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Specificity of insect-plant associations and their role in the formation of plant defenses and speciation

Ph.D. Thesis

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Annotation

The aim of this dissertation is to investigate what role insect-plant interactions play in the formation of host-plant defenses and in the diversification of both groups. We show that various groups of herbivore respond differently to host-plant defenses. Therefore plant defenses diversify into suites of complementary traits, as individual traits fail to provide protection against specialized herbivores. Further, we identify what levels of host-phylogeny shape the food-web structure of insect herbivores. We show that specialized herbivores are affected mainly by the terminal parts of the host-phylogeny. In contrast, more polyphagous guilds are affected mainly by the mid-levels of the host phylogeny because the effects of terminal or deeper phylogeny seem to be surpassed by other factors in more generalist insect species. In the last chapter, we show how specialized insect-plant interactions generated by tight insect-plant coevolution can influence the speciation in plants over environmental gradients.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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List of papers, manuscripts, and author's contribution

The thesis is based on the following papers (listed chronologically):

I. Volf, Martin*, Jan Hrcek, Riitta Julkunen-Tiitto, and Vojtech Novotny. "To each its own: differential response of specialist and generalist herbivores to plant defence in willows". *Journal of Animal Ecology* 84, no. 4 (2015): 1123-1132. (IF= 4.827)   *Martin Volf sampled the insect and plant data, participated in the secondary metabolite analysis, measured the physical traits, did the molecular analysis, reconstructed the host-plant phylogeny, did the statistical analysis of the data, formulated the hypotheses, wrote the first draft of the manuscript.*

II. Volf, Martin*, Riitta Julkunen-Tiitto, Jan Hrcek, and Vojtech Novotny. "Insect herbivores drive the loss of unique chemical defense in willows". *Entomologia Experimentalis et Applicata* 156, no. 1 (2015): 88-98. (IF= 1.442)
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III. Volf*[+], Martin, Simon T Segar[+], Scott E Miller, Brus Isua, Mentap Sisol, Gibson Aubona, Legi Sam, Petr Šimek, Martin Moos, Juha-Pekka Salminen, Jan Zima, George D Weiblen and Vojtech Novotny. "Dynamic plant defenses of sympatric *Ficus* species structure local larval leaf-chewer communities". (Manuscript)
Martin Volf participated in the secondary metabolite analysis, measured part of the physical traits, did part of the molecular analysis, reconstructed the host-plant phylogeny, did part of the statistical analysis of the data, helped to formulate the hypotheses, wrote the first draft of the manuscript together with Simon T Segar.

IV. Volf*, Martin, Petr Pyszko, Tomolazu Abe, Martin Libra, Nela Kotásková, Martin Šigut, Rajesh Kumar, Ondřej Kaman, Philip T Butterill, Jan Šipoš, Haruka Abe, Hiroaki Fukushima, Pavel Drozd, Naoto Kamata, Masashi Murakami and Vojtech Novotny. "Phylogenetic diversity of host plants drives insect-plant food web structure". (Manuscript)

Martin Volf sampled part of the insect and plant data, did the molecular analysis, reconstructed the host-plant phylogeny, did the statistical analysis of the data, formulated the hypotheses, wrote the first draft of the manuscript.

V. Segar*[+], T Simon, Martin Volf [+], Jan Zima Jnr., Brus Isua, Mentap Sisol, Legi Sam, Katerina Sam, Daniel Souto-Vilarós and Vojtech Novotny. "Speciation in a keystone plant genus is driven by elevation: a case study in New Guinean *Ficus*". (Manuscript)

Martin Volf did part of the molecular analysis, , did part of the statistical analysis of the data, helped to formulate the hypotheses, wrote the first draft of the manuscript together with Simon T Segar.

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Co-author agreement:

Vojtěch Novotný, the supervisor of Ph.D. thesis and co-author of all presented papers and manuscripts, fully acknowledges the major contribution of Martin Volf in all presented papers.

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Prof. RNDr. Vojtěch Novotný, CSc.

Simon T. Segar, the corresponding author of "Speciation in a keystone plant genus is driven by elevation: a case study in New Guinean *Ficus*" presented in Chapter IV, acknowledges the major contribution of Martin Volf in the presented manuscript.

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Simon T. Segar, Ph.D.

Contents

Volf, Martin*, Jan Hrcek, Riitta Julkunen-Tiitto, and Vojtech Novotny. "To each its own: differential response of specialist and generalist herbivores to plant defence in willows". *Journal of Animal Ecology* 84, no. 4 (2015): 1123-1132.

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Curriculum vitae of the author

Introduction

1.1 History of Insect-Plant Interactions and Coevolution

Herbivorous insects and vascular plants represent two of the most numerous groups of multi-cellular organisms driving major ecological processes in many terrestrial habitats (Schoonhoven, van Loon & Dicke 2005; Basset *et al.* 2012; Hamilton *et al.* 2013). They owe their great abundance and diversity of species to a long shared history of interactions and coevolution (Ehrlich & Raven 1964; Farrell, Dussourd & Mitter 1991; Janz 2011).

The first arthropods began utilizing plants as a food source in the Early Devonian period, only several million years after vascular plants colonized terrestrial habitats (Steemans *et al.* 2009; Labandeira 2013). This led to a progressive radiation in the diversity of arthropods and their feeding habits. The pioneer arthropod herbivores were sap-suckers, stem-borers, and consumers of spores (Labandeira 2007). Thalli, which evolved in leaves some time later, started to be consumed only shortly after that in the Middle Devonian (Labandeira 2013). All modern herbivore guilds, with the possible exception of leaf-miners, were present by the Late Carboniferous, more than 300 million years ago (Labandeira 2013).

Proliferation of herbivore lineages and guilds has increased herbivory pressure on plants and led to an arms-race between plants and insects lasting hundreds of million years (Ehrlich & Raven 1964; Janz 2011). In their coevolutionary theory, Ehrlich & Raven (1964) proposed that the genesis of novel defensive traits allows plants to escape herbivory leading to speciation of respective plant lineages. The novel defense is eventually overcome by herbivores allowing them to colonize the plant lineage defended by it. The herbivores speciate and the process starts over. In turn, the coevolutionary process should generate i) increased diversification of both plants and insect herbivores and ii) escalation of defenses during the course of plant evolution (Ehrlich & Raven 1964; Vermeij 1994; Janz 2011).

Ehrlich & Raven´s (1964) theory was proposed as a process based on pair-wise interactions. However pair-wise interactions resulting in co-cladogenesis of plants and insects appear to be scarce, despite thorough searches by many researchers (Farrell & Mitter 1990; Futuyma 2000). In fact, the best examples of insect-plant co-cladogenesis have been documented only for a couple of highly specialized insect-plant systems, such as *Agaonidae* fig wasps or *Prodoxidae* yucca moths (Pellmyr 2003; Cruaud *et al.* 2012), which do not represent typical herbivores.

There may be several explanations for scant evidence of insect plant co-cladogenesis. First, many plant lineages are probably much older than the insect lineages interacting with them which largely limits the potential of insects to affect plant evolution in such cases (Vane-Wright 2004; Magallon & Sanderson 2005). Second, insect adaptation probably does not require co-speciation with their hosts (Thompson 1994). Third, the most important factor limiting co-cladogenesis probably is that the evolution of host-plants is usually affected by interactions with entire insect communities rather than individual insect species resulting in complex interactions and patterns different from correspondence of herbivore and host phylogenies (Janzen 1980; Janz 2011).

1.2. Diffuse Coevolution and its Impacts on Host-Plant Defensive Patterns

Terrestrial plants support diverse insect communities and a single tree can harbor dozens of insect herbivore species from multiple taxa (Novotny *et al.* 2006; Novotny *et al.* 2010). These include herbivores from several guilds utilizing various feeding strategies (Novotny *et al.* 2010). Plant evolution is thus shaped by multiple interactions with diverse herbivore communities composed of species with a broad variety of life-histories resulting in diffuse coevolution rather than

strict coevolution between pairs of plant and insect species (Janzen 1980; Futuyma 2000).

Herbivores with various life-histories frequently exhibit different responses to plant traits and defences (Schoonhoven, van Loon & Dicke 2005; Roslin & Salminen 2008; Ali & Agrawal 2012). Certain life-history traits of insects are especially prominent in forming insect responses. Insect specialization is one such trait. Whereas unspecialized insects are often excluded from plants defended by unique or highly toxic secondary metabolites (Becerra 1997; Agrawal 2005; Volf *et al.* 2015b), insect specialists have repeatedly evolved mechanisms to overcome toxic or deterrent effects of such defences (Denno, Larsson & Olmstead 1990; Treutter 2006). Physical defensive traits, such as leaf toughness, that reduce herbivore feeding efficiency resulting in a prolonged feeding period , thus increasing specialist mortality through enhanced risk of predation or parasitism, may therefore be more effective against specialized insects (Richards *et al.* 2010; Dimarco, Nice & Fordyce 2012). Body size is another life-history trait affecting insect responses to host-plant traits. Feeding on some plant parts, such as xylem fluids with large negative pressure, require large body sizes to achieve efficient feeding preventing small herbivores from utilizing this source (Novotny & Wilson 1997; Schoonhoven, van Loon & Dicke 2005). Small herbivores are also more severely affected by some physical traits such as trichomes (Agrawal 2005). On the other hand, small sucking insects are sometimes better in avoiding tissues with high content of defensive chemicals (Schoonhoven, van Loon & Dicke 2005).

Differential responses of insect herbivores constrains the ability of plants to develop defensive traits that are universally effective against herbivores (Koricheva, Nykanen & Gianoli 2004; Volf *et al.* 2015a). Maintaining an effective protection against herbivores thus requires several complementary defensive traits (Koricheva, Nykanen & Gianoli 2004; Volf *et al.* 2015a). This may lead to diversification of host-plant defenses into suites of complementary traits and the

formation of defensive syndromes (Agrawal & Fishbein 2006; Agrawal 2007; Volf *et al.* 2015a). In turn, plant defensive traits are usually mutually independent or positively correlated, as observed by Agrawal & Fishbein (2006) or Hattas *et al.* (2011). Trade-offs between individual defensive traits, once suggested to be common among plant defences (Rehr, Feeny & Janzen 1973), may thus be expected to occur mainly under specific conditions, such as in low nutrient environments or in the case of negative dependence in metabolic pathways (Agrawal, Salminen & Fishbein 2009; Sampedro, Moreira & Zas 2011). Diffuse coevolution and herbivory pressure by diverse communities of differentially responding herbivores may thus further support the diversification of plant defences during the course of plant evolution (Volf *et al.* 2015a).

1.3. Diversification and Escalation of Host-Plant Defences

In their coevolutionary theory, Ehrlich & Raven (1964) proposed that the arms-race between plants and herbivorous insects leads to diversification of defensive traits driven by the strong impact of novel traits on herbivores. It is true that insects act as a selective pressure promoting increased plant defense (Benderoth *et al.* 2006), and many novel defensive traits appear during the course of plant evolution (Fucile, Falconer & Christendat 2008; Kliebenstein & Osbourn 2012). However, it seems that the evolution of host-plant defenses may follow more complex trajectories than simple unidirectional diversification (Agrawal & Fishbein 2008; Kursar *et al.* 2009; Volf *et al.* 2015b)

The evolution of plant defenses was studied in several systems (Agrawal & Fishbein 2008; Becerra, Noge & Venable 2009; Kursar *et al.* 2009; Agrawal *et al.* 2012; Pearse & Hipp 2012; Volf *et al.* 2015b). There is strong evidence for the diversification of secondary metabolites over large temporal and spatial scales in the genus

Asclepias, Bursera, and *Quercus* (Agrawal & Fishbein 2008; Becerra, Noge & Venable 2009; Pearse & Hipp 2012). However, diversification does not seem to universally apply to all defensive traits even in these systems. For example, the presence of cardenolides appears to have decreased with phylogenetic diversification in *Asclepias* (Agrawal & Fishbein 2008). Interestingly, several studies did not find patterns indicating diversification of plant defensive mechanisms when considering only plants growing in sympatry. They recovered labile and divergent defences among closely related species sometimes leading to a loss of defense diversity (Becerra 2007; Kursar *et al.* 2009; Volf *et al.* 2015b).

The reduction or loss of secondary metabolites is expected if they become ineffective in anti-herbivore defense or too costly, which may explain the loss of secondary metabolite diversity in some cases (Volf *et al.* 2015b). For example, in the case of willows, several groups of herbivores have adapted to salicylates, a specialized defence of willows, making them rather ineffective (Denno, Larsson & Olmstead 1990; Soetens, Rowell-Rahier & Pasteels 1991; Volf *et al.* 2015b). Not only do these herbivores seem to be able to overcome their negative effects to some extent, but also they are able to use them for sequestering salicyl-aldehyde which they use as a protection from invertebrate predators (Pasteels *et al.* 1983; Rowell-Rahier & Pasteels 1986).

These contrasting findings suggesting divergence in defences among plants growing in sympatry could be a result of different selection pressures on large-scale and local levels. Reported intergeneric differences in defensive chemicals among closely related plants are frequently generated by the production of various derivatives of a similar origin, representing tweaks to existing metabolic pathways rather than radical new changes (Wink 2003; Agrawal *et al.* 2012). Specialist herbivores have been reported to be able to overcome a variety of secondary metabolites of similar origin (Denno, Larsson & Olmstead 1990; Nishida 1994). In such cases a completely different

form of protection may be effective against specialists, possibly enforcing divergence of defensive traits (Volf *et al.* 2015a). When exposed to various herbivores in a local community, divergence in defences among congeneric plants may thus be an ideal strategy in order to escape herbivory from specialized insects (Becerra 2007). Moreover, divergence in defensive strategies among closely related plants may also help plants to escape herbivores targeting related host species.

Many herbivores tend to feed on related host-plants and host shifts are more common among closely related plants than among distantly related lineages (Janz & Nylin 1998; Winkler & Mitter 2008). This results in many insects being phylogenetically conservative in their food choice (Futuyma & Agrawal 2009). Closely related hosts are thus likely to share many herbivores which can potentially increase herbivory damage to them (Becerra 2007). It has been shown that host-shifts among host-plants are driven by similarities in plant defences and palatability rather than phylogenetic relatedness in cases where these traits were uncorrelated with plant phylogeny (Becerra 1997; Wahlberg 2001). In turn, divergence in defensive mechanisms could help closely related plants growing in sympatry to reduce the probability of host-shifts and to avoid sharing large pools of herbivores, thereby possibly reducing the potential population size of herbivores and herbivory damage. There are some indications that such strategies might occur among plants in natural communities driving local communities of co-occurring related species towards divergence in their defenses (Becerra 2007).

1.4. The Role of Host-Plants in Diversification of Insect Herbivores

Major speciation events in plants have supported speciation in insects (Winkler & Mitter 2008). Derived and diverse plant lineages harbor

more speciose insect communities as a result (Wiegmann, Regier & Mitter 2002; Janz, Nylin & Wahlberg 2006). Plant speciation has also led to diversification of their traits producing great variation in the palatability of individual plant species or lineages to herbivores as shown above (Schoonhoven, van Loon & Dicke 2005; Agrawal & Fishbein 2008; Futuyma & Agrawal 2009). The diversification and divergence of both host-plant lineages and defences sometimes required very elaborate insect adaptations to cope with them (Ehrlich & Raven 1964; Janz 2011). However, once defences were overcome the availability of the new niche usually triggered insect speciation. For example, the ability to sequester highly toxic cardenolides found in latex of *Asclepias* plants probably spurred speciation in *Tetraopes* making this genus of long-horn beetles the most species-rich group of herbivores associated with milk-weeds (Farrell 2001). Such processes have generated an enormous diversity of insect herbivores, and arthropods in general, with recent global estimates around 6.1 millions of arthropod species (Basset *et al.* 2012; Hamilton *et al.* 2013). In turn, insect taxa feeding on plants are generally more speciose than their counterparts exploiting different food sources (Mitter, Farrell & Wiegmann 1988). Cynipids can used as illustrative example. Utilizing plants as a food source has led to the diversification of gall-forming species which now represent ca 85% percent of Cynipidae with parasitoids accounting only for 15% of the family richness (Liljebland & Ronquist 1998).

There is no doubt that high diversity of host-plants is also one of the key factors maintaining hot-spots of extant insect diversity, such as those in lowland tropical forests (Novotny *et al.* 2006). The positive effect of plant diversity on insect diversity is further supported by high specialization of herbivores (Dyer *et al.* 2007). Many studies have recorded rather high insect specialization in general (Novotny *et al.* 2004; Schoonhoven, van Loon & Dicke 2005). For example, the majority of caterpillars randomly picked from the vegetation in a secondary rain forest in New Guinea feed on one to three host-plants

and have 90% of their populations concentrated on a single host-plant species (Novotny *et al.* 2004). Super-polyphagous species feeding on dozens of host-plants from various lineages are rare even among herbivores considered to be generalists and frequently represent only a small portion of herbivore community (Novotny *et al.* 2004; Forister *et al.* 2015). In turn, most extant insect herbivores seem to be specialists showing some level of phylogenetic conservatism with a strong preference for confamilial or congeneric hosts (Novotny *et al.* 2002; Forister *et al.* 2015). Individual herbivore species are thus often confined only to subsets of such plant communities which may promote the overall level of specialization of insect assemblages and, in turn, total insect diversity in the community (Novotny *et al.* 2004).

However, the specific role of host-plant diversity is likely to differ among guilds of insect herbivores according to their specialization. The levels of specialization and phylogenetic conservatism in their host choice differ among herbivore guilds (Novotny et al. 2010). The spectrum of insect specialization ranges from polyphagous root-chewing larvae feeding often on multiple plant families through leaf-chewing larvae often feeding on several congeneric or confamilial hosts to miners and gallers, typically specialized on a single host-plant species and seldom shared even among congeneric hosts (Novotny *et al.* 2010; Forister *et al.* 2015). This range of specialization means that food-web structure and species richness of individual herbivorous guilds may be driven by plant speciation events of different ages. Recent events are likely to affect mainly the most specialized guilds, such as miners and gallers. On the other hand, herbivores from less specialized guilds are often shared between related hosts and appear to respond mainly to deeper phylogenetic relationships of their host-plants (Novotny *et al.* 2004; Futuyma & Agrawal 2009). In turn, high species diversity of host-plants should support high diversity of specialized guilds, whereas the diversity of more polyphagous herbivores may be maintained mainly

by diversity of higher phylogenetic or taxonomical lineages, such as families.

1.5. The Role of Specialized Mutualisms in Plant Speciation

Phytophagous insects often have antagonistic relationships with plants as outlined in the previous chapters, but intimate insect-plant interactions have given rise also to specialized herbivores which became involved in tight pollination mutualisms (Cruaud *et al.* 2012). *Agaonidae* fig wasps and *Prodoxidae* yucca moths are two examples of phytophagous insects with seed or flower eating larvae that became one of the most specialized pollinators involved in tight coevolution with their host-plants (Pellmyr 2003; Cruaud *et al.* 2012). Pollinating *Agaonidae* and *Prodoxidae* play an irreplaceable role in the pollination of fig trees and yuccas and their larvae feed exclusively on the flowers of their hosts. In fact, most fig and yucca species are pollinated by only one fig wasp or yucca moth species, respectively, and most fig wasps and yucca moths are associated with just a single host species (Pellmyr 1997; Cook & Rasplus 2003; Pellmyr 2003; Cook & Segar 2010).

Interactions between these herbivores and their host-plants range from obligate mutualism and commensalism to antagonism. The close relatives of these two groups have antagonistic relationships with their hosts, either as gallers in case of both *Agaonidae* and *Prodoxidae* or as parasitoids of pollinators in case of some *Agaonidae* (Weiblen 2001; Pellmyr 2003). The larvae of pollinating *Agaonidae* and *Prodoxidae* species also have some negative effects on the host as they feed on the seeds. However, this means that seed eating species can finish their development only in pollinated flowers (Weiblen 2001; Pellmyr 2003). This leads to very specific mutualisms characterized by conflicts over seed resources as the consumption of too many or too few seeds by pollinators could drive

the mutualism toward extinction (Pellmyr 1997; Weiblen 2001). Each partner is thus entirely dependent on the other for reproduction (Pellmyr 2003; Cruaud *et al.* 2012).

Host recognition plays a key role in such systems (Hossaert-McKey *et al.* 2010). In case of *Ficus*, the importance of accurate host recognition is further pronounced by the short life-span of fig wasps reaching couple of days at the most (Abdurahiman & Joseph 1976). Moreover, once the pollinating female enters the fig it loses its wings and is unable to leave it (Galil & Eisikowitch 1968). Wrong host recognition thus has severe consequences. This probably further promotes the intimacy and selection in *Ficus-Agaonidae* system. Most fig wasps recognize their host based on chemicals with volatile compounds playing the prominent role (Hossaert-McKey *et al.* 2010; Cruaud *et al.* 2012). One could expect that the compounds responsible for host recognition would be very specific in such highly specialized system. Surprisingly, species specific chemicals are involved in host recognition rather rarely (Chen *et al.* 2009). The majority of fig wasps recognize their *Ficus* hosts based on the relative concentration of individual volatiles in mixes of common volatile compounds which are characteristic for individual *Ficus* species (Proffit *et al.* 2009).

Fig wasps use the chemical cues for very effective recognition of their hosts and can efficiently pollinate figs over large distances in continuous habitats such as lowland tropical forests or deserts (Nason, Herre & Hamrick 1996; Ahmed *et al.* 2009). In turn *Ficus* trees with their ca 750 species are wide spread in various habitats across tropics and provide many ecosystem services (Cruaud *et al.* 2012). *Ficus* species represent one of the key genera in forest communities supporting extremely species rich communities of herbivorous insects from several guilds (Novotny *et al.* 2005). Being one of the most important plant genus for tropical frugivores, *Ficus* trees also provide important food source for broad variety 270 of vertebrates with some of them being dependent on fig consumption

(Howe & Smallwood 1982; Shanahan *et al.* 2001) The fig-pollinator mutualism is therefore ecologically important in most tropical ecosystems (Shanahan *et al.* 2001).

Being very plastic, *Ficus* species are over-represented amongst plant species with wide elevational ranges and represent one of the key genera in forest communities along elevational gradients (Novotny *et al.* 2005). Given that wasp-mediated gene flow between populations of *Ficus* in continuous lowland habitats can cover tens to hundreds of kilometers (Nason, Herre & Hamrick 1996; Ahmed *et al.* 2009) we might expect to see a similar pattern in montane populations. However, environmental conditions vary dramatically across altitudinal gradients leading to local adaptation and limitations to pollinator dispersal. Above canopy winds, allowing long-range dispersal of wasps in lowland habitats, are likely to be a less effective method of pollinator dispersal to higher elevations. This may be especially true for understory tree species whose pollinators tend to disperse in the forest understory too (Harrison 2003). Moreover, fig wasps have been shown to be strongly affected by environmental factors. These tiny insects are particularly sensitive to changes in temperature (Jevanandam, Goh & Corlett 2013). Fig wasps may be unable to cross the gradual temperature gradient found between lower and upper elevations. This can be thus expected to limit gene-flow between lowland and highland populations of fig trees strongly contributing to the speciation of *Ficus* along elevational gradients.

There are closely related sister species or subspecies of lowland and highland fig trees (Berg, Corner & Nooteboom 2005). Highland populations are often smaller in stature and have fruits with prominent pubescence. Similarly *F. dammaropsis* has baseball sized fruits in the lowlands which are covered with open bracts, in contrast to highland populations, which have substantially larger fruits that are generally smoother and have the bracts closed (Segar, *personal communication*). However, the taxonomic status of the highland and lowland varieties is largely uncertain (Berg, Corner & Nooteboom

2005) as speciation and gene-flow in *Ficus* along elevational gradients has been rarely studied. We are thus possibly overlooking one of the factors which may significantly promote *Ficus* diversity.

1.6. Aims and Scope

Insect-plant coevolution has generated an enormous diversity of plants, insects, their traits and strategies (Ehrlich & Raven 1964; Farrell, Dussourd & Mitter 1991; Janz 2011). Both insects and plants have become fundamental elements of most terrestrial habitats. Herbivory and insect-plant coevolution in a broad sense are therefore key factors shaping the nature of numerous ecosystems (Fine, Mesones & Coley 2004; Schoonhoven, van Loon & Dicke 2005; Basset *et al.* 2012; Hamilton *et al.* 2013). Still, we need a better understanding of several aspects of insect-plant coevolution as shown in the previous chapters. The aim of this dissertation is to identify some of the patterns underlying insect-plant coevolution and to contribute to our understanding of insect-plant interactions.

In **Chapter I** we analyze the response of assemblages of specialized and generalized insect herbivores to host-plant traits. We investigate whether we can detect any characteristic response by these herbivores and discuss the implications of differential insect response for the evolution of host-plant defences. We show that assemblages of insects with different levels of specialization exhibit various responses to host-plant traits which probably prevents plants from evolving a universal defense based on individual traits and leads to formation of defensive syndromes consisting of complementary defenses.

In **Chapter II** we investigate the defensive patterns in a local community of *Salix* species. We focus on the role of salicylates, a *Salix* specific defence, in *Salix* protection. We investigate the connection between salicylate investment costs, the effects salicylates

have on insect herbivores, and salicylate diversification in willows. We show that the diversification of host-plant defenses is highly dependent on their relative costs and benefits and that low effectiveness against specialist herbivores may lead to loss of defensive traits.

Chapter III is focused on the evolution of host-plant defences in a lowland *Ficus* species growing in sympatry. We extend previous food web studies across *Ficus* to include not only insect herbivore data but also trait and plant chemistry data in a phylogenetic context. We investigate the trade-offs between several key defensive traits and palatability as well as their effects on specialist and generalist caterpillar assemblages. We focus on the role of diversification or divergence of defensive traits in a community of closely related plants growing in sympatry from the perspective of their protection from local pools of insect herbivores. We show that related species of sympatric *Ficus* species differ in several key defensive traits which may help them to reduce pressure by specialized herbivores. Further, we show that differential response of herbivores to host-plant traits may lead to divergence in evolutionary trajectories between various defensive traits.

In **Chapter IV** we identify the role of host-plant phylogenetic diversity in supporting insect specialization and diversity. Utilizing a plot based approach, we reconstruct insect-plant food webs and host-plant phylogenies for three guilds of herbivores from three sites with various host-plant diversity. We reconstruct food-webs for communities with various levels of host-plant phylogenies collated to reveal which level of host-plant phylogeny plays the key role in insect specialization. Further, we analyze whether monophyletic plant lineages share more herbivore species that randomly selected pool of plants. Our results suggest the high importance of host-plant phylogeny in modulating insect-plant food-web structure revealing non-trivial dependence of insect specialization on host-plant phylogenetic composition. The specific role of host-plant diversity is

likely to differ depending on the level of specialization of respective insect guild.

Chapter V focuses on plant speciation. Using microsatellite data, we examine gene-flow in *Ficus* species along an altitudinal gradient in Papua New Guinea and its possible implications for *Ficus* speciation. We show that there are barriers for gene-flow between lowland and highland *Ficus* populations and discuss the role of *Ficus* specialized insect pollinators and vertebrate seed dispersers in this pattern.

References

Abdurahiman, U. & Joseph, K. (1976) Observations on the biology and behaviour of Ceratosolen marchali Mayr (Agaonidae, Chalcidoidea, Hymenoptera). *Entomon,* **1,** 115-122.

Agrawal, A.A. (2005) Natural selection on common milkweed (*Asclepias syriaca*) by a community of specialized insect herbivores. *Evolutionary Ecology Research,* **7,** 651-667.

Agrawal, A.A. (2007) Macroevolution of plant defense strategies. *Trends in Ecology & Evolution,* **22,** 103-109.

Agrawal, A.A. & Fishbein, M. (2006) Plant defense syndromes. *Ecology,* **87,** S132-S149.

Agrawal, A.A. & Fishbein, M. (2008) Phylogenetic escalation and decline of plant defense strategies. *Proceedings of the National Academy of Sciences of the United States of America,* **105,** 10057-10060.

Agrawal, A.A., Petschenka, G., Bingham, R.A., Weber, M.G. & Rasmann, S. (2012) Toxic cardenolides: chemical ecology and coevolution of specialized plant-herbivore interactions. *New Phytologist,* **194,** 28-45.

Agrawal, A.A., Salminen, J.P. & Fishbein, M. (2009) Phylogenetic trends in phenolic metabolism of milkweeds (Asclepias): evidence for escalation. *Evolution,* **63,** 663-673.

Ahmed, S., Compton, S.G., Butlin, R.K. & Gilmartin, P.M. (2009) Wind-borne insects mediate directional pollen transfer between desert fig trees 160 kilometers apart. *Proceedings of the National Academy of Sciences,* **106,** 20342-20347.

Ali, J.G. & Agrawal, A.A. (2012) Specialist versus generalist insect herbivores and plant defense. *Trends in Plant Science,* **17,** 293-302.

Basset, Y., Cizek, L., Cuenoud, P., Didham, R.K., Guilhaumon, F., Missa, O., Novotny, V., Odegaard, F., Roslin, T., Schmidl, J., Tishechkin, A.K., Winchester, N.N., Roubik, D.W., Aberlenc, H.P., Bail, J., Barrios, H., Bridle, J.R., Castano-Meneses, G., Corbara, B., Curletti, G., da Rocha, W.D., de Bakker, D., Delabie, J.H.C., Dejean, A., Fagan, L.L., Floren, A., Kitching, R.L., Medianero, E., Miller, S.E., de Oliveira, E.G., Orivel, J., Pollet, M., Rapp, M., Ribeiro, S.P., Roisin, Y., Schmidt, J.B., Sorensen, L. & Leponce, M. (2012) Arthropod Diversity in a Tropical Forest. *Science,* **338,** 1481-1484.

Becerra, J.X. (1997) Insects on plants: Macroevolutionary chemical trends in host use. *Science,* **276,** 253-256.

Becerra, J.X. (2007) The impact of herbivore-plant coevolution on plant community structure. *Proceedings of the National Academy of Sciences of the United States of America,* **104,** 7483-7488.

Becerra, J.X., Noge, K. & Venable, D.L. (2009) Macroevolutionary chemical escalation in an ancient plant– herbivore arms race. *Proceedings of the National Academy of Sciences of the United States of America,* **106,** 18062–18066.

Benderoth, M., Textor, S., Windsor, A.J., Mitchell-Olds, T., Gershenzon, J. & Kroymann, J. (2006) Positive selection driving diversification in plant secondary metabolism. *Proceedings of the National Academy of Sciences of the United States of America,* **103,** 9118-9123.

Berg, C.C., Corner, E.J.H. & Nooteboom, H. (2005) *Flora Malesiana. Series I, Seed plants. Volume 17, Part 2: Moraceae (Ficus).* Nationaal Herbarium Nederland.

Cook, J.M. & Rasplus, J.-Y. (2003) Mutualists with attitude: coevolving fig wasps and figs. *Trends in Ecology & Evolution,* **18,** 241-248.

Cook, J.M. & Segar, S.T. (2010) Speciation in fig wasps. *Ecological Entomology,* **35,** 54-66.

Cruaud, A., Ronsted, N., Chantarasuwan, B., Chou, L.S., Clement, W.L., Couloux, A., Cousins, B., Genson, G., Harrison, R.D., Hanson, P.E., Hossaert-Mckey, M., Jabbour-Zahab, R., Jousselin, E., Kerdelhue, C., Kjellberg, F., Lopez-Vaamonde, C., Peebles, J., Peng, Y.Q., Pereira, R.A.S., Schramm, T., Ubaidillah, R., van Noort, S., Weiblen, G.D., Yang, D.R., Yodpinyanee, A., Libeskind-Hadas, R., Cook, J.M., Rasplus, J.Y. & Savolainen, V. (2012) An extreme case of plant-insect codiversification: Figs and fig-pollinating wasps. *Systematic Biology,* **61,** 1029-1047.

Denno, R.F., Larsson, S. & Olmstead, K.L. (1990) Role of enemy-free space and plant quality in host-plant selection by willow beetles. *Ecology,* **71,** 124-137.

Dimarco, R.D., Nice, C.C. & Fordyce, J.A. (2012) Family matters: effect of host plant variation in chemical and mechanical defenses on a sequestering specialist herbivore. *Oecologia,* **170,** 687-693.

Dyer, L.A., Singer, M.S., Lill, J.T., Stireman, J.O., Gentry, G.L., Marquis, R.J., Ricklefs, R.E., Greeney, H.F., Wagner, D.L., Morais, H.C., Diniz, I.R., Kursar, T.A. & Coley, P.D. (2007) Host specificity of Lepidoptera in tropical and temperate forests. *Nature,* **448,** 696-U699.

Ehrlich, P.R. & Raven, P.H. (1964) Butterflies and plants - a study in coevolution. *Evolution,* **18,** 586-608.

Farrell, B.D. (2001) Evolutionary Assembly of the Milkweed Fauna: Cytochrome Oxidase I and the Age of TetraopesBeetles. *Molecular Phylogenetics and Evolution,* **18,** 467-478.

Farrell, B.D., Dussourd, D.E. & Mitter, C. (1991) Escalation of plant defense - do latex and resin canals spur plant diversification. *American Naturalist,* **138,** 881-900.

Farrell, B.D. & Mitter, C. (1990) Phylogenesis of insect plant interactions - have Phyllobrotica leaf beetles (Chrysomelidae) and the Lamiales diversified in parallel. *Evolution,* **44,** 1389-1403.

Fine, P.V.A., Mesones, I. & Coley, P.D. (2004) Herbivores promote habitat specialization by trees in amazonian forests. *Science,* **305,** 663-665.

Forister, M.L., Novotny, V., Panorska, A.K., Baje, L., Basset, Y., Butterill, P.T., Cizek, L., Coley, P.D., Dem, F., Diniz, I.R., Drozd, P., Fox, M., Glassmire, A.E., Hazen, R., Hrcek, J., Jahner, J.P., Kaman, O., Kozubowski, T.J., Kursar, T.A., Lewis, O.T., Lill, J., Marquis, R.J., Miller, J.S., Morais, H.C., Murakami, M., Nickel, H., Pardikes, N.A., Ricklefs, R.E., Singer, M.S., Smilanich, A.M., Stireman, J.O., Villamarín-Cortez, S., Vodka, S., Volf, M., Wagner, D.L., Walla, T., Weiblen, G.D. & Dyer, L.A. (2015) The global distribution of diet breadth in insect herbivores. *Proceedings of the National Academy of Sciences,* **112,** 442-447.

Fucile, G., Falconer, S. & Christendat, D. (2008) Evolutionary Diversification of Plant Shikimate Kinase Gene Duplicates. *Plos Genetics,* **4**.

Futuyma, D.J. (2000) Some current approaches to the evolution of plant herbivore interactions. *Plant Species Biology,* **15,** 1-9.

Futuyma, D.J. & Agrawal, A.A. (2009) Macroevolution and the biological diversity of plants and herbivores. *Proceedings of the National Academy of Sciences of the United States of America,* **106,** 18054-18061.

Galil, J. & Eisikowitch, D. (1968) On the pollination ecology of Ficus sycomorus in East Africa. *Ecology***,** 259-269.

Hamilton, A.J., Novotny, V., Waters, E.K., Basset, Y., Benke, K.K., Grimbacher, P.S., Miller, S.E., Samuelson, G.A., Weiblen, G.D., Yen, J.D.L. & Stork,

N.E. (2013) Estimating global arthropod species richness: refining probabilistic models using probability bounds analysis. *Oecologia,* **171,** 357-365.

Harrison, R.D. (2003) Fig wasp dispersal and the stability of a keystone plant resource in Borneo. *Proceedings of the Royal Society of London B: Biological Sciences,* **270,** S76-S79.

Hattas, D., Hjalten, J., Julkunen-Tiitto, R., Scogings, P.F. & Rooke, T. (2011) Differential phenolic profiles in six African savanna woody species in relation to antiherbivore defense. *Phytochemistry,* **72,** 1796-1803.

Hossaert-McKey, M., Soler, C., Schatz, B. & Proffit, M. (2010) Floral scents: their roles in nursery pollination mutualisms. *Chemoecology,* **20,** 75-88.

Howe, H.F. & Smallwood, J. (1982) Ecology of seed dispersal. *Annual Review of Ecology and Systematics,* **13,** 201-228.

Chen, C., Song, Q., Proffit, M., Bessière, J.M., Li, Z. & Hossaert-McKey, M. (2009) Private channel: a single unusual compound assures specific pollinator attraction in Ficus semicordata. *Functional Ecology,* **23,** 941-950.

Janz, N. (2011) Ehrlich and Raven Revisited: Mechanisms Underlying Codiversification of Plants and Enemies. *Annual Review of Ecology, Evolution, and Systematics*, pp. 71-89.

Janz, N. & Nylin, S. (1998) Butterflies and plants: A phylogenetic study. *Evolution,* **52,** 486-502.

Janz, N., Nylin, S. & Wahlberg, N. (2006) Diversity begets diversity: host expansions and the diversification of plant-feeding insects. *Bmc Evolutionary Biology,* **6**.

Janzen, D.H. (1980) When is it coevolution? *Evolution,* **34,** 611-612.

Jevanandam, N., Goh, A.G. & Corlett, R.T. (2013) Climate warming and the potential extinction of fig wasps, the obligate pollinators of figs. *Biology letters,* **9,** 20130041.

Kliebenstein, D.J. & Osbourn, A. (2012) Making new molecules - evolution of pathways for novel metabolites in plants. *Current Opinion in Plant Biology,* **15,** 415-423.

Koricheva, J., Nykanen, H. & Gianoli, E. (2004) Meta-analysis of trade-offs among plant antiherbivore defenses: Are plants jacks-of-all-trades, masters of all? *American Naturalist,* **163,** 64-75.

Kursar, T.A., Dexter, K.G., Lokvam, J., Pennington, R.T., Richardson, J.E., Weber, M.G., Murakami, E.T., Drake, C., McGregor, R. & Coley, P.D. (2009) The evolution of antiherbivore defenses and their contribution to species coexistence in the tropical tree genus Inga. *Proceedings of the National Academy of Sciences,* **106,** 18073-18078.

Labandeira, C. (2007) The origin of herbivory on land: initial patterns of plant tissue consumption by arthropods. *Insect Science,* **14,** 259-275.

Labandeira, C.C. (2013) A paleobiologic perspective on plant–insect interactions. *Current Opinion in Plant Biology,* **16,** 414-421.

Liljebland, J. & Ronquist, F. (1998) A phylogenetic analysis of higher-level gall wasp relationships (Hymenoptera: Cynipidae). *Systematic Entomology,* **23,** 229-252.

Magallon, S.A. & Sanderson, M.J. (2005) Angiosperm divergence times: The effect of genes, codon positions, and time constraints. *Evolution,* **59,** 1653-1670.

Mitter, C., Farrell, B. & Wiegmann, B. (1988) The Phylogenetic Study Of Adaptive Zones - Has Phytophagy Promoted Insect Diversification. *American Naturalist,* **132,** 107-128.

Nason, J.D., Herre, E.A. & Hamrick, J.L. (1996) Paternity analysis of the breeding structure of strangler fig populations: evidence for substantial long-distance wasp dispersal. *Journal of Biogeography,* **23,** 501-512.

Nishida, R. (1994) Sequestration of plant secondary compounds by butterflies and moths. *Chemoecology,* **5,** 127-138.

Novotny, V., Basset, Y., Miller, S.E., Weiblen, G.D., Bremer, B., Cizek, L. & Drozd, P. (2002) Low host specificity of herbivorous insects in a tropical forest. *Nature,* **416,** 841-844.

Novotny, V., Drozd, P., Miller, S.E., Kulfan, M., Janda, M., Basset, Y. & Weiblen, G.D. (2006) Why are there so many species of herbivorous insects in tropical rainforests? *Science,* **313,** 1115-1118.

Novotny, V., Miller, S.E., Baje, L., Balagawi, S., Basset, Y., Cizek, L., Craft, K.J., Dem, F., Drew, R.A.I., Hulcr, J., Leps, J., Lewis, O.T., Pokon, R., Stewart, A.J.A., Samuelson, G.A. & Weiblen, G.D. (2010) Guild-specific patterns of species richness and host specialization in plant-herbivore food webs from a tropical forest. *Journal of Animal Ecology,* **79,** 1193-1203.

Novotny, V., Miller, S.E., Basset, Y., Cizek, L., Darrow, K., Kaupa, B., Kua, J. & Weiblen, G.D. (2005) An altitudinal comparison of caterpillar (Lepidoptera) assemblages on Ficus trees in Papua New Guinea. *Journal Of Biogeography,* **32,** 1303-1314.

Novotny, V., Miller, S.E., Leps, J., Basset, Y., Bito, D., Janda, M., Hulcr, J., Damas, K. & Weiblen, G.D. (2004) No tree an island: the plant–caterpillar food web of a secondary rain forest in New Guinea. *Ecology Letters,* **7,** 1090-1100.

Novotny, V. & Wilson, M.R. (1997) Why are there no small species among xylem-sucking insects? *Evolutionary Ecology,* **11,** 419-437.

Pasteels, J.M., Rowell-Rahier, M., Braekman, J.C. & Dupont, A. (1983) Salicin from host plant as precursor of salicylaldehyde in defensive secretion of Chrysomeline larvae. *Physiological Entomology,* **8,** 307-314.

Pearse, I.S. & Hipp, A.L. (2012) Global patterns of leaf defenses in oak species. *Evolution,* **66,** 2272-2286.

Pellmyr, O. (1997) Pollinating seed eaters: why is active pollination so rare? *Ecology,* **78,** 1655-1660.

Pellmyr, O. (2003) Yuccas, yucca moths, and coevolution: a review. *Annals of the Missouri Botanical Garden,* 35-55.

Proffit, M., Chen, C., Soler, C., Bessière, J.M., Schatz, B. & Hossaert-McKey, M. (2009) Can chemical signals, responsible for mutualistic partner encounter, promote the specific exploitation of nursery pollination mutualisms?–The case of figs and fig wasps. *Entomologia Experimentalis et Applicata,* **131,** 46-57.

Rehr, S.S., Feeny, P.P. & Janzen, D.H. (1973) Chemical defence in central-american non-ant-acacias. *Journal of Animal Ecology,* **42,** 405-416.

Richards, L.A., Dyer, L.A., Smilanich, A.M. & Dodson, C.D. (2010) Synergistic effects of amides from two Piper species on generalist and specialist herbivores. *Journal of Chemical Ecology,* **36,** 1105-1113.

Roslin, T. & Salminen, J.P. (2008) Specialization pays off: contrasting effects of two types of tannins on oak specialist and generalist moth species. *Oikos,* **117,** 1560-1568.

Rowell-Rahier, M. & Pasteels, J.M. (1986) Economics of chemical defense in Chrysomelinae. *Journal of Chemical Ecology,* **12,** 1189-1203.

Sampedro, L., Moreira, X. & Zas, R. (2011) Costs of constitutive and herbivore-induced chemical defences in pine trees emerge only under low nutrient availability. *Journal of Ecology,* **99,** 818-827.

Shanahan, M., So, S., Compton, S.G. & Corlett, R. (2001) Fig-eating by vertebrate frugivores: a global review. *Biological Reviews of the Cambridge Philosophical Society,* **76,** 529-572.

Schoonhoven, L.M., van Loon, J.J.A. & Dicke, M. (2005) *Insect-plant biology*. Oxford University Press, New York.

Soetens, P., Rowell-Rahier, M. & Pasteels, J.M. (1991) Influence of phenolglucosides and trichome density on the distribution of insects herbivores on willows. *Entomologia Experimentalis et Applicata,* **59,** 175-187.

Steemans, P., Le Herisse, A., Melvin, J., Miller, M.A., Paris, F., Verniers, J. & Wellman, C.H. (2009) Origin and radiation of the earliest vascular land plants. *Science,* **324,** 353-353.

Thompson, J.N. (1994) *The Coevolutionary Process*. University of Chicago Press, Chicago.

Treutter, D. (2006) Significance of flavonoids in plant resistance: a review. *Environmental Chemistry Letters,* **4,** 147-157.

Vane-Wright, D. (2004) Entomology - Butterflies at that awkward age. *Nature,* **428,** 477-+.

Vermeij, G.J. (1994) The evolutionary interaction among species - selection, escalation, and coevolution. *Annual Review of Ecology and Systematics,* **25,** 219-236.

Volf, M., Hrcek, J., Julkunen-Tiitto, R. & Novotny, V. (2015a) To each its own: differential response of specialist and generalist herbivores to plant defence in willows. *Journal of Animal Ecology,* **84,** 1123-1132.

Volf, M., Julkunen-Tiitto, R., Hrcek, J. & Novotny, V. (2015b) Insect herbivores drive the loss of unique chemical defense in willows. *Entomologia Experimentalis et Applicata,* **156,** 88-98.

Wahlberg, N. (2001) The phylogenetics and biochemistry of host-plant specialization in Melitaeine butterflies (Lepidoptera : Nymphalidae). *Evolution,* **55,** 522-537.

Weiblen, G.D. (2001) Phylogenetic relationships of fig wasps pollinating functionally dioecious Ficus based on mitochondrial DNA sequences and morphology. *Systematic Biology,* **50,** 243-267.

Wiegmann, B.M., Regier, J.C. & Mitter, C. (2002) Combined molecular and morphological evidence on the phylogeny of the earliest lepidopteran lineages. *Zoologica Scripta,* **31,** 67-81.

Wink, M. (2003) Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry,* **64,** 3-19.

Winkler, I.S. & Mitter, C. (2008) The phylogenetic dimension of insect-plant interactions: a review of recent evidence. *Specialization, Speciation, And Radiation: The Evolutionary Biology of Herbivorous Insects*, pp. 240-263.

Chapter I

To each its own: differential response of specialist and generalist herbivores to plant defence in willows

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Summary

1. Plant–insect food webs tend to be dominated by interactions resulting from diffuse co-evolution between plants and multiple lineages of herbivores rather than by reciprocal co-evolution and co-cladogenesis. Plants therefore require defence strategies effective against a broad range of herbivore species. In one extreme, plants could develop a single universal defence effective against all herbivorous insects, or tailor-made strategies for each herbivore species. The evolution and ecology of plant defence has to be studied with entire insect assemblages, rather than small subsets of pairwise interactions.

2. The present study examines whether specialists and generalists in three coexisting insect lineages, forming the leaf-chewing guild, respond uniformly to plant phylogeny, secondary metabolites, nutrient content and mechanical antiherbivore defences of their hosts, thus permitting universal plant defence strategies against specialized and generalist folivorous insects from various taxa.

3. The extensive data on folivorous assemblages comprising three insect orders and 193 species are linked with plant phylogeny, secondary chemistry (salicylates, flavonoids and tannins), leaf morphological traits [specific leaf area (SLA) and trichome coverage], nutrient (C : N) content and growth form of eight willow (*Salix*) and one aspen (*Populus*) species growing in sympatry.

4. Generalists responded to overall host plant chemistry and trichomes, whilst specialists responded to host plant phylogeny and secondary metabolites that are unique to willows and that are capable of being utilized as an antipredator protection. We did not find any significant impact of other plant traits, that is SLA, C : N ratio, flavonoids, tannins and growth form, on the composition of leaf-chewing communities.

5. Our results show that the response to plant traits is differential among specialists and generalists. This finding constrains the ability of plants to develop defensive traits universally effective against herbivores and may lead to diversification of plant defensive mechanisms into several complementary syndromes, required for effective protection against generalists and specialists from multiple insect taxa comprising most leaf-chewing assemblages. These results point to the necessity of broad studies of plant–herbivore interactions, across multiple insect taxa and guilds.

Key-words: community structure, defensive traits, herbivory, leaf-chewing guild, life history, salicylates, *Salix*, specialization

Introduction

Ehrlich & Raven (1964) suggested that herbivorous insects and their host plants co-evolve, leading to the genesis of

novel plant defences followed by the origin of specialized herbivores able to overcome the enhanced protection. Although there are some well-studied examples showing tight co-evolution and co-cladogenesis (Farrell & Mitter 1990; Cruaud *et al.* 2012), the majority of plant–insect interactions result from diffuse co-evolution between

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plants and insect assemblages (Janz 2011) where host switches are common even in the systems with high consumer specialization (Wilson *et al.* 2012). As a result, host plant defensive traits tend to be better predictors of insect community composition than host phylogeny per se (Becerra 1997), although plant traits governing insect food choice often differ among herbivores (Koricheva, Nykänen & Gianoli 2004).

Herbivores with different levels of specialization are frequently affected by different plant characteristics (Ali & Agrawal 2012). Whereas unspecialized insects are often excluded from plants defended by unique or highly toxic secondary metabolites (Becerra 1997; Agrawal 2005), insect specialists have repeatedly evolved mechanisms to overcome toxic or deterrent effects of such defences (Denno, Larsson & Olmstead 1990; Treutter 2006). Physical defensive traits, such as trichomes or leaf toughness, may be more effective against specialists as they reduce feeding efficiency (Dimarco, Nice & Fordyce 2012). Plant defences affecting generalists tend to increase mortality, whereas those affecting specialists prolong time needed for feeding, increasing the mortality indirectly through enhanced risk of predation or parasitism (Richards *et al.* 2010). The importance of nitrogen content also differs between specialist and generalist herbivores. In specialized insects that are able to cope with toxins, nitrogen is often an important determinant of host preference as its high content may help to overcome the impact of traits lowering feeding efficiency. In contrast, generalist insects may not be able to fully overcome toxic host chemistry and are thus prevented from benefiting from high nitrogen content (Coley, Bateman & Kursar 2006).

