The Genetics of Species Interactions in Model and Natural Ecosystems

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

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Abstract

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Within an ecological community, interactions between species in a community occur directly, through physical contact, and indirectly, via other species or through abiotic environmental modification. Genetic variation within a species has the ability to alter the outcome of interactions between species. In other words, the specific genotypes of the interacting individuals are important for the outcome of the interaction.

In this thesis, I begin by showing that indirect interactions between a soil rhizobacteria (*Pseudomonas aeruginosa*) and a parasitoid wasp (*Aphidius rhopalosiphum*) are mediated by genotypic interactions between the two linking species, aphids (*Sitobion avenae*) and barley (*Hordeum vulgare*). This means that the magnitude and direction of the indirect effect of rhizobacteria (presence/absence) on the wing size of the parasitoid wasp was different, depending on the combination of interacting aphid and barley genotypes. If such interactions were to have an evolutionary effect, there would need to be non-random association between the aphid and barley genotypes. In the next chapter, I demonstrated that different aphid genotypes actively choose (no effect on reproductive rate) to colonize particular barley genotypes. I then showed that host preference of an aphid genotype could be altered by the presence, and sometimes genotypic identity, of another competing aphid genotype. I confirmed that these interactions were indirect, via the plant, by showing that aphid growth rate can be reduced when a plant is pre-conditioned with a different aphid genotype. Further investigation, using microarrays, showed that the different aphid genotypes induced differential gene expression in a single barley genotype. Many of these sequences belonged to known plant defense pathways and suggest a possible mechanism for the observed genotypic interactions between aphid and barley.

In order to further understand the influence of within-species genetic variation on species interactions there is a need to consider these interactions in a natural system. I therefore investigated the influence of genetic variation within a single tree species on the associated plant and invertebrate communities in a complex, natural tropical ecosystem. I found that more closely related trees were host to more similar communities of epiphytic plants, leaf litter invertebrates around the base of the tree and trunk-dwelling invertebrates. This shows that even in a highly diverse, naturally occurring ecosystem the effect of genetic variation within a species can be an important factor for the structure of associated communities of both plants and animals.

Research on the influence of within-species genetic variation on species interactions at a community level has wide applications for understanding how communities and ecosystems function, which can benefit agriculture, disease management and conservation practices.

Declaration

I, Sharon E. Zytynska declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright statement

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Chapter 1. The genetics of species interactions in model and natural ecosystems

THESIS INTRODUCTION

Species interactions in an ecological community

An ecological community is a complex system that consists of all the interacting organisms (biotic factors) in an area, whereas an ecosystem is described when all the biotic and abiotic (e.g. light, precipitation, pH) factors are considered together. Within a community, organisms are not independent entities since interactions occur between individuals of the same species (intraspecific interactions), and individuals from different species (interspecific interactions). Interactions can occur through: antagonism, e.g. predator-prey relationships, the transfer of energy through trophic levels; parasitism, e.g. a parasitoid wasp lays an egg in an aphid, which is then consumed by the growing wasp larva, this harms the host to the benefit of the parasitoid (Shirota et al. 1983); commensalism, e.g. some insectivorous bird species follow livestock that disturb insects whilst feeding in long grass, which benefits the birds with no effect on the livestock (Kushlan 1981); mutualism, e.g. pollination of yucca by yucca moths, which benefits both the plant and the moth (Riley 1892); and competition, e.g. an invasive species decreases the abundance of a native species of which the outcome depends on which species has the superior ability to use the resources and alters community dynamics (Sakai et al. 2001).

Interactions between different species in a community can occur directly, through physical contact, and indirectly, via other species or through abiotic environmental

modification. In this thesis, I consider indirect interactions between species as those that are mediated by other species in a community, rather than via abiotic effects (sensu Wootton 1994). For an indirect interaction to occur there needs to be at least three species in the community due to the inherent nature of these interactions. Indirect interactions can be created through chains of direct interactions or by the interaction between two species being modified by the presence of another species (Wootton 1994). Indirect interactions can cause effects through changes in the population densities of species' (density-mediated indirect effects) or alternatively they can cause an effect on the behaviour of a species (trait-mediated indirect effects), or both density- and trait-mediated indirect effects can occur at the same time (Müller and Godfray 1999, Werner and Peacor 2003). An example of a trait-mediated indirect interaction is when a predator causes a prey species to reduce its activity, or increase its hiding, which in turn alters competitive relationships of other species independent of the effect of density from alternative prey removal (Werner and Peacor 2003). These density- and trait-mediated indirect effects can create strong trophic cascades that have the potential to strongly influence the surrounding community (Werner and Peacor 2003).

In natural systems, indirect interactions can create effects such as exploitative competition (ability to gather biotic resources e.g. two predators compete for one prey species), trophic cascades (energy transfer e.g. plant-herbivore-predator interactions), apparent competition (indirect competition between species mediated by a third e.g. two prey species that are consumed by one predator species), indirect mutualism (a consumer-resource linked interaction combined with exploitative or interference competition) and interaction modification (a third species can modify an interaction between two other species) (Figure 1.1; Wootton 1994).

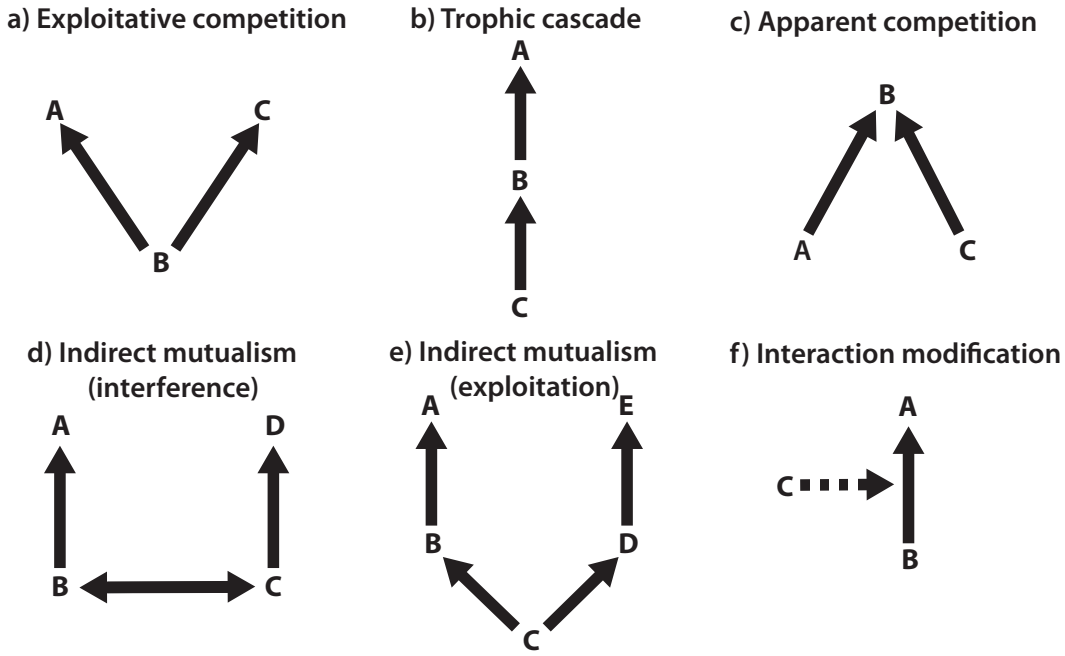

Figure 1.1. Effects of indirect interactions (modified from Wootton 1994)
Horizontal arrows show interference competition (arrows show impacted species); vertical arrows show consumer-resource interactions (arrows determine direction of energy flow). (a) Exploitative competition, (b) trophic cascade, (c) apparent competition, (d) indirect mutualism involving interference competition, (e) indirect mutualism involving exploitative competition, (f) interaction modification

At the community-level, indirect interactions between species are thought to stabilize the community structure as they create links between species that may not actually encounter each other physically (Wootton 1994, Miller and Travis 1996). Empirical work on interaction food webs shows that the number of indirect interaction effects increases exponentially per species, with each addition of a new species (Menge 1995). Thus, the removal of one species will reduce the number of indirect interactions and potentially reduce the stability of the system. For an indirect interaction to have a detectable effect within a community there needs to be strong direct interactions between the species involved. Weak direct interactions between species in a community may occur if there is a large amount of environmental variation (Wootton 1994). It is expected that the longer the chain of interactions, leading to the indirect interaction, the

weaker the effect will be due to diffusion of the effect (Dodds and Nelson 2006). Even if there are many weak interactions, resulting from large variation in the magnitudes and direction of the interactions in a community, these may actually prove strong at a local scale and enhance stabilization in a community (Berlow 1999).

Understanding the role a species has in a community will enable more accurate predictions of the health of a community. For example, if the species was a true keystone species and became extinct it is expected that the whole community would suffer through reduced diversity, and potentially collapse; however, the extinction of a minor member of a community could cause relatively little effect. In the area of conservation management, the consideration of the strength of interactions, both direct and indirect, between species in a community rather than just monitoring particular charismatic species groups will provide more insight into community health and allow more efficient management programs to be devised (Mills et al. 1993). Work on indirect interactions is also important to be able to further quantify the effects an introduced or invasive species will have on a particular ecological community (Wootton 1994). A model competition study by Case (1990) showed that the invasion success rate of a superior invader plant over a resident inferior species halved when the number of species in the community was increased from one to seven. This study demonstrated that a greater number of strongly interacting resident species in the community reduced the invasion success of species with similar functionality in the community. A more recent meta-analysis study by Levine et al. (2004) concluded that species interactions within a multi-trophic community do not reduce the invasion success but rather reduce the abundance of the invader species once it has become established. In this meta-analysis was found that the below-ground community could act either as a mutualist or parasite on the invader species depending on the invader's identity, thus in some cases

strongly reducing invasion success but in other increasing it (Levine et al. 2004). This highlights how the other species in a community, and the interactions between them, can influence the survivability of particular species of interest.

Species interactions and coevolution

Coevolution occurs when a trait in one population affects the evolutionary trajectory of another population, which in turn also promotes evolutionary change in the first population (Janzen 1980). Species interactions do not necessarily result in coevolution between those species, but they can (Janzen 1980). Interactions between species can sometimes produce very strong coevolutionary relationships, where each species depends on the other to survive and reproduce, such as in the yucca-yucca moth mutualism (Riley 1892). Since coevolution between two species strengthens the interaction between them it is an important factor in the dynamics of ecological communities (Kopp and Gavrilets 2006). However, before coevolution will arise, there is a requirement for each species to have an effect on the other's performance and there needs to be sufficient genetic variation at the loci affecting the trait to allow adaptation to occur (Espinosa and Fornoni 2006). Indirect interactions can enhance or decrease the response of one species to another, which means indirect interactions have the potential to promote coevolution between species (Miller and Travis 1996). Thus, in an interacting ecological community, diffuse coevolution is more important than classic co-evolution - a change in the interaction between two species is expected to affect more than just those species involved and the evolution of a species is expected to be influenced by the numerous interactions it experiences with all the other species in the community (Janzen 1980, Fox 1988). Populations of a species residing within different communities are likely to experience different evolutionary pressures due to the different interactions they have experienced, since community assembly (both species

and genetic) will vary with a number of factors including geographical location (Kersch and Fonesca 2005, Thompson and Fernandez 2006).

Introducing community genetics

Within an ecological community, a variety of species exist and interact, but within each species there is also variation (genetic and non-genetic), which means individuals from the same species will not always respond to stimuli in the same way. The discipline of community genetics was posited by Antonovics (1992), resulting from discussions with Dr. Jim Collins, and was defined as 'the study of the genetics of species interactions and their ecological and evolutionary consequences'. Overall, this area combines the fields of community ecology and evolutionary genetics (Collins 2003, Neuhauser et al. 2003). The aim of community genetics is to understand the role that within-species genetic variation has on the relationship between interacting species and, ultimately, the whole interacting ecological community. As Whitham et al. (2003) state 'community genetics recognizes the simple, but messy, truth that organisms do not live in a vacuum'. There are two approaches to studying community genetics (Figure 1.2). The first approach investigates the influence of genetic interactions between species within the community on a focal species (Tétard-Jones et al. 2007; Figure 1.2a) and the second considers the effect of genetic variation within a focal species on the surrounding community structure of particular groups of species (Whitham et al. 2006, Johnson and Stinchcombe 2007; Figure 1.2b).

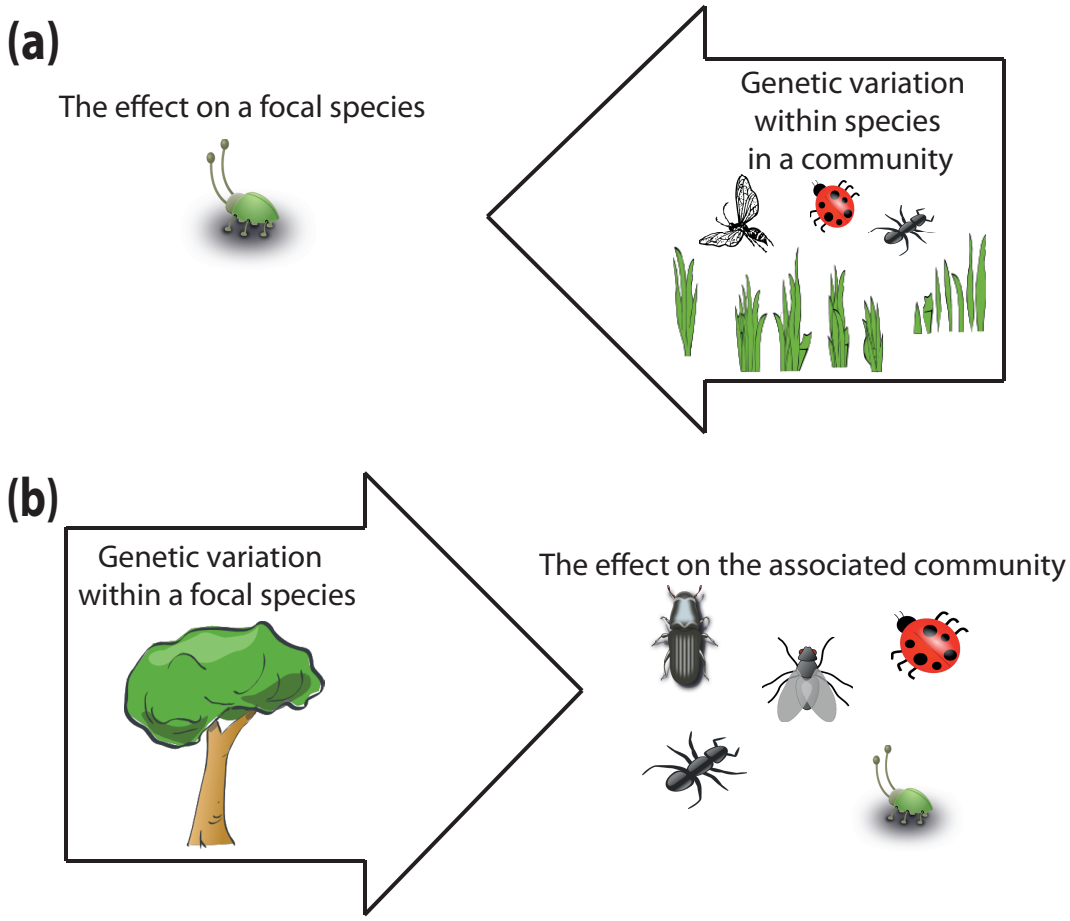

Figure 1.2. Two approaches to studying community genetics

(a) The influence of genetic interactions between species in the surrounding community on a focal species, (b) the influence of genetic variation within a focal species on the surrounding ecological community (Images courtesy of Clker.com, online royalty free public domain clip art).

These ideas originate from the extended phenotype theory (Dawkins 1982) where an expressed gene in one individual is expected to influence more than just the phenotype of the individual itself, it will also have an effect on those which interact with the individual. This implies that genetically-based traits can pre-dispose an individual to interact with particular organisms or lead to the display of specific behaviours that affect other interacting organisms. Studies within the area of community genetics also investigate systems in equilibrium, where co-evolution between naturally interacting

species has shaped the interactions (Whitham et al. 2003), or in non-equilibrium systems, where species have been interacting for a short period of time, for example agricultural systems involving transgenic crops (Neuhauser et al. 2003). Ultimately, the use of community genetics frameworks can improve our understanding of evolution within multi-species systems (Collins 2003).

Genetic variation within a species influences species interactions

Genotypes within a species often vary in their response to an interaction with another species, such that some genotypes will experience a greater effect than others from the interacting species. *Poecilia reticulate* (guppy) genotypes show variation in anti-predator response behaviour, which means some genotypes will more often 'freeze' in the presence of a predator whereas others will perform 'agitated swimming'; this is expected to alter their chance of being predated and therefore influence fitness of the genotypes (Bleakley et al. 2006). Different genotypes within a species also vary in their resistance to disease; for example, within pea aphids (*Acyrthosiphon pisum*) clonal variation exists for resistance to parasitoids and fungal pathogens (Ferrari et al. 2001).

Genotypic variation within a species can also affect the fitness of interacting species. When the genotype of one individual influences the phenotype of a second individual, this is described as an indirect genetic effect (IGE; sensu Moore et al. 1997). For particular genotypes to have specific effects on another individual the underlying genetic differences between genotypes need to be expressed and these also need to cause a detectable change in the phenotype of the second individual. The genetic effect is described as indirect from the interacting individual since it does not originate from the individual itself. IGEs are known to occur between individuals within a species, as shown in *Arabidopsis thaliana* (Mutic and Wolf 2007) and can span across species,

resulting in an interspecific indirect genetic effect (IIGE; Whitham et al. 2006); for example, melon aphid longevity and fecundity is influenced by the host chrysanthemum cultivar (Bethke et al. 1998). These effects can also influence indirect interactions, such that the genetic effect originates from a species that is indirectly interacting with the focal species. This has been demonstrated in a system with ladybirds and aphids that have either fed on potato plants expressing snowdrop lectin, an aphid resistance gene (*Galanthus nivalis* agglutinin; GNA), or potato plants which do not express this gene (Birch et al. 1999). In this study, ladybirds feeding on aphids that were reared on snowdrop-lectin-expressing potato exhibited reduced longevity, egg viability and fecundtiy compared to those feeding on aphids reared on control plants (Birch et al. 1999). This shows that a single expressed gene in a plant can indirectly affect a species, which is two links away in a chain of direct interactions. Therefore, IIGE's can have an effect on directly and indirectly interacting species.

When the phenotype of a particular genotype alters within different environments it is termed phenotypic plasticity (West-Eberhard 1989). This environment can be abiotic (e.g. pH, temperature) or it can biotic, from interactions with other organisms. Phenotypic plasticity can create variation in both the direction and magnitude of ecological interactions, since not all individuals will respond to the same stimuli in a similar manner (Fordyce 2006). If there is genetic variation within a species for phenotype plasticity, i.e. different genotypes show variation in responses to the environment, a genotype-by-environment (GxE) interaction is obtained (Falconer 1952; Figure 1.3). If all genotypes within a species respond to a change in environment in the same way then no interaction term will occur.

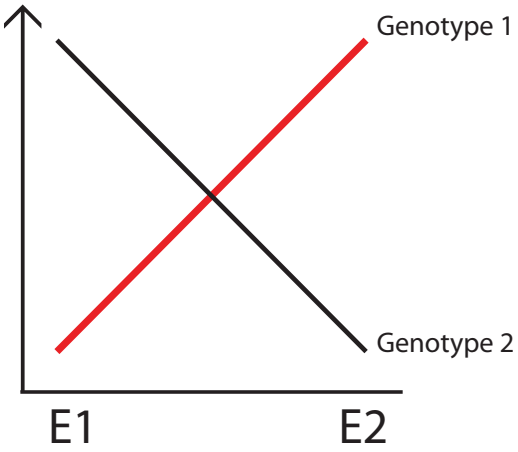

Figure 1.3. Genotype-by-environment (GxE) interaction
These interactions occur when genotypes differ in their response to environmental change. E1 and E2 represent two different environments, which can be abiotic or biotic.

Following a reciprocal experimental method with clones of the plant *Borrichia frutescens* on different islands, Stiling and Rossi (1996) showed that plant stem-tip diameter and nitrogen content were influenced by a GxE interaction between plant clone and 'receptor' island. The authors also showed that these GxE interactions influenced traits associated with a galling midge, which oviposits on the plants, including gall diameter, gall abundance and the number of parasitized midges. This study demonstrated the influence GxE interactions can have on the species themselves (*Borrichia* plants), directly interacting species (galling midges), and indirectly interacting species (parasitoids) (Stiling and Rossi 1996). GxE interactions between evening primrose genotype and abiotic habitat have been shown to influence the structure of the arthropod community found on the plants, thus the associated arthropod community is a function of both the plant genotype and environment combined (Johnson and Agrawal 2005). The environment may also be a biotic factor such as the presence of another species; for example, *Rhopalosiphum padi* aphid genotypes show variation in fecundity and longevity due to the environment of the presence or absence

of endosymbiotic fungi (*Neotyphodium coenophialum*) within the host plants (Bieri et al. 2009).

When the biotic environment is comprised of different genotypes from another species, a genotype-by-genotype (GxG) interaction between the two species is established, i.e. the environment term from the GxE interaction is replaced by 'genotype' from the second species. A GxG interaction between individuals belonging to different species could also be described as a case of a reciprocal or two-way IIGE if it has an effect on both species involved. In 1984, Service demonstrated that fitness traits of an aphid (*Uroleucon rudbeckiae*) and plant (*Rudbeckia laciniata)* were influenced by GxG interactions between these species. This means the specific combination of interacting genotypes is important for the outcome of the interaction. These interactions are also known to occur in a host-parasite system involving the freshwater crustacean, *Daphnia magna,* and a bacterial parasite, *Pasteuria ramosa* (Carius et al. 2001). Unless the interacting species reside in a two-species community, it is expected that they will also experience interactions (direct and indirect) with other species. The presence of another species in the community can alter the outcome of the GxG interaction between two species, creating a genotype-by-genotype-by-environment (GxGxE) interaction (Figure 1.4). Tétard-Jones et al. (2007) used an aphid-barley model system to show that the addition of rhizobacteria into the system altered the outcome of the genotypic interactions between the aphid and barley. They demonstrated that 31.8% of the variation in aphid performance and 42.4% in barley performance was explained by the GxGxE interaction between aphid genotype, barley genotype and environment of presence/absence of rhizobacteria.

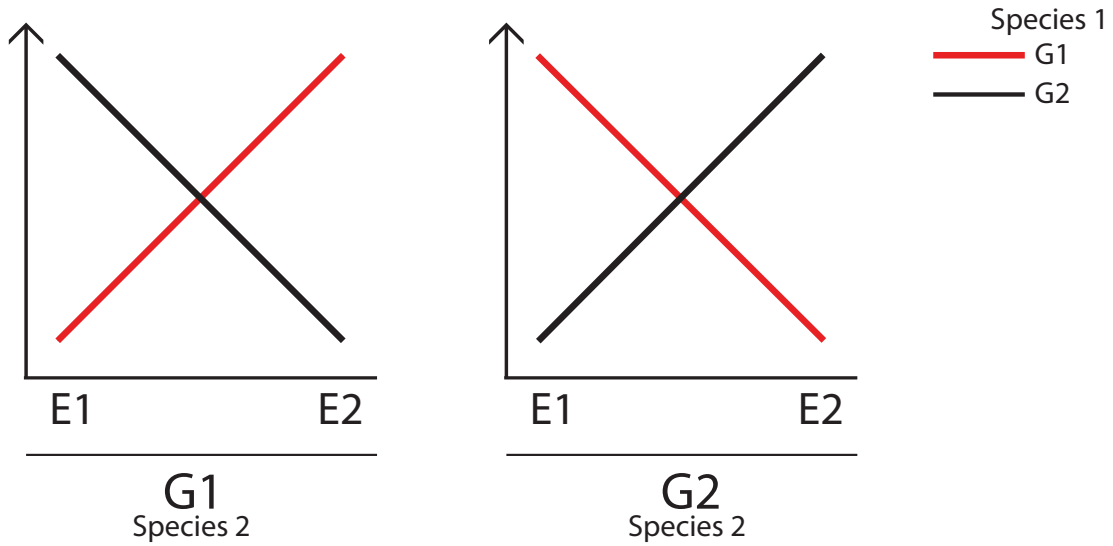

Figure 1.4. Genotype-by-Genotype-by-Environment (GxGxE) interaction
These interactions occur when genotypes (G1 and G2) from species 1 respond differently to a change in environment (E1 and E2) dependant on the genotype of the interacting species (G1 and G2 from species 2).

Phenotypic plasticity can mediate evolutionary changes between interacting species, since different genotypes within a species can respond in a variety of ways to the interaction stimulus (Fordyce 2006). This can also lead to an increase in phenotypic diversity within and between populations, especially if there is genetically-based variation for phenotypic plasticity (i.e. GxE or GxG interactions) in a population. If a response to an ecological interaction is unique and common within a population there is potential for this to cause genetically-based changes in this population, through adaptative evolution (Fordyce 2006). However, high genotypic diversity within a population will increase the effects of GxG or GxE interactions and reduce the rate of adaptive evolution, due to the number of different interactions among genotypes diffusing the effect of each single interaction (Fordyce 2006). If assortative association occurred between particular genotypes of interacting species, i.e. two genotypes were more often observed interacting with each other than with other genotypes, the rate of adaptive evolution may be increased.

At the community-level, within-species genetic variation in a number of plant species has been shown to influence the surrounding ecological community structure of a variety of species groups, indicating that genetically-based traits within particular species can have extended phenotype effects on other species residing in the community. In cottonwood trees, more closely related trees are host to more similar communities of leaf-modifying arthropods at both a local and a regional scale (Whitham et al. 2003, Bangert et al. 2008). The influence of genetic variation on invertebrate community structure has also been demonstrated in willow trees (Fritz and Price 1988, Hochwender and Fritz 2004), goldenrods (Maddox and Root 1990), rabbitbrush (Floate et al. 1996), eucalyptus (Dungey et al. 2000, Barbour et al. 2009) and evening primrose (Johnson et al. 2006). Although invertebrates are, by far, the most studied group of organisms in this area, other work has shown that genetically-based chemical diversity in Scots Pine influences the understory plant community structure (Iason et al. 2005) and genetic variation in an oak hybrid population influences the community of endophytic fungi in the tree leaves (Gaylord et al. 1996). Studies on the cottonwood system, have also shown that beavers preferentially harvest particular genotypes of trees (Bailey et al. 2004) and this has a knock-on effect on sawfly density, which are more abundant on browsed than unbrowsed trees (Bailey et al. 2006).

Genetic variation within the cottonwood tree hybrid system also indirectly influences other communities, such as nearby aquatic fauna (LeRoy et al. 2006). These effects are weaker than observed for the arthropod communities directly interacting with the same trees. This is not unexpected and supports a genetic diffusion hypothesis that due to cascading effects the influence of tree genotype is weaker when a community depends on the tree to a lesser extent (LeRoy et al. 2006). Within-species genetic variation has also been shown to influence ecosystem processes such as nutrient release of carbon,

nitrogen and sulfur (Schweitzer et al. 2004, Madritch et al. 2006). These studies highlight the importance of considering within-species genetic variation when investigating ecological interactions and community dynamics.

Genetic variation within a species can therefore influence the phenotypes of interacting species, the structure of the associated ecological communities and potentially drive evolution within an interacting species. The association of particular species, such as herbivores on a specific host plant species or arthropods on a particular genotype of tree, could occur through passive or active mechanisms. For example, orchid colonization of oak trees (*Quercus sp.*) in Mexico is limited to two of five tree species due to inhibitory chemicals in the bark, which reduce germination success in three of the tree species (Frei and Dodson 1972). This is an example of a passive mechanism influencing the species that interact, since the orchid species experience differential survival due to bark substrates once a seed has already settled. Alternatively, in a two-choice disc test, potato aphids (*Aulacorthum solani*), onion thrips (*Thrips tabaci*) and two-spotted spider mites (*Tetranychus urticae*) all show host preference against plants expressing snowdrop lectin (GNA), an aphid resistance gene (Rovenska and Zemek 2006). The numbers of aphids and thrips found on the control plant discs were significantly greater than those on the GNA-expressing plant discs even after only one hour, suggesting the invertebrates actively move to these discs rather than this occurring through a reduction in fitness (reproductive success).

Host choice and association

Host choice behaviour can maintain interactions between species, and genotypes within species, by creating associations between specific individuals and reducing random associations between individuals within a community. Understanding host choice

behaviour and preference could enhance the predictability of species interactions and evolutionary implications in a community (Gorur et al. 2007). In an experimental system, pea aphid (*Acyrthosiphon pisum*) preference for particular host plant species differed among host races with each host race preferentially colonizing their plant species of origin but exhibiting variation in preference for another seven plant host species (Ferrari et al. 2006). A number of insect species are also vectors for many economically important diseases of crop plant, increasing the importance of understanding host choice preference in insect-plant interactions. For example, a study on grape (*Vitis sp.*) demonstrated that the glassy-winged sharpshooter (*Homalodisca vitripennis*), which transmits the disease causing bacterium *Xylella fastidiosa*, exhibits host preference for particular grape genotypes and preferentially colonizes the 'Chardonnay' genotype, which is an economically important genotype for wine production (Fritschi et al. 2007).

In plants, host preference often occurs due to post-dispersal passive mechanisms, for example seedling establishment and germination may be influenced by a variety of abiotic and biotic factors that differ among microclimates created by different species or genotypes within a species. In a tropical natural ecosystem, a number of vascular epiphyte species show host preference for certain host tree species (Laube and Zotz 2006). The data within the study by Laube and Zotz (2006) suggests there is variation within a tree species for epiphyte abundance, although the effect of within-species genetic variation was not been tested. Host preference can also occur indirectly, for example, the composition of the non-vascular epiphyte community on particular trees is correlated with the abundance of the vascular epiphyte, *Tillandsia useneoides* (Callaway et al. 2001). When the authors grew *T. useneoides* with extracts from different non-vascular epiphytes they found that extracts from *Cryptothecia rubrocincta*

lichen reduced plant survival and leaf length, whereas extracts from *Parmotrema sp.* lichens increased survival and leaf length. Both these lichens are found in different abundances on different tree hosts suggesting a route of indirect host preference from tree to lichen to vascular epiphyte.

Ecological specialization

Host preference can lead to host-associated differentiation (HAD), such that particular species or genotypes within a species become associated with a specific host leading to adaptation to that host and genetic differentiation between the species or genotypes. There is an increasing amount of theoretical and empirical evidence suggesting that genetic differentiation between host races or phytophagous insects could lead to ecological specialization and speciation in sympatry (Stirman et al. 2005). Host-associated genetic differentiation has been documented in the apple maggot fly (Feder 1998), pea aphids (Via et al. 2000), a bud-galling herbivore mite (*Aceria parapopuli*; Evans et al. 2008) and a number of galling insects associated with goldenrods (Stireman et al. 2005). However, the use of a universal host in a population, i.e. one that all genotypes will readily colonize, will reduce the effects of host-associated genetic differentiation and therefore reduce the chance of speciation (Ferrari et al. 2006). Within pea aphids, host races are genetically adapted to their plant of origin as indicated by the finding that aphid fecundity and longevity is not increased on an alternate host plant even after three generations of experience (Via 1991). In a study on *Sitobion avenae* cereal aphids it was found that experience on an alternate host plant for only single generation was sufficient to increase aphid performance on that plant, although there was variation among clones for this effect (De Barro et al. 1995). This indicates that in these cereal aphids there was enough variation and gene flow between the aphids to reduce any effect due to adaption to the plant of origin, however, gene flow between

the pea aphid host races seems to have been restricted resulting in host-associated genetic differentiation between them. HAD can also influence the rest of the interacting community through cascading effect, such as parasitoids (Stireman et al. 2006). Stireman et al. looked at two different parasitoid species, which use different host insects, on two sympatric goldenrod species. They found that there was morphologically cryptic genetic differentiation within each parasitoid species, dependent on which plant the host insect was collected from; one parasitoid species exhibited distinct genetic differences, suggesting long-term effects of cascading-HAD, whereas the other exhibited subtle differences suggesting these populations have only recently diverged and gene flow still occurs between them.

The link of between host-associated genetic differentiation, ecological specialization and, ultimately, speciation requires the potential for evolutionary change within the physiological, morphological or behavioural traits associated with the restricted resource use (Caillaud and Via 2000). Ecological speciation can be defined as 'the process by which barriers to gene flow evolve between populations as a result of ecologically-based divergent selection' and requires a source of divergent selection, reproductive isolation and a genetic mechanism which links selection and reproductive isolation (Matsubayashi et al. 2010). Two host races of pea aphids, genetically adapted to two different host plants, exhibit an unwillingness to feed on the alternate host plant, which could lead to reproductive isolation and speciation, via assortative mating within each host race, i.e. a member of one host race is more likely to mate with a member of the same host race rather than the other (Caillaud and Via 2000). These authors show that nutritional deficiencies are not reducing aphid fitness on the alternative host plant, rather it is the rejection of the plant prior to phloem location which leads to reduced aphid fitness when restricted to this plant. However, the potential for differences in

plant defenses cannot be ignored as a potential cause for rejection of the alternate host (Caillaud and Via 2000).

Interactions between species, and between genotypes across species, can enhance ecological speciation by creating ecologically-based divergent selection (Matsubayashi et al. 2010). Therefore, by further investigating the genetics of species interactions in a community, aspects such as community structure, dynamics and the evolution of species within a multi-species assemblage can be better understood.

THESIS RATIONALE

This thesis follows a community genetics framework to further understand the influence within-species genetic variation can have on the interactions between species and on associated communities. I use two complementary approaches, as discussed in the thesis introduction (Figure 1.2), to investigate community genetics in model and natural ecosystems.

Model ecosystem

I begin in chapter 2 by determining if GxGxE interactions between three species in a model aphid-barley-rhizobacteria community can influence a fourth species. Previous work has shown that rhizobacteria can alter the GxG interactions between aphid and barley genotypes (Tétard-Jones et al. 2007). In this paper, we show that the indirect ecological effect (IEE) of a plant-growth-promoting rhizobacteria on a parasitoid wasp is influenced by genetic interactions between barley and aphid genotypes. This work was published in Ecology (June 2010) entitled 'Community genetic interactions mediate indirect ecological effects between a parasitoid wasp and rhizobacteria'. This paper showed that GxG interactions between the aphid and barley mediate interactions between other species in this community, but it is unknown how or if these interactions are maintained across generations.

Previous work has indicated that different aphid genotypes show preference for particular host plant species (Via 1991, De Barro et al. 1995, Nikolakakis et al. 2003, Ferrari et al. 2006, Gorur et al. 2007). In chapter 3, I used choice and no-choice experiments to investigate the preference of the different aphid genotypes to six barley genotypes, and showed that different aphid genotypes preferentially colonize different barley genotypes. If particular aphid genotypes actively colonize (i.e. not a function

differential aphid growth rate on barley genotype) particular genotypes of barley then this can lead to assortative associations between the aphid and barley genotypes, and suggests a mechanism by which GxG interactions could be maintained between these species.

