a significant interaction of dietary effect and time ($F_{\text{diet} \times \text{time}} = 3.32$, d.f. = 35, $P < 0.001$), with increasing feeding on alfalfa and alfalfa extract over time, and decreasing feeding on clover, clover extract, and control solutions over time (Fig. 1a). For genotype C1, the clover specialist, there was a significant main effect of diet ($F_{\text{diet}} = 75.72$, d.f. = 5, $P < 0.001$), again with more probing by nymphs on the native host (clover) and clover extract than on alfalfa, alfalfa extract, or control solutions. There was a significant interaction of diet and time ($F_{\text{diet} \times \text{time}} = 3.92$, d.f. = 35, $P < 0.001$), with increasing feed-

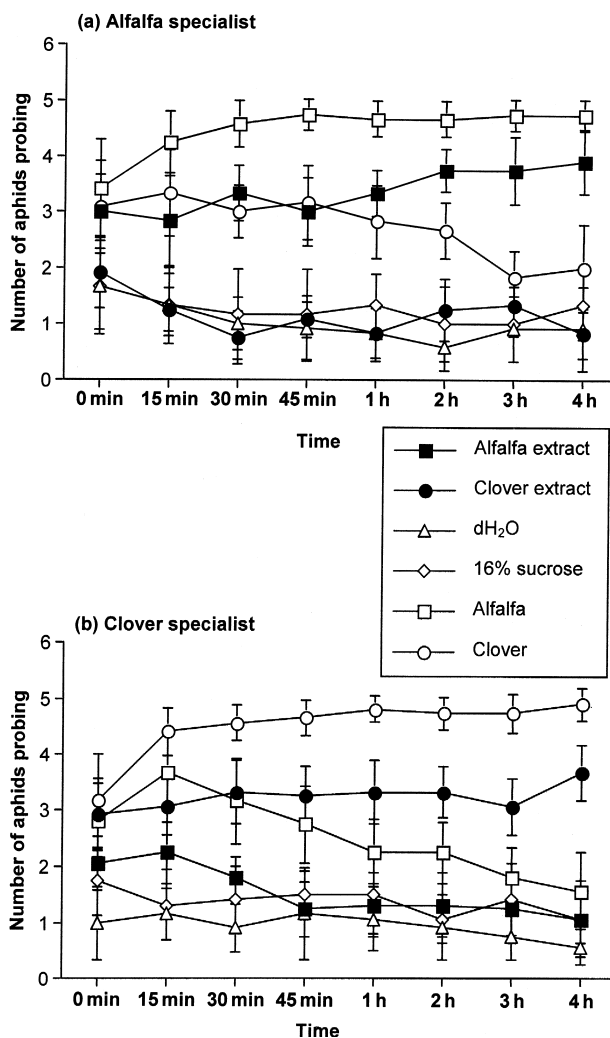


Fig. 1. Feeding behaviour of (a) alfalfa specialist (A1) and (b) clover specialist (C1) nymphs on diet chambers. Bars: 95% confidence intervals. Each data point is the mean of 12 replicates, each replicate containing five aphids. For A1, $F_{\text{diet}} = 87.97$, d.f. = 5, $P < 0.001$; $F_{\text{diet} \times \text{time}} = 3.32$, d.f. = 35, $P < 0.001$. For C1, $F_{\text{diet}} = 75.72$, d.f. = 5, $P < 0.001$; $F_{\text{diet} \times \text{time}} = 3.92$, d.f. = 35, $P < 0.001$.

ing on clover, sustained high feeding on clover extract over time, and decreasing feeding on alfalfa, alfalfa extract, and control solutions over time (Fig. 1b).

Bioassay 1. adult larviposition in diet chambers

For genotype A1, the alfalfa specialist, there was a significant main effect of diet on offspring production ($F_{\text{diet}} = 13.86$, d.f. = 5, $P < 0.001$). The alfalfa extract was as good a stimulant for larviposition by genotype A1 as the alfalfa foliage itself, while no significant difference in larviposition by genotype A1 was detected among the other diets (clover, clover extract, water, 16% sucrose), which were significantly lower (Fig. 2). For genotype C1, the clover specialist, there was also a significant diet effect on larviposition ($F_{\text{diet}} = 29.55$, d.f. = 5, $P < 0.001$). The clover extract was as good a stimulant for larviposition by genotype C1 as the clover foliage itself, while no significant difference in larviposition by genotype C1 was detected among the other diets (clover, clover extract, water, 16% sucrose), which were significantly lower (Fig. 2).