The effectiveness of defensive traits against specialist and generalist herbivores, which often use different mechanisms to overcome plant defence, in combination with plant tolerance to herbivore damage may shape host plant defensive patterns (Ali & Agrawal 2012). For instance, the defence of individual *Piper* species appears to be dependent on either secondary metabolites, physical traits or protection by ants (Fincher *et al.* 2008). Such a strategy can be effective only if various herbivore groups, such as specialists and generalists, respond uniformly to a particular plant defence. In contrast, defensive traits were uncorrelated or correlated positively among *Asclepias* species (Agrawal & Fishbein 2006), suggesting that effective protection can be maintained by a set of defensive traits forming a complex defence against multiple herbivores with different levels of specialization (Koricheva, Nykänen & Gianoli 2004).

A variable impact of particular defensive traits on specialist and generalist herbivores has been amply demonstrated (e.g. Richards *et al.* 2010; Reudler *et al.* 2011). However, it is important to quantify this impact at the level of the entire herbivore assemblage, as insect–plant food webs tend to be dominated by interactions resulting from diffuse co-evolution rather than from pairwise co-evolution with single plant and herbivore species

(Janzen 1980; Futuyma 2000). There is an increasing body of literature focused on explaining host plant defences, but most studies relate these defences to a single herbivore species or lineage, representing a small subset of herbivore assemblages, or study only herbivory damage, lacking insect data completely (Becerra 1997; Agrawal, Lajeunesse & Fishbein 2008; Pearse & Hipp 2012; Schuldt *et al.* 2012; Peñuelas *et al.* 2013). Although studying model insect species and their impact on host plants provides valuable information on pairwise insect–plant interactions, it may be hard to apply these findings to species-rich communities dominated by diffuse interactions. To our knowledge, there is only one study analysing responses to defensive traits by a diverse insect assemblage within a phylogenetic context (Lavandero *et al.* 2009), but this study used regional host plant data collated from the literature rather than species locally coexisting on each plant species.

Salix, one of the few species-rich genera of woody plants in Europe, is an excellent model for the analysis of defensive traits and their impact on herbivorous assemblages. Willows are widely distributed trees and shrubs with diverse defensive mechanisms, hosting rich communities of herbivorous insects consisting of species from several lineages with different levels of specialization. Leaves of willows are defended by trichomes and common secondary metabolites, such as flavonoids and condensed tannins, but also by salicylates. Salicylates are phenolic glycosides that are characteristic of, and to a large extent unique to, the Salicaceae family (Julkunen-Tiitto 1989). Salicylates function mainly as a defence against herbivores. They have been found to be effective against unspecialized herbivores, deterring them from feeding and increasing larval mortality (Matsuki & Maclean 1994; Kolehmainen *et al.* 1995). Despite this defence, willows harbour numerous insect species ranging from generalists to specialists, including well-adapted herbivores that can even use salicylates as a source of energy or protection against invertebrate predators (Rowell-Rahier & Pasteels 1986; Denno, Larsson & Olmstead 1990).

Using species-rich communities of herbivores associated with *Salix* hosts, we analyse the effect of phylogenetic distance and plant traits on willow specialists and generalists from a leaf-chewing guild. We test whether assemblages of specialists and generalists from three coexisting insect lineages respond uniformly to plant phylogeny, secondary metabolites, mechanical antiherbivore defences, nutrient content and growth form of their hosts, thus permitting universal plant defence strategies against various folivorous insects with different levels of specialization and life history.

Material and methods

HOST PLANTS AND STUDY SITES

Willows are usually divided into species defended mainly quantitatively by tannins and those defended qualitatively by salicylates

(Julkunen-Tiitto 1989). The eight species of trees and shrubs from the genus *Salix* (*S. aurita, S. caprea, S. cinerea, S. fragilis, S. pentandra, S. purpurea, S. rosmarinifolia* and *S. viminalis*) studied here were selected to represent both of these groups. Further, *Populus tremula* was studied as an outgroup (Table 1).

Our study was carried out within a 10 × 10 km area comprising lowland wetlands and wet meadows, situated in South Bohemia, Czech Republic (48°51′58″–48°59′45″N, 14°26′20″–14°35′48″E). All host plants studied represented mature trees and mature shrubs. Shaded plants were excluded since their life-history traits (including leaf chemistry) could be significantly different from plants exposed to sunlight. We also avoided plants which had obviously experienced browsing by herbivores or damage from other sources prior to the sampling as these factors can cause significant changes in plant traits (Nakamura *et al.* 2005; Ohgushi 2005). All host plant traits (described below) were measured for two to seven plant individuals per species (a total of 44 plants), and means were used to characterize each species (Table 1).

PHYSICAL TRAITS

We measured trichome density and specific leaf area (SLA), a surrogate for leaf thickness and toughness (Groom & Lamont 1999), as measures of leaf morphology with a possible impact on leaf-chewing insects. Leaves from the central part of shoot were used for the measurement, avoiding apical leaves. Trichome density was estimated as the average percentage of surface area (0·5 cm square) covered by trichomes for mature leaves; values for dorsal and ventral sides were averaged. SLA was calculated as the area per unit mass of a dried leaf disc of known diameter. Leaf discs were cut, avoiding the central vein, and air-dried to constant weight. Three leaf discs were obtained every 2 weeks throughout the 2010 vegetation season (10 sampling occasions) for each of the 44 plant individuals examined. The obtained values were used to estimate the mean SLA.

CHEMICAL ANALYSIS

For chemical analyses, samples of leaf lamina were cut (avoiding primary and secondary leaf veins) and air-dried at room temperature immediately after collection. We used the central parts of the leaf blade, avoiding both apical and basal part. Total carbon and nitrogen content was determined by dry combustion with a Carlo Erba NC 2500 element analyser (Carlo Erba, Milano, Italy) using 30 mg of dried and homogenized leaf material. Tissue sampling was repeated six times throughout the 2010 vegetation season. For all nine study species, we sampled three individuals to estimate total N and C content over the entire vegetation season. The obtained C and N contents were used to estimate the mean C : N ratio.

The contents of salicylates, flavonoids and condensed tannins were analysed from 5 to 9 mg samples from leaves sampled at the beginning of June when insect density in the study area reaches its peak. Phenolic compounds were extracted with methanol as described in Nybakken, Horkka & Julkunen-Tiitto (2012). Extracts were dried and kept in a −20°C freezer. Before the analysis, dried samples were redissolved in 600 μl of 1 : 1 methanol–water solution. We used 20 μl of redissolved samples for HPLC analysis of salicylates and flavonoids following Nybakken, Horkka & Julkunen-Tiitto (2012). Compounds were separated using a Zorbax SBC18 (4·6 × 60 mm) HPLC column (Agilent Technologies, Santa Clara, CA, USA) employing a water/methanol gradient (Julkunen-Tiitto & Sorsa 2001). Salicylate and flavonoid contents were measured based on the absorbance at 270 nm and 320 nm, respectively. Retention times and spectra compared to standards were used to identify the compounds.

Soluble condensed tannins were determined by the acid–butanol assay according to the method of Hagerman (2002) from an aliquot of the HPLC sample and insoluble condensed tannins from room-dried tissue residues. After hydrolyses, absorbance values at 550 nm were measured (Spectronic 20 Genesys TM spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). The condensed tannin content was calculated based on a calibration curve obtained for *S. purpurea* leaf tannins.

HOST PLANT PHYLOGENY RECONSTRUCTION

We chose four loci that are usually variable at genus level for host plant phylogeny reconstruction: matK, ITS, trnT-trnL and ADH. We used standard procedures, reaction conditions and primer sequences for DNA extraction and PCR amplification, which were the same as those used in the original studies employing these markers (White *et al.* 1990; Taberlet *et al.* 1991; Cronn *et al.* 2002; Selosse, Bauer & Moyersoen 2002; Savage & Cavender-Bares 2012). Since multiple copies of ADH were present in each individual except *S. viminalis*, the ADH PCR products were cloned to separate potential paralogs and hybrid sequences. Some *S. cinerea* and *S. fragilis* individuals exhibited a hybrid origin for some of their ADH sequences. This trend was pronounced in

Table 1. Host plant traits for the tree species studied. The means from individual plants ± standard deviation are reported. Numbers in brackets show number of trees measured for defensive traits per species

	Growth form	Specific leaf area (cm² g⁻¹)	Trichome cover (%)	Flavonoids (mg g⁻¹)	Salicylates (mg g⁻¹)	Tannins (mg g⁻¹)	Carbon (mg g⁻¹)	Nitrogen (mg g⁻¹)
Salix aurita (4)	Shrub	144·8 ± 27·0	19 ± 3·0	29·5 ± 4·4	0·0	194·8 ± 44·4	45·4 ± 1·1	3·05 ± 0·66
Salix caprea (6)	Tree	146·3 ± 31·8	26 ± 3·5	10·6 ± 2·2	0·0	138·8 ± 43·0	43·8 ± 1·4	2·51 ± 0·62
Salix cinerea (7)	Shrub	131·5 ± 38·9	21 ± 2·0	15·0 ± 2·5	0·0	159·1 ± 61·3	45·6 ± 1·9	2·93 ± 0·58
Salix fragilis (6)	Tree	134·8 ± 32·7	0	25·5 ± 7·2	27·8 ± 10·0	51·9 ± 33·2	44·0 ± 1·0	2·98 ± 0·60
Salix pentandra (3)	Tree	118·5 ± 39·6	0	60·6 ± 10·5	41·8 ± 20·3	190·7 ± 34·3	45·2 ± 2·0	2·56 ± 0·75
Salix purpurea (5)	Shrub	141·2 ± 39·8	0	21·3 ± 2·6	164·8 ± 36·5	42·7 ± 56·5	44·1 ± 0·8	2·74 ± 0·89
Salix rosmarinifolia (2)	Shrub	125·3 ± 29·9	14 ± 4·0	20·9 ± 1·2	169·0 ± 32·2	133·4 ± 82·1	45·5 ± 0·6	2·47 ± 0·91
Salix viminalis (4)	Shrub	165·8 ± 29·8	36 ± 7·3	16·0 ± 3·4	0·0	137·4 ± 35·6	46·0 ± 3·6	3·57 ± 0·62
Populus tremula (5)	Tree	144·5 ± 76·9	0	33·8 ± 8·3	19·4 ± 14·9	38·2 ± 35·6	43·3 ± 2·8	2·51 ± 0·70

individuals sharing a site with their sibling species. Their sequences therefore did not form monophyletic lineages and the position of a proportion of the sequences was reconstructed with high support as an internal group within the sibling species. Since willow species are known to frequently hybridize with their sibling species (Skvortsov 1968), these sequences were considered of hybrid origin and removed from analysis.

Sequences were assembled and edited using GENEIOUS 5.4 (Drummond *et al.* 2011). Trees for individual genes were not in conflict, allowing us to reconstruct the host plant phylogeny based on a matrix with all four loci combined. Host plant phylogeny was reconstructed using Bayesian inference in MRBAYES 3.1.2 (Ronquist & Huelsenbeck 2003). A GTR substitution model selected using Akaike Information Criterion (AIC) was used for Bayesian analysis with a flat Dirichlet prior probability density for the distribution of substitution rates and stationary nucleotide frequencies. Sampling was carried out every 10^3 generations for 10^7 generations. The first 25% of generations were discarded as burn-in, and the results were summarized with a 50% majority-rule consensus tree.

INSECT SAMPLING

We focused on externally feeding and semi-concealed leaf-chewing insects as this guild often inflicts the most damage among insect herbivores (Schoonhoven, van Loon & Dicke 2005). This guild also includes various distantly related insect lineages (e.g. Coleoptera, Lepidoptera and Hymenoptera). All leaf-chewing insects were sampled during the 2008–2011 vegetation seasons (April–September) at 1-week intervals by sweeping the foliage and by manual searching, and our samples also included leaf-tying and leaf-rolling herbivores. We kept the time of sampling events constant, with 3 min of sweeping and 3 min of manual searching per inspection. Due to variation in willow population densities, the sampling effort was not completely balanced. For most tree species, the total sampling effort was 200–400 min; however, for *S. pentandra*, which is locally rare, total sampling effort was only 100 min. Insect larvae were reared to adults for identification. Dead larvae were morphotyped based on photographs or discarded when morphotyping proved impossible.

STATISTICAL ANALYSES

Insect abundance was standardized as the number of insects obtained per sampling time (in minutes). Singletons and doubletons were excluded from analyses. The number of host plants used by individual insect species was analysed by ANOVA to compare host specificity among insect lineages. We compared the host plant specificity of the examined herbivores by ANOVA using Kullback–Leibler distances to remove the bias due to total observation frequencies (Blüthgen, Menzel & Blüthgen 2006). The impact of chemical composition and host plant phylogeny on insect communities was quantified by two analyses – a Mantel test and a multivariate analysis. We used a Mantel test to examine how insect community similarity reflects willow phylogeny and secondary metabolite dissimilarity (representing overall willow chemical defence) and multivariate ordination to analyse the role of individual plant traits in forming insect communities.

The similarity of herbivore communities between willow species employed in the Mantel test and partial Mantel test (with phylogenetic distance used as covariate) was estimated using the Bray–Curtis index, computed in ESTIMATES 8.2 (Colwell 2006). A chemical dissimilarity matrix was obtained using UPGMA with Euclidean distances based on log-transformed mean concentrations of individual salicylates and flavonoids, and log-transformed total concentration of condensed tannins. For visualization, the similarities of host plant chemistry and insect communities were also analysed by PCA employing the same data. The phylogenetic distances (in substitutions per base) between host plants were based on the mean branch lengths derived from the Bayesian phylogeny. We chose to use a one-sided Mantel test, as we expected that both chemically similar host plants and closely related host plants will support similar insect communities. Following the whole community analysis, separate similarity matrices for Coleoptera, Lepidoptera, Hymenoptera, generalist herbivores and Salicaceae specialists (i.e. species feeding only on Salicaceae; based on Smreczyński 1966, 1972, 1974; Lacourt 1999; Warchalowski 2003; Macek *et al.* 2007; Kopelke 2007a,b; Macek *et al.* 2008; Macek, Prochazka & Traxler 2012) were computed and analysed as above.

The effect of individual defensive traits on insect communities was analysed using multivariate ordination analyses conducted in CANOCO for Windows 4.56 (ter Braak & Smilauer 2002). The impact of total salicylate, flavonoid and condensed tannin concentrations, trichomes, SLA, C : N ratio and plant growth form on herbivore community composition harboured by different host plant species was analysed by redundancy analysis (RDA) with host plant phylogeny used as a covariate. Phylogenetic distances between plant species were transformed from the ultrametric tree into coordinates using PCoA. Since the values of host plant traits were not available for all plant individuals from which insects were sampled or for all periods of the season, we used mean values for plant species as individual data points. In this analysis, the insect data obtained from conspecific plant individuals were combined into one data point and variables best explaining their variability were selected by forward selection under 9999 permutations.

Results

HOST PLANT TRAITS AND PHYLOGENY

Host plant phylogenetic distance and chemical dissimilarity were not correlated ($r = 0.15$, d.f. = 43, $P = 0.334$) (Fig. S1, Supporting information). The plant species studied here differed widely with respect to their morphological and chemical traits (Tables 1 and S1, Supporting information). In particular, large differences were found in trichome density and salicylate content and composition. Most of the salicylate and flavonoid compounds found were unique to a single plant species (Fig. S2, Supporting information), which resulted in a high level of reconstructed variability in chemical composition. PCA analysis revealed four distinctive groups of willows with assorted chemical profiles: (I) *S. ix purpurea* and *S. rosmarinifolia* with very high salicylate content; (II) *S. aurita*, *S. caprea*, *S. cinerea* and *S. viminalis* containing no salicylates; (III) *S. fragilis* and *P. tremula* with moderate salicylate content; and (IV) *S. pentandra* with chemical profile distinctively different from other studied host plants (Fig. 1b).
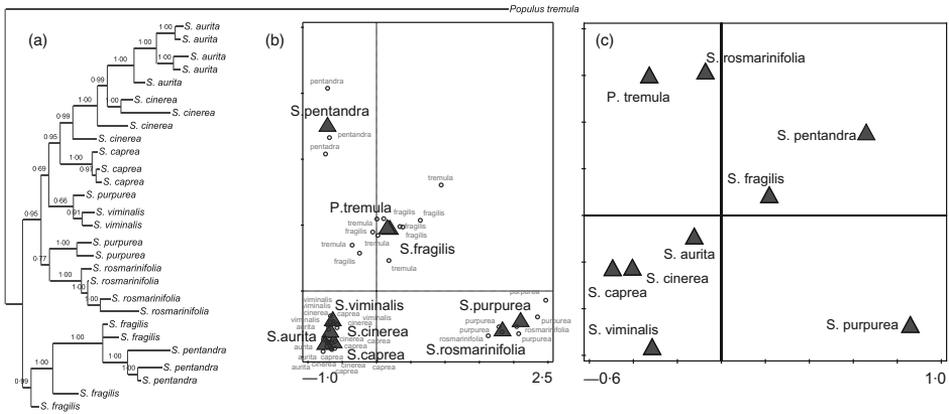
Fig. 1. Host plant phylogeny as reconstructed by Bayesian inference (a), chemical similarity (b) and insect community similarity (c) as reconstructed by PCA. The support of clades in reconstructed phylogeny is characterized by posterior probabilities. Host plant species are marked by triangles in PCA diagrams. Tree individuals included in the PCA diagram of chemical dissimilarity are marked by dots and labelled by species names.

Willow sequences were rather conservative, with a low proportion of informative sites. The proportion of informative sites in matK was extremely low, and we excluded this marker from further analyses. ADH was the most informative marker, although its use was limited by the presence of hybrid sequences requiring cloning. Bayesian inference provided a topology supporting the traditional willow taxonomy and suggesting monophyly of both examined willow subgenera, *Salix* and *Vetrix* (Skvortsov 1968) (Fig. 1a). However, support for some of the clades was quite low. The most ambiguous grouping is that of *S. viminalis* as it is often reconstructed as a sister species to *S. caprea, S. cinerea* and *S. autita* group or forms a monophyletic group with *S. purpurea* and *S. rosmarinifolia*.

INSECT COMMUNITIES

We collected 7786 individuals of leaf-chewing insects from 192 species, representing three insect orders – Coleoptera, Hymenoptera and Lepidoptera. Salicaceae specialists included 28 Coleoptera, 49 Hymenoptera and 29 Lepidoptera species. Generalists included 30 Coleoptera, 4 Hymenoptera and 52 Lepidoptera species (Table S2, Supporting information). We found significant difference in host breadth with examined orders ($F_{2,109} = 8.46$, $P < 0.001$) with Hymenoptera being the most host specific and Coleoptera and Lepidoptera being moderately and the least host specific, respectively. Hymenoptera also included the highest proportion of Salicaceae specialists, that is species feeding only on Salicaceae (96%), whereas the specialist proportion was moderate in Coleoptera (49%) and lowest in Lepidoptera (32%). The PCA analysis pointed to rela-

tively smaller variability in herbivorous insect community structure on low salicylate willows compared to large interspecific variability in herbivore community structure on high salicylate willows (Fig. 1c).

IMPACT OF HOST PLANT TRAITS ON INSECT COMMUNITIES

Both total chemical dissimilarity ($r = 0.37$, d.f. = 43, $P = 0.027$) and phylogenetic distance ($r = 0.38$, d.f. = 43, $P = 0.028$) exhibited a significant impact on insect community similarity (Table 2). When specialists and generalists were analysed separately, generalist community structure was significantly affected by chemical dissimilarity, whereas specialist community structure was affected by phylogenetic distance (Table 2, Fig. 2).

Redundancy analysis revealed significant impact of host plant phylogeny on community structure of leaf-chewing herbivores on willows ($F_1 = 1.3$, $P = 0.044$). However, when other plant traits were added into analysis, phylogeny had no effect on insect communities ($F_3 = 1.3$, $P = 0.178$) as chemical and physical plant traits explained insect community structure better. RDA analysing the impact of individual physical and chemical plant traits, including host plant phylogeny as a covariate, showed significant effects of total salicylate content and trichome cover on herbivore communities (Fig. 3, Table 3). Salicylates exhibited a significant effect on the whole leaf-chewer communities ($F_1 = 1.5$, $P = 0.031$) and Salicaceae specialists ($F_1 = 1.5$, $P = 0.043$). Some Salicaceae specialists exhibited a strong positive response to salicylates, whereas the majority of specialists showed a weak negative or nearly no response to these secondary metabolites

(Fig. S3, Supporting information). Trichomes played a significant role in structuring assemblages of generalists ($F_1 = 1.80$, $P = 0.035$).

In the analysis comparing the response of insect orders to defensive traits, Coleoptera responded to host plant chemical dissimilarity and salicylate content and Hyme-noptera to trichomes (Tables S3 and S4, Supporting information).

Discussion

In this study, we analysed whether assemblages of specialists and generalists from three coexisting insect orders respond uniformly to host plant traits. Our results suggest

Table 2. Impact of host plant chemical dissimilarity and phylogenetic distance on insect community similarity (based on Bray–Curtis index). The results of one-sided Mantel tests and partial Mantel tests (with phylogenetic distance used as covariate) are shown for whole leaf-chewing insect communities, Salicaceae specialists and generalist herbivores

	P/r		
	Whole community	Specialists	Generalists
Phylogenetic distance	**0·028/0·38**	**0·023/0·45**	0·103/0·24
Chemical dissimilarity	**0·027/0·38**	0·078/0·23	**0·004/0·54**
Chemical diss. (par. Mantel)	**0·049/0·34**	0·107/0·18	**0·007/0·52**

Significant ($P < 0.05$) results are highlighted in bold, d.f. = 43 for Mantel tests and d.f. = 42 for partial Mantel tests.

Table 3. Impact of host plant traits on insect community structure analysed by RDA. The results are shown for whole leaf-chewing insect communities, Salicaceae specialists and generalist herbivores

	P/F		
	Whole community	Specialists	Generalists
Salicylates	**0·032**/1·50	**0·040**/1·27	0·059/1·37
Flavonoids	0·523/0·96	0·517/0·90	0·497/1·01
Tannins	0·305/1·16	0·265/1·16	0·406/1·08
Trichomes	0·224/1·18	0·367/1·11	**0·035**/1·80
Specific leaf area	0·489/0·98	0·498/0·98	0·429/1·07
C : N	0·507/0·98	0·500/0·99	0·455/1·02
Growth form	0·180/1·31	0·501/1·00	0·332/1·30

Significant results are in bold.
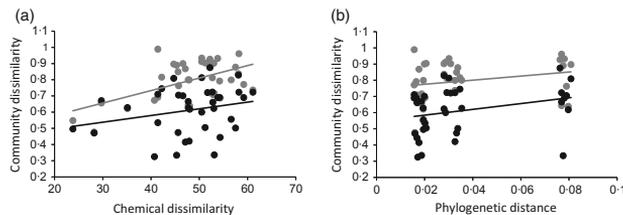
Fig. 2. Impact of host plant chemical similarity (a) and phylogenetic distance (b) on similarity of specialist (black) and generalist (grey) communities on Salicaceae. Community similarity is based on Bray–Curtis index. Chemical dissimilarity exhibited a significant impact on generalists, whereas phylogenetic distance affected communities of specialists when analysed by partial Mantel test ($r = 0.52$, $P = 0.007$ and $r = 0.45$, $P = 0.023$, respectively).
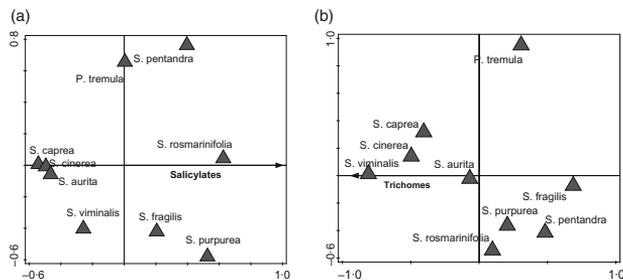
Fig. 3. RDA ordination diagram of specialist and generalist community responses to host plant traits. Host plant phylogeny was used as a covariate. Whereas specialists (a) responded to salicylates ($F_1 = 1.5$, $P = 0.043$), generalists (b) rather responded to trichomes ($F_1 = 1.80$, $P = 0.035$). Host plant species are marked by triangles.

that specialists and generalists exhibit a specific response to host plant traits, which may play a major role in forming host plant defences.

There were major differences in the importance of host plant phylogeny for community structure of examined insect lineages. These differences appeared to be linked to the level of specialization of respective herbivores. Host plant phylogeny had a significant effect on the specialist component of the leaf-chewing community, that is those species feeding exclusively on Salicaceae. However, although some lineages of insect specialists are known to have diversified on Salicaceae, their phylogenetic conservatism probably does not result from co-speciation. For instance, *Chrysomela* leaf beetles, ancestrally associated with Salicaceae, show multiple reversals back to Salicaceae from other hosts, suggesting there is a higher chance that host shifts will occur to plants that had been used by the lineage in the past than shifts to novel host plants (Termonia *et al.* 2001). This pattern of host shifting back and forth between the same plant lineages may result in phylogenetic conservatism, as was observed here (Janz, Nyblom & Nylin 2001). However, correlations between plant phylogeny and insect community structure may be observed only in specialist lineages which do not include numerous monophages since such species cannot contribute to the relationships between plant phylogenetic distance and community similarity, as demonstrated in this study by the absence of significant effects of plant phylogeny on Hymenoptera in this study. The congruence of host plant phylogeny and insect community structure is also frequently an outcome of conservative evolution of plant traits important for insect host preference (Becerra 1997). Although sometimes conserved on higher phylogenetic level, these traits are often variable within plant genera (Julkunen-Tiitto 1989; Fincher *et al.* 2008). Host plant phylogeny is thus expected to play a minor role as a determinant of community assembly of herbivores on large plant genera, particularly for generalists that respond predominately to host plant defences.

Previous studies showed that secondary metabolite profile may differ among willow genotypes (Hochwender & Fritz 2004). Nevertheless, this variability among willow genotypes is generally considered to be smaller than differences among species (Nyman & Julkunen-Tiitto 2005). Although our results show that differences in secondary metabolite profile can be rather small, especially among salicylate poor species, willows examined in this study formed several chemically well-defined groups that have significant impacts on insect communities.

Specialists and generalists differed in their responses to host plant chemical profiles. Generalists were affected by total host plant chemistry, whereas specialists were affected only by secondary metabolites unique to willows. The impact of total chemical dissimilarity (based on salicylates, flavonoids and tannins) on generalists shows that willow secondary metabolites have a strong impact on less adapted groups and suggests that a degree of adaptation

is required in order to overcome chemical defences of willows. On the other hand, the lack of significant effect of individual groups of secondary metabolites revealed by RDA suggests that none of these secondary metabolite groups might be effective enough to affect generalist communities when employed alone.

Multivariate analysis highlights the importance of salicylates in host plant preference by specialists, indicated by their significant impact on specialist community structure. Although high salicylate content had a slightly negative impact on some Salicaceae specialists, certain specialists showed strong positive response to the secondary metabolites. The *Phratora* leaf beetles, which utilize salicylates and use them for protection against invertebrate predators (Rowell-Rahier & Pasteels 1986), were among the species with strongest positive response. Other specialists showing positive response to salicylates may use them as an extra source of energy which explains their faster growth on willows with high salicylate content (Matsuki & Maclean 1994). There is also a phagostimulating effect of salicylates on some specialists (Kolehmainen *et al.* 1995), which may interfere with preference for high nitrogen content of host plants, possibly explaining why the C : N ratio had no effect on specialist assemblages on willows. On the other hand, willow secondary metabolites are known to be effective against generalists by increasing their mortality (Matsuki & Maclean 1994). For plants with toxic secondary metabolites, generalist food choice is thus likely to be governed by secondary metabolite content rather than C : N ratio, as observed here. In summary, although secondary metabolites of willows influence both generalist and specialist community structure, the response of these two herbivore groups to willow chemistry differs.

Growth form and plant architecture may play an important role in forming herbivorous insect communities, perhaps related to predation and parasitism risk (Lavandero *et al.* 2009; Sipos & Kindlmann 2013). However, we did not find any significant effect of plant growth form on leaf-chewing insect communities associated with willows. Since many studies reporting significant impact of host plant architecture focused on plants with less pronounced chemical defence (Marquis, Lill & Piccinni 2002; Sipos & Kindlmann 2013), our findings may indicate a lesser role of plant architecture in structuring insect communities on chemically well-defended plants. Large interspecific differences in defensive traits may be more important for structuring insect communities in such cases. However, it would require further analysis incorporating the third trophic level to confirm this.

Some studies reported pronounced impacts of physical defences on specialists (Dimarco, Nice & Fordyce 2012), but our study indicates in contrast that trichomes affected the community structure of generalists. Moreover, some of our results suggest that insect response is not directly connected with the level of specialization as trichomes also affected Hymenoptera, which included almost only specialized insects. We suggest that life-history traits other

than specialization may be more important determinants of insect response to trichomes. Since trichomes influence mainly small insects (Agrawal 2005), body size and traits correlated with it (e.g. size of mandibles or ovipositor length) could be such factors. The second examined physical trait, SLA, had no impact on insect assemblages, probably because of low variability in this trait among examined willow species.

Differential response by herbivores to defensive traits may restrain plants from developing a universal antiherbivore defence. This may lead to defensive trait diversification. In the case of willows, specialized insects were able to adapt to salicylates and reach high densities on salicylate-rich hosts. Although salicylates play a significant role in structuring insect communities, their protective value against specialized herbivores appears to be low. Maintaining an effective defence thus probably requires several defensive mechanisms, such as chemical defence and trichomes which affect both generalists and some specialists. These findings suggest plant defensive traits to be mutually independent or positively correlated, as observed by Agrawal & Fishbein (2006) or Hattas *et al.* (2011). Trade-offs between individual defensive traits may be expected only given specific conditions, such as in low nutrient environments or in the case of negative dependence in metabolic pathways (Agrawal, Salminen & Fishbein 2009; Sampedro, Moreira & Zas 2011).

Our analysis revealed a significant response to host plant traits by assemblages of insect species sharing similar levels of specialization. With a systematic response by specialists and generalists, an effective plant defence may be based on a relatively small number of defensive traits since each defensive trait is likely to affect multiple herbivore species with similar levels of specialization. In the case of willows, a combination of physical defence and secondary metabolites probably provides good protection against a large proportion of both specialists and generalists.

It appears that defensive syndromes are not phylogenetically conserved. Related species thus often exhibit diverging strategies relying on different traits in their protection against insects (Agrawal & Fishbein 2006; Fincher *et al.* 2008). This pattern could be tentatively identified also in willows characterized by large interspecific variation in salicylate concentration and trichome density – two defensive traits with the most pronounced impact on herbivores. It may be advantageous for sibling species to use different mechanisms for their defence, as this may lower the number of host shifts by insect herbivores between related plant species. These shifts would otherwise be likely due to phylogenetic conservatism. Colonization from phylogenetically more distant host plants remains less likely, as many plant traits important for insect preference, such as plant phenology, are phylogenetically rather conservative (Davies *et al.* 2013). This suggests that herbivore pressure may lead towards divergence in defensive syndromes between closely related plant species, or

bias community assembly towards chemical heterogeneity as reported by Becerra (2007), which may help plants to escape herbivory.

In summary, our results show that the response to plant traits by herbivores differs systematically among insects with different levels of specialization. This constrains the ability of plants to develop defensive traits that are universally effective against a broad range of herbivores and may lead to diversification of plant defensive mechanisms into several complementary syndromes, required for maintaining effective protection against diverse insect communities. These findings suggest that plant defences should be considered from the perspective of diffuse impact from a broad range of insect species rather than as a result of reciprocal co-evolution with a particular insect lineage. Further, the impact of the next trophic level, predators and parasitoids, on herbivores, as well as induced plant defences, is among other important factors potentially modifying plant–herbivore interactions (Ohgushi 2005; Wilson *et al.* 2012). Studying these factors is required for comprehensive understanding of the insect–plant associations and the resulting host plant defensive patterns.

Acknowledgements

Data accessibility

Host plant chemistry and insect community data are included in the supporting information (Tables S1 and S2, Supporting information). Host plant nucleotide sequences are available from the European Nucleotide Archive: www.ebi.ac.uk/ena/data/view/LN734767-LN734821.

References

Agrawal, A.A. (2005) Natural selection on common milkweed (*Asclepias syriaca*) by a community of specialized insect herbivores. *Evolutionary Ecology Research*, 7, 651–667.

Agrawal, A.A. & Fishbein, M. (2006) Plant defense syndromes. *Ecology*, 87, S132–S149.

Agrawal, A.A., Lajeunesse, M.J. & Fishbein, M. (2008) Evolution of latex and its constituent defensive chemistry in milkweeds (*Asclepias*): a phylogenetic test of plant defense escalation. *Entomologia Experimentalis et Applicata*, 128, 126–138.

Agrawal, A.A., Salminen, J.P. & Fishbein, M. (2009) Phylogenetic trends in phenolic metabolism of milkweeds (Asclepias): evidence for escalation. *Evolution*, 63, 663–673.

Ali, J.G. & Agrawal, A.A. (2012) Specialist versus generalist insect herbivores and plant defense. *Trends in Plant Science*, 17, 293–302.

Becerra, J.X. (1997) Insects on plants: macroevolutionary chemical trends in host use. *Science*, 276, 253–256.

Becerra, J.X. (2007) The impact of herbivore–plant coevolution on plant community structure. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 7483–7488.

Blüthgen, N., Menzel, F. & Blüthgen, N. (2006) Measuring specialization in species interaction networks. *BMC Ecology*, **6**, 9.

ter Braak, C.J.F. & Smilauer, P. (2002) *CANOCO Reference Manual and CanoDraw for Windows User's Guide: Software for Canonical Community Ordination (Version 4.5)*. Biometris, Ithaca, NY.

Coley, P.D., Bateman, M.L. & Kursar, T.A. (2006) The effects of plant quality on caterpillar growth and defense against natural enemies. *Oikos*, **115**, 219–228.

Colwell, R.K. (2006) Statistical estimation of species richness and shared species from samples. Version 8. Available from http://www.purtl.oclc.org/estimates.

Cronn, R.C., Small, R.L., Haselkorn, T. & Wendel, J.F. (2002) Rapid diversification of the cotton genus (*Gossypium : Malvaceae*) revealed by analysis of sixteen nuclear and chloroplast genes. *American Journal of Botany*, **89**, 707–725.

Cruaud, A., Ronsted, N., Chantarasuwan, B., Chou, L.S., Clement, W.L., Couloux, A. *et al.* (2012) An extreme case of plant-insect codiversification: figs and fig-pollinating wasps. *Systematic Biology*, **61**, 1029–1047.

Davies, T.J., Wolkovich, E.M., Kraft, N.J., Salamin, N., Allen, J.M., Ault, T.R. *et al.* (2013) Phylogenetic conservatism in plant phenology. *Journal of Ecology*, **101**, 1520–1530.

Denno, R.F., Larsson, S. & Olmstead, K.L. (1990) Role of enemy-free space and plant quality in host-plant selection by willow beetles. *Ecology*, **71**, 124–137.

Dimarco, R.D., Nice, C.C. & Fordyce, J.A. (2012) Family matters: effect of host plant variation in chemical and mechanical defenses on a sequestering specialist herbivore. *Oecologia*, **170**, 687–695.

Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Duran, C. *et al.* (2011) Geneious v5.4.

Ehrlich, P.R. & Raven, P.H. (1964) Butterflies and plants – a study in coevolution. *Evolution*, **18**, 586–608.

Farrell, B.D. & Mitter, C. (1990) Phylogenesis of insect plant interactions – have Phyllobrotica leaf beetles (Chrysomelidae) and the Lamiales diversified in parallel. *Evolution*, **44**, 1389–1403.

Fincher, R.M., Dyer, L.A., Dodson, C.D., Richards, J.L., Tobler, M.A., Searcy, J. *et al.* (2008) Inter- and intraspecific comparisons of antiherbivore defenses in three species of rainforest understory shrubs. *Journal of Chemical Ecology*, **34**, 558–574.

Futuyma, D.J. (2000) Some current approaches to the evolution of plant herbivore interactions. *Plant Species Biology*, **15**, 1–9.

Groom, P.K. & Lamont, B.B. (1999) Which common indices of sclerophylly best reflect differences in leaf structure? *Ecoscience*, **6**, 471–474.

Hagerman, A. (2002) *The Tannin Handbook*. Miami University, Oxford OH 45056. www.users.muohio.edu/hagermae/.

Hattas, D., Hjältén, J., Julkunen-Tiitto, R., Scogings, P.F. & Rooke, T. (2011) Differential phenolic profiles in six African savanna woody species in relation to antiherbivore defense. *Phytochemistry*, **72**, 1796–1803.

Hochwender, C.G. & Fritz, R.S. (2004) Plant genetic differences influence herbivore community structure: evidence from a hybrid willow system. *Oecologia*, **138**, 547–557.

Janz, N. (2011) Ehrlich and raven revisited: mechanisms underlying codiversification of plants and enemies. *Annual Review of Ecology, Evolution, and Systematics*, **42**, 71–89.

Janz, N., Nyblom, K. & Nylin, S. (2001) Evolutionary dynamics of host–plant specialization: a case study of the tribe Nymphalini. *Evolution*, **55**, 783–796.

Janzen, D.H. (1980) When is it coevolution? *Evolution*, **34**, 611–612.

Julkunen-Tiitto, R. (1989) Phenolic constituents of *Salix* – a chemotaxonomic survey of further Finnish species. *Phytochemistry*, **28**, 2115–2125.

Julkunen-Tiitto, R. & Sorsa, S. (2001) Testing the effects of drying methods on willow flavonoids, tannins, and salicylates. *Journal of Chemical Ecology*, **27**, 779–789.

Kolehmainen, J., Julkunen-Tiitto, R., Roininen, H. & Tahvanainen, J. (1995) Phenolic glucosides as feeding cues for willow-feeding leaf beetles. *Entomologia Experimentalis et Applicata*, **74**, 235–243.

Kopelke, J.P. (2007a) The European species of the genus Phyllocolpa, part I: the leucosticta-group (Insecta, Hymenoptera, Tenthredinidae, Nematinae). *Senckenbergiana Biologica*, **87**, 75–109.

Kopelke, J.P. (2007b) The European species of the genus Phyllocolpa, part II: the leucapsis-group (Insecta, Hymenoptera, Tenthredinidae, Nematinae). *Senckenbergiana Biologica*, **87**, 149–161.

Koricheva, J., Nykänen, H. & Gianoli, E. (2004) Meta-analysis of trade-offs among plant antiherbivore defenses: are plants jacks-of-all-trades, masters of all? *The American Naturalist*, **163**, 64–75.

Lacourt, J. (1999) *Répertoire des Tenthredinidae ouest-paléarctiques (Hymenoptera, Symphyta)*. Société entomologique de France, Paris.

Lavandero, B., Labra, A., Ramirez, C.C., Niemeyer, H.M. & Fuentes-Contreras, E. (2009) Species richness of herbivorous insects on Nothofagus trees in South America and New Zealand: the importance of chemical attributes of the host. *Basic and Applied Ecology*, **10**, 10–18.

Macek, J., Prochazka, J. & Traxler, L. (2012) *Noční motýli III*. Academia, Praha.

Macek, J., Dvorak, J., Traxler, L. & Cervenka, V. (2007) *Noční motýli I*. Academia, Praha.

Macek, J., Dvorak, J., Traxler, L. & Cervenka, V. (2008) *Noční motýli II*. Academia, Praha.

Marquis, R.J., Lill, J.T. & Piccinni, A. (2002) Effect of plant architecture on colonization and damage by leaftying caterpillars of *Quercus alba*. *Oikos*, **99**, 531–537.

Matsuki, M. & Maclean, S.F. (1994) Effects of different leaf traits on growth rates of insect herbivores on willows. *Oecologia*, **100**, 141–152.

Nakamura, M., Utsumi, S., Miki, T. & Ohgushi, T. (2005) Flood initiates bottom-up cascades in a tri-trophic system: host plant regrowth increases densities of a leaf beetle and its predators. *Journal of Animal Ecology*, **74**, 683–691.

Nybakken, L., Horkka, R. & Julkunen-Tiitto, R. (2012) Combined enhancements of temperature and UVB influence growth and phenolics in clones of the sexually dimorphic *Salix myrsinifolia*. *Physiologia Plantarum*, **145**, 551–564.

Nyman, T. & Julkunen-Tiitto, R. (2005) Chemical variation within and among six northern willow species. *Phytochemistry*, **66**, 2836–2843.

Ohgushi, T. (2005) Indirect interaction webs: herbivore-induced effects through trait change in plants. *Annual Review of Ecology, Evolution, and Systematics*, **36**, 81–105.

Pearse, I.S. & Hipp, A.L. (2012) Global patterns of leaf defenses in oak species. *Evolution*, **66**, 2272–2286.

Peñuelas, J., Sardans, J., Llusia, J., Silva, J., Owen, S.M., Bala-Ola, B. *et al.* (2013) Foliar chemistry and standing folivory of early and late-successional species in a Bornean rainforest. *Plant Ecology & Diversity*, **6**, 245–256.

Reudler, J.H., Biere, A., Harvey, J.A. & Van Nouhuys, S. (2011) Differential performance of a specialist and two generalist herbivores and their parasitoids on *Plantago lanceolata*. *Journal of Chemical Ecology*, **37**, 765–778.

Richards, L.A., Dyer, L.A., Smilanich, A.M. & Dodson, C.D. (2010) Synergistic effects of amides from two *Piper* species on generalist and specialist herbivores. *Journal of Chemical Ecology*, **36**, 1105–1113.

Ronquist, F. & Huelsenbeck, J.P. (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.

Rowell-Rahier, M. & Pasteels, J.M. (1986) Economics of chemical defense in Chrysomelinae. *Journal of Chemical Ecology*, **12**, 1189–1203.

Sampedro, L., Moreira, X. & Zas, R. (2011) Costs of constitutive and herbivore-induced chemical defences in pine trees emerge only under low nutrient availability. *Journal of Ecology*, **99**, 818–827.

Savage, J.A. & Cavender-Bares, J. (2012) Habitat specialization and the role of trait lability in structuring diverse willow (genus *Salix*) communities. *Ecology*, **93**, S138–S150.

Schoonhoven, L.M., van Loon, J.J.A. & Dicke, M. (2005) *Insect-Plant Biology*. Oxford University Press, New York, NY.

Schuldt, A., Bruelheide, H., Durka, W., Eichenberg, D., Fischer, M., Kröber, W. *et al.* (2012) Plant traits affecting herbivory on tree recruits in highly diverse subtropical forests. *Ecology Letters*, **15**, 732–739.

Selosse, M.A., Bauer, R. & Moyersoen, B. (2002) Basal hymenomycetes belonging to the Sebacinaceae are ectomycorrhizal on temperate deciduous trees. *New Phytologist*, **155**, 183–195.

Sipos, J. & Kindlmann, P. (2013) Effect of the canopy complexity of trees on the rate of predation of insects. *Journal of Applied Entomology*, **137**, 445–451.

Skvortsov, A.K. (1968) *Willows of the USSR: A Taxonomic and Geographic Revision*. Nauka, Moscow.

Smreczyński, S. (1966) *Klucze do oznaczania owadów polski. XIX: Chrząszcze – Coleoptera Zeszyt 98 b, Ryjkowce – Curculionidae, podrodziny Otiorhynchinae, Brachyderinae*. Państvove wydawnictwo naukove, Warszawa.

Smreczyński, S. (1972) *Klucze do oznaczania owadów polski. XIX: Chrząszcze – Coleoptera. Zeszyt 98 d, Ryjkowce – Curculionidae, podrodzina Curculioninae, plemiona Dryophthorini, Cossonini, Bagoini, Tanysphyrini, Notarii, Smicronychini, Ellescini, Acaliptini, Tychiini,*

Anthonomini, Curculiononi, Pissodini, Magdalini, Trachodini, Rhynchophorini, Cryptorhynchini. Państvove wydawnictwo naukove, Warszawa.

Smreczyński, S. (1974) *Klucze do oznaczania owadów polski. XIX: Chrząszcze – Coleoptera. Zeszyt 98 e, Ryjkowce – Curculionidae, podrodzina Curculioninae, plemiona Barini, Coryssomerini, Ceutorhynchini.* Państvove wydawnictwo naukove, Warszawa.

Taberlet, P., Gielly, L., Pautou, G. & Bouvet, J. (1991) Universal primers for amplification of three noncoding regions of chloroplast DNA. *Plant Molecular Biology*, **17**, 1105–1109.

Termonia, A., Hsiao, T.H., Pasteels, J.M. & Milinkovitch, M.C. (2001) Feeding specialization and host-derived chemical defense in Chrysomeline leaf beetles did not lead to an evolutionary dead end. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 3909–3914.

Treutter, D. (2006) Significance of flavonoids in plant resistance: a review. *Environmental Chemistry Letters*, **4**, 147–157.

Warchalowski, A. (2003) *Chrysomelidae: The Leaf Beetles of Europe and the Mediterranean Area.* Natura optima dux Foundation, Warszawa.

White, T.J., Bruns, T.D., Lee, S.B. & Taylor, J.W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR – Protocols and Applications – A Laboratory Manual* (eds N. Innis, D. Gelfand, J. Sninsky & T. White), pp. 315–322. Academic Press, New York, NY.

Wilson, J.S., Forister, M.L., Dyer, L.A., O'Connor, J.M., Burls, K., Feldman, C.R. *et al.* (2012) Host conservatism, host shifts and diversification across three trophic levels in two Neotropical forests. *Journal of Evolutionary Biology*, **25**, 532–546.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Correlation between host-plant phylogeny and chemical profile.

Fig. S2. Number of compounds shared by studied host-plant species.

Fig. S3. Species response curves of Salicylate specialists to salicylate content as analyzed by RDA

Table S1. List of secondary metabolites (mg g^{-1}) found in examined host-plants.

Table S2. List of insect individuals sampled on individual tree species.

Table S3. Impact of host-plant chemical dissimilarity and phylogenetic distance on insect community similarity (based on Bray–Curtis index).

Table S4. Impact of host-plant traits on insect community structure analyzed by RDA.

Supporting Information
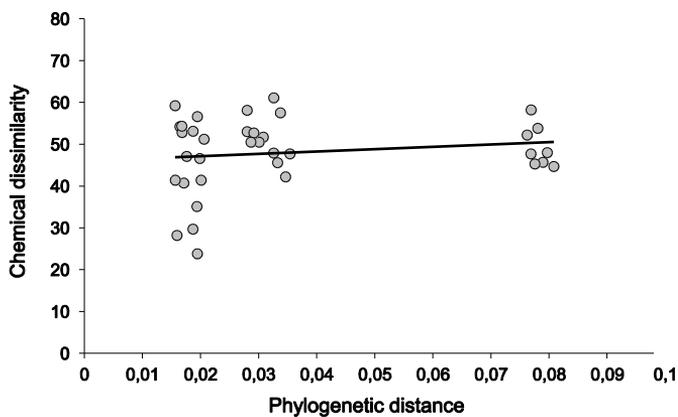
Figure S1. Correlation between host-plant phylogeny and chemical profile. Host-plant phylogenetic distance was uncorrelated with secondary metabolite dissimilarity when analyzed by Mantel test (r=0.15, p=0.334).
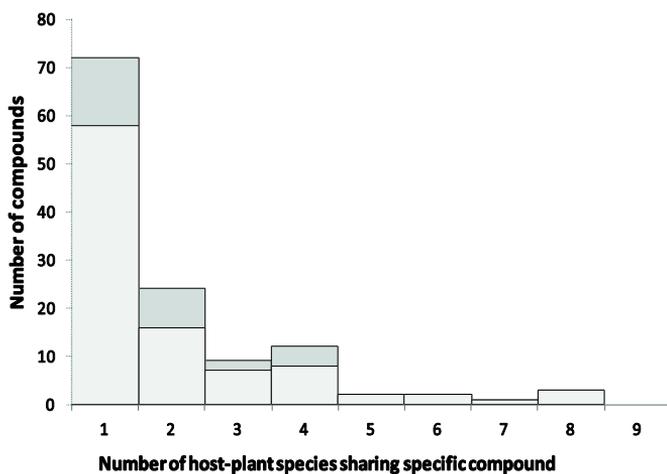
Figure S2. Number of compounds shared by studied host-plant species. Light grey represents flavonoids, dark grey represents salicylates.
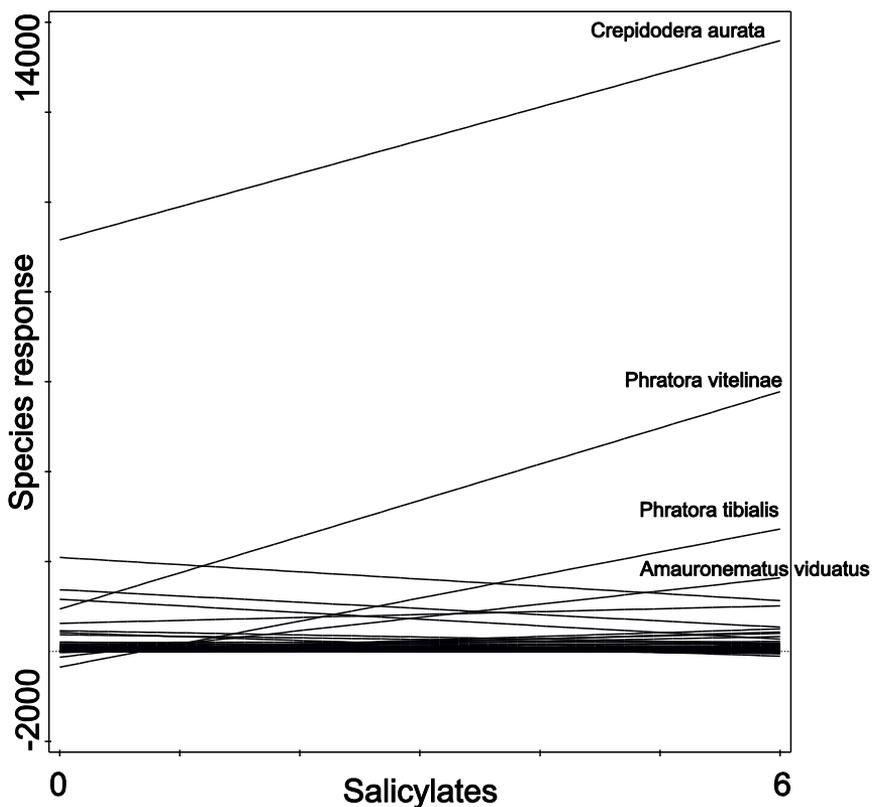
Figure S3. Species response curves of Salicylate specialists to salicylate content as analyzed by RDA. Some of Salicaceae specialists exhibited strong positive response to salicylates whereas majority of specialists showed weak negative or nearly no response to these secondary metabolites. Species with strong positive response are labeled with their names.