Chapter 3 only considered host preference when an aphid genotype was reared alone; however, aphids are unlikely to exist in single genotype populations in nature. Therefore, in chapter 4, I carried out another experiment that investigated the effect of intraspecific competition between aphid genotypes on host genotype preference. This work showed that host genotype preference of an aphid could be altered when reared in competition with a second aphid genotype. There was co-existence of aphid genotypes on each plant suggesting that direct competition between genotypes did not explain all the effects seen. Indirect competition via plant-mediated traits is common between phytophagous insect species (Denno et al. 1995) and could provide a mechanism for the change in host preference seen by the aphid genotypes.

In my final model ecosystem paper (chapter 5), I confirmed that the interaction between aphid genotypes occurs via plant-mediated traits. The second part of this paper used microarrays to investigate induced gene expression in a single barley genotype due to exposure to different aphid genotypes. Differential expression of genes has been demonstrated within different barley genotypes due to aphid exposure (Delp et al. 2009) and we show that different aphid genotypes induced differential gene expression in a single barley genotype.

Natural ecosystem

The first four papers in this thesis investigated species interactions in a model ecosystem, and followed a gradually more reductionist pathway ending with identifying

particular gene pathways that may be involved in GxG interactions between an aphid and barley. Using this approach, a number of genotypes were experimentally manipulated to be able to quantify the amount of phenotypic variation in a species that is explained by interactions with other species in the system (see Figure 1.2a). These experiments allow the importance of species interactions to be determined, but because they are lab studies they may not reflect the true impact of these interactions in a natural system (Carpenter 1996). In order to further understand the importance of species interactions and community genetics in natural systems one must approach the questions in an alternative way to that used in model systems (Figure 1.2b).

In chapter 6, I considered the association between within-species genetic variation in a tree and the associated invertebrate and plant communities. The literature on community genetics is thus far restricted to temperate species (Whitham et al. 2006), which are often in ecosystems with limited species diversity. Chapter 6 of this thesis investigated the influence of genetic variation within a tropical tree in a complex rainforest system in Belize, Central America on communities of vascular epiphytes and terrestrial invertebrates. This paper has been accepted for publication in the Philosophical Transaction of the Royal Society: Biological Sciences (community genetics special issue) entitled 'Genetic variation in a tropical tree species influences the associated epiphytic plant and invertebrate communities in a complex forest ecosystem'. In this paper, we showed that more closely related trees were host to more similar communities of vascular epiphytic plants, leaf-litter invertebrates and trunk-dwelling invertebrates.

Prior to the genetic study for chapter 6, I conducted an investigation to determine whether the Amplified Fragment Length Polymorphism (AFLP) genetic profiles obtained from two tissues of the tree (leaf and cambium) differed. This work was

undertaken since it has previously been suggested that a genetic study should use only a single tissue throughout (Colpaert et al. 2005), due to potential discrepancies between profiles obtained from different plant tissues in a single individual (Donini et al. 1997). We found there to be no differences between using cambium and leaf tissue, but discuss the merits of using each tissue plus the benefit of using both tissues in a single genetic study, in chapter 7.

The final thesis discussion chapter brings together all the work presented in this thesis and shows how my research has contributed to the area of community genetics. I also discuss the potential applications of community genetics research.

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Chapter 2. Community genetic interactions mediate indirect ecological effects between a parasitoid wasp and rhizobacteria

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PERSONAL CONTRIBUTION

I carried out this experiment with a final year undergraduate student (Sarah Fleming) and support from Richard Preziosi. I statistically analysed the data, wrote the initial drafts of the paper and then refined it with R. Preziosi, alongside comments from C. Tétard-Jones and M. Kertez. Additionally, C.Tétard-Jones had completed preliminary experiments with this system and M. Kertesz provided the rhizobacteria strain.

ABSTRACT

Indirect ecological effects (IEEs) clearly influence species dynamics and abundance, yet relatively little is known about how they influence the evolution of species involved. While genetic variation in the species causing and responding to the IEE has obvious effects, the influence of genetic variation in intermediate species remains unexamined. Given the often counterintuitive responses of populations to IEEs this seems a significant omission. Following a community genetics approach, we used a model tetra-trophic system (parasitoid wasp, aphid, barley and rhizobacteria) to investigate the effect of genetic interactions within the two linking species (aphids and barley) on the IEE of rhizobacteria on wasps. We show that 12.4% of the variation in wasp size, a proxy for fitness, is explained by higher-order interactions between aphid genotype, barley genotype and presence or absence of rhizobacteria ($Genotype_{Barley}xGenotype_{Aphid}xEnvironment_{Rhizobacteria}$). Thus, the IEE of rhizobacteria on the parasitoid wasp is influenced by the specific combination of aphid and barley genotypes that mediate the interactions. In some cases changes in the genotypes of the intermediate species completely reverses the effect of rhizobacteria on wasp size. Our work demonstrates that within-species genetic variation is important in shaping IEEs in communities, an essential component of community evolutionary processes.

INTRODUCTION

Indirect interactions in a community often occur through linked chains of direct interactions. When one species influences another through mutual interactions with a third (or more) species it is called an indirect ecological effect (IEE; Wootton 1994, Astles et al. 2005). A simple way to study IEEs is to look at trophic systems, where many species are linked through energy transfer. Trophic systems also provide a more realistic experimental approach to studying communities than using a single trophic level. Within trophic systems, indirect interactions are known to often have strong effects and can cause unexpected results in species' responses to interactions with others (Wootton 1994, Miller and Travis 1996, Berlow 1999). For example, a predator is expected to reduce prey abundance but in some cases it can cause an increase in prey abundance, potentially through reducing competition between different prey species. Such 'unexpected effects' are thought to be relatively common but will only become apparent when the positive indirect effects (increasing abundance through decreasing competition) outweigh the direct negative effects (mortality through predation) (Sih et al. 1985). Further, empirical work on interaction food webs shows that indirect interaction effects increase exponentially per species with each addition of a new species (Menge 1995). Studying indirect interactions between species will therefore develop our understanding of community composition and dynamics further than studies that focus on direct interaction effects.

The majority of work on indirect interactions in communities has investigated how the change in abundance of a species influences the abundance of other species in the community (Wootton 1994, Muller and Godfray 1999). This work allows the impact of species extinctions or introductions to be predicted, however, it does not tell us how IEEs could influence a trophic community through evolutionary processes. Evolution

within a species is a change in allele frequency over time, it can occur when there is genetic variation for a trait and selective forces act on that trait, due to some genotypes being superior in the current environment than others (Mopper 1996). The environment experienced by an individual will include biotic factors, such as the phenotype (or expressed genome) of interacting individuals, as well as abiotic factors. Indirect effects, including IEEs, are expected to be influential in the evolution of species within a community but only when they have strong ecological effects (Miller and Travis 1996). To understand more about the evolutionary influences of indirect effects within a community we must first understand how genetic variation can affect indirect interactions between species. The field of community genetics aims to understand the impact of within-species genotypic variation on species interactions and community structure.

Previous community genetics work using model communities has shown that there is genetic variation in response to an IEE in a tri-trophic system (ladybirds exhibited variation in responses when fed on clonal aphids, which were raised on two different host plants) and suggested that there is potential for IEEs to be a strong selective force in a community (Astles et al. 2005). It has also been demonstrated that within-species genetic variation is important for directly interacting species (Service 1984, Mopper 1996) and the effect can be mediated by the presence or absence of a third species in the community (Tetard-Jones et al. 2007). Thus, there is clear evidence showing that genetic variation within a species will affect directly interacting species; however, we do not know to what extent genetic variation, and genotypic interactions, within intermediate species will affect the outcome of indirect interactions. This is an important omission seeing that indirect interactions can be strong selective forces when they have a strong ecological effect.

If genetic variation within a species influences IEEs, this will lead to different outcomes in different populations potentially influencing the evolutionary trajectory of a species differently among populations. The number of genotypes in a population is only a subset of the available genotypes for a particular species, leading to differences in genotypic diversity between populations. Since this is true for every species in a community, it means that the genotype combinations among interacting species will be different between populations producing a number of different responses to an IEE. The overall response of a population (positive, negative or negligible) is determined by the frequency of the genotypes within a population and the resulting frequency of each inter-specific genotypic interaction. For example, two populations of a species may reside in different communities with similar species diversity, but every species within this community has a different subset of genotypes than other communities. The resulting response of the species to an IEE is likely to differ in both direction and magnitude depending on which genotypes of each species are present.

Here, we have used a quantitative community genetics approach to quantify the magnitude of within-species genetic variation on an IEE. By using quantitative genetics methods, phenotypic variation in a species can be partitioned out into the effects of its own genotype, the genotype of other species and the interactions between these genotypes assuming the abiotic environment is controlled. We use a multi-species model ecosystem, which allows manipulation of numerous genotypes of different species, through use of clonal species and homozygous lines. We investigated the effect of indirect interactions using a tetra-trophic system of a plant-growth-promoting rhizobacteria, barley, an aphid and a parasitoid wasp. We show that the IEE between the rhizobacteria and the parasitoid is mediated by genotypic interactions between the two intermediate species (barley and aphids). This study is the first to focus on the influence of genotypic interactions using a system involving four trophic levels.

MATERIALS AND METHODS

Experimental design

A fully factorial design was used consisting of four barley genotypes, four aphid genotypes, rhizobacterial treatment (addition of rhizobacteria versus no addition of rhizobacteria), producing 32 treatments. Each treatment was replicated 5 times, giving a total of 160 plants. Plants were grown in a randomized design.

Rhizobacteria preparation

Single colonies of *Pseudomonas aeruginosa* 7NSK2, known to have plant-growth-promoting effects (Iswandi et al. 1987), were chosen from a streak plate and grown aerobically in LB medium (Sambrook et al. 1989) for 24 hours at 30°C. The bacteria were harvested by centrifugation (13000rpm for 13 minutes), washed three times in 10mM $MgSO_4$ and resuspended at $A_{600} = 1$, using 10mM $MgSO_4$ as a diluent. The suspension was used directly for root inoculation of seedlings.

Plant material

Hordeum vulgare accessions Morex, Steptoe, BCD47 and Baronesse (originally from P.Hayes, Oregon State University) were used, which had been grown in a common environment with genotypes isolated from each other to ensure selfing occurred prior to the experiment. The seeds were sterilized by soaking in 10% NaOCl for ten minutes, on a rotary shaker, and then washed six times in sterilised distilled water. The seeds were germinated by placing them between two pieces of filter paper that had been soaked in sterilised distilled water, and then kept in the dark at 23°C for 5 days. Seedlings of uniform shoot and root length were chosen for the experiment. Seedling roots were inoculated with bacteria by submerging in bacterial suspension for 1 hour (control

plants were submerged in 10mM $MgSO_4$) and then seedlings were then transplanted into 10cm pots containing heat sterilized horticultural sand, which had been watered with tap water the previous day. Each pot contained a single plant for the duration of the experiment. The experiment was undertaken in a glasshouse at the Firs Experimental Research Station, University of Manchester using supplemental mercury lights to provide a 16:8 L:D regime and with a daily temperature range of 15-25°C. Plants were watered with 40ml full strength Hoaglands solution (Hoagland and Arnon 1950) twice a week for the first three weeks and once a week until the end of the experiment. If the sand was dry between Hoaglands applications, tap water was given. Three days prior to aphid introduction, plastic tubes with mesh tops and mesh windows were placed around each plant to isolate it from the others (described in Tetard-Jones et al. 2007).

Aphids

Sitobion avenae genotypes HF92a, H1, CLO7 and DAV95 were supplied by Rothamsted Research, Harpenden, UK. Aphids were reared on *Hordeum vulgare* accession B83 prior to the study in isolation cages. Eleven days post-transplantation of the barley, one adult aphid was placed on the corresponding plants. When nymphs were produced, all aphids but two nymphs were removed from the plant and the nymphs allowed to develop and reproduce. This process was done to remove the effect of maternal rearing environment. Aphid numbers were counted to assess aphid fitness but those data are not reported here as we found a similar effect to that already described in Tetard-Jones et al. (2007).

Parasitoid measurements

Aphidius rhopalosiphi wasps were supplied by M. Torrance, Rothamsted Research, Harpenden, UK. The parasitoid wasps were reared, in a large population, on aphid

genotype DAV95 (also used as an experimental genotype) and barley genotype B83 (not used as an experimental genotype) prior to the study, grown in John Innes compost No.3. The results show no bias towards aphid DAV95 used to rear the parasitoid wasps and therefore we assume there are negligible conditioning effects from using this genotype in the experiment. A single randomly mated female parasitoid was placed onto each plant 40 days after seedling transplantation (29 days after aphid introduction). Mummified aphids were collected from each plant, within one week of releasing the parasitoid, and kept in glass vials until the new parasitoids emerged. Up to 8 emerged females parasitoids were dropped in ethanol and then the wings removed using tweezers and mounted on glass slides to allow measurements to be taken. Wing morphological measurements were taken of costal vein length (Figure 2.1) by taking digital photographs using a light microscope with mounted camera (Leica, Wetzlar, Germany). Costal vein length was measured using software Image J (Abramoff et al. 2004). Wing size is used as a measure of parasitoid fitness, since it is a general measure of body size which correlates positively with fitness traits such as egg load and fat reserves (Visser 1994, Ellers et al. 1998, Kolliker-Ott et al. 2003).

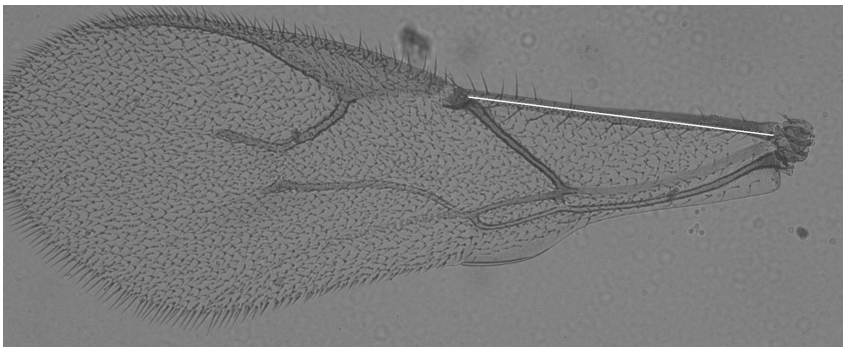

Figure 2.1. Diagram indicating measured costal vein on a parasitoid wasp (white line)

Data Analysis

A generalized linear model (GZLM) was used to analyse the effect of variation within the Rhizobacteria-Barley-Aphid system on costal vein length in emerged parasitoids. Mean parasitoid wing sizes among the treatments were calculated and analysis was performed on this data. A model with linear scale response, identity link function, normal distribution, maximum likelihood scale parameter estimate and a robust covariance matrix estimator was used. The Wald chi-square statistics was used to analyse the significance of each term in the model. Variance component analysis (Maximum Likelihood method) was used to determine the amount of variance explained by each term in the model. For the VCA we used each parasitoid as an individual data point, in order to further understand the effect the GxGxE interaction is having on the parasitoid population; rhizobacteria was a fixed effect, with barley and aphid being random effects in the analysis. Analyses were performed using SPSS v14.0 for Windows. Pixel length was used in the analysis to reduce the error associated with converting the data into length measurements. However, pixel data was converted into millimetres using a micrometer calibration slide on the microscope for result interpretation.

RESULTS

A total of 338 female parasitoid wasp wings, across all 32 treatment groups, were collected and measured. The wasps measured had a mean costal vein length of 0.808mm and length ranged from 0.529mm to 0.983mm, following a normal distribution. Within each treatment the results followed a normal distribution. The costal vein length (wing size) of the parasitoid was significantly affected by the GxGxE interaction term between the aphid, barley and rhizobacteria treatments (Wald Chi-square=37.574, $p<0.001$; Table 2.1).

Table 2.1. Results from a generalized linear model (GZLM) and variance component analysis of the effect of aphid genotype, barley genotype, rhizobacteria environment, and interactions between these factors, on parasitoid wing costal vein length.

Source of Variation	Effect	Wald Chi-square	df	Significance (p-value)	% variance explained
Rhizo	Main	1.703	1	0.192 (NS)	-
Barley	Main	11.232	1	0.011 *	0.0
Aphid	Main	51.224	3	<0.001 ***	11.4
Barley * Rhizo	GxE	16.474	3	0.001 ***	0.8
Aphid * Rhizo	GxE	6.405	3	0.094 (NS)	0.0
Barley* Aphid	GxG	26.161	9	0.002 **	0.0
Barley * Aphid * Rhizo	GxGxE	37.574	9	<0.001 ***	12.4

Notes: Results for the full GZLM model are shown. Variance component analysis was performed on data using individual parasitoids and a maximum-likelihood method. Key to abbreviations: G, genotype or the specific genetic makeup of a particular individual, e.g., a particular clone of aphid; E, environment, the environment experienced by an individual, e.g., the presence or absence of rhizobacteria.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS = nonsignificant ($P > 0.05$).

Parasitoid wing size is influenced by whether or not rhizobacteria are present in the system, with the direction of the IEE being dependent on the combination of aphid genotype and barley genotype (Figure 2.2). For example, parasitoids emerging from CLO7 aphids grown on Morex barley are larger when rhizobacteria are present than when absent. However, parasitoids emerging from CLO7 aphids grown on BCD47

barley are smaller when rhizobacteria are present than when absent. The largest negative effect of the presence of rhizobacteria on parasitoid wing size was when parasitoids emerged from HF92a aphids raised on Baronesse barley; the mean parasitoid wasp costal vein length was 0.190mm greater when no rhizobacteria were present than when rhizobacteria were present. The largest positive effect of rhizobacterial presence on parasitoid wasp size was with DAV95 aphids reared on BCD47 barley, when the presence of rhizobacteria increased costal vein length by 0.125mm. The variance component analysis shows that the GxGxE interaction between rhizobacteria, aphid and barley is explaining 12.4% of the variation in parasitoid wing size (Table 2.1).

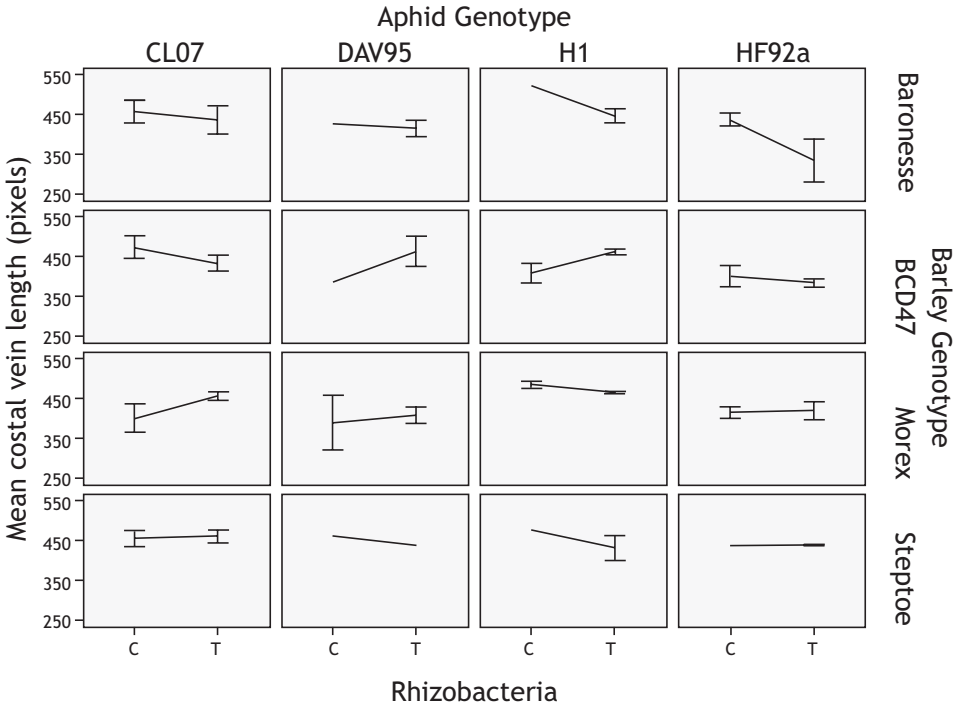

Figure 2.2. Phenotypic response of the parasitoid wasp to the IEE of rhizobacteria is dependant on the combination of aphid and barley genotypes in the model
Measured trait is parasitoid wing costal vein length (pixels); each separate graph shows untreated control with rhizobacteria on the left (C), and treated on the right (T). Each row is the result for a different barley genotype and each column for a different aphid genotype. Error bars represent ±1 standard error. For each treatment with a single plant replicate, the data is the average of either 4 or 5 parasitoids emerged from the one plant, except for treatment Control-H1-Baronesse, which is from a single parasitoid.

DISCUSSION

Indirect ecological effects can be highly influential on the phenotype of higher organisms in a trophic system. In this study, we showed that the wing costal vein length of a parasitoid wasp is affected by GxGxE interactions between the three species below it in a tetratrophic system (an aphid, barley and rhizobacteria). This means that the interaction between aphid genotype, barley genotype and presence or absence of rhizobacteria is having a strong effect on the phenotypic variation of parasitoid wing size, in our model system; we show that 12.4% of the variation in parasitoid wing size is explained by the GxGxE interaction. Both the aphid and barley genotypes we used are clonal and thus natural sexually reproducing populations may show smaller interaction effects because of greater heterozygosity within species and a reduced genetic covariance between species.

Although parasitoid genetics were not included in our model, it is expected that this will explain much of the remaining variation in wing length since body size in a related parasitoid species, *Aphidius ervi,* has been shown to have a broad sense heritability of 30% in females and 60% in males (Gilchrist 1996). There are many possible mechanisms for the influence of the GxGxE interaction between rhizobacteria, barley and aphid on parasitoid wing size. The rhizobacterium we used is a plant-growth-promoting bacterium and may differentially affect the barley growth across genotypes, also the different barley genotypes may respond differently to aphid attack and differently between aphid genotypes. This may all influence the aphid population through aphid size, number or aphid physiology, which can in turn affect the growth of the parasitoid in the aphid host. However, the mechanisms for the effect of the 3-way interaction on parasitoid wasp size have not been determined.

Effect of genetic variation on IEEs

We have shown that an IEE can span across two species but, importantly, we show that the effect of the IEE is mediated by the genotypic interactions within the intermediate species. It is notable that genotypic changes in the intermediate species can completely reverse the effect of rhizobacteria on the parasitoid wasp. This suggests a mechanism by which IEEs might produce differential results among a number of different populations. As we show, IEEs can produce positive and negative responses dependent on which genotypes are interacting and the relative frequencies of these responses (determined by the relative frequencies of the genotypes) will produce the overall response of a population. In our system, if the majority of IEEs in a population produced positive effects this would result in a larger mean parasitoid costal vein length than in a population dominated by negative effects. Indirect interactions often strengthen community structure (Menge 1995, Berlow 1999) and we show they may be highly influenced by genetic variation within intermediate species, therefore we argue that understanding genetic diversity at all levels is highly important when considering community structure or dynamics.

Potential evolutionary effect of IEEs

It has been shown that there is genetic variation for a response to an IEE (Astles et al. 2005), which means that there is the potential for IEEs to alter the evolutionary trajectory of a species. In addition, we found that genetic variation in the intermediate species mediated the effect of the IEE, so potentially the interspecific genotypic interactions between the aphids and barley, in our system, will affect the IEE of the rhizobacteria and alter the evolutionary trajectory of the parasitoid wasp. Within a population, if the majority of aphid-barley combinations influenced the IEE to produce a positive response in the parasitoid (larger wing size) then the mean size of parasitoid

wing may be expected to increase over the next generations. However, irrespective of the fact that interactions between the aphids and barley strongly influence the morphology, and thus the fitness of the parasitoid (Visser 1994, Ellers et al. 1998, Kolliker-Ott et al. 2003), if there is no selective effect there will be no resulting change in the species over time. For an evolutionary trajectory to be altered by these interspecific interactions there would need to be a non-random association of genotypes in the community. Random associations of genotypes would cause any effects to average out over time, although they might occasionally have a strong effect. A non-random association of genotypes might occur through differential survival or through assortative association. For example, non-random association of aphid and barley genotypes might occur either due to differential survival of aphid genotypes on different barley genotypes (Tetard-Jones et al. 2007), through aphid genotypes preferring different barley genotypes as hosts or as an influence of parasitoids attacking different aphid genotypes depending on barley host. In either case a genetic correlation would be established between the two species. Given current agricultural strategies of planting large areas with a small number of barley genotypes such associations appear entirely plausible. The extent to which such associations occur in more complex natural communities is unclear as they are rarely examined. However, random association in natural communities cannot be assumed, as demonstrated by the preference or host associated differences (HAD) for a number of phytophagous insects to different goldenrod genotypes (Stireman et al. 2005). Furthermore, the recent discovery that insect-host genetic differences promote parasitoid cryptic diversity, through cascading HAD, suggests that the evolutionary trajectory of the parasitoid will be influenced by genotypic interactions between interacting species (Stireman et al. 2006).

In conclusion, our study shows that parasitoid morphology, a surrogate for fitness, is dependent to a considerable extent on the GxGxE interaction between the aphid, barley

and rhizobacteria. The response of the parasitoid to the IEE of rhizobacteria presence or absence differs due to the genotypic combinations of the intermediate species and in a natural population this means the response will be dependent on the frequency of different genotypes. However, for this to have an evolutionary effect there need to be a selective force acting on the parasitoid, determined by the biotic environment of the expressed genomes of interacting species and a non-random association of the genotypes. There is the potential for non-random association of genotypes in certain systems, however, it is still unclear to what extent this can be generalised.

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Chapter 3. Genetic interactions influence host choice behaviour in a plant-insect system

Sharon E. Zytynska and Richard F. Preziosi

PERSONAL CONTRIBUTION

I designed, carried out the experiment and statistically analysed the data, with support from Richard Preziosi. In addition, I wrote the paper alongside comments from Richard Preziosi.

ABSTRACT

Phytophagous insects generally feed on a restricted range of host plants, using a number of different sensory and behavioural mechanisms to locate and recognize their host plants. Phloem-feeding aphids have been shown to exhibit genetic variation for host preference of different plant species, often with an aphid preferring and performing better on their host of origin. In addition, genetic variation within a host plant species can alter an aphid species' preference and acceptance. It is unknown, however, if different aphid genotypes exhibit host preference for different plant genotypes. Host preference can occur through influences on aphid reproductive rate (passive choice) or through aphids colonizing particular plants independent of reproductive rate effects (active choice). In this study, we demonstrate that different aphid genotypes show both active and passive choice for different barley genotypes, with one aphid genotype exhibiting preference through mainly passive choice and the other genotypes exhibiting primarily active choice for different barley genotypes. In a community context, such associations between aphid and barley genotypes could have evolutionary effects on the surrounding interacting community, especially in ecosystems of limited species and genetic diversity.

INTRODUCTION

Phytophagous insects constitute approximately one-quarter of all described species on this planet (Strong et al. 1984). These insects often feed on a restricted range of host plants, for example plants belonging to the same species, genus or subtribe (Lewisohn et al. 2005). Phytophagous insects locate and recognize host plants using a number of different sensory and behavioural mechanisms (Chapman 2003, Powell et al. 2006). Phloem-feeding phytophagous insects, such as aphids, use specialized mandibles (stylets) to feed on the phloem sap within the plant, creating a unique interaction between these insects and their host plants (Powell et al 2006). Aphids use a number of different pre- and post-stylet insertion cues to detect an acceptable host plant and Powell et al. (2006) stated that 'acceptance of an aphid to a host plant is defined as when the aphid reproduces'. Thus, if an aphid does not reproduce on a plant host after sufficient time then it can be assumed this plant is not a suitable host. An aphid will use a combination of different cues to determine whether a plant is an acceptable host. When an aphid lands on a plant it will first assess visual (e.g. colour) and surface cues (e.g. epicuticular waxes or texture) of the plant, followed by probing of the epidermis cells with the stylet, after which an aphid will penetrate the mesophyll or parenchyma cells with the stylet, then the sieve element, finally inserting the stylet into the phloem and if the host plant seems suitable there will be sustained ingestion of the sap (Powell et al. 2006). The factors an aphid is assessing include the plant's nutritional quality (e.g. amino acid, protein or carbohydrate content) and secondary metabolites, which can enhance aphid acceptance or decrease it (e.g. plant defense chemicals) (Chapman 2003, Powell et al. 2006).

Aphid host preference is the outcome of a combination of factors, such as host acceptance, where an aphid will only reproduce on a suitable host, and 'differential rate

of departure' where aphids on suitable host plants do not leave the plant in search of a better host (Powell et al. 2006). Thus, preferred host plants will harbour greater numbers of aphids than un-preferred hosts due to both increased reproductive success and reduced rate of departures on preferred host plants. A number aphid species exhibit variation in host preference and performance on different host plants (Via 1991, De Barro et al. 1995, Nikolakakis et al. 2003, Ferrari et al. 2006, Gorur et al. 2007). For example, pea aphids (*Acyrthosiphon pisum*) show preference for their plant of origin but also exhibit clonal variation in their preference for alternative host plants (Ferrari et al. 2006). Different host plant species are generally physically and chemically very different from each other, and therefore aphid choice for a particular host can often be made relatively quickly using chemical cues prior to stylet insertion, often confirmed by probing of the plant tissues (Powell et al. 2006).

Within a plant species, there are multiple genotypes that an aphid may encounter and these genotypes are likely to be more similar to each other than plants from different species but they will still exhibit variation. The bird cherry-oat aphid (*Rhopalosiphum padi*) has been shown to induce differential expression of several aphid-resistance genes within closely related genotypes of barley (*Hordeum vulgare*) (Delp et al. 2009). These induced defense responses in the plant also reduced aphid performance, and show how there can be genetic variation within a plant for response to aphid attack. Aphids also respond to genetic differences in a plant species. Rovenska and Zemek (2006), showed that the potato aphid (*Aulacorthum solani*) could discriminate between transgenic potato plants (expressing the aphid-resistant snowdrop lectin gene) and control potato plants, with the aphids preferentially colonizing the control plants. Therefore, it is known that different aphid genotypes prefer different host plant species, and there is potential for

aphids to discriminate between different plant genotypes. It is unknown, however, if different aphid genotypes show host preference for different plant genotypes.

There are two potential mechanisms for aphid host preference. The first occurs through an effect of the plant on aphid reproductive performance, such that aphids feeding on one plant genotype experience reduced reproductive success compared to when feeding on another plant genotype (passive choice). This would appear as if more aphids were colonizing the plant genotypes that infer a higher fitness, however the effect is passive through differential reproductive performance of the aphids on the plants. On the other hand, the effect on reproductive performance may be insignificant and the aphids actively choose a particular plant genotype, as it is more 'attractive' than another (active choice). This 'attraction' can occur via particular cues the aphid uses to select a host plant and can result in some nutritionally suitable host plants not being colonized due to the absence, or presence, of specific cues (Powell et al. 2006). For example, *Myzus persicae* aphids performed similarly on both sweet pepper plants and tobacco plants, but aphids originating from tobacco-growing regions preferentially colonized tobacco plants and those originating from regions where no tobacco is grown preferred pepper plants (Nikolakakis et al. 2003). It may seem reasonable to assume that an individual would choose the host plant that infers the highest fitness (i.e. the greatest reproductive rate) but it has been shown that there can be little correlation between host preference and fecundity suggesting this assumption could be wrong (McCauley et al. 1990).

In this study, we conducted no-choice and choice experiments for a number of aphid genotypes to different barley genotypes to determine if there was host preference among the different aphid genotypes for particular barley genotypes. The no-choice experiments were used to determine the reproductive rate of the aphids on each barley genotypes (passive choice), and the choice experiments indicate preference for

particular barley genotypes (active choice). Since aphid acceptance can often only be confirmed after reproduction has occurred on a host plant (Powell et al. 2006) we counted the number of aphids on the plants, in the choice experiment, after one day (no reproduction) and two weeks (approximately one generation). We present results to show that there is differential aphid preference to different barley genotypes and aphid choice can be active (independent of effects on aphid reproductive rate) and passive (due to effects on aphid reproductive rate), dependent on genotype combination of aphid and barley.

MATERIALS AND METHODS

Aphids and barley

Four aphid genotypes (*Sitobion avenae*; CLO7, DAV95, H1 and HF92a) and six barley genotypes (*Hordeum vulgare*; Baronesse, BCD47, Blenheim, Morex, Oregon Wolf Barley Recessive and Steptoe) were used in this experiment. Experimental barley seed was harvested from plants grown in a common glasshouse environment, but separated by genotype to ensure no crossing occurred among genotypes. The seed used in the experiment was from the same harvest, in order to reduce the chance of environmentally induced variation within the genotypes. The aphid lines were kept in asexual reproduction (clonal lines) and were reared on a generic batch of *Hordeum vulgare* seed. Experimental aphid populations were reared asexually from approximately ten starting aphids. In natural systems, these aphids reproduce sexually during winter months and asexually during summer months (Aminu-Kano 1991).

No-choice experiment

Barley seed was germinated by placing seeds between two layers of filter paper, moistened with sterilized distilled water, and kept in a dark growth chamber at 21°C for 6 days. Barley seedlings were transplanted into 15cm diameter plant pots containing John Innes Compost No. 3. Six seedlings of the same genotype were planted 2cm from the edge of the pot in a symmetrical design to ensure uniformity across pots. Each pot was covered in black nylon mesh, supported by a wire frame, to ensure no aphids could escape or no other aphid could invade. Prior to aphid infestation, a 2cm layer of sand was added to the pots to ensure an even surface for aphid movement. Six adult apterous aphids from a single genotype were placed on a 3cm diameter disc of filter paper and carefully placed in the center of the pot. Three repeats were made for each treatment of barley and aphid combination, with a total of 72 pots. Pot order was randomized across the experiment. The no-choice experiment was undertaken in a single growth chamber

at 21˚C 16:8 light:dark regime. Aphid number was counted after 2 weeks. A two-way ANOVA (JMP v8.0.2) was used to determine if aphid per capita growth rate was influenced by aphid genotype, barley genotype and the interaction between aphid and barley genotypes. Aphid per capita growth rate was calculated as (Agrawal 2004):

$$\frac{\left(\ln N_2 - \ln N_1\right)}{t} \quad (1)$$

where, N_2 is the final aphid number, N_1 is the initial aphid number and t is the number of days spent on the plant. Standard least squares post hoc tests were used to determine the significance of each aphid-barley genotypic combination (JMP v8.0.2).

Choice experiment

The experiment was set up as for the no-choice experiment. Each plant pot contained six barley seeds, one of each genotype. The seedlings were planted 2cm from the edge of the pot in a symmetrical design to ensure uniformity across pots and no bias for aphid choice. The different seedling genotypes were planted in a random order, within the pot, so no two genotypes were always next to each other. Six apterous aphids, from the same genotype, were placed on a 3cm diameter disc of filter paper and carefully placed in the center of the pot. Ten repeats were made per aphid genotype, with a total of 40 pots. Pot order was randomized across the experiment. The pots were maintained at The Firs Botanical Grounds, The University of Manchester in a glasshouse at 18-22°C, 16:8 light:dark regime. Aphid numbers on each barley plant were counted after 1 day and 2 weeks. The results were analysed using a G-test (Sokal and Rohlf 2000) to determine if the aphids colonized each plant within a pot at random. The expected number of aphids per plant, if random colonization occurred, is one-sixth of the total aphids per pot since there were six plants per pot. Additionally, correlations were made using Pearson's correlation coefficient of the number of aphids on each barley genotype between the choice and no-choice test. A positive relationship would indicate an association between

reproductive rate on host preference, whereas no correlation between aphid numbers suggests that aphid preference is not associated with reproductive differences on the barley genotypes.