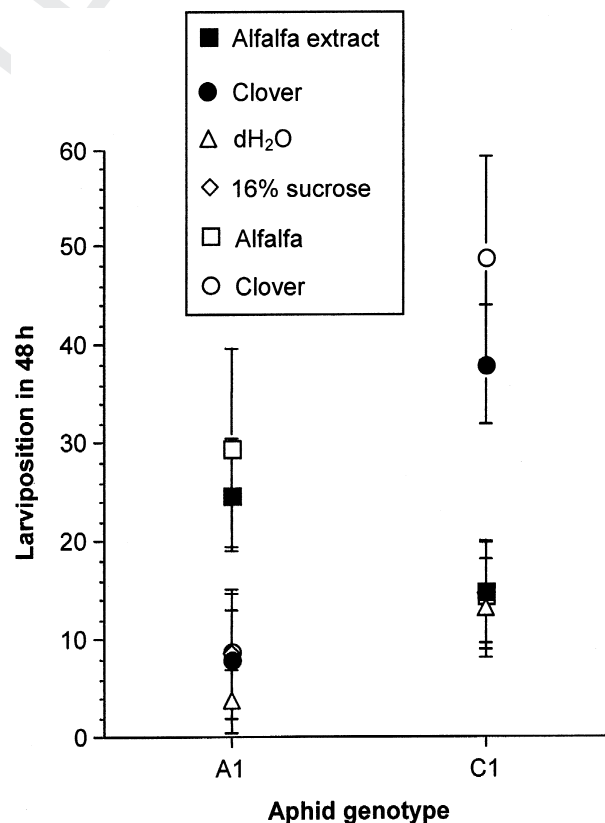


Fig. 2. Larviposition of alfalfa specialist (A1) adults ($F_{\text{diet}} = 13.86$, d.f. = 5, $P < 0.001$) and clover specialist (C1) adults ($F_{\text{diet}} = 29.55$, d.f. = 5, $P < 0.001$) on diet chambers, in a 48-h period. Bars: 95% confidence intervals. Each data point represents the mean of nine replicates, each replicate containing five adult aphids.

Bioassay 2. feeding behaviour of adults on extract-treated plants

For genotype A1, the alfalfa specialist, there was a significant plant effect ($F_{\text{plant}} = 190.55$, d.f. = 1, $P < 0.001$), with genotype A1 probing more on alfalfa plants than on clover plants, whether they had no extract, a non-host extract (from clover), or a host extract (from alfalfa) (Fig. 3a). There was a significant interaction between plant and extract ($F_{\text{plant} \times \text{extract}} = 16.20$, d.f. = 2, $P < 0.001$), with genotype A1 feeding on clover plants treated with the alfalfa extract more than on untreated clover. A comparable pattern was observed for genotype C1, the clover specialist (Fig. 3b). Genotype C1 probed more on clover than on alfalfa, whether the plants had no extract, a clover extract, or an alfalfa extract ($F_{\text{plant}} = 91.37$, d.f. = 1,

$P < 0.001$). Also, genotype C1 adults fed on alfalfa plants treated with a clover extract almost as much as on clover plants themselves ($F_{\text{plant} \times \text{extract}} = 16.66$, d.f. = 2, $P < 0.001$).

Discussion

Mechanisms of host-plant specialisation in pea aphids

Previous work on the proximate mechanisms of host-plant specialisation in the pea aphid suggested that plant chemistry plays a key role in host acceptance (Caillaud & Via, 2000). Here, this hypothesis was tested by presenting specialised aphid genotypes with aqueous extracts instead of whole plants, and the type of chemical cues that may be involved were investigated by applying these aqueous extracts on whole plants. When included in diet chambers, the extracts of the favoured host plant were shown to stimulate feeding behaviour by nymphs and larviposition by adults, while extracts of the non-host plant elicited a response similar to that recorded on water and sucrose, known to be a neutral and a weak stimulant respectively (Campbell *et al.*, 1986) (Figs 1 and 2). Importantly, host extracts applied on non-host foliage made non-hosts acceptable, while non-host extracts applied on host foliage did not reduce the acceptability of the favoured host plant (Fig. 3). Thus, each specialised genotype appears to recognise compounds specific to its favoured host plant, which triggers feeding and hence high fitness on that plant (Caillaud & Via, 2000). Because the host-specific compounds are not detected on non-host plants, the aphid rejects the non-host plants and either leaves them or starves to death (Caillaud & Via, 2000). The results establish clearly that differences in plant acceptance between the alfalfa and clover host races are chemically mediated. Moreover, plant acceptance depends on the presence/absence of host-specific chemicals that have a *stimulatory* effect, not on the presence of toxins or deterrents in the non-host plants. This suggests that plant specialisation depends on host plant chemistry rather than on non-host chemistry, and that a recognition system is involved.