Table S1. List of secondary metabolites (mg/g) found in examined host-plants according to their retention time. Following number of host-plants was analyzed per species: *Salix aurita* (4), *Salix caprea* (6),   *Salix cinerea* (7), *Salix fragilis* (6), *Salix   pentandra*   (3),   *Salix purpurea* (5), *Salix rosmarinifolia* (2), *Salix viminalis* (4), *Populus. tremula* (5).

	RT (min)	*S. aurita*	*S. caprea*	*S. cinerea*	*S. fragilis*	*S. pentandra*	*S. purpurea*	*S. rosmarinifolia*	*S. viminalis*	*P. tremula*
Salicylates										
salicyl alcohol-diglucoside	2.00	-	-	-	2.386	-	-	-	-	-
salicin	2.29	-	-	-	1.161	3.231	13.999	15.047	-	2.847
salicyl alcohol	3.89	-	-	-	0.089	-	2.165	3.763	-	-
2`-O-acetylsalicin	9.15	-	-	-	-	5.603	-	-	-	-
salicortin	13.29	-	-	-	3.468	-	70.265	88.099	-	3.98
acetyl-salicortin	19.48	-	-	-	-	21.144	-	-	-	2.227
HCH-salicortin	22.64	-	-	-	0.608	-	-	-	-	-
tremuloidin	24.26	-	-	-	4.599	-	-	-	-	-
HCH-acetyl-salicortin	26.78	-	-	-	-	10.658	-	-	-	-
cinnamoyl acetyl-salicortin	28.42	-	-	-	-	0.341	-	-	-	-
cinnamoyl salicylate 1	30.30	-	-	-	-	-	-	4.137	-	-
cinnamoyl tremuloidin	30.31	-	-	-	3.212	-	-	-	-	-
cinnamoyl salicylate 2	31.36	-	-	-	-	-	1.898	-	-	-
cinnamoyl salicylate 3	31.67	-	-	-	-	-	1.457	-	-	-
cinnamoyl salicortin	31.75	-	-	-	-	-	-	-	-	0.617
tremulacin derivative 1	32.32	-	-	-	0.035	-	-	-	-	-
tremulacin	32.75	-	-	-	10.33	-	60.361	47.421	-	9.773
disalicortin	36.30	-	-	-	-	-	4.349	3.327	-	-

	RT (min)	*S. aurita*	*S. caprea*	*S. cinerea*	*S. fragilis*	*S. pentandra*	*S. purpurea*	*S. rosmarinifolia*	*S. viminalis*	*P. tremula*
cinnamoyl tremulacin	36.58	-	-	-	0.08	-	-	-	-	-
tremulacin derivative 2	38.57	-	-	-	0.96	-	-	-	-	-
HCH-tremulacin derivative 1	40.19	-	-	-	-	-	0.702	0.507	-	-
HCH-tremulacin derivative 2	40.57	-	-	-	-	-	0.801	0.537	-	-
cinnamoyl salicylate 4	42.95	-	-	-	-	-	-	0.081	-	-
ditremulacin derivative 1	44.63	-	-	-	-	-	2.039	1.059	-	-
ditremulacin derivative 2	44.90	-	-	-	-	-	2.138	0.999	-	-
ditremulacin derivative 3	46.13	-	-	-	-	-	0.417	0.224	-	-
ditremulacin derivative 4	47.86	-	-	-	0.24	-	1.329	2.081	-	-
tremulacin derivative 3	49.48	-	-	-	0.595	0.769	1.299	1.758	-	-
Flavonoids										
protocatechuic acid	2.09	0.036	0.037	0.134	-	-	-	-	0.078	-
p-OH-cinnamic acid derivative 1	3.71	-	0.105	-	-	-	-	-	-	-
chlorogenic acid derivative 1	3.82	-	-	0.215	-	-	-	-	-	-
cinnamic acid derivative 1	4.86	-	-	-	-	-	-	-	-	1.094
neochlorogenic acid	6.19	0.119	0.373	0.515	3.067	14.218	-	0.748	0.266	5.349
p-OH-cinnamic acid derivative 2	8.31	0.221	0.187	0.304	0.431	1.54	-	0.263	0.358	0.769
eriodictyol diglycoside 1	8.57	-	-	-	-	-	2.086	-	-	-
p-OH-cinnamic acid derivative 3	8.73	-	0.266	-	0.347	-	-	0.299	0.056	0.387
(+)-catechin	9.71	7.785	0.584	4.184	-	-	1.371	1.861	2.838	1.344
chlorogenic acid	9.87	2.605	-	1.266	8.861	23.361	-	-	0.736	2.459
quercetin triglycoside 1	10.48	-	-	-	-	-	-	-	-	0.312
eriodictyol diglycoside 2	12.02	-	-	-	-	-	0.601	-	-	-

	RT (min)	*S. aurita*	*S. caprea*	*S. cinerea*	*S. fragilis*	*S. pentandra*	*S. purpurea*	*S. rosmarinifolia*	*S. viminalis*	*P. tremula*
flavonoid diglucoside	12.06	-	-	-	-	-	-	-	-	0.405
p-OH-cinnamic acid glucoside	12.12	0.323	-	0.045	0.328	0.681	-	-	-	-
dihydromyricetin	12.14	-	-	-	-	-	-	-	1.37	-
dihydroquercetin	12.86	-	-	-	-	2.39	-	-	-	-
dihydrokaempferol	13.92	-	-	-	-	-	0.14	-	-	-
chlorogenic acid derivative 2	14.13	-	-	-	2.181	-	-	-	0.197	-
quercetin triglycoside 2	16.03	-	-	-	-	-	-	-	0.108	-
eriodictyol 7-glucoside	17.28	-	-	-	-	-	2.444	-	-	-
p-OH-cinnamic acid derivative 4	17.30	-	-	-	0.035	-	-	-	-	-
chrysoeriol derivative 1	17.52	-	-	0.057	-	-	-	-	-	-
luteolin glycoside 1	17.90	-	-	-	0.08	-	-	-	-	-
myricetin 3-galactoside	17.95	-	-	0.197	-	0.621	-	-	0.645	-
quercetin diglycoside 1	18.15	0.393	-	-	-	-	-	-	-	2.194
myricetin 3-glucoside	18.19	-	0.011	0.007	-	2.348	-	-	-	0.34
quercetin diglycoside 2	18.27	0.066	-	-	0.493	-	-	-	0.067	-
quercetin diglycoside 3	18.69	-	-	-	0.905	-	-	-	0.099	0.998
quercetin diglycoside 4	18.99	-	-	-	-	-	-	-	0.136	-
myricetin 3-arabinoside	19.34	-	-	-	-	-	-	-	0.051	-
luteolin glycoside 2	19.34	0.205	0.115	-	-	-	-	-	-	-
luteolin glycoside 3	19.67	0.356	0.237	-	-	-	-	-	-	-
luteolin 5-glucoside	19.70	-	-	-	0.905	-	-	7.116	-	-
quercetin glycoside 1	20.04	-	-	-	-	-	-	-	0.055	-
quercetin triglycoside 3	20.10	-	-	-	-	-	-	-	0.05	0.352

	RT (min)	*S. aurita*	*S. caprea*	*S. cinerea*	*S. fragilis*	*S. pentandra*	*S. purpurea*	*S. rosmarinifolia*	*S. viminalis*	*P. tremula*
naringenin 7-glucoside	20.12	-	-	-	-	-	0.501	-	-	-
myricitrin	20.14	-	-	-	-	0.457	-	-	-	-
luteolin 7-glucoside	20.44	1.275	0.307	0.313	0.117	-	5.559	5.563	-	-
hyperin	20.60	-	-	-	-	3.029	-	-	-	1.391
kaempferol glycoside derivative 1	20.61	-	-	-	1.508	-	-	-	-	-
quercetin glycoside 2	20.65	-	-	-	-	-	-	-	0.099	-
luteolin glycoside 4	20.82	0.354	-	-	-	-	-	-	-	-
isorhamnetin glycoside 1	20.85	-	-	-	1.548	-	-	-	-	-
quercetin 3-glucoside	21.08	2.311	0.406	1.067	2.558	7.096	0.615	-	2.272	11.884
luteolin glycoside 5	21.33	-	-	-	0.034	-	1.743	-	-	-
cinnamic acid derivative 2	21.53	-	-	-	0.104	-	-	-	-	-
quercetin glycoside 3	21.56	-	0.051	-	-	-	-	-	-	-
myricetin glycoside	21.77	-	-	-	-	-	-	-	0.075	-
quercetin 3-arabinopyranoside	21.93	-	-	0.013	-	2.637	-	-	0.425	0.594
chlorogenic acid derivative 3	21.95	-	-	-	0.132	-	-	-	-	-
apigenin 5-glucoside	22.14	-	-	-	-	-	-	0.467	-	-
luteolin glycoside 6	22.34	3.951	1.95	-	-	-	-	-	-	-
apigenin 7-glucoside	22.62	-	-	0.313	-	-	0.483	0.342	0.05	-
p-OH-cinnamic acid derivative 5	22.66	-	-	-	-	-	-	0.122	-	-
quercetin 3-arabinofuranoside	22.75	-	-	-	-	1.03	-	-	-	-
luteolin glycoside 7	22.79	6.964	3.864	-	-	-	-	-	-	-
methyl-luteolin glycoside 1	23.28	-	-	-	0.083	-	0.601	-	-	-

Compound	RT									
methyl-luteolin glycoside 2	23.49	0.13	-	0.073	0.08	-	-	-	-	-
quercitrin	23.66	-	-	-	0.222	0.707	-	-	1.609	-
kaempferol glycoside derivative 2	23.91	-	-	-	-	-	-	-	-	0.231
kaempferol 3-glucoside	24.09	-	-	-	0.064	0.395	-	-	0.109	2.763
methyl-luteolin 5-glucoside	24.22	1.372	0.489	2.38	-	-	-	0.325	-	-
chrysoeriol glycoside	24.32	-	-	-	-	-	3.455	-	-	-
isorhamnetin derivative 1	24.70	-	-	-	-	-	-	-	0.784	-
isorhamnetin glycoside 2	24.72	-	-	-	1.001	-	-	-	-	-
methyl-luteolin glycoside 3	24.76	-	-	-	-	-	0.447	-	-	-
isorhamnetin derivative 2	25.14	-	-	-	-	-	-	-	0.909	0.162
kaempferol glycoside derivative 3	25.20	-	-	-	-	0.111	-	-	-	-
kaempferol 3-arabinoside	25.27	-	-	-	1.126	-	-	-	0.064	-
p-OH-cinnamic acid derivative 6	25.63	-	0.006	-	-	-	-	-	-	-
salipurposide	25.84	-	-	-	-	-	0.354	-	-	-
methyl-luteolin glycoside 4	26.11	-	0.071	0.006	-	-	0.74	1.272	-	-
isorhamnetin rhamnoside	27.01	-	-	-	-	-	-	-	0.633	-
isorhamnetin derivative 3	27.18	-	-	-	0.212	-	-	-	-	-
p-OH-cinnamic acid derivative 7	27.55	0.004	-	-	-	-	-	-	-	0.015
apigenin derivative 1	27.61	-	-	-	-	-	0.153	-	-	-
luteolin aglycon derivative 1	27.83	-	-	-	-	-	0.355	-	-	-
p-OH-cinnamic acid derivative 8	28.15	-	-	-	-	-	-	-	-	0.017
apigenin derivative 2	29.33	-	-	-	-	-	-	0.026	-	-
methyl-luteolin aglycon	29.74	0.064	0.323	-	-	-	-	-	-	-

	RT (min)	*S. aurita*	*S. caprea*	*S. cinerea*	*S. fragilis*	*S. pentandra*	*S. purpurea*	*S. rosmarinifolia*	*S. viminalis*	*P. tremula*
luteolin glycoside 8	30.60	-	-	-	0.048	-	-	-	-	-
apigenin derivative 3	34.60	-	-	-	-	-	-	-	-	0.078
luteolin aglycon derivative 2	35.30	0.022	0.007	-	-	-	-	-	-	-
monocoumaroyl flavonol	37.61	-	0.016	-	-	-	-	-	-	-
monocoumaroyl astragalin	38.31	0.944	1.16	4.092	-	-	-	0.159	1.899	-
chrysoeriol derivative 2	39.07	-	-	-	-	-	-	-	-	0.048
apigenin derivative 4	39.58	-	-	0.016	-	-	-	-	-	-
apigenin derivative 5	42.99	-	-	0.005	-	-	-	-	-	-
methyl-apigenin derivative 1	43.03	-	-	-	-	-	-	-	-	0.107
methyl-apigenin derivative 2	43.71	-	-	-	-	-	-	-	-	0.434
methyl-apigenin derivative 3	44.61	-	-	-	-	-	-	-	-	0.024
dicoumaroyl flavonol	46.61	0.01	0.016	0.104	-	-	-	-	-	-
methyl-apigenin derivative 4	47.99	-	-	-	-	-	-	-	-	0.054
Condensed tannins		194.8	138.8	159.1	51.9	190.7	42.7	133.4	137.4	38.2

Table S2. List of insect individuals sampled on idividual tree species. Species trated in this study as Salicaceae specialists are in bold. Following number of host-plants was sampled per species: *S. aurita* (6), *S. caprea* (10), *S. cinerea* (14),   *S. fragilis* (12), S. *pentandra* (3), *S. purpurea* (6), *S. rosmarinifolia* (2), *S. viminalis* (8), *P. tremula* (10).

Species	*S. aurita*	*S. caprea*	*S. cinerea*	*S. fragilis*	*S. pentandra*	*S. purpurea*	*S. rosmarinifolia*	*S. viminalis*	*P. tremula*	TOTAL
Coleoptera										
Agelastica alni	-	1	-	5	1	-	1	-	-	8
Anaesthetis testacea	-	-	-	-	-	-	-	3	-	3
Archarius crux	13	5	1	20	-	6	3	2	3	53
Archarius salicivorus	2	6	6	1	-	1	3	3	1	23
Byctiscus betulae	-	-	1	1	-	-	-	-	-	2
Byctiscus populi	-	-	-	-	-	-	-	-	3	3
Chrysomela populi	-	-	-	-	-	-	6	-	16	22
Chrysomela tremula	-	-	-	-	-	-	2	-	-	2
Chrysomela vigintipunctata	-	-	-	1	-	-	-	-	-	1
***Chrysomela vigintipunctata* larva**	-	-	-	-	2	3	-	-	-	5
Clytra laeviscula	3	10	13	-	-	-	-	4	-	30
Crepidodera aurata	133	357	109	1433	21	190	162	220	111	2736
Crepidodera aurea	47	36	12	8	2	1	19	6	344	475
Crepidodera fulvicornis	68	8	148	61	29	9	1	8	13	345
Cryptocephalus bipunctatus	1	-	-	-	-	-	1	-	-	2
Cryptocephalus decemmaculatus	-	1	8	-	-	-	-	9	-	18
Cryptocephalus fulvus	-	2	-	-	-	-	-	-	-	2
Cryptocephalus labiatus	-	2	-	-	-	-	-	-	-	2

Species	S. aurita	S. caprea	S. cinerea	S. fragilis	S. pentandra	S. purpurea	S. rosmarinifolia	S. viminalis	P. tremula	TOTAL
Cryptocephalus ocellatus	-	2	-	-	-	-	-	-	-	2
Cryptocephalus pusillus	4	-	-	-	-	-	-	1	-	5
Dorytomus affinis	-	-	-	-	-	-	-	-	5	5
Dorytomus ictor	-	-	-	-	-	-	1	-	-	1
Dorytomus melanophtalmus	3	5	1	11	-	16	1	2	1	40
Dorytomus rufatus	1	34	1	1	-	1	-	11	4	53
Dorytomus taeniatus	5	13	2	2	-	-	3	3	13	41
Dorytomus tortrix	-	2	-	-	-	-	-	-	2	4
Ellescus bipunctatus	5	2	1	4	1	-	-	1	-	14
Ellescus scanius	-	1	2	1	-	1	1	-	9	15
Galerucella lineola	-	-	-	1	-	3	-	1	-	5
Isochnus populicola	-	-	-	4	5	-	-	-	-	9
Lagria hirta	1	-	-	-	-	-	-	-	-	1
Lochmaea capreae	13	47	9	1	-	-	408	43	10	531
Lochmaea capreae larva	16	-	56	-	-	-	234	145	-	451
Luperus flavipes	-	5	2	-	-	1	-	-	4	12
Luperus longicornis	-	-	-	1	-	-	2	-	-	3
Magdalis linearis	-	1	-	-	-	-	-	-	-	1
Magdalis ruficornis	-	-	-	-	-	-	1	-	-	1
Oberea oculata	1	-	-	-	-	-	-	-	-	1
Phratora tibialis	1	-	-	-	-	127	-	-	-	128
Phratora tibialis larva	-	-	-	-	-	19	-	-	-	19
Phratora vitellinae	2	6	-	437	-	47	97	3	9	601

Species	S. aurita	S. caprea	S. cinerea	S. fragilis	S. pentandra	S. purpurea	S. rosmarinifolia	S. viminalis	P. tremula	TOTAL
Phratora vitellinae larva	-	-	-	86	-	1	-	65	3	155
Phyllobius arborator	-	2	-	1	-	-	-	-	1	4
Phyllobius argentatus	1	-	2	-	-	-	1	-	16	20
Phyllobius oblongus	1	-	-	2	-	-	-	-	1	4
Phyllobius pomaceus	-	-	-	-	-	-	12	-	-	12
Phyllobius pyri	5	4	-	1	1	-	1	1	56	69
Phyllobius vespertinus	-	-	-	1	-	-	-	-	-	1
Phyllobius viridicollis (clorupus)	-	-	11	-	-	-	1	-	4	16
Phyllopertha horticola	2	-	1	2	1	-	8	2	2	18
Plagiodera versicolora	4	2	1	123	10	3	1	14	-	158
Plagiodera versicolora larva	-	-	-	19	1	-	-	20	-	40
Polydrusus cervinus	1	-	2	-	-	-	-	-	2	5
Polydrusus formosus	8	34	12	6	-	-	20	1	26	107
Polydrusus picus	-	-	-	-	-	-	-	-	2	2
Polydrusus ruficornis	-	1	-	-	-	-	1	-	-	2
Rhampus pulicarius	3	43	42	11	9	18	2	48	3	179
Smaragdina salicina	-	1	-	-	-	-	-	-	-	1
Tachyerges decoratus	-	-	1	16	-	12	-	2	-	31
Tachyerges salicis	3	1	3	7	-	-	2	1	-	17
Tachyerges stigma	6	3	4	5	-	9	3	6	1	37
Temnocerus tomentosus	1	2	14	2	-	-	7	4	4	34
Tetrops praeustus	-	-	1	-	-	-	-	-	-	1

Hymenoptera

Allantus togatus	1	-	-	3	-	-	3	-	-	7
Amauronematus alpicola	-	-	-	-	-	5	-	-	-	5
Amauronematus fasciatus	-	1	-	-	-	-	-	-	-	1
Amauronematus humeralis	3	4	1	-	-	-	-	-	-	8
Amauronematus humilis	-	-	-	-	-	-	-	2	-	2
Amauronematus hystrio	4	-	1	-	-	-	-	-	-	5
Amauronematus leucolaenus	2	-	-	-	-	-	-	-	-	2
Amauronematus longisera	3	-	-	-	-	-	-	-	-	3
Amauronematus longiserra	-	-	-	-	-	-	3	-	-	3
Amauronematus mimus	8	1	-	-	-	-	-	-	-	9
Amauronematus new1	1	-	4	-	-	-	-	-	-	5
Amauronematus new2	-	-	1	-	-	-	-	-	-	1
Amauronematus palipes	-	-	2	-	-	-	-	-	-	2
Amauronematus puniceus	-	-	-	3	-	-	-	-	-	3
Amauronematus sp1	-	-	-	-	-	2	-	-	-	2
Amauronematus sp2	-	-	-	-	-	2	-	-	-	2
Amauronematus sp3	-	-	-	-	-	2	-	-	-	2
Amauronematus sp4	-	-	-	-	-	1	-	-	-	1
Amauronematus sp5	-	-	-	-	-	1	-	-	-	1
Amauronematus viduatoides	8	1	1	-	-	-	-	-	-	10
Amauronematus viduatus	1	1	-	-	6	27	55	2	-	92
Amauronematus vittatus	3	2	-	-	-	1	-	6	-	12
Ametastegia perla	-	-	1	1	-	-	-	-	-	2

Species	S. aurita	S. caprea	S. cinerea	S. fragilis	S. pentandra	S. purpurea	S. rosmarinifolia	S. viminalis	P. tremula	TOTAL
Arge enodis	-	3	-	2	-	-	-	1	1	7
Arge ustulata	-	-	1	-	-	-	-	2	-	3
Empria immersa	1	-	-	-	-	-	-	-	-	1
Nematus bergmanni	1	1	9	8	-	1	-	7	2	29
Nematus bipartitus	-	1	-	1	-	-	-	-	-	2
Nematus hypoxanthus	-	1	2	1	-	1	27	3	-	35
Nematus melanaspis	-	-	-	-	-	4	-	4	-	8
Nematus melanocephalus	-	-	-	5	-	-	-	-	-	5
Nematus nigricornis	-	-	-	-	-	-	-	-	4	4
Nematus oligospilus	-	1	-	-	-	-	-	1	-	2
Nematus pavidus	8	-	-	-	-	-	-	-	-	8
Nematus respondens	-	-	-	3	-	-	-	-	-	3
Nematus salicis	-	-	3	2	-	-	-	-	-	5
Nematus sp1	-	-	-	-	5	-	-	-	-	5
Nematus sp2	-	-	-	-	-	-	-	1	-	1
Phyllocolpa alienata	29	-	-	-	-	-	-	-	-	29
Phyllocolpa leucapsis	-	-	70	-	-	-	-	-	-	70
Phyllocolpa leucosticta	-	13	-	-	-	-	-	-	-	13
Phyllocolpa oblita	1	-	-	18	-	2	-	-	-	21
Phyllocolpa polita	-	-	-	-	-	10	-	-	-	10
Phyllocolpa scotaspis	-	-	-	-	-	-	-	2	-	2
Pristiphora confusa	-	-	-	-	-	-	-	3	-	3
Pristiphora lanifica	1	-	-	-	-	-	-	-	-	1

Species	S. aurita	S. caprea	S. cinerea	S. fragilis	S. pentandra	S. purpurea	S. rosmarinifolia	S. viminalis	P. tremula	TOTAL
Pristiphora sp1	-	-	-	-	-	1	-	-	-	1
Pristiphora sp2	-	-	-	-	-	-	5	-	-	5
Pristiphora subopaca	-	-	-	-	-	-	-	2	-	2
Rhogogaster chlorosoma	-	1	-	10	-	-	-	-	-	11
Tenthredo livida	-	-	-	-	-	-	-	1	-	1
Tentredo fagi	-	-	-	-	-	-	-	-	1	1
Trichiocampus grandis	-	-	-	-	-	-	-	-	1	1
Lepidoptera										
Acleris hastiana	1	-	1	-	-	-	4	1	1	8
Acronicta aceris	-	-	-	-	-	-	-	1	-	1
Acronicta alni	-	-	-	-	-	-	-	-	1	1
Acronicta auricoma	-	-	-	1	-	-	1	-	-	2
Acronicta megacephala	1	-	-	-	-	-	-	-	3	4
Acronicta psi	1	-	-	-	-	-	-	-	-	1
Agonopterix conterminella	3	1	2	-	-	-	1	1	-	8
Agonopterix ocellana	1	-	6	7	-	2	3	2	-	21
Agriopis aurantiaria	-	-	-	-	-	1	-	2	-	3
Agriopis marginaria	-	1	-	-	-	-	-	-	-	1
Agrochola lota	-	-	1	1	-	-	-	1	-	3
Alsophila aescularia	1	-	-	-	-	-	-	-	-	1
Amphipyra berbera	-	-	-	-	-	-	-	1	-	1
Amphipyra perflua	-	2	-	1	-	-	-	-	1	4
Amphipyra pyramidea	-	-	-	1	-	-	-	-	-	1

Species	S. aurita	S. caprea	S. cinerea	S. fragilis	S. pentandra	S. purpurea	S. rosmarinifolia	S. viminalis	P. tremula	TOTAL
Anacampsis populella	1	2	2	1	-	1	1	2	20	30
Apatura iris	-	2	-	-	-	-	-	-	1	3
Apocheima pilosaria	-	1	-	-	1	-	-	-	1	3
Apotomis capreana	-	1	1	-	-	-	-	-	-	2
Archiearis notha	-	-	-	-	-	-	-	-	2	2
Bena prasina	-	-	-	1	-	-	-	-	-	1
Biston betularius	-	-	-	2	-	-	-	-	-	2
Biston stratarius	-	-	-	1	-	-	-	-	-	1
Cabera exanthemata	21	5	27	1	1	3	11	8	9	86
Callimorpha dominula	-	-	-	-	-	-	3	-	-	3
Celypha lacunana	-	-	1	-	-	-	2	1	-	4
Clostera anachoreta	-	-	1	-	-	-	-	-	-	1
Clostera curtula	-	-	-	-	1	1	2	-	-	4
Clostera pigra	-	-	-	3	1	-	18	1	5	28
Colobochyla salicalis	-	-	-	-	-	-	1	-	-	1
Colotois pennaria	-	-	-	-	-	-	-	1	-	1
Conistra vaccinii	1	-	2	-	-	-	-	2	-	5
Cosmia trapezina	-	1	1	1	-	-	-	2	3	8
Cyclophora pendularia	-	-	4	-	-	-	1	-	-	5
Earias clorana	3	1	7	3	-	-	1	2	-	17
Ectropis crepuscularia	-	-	-	-	-	-	1	2	1	4
Ematurga atomaria	-	-	2	-	-	-	3	-	-	5
Erannis defoliaria	-	-	-	1	-	-	-	-	1	2

Eudia pavonia	-	-	-	-	-	-	2	-	-	2
Eupithecia subfuscata	-	-	-	-	-	-	4	-	-	4
Euproctis similis	1	3	-	-	-	-	-	1	-	5
Eupsilia transversa	-	-	-	-	-	-	-	1	-	1
Gelechia sororculella	-	1	1	-	-	-	-	-	-	2
Gypsonoma dealbana	-	-	1	-	-	-	-	-	-	1
Hydria undulata	-	-	-	1	-	-	-	-	-	1
Hydriomena autumnalis	-	1	-	-	-	-	-	-	-	1
Hydriomena furcata	-	1	-	-	-	-	-	-	-	1
Hypomecis punctinalis	-	-	-	1	-	-	-	-	-	1
Ipimorpha retusa	1	-	1	1	1	2	-	-	2	8
Ipimorpha subtusa	-	-	-	-	1	-	-	-	-	1
Laothoe populi	-	-	-	-	-	-	-	-	1	1
Lithophane socia	-	5	1	5	5	-	1	2	-	19
Lobophora halterata	-	-	-	-	-	-	-	1	3	4
Lomaspilis marginata	8	6	15	9	1	7	1	18	8	73
Lymantria dispar	-	1	-	-	-	-	-	-	1	2
Notodonta ziczac	-	-	-	1	1	1	2	-	-	5
Operophtera brumata	9	18	46	10	10	10	2	60	2	167
Orgyia antiqua	-	-	-	-	-	-	-	-	1	1
Orthosia cerasi	-	-	-	-	-	-	-	1	-	1
Orthosia cruda	1	-	-	-	-	-	-	-	-	1
Orthosia gothica	-	1	1	-	-	-	-	1	1	4
Orthosia incerta	1	-	1	2	1	2	-	-	2	9

Species	S. aurita	S. caprea	S. cinerea	S. fragilis	S. pentandra	S. purpurea	S. rosmarinifolia	S. viminalis	P. tremula	TOTAL
Orthosia munda	1	-	-	-	-	-	-	-	-	1
Orthosia opima	-	-	-	-	-	-	1	-	-	1
Orthosia populi	-	-	-	-	-	-	-	-	2	2
Pandemis heparana	-	-	1	-	-	-	-	-	-	1
Parastichtis suspecta	-	-	-	-	-	-	-	2	-	2
Phalera bucephala	-	1	16	-	-	-	-	4	1	22
Pheosia tremulae	-	-	-	-	-	-	-	1	3	4
Pseudosciaphila branderiana	-	-	-	-	-	-	-	-	1	1
Pterapherapteryx sexalata	-	-	2	-	-	-	-	-	-	2
Pterostoma palpinum	-	1	-	1	-	-	-	2	1	5
Ptilodon capucina	-	1	-	-	-	-	-	1	-	2
Ptycholoma lecheanum	-	1	-	-	-	-	-	-	-	1
Rhyparia purpurata	-	-	-	-	-	-	-	1	-	1
Sciota hostilis	-	-	-	-	-	-	1	-	1	2
Scoliopteryx libatrix	5	1	4	1	-	7	4	5	-	27
Selenia tetralunaria	-	2	-	-	-	-	-	1	-	3
Semiothisa alternaria	-	1	2	-	-	1	-	1	-	5
Semiothisa notata	-	-	2	-	-	5	-	9	-	16
Tethea or	-	-	-	-	-	-	-	-	5	5
Trichopteryx carpinata	-	-	-	1	-	-	-	-	-	1

Table S3. Impact of host-plant chemical dissimilarity and phylogenetic distance on insect community similarity (based on Bray-Curtis index). The results of one-sided Mantel tests and partial Mantel tests (with phylogenetic distance used as covarite) are shown for Coleoptera, Lepidoptera, and Hymenoptera. Significant ($p<0.05$) results are highlighted in bold, d.f.=43 for Mantel tests and d.f.=42 for partial Mantel tests.

	p / r		
	Coleoptera	**Lepidoptera**	**Hymenoptera**
Phylogenetic distance	**0.033 / 0.35**	0.095 / 0.41	0.114 / 0.15
Chemical dissimilarity	**0.033 / 0.33**	**0.049 / 0.36**	0.223 / 0.01
Chemical diss. (par. Mantel)	**0.048 / 0.30**	0.057 / 0.33	0.227 / -0.01

Table S4. Impact of host-plant traits on insect community structure analyzed by RDA. The results are shown for Coleoptera, Lepidoptera, and Hymenoptera. Significant results are in bold.

	p /F		
	Coleoptera	**Lepidoptera**	**Hymenoptera**
Salicylates	**0.035** / 1.70	0.251 / 1.39	0.280 / 1.31
Flavonoids	0.378 / 1.17	0.360 / 1.22	0.322 / 1.29
Tannins	0.274 / 1.27	0.256 / 1.27	0.150 / 1.46
Trichomes	0.256 / 1.35	0.093 / 1.32	**0.041** / 1.55
SLA	0.541 / 0.82	0.519 / 0.85	0.480 / 0.95
C:N	0.293 / 1.18	0.283 / 1.22	0.242 / 1.30
Growth-form	0.374 / 1.14	0.328 / 1.22	0.252 / 1.23

Chapter II

Insect herbivores drive the loss of unique chemical defense in willows

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Abstract
Throughout the course of their evolution, plants have acquired a wide range of chemical and mechanical defenses to protect against herbivores. Ehrlich & Raven's coevolutionary theory suggests that this diversification of defensive traits is driven by the strong impact of novel traits on insect herbivores. However, the impact of plant defenses on insects is difficult to compare between related plant species due to variation in environmental and biotic conditions. We standardized these factors as far as possible by analyzing the effects of chemical and mechanical defensive traits on insects in a local community of 11 Salicaceae species growing in sympatry, and their leaf-chewing herbivores. Defensive traits (salicylates, flavonoids, tannins, trichomes, and leaf toughness) were generally not inter-correlated, with the exception of a negative correlation between salicylates and trichomes. The content of salicylates, a novel group of defensive metabolites in the Salicaceae, was correlated with low herbivore diversity and high host specificity. Despite these effects, the phylogeny of the studied species shows loss of salicylates in some *Salix* species instead of their further diversification. This could be due to salicylates not decreasing the overall abundance of herbivores, despite accounting for up to 22% of the dry leaf mass and therefore being costly. The defense of low-salicylate willow species is thus probably maintained by other defensive traits, such as trichomes. Our study shows that the balance between costs and benefits of defensive traits is not necessarily in favor of novel compounds and illustrates a process, which may lead to the reduction in a defensive trait.

Introduction

In their coevolutionary theory, Ehrlich & Raven (1964) proposed that an arms race between plants and herbivorous insects leads to the continued diversification of defensive traits, driven by the strong impact of novel traits on herbivores. The insects act as a selective pressure promoting increased plant defense (Benderoth et al., 2006), and many novel defensive traits appear during the course of plant evolution (Fucile et al., 2008; Kliebenstein & 2012). Although the evolution of plant defenses was studied in several systems (e.g., Agrawal & Fishbein, 2008; Becerra et al., 2009; Kursar et al., 2009; Agrawal et al., 2012), explaining the evolution of plant

secondary metabolites in the coevolutionary process requires further attention as different groups of secondary metabolites exhibit different evolutionary patterns. For example, diversification of secondary metabolites has been found in the genus *Bursera* (Becerra et al., 2009). On the other hand, the support for theoretical predictions of ever-expanding and diversifying defenses in *Asclepias* spp. is more equivocal, as the presence of cardenolides appears to have decreased with phylogenetic diversification (Agrawal & Fishbein, 2008).

The reduction or loss of secondary metabolites is expected, if they become ineffective in anti-herbivore defense or too costly. The benefits of defensive traits are defined by a combination of their anti-herbivore efficacy and the impact of herbivores on unprotected plants. Previous studies analyzed this cost–benefit balance by focusing on the overall abundance of herbivores and/or plant damage caused by them, while paying little attention to the herbivore species causing it (Coley et al., 2005; Agrawal &

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Fishbein, 2008). We suggest that it is important to analyze herbivore community composition and life history traits, as individual defensive traits may have different effects on specialist and generalist herbivores (Ali & Agrawal, 2012). In this study, we focus on the relationships between host plant defensive traits and the composition, population density, and host specificity of their leaf-chewing insects, ecologically one of the key herbivore guilds (Schoonhoven et al., 2005).

Biological interactions, including herbivory, are geographically variable; herbivore specialization varies with latitude and plants may exhibit different defensive traits when exposed to different pools of herbivores (Dyer et al., 2007; Christensen et al., 2014). Filtering geographical variation also minimizes differences in temperature, rainfall, and abundance of natural enemies, which all drive insect abundance (Connahs et al., 2011; Kozlov et al., 2013). The interplay of individual defense traits and their impact on herbivores can thus be best understood by studying co-occurring species from plant lineages with diverse chemical and morphological modes of protection. The genus *Salix*, a species-rich lineage with numerous shrub and tree species often occurring sympatrically, is an excellent model for such studies. Some species of the genus are protected by trichomes and tough leaves, which restrict herbivores from feeding and erode their mandibular jaws (Raupp, 1985; Zvereva et al., 1998), as well as by various secondary metabolites such as salicylates, flavonoids, and condensed tannins.

Salicylates are characteristic secondary metabolites of the Salicaceae; they are a family of compounds derived from salicyl alcohol and reach their highest diversity in the Salicaceae. As well as flavonoids and condensed tannins, salicylates have been repeatedly reported to have a detrimental impact on insect herbivores (Matsuki & Maclean, 1994; Kopper et al., 2002; Pearse, 2011). The anti-herbivorous function of salicylates is well recognized and their reported impacts on generalist herbivores include deterrent effects, retarded larval growth, and increased mortality (Matsuki & Maclean, 1994; Kolehmainen et al., 1995). Nevertheless, the distribution of salicylates among willows is not equal and it is well established that tissues of some willow species contain very low or zero concentrations of these secondary metabolites (Julkunen-Tiitto, 1989; Nyman & Julkunen-Tiitto, 2005). As the reduction in secondary metabolites can be a result of ineffectiveness against herbivores, it is remarkable that despite the anti-herbivorous effects of salicylates, certain specialist herbivores are known to be able to sequester salicylates and use them for protection against predators (Pasteels et al., 1983; Denno et al., 1990).

Here, we examine the defensive trait pattern of co-occurring willow species and the impact of these traits on associated leaf-chewing herbivores. First, we test whether any of the studied defensive traits are correlated and thus form distinct defense syndromes. Second, we test effectiveness of the salicylates and other defensive traits against herbivores, as indicated by their impact on herbivore abundance, species richness, and specialization. Third, we examine whether the presence, high content, and high diversity of salicylates are ancestral or derived characters among willows to explore the processes of origin and loss of host plant defensive traits.

Materials and methods

Host plants

The study was carried out within a 10 × 10 km area in South Bohemia, Czech Republic (48°51′58″–48°59′45″N, 14°26′20″–14°35′48″E) representing lowland wet meadows. This approach allowed sampling from an area with as far as possible similar abiotic conditions and with all host plants potentially available for colonization from the same pool of herbivore species.

The insects were sampled on eight willow species (out of nine growing in the area), two of their hybrids, and *Populus tremula* L., a related species of *Salix* spec. (Salicaceae) (Table 1). Shaded plants were excluded, as their traits and leaf chemistry could be significantly different. We avoided immature plants and plants that had obviously experienced browsing by herbivores or damage from other sources prior to the sampling, as these factors can cause significant changes in plant traits due to induced defense (Nakamura et al., 2005). All defensive traits were measured for 2–7 plant individuals per species (a total of 48 plants) and means were used as estimates for each species. Only two individuals of *Salix rosmarinifolia* L. and *S. viminalis* × *purpurea* were used for measuring plant traits, as other individual plants growing at our field sites were probably their clones and thus including them into analysis would not have provided additional information on trait variability. Two additional Central European lowland species (*Salix myrsinifolia* L. and *Salix alba* L.) were included in the phylogenetic analysis to make it more robust and the evolutionary trends of defensive traits more informative. Taxonomically, the studied willow species represent two subgenera of the genus *Salix*, viz., *Vetrix* and *Salix* sensu stricto (Skvortsov, 1968).

Leaf morphology

Trichome density and specific leaf area (SLA), a surrogate for leaf thickness and toughness (Groom & Lamont, 1999), were measured as parameters of leaf morphology

with possible impact on leaf-chewing insects. Trichome density was estimated as average trichome coverage (%) per 5 mm^2 area of mature leaf surface and values for dorsal and ventral side were combined.

Leaf disks of known diameter (not containing central vein) were cut and dried to a constant weight and the SLA was calculated as weight per area of the dried leaf disk. Leaf disks were sampled 10×, at 14-day intervals throughout the whole 2010 vegetative season. In total, 30 leaf disks were obtained from each plant individual.

Chemical analysis
Samples for chemical analysis were dried immediately after collection and kept in silica gel. The content (mg g^{-1}) of salicylates, flavonoids, and condensed tannins was analyzed from 5 to 9 mg of young leaves (avoiding primary and secondary leaf veins) sampled in early June. We used samples obtained in early June for the analysis of defensive trait impact on herbivores as salicylate and flavonoid concentration and diversity in young leaves tend to be higher than in leaves obtained in summer. Nevertheless, we also measured samples obtained at the beginning of August to estimate seasonal variability.

Phenolic compounds were extracted with methanol as described in Nybakken et al. (2012). Extracts were dried and kept in a freezer at −20 °C. Before the analysis, dried samples were re-dissolved in 600 μl methanol:water (1:1). We used 20 μl of re-dissolved samples for high-performance liquid chromatography (HPLC) analysis of salicylates and flavonoids following Nybakken et al. (2012). Compounds were separated using a Zorbax SBC18 (4.6 × 60 mm) HPLC column (Agilent Technologies, Waldbronn, Germany) employing a water/methanol gradient (Julkunen-Tiitto & Sorsa, 2001). Salicylate and flavonoid content was measured based on the absorbance at 220 and 320 nm, respectively. Retention times and spectra compared with those of standards were used to identify the compounds.

Soluble condensed tannins were measured using an acid-butanol assay starting with an aliquot of the HPLC sample and following the methods of Hagerman (2002). Insoluble condensed tannins were measured from tissue residues dried at room temperature. After hydrolysis, absorbance values at 550 nm were measured (Spectronic 20 Genesys spectrophotometer; Thermo Fisher Scientific, Waltham, MA, USA). The condensed tannin content was calculated based on equivalents of *Betula nana* L. leaf tannins.

Limited sampling can lead to underestimation of secondary metabolite diversity. Therefore, we reconstructed secondary metabolite accumulation curves for two willow species (*Salix cinerea* L. and *Salix fragilis* L., representing

low- and high salicylate willow lineages) well represented in our sampling to estimate number of plant individuals needed for reliable secondary metabolite diversity analysis. The accumulation curves were based on Mao Tau index for the number of individuals, computed in EstimateS 8.2 (Colwell, 2006).

Host plant phylogeny reconstruction
Three loci were used for host plant phylogeny reconstruction: ITS, trnT-trnL, and ADH. Standard procedures for DNA extraction and PCR amplification with reaction conditions and primer sequences identical to those used in the original studies employing these markers were used (Taberlet et al., 1991; Cronn et al., 2002; Savage & Cavender-Bares, 2012). As multiple copies of ADH were present in each individual except *Salix viminalis* L., the ADH PCR products were cloned to separate potential paralogs and hybrid sequences. *Populus tremula* partial ADH gene sequence, accession number AJ842900 (Ingvarsson, 2005), was downloaded from GenBank.

A proportion of *S. alba*, *S. cinerea*, *S. fragilis*, and *S. myrsinifolia* individuals exhibited hybrid origin of some of their ADH sequences. This trend was pronounced in individuals growing on the same site as their sibling species. Their sequences therefore did not form monophyletic lineages and the position of a proportion of them was reconstructed with high support as an internal group within the sibling species. As these species are known to frequently hybridize with their sibling species (Skvortsov, 1968), these sequences were considered of hybrid origin and such individuals were removed from analysis.

Sequences were assembled and edited using Geneious 5.4 (Drummond et al., 2011). Trees for individual genes were not in conflict, allowing us to reconstruct the host plant phylogeny based on a matrix with all examined loci combined. Host plant phylogeny was reconstructed using the Bayesian inference in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The generalized time reversible substitution model (GTR) selected using Akaike's information criterion (AIC) was used for Bayesian analysis with a flat Dirichlet prior probability density for the distribution of substitution rates and stationary nucleotide frequencies. Sampling was carried out every 10^3 generations for 10^7 generations, the first 25% of all generations were discarded as 'burnin' and the results were summarized with a 50% majority-rule consensus tree.

Insect sampling
In this study, we focused on leaf-chewing insects as one of the herbivore guilds causing the highest damage to willows. Sampling herbivores from one guild minimized the differences in feeding of examined herbivores, making the

results for different herbivores comparable. All adult and larval leaf-chewing insects were sampled during the 2008–2011 growing seasons, from the end of April to the end of September, at ca. 1-week intervals from the same tree individuals used for analysis of defense traits and their nearby conspecifics growing at the same locality (ca. 100 plants in total). Insects were sampled by sweeping and manually searching the foliage for free feeding as well as semi-concealed herbivores (leaf-tiers and leaf-rollers). Immature stages were reared to adults for identification. Dead larvae were morphotyped based on photographs, or discarded in cases when safe morphotyping proved to be impossible.

The sampling effort was equal for all plant individuals, represented by inspections that consisted of 3 min of sweeping and 3 min of manual searching. The sampling effort for different species was not completely balanced due to variation in willow population densities. For most species, the total sampling effort was 200–400 min; however, for the rare species *Salix pentandra* L. and *S. viminalis × purpurea* it was only 100 min.

Statistical analysis

All plant species, including willow hybrids and the single poplar species, were included in the analyses of defensive trait impact on insect diversity and population density. Willow hybrids were also included in the analyses of insect specialization on willows. These analyses are not focused on host plant evolutionary history and so the hybrid host plants can be considered independent data points with different defensive and herbivore traits. Both hybrids and the poplar species were excluded from the analysis of *Salix* defensive trait correlations, as *S. alba × fragilis* and *S. purpurea × viminalis* defensive traits patterns are products of hybridization, rather than evolution. Only non-

hybrid willow species containing salicylates were used in the analysis of salicylate content and salicylate diversity correlation.

Insect abundance was expressed as population density, i.e., the number of insects sampled per unit sampling time (in min). The diversity of herbivore communities on individual willow species was estimated by species accumulation curves based on Mao Tau index, computed in EstimateS 8.2 (Colwell, 2006), plotted against the number of tree inspections. The number of species found during 40 tree inspections (corresponding to the lowest number of inspections per tree species, achieved for *S. pentandra*) was used to quantitate the herbivore species diversity of each tree species.

The impact of salicylate diversity (measured as Simpson's index of individual salicylate components), salicylate concentration, condensed tannin concentration, flavonoid concentration, trichome density, and SLA on herbivorous insect diversity and population density was analyzed by linear regression, using all nine plant species and hybrids. A phylogenetic generalized least-squares (PGLS) model was employed to test this correlation within a phylogenetic context. The optimal model of evolution was selected between Brownian and Ornstein–Uhlenbeck models, using AIC. The test was performed using nlme 3.1 and ape 2.6 packages in R 2.10.1 (R Development Core Team, 2009; Pinheiro et al., 2010). Before analysis, all predictors were log-transformed to normalize their distributions.

Linear regression was used to test correlations between defensive traits within the genus *Salix*. All variables were log-transformed and all eight *Salix* species were used as individual data points. Phylogenetic generalized least-squares were employed and as optimal model of evolution

Table 1 Mean ($\pm$ SE) values of defensive traits in studied willow species and hybrids (Salicaceae)

Host plant species	Salicylate group[1]	Salicylates (mg g^{-1})	Salicylate diversity (Simpson's index)	Flavonoids (mg g^{-1})	Tannins (mg g^{-1})	SLA (cm^2 g^{-1})	Trichome cover (%)
Salix (*Vetrix*) *aurita*	LS	0.0	0	29.5 $\pm$ 1.1	196.7 $\pm$ 45.0	144.8 $\pm$ 27.0	19 $\pm$ 3.0
S. (*Vetrix*) *caprea*	LS	0.0	0	10.6 $\pm$ 1.0	139.8 $\pm$ 37.7	146.3 $\pm$ 31.8	26 $\pm$ 3.5
S. (*Vetrix*) *cinerea*	LS	0.0	0	15.0 $\pm$ 2.5	160.5 $\pm$ 62.2	131.5 $\pm$ 38.9	21 $\pm$ 2.1
S. (*Salix*) *fragilis*	HS	27.8 $\pm$ 9.4	0.79 $\pm$ 0.09	25.5 $\pm$ 6.5	51.9 $\pm$ 44.1	134.8 $\pm$ 32.7	0
S. (*Salix*) *pentandra*	HS	41.8 $\pm$ 21.5	0.65 $\pm$ 0.2	60.6 $\pm$ 6.3	192.2 $\pm$ 34.7	118.5 $\pm$ 39.6	0
S. (*Vetrix*) *purpurea*	HS	164.8 $\pm$ 19.1	0.67 $\pm$ 0.06	21.3 $\pm$ 1.4	42.6 $\pm$ 59.2	141.2 $\pm$ 39.8	0
S. (*Vetrix*) *rosmarinifolia*	HS	169.0 $\pm$ 42.0	0.64 $\pm$ 0.07	20.9 $\pm$ 0.4	134.3 $\pm$ 82.9	125.3 $\pm$ 29.9	14 $\pm$ 1.9
S. (*Vetrix*) *viminalis*	LS	0.0	0	16.0 $\pm$ 3.4	138.5 $\pm$ 35.9	165.8 $\pm$ 29.8	36 $\pm$ 7.3
S. alba × fragilis	LS	3.4 $\pm$ 1.9	0.49 $\pm$ 0.08	27.3 $\pm$ 4	127.3	105.3	2 $\pm$ 0.8
S. viminalis × purpurea	LS	2.6 $\pm$ 1.3	0	33.1 $\pm$ 3.8	112.3	160.4	0
Populus tremula L.	–	19.4 $\pm$ 20.7	0.34 $\pm$ 0.32	33.8 $\pm$ 5.9	37.9 $\pm$ 35.0	144.5 $\pm$ 76.9	0

SLA, specific leaf area.
[1]'LS' and 'HS' categories indicate willows with, respectively, low and high salicylate concentration and diversity.

was selected as above to test for correlation between defensive traits within a phylogenetic context.