Active and passive aphid choice definitions

In this paper, aphid choice is defined as being either active or passive. The results from the no-choice and choice experiments were compared to determine of the aphid preference observed was due to passive or active host choice. Passive choice demonstrates an aphid genotype's ability to reproduce on a plant, whereas active choice is a decision made by the aphid to accept or reject a plant irrespective of reproductive rate effect.

Passive choice was assumed if the aphid genotype was observed in greater or fewer numbers on the barley genotype in the no-choice test, indicating an influence of the barley genotype on aphid reproductive rate. Further, if this corresponded with aphid preference in the choice test then the result must be, at least partially, influenced by passive choice. Active choice was assumed if the aphid genotype was found in higher/lower proportions on a particular plant genotype in the choice experiment, which could not be explained by the effect of barley genotype on aphid reproductive rate. A combination of active and passive choice was also considered, where there was an effect of the barley genotype on aphid growth rate (no-choice experiment) but the magnitude of this passive effect could not fully explain the results from the choice experiment.

In the results section, aphid preference is described as 'like' or 'dislike', where 'like' means more aphids than expected were found on a particular plant genotype and 'dislike' means fewer than expected were found on a particular plant genotype, in the choice experiment.

RESULTS

No-choice experiment

In the no-choice, experiment aphid growth rate was significantly different between aphid genotypes ($F_{3,48} = 24.765$, $p<0.0001$), among barley genotypes ($F_{5, 48} = 13.01$, $p<0.0001$), and there was a significant interaction between aphid and barley genotypes ($F_{15, 48} = 2.09$, $p = 0.03$; Figure 3.1). DAV95 and H1 aphid genotypes had lower overall mean aphid number per pot (150.7 and 130.3, respectively) than CLO7 and HF92a genotypes (203.1 and 245.3, respectively). Aphid genotypes DAV95 and H1 showed reduced aphid growth rate on barley genotype OWBrec compared to other barley genotypes (Figure 3.1). CLO7 and HF92a aphids did not show a significant reduction from average on OWBrec, although aphid growth rate was at the lower end of the range for these aphid genotypes. DAV95 and H1 aphids also showed an increased growth rate on Morex and Blenheim barley, with HF92a aphids also having increased growth rate on Morex (Figure 3.1)

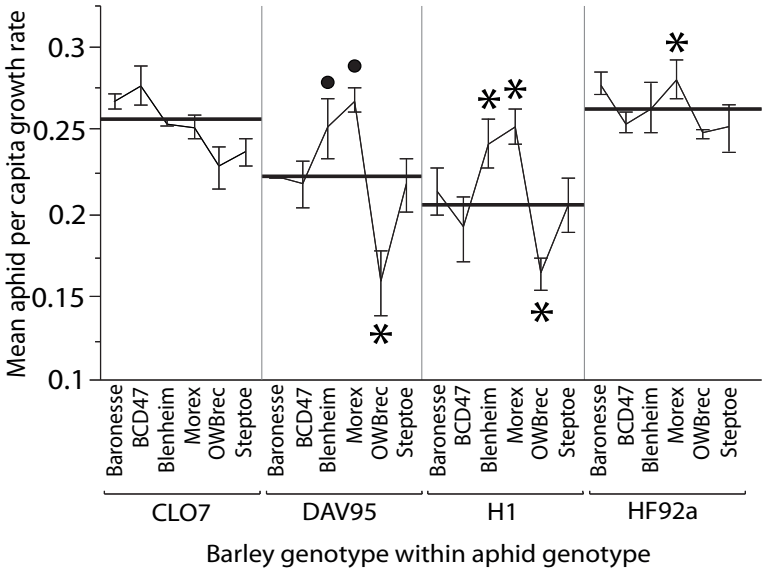


Figure 3.1. No choice experiment results from week 2 (aphid per capita growth rate), for each aphid-barley genotype combination

Error bars represent ±1S.E. Horizontal lines indicate overall mean number of aphids across all barley genotypes, within aphid genotype. Stars indicate aphid-barley combinations with significant deviation from the mean at $p<0.05$, and dots a trend for significant deviation from the mean with $p<0.10$

Choice experiment

In the choice experiment, we found a significant effect of barley genotype on the number of aphids observed on the plants after two weeks (G-test = 161.4, $p < 0.0001$), thus different aphid genotypes were found more often on different barley genotypes, but no significant overall effect was found after one day (G-test = 10.52, $p = 0.786$).

After one day there was no aphid reproduction, since the number of aphids per pot was equal to the number added the previous day. At week two, there was a correlation between aphid numbers on the different barley genotypes in the choice and no-choice experiments for CLO7 aphids, indicating a large effect of reproductive performance on these aphids (passive choice) in host preference (Table 3.1). The other three aphid genotypes did not have an association between the numbers of aphids on each barley genotype in the choice compared to the no-choice experiments, indicating more active host choice (Table 3.1).

Table 3.1. Correlation values for total aphid number in the choice compared to the no-choice experiment, across barley genotypes.

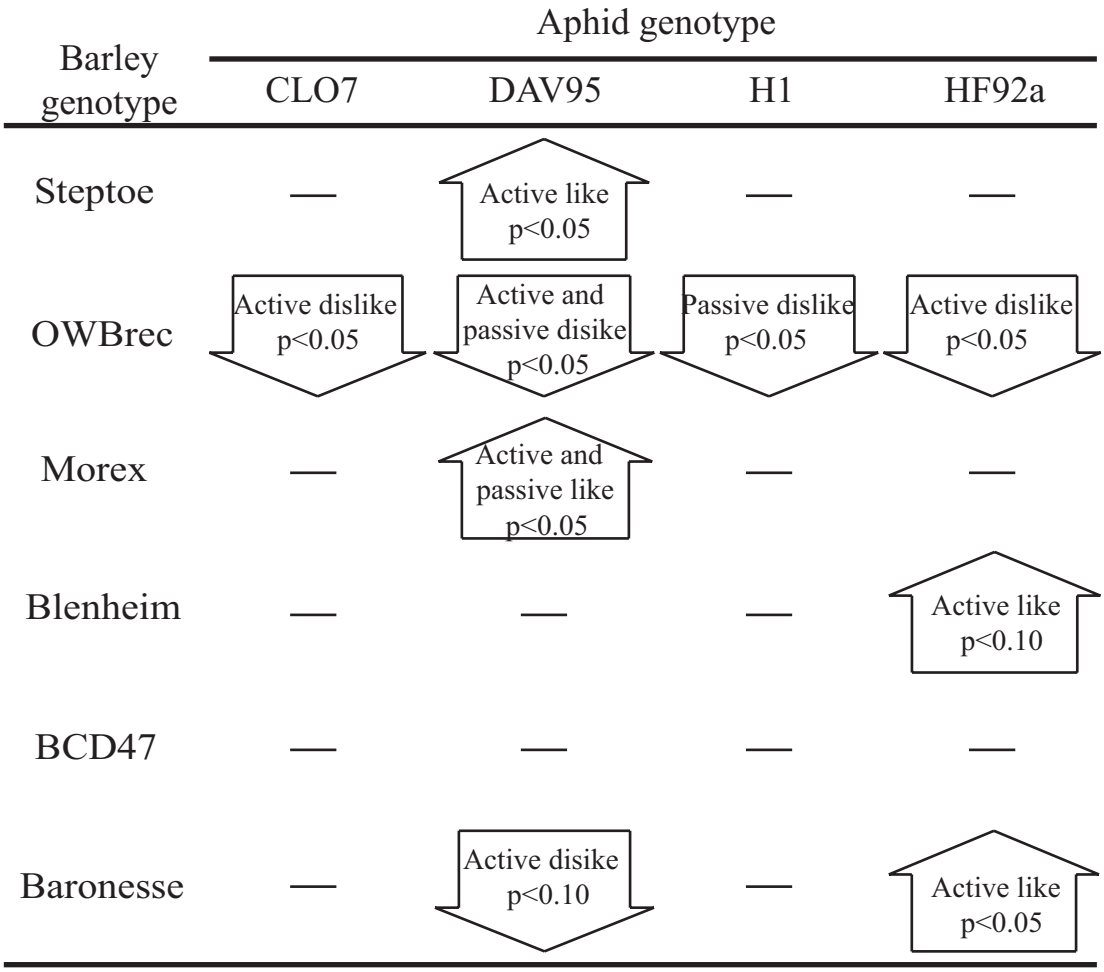
Aphid Genotype	r	p-value
CLO7	0.892	0.017 *
DAV95	0.535	0.274 NS
H1	0.315	0.543 NS
HF92a	0.608	0.201 NS

Notes. Pearson's correlation coefficient (r). Significance * = $p<0.05$, NS = $p>0.05$

There was considerable variation among aphid genotypes for barley genotype preference, both for active/passive choice and like/dislike (Table 3.2; see Methods section for definitions of active/passive choice and like/dislike). Aphid genotype CLO7 exhibited dislike to barley genotype OWBrec in the choice experiment after one day and

two weeks (Figure 3.2). In the no-choice experiment aphid growth rate was not reduced compared to the mean for CLO7 aphids over all barley genotypes (Figure 3.1), therefore, CLO7 aphids had an active dislike of barley OWBrec (Table 3.2). CLO7 aphids showed no preference for any of the other five barley genotypes. Similarly, H1 aphids only showed preference against barley genotype OWBrec (Figure 3.2), although here it was described as passive dislike (Table 3.2) since H1 aphids also had reduced reproductive performance on OWBrec in the no-choice experiment (Figure 3.1) and further, showed no dislike after one day in the choice experiment (Figure 3.2a). Aphid genotype DAV95 showed active and passive dislike for OWBrec (Table 3.2) since fewer aphids were found on OWBrec plants in the choice experiment after both one day and two weeks (Figure 3.2), and additionally had reduced performance on OWBrec in the no-choice experiment (Figure 3.1). DAV95 aphids also showed a trend ($p<0.10$) for active dislike to Baronesse (Figure 3.2b), with no reduced performance in the no-choice experiment on this barley (Figure 3.1). Significant active like ($p<0.05$) was also shown by DAV95 for barley genotype Steptoe (Table 3.2, Figure 3.2). In addition, these aphids showed both active and passive like for barley Morex, since DAV95 aphids showed a trend ($p<0.10$) for increased reproduction on Morex in the no choice test (Figure 3.1) as well as greater than expected numbers in the choice test (Figure 3.2). Aphid genotype HF92a showed active dislike for barley genotype OWBrec (Figure 3.2), as it did not have a significantly ($p > 0.10$) reduced performance on this barley genotype in the no-choice experiment (Figure 3.1) and fewer aphids were counted on OWBrec at day 1 in the choice experiment (Figure 3.2a). HF92a aphids also showed active like for Baronesse ($p<0.05$) and Blenheim ($p<0.10$) barley genotypes (Table 3.2). The HF92a aphids also had increased reproductive performance on barley Morex (Figure 3.1) but this did not relate to an increase in these aphids on Morex in the choice test (Figure 3.2).

Table 3.2. Host preference of different aphid genotypes on different barley genotypes



Notes. Aphid genotypes are in columns and barley genotypes in the rows. A dash (-) means there was no departure from the expected proportions. 'Like' means more aphids than expected were observed on the barley genotype and 'dislike' means fewer aphids than expected were observed. Arrows indicate like (upwards) and dislike (downwards). Passive choice is defined as the aphid also showing reduced performance in the no-choice experiment, active choice is defined as when there is no performance effect in the no-choice experiment but deviation from expectations is noted in the choice experiment - inferred from both day 1 data (no aphid reproduction) and week 2 data (approximately one generation).

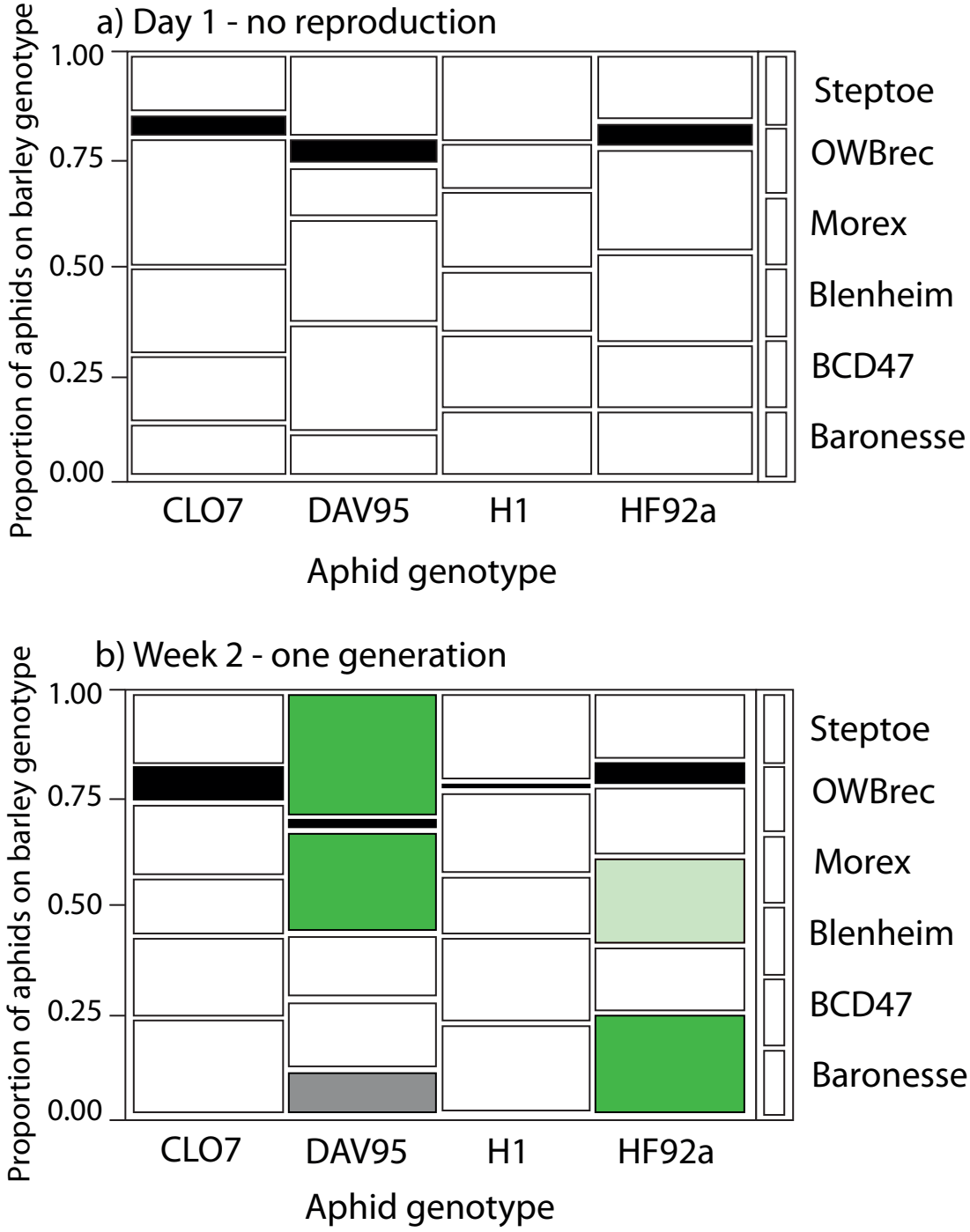


Figure 3.2. Choice experiment results after (a) 1 day and (b) 2 weeks, showing proportion of aphids (columns) on each barley genotype (rows) weighted by pot
Black colour indicates the combinations of aphid and barley that had significantly lower proportion of aphids than expected at random ($p<0.05$) and grey colour indicates a trend ($p<0.10$). Green colour indicates those combinations where a greater proportion of aphids were found on the barley than expected at random ($p<0.05$) and light green indicates a trend ($p<0.10$). Column on the right shows the expected proportion of aphids on each barley genotype if there was a random distribution across the plants.

DISCUSSION

This paper demonstrates that different aphid genotypes preferentially feed on different host plant genotypes. This indicates that there is an interaction between aphid genotype and barley genotype for aphid host preference. By comparing results from a no-choice test, where aphids were presented with a single barley genotype, and a choice test, where each aphid genotype could choose between six different barley genotypes, we were able to show that host preference can occur through active and passive choice. Our results indicate that passive choice (through barley genotypic effects on reproductive performance) explained up to 79% of the variation in host preference for CLO7 aphids, but no significant correlation was seen between aphid growth rate in the choice and no-choice experiments for any of the other three aphid genotypes. This suggests that active choice of an aphid, rather than passive choice, is more important for overall variation in observed aphid number on particular barley genotypes.

Pea aphids have been shown to initiate reproduction after only 30 minutes on a plant (Caillaud and Via 2000), and other aphid species will initiate reproduction within six hours (Powell et al. 2006). In this study, after one day there was no reproduction, which could indicate that initiation of reproduction occurs through alternative mechanisms in *S. avenae* aphids than other species. This is possible since *S. avenae* aphids have been documented to produce offspring even in the absence of plant tissue, whereas other species such as *Aphis fabae* or *Myzus persicae* required the presence of plant tissue for reproduction (Lowe 1985). The lack of reproduction after one day could also be an artifact of the experimental design, since the aphids were placed on a disc of filter paper in the middle of the pot, with the barley plants growing around the edge of the pots meaning the aphids had to move toward a barley plant, and around to subsequent barley plants before the final host choice decisions were made. Since we used apterous aphids

the movement between plants would occur through walking, which is potentially more time consuming compared to flying, as would be used by the dispersal alate morphs.

We used apterous (wingless) aphids in these experiments to understand further the importance of aphid choice and their interaction with barley host plant genotypes in an unstressed environment; winged, dispersal aphid morphs are produced when a mother aphid has experienced high aphid density or low quality plants. Even without wings, adult aphids can still move relatively large distances from plant to plant and will often only stay on a single plant for two days before moving on (Vickerman and Wratten 1979). This shows that movement across a local area may be more influenced by host plant choice of non-winged aphids than winged ones, which are more suited to long-distance dispersal. This is important since host choice within a population, i.e. between genotypes within a host plant species, is likely to occur at more local scale than the choice of host plant species by dispersal morphs.

After one day (no reproduction) in the choice experiment, the only significant effect was host preference against OWBrec for three aphid genotypes (CLO7, DAV95 and HF92a); there was no significant preference for any barley genotype after 1 day. This may indicate that OWBrec barley has a particular trait that can be easily detected by an aphid to deter it from settling on the plant, whereas, the other barley genotypes may have more subtle factors determining aphid acceptance which only become apparent after aphids have had more long-term stylet penetration and feeding events (Powell et al. 2006). Over the whole experiment, we found the preference against OWBrec occurred with different magnitudes and potentially different mechanisms across aphid genotype. DAV95 aphids showed active and passive dislike, CLO7 and HF92a showed active dislike and H1 showed passive dislike. It is unlikely that a single mechanism can

explain the effects seen. Possible pre-stylet insertion mechanisms, which deter aphid settlement, could be through visual, chemical or physiological cues, such as color, waxes, hairs or epidermis chemicals (Caillaud and Via 2000, Powell et al. 2006). Although aphids did not preferentially colonize this barley genotype in the choice experiment, in the no-choice experiment aphid reproductive rate was significantly reduced, compared to an average over all barley genotypes, in only two of the four aphids genotypes (H1 and DAV95). Therefore, when presented with no other host plant genotype it is likely the aphid populations would still persist on OWBrec barley plants.

After two weeks, there was only one barley genotype (BDC47) that did not induce a response from any aphid genotype. This barley genotype was therefore colonized at random by all aphid genotypes and may be a universally accepted plant that is neither liked nor disliked by the aphids. In an ecosystem with mixed barley host plant genotypes, this particular genotype may act as a suppressor for speciation within this aphid species (Ferrari et al. 2006). Significant preferences for a barley genotype among the aphid genotypes were seen in aphid DAV95 for barley genotypes Morex and Steptoe, HF92a for barley genotype Baronesse and to a lesser degree HF92a on Blenheim. For DAV95 and HF92a aphids there is no crossover of aphid preference for a barley genotype and therefore when grown together in a genotypically diverse plant community there is potential for strong assortative association (non-random association) to occur. In addition, on barley genotype Baronesse, HF92a showed active like and DAV95 showed a strong trend for active dislike, which further separates the preferences of these two aphid clones. Neither H1 nor CLO7 showed increased preference for any barley genotype, indicating they are more generalist aphid clones than DAV95 or HF92a, and in a high diversity community would be found on a number of different genotypes.

Our work demonstrates that aphids do not choose to reproduce on the barley genotype that would infer the highest fitness. Only one aphid genotype (CLO7) was found more often on the barley genotype it has the greatest reproductive performance on, and another aphid genotype (H1) was found only at the expected proportions, in the choice experiment, on barley genotype Morex that inferred the highest fitness for these aphids in the no-choice experiment. This supports previous work which showed that aphids do not chose specific host plant species that provide the greatest reproductive environment (McCauley 1990) and our work furthers this showing that, within host plant species, aphids do not chose plant genotypes that would infer the highest fitness. Before we can fully understand aphid-plant relationships, the mechanisms and implications of aphid active choice must be further investigated.

Previous work has demonstrated that genetically based interactions between species can influence the phenotypes of both the directly and indirectly interacting individuals (Service 1984, Carius et al. 2001, Tétard-Jones et al. 2007, Zytynska et al. 2010). For any evolutionary effect of these inter-specific interactions to occur there is a need for non-random association of genotypes from each species across many generations. Here, we have shown that different aphid genotypes preferentially chose or avoid specific barley genotypes and therefore these interactions have the potential to influence the structure and dynamics of the communities these species live in. With current agricultural strategies of planting large areas with a single genotype of crop plant, there is a high likelihood these genetically-based interactions between plant and aphid are influencing the evolutionary trajectories of higher trophic species such as parasitoids, through cascading host-associated differences (Stireman et al. 2006). These effects may also be influential in other low diversity systems, where the impact of a single interspecific interaction will be greater than in a system with high species and genotypic diversity.

In conclusion, we have demonstrated that there is genetic variation within an aphid species for host preference of different barley genotypes. This has been shown to occur by both active choice of the aphid and passive choice through effects on reproductive performance. Our results indicate that aphid choice behavior could lead to assortative associations of aphid genotypes and barley genotypes, and the influence of these genetically-based interactions between aphid and plant could have evolutionary effects for the rest of the interacting community. However, the use of even a single host genotype by all aphid genotypes, such as BCD47 in this study, could decrease the effect on higher trophic organisms. These results have strong implications for low-diversity ecosystems where both species diversity and genetic diversity are reduced, leading to stronger interspecific interactions between different genotypes across species.

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Chapter 4. Intraspecific competition alters host choice behaviour in a plant-insect system

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PERSONAL CONTRIBUTION

I designed, carried out the experiment and statistically analysed the data, with support from Richard Preziosi. In addition, I wrote the paper alongside comments from Richard Preziosi.

ABSTRACT

Competition between phytophagous insects can be an important force in insect communities, especially in sessile species that feed on forbs and grasses. Within phloem-feeding insect species, competition can be direct and indirect. Intra-specific indirect competition can occur through plant-mediated traits, such as phloem composition changes or plant-induced defense response. It is known that phloem-feeding aphids preferentially colonize particular host plant species and host plant genotypes, due to differences among plants for particular traits. Therefore, plant-mediated traits could influence both host choice behaviour and competition between aphids. It is, however, unknown if competition with another aphid genotype can alter host choice behaviour. We investigated the influence of intra-specific competition on host choice behaviour of an aphid (*Sitobion avenae*) for different barley genotypes. We found that all but one aphid genotype altered their host choice behaviour when reared alongside another aphid genotype. One aphid genotype showed variation in host choice behaviour when reared with different aphid genotypes, indicating that host choice of barley genotypes is dependant on the genotypic identity of the competing aphid. Our work demonstrates that within a complex ecological community an individual's behaviour can be influenced by interactions with other genotypes within the same species, as well as interactions with genotypes of other species, such as the host plant.

INTRODUCTION

Competition between phyophagous insects has been found to be an important force in insect communities (Denno et al. 1995). It has also been stated that this force is greater between closely related, sessile and aggregative species that feed on forbs and grasses (Denno et al 1995). In phloem-feeding aphids, individuals can compete directly, through physical fighting, and indirectly, via plant-mediated traits. Direct competition has been documented in *Astegopteryx minuta,* where aphids fight to gain feeding sites in bamboo (Foster 1996) and in palm aphids, *Cerataphis brasilinsis* (Howard et al. 1998). However, examples of interference competition (active fighting between individuals), such as these, are rare. Indirect interactions between the competing aphids through plant-mediated effects are expected to be more frequent than by interference competition between the aphids (Denno et al. 1995).

Indirect competitive interactions between aphids for the plant phloem sap resource may be strong because it is a poor source of essential amino acids required for aphid growth (Douglas 2006), thus leading to exploitative competition mediated through the host plant (Wootton 1994). Aphids also acquire essential amino acids from primary symbiotic bacteria (*Buchnera aphidicola*), which synthesize essential amino acids from the phloem-sap abundant amino-acid glutamic acid (Douglas et al. 2001). To enhance this, a number of aphid species can induce the plant to alter the amino acid content of the phloem sap in a plant when feeding, to increase essential amino acid concentrations (Telang et al. 1999; Sandstrom et al. 2000; Petersen and Sandstrom 2001). The change in phloem could be caused by either increased transport of amino acids to the feeding area or by induction of the plant to synthesise more essential amino acids. For example, aphid galls induced by *Geoica sp.* are able to divert normal phloem transport, reducing the phloem available to and reducing the reproductive success of a competing aphid

species (*Forda formicaria*), leading to exploitative competition between these species (Inbar et al. 1995). Additionally, *Melanocallis caryaefoliae* aphids are able to alter the local composition and profile of the phloem, within pecan plants (Petersen and Sandstrom 2001). This behaviour can be inhibited by prior feeding of *Monellia caryella*, an aphid species that does not alter phloem composition, resulting in reduced reproductive rate of *M. caryaefoliae* and thus, exploitative competition between these aphid species.

Indirect competition mediated via plant traits can also occur through induced plant defenses. There have been a number of studies that have identified genes and gene pathways involved in induced plant defense against phloem-feeding aphids (see Walling 2000, Goggin 2007, Smith and Boyko 2007, Delp et al. 2009). Induced plant defenses may influence intra-specific competition between aphids, as particular genotypes could induce a stronger defense response in the plant, which alters the fitness or choice of other genotypes (Dicke and Hilker 2003). For example, pre-conditioning of a plant with one genotype of an aphid is known to influence the feeding behaviour of another aphid genotype due to the differentially induced expression of an aphid resistance gene by aphids that the plant was either resistant or susceptible to (Hays et al. 1999).

Competition between two closely related aphid sub-species, *Myzus persicae s.s*. and *Myzus persicae nicotianae,* has been shown to exclude *M. persicae nicotianae* from sweet pepper plants so that this sub-species is limited to tobacco host plants (Tapia et al. 2008). *M. persicae nicotianae* was able survive on sweet pepper in laboratory experiments and therefore competitive exclusion, through either interference competition or exploitative competition, leading to competitive superiority of *Myzus persicae s.s* is expected to, in part, explain why *Myzus persicae nicotianae* is not found

on sweet-pepper plants in the field (Tapia et al. 2008). These interactions have also led to host choice differences between these aphid sub-species, with adaptation occurring in *Myzus persicae nicotianae* to the tobacco host plant resulting in a preference to colonise this host plant above sweet pepper (Vargas et al. 2005). Host preference has been documented among pea aphid genotypes (*Acyrthosiphon pisum*), where genetically differentiated host races exhibit strong preferences for their host plant species from which they were collected (Ferrari et al. 2006). Within a species, different genotypes of *Sitobion avenae* aphids have also been shown to have preference for particular barley plant genotypes (Chapter 3). These host preferences are expected to be mediated by plant traits such as phloem composition and induced defense responses, which are also expected to be involved in competition between aphids, but it is unknown how intraspecific competition can influence host preference.

In a natural system, aphids are expected to exist in genotypically diverse populations and therefore are expected to encounter intraspecific competition within a population feeding on the same host plant. In this study, we ask whether host plant genotype choice of a *Sitobion avenae* aphid genotype is altered due to competition with another aphid genotype of the same species, and further, if the identity of the competing aphid genotype is important for host choice preference. We therefore aim to determine if intraspecific genotypic interactions among the aphids influences aphid choice of barley genotypes. We counted the aphids on each plant after one day (no reproduction) and after two weeks, which corresponds with approximately one generation.

MATERIALS AND METHODS

Aphids and barley

Four aphid genotypes (*Sitobion avenae*; CLO7, DAV95, H1 and HF92a) and six barley genotypes (*Hordeum vulgare*; Baronesse, BCD47, Blenheim, Morex, Oregon Wolf Barley Recessive and Steptoe) were used in this experiment. The aphid lines are kept in asexual reproduction (clonal lines) and were reared on a generic batch of *Hordeum vulgare* seed. This aphid is a non-symptomatic aphid of barley, that is, it reduces plant growth rates but does not cause significant visual effects on the leaf material. The aphids were originally obtained from Rothamsted Research Centre, UK. Experimental barley seed was harvested from plants grown in a common glasshouse environment, but separated by genotype to ensure no crossing occurred among genotypes. The barley seed was originally obtained from P.Hayes, Oregon State University.

Experimental design

Each treatment consisted of a choice pot containing six barley genotypes, six aphids from a single aphid genotype or three individuals from each of two aphid genotypes placed in the middle of the pot and the aphids were allowed to reproduce for two weeks. A non-factorial design was used since not all pairs of aphid genotypes could be used; two genotypes are black (CLO7 and HF92a) and two genotypes are green (H1 and DAV95), therefore, only the easily observable aphid combinations were used, consisting of one green genotype and one black genotype (CLO7-DAV95, CLO7-H1, HF92a-DAV95, HF92a-H1).

Experimental set-up

Barley seed was germinated by placing seeds between two layers of filter paper, moistened with sterilized distilled water, and kept in a dark growth chamber at 21°C for 6 days. Barley seedlings were transplanted into 15cm diameter plant pots containing John Innes Compost No. 3. Each plant pot contained six barley seedlings, one of each genotype. The seedlings were planted 2cm from the edge of the pot in a symmetrical design to ensure uniformity across pots and no bias for aphid choice. The different seedling genotypes were planted in a random order, so no two genotypes were always next to each other in a pot. Pot order and position in the glasshouse was randomized across the experiment. Each pot was covered in black nylon mesh, supported by a wire frame, to ensure no aphids could escape or no other aphid could invade. Prior to aphid infestation, a 2cm layer of sand was added to the pots to ensure an even surface for aphid movement. Six adult aphids from a single genotype or three aphids from each of two genotypes were placed on a 3cm diameter disc of filter paper and carefully placed in the center of the pot; ten replicate pots were made per aphid genotype or combination of genotypes. Pots were maintained in a glasshouse at The Firs Botanical Grounds, The University of Manchester at 18-22°C, 16:8 light:dark regime. Aphid numbers on each barley plant were counted after one day (no reproduction) and two weeks (approximately one generation).

Statistical Analysis

A two-way ANOVA (JMP 8.0.2) was used to analyze the effect of aphid genotype and presence or absence of a second genotype on aphid number (mean aphid number of each genotype per pot). Host choice results were analyzed using G-tests for heterogeneity in JMP 8.0.2. Aphid number per plant was used and the analysis was blocked by pot, so that within each pot the number of aphids per plant was compared to

the total number of aphids in that pot. This tests whether the ratio of aphids on each plant genotype within the pots was similar across treatments (aphid genotype pair). We tested the null hypotheses that (a) the proportion of aphids on each barley genotype (per pot) does not differ in the presence of another aphid genotype, and (b) the proportion of aphids on each barley genotype (per pot) does not differ due to the identity of the other aphid genotype. Aphid number was adjusted for density effects, as when the aphids were grown alone there were six initial aphids and when grown in pairs there were three initial aphids. The number of aphids was halved for the single-aphid treatment when comparing treatments allowing for aphid starting density to be consistent. Adjusted aphid number, with the data blocked by pot, was used rather than aphid proportion on each plant since a G-test was used to analyse the data, which requires frequency data.

RESULTS

Aphid number, within pot, differed among aphid genotypes at week 2 ($F_{3, 113}$=11.93, P<0.0001) but this was not altered due to being reared alone or in a pair ($F_{1, 113}$=0.55, p=0.462). Thus, there was no affect on total aphid number per pot, due to the presence of another aphid genotype.

After one day, there was no influence of the presence or genotypic identity of another aphid genotype on the host preference of any aphid genotype. After two weeks, aphid genotype CLO7 did not show any change in host preference to the presence of another aphid genotype (H1 and DAV95; presence-absence: G-test = 4.608, p=0.46) or the identity of the competing aphids (genotypic identity: G-test = 2.29, p=0.81) (Table 4.1). CLO7 aphids were observed in similar proportions on each barley genotype when reared with a competing aphid genotype as those observed when reared alone (Figure 4.1a). HF92a aphids altered their preference in the presence of the other aphid genotypes (H1 and DAV95; presence-absence: G-test = 13.82, p=0.017; Table 4.1) and were more often observed on barley genotype Blenheim when reared with a competing aphid genotype as opposed to alone (Figure 4.1b). The change in host preference of HF92a aphids was not influenced by the genotypic identity of the competitor (Table 4.1). Aphid genotype DAV95 showed a strong change in host preference when reared in competition with another aphid genotype (CLO7 and HF92a; presence-absence: G-test = 34.83, p<0.0001; Table 4.1). There was a trend for this preference to alter depending on the identity of the competing aphid (genotypic identity: G-test = 9.89, p=0.07) (Table 4.1), with more DAV95 aphids moving away from barley genotypes BCD47 and Blenheim when competing with CLO7 than with HF92a aphids (Figure 4.1c). The genotypic identity of the competing aphid was a strong effect for aphid genotype H1 (genotypic identity: G-test = 24.55, p=0.0002; Table 4.1), with H1 aphids showing a

different pattern of host preference when reared in competition with CLO7 compared to HF92a aphids (Figure 4.1d).

Overall, host choice of aphid CLO7 was not influenced by either the presence of another aphid or the identity of the competing aphid. Host choice behavior of aphid HF92a was influenced by the presence of another aphid genotype but the identity of the competing aphid was not important. Host choice of DAV95 was strongly influenced by the presence of another aphid genotype but the pattern seen when DAV95 was competed with CLO7 or HF92a is similar and therefore genotypic identity was only marginally important. Host choice of H1 was influenced strongly by the genotypic identity of the competing aphid.