It is hypothesised that these host-specific stimulants are allelochemicals, not plant nutrients. Primary plant substances, notably sugars and amino acids, are known to affect host-plant acceptance by insects, but they occur in all plants and their concentrations vary so much with plant developmental stage, age, physiological condition, and environmental factors, that they are seen as unlikely sole determinants of host specificity (Schoonhoven *et al.*, 1998). In the case of pea aphids, several studies have shown that plant composition in nutrients alone cannot explain host specialisation (Febvay *et al.*, 1988a,b; Rahbé *et al.*, 1988; Sandstrom & Petersson, 1994). Moreover, Sandstrom and Petersson (1994) showed that the type and amount of amino acids in alfalfa and red clover were very similar. In contrast, plant allelochemicals have been shown to exert strong influence on host use in the pea aphid (Wegorek & Krzymanska, 1972, 1976; Krzymanska *et al.*, 1983; Dreyer *et al.*, 1985,

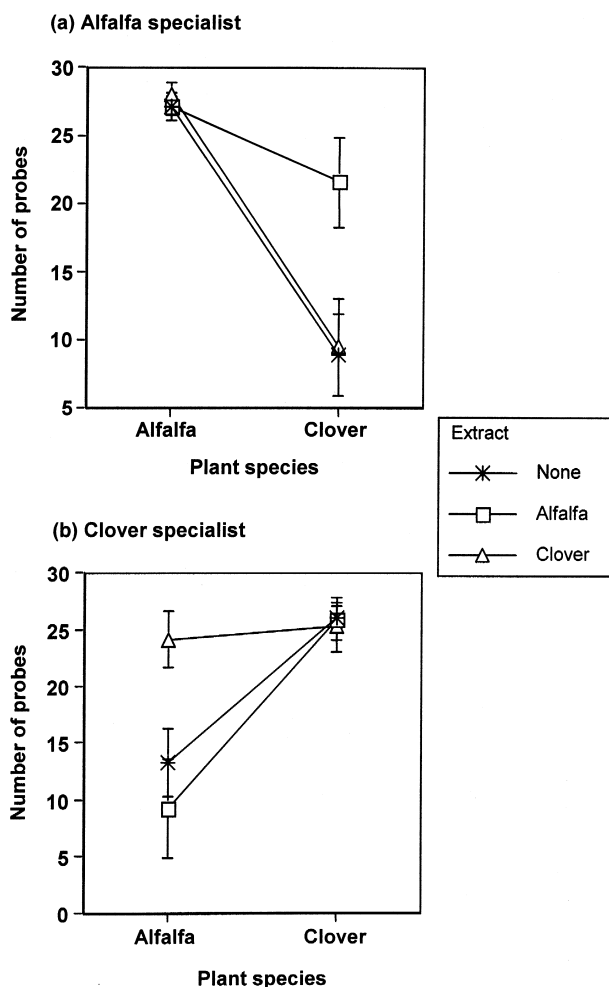


Fig. 3. Feeding behaviour of (a) alfalfa specialist (A1) and (b) clover specialist (C1) adults on extract-treated foliage. Bars: 95% confidence intervals. For A1, $F_{\text{plant}} = 190.55$, d.f. = 1, $P < 0.001$; $F_{\text{extract}} = 14.98$, d.f. = 2, $P < 0.001$; $F_{\text{plant} \times \text{extract}} = 16.20$, d.f. = 2, $P < 0.001$. For C1, $F_{\text{plant}} = 91.37$, d.f. = 1, $P < 0.001$; $F_{\text{extract}} = 13.17$, d.f. = 2, $P < 0.001$; $F_{\text{plant} \times \text{extract}} = 16.66$, d.f. = 2, $P < 0.001$.