In the analysis of salicylate impact on insect specialization, we divided willows into two groups – species with high salicylate diversity and concentration (high salicylate, 'HS' species) and species with low salicylate diversity and concentration (low salicylate, 'LS' species; Table 1). We used three host specificity indices, each measuring a different aspect of insect specialization. (1) The proportion of Salicaceae specialists, i.e., species feeding only on Salicaceae (based on Smreczyński, 1966, 1972; Lacourt, 1999; Warchalowski, 2003; Macek et al., 2007, 2008, 2012), was estimated for each *Salix* species and compared between those with low and high salicylate content by ANOVA with arc-sin data transformation. (2) Herbivore specialization on plant species containing salicylates was estimated for each herbivore as the Salicilate Specificity Index (SSI), measuring its distribution between high (HS) and low (LS) salicylate species as follows: mean density per HS species/(mean density per HS species + mean density per LS species) (see Table 1). The SSI values range from 1 for complete HS specialists, through 0.5 for herbivores indifferent to salicylate content, to 0 for complete LS specialists. The mean SSI values of their individual herbivores were compared between HS and LS willow species. (3) Host-range breadth within the Salicaceae was based on our sampling of herbivores. It was measured quantitatively using Simpson's index, capturing the density distribution for each herbivore species among the studied willow species. The herbivore community on each willow species was characterized by the mean host-range breadth, calculated as average value of host-range breadth for all its constituent species. Resulting values of specialization were compared between communities on willows with high and low salicylate content by ANOVA with arcsine data transformation applied to frequency values. Singletons and doubletons were excluded as uninformative from all host specificity analyses.

Results

Host plant phylogeny and defensive traits

The phylogram reconstructed based on Bayesian inference suggests monophyly of both examined willow subgenera, *Salix* and *Vetrix* (Figure 1). However, support for some clades is low, which complicates our interpretation of how defensive traits might have evolved. The most ambiguous is the position of *S. viminalis*, which often forms a monophyletic group with *Salix purpurea* L. and *S. rosmarinifolia*.

There was large interspecific variability in willow defensive traits (Table 1). Flavonoids and condensed tannins were found in leaves of all studied host plants, and the content of salicylates varied from 0 to 22% of leaf dry mass in young leaves among species (Figures 1A and S1, Table S1). The highest salicylate content and diversity was found in the leaves of rather basal *S. rosmarinifolia* and *S. purpurea*. Moderate diversity and content was found in *S. fragilis*, *S. pentandra*, and *P. tremula*, suggesting with high support at least two independent losses of salicylates (Figure 1B, Table 1).

In total, 108 flavonoid and 28 salicylate compounds and their derivatives were found (Table S1). Flavonoids and salicylates exhibited a high proportion of species-specific compounds, 56 and 46%, respectively (Figure S1). No compound was shared by all willow species. We observed
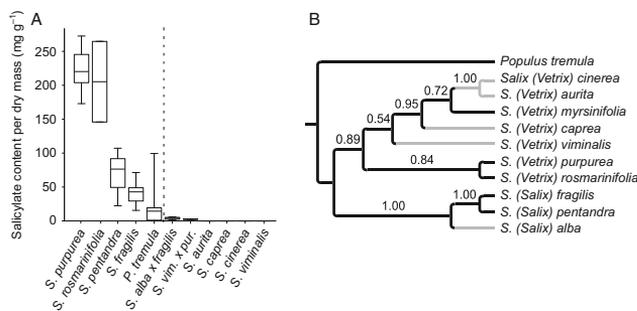

Figure 1 Salicylate content in studied *Salix* and *Populus* host plant species and hybrids and the distribution of salicylates throughout the willow phylogeny. (A) Salicylate content in young leaves. The boxes indicate the first to third quartiles with the medians as thick horizontal lines, the whiskers indicate ranges. The dashed line separates the five species with high salicylate content (on the left) from species with low salicylate content. (B) Phylogeny as reconstructed by Bayesian inference. The support of clades is characterized by posterior probabilities. Grey indicates lineages with low salicylate content, as estimated from our measurements and the literature (Julkunen-Tiitto, 1989).
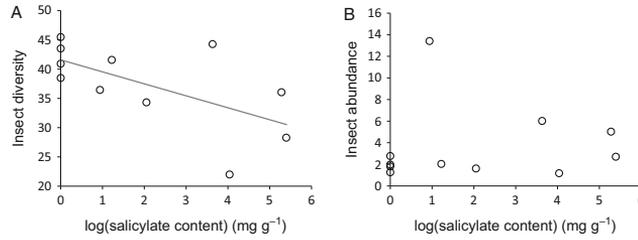
Figure 2 Impact of salicylates on insect communities. The correlation of salicylate content (mg g^{-1}) with the (A) diversity ($F_{2,9}$ = 7.37, P = 0.043) and (B) density ($F_{2,9}$ = 0.03, P = 0.87) of leaf-chewing insects. As measure of insect density was taken the number of insect individuals divided by sampling effort, expressed as the time (min) used for sampling insects. The diversity of herbivore communities was estimated using species accumulation curves based on Mao Tau index.

an increase in salicylates and flavonoids and a decrease in condensed tannins during the season. Nevertheless, the difference was quantitative rather than qualitative and the relative differences between species remained very similar (Table S2). The accumulation curves revealed that secondary metabolite analysis of a relatively low number of willow individuals is necessary to reach a plateau of diversity of willow secondary metabolites, making the estimates based even on only three plant individuals satisfactory (Figure S2).

Defensive traits were not correlated between plant species (Table S3) except for a negative correlation between salicylate content and trichome density ($F_{1,6}$ = 5.52, P = 0.043 for linear regression, $F_{1,6}$ = 4.98, P = 0.035 for PGLS) and salicylate diversity and trichome density ($F_{1,6}$ = 20.29, P = 0.004 for linear regression, $F_{1,6}$ = 18.36, P = 0.003 for PGLS).

Herbivore diversity and population density

We collected 9 196 individuals from 201 species of leaf-chewing insects, representing adult beetles and their larvae, caterpillars, and sawfly larvae from a total of 21 families

(Table 2). From the host plant defense traits studied, only salicylate content had a significant negative impact on herbivore diversity ($F_{2,9}$ = 7.37, P = 0.043 for linear regression, $F_{2,9}$ = 7.98, P = 0.020 for PGLS; Figure 2, Table S4), whereas the impact of salicylate diversity (measured as Simpson's index of individual salicylate components) was not significant ($F_{2,9}$ = 2.46, P = 0.15 for linear regression, $F_{2,9}$ = 1.74, P = 0.23 for PGLS). Neither salicylate content nor any other defensive trait exhibited a significant effect on overall insect herbivore density (Figure 2, Table S4). We carried out separate analyses of effect on insect density for all 28 salicylates and their derivatives, but none of the examined compounds exhibited a significant or marginally significant impact on herbivore density or diversity.

Insect specialization

We found salicylates to influence insect specialization. Communities harbored by willows with high salicylate content exhibited a higher ratio of Salicaceae specialists to generalists (Figure 3). On high-salicylate willows, herbivores were to some extent specialized on these high-salicy-

Table 2 The total number of species/ Salicaceae-specialist species (feeding only on Salicaceae) sampled from insect herbivore families

| Lepidoptera		Coleoptera		Hymenoptera	
Arctiidae	3/0	Attelabidae	3/1	Argidae	2/1
Depressariidae	2/2	Cerambycidae	2/0	Tenthredinidae	52/49
Drepanidae	1/1	Chrysomelidae	24/12		
Gelechiidae	2/2	Curculionidae	30/17		
Geometridae	27/6	Scarabaeidae	1/0		
Lymantridae	4/0	Tenebrionidae	1/0		
Noctuidae	25/6				
Nolidae	2/1				
Notodontidae	8/4				
Pyralidae	1/1				
Saturniidae	1/1				
Sphingidae	1/1				
Tortricidae	7/2				
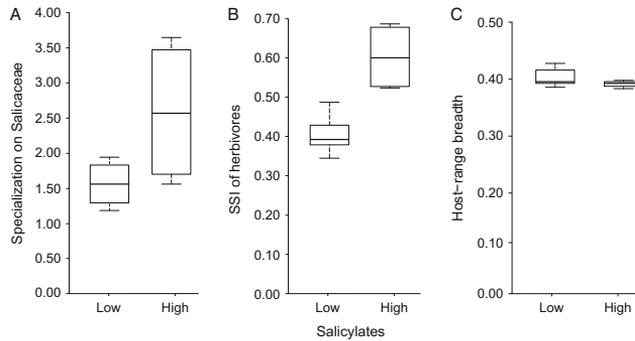
Figure 3 Impact of salicylates on insect specialization. (A) Ratio of Salicaceae specialist-to-generalist species, (B) SSI (i.e., Salicylate Specificity Index, indicating specialization on willow species containing salicylates), and (C) host specificity of herbivore species (host-range breadth) on willows with high and low salicylate content. Ratio of specialist-to-generalist species (A) and SSI (B) were significantly different on host species with high vs. low salicylate content ($F_{1,7} = 7.08$, $P = 0.029$ and $F_{1,7} = 72.14$, $P = 0.002$, respectively), whereas there was no difference in host-range breadth (C; $F_{1,7} = 1.54$, $P = 0.25$). The boxes indicate the first to third quartiles with the medians as thick horizontal lines, the whiskers indicate ranges.

late hosts (SSI>0.5), and vice versa, the herbivore species on low salicylate willows were specialized on low-salicylate hosts (SSI<0.5; Figure 3). In either case, this specialization was not absolute, as indicated by the SSI values ≫0 and ≪1, respectively. Separate analyses for Coleoptera, Lepidoptera, and Hymenoptera produced a similar trend of SSI (Figure S3). On the other hand, the host specificity of herbivore species within the examined set of host plants did not differ between herbivore communities from willows with low and high salicylate content (Figure 3).

Within the studied set of eight *Salix* species, generalist herbivores used the same number of host species as Salicaceae specialists ($F_{1,7} = 2.16$, $P = 0.15$). However, we found major differences between Coleoptera, Lepidoptera, and Hymenoptera when insect lineages were analyzed separately. Salicaceae specialists used significantly more host species than generalists within both Coleoptera and Lepidoptera (Figure S4), whereas the generalists were almost entirely lacking in Hymenoptera (Table 2).

Discussion

Salicylates, which represent a group of secondary metabolites unique for Salicaceae, had the most pronounced impact of all examined defensive traits, affecting diversity of leaf-chewing herbivores and some characteristics of host specialization. Communities on willows with high salicylate content were most specialized, with the majority of herbivores being known to feed solely on the Salicaceae family and preferring a diet containing salicylates. The

high number of species feeding only on Salicaceae suggests that a certain level of specialization is needed to overcome high salicylate content.

Salicylates have been reported to have various negative effects on generalist herbivores (Matsuki & Maclean, 1994; Rank et al., 1998). However, only a few previous studies attempted to examine the effect of willow salicylates at insect community level (Topp et al., 2002). We demonstrate that high salicylate content causes partial exclusion of generalist herbivores, resulting in less diverse communities on salicylate-rich hosts. Salicylates therefore pose an effective feeding barrier for many generalist species otherwise common on willows lacking these secondary metabolites.

None of the other examined defensive traits had a significant impact on leaf-chewing insects, although their detrimental effect on certain insect species' feeding or survival has been repeatedly recorded (Raupp, 1985; Zvereva et al., 1998; Kopper et al., 2002; Lahtinen et al., 2006). Similar situations in which recognized defensive traits, such as trichomes or latex outflow, do not affect insect population densities have been recorded before (Basset & Novotny, 1999). The lack of any impact may indicate that certain herbivores are able to cope with specific defensive traits and hence compensate for partial exclusion of non-adapted species under field conditions.

Salicylates thus play a major role in forming insect communities on willows. Pronounced impact of salicylates, especially when compared with the effect of other examined defensive traits, implies that some insect species have

not been able to adapt to salicylates. This could be due to the scarce distribution of salicylates among plants, which is in congruence with the biochemical barrier theory (Jones & Lawton, 1991), suggesting that generalists are excluded from insect communities harbored by plants with unique or highly toxic secondary metabolites, as found by previous studies (Becerra, 1997; Agrawal, 2005).

Salicylate-rich willows harbored a higher proportion of specialists feeding only on the Salicaceae family, showing that high salicylate content narrows the total host range of associated herbivores. On the other hand, salicylates did not have any negative effect on insect relative host ranges within the examined set of willow species. The willows with high salicylate content thus harbored insects feeding on the same proportion of examined willow species as their salicylate-poor relatives. Moreover, Salicaceae specialists in both Coleoptera and Lepidoptera used more willow species, within the examined set of willows, than herbivores feeding also on other families. This implies that Salicaceae specialists are better at using a variety of willows, whereas generalists may be confined only to particular willow species.

Total insect density was unaffected by salicylate content and diversity. Specialist willow herbivores have even been reported to benefit from salicylates, using them for the production of defensive compounds (Pasteels et al., 1983; Denno et al., 1990) or possibly as a source of glucose (Rowell-Rahier & Pasteels, 1986; Rank et al., 1998). These specialists can thus reach very high population densities on willows with high salicylate content. They can be much more abundant on high salicylate species than generalist insect herbivores on willows with no salicylates. This would explain the observed situation in which there is no significant impact of salicylates on the total insect abundance.

The low protective value of salicylates against specialized insects is also suggested by other local studies throughout Europe and North America, which found high population densities of specialized herbivores on willows with high salicylate content (Denno et al., 1990; Kolehmainen et al., 1995; Martinsen et al., 1998). The specialists' density appears to be driven by nitrogen content or leaf quality in such cases (Nakamura et al., 2005). Salicylates thus appear to be to a large extent ineffective against the majority of specialized herbivores associated with willows throughout their geographic ranges.

Production of salicylates requires a large investment of energy, as the total salicylate content can reach up to 22% of dry leaf mass in the early stages of leaf development. In *S. purpurea* and *S. rosmarinifolia*, the energy allocated to salicylates seems to be higher than the allocation to condensed tannins and flavonoids combined (Gershenzon,

1994). Such considerable investment has lead to a trade-off between salicylate production and plant growth (Osier & Lindroth, 2001). Although a high concentration of salicylates negatively influences communities of generalist herbivores, it has no impact or even a positive influence on specialists and no overall impact on total herbivore abundance.

Low protective value of salicylates against specialized herbivores and the high energy allocation required for their synthesis may have led to the loss of salicylates in some willow lineages. Although some willow species contain very low to zero concentrations of salicylates (Julkunen-Tiitto, 1989; Nyman & Julkunen-Tiitto, 2005), the possession of salicylates appears to be an ancestral state within the genus *Salix*, with salicylates being widespread among poplars, the sister genus of willows (Palo, 1984; Leskinen & Alstrom-Rapaport, 1999). The absence of salicylates in some of the derived lineages within the genus *Salix* thus appears to be secondary. Our interpretation of salicylate evolution within the genus *Salix* is complicated by low support for some clades and the limited extent of our dataset. Additional plant species would be required to describe salicylate evolution in willows. Although we cannot document the exact course of salicylate evolution, our results, along with previous studies documenting the lack of salicylates in many willow species (Julkunen-Tiitto, 1989; Nyman & Julkunen-Tiitto, 2005), suggest that willows lost salicylates repeatedly during their evolution – at least 2 or 3× during the evolution of the willow species we studied.

A negative correlation of content and diversity of salicylates with density of trichomes suggests that willows lacking salicylates may rely more on mechanical defenses. Although we failed to find any negative correlation between trichome density and insect population density in this study, willow trichomes have been reported to influence both Salicaceae specialists and generalists, making them potentially effective against a broad spectrum of herbivores (Matsuki & Maclean, 1994; Zvereva et al., 1998).

Moreover, divergence in defensive traits may help related species growing in sympatry to avoid herbivory. As many insect herbivores are phylogenetically conservative in their food choice (Schoonhoven et al., 2005), host-shifts among related plants with similar defense are very likely. Large interspecific differences among sibling species may make these shifts less common. Induced defense, pronounced in many willow species (e.g., Nakamura et al., 2005), which also increases variation in plant defense, may play a similar role. In turn, related species often exhibit diverging defense strategies (e.g., Agrawal & Fishbein, 2008; Fincher et al., 2008) and herbivory was reported to bias community assembly toward chemical heterogeneity

(Becerra, 2007). In willows, benefits brought by a large variation in defensive traits may have resulted in the observed situation in which some species are defended by salicylates and some by trichomes, which may be another factor driving the divergence in willow defenses and in turn enhancing the selection against salicylates in some species.

In conclusion, our results show that salicylates do not lower insect abundance in the local communities we studied. We suggest that the lack of effect on insect herbivore abundance may be one of the factors driving the loss of host plant defensive traits. In the case of willows, several lineages of highly specialized leaf-chewing herbivores were able to adapt to salicylates, making the required high energetic allocation to this defensive trait costly. Diversification of salicylates might have met a dead end and certain willow lineages may presumably use the energy saved for maintaining other strategies of defense. Although plant defenses have probably diversified during the course of plant evolution (Becerra et al., 2009), our results suggest that certain defensive traits might be lost or reduced in insect-plant systems with high insect specialization and high defense costs, as in willows or *Asclepias* spp. (Agrawal & Fishbein, 2008). These findings thus illustrate that evolution of plant defensive traits is a dynamic process rather than a simple directional trend.

Acknowledgements

References

Agrawal AA (2005) Natural selection on common milkweed (*Asclepias syriaca*) by a community of specialized insect herbivores. Evolutionary Ecology Research 7: 651–667.

Agrawal AA & Fishbein M (2008) Phylogenetic escalation and decline of plant defense strategies. Proceedings of the National Academy of Sciences of the USA 105: 10057–10060.

Agrawal AA, Hastings AP, Johnson MT, Maron JL & Salminen J-P (2012) Insect herbivores drive real-time ecological and evolutionary change in plant populations. Science 338: 113–116.

Ali JG & Agrawal AA (2012) Specialist versus generalist insect herbivores and plant defense. Trends in Plant Science 17: 293–302.

Basset Y & Novotny V (1999) Species richness of insect herbivore communities on *Ficus* in Papua New Guinea. Biological Journal of the Linnean Society 67: 477–499.

Becerra JX (1997) Insects on plants: macroevolutionary chemical trends in host use. Science 276: 253–256.

Becerra JX (2007) The impact of herbivore-plant coevolution on plant community structure. Proceedings of the National Academy of Sciences of the USA 104: 7483–7488.

Becerra JX, Noge K & Venable DL (2009) Macroevolutionary chemical escalation in an ancient plant-herbivore arms race. Proceedings of the National Academy of Sciences of the USA 106: 18062–18066.

Benderoth M, Textor S, Windsor AJ, Mitchell-Olds T, Gershenzon J & Kroymann J (2006) Positive selection driving diversification in plant secondary metabolism. Proceedings of the National Academy of Sciences of the USA 103: 9118–9123.

Christensen S, Heimes C, Agerbirk N, Kuzina V, Olsen CE & Hauser TP (2014) Different geographical distributions of two chemotypes of *Barbarea vulgaris* that differ in resistance to insects and a pathogen. Journal of Chemical Ecology 40: 491–501.

Coley PD, Lokvam J, Rudolph K, Bromberg K, Sackett TE et al. (2005) Divergent defensive strategies of young leaves in two species of *Inga*. Ecology 86: 2633–2643.

Colwell RK (2006) Statistical Estimation of Species Richness and Shared Species from Samples, version 8. Available at: http://wwwpurtloclcorg/estimates.

Connahs H, Aiello A, Van Bael S & Rodriguez-Castaneda G (2011) Caterpillar abundance and parasitism in a seasonally dry versus wet tropical forest of Panama. Journal of Tropical Ecology 27: 51–58.

Cronn RC, Small RL, Haselkorn T & Wendel JF (2002) Rapid diversification of the cotton genus (*Gossypium*: Malvaceae) revealed by analysis of sixteen nuclear and chloroplast genes. American Journal of Botany 89: 707–725.

Denno RF, Larsson S & Olmstead KL (1990) Role of enemy-free space and plant quality in host-plant selection by willow beetles. Ecology 71: 124–137.

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A et al. (2011) Geneious v54. Available at: http://www.geneious.com.

Dyer LA, Singer MS, Lill JT, Stireman JO, Gentry GL et al. (2007) Host specificity of Lepidoptera in tropical and temperate forests. Nature 448: 696–699.

Ehrlich PR & Raven PH (1964) Butterflies and plants – a study in coevolution. Evolution 18: 586–608.

Fincher RM, Dyer LA, Dodson CD, Richards JL, Tobler MA et al. (2008) Inter- and intraspecific comparisons of antiherbivore

defenses in three species of rainforest understory shrubs. Journal of Chemical Ecology 34: 558–574.

Fucile G, Falconer S & Christendat D (2008) Evolutionary diversification of plant shikimate kinase gene duplicates. PLoS Genetics 4: e1000292.

Gershenzon J (1994) The cost of plant chemical defense against herbivory: a biochemical perspective. Insect-Plant Interactions, Vol. 5 (ed. by EA Bernays), pp. 105–173. CRC Press, Boca Raton, FL, USA.

Groom PK & Lamont BB (1999) Which common indices of sclerophylly best reflect differences in leaf structure? Ecoscience 6: 471–474.

Hagerman AE (2002) The Tannin Handbook. Miami University, Oxford, OH, USA.

Ingvarsson PK (2005) Nucleotide polymorphism and linkage disequilibrium within and among natural populations of European Aspen (*Populus tremula* L., Salicaceae). Genetics 169: 945–953.

Jones CG & Lawton JH (1991) Plant chemistry and insect species richness of British umbellifers. Journal of Animal Ecology 60: 767–777.

Julkunen-Tiitto R (1989) Phenolic constituents of *Salix* – a chemotaxonomic survey of further Finnish species. Phytochemistry 28: 2115–2125.

Julkunen-Tiitto R & Sorsa S (2001) Testing the effects of drying methods on willow flavonoids, tannins, and salicylates. Journal of Chemical Ecology 27: 779–789.

Kliebenstein DJ & Osbourn A (2012) Making new molecules – evolution of pathways for novel metabolites in plants. Current Opinion in Plant Biology 15: 415–423.

Kolehmainen J, Julkunen-Tiitto R, Roininen H & Tahvanainen J (1995) Phenolic glucosides as feeding cues for willow-feeding leaf beetles. Entomologia Experimentalis et Applicata 74: 235–243.

Kopper BJ, Jakobi VN, Osier TL & Lindroth RL (2002) Effects of paper birch condensed tannin on whitemarked tussock moth (Lepidoptera: Lymantriidae) performance. Environmental Entomology 31: 10–14.

Kozlov MV, van Nieukerken EJ, Zverev V & Zvereva EL (2013) Abundance and diversity of birch-feeding leafminers along latitudinal gradients in northern Europe. Ecography 36: 1138–1149.

Kursar TA, Dexter KG, Lokvam J, Pennington RT, Richardson JE et al. (2009) The evolution of antiherbivore defenses and their contribution to species coexistence in the tropical tree genus *Inga*. Proceedings of the National Academy of Sciences of the USA 106: 18073–18078.

Lacourt J (1999) Répertoire des Tenthredinidae Ouest-Paléarctiques (Hymenoptera, Symphyta). Société Entomologique de France, Paris, France.

Lahtinen M, Kapari L, Haukioja E & Pihlaja K (2006) Effects of increased content of leaf surface flavonoids on the performance of mountain birch feeding sawflies vary for early and late season species. Chemoecology 16: 159–167.

Leskinen E & Alstrom-Rapaport C (1999) Molecular phylogeny of Salicaceae and closely related Flacourtiaceae: evidence from 5.8 S, ITS 1 and ITS 2 of the rDNA. Plant Systematics and Evolution 215: 209–227.

Macek J, Dvorak J, Traxler L & Cervenka V (2007) Noční Motýli I. Academia, Prague, Czech Republic.

Macek J, Dvorak J, Traxler L & Cervenka V (2008) Noční Motýli II. Academia, Prague, Czech Republic.

Macek J, Prochazka J & Traxler L (2012) Noční Motýli III. Academia, Prague, Czech Republic.

Martinsen GD, Driebe EM & Whitham TG (1998) Indirect interactions mediated by changing plant chemistry: beaver browsing benefits beetles. Ecology 79: 192–200.

Matsuki M & Maclean SF (1994) Effects of different leaf traits on growth rates of insect herbivores on willows. Oecologia 100: 141–152.

Nakamura M, Utsumi S, Miki T & Ohgushi T (2005) Flood initiates bottom-up cascades in a tri-trophic system: host plant regrowth increases densities of a leaf beetle and its predators. Journal of Animal Ecology 74: 683–691.

Nybakken L, Horkka R & Julkunen-Tiitto R (2012) Combined enhancements of temperature and UVB influence growth and phenolics in clones of the sexually dimorphic *Salix myrsinifolia*. Physiologia Plantarum 145: 551–564.

Nyman T & Julkunen-Tiitto R (2005) Chemical variation within and among six northern willow species. Phytochemistry 66: 2836–2843.

Osier TL & Lindroth RL (2001) Effects of genotype, nutrient availability, and defoliation on aspen phytochemistry and insect performance. Journal of Chemical Ecology 27: 1289–1313.

Palo RT (1984) Distribution of birch (*Betula* spp.), willow (*Salix* spp.), and poplar (*Populus* spp.) secondary metabolites and their potential role as chemical defense against herbivores. Journal of Chemical Ecology 10: 499–520.

Pasteels JM, Rowell-Rahier M, Braekman JC & Dupont A (1983) Salicin from host plant as precursor of salicylaldehyde in defensive secretion of chrysomeline larvae. Physiological Entomology 8: 307–314.

Pearse IS (2011) The role of leaf defensive traits in oaks on the preference and performance of a polyphagous herbivore, *Orgyia vetusta*. Ecological Entomology 36: 635–642.

Pinheiro J, Bates D, DebRoy S, Sarkar D & Team RDC (2010) nlme: Linear and Nonlinear Mixed Effects Models, R package version 3.1-97. R Foundation for Statistical Computing, Vienna, Austria.

R Development Core Team (2009) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Rank NE, Kopf A, Julkunen-Tiitto R & Tahvanainen J (1998) Host preference and larval performance of the salicylate-using leaf beetle *Phratora vitellinae*. Ecology 79: 618–631.

Raupp MJ (1985) Effects of leaf toughness on mandibular wear of the leaf beetle, *Plagiodera versicolora*. Ecological Entomology 10: 73–79.

Ronquist F & Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Rowell-Rahier M & Pasteels JM (1986) Economics of chemical defense in Chrysomelinae. Journal of Chemical Ecology 12: 1189–1203.

Savage JA & Cavender-Bares J (2012) Habitat specialization and the role of trait lability in structuring diverse willow (genus *Salix*) communities. Ecology 93: S138–S150.

Schoonhoven LM, van Loon JJA & Dicke M (2005) Insect-Plant Biology, 2nd edn. Oxford University Press, Oxford, UK.

Skvortsov AK (1968) Willows of the USSR: A Taxonomic and Geographic Revision. Nauka, Moscow, Russia.

Smreczyński S (1966) Klucze do Oznaczania Owadów Polski XIX: Chrząszcze – Coleoptera Zeszyt 98 b, Ryjkowce – Curculionidae, Podrodziny Otiorhynchinae, Brachyderinae. Państwowe Wydawnictwo Naukove, Warsaw, Poland.

Smreczyński S (1972) Klucze do Oznaczania Owadów Polski XIX: Chrząszcze – Coleoptera. Zeszyt 98 d, Ryjkowce – Curculionidae, Podrodzina Curculioninae, Plemiona Dryophthorini, Cossonini, Bagoini, Tanysphyrini, Notarii, Smicronychini, Ellescini, Acaliptini, Tychiini, Anthonomini, Curculiononi, Pissodini, Magdalini, Trachodini, Rhynchophorini, Cryptorhynchini. Panstvove Wydawnictwo Naukove, Warsaw, Poland.

Taberlet P, Gielly L, Pautou G & Bouvet J (1991) Universal primers for amplification of three noncoding regions of chloroplast DNA. Plant Molecular Biology 17: 1105–1109.

Topp W, Kulfan J, Zach P & Nicolini F (2002) Beetle assemblages on willow trees: do phenolic glycosides matter? Diversity and Distributions 8: 85–106.

Warchalowski A (2003) Chrysomelidae: The Leaf Beetles of Europe and the Mediterranean Area. Natura Optima Dux Foundation, Warsaw, Poland.

Zvereva EL, Kozlov MV & Niemela P (1998) Effects of leaf pubescence in *Salix borealis* on host-plant choice and feeding behaviour of the leaf beetle *Melasoma lapponica*. Entomologia Experimentalis et Applicata 89: 297–303.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Number of flavonoids and salicylates found in examined *Salix* and *Populus* host plants. Grey indicates species-unique compounds.

Figure S2. Secondary metabolite diversity accumulation curves for *Salix cinerea* (black) and *Salix fragilis* (gray) based on Mao Tau index plotted against number of individuals analyzed.

Figure S3. Specialization on diet containing salicylates by examined insect taxa on willows with high and low salicylate content. (A) Coleoptera ($F_{1,41}$ = 32.39, P<0.001), (B) Lepidoptera ($F_{1,39}$ = 11.30, P = 0.010), and (C) Hymenoptera ($F_{1,32}$ = 15.79, P = 0.004). The boxes indicate the first to third quartiles with the median as thick horizontal lines, the whiskers indicate ranges.

Figure S4. Number of willow host plant species used by Salicaceae specialists and generalists. There was no difference in the number of willow species used between Salicaceae specialists and generalists ($F_{1,114}$ = 2.02, P = 0.16), but separate analyses for Coleoptera and Lepidoptera revealed more willow hosts in Salicaceae specialists than in generalists (Coleoptera: $F_{1,41}$ = 7.699, P<0.01; Lepidoptera: $F_{1,39}$ = 6.066, P = 0.018). Hymenoptera, including highly specialized sawflies, exhibited the narrowest host spectra. For Hymenoptera the specialist/generalist spectrum breadth difference was not analyzed, as there was only one generalist species with more than two individuals present in our samples. The boxes indicate the first to third quartiles with the medians as thick horizontal lines, the whiskers indicate ranges.

Table S1. Mean concentrations (mg g^{-1}) of secondary metabolites found in examined *Salix* and *Populus* host plant species and hybrids.

Table S2. Seasonal variability in secondary metabolite content (mg g^{-1}) and salicylate diversity (Simpson's index). The samples for analysis were obtained in early June ('spring') and early August ('summer').

Table S3. Correlation between defensive traits of studied willow species ($F_{1,6}$/P values, based on PGLS and linear regression). The lower triangle shows values obtained from analysis using phylogenetic generalized least squares, the upper triangle shows values obtained from simple linear regression.

Table S4. Impact of *Salix* defensive traits on leaf-chewing insect diversity and density. Table includes $F_{2,9}$/P-values, based on simple linear regression (Linear) and phylogenetic generalized least squares (PGLS).

Supporting Information
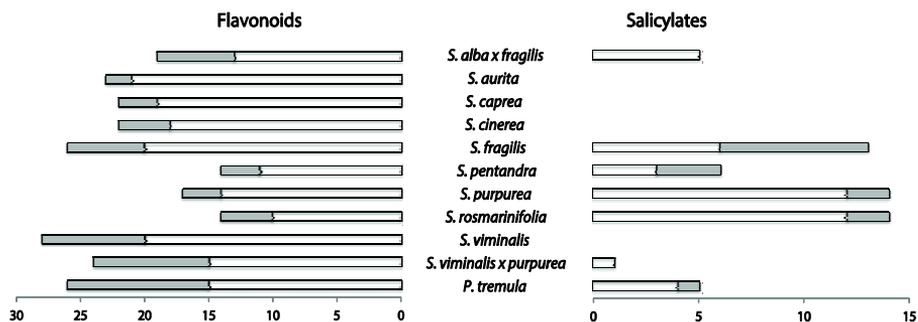
Figure S1. Number of salicylates and flavonoids found in examined host-plants. Grey color indicates species-unique compounds.
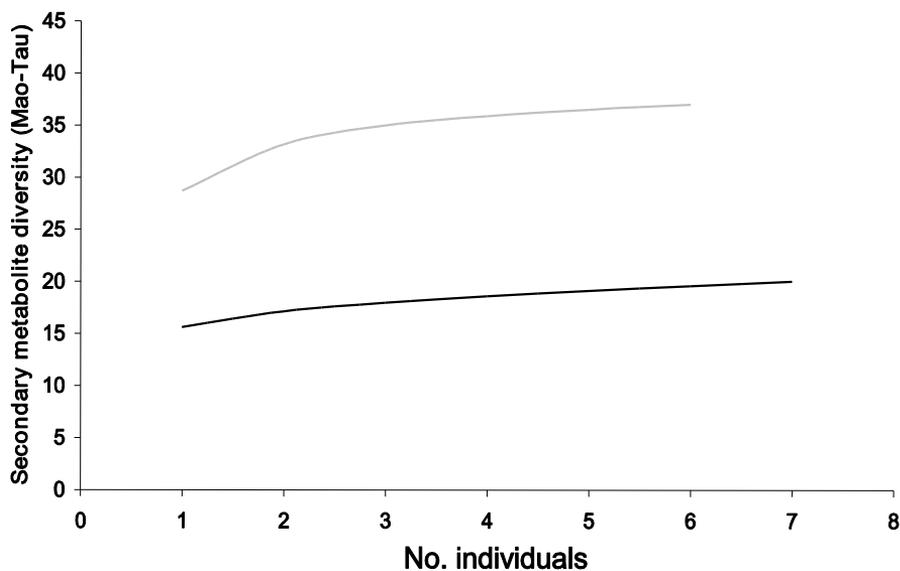

Figure S2. Secondary metabolite diversity accumulation curves for *Salix cinerea* (black) and *S. fragilis* (grey) based on Mao Tau index plotted against number of individuals analyzed.
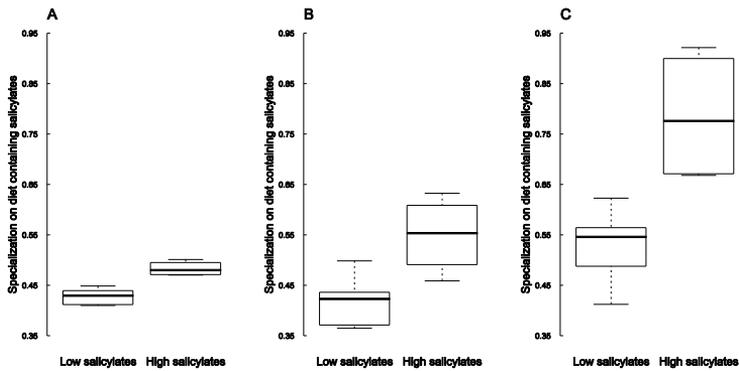
Figure S3. Specialization on diet containing salicylates by examined insect taxa on willows with high and low salicylate content. A: Coleoptera ($F_{(1,41)}$=32.39, p<0.001), B: Lepidoptera ($F_{(1,39)}$=11.30, p=0.010), C: Hymenoptera ($F_{(1,32)}$=15.79, p=0.004). The box shows the first to third quartile with the median as a horizontal line, the whiskers show range.
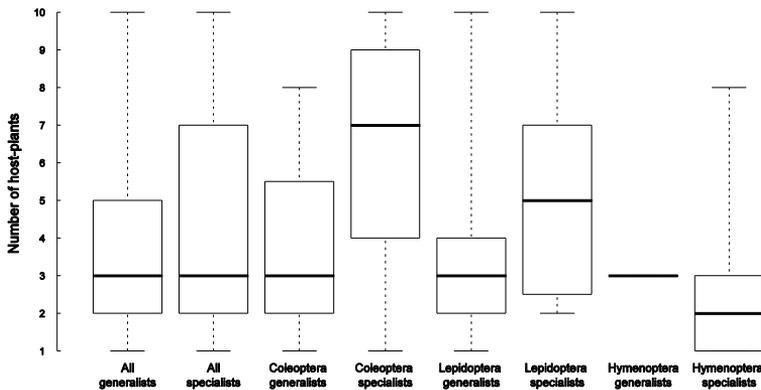

Figure S4. Number of willow host plant species used by Salicaceae specialists and generalists. There was no difference in the number of used willow species between Salicaceae specialists and generalists ($F_{(1,114)}$= 2.02, p=0.158), but separate analysis for Coleoptera and Lepidoptera revealed significantly high number of willow hosts in Salicaceae specialists than generalists (D) (Coleoptera: $F_{(1,41)}$=7.6989, p>0.01; Lepidoptera: $F_{(1,39)}$=6.066, p=0.018). Hymenoptera, including highly specialized sawflies, exhibited the narrowest host-spectra. For Hymenoptera the specialist/generalist spectra breadth difference was not analysed, since there was only one generalist species with more than two individuals present in our samples. The box shows the first to third quartile with the median as a horizontal line, the whiskers show range.

Table S1. List of secondary metabolites found in examined host-plants. Values indicate mean concentrations (mg/g).

	S. alb x fra	S. aur	S. cap	S. cin	S. fra	S. pen	S. pur	S. ros	S. vim	S. vim x pur	P. tre
Salicylates											
2`-O-acetylsalicin	-	-	-	-	-	5.603	-	-	-	-	-
acetyl-salicortin	-	-	-	-	-	21.144	-	-	-	-	2.227
cinnamoyl acetyl-salicortin	-	-	-	-	-	0.341	-	-	-	-	-
cinnamoyl salicylate 1	-	-	-	-	-	-	-	4.137	-	-	-
cinnamoyl salicylate 2	-	-	-	-	-	-	1.898	-	-	-	-
cinnamoyl salicylate 3	-	-	-	-	-	-	1.457	-	-	-	-
cinnamoyl salicylate 4	-	-	-	-	-	-	-	0.081	-	-	-
cinnamoyl salicortin	-	-	-	-	-	-	-	-	-	-	0.617
cinnamoyl tremulacin	-	-	-	-	0.080	-	-	-	-	-	-
cinnamoyl tremuloidin	-	-	-	-	3.212	-	-	-	-	-	-
disalicortin	-	-	-	-	-	-	4.349	3.327	-	-	-
ditremulacin derivative 1	-	-	-	-	-	-	2.039	1.059	-	-	-
ditremulacin derivative 2	-	-	-	-	-	-	2.138	0.999	-	-	-
ditremulacin derivative 3	-	-	-	-	-	-	0.417	0.224	-	-	-
ditremulacin derivative 4	-	-	-	-	0.240	-	1.329	2.081	-	-	-
HCH-acetyl-salicortin	-	-	-	-	-	10.658	-	-	-	-	-
HCH-salicortin	-	-	-	-	0.608	-	-	-	-	-	-
HCH-tremulacin derivative 1	-	-	-	-	-	-	0.702	0.507	-	-	-
HCH-tremulacin derivative 2	-	-	-	-	-	-	0.801	0.537	-	-	-

	S. alb x fra	S. aur	S. cap	S. cin	S. fra	S. pen	S. pur	S. ros	S. vim	S. vim x pur	P. tre
salicin	0.680	-	-	-	1.161	3.231	13.999	15.047	-	1.636	2.847
salicortin	0.001	-	-	-	3.468	-	70.265	88.099	-	-	3.980
salicyl alcohol	-	-	-	-	0.089	-	2.165	3.763	-	-	-
salicyl alcohol-diglucoside	1.453	-	-	-	2.386	-	-	-	-	-	-
tremulacin	0.138	-	-	-	10.330	-	60.361	47.421	-	-	9.773
tremulacin derivative 1	0.001	-	-	-	0.960	-	-	-	-	-	-
tremulacin derivative 2	-	-	-	-	0.035	-	-	-	-	-	-
tremulacin derivative 3	-	-	-	-	0.595	0.769	1.299	1.758	-	-	-
tremuloidin	-	-	-	-	4.599	-	-	-	-	-	-
Flavonoids											
(+)-catechin	-	7.785	0.584	4.184	-	-	1.371	1.861	2.838	1.214	1.344
apigenin 5-glucoside	-	-	-	-	-	-	-	0.467	-	-	-
apigenin 7-glucoside	-	-	-	0.313	-	-	0.483	0.342	0.050	0.349	-
apigenin derivative 1	-	-	-	-	-	-	-	0.026	-	-	-
apigenin derivative 2	-	-	-	-	-	-	-	-	-	-	0.078
apigenin derivative 3	-	-	-	-	-	-	0.153	-	-	-	-
apigenin derivative 4	-	-	-	0.016	-	-	-	-	-	-	-
apigenin derivative 5	-	-	-	0.005	-	-	-	-	-	-	-
chlorogenic acid	8.132	2.605	-	1.266	8.861	23.361	-	-	0.736	-	2.459
chlorogenic acid derivative 1	-	-	-	-	2.181	-	-	-	0.197	-	-
chlorogenic acid derivative 2	0.150	-	-	-	0.132	-	-	-	-	-	-

	S. alb x fra	S. aur	S. cap	S. cin	S. fra	S. pen	S. pur	S. ros	S. vim	S. vim x pur	P. tre
chlorogenic acid derivative 3	-	-	-	0.215	-	-	-	-	-	-	-
chrysoeriol derivative 1	-	-	-	0.057	-	-	-	-	-	-	-
chrysoeriol derivative 2	-	-	-	-	-	-	-	-	-	-	0.048
chrysoeriol glycoside	-	-	-	-	-	-	3.455	-	-	3.884	-
cinnamic acid derivative 1	0.134	-	-	-	-	-	-	-	-	-	-
cinnamic acid derivative 2	0.019	-	-	-	0.104	-	-	-	-	-	-
cinnamic acid derivative 3	-	-	-	-	-	-	-	-	-	-	1.094
dicoumaroyl flavonol	-	0.010	0.016	0.104	-	-	-	-	-	-	-
dihydromyricetin	-	-	-	-	-	-	-	-	1.370	-	-
dihydroquercetin	-	-	-	-	-	2.390	-	-	-	-	-
dihydrokaempferol	-	-	-	-	-	-	0.140	-	-	-	-
eriodictyol 7-glucoside	-	-	-	-	-	-	2.444	-	-	5.790	-
eriodictyol aglycon derivative 1	-	-	-	-	-	-	-	-	-	1.219	-
eriodictyol aglycon derivative 2	-	-	-	-	-	-	-	-	-	1.640	-
eriodictyol derivative 1	-	-	-	-	-	-	-	-	-	0.752	-
eriodictyol derivative 2	-	-	-	-	-	-	-	-	-	0.249	-
eriodictyol diglycoside 1	-	-	-	-	-	-	2.086	-	-	1.504	-
eriodictyol diglycoside 2	-	-	-	-	-	-	0.601	-	-	1.524	-
eriodictyol diglycoside 3	-	-	-	-	-	-	-	-	-	2.361	-
eriodictyol glycoside	-	-	-	-	-	-	-	-	-	0.483	-
flavonoid diglucoside	-	-	-	-	-	-	-	-	-	-	0.405
hyperin	-	-	-	-	-	3.029	-	-	-	-	1.391

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11
isorhamnetin aglycon derivative 1	0.104	-	-	-	-	-	-	-	-	-	-
isorhamnetin aglycon derivative 2	0.703	-	-	-	-	-	-	-	-	-	-
isorhamnetin derivative 1	-	-	-	-	0.212	-	-	-	-	-	-
isorhamnetin derivative 2	-	-	-	-	-	-	-	-	0.784	0.806	-
isorhamnetin derivative 3	-	-	-	-	-	-	-	-	0.909	-	0.162
isorhamnetin derivative 4	0.473	-	-	-	-	-	-	-	-	-	-
isorhamnetin glycoside 1	0.866	-	-	-	1.548	-	-	-	-	-	-
isorhamnetin glycoside 2	-	-	-	-	1.001	-	-	-	-	-	-
isorhamnetin rhamnoside	2.152	-	-	-	-	-	-	-	0.633	-	-
kaempferol 3-arabinoside	-	-	-	-	1.126	-	-	-	0.064	-	-
kaempferol 3-glucoside	-	-	-	-	0.064	0.395	-	-	0.109	-	2.763
kaempferol 3-rhamnoside	-	-	-	-	-	-	-	-	-	0.401	-
kaempferol glycoside derivative 1	-	-	-	-	1.508	-	-	-	-	-	-
kaempferol glycoside derivative 2	-	-	-	-	-	-	-	-	-	-	0.231
kaempferol glycoside derivative 3	-	-	-	-	-	0.111	-	-	-	-	-
luteolin 5-glucoside	-	-	-	-	0.905	-	-	7.116	-	-	-
luteolin 7-glucoside	-	1.275	0.307	0.313	0.117	-	5.559	5.563	-	1.018	-
luteolin aglycon derivative 1	-	-	-	-	-	-	-	-	-	0.353	-
luteolin aglycon derivative 2	-	-	-	-	-	-	0.355	-	-	1.072	-
luteolin aglycon derivative 3	-	0.022	0.007	-	-	-	-	-	-	-	-
luteolin glycoside 1	-	-	-	-	0.034	-	1.743	-	-	0.718	-
luteolin glycoside 2	-	-	-	-	0.080	-	-	-	-	-	-
luteolin glycoside 3	-	-	-	-	0.048	-	-	-	-	-	-

	S. alb x fra	S. aur	S. cap	S. cin	S. fra	S. pen	S. pur	S. ros	S. vim	S. vim x pur	P. tre
luteolin glycoside 4	-	0.205	0.115	-	-	-	-	-	-	-	-
luteolin glycoside 5	-	0.356	0.237	-	-	-	-	-	-	-	-
luteolin glycoside 6	-	3.951	1.950	-	-	-	-	-	-	-	-
luteolin glycoside 7	-	6.964	3.864	-	-	-	-	-	-	-	-
luteolin glycoside 8	-	0.354	-	-	-	-	-	-	-	-	-
luteolin glycoside 9	-	-	-	-	-	-	-	2.150	-	-	-
methyl-apigenin derivative 1	-	-	-	-	-	-	-	-	-	-	0.107
methyl-apigenin derivative 2	-	-	-	-	-	-	-	-	-	-	0.434
methyl-apigenin derivative 3	-	-	-	-	-	-	-	-	-	-	0.024
methyl-apigenin derivative 4	-	-	-	-	-	-	-	-	-	-	0.054
methyl-luteolin 5-glucoside	-	1.372	0.489	2.380	-	-	-	0.325	-	-	-
methyl-luteolin aglycon	-	0.064	0.323	-	-	-	-	-	-	-	-
methyl-luteolin glycoside 1	-	-	-	-	0.083	-	0.601	-	-	-	-
methyl-luteolin glycoside 2	-	0.130	-	0.073	0.080	-	-	-	-	0.229	-
methyl-luteolin glycoside 3	-	-	0.071	0.006	-	-	0.740	1.272	-	0.158	-
methyl-luteolin glycoside 4	-	-	-	-	-	-	0.447	-	-	-	-
monocoumaroyl astragalin	-	0.944	1.160	4.092	-	-	-	0.159	1.899	-	-
monocoumaroyl flavonol	-	-	0.016	-	-	-	-	-	-	-	-
myricetin 3-arabinoside	-	-	-	-	-	-	-	-	0.051	-	-
myricetin 3-galactoside	-	-	-	0.197	-	0.621	-	-	0.645	-	-
myricetin 3-glucoside	-	-	0.011	0.007	-	2.348	-	-	-	-	0.340
myricetin glycoside	-	-	-	-	-	-	-	-	0.075	-	-

	S. alb x fra	S. aur	S. cap	S. cin	S. fra	S. pen	S. pur	S. ros	S. vim	S. vim x pur	P. tre
myricitrin	-	-	-	-	-	0.457	-	-	-	-	-
naringenin 7-glucoside	-	-	-	-	-	-	0.501	-	-	2.058	-
neochlorogenic acid	9.406	0.119	0.373	0.515	3.067	14.218	-	0.748	0.266	-	5.349
p-OH-cinnamic acid derivative 1	0.451	0.221	0.187	0.304	0.431	1.540	-	0.263	0.358	-	0.769
p-OH-cinnamic acid derivative 2	0.425	-	0.266	-	0.347	-	-	0.299	0.056	-	0.387
p-OH-cinnamic acid derivative 3	-	0.004	-	-	-	-	-	-	-	-	0.015
p-OH-cinnamic acid derivative 4	-	-	0.006	-	-	-	-	-	-	-	-
p-OH-cinnamic acid derivative 5	-	-	0.105	-	-	-	-	-	-	-	-
p-OH-cinnamic acid derivative 7	-	-	-	-	0.035	-	-	-	-	-	-
p-OH-cinnamic acid derivative 8	-	-	-	-	-	-	-	-	-	-	0.017
p-OH-cinnamic acid derivative 9	-	-	-	-	-	-	-	0.122	-	-	-
p-OH-cinnamic acid glucoside	0.267	0.323	-	0.045	0.328	0.681	-	-	-	-	-
protocatechuic acid	-	0.036	0.037	0.134	-	-	-	-	0.078	-	-
quercetin 3-glucoside	0.820	2.311	0.406	1.067	2.558	7.096	0.615	-	2.272	1.543	11.884
quercetin 3-arabinopyranoside	1.321	-	-	0.013	-	2.637	-	-	0.425	-	0.594
quercetin 3-arabinofuranoside	-	-	-	-	-	1.030	-	-	-	-	-
quercetin aglycon	0.584	-	-	-	-	-	-	-	-	-	-
quercetin derivative 1	0.160	-	-	-	-	-	-	-	-	-	-
quercetin diglycoside 1	-	0.393	-	-	-	-	-	-	-	-	2.194
quercetin diglycoside 2	-	-	-	-	0.905	-	-	-	0.099	-	0.998
quercetin diglycoside 3	-	-	-	-	-	-	-	-	0.136	-	-
quercetin diglycoside 4	0.394	0.066	-	-	0.493	-	-	-	0.067	-	-

quercetin glycoside 1	-	-	0.051	-	-	-	-	-	-	-	-
quercetin glycoside 2	-	-	-	-	-	-	-	-	0.055	-	-
quercetin glycoside 3	-	-	-	-	-	-	-	-	0.099	-	-
quercetin triglycoside 1	-	-	-	-	-	-	-	-	0.108	-	-
quercetin triglycoside 2	-	-	-	-	-	-	-	-	0.050	-	0.352
quercetin triglycoside 3	-	-	-	-	-	-	-	-	-	-	0.312
quercitrin	0.765	-	-	-	0.222	0.707	-	-	1.609	2.296	-
rhamnetin aglycon derivative	-	-	-	-	-	-	-	-	-	1.501	-
salipurposide	-	-	-	-	-	-	0.354	-	-	-	-
Condensed tannins	127.260	196.726	139.765	160.468	51.911	192.185	42.617	134.299	138.526	112.270	37.895

Table S2. Seasonal variability in secondary metabolite content. The samples for analysis were obtained in early June ("spring") and early August ("summer")

Host-plant species	Salicylates spring (mg/g)	Salicylates summer (mg/g)	Flavonoids spring (mg/g)	Flavonoids summer (mg/g)	Tannins spring (mg/g)	Tannins summer (mg/g)	Salicylates spring (Simpson)	Salicylates summer (Simpson)
Salix (Vetrix) aurita	0.0	0.0	29.5±1.1	21.4±2.3	196.7±45.0	212.5±51.9	0	0
S. (Vetrix) caprea	0.0	0.0	10.6±1.0	6.7±1.4	139.8±37.7	146.2±37.0	0	0
S. (Vetrix) cinerea	0.0	0.0	15.0±2.5	12.6±4.1	160.5±62.2	202.1±48.3	0	0
S. (Salix) fragilis	27.8±9.4	13.8±7.8	25.5±6.5	15.7±7.8	51.9±44.1	55.3±38.0	0.79±0.09	0.45±0.12
S. (Salix) pentandra	41.8±21.5	14.9±10.4	60.6±6.3	49.4±23.8	192.2±34.7	217.9±25.8	0.65±0.2	0.58±0.11
S. (Vetrix) purpurea	164.8±19.1	106.6±45.2	21.3±1.4	24.0±5.9	42.6±59.2	71.3±60.5	0.67±0.06	0.64±0.09
S. (Vetrix) rosmarinifolia	169.0±42.0	132.9±11.0	20.9±0.4	20.4±3.9	134.3±82.9	149.1±46.5	0.64±0.07	0.62±0.03
S. (Vetrix) viminalis	0.0	0.0	16.0±3.4	11.9±4.3	138.5±35.9	121.2±40.3	0	0
S. alba x fragilis	3.4±1.9	0.7±0.9	27.3±4	21.0±6.3	127.3±37.1	133.2±52.0	0.49±0.08	0.24±0.27
S. viminalis x purpurea	2.6±0.7	0.7±0.3	30.5±3.8	35.8±5.0	112.3±24.2	137.0±30.8	0	0
Populus tremula	19.4±20.7	6.0±5.8	33.8±5.9	20.4±5.6	37.9±35.0	57.6±32.1	0.34±0.32	0.38±0.25

Table S3. Correlation between defensive traits of studied willow species. The lower triangle shows values obtained from analysis using phylogenetic generalized least squares, the upper triangle obtained from simple linear regression. Significant values are in bold.