Table 4.1. Summary of week 2 results showing the effect of the presence of another aphid genotype and the identity of the competing genotype

	Presence/absence		Genotypic identity	
Aphid	**G-test result**	**p-value**	**G-test result**	**p-value**
CLO7	4.61	0.455 NS	2.29	0.810 NS
DAV95	34.83	<0.001 ***	9.89	0.070 •
H1	6.10	0.297 NS	24.55	<0.001 ***
HF92a	13.82	0.017 *	5.76	0.330 NS

Notes. G-test result tested against a χ^2 distribution. Presence/absence of other genotype. Significance: NS = not significant, • = $p<0.10$, * = $p<0.05$, *** = $p<0.001$

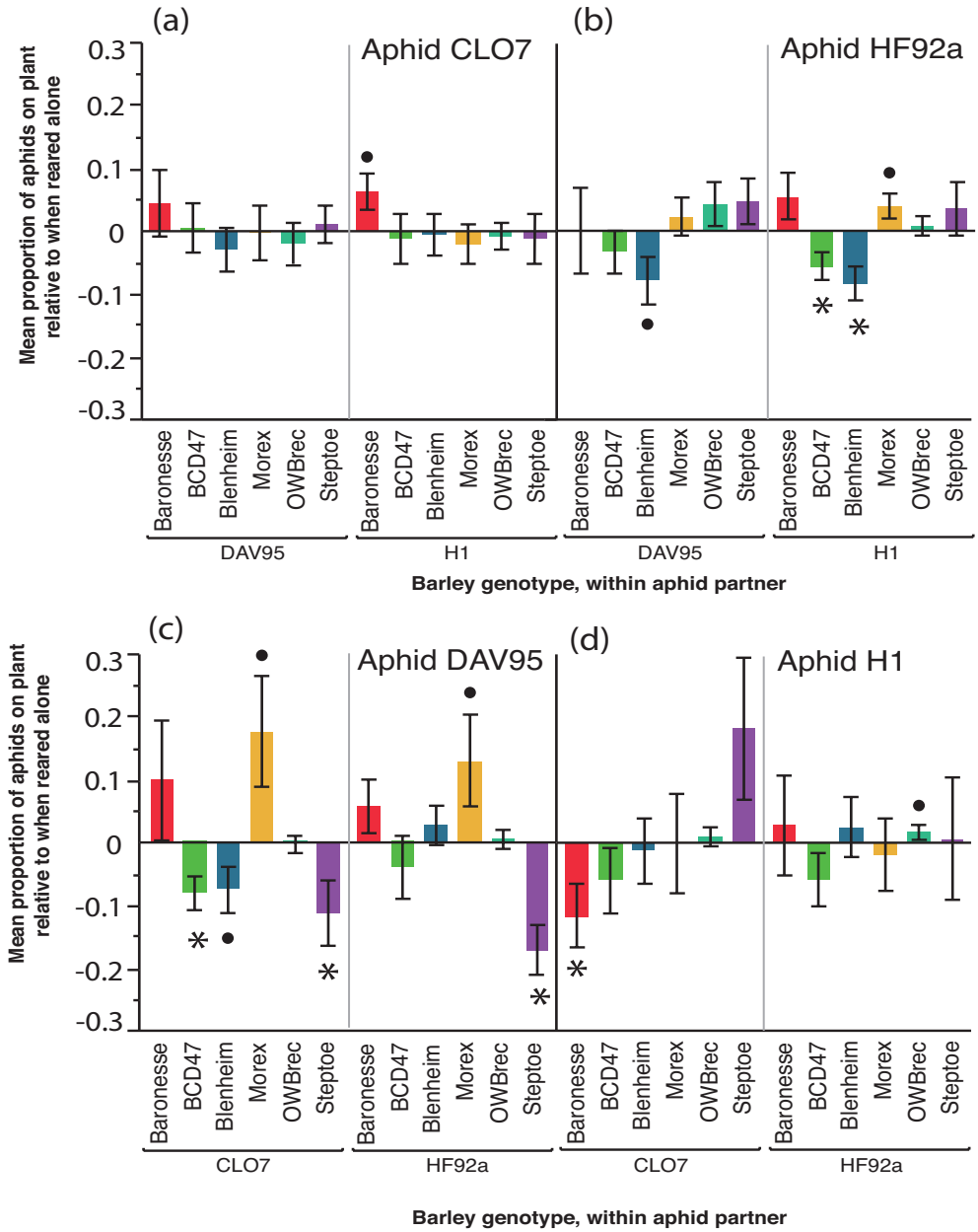

Figure 4.1. The proportion of aphids (a) CLO7, (b) HF92a, (c) DAV95 and (d) H1 on each barley genotype, when in the presence of two different aphid genotypes relative to when the aphid is reared alone. (a) host choice of CLO7 is not influenced by the presence of another aphid, or the identity of the aphid; (b) host choice of HF92a is influenced by the presence of another aphid but the pattern is similar when HF92a is in the presence of either aphid; (c) host choice of DAV95 is highly influenced by the presence of another aphid but DAV95 responds similarly to both other aphids, indicating identity of the other aphid is not a large effect; (d) H1 host choice is influenced by the presence of another aphid and the identity is important as host choice is more influenced when it is in the presence of CLO7 than HF92a. Error bars represent ±1 standard error. Significant deviations are indicated by a star (*) if $p<0.05$, and a dot (•) if $0.05 < p < 0.10$.

DISCUSSION

We have shown that aphid host choice behavior for particular barley genotypes can be altered due to the presence and, in some cases, the identity, of another aphid genotype from the same species. This shows that *Sitobion avenae* genotypes respond differently to intra-specific competition, in their choice of host barley genotypes and with varying strengths.

Aphid genotype CLO7 had the strongest effect on the other genotypes, altering the host choice of another aphid genotype to a greater extent than any other. Interestingly, this genotype was the least affected by being in competition with another aphid genotype. These results may indicate that CLO7 aphids induce a greater defense response in a plant than do the other genotypes, and therefore it can tolerate such environments but the other genotypes cannot. This has been indicated in another aphid species, where one aphid biotype induces a plant defense response, which reduces the reproductive performance of another aphid biotype but not itself (Hays et al. 1999). However, our study is the first to show that intra-specific competition can alter host choice behavior of an aphid genotype.

DAV95 aphids changed their host preference the most out of all the aphid genotypes tested, in the presence of another aphid genotype. These aphids were found in significantly lower proportions on barley genotype Steptoe when reared in the presence of another aphid compared to when reared alone. This indicates that the competing aphid genotype has created an environment on the Steptoe barley that cannot be tolerated by DAV95 and therefore DAV95 either moves away from this genotype onto another genotype or its reproductive performance on this genotype is reduced. Since aphid reproductive rate was not influenced across the whole pot due to the presence of

another genotype we suggest that the aphids are actively moving to seek suitable hosts. Indeed, DAV95 is found at greater numbers on barley genotype Morex when in the presence of another aphid genotype suggesting it is moving to this barley genotype from Steptoe. In our experiment, the barley genotypes were randomized within the pot, so two genotypes were never consistently next to each other. This indicates that when an aphid moves from a host plant to another, it uses specific cues to find another suitable host and will not just move to the closest neighbour plant. An alternative explanation for the pattern seen is that the presence of another aphid genotype improved the environment on the Morex barley genotype, thereby increasing aphid reproductive performance and impaired the environment on Steptoe barley genotype, thereby decreasing aphid performance (Petersen and Sandstrom 2001).

When H1 aphids were reared in the presence of another genotype, these aphids altered their host preference differently due to the identity of the competing aphid genotype. When competing with CLO7 aphids, H1 aphids were found less often on Baronesse barley and more often on Steptoe. However, when competing with HF92a aphids the H1 aphids were found more often on OWBrec and there was a trend for fewer to be present on barley BCD47. This shows that CLO7 aphids and HF92a aphids are potentially inducing different responses in the plants, which affect H1 aphids in varying ways.

There was no observation of physical fighting between the aphid genotypes, and no displacement, since aphids were found co-existing on the plants. The effects observed in this study are due to differences in the proportion of the total number of aphids on each barley genotype, rather than host-specificity between an aphid genotype and a host plant genotype. We therefore expect the mechanisms for the competition observed are via

plant-mediated traits, rather than direct competition between the aphids. Indirect competition between the aphid genotypes may occur through changes in the phloem composition (Petersen and Sandstrom 2001, Messina et al. 2002) or plant induced defenses (Hays et al. 1999, Walling 2000, Goggin 2007, Delp et al. 2009). Competition through plant-induced defenses could also occur via the release of volatiles by the plant (Du et al. 1998, Walling 2000). These volatiles can induce a defense response in neighbouring plants (Paré and Tumlinson 1999) and can alter aphid host plant acceptance (Ninkovic et al. 2002). In a natural community, these plant volatiles will also attract predators and parasitoids and could lead to apparent competition between aphid genotypes.

In this study, there was a greater effect of the black aphid clones (CLO7 and HF92a) on the host choice behavior of the green aphid clones (DAV95 and H1), which could be mediated by a bacterial symbiont. The green aphid clones are known to harbor the secondary facultative symbiont, *Regiella insecticola,* whereas the two black aphid clones do not (J. Ferrari, pers. comm.). Facultative secondary bacterial symbionts within aphids have been linked to host-plant species use, with a γ-proteobacterium increasing pea aphid fitness on white clover (Tsuchida et al. 2004) and *R. insecticola* reducing acceptance and performance of a number of aphid clones on *Vicia fabae* (Ferarri et al. 2007). This study indicates that the interaction between aphid and symbiont may influence its competitive ability with other aphid genotypes, although the mechanisms for this are unclear. Further work needs to be done to fully understand the influence of the symbiont on intra-specific competition and its affect on the host choice behaviour of the competing aphid. It is unlikely that aphid symbionts will explain all the variation observed in this study since differences were also seen between the aphid clones that shared a symbiont status (presence/absence of *Regiella insecticola*).

Understanding how intraspecific competition between aphid genotypes influences host-plant genotype choice of an aphid is important to further unravel the complex interactions between plants and phloem-feeding insects. Intra-specific competition between aphids could enhance isolation and divergence of populations through competitive exclusion or reinforcing host choice differences between aphids (Rundle and Nosil 2005, Matsubayashi et al. 2010). Consider an extreme example where two aphid genotypes exhibit strong host preferences for two different host plant genotypes, when these two aphids are grown in competition the host choice of each aphid is not altered and these two aphids still preferentially colonize different host plant genotypes. This would reinforce the original host choice and enhance the isolation and divergence between these aphid genotypes. On the other hand, if aphid genotypes show host preference for different plant genotypes but alter their preference due to competition with another aphid, dependant on the particular genotypic identity of the competing aphid, then the outcome of the host choice will become less predictable. The numbers of each aphid genotype on each host plant genotype will differ depending on the genotypic identity of the surrounding aphid community and thus lead to reduced isolation and divergence between these genotypes.

Overall, the aphid genotypes within this study did alter their host preference due to being in competition with another aphid genotype. Only one aphid genotype (CLO7) showed no change in preference, but this genotype has also been shown in previous studies to exhibit little host preference (chapter 3). Therefore, for the *Sitobion avenae* clones studied here, it is suggested that the change in host choice preference due to intraspecific competition may reduce non-random associations between the aphid and barley genotypes, in a genotypically diverse population. A previous study by Zytynska et al. (2010) showed that genetic interactions between *Sitobion avenae* genotypes

mediate indirect interactions between rhizobacteria in the soil and a paraitoid wasp. The authors suggest that these interactions could be maintained through non-random assortative association between the aphid and barley genotypes and lead to evolutionary change in the parasitoid wasp. However, the present study suggests that in a genotypically diverse community, these non-random associations could be broken down due to intra-specific competition between aphid genotypes.

Finally, understanding the influence of intraspecific competition on plant-insect interactions can provide economic benefits since many phytophagous insects transmit diseases that can devastate agricultural crops. As more is known about the interactions between insects and plants, including the ecology and underlying genetic mechanisms, strategies for reducing crop damage can be found, which reduce reliance on environment-damaging pesticides.

In conclusion, we have shown that intraspecific competition between aphid genotypes can alter the host plant genotype preference of an aphid, and the magnitude of this effect is dependent on the competing aphid genotypes. The competition between aphid genotypes is likely to be mediated through plant traits such as induced defenses and shows that, within a community, interactions between genotypes of the same species can influence their interactions with another species through behaviour modification. Our work demonstrates how genetic diversity within a community can influence the behaviour of a species. Thus, at the community level, an individual will be influenced by its own genotype, the genotype of conspecific interacting neighbours and the genotypes of interacting individuals from other species.

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Chapter 5. Intraspecific genetic variation in a phytophagous insect affects gene expression in a single plant genotype

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PERSONAL CONTRIBUTION

I designed and carried out the experiments in this paper. Technicians at the Genomic Technologies Core Facility at The University of Manchester performed the microarray hybridization. I statistically analysed the data for the first experiment and Violaine Jourdie analysed the fold-change and PPLR values for microarray data. I then collated the results and wrote this paper, with comments from the other authors.

ABSTRACT

Phytophagous insects are highly species diverse and this is partly attributed to the diversity of plant defense chemicals used to counter insect attack. A number of gene expression studies have shown the wide range of genes in plants that are induced by insect feeding. Phloem feeding insects, such as aphids, often induce pathogenic defense responses in the plant, as well as wounding defense responses, due to their piercing style of feeding. Interactions between plant genotypes and aphid genotypes show that genetically-based interactions between these species can influence both plant and insect fitness. Here I show that preconditioning of a single barley genotype with particular *Sitobion avenae* aphid genotypes can reduce the reproductive rate of other aphid genotypes from the same species. Insect induced gene expression profiles in a plant are known to differ due to plant genotype, and here we show that different aphid genotypes also induce differential gene expression in a single plant genotype. Differential induction of plant defense genes by different aphid genotypes could influence other herbivores feeding on the plants, as well as higher trophic organisms, such as parasitoid wasps.

INTRODUCTION

The high species diversity of phytophagous insects has been partly attributed to the diversity of chemicals used by a plant for defense (Futuyama and Agrawal 2009). Plant defenses can reduce insect fitness and therefore create strong selective pressures on the feeding insects. The high diversity of chemicals that plants can use in defense against phytophagous insects leads to many different ecological niches being available for colonization by phytophagous insects. This variety of different chemical defense strategies in plants means that host-plant shifts in insects often occur between plants with similar chemistry irrespective of the plant phylogenetic relationships, (Ehrlich and Raven 1964).

Plants actively respond to insect attack through the induction of toxins and defensive proteins (Howe and Jander 2008). A plant can employ a number of defensive strategies and these will also affect other herbivores that are feeding on the plant (Chen 2008). Different attackers (i.e. different herbivore species, species morphs or developmental stages) are expected to induce different responses in the plant, often due to the feeding behaviour, and this results in some insects inducing much stronger defense responses than others (Agrawal 2000). Initial colonization of a plant by different herbivore species has been shown to influence the subsequent colonization by other herbivores and species richness at the community-level (Van Zandt et al. 2004). This indicates that the induction of plant chemical defenses by particular species could alter community structure and dynamics. Community-level effects of differential gene expression have been studied in a field experiment on *Brassica oleracea* (Broekgaarden et al. 2010). Two *Brassica oleracea* cultivars were used that differed in gene expression of a number of defense related sequences. Plants from the different cultivars hosted different herbivore communities, with the overall number of herbivores lower in the cultivar that

had reduced expression of a number of plant defense related genes, thus indicating that differential gene expression between these cultivars influenced the herbivore community composition (Broekgaarden et al. 2010).

Two main groups of phytophagous insects are the chewing insects (e.g. caterpillars) and the sap-feeding insects (e.g. aphids). Sap-feeding insects use modified mouthparts (stylets) to feed on the plant phloem or xylem sap, thus creating a unique pathway between the insect and plant (Powell et al. 2006). This results in these insects inducing a pathogen-like response, more often induced by bacteria, fungi or viruses, as well as a general wounding response, which is also strongly induced by chewing insects (Walling 2000, Thompson and Goggin 2006, Howe and Jander 2008). A number of gene expression studies have demonstrated that phloem-feeding aphids induce expression of a wide variety of genes in a plant, including genes related to mechanical wounding, pathogenesis, metabolism, oxidative stress, signaling, cell wall modification, senescence and insect digestion (Moran et al. 2002, Voelckel et al. 2004, Zhu-Salzman et al. 2004, De Vos et al. 2005, Divol et al. 2005, Couldridge et al. 2007, Kuśnierczyk et al. 2008, Delp et al. 2009). Additionally, plants release volatile chemicals after experiencing damage by phytophagous insects to attract parasitoids (Du et al. 1998) and predators (Takabayashi and Dicke 1996) to the plant. These chemicals can also induce a defense response in neighbouring undamaged plants (Paré and Tumlinson 1999). Phloem-feeding aphids may also defend against the plant by releasing salivary enzymes and other compounds that can reduce the harmful effects of the plant defensive chemicals (Miles 1999). These genome-based interactions between plant and insect could lead to coevolution between the interacting species, although the presence of an interaction does not infer coevolution (Janzen 1980).

In pea aphids, there is variation among aphid genotypes for preference to different host plants (Ferrari et al. 2006), indicating that differences between plant species can influence insect colonization. Within a plant species, defense-related genes induced in response to *Rhopalosiphum padi* aphids have been shown to differ between resistant and susceptible barley genotypes (Delp et al. 2009). Further, Rovenska and Zemek (2006) showed that potato aphids (*Aulacorthum solani*) preferentially colonize control potato plants over transgenic potato plants (expressing the aphid resistant snowdrop lectin gene), suggesting the aphids could distinguish between the plants through the differential expression of a single gene. If different aphid genotypes induce responses that vary among plant genotypes that also reduces aphid reproductive potential, this would create genotype-by-genotype (GxG) interactions between the insect and plant based at the transcriptome, or gene expression level. Interspecific GxG interactions, where the specific combination of the interacting genotypes from each species is important to the outcome of the interaction, are known to influence fitness traits of both the aphids and host plant (Service 1984). Interactions between plant and aphid genotypes at the gene expression level could lead to a mechanism for the basis of observed GxG interactions. However, the effect of aphid genotype on the expression of induced defenses in plants is unknown. Hays et al. (1999) demonstrated that different aphid genotypes could potentially induce differential expression of plant defense genes. Hays et al. found that pre-conditioning a plant containing an aphid resistance gene with an aphid genotype that the plant was resistant to, influenced the probing and feeding behaviour of a second aphid genotype. However, pre-conditioning the plant with an aphid genotype that the plant was susceptible to, did not affect the feeding behaviour of a second aphid genotype. This was hypothesized to occur due to the expression of the aphid resistance gene in the plant, induced by an aphid genotype the plant was resistant to but not by the aphid genotype the plant was susceptible to.

Previous work (Chapter 3) has shown that different *Sitobion avenae* aphid genotypes show active preference for particular barley (*Hordeum vulgare*) genotypes. This suggests there are detectable differences between these plants, or differences in aphid detection ability, influencing the choice of different aphid genotypes. Furthermore, aphid genotype preference could be altered if an aphid genotype experienced competition with another aphid genotype (Chapter 4). In chapter 4, there was co-existence of aphid genotypes on the plant genotypes and no observation of physical fighting was seen. It is therefore hypothesized that these interactions are occurring through plant-mediated traits induced by the aphids and which influence the palatability or fitness differently among the aphid genotypes.

In this paper, we investigated whether pre-conditioning of a single barley genotype with particular *Sitobion avenae* aphid genotypes influences the reproductive potential of different aphid genotypes. This was to confirm that the interaction between the aphid genotypes was indirect, through plant-mediated traits, as opposed to direct competition between the aphids. We also investigated gene expression in a single barley genotype using microarrays, to see if different aphid genotypes induce differential gene expression in the plant. Finally, we compare these findings to determine if there is a relationship between the effect on aphid growth rate of pre-conditioning a plant with another aphid gentoype and the up-regulated genes induced in the plant by the aphids.

MATERIAL AND METHODS

Aphids and barley

A single barley genotype, Steptoe, was used for all experiments (seed originally obtained from P.Hayes, Oregon State University). Four aphid genotypes were used (CLO7, DAV95, H1 and HF92a), originally obtained from Rothamsted Research, UK. The aphid lines are kept in asexual reproduction (clonal lines) and were reared on a generic batch of *Hordeum vulgare* seed. Experimental barley seeds were harvested from plants grown in a common glasshouse environment, but separated by genotype to ensure no crossing occurred among genotypes. Barley seeds used in this experiment were taken from a single harvest to minimize differences from seed stocks grown in different years.

Do aphid induced plant traits affect aphid reproductive rate?

Experimental design. In this experiment, plants were first exposed to one of the four aphid genotypes (from here on called the pre-conditioning genotype). After five days these pre-conditioning aphids were removed and either the same or another aphid genotype was introduced to the plant. The experimental design was fully factorial, with all aphid genotypes being reared on plants that had been pre-conditioned by all other genotypes, including its own (12 treatments). Twenty repeats were made for each treatment.

Experimental set-up. The barley seeds were germinated by placing the seeds between two layers of filter paper, in a petri dish, moistened with sterilized distilled water. The petri dishes containing the seeds were placed in a dark growth chamber at 21ºC for five days. The barley seedlings were then transplanted into 4cm diameter pots (one seed per

pot) containing John Innes Compost No.3. After seven days, four aphids (3rd and 4th instar) were placed on a plant and each pot was covered using a plastic tube with a mesh top and mesh window, to isolate each plant from the others. The pre-conditioning aphids were left on the plants for five days, after which all aphids were carefully removed with a fine paintbrush and the plant checked carefully to ensure it was free of aphids. Two adult aphids were then placed on the plants and allowed to reproduce. The experiment was maintained at The Firs Botanical Grounds, The University of Manchester in a glasshouse at 18-25°C, 16:8 light:dark regime. Total aphid number was counted 14 days after the second aphids were introduced to the plants. Since equal numbers of aphids were introduced to the plants for a specific number of days, the final aphid number can also be considered a growth rate, comparable between aphid genotypes.

Data analysis. The data was analysed using a uni-variate ANOVA (JMP 8.0.2) to determine the influence of the pre-conditioning aphid genotype on the number of post-exposure aphids. Additional chi-square tests were used to determine if the number of aphids on the plants was reduced when the plants were pre-conditioned with a different aphid genotype (observed number) compared to the number of aphids on plants pre-conditioned with the same genotype of aphid (expected number).

Do aphid genotypes induce differential gene expression?

Experimental design. In this experiment, barley plants from a single genotype were exposed to four different aphid genotypes (CLO7, DAV95, H1 and HF92a) and there was a 'no-aphid' control. The aphids remained on the plant for five days after which the plant leaf material was harvested and the RNA extracted. The RNA was pooled from plants within the same treatment and plant gene expression analysed using microarrays.

Experimental set-up. The barley seeds were sterilized by soaking in 10% NaOCl for 10 minutes on a rotary shaker and then washed six times in sterilized distilled water. The seeds were germinated by placing between two layer of filter paper moistened with sterilized distilled water and placing in a dark growth chamber at 21ºC for five days. The seedlings were transplanted into 4cm pots filled with autoclaved horticultural grade sand, watered with sterilized distilled water. The experiment was undertaken in an experimental growth chamber at 21°C, 16:8 light:dark regime. Each pot contained one seedling and was covered using a plastic tube with a mesh top and mesh window. After two days, five 2nd or 3rd instar aphids were introduced to the plants. There were five treatments: four aphid genotypes (CLO7, DAV95, H1 and HF92a) and no aphids, with 15 repeats per treatment. In order to control for density effects across aphid genotypes, the plants were checked daily to ensure no aphid reproduction had occurred. The plants were watered with 40ml of autoclaved Hoaglands solution (Hoagland and Arnon 1950) on the second, fourth and sixth day. Five days after aphid infestation (day 7), the plants were harvested and the leaf material immediately submersed in liquid nitrogen. RNA was extracted from the leaf material using a Qiagen RNeasy® (Qiagen, Hilden, Germany) kit following the guidelines in the manual. One extraction was made per plant and the RNA quality was checked to ensure successful extractions. For each treatment (CLO7, DAV95, H1, HF92a, NoAphids), the RNA from five plants was randomly pooled resulting in three pooled biological repeats for each of the five treatments. Each sample was hybridized to a microarray chip (GeneChip Barley1 22k genome array, Affymetrix, Santa Clara, CA, USA) at the Genomic Technologies Core Facility at The University of Manchester. A total of 15 microarray hybridizations were performed.

Data analysis. Microarray data were analysed using the puma package implemented in Bioconductor (Pearson et al. 2009). This software uses a Bayesian approach (multi-mgMOS method; Liu et al. 2005) to associate credibility intervals with expression levels. Therefore, it does not calculate p-values, but rather the probability of a positive log ratio (PPLR), which gives a ranking of the significance of differential expression of probes but does not actually calculate a false-discovery rate (Liu et al. 2006). Fold changes (FC), i.e. the ratio between expression levels, were calculated from the multi-mgMOS normalized expression data. A probe was considered differentially expressed if PPLR < 0.15 or PPLR > 0.85, and FC < 0.5 (down-regulated) or FC > 2 (up-regulated). Information concerning target description, gene symbol, gene title, pathway, GO biological process term, GO molecular function and GO cellular component term was looked up for each probe in the NetAffx™ Analysis Center database (https://www.affymetrix.com). In addition, the sequences were also annotated using the Barley1.77 BEST BLASTX (www.harvest-web.org/barley1chip) and UniProt (www.uniprot.org).

Within this paper, we describe an increased gene expression in one treatment to another as up-regulation of the particular sequence. Thus, a decrease in gene expression from one treatment to another is described as down-regulation

RESULTS

Do aphid induced plant traits affect aphid reproductive rate?

Final aphid number was influenced by the genotype of the aphid counted (post-conditioning aphid: $F_{3, 301}$=7.03, p<0.0001). The genotype of the first aphid, used to pre-condition the plant, did not significantly influence the final aphid number as a main effect but there was a significant interaction effect between pre- and post-conditioning aphid genotype (Interaction effect: $F_{9,301}$=2.93, p=0.0023; Figure 5.1). Thus, the effect of post-conditioning aphid genotype (the aphid counted) was dependant on the genotype of the pre-conditioning aphid. The number of CLO7 aphids on the plants was reduced when the plants were pre-conditioned with a different aphid type (mean number of aphids: χ^2 = 34.89, p<0.0001, df=3; Figure 5.1), with aphid genotypes DAV95 and H1 causing the greatest effect (Table 5.1). The number of H1 and DAV95 aphids was reduced (mean number of aphids; DAV95 χ^2 = 13.89, p=0.003 df=3; H1 χ^2 = 10.55, p=0.014, df=3; Figure 5.1) due to pre-conditioning of the plants with aphid genotypes CLO7 and HF92a (Table 5.1). There was a trend for the mean number of HF92a aphids to alter when averaging across all aphid genotypes (χ^2 = 6.38, p=0.095 df=3; Figure 5.1), although this affect is primarily due to the reduced number of aphids on plants that have been pre-conditioned with H1 aphids (Table 5.1).

Table 5.1. Mean numbers of aphids on plants pre-conditioned with each aphid genotype

	Mean number of aphids			
Pre-conditioning aphid genotype	CLO7	DAV95	H1	HF92a
CLO7	**99.0**	75.6*	79.1*	126.4
DAV95	65.8*	**106.1**	113.5	112.5
H1	53.1*	113.2	**100.7**	92.4*
HF92a	83.5	83.8*	79.9*	**117.9**

Notes: Expected number of aphids is equal to the number observed when the plant is pre-conditioned with the same aphid genotype (bold). * p < 0.05

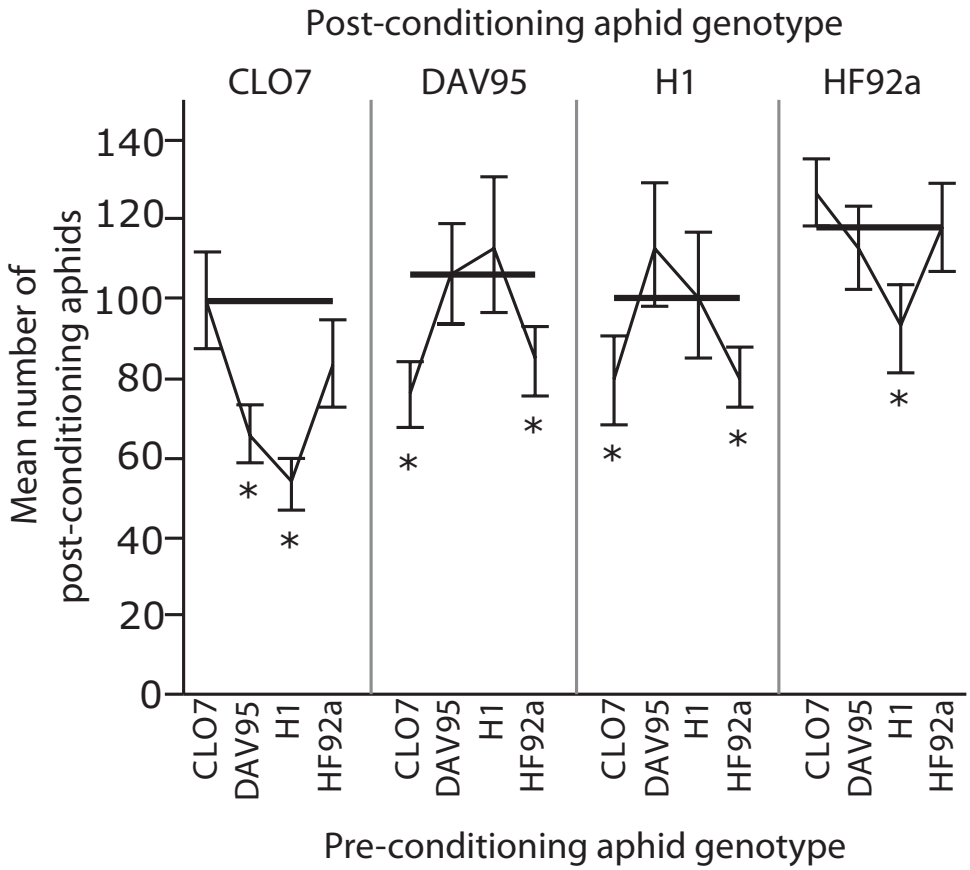

Figure 5.1. The number of aphids from the post-conditioning genotype (top axis), when Steptoe barley has been pre-conditioned with another aphid genotype (bottom axis). Stars (*) indicates significant deviation from the number of aphids when pre- and post-conditioning aphid are the same (black bars)

Aphid induced gene expression in plants

One microarray chip was discarded due to poor quality, which resulted in three replicates for each treatment except for the 'no aphid' treatment. From 22,840 sequences, aphid genotypes DAV95, HF92a, CLO7 and H1 induced the up-regulation of 197, 122, 93 and 55 sequences in the plant, respectively, compared to when plants were not infested with aphids ('no aphid' treatment). Thirty-four of these sequences were up-regulated by all aphid genotypes, compared to 'no aphid' plants, which included 16 sequences related to the jasmonate and lipoxygenase pathways and (Table 5.2). Aphid exposure reduced the expression of 19 sequences in the plants, including two blue-copper-binding protein homologs.

Table 5.2. Sequences up-regulated in the plant due to exposure to aphids, compared to plant not exposed to aphids

Description	Contig	Fold change	
Jasmonate pathway			
23 kDa jasmonate-induced protein	rbaal17b01_s_at	276.16	
24 kDa jasmonate-induced protein	rbags15p13_s_at	158.70	§
Jasmonate induced protein	Contig2900_at	286.84	§
Jasmonate induced protein	Contig6155_at	52.36	§
Jasmonate induced protein	Contig2899_s_at	12.38	§
Jasmonate induced protein	HX05K09r_at	2.58	
Jasmonate induced protein	Contig1675_s_at	2389.40	§
Jasmonate induced protein	Contig1679_s_at	15.18	§
Jasmonate induced protein	Contig1686_at	2.52	§
Putative 32.7 kDa jasmonate-induced protein	Contig7887_at	3.53	§
Putative 32.6 kDa jasmonate-induced protein	Contig7886_at	2.97	
Allene oxide synthase	Contig3097_at	2.37	§
Allene oxide synthase	Contig3096_s_at	2.31	
Lipoxygenase pathway			
Lipoxygenase 2 (LoxC)	HY03N19u_s_at	210.78	§
Lipoxygenase 2 (LoxC)	HI02E21u_s_at	34.29	§
Methyljasmonate-inducible lipoxygenase 2	Contig2305_at	3.82	
Lipase			
Lipase-like protein	Contig2631_at	13.14	§
Lipase, putative	Contig13413_at	11.83	§
Lipase, putative	Contig6611_at	2.35	
Thionin			
Thionin precursor, leaf	Contig1580_x_at	952.13	§
Barley mRNA for leaf-specific thionin	Contig1570_s_at	498.03	§
Others			
Sucrose:sucrose 1-fructosytransferase	Contig4521_s_at	6.85	§
O-methyltransferase	Contig6251_at	4.78	§
Lipid transfer protein	Contig3780_x_at	2.27	
Putative acid phosphatase	Contig2427_at	5.87	
Putative aminotransferase	Contig7739_at	2.58	
Putative aminotransferase	rbags11h24_s_at	2.42	§
Putative proteinase inhibitor	Contig50_x_at	3.66	
Putative proteinase inhibitor	Contig34_s_at	2.48	
Trypsin inhibitor (Bowman-Birk)	Contig2088_s_at	5.16	
Unknown	Contig15777_at	5.40	
Unknown	HB20B10r_at	4.65	
Unknown	Contig11993_at	4.52	
Unknown	HVSMEb0002K02r2_s_at	2.18	

Notes. Comparison between 'no aphids' and the average across all aphid genotypes used in the experiment.

§ = also differentially induced by different aphid genotypes

Do aphid genotypes induce differential gene expression?

From the 22,840 sequences on the gene-chip, 1004 were differentially induced in the plant by at least one aphid genotype compared to the others, including 18 sequences that were up-regulated by all aphid genotypes in the plant (Table 5.2). Differential expression of a sequence is defined as when the expression of a sequence in the plant due to exposure to one aphid genotype, compared to another aphid genotype, is increased or decreased by at least 2-fold (FC=2 or 0.5); the PPLR value for the comparison must also be significant. Many of the 1004 differentially expressed sequence probes were described as "hypothetical protein", "expressed protein", "unknown protein" or "none", and were discarded since, for this study, they provided no information on the expression of plant defense genes induced by aphid feeding. Ribosomal proteins, histones, transposons and retrotransposons were also removed, leaving 203 sequences. The complete list of the 203 regulated sequences, and the fold-change between aphid genotypes can be accessed as supplementary material (Table S5.1). The annotated sequences belonged to a number of different groups including abscisic acid, amino acid related, auxin, bet vI allergen, beta-glucosidase, blue-copper-binding proteins, caleosin, cell maintenance, cell wall modification, chitinase, cytochrome c, cytochrome P450, fasciclin domain containing proteins, GDSL-like proteins, gibberellin, glycosyls, hordeins, jamonate pathway, kinases, lipases, lipid-transfer proteins, lipoxygenase pathway, methlytransferase, peroxidases, phenylpropanoid biosynthesis, phosphatases, proteases, reactive oxygen species, resource allocation, shikimate pathway, thionins and ubiquitin.

Among these 203 sequences, DAV95 induced the up-regulation of 49.7 sequences in the plant compared to the other aphid genotypes, whereas aphid H1 only induced increased expression of 17.3 sequences in the plant over the other aphid genotypes (Table 5.3). The expression profiles induced in the plant by aphid genotypes CLO7 and

HF92a were most similar and DAV95 induced the least similar expression profile in the plant compared to the other aphid genotypes (Figure 5.2). CLO7 and DAV95 aphids produced the most different expression profiles compared to plants that were not exposed to aphids (Euclidean distances of 31.91 and 31.95, respectively). Aphid genotype H1 induced the regulation of a number of genes in common with all the other aphid genotypes.

