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Divergent evolution in antiherbivore defences within species complexes at a single Amazonian site

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Summary

1. Classic theory in plant–insect interactions has linked herbivore pressure with diversification in plant species. We hypothesize that herbivores may exert divergent selection on defences, such that closely related plant species will be more different in defensive than in non-defensive traits.

2. We evaluated this hypothesis by investigating two clades of closely related plant species coexisting at a single site in the Peruvian Amazon: *Inga capitata* Desv. and *Inga heterophylla* Willd. species complexes. We compared how these lineages differ in the suite of chemical, biotic, phenological and developmental defences as compared to non-defensive traits that are related to habitat use and resource acquisition. We also collected insect herbivores feeding on the plants.

3. Our data show that sister lineages within both species complexes are more divergent in antiherbivore defences than in other non-defensive, functional traits. Moreover, the assemblages of herbivore communities are dissimilar between the populations of coexisting *I. capitata* lineages.

4. *Synthesis.* Our results are consistent with the idea that for the *I. capitata* and *I. heterophylla* species complexes, interactions with their natural enemies may have played a significant role in their phenotypic divergence and potentially in their diversification and coexistence. It also suggests that defensive traits are evolutionary labile.

Key-words: herbivores, *Inga*, Peru, plant defences, plant–herbivore interactions, trait divergence, tropical rain forests

Introduction

The arms race between plants and insect herbivores has been invoked as one of the main mechanisms driving trait diversification and coevolution for both groups (Becerra 1997; Thompson 1988, Becerra, Noge & Venable 2009; Futuyma & Agrawal 2009; Kursar *et al.* 2009; Agrawal *et. al.* 2012). A fundamental prediction of this theory is that herbivores drive the evolution of plant antiherbivore defences faster than for other traits (Thompson 2005; Kursar *et al.* 2009). Testing this hypothesis requires demonstrating that sister species are more different in antiherbivore defences than in traits related to adaptations to other extrinsic factors, such as the abiotic environment. However, studies testing this idea are surprisingly few (e.g. Agrawal *et al*. 2009). Consequently, in this study, we combine data on plant functional traits and insect herbivores to compare patterns of divergence in two groups of closely related species coexisting at a single site.

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The coevolutionary theory of plant–herbivore interactions suggests that the production of defences against insects has played a dominant role in host and enemy radiations (Ehrlich & Raven 1964). Specifically, this theory predicts a tight correlation between plant relatedness and plant defences. Although widely accepted, relatively few studies have tested this, and some even question the fundamental assumptions of this theory. For example, Becerra (1997) found only a weak relationship between the phylogenetic hypothesis and chemical similarity for the species of *Bursera,* common trees in the dry forests of Mexico. Likewise, Kursar *et al.* (2009) found a weak correlation between phylogenetic distances and chemical distances within the Neotropical tree genus, *Inga.* This lack of phylogenetic signal in the expression of secondary metabolites suggests divergent selection on antiherbivore defences, such that closely related species are not necessarily similar in defences. This should make it more difficult for herbivores to track hosts over evolutionary time thereby reducing herbivore pressure on plants.

Although the role of the physical environment on trait divergence has received considerable attention (Anacker &

Strauss 2014), the role of defensive traits in plant diversification is not well understood (Futuyma & Agrawal 2009). Yet, several studies have provided indirect evidence for the significance of defensive traits in evolutionary diversification by showing a relationship between variation in these traits and species diversity (Farrell, Dussourd & Mitter 1991; Becerra 1997; Agrawal & Fishbein 2008; Agrawal, Salminen & Fishbein 2009; Agrawal *et al.* 2009; Kursar *et al.* 2009). A better understanding of the relative importance of defensive traits in phenotypic diversity and species divergence will require examining differences in defensive and non-defensive traits simultaneously between recently diverged species or populations at an incipient state of divergence (Futuyma & Agrawal 2009, Fine *et al.* 2013).