	Salicylate content (mg/g) $F_{(6)}$ / p	Salicylate Diversity (Simpson) $F_{(6)}$ / p	Flavonoid content (mg/g) $F_{(6)}$ / p	Tannin content (mg/g) $F_{(6)}$ / p	SLA (cm^2/g) $F_{(6)}$ / p	Trichome density (%) $F_{(6)}$ / p
Salicylate cont.	----------------	1.91 / 0.301	1.22 / 0.319	1.35 / 0.298	0.01 / 0.951	**7.37 / 0.042**
Salicylate div.	1.71 / 0.369	----------------	2.57 / 0.160	3.35/ 0.117	3.37 / 0.116	**20.29 / 0.004**
Flavonoid cont.	1.79 / 0.245	0.26 / 0.777	--------------	0.35 / 0.579	0.86 / 0.396	3.00 / 0.143
Tannin cont.	2.84 / 0.136	2.97 / 0.127	0.21 / 0.816	--------------	0.15 / 0.718	2.08 / 0.215
SLA	>0.01 / 0.999	3.30 / 0.108	2.09 / 0.205	0.13 / 0.880	-------------	0.19 / 0.678
Trichome den.	**7.98 / 0.020**	**18.36 / 0.003**	4.19 / 0.073	4.34 / 0.066	0.47 / 0.647	-------------

Table S4. Impact of *Salix* defensive traits on leaf-chewing insect diversity and density. Table includes results of simple linear regression and phylogenetic generalized least squares (PGLS). Significant values are in bold.

	Diversity		Density	
	$F_{(9)}$ / p	PGLS $F_{(9)}$ / p	$F_{(9)}$ / p	PGLS $F_{(9)}$ / p
Salicylate content (mg/g)	**5.52 / 0.043**	**4.98 / 0.035**	0.03 / 0.866	0.06 / 0.945
Salicylate diversity (Simpson)	2.46 / 0.152	1.74 / 0.230	0.14 / 0.719	0.14 / 0.719
Flavonoid content (mg/g)	3.34 / 0.101	2.83 / 0.111	0.20 / 0.668	0.35 / 0.712
Tannin content (mg/g)	0.17 / 0.687	0.05 / 0.951	0.12 / 0.738	0.50 / 0.621
Specific leaf area (cm^2/g)	0.42 / 0.683	0.60 / 0.570	1.55 / 0.244	1.45 / 0.286
Trichome density (%)	3.47 / 0.095	2.68 / 0.123	1.03 / 0.336	1.53 / 0.268

Chapter III

Dynamic plant defenses of sympatric *Ficus* species structure local larval leaf-chewer communities

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Abstract

Speciose plant genera harbour a large diversity of insect herbivores, and such systems are important for understanding the genesis of insect diversity and the evolution of host-plant defenses. Previous studies focusing on large plant genera have generally suggested a diversification of host-plant defenses on large scales or their divergence within sympatric communities. Here we bring together data on plant phylogeny, plant chemistry and insect herbivore communities for *Ficus*, a hyper-diverse plant genus, to investigate the

evolutionary patterns in defensive traits and their interplay with herbivore communities. We show that defensive traits can follow several evolutionary trajectories within one system. Whereas some defensive traits were conserved, several key defenses showed divergence among closely related species of sympatric *Ficus*. Both conserved and divergent traits had a significant impact on the associated larval leaf-chewer communities. We revealed a strong effect of cysteine protease activity on the community structure and abundance of larval leaf-chewers, suggesting that this proteolytic enzyme is of high defensive value in *Ficus*. Several other traits also had strong effects on larval leaf-chewers, but they often impacted only certain larval leaf-chewer taxa rather than the entire community. In particular, the specialist Choreutidae and generalist Pyraloidea responded to plant traits differently with Choreutidae responding to protease activity, Specific Leaf Area (SLA), and trichome density and Pyraloidea responding solely to C:N. Maintaining an effective protection against herbivores probably requires several complementary defensive traits, because specialist and generalist herbivore taxa respond to plant defenses in different ways. This suggestion is supported by the lack of negative correlations between the traits measured here. Further, plant defenses composed of multiple complementary traits with various evolutionary histories may be harder for herbivores to adapt to. Given the high phylogenetic conservatism of many herbivores, trait divergence seems to be especially beneficial to closely related plants growing in sympatry. However, both diversification and conservatism of traits over evolutionary time should play an important role in such systems. Both wide scale and local processes can potentially shape the evolution of plant defensive traits into a dynamic system, with the evolution of traits following periods of conservatism and divergence.

Key words

Coevolution, defensive traits, divergence, diversification, *Ficus*, herbivory, protease, secondary metabolites

Introduction

Insects started using plants as a food source shortly after the first vascular plants colonized terrestrial habitats, resulting in a burst of adaptive radiation (Steemans et al. 2009). Both these diverse groups have been interacting ever since and their complex evolutionary dynamics may have amplified the diversity and phenotypic adaptations seen in both groups through an 'arms-race' mechanism (Ehrlich and Raven 1964, Farrell et al. 1991, Janz 2011). As with any ongoing evolutionary process the outcome of insect-plant coevolution is highly scale dependent. Broad scale phylogenetic studies of several herbivorous insect orders show general phylogenetic congruence between herbivores and host-plants (Ehrlich and Raven 1964, Janz and Nylin 1998) whilst studies taking a finer approach tend to find less clear-cut patterns (Janz et al. 2001, Nyman et al. 2006). Theories on the macroevolution of host-plant defenses suggest diversification and escalation in plant defensive traits in response to insect adaptation (Ehrlich and Raven 1964, Vermeij 1994). Such an escalation of host-plant defenses has been found in several plant lineages by studies compiling data on focal plant taxa on large spatial scales (Agrawal and Fishbein 2008, Becerra et al. 2009, Pearse and Hipp 2012). On the other hand, labile and divergent defences were found among closely related species growing in sympatry (Becerra 2007, Kursar et al. 2009, Salazar et al. 2016). Local plant communities are potentially exposed to a shared pool of potential herbivores. It is thus possible that different evolutionary processes act across large and local scales among closely related plant species.

Insects tend to feed on multiple closely related hosts and are generally phylogenetically conservative in their food choice (Futuyma and Agrawal 2009, Cagnolo et al. 2011, Volf et al. 2015a). Divergent defences may thus help closely related plants growing in sympatry to escape herbivory as they decrease the pool of herbivores shared among closely related hosts (Becerra 2007, Salazar et al. 2016). Moreover, intrageneric differences phytochemical composition are frequently generated by the production of various chemical compounds of a similar metabolic origin, representing tweaks to existing metabolic pathways rather than radical new changes (Wink 2003, Agrawal et al. 2012). Specialist herbivores can overcome a variety of secondary metabolites of the same origin (Denno et al. 1990, Nishida 1994). In such cases an ability to mix between a pool of various defensive traits may be beneficial (Kursar et al. 2009, Janz 2011, Volf et al. 2015a). Divergence in defences among congeneric plants growing in sympatry may thus be an ideal strategy to escape herbivory (Becerra 2007). From an evolutionary perspective this is expected to result in a lack of congruence between host and insect phylogenies at a lower level, as has been frequently reported (e.g. Futuyma and McCafferty 1990, Becerra and Venable 1999).

The formation of defenses can be further affected by the differential response of various groups of herbivores to host-plant traits (Ali and Agrawal 2012, Volf et al. 2015a). Whereas some defences are effective against specialists, others may be effective against generalists (Ali and Agrawal 2012, Volf et al. 2015a). Individual defensive traits are thus exposed to different selective pressures and may display a range of evolutionary histories (Agrawal and Fishbein 2008). Suites of defenses are often mutually independent or follow similar trajectories, forming defensive syndromes consisting of positively correlated complementary traits (Agrawal and Fishbein 2006) providing protection against a broad spectrum of herbivores (Koricheva et al. 2004, Volf et al. 2015a). Such patterns can be best identified in species rich communities comprising closely related

plants. Rainforest assemblages of *Ficus* species in Papua New Guinea represent an excellent model system. This pantropical genus is extraordinarily species rich, with over 800 species, 150 of which occur in Papua New Guinea (Berg and Corner 2005, Cruaud et al. 2012). In PNG, *Ficus* represents one of key genera in forest communities as it is locally rich in species, with up to 40 coexisting species, reaches high biomass and supports species rich communities of herbivorous insects from several guilds (Novotny *et al.* 2010). *Ficus* species have acquired a broad range of chemical and physical defences against these pecies rich communities of herbivores. These include traits common among a variety of plants, such as polyphenols, terpenoids and physical defences such as trichomes or tough leaves (Basset and Novotny 1999). *Ficus* species also produce latex that serves as a physical defense as well as a transport system for various specialized defenses such as alkaloids or cysteine proteases interfering with insect digestion and significantly increasing larval mortality (Konno *et al.* 2004).

In this study, we examine anti-herbivore defensive patterns in 21 species of *Ficus* growing in sympatry in a rainforest community in PNG. We test these traits for phylogenetic signal, particularly whether they are over-dispersed among closely related species. We also examine whether defensive traits correlate with each other and form defensive syndromes. Finally, we test the effects of *Ficus* defences on larval leaf-chewer communities and analyze their impact on both specialists and generalist. We predict (i) divergence in defense traits among closely related *Ficus* rather than diversification of host-plant defenses in more derived species, (ii) differential response of specialists and generalists to individual defense traits, leading to the formation of suites of complementary defensive traits.

Methods

Sampling and Insect Data

We sampled the 21 Ficus species surveyed by Novotny *et al.* (2010) for insect herbivores. We also sampled three additional species included in that study, but lacking detailed insect herbivore data (*F. virens, F. hahliana* and *F. congesta*). For the analysis of triterpenes and physical defensive traits we sampled five individuals per species. We collected two leaf discs per leaf for 10 young, but fully expanded leaves for each individual, avoiding the central vein. We avoided trees with high rates of herbivory or obvious physical damage and maintained >10 m distance between trees, avoiding obviously clonal individuals. The same caveats were used for selecting trees for the analysis of cysteine protease but in this case we sampled several whole leaves (both expanding and fully expanded) and latex. Latex was sampled by cutting the main stem of each leaf and letting latex flow into a 2ml collection tube for 30 seconds. All leaf and latex samples were stored on ice in the field and were not allowed to exceed a temperature of 2˚C before being returned to the New Guinea Binatang Research Centre for storage at -20˚C. All samples were subsequently transported by air and in ice boxes to the University of Goroka for further analysis; the temperature inside the ice boxes never exceeded 4˚C.

The insect data were taken from Novotny *et al.* (2010) sampled in Madang Province, PNG (Table 1). We focused on leaf-chewing larvae (including 112 Lepidoptera, 2 Coleoptera species) as a guild that is well represented on our focal *Ficus* species and inflicts a large amount of damage to *Ficus* leaves The larval leaf-chewer guild harboured by *Ficus* included Lepidoptera from several families. We focused mainly on Pyraloidea, a relativly polyphagous group feeding on several plant taxa, and Choreutidae, which are mostly Moraceae specialists (Novotny *et al.* 2002).

Table 1. Summary of insect community data including total abundance of leaf-chewing larvae, total number of their species, number of Choreutidae species and number of Pyraloidea species, all per 1,500 m^2 of foliage sampled from each *Ficus* species in Madang Province, PNG. The data were taken from Novotny *et al.* (2010).

Species	Total ab.	Total sp.	Choreutidae	Pyraloidea
Ficus aurantiacafolia	53	26	6	3
Ficus botryocarpa	136	24	6	8
Ficus congesta	NA	NA	NA	NA
Ficus conocepholia	302	27	4	11
Ficus copiosa	220	29	6	10
Ficus dammaropsis	121	12	6	8
Ficus gul	133	7	3	4
Ficus hahliana	NA	NA	NA	NA
Ficus hispidioides	204	22	4	7
Ficus mollior	42	16	5	8
Ficus nodosa	157	36	5	14
Ficus pachyrrhachis	239	16	6	2
Ficus phaeosyce	234	17	3	9
Ficus pungens	95	31	7	8
Ficus rubrivestimenta	39	19	7	5
Ficus septica	159	21	5	9
Ficus subtrinervia	79	14	5	6
Ficus trachypison	137	20	2	10
Ficus variegata	249	35	5	12
Ficus virens	NA	NA	NA	NA
Ficus wassa	204	38	7	11

Phylogeny Reconstruction

Host-plant phylogeny was reconstructed using four loci: ITS, ETS, G3PD, and GBSSI. We used sequences from Cruaud *et al.* (2012) downloaded from GenBank if available. For species not included in the analysis of Cruaud *et al.* (2012), air-dried leaf discs were used to obtain host-plant DNA. We used standard procedures, reaction conditions and primer sequences for DNA extraction and PCR

amplification, which were the same as those used in the original studies employing these markers (Mason-Gamer et al. 1998, Cronn et al. 2002, Ronsted et al. 2008). Sequences were assembled and edited using Geneious 5.4 (Drummond et al. 2011). Host-plant phylogeny was reconstructed using Bayesian inference as implemented in BEAST v2.1.3 (Drummond et al. 2012). The following substitution models selected based on BIC computed in JModelTest 2 (Darriba et al. 2012) were used for individual loci: ITS: GTR+I+G, ETS: HKY+I+G, G3PD: GTR+I+G, GBSSI: HKY+I+G. We used section level constraints based on Cruaud *et al.* (2012). Sampling was carried out every 10^3 generations for 10^7 generations, the first 10% of all generations were discarded as 'burnin' and the results were summarized with a maximum clade credibility tree. Further we used constraints based on microsatellite data, in case of section Sycocarpus, which has undergone a rapid radiation in PNG. We selected 11 microsatellite loci previously published for the genus *Ficus* (Garcia *et al.* 2012, Moe & Weiblen 2011), which were amplified in three multiplex sets. The phylogenetic relationships between the species in section Sycocarpus were visualized by plotting neighbor joining trees using Nei's distance as implemented in BAPS v5.4 (Corander *et al.* 2004). We used the 'clustering of groups of individuals' method, assigning the five individuals from each species to a group and setting k to 20 to derive the distance matrix.

Physical Traits

We measured trichome density and specific leaf area (SLA), a surrogate for leaf thickness and toughness, which is also frequently correlated with water content (Groom and Lamont 1999), as parameters of leaf morphology with a possible impact on leaf-chewing insects. The physical traits were measured using leaf discs. Leaf discs were cut (avoiding the central vein) and air dried to a constant weight. The total number of trichomes per 10 mm^2 and their average length was measured on five leaf discs per individual using

ImageJ (ver.1.48). Values for dorsal and ventral sides of the discs were combined. SLA was calculated as the area per unit mass of twenty dried leaf disc of known diameter for each individual.

Triterpenes

Approximately 50 mg of dried powdered sample was ground with 1 ml of methanol in a TissueLyser LT (Dynex Technologies, Bustehrad, Czech Republic) at 30 Hz for 2 min. After centrifugation (10000 rpm) at 8°C for 10 min, a 100 µl of the supernatant´s aliquot was mixed with 200 µl of methanol containing 0.1% formic acid. Terpenoids were measured on a Dionex Ultimate 3000 LC system equipped with an Open XRS autosampler and coupled to a Q Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). A reversed phase Kinetex C18 100AHPLC column, 150mm x 2.1 mm i.d., 2.6µm (Phenomenex, Torrance, CA, USA) was used for separation of analytes. A positive atmospheric pressure ionization mode (APCI) and a combined full scan mass range (250 – 625 Da) and a data dependent tandem MS^2 scan modes were used. Acquired data were processed using Xcalibur 2.1 Software (Thermo Fisher Scientific) and the terpenoid data were further mined by means of the Metabolite Mapper platform, which was developed in-house.

Protease

We analysed the protease activity of both leaves and latex using the methods of Konno *et al.* (2004) and Agrawal *et al.* (2008). We modified the methods to deal with solidified latex by adding 50ul of sodium phosphate buffer to the crude latex and and centrifuging for 3000 rpm for 10 minutes at 4˚C, the supernatants were centrifuged again at 3500 rpm for 30 minutes at 4 ˚C. The gums were discarded and a 20 µl of latex supernatants was used for the reaction and

another 20 µl were used for the control (terminated immediately with trichloroacetic acid as described in Konno et al., 2004).

Statistical Analysis

The phylogenetic signal and anti-signal of each of our traits were measured using a statistical comparison of the observed variance in phylogenetic independent contrasts across the tree to a randomly generated set of values derived from swapping tip labels randomly across the tree. We measured phylogenetic signal from the root of the tree as well as from internal nodes representing the major bifurcations in our tree (see Table 2 for a list of the nodes).

Table 2. Phylogenetic signal and anti-signal in studied traits as measured using a statistical comparison of the observed variance in PICs across the tree to a randomly generated set of values derived from swapping tip labels randomly across the tree. Table shows *p*-values for individual traits at several nodes of the phylogeny: protease activity in latex (Prot.Latex), protease activity in leaves (Prot.Leaves), triterpene total content (Trit.Cont), triterpene diversity (Trit.Div), trichome density (Tric.Den), trichome length (Tric.Len), C:N, and specific leaf area (SLA). Significant values are in bold.

Node	Prot. Latex	Prot. Leaves	Trit. Cont	Trit. Div	Tric. Den.	Tric. Len	C:N	SLA
II (Root)	**0.021**	**0.036**	**0.047**	**0.013**	0.79	0.19	**0.031**	0.434
XVI (Sect. Sycidium)	0.825	0.887	0.5125	0.826	0.1285	0.97	**0.01**	0.681
XIII (Sect. Adenosperma)	0.169	0.479	0.835	0.8225	0.495	0.1765	0.832	0.172
V (Sect. Sycocarpus)	0.337	**0.043**	**0.011**	**0.007**	0.619	0.209	**0.029**	0.554
III (Sub-gen. Sycomorus)	0.054	**0.039**	0.07	0.111	0.776	0.22	0.37	0.192

To reconstruct macroevolutionary patterns in the traits, we fitted likelihood models of evolution for each trait across the phylogeny using a set of commonly used models (Brownian motion, Brownian motion with a trend, Pagels Lambda, Delta and Kappa, Early Burst, Ornstein Uhlenbeck and the non-phylogenetic white noise model). These models were implemented using the 'FitContinous' function in

the R package 'Geiger' (Harmon *et al.* 2008). We used the default bounds for each model and compared the models using their AIC weights.

To further examine evolution of individual traits through time, we plotted the values of trait disparity through time (DTT) from root to tips using the function 'dtt' in the R package 'Geiger' (Harmon *et al.* 2008). We used the average square distance metric to calculate trait disparity and created a null distribution of DTT with 95% confidence intervals using 1,000 simulations under Brownian motion.

The larval leaf-chewer community similarity across studied *Ficus* species was analyzed using principal component analysis (PCA) with individual *Ficus* species used as samples. The effects of host-plant traits on larval leaf-chewer communities were analysed by canonical correspondence analysis (CCA) using species means of traits as explanatory variables and individual *Ficus* species as samples. The traits significantly explaining community structure were selected using forward selection. We ran separate analyses for i) the whole larval leaf-chewer community, ii) Pyraloidea, and iii) Choreutidae. The traits with significant effects were then used to partition the variance in larval leaf-chewer community structure explained by traits, host phylogeny and both traits and phylogeny. To analyze the impact of host-plant phylogeny on larval leaf-chewer communities, we transformed the patristic distances derived from the ultrametric tree into axes of coordinates using principal coordinate analysis (PCoA). The axes with significant effects on larval leaf-chewer communities were selected using forward selection and used in the following analyses as explanatory variables or covariables. First, the relative contribution of host-plant traits and phylogeny in explaining variability in larval leaf-chewer communities was analyzed by CCA and variance partitioning. Only host-plant traits and phylogenetic axes with significant effects on larval leaf-chewer community were included in this analysis. Second, we analyzed the effect of host-plant traits in a phylogenetic context using forward selection with

significant phylogenetic axes used as covariables. All multivariate analyses were conducted in CANOCO 5 (Ter Braak and Smilauer 2012).

We used separate Phylogenetic Generalised Least Squares regression (PGLS) to further account for effects on insect communities by following various models of evolution (Brownian motion, Brownian motion with a trend, Pagels Lambda, Delta and Kappa, Early Burst, Ornstein Uhlenbeck and the non-phylogenetic white noise model). We modeled the relationship between our traits (response variable) and larval leaf-chewer abundance. We analysed the i) entire larval leaf-chewer community, ii) only Pyraloidea and iii) only Choreutidae.

Results

The studied plant traits showed high interspecific variability (Table S1, Figure 1). The traits were generally positively correlated or uncorrelated. The only significant negative correlation we found was between triterpene total content and diversity (Table 3). At the root of the tree there was significant phylogenetic signal in all chemical traits (C:N, protease latex, protease leaves, triterpene diversity and triterpene total content). No phylogenetic signal was detected in the physical defensive traits (SLA, trichome length and trichome density). Our community consists of *Ficus* species from several sections, the largest of which are Sycidium (node XVI) and Sycocarpus (node V). The only trait with phylogenetic signal in section Sycidium is C:N, whilst trichome length shows phylogenetic anti-signal. In section Sycocarpus, triterpene diversity and total content have phylogenetic signal. If we go further back in the tree and include section Adenosperma (node XIII) as well as Sycomorus (node XII) the only traits with phylogenetic signal are protease latex and protease leaves (node III, sub-genus Sycomorus) (Fig. 1, Table 2).
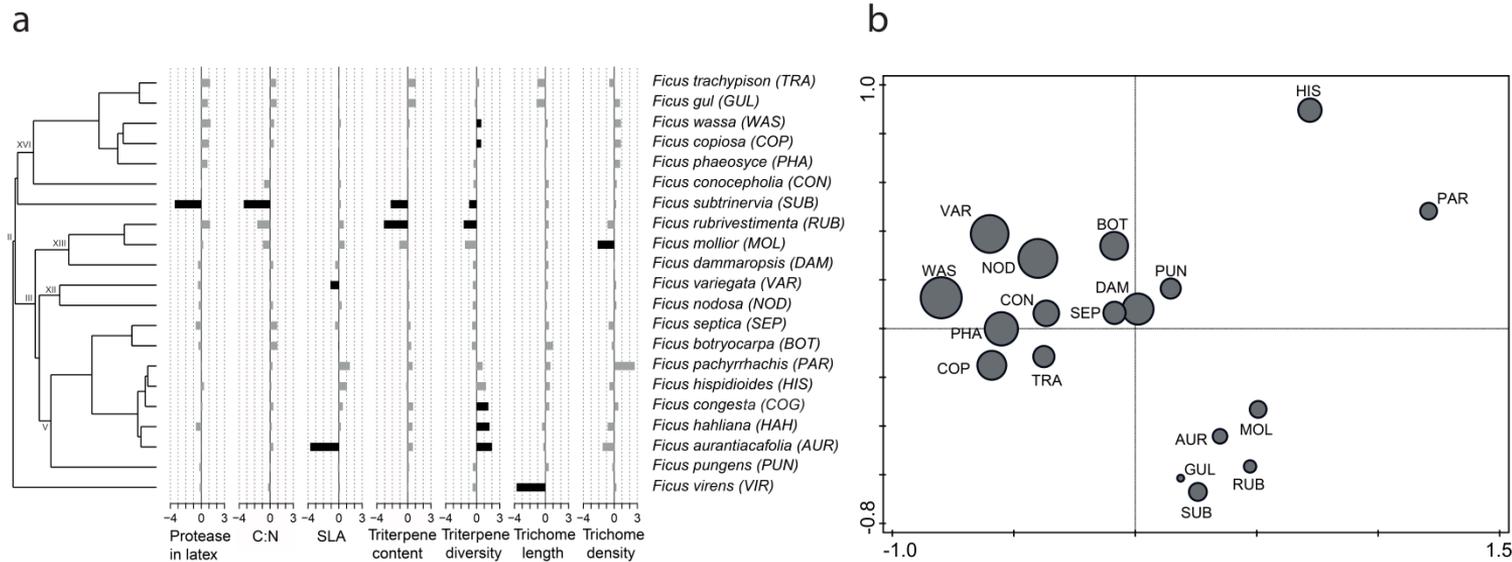
Figure 1. The distribution of host-plant traits across the phylogeny of studied *Ficus* species (a) and the similarity of their larval leaf-chewer communities (b). Host-plant traits were mapped along the phylogeny of 21 *Ficus* species. Important nodes representing the root (II), sub-genus Sycomorus (III), section Sycocarpus (V), section Sycomorus (XII), section Adenosperma (XIII), and section Sycidium (XVI) are labeled with roman numerals. Species traits with significant deviation in local Moran's index are in black. Data are centered and scaled by trait. Community similarity was analysed using PCA including all leaf-chewing larvae. Dot size reflects the larval leaf-chewer abundance.

Table 3. Correlations between the traits studied as analyzed by phylogenetic least squares regression. Values show p / t for protease activity in latex, protease activity in leaves, triterpene total content, triterpene diversity (Shannon), trichome density and average length, C:N, specific leaf area.

	Prot. Latex	Prot. Leaves	Trit. Cont	Trit. div	Tric. Den	Tric. Len	C:N	SLA
Prot.Latex	-	**0.007 / 3.04**	0.188 / 1.37	0.378 / -0.90	0.277 / 1.12	0.545 / 0.62	**0.002 / 3.59**	0.116 / 1.65
Prot.Leaves	**0.007 / 3.04**	-	0.502 / 0.69	0.462 / -0.75	0.199 / -1.33	0.762 / 0.31	**0.026 / 2.41**	0.444 / 0.78
Trit.Cont	0.188 / 1.37	0.502 / 0.69	-	**<0.001 / -4.93**	0.099 / -1.73	0.893 / 0.14	**0.005 / 3.21**	0.314 / 1.03
Trit.div	0.378 / -0.90	0.462 / -0.75	**<0.001 / -4.93**	-	0.104 / 1.71	0.827 / 0.22	**0.006 / -3.11**	0.186 / -1.37
Tric.Den	0.277 / 1.12	0.199 / -1.33	0.099 / -1.73	0.104 / 1.71	-	0.798 / -0.26	0.181 / -1.39	0.489 / -0.71
Tric.Len	0.545 / 0.62	0.762 / 0.31	0.893 / 0.14	0.827 / 0.22	0.798 / -0.26	-	0.111 / 1.67	0.484 / -0.71
C:N	**0.002 / 3.59**	**0.026 / 2.41**	**0.005 / 3.21**	**0.006 / -3.11**	0.181 / -1.39	0.111 / 1.67	-	0.690 / 0.40
SLA	0.116 / 1.65	0.444 / 0.78	0.314 / 1.03	0.186 / -1.37	0.489 / -0.71	0.484 / -0.71	0.690 / 0.40	-

Protease latex was best modeled with Brownian motion, protease leaves with a delta model (delta>1), C:N with a Brownian model, SLA with a non-phylogenetic white noise model, triterpene total with a delta model (delta>1), triterpene diversity with a delta model (delta>1) and trichome length and trichome density with a white noise model (Table S2). Our DTT plots and simulations under Brownian motion generally agree with the macroevolutionary models selected above. Protease latex and C:N show little deviation from Brownian motion, whilst the disparity in trait values increases at the tips (protease leaves) or in the middle of the phylogeny (triterpene diversity and triterpene total content). For the physical defensive traits - SLA and trichome length - we also see an increase in trait disparity at the tips of the tree, the plot for trichome density suggests extreme trait disparity at all levels of the phylogeny (Figure 2).
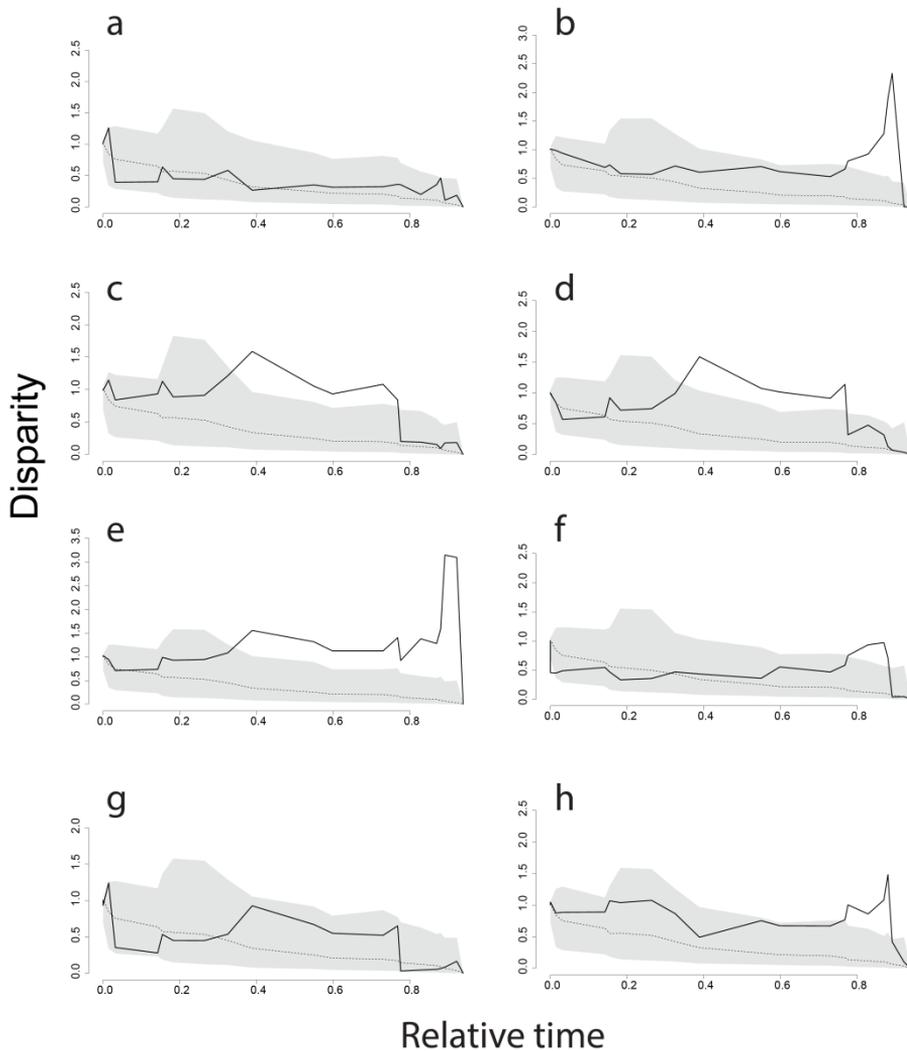
Figure 2. Mean disparity through time (DTT) for studied traits (solid line). Plots show disparity in protease activity in latex (a), protease activity in leaves (b), triterpene content (c), triterpene diversity measured as Shannon index (d), trichome density (e), trichome length (f), C:N (g), and specific leaf area (h). The dashed line indicates the median DTT based on 10 000 simulations of character evolution on the phylogeny of studied *Ficus* species under Brownian motion. The grey shaded area indicates the 95% DTT range for the simulated data.

Ficus species with strong anti-herbivore protection, such as *F. subtrinervia*, *F. rubrivestimenta*, *F. aurantiacafolia* and *F. mollior*, harbored distinct herbivore communities characterized by low abundance of larval leaf-chewers (Fig. 1). CCA revealed significant effects of protease activity in latex (p=0.002) and trichome density (p=0.024) on larval leaf-chewer communities. The response of specialist Choreutidae and generalist Pyraloidea to host-plant traits differed. Whereas Choreutidae responded to protease activity in latex (p=0.001), SLA (p=0.015) and trichome density (p=0.043), Pyraloidea responded only to C:N (p=0.022). The results of analysis for whole larval leaf-chewer community and Choreutidae changed when the phylogeny was included. Only SLA had a significant effect on the whole larval leaf-chewer community (p=0.017) and there was no significant effect of host traits on Choreutidae community when the host phylogeny was used as a covariable. The results for Pyraloidea remained unchanged. On the whole, both host traits and phylogeny (and their covariation) contributed to the explained variability in composition of whole larval leaf-chewer communities explaining 14.9% of the variability in the data. Host phylogeny and its covariation with host traits explained high proportion of variability in composition of Choreutidae communities (39.5%), but there was no variability explained solely by the traits themselves. On the other hand, phylogeny had no effect on communities of Pyraloidea which were affected solely by C:N which explained 8.3% of variability in their composition (Figure 3).
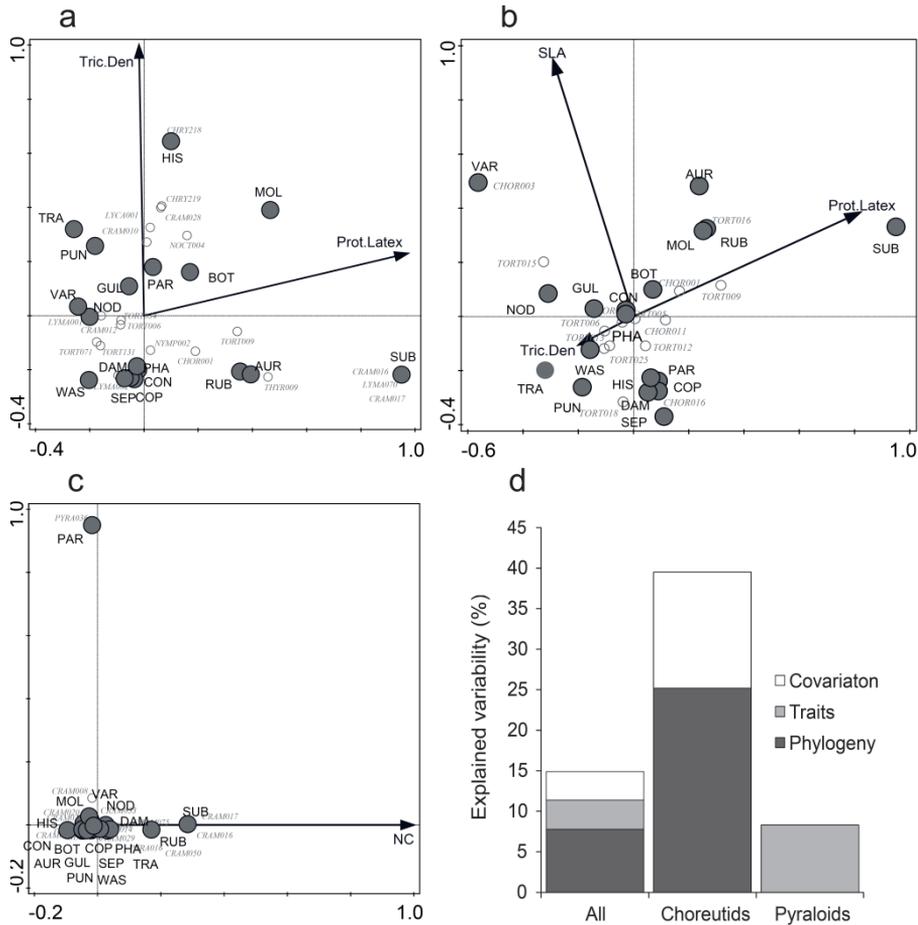
Figure 3. Response of the whole larval leaf-chewer community (a), Choreutidae (b) and Pyraloidea (c) to host-plant traits and relative contribution of host-plant traits and phylogeny to the explained variability in the community structure (d). The response of insect communities to the host-plant traits was analyzed using CCA. The relative contribution of host-plant traits to the explained variability in the community structure was analyzed by CCA and variance partitioning with phylogeny decomposed into axes by PCoA. See Fig.1 for codes of *Ficus* species.

PGLS analysis for the whole larval leaf-chewer community revealed that only protease latex has a significant relationship with larval leaf-chewer abundance (t=-2.669 p=0.016). When considering only Choreutidae no single variable had a significant effect on larval leaf-chewer abundance. For Pyraloidea there was a significant negative relationship between protease latex (t=-2.394, p=0.028) and a

significant positive relationship with triterpene diversity (t=2.256, p=0.038).

Discussion

In this study, we focused on the evolution of defensive traits in a community of closely related *Ficus* species from the perspective of protection from a local pool of insect herbivores. Previous studies focusing on large plant genera have generally suggested either diversification of host-plant defenses (Agrawal and Fishbein 2008, Becerra et al. 2009, Pearse and Hipp 2012) or their divergence (Kursar et al. 2009, Volf et al. 2015b, Salazar et al. 2016). We show that defensive traits can follow several evolutionary trajectories within one system. Whereas protease activity in latex and C:N were conserved, other key traits showed divergence among closely related species of sympatric *Ficus* and their evolution had differed from Brownian motion. The divergent traits showed a peak of disparity mainly at terminal levels of the host phylogeny (protease activity in leaves, SLA and trichome length), mid levels of the host phylogeny (triterpene content and diversity) or both (trichome density). This was epitomized by the convergence of *Ficus* species from different sections on to similar defensive strategies (e.g. *F. subtrinervia* and *F. rubrivestimenta*) and the divergence of closely related species (e.g. *F. hahliana* and *F. aurantiacafolia*). Notably, most of the traits were not strictly conserved or divergent but showed dynamic increase or decrease in disparity over time as illustrated by traits showing deep level conservatism (e.g. at the section level) followed by divergence at the tips of the tree. Such processes seem to shape the evolution of plant defensive traits into a dynamic system with the evolution of traits following periods of diversification, divergence and sometimes decline (Agrawal and Fishbein 2008, Janz 2011).

Both conserved traits and traits showing divergence significantly affected the community structure of herbivores. The resulting community structure of associated larval leaf-chewers reflected both conservatism and divergence in trait evolution. For example, the larval leaf-chewer communities associated with *F. subtrinervia*, *F. rubrivestimenta*, *F. aurantiacafolia* and *F. mollior* were quite distinct from those of the other *Ficus* species and low in insect abundance, which is likely to be driven by the shared defensive trait values through phylogenetic conservatism (*F. mollior* and *F. rubrivestimenta*) or convergence (*F. subtrinervia* and *F. aurantiacafolia*). We found major differences in response to host traits due to various life-histories of examined herbivores, mainly of Pyraloidea and Choreutidae. Pyraloidea are generalists with only 20% of local species feeding exclusively on *Ficus* and 85% of individuals found on other host-plants. On the other hand, Choreutidae are *Ficus* specialists with 63% of local species feeding exclusively on *Ficus* and only 19% of individuals found on other hosts (Novotny et al. 2002, Novotny et al. 2010).

Each caterpillar taxon was affected by a different suite of plant traits. The activity of cysteine protease in *Ficus* latex had the broadest impact. Our results suggest that cysteine proteases play a prominent role in *Ficus* defenses. This is in line with previous findings showing that cysteine proteases interfere with digestion of plant tissue and processes at the perithrophic membrane, increasing caterpillar mortality and reducing caterpillar growth rate (Konno *et al.* 2004). Physical traits such as trichomes have been shown to mainly affect small insects and some specialist taxa, possibly lowering their feeding efficiency (Agrawal 2005, Volf et al. 2015a). In our case, trichomes and SLA affected the community structure of the whole larval leaf-chewer assemblage and assemblages of specialized Choreutidae, but had only a minor effect on generalist Pyraloidea. It has been suggested that specialists, which are often able to overcome host defenses, should choose their hosts according to their nutrient quality

(Denno et al. 1990, Coley et al. 2006). Surprisingly, C:N had only limited effects on Choreutidae and affected mainly generalist Pyraloidea. High nitrogen content may thus compensate for negative effects of host defences for these generalist. Triterpenes, as a rather generalized form of chemical defense, did not show a strong impact on larval leaf-chewers except for a positive correlation between their diversity and Pyraloidea abundance. This was probably due to the positive correlation between triterpene diversity and nitrogen content which was one of the main predictors of Pyraloidea community structure.

The significant effects of both conserved traits and traits showing divergence suggests that host-plants rely on complementary defenses with various evolutionary history. This is further supported by a notable lack of negative correlations between studied traits. The only exception was the negative correlation between content and diversity of triterpenes suggesting that there may be a trade-off in metabolic pathways in order to produce large concentration or diversity of these secondary metabolites. This is in line with recent studies showing that trade-offs between defenses occur mainly under specific conditions, such as in case of negative dependence in metabolic pathways or in low nutrient environments (Agrawal et al. 2009, Sampedro et al. 2011). Rare negative correlations between defenses probably also reflect differential response to various herbivores in the studied community. Given the differential response of Pyraloidea and Choreutidae, maintaining an effective protection against herbivores may require several complementary defensive traits. This was suggested also by previous studies of diverse herbivore communities (Koricheva et al. 2004, Volf et al. 2015a). Further, employing several complementary defenses with different evolutionary histories could strengthen host protection as it may be harder for herbivores to adapt to defenses that follow a range of evolutionary trajectories. This may lead to the formation of host-plant defenses into suites of complementary traits with various function, rather than to the

formation of anti-herbivore protection based on single traits showing negative correlations and trade-offs (Agrawal and Fishbein 2006, Agrawal 2007).

The disparity recovered in several of the studied traits may help closely related hosts growing in sympatry to escape herbivory from insects which are phylogenetically conservative in their food-choice. Many herbivores tend to feed on related host-plants and host shifts are more common between closely related plants than between distantly related lineages (Janz and Nylin 1998, Winkler and Mitter 2008, Futuyma and Agrawal 2009). Our results show a moderate effect of host-plant phylogeny on whole larval leaf-chewer community and its strong effect on assemblages of specialized Choreutidae. In the case of Choreutidae phylogeny explained a substantial proportion of community variability whereas the response to host-plant traits was weaker and resulted mainly from the effects of covariation between traits and host phylogeny (e.g. phylogenetically conserved defensive traits like cysteine protease). On the other hand, there was no sign of phylogenetic conservatism in food choice of generalist Pyraloidea that exclusively followed host-plant traits. A high diversity of defenses in local communities can serve as a good protection against specialist (Salazar et al. 2016). Here we suggest that divergence in defenses may allow closely related hosts to lower the likelihood of host-shifts by generalist herbivores that follow trait similarity rather than host phylogeny. This may reduce the potential population size of herbivores and herbivory related damage. Herbivory pressure thus probably drives some plant communities towards divergence in their defenses (Becerra 2007, Kursar et al. 2009, Salazar et al. 2016).

In conclusion, our results suggest that individual defensive traits may follow various evolutionary trajectories, such as conservatism or divergence. Divergence in defenses rather than accumulation of their diversity is in line with findings of previous studies focusing on local plant communities (Kursar et al. 2009, Volf et al. 2015b, Salazar et al.

2016). On the other hand, this is in contrast with results of studies focusing on several plant genera on large spatial scales that found diversification of defenses as predicted by Ehrlich and Raven´s theory (Ehrlich and Raven 1964, Agrawal and Fishbein 2008, Becerra et al. 2009, Pearse and Hipp 2012). We suggest that defensive traits may follow both evolutionary trajectories with the relative importance of diversification and divergence potentially differing between large and local-scales, for example in local communities of closely related plants. When considering plant lineages on large temporal and spatial scales, the evolution of novel defenses may probably help them to escape-and-radiate leading to diversification of their defensive mechanism as a whole. However, any defensive strategy will decrease in efficiency as specialized insects accumulate with time (Janz 2011, Volf et al. 2015b). In such a situation, the divergence in defenses and formation of several defensive strategies including both conserved and divergent traits may help plants to escape herbivory. This seems be especially beneficial to plants growing in sympatry which are exposed to the same herbivores, as defenses based on similar traits would lead to sharing herbivores among multiple related hosts. Such processes seem to shape the evolution of plant defensive traits into a dynamic system with the evolution of traits following periods of diversification and divergence (Janz 2011) as illustrated by studied traits showing deep level conservatism (e.g. at the section level) followed by divergence at the tips of the tree.