Table 5.3. The number of up- and down-regulated sequences induced by each aphid genotype.

		Up-regulated				
	Aphid Genotype	CLO7	DAV95	H1	HF92a	Average
Down-regulated	CLO7	-	51	11	26	29.3
	DAV95	48	-	29	27	34.6
	H1	38	49	-	39	42.0
	HF92a	26	49	12	-	29.0
	Average	37.3	49.7	17.3	30.7	

Notes. Aphid genotype up-regulating is in columns and down-regulating is in rows. Data from comparisons among plant infestation with four aphid genotypes i.e. if a sequence is up-regulated then it is up-regulated relative to at least one other aphid genotype.

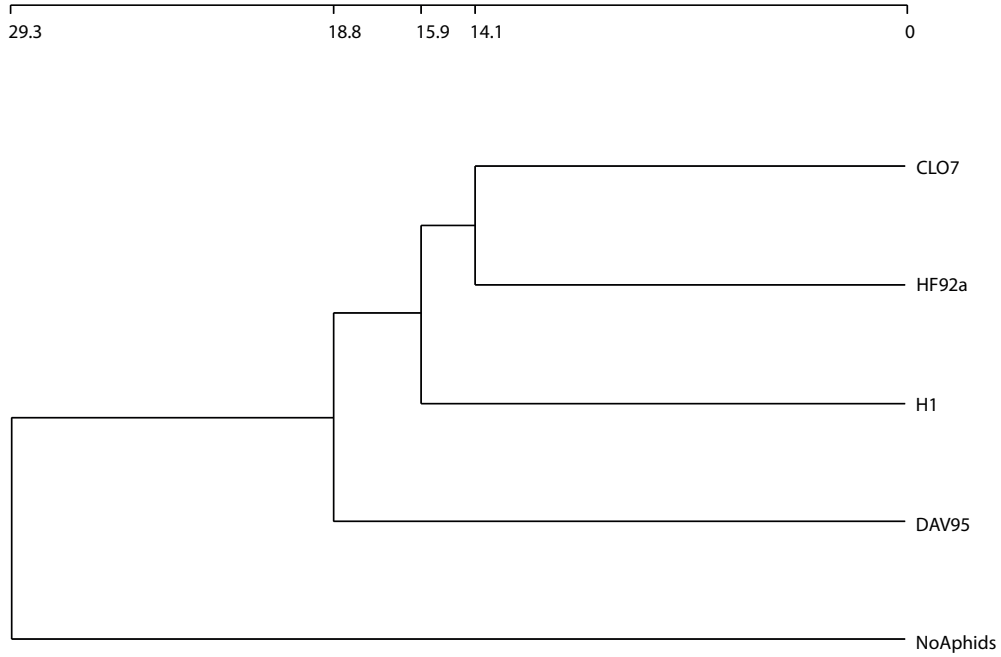

Figure 5.2. The Euclidean distance relationship between expression profiles induced by the four aphid genotypes. Data from the 203 differentially expressed sequences with respect to aphid treatment.

Each aphid genotype induced greater expression of specific sequences above all other genotypes and these are described as unique sequences. A unique sequence is therefore one that is up-regulated in the plant due to exposure to only one of the four aphid genotypes. DAV95 aphids induced the up-regulation of 23 unique sequences, whereas CLO7, HF92a and H1 induced up-regulation of only six, six and three unique sequences, respectively (Supplementary Figure S5.1). The six unique sequences up-regulated by barley plants infested by CLO7 aphids, included a putative cinnamoyl-CoA reductase (up-regulated 2.5-4.5 fold) and reduced expression of a peroxidase was detected (Table 5.4). DAV95 aphids induced greater expression of 23 unique sequences in the barley, including five thionin related sequences (up-regulated 2.1-5.1 fold) (Table 5.4). DAV95 aphids also induced reduced expression of a probable UDP-arabinose-4-epimerase (Table 5.4). The plants infested with H1 aphids showed up-regulation of only three unique sequences including a bacterial-induced peroxidase precursor and a low affinity nitrate transporter (Table 5.4). However, plants infested with H1 aphids did have reduced expression (down-regulation) of 11 unique sequences, of which five are related to the jasmonate pathway (Table 5.4). Plants infested with HF92a aphids did not show reduced expression of any unique sequence, but did show up-regulation of six unique sequences including a putative blue-copper-binding protein (up-regulated 2.6-5.7 fold) and sucrose-1-fructosytransferase (up-regulated 2.5-3.3 fold) (Table 5.4).

Table 5.4. Sequences differentially expressed in barley genotype Steptoe due to exposure to a specific *Sitobion avenae* aphid genotype (unique sequences).

Contig	Description	Fold change		
Aphid genotype CLO7		DAV95	H1	HF92a
Contig17213_at	Putative cinnamoyl-CoA reductase	4.5	2.5	3.0
Contig8550_s_at	Putative GDSL-like lipase/acylhydrolase protein	3.1	2.7	3.1
Contig3811_at	Galactinol synthase 3, putative, expressed	4.0	3.0	4.0
Contig3776_at	Putative lipid transfer protein	3.7	3.5	2.0
EBed07_SQ003_I06_at	Gamma-hordein 3	3.3	3.4	2.7
HT07A18u_x_at	Adenylate kinase	3.1	2.3	3.0
Contig2114_s_at	Peroxidase	0.34	0.27	0.27

Contig	Description	Fold change		
Aphid genotype DAV95		CLO7	H1	HF92a
Contig24511_at	Caleosin	3.5	3.0	3.4
HS05I21u_at	Putative laccase-15 precursor	3.2	3.0	2.6
Contig1267_at	S-adenosylmethionine synthetase	3.0	2.6	2.7
Contig5434_at	Cysteine-type endopeptidase inhibitor activity	3.2	3.5	2.2
Contig1039_at	Glycine-rich cell wall structural protein	2.3	2.6	2.2
Contig10778_at	Polygalacturonase isoenzyme 1 beta subunit	5.4	2.7	3.9
HVSMEn0022N15r2_at	Cytochrome P450-like protein	3.2	2.1	3.0
Contig5834_at	Probable hydroxymethylglutaryl-CoA reductase	5.4	2.5	2.9
HB26H24r_x_at	Beta-hordein	2.8	2.8	3.6
HVSMEi0004O10r2_at	Putative gamma 2 hordein	3.5	4.3	3.3
HT07A18u_at	Adenylate kinase	2.0	4.5	3.7
Contig25448_at	Serine/threonine kinase-like protein	4.5	2.3	2.6
HT07L09u_x_at	Lipid transfer protein precursor 1	2.8	2.6	2.4
HVSMEh0099O01f_s_at	Proteinase inhibitor	2.5	3.0	2.5
Contig3371_at	C13 endopeptidase NP1 precursor	3.7	2.1	3.2
Contig3782_x_at	Putative lipid transfer protein	2.6	2.1	2.0
Contig18242_s_at	Putative lipid transfer protein	2.6	2.1	2.6
Contig4478_at	Similar to copper chaperone protein	3.4	2.7	2.6
Contig796_at	Alpha-hordothionin	2.5	2.5	2.3
HB29O17r_x_at	Alpha-hordothionin	3.6	2.8	3.4
Contig1579_s_at	Leaf-specific thionin DB4	2.4	2.0	2.6
Contig1582_x_at	Leaf-specific thionin	2.1	5.7	3.2
Contig1583_at	Leaf-specific thionin	4.9	5.1	2.5
Contig5788_at	Probable UDP-arabinose 4-epimerase 1	0.15	0.19	0.17
Aphid genotype H1		CLO7	DAV95	HF92a
HVSMEf0014C12r2_at	Fasciclin domain containing protein	2.0	3.7	4.7
HVSMEf0021D01r2_x_at	Bacterial-induced peroxidase precursor	3.9	4.8	3.6
Contig22626_at	Low affinity nitrate transporter	2.0	2.5	2.6
Contig2900_at	Jasmonate induced protein	0.22	0.37	0.26
HX05K09r_at	Jasmonate induced protein	0.44	0.41	0.45
Contig1675_s_at	23 kDa jasmonate-induced protein	0.42	0.42	0.49
Contig1679_s_at	23 kDa jasmonate-induced protein	0.14	0.23	0.16
Contig1686_at	23 kDa jasmonate-induced protein	0.14	0.21	0.26
Contig13413_at	Putative phospholipase protein	0.30	0.28	0.17
Contig2631_at	Putative phospholipase protein	0.15	0.29	0.18
HY03N19u_s_at	Lipoxygenase 2	0.16	0.28	0.15
Contig6251_at	O-methyltransferase 3	0.22	0.31	0.10
rbags11h24_s_at	Putative aminotransferase	0.30	0.48	0.27
Contig1570_s_at	Barley mRNA for leaf-specific thionin	0.07	0.05	0.09
Aphid genotype HF92a		CLO7	DAV95	H1
Contig13477_at	Putative blue copper-binding protein	4.1	5.7	2.6
Contig20099_at	Putative protein kinase	2.4	5.4	2.4
HV_CEb0001H04r2_at	Putative protein kinase Xa21, receptor type	7.6	4.7	3.8
Contig4521_s_at	Sucrose-1-fructosytransferase	2.5	2.7	3.3
Contig3547_s_at	O-methyltransferase	3.2	3.9	2.5
Contig504_x_at	Putative lipid transfer protein	3.3	3.0	4.5

Notes. Fold change expressed as up-regulation (>2) or down-regulation (<0.5) by focal aphid genotype, compared to other aphid genotypes (in columns). Significance of fold changes was calculated using PPLR values (i.e. significant if PPLR>0.85 or PPLR<0.15); only sequences with significant fold changes are presented.

Does the gene expression data explain the phenotypic experiment data?

There was no correlation between the number of aphids of the post-conditioning genotype, in the first experiment, and the number of up-regulated sequences, in the plant, induced by the pre-conditioning aphid genotype (Pearson's r = -0.194, p=0.47). Therefore, an increased number of up-regulated sequences by the pre-conditioning aphid genotype did not decrease final aphid number of the post-conditioning genotype.

A number of sequences were differentially expressed when comparing particular genotype comparisons (Supplementary Figure S5.1). Pre-conditioning of the plants with CLO7 aphids reduced the number of DAV95 and H1 aphids on the plants, but not the number of HF92a aphids (Figure 5.1). Compared to both DAV95 and H1 aphids, CLO7 aphids induced the up-regulation of four sequences including a chalcone synthase and lipoxygenase that were also up-regulated by HF92a aphids (Table 5.5). Additionally, CLO7 aphids increased the expression of 19 sequences compared to DAV95 aphids and seven sequences compared to H1 aphids, of which three of these were also up-regulated by HF92a compared to H1 (Table 5.5). Pre-conditioning of plants with HF92a aphids reduced the growth rate of DAV95 and H1 aphids, but not of CLO7 aphids (Figure 5.1). Apart from the sequences up-regulated by both HF92a and CLO7, HF92a induced greater expression of four sequences compared to both DAV95 and H1, seven sequences compared to DAV95 aphids and eight sequences compared to H1, of which five were also up-regulated by CLO7 aphids (Table 5.5). When plants were pre-conditioned with DAV95 the only aphid genotype to have reduced growth rate was CLO7, despite DAV95 inducing the up-regulation of many more unique genes in the plant. DAV95 aphids induced the up-regulation of seven sequences compared to CLO7, of which two were also up-regulated by H1 aphids compared to CLO7 (Table 5.5). Pre-conditioning of a plant with H1 aphids reduced the growth rate of CLO7 and HF92a aphids, but not DAV95 aphids (Figure 5.1). Two sequences were up-regulated by H1

aphids compared to both CLO7 and HF92a, four sequences in the plant compared to just CLO7, of which two of these are also up-regulated by DAV95 aphids and one sequence compared to just HF92a (Table 5.5).

Table 5.5. Putative sequences up-regulated by one aphid genotype which may reduce growth rate of a different aphid genotype.

Contig	Description		Fold change	
Aphid genotype CLO7			**DAV95**	**H1**
Y09233_at	Chalcone synthase 2	‡	4.24	4.29
Contig2519_x_at	Endochitinase 2 precursor		2.15	2.54
Contig9308_s_at	Putative cytochrome P450		7.41	4.38
HI02E21u_s_at	Lipoxygenase 2 (LoxC)	‡	5.15	8.38
HV11O04r_at	Glutamine-dependent asparagine synthetase		7.03	
Contig3235_at	Methylenetetrahydrofolate reductase		2.26	
Contig3237_s_at	Methylenetetrahydrofolate reductase		2.94	
Contig16509_at	Putative phosphoribosylformylglycinamidine synthase		3.81	
Contig3421_at	Casein kinase II		2.09	
Contig11835_at	Protein kinase		2.15	
Contig22980_at	Protein receptor-like protein kinase 5 precursor		2.13	
Contig4376_at	Senescence-associated protein-like		2.16	
HVSMEn0009M15f_s_at	Senescence-associated protein-like		2.43	
Contig10361_at	Iron/ascorbate-dependent oxidoreductase		2.14	
Contig18012_at	Protein Ser/Thr protein phosphatase family protein		6.33	
Contig88_x_at	CI2c endopeptidase inhibitor		4.20	
Contig3775_s_at	Lipid-transfer protein		3.79	
Contig6685_at	Putative Serine Carboxypeptidase homologue		2.14	
Contig7374_at	Magnesium transporter CorA-like family protein		2.02	
Contig613_s_at	Putative malate dehydrogenase		2.22	
Contig20244_at	Putative thioredoxin		2.15	
Contig1086_at	Polyubiquitin		3.60	
HI04D03u_at	Ubiquitin carboxyl-terminal hydrolase, family 1		4.68	
Contig5994_at	Arginine decarboxylase	‡		2.19
HU05I16u_at	Protein xylanase inhibitor			2.41
Contig12571_at	Protein cytochrome P450			2.93
Contig15712_s_at	Cytokinin-O-glucosyltransferase 2	‡		2.41
rbags15p13_s_at	23kDa jasmonate-induced protein	‡		3.62
Contig6155_at	Jasmonate induced protein			2.57
Contig25762_at	Lipid-transfer protein			3.31
Aphid genotype HF92a			**DAV95**	**H1**
Contig10435_at	Xylan synthase protein		3.41	2.56
M36941_at	C hordein		4.01	2.67
Contig3097_at	Allene oxide synthase		2.47	2.87
HI02E21u_s_at	Lipoxygenase 2 (LoxC)	†	6.34	10.32
Contig3774_s_at	Lipid-transfer protein		2.34	2.69
HV11O04r_s_at	Glutamine-dependent asparagine synthetase 1		2.45	
Contig7036_at	Similar to blue copper-binding protein		2.61	
Contig6782_at	Fasciclin domain containing protein		2.50	
Contig4986_at	Allene oxide cyclase		2.11	
Contig13973_at	Protein kinase calcineurin-B-like		2.03	
Contig3829_at	Serine/threonine Kinase		3.86	
Contig955_s_at	Lipid transfer protein precursor 2		2.24	
Contig5038_at	O-methyltransferase		3.52	
Contig12199_at	Putative peroxidase		2.24	
HU03I15u_s_at	Protein phosphatase		2.39	

Contig13772_at	L-ascorbate oxidase		5.16	
Contig5714_s_at	Protein shikimate kinase, putative, expressed		3.18	
Contig5994_at	Arginine decarboxylase	†		2.79
Y09233_at	Chalcone synthase 2	†		2.35
Contig15987_at	1-aminocyclopropane-1-carboxylate oxidase 2			2.08
Contig15712_s_at	Cytokinin-O-glucosyltransferase 2	†		2.03
rbags15p13_s_at	23kDa jasmonate-induced protein	†		3.70
rbags10g12_at	Protein methylthioribose kinase			2.81
Contig16993_at	Serine/threonine-protein kinase			2.51
Contig88_x_at	CI2c endopeptidase inhibitor	†		5.37

Aphid genotype DAV95			**CLO7**	
rbaal24l19_at	Acetolactate synthase small subunit		2.55	
Contig22353_at	Chitinase		2.00	
Contig14804_at	Protein cytochrome P450	§	2.07	
Contig21640_at	Glutathione S-transferase TSI-1		3.44	
Contig24771_at	O-methyltransferase		2.33	
HV_CEa0002I10r2_at	Glucose-6-phosphate isomerase		3.07	
Contig254_x_at	Alpha-hordothionin	§	2.25	

Aphid genotype H1			**CLO7**	**HF92a**
Contig14804_at	Protein cytochrome P450	Δ	2.66	2.58
Contig15886_at	Defensin thionin		3.96	3.47
Contig2114_s_at	Peroxidase precursor	Δ	3.71	
Contig9791_at	Mannose-6-phosphate isomerase		2.49	
Contig254_x_at	Alpha-hordothionin	Δ	2.35	
HVSMEb0005M08r2_at	Polyubiquitin		2.66	
Contig16998_at	Ca dependent protein kinase			2.03

Notes. Fold change expressed as up-regulation (>2) or down-regulation (<0.5) by focal aphid genotype, compared to other aphid genotypes (in columns). Significance of fold changes was calculated using PPLR values (i.e. significant if PPLR>0.85 or PPLR<0.15); only sequences with significant fold changes are presented.

‡ also up-regulated by HF92a aphids,

† also up-regulated by CLO7 aphids,

§ also up-regulated by H1 aphids,

Δ also up-regulated by DAV95 aphids

DISCUSSION

This paper shows that aphid growth rate can be reduced when a single genotype of barley (Steptoe) is pre-conditioned with a different aphid genotype. These results concur with Hays et al. (1999), who showed that pre-conditioning of a plant with one aphid biotype affects the probing and feeding behaviour of a second aphid biotype. In the present study, not every aphid genotype influenced the growth rate of all other aphid genotypes, indicating there is variation for these traits depending on the genotype of both aphids. The growth rate of three of the four aphid genotypes was reduced after pre-conditioning of the plant with two other aphid genotypes, which means that each of these aphid genotypes are not influenced by one aphid genotype. Additionally, one aphid genotype (HF92a) only showed reduced numbers on plants that were pre-conditioned by a single other genotype (H1). By carefully removing all aphids from the plants before introducing the second genotype we have shown that the effect of one aphid on another in a single barley genotype is likely to occur via plant-mediated traits such as induced defense response. The second part of this study showed that gene expression within a single genotype of barley is altered due to exposure to different aphid genotypes. This experiment confirmed that the different aphid genotypes induced differential expression of plant defense-related genes in a single genotype of barley.

A number of studies have identified many different genes that are induced in a plant due to aphid feeding (Moran et al. 2002, Voelckel et al. 2004, Zhu-Salzman et al. 2004, De Vos et al. 2005, Divol et al. 2005, Couldridge et al. 2007, Kuśnierczyk et al. 2008, Delp et al. 2009). Differences in induced gene expression by an aphid on susceptible and resistant barley genotypes show that there is genetically based differences between the plant genotypes related to defense responses to aphids (Delp et al. 2009). We have shown that different aphid genotypes induce differential gene expression in a single

plant genotype. A total of 1004 sequences, from the 22 840 on the gene chip, were differentially expressed in the plant due to being exposed to different aphid genotypes. This means that genetic variation within *Sitobion avenae* aphids can alter the gene expression pattern in a single plant genotype.

Only thirty-four sequences were up-regulated (increased expression) in the plant by all aphid genotypes (average of data from all aphid genotype treatments) compared to plants which had not been exposed to aphids. Eighteen of these were also differentially induced by different aphid genotypes, indicating these sequences are up-regulated in the plant by exposure to all aphid genotypes, but some aphid genotypes induced greater expression than others. Of these sequences, a number were related to the well-known wound-responsive jasmonate and lipoxygenase pathways (Walling et al. 2000, Feussner and Wasternack 2002, Turner et al. 2002, Howe and Jander 2008). Therefore, this study highlights how much information can be lost by using only a single aphid clone or by calculating the average expression level across a number of aphid genotypes.

From the 1004 differentially expressed sequences, only 203 could be annotated due to lack of information. We used the fold change (or ratio) in expression levels of these sequences comparing plants exposed to one genotype compared to another to detect the differences in induced responses by the different aphid genotypes. To reduce the chance of false positive results, we only considered fold changes of two or above (less than 0.5 if considering reduced expression), and further only those with a significant PPLR (probability of positive log ratio; Liu et al. 2006) were included. Therefore the sequences in this study, which were differentially induced by different aphid genotypes, are expected, in some way, to be involved in the interaction between these phytophagous insects and the plant. The use of microarray data can, however,

sometimes mislead the functional understanding of the traits involved, since annotations are described as opposed to traits (Kant and Baldwin 2007). Thus, the results presented in this paper are only a step forward in understanding how within-species genetic variation in a phytophagous insect species can alter gene expression within a single genotype of plant. Further in-depth work, involving techniques such as quantitative RT-PCR, would need to be used to identify specific candidate genes involved in plant defense against particular aphid genotypes.

The aphid genotypes CLO7 and HF92a induced the most similar expression profiles in the plant, and in line with this, neither had an effect on the other's growth rate in the primary experiment. DAV95 aphids induced the least similar profile to any other aphid genotype and they also induced increased expression of the greatest number of unique sequences in the single plant genotype tested. A unique sequence is described as one that is up-regulated in the plant due to the exposure to a single aphid genotype. The increased number of up-regulated unique sequences in DAV95 did not relate to an effect on other aphid genotypes since pre-conditioning of a plant with DAV95 aphids only reduced the growth rate of CLO7 aphids, whereas all the other aphid genotypes had an effect on two of the other aphid genotypes. The results showed that the number of up-regulated genes induced by the pre-conditioning aphid genotype is not correlated with aphid number on a plant, of the second aphid genotype. This indicates that specific genes in the plant are having an effect between aphid genotypes, rather than an overall increase in the diversity of plant defense responses induced by particular aphid genotypes. These results are not unexpected since the response of the plant to different genotypes within an aphid species is expected to be relatively similar as they all belong to the same species. The differences between the genotypes will be due to subtle differences in, for example, the behaviour of feeding, such as probing or stylet insertion,

and potentially due to genetic differences in saliva enzymes, although this is still to be empirically tested (Miles 1999, Howe and Jander 2008).

No single genotype of aphid induced a response in the plant that reduced the growth rate of all other genotypes and this can be explained in two different ways. The first is that the genes of interest are those up-regulated by an aphid genotype only compared to the aphid genotype or genotypes that also show reduced growth rate. Alternatively, the second way these results can be explained is if an aphid genotype increases the expression of a gene in the plant relative to all other aphid genotypes, but one (or more) of the aphid genotypes has tolerance to the related defense in the plant. This would result in the fitness of the aphid exhibiting tolerance not being reduced due to the induced expression of that gene by the pre-conditioning aphid. Genetic polymorphism in the aphid population could be maintained through these competitive interactions between aphid genotypes, since no single aphid genotype will be superior relative to all others, even on a single plant genotype, in a genotypically diverse aphid population.

In this study, two of the four aphid clones (DAV95 and H1) are host to a secondary facultative bacterial symbiont (*Regiella insecticola*; J. Ferrari pers. comm.). From the primary experiment results, it shows that the two aphid genotypes hosting the symbiont have reduced numbers when a plant is pre-conditioned with either of the two other non-symbiont hosting aphid genotypes. A similar effect was found vice-versa, with the symbiont hosting aphid genotypes reducing the numbers of the non-symbiont aphid genotypes, although the number of HF92a aphids was not reduced when a plant was pre-conditioned with DAV95 aphids. The effect of symbiont presence/absence on plant gene expression was not investigated in this study it shows that there is a potential influence of aphid symbiont on induced defense responses in the plant. Aphid

facultative bacterial symbionts have been linked to host-plant use (Tsuchida et al. 2004) and the acceptance and performance of aphids on a plant (Ferarri et al. 2007), suggesting these symbionts may mediate the effect of aphid genotype on induced plant defenses.

Plants exposed to DAV95 aphids show increased expression of many thionin related sequences in the plant relative to the other aphid genotypes. These include alpha- and beta- thionins and defensins (gamma-hordothionins) and are thought to be involved in plant defense against pathogens, acting directly on the membranes, and insects, through inhibiting insect digestive enzymes (Stec 2006, Gorjanovic 2009). Additionally, plants exposed to DAV95 aphids show reduced expression of UDP-arabinose-4-epimerase relative to the other aphid genotypes. This gene is related to cell wall synthesis (Zhang et al. 2005) and indicates that DAV95 aphids cause less mechanical damage than do the other aphid genotypes. Plants exposed to H1 aphids show increased expression of a bacterial-induced peroxidase precursor, which suggests it is invoking a more pathogenic response than a wounding response in the plant. Additionally, H1 aphids induce a much lower expression of many jasmonate pathway proteins compared to the other aphid genotypes, which may suggest it also induces less of a general wounding response in the plant. The reduction of jasmonate related proteins in the plant after H1 exposure may leave the plant susceptible to insect attack; however, pre-conditioning a plant with H1 aphids reduces the number of CLO7 and HF92a aphids. Therefore, any reduction in the expression of jasmonate proteins is likely to influence only the H1 aphids since as soon as alternative aphid genotypes attack the plant they will induce the up-regulation of these genes. Aphid genotypes CLO7 and HF92a do not induce the expression of particular pathways in the plants, but relative to both H1 and DAV95 aphids (the genotypes affected by pre-conditioning with these genotypes) these aphids induce a

greater expression of a lipoxygenase sequence in the plant (increasing the expression by up to 10-fold). The lipoxygenase pathway, induced through tissue wounding, is a known plant defense pathway against insect attack and is related to the jasmonate pathway (Feussner and Wasternack 2002).

The interactions between an aphid and plant are complex and I have shown that there is plasticity in a single genotype of plant due to the biotic environment of the aphid genotype it is exposed to. This work suggests that genotype-by-genotype (GxG) interactions between phytopagous insects and their plant hosts may be mediated by the induction of differential gene expression profiles in the plant by the different insect genotypes. The interaction between barley genotypes and *Sitobion avenae* aphid genotypes can also be altered by the addition of rhizobacteria in the soil (Tétard-Jones et al. 2007). A potential mechanism for this is that rhizobacteria prime plants by inducing a defense response, creating a new environment for the feeding aphid (Ahn et al. 2007, Van Wees et al. 2008). This could lead to the observed genotype-by-genotype-by-environment (GxGxE) interaction between aphid genotype, barley genotype and presence/absence of rhizobacteria, if there was genotypic variation in the response of both aphids and barley to the change in environment by the rhizobacteria. The GxG interactions between these aphids and barley are also known to influence the indirect interactions between the rhizobacteria and a parasitoid wasp, which uses the aphid as a host (Zytynska et al. 2010). These studies indicate that plant-mediated changes and genotypic variation among the interacting species can have wide-ranging effects for the rest of the community. Changes in gene expression within a plant have been shown to influence the community of herbivores found on the plant, and these changes in expression may be induced by specific herbivores in that community (Agrawal 2000, Van Zandt et al 2004, Broekgaarden et al. 2010). Understanding the complex

interactions between phytophagous insects, their plant hosts and the associated ecological community, will enable more efficient control of disease-spreading insects on economically important crop plants, without the use of environmentally damaging pesticides.

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SUPPLEMENTARY DATA

Table S5.1. List of 203 significantly differentially expressed sequences in barley genotype 'steptoe' due to exposure to different aphid genotypes

		Fold change (>2 up-regulation, <0.5 down-regulation)											
		CLO7			DAV95			H1			HF92a		
Description	**Contig**	**DAV95**	**H1**	**HF92a**	**CLO7**	**H1**	**HF92a**	**CLO7**	**DAV95**	**HF92a**	**CLO7**	**DAV95**	**H1**
Abscisic acid													
Abscisic acid-induced protein ABA7	Contig6276_s_at						**3.09**					0.32	
Amino acids													
Acetolactate synthase small subunit	rbaal24l19_at	0.39			**2.55**								
Arginine decarboxylase	Contig5994_at		**2.19**					0.46		0.36			**2.79**
Cysteine-type endopeptidase inhibitor activity	Contig5434_at	0.31			**3.24**	**3.49**	**2.19**		0.29			0.46	
Glutamine synthase root isozyme 3	HS09M17u_s_at	**2.11**			0.47	0.47			**2.12**				
Glutamine-dependent asparagine synthetase	HV11O04r_at	**7.03**			0.14								
Glutamine-dependent asparagine synthetase 1	HV11O04r_s_at						0.41					**2.45**	
S-adenosylmethionine synthetase	Contig1267_at	0.34			**2.95**	**2.56**	**2.70**		0.39			0.37	
Tryptophan decarboxylase	Contig23667_at		0.44			0.13		**2.28**	**7.52**				
Methylenetetrahydrofolate reductase	Contig3235_at	**2.26**			0.44								
Methylenetetrahydrofolate reductase	Contig3237_s_at	**2.94**			0.34								
Auxillin													
Putative auxillin	Contig1907_s_at	**6.51**		**2.85**	0.15						0.35		
Auxin													
Auxin-responsive protein	Contig9343_at	0.36			**2.79**		**2.42**					0.41	
Putative auxin-responsive protein IAA2	HVSMEb0011L05r2_x_at			0.42						0.32	**2.38**		**3.16**
Bet v I allergen													
Bet v I allergen family	Contig2773_s_at					**2.27**			0.44				
Beta-glucosidase													
Beta-glucosidase	Contig2347_at	**12.05**		**3.15**	0.08						0.32		
Blue copper-binding													
Blue copper-binding protein homolog	Contig3208_at					0.36			**2.82**				
Blue copper-binding protein homolog	Contig3211_at	**6.12**			0.16	0.16			**6.40**	**2.01**			0.50
Blue copper-binding protein II	Contig13477_at			0.24			0.18			0.39	**4.15**	**5.66**	2.59
Similar to blue copper-binding protein	Contig7036_at						0.38					**2.61**	
Caleosin													
Caleosin	Contig24511_at	0.29			**3.46**	**3.02**	**3.36**		0.33			0.30	

Description	Contig	Fold change (>2 up-regulation, <0.5 down-regulation)											
		CLO7			DAV95			H1			HF92a		
		DAV95	H1	HF92a	CLO7	H1	HF92a	CLO7	DAV95	HF92a	CLO7	DAV95	H1
Cell wall modification													
Glycine-rich cell wall structural protein	Contig1039_at	0.43			**2.30**	**2.60**	**2.21**		0.38			0.45	
Pectinesterase	Contig13318_at	**4.90**		**2.03**	0.20						0.49		
Polygalacturonase	Contig21718_at	0.46			**2.16**		**2.87**					0.35	
Polygalacturonase isoenzyme 1 beta subunit	Contig10778_at	0.19			**5.37**	**2.68**	**3.93**		0.37			0.25	
Protein xylanase inhibitor, putative, expressed	HU05I16u_at		**2.41**					0.41					
Putative glycine-rich cell wall structural protein 2 precursor	Contig13783_at		**2.70**	**3.51**				0.37			0.29		
Xylan synthase protein, putative, expressed	Contig10435_at						0.29			0.39		**3.41**	**2.56**
UDP-galactose 4-epimerase-like protein	Contig5788_at	**6.65**			0.15	0.19	0.17		**5.18**			**5.97**	
UDP-glucose dehydrogenase, putative	HVSMEl0011E20r2_at		**2.48**			**3.12**		0.40	0.32				
UDP-glucuronic acid decarboxylase, putative	Contig2031_s_at	**2.69**			0.37	0.42			**2.36**				
UTP-glucose-1-phosphate uridylyltransferase, putative	Contig19088_at			**2.44**		0.21			**4.66**	**2.88**	0.41		0.35
Chalcone													
Chalcone synthase 2	Y09233_at	**4.24**	**4.29**		0.24			0.23		0.43			**2.35**
Chitinase													
Chitinase	Contig22353_at	0.50			**2.00**								
Chitinase	Contig22353_x_at	0.28		0.31	**3.62**	**2.54**			0.39	0.44	**3.26**		**2.28**
Endochitinase 2 precursor (CHI-26)	Contig2519_x_at	**2.15**	**2.54**		0.47			0.39					
Protein glycosyl hydrolases family 16, putative	HVSMEf0011A08r2_at			0.30						0.27	**3.38**		**3.67**
Protein glycosyl hydrolases, putative, expressed	Contig15050_at						**2.06**					0.48	
Cytochrome C													
Cytochrome b5 reductase	Contig3972_at	**2.28**			0.44	0.46			**2.18**				
Cytochrome c oxidase subunit	S0000800001B06F1_at						**2.41**					0.41	
Putative cytochrome c1 precursor	Contig3118_at	**2.18**			0.46	0.50			**2.00**				
Cytochrome P450													
Cytochrome P450	HVSMEf0019N09r2_at	0.21		0.22	**4.71**						**4.60**		
Cytochrome P450-like protein	HVSMEn0022N15r2_at	0.31			**3.24**	**2.06**	**3.00**		0.48			0.33	
Protein cytochrome P450, putative, expressed	Contig12571_at		**2.93**					0.34					
Protein cytochrome P450, putative, expressed	Contig14804_at	0.48	0.38		**2.07**			**2.66**		**2.58**			0.39
Putative cytochrome P450	Contig12509_at					**2.78**	**4.21**		0.36			0.24	
Putative cytochrome P450	Contig22442_at	0.22	0.11		**4.59**		**15.92**	**9.25**		**32.06**		0.06	0.03
Putative cytochrome P450-related protein	Contig9308_s_at	**7.41**	**4.38**		0.13			0.23					

		Fold change (>2 up-regulation, <0.5 down-regulation)											
		CLO7			DAV95			H1			HF92a		
Description	**Contig**	**DAV95**	**H1**	**HF92a**	**CLO7**	**H1**	**HF92a**	**CLO7**	**DAV95**	**HF92a**	**CLO7**	**DAV95**	**H1**
Ethylene pathway													
Protein 1-aminocyclopropane-1-carboxylate oxidase 2	Contig15987_at									0.48			**2.08**
Fasciclin													
Fasciclin domain containing protein, expressed	Contig6782_at						0.40					**2.50**	
Fasciclin domain containing protein, expressed	HVSMEf0014C12r2_at		0.50			0.27		**2.01**	**3.66**	**4.72**			0.21
GDSL-like protein													
GDSL-like Lipase/Acylhydrolase family protein	Contig3996_at	0.49		0.17	**2.06**					0.26	**5.73**		**3.89**
GDSL-like lipase/acylhydrolase, putative, expressed	Contig14709_at					0.47			**2.13**				
GDSL-like lipase/acylhydrolase, putative, expressed	Contig8550_s_at	**3.09**	**2.75**	**3.09**	0.32			0.36			0.32		
GDSL-like lipase/acylhydrolase, putative, expressed	EBro04_SQ004_O07a_at	0.26			**3.84**		**2.60**					**0.38**	
GDSL-like lipase/acylhydrolase, putative, expressed	HR01B05u_at						**2.56**					**0.39**	
GDSL-like lipase/acylhydrolase, putative, expressed	rbasd11p06_at	0.34			**2.95**		**3.08**					**0.32**	
Serine esterase family protein, putative, expressed	Contig6620_at	0.31			**3.24**		**2.79**					**0.36**	
Serine esterase family protein, putative, expressed	HW09E23u_at					0.32			**3.13**				
Gibberellin													
Protein gibberellin receptor GID1L2, putative, expressed	HVSMEg0016J19r2_s_at	0.37			**2.69**	**2.75**			0.36				
Glycosyl													
Cytokinin-O-glucosyltransferase 2, putative	Contig15712_s_at		**2.41**					0.42		0.49			**2.03**
Glycosyl transferase protein A-like	HF11O19r_at	**4.95**		**5.58**	0.20					**3.76**	0.18		0.27
Hordein													
B-hordein	Contig765_x_at			**2.61**							0.38		
B-hordein	HB26H24r_x_at	0.35			**2.88**	**2.76**	**3.57**		0.36			0.28	
C hordein	M36941_at						0.25			0.38		**4.01**	**2.67**
Gamma-hordein 3	EBed07_SQ003_I06_at	**3.35**	**3.44**	**2.67**	0.30			0.29			0.37		
Gamma-hordein 1 precursor	HVSMEi0004O10r2_at	0.29			3.47	4.27	3.27		0.23			0.31	
Jasmonate pathway													
23kDa jasmonate-induced protein	Contig1675_s_at		**2.41**			**2.37**		0.42	0.42	0.49			**2.06**
23kDa jasmonate-induced protein	Contig1679_s_at		**7.07**			**4.43**		0.14	0.23	0.16			**6.15**
23kDa jasmonate-induced protein	Contig1686_at		**7.23**			**4.75**		0.14	0.21	0.26			**3.91**
23kDa jasmonate-induced protein	rbags15p13_s_at		**3.62**					0.28		0.27			**3.70**
Allene oxide cyclase	Contig4986_at						0.47					**2.11**	
Allene oxide synthase	Contig12918_at						**2.34**					0.43	