Here, we examine the contribution of plant–insect interactions to divergence among species by determining variation in functional traits and herbivore communities within two clades of closely related species coexisting at a single site in the Peruvian Amazon: *Inga capitata* Desv. and *Inga heterophylla* Willd. species complexes. The taxa in each complex are considered a single species based on the morphological traits of reproductive individuals (Pennington 1997). Our field observations of subtle differences within each clade in colour of the expanding leaves (see Appendix S1 in Supporting Information), number of leaflets and stipule morphology have motivated the present characterization of trait divergence within these clades. In fact, plastid DNA analyses distinguish each member as a different evolutionarily significant unit (ESU) and as sister taxa (Kursar *et al.* 2009, Fig. S2). Based on these analyses, *I. capitata* comprises three ESUs and *I. heterophylla* two ESUs. Four of the ESUs co-occur in terra firme habitats, often within metres of each other, whereas one *I. capitata* ESU species (cap2) occurs primarily in the nearby floodplains.

In order to achieve a comprehensive analysis, we collected data on many different defensive traits including chemical, biotic, phenological and developmental defences, as well as on insect herbivores. We also collected data on non-defensive traits that are related to habitat use and resource acquisition. Our previous studies on the genus *Inga* suggest that defences evolve rapidly (Kursar *et al.* 2009). Specifically, we expect the ESUs within an *Inga* lineage to be more similar with respect to non-defence traits such as primary metabolites and resource acquisition traits. In contrast, the observation that antiherbivore traits show a greater difference among relatives than for non-defence traits would support the key role of herbivores in shaping divergence and niche separation in their host plants.

Materials and methods

STUDY SITE

This study was conducted at Los Amigos Biological Station (Spanish acronym: CICRA, Centro de Investigación y Capacitación Rio Los Amigos). Los Amigos is located in the south-eastern Peruvian Amazon, in the Madre de Dios Department at 12°34′9″ S, 70°6′0.40″ W, 268 m.a.s.l. Los Amigos covers 453 ha of lowland Amazonian forest and consists of a mosaic of terra firme and floodplain forests. Mean annual rainfall is between 2700 and 3000 mm, and the mean monthly temperature ranges from 21 to 26 °C (Pitman 2007).

STUDY SPECIES

Inga capitata comprises three phenotypically divergent ESUs: cap1, cap2 and cap3 (Kursar *et al.* 2009). In addition, they present different habitat preferences, with cap1 and cap3 showing a preference for terra firme and cap2 for floodplains. The *I. heterophylla* species complex includes two phenotypically different lineages: het1 and het2 (Kursar *et al.* 2009), both on terra firme. For those ESUs found in terra firme, one ESU often is metres away from another and no intermediates were observed. The study plants were widely distributed within their respective habitat types; aside from as noted above, inspection of the location data and our field observations showed no tendency for the study species to be clumped or restricted to certain habitats (e.g. preference for treefall light gaps).

CENSUSES AND LEAF TRAITS

In the present study, antiherbivore defences are defined as those plant traits that have been selected in response to herbivory. These include developmental defences (leaf expansion rate; Kursar & Coley 2003), biotic defences (leaf-defending ants and the area of extrafloral nectaries; Koptur 1984; Brenes-Arguedas, Coley & Kursar 2008), phenological defences (the timing and synchrony of young leaf production, Aide 1993; Kursar & Coley 2003) and chemical defences (phenolics and non-protein amino acids; Coley *et al.* 2005). This set of defence traits was measured only on expanding leaves because more that 80% of the damage accrued during a leaf's lifetime happens during the short period (1–3 weeks) of leaf expansion (Coley & Aide 1991; Kursar & Coley 2003; Brenes-Arguedas *et al.* 2006). Therefore, young leaf defences are under strong natural selection by herbivores.

Traits under selection from the physical environment are considered here as non-defence traits. These traits were measured only on mature leaves. These include leaf mass per area (LMA), leaf nitrogen content, area per leaflet, number of leaflets per leaf, and the presence or absence of wings. These include some of the key ecophysiological attributes that correlate with photosynthetic capacity and transpiration, with habitat type such as light availability and with resources such as soil nutrient content (Cornelissen *et al.* 2003; Wright *et al.* 2004; Fujita, van Bodegom & Witte 2013). Although, in principle, the LMA and leaf nitrogen of mature leaves can