Acknowledgements

References

Agrawal, A. A. 2005. Natural selection on common milkweed (*Asclepias syriaca*) by a community of specialized insect herbivores. Evolutionary Ecology Research **7**:651-667.

Agrawal, A. A. 2007. Macroevolution of plant defense strategies. Trends In Ecology & Evolution **22**:103-109.

Agrawal, A. A., and M. Fishbein. 2006. Plant defense syndromes. Ecology **87**:S132-S149.

Agrawal, A. A., and M. Fishbein. 2008. Phylogenetic escalation and decline of plant defense strategies. Proceedings Of The National Academy Of Sciences Of The United States Of America **105**:10057-10060.

Agrawal, A. A., G. Petschenka, R. A. Bingham, M. G. Weber, and S. Rasmann. 2012. Toxic cardenolides: chemical ecology and coevolution of specialized plant-herbivore interactions. New Phytologist **194**:28-45.

Agrawal, A. A., J. P. Salminen, and M. Fishbein. 2009. Phylogenetic trends in phenolic metabolism of milkweeds (Asclepias): evidence for escalation. Evolution **63**:663-673.

Ali, J. G., and A. A. Agrawal. 2012. Specialist versus generalist insect herbivores and plant defense. Trends In Plant Science **17**:293-302.

Basset, Y., and V. Novotny. 1999. Species richness of insect herbivore communities on *Ficus* in Papua New Guinea. Biological Journal Of The Linnean Society **67**:477-499.

Becerra, J. X. 2007. The impact of herbivore-plant coevolution on plant community structure. Proceedings Of The National Academy Of Sciences Of The United States Of America **104**:7483-7488.

Becerra, J. X., K. Noge, and D. L. Venable. 2009. Macroevolutionary chemical escalation in an ancient plant− herbivore arms race. Proceedings Of The National Academy Of Sciences Of The United States Of America **106**:18062−18066.

Becerra, J. X., and D. L. Venable. 1999. Macroevolution of insect–plant associations: the relevance of host biogeography to host affiliation. Proceedings of the National Academy of Sciences **96**:12626-12631.

Berg, C., and E. Corner. 2005. Moraceae (Ficus). Flora Malesiana, Series I (Seed plants,) Volume 17/Part 2. National Herbarium of the Netherlands, Leiden.

Cagnolo, L., A. Salvo, and G. Valladares. 2011. Network topology: patterns and mechanisms in plant-herbivore and host-parasitoid food webs. Journal Of Animal Ecology **80**:342-351.

Coley, P. D., M. L. Bateman, and T. A. Kursar. 2006. The effects of plant quality on caterpillar growth and defense against natural enemies. Oikos **115**:219-228.

Corander, J., P. Waldmann, P. Marttinen, and M. J. Sillanpää. 2004. BAPS 2: enhanced possibilities for the analysis of genetic population structure. Bioinformatics **20**:2363-2369.

Cronn, R. C., R. L. Small, T. Haselkorn, and J. F. Wendel. 2002. Rapid diversification of the cotton genus (*Gossypium* : *Malvaceae*) revealed by analysis of sixteen nuclear and chloroplast genes. American Journal Of Botany **89**:707-725.

Cruaud, A., N. Ronsted, B. Chantarasuwan, L. S. Chou, W. L. Clement, A. Couloux, B. Cousins, G. Genson, R. D. Harrison, P. E. Hanson, M. Hossaert-Mckey, R. Jabbour-Zahab, E. Jousselin, C. Kerdelhue, F. Kjellberg, C. Lopez-Vaamonde, J. Peebles, Y. Q. Peng, R. A. S. Pereira, T. Schramm, R. Ubaidillah, S. van Noort, G. D. Weiblen, D. R. Yang, A. Yodpinyanee, R. Libeskind-Hadas, J. M. Cook, J. Y. Rasplus, and V. Savolainen. 2012. An extreme case of plant-insect codiversification: Figs and fig-pollinating wasps. Systematic Biology **61**:1029-1047.

Darriba, D., G. L. Taboada, R. Doallo, and D. Posada. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nature methods **9**:772-772.

Denno, R. F., S. Larsson, and K. L. Olmstead. 1990. Role of enemy-free space and plant quality in host-plant selection by willow beetles. Ecology **71**:124-137.

Drummond, A. J., B. Ashton, S. Buxton, M. Cheung, A. Cooper, C. Duran, M. Field, J. Heled, M. Kearse, S. Markowitz, R. Moir, S. Stones-Havas, S. Sturrock, T. Thierer, and A. Wilson. 2011 Geneious v5.4.

Drummond, A. J., M. A. Suchard, D. Xie, and A. Rambaut. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Molecular biology and evolution **29**:1969-1973.

Ehrlich, P. R., and P. H. Raven. 1964. Butterflies and plants - a study in coevolution. Evolution **18**:586-608.

Farrell, B. D., D. E. Dussourd, and C. Mitter. 1991. Escalation of plant defense - do latex and resin canals spur plant diversification. American Naturalist **138**:881-900.

Futuyma, D. J., and A. A. Agrawal. 2009. Macroevolution and the biological diversity of plants and herbivores. Proceedings Of The National Academy Of Sciences Of The United States Of America **106**:18054-18061.

Futuyma, D. J., and S. S. McCafferty. 1990. Phylogeny and the evolution of host plant associations in the leaf beetle genus Ophraella (Coleoptera, Chrysomelidae). Evolution:1885-1913.

Groom, P. K., and B. B. Lamont. 1999. Which common indices of sclerophylly best reflect differences in leaf structure? Ecoscience **6**:471-474.

Harmon, L. J., J. T. Weir, C. D. Brock, R. E. Glor, and W. Challenger. 2008. GEIGER: investigating evolutionary radiations. Bioinformatics **24**:129-131.

Janz, N. 2011. Ehrlich and Raven Revisited: Mechanisms Underlying Codiversification of Plants and Enemies. Pages 71-89 Annual Review of Ecology, Evolution, and Systematics.

Janz, N., K. Nyblom, and S. Nylin. 2001. Evolutionary dynamics of host-plant specialization: a case study of the tribe Nymphalini. Evolution **55**:783-796.

Janz, N., and S. Nylin. 1998. Butterflies and plants: A phylogenetic study. Evolution **52**:486-502.

Konno, K., C. Hirayama, M. Nakamura, K. Tateishi, Y. Tamura, M. Hattori, and K. Kohno. 2004. Papain protects papaya trees from herbivorous insects: role of cysteine proteases in latex. Plant Journal **37**:370-378.

Koricheva, J., H. Nykanen, and E. Gianoli. 2004. Meta-analysis of trade-offs among plant antiherbivore defenses: Are plants jacks-of-all-trades, masters of all? American Naturalist **163**:64-75.

Kursar, T. A., K. G. Dexter, J. Lokvam, R. T. Pennington, J. E. Richardson, M. G. Weber, E. T. Murakami, C. Drake, R. McGregor, and P. D. Coley. 2009. The evolution of antiherbivore defenses and their contribution to species coexistence in the tropical tree genus Inga. Proceedings of the National Academy of Sciences **106**:18073-18078.

Mason-Gamer, R. J., C. F. Weil, and E. A. Kellogg. 1998. Granule-bound starch synthase: structure, function, and phylogenetic utility. Molecular Biology and Evolution **15**:1658-1673.

Nishida, R. 1994. Sequestration of plant secondary compounds by butterflies and moths. Chemoecology **5**:127-138.

Novotny, V., Y. Basset, S. E. Miller, P. Drozd, and L. Cizek. 2002. Host specialization of leaf-chewing insects in a New Guinea rainforest. Journal Of Animal Ecology **71**:400-412.

Novotny, V., S. E. Miller, L. Baje, S. Balagawi, Y. Basset, L. Cizek, K. J. Craft, F. Dem, R. A. I. Drew, J. Hulcr, J. Leps, O. T. Lewis, R. Pokon, A. J. A. Stewart, G. A. Samuelson, and G. D. Weiblen. 2010. Guild-specific patterns of species richness and host specialization in plant-herbivore food webs from a tropical forest. Journal Of Animal Ecology **79**:1193-1203.

Nyman, T., B. D. Farrell, A. G. Zinovjev, and V. Vikberg. 2006. Larval habits, host-plant associations, and speciation in nematine sawflies (Hymenoptera : Tenthredinidae). Evolution **60**:1622-1637.

Pearse, I. S., and A. L. Hipp. 2012. Global patterns of leaf defenses in oak species. Evolution **66**:2272-2286.

Ronsted, N., G. Weiblen, W. Clement, N. Zerega, and V. Savolainen. 2008. Reconstructing the phylogeny of figs (Ficus, Moraceae) to reveal the history of the fig pollination mutualism. Symbiosis (Rehovot) **45**:45.

Salazar, D., A. Jaramillo, and R. J. Marquis. 2016. The impact of plant chemical diversity on plant–herbivore interactions at the community level. Oecologia:1-10.

Sampedro, L., X. Moreira, and R. Zas. 2011. Costs of constitutive and herbivore-induced chemical defences in pine trees emerge only under low nutrient availability. Journal Of Ecology **99**:818-827.

Steemans, P., A. Le Herisse, J. Melvin, M. A. Miller, F. Paris, J. Verniers, and C. H. Wellman. 2009. Origin and radiation of the earliest vascular land plants. Science **324**:353-353.

Ter Braak, C. J., and P. Smilauer. 2012. Canoco reference manual and user's guide: software for ordination, version 5.0.

Vermeij, G. J. 1994. The evolutionary interaction among species - selection, escalation, and coevolution. Annual Review of Ecology and Systematics **25**:219-236.

Volf, M., J. Hrcek, R. Julkunen-Tiitto, and V. Novotny. 2015a. To each its own: differential response of specialist and generalist herbivores to plant defence in willows. Journal Of Animal Ecology **84**:1123-1132.

Volf, M., R. Julkunen-Tiitto, J. Hrcek, and V. Novotny. 2015b. Insect herbivores drive the loss of unique chemical defense in willows. Entomologia Experimentalis Et Applicata **156**:88-98.

Wink, M. 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry **64**:3-19.

Winkler, I. S., and C. Mitter. 2008. The phylogenetic dimension of insect-plant interactions: a review of recent evidence. Pages 240-263 Specialization, Speciation, And Radiation: The Evolutionary Biology Of Herbivorous Insects.

Supporting information

Table S1. Species codes and traits. The table shows species mean and standard deviations for protease activity in latex (ΔA280), protease activity in leaves (ΔA280), triterpene total content, triterpene diversity (Shannon), trichome density and average length, C:N, specific leaf area.

Species	Prot.Latex	Prot.Leaves	Trit.Cont	Trit.Div	Tric.Den	Tric.Len	C:N	SLA
Ficus aurantiacafolia	0.161±0.113	0.025±0.033	24.18±0.31	0.73±0.16	3.56±4.75	68.44±17.08	18.5±0.7	235.1±26.7
Ficus botryocarpa	0.127±0.108	0.034±0.034	22.52±0.24	2.04±0.21	123.92±17.33	69.61±5.63	16.7±2.1	173.3±42.6
Ficus congesta	0.1±0.176	0.016±0.011	24.26±0.44	1.18±0.26	76.23±80.53	98.43±11.77	18.6±1.1	156.1±25
Ficus conocepholia	0.057±0.05	0.02±0.017	22.29±0.16	2.29±0.28	11.24±6.18	87.33±44.07	15.1±1.5	174.8±29.5
Ficus copiosa	0.051±0.038	0.021±0.011	23.95±1.71	2.46±0.8	0.44±0.3	70.65±46.68	17.6±2.1	126.6±28.2
Ficus dammaropsis	0.048±0.054	0.024±0.013	22.9±0.34	1.91±0.31	2.2±2.89	55.52±61.68	19.3±0.4	120.4±46.2
Ficus gul	0.067±0.074	0.027±0.023	22.44±0.85	1.84±0.83	108.75±41.69	148.6±47.95	17.8±2.2	174.6±41.3
Ficus hahliana	0.067±0.057	0.005±0.003	24.05±0.23	1.07±0.2	21.12±11.41	165±22.11	19.4±1.6	137.8±26.5
Ficus hispidioides	0.138±0.175	0.016±0.02	23.44±0.39	1.33±0.12	276.68±80.07	102.15±14.01	19.3±2.8	101.2±13.4
Ficus mollior	0.215±0.167	0.032±0.023	22.61±0.1	2.57±0.11	194.48±33.81	105.53±14.05	17.5±0.5	193.2±16.3
Ficus nodosa	0.023±0.014	0.007±0.006	23.18±0.82	1.62±0.38	73.97±36.68	24.48±3.64	19.1±2.1	195.4±67.1
Ficus pachyrrhachis	0.093±0.04	0.021±0.014	24.05±0.26	1.48±0.21	130.6±44.18	119.42±7.23	17.7±1	120.3±30.4
Ficus phaeosyce	0.057±0.037	0.023±0.022	23.86±0.6	1.8±0.28	15.5±10.98	136.87±35.53	19.6±1.1	172±9.6
Ficus pungens	0.044±0.025	0.013±0.014	23.02±0.86	1.55±0.16	156.4±73.43	92.52±17.22	16.8±2.9	126.3±32.5
Ficus rubrivestimenta	0.152±0.119	0.046±0.047	24.98±1.6	1.07±0.91	7.12±4.22	82.3±24.51	23.9±3.1	207.9±40.8

Species	Prot.Latex	Prot.Leaves	Trit.Cont	Trit.Div	Tric.Den	Tric.Len	C:N	SLA
Ficus septica	0.042±0.04	0.04±0.043	21.85±0.79	1.98±0.3	1.7±2.88	18.55±69.22	16.6±2.8	104.1±5.1
Ficus subtrinervia	0.302±0.355	0.047±0.037	25.03±0.22	1.08±0.07	0.07±0.16	99.3±40.54	27.8±4.2	171.8±30.7
Ficus trachypison	0.028±0.02	0.014±0.01	21.69±0.65	2.5±0.39	176.4±47.54	48.1±8.05	17.1±1.1	142.4±43.1
Ficus variegata	0.015±0.011	0.014±0.012	23.11±0.69	1.65±0.47	86.24±115.67	34.81±15.68	17.4±1.6	274.9±82.6
Ficus virens	0.145±0.099	0.035±0.019	22.13±1.1	2.37±0.68	9.4±7.78	285.55±37.92	23.1±2.5	149.3±25.2
Ficus wassa	0.009±0.005	0.034±0.026	21.91±0.47	2.53±0.32	0.48±0.4	70.36±13.71	17.8±2.1	158.9±40.2

Table S2. Selected evolutionary models, likelihood scores and AIC weights for individual traits.

Trait	Fit	AIC Weight	Model
Protease Latex	-50.732	0.257	Brownian Motion
Protease Leaves	-123.875	0.221	Delta
C:N	101.612	0.305	Brownian Motion
SLA	220.634	0.693	White Noise
Triterpene Content	27.495	0.338	Delta
Triterpene Diversity	35.402	0.369	Delta
Trichome Length	233.207	0.413	White Noise
Trichome Density	246.953	0.706	White Noise

Chapter IV

Phylogenetic diversity of host plants drives insect-plant food web structure

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Abstract

Herbivorous insects owe their broad diversification to coevolution with plants. Diversity of herbivorous insects is further promoted by their generally high specialization. We investigate the effects of host-plant diversity on insect specialization and food-webs using herbivore assemblages studied in temperate forests of floristically diverse Japan and the less diverse Czech Republic. Sampling from a canopy crane, a cherry picker and felled trees allowed a complete census of plant-herbivore interactions within three 0.1 ha plots for leaf-chewing larvae, miners and gallers. We reconstructed insect-plant food webs and host-plant phylogenies for individual guilds and sites. Larval leaf-chewers exhibited substantial levels of generality at all three sites, whereas gallers and miners were almost exclusively

monophagous. Our results suggest a high impact of host-plant phylogeny on insect food-web structure. Leaf-chewer generality dropped rapidly when older (20-80 myr) lineages were collated. This shows that leaf-chewer generality has been maintained by feeding on confamiliar hosts while a few insect herbivores were shared between more distant plant lineages. In contrast to leaf-chewing larvae, miner and galler abundances were correlated mainly to the terminal nodes of the host phylogeny and their generality dropped immediately after collating congeneric hosts. Despite the effects of host-plant phylogeny on insect specialization, network specialization (H_2') of the food-webs on monophyletic plant lineages showed high variability and was almost identical to the H_2' on randomly selected plants. This illustrates large differences between monophlyletic host lineages and suggests that it is probably not monophyly itself but the age of divergence which drives insect specialization. In summary, our results suggest that whereas the specialization and abundance of monophagous guilds seems to be affected mainly by the terminal parts of the plant phylogeny and number of host species, the food-web structure of more generalist guilds, such as leaf-chewer larvae, is affected mainly by the of diversity of deeper plant lineages at individual sites. Further, we show that not all levels of host-plant phylogeny are equal; some play a more prominent role in structuring insect-plant food-webs than others.

Key Words

Diversity, food-webs, gallers, herbivory, leaf-chewers, miners, phylogeny, specialization

Introduction

Arthropods, recently estimated at around 6.1 million species, are one of the most specious animal taxa (Hamilton et al. 2013). Arthropods, and insects in particular, owe their enormous diversity to a large extent to their interactions with plants and a long history of insect-plant coevolution (Futuyma and Agrawal 2009). Major speciation events in plant evolution, such as radiation of angiosperms, have supported speciation in insects (Winkler and Mitter 2008) and insect taxa feeding on plants are generally more species-rich than their counterparts exploiting different food sources (Mitter et al. 1988). In turn, host-plant diversity seems to be one of the main factors driving global diversity of insects (Novotny et al. 2006).

The high diversity of herbivores is driven not only by the diversity of plants but also by high level of specialization of many insect herbivores (Dyer et al. 2007, Forister et al. 2015). For example, the majority of caterpillars randomly picked from the vegetation in a secondary rain forest in New Guinea feed on up to three host plants and have 90% of their population concentrated on a single host plant species (Novotny et al. 2004). Some level of specialization can be often detected even among polyphagous species with many of them showing a preference for congeneric or confamiliar hosts (Novotny et al. 2002, Novotny et al. 2004). Herbivores tend to feed on related host plants and host shifts are more common between closely related plants than between distantly related plant lineages (Janz and Nylin 1998, Winkler and Mitter 2008). This results in a majority of insects being phylogenetically conservative in their food-choice (Futuyma and Agrawal 2009). Host plant phylogeny is thus usually one of the reliable predictors of insect food-choice, specialization and community structure (Winkler and Mitter 2008, Volf et al. 2015a).

Insects also respond to a plethora of plant traits, some of them with low phylogenetical signal (Futuyma and Agrawal 2009, Volf et al. 2015a). Variability in functional traits may lead to large variability in

the susceptibility of even related plant species or lineages to herbivores (Agrawal and Fishbein 2008, Futuyma and Agrawal 2009). In several cases, it has been shown that host-shifts among host-plants have followed similarities in plant defences and palatability rather than their phylogeny, particularly in cases where these traits were uncorrelated with plant phylogeny (Becerra 1997, Wahlberg 2001). Secondary metabolites in particular have often shown low phylogenetic signal as chemical profiles varied considerably among congeneric species from *Bursera*, *Inga*, and *Salix* (Becerra 2007, Kursar et al. 2009, Volf et al. 2015b). On the other hand, several important plant traits, such as plant phenology, are generally conserved on higher phylogenetic levels (Davies et al. 2013). The patterns of insect specialization thus usually result from an interplay of both host-phylogeny and functional traits (Volf et al. 2015a) with their relative importance possibly depending on the phylogentic level considered.

The levels of specialization and phylogenetic conservatism in host choice differs among herbivore guilds, ranging from polyphagous root-chewing larvae feeding often on multiple plant families through to leaf-chewing larvae often feeding on several congeneric or confamiliar hosts to miners and gallers, typically specialized on a single host-plant species (Novotny et al. 2010, Forister et al. 2015). This range of host-plant choice means that species richness of individual herbivorous guilds may be driven by plant speciation events of different ages. Deeper plant phylogeny is likely to play an important role in driving the diversity of more polyphagous guilds as closely related hosts are likely to share insects whereas the communities harbored by distantly related hosts will differ. In contrast, highly specialized guilds may respond to the most recent plant diversification, e.g. on the level of species (Futuyma and Agrawal 2009).

The majority of previous studies have sampled herbivore communities from a taxonomically stratified selection of local plant

species, each sampled with constant sampling effort, rather than contiguous vegetation plots. While this protocol is suitable for the analysis of feeding preferences among plant species, it does not take into account differences in plant abundance in real vegetation, providing thus a biased assessment of herbivore species richness and host specificity (Novotny et al. 2004) and does not allow quantitative analysis of food-web structure (Godfray et al. 1999). Here we utilize a plot based approach to analyze herbivore-plant food-web structure in three 0.1 ha plots in the Czech Republic and Japan, representing lowland forests with varying host-plant diversity. We focus on three herbivore guilds – larval leaf-chewers, miners, and gallers characterized by various levels of specialization. We examine the role of host-plant phylogeny in structuring herbivore-plant food-webs. In particular, we identify what levels of host phylogeny are the most important for herbivore food-choice in individual guilds and vegetation types. We compare the characteristics of real food-webs to those generated by random host plant choice to reveal relationships between insect specialization and plant phylogeny. We expect that i) food-web characteristics differ among guilds but not study sites , ii) leaf-chewer food-webs reflect deeper plant phylogeny while those of miners and gallers reflect plant diversity on the species level.

Methods

Herbivore Sampling

We focused on three herbivore guilds with various level of specialization – leaf-chewing insect larvae, mining insects, and galling arthropods (including mites). All herbivores were as far as possible completely sampled from plants with DBH>5cm at one 0.1 ha plot in Japan (Tomakomai) and two 0.1 ha plots in the Czech Republic (Lanzhot and Mikulcice) in broadleaf lowland forests (Table 1). The Tomakomai plot had markedly higher plant

diversity, both on the species and familial levels, than the two Czech plots. While the three plots had a comparable leaf area, the number of trees was higher in Tomakomai than the two remaining plots (Table 1). The general differences in diversity were retained even when the tree communities from Tomakomai and Mikulcice were rarefied to same number of stems as in Lanzhot. The rarefaction resulted in 14±1.6 species, 9±0.9 families in Tomakomai and 7±0.5 species, 6±0.4 families in Mikulcice vs. 8 species and 6 families in Lanzhot. All plots were dominated by *Acer* (*Sapindaceae*), *Carpinus* (*Betulaceae*), *Fraxinus* (*Oleaceae*), *Quercus* (*Fagaceae*), and *Tilia* (*Malvaceae*). In the Tomakomai plot, *Magnoliaceae* and *Cercidiphyllaceae* were also important (Table S1).

Table 1. Study site characteristics: latitude, longitude, altitude, mean annual temperature and rainfall, the number of plant individuals (N_i), species (N_s) and families (N_f) with DBH ≥5cm, total sampled leaf area (m^2), and the number of individuals (N_i) and species (N_s) of leaf-chewing larvae, miners and gallers.

Site	Tomakomai, JP	Lanzhot, CZ	Mikulcice, CZ
Latitude	42° 43' N	48° 48' N	48°41'N
Longitude	141° 36'E	17° 5'E	16°56'E
Altitude (m asl)	90	152	164
T (^{o}C)	5.6	9.0	9.0
Rainfall (mm)	1,161	525	525
Tree (Ni/Ns/Nf)	81/19/11	32/8/6	53/7/6
Leaf area (m^2)	1301	1333	1137
Chewers (N_i/N_s)	8707 / 181	6152 / 162	2341 / 91
Miners (N_i/N_s)	219 / 29	5956 / 35	4763 / 12
Gallers (N_i/N_s)	525560 / 45	339885 / 55	1636525 / 34

Canopies were accessed from a canopy crane in Tomakomai. In Lanzhot, canopies were accessed from an elevated truck-mounted work platform (cherry-picker) with 4WD and a 40 m arm with several joints allowing efficient access to the canopies of all trees within the plot. In Mikulcice, herbivores were sampled from the canopies of

felled trees immediately upon felling. These methods all resulted in ca 80% of canopy foliage being sampled in all plots. The sampling was carried out in 2013 and 2014. Insects were sampled during the peak of herbivore abundance in Tomakomai and Mikulcice (mid May -mid June and late May - mid July, respectively). Lanzhot plot was sampled from mid May to August. Some species of galling mites were extremely abundant on certain trees, making sampling of individual galls impossible. In such cases, we selected three to five branches with 100-500 leaves each, calculated the mean number of galls per leaf for each branch and used these values to estimate total galler abundance on the respective tree. All herbivores sampled were morphotyped and reared to adults in plastic containers or zip-lock bags for further identification by specialists. Dead caterpillars were preserved for DNA barcoding. DNA barcoding of Lanzhot and Mikulcie specimens was performed at the Biodiversity Institute of Ontario, University of Guelph and at the University of Ostrava. Voucher specimens are deposited at the University of Chiba, the Biology Center of the Czech Academy of Sciences and the University of Ostrava.

The leaf area sampled in Mikulcice was estimated from a randomly selected subsample of leaves from each tree that was weighed, leaves were then arranged within a 50x50 cm white frame, photographed, and the leaf area calculated in ImageJ 1.48. The canopies of individual trees were defoliated, the total foliage biomass weighed and converted to leaf area using the data from the 50x50cm frames. The trees in Tomakomai and Lanzhot could not be defoliated so that the number of leaves on individual trees was estimated visually. The estimates were done independently by two persons and the mean value was used. The estimates were conducted separately for each branch and were then integrated to an estimate for the entire tree. A subsample of leaves from each tree was photographed in a 50x50 cm frame, their area calculated in ImageJ 1.48. and used to convert leaf numbers to leaf area for each tree.

Host-plant phylogeny reconstruction

Host-plant phylogeny was reconstructed using four loci: ITS, matK, rbcL, and trnL-trnF. Sequences were downloaded from GenBank where available (Table S2). If not available, air-dried leaf discs were used to obtain host-plant DNA. We used standard procedures, reaction conditions and primer sequences for DNA extraction and PCR amplification, which were the same as those used in the original studies employing these markers (Taberlet et al. 1991, Fay et al. 1997, Cronn et al. 2002). Sequences were assembled and edited using Geneious 5.4 (Drummond et al. 2011). Host plant phylogeny was reconstructed using Bayesian inference as implemented in BEAST v2.1.3 (Drummond et al. 2012). The following substitution models were selected based on BIC computed in JModelTest 2 (Darriba et al. 2012) and were used for individual loci: ITS: GTR+I+G, matK: GTR+G, rbcL: TPM3+I+G, trnL-trnF: TVM+G. The topology was constrained using Phylomatic 3 (Webb and Donoghue 2005). A log-normal relaxed molecular clock, following Bell, Soltis & Soltis (2010), with dating based on Wikström, Savolainen & Chase (2001) was used for time-calibrating the phylogeny. Sampling was carried out every 10^3 generations for 10^7 generations, the first 10% of all generations were discarded as 'burnin' and the results were summarized with a 50% majority-rule consensus tree. The reconstructed host-plant phylogeny (Fig. S1) was used in following analyses examining effects of host-plant phylogeny on insect-plant food-web structure.

Statistical analysis

Quantitative herbivore-plant food-webs were reconstructed using bipartite package (Dormann et al. 2008) in R 3.0.2 (R Development Core Team 2014). We focus mainly on two quantitative measures of specialization – generality and network specialization (H_2').

Generality is a weighted mean number of host species per herbivore (Tylianakis et al. 2007). H_2' is derived from Shannon entropy and characterizes the degree of specialization among two parties in the entire network (Blüthgen et al. 2006). Firstly, we reconstructed food-webs for individual herbivore guilds associated with host-plant species at individual sampling sites and measured their characteristics. Secondly, we also explored the effect of plant phylogeny on food web structure. We collated insect data from host lineages based on their age of divergence and reconstructed associated food-webs. We started with species-level time-calibrated host-plant phylogeny. Then we successively collated plant lineages younger than 5, 20, 50, 80, 100, and 150 million years, respectively, and measured generality for herbivore assemblages on individual plant lineages at each of these steps (see Table S3 for more details on the collated lineages). These results were compared with those obtained when the same number of plant species collated at random, to account for the effects of food-web size on the studied web parameters. We ran 100 randomizations per each step.

Thirdly, we reconstructed food-webs associated with all possible monophyletic subsets of the plant-community, from pairs of sister species to the entire community. We compared H_2' of these food-webs with those for food-webs on randomly selected subsets of host-plant species, ranging from two plant species to the entire plant community. We run 100 randomizations per each size of the random subset.

The abovementioned procedures provided a distribution of food-web generality and H_2' values for a random subsets of plant species. However, these randomizations were not completely independent from the observed food-web characteristics. The observed and random data converged on the same final point when all host-plants were included in the analysis of both randomly selected and collated species. This prevented any statistical inference based on the comparisons of observed and random data distributions.

Finally, we analyzed the correlations between the plant phylogeny and herbivore abundance associated with host-plant species using the Tomakomai data, which represented the most diverse plot with enough individual host-plant species and lineages for meaningful analysis. The abundances were standardized by leaf area sampled. We used a phylogenetic signal-representation (PSR) curve, built upon phylogenetic eigenvectregression (PVR) in PVR package in R (Diniz-Filho et al. 2012, Diniz Filho et al. 2012), to visualize the correlations between herbivore abundances and various levels of host-plant phylogeny. Sequential PVR models were fitted after successively increasing the number of eigenvectors and their R^2 was plotted against the accumulated eigenvalues.

Results

In total, we sampled 3,683 m^2 of foliage from the three plots. The Tomakomai plot harbored the most diverse community of herbivores with 255 species of leaf-chewing larvae, miners and gallers, followed by Lanzhot (252 species) and Mikulcice (137 species, Table 1). Communities of leaf-chewing insect larvae were dominated by Lepidoptera and Hymenoptera at all sites, with some Coleoptera larvae present in Tomakomai. Miners included Lepidoptera, Coleoptera, Hymenoptera and Diptera. The gall community was strongly dominated by galling mites (*Eriophyidae*), representing up to 97% of the galler abundance, followed by Diptera, Hymenoptera and Hemiptera.

Leaf-chewing insect larvae were the least specialized of the tree studied guilds with an average species using 2-4 hosts, resulting in food-web generality ranging from 1.4 to 3.6 at individual sites (Table 2). Still, 16% (Tomakomai), 25% (Lanzhot) and 38% (Mikulcice) species of leaf-chewing larvae fed on a single host species even when excluding singletons and doubletons. The generality of leaf-chewing larvae decreased in the same order as plant diversity in the studied plots, from the highest values in Tomakomai to the lowest in

Mikulcice. On the other hand, vulnerability did not follow trends in host diversity and was high in Lanzhot, moderate in Tomakomai and low in Mikulcice (Table 2).The food-web in Mikulce was dominated by a single monophagous species of Hymenoptera which further contributed to low generality and vulnerability at this site (Fig. 1).

Table 2. Food-web characteristics for larval leaf-chewers, miners and gallers at individual sites.

Herbivore guild	Generality	Vulnerability	H'_2
Larval leaf-chewers			
Tomakomai	3.651	13.376	0.460
Lanzhot	2.465	16.045	0.503
Mikulcice	1.434	7.038	0.703
Miners			
Tomakomai	1.069	2.191	0.970
Lanzhot	1.041	2.676	0.978
Mikulcice	1.000	1.850	1.000
Gallers			
Tomakomai	1.000	1.863	1.000
Lanzhot)	1.047	1.673	0.960
Mikulcice	1.000	1.448	1.000

Miners and gallers were much more specialized when compared to leaf-chewing larvae, with almost all miner and galler species being monophagous at all studied sites. The only exceptions were the six miner and ten galler species which were shared among closely related (mostly congeneric) host-plants (Fig. 1). Miner and galler generality thus ranged only from 1.000 to 1.069 and was independent of plant diversity (Table 2). The number of miner and galler species per host-plant was lower when compared to leaf-chewing larvae and resulted in vulnerability from 1.448 to 2.676.
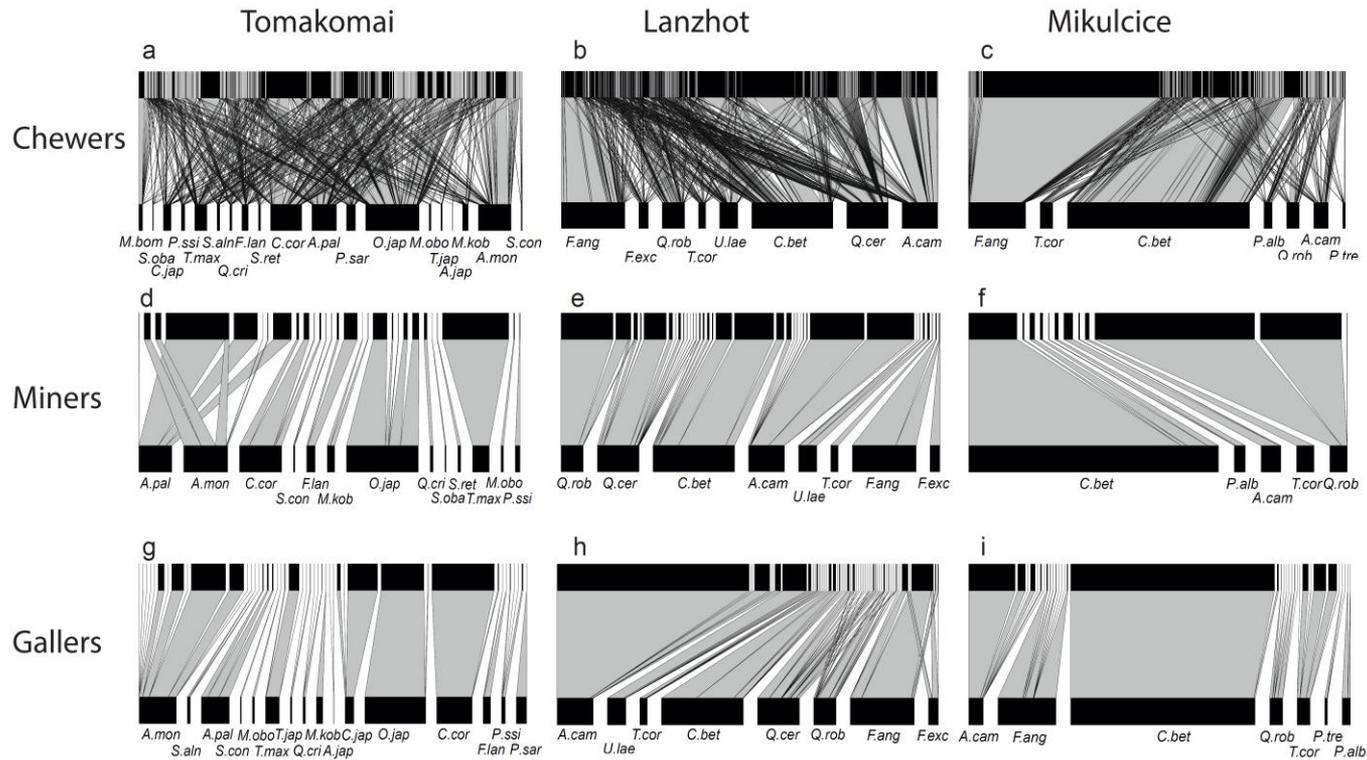
Figure 1. Food-webs showing trophic associations of larval leaf-chewers, miners, and gallers in Tomakomai, Lanzhot, and Mikulcice. The widths of species blocks reflect their relative abundance in the community, based on leaf area for plant species and the number of individuals for herbivore species (see table S1 for used abbreviations of host-plant species names).

The analysis of food-webs revealed a non-linear decrease in generality when collating progressively older plant lineages. In larval leaf-chewers from Tomakomai, there was a very small decrease in generality after collating the most closely related plants from young lineages, viz. congeneric species from *Acer* and *Tilia* that separated <5 myr ago (Fig. 2a-c). Collating older plant lineages resulted in a major drop in food-web generality for lineages 5 - 20 myr old, including the older speciation events of congeneric species (*Acer*, *Magnolia*) and the separation of the Betulaceae genera (Fig. 2a). The much simpler phylogenetic structure of the Lanzhot and Mikulcice communities did not allow for detailed analysis, but a major drop in generality was noted when collating plant lineages 20 - 80 myr old (Fig. 2b-c), particularly in comparison with randomized data. Miners and gallers were often monophagous which resulted in the generality of their food-webs being constantly 1.0 in the case of Tomakomai gallers and Mikulcice miners and gallers. Communities of Tomakomai miners and Lanzhot miners and gallers included some species feeding on more than one host-plant species. In this case, there was a large decrease in generality after collating closely related plants from young lineages with this trend being more pronounced in gallers (Fig. 2d-f).

The H_2' of the food-webs associated with monophyletic subsets of plant communities showed rather large variability in most cases (Fig. 3). Some of the monophyletic lineages showed low H_2' when compared to random data whereas other monophyletic lineages showed H_2' values close to random or even above the mean of random data. This resulted in H_2' of the monophyletic lineages following a random distribution on the whole. Miners and gallers were often monophagous which resulted in the H_2' of their food-webs being constantly 1.0 in case of Tomakomai gallers and Mikulcice miners and gallers. The remaining miner and galler communities showed similar trends to larval leaf-chewers but with generally much higher H_2' values (Fig. 3d-f).
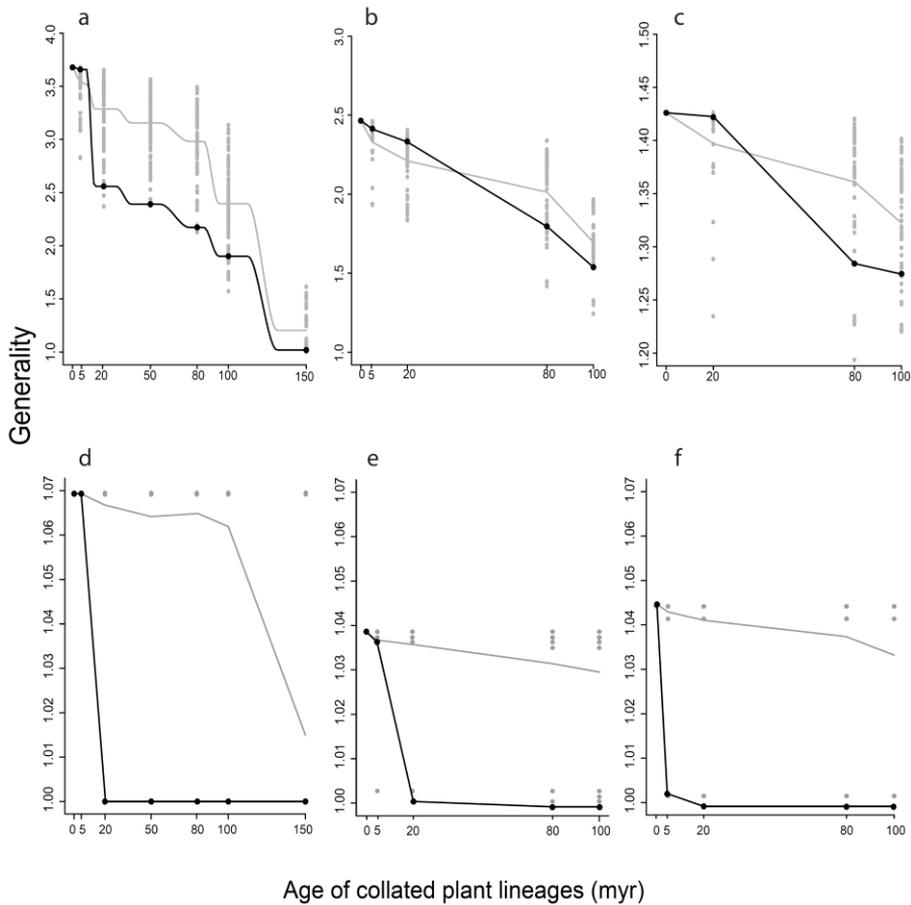
Figure 2. Effects of collating plant lineages on the generality of larval leaf-chewers in Tomakomai, Lanzhot, and Mikulcice (a-c), miners in Tomakomai and Lanzhot (d-e), and gallers in Lanzhot (f). Other miners and gallers were exclusively monophagous and their generality was constantly 1.0 (not shown in the figure). Data were obtained in analyses successively collating insect data from plant lineages younger than 5, 20, 50, 80, 100, and 150 million years (black), or at random (grey). The curves were modeled using a loess smoother with α selected based on AIC in Fig. 3a. There were not enough data points in other cases for using loess smoother reliably. In these cases the means were connected by straight lines.
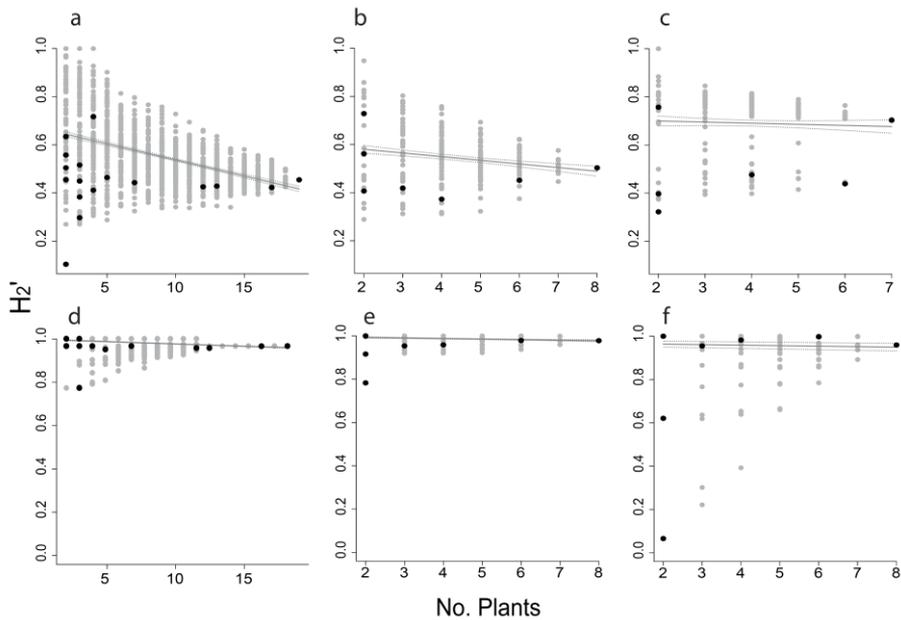
Figure 3. H$_2$' of monophyletic plant lineages (black) and randomly selected host samples (grey) shown for larval leaf-chewers in Tomakomai, Lanzhot and Mikulcice (a-c), miners in Tomakomai and Lanzhot (d-e), and gallers in Lanzhot (f). Other gallers and miners were exclusively monophagous and their H$_2$' was constantly 1.0 (not shown in the figure). Grey lines with confidence bands show linear trends in random data.
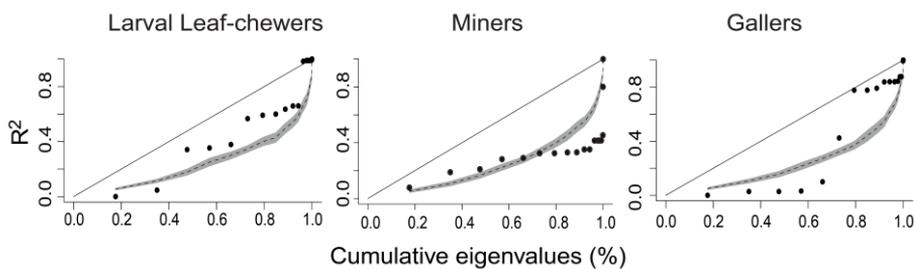
Figure 4. PSR curves showing the correlations between the host-plant phylogeny and larval leaf-chewer, miner and galler abundance in Tomakomai. The points represent eigenvectors standing for individual nodes of the phylogeny showing how R^2 increases with different node depths. The dashed line represents the curve expected under a null model and its confidence interval

PSR curves showed guild-specific differences in correlation between Tomakomai herbivore abundances and the depth of the corresponding eigenvectors. There was an almost linear increase in R^2 with decreasing depth of eigenvectors in the case of larval leaf-chewer abundances. On the other hand, miner and galler abundances showed a low correlation with the lower and mid-levels of host-plant phylogeny and a steep increase in the R^2 of the shallow eigenvectors, suggesting strong effects of species level phylogeny (Fig. 4).

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Discussion

Insect specialization is one of the key factors maintaining a high diversity of insect herbivores (Novotny et al. 2006, Dyer et al. 2007). In this study we examined the role of host-plant phylogenetic diversity and abundance in supporting insect specialization and structuring insect-plant food-webs in three temperate forest communities in Japan and Central Europe. Our results suggest a high impact of host-plant phylogeny on insect specialization, in line with many previous studies (Futuyma and Agrawal 2009, Cagnolo et al. 2011, Volf et al. 2015a). However, we show that not all levels of host-plant phylogeny are equal; some play a more prominent role in structuring insect-plant food-webs than others. Further, we suggest that these trends are guild-dependent so that the importance of host-plant phylogeny in maintaining herbivore communities differs among herbivore guilds with varying degrees of specialization.

Insects tend to feed on closely related hosts (Futuyma and Agrawal 2009, Cagnolo et al. 2011, Volf et al. 2015a). For example, there were large similarities in the Lepidoptera community composition among congeneric hosts in lowland tropical forest in Papua New Guinea whereas the overlap decreased steeply towards zero with increasing phylogenetic distance between the hosts (Novotny et al. 2002). Here, we utilized a new approach of collating insect data associated with plant lineages younger than the specified age of

divergence to identify the relative importance of different stages of host-plant phylogeny for the generality of insect-plant food-webs. Our results revealed a major drop in food-web generality of leaf-chewing larvae when collating plant lineages with ages of divergence 5-20 myr in case of diverse Tomakomai community. This corresponded to the separation of some congeneric species (in *Acer* and *Magnolia)* and also *Betulaceae* genera; all these lineages represented substantial parts of the community. Collating younger lineages such as congeneric species in *Tilia* and some *Acer* species, as well as collating older lineages representing different general families or orders, did not lead to large drops in generality, especially when compared to random data. Collating Euasterids and Eurosids into a single eudicot species pool resulted in the final major drop in generality as large proportion of species from Geometridae (42%), Noctuidae (50%) and Tortricidae (45%), as the most species rich Lepidoptera families in our data, fed exclusively either on Eurosids or Euasterids. This could suggest that radiation of lineages such as Euasterids and Eurosids were not only some of the major drivers of insect speciation (Winkler and Mitter 2008) but also that they are important drivers of insect food-choice in present day communities due to large differences between host plant traits from such distant lineages. However, this may also be an artifact of the sample size as a as similar drop was observed in the random data.

The composition of both the Lanzhot and Mikulcice plant communities was species poor and there were very few congeneric and confamiliar hosts present to test these patterns. There was thus only a limited drop in generality when collating young plant lineages. The major drop in generality was shifted towards deeper phylogenetic relationships – mainly to the division into orders with the divergence of Fagales playing a major role. On the whole, our results suggest the important role of mid-level host phylogeny in structuring food-webs of larval leaf-chewers, although the identity and specific depth of the

important phylogenetic nodes may differ among sites depending on the host community composition.

Although many larval leaf-chewers fed on confamiliar hosts, we found only a limited decrease in generality when collating the closest congeneric relatives among host-plants. The decrease in generality when collating food-webs associated with closely related congenerics was remarkably lower than in the random data suggesting a relatively low number of insects being shared among the closest relatives. This may reflect large differences in traits among congeneric plants growing in sympatry (Becerra 2007, Kursar et al. 2009, Volf et al. 2015b). This appears to be the case for poplars, relying on species-specific defences (Palo 1984), which were the only congeneric plants in the Mikulcice community. Divergent defences may help closely related plants to escape herbivory in such cases as they decrease the pool of herbivores shared among closely related hosts (Becerra 2007). This leaves older diversification, on the intermediate levels of host-plant phylogeny, more important that terminal phylogeny which is possibly over ridden by other factors as insect host choice follows similarities in plant defence and palatability rather than their phylogeny in cases where the two are uncorrelated (Becerra 1997, Wahlberg 2001). However, further analyses including larger numbers of closely related hosts would be needed to verify this conclusion.

Miners and gallers are more specialized than larval leaf-chewers and their food-webs are highly compartmentalized (Novotny et al. 2010, Cagnolo et al. 2011, Forister et al. 2015). Although they can to some extend bypass toxic plant defenses, other aspects, mainly metabolic factors, drive their food-choice and their host-shifts are generally rare even among closely related hosts (Stone et al. 2009). In our case, miners and gallers were often monophagous or feeding on pairs of closely related congeneric hosts. This was reflected by their food-web generality which was either constantly 1.0 despite collating plant lineages or dropped immediately after collating congeneric hosts.