		Fold change (>2 up-regulation, <0.5 down-regulation)											
		CLO7			DAV95			H1			HF92a		
Description	**Contig**	**DAV95**	**H1**	**HF92a**	**CLO7**	**H1**	**HF92a**	**CLO7**	**DAV95**	**HF92a**	**CLO7**	**DAV95**	**H1**
Jasmonate pathway													
Allene oxide synthase	Contig3097_at						0.40			0.35		**2.47**	**2.87**
Glutathione S-transferase	Contig2976_at					**2.19**			0.46				
Glutathione S-transferase TSI-1	Contig21640_at	0.29			**3.44**								
Glutathione-S-transferase 19E50	HVSMEa0001A19r2_at						**2.33**					0.43	
Jasmonate induced protein	Contig2899_at		**6.23**	**2.76**		**3.08**		0.16	0.32		0.36		
Jasmonate induced protein	Contig2899_s_at	**2.02**	**3.59**		0.49			0.28		0.37			**2.67**
Jasmonate induced protein	Contig2900_at		**4.46**			**2.69**		0.22	0.37	0.26			**3.90**
Jasmonate induced protein	Contig6155_at		**2.57**					0.39					
Jasmonate induced protein	HX05K09r_at		**2.27**			**2.44**		0.44	0.41	0.45			**2.22**
Probable hydroxymethylglutaryl-CoA reductase	Contig5834_at	0.18			**5.42**	**2.52**	**2.85**		0.40			0.35	
Putative 32.6 kDa jasmonate-induced protein	Contig7886_at		**5.56**	**4.75**		**3.97**	**3.39**	0.18	0.25		0.21	0.29	
Putative 32.7 kDa jasmonate-induced protein	Contig7887_at		**2.51**	**3.99**		**2.40**	**3.81**	0.40	0.42		0.25	0.26	
Putative glutathione S-transferase	Contig9944_at			0.41						0.49	**2.43**		**2.03**
Kinase													
Adenylate kinase	HT07A18u_at	0.49			**2.04**	**4.48**	**3.69**		0.22			0.27	
Adenylate kinase	HT07A18u_x_at	**3.10**	**2.31**	**2.95**	0.32			0.43			0.34		
Ca dependent protein kinase	Contig16998_at									**2.03**			0.49
Casein kinase II	Contig3421_at	**2.09**			0.48								
Protein Casein Kinase, expressed	Contig12162_at	**6.29**		**2.46**	0.16						0.41		
Protein cyclin G-associated kinase, putative, expressed	Contig12858_at		**2.48**	**2.75**				0.40			0.36		
Protein diacylglycerol kinase, putative, expressed	Contig15531_s_at					0.36			**2.79**				
Protein kinase	Contig11835_at	**2.15**			0.47								
Protein kinase calcineurin-B-like	Contig13973_at						0.49					**2.03**	
Protein kinase, putative	Contig20099_at			0.41			0.18			0.41	**2.44**	**5.42**	**2.42**
Protein methylthioribose kinase, putative, expressed	rbags10g12_at									0.36			**2.81**
Protein receptor-like kinase, putative, expressed	Contig20397_at			**2.76**							0.36		
Protein receptor-like protein kinase 5 precursor, putative	Contig22980_at	**2.13**			0.47								
Protein receptor-like protein kinase 5 precursor, putative	Contig24926_at			0.36			0.37				**2.76**	**2.68**	
Protein uridine/cytidine kinase-like 1, putative, expressed	Contig8911_at					0.41			**2.42**				
Putative protein kinase	Contig10013_at	**3.38**		**2.38**	0.30	0.36			**2.75**		0.42		
Putative protein kinase Xa21, receptor type precursor	HV_CEb0001H04r2_at			0.13			0.21			0.26	**7.64**	**4.74**	**3.84**

Description	Contig	Fold change (>2 up-regulation, <0.5 down-regulation)											
		CLO7			DAV95			H1			HF92a		
		DAV95	H1	HF92a	CLO7	H1	HF92a	CLO7	DAV95	HF92a	CLO7	DAV95	H1
Kinase													
Putative sphingosine/diglycerol kinase	Contig11377_s_at					0.46			**2.17**				
Serine/threonine Kinase	Contig3829_at						0.26					**3.86**	
Serine/threonine kinase-like protein	Contig25448_at	0.22			**4.50**	**2.26**	**2.62**		0.44			0.38	
Serine/threonine kinase-like protein	Contig7366_at			0.50							**2.01**		
Serine/threonine-protein kinase	Contig16993_at									0.40			**2.51**
Lipase													
Lipase-like protein	Contig2631_at		**6.84**			**3.47**		0.15	0.29	0.18			**5.64**
Lipase-like protein	HA05i14r_at			0.30							**3.38**		
Lipase, putative	Contig13413_at		**3.30**			**3.61**		0.30	0.28	0.16			**6.07**
Lipid transfer protein													
Lipid transfer protein precursor 1	HT07L09u_x_at	0.36			**2.79**	**2.59**	**2.39**		0.39			0.42	
Lipid transfer protein precursor 2	Contig955_s_at						0.45					**2.24**	
Lipid transfer protein precursor 3	Contig3259_at	0.33			**3.06**	**2.40**			0.42				
Lipid transfer protein family	Contig18242_s_at	0.38			**2.61**	**2.11**	**2.56**		0.47			0.39	
Lipid transfer protein family	Contig3774_s_at						0.43			0.37		**2.34**	**2.69**
Lipid transfer protein family	Contig3775_s_at	**3.79**			0.26								
Lipid transfer protein family	Contig3776_at	**3.72**	**3.51**	**2.02**	0.27			0.28			0.50		
Lipid transfer protein family	Contig3782_x_at	0.38			**2.63**	**2.11**	**2.03**		0.47			0.49	
Lipid transfer protein family	Contig245_s_at	0.40		0.42	**2.50**						**2.38**		
Lipid transfer protein family	HT02L01r_at			0.41			0.19				**2.44**	**5.26**	
Lipid transfer protein family	EBro04_SQ001_J04_at	0.41			**2.42**		**2.40**					0.42	
Lipid transfer protein family	EBed02_SQ002_E13_x_at			0.48							**2.10**		
Lipid transfer protein family	Contig504_x_at			0.31			0.34			0.22	**3.26**	**2.98**	**4.54**
Lipid transfer protein family	Contig25762_at		**3.31**					0.30					
Lipoxygenase pathway													
Lipoxygenase 2 (LoxC)	HI02E21u_s_at	**5.15**	**8.38**		0.19		0.16	0.12		0.10		**6.34**	**10.32**
Lipoxygenase 2 (LoxC)	HY03N19u_s_at		**6.34**			3.61		0.16	0.28	0.15			**6.59**
Senescence-associated protein-like	Contig4376_at	**2.16**			0.46								
Senescence-associated protein-like	HVSMEn0009M15f_s_at	**2.43**			0.41								
Methyltransferase													
O-methyltransferase	Contig24771_at	0.43			**2.33**								

Description	Contig	Fold change (>2 up-regulation, <0.5 down-regulation)											
		CLO7			DAV95			H1			HF92a		
		DAV95	H1	HF92a	CLO7	H1	HF92a	CLO7	DAV95	HF92a	CLO7	DAV95	H1
Methyltransferase													
O-methyltransferase	Contig3547_s_at			0.31			0.26			0.39	**3.20**	**3.87**	**2.55**
O-methyltransferase	Contig5038_at						0.28					**3.52**	
O-methyltransferase	Contig6251_at		**4.66**	0.45		**3.25**	0.31	0.21	0.31	0.10	**2.24**	**3.21**	**10.43**
O-methyltransferase	Contig6344_at	0.30			**3.28**		**2.18**					0.46	
O-methyltransferase	Contig6344_s_at			0.44						0.42	**2.26**		**2.36**
Oxidase													
Protein laccase-15 precursor, putative, expressed	HS05I21u_at	0.31			**3.24**	**2.99**	**2.58**		0.33			0.39	
Oxidoreductase													
Iron/ascorbate-dependent oxidoreductase	Contig10361_at	**2.14**			0.47								
NADH-ubiquinone oxidoreductase chain 3	HVSMEk0018C05r2_at						**2.19**					0.46	
Protein oxidoreductase, putative, expressed	EBem05_SQ004_I01_at			**2.13**							0.47		
Peroxidase													
Bacterial-induced peroxidase precursor	HVSMEf0021D01r2_x_at		0.26			0.21		**3.91**	**4.77**	**3.60**			0.28
Peroxidase precursor	Contig19644_at					0.17			**5.74**	**4.13**			0.24
Peroxidase precursor	Contig2114_s_at	0.34	0.27	0.27	**2.92**			**3.71**			**3.76**		
Peroxidase precursor	Contig25242_at						**2.48**					0.40	
Putative peroxidase	Contig12199_at						0.45					**2.24**	
Phenylpropanoid synthesis													
Putative cinnamoyl-CoA reductase	Contig17213_at	**4.50**	**2.55**	**3.01**	0.22			0.39			0.33		
Phosphatase													
Protein inositol-1-monophosphatase, putative, expressed	Contig20457_at	**2.44**			0.41	0.37			**2.70**				
Protein phosphatase	HU03I15u_s_at						0.42					**2.39**	
Protein phosphatase 2C	Contig22065_at	0.35			**2.89**	**5.06**			0.20	0.35			**2.84**
Protein Ser/Thr protein phosphatase family protein, putative	Contig18012_at	**6.33**			0.16								
Putative Ser/Thr protein phosphatase	Contig5441_at	**2.68**			0.37	0.31			**3.22**				
Polygalacturonase													
Polygalacturonase	Contig13474_at					0.44			**2.25**				
Protease													
BTI-CMe4 protein	Contig4_at			0.25							**4.02**		
C13 endopeptidase NP1 precursor	Contig3371_at	0.27			**3.68**	**2.10**	**3.19**		0.48			0.31	
C13 endopeptidase NP1 precursor	S0001100113B11F2_x_at		**2.22**	**2.63**				0.45			0.38		

Description	Contig	Fold change (>2 up-regulation, <0.5 down-regulation)											
		CLO7			DAV95			H1			HF92a		
		DAV95	H1	HF92a	CLO7	H1	HF92a	CLO7	DAV95	HF92a	CLO7	DAV95	H1
Protease													
CI2c endopeptidase inhibitor	Contig88_x_at	**4.20**			0.24					0.19			5.37
Protein peptidase M50 family protein, putative	Contig5436_s_at					0.07			**14.38**	**7.30**			0.14
Protein peptidase, cysteine peptidase active site, putative	HK05B20r_at	0.38			**2.61**		**3.45**					0.29	
Proteinase inhibitor	HVSMEh0099O01f_s_at	0.41			**2.45**	**3.03**	**2.47**		0.33			0.40	
Putative Serine Carboxypeptidase homologue, expressed	Contig6685_at	**2.14**			0.47								
Thiol protease SEN102 precursor - cysteine proteinase	EBma01_SQ002_F07_s_at					**2.81**			0.36				
Reactive oxygen species													
L-ascorbate oxidase	Contig13772_at						0.19					**5.16**	
Lactoylglutathione lyase-like	HW08E22u_at			0.41							**2.43**		
Similar to copper chaperone related protein	Contig4478_at	0.30			**3.35**	**2.70**	**2.61**		0.37			0.38	
Resource allocation													
Galactinol synthase 3, putative, expressed	Contig3811_at	**4.00**	**2.99**	**4.03**	0.25			0.33			0.25		
Glucose-6-phosphate isomerase	HV_CEa0002I10r2_at	0.33			**3.07**								
Glyceraldehyde-3-phosphate dehydrogenase, putative	HS06K05u_s_at	**2.07**			0.48	0.46			**2.15**				
Low affinity nitrate transporter	Contig22626_at		0.49			0.40		**2.05**	**2.52**	**2.62**			0.38
Magnesium transporter CorA-like family protein, putative	Contig7374_at	**2.02**			0.49								
Mannose-6-phosphate isomerase	Contig9791_at		0.40					**2.49**					
Protein phosphoglycerate mutase, putative, expressed	Contig17239_s_at					0.48			**2.10**				
Protein phosphoribosylformylglycinamidine synthase	Contig16509_at	**3.81**			0.26								
Putative aminotransferase	rbags11h24_s_at		**3.35**			**2.09**		0.30	0.48	0.27			**3.67**
Putative CorA-like Mg2+ transporter protein	Contig10636_at		**2.10**	**2.65**		0.42		0.48	**2.38**		0.38		
Putative malate dehydrogenase	Contig613_s_at	**2.22**			0.45								
Sucrose 1-fructosytransferase	Contig4521_s_at			0.40			0.37			0.30	**2.50**	**2.71**	**3.33**
Shikimate pathway													
Protein shikimate kinase, putative, expressed	Contig5714_s_at						0.31					**3.18**	
Thionin													
Alpha-hordothionin	Contig254_x_at	0.45	0.42		**2.25**			**2.35**					
Alpha-hordothionin	Contig796_at	0.40			**2.49**	**2.53**	**2.30**		0.39			0.43	
Alpha-hordothionin	HB29O17r_x_at	0.28			**3.58**	**2.78**	**3.38**		0.36			0.30	
Barley mRNA for leaf-specific thionin	Contig1570_s_at		**15.01**			**21.61**		0.07	0.05	0.08			11.79
Defensin thionin	Contig15886_at		0.25					**3.96**		**3.47**			0.29

		Fold change (>2 up-regulation, <0.5 down-regulation)											
		CLO7			DAV95			H1			HF92a		
Description	**Contig**	**DAV95**	**H1**	**HF92a**	**CLO7**	**H1**	**HF92a**	**CLO7**	**DAV95**	**HF92a**	**CLO7**	**DAV95**	**H1**
Thionin													
Defensin thionin	Contig25366_at	0.32			**3.13**		**2.82**					0.35	
Defensin thionin	EBed01_SQ003_C19_at	0.19		0.31	**5.18**	**3.38**			0.30	0.48	**3.18**		**2.08**
Defensin thionin, Amylase inhibitor-like protein	Contig3217_s_at						**2.27**					0.44	
Gamma-hordothionin	Contig1763_s_at			0.47							**2.13**		
Gamma-thionin	Contig371_x_at			0.26							**3.85**		
Gamma-thionin family	Contig22779_s_at	0.26			**3.82**		**2.59**					0.39	
Leaf-specific thionin	Contig1582_x_at	0.48			**2.09**	**5.68**	**3.18**		0.18			0.31	
Leaf-specific thionin	Contig1583_at	0.20			**4.93**	**5.08**	**2.49**		0.20			0.40	
Leaf-specific thionin DB4	Contig1579_s_at	0.41			**2.41**	**2.02**	**2.60**		0.49			0.38	
Leaf-specific thionin precursor	Contig2653_s_at	0.15			**6.53**		**2.27**					0.44	
Putative thionin	rbags16e08_at					**2.13**			0.47				
Putative thioredoxin	Contig20244_at	**2.15**			0.47								
Thionin precursor, leaf	Contig1580_x_at		**6.35**	**2.32**		**6.66**	**2.43**	0.16	0.15		0.43	0.41	
Ubiquitin													
Polyubiquitin	Contig1086_at	**3.60**			0.28								
Polyubiquitin	HVSMEb0005M08r2_at		0.38					**2.66**					
Ubiquitin carboxyl-terminal hydrolase	Contig10844_at	**4.00**		**2.27**	0.25						0.44		
Ubiquitin carboxyl-terminal hydrolase	Contig13732_at	**2.82**		**2.28**	0.35						0.44		
Ubiquitin carboxyl-terminal hydrolase, family 1, putative	HI04D03u_at	**4.68**			0.21								
Ubiquitin carrier protein	Contig9637_at						**2.26**					0.44	
Ubiquitin carrier protein	HVSMEm0014A16r2_s_at	**2.13**			0.47	0.48			**2.10**				
Ubiquitin precursor	Contig1106_s_at					0.47			**2.14**				
Ubiquitin-like protein	Contig12160_at					0.47			**2.14**				

Notes. Fold changes (FC) presented are those significant at 0.15< PPLR <0.85; PPLR = probability of a positive log ratio (Liu et al. 2006). Up-regulated sequences are shown in bold text (FC>2), down-regulated in regular text (FC<0.5).

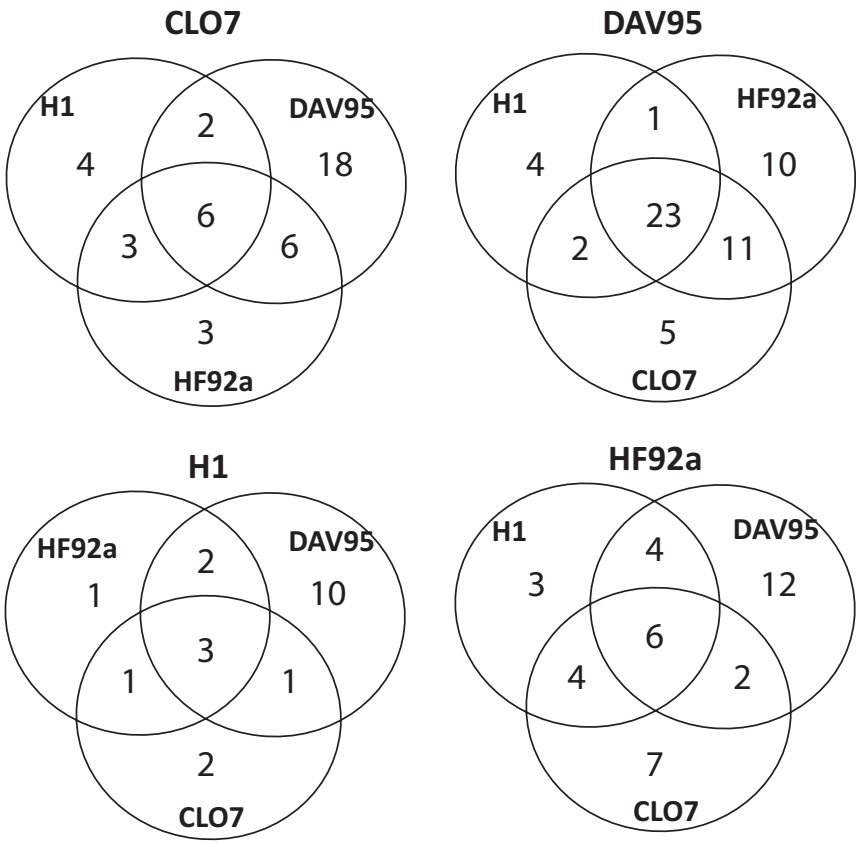

Figure S5.1. Venn diagrams of the number of up-regulated sequences induced in a plant by an aphid genotype compared to the other aphid genotypes.

For example, the top-left diagram shows that aphid genotype CLO7 induces the up-regulation of a total of 42 sequences in the plant, of which six (centre) are up-regulated by only CLO7, i.e. CLO7 induces greater expression of these six sequences compared to DAV95, H1 and HF92a. Additionally, for example, CLO7 aphids induce the up-regulation of two sequences compared to H1 and DAV95 (upper section), therefore expression of these sequences are similar in CLO7 and HF92a.

Chapter 6. Genetic variation in a tropical tree species influences the associated epiphytic plant and invertebrate communities in a complex forest ecosystem

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PERSONAL CONTRIBUTION

I collected and genotyped all genetic samples for this paper and the AFLP genotyping was conducted at Jodrell Laboratory, Kew Gardens, with help from Robyn Cowan. Alongside Waldo Etherington, I collected the epiphyte data by using tree climbing techniques. I identified the orchids to species, after spending a week with Brendan Sayers at the Dublin Botanical Grounds, who taught me orchid identification of the common species in Las Cuevas, Belize. I collected the invertebrate data, supported by a team of undergraduate field-course students (H. Eyland, B. Hurst, R. Robinson and R. Sweeney), who as part of their field-course project identified the individuals into morphospecies. In the UK, David Penney identified the invertebrate morpospecies into families. I performed all statistical analyses and wrote the paper, with support and comments from the other authors.

ABSTRACT

Genetic differences amongst tree species, their hybrids and within tree species are known to influence associated ecological communities and ecosystem processes in areas of limited species diversity. The extent to which this same phenomenon occurs based on genetic variation within a single tree species, in a diverse complex ecosystem such as a tropical forest, is unknown. The level of biodiversity and complexity of the ecosystem may reduce the impact of a single tree species on associated communities. We assessed the influence of within-species genetic variation in the tree *Brosimum alicastrum* (Moraceae) on associated epiphytic and invertebrate communities in a neotropical rainforest. We found a significant positive association between genetic distance of trees and community difference of the epiphytic plants growing on the tree, the invertebrates living amongst the leaf litter around the base of the tree, and the invertebrates found on the tree trunk. This means that the more genetically similar trees are host to more similar epiphyte and invertebrate communities. Our work has implications for whole ecosystem conservation management, since maintaining sufficient genetic diversity at the primary producer level will enhance species diversity of other plants and animals.

INTRODUCTION

Within an ecological community many species exist together and both direct and indirect interactions occur between species. Species interact with each other by a variety of means (e.g. competition, predation, mutualism, commensalism and parasitism) and each species in a community will experience numerous interactions with many other species. Additional complexity occurs when the specific combinations of genotypes of the interacting species influence the outcome of direct and indirect species interactions (Service 1984, Aarssen and Turkington 1985, Kassen and Rainey 2004, Proffitt et al. 2005, Tétard-Jones et al. 2007, Zytynska et al. 2010). It is therefore understood that genetic variation within a species and genotypic interactions between different species can have strong effects on another species in a community. However, experiments that quantify the effect of another species' genotype on the phenotype of a focal species are often limited to experimental communities with a small number of species due to complexity and cost. This work can create unnatural genotypic combinations, minimizes abiotic (and other biotic) variation and may not reflect the true influence of these interactions in a natural ecosystem (Carpenter 1996).

An alternative approach to understanding how genetic variation within a species influences the associated community is to consider genetic variation in a focal species, and determine how this is associated with the surrounding community structure of particular species groups. Within temperate ecosystems, genetic variation within a dominant plant species can influence the structure of the associated invertebrate (Fritz and Price 1988, Maddox and Root 1990, Floate et al. 1996, Stiling and Rossi 1996, Dungey et al. 2000, Whitham et al. 2003, Hochwender and Fritz 2004, Johnson et al. 2006, Bangert et al. 2008, Barbour et al. 2009), plant (Iason et al. 2005), endophytic fungi (Gaylord et al. 1996) and vertebrate (Bailey et al. 2004, Muller et al. 2006)

communities. An important factor in these interactions has been found to be plant biochemistry. Many plant species accumulate specific chemicals (e.g. phenols, monoterpenes and tannins) in their leaves, which reduce herbivory and can influence the chemistry of the surrounding soil when the leaves drop to the ground. Chemical diversity in Scots pine has been found to influence the surrounding species richness of understory plants (Iason et al. 2005) and leaf tannin concentration has been demonstrated as having a large effect on the associated animal communities of cottonwood trees (Driebe and Whitham 2000, Whitham et al. 2003, Bailey et al. 2004, Bangert et al. 2006, Muller et al. 2006, Whitham et al. 2006). Leaf decomposition rates and leaf litter nutrient concentrations are also influenced by plant genetic variation (LeRoy et al. 2006, Madritch 2006, Crutsinger et al. 2009), through variation in leaf chemistry. These factors will, in turn, influence ecosystem processes such as nitrogen mineralization (Whitham et al. 2006) and can affect indirectly interacting communities, such as aquatic fauna (LeRoy et al. 2006).

There is a vast amount of evidence showing that plant genotype, especially for tree species, can have a strong effect on associated species, and community and ecosystem processes. However, thus far, all the studies are from temperate regions of the world where species diversity and ecosystem complexity is low compared to the species rich tropical regions. Within-species genetic variation is expected to have a greater impact on the surrounding ecosystem when species diversity and genetic diversity is limited. To what extent these effects will be important in an ecosystem with high species and genetic diversity is unknown. In a genetically diverse population of trees, a genetic mosaic of ecosystem function could arise which means that the environment (biotic and abiotic) around a single tree differs due to tree genotype (Lindroth et al. 2007, Madritch et al. 2009). This leads to a heterogeneous environment, and thus, different tree

genotypes within a diverse ecosystem could still differentially influence the structure of associated communities and even the coevolution of species (Madritch et al. 2009). In this study, we investigated the effect of within-species genetic variation in a tropical tree species on both plant and invertebrate communities in a diverse complex natural tropical ecosystem.

Epiphytic plants are highly diverse and abundant in tropical forests, and they use the trees as hosts for structural support to gain access to light. Previous work has found that epiphyte species have some preference for different host species, but no particular host trait was found to explain the associations observed (Callaway et al. 2002). A recent study showed that 80% of epiphyte species actually showed a random distribution among tree hosts in a tropical forest; however, this means that 20% of the epiphyte species studied showed host preference for (i.e. non-random association with) certain tree species (Laube and Zotz 2006). Within a species, individual trees vary in the abundance of epiphytes they are host to and this can only partially be explained by tree size and branch area (Zimmerman and Olmsted 1992). It is plausible that genetically-based traits within a tree species will influence which epiphyte species will grow on them and therefore will influence the community structure of the epiphytic flora.

Invertebrates are one of the most species diverse groups of organisms in a tropical forest and previous work has demonstrated that tree genotype can influence invertebrate community structure. Within a tropical forest ecosystem, there are numerous different habitats around a tree in which invertebrates may live, including tree leaves, canopy branches, in epiphytic plants, under bark, on the trunk, on rotting areas, as well as around the base of the tree in the soil and leaf litter. These habitats are likely to contain different invertebrate communities and the extent to which a single tree influences these

communities may differ, leading to variation in the strength of effect of tree genetic variation on these different associated communities.

In this study, we investigated the influence of genetic variation within a single tree species (*Brosimum alicastrum*: Moraceae) on the epiphytic floral community and three different invertebrate faunal communities, in a tropical forest ecosystem. This is the first study to date in which community genetic approaches have been used in an attempt to understand how genetic variation within a single tree species can influence associated communities in a complex and diverse tropical ecosystem. We aim to determine whether the influence of tree genotype on associated ecological communities is still measurable in a complex tropical system or if the complexity of the system overcomes the effect because of the increase in the number of species interactions expected in tropical systems.

MATERIALS AND METHODS

Study site and tree species

This study was undertaken in the Chiquibul Forest around Las Cuevas Research Station, Belize, Central America. The sample area was approximately 4km^2. The tree species studied was the breadnut, *Brosimum alicastrum* (Moraceae), which is a common tree species in this area. It can grow up to 40m in height and produces large nutritious fruits on which many animals and birds feed (Rocas 2003). Geographic location was recorded using a GPS unit and the location confirmed at least once over two separate field seasons. The GPS coordinates were converted to digital coordinates, then converted to radians before using the spherical law of cosine to calculate the distance between each pair of trees:

$$\text{distance} = a\cos[\sin(lat_1).\sin(lat_2) + \cos(lat_1).\cos(lat_2).\cos(long_2 - long_1)].6371 \qquad (1)$$

Genetic analysis

Genetic material (leaf or cambium) was sampled from each tree used in this study, and preserved in silica gel (Chase and Hills 1991). DNA was extracted using a modified CTAB method (Doyle and Doyle 1987) and eluted DNA was cleaned using spin columns (Sigma-Alrich). Amplified fragment length polymorphisms (AFLP; Vos et al. 1995) were used to calculate Nei's genetic identity using TFPGA (*Tools For Population Genetics Analysis*, M. Miller) between each pair of trees in the study population. For the AFLPs, EcoR1 and Mse1 restriction enzymes, and three selective primer combinations were used (ACA-CTC, ACG-CTC and AGC-CAT) producing 119 scorable loci. The AFLP profiles were visualized using an ABI Prism 3100 and analysed using Genotyper v2.0 software (Applied Biosystems). A 5% error rate was used when analysing the AFLP bands, to ensure differentiation between trees was not overestimated.

Epiphyte community

The canopy bromeliad and orchid epiphyte community was surveyed on 53 *Brosimum alicastrum* trees (Figure S6.1) during June-July 2008. The trees were accessed using ropes and harnesses, in order to get high into the canopy. Three branches per tree were chosen, generally the first branch (closest to the ground), the highest accessible branch and another in between these. This method does not restrict the branches to a specific elevation, but it does provide an overall survey for plants across the canopy. Further, branch size was not restricted to similar sized branches and therefore branch surface area was calculated to control for these differences among trees, as larger branches are expected to host a greater number of epiphytes. To calculate branch surface area each branch was measured in three sections (inner, middle and outer). The diameter and length of each section was measured and used to calculate surface area using formula for a cylinder (inner and middle) and a cone (outer), ignoring the end surfaces. These measurements were added together for each tree and a matrix created of surface area difference between every pair of trees.

Each epiphyte individual was counted and identified to species where possible and photographs were taken of the majority of sampled plants in order to confirm identification later. A subset of the epiphytic plants was sampled for genetic analysis and we used barcoding techniques to sequence the *matK* gene to confirm field identification; reference samples were obtained from B. Sayers at the Botanical Gardens, Dublin, Ireland.

Similarity indices were used to calculate community structure similarity/distance between each pair of trees in CAP4 (*Community Analysis Package 4,* Version 4.1.3, Pisces Conservation Ltd., 2007). For presence/absence data the Rogers and Tanimoto (RT) similarity index (see Zuur et al. 2007) was used:

$$\mathrm{RT} = \frac{a + d}{a + 2b + 2c + d} \quad (2)$$

where a = species present on both trees, d = species absent on both trees, b and c = present on only one tree. This index includes circumstances when an epiphyte species known to grow on another tree in the population, is not found on either tree being compared (double absence). This is an improved index for comparisons over small geographic distances whereas indices such as the Bray-Curtis index ignore double absences. There was no relationship between geographic distance and the number of double absences (Mantel test, r = -0.075, p=0.10), indicating the absence of a species on two trees is informative rather than an artifact of variation due to geographic variation; the scale of this study is very small and therefore variation due to geographic location is reduced to a minimum. Further, we believe these trees have been studied sufficiently such that the majority of species will have been recorded and false positive rate is low. For the abundance data, Mean Character Difference (MCD) or Czekanowski index (see Ramirez-Trejo et al. 2004) was used:

$$\mathrm{MCD} = \frac{1}{n}\sum |\, s_1 - s_2 \,| \quad (3)$$

where s_1 and s_2 are species abundances from tree 1 and tree 2. This measure also uses the absence of a species on both trees as information, as for the presence/absence RT index.

We used Mantel and partial Mantel tests to statistically analyse the data. Mantel tests use pairwise matrices to test the correlative relationship between two variables, and partial Mantel tests allow for one or more other matrices to be kept constant in the analysis. Here, we analysed the effect of genetic distance between the trees on epiphyte

community difference, whilst controlling for geographic distance and branch surface area. The RT similarity index produces results, which range from 0 to 1, therefore by calculating '1-RT' we can compare this index with the MCD measure and genetic distance to give positive correlation results when significant.

Invertebrate community

The invertebrate community was surveyed on and around 30 *Brosimum alicastrum* trees (Figure S6.1) during July 2009. Tree number was reduced compared to the epiphyte work due to time constraints in the field, tree size (large trees with buttress roots were excluded) and accessibility to the base of the tree. Tree size was measured (diameter at breast height; dbh) for every tree.

We used three different methods to collect invertebrates: 1) leaf litter collection, 2) pitfall traps and 3) trunk traps. The leaf litter was collected once from the base of each tree using an empty plastic tub to regulate the volume of leaf litter collected. The litter was then sifted through leaf by leaf to collect all invertebrates living within it. The invertebrates were preserved in 70% ethanol for identification. Two pitfall traps were set up at the north and south sides of the tree base, using plastic pint cups and polystyrene plates as a cover. A small amount of water with a drop of washing up liquid was placed in the bottom of each cup to reduce error from escapees. The pitfall traps were emptied daily for three days and collected invertebrates were preserved in 70% ethanol. Trunk traps were set on each tree at breast height by wrapping the trunk in one tight layer of black plastic, over the top of this a slightly smaller width of black plastic was wrapped loosely around and the gaps were filled with twigs and leaves. After three days, the trunk traps and invertebrates within were collected. All invertebrates were collected, stored in 70% ethanol and identified to morphospecies.

The Jaccard's (JA) similarity index was used to measure invertebrate community similarity between each pair of trees, using presence/absence data (see Zuur et al. 2007):

$$\text{JA} = \frac{a}{a+b+c} \quad (4)$$

where a = species present on both trees, b and c = present on only one tree. The JA index does not use 'double absence' information, when a species is absent from both trees. In this case, this is preferable since sampling for these communities was not exhaustive (Legendre and Legendre 1998). Using double absence information would lead to increased false positive rates due to the reduced sampling effort for invertebrates. We still assume geographic distance between the trees is small enough to not increase false positive discovery rate. The JA index gives a range of results from 0 to 1, allowing '1-JA' to be used as a dissimilarity index rather than a similarity index. This means the results obtained are comparable to the epiphyte community analyses. Partial Mantel tests were used to analyse the association of genetic distance between trees and invertebrate community dissimilarity (1-JA), whilst controlling for geographic distance and tree size difference. Mantel tests were used to assess the influence of geographic distance/genetic distance/tree size on the invertebrate communities.

RESULTS

Epiphyte community

We surveyed 2142 individuals of 46 orchid and 17 bromeliad species on the 53 *Brosimum alicastrum* trees. Of the trees sampled, the mean genetic distance between trees from AFLP data was 0.316 (range of 0.097-0.702). The average difference in branch surface area between trees was 3.8m^2 (smallest branch area was 0.2m^2 and the largest 22m^2, average branch area was 3.5m^2). The largest geographic distance between trees was 2118m, smallest was 1m. The most abundant epiphyte species in our sample was the orchid *Christensonella uncata* (Lindl.).