1987), as well as in other aphid species (Nault & Styer, 1972; Chen *et al.*, 1999). For instance, Nault and Styer (1972) showed that sinigrin, a crucifer-specific glycoside, stimulated feeding of crucifer-specialist aphid species when applied on non-crucifer foliage. Thus, plant allelochemicals are more likely than plant nutrients to be host recognition cues for pea aphids.

Recognition of host-specific stimulants, plant fidelity, and reproductive isolation

Recognition mechanisms influence the chance of encountering and/or accepting a potential mate and have long been described as key aspects of pre-mating isolation and speciation. Recognition signals include general visual perception of body size or ornamentation, acoustic signals, pheromone recognition, kin recognition, or recognition between male and female gametes (Palumbi, 1998; Panhuis *et al.*, 2001). In the pea aphid, plant chemistry may be a recognition signal and may act as an important proxy for mate recognition. It is known that the most specialised aphids discriminate quickly between their favoured host plants and non-host plants. Following a brief penetration of the plant tissues with their stylets, aphids abandon non-host plants or feed and accumulate on their favoured host plant (Caillaud & Via, 2000). The results presented here suggest further that chemical information is gathered during this brief penetration and that plant chemicals are used as cues to recognise the *right* plant habitat by the alfalfa and clover host races. In doing so, these chemicals also determine on which plant aphids will settle and thus the spatial location of males and sexual females at the time of mating. Therefore, for the most specialised genotypes A1 and C1, recognition of host-specific chemicals results incidentally in assortative mating within each plant habitat. Because previous work has shown that many other genotypes exhibit behaviour on a whole plant that is comparable to that of genotypes A1 and C1 (Caillaud & Via, 2000), the present study suggests that host-specific chemicals contribute to gene flow restriction between alfalfa and clover populations. These chemicals could also have played some role in the evolution of genetic divergence between alfalfa and clover populations. As recognition of host chemistry improved due to fitness effects of recognising the right host quickly, assortative mating became stronger, which facilitated host specialisation and increased the accuracy of recognition of the right host.

The finding that host-specific stimulants rather than either toxins or deterrents currently restrict gene flow between host-associated populations of the pea aphid may be very significant. In another system, the moth *Yponomeuta*, which has evolved reproductive isolation on different host plants, host-plant specific stimulants are also involved (review by Menken & Roessingh, 1998). Dulcitol stimulates feeding in *Yponomeuta* species associated with *Celastraceae* while sorbitol elicits feeding in species associated with *Rosaceae*. As in the case of the pea aphid, a

rosaceous non-host can be rendered acceptable to the *Celastraceae* specialist *Y. cagnagellus* by impregnating its foliage with dulcitol (Menken & Roessingh, 1998). It should be expected that the recognition of host-specific stimulants play a particularly important role when divergence, and eventually speciation, is driven by the use of different host plants. Compared with deterrents and toxins, stimulants may have a more immediate effect on insect fitness. Del Campo and Renwick (2000) showed that larvae of *Manduca sexta* raised on a diet containing the host-specific stimulant Indiosine D had increased growth rate and fecundity compared with larvae raised on the standard diet used for *M. sexta*, presumably because of their increased ingestion rate in the presence of chemical stimulant (Del Campo & Renwick, 2000; Del Campo *et al.*, 2001). Thus, host-specific stimulants not only arrest the insect rapidly on a particular plant, which prevents potential encounters with either natural enemies or mates specialised to the alternate host, but they also increase ingestion and insect fitness *immediately*. Host shifts and the evolution of insect-plant interactions are usually thought to be driven by the loss of sensitivity to plant deterrents or the gain in the ability to detoxify toxins (Ehrlich & Raven, 1964; Jermy, 1984; Schoonhoven *et al.*, 1998; Chapman, 1999). This work suggests that host shifts, and the evolution of reproductive isolation as a result of this host shift, may also occur when a particular chemical becomes co-opted as a recognition cue.

Conclusion

Within-species genetic divergence must be studied in order to understand the role of chemo-reception in the evolution of host specialisation and ecological speciation. This study suggests that variation in plant acceptance between host races of the pea aphid is due to variation in peripheral chemo-sensory reception of a chemical feeding stimulant and/or central integration of neuronal inputs encoding plant quality. Thus, the pea aphid system provides the opportunity to establish more clearly the role of chemo-sensory mechanisms and recognition systems in ecological speciation of herbivorous insects. The isolation and identification of host-plant specific stimulants from alfalfa and red clover are currently in progress.

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