Miners and gallers have been strongly affected by host-plant evolution and diversification due to their intimate interactions with host-plants, although the majority of miner and galler lineages radiated well after their hosts (Leppänen et al. 2012). For example, the colonization of species rich host-plant lineages has led to the radiation in some groups, leading to gallers and miners being in general well represented on species rich plant groups (Nyman et al. 2006, Stone et al. 2009). Our results revealed only a limited correlation between lower and mid-levels of host-plant phylogeny and Tomakomai miner and galler abundances suggesting large interspecific differences in galler and miner abundance within the plant families or genera examined. On the other hand, there was steep increase in the correlation between their abundances and terminal nodes of host-phylogeny, especially when compared to larval leaf-chewers which showed an almost linear increase with decreasing depth of host-plant phylogeny, which further stresses the role of terminal plant relationships in structuring miner and galler communities and food-webs.

Despite the effect of host-plant phylogeny on insect specialization, closely related hosts represented by monophyletic lineages of various sizes did not have lower H_2' than expected by chance. Most insect herbivores are specialists and maintain the majority of their populations on a limited number of related hosts (Novotny et al. 2002, Novotny et al. 2004, Forister et al. 2015). This should result in lower H_2' of food-webs associated with monophyletic hosts, as the most closely related subsets of the host community of a given size, compared to randomly selected samples. However, the H_2' of monophyletic host lineages followed a random distribution, showing no trend on the whole. This was due to the large variability in their H_2'. For example, whereas *Fraxinus lanuginosa* and *Syringa reticulata* had lower H_2' than any random combination of hosts, *Tilia maxomowiziana* and *T. japonica* showed H_2' values close to the mean of random data. This large variability between lineages with several

congeneric species showing high H_2' suggests that food-web structure depends on the specifics of plant phylogenetic structure, such as the age of divergence, determining the decrease in the number of lineages retained at each age, as well as the herbivores.

There is no doubt that the high diversity of host-plants is one of the key factors maintaining hot-spots of insect diversity, such as those in lowland tropical forests (Novotny et al. 2006). Our results suggest that the specific role of plant phylogenetic diversity in maintaining insect-plant food-web structure, and in turn insect diversity, differs among herbivore guilds based on their specialization. Changes in phylogenetic diversity acting at taxonomic levels higher than the species level are likely to mainly affect the specialization and diversity of less specialized guilds. On the other hand, high host diversity at the species level and presence of certain host species drives specialization of herbivores from specialized guilds, such as miners and gallers. This can be illustrated by plant communities with relatively low phylogenetic diversity, such as willow dominated wetlands. The predominance of closely related hosts seems to result, to large extent, in a shared pool of leaf-chewing herbivores feeding on many hosts in the community (Volf et al. 2015a). Leaf-chewer specialization in communities of closely related hosts than seems to be driven mainly by functional traits rather than host phylogeny (Becerra 1997, Kursar et al. 2009, Volf et al. 2015b). On the other hand, such communities may support a high diversity of specialized guilds, such as gallers (Nyman et al. 2006) which respond mainly to the terminal nodes of the host phylogeny and are seldom shared even between closely related hosts (Novotny et al. 2010, Forister et al. 2015).

In conclusion, our results demonstrate the high importance of host-plant phylogeny in modulating insect-plant food-web structure, revealing non-trivial dependence of insect specialization on host-plant phylogenetic composition. The specific role of host-plant diversity is likely to differ depending on the level of specialization of the

respective insect guild. Further, previous studies showed a predominant role of plant traits in structuring insect communities if they were uncorrelated with the host-plant phylogeny (Becerra 1997, Wahlberg 2001). It would be highly interesting to unravel the specific role of the interaction between host-plant traits and various levels of host-plant phylogeny in structuring insect-plant food-webs, as some results suggest that this interaction may differ depending on the levels of host phylogeny included in the analysis.

Acknowledgements

References

Agrawal, A. A., and M. Fishbein. 2008. Phylogenetic escalation and decline of plant defense strategies. Proceedings of the National Academy of Sciences of the United States of America **105**:10057-10060.

Becerra, J. X. 1997. Insects on plants: Macroevolutionary chemical trends in host use. Science **276**:253-256.

Becerra, J. X. 2007. The impact of herbivore-plant coevolution on plant community structure. Proceedings of the National Academy of Sciences of the United States of America **104**:7483-7488.

Bell, C. D., D. E. Soltis, and P. S. Soltis. 2010. The age and diversification of the angiosperms re-revisited. American Journal of Botany **97**:1296-1303.

Blüthgen, N., F. Menzel, and N. Blüthgen. 2006. Measuring specialization in species interaction networks. BMC ecology **6**:1.

Cagnolo, L., A. Salvo, and G. Valladares. 2011. Network topology: patterns and mechanisms in plant-herbivore and host-parasitoid food webs. Journal of Animal Ecology **80**:342-351.

Cronn, R. C., R. L. Small, T. Haselkorn, and J. F. Wendel. 2002. Rapid diversification of the cotton genus (*Gossypium* : *Malvaceae*) revealed by analysis of sixteen nuclear and chloroplast genes. American Journal of Botany **89**:707-725.

Darriba, D., G. L. Taboada, R. Doallo, and D. Posada. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nature methods **9**:772-772.

Davies, T. J., E. M. Wolkovich, N. J. Kraft, N. Salamin, J. M. Allen, T. R. Ault, J. L. Betancourt, K. Bolmgren, E. E. Cleland, and B. I. Cook. 2013. Phylogenetic conservatism in plant phenology. Journal of Ecology **101**:1520-1530.

Diniz-Filho, J. A. F., L. M. Bini, T. F. Rangel, I. Morales-Castilla, M. Á. Olalla-Tárraga, M. Á. Rodríguez, and B. A. Hawkins. 2012. On the selection of phylogenetic eigenvectors for ecological analyses. Ecography **35**:239-249.

Diniz Filho, F., J. Alexandre, T. F. Rangel, T. Santos, and L. Mauricio Bini. 2012. Exploring patterns of interspecific variation in quantitative traits using sequential phylogenetic eigenvector regressions. Evolution **66**:1079-1090.

Dormann, C. F., B. Gruber, and J. Fründ. 2008. Introducing the bipartite package: analysing ecological networks. interaction **1**:0.2413793.

Drummond, A. J., B. Ashton, S. Buxton, M. Cheung, A. Cooper, C. Duran, M. Field, J. Heled, M. Kearse, S. Markowitz, R. Moir, S. Stones-Havas, S. Sturrock, T. Thierer, and A. Wilson. 2011 Geneious v5.4.

Drummond, A. J., M. A. Suchard, D. Xie, and A. Rambaut. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Molecular biology and evolution **29**:1969-1973.

Dyer, L. A., M. S. Singer, J. T. Lill, J. O. Stireman, G. L. Gentry, R. J. Marquis, R. E. Ricklefs, H. F. Greeney, D. L. Wagner, H. C. Morais, I. R. Diniz, T. A. Kursar, and P. D. Coley. 2007. Host specificity of Lepidoptera in tropical and temperate forests. Nature **448**:696-U699.

Fay, M. F., S. M. Swensen, and M. W. Chase. 1997. Taxonomic affinities of Medusagyne oppositifolia (Medusagynaceae). Kew Bulletin:111-120.

Forister, M. L., V. Novotny, A. K. Panorska, L. Baje, Y. Basset, P. T. Butterill, L. Cizek, P. D. Coley, F. Dem, I. R. Diniz, P. Drozd, M. Fox, A. E. Glassmire, R. Hazen, J. Hrcek, J. P. Jahner, O. Kaman, T. J. Kozubowski, T. A. Kursar, O. T. Lewis, J. Lill, R. J. Marquis, J. S. Miller, H. C. Morais, M. Murakami, H. Nickel, N. A. Pardikes, R. E. Ricklefs, M. S. Singer, A. M. Smilanich, J. O. Stireman, S. Villamarín-Cortez, S. Vodka, M. Volf, D.

L. Wagner, T. Walla, G. D. Weiblen, and L. A. Dyer. 2015. The global distribution of diet breadth in insect herbivores. Proceedings of the National Academy of Sciences **112**:442-447.

Futuyma, D. J., and A. A. Agrawal. 2009. Macroevolution and the biological diversity of plants and herbivores. Proceedings of the National Academy of Sciences of the United States of America **106**:18054-18061.

Godfray, H. C. J., O. T. Lewis, and J. Memmott. 1999. Studying insect diversity in the tropics. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences **354**:1811-1824.

Hamilton, A. J., V. Novotny, E. K. Waters, Y. Basset, K. K. Benke, P. S. Grimbacher, S. E. Miller, G. A. Samuelson, G. D. Weiblen, J. D. L. Yen, and N. E. Stork. 2013. Estimating global arthropod species richness: refining probabilistic models using probability bounds analysis. Oecologia **171**:357-365.

Janz, N., and S. Nylin. 1998. Butterflies and plants: A phylogenetic study. Evolution **52**:486-502.

Kursar, T. A., K. G. Dexter, J. Lokvam, R. T. Pennington, J. E. Richardson, M. G. Weber, E. T. Murakami, C. Drake, R. McGregor, and P. D. Coley. 2009. The evolution of antiherbivore defenses and their contribution to species coexistence in the tropical tree genus Inga. Proceedings of the National Academy of Sciences **106**:18073-18078.

Leppänen, S. A., E. Altenhofer, A. D. Liston, and T. Nyman. 2012. Phylogenetics and evolution of host-plant use in leaf-mining sawflies (Hymenoptera: Tenthredinidae: Heterarthrinae). Molecular Phylogenetics and Evolution **64**:331-341.

Mitter, C., B. Farrell, and B. Wiegmann. 1988. The Phylogenetic Study of Adaptive Zones - Has Phytophagy Promoted Insect Diversification. American Naturalist **132**:107-128.

Novotny, V., Y. Basset, S. E. Miller, G. D. Weiblen, B. Bremer, L. Cizek, and P. Drozd. 2002. Low host specificity of herbivorous insects in a tropical forest. Nature **416**:841-844.

Novotny, V., P. Drozd, S. E. Miller, M. Kulfan, M. Janda, Y. Basset, and G. D. Weiblen. 2006. Why are there so many species of herbivorous insects in tropical rainforests? Science **313**:1115-1118.

Novotny, V., S. E. Miller, L. Baje, S. Balagawi, Y. Basset, L. Cizek, K. J. Craft, F. Dem, R. A. I. Drew, J. Hulcr, J. Leps, O. T. Lewis, R. Pokon, A. J. A. Stewart, G. A. Samuelson, and G. D. Weiblen. 2010. Guild-specific patterns of species richness and host specialization in plant-herbivore food webs from a tropical forest. Journal of Animal Ecology **79**:1193-1203.

Novotny, V., S. E. Miller, J. Leps, Y. Basset, D. Bito, M. Janda, J. Hulcr, K. Damas, and G. D. Weiblen. 2004. No tree an island: the plant–caterpillar

food web of a secondary rain forest in New Guinea. Ecology Letters **7**:1090-1100.

Nyman, T., B. D. Farrell, A. G. Zinovjev, and V. Vikberg. 2006. Larval habits, host-plant associations, and speciation in nematine sawflies (Hymenoptera : Tenthredinidae). Evolution **60**:1622-1637.

Palo, R. T. 1984. Distribution of birch (*Betula* spp), willow (*Salix* spp), and poplar (*Populus* spp) secondary metabolites and their potential role as chemical defense against herbivores. Journal of Chemical Ecology **10**:499-520.

R Development Core Team. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Stone, G. N., A. Hernandez-Lopez, J. A. Nicholls, E. Di Pierro, J. Pujade-Villar, G. Melika, and J. M. Cook. 2009. Extreme host plant conservatism during at least 20 million years of host plant pursuit by oak gallwasps. Evolution **63**:854-869.

Taberlet, P., L. Gielly, G. Pautou, and J. Bouvet. 1991. Universal primers for amplification of three noncoding regions of chloroplast DNA. Plant Molecular Biology **17**:1105-1109.

Tylianakis, J. M., T. Tscharntke, and O. T. Lewis. 2007. Habitat modification alters the structure of tropical host–parasitoid food webs. Nature **445**:202-205.

Volf, M., J. Hrcek, R. Julkunen-Tiitto, and V. Novotny. 2015a. To each its own: differential response of specialist and generalist herbivores to plant defence in willows. Journal of Animal Ecology **84**:1123-1132.

Volf, M., R. Julkunen-Tiitto, J. Hrcek, and V. Novotny. 2015b. Insect herbivores drive the loss of unique chemical defense in willows. Entomologia Experimentalis et Applicata **156**:88-98.

Wahlberg, N. 2001. The phylogenetics and biochemistry of host-plant specialization in Melitaeine butterflies (Lepidoptera : Nymphalidae). Evolution **55**:522-537.

Webb, C. O., and M. J. Donoghue. 2005. Phylomatic: tree assembly for applied phylogenetics. Molecular Ecology Notes **5**:181-183.

Wikström, N., V. Savolainen, and M. W. Chase. 2001. Evolution of the angiosperms: calibrating the family tree. Proceedings of the Royal Society of London B: Biological Sciences **268**:2211-2220.

Winkler, I. S., and C. Mitter. 2008. The phylogenetic dimension of insect-plant interactions: a review of recent evidence. Pages 240-263 Specialization, Speciation, and Radiation: The Evolutionary Biology of Herbivorous Insects.

Supporting information
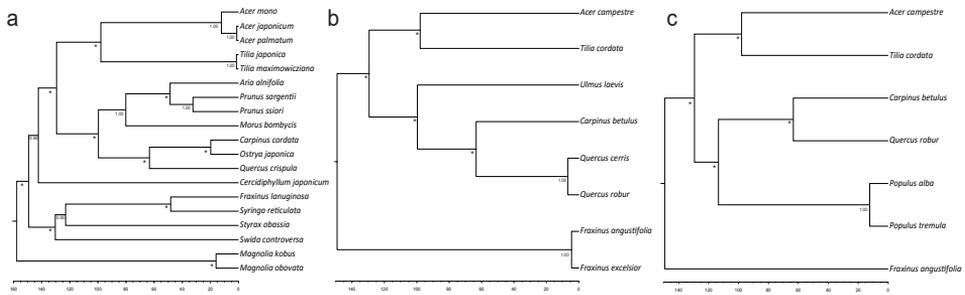
Figure S1. Phylogenies of host-plant communities in Tomakomai (a), Lanzhot (b), and Mikulcice (c). Host-plant phylogenies was reconstructed using ITS, matK, rbcL, and trnL-trnF loci by Bayesian inference. Nodes are labeled by posterior probabilities or by asterisks if constrained using Phylomatic 3 (Webb & Donoghue 2005). Scale axis shows time calibration (in millions of years) with dating based on Wikström, Savolainen & Chase (2001).

Table S1. List of host-plant species, their abbreviations used in Figure 1 and number of harboured individuals (N_i) and species (N_s) of leaf-chewing larvae, miners and gallers.

Full name	Abbreviation	Leaf-chewer (Ni/Ns)	Miner (Ni/Ns)	Galler (Ni/Ns)
Tomakomai				
Acer japonicum	A.jap	3/3	0/0	21/2
Acer mono	A.mon	1263/48	148/5	159065/13
Acer palmatum	A.pal	787/54	927/4	7286/3
Carpinus cordata	C.cor	1560/64	83/4	16/2
Cercidiphyllum japonicum	C.jap	56/23	141/2	149471/2
Fraxinus lanuginosa	F.lan	1511/49	141/2	77897/3
Magnolia kobus	M.kob	42/13	5/2	109/2
Magnolia obovata	M.obo	17/3	6/1	2/1
Morus bombycis	M.bom	271/17	0/0	1/1
Ostrya japonica	O.jap	2391/84	233/5	0/0
Prunus sargentii	P.sar	110/22	0/0	102289/2
Prunus ssiori	P.ssi	123/22	3/1	2/1
Quercus crispula	Q.cri	186/37	28/1	3282/4
Sorbus alnifolia	S.aln	54/20	0/0	44/3
Styrax obassia	S.oba	48/11	1/1	0/0
Swida controversa	S.con	5/4	6/2	3/1
Syringa reticulata	S.ret	123/14	2/1	0/0
Tilia japonica	T.jap	45/10	0/0	963/2
Tilia maximowicziana	T.max	112/35	607/1	25107/3
Lanzhot				
Acer campestre	A.cam	1888/83	2284/9	255900/7
Carpinus betulus	C.bet	845/81	1431/8	6860/7
Fraxinus angustifolia	F.ang	532/37	21/2	30545/7
Fraxinus excelsior	F.exc	74/20	10/1	4174/4
Quercus cerris	Q.cer	2333/91	745/13	8162/16
Quercus robur	Q.rob	184/43	1411/3	553/14
Tilia cordata	T.cor	93/24	8/1	288/5
Ulmus laevis	U.lae	203/34	86/3	33468/5

Mikulcice

Acer campestre	A.cam	65/22	149/2	330902/8
Carpinus betulus	C.bet	563/55	857/4	1144759/3
Fraxinus angustifolia	F.ang	1573/35	0/0	13370/9
Populus alba	P.alb	22/14	60/1	476/2
Populus tremula	P.tre	8/5	0/0	168/3
Quercus robur	Q.rob	88/19	3637/3	758/9
Tilia cordata	T.cor	22/10	60/1	146092/7

Table S2. GenBank Sequences used for host-plant phylogeny reconstruction.

Species	Accession Number	Authors
ITS		
Acer campestre	DQ238434.1	Grimm,G.W., Renner,S.S., Stamatakis,A. and Hemleben,V. "A nuclear ribosomal DNA phylogeny of Acer inferred with maximum likelihood, splits graphs, and motif analysis of 606 sequences". Evol Bioinform Online 2, 279-294 (2006)
Acer japonicum	DQ238397.1	Grimm,G.W., Renner,S.S., Stamatakis,A. and Hemleben,V. "A nuclear ribosomal DNA phylogeny of Acer inferred with maximum likelihood, splits graphs, and motif analysis of 606 sequences". Evol Bioinform Online 2, 279-294 (2006)
Acer mono	DQ238453.1	Grimm,G.W., Renner,S.S., Stamatakis,A. and Hemleben,V. "A nuclear ribosomal DNA phylogeny of Acer inferred with maximum likelihood, splits graphs, and motif analysis of 606 sequences". Evol Bioinform Online 2, 279-294 (2006)
Acer palmatum	JF980312.1	Oh,D.-J., Song,G.-P., Choi,S.-A., Ko,M.-S., Yim,E.-Y., Park,S.-H. and Jung,Y.-H. "Genetic analysis of plants distributed in Jeju Island"
Aria alnifolia	FJ796908.1	Li,Q., Guo,W., Liao,W., Macklin,J.A. and Li,J. "Generic limits of Pyrinae: Insights from Sequences of Nuclear Ribosomal DNA"
Carpinus betulus	HM235960.1	Lefort,F., Roemer,J. and Crovadore,J. "Direct DNA amplification from plant infected tissues"
Carpinus cordata	FJ011713.1	Yoo,K.-O. and Wen,J., "Phylogeny of subfamily Coryloideae (Betulaceae) based on seven nuclear and plastid markers"
Cercidiphyllum japonicum	AF147756.1	Zhang,Q., Shi,S., Huang,Y., Tan,F., Jin,H. and Chang,H., "The analyses of ITS sequences from Hamamelidineae and its phylogenetic significance"
Fraxinus angustifolia	EU314820.1	Wallander,E. "Systematics of Fraxinus (Oleaceae) and evolution of dioecy". Plant Syst. Evol. 273 (1-2), 25-49 (2008)
Fraxinus excelsior	EU314847.1	Wallander,E. "Systematics of Fraxinus (Oleaceae) and evolution of dioecy". Plant Syst. Evol. 273 (1-2), 25-49 (2008)
Fraxinus lanuginosa	EU314857.1	Wallander,E. "Systematics of Fraxinus (Oleaceae) and evolution of dioecy". Plant Syst. Evol. 273 (1-2), 25-49 (2008)
Morus bombycis	AY345151.1	Weiguo,Z., Yile,P. and Zhifang,Z. "Phylogeny and systematics of Morus as determined by sequence analysis of ITS".
Ostrya japonica	FJ011754.1	Yoo,K.-O. and Wen,J., "Phylogeny of subfamily Coryloideae (Betulaceae) based on seven nuclear and plastid markers"
Populus alba	JQ898650.1	Zhao,J.P., Diao,S., Zhang,B.Y., Niu,B.Q., Wang,Q.L., Wan,X.C. And Luo,Y.Q "Phylogenetic Analysis and Molecular Evolution Patterns in the MIR482-MIR1448 Polycistron of Populus L". PLoS ONE 7 (10), E47811 (2012)
Populus tremula	KC485108.1	Feng,J., Jiang,D., Shang,H., Zhao,C., Liu,J. and Mao,K. "Barcoding poplars (Populus L.) from Western china". PLoS ONE 8 (8), E71710 (2013)
Prunus sargentii	AF179512.1	Lee,S. and Wen,J. " A phylogenetic analysis of Prunus and the Amygdaloideae (Rosaceae)using ITS sequences of nuclear ribosomal DNA". Am. J. Bot. 88 (1), 150-160 (2001)
Quercus cerris	FM243864.1	Denk,T. and Grimm,G.W. "The oaks of western Eurasia: traditional classifications and evidence from two nuclear markers"
Quercus robur	EU628560.1	Simeone,M.C. and Papini,A. "Systematic relationships of two west-Asiatic oaks, Quercus iberica M. Bieb. and Quercus macranthera Fisch. & Mey. ex Hohen., inferred from a multi-species oak molecular phylogeny"
Styrax obassia	AF327479.1	Fritsch,P.W. "Phylogeny and biogeography of the flowering plant genus Styrax (Styracaceae) based on chloroplast DNA restriction sites and DNA sequences of the internal transcribed spacer region". Mol. Phylogenet. Evol. 19 (3), 387-408 (2001)

Species	Accession Number	Authors
Swida controversa	JF980315.1	Oh,D.-J., Song,G.-P., Choi,S.-A., Ko,M.-S., Yim,E.-Y., Park,S.-H. and Jung,Y.-H. "Genetic analysis of plants distributed in Jeju Island"
Syronga reticulata	AF297080.1	Li,J., Zhang,D. and Alexander,J.H. III. "Systematics and classification of tree lilacs inferred from morphology and DNA sequences"
Tilia maximowicziana	KF445420.1	Melosik,I., Ciupinska,M., Winnicka,K. and Koukoulas,G. "Species/geographic boundaries and evolutionary interrelationships of cultivated linden-trees (Tilia L.) based on morphological and nrDNA ITS characteristics". J Biodivers Environ Sci 5 (5), 90-118 (2014)
Ulmus laevis	KC539599.1	Neubig,K.M., Herrera,F., Manchester,S.R., Germain-Aubrey,C., Abbott,J.R. and Whitten,W.M. "Building the elm tree: expanded phylogenetics of Ulmaceae using DNA data, fossils, biogeography and dating"

matK

Species	Accession Number	Authors
Acer japonicum	AB872515.1	Nakadai,R., Murakami,M. and Hirao,T. "Effects of phylogeny, leaf traits, and the altitudinal distribution of host plants on herbivore assemblages on congeneric Acer species". Oecologia 175 (4), 1237-1245 (2014)
Acer palmatum	AB038174.1	Kita,Y. and Kato,M. "Phylogenetic relationships of the aquatic angiosperm family Podostemaceae inferred from matK sequence data"
Aria alnifolia	DQ860451.1	Campbell,C.S., Evans,R.C., Morgan,D.R., Dickinson,T.A. and Arsenault,M.P. "Phylogeny of subtribe Pyrinae (formerly the Maloideae, Rosaceae): Limited resolution of a complex evolutionary history". Plant Syst. Evol. 266 (1-2), 119-145 (2007)
Carpinus cordata	AY211986.1	Yoo,K.-O. and Wen,J., "Phylogeny of subfamily Coryloideae (Betulaceae) based on seven nuclear and plastid markers"
Cercidiphyllum japonicum	AB490219.1	Kokubun,H., Watanabe,H., Koizumi,M., Hashida,H. and Ando,T. "Intraspecific phylogeographical structure reflecting a Tertiary event"
Fraxinus excelsior	AM933427.1	Besnard,G., Rubio de Casas,R., Christin,P.A. and Vargas,P. "Phylogeny of Olea (Oleaceae) based on plastid and nuclear-ribosomal DNA sequences: impact of tertiary climatic shifts on its diversification"
Magnolia kobus	JX280396.1	Song,E. and Kim,S.
Morus bombycis	GU145560.1	Venkateswarlu,M., Ravikumar,G. and Nair,V.C. "Testing candidate plant barcode regions in 13 Morus species"
Ostrya japonica	AY212005.1	Yoo,K.-O. and Wen,J., "Phylogeny of subfamily Coryloideae (Betulaceae) based on seven nuclear and plastid markers"
Prunus sargentii	KF154805.1	Shi,S., Li,J., Sun,J., Yu,J. and Zhou,S. "Phylogeny and Classification of Prunus sensu lato (Rosaceae)". J Integr Plant Biol (2013)
Quercus crispula	AB727873.1	Liu,H. and Harada,K. "Geographic distribution and the origin of newly occurred chloroplast C- type in Quercus species in the northeastern Japan"
Swida controversa	U96893.1	Xiang,Q., Soltis,D. and Soltis,P. "Phylogenetic relationships of Cornaceae and close relatives inferred from matK and rbcL sequences". Am. J. Bot. 85 (2), 285 (1998)
Syringa reticulata	JN590998.1	Li,J., Goldman-Huertas,B., DeYoung,J. and Alexander,J. "Phylogenetics of Syringa: Evidence from nuclear and plastid DNA sequence data"

rbcL

Species	Accession Number	Authors
Acer campestre	DQ978399.1	Renner,S.S., Beenken,L., Grimm,G.W., Kocyan,A. and Ricklefs,R.E. "The evolution of dioecy, heterodichogamy, and labile sex expression in Acer". Evolution 61 (11), 2701-2719 (2007)
Acer japonicum	AB872548.1	Nakadai,R., Murakami,M. and Hirao,T. "Effects of phylogeny, leaf traits, and the altitudinal distribution of host plants on herbivore assemblages on congeneric Acer species". Oecologia 175 (4), 1237-1245 (2014)

Species	Accession Number	Authors
Acer mono	DQ978416.1	Renner,S.S., Beenken,L., Grimm,G.W., Kocyan,A. and Ricklefs,R.E. "The evolution of dioecy, heterodichogamy, and labile sex expression in Acer". Evolution 61 (11), 2701-2719 (2007)
Acer palmatum	DQ978421.1	Renner,S.S., Beenken,L., Grimm,G.W., Kocyan,A. and Ricklefs,R.E. "The evolution of dioecy, heterodichogamy, and labile sex expression in Acer". Evolution 61 (11), 2701-2719 (2007)
Carpinus betulus	AY263928.1	Li,R.-Q., Chen,Z.-D., Lu,A.-M., Soltis,D.E. and Soltis,P.S. "High Resolution and Stability Testing of Fagales Phylogenetic Trees Based on Multiple DNA Sequences from Three Genomes"
Carpinus cordata	KF418945.1	Xiang,X.-G., Wang,W., Li,R.-Q., Lin,L., Liu,Y., Zhou,Z.-K., Li,Z.-Y. and Chen,Z.-D. "The interplay of diaspores and environments triggers increased diversification of Fagales in the Paleogene". Perspect. Plant Ecol. Evol. Syst. 16 (3), 101-110 (2014)
Cercidiphyllum japonicum	L11673.1	Olmstead,R.G., Michaels,H.J., Scott,K.M. and Palmer,J.D. "Monophyly of the Asteridae and identification of their major lineages inferred from DNA sequences of rbcL". Ann. Mo. Bot. Gard. 79, 249-265 (1992)
Fraxinus angustifolia	HG765055.1	Laiou,A., Mandolini,L.A., Piredda,R., Bellarosa,R. and Simeone,M.C. "DNA barcoding as a complementary tool for conservation and valorisation of forest resources". Zookeys 365, 197-213 (2013)
Fraxinus excelsior	FJ395592.1	James,K.E., Rumsey,F., Spencer,M., Carine,M., Vogel,J.C. And Schneider,H. "Barcoding Darwin's meadow: high-throughput DNA barcoding from specimen to sequence".
Magnolia kobus	AY743438.1	Pirie,M.D., et.al. "Phylogeny reconstruction and molecular dating in four Neotropical genera of Annonaceae: the effect of taxon sampling in age estimations".REGNUM VEGETABILE 143: 149-174 (2005)
Ostrya japonica	KF418957.1	Xiang,X.-G., Wang,W., Li,R.-Q., Lin,L., Liu,Y., Zhou,Z.-K., Li,Z.-Y. and Chen,Z.-D. "The interplay of diaspores and environments triggers increased diversification of Fagales in the Paleogene". Perspect. Plant Ecol. Evol. Syst. 16 (3), 101-110 (2014)
Populus alba	HM850277.1	Schaefer,H., Hardy,O.J., Silva,L., Barraclough,T.G. and Savolainen,V. "Testing Darwin's naturalization hypothesis in the Azores" . Ecol. Lett. 14 (4), 389-396 (2011)
Populus tremula	AJ418827.1	Chase,M.W. "When in doubt, put it in Flacourtiaceae: a molecular phylogenetic analysis based on plastid rbcL DNA sequences"
Prunus sargentii	AY052515.1	Jung,Y.H., Han,S.H., Oh,Y.S. and Oh,M.Y. "Prunus sargentii (34p4p3) sequence of rbcL gene"
Quercus cerris	AB125017.1	Kamiya,K., Harada,K., Ogino,K., Mahani,M.C. and Latiff,A. "Phylogeny and genetic variation of Fagaceae in tropical montane forests".
Quercus robur	AB125025.1	Kamiya,K., Harada,K., Ogino,K., Mahani,M.C. and Latiff,A. "Phylogeny and genetic variation of Fagaceae in tropical montane forests".
Styrax obassia	AF396158.1	Fritsch,P.W., Morton,C.M., Chen,T. and Meldrum,C. "Phylogeny and biogeography of the Styracaceae".
Swida controversa	AF190433.1	Xiang,Q.-Y., Soltis,D.E., Soltis,P.S., Manchester,S.R. and Crawford,D.J. "Timing the Eastern Asian-Eastern North American Floristic Disjunction: Molecular Clocks Corroborate Paleontological Evidence".
Tilia cordata	KP088885.1	Dong,W., Xu,C., Li,C., Sun,J., Zuo,Y., Shi,S., Cheng,T., Guo,J. And Zhou,S. "ycf1, the most promising plastid DNA barcode of land plants". Sci Rep 5, 8348 (2015)
Ulmus laevis	KC539702.1	Neubig,K.M., Herrera,F., Manchester,S.R., Germain-Aubrey,C., Abbott,J.R. and Whitten,W.M. "Building the elm tree: expanded phylogenetics of Ulmaceae using DNA data, fossils, biogeography and dating".
trnL-trnF		
Acer campestre	AF401189.1	Tian,X., Guo,Z.-H. and Li,D.-Z. "Phylogeny of Aceraceae based on ITS and trnL-F data sets".

Species	Accession Number	Authors
Acer japonicum	AJ413167.1	Pfosser,M.F., Guzy-Wrobelska,J., Sun,B.Y., Stuessy,T.F.,Sugawara,T. and Fujii,N. "The origin of species of Acer (Sapindaceae) endemic to Ullung Island, Korea". Syst. Bot. 27 (2), 351-367 (2002)
Acer mono	JN102147.1	Oh,D.-J., Song,G.-P., Choi,S.-A., Ko,M.-S., Yim,E.-Y., Park,S.-H. and Jung,Y.-H. "Genetic analysis of plants distributed in Jeju Island"
Acer palmatum	AJ413165.1	Pfosser,M.F., Guzy-Wrobelska,J., Sun,B.Y., Stuessy,T.F.,Sugawara,T. and Fujii,N. "The origin of species of Acer (Sapindaceae) endemic to Ullung Island, Korea". Syst. Bot. 27 (2), 351-367 (2002)
Aria alnifolia	DQ863223.1	Campbell,C.S., Evans,R.C., Morgan,D.R., Dickinson,T.A. And Arsenault,M.P. "Phylogeny of subtribe Pyrinae (formerly the Maloideae, Rosaceae): Limited resolution of a complex evolutionary history". Plant Syst. Evol. 266 (1-2), 119-145 (2007)
Carpinus betulus	FJ012011.1	Yoo,K.-O. and Wen,J., "Phylogeny of subfamily Coryloideae (Betulaceae) based on seven nuclear and plastid markers".
Carpinus cordata	AY211400.1	Yoo,K.-O. and Wen,J., "Phylogeny of subfamily Coryloideae (Betulaceae) based on seven nuclear and plastid markers".
Cercidiphyllum japonicum	AM397171.1	Worberg,A., Quandt,D., Barniske,A.M., Loehne,C., Hilu,K.W. And Borsch,T. "Phylogeny of basal eudicots: Insights from non-coding and rapidly evolving DNA". Org. Divers. Evol. 7 (1), 55-77 (2007)
Fraxinus excelsior	AY911646.1	Harbourne,M.E., Douglas,G.C., Waldren,S. and Hodkinson,T.R. "Characterization and primer development for amplification of chloroplast microsatellite regions of Fraxinus excelsior". J. Plant Res. 118 (5), 339-341 (2005)
Magnolia kobus	AY743457.1	Pirie,M.D., et.al. "Phylogeny reconstruction and molecular dating in four Neotropical genera of Annonaceae: the effect of taxon sampling in age estimations".REGNUM VEGETABILE 143: 149-174 (2005)
Magnolia obovata	AB570261.1	Wu,Y., Zhu,S. and Komatsu,K. "Identification of herbal drugs by DNA sequences".
Morus bombycis	JN006417.1	Chen,R.-F., Yu,M.-D., Zhang,Z., Xu,L., Wang,X.-L. and Tang,Z. "Morus ITS, trnL-F, rps16 Sequence and Phylogenetic Analysis of Mulberry Resources". Zhongguo Nong Ye Ke Xue 44 (8), 1553-1561 (2011)
Ostrya japonica	AY211421.1	Yoo,K.-O. and Wen,J., "Phylogeny of subfamily Coryloideae (Betulaceae) based on seven nuclear and plastid markers".
Prunus sargentii	AF429919.1	Jung,Y.H., Han,S.H., Oh,Y.S. and Oh,M.Y.
Quercus cerris	HM770073.1	Paule,J., Newbury,H.John. and Ford-Lloyd,B.V. "CpDNA variation in Carpathian oaks (Quercus L.)"
Quercus crispula	AB727893.1	Liu,H. and Harada,K. "Geographic distribution and the origin of newly occurred chloroplast C-type in Quercus species in the northeastern Japan".
Quercus robur	HM770066.1	Paule,J., Newbury,H.John. and Ford-Lloyd,B.V. "CpDNA variation in Carpathian oaks (Quercus L.)"
Styrax obassia	AB237440.1	Iwasaki,T., Aoki,K., Seo,A. and Murakami,N. "Intraspecific sequence variation of chloroplast DNA among the component species of deciduous broad-leaved forests in Japan". J. Plant Res. 119 (5), 539-552 (2006)
Syringa reticulata	JN591017.1	Li,J., Goldman-Huertas,B., DeYoung,J. and Alexander,J. "Phylogenetics of Syringa: Evidence from nuclear and plastid DNA sequence data".
Ulmus laevis	KC539736.1	Neubig,K.M., Herrera,F., Manchester,S.R., Germain-Aubrey,C., Abbott,J.R. and Whitten,W.M. "Building the elm tree: expanded phylogenetics of Ulmaceae using DNA data, fossils, biogeography and dating".

Table S3. Grouping of collated plant lineages following time-calibrated phylogeny. Letters indicate plants collated in one group at individual steps.

Tomakomai

Species	0 mya	5 mya	20 mya	50 mya	80 mya	100 mya	150 mya
A. japonicum		A	A	A	A	A	A
A. mono			A	A	A	A	A
A. palmatum		A	A	A	A	A	A
A. alnifolia				F	F	C	A
C. cordata			C	C	C	C	A
C. japonicum							A
F. lanuginosa				E	E	E	A
M. kobus			D	D	D	D	D
M. obovata			D	D	D	D	D
M. bombycis					F	C	A
O. japonica			C	C	C	C	A
P. sargentii				F	F	C	A
P. ssiori				F	F	C	A
Q. crispula					C	C	A
S. obassis							A
S. controversa							A
S. reticulata				E	E	E	A
T. japonica		B	B	B	B	A	A
T. maximowicziana		B	B	B	B	A	A

Lanzhot

Species	0 mya	5 mya	20 mya	50 mya	80 mya	100 mya	150 mya
A. campestre						C	A
C. betulus					B	B	A
F. angustifolia		A	A	A	A	A	A
F. excelsior		A	A	A	A	A	A
Q. cerris			B	B	B	B	A
Q. robur			B	B	B	B	A
U. laevis						B	A
T. cordata						C	A

Mikulcice

Species	0 mya	5 mya	20 mya	50 mya	80 mya	100 mya	150 mya
A. campestre						C	A
C. betulus					B	B	A
F. angustifolia							A
P. alba			A	A	A	A	A
P. tremula			A	A	A	A	A
Q. robur					B	B	A
T. cordata						C	A

Chapter V

Speciation in a keystone plant genus is driven by elevation: a case study in New Guinean *Ficus*

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Abstract

Much of the world's insect and plant biodiversity is found in tropical and subtropical 'hotspots', which often include long elevational gradients. These gradients may function as 'diversity pumps' and contribute to both regional and local species richness. Climactic conditions on such gradients often change rapidly along short vertical distances, and may result in local adaptation and high levels of population genetic structure in plants and insects. We investigated the population genetic structure of two species of *Ficus* (Moraceae) along a continuously forested elevational gradient in Papua New Guinea. This speciose plant genus is pollinated by tiny, species specific and highly co-evolved chalcid wasps (Agaonidae). Our results from six elevations (200 m-2,700 m a.s.l.) (representing the entire altitudinal range of *Ficus*) and 10 polymorphic microsatellite loci show that strong barriers to gene flow exist between 1,200 m and 1,700 m a.s.l. Whereas lowland populations are panmictic across distances over 70 km, highland/lowland subpopulations can be disjunct over 4 km. We suggest that the limited gene flow between populations of montane

Ficus maybe driven by environmental limitations on pollinator or seed dispersal in combination with local adaptation of *Ficus* populations. Such a mechanism may have wider implications for plant and pollinator speciation across long and continuously forested elevational gradients if more generalist insect pollinators and vertebrate seed dispersers also respond to elevational changes in climate and form subpopulations based on elevation.

Key Words

Geneflow, Mountains, Mutualism, Papua New Guinea, Pollination

Introduction

Many of the world's biodiversity 'hotspots' include long tropical or subtropical elevational gradients (Myers et al. 2000, Mittermeier et al. 2004). Rapidly changing environmental conditions along such elevational gradients can lead them to function as 'diversity pumps' which may contribute to the origin of a large proportion of the world's biodiversity (Robin et al. 2010, Schultheis et al. 2012, Toussaint et al. 2014). Phylogeographic studies of insects indicate that the formation of species in parapatry; where species ranges abut but do not overlap (Gavrilets 2004) in montane habitats can create speciation 'cradles' that fuel lowland diversity (Hall 2005). Studies of plant communities also reveal high levels of species turnover at mid-elevations in large, species rich tropical families (e.g. Burger 1995). Local adaptation (and the filtering of maladapted genotypes) and limitations to insect mediated gene flow are likely to be especially important in insect pollinated flowering plants flowering plants (Reis et al. 2015), which represent the majority of tropical forest plants. Given that over 60% of described eukaryote species in terrestrial ecosystems is either a plant or an insect feeding on a plant (Price

2002) it is not unreasonable to suggest that understanding the mechanisms of speciation in plants along elevational gradients is an important research goal, especially given our rapidly changing climate.

Montane habitats surrounded by lowland forest (called "sky islands") have a clear role in promoting peripatric speciation in the hyper-diverse Australasian region (Toussaint et al. 2013), with species of montane origin feeding back into the overall lowland species pool. The regions highlands provide an excellent natural laboratory in which to investigate ongoing or incipient speciation. Within the wider region, Papua New Guinea (PNG) is recognized as being particularly biodiverse. Indeed, 5% of the world's animal and plant species are found in PNG, an area representing 0.5% of the world's total land area, and two thirds of these species are endemic. The country is also known for its dramatic and geologically active topography which may contribute considerably to its high levels of endemism and biodiversity. Many ecologically important plant genera have diversified considerably in PNG, acting as important host islands for insect herbivores (Weiblen et al. 2001, 2006, Novotny et al. 2010). One such species rich genus is *Ficus* (Moraceae). This pantropical genus is extraordinarily species rich, containing over 800 species, 157 of which occur in PNG (Berg and Corner 2005, Cruaud et al. 2012). In PNG, *Ficus* species are over-represented amongst plant species with wide elevational ranges and represent one of the key genera in forest communities along elevational gradients (Novotny et al. 2005). Pollination in *Ficus* is performed exclusively by wasps in the chalcid family Agaonidae (Wiebes 1979). These tiny wasps are usually species specific and can act as effective pollinators over tens to hundreds of kilometers in continuous habitats such as rainforests or deserts (Nason et al. 1996, Ahmed et al. 2009), whilst seed dispersal is carried out by a wide range of vertebrates, including bats and birds (Shanahan et al. 2001). As such, both fig pollen and seeds can be transported over large distances in homogeneous environments.

However, little is known about gene flow in *Ficus* along ecological gradients, for instance between populations of *Ficus* species with wide elevational ranges. Whilst there are documented examples of lowland and highland varieties or subspecies in at least three sections of *Ficus* (Berg and Corner 2005) several examples of extremely close relatives occupying lowland and highland habitats can be found within the Papuan species in section Sycocarpus (which is pollinated by wasps from the genus *Ceratosolen*). The Papuan species of Sycocarpus are relatively recent in origin (around 15 MY) (Cruaud et al. 2012) and form something of a species complex with some species still capable of hybridizing (Moe and Weiblen 2012). We studied gene flow in two species of *Ficus* from section Sycocarpus, *F. arfakensis* King and *F. hahliana* Diels, along a continuously forested elevational gradient from 200 m a.s.l. to the altitudinal limit of their distribution at 2,700 m a.s.l. in Papua New Guinea's Central Range,.

Given that wasp-mediated gene flow between populations of *Ficus* in continuous lowland habitats can cover tens to hundreds of kilometers (Nason et al. 1996, Ahmed et al. 2009) we might expect to see a similar pattern in montane populations. This would be evidenced by panmixia in the populations of both species studied here. However, environmental conditions vary dramatically across elevational gradients and may lead to limitations on pollinator and/or seed dispersal and even local adaptation followed by phenotypic isolation. This would result in genetic structure corresponding to gradual or sudden changes in vertical distance, so called subpopulations. We expect genetic diversity in *Ficus* to decrease with elevation. This is because lowland populations are connected to a large gene pool through long distance wasp migration (Nason et al. 1996), whereas highland allelic diversity would be a nested subset of lowland diversity if vertical transmission is limited (mountains acting as bottlenecks). Mechanistically, the above canopy winds that facilitate long-range dispersal of wasps in lowland habitats are likely to be a less effective method of pollinator dispersal to higher elevations. This

may be especially true for understory tree species (Harrison 2003) like *F. arfakensis* and *F. hahliana*. Furthermore, major genetic bottlenecks may occur at climatic interfaces, e.g. at the 'cloud layer' (the site of near constant cloud immersion resulting from relief precipitation), limiting gene flow between elevations and exacerbating the genetic disparity between adjacent populations and allowing subpopulation specific alleles to accumulate.

Methods

Survey of Ficus diversity

A detailed survey was carried out at each of six study sites along an elevational gradient focused on Mt Wilhelm in Papua New Guinea (see Table 1 and Fig. S1 for site locations). At each elevational site teams of researchers and paraecologists tagged all *Ficus* trees having a d.b.h (diameter at breast height) greater than 1 cm within ten 500 x 10m transects, transects were located at least 200 m from each other. Each tree was identified to species and given a unique tree identifier number.

Focal species, plant tissue collection and genotyping

F. arfakensis is present at four sites (between 200-1,700 m) and we sampled an average of 15 individual trees per site, whilst *F. hahliana* is present at six sites (between 200-2,700 m) and we sampled an average of 8 individual trees per site (Table 1). Leaf tissue was collected only from male trees large enough to bear fruit, at least 20 m was left between individuals and clonal individuals were avoided. We initially aimed to sample one tree per transect. We selected only male trees so that pollinating wasps could be subsequently collected and associated with a given host tree (Souto-Vilarós et al., in prep). Our selective sampling criteria and the naturally low density of mature trees meant that we effectivly sampled haphazardly across

transects at each site, GPS location and voucher specimens were collected for a subset of the trees. The distance between trees sampled at a given site therefore ranged from 20 m to 1 km but was always less than the distance between sites, so that sampling at each site was representative of the local subpopulation. We had to relocate our 1,700 m sampling site during the project (Table 1) due to land ownership disputes at our original site. Furthermore, we also sampled one lowland population of both species from Ohu village (145°41' E, 5°14' S) near Madang (around 70 km North East of our transect).

Table 1. Names of sample sites, their elevation (m a.s.l), their GPS coordinates, distance in a straight line to Kausi, the transect site with the lowest elevation (DLE), number of sampled *Ficus hahliana* and *F. arfakensis* individuals, and size of *F. hahliana* syconia.

Site	Elev. (m)	Latitude (S)	Longitude (E)	DLE (km)	*Ficus* N (hah./arf.)	Syconia (cm3)
Ohu	200	05°14' 00"	145°41' 00"	70	4/1	NA
Kausi	200	05°44'33"	145°20'01"	0	10/14	0.99±0.03
Numba	700	05°44'14"	145°16'12"	7	5/15	0.89±0.02
Memeku	1,200	05°43'18"	145°16'17"	7	10/13	1.17±0.03
Bananumbu	1,700	05°45'21"	145°14'11"	11	5/5	NA
Degenumbu	1,700	05°45'45"	145°11'55"	15	10/10	2.88±0.44
Sinopass	2,200	05°45'34"	145°10'49"	17	5/0	4.48±0.43
Br. Sawmill	2,700	05°48'57"	145°09'02"	22	5/0	5.45±0.28

Ficus arfakensis has a recorded range of up to 1,600 m in elevation (Berg and Corner, 2005). It is widespread in PNG. As with many members of section Sycocarpus, *F. arfakensis* grows as a small understory tree and is often locally abundant in secondary forest (Berg and Corner, 2005). *Ficus hahliana,* described by Berg and Corner (2005) as a lowland species, is often found close to rivers throughout PNG. Morphologically *F. hahliana* is easily confused with *F. bernaysii* King (up to 1,800 m). Both species form a species complex including also the highland species *F. iodotricha* Diels (700-

2,900 m). In order to provide context we genotyped four individuals of the latter two species.

Leaf discs (collected using a cork borer of 2.4cm in diameter) were dried in the field in zip-lock plastic bags containing two table spoons of colour indicating silica gel, which was replaced when necessary. All samples were then stored at -20 ˚C until needed for analysis. We isolated DNA from one leaf disc per individual using Invisorb Spin Plant Mini Kits (STRATEC Molecular, Germany). Due to polyphenol and secondary metabolite carry over through the spin column in some samples (in particular for *F. hahliana*), we also extracted DNA using a modified CTAB protocol (Doyle and Doyle 1987) with an additional cleaning step through a silica spin column or agarose gel. This removed all traces of contaminants yielding highly concentrated and pure DNA as measured by both Qubit Fluorometer (Invitrogen, OR, USA) and NanoDrop (Thermo Scientific, DE, USA). Because the syconia of *F. hahliana* clearly vary in size with elevation, we collected a total of 590 mature syconia across six sites (Table 1) and measured both height and width to the nearest 0.01mm using Vernier calipers. Volume (cm^3) was calculated using a standard cone volume formula: $= \pi r^2 \frac{h}{3}$.

We selected 11 microsatellite loci previously published for the genus *Ficus* (Moe and Weiblen 2011, Garcia et al. 2012), which were amplified in three multiplex sets (Table 2). Each PCR reaction was composed of 4µl of Multiplex PCR Master Mix (QIAGEN), 0.2 µM of each primer, 1 µl Q-solution (QIAGEN), approximately 20-50 ng of template DNA and filled with PCR H$_2$O to the total volume of 10 µl. Conditions for the PCR reactions were: 15 min of 94 °C, followed by 35 cycles of 94 °C (30 s), 54 °C (90 s), 72 °C (60 s), with final elongation at 60 °C for 30 min. Genotypes were scored using the software Genemapper 3.7 (Applied Biosystems).