We found that as genetic distance between trees increased the epiphyte community difference between trees also increased, both for species presence/absence (partial Mantel test, '1-RT', r = 0.216, p = 0.025; Table 6.1) and abundance data (partial Mantel test, MCD, r = 0.210, p = 0.035; Figure 6.1). Geographic distance had a significant effect on both genetic distance (Mantel test, r=0.116, p=0.02) and community difference (Mantel test, MCD, r = 0.147, p = 0.009; Figure 6.1); however, the correlation was weaker than between genetic distance and epiphyte community difference. Branch surface area difference between the trees had a strong effect on epiphyte community difference (Mantel test, MCD, r = 0.514, p = 0.003; Table 6.1) and species richness increased as branch surface area increased (Pearson's, r=0.445, p=0.0008).

Table 6.1. Summary of results from Mantel and partial Mantel tests for the epiphyte community data

Correlation (matrices held constant)	Presence-absence	Abundance
Genetic distance to epiphyte community (geo + area)	**r = 0.216 (0.025)***	**r = 0.210 (0.035)***
Genetic distance to epiphyte community	r = 0.222 (0.022)*	r = 0.206 (0.057)^{NS}
Genetic distance to geo	r = 0.116 (0.020)*	
Epiphyte community to geo	r = 0.095 (0.040)*	r = 0.147 (0.009)**
Epiphyte community to area	r = 0.275 (0.016)*	r = 0.514 (0.003)**

Notes. r = pearsons correlation, calculated through mantel tests. Geo = geographic distance between trees, area = surface area difference between trees, genetic distance = Nei's genetic distance between trees, epiphyte community = community difference between trees. The first row, in bold, indicates the correlation between epiphyte community and genetic distance, with the appropriate matrices held constant in a partial Mantel test. In the first column, the matrices held constant in a partial mantel test are indicated in parentheses. The numbers in parentheses in the presence/absence and abundance columns indicate the p-value for the Mantel test, calculated using 9999 permutations.

* P < 0.05; ** P < 0.01; *** P < 0.001; NS = nonsignificant (P >0.05).

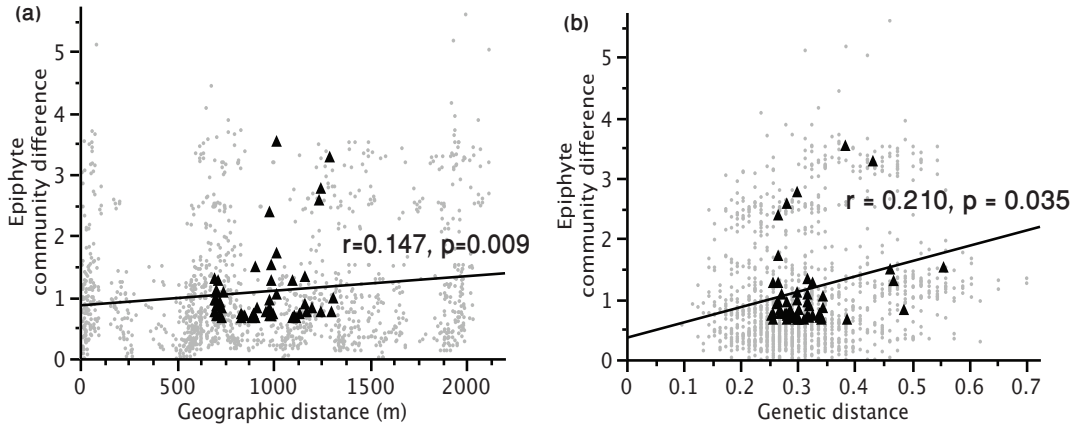

Figure 6.1. The relationship between epiphyte community distance (abundance data) and a) geographic distance, b) genetic distance.

Dots in grey show data for every pairwise combination among the 53 trees, with the average for each tree shown as a black triangle.

Invertebrate communities

We surveyed 1933 invertebrate individuals from 30 *Brosimum alicastrum* trees from the leaf litter, trunk and pitfall samples. For these trees, the mean genetic distance from AFLP data was 0.304 (range of 0.097-0.702), the largest tree dbh was 82.5cm and smallest 11.5cm (mean size difference between trees was 20cm) and the greatest geographic distance between two trees was 2170.8m (the two closest trees were 1m apart). The pitfall traps produced the greatest number of individuals at 1084 from 83 morphospecies, the leaf litter community consisted of 349 individuals from 55 morphospecies and the trunk traps contained 500 individuals from 43 morphospecies (Table S6.1). The main invertebrate taxa in our collections were spiders (Araneae), crickets (Orthoptera), beetles (Coleoptera) and ants (Hymenoptera: Formicidae).

Our results show variation in significance values and strength of effect of tree genetic variation across the different invertebrate communities (Table 6.2). We found that the leaf litter invertebrate community was influenced by tree genetic distance, with more closely related trees having the most similar leaf litter communities (partial Mantel test, $r = 0.221$, $p = 0.005$; Figure 6.2). Trunk invertebrate community dissimilarity was also significantly positively correlated with tree genetic distance, but less strongly than for the leaf litter data (partial Mantel test, $r = 0.138$, $p = 0.05$; Figure 6.2). The pitfall invertebrate community was not influenced by genetic distance between trees (partial Mantel test, $r = 0.072$, $p = 0.263$). Geographic distance between the trees was held constant when analysing all data sets, as it significantly influenced tree genetic distance (Mantel test, $r = 0.233$, $p = 0.001$). Geographic distance was not significantly associated with any of the invertebrate communities (Table 6.2). Tree size was not significantly correlated with the community structure of any invertebrate community or tree genetic distance, and therefore was not controlled for in the main analyses.

Table 6.2. Summary of results from Mantel and partial Mantel tests for the invertebrate data

Correlation (matrices held constant)	Invertebrate community
Genetic distance to geographic distance	r = 0.233 (0.001)***
Genetic distance to tree size	r = 0.147 (0.068) ^{NS}
Leaf litter community to genetic distance (geo)	**r = 0.221 (0.005)****
Leaf litter community to geographic distance	r = 0.074 (0.108) ^{NS}
Leaf litter community to tree size	r = 0.017 (0.402)^{NS}
Trunk community to genetic distance (geo)	**r = 0.138 (0.050)***
Trunk community to geographic distance	r = 0.025 (0.322)^{NS}
Trunk community to tree size	r = -0.006 (0.492)^{NS}
Pitfall community to genetic distance (geo)	**r = 0.072 (0.263)^{NS}**
Pitfall community to geographic distance	r = 0.068 (0.141)^{NS}
Pitfall community to tree size	r = -0.104 (0.117) ^{NS}

Notes. r = pearsons correlation, calculated through mantel tests. The rows in bold are the main correlations between invertebrate community and genetic distance. Geo = geographic distance between trees, tree size = dbh difference between trees. In the first column, the matrices held constant in a partial Mantel test are indicated in parentheses. In the second column, the numbers in parentheses indicate the p-value from the Mantel test, calculated from 9999 permutations.

* P < 0.05; ** P < 0.01; *** P < 0.001; NS = nonsignificant (P >0.05).

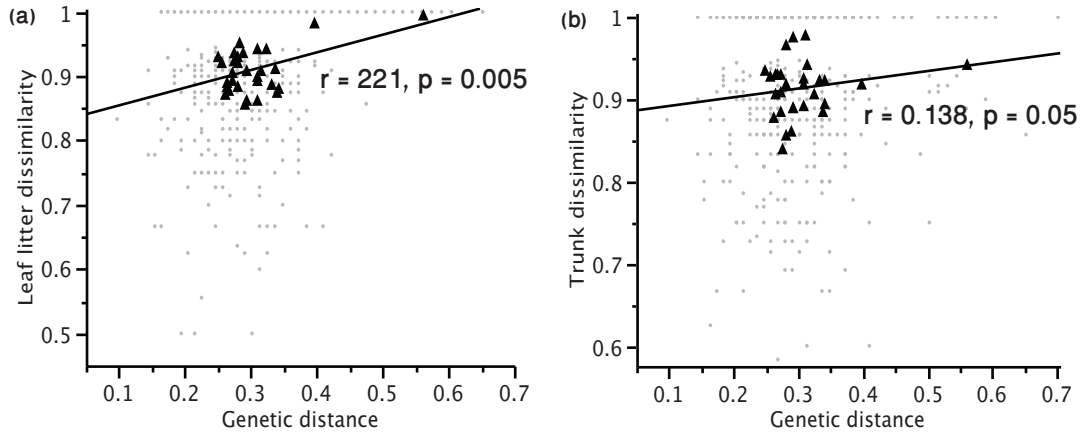

Figure 6.2. Graphs showing the relationship between genetic distance and a) leaf litter community dissimilarity (1-Jaccard's), b) trunk community dissimilarity (1-Jaccard's) for every pair of trees (grey dots) and the average for each tree (black triangles).

DISCUSSION

We demonstrated that *Brosimum alicastrum* trees, which are genetically more similar host more similar communities of epiphytic plants, leaf litter invertebrates and trunk dwelling invertebrates. Our work shows that genetic variation within a single tree species influences associated plant and animal communities, in a complex natural tropical forest ecosystem. This study significantly furthers our knowledge of community genetics concepts because it demonstrates that the extended phenotype phenomenon occurs not only in ecosystems dominated by a small number of tree species but also in a diverse tropical forest where over 300 tree species can be found (Bridgewater et al. 2006). We have shown that even in a complex environment, genes within a tree species are important for the dependant plant and animal communities. By using AFLP markers for this study we were able to detect small genetic differences between these trees. Although AFLP markers are often considered neutral, we have shown that the differences in AFLP profiles among these trees are related to community differences in the interacting species. Therefore, it is likely that one or more of the loci are in linkage disequilibrium with a gene, or number of genes, which influence the communities. In systems where there is no linkage disequilibrium between markers and genes of effect then relationships between genotype and associated communities might not be detected. In contrast, systems with high linkage disequilibrium (e.g. hybridizing systems) might show large and easily detectable associations following an apparent genetic similarity rule (sensu Bangert et al. 2006).

We have shown that the epiphytic plant community on a tree is influenced by the individual genetic make-up of that tree. This indicates that there are genetically based traits within this tree species that determine which epiphyte species can grow on different host tree individuals. The possible mechanisms for epiphyte 'host-preference'

are unlikely to be through active choice since most seeds are wind-dispersed and adult plants do not actively move. Host preference will most likely act through differential seedling survival on a tree during seed germination or plant growth. Such mechanisms might include the inhibition of seed germination by bark substrates (Frei and Dodson 1972) or a requirement for certain mycorrhizal fungal strains to be present on the tree bark, specifically for orchid seed germination (Arditti 1967, Otero et al. 2002). For both bromeliad and orchid epiphyte species, early growth could be influenced by nutrient run-off from the tree and the presence of other flora and fauna on the tree host, for example moss or lichen abundance (Callaway et al. 2001).

We detected an effect of geographic distance on tree genetic distance, indicating that the trees located near to each other are more likely to be genetically similar. However, the effect of geographic distance on tree genetic distance was relatively small compared to the effect of genetic distance on the epiphyte community. As expected, we also found a small influence of geographic distance on epiphyte community difference (Zotz and Vollrath 2003); however, this effect is again smaller than the effect of genetic distance on the epiphyte community. By controlling for geographic distance between each pair of trees in our analyses we have attempted to reduce any error associated with varying forest habitats, potential strong abiotic environmental differences and tree genetic population structure. Since our sample area was relatively small (approximately $4km^2$), including geographic distance in the model was considered sufficient. We also, as expected, found a significant effect of tree branch area sampled, which simply means that the larger branches were host to more species and more individuals of epiphytes. This effect is due to tree age since older trees have had longer for epiphyte colonization (Zotz and Vollrath 2003).

For our invertebrate study we chose to use three different sampling techniques to investigate the invertebrate fauna associated with the tree: leaf litter collection, trunk traps and pitfall traps. Tree size (diameter at breast height) did not influence the results from any of our sampling methods. Tree genetic distance was significantly correlated with geographic distance, more so than for the larger sample of trees used in the epiphyte study. This shows a greater degree of population genetic structure for this subset of 30 *Brosimum alicastrum* trees; however, we still consider the inclusion of geographic distance into the analyses to be sufficient to control for the effect.

We found that the community structure of invertebrates living within the leaf litter was relatively strongly associated with tree genetic distance, the trunk invertebrate community was less associated, but still significantly so, but the pitfall samples were not. The pattern we see here may be due to the different species found in these communities; for example, the leaf litter community might be more sedentary than the pitfall community, which need to be moving in order to fall into the trap. Indeed, we found more Diptera, Hymenoptera, Coleoptera and Orthoptera in the pitfall samples than in the leaf litter samples, which supports the idea that these communities differ in the movement capabilities of their inhabitants. Pitfall traps are notoriously inefficient at sampling the entire faunal component of a region, but we do not consider this an important effect in this instance because our pitfall samples contained almost as many different morphospecies as the other two sampling techniques combined. The strong difference in the effect of tree genetic variation on the leaf litter and pitfall communities is therefore potentially a result of sedentary leaf litter invertebrates depending much more on the individual tree, and its direct surroundings (including living within its fallen leaves), than transient individuals which were opportunistically caught as they passed by the tree. The trunk community may consist of both more sedentary trunk

dwelling species, those species moving up and down the trunk, and possibly also temporary visitors of winged species that may not be expected to be directly associated with a single tree species. This would explain the pattern detected of the trunk community being only moderately influenced by tree genetics (in between the leaf litter and pitfall results). Potential mechanisms for these effects could include food availability, tree or bark structure, presence of other animals and plants, or phytochemical differences in leaves and leaf litter decomposition. Leaf litter invertebrates will depend more on the phytochemistry of the leaves, which are falling around the tree, and the subsequent decomposition of these, more so than trunk invertebrates or transient species. It has been shown in other plant species that chemical composition of the leaves and soil chemical composition is related to plant genotype (LeRoy et al. 2006, Madritch et al. 2006, Crutsinger et al. 2009). This may explain the stronger interaction seen, in our study, between the leaf litter invertebrates and tree genetics, than for the trunk or pitfall communities.

In complex ecosystems, the number of interactions experienced by one species is expected to be high and therefore the impact of each single interaction will be reduced. We determined that tree genotype explains approximately 4.6% of the variation in epiphyte community structure, 4.8% of the variation in the leaf litter invertebrate community structure and 3.7% of the variation in the trunk invertebrate community. In such a complex ecosystem, with numerous interactions between many species, we consider this effect to be biologically significant. The detection of a significant effect within a naturally high diversity ecosystem would suggest that in a low diversity or experimental common garden system the variation explained would be much higher. These effects of tree genotype on epiphyte and invertebrate communities were also detectable even though a strong direct interaction is unlikely. Epiphytes are not parasitic

and it is expected that most strong interactions with the tree will be indirect, although there may be a strong direct interaction at seed germination stage through bark substrates. Also, the invertebrate community was sampled by such methods that any species could potentially be trapped. Many previous community genetics studies have focused on directly interacting invertebrate communities, such as gall-inducing or leaf-folding arthropods (Whitham et al. 2006), which are more likely to be strongly influenced by variation among individual tree hosts. This suggests that the effect of tree genotype, even in a complex system, is likely to be important for the many other associated species in this tropical ecosystem that interact more strongly with this tree species, such as parasitic plants and animals. Furthermore, species that depend on the epiphytic plants for shelter or the invertebrates for food may also be influenced indirectly by tree genetic variation through the epiphytes and invertebrates.

This work supports the idea of conserving a minimal viable interacting population (MVIP; Whitham et al. 2003) as opposed to concentrating conservation efforts on minimal viable populations (MVP) of a species. The level of genetic variation required for maintaining a single tree species may be considerably lower than the level required to maintain the associated communities of plants and animals. Therefore, future conservation efforts or reintroductions of tree species should consider the associated communities when determining the level of genetic diversity to be conserved.

In conclusion, we have shown that genetic variation within a single tropical tree species can have extended phenotype effects on associated ecological communities in a complex and highly diverse natural ecosystem. This, in turn, is likely to influence the community structure of other species such as amphibians, birds and mammals and we suggest that whole ecosystem conservation may be possible through conserving genetic diversity at the primary producer level, even in complex ecosystems.

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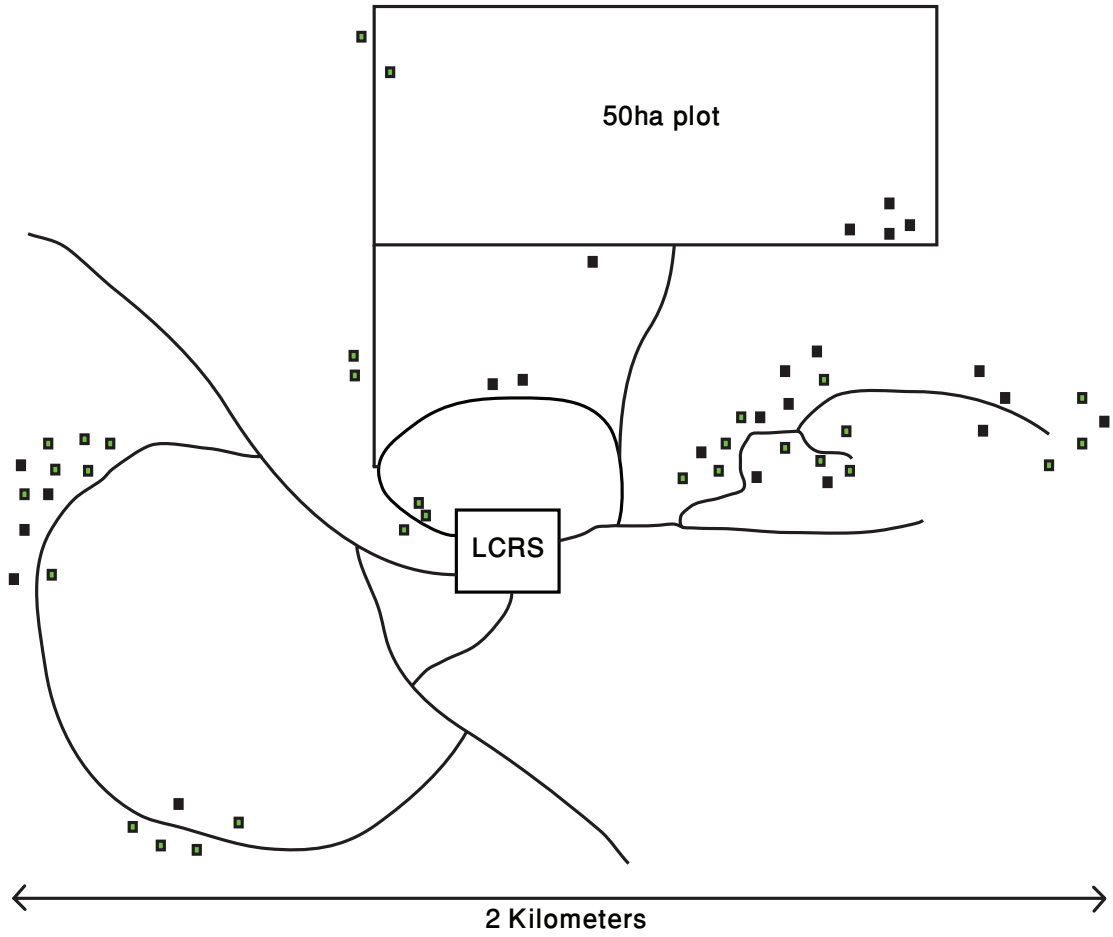

Figure S6.1. Map showing the location of the genotypes trees in Las Cuevas Research Station (LCRS), Belize.

Each square represents a genotyped tree (all were used in the epiphyte study and the squares with a green centre represent the trees used in the invertebrate study)

Table S6.1. Number of invertebrate morphospecies within different taxa sampled from each sampling technique.

	Number of morphospecies		
Taxa	**Leaf Litter**	**Trunk**	**Pitfall**
Araneae	8	9	11
Orthoptera	4	8	8
Coleoptera	6	1	11
Hymenoptera (Formicidae)	5	4	8
Blattodea	5	2	7
Myriapoda	5	5	4
Heteroptera	4	0	5
Lepidotera (larvae)	2	1	4
Chilopoda	3	4	0
Opiliones	2	1	4
Diptera	0	0	4
Hymenoptera (non-formicine)	1	0	3
Gastropoda	1	2	1
Isopoda	1	2	1
Psocoptera (bark)	2	0	1
Dermaptera	1	1	1
Homoptera	0	0	3
Protura	1	1	1
Archaeognatha	0	1	1
Annelida	1	0	1
Acri	1	0	1
Pseudoscorpiones	1	0	1
Psocoptera (book)	0	0	1
Collembola	0	0	1
Scorpiones	0	1	0
Isoptera	1	0	0
Total	**55**	**43**	**83**

Chapter 7. Comparing the use of leaf and cambium tissue for a single genetic study using amplified fragment length polymorphisms (AFLP)

Sharon E. Zytynska, Michael F. Fay and Richard F. Preziosi

PERSONAL CONTRIBUTION

I collected all the leaf and cambium samples, with tree climbing help from Waldo Etherington. I also extracted all the DNA samples, performed the AFLP analysis and analysed the data. The AFLP laboratory work was undertaken at the Jodrell Laboratory, Kew Gardens who were my PhD CASE sponsor. I wrote this paper with support from Richard Preziosi.

ABSTRACT

Amplified fragment length polymorphisms (AFLPs) are a useful molecular tool for studying species with little available genetic information. A method for extracting DNA, suitable for AFLP analysis, from the cambium tissue of tropical trees has been previously described. Cambium tissue can be advantageous, as a source of DNA, over the more commonly used leaf tissue because it contains fewer contaminants, such as endophytic fungi or plant defence chemicals that can inhibit downstream PCR. Additionally, cambium tissue can be easier to sample than leaf material, especially for tall trees. However, cambium sampling requires the bark of the tree to be damaged and therefore increases the risk of introducing disease. The use of both leaf and cambium tissue in a study can increase the sample size, but it has previously been suggested that only a single tissue type should be used within a genetic study. This is due to potential differences in the AFLP profiles obtained from DNA of different tissues within the same individual. In this study, we show that within three tropical tree species, there is no significant difference between AFLP profiles obtained from either cambium or leaf tissue from the same tree, with average reproducibility of the AFLP profiles ranging from 89.1% to 97.4%, depending on tree species. Furthermore, the genetic relationship between the trees studied does not differ dependent on tissue type used. This means that DNA extracted from both cambium and leaf tissues can be used interchangeably in a single genetic study.

INTRODUCTION

Genetic studies using amplified fragment length polymorphisms (AFLP) use universal primers and thus require no previous investment in primer design and synthesis (Vos et al. 1995). The use of AFLPs is beneficial when little is known about a species, however universal primers with broad applicability across numerous taxa can also result in the DNA of contaminants (for example endophytic fungi within leaf samples) being amplified alongside that of the target individual (Saar et al. 2001). Furthermore, AFLPs require substantial amounts of good quality DNA to ensure complete digestion of the whole genome by restriction enzymes (Blears et al. 1998). This means that the choice of tissue used to extract the DNA from, is important in order to minimise the chance of contamination from non-target organisms and to ensure sufficient DNA for the analysis is obtained.

Colpaert et al. (2005) described a method for extracting DNA from the cambium tissue of tropical trees, which was suitable for AFLP analysis. Cambium tissue is a source of DNA from undifferentiated cells, found on the inner surface of the bark. The cambial tissue of a tree is protected by the bark and therefore contains fewer biotic contaminants, such as microorganisms, endophytic fungi or tiny invertebrates (Colpaert et al. 2005). Cambium also contains fewer defensive chemicals, such as phenols or tannins, due to the reduced risk of herbivory compared to leaf material, currently the most commonly used source of DNA from plants. Plant defensive chemicals can inhibit downstream PCR (polymerase chain reaction) success and therefore cambium tissue is potentially a better source of DNA than leaf material for genetic analysis (Colpaert et al. 2005). Furthermore, the leaves of trees can often be inaccessible if the tree is tall whereas cambium tissue is easily obtained at ground level. Alternatively, difficulties in identifying the cambium zone, thick bark and a porous and spongy cambium can reduce the sampling efficiency and the yield of DNA recovered (Colpaert et al. 2005).

Cambium sampling also requires the bark to be damaged and this could introduce disease to the tree (Pearce 1996). Therefore, in many circumstances neither leaf nor cambium tissue is the ultimate preferred choice. The use of both cambium and leaf tissue in a genetic study of tropical trees would be advantageous and increase the sample size, since cambium tissue can be used when leaf material is not available or when biotic contamination of leaf material is a concern. This may be a greater concern for tropical trees, where species diversity at all levels is high. However, endophytic fungi have been found in all woody plants examined for them, suggesting the concern of biotic contamination of leaf material could be well-founded, even for studies of trees within temperate regions (Saikkonen et al. 1998). Alternatively, if cambium cannot be reliably collected then leaf material can be used, for example when the outer bark is too thick or the cambium layer is not easily distinguishable resulting in low DNA yield (Colpaert et al. 2005). It has previously been suggested that AFLP profiles could differ between tissues within a single individual. This was found in a study on wheat, where differences in DNA methylation between seed and leaf tissues were assumed to explain observed differences in AFLP profiles (Donini et al. 1997). We suggest that due to the nature of the undifferentiated cambium cells these concerns are unfounded and the use of both tissues as a source of DNA can benefit a genetic study of tropical trees.

In this study we compared the yield and purity of DNA obtained from leaf and cambium tissues within three tropical tree species. We then investigated whether the AFLP profiles obtained from different tissues within the same tree were more similar than samples from tissues among different trees. Further, we investigated whether the genetic relationship among the trees is dependant on tissue sample used. If the samples within a tree are more similar to each other than to any sample from another tree, this shows that both leaf and cambium DNA can be used interchangeably a single study.

MATERIALS AND METHODS

Study area and tree species

The samples were collected at Las Cuevas Research Station within the Chiquibul National Park of Belize, Central America in 2007-2008. The tree species we investigated were the breadnut (*Brosimum alicastrum*, Moraceae), mahogany (*Swietenia macrophylla*, Meliaceae) and kapok, (*Ceiba pentandra*, Malvaceae). The study area was approximately 4km^2 in broadleaf, deciduous forest (Penn et al. 2004).

Tissue collection and preservation

Leaf tissue was collected from each tree using rope-climbing techniques (Perry 1978). Once collected, the surface of each leaf was wiped using disposable tissue soaked in 70% isopropanol, to reduce the potential for contamination from surface biota. Each leaf was cut into thin strips and dried using grade12 silica gel (Sigma-Aldrich) in airtight plastic bags (Chase and Hills 1991). A small amount of self-indicating silica gel was added to each bag to show when the silica had become saturated; in which case the silica gel was changed. Cambium tissue was collected from the trees using a leather punch (according to the methods in Colpaert et al. 2005). To reduce the chance of disease introduction the plug of outer bark was replaced into the tree after cambium collection and all equipment was cleaned using 70% isopropanol before and after sampling, which also reduced the chance of contamination between samples. Cambial discs were dried using grade12 silica in O-ring tubes (Anachem), with two crystals of self-indicating silica gel to indicate if the silica gel became saturate. The silica gel was replaced once, one day after the initial collection.

DNA extraction

For DNA extraction we used 0.03g of dried leaf and 0.06g of dried cambium tissue. Each sample was ground into a fine powder using a mixer-mill. The cambium tissue was tough when dried and therefore we cut it up into small cubes with a scalpel before grinding, to ensure a powder was obtained. DNA was extracted using a modified 2×CTAB method (Doyle and Doyle, 1987): the extraction buffer (100mM Tris-HCl, pH 8, 1.4M NaCl, 20mM EDTA, 2% CTAB (hexadecyltrimethylammonium bromide), 2% PVP, plus 0.4% mercaptoethanol added just prior to use) was heated to 65ºC and then 750µl was added to the powdered tissue (in a 2ml tube), vortexed to mix and incubated in a water bath at 65ºC for 45mins. Then 750µl of 24:1 chloroform:isoamyl alcohol was added, and the samples rocked (to ensure continual mixing) at 140rpm at room temperature for 60mins. The samples were then centrifuged at 9000rpm for 15mins, and the upper supernatant layer was carefully pipetted off and placed in a fresh 1.5ml tube. To this -20ºC isopropanol was added (at a ratio of 2:3 isopropanol:supernatant) and samples placed into a -20ºC freezer overnight for DNA precipitation. The following day, the samples were centrifuged at 13000rpm for 10mins to pellet the DNA, the supernatant was removed and the pellet washed in 70% ethanol for 5mins. The samples were then centrifuged at 13000rpm for a further 10mins, all the liquid removed and the pellet allowed to dry in a fume cupboard at room temperature for at least 2 hours. The pellet was resuspended in 0.1TE buffer (10mM Tris, 1mM EDTA) and placed in a fridge overnight. The samples were then cleaned using spin columns (Sigma-Aldrich), where 500µl of the resuspended DNA was added to a binding buffer and spun through a spin column, which leaves the DNA bound to the silica membrane of the column. The membrane was then washed using a wash buffer containing ethanol to keep the DNA bound to the membrane and finally the DNA was recovered and eluted in 50µl elution buffer.

AFLP analysis

DNA preparation. A NanoDrop® ND-1000 spectrophotometer was used to analyse the purity (ratios of absorbance at 260nm/280nm and 260nm/230nm) and quantity of DNA in the extractions. DNA yield was calculated as the amount of DNA (µg) per gram of dry tissue material used in the extraction (0.03g for leaf material and 0.06g for cambium material). The volume required for 500ng of DNA was calculated and aliquoted into a 200µl tube and the liquid evaporated off in an oven at 60°C. The dried DNA was resuspended in 5.5 µl of sterilised double-distilled (SDD) water. If the quantity of DNA was <12ng/µl (i.e. less than 500ng in the entire extraction) then the extraction was deemed a failure and a second extraction was carried out to determine if the failure was due to the extraction process or lack of DNA in the sample.

Restriction ligation. *Mse1* and *EcoR1* restriction enzymes were used. Adaptor pairs were denatured at 95°C for 5mins and cooled for 10mins at room temperature. Then 1.1µl of 10x T4 ligase buffer, 1.1µl of 0.5M NaCl, 0.55µl of 1mg/ml BSA, 0.02µl of 50U/µl Mse1, 0.2µl of 25U/µl EcoR1, 0.05µl of 20U/µl ligase, 1µl of each of the denatured adaptor pairs and 0.48µl of SDD water were added to each sample of 5.5µl SDD water containing 500ng of DNA. The samples were incubated in a PCR machine at 37°C for 60 minutes and then 5µl of each sample was run on a 1.5% agarose gel to check it had worked. A positive result shows a faint smear down the gel. The restriction ligation samples were then diluted 1:20 using 0.1M TE buffer.

Pre-selective amplification. For each sample, 7.5µl of AFLP Core Mix (Applied Biosystems) and 0.5µl of regular genome size pre-selective primer pairs (Applied Biosystems) was added to 2µl of diluted restriction ligation product. The pre-selective primer pairs were not denatured during this stage. The samples were amplified using a

Gene Amp PCR System 9700 (heated at 72°C for 2min, then for 20 cycles run at 94°C for 20s, 56°C for 30s, 72°C for 2min, after which the samples were held at 60°C for 30mins before cooling to 4°C). 5µl of the pre-selective products were run on a 1.5% agarose gel using electrophoresis at 100V for 30mins to determine success. A positive reaction would show a smear in the range of 100-1500bp. Samples were then diluted 1:20 using 0.1M TE buffer.

Selective Amplification. To 1.5µl of diluted pre-selective amplification product, 7.5µl of AFLP Core Mix, 0.5µl of the *MseI* primer (Cxx) and 0.5µl of the *EcoR1* primer (dye + Axx) were added (Table 7.1). The samples were amplified using a Gene Amp PCR System 9700 (heated at 94°C for 2min, then for 10 cycles run at 94°C for 20s, 66°C for 30s, 72°C for 2mins, followed by 20 cycles of 94°C for 20s, 56°C for 30s, 72°C for 2min, after which the samples were held at 60°C for 30mins before cooling to 4°C). A preliminary primer trial was done on four different primer combinations of each dye for two samples for each tree species. From these results the final three primer combinations (one FAM™, one JOE™ and one NED™) were chosen by which produced the greatest number of bands.

Table 7.1. List of Selective amplification primers used for each species.

Species	FAM (Blue)	JOE (Green)	NED (Yellow)
Brosimum alicastrum	ACA/CTC	ACG/CTC	AGC/CAT
Swietenia macrophylla	ACT/CAG	AGG/CAT	AGC/CAC
Ceiba pentandra	ACT/CTG	ACG/CTC	ACC/CAT

Notes. Axx is the EcoR1 primer and Cxx is the Mse1 primer. FAM, JOE and NED are the fluorescent dyes used on the primers to enable the use of a genetic analyser (Applied Biosystems).

Visualising the AFLP bands. Samples were multiplexed and run on a Genetic Analyser ABI Prism 3100 (Applied Biosystems). For each well in the sequencing plate 10µl HiDi and 0.2µl Genescan™ 500 Rox™ Size Standard were added. To this, the undiluted selective amplification products were added (0.8µl of the FAM™ or JOE™ primer combinations or 1.0µl of the NED™ primer combination). The samples were denatured at 95°C for 5 minutes and cooled on ice for 10 minutes before being transferred to the genetic analyser.

AFLP analysis. The results were analysed using Genotyper 2.0 (Applied Biosystems), peaks were automatically assigned but individually checked by hand to ensure correct assignment. A binary matrix was created for band presence (1) and absence (0) for each sample. A 5% error rate was used to eliminate spurious bands, which would cause an over estimation of differentiation between individuals. The results were analysed using CAP4 (*Community Analysis Package 4,* Version 4.1.3, Pisces Conservation Ltd., 2007); the similarity between each pair of samples was calculated using the simple matching index (*a/N*) where, *a* is the number of bands shared by two samples and *N* is the total number of bands. The similarity value between the leaf and cambium sample within a tree was compared to the average similarity value between the cambium sample of that tree and the leaf samples of all other trees; thus comparing the similarity of tissue samples within a tree to those between trees. This data was analysed using a one-way ANOVA and graphically presented in a boxplot, using JMP® 8.0.2. Clustering dendrograms (average linkage) were constructed in CAP4 to determine if the tissue samples within a tree clustered more often than those between trees. This was see if the relationship among the trees differed depending on tissue type. The reproducibility of the AFLP profiles between leaf and cambium tissue was calculated as the average number of shared bands between samples, divided by the total number of loci.

RESULTS

A total of 49 *Brosimum alicastrum* trees were sampled, producing 136 loci (bands) from the AFLP analysis, of which 19 were monomorphic (i.e. every sample produced a band for this locus). Forty-one trees were sampled from *Swietenia macrophylla* and these samples produced 119 loci from AFLP analysis with 21 monomorphic loci. Only seven *Ceiba pentandra* trees were sampled, which produced 99 loci from AFLP analysis, with 32 monomorphic bands. From observation, the cambium of *Brosimum alicastrum* was more distinct than for the other tree species, *Swietenia macrophylla* possessed a spongy cambium layer and *Ceiba pentandra* trees often had thick bark resulting in greater difficulty in collecting cambium samples from this tree species.

DNA yield and purity

The DNA yield obtained from the samples differed between tissue type ($F_{2,235}$=16.76, $p<0.0001$) and tree species ($F_{1,235}$=50.30, $p<0.0001$) (Figure 7.1). Overall the leaf samples produced greater yield of DNA per gram of dried tissue, although samples from *Ceiba pentandra* produced on average low yields of DNA. The DNA purity was consistent between leaf and cambium samples within tree species, indicating little contamination among the samples (Table 7.2). The leaf and cambium samples from *Ceiba pentandra* had reduced ratios of 260/230nm suggesting a greater salt content (potentially from EDTA) than samples from the other tree species.

Table 7.2. DNA purity from leaf and cambium samples

	Protein contamination (260/280)		Salt contamination (260/230)	
Tree species	Cambium	Leaf	Cambium	Leaf
Brosimum alicastrum	1.81 ± 0.03	1.77 ± 0.02	1.98 ± 0.06	1.80 ± 0.08
Swietenia macrophylla	1.74 ± 0.02	1.75 ± 0.02	1.54 ± 0.06	1.70 ± 0.06
Ceiba pentandra	1.76 ± 0.12	1.74 ± 0.12	1.10 ± 0.11	1.06 ± 0.11

Notes. Results are the calculated ratio of absorbance from different wavelengths (230nm, 260nm and 280nm). Expected ratios of 260/280 = ~1.8 and 260/230 = 2.0 for pure DNA.

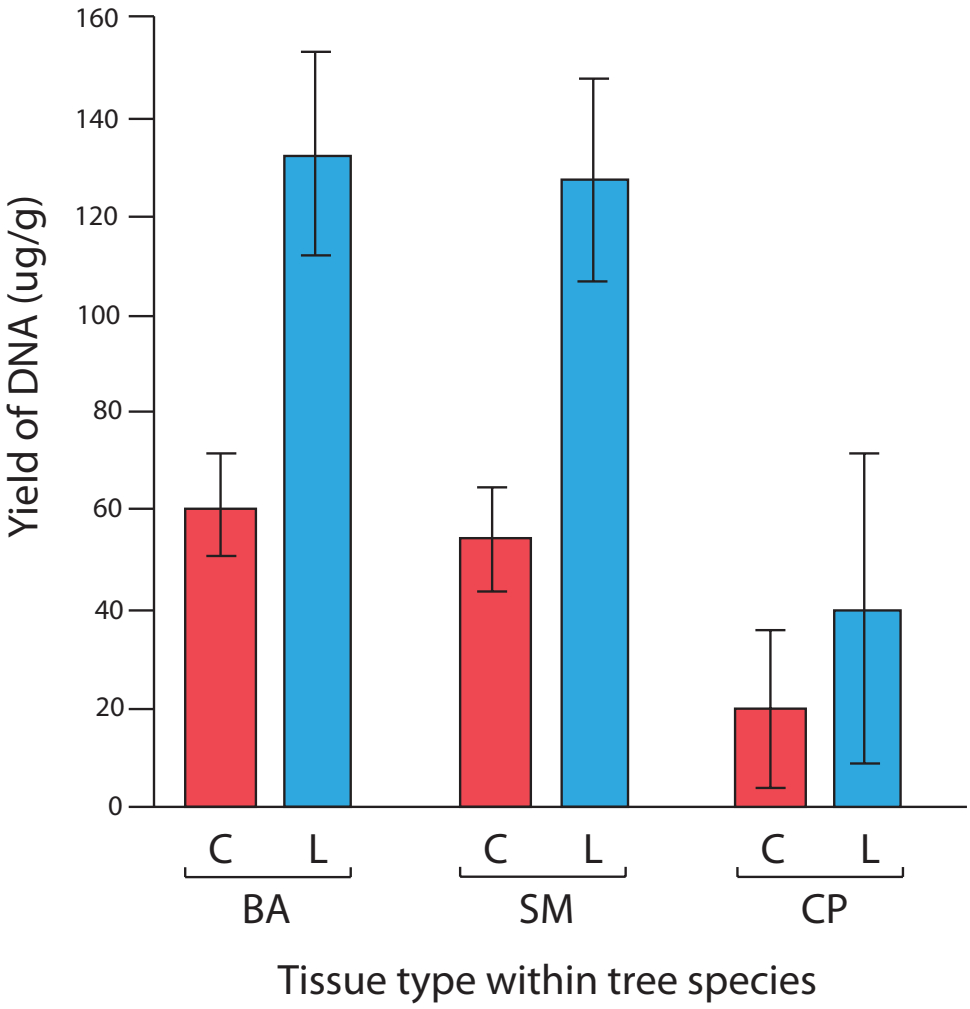

Figure 7.1 DNA yield (µg) obtained from 1g of dried tissue: C = cambium, L = leaf. Error bars represent ± 1 standard error. Species: BA = *Brosimum alicastrum*, SM = *Swietenia macrophylla*, CP = *Ceiba pentandra*.

AFLP profile similarity within tree

The AFLP profiles obtained from samples of different tissues (leaf and cambium) within the same tree were more similar than those obtained from samples of the different tissues from different trees ($F_{1, 188} = 560.1$, $p<0.0001$; Figure 7.2). There was also a main effect of tree species ($F_{2, 188} = 36.1$, $p<0.0001$), with *Ceiba pentandra* samples showing the least amount of similarity between tissue samples within a tree; this tree also had the lowest sample size.

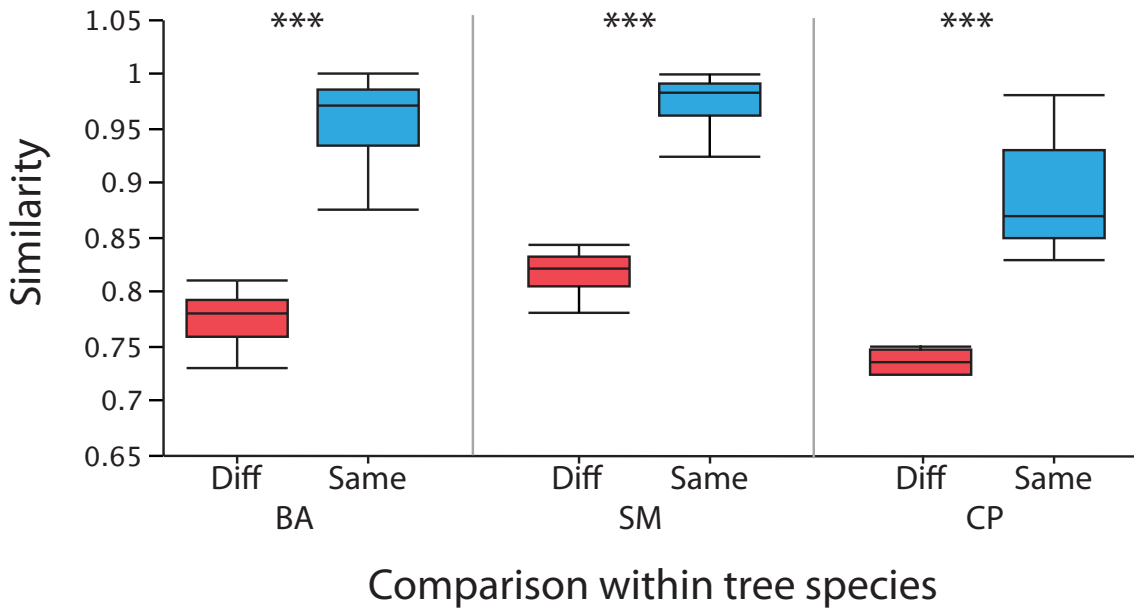

Figure 7.2. The distribution of similarity values (simple matching) between AFLP profiles of cambium and leaf tissue samples within the same tree (same) or different trees (diff), on the three tree species studied (BA: *Brosimum alicastrum*, SM: *Swietenia macrophylla*, CP: *Ceiba pentandra*). This shows that AFLP profiles are more similar from the different tissues within the same tree than among trees.

Does the genetic relationship between trees depend on tissue type?

The leaf and cambium samples from the same tree cluster together for all trees within *Brosimum alicastrum* (Figure 7.3) and *Swietenia macrophylla* (Figure 7.4). All but one tree for *Ceiba pentandra* has samples from the two tissue samples clustering more closely together than with any other sample (Figure 7.5). Therefore, there is no change in the relationship between trees due to tissue type. Within *Brosimum alicastrum* (n=49), there were five trees for which the leaf and cambium AFLP profiles did not differ at all, and the greatest number of mismatching bands (i.e. where one sample shows presence of a band, or locus, and the other does not) was 17. The mean number of mismatched bands for *Brosimum alicastrum* was 5.44±0.64 (mean ± standard error), from a total of 136 loci. Thus, reproducibility of the AFLP profiles was on average 96%. Within *Swietenia macrophylla* there were eight trees for which the AFLP profiles

did not differ between the leaf and cambium samples, and the greatest number of mismatched bands was 15. The mean number of mismatched bands was 3.15±0.52 (mean ± standard error), out of a possible 119 loci, which means the reproducibility of the AFLP profiles between leaf and cambium tissues was 97.4%. Within *Ceiba pentandra* there were no trees that had completely matching AFLP profiles from the leaf and cambium samples, although only one tree did not have clustering leaf and cambium samples (Figure 7.5). The greatest number of mismatching bands between the leaf and cambium samples for this tree species was 15 between the clustering samples within a tree and 17 between the non-clustering sample (overall mean ± standard error = 10.86±1.96). The reproducibility of AFLP profiles between leaf and cambium samples within *Ceiba pentandra* was 89.1%.

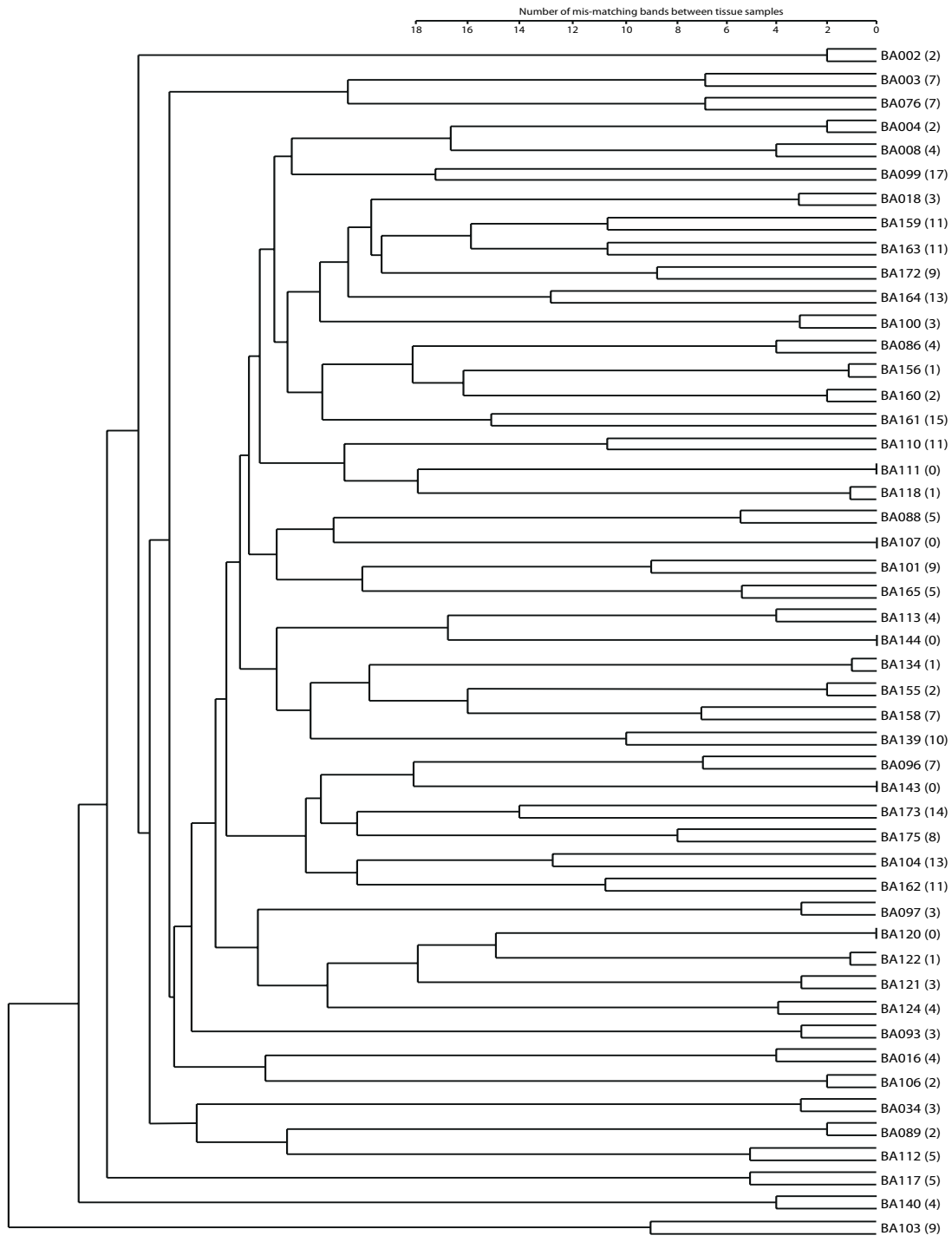

Figure 7.3. Clustering relationship of leaf and cambium samples within 49 individual *Brosimum alicastrum* (BA) trees. BA numbers correspond to individual tree numbers and the x-axis (top) shows the numbers of mismatched bands (i.e. those present in one tissue sample but not in the other). Numbers of mismatch bands within a tree are given in parentheses down the right-hand side. Leaf and cambium samples cluster within tree. The cambium and leaf tissue samples within five trees (BA111, BA107, BA144, BA143 and BA120) produced exactly the same AFLP fingerprint, indicated by zero mismatched bands. Greatest difference between tissue samples was for tree BA099, with 17 mismatched bands.

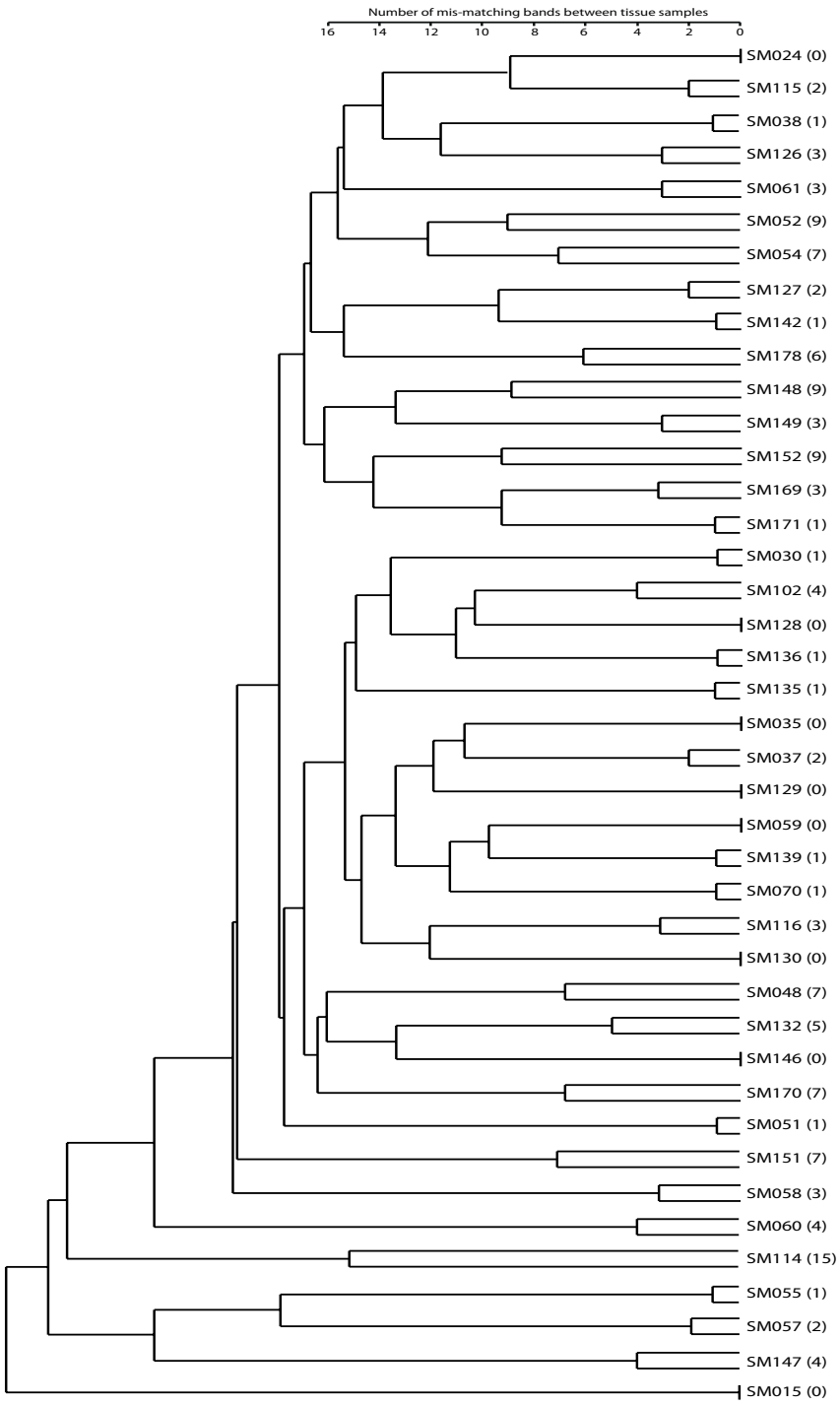

Figure 7.4. Clustering relationship of leaf and cambium samples within 41 individual *Swietenia macrophylla* (SM) trees. SM numbers correspond to individual tree numbers and the x-axis (top) shows the numbers of mismatched bands (i.e. those present in one tissue sample but not in the other). Numbers of mismatch bands within a tree are given in parentheses down the right-hand side. Leaf and cambium samples cluster within tree. The cambium and leaf tissue samples within eight trees (SM024, SM128, SM035, SM129, SM059, SM130, SM146 and SM015) produced exactly the same AFLP fingerprint, indicated by zero mismatched bands. Greatest difference between tissue samples was for tree SM114, with 15 mismatched bands.

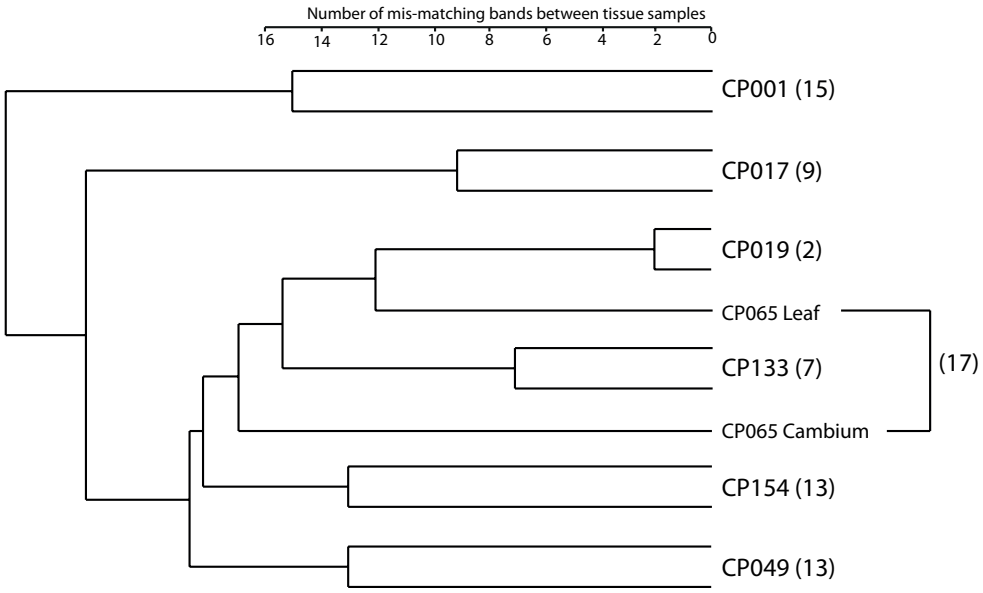

Figure 7.5. Clustering relationship of leaf and cambium samples within seven individual *Ceiba pentandra* (CP) trees. CP numbers correspond to individual tree numbers and the x-axis (top) shows the numbers of mismatched bands (i.e. those present in one tissue sample but not in the other). Numbers of mismatch bands within a tree are given in parentheses down the right-hand side. Tissue samples within six trees are more similar to each other than any other samples. The leaf and cambium samples from tree CP065 did not cluster together, with 17 band mismatches between them. The most similar leaf and cambium samples were from tree CP019, with only two band mismatches.

DISCUSSION

This paper demonstrated that DNA samples extracted from the leaf and cambium tissue of the same tree produced more similar AFLP profiles to each other than to any other sample. The genetic relationship between the trees was not altered within *Brosimum alicastrum* and *Swietenia macrophylla* when AFLP analysis was performed using different tissue samples. This shows that leaf and cambium tissue can be used interchangeably within a single genetic study without introducing significant error to the results. The samples within *Ceiba pentandra* showed a similar pattern, however, the samples of one tree did not cluster together indicating a potential route for error when using both tissues in a single study. Possible reasons for the differences observed include the reduced sample size of trees of this species, the low yield of DNA recovered and the numbers of AFLP markers were lower compared to the other tree species. Previous work has suggested the use of a single tissue type throughout a genetic study (Colpaert et al. 2005) due to potential differences in DNA methylation between tissues (Donini et al. 1997). We have shown that these concerns are unfounded within the tropical tree species studied in this paper and suggest the results are widely applicable since the tree species studied reside in different tree families and orders.

The DNA yield and quality obtained from both cambium and leaf samples for each tree sample were sufficient for AFLP analysis. The leaf samples collected from *Ceiba pentandra* produced lower yields of DNA than the samples from *Brosimum alicastrum* and *Swietenia macrophylla*. Reduced DNA yield from *C. pentandra* samples may have been caused by the formation of mucilage in leaf DNA extractions and this can also potentially reduce the performance of AFLP reactions (Bayer et al. 1999). Further, the low yield of DNA from *C. pentandra* cambium samples may be a result of the presence of a thick outer bark, reducing the efficiency of cambium sampling (Alvarado et al.

2003). The cambium and leaf samples from *C. pentandra* also showed an increase in salt concentration, potentially due to EDTA contamination, which may reduce the success of downstream PCR although since the total DNA was dried prior to AFLP analysis this is unlikely to have influenced the results. The DNA extraction method used in this study is good for removing unwanted plant phenols and polysaccharides, and the additional spin-column DNA cleaning stage will remove any remaining contaminants. Therefore, by following these methods the quality of the DNA recovered will be maximised.

The reproducibility of the AFLP profiles between the different tissues (leaf and cambium) was 97.4% in *S. macrophylla*, 96% in *B. alicastrum*, down to 89.1% in *C. pentandra*. These results are comparable to those found by Huy et al. (1996) who estimated reproducibility at 95-98.5% for AFLP profiles for bacterial samples. The reduced success of *C. pentandra* samples is likely to be related to the low yield of DNA obtained, the low number of trees sampled and the reduced number of polymorphic loci compared to the other tree species. This meant that even with the same number of mismatched bands between samples within tree, as some of the samples within trees for the other species studied, the lack of differentiation across all samples in *C. pentandra* caused them to not cluster. Mismatched bands (differences between AFLP profiles) may have been produced through differences in the quality of the DNA or during the extraction procedure (Jones et al. 1997). Due to the complex nature of the AFLP method, small discrepancies can occur through experimental error and create the differences observed. The use of an automated sequencer allows for weak bands to be detected, however, if the signal is particularly weak then the band will not be observed since it will fall below the baseline error rate. In this case, the strength of the remaining bands will also be reduced and the sample would have to be run again. Mismatched

bands could also occur through incomplete amplification during AFLP analysis or due to degradation of the template DNA (Jones et al. 1997, Blears et al. 1998).

Within a single study the use of both cambium and leaf tissue will enable a greater sample number of trees to be studied. Within a tropical forest the canopy of a tree can be high and the height of the first branch is often out of reach, requiring techniques such as tree climbing with ropes and harnesses to be used to collect leaf material, which can incur greater risk. In addition, tropical forests can be difficult to navigate and transport of climbing equipment can be costly. Leaf material can contain more biotic contaminants, such as endopathogenic bacteria (Saar et al. 2001), or higher levels of plant defensive chemicals that can inhibit downstream PCR, compared to cambium material. The use of cambium is therefore beneficial since it can be accessed at ground level with minimal equipment and the risk of disease introduction through cambium sampling can be reduced by thorough cleaning equipment with 70% isopropanol before and after every sample. However, there is no study to date that quantitatively assesses the risk of disease introduction following cambium sampling in tropical forests, where optimal conditions for pathogenic growth can occur. We suggest using leaf material where possible, but collecting cambium tissue if leaf material is inaccessible or contaminated. The cambium samples in this study were collected in 2007-2008 and these trees have been returned to in subsequent years, with no obvious incidence of disease (S. Zytynska pers. obs.). Therefore, over a time-scale of 3-4 years the collection of cambium tissue from these trees has not resulted in tree death or disease.

We dried and stored both the leaf and cambium material in silica gel (Chase and Hills 1991), with successful DNA recovery. This method for preservation and storage is also suitable for air-transportation between countries, which is advantageous when studying

the genetics of tropical trees. AFLPs are useful molecular tools and can be used in population and conservation genetics, systematics, biodiversity surveys, pathtyping and QTL mapping (Mueller and Wolfenbarger 1999). In addition, AFLPs require good quality DNA (Blears et al. 1998) and therefore the DNA recovered in this study will also be useful for other genetic analysis techniques such as microsatellites and sequencing.

In conclusion, we have shown that leaf tissue and cambium tissue can be used as a source of DNA for genetic analysis. Furthermore, we showed that a combination of these tissues could be used in a single genetic study of tree populations without compromising the accuracy of the phylogenetic relationships obtained among the trees studied.

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Chapter 8. The genetics of species interactions in model and natural ecosystems

THESIS DISCUSSION

Chapter discussion

Within the chapters of this thesis I demonstrate the use of model and natural ecosystems to further understand the influence of within-species genetic variation on species interactions following community genetics approaches. Two complementary approaches were used (see Figure 1.2). The first approach estimates the amount of phenotypic variation in a focal species that is explained by genetic interactions between the other species in a community, and this was followed in chapters 2-5. The second approach aims to determine if genetic variation within a focal species influences the surrounding ecological community and was followed in chapter 6. The second method was used since it is important to understand how within-species genetic variation influences naturally occurring communities, where the first method is unsuitable. Chapter 7 discussed the use of leaf and cambium tissue in a single genetic study, which allowed a greater sampling size, since even when using tree climbing techniques the leaves of a tropical tree are occasionally out of reach.

I began, in chapter 2 by showing that genetic interactions between aphids and barley mediate the indirect ecological effect (IEE) of rhizobacteria in the soil on the wing size (a proxy for fitness) of a parasitoid wasp. This paper showed that the direction and magnitude of the effect can be reversed dependent on the particular genotypes of aphid and barley interacting in the system and the interactions can explain up to 12.4% of the variation in parasitoid wing size. Therefore, indirect interactions within a community can have strong effects, even when two species do not physically meet each other and

are three links away in a direct interaction chain. In this paper, we suggest that these interactions have the potential to create evolutionary change in the parasitoid if there are non-random associations between the aphid and barley genotypes, which persist over a number of generations.

I progressed by showing that different aphid genotypes exhibit host preference for particular barley genotypes (chapter 3) and that this can occur through both passive (differential survival through affects on reproductive rate) and active (no effect on reproductive rate but migration to particular hosts) mechanisms. This indicates there is non-random association between the aphid and barley genotypes. The strength of host preference was also shown to differ among aphid genotypes and due to the presence of another aphid genotype, through intra-specific competition assumed to occur via plant-mediated traits (chapter 4). Within these chapters I showed that one aphid genotype (CLO7) did not exhibit active host choice among the barley genotypes, with host choice being explained by passive means, i.e. differing reproductive rates. In addition, this genotype also showed no change in host preference when reared in competition with another aphid genotype. The reproductive rate of CLO7 aphids was reduced when a Steptoe plant was pre-conditioned with DAV95 or H1 aphids (chapter 5), and these aphids also induced the up-regulation of a number of sequences in the plant compared to CLO7. The up-regulation of these genes may alter the reproductive rate of CLO7 aphids on Steptoe but they were found at in the same proportion on Steptoe when grown with the other aphids in a choice test, as when reared alone, suggesting these sequences do not alter the attractiveness of the plant to CLO7 aphids. Since the microarray experiments in chapter 5 were only performed with a single aphid genotype, the effect of differentially induced sequences by one aphid genotype over another affecting the host preference of another aphid genotype can only be speculative. Aphids from the H1

genotype also showed little host preference when reared alone but altered their host preference when reared in competition with another aphid genotype, dependent on the genotypic identity of the other aphid (HF92a or CLO7; chapter 4). These aphids also induced the up-regulation of the fewest sequences in the plant compared to the other aphid genotypes, and were shown to induce down-regulation of sequences from the jasmonate pathway (chapter 5). This could potentially explain the differences in host choice behaviour of H1 aphids when grown alone to when grown in competition with other aphids, since this aphid may not be able to tolerate the high induction of certain defense pathways in the plant. This finding is supported by the first experiment in chapter 5, which showed that pre-conditioning of a plant with CLO7 or HF92a aphids also reduced the number of H1 aphids on these plants, suggesting the induction of plant defenses that reduced the reproductive rate of H1 aphids. The effect on reproductive rate of an aphid would constitute a passive mechanism for host choice, where fewer aphids are present because fewer offspring are produced. However, active choice was deemed a strong factor in aphid host choice, suggesting the presence of HF92a and CLO7 aphids also decreased the attractiveness or palatability of particular barley genotypes for H1 aphids.

Overall chapters 2-5 show that genetic interactions between aphids and barley can have strong effects on interacting species, and there is potential for these effects to be maintained through host choice behaviour of the different aphid genotypes to different barley genotypes. For example, HF92a aphids show active choice for barley genotype Baronesse, whereas DAV95 aphids show active choice against this genotype, potentially enhancing the separation of these aphid genotypes. However, intra-specific competition between the aphid genotypes can alter the host choice of particular aphid genotypes, thereby enhancing mixing of the genotypes and encouraging gene flow

between them. As an aphid genotype experiences a greater number of interactions (intra- and inter-specific), the predictability of the outcome of each individual interaction is likely to decrease as the effect of a new interaction can either reinforce or undermine the effect of the first. The research within this thesis suggests that in *Sitobion avenae* aphids the genetic interactions between aphids and barley could be maintained through assortative associations between the genotypes, but within a high diversity system these associations are unlikely to be strong enough to produce an evolutionary effect in higher trophic organisms, such as a parasitoid wasp. However, in a different aphid species with greater genetic differentiation between genotypes, for example the pea aphid (*Acyrthosiphon pisum*), or between different plant host species rather than host plant genotypes, these effects may prove much stronger and influence the evolutionary trajectories of associated species.

In order to further understand the importance of within-species genetic variation on species interactions, there is a need to study this in natural systems. Work on model systems shows how genetically-based species interactions can influence other species in a community, but often abiotic factors are controlled and the genotypes used are highly differentiated to maximize the effects observed. Furthermore, genotypes are manipulated to enable a superior experimental design resulting in a number of non-naturally occurring interactions. Previous work, in naturally occurring systems, has shown the effect of within-species genetic variation in a number of plant species on a variety of different ecological communities (see Introduction and chapter 6 introduction). However, these have all been restricted to temperate systems where often the plant is a dominant species in an area of limited species diversity. I considered the effect of within-species genetic variation using amplified fragment length polymorphisms (AFLP) in a single tree species on the surrounding plant and

invertebrates of a common, but not dominant, tree in a complex tropical forest ecosystem (chapter 6). In this paper, we showed that more closely related trees were host to more similar communities of vascular epiphytes and invertebrates. This shows that even in a highly diverse, naturally occurring ecosystem the effect of genetic variation within a species can be an important factor for the structure of associated communities of both plants and animals.

Applications of community genetics

Research in the area of community genetics has potential applications for agriculture, biocontrol, disease management and conservation, in addition to furthering our understanding of the role of within-species genetic variation in species interaction, microevolution in multi-species systems and ecological speciation.

In an agricultural rice system in China, disease severity of rice blast (a major rice disease) was reduced by 94%, yield increased by 89%, and the use of fungicide reduced, when disease-susceptible genotypes of rice were grown in a mixed-crop with disease-resistance genotypes, compared to when grown in monoculture (Zhu et al. 2000). Thus, the affect of a pathogen on the disease-susceptible rice genotypes was decreased due to indirect intra-specific interactions with the disease-resistant rice genotype. Understanding species interactions between plants, herbivores and their predators and parasitoids can also benefit in agriculture, when assessing the impact of genetically-modified (GM) crop introduction (Poppy and Sutherland 2004) or considering the effectiveness of a new biological control strategy (Roderick & Navajas 2003). The control and management of many diseases can also benefit from further understanding of how genetic variation can affect species interactions. For example, there are strong genotype-by-genotype (GxG) interactions between the malaria parasite (*Plasmodium*

falciparum) and its mosquito vector (*Anopheles gambiae*) for the proportion of mosquitoes infected and the intensity of infection (Lambrechts et al. 2005). Further evidence suggests that humans with the sickle-cell trait present fewer symptoms of malaria, although they still harbour the parasite, and this trait may enhance future immunity of the individual to the disease (Kiwanuka et al. 2009). Therefore, complex genetic interactions between the malaria parasite, mosquito vector and human susceptibility can contribute to the disease impact.

The maintenance of genetic variation within a species can also enhance conservation efforts, since small populations with low genetic diversity can suffer inbreeding depression and accumulation of deleterious alleles (Frankham 1995). Population size reduction can occur through anthropogenic habitat destruction and fragmentation, as well as a consequence of natural disasters and climate change. As previously discussed, community genetics research has shown that genetic variation within a tree species can influence the associated plant and animal communities. Conservation of genetic variation within these tree species in an area could enhance the species diversity in the surrounding community and be achieved through maintaining a minimal viable interacting population (MVIP; Whitham et al. 2003, Bangert et al. 2005). The MVIP is the population size required to maintain sufficient genetic diversity that is required for stable populations of the other interacting populations within the community (Whitham et al. 2003). Using the MVIP could also benefit management strategies when considering species reintroductions, since the genetic diversity of the reintroduced population should be considered to ensure the survival of the population itself and to enhance the diversity of the interacting community.

Indirect interactions within a community are expected to strengthen community structure (Wootton 1994, Miller and Travis 1996), and genetic variation within a species for phenotypic plasticity (GxE or GxG interactions) can maintain genotypic diversity within a population. This suggests that maintaining genetic diversity within species in a community can enhance community stability and further, genetic diversity within dominant species can enhance species diversity of a community.

Conclusion

This thesis has furthered our knowledge of how genetic interactions between species could affect the surrounding community through behavioural mechanisms, such as host choice behaviour. It has also shown, for the first time, that different genotypes of an aphid can induce differential gene expression of numerous plant defense related sequences in a single genotype of plant. Finally, it has shown that even in a complex, diverse tropical ecosystem, where numerous interactions occur, the genetic variation within a single tree can influence the community structure of associated plants and invertebrates. Research in community genetics has wide applications for understanding how communities and ecosystems function, which can benefit agriculture, disease management and conservation practices.

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