Bayesian analysis of population structure was performed to determine i) the proportion of the sampled genome of an individual that came from each subpopulation using STRUCTURE v2.3.4 (Pritchard et al. 2000) as well as the number of subpopulation clusters using both STRUCTURE and BAPS v5.4 (Corander et al. 2004) and ii) the hierarchical clustering of individuals using BAPS. In STRUCTURE we used the admixture model with the default settings and a burnin of 10,000 and 1,000,000 reps. We estimated k (the number of allelic clusters in our data set) using Evanno's ΔK (Evanno et al. 2005), using 10 replicates for each value of k between one and six. We used STRUCTURE Harvester (Earl and von Holdt 2012) to compare ΔK and CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007) (using the 'full search' algorithm) and Distruct v1.1 (Rosenberg 2003) to summarise and plot the output. In BAPS we grouped individuals using the 'clustering of groups of individuals' and 'clustering of individuals' to assign populations and individuals to clusters with k set to 100, the number of clusters (k) was determined using maximum likelihood. The relationships between the clusters of individuals were visualized by plotting neighbor joining trees using Nei's distance. A species level neighbor joining tree was estimated for the *F. hahliana* complex.

To compare genetic variation within and between the major subpopulations we used AMOVA (analysis of molecular variance) (Excoffier et al. 1992). We performed AMOVA of pair-wise Euclidean distances using the 'poppr.amova' function in the R package 'poppr' (Kamvar et al. 2014). We tested the significance of genetic structure at each level (within individuals, within subpopulations and between subpopulations) using the 'randtest' function in the package 'ade4'(Dray et al. 2007). Individuals were grouped to subpopulations using the clusters derived by ΔK as the most conservative estimate of k, and this gave the same conclusions as using the BAPS subpopulation clusters (results not shown).

Table 2. Genetic diversity over 10 microsatellite loci in the two *Ficus* species studied. values are shown for *F. hahliana*/*F. arfakensis:* Na = Number of alleles, Ho o= bserved heterozygosity, He = expected heterozygosity, F = fixation index. Source studies: Garcia et al. (2012), Moe & Weiblen (2012).

Locus	Source	Na	Ho	He	F
Micr2(CA)	Garcia et al.	16/6	0.60/0.23	0.89/0.60	0.32/0.62
Sur1(GA)	Garcia et al.	3/3	0.19/0.13	0.21/0.12	0.07/-0.06
Car10(TG)	Garcia et al.	5/3	0.30/0.58	0.42/0.53	0.28/-0.11
Sur2(AG)	Garcia et al.	1/4	0.00/0.40	0.00/0.36	0.00/-0.12
Car11(CA)	Garcia et al.	10/9	0.53/0.59	0.80/0.76	0.34/0.23
Micr3(CT)	Garcia et al.	1/4	0.00/0.12	0.00/0.48	0.00/0.74
P211(GA)	Moe & Weiblen	5/8	0.33/0.61	0.67/0.72	0.50/0.15
B83(AG)	Moe & Weiblen	11/8	0.35/0.83	0.77/0.67	0.55/-0.25
B47(GAA)	Moe & Weiblen	14/7	0.22/0.37	0.63/0.46	0.65/0.20
P215(ATGT)	Moe & Weiblen	13/10	0.51/0.73	0.89/0.79	0.43/0.07
Mean		9.6/6.2	0.38/0.46	0.66/0.55	0.39/0.15

We summarised the F_{st} values within and between each ∆K cluster and conducted more detailed analyses of population genetic parameters between BAPS subpopulation clusters to describe the finer scale differences along the gradient, given that the mid-elevation subpopulations of each species may represent contact zones which contained a number of private alleles. We calculated genetic diversity parameters using GeneAlex (Peakall and Smouse 2006), i.e. the number of alleles per locus, the observed and expected heterozygosities and the fixation index - an analogue to the inbreeding coefficient expressing the probability that two alleles in an individual are identical by descent (Hedrick 2005). We used 'poppr' (Kamvar et al. 2014) to calculate the number of private alleles in each subpopulation. Differences in pairwise F_{st} values between subpopulations of both species from different elevations were visualized using parametric smoothing as implemented in the R package 'loess', the smoothing parameter was selected using AIC.

We recognize that using only a limited number of individuals and loci can influence estimates of genetic distance and clustering inferences. We therefore tested the power of our data to detect non-homogenous subpopulations using the software 'Powsim' v4.1 (Ryman and Palm 2006). We stress that our main aim was quite simple, to detect non-homogeneity and assign major genetic clusters. We tested the power of our data to detect the two major subpopulations inferred using STRUCTURE at an F_{st} threshold of 0.025 for both species. We used the default MCMC chain settings but set N_e to 2,000 and t to 100 to give the desired F_{st} threshold of 0.025. We used 100 replicates in each case. Power was assessed as the proportion of significances according to both the Chi-square test and Fishers exact test. We also estimated α (the chance of a Type I error) by setting the F_{st} threshold to 0 and sampling directly from the base population.

Results

In our field surveys we identified 12,500 individuals from 73 species, around 45% of the country's 157 *Ficus* species. The patterns in species richness and species turnover with elevation are summarised in Fig. 1. In total we genotyped 58 individuals of *F. arfakensis* and 49 individuals of *F. hahliana* for 11 microsatellite loci. In *F. arfakensis* three loci (Car9, Micr3 and Sur2) were either monomorphic or failed to amplify, so that eight polymorphic loci were used for the analysis. In *F. hahliana* locus Car9 was monomorphic, but the remaining 10 loci were polymorphic and were included in the analyses.

We used STRUCTURE to estimate the proportion of the sampled genome of an individual that came from each subpopulation. For *F. arfakensis* we identified two clusters using ΔK (Mean L(K)=-1143.8, ΔK =83.1) and three clusters using L(K) (Mean L(K)=-1039.0, ΔK =6.6) (Fig 2). For *F. hahliana* we identified two clusters of genotypes using both ΔK (Mean L(K)=-896.3, ΔK=2,730.6) and L(K). For *F.*

arfakensis in particular it was difficult to rule out the existence of more than two clusters of genotypes. Given the nested structure of our data set and non-homogenous gene flow (mid-elevation sites represent a mixture of lowland and highland alleles, but the highlands contain a subset of these) we consider the ΔK clusters to represent the major genetic divisions.
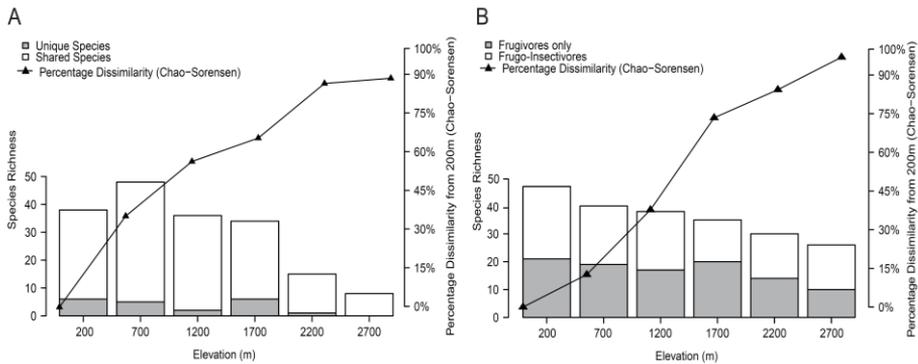
Figure 1. The species richness of *Ficus* for each elevation (bars, left axis) and percentage dissimilarity in comparison to the 200 m site calculated using the Chao-Sorensen distance (line, right axis). Bars are partitioned into species unique only to that elevation (grey) and species shared across more than one elevation (white) (a). The species richness of birds with at least a partially frugivorous diet for each elevation (bars, left axis) and percentage dissimilarity in comparison to the 200 m site calculated using the Chao-Sorenson Abundance based distance (line, right axis) (b). Bars are partitioned into bird species that are purely frugivorous (grey) and species that also eat insects (white) – data based on point count survey by Sam & Koane (2014).

We used BAPS to cluster sampling sites into subpopulations based on their genotypes, and indeed we recovered four clusters for *F. arfakensis* (cluster one: Ohu, cluster two: 200m and 700m, cluster three: 1,200 m and cluster four: 1,700 m) and three clusters for *F. hahliana* (cluster one: Ohu, 200 m, 700 m, 1,200 m, cluster two: 1,700 m and cluster three: 2,200 m and 2,700 m). Our final level of clustering addressed individual genotypes, and we showed a clear

contact zone at 1,200 m for *F. arfakensis*, with some individuals showing the strongest affinity to the 1,700 m cluster, whilst others grouped with genotypes more common at 200 m and 700 m (Fig 3).
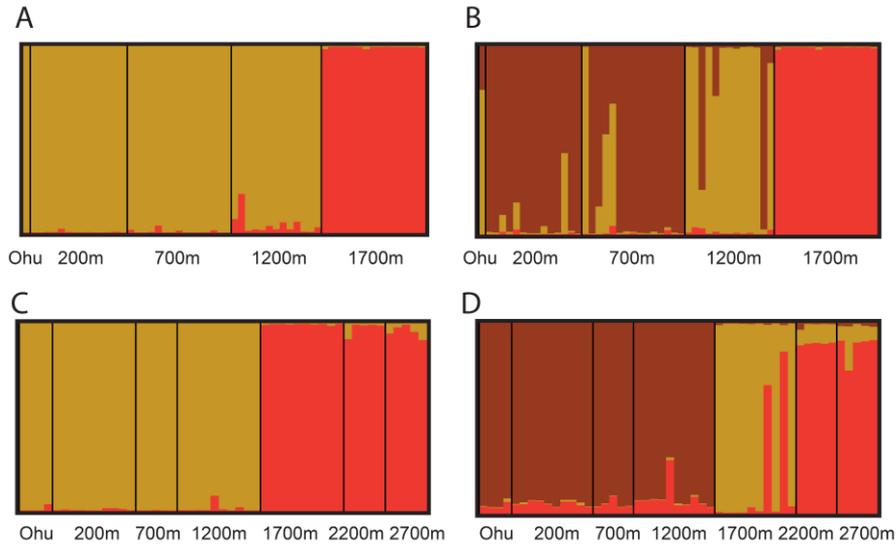
Figure 2. The proportion of the sampled genome of each individual originating from each sub-population as derived by STRUCTURE *F. hahliana* (k=2) (a), *F. hahliana* (k=3) (b), *F. arfakensis* (k=2) (c) and *F. arfakensis* (k=3) (d).

We used AMOVA to test for panmixia across all individuals from each species and to test for hierarchical structure in genetic variation. We found significant genetic structure at the within individual level in each species so that the null hypothesis of panmixia was rejected. This is evidenced by the fact that for both *F. arfakensis* and *F. hahliana* the variance explained at the within individual level was significantly less than the value obtained through permutation (Phi-individuals -total=0.549, p<0.001 and Phi-individuals-total=0.306, p<0.001 respectively). However, in both cases it explained a considerable amount of genetic variation (45% in *F. arfakensis* and 69% in *F. hahliana*).
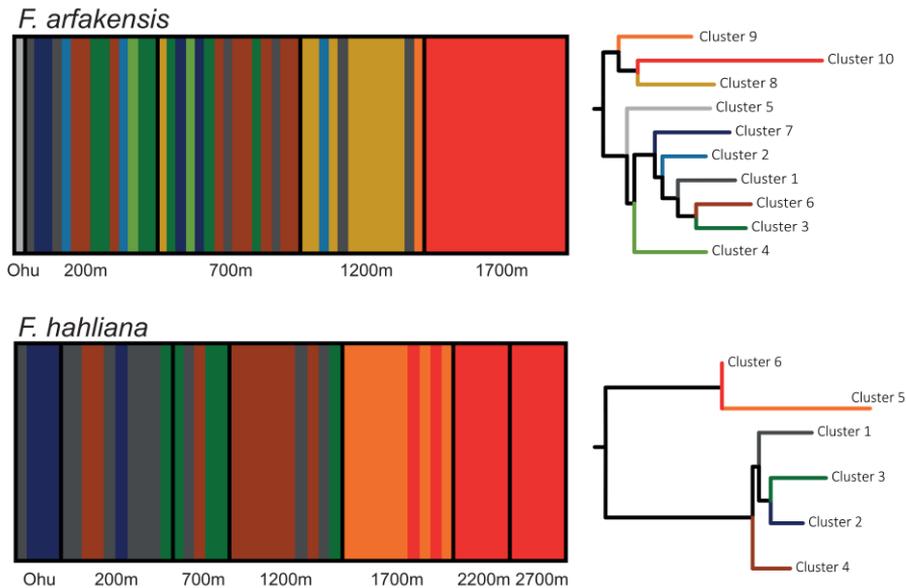
Figure 3. Clusters resulting from the distribution of alleles amongst individuals for *F. arfakensis* and (a) *F. hahliana* (left hand side) (b) and neighbor joining trees estimated using Nei's distances colored according to cluster (right hand side).

For both species genetic variation between subpopulations was much greater than within subpopulations, suggesting that subpopulations represent biologically meaningful groups with some limitations to gene flow between them. In *F. arfakensis* genetic variation was significantly greater than under null expectations for both between and within populations (Phi-population-total=0.360, p<0.001 and Phi-individuals -population=0.295, p<0.001) but genetic variation between populations explained almost twice as much of the total variance (36%) than the variation within populations (19%). For *F. hahliana* genetic variation between populations was large (Phi-population-total=0.326, p<0.001) whilst genetic variation within populations was very low (Phi- individuals -population=-0.030, p=0.764).
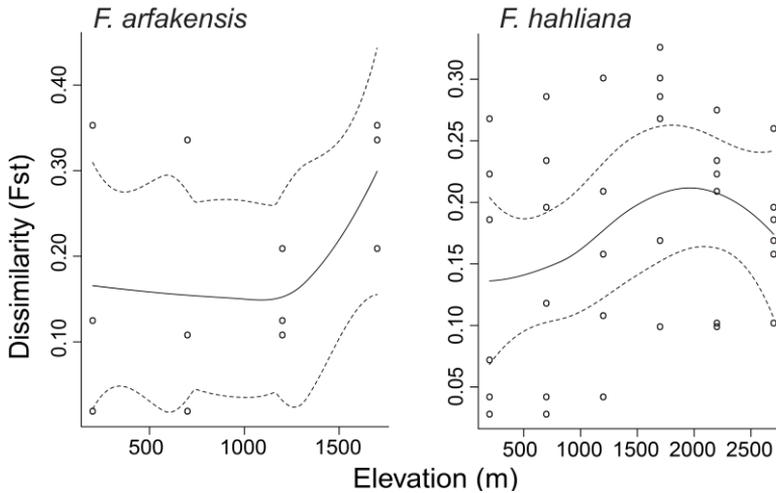
Figure 4. Pairwise dissimilarity between all remaining subpopulations of *F. arfakensis* and *F. hahliana* from different elevations based on F_{st} with a curve and 95% confidence interval fitted with loess smoothing.

Pairwise F_{st} values between elevational sites ranged from 0.019 to 0.353 in *F. afarkensis* and from 0.028 to 0.326 in *F. hahliana*. In general, the F_{st} values between subpopulations within 'lower' or 'higher' clusters (as defined by ΔK) were lower than F_{st} values between subpopulations from a different cluster. The mean F_{st} value within lowland/highland subpopulations of *F. afarkensis* was 0.084 and 0.068/0.123 in *F. hahliana*, whilst the mean F_{st} values between subpopulations was 0.299 in *F. afarkensis* and 0.244 in *F. hahliana*. This can is shown by the sharp increase in pairwise differences in F_{st} values with elevation found in both species (Fig. 4). It is notable that genetic diversity decreases with elevation. Although private alleles could be found for each subpopulation, they are more dominant in the lowlands, suggesting a bottle neck effect (Table 3). Our power analysis suggested that we employed a suitable number of loci and individuals to test our simple hypothesis of non-homogeneity in both species. For *F. arfakensis* the power to detect population differentiation at an F_{st} of 0.025 was 98% using the Chi-square test

and 97% using Fishers exact test (α=4% and 2%) and for *F. hahliana* it was 93% using the Chi-square test and 93% using Fishers exact test (α=4% and 4%).

Table 3. Genetic diversity of the *Ficus* studied over the three subpopulations. Following values are shown for *F. hahliana*/*F. arfakensis*: Na Number of alleles, Ho observed heterozygosity, He expected heterozygosity, F fixation index, Pa number of private alleles, %Pa proportion of private alleles. *F. hahliana* (cluster one: 200 m, 700 m, 1,200 m, cluster two: 1,700 m and cluster three: 2,200 m and 2,700 m) and four clusters for *F. arfakensis* (cluster one: 200 m and 700 m, cluster two: 1,200 m and cluster three: 1,700 m).

	Na	Ho	He	F	Pa	%Pa
Cluster 1	4.27/5.73	0.42/0.25	0.4/0.42	-0.05/0.42	24/21	0.4/0.26
Cluster 2	3.00/4.50	0.382/0.34	0.368/0.43	-0.038/0.22	7/15	0.11/0.19
Cluster 3	2.64/2.27	0.473/0.21	0.426/0.24	-0.109/0.15	2/5	0.03/0.06

The syconia of *F. hahliana* generally increase in size with elevation and form groups that overlap with the genetic clusters recovered, with the largest divide being between 1,200 m and 1,700 m (Table 1).

Discussion

We demonstrate that strong barriers to gene flow exist between 1,200 m and 1,700 m for two species of *Ficus*. Our results show that distinct lowland and highland subpopulations exist for *F. arfakensis* and *F. hahliana* growing along a continuously forested elevational gradient in Papua New Guinea. Indeed, most lowland (below 1,200 m) individuals of *F. arfakensis* are more similar to those found 70 km away than those from a population less than 4 km away, but separated by 500m in elevation. For *F. arfakensis,* at least, these sub-populations are not likely to represent isolated genetic entities (a proportion of alleles are usually shared between peripatric populations along the entire transect and three loci are invariable only

in this species). The 1,200 m population contains alleles that are otherwise unique to both the lower and higher populations, often in the form of heterozygote individuals suggesting that this population is a contact zone. Genetic diversity also drops considerably at 1,700 m, but allele frequency is consistent across two separate 1,700 m sites, suggesting genuinely limited gene flow to this elevation that has resulted in low genetic diversity. However, there are also a proportion of private lowland and highland alleles for each species and clear genetic and morphological distinctions between lowland and highland *F. hahliana*, which may represent a case of recent divergence into two species (likely to be sisters given our current sampling, Fig S2). Indeed, we suggest that the highland populations should be referred to as *F.* cf *hahliana* form hereon. The most obvious limitations of our work are the relatively low number of individuals and loci sampled. Furthermore, our sampling strategy includes only one elevational transect. However, we detected relatively high levels of allelic diversity (see Table 1) among a relatively small amount of individuals, which allows us to consider the results trustworthy, even with the use of a moderate number of loci (Kalinowski 2002). This is supported by the results of our power analysis, which suggests that 8-10 polymorphic loci is enough to detect large genetic structure given the number of individuals and variability of the loci used. Indeed, using a lower number of more variable loci gave more power in *F. arfakensis* than *F. hahliana*. For more detailed analyses of fine scale genetic structure and hybridization we would suggest increasing the number of loci used, because a low number of loci may overestimate genetic distances (Kalinowski 2002), the loci used by Moe and Weiblen (2012) show considerable promise here. It would be very useful to include additional elevational gradients, but this would require a considerable amount of extra funding given the costs and practicality of working at one of the world's only fully forested elevational study sites.

Our findings suggest that there are at least two occurrences of limitations to gene flow at our study site. This is in contrast to two previous studies on the genetic structure of *Ficus* populations, which have demonstrated high levels of gene flow across large areas of lowland rainforest (Nason et al. 1996) and desert (Ahmed et al. 2009). In both previous studies dispersing wasps not only face environmentally homogenous habitats, but are also aided by strong above canopy winds. In the latter case the dispersing wasp is congeneric with the pollinators of *F. arfakensis* (*Ceratosolen solitarius*, Weibes) and *F. hahliana* (*C. hooglandii*, Weibes). To our knowledge, our study represents the first study of gene flow between *Ficus* populations along an elevational gradient, where environmental conditions change rapidly with vertical distance. It is likely that fig wasp dispersal is important in explaining the observed results. These tiny insects are particularly sensitive to changes in temperature (Jevanandam et al. 2013) and may be unable to cross the 15˚C temperature gradient found between lower and upper elevations. This hypothesis is supported by the occurrence of two species of pollinator associated with *F. sur* in West Africa that are also segregated by elevation. It is possible that vertebrate seed dispersers also have limited ranges, with many endemic birds and mammals having restricted elevational ranges (Winter 1997). Indeed, we see a strong turnover in bird community structure around 1,200-1,700 m along our gradient (Fig. 1), with distinct highland and lowland communities potentially limiting the vertical distance that seeds can be dispersed (Sam and Koane 2014). However, some degree of limited wasp dispersal is required in both scenarios because long distance pollen dispersal can mask even highly limited seed dispersal.

Local adaptation in *Ficus* itself may also play a role in reducing gene flow, especially if this is linked to changes in fruit morphology that prevent maladaptation through the exchange of genetic material from higher or lower elevations. Indeed, both *F. arfakensis* and *F. hahliana* exhibit a degree of morphological variation along the gradient with

respect to fig size, figs being larger at higher elevations (Table 1). Observations from other *Ficus* species demonstrate even more extreme morphological variation with elevation than species examined in this study. For example, *F. dammaropsis* Diels has cricket ball/baseball sized fruits in the lowlands which are covered with open bracts, in contrast highland populations have substantially larger fruits which are generally smoother and have the bracts closed. There are also well documented instances of highland and lowland varieties or subspecies of *Ficus*, for example *F. trichocerasa subsp. trichocerasa* Diels is found mainly up to elevations of 1,400 m but grades slowly into *subsp. pleioclada* (Diels) C.C. Berg in higher elevations up to 2,600 m (Berg 2004). Furthermore, *F. wassa* Roxb. is similarly found in two varieties, *var. wassa* Roxb. in lowlands (up to 1,300 m) and *var. nubigena* Diels in the highlands (1,300-3,000 m) (Berg and Corner, 2005). The lowland variety grows as a tree up to 15m and has red figs at maturity, the highland variety has a scadent, scrambling habit, growing up to 3m and bearing greyish white figs when ripe. Despite these apparently important ecological differences neither variety can be separated on the mostly vegetative characters listed in Berg and Corner (2005). The extent to which this variation is genetic or environmental is yet to be established in these species. Indeed members of the genus *Ficus* can display high levels of phenotypic plasticity (Harrison 2005). We suggest that additional detailed morphological studies are required across several species found at this site to assess the true degree of variation observed, and that these should be conducted in conjunction with more detailed and wide scale population genetic studies of both figs and their pollinating wasps.

It has long been recognized that species turnover (or beta diversity) along elevational gradients is usually high, whilst community level nestedness is low. However, peripatric species are often close relatives, suggesting that speciation is facilitated by local adaptation and decreased gene flow. Whilst our study addresses gene flow in a

specialized pollination mutualism we suggest that it may have wider implications for less specialized systems because any level of specialization in pollination or seed dispersal may lead to potential isolation. Furthermore, whilst insect herbivores (Craft et al. 2010) and pollinators (Nason et al. 1996, Ahmed et al. 2009) of *Ficus* show low levels of population structure in lowland habitats we have little understanding of how pollinator and insect herbivore populations are structured along elevational gradients, but turnover of species within genera appears likely (Novotny et al. 2005). *Ficus* species represent one of the key genera in forest communities supporting extremely species rich communities of herbivorous insects from several guilds (Novotny et al. 2005). Being one of the most important plant genera for tropical frugivores, *Ficus* also provides important food source for a broad variety of vertebrates with some of them being dependent on fig consumption (Shanahan et al. 2001). Divergence in *Ficus* populations and associated variation in their traits, fruit morphology and phenology are thus likely to have pronounced effects on numerous associated organisms. It would certainly be valuable to conduct further studies along tropical elevational gradients to investigate the population genetic structure of additional plant species and its correlation with a structure of associated communities of other organisms. We suggest that such an approach would be a useful step in understanding the processes of speciation in some of the world's most biodiverse hotspots.

Acknowledgments

References

Ahmed, S. et al. 2009. Wind-borne insects mediate directional pollen transfer between desert fig trees 160 kilometers apart. - Proc. Natl Acad. Sci. USA 106: 20342–   20347.

Berg, C. C. 2004. Flora Melesiana Precursor for the treatment of Moraceae Ficus 6: Ficus Subgenus Sycomorus. - Blumea 49: 155–200.

Berg, C. C. and Corner, E. J. H. 2005. Flora Malesiana. - Nationaal Herbarium Nederland.

Burger, W. 1995. Montane species-limits in Costa Rica and evidence for local speciation on altitudinal gradients. - Biodiversity and conservation of Neotropical montane forests. Proceedings of a symposium, New York Botanical Garden, 21-26 June 1993.: 127–133.

Corander, J. et al. 2004. BAPS 2: enhanced possibilities for the analysis of genetic population structure. - Bioinformatics 20: 2363–2369.

Craft, K. J. et al. 2010. Population genetics of ecological communities with DNA barcodes: An example from New Guinea Lepidoptera. - Proc. Natl Acad. Sci. USA 107: 5041–5046.

Cruaud, A. et al. 2012. An Extreme Case of Plant-Insect Co-Diversification: Figs and Fig-Pollinating Wasps. - Syst. Biol. 61: 1029–1047.

Doyle, J. J. and Doyle, J. L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. - Phytochem. Bull. 19: 11–15.

Dray, S. et al. 2007. The ade4 package: implementing the duality diagram for ecologists. - J. Stat. Softw. 22: 1–20.

Earl, D. A. and vonHoldt, B. M. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. - Conserv. Genet. Resour. 4: 359–361.

Evanno, G. et al. 2005. Detecting the number of clusters of individuals using the software structure: a simulation study. - Mol. Ecol. 14: 2611–2620.

Excoffier, L. et al. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction   data. - Genetics 131: 479–491.

Garcia, M. et al. 2012. Portable microsatellite primers for Ficus (Moraceae). - Am. J. Bot. 99: e187–e192.

Gavrilets, S. 2004. Fitness Landscapes and the Origin of Species. - Princeton University Press.

Hall, J. P. 2005. Montane speciation patterns in Ithomiola butterflies (Lepidoptera: Rhiodinidae). - Proc. Roy. Soc. B Biol. Sci. 272: 2457–2466.

Harrison, R. D. 2003. Fig wasp dispersal and the stability of a keystone plant resource in Borneo. - Proc. Roy. Soc. B Biol. Sci. 270: S76–S79.

Harrison, R. D. 2005. Figs and the diversity of tropical rainforests. - Bioscience 55: 1053–1064.

Hedrick, P. W. 2005. Genetics of Populations. - Jones and Bartlett.

Jakobsson, M. and Rosenberg, N. A. 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. - Bioinformatics 23: 1801–1806.

Jevanandam, N. et al. 2013. Climate warming and the potential extinction of fig wasps, the obligate pollinators of figs. - Biol. Lett. 9: 20130041–20130041.

Kalinowski, S. T. 2002. Evolutionary and statistical properties of three genetic distances. - Mol. Ecol. 11: 1263–1273.

Kamvar, Z. N. et al. 2014. *Poppr* : an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. - PeerJ 2: e281.

Mittermeier, R. A. et al. 2004. Hotspots revisited: Earth's Biologically Richest and Most Threatened Ecoregions. - CEMEX.

Moe, A. M. and Weiblen, G. D. 2011. Development and characterization of microsatellite loci in dioecious figs (*Ficus*, Moraceae). - Am. J. Bot. 98: 25–27.

Moe, A. M. and Weiblen, G. D. 2012. Pollinator-Mediated Reproductive Isolation Among Dioecious Fig Species (*Ficus*, Moraceae). - Evolution 66: 3710–3721.

Myers, N. et al. 2000. Biodiversity hotspots for conservation priorities. - Nature 403: 853–858.

Nason, J. D. et al. 1996. Paternity analysis of the breeding structure of strangler fig populations: Evidence for substantial long-distance wasp dispersal. - J. Biogeogr. 23: 501–512.

Novotny, V. et al. 2005. An altitudinal comparison of caterpillar (Lepidoptera) assemblages on Ficus trees in Papua New Guinea. - J. Biogeogr. 32: 1303–1314.

Novotny, V. et al. 2010. Guild-specific patterns of species richness and host specialization in plant–herbivore food webs from a tropical forest. - J. Anim. Ecol. 79: 1193–1203.

Peakall, P. and Smouse, P. E. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. - Mol. Ecol. Notes. 6: 288–295.

Price, P. W. 2002. Resource-driven terrestrial interaction webs. - Ecol. Res. 17: 241–247.

Pritchard, J. K. et al. 2000. Inference of population structure using multilocus genotype data. - Genetics 155: 945–959.

Reis, T. S. et al. 2015. Elevation as a barrier: genetic structure for an Atlantic rain forest tree (*Bathysa australis*) in the Serra do Mar mountain range, SE Brazil. - Ecol. Evol. 5: 1919–1931.

Robin, V. V. A. et al. 2010. Ancient geographical gaps and paleo-climate shape the phylogeography of an endemic bird in the sky islands of southern India. - PLoS ONE 5: e13321.

Rosenberg, N. A. 2003. distruct: a program for the graphical display of population structure: PROGRAM NOTE. - Mol. Ecol. Notes. 4: 137–138.

Ryman, N. and Palm, S. 2006. POWSIM: a computer program for assessing statistical power when testing for genetic differentiation. - Mol. Ecol. Notes. 6: 600–602.

Sam, K. and Koane, B. 2014. New avian records along the elevational gradient of Mt. Wilhelm, Papua New Guinea. - Bull. B.O.C. 134: 116–133.

Schultheis, A. S. et al. 2012. Phylogeography and species biogeography of montane great basin stoneflies. - Mol. Ecol. 21: 3325–3340.

Shanahan, M. et al. 2001. Fig-eating by vertebrate frugivores: a global review. - Biol. Rev. 76: 529–572.

Toussaint, E. F. A. et al. 2013. Australasian sky islands act as a diversity pump facilitating peripheral speciation and complex reversal from narrow endemic to widespread ecological supertramp. - Ecol. Evol. 3: 1031–1049.

Toussaint, E. F. A. et al. 2014. The towering orogeny of New Guinea as a trigger for arthropod megadiversity. - Nature Commun. 5: 1–10.

Weiblen, G. D. et al. 2001. Pollination and parasitism in functionally dioecious figs. - Proc. Roy. Soc. B Biol. Sci. 268: 651–659.

Weiblen, G. D. et al. 2006. Phylogenetic dispersion of host use in a tropical insect herbivore community. - Ecology 87: S62–S75.

Wiebes, J. T. 1979. Coevolution of figs and their insect pollinators. - Annu. Rev. Ecol. Syst. 10: 1–12.

Winter, J. W. 1997. Responses of non-volant mammals to late Quaternary climatic changes in the wet tropics region of north-eastern Australia. - Wildl. Res. 24: 493–511.

Supporting information
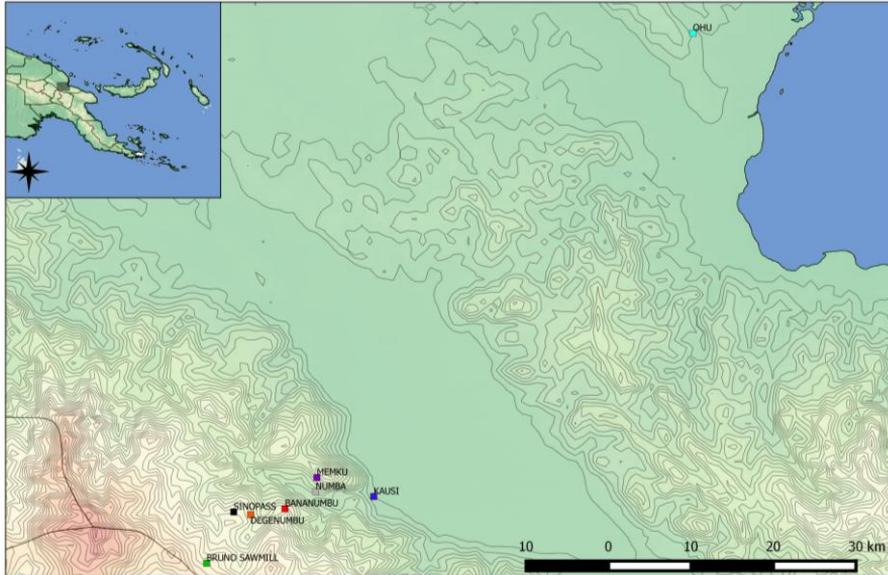
Figure S1. A map of our sampling sites, contour lines are given every 100 m. Note that sampling at 1,700 m was spread across two sites (Bananumbu and Degenumbu).
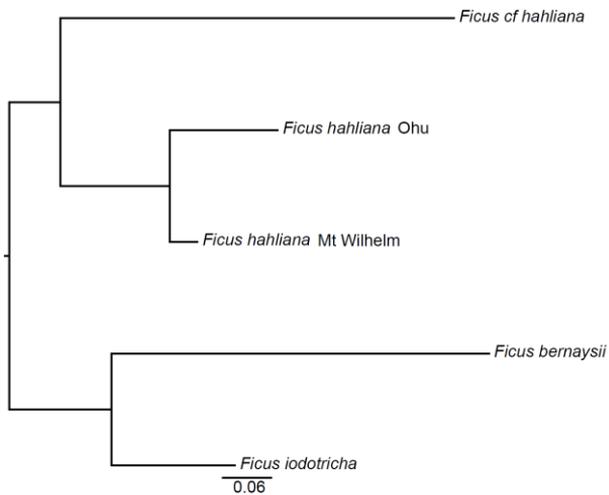

Figure S2. A neighbor joining tree constructed using Nei's distances derived from a 'clustering of groups of individuals' analysis as implemented in BAPS (Corander et al., 2004).

Plate S1. *F. iodotricha* (2,200 m, above) and *F. hahliana* (2,700 m, below). Note the difference in how the petiole joins the leaf, the coloration of the veins and the persistent stipules of *F. hahliana*. *F. iodotricha* as described here matches GW2135 from Chimbu Province in PNG (http://ng.atrium-biodiversity.org/).

Plate S2. *Ficus hahliana* 700 m above, 2,200 m below. Note the reduced leaf asymmetry and stem pubescence in high elevation *F. hahliana* but the otherwise similar presentation of the leaves.

Summary

The aim of this dissertation was to identify patterns underlying insect-plant coevolution and their role in the diversification of host-plant defenses, insect specialization, and the speciation of both plants and insects.

Theories on the macroevolution of host-plant defenses have suggested diversification and escalation in plant defensive traits in response to insect adaptation (Ehrlich and Raven 1964, Vermeij 1994). We show that the diversification of host-plant defenses is highly dependent on their relative costs and benefits and that low effectiveness against specialist herbivores may lead to a loss of defensive traits (Volf et al. 2015b). The effectiveness of individual defenses is highly dependent on the level of specialization of respective herbivores, as assemblages of insects with different levels of specialization exhibit various responses to host-plant traits (Volf et al. 2015a). This seems to prevent plants from evolving a universal defense based on individual traits and leads to the formation of defensive syndromes consisting of complementary defenses (Koricheva et al. 2004, Agrawal and Fishbein 2006).

We suggest that the effectiveness of host-plant defenses can be further increased if protection is maintained by defensive traits with various evolutionary histories. Using the plant genus *Ficus* as a model group we showed that defensive traits can follow several evolutionary trajectories within one system, making the resulting suites of defenses potentially harder for insect herbivores to adapt to. Several previous studies have found labile and divergent defences among closely related plant species growing in sympatry (Becerra 2007, Kursar et al. 2009, Salazar et al. 2016). We have also recovered rather high divergence in several defensive traits among closely related *Ficus* species in Papua New Guinea (PNG). Such divergence in defenses among congeneric plants growing in sympatry may be an ideal strategy to escape herbivory (Becerra 2007), as it may reduce the number of herbivores shared by closely related plants. From an evolutionary perspective it may also reduce the number of host-shifts

among closely related plants which would otherwise be likely due to the high phylogenetic conservatism of many insect herbivores (Futuyma and Agrawal 2009).

The majority of insect herbivores are phylogenetically conservative in their food-choice (Futuyma and Agrawal 2009). The evolutionary history of many herbivorous insect groups consists of frequent minor hosts shifts, for example, 50% of the speciation events investigated by Winkler and Mitter (2008) involved host shifts to congeneric species. Whilst major shifts occur less frequently, with fewer than 20% of speciation events involving host shifts to different families (Mitter & Farrell 1991), they may be more significant, as they have the potential to open up new adaptive zones (Janz & Nylin 1998; Winkler & Mitter 2008). In this dissertation we aimed to identify the role of host-plant phylogeny in maintaining insect specialization and food-web structure, and what level of phylogeny was most important in this respect. Our results suggest that the impact of host-plant phylogeny on insect food-web structure differs between herbivore guilds. We showed that leaf-chewer generality was maintained mainly by feeding on confamiliar or conordinal hosts (with an age of divergence between 20-80 mya) while few insect herbivores were shared between more distant plant lineages. In contrast to leaf-chewing larvae, miner and galler abundances were correlated mainly to the terminal nodes of the host phylogeny and their generality was maintained by feeding on congeneric hosts (younger than 20 mya). Our results thus suggest that whereas the specialization and abundance of monophagous guilds seems to be affected mainly by the terminal parts of the plant phylogeny and number of host species, the food-web structure of more generalist guilds, such as leaf-chewer larvae, is affected mainly by the of diversity of deeper plant lineages at individual sites.

Though we show that host-plant phylogeny plays important role in insect food-choice, we did not find much support for strict insect-plant coevolution even in case of the most specialized herbivore

guilds studied here. We suggest that diffuse coevolution involving more relaxed interactions is more frequent (Volf et al. 2015a). Strict insect-plant coevolution, in the sense of reciprocal induction of trait change (Janz and Nylin 2008), probably requires a special setting and has been found only in some insect-plant systems (Pellmyr 2003, Cruaud et al. 2012). Unlike most insect-plant assemblages, which involve multiple interacting species, intimate interactions between these highly coadapted partners can lead to direct reciprocal effects on both partners. This is suggested by our results and several examples of speciation in *Ficus* along an elevational gradient in PNG.

In summary, our results suggest that the processes shaping the insect-plant relationships are strongly depended on the level of their specialization and intimacy. We show that the specialization of these interactions is often generated through an interplay of host-plant phylogeny and trait variability, with their relative importance being different among herbivore guilds. It would be highly desirable to unravel the relative importance of host-plant phylogeny and traits changes across the plant and insect phylogenies under consideration, as these factors are undoubtedly amongst the main drivers of insect and plant diversification.

References

Agrawal, A. A., and M. Fishbein. 2006. Plant defense syndromes. Ecology **87**:S132-S149.

Becerra, J. X. 2007. The impact of herbivore-plant coevolution on plant community structure. Proceedings of the National Academy of Sciences of the United States of America **104**:7483-7488.

Cruaud, A., N. Ronsted, B. Chantarasuwan, L. S. Chou, W. L. Clement, A. Couloux, B. Cousins, G. Genson, R. D. Harrison, P. E. Hanson, M. Hossaert-Mckey, R. Jabbour-Zahab, E. Jousselin, C. Kerdelhue, F. Kjellberg, C. Lopez-Vaamonde, J. Peebles, Y. Q. Peng, R. A. S. Pereira,

T. Schramm, R. Ubaidillah, S. van Noort, G. D. Weiblen, D. R. Yang, A. Yodpinyanee, R. Libeskind-Hadas, J. M. Cook, J. Y. Rasplus, and V. Savolainen. 2012. An extreme case of plant-insect codiversification: Figs and fig-pollinating wasps. Systematic Biology **61**:1029-1047.

Ehrlich, P. R., and P. H. Raven. 1964. Butterflies and plants - a study in coevolution. Evolution **18**:586-608.

Futuyma, D. J., and A. A. Agrawal. 2009. Macroevolution and the biological diversity of plants and herbivores. Proceedings of the National Academy of Sciences of the United States of America **106**:18054-18061.

Janz, N., and S. Nylin. 2008. The Oscillation Hypothesis of Host-Plant Range and Speciation. Pages 204-215 Specialization, Speciation, And Radiation: The Evolutionary Biology Of Herbivorous Insects.

Koricheva, J., H. Nykanen, and E. Gianoli. 2004. Meta-analysis of trade-offs among plant antiherbivore defenses: Are plants jacks-of-all-trades, masters of all? American Naturalist **163**:64-75.

Kursar, T. A., K. G. Dexter, J. Lokvam, R. T. Pennington, J. E. Richardson, M. G. Weber, E. T. Murakami, C. Drake, R. McGregor, and P. D. Coley. 2009. The evolution of antiherbivore defenses and their contribution to species coexistence in the tropical tree genus Inga. Proceedings of the National Academy of Sciences **106**:18073-18078.

Pellmyr, O. 2003. Yuccas, yucca moths, and coevolution: a review. Annals of the Missouri Botanical Garden:35-55.

Salazar, D., A. Jaramillo, and R. J. Marquis. 2016. The impact of plant chemical diversity on plant–herbivore interactions at the community level. Oecologia:1-10.

Vermeij, G. J. 1994. The evolutionary interaction among species - selection, escalation, and coevolution. Annual Review of Ecology and Systematics **25**:219-236.

Volf, M., J. Hrcek, R. Julkunen-Tiitto, and V. Novotny. 2015a. To each its own: differential response of specialist and generalist herbivores to plant defence in willows. Journal Of Animal Ecology **84**:1123-1132.

Volf, M., R. Julkunen-Tiitto, J. Hrcek, and V. Novotny. 2015b. Insect herbivores drive the loss of unique chemical defense in willows. Entomologia Experimentalis Et Applicata **156**:88-98.

Appendix

Martin Volf
Date of Birth: 2.9. 1987

Education
2012 - present
Ph.D. in Entomology, University of South Bohemia
Supervisor: prof. RNDr. Vojtěch Novotný CSc
Evolution of host-plant defensive traits and their impact on herbivorous insect in the temparate zone and the tropics.
2010 - 2012
M.Sc. in Zoology, University of South Bohemia. Honours. Awarded by the Dean's and the University President's prizes.
Supervisor: M.Sc. Jan Hrček Ph.D.
The impact of deffensive host-plant traits on community structure of herbivorous insects on willows
2007 - 2010
B.Sc. in Biology, University of South Bohemia. Honours.
Supervisor: M.Sc. Jan Hrček. Ph.D.
Community structure of leaf-chewing insect on willows
2015
RNDr. in Zoology
Supervisor: prof. RNDr. Vojtěch Novotný CSc
Insect herbivores drive the loss of unique chemical defense in willows

Specialization
Insect-plant relationships
Evolution of plant defensive traits
Latitudinal trends in herbivory and insect diversity

Currently involved in following projects:
Evolutionary trends in *Ficus* defences and their impact on herbivore community assembly
The role of pollinating insects in *Ficus* speciation along altitudinal gradients
Explaining latitudinal trends in global biodiversity: why are there more species of insect herbivores in tropical than temperate forests?

Publications

Volf, M., Hrcek, J., Julkunen-Tiitto, R., & Novotny, V. (2015). To each its own: differential response of specialist and generalist herbivores to plant defence in willows. Journal of Animal Ecology, 84, 1123–1132.

Volf, M., Julkunen-Tiitto, R., Hrcek, J., & Novotny, V. (2015). Insect herbivores drive the loss of unique chemical defense in willows. Entomologia Experimentalis et Applicata, 156, 88–98.

Volf, M.,Redmond, C., Albert, Á.J., Le Bagousse-Pinguet, Y., Biella, P., Götzenberger, L., Hrázský, Z., Janeček, Š., Klimešová, J., Lepš, J., Šebelíková, L., Vlasatá, T., de Bello, F. (2016) Effects of long- and short-term management on the functional structure of meadows through species turnover and intraspecific trait variability. Oecologia. 1-10.

Forister, M.L., Novotny, V., Panorska, A.K., Baje, L., Basset, Y., Butterill, P.T., Cizek, L., Coley, P.D., Dem, F., Diniz, I.R., Drozd, P., Fox, M., Glassmire, A., Hazen, R., Hrcek, J., Jahner, J.P., Kaman, O., Kozubowski, T.J., Kursar, T.A., Lewis, O.T., Lill, J., Marquis, R.J., Miller, S.E., Morais, H.C., Murakami, M., Nickel, H., Pardikes, N., Ricklefs, R.E., Singer, M.S., Smilanich, A.M., Stireman, J.O., Villamarín-Cortez, S., Vodka, S., Volf, M., Wagner, D.L., Walla, T., Weiblen, G.D., Dyer, L.A. "The global distribution of diet breadth in insect herbivores." *Proceedings of the National Academy of Sciences* 112.2 (2015): 442-447.

Professional Appointments

2011 – one month research stay at University of Eastern Finland, Riitta Julkunen-Tiitto lab
   project: HPLC analysis of Salix secondary metabolites
   – two month research stay at University of Minnesota, Jeannine Cavender-Bares lab
   project: reconstruction of Salix phylogeny

2012 - present **–** Postgraduate fellowship at Biology Center, Czech Academy of Sciences

2014 – three month stay at Tomakomai Experimental Forest,   Hokkaido, Japan
   project: field survey of herbivore communities using canopy crane in collaboration with University of Tokyo and Chiba University

2015 – two month stay at Tomakomai Experimental Forest, Hokkaido, Japan

> *project: field survey of herbivore communities using canopy crane in collaboration with University of Tokyo and Chiba University*

2016 – short term stay as a research assistant at Smithsonian Tropical Resaerch Institution (hosted by Yves Basset)

2016 - present – Predoctoral fellowship at the Smithsonian Institution (hosted by Kristina A. Teixeira)

> *project: "Why are there so many insect herbivore species in tropical rainforests?"*

Conferences

International

- 11th INTECOL Congress, 2013, London (oral): Insect Herbivore Adaptation Drives the Loss of Unique Chemical Defense in Willows.
- European Conference of Tropical Ecology, 2016, Göttingen (oral): Divergent chemical syndromes in species rinch plant genera: the case in Ficus local community in Papua New Guinea
- ESA Annuan Meeting, 2016, Fort Lauderdale (oral): Divergent chemical syndromes in species rich plant genera: the case in *Ficus* local community in Papua New Guinea

Local

- Zoology Days 2012, Olomouc (oral): Struktura společenstev herbivorního hmyzu na vrbách je určena obsahem salicylátů v listech.
- Zoology Days 2013, Brno (oral): Insect herbivore adaptation drives the loss of unique chemical defense in willows. *Awarded with the prize of Czech Entomological Society and 2nd prize for the best student talk.*
- Zoology Days 2014, Ostrava (oral): Výzkum latitudinálních trendů diverzity herbivorního hmyzu - Jaké faktory určují jeho diverzitu?
- Zoology Days 2015, Brno (oral): The effect of environmental changes on specialized pollinators limits geneflow in New Guinean *Ficus* species along altitudinal gradients
- Zoology Days 2016, České Budějovice (oral): Divergent defensive syndromes in Ficus species growing in sympatry
- Zoology Days 2016, České Budějovice (oral): Host-plant phylogenetic diversity drives insect-plant food-web structure. *Awarded with the prize of Czech Entomological Society and 1st prize for the best student talk*.

Student supervision

- Martin Libra (M.Sc. thesis), 2013-2015. *Diversity of gall-inducing arthropods in two different host communities in temperate forests.*
- Markéta Tahadlová (B.Sc. thesis, supervised as a cosupervisor with Jakub Těšitel) 2013-2015. *The interactions of hemiparasitic plants with invertebrate herbivores.*
- Jan Kadlec (B.Sc. thesis) 2014-present. *Host-preference of willow associated gallers.*

Teaching

2013 - Field courses in entomology and ecology**,** Practicals in invertebrate biology

2014 - Practicals in insect systematic**,** Field courses in entomology and ecology

2015 - Field course in Alpine zoology

2016 - Field course in vertebrate zoology

Skills

Statistics: ANOVA, GLM, LME, multivariate, phylogenetic comparative methods (PGLS, PGR, PSR), data visualization, food-web analysis.

Molecular methods: DNA extraction, PCR, nested PCR, multiplex PCR, preparation for sequencing, DNA cloning, sequence processing, phylogenetics.

Chemical analysis: Basics of HPLC.

Field work: Extensive experience in insect sampling and insect rearing gained when leading projects focused on i) sampling of complete communities of leaf-chewers, miners, gallers, mezophyll-suckers, ants, and spiders from tree canopy at 0.1ha plots in Czech Republic and Japan, and ii) communities of leaf-chewing insects harbored by willows.

Identification skills: good general knowledge of invertebrates, specialized in Coleoptera (Buprestidae, Carabidae). Experience in Salicaceae identification.

Software:

Phylogenetics and sequence processing: Geneious, BioEdit, JModelTest, Beast, MrBayes, PAUP, FigTree, TreeGraph

Statistics and community analysis: R, CANOCO, Statistica, EstimateS, Past

Graphical tools: Adobe Photoshop, Adobe Illustrator, GIMP, ImageJ

Others: Microsoft Office

International courses and workshops
2009 – Field course on Tropical Ecology at Binatang Research Centre, PNG
2015 – University Course on Molecular Analysis of Trophic Interactions (MATI) at University of Innsbruck, Austria

Specificity of insect-plant associations and their role in the formation of plant defenses and speciation
Ph.D. Thesis Series, 2016, No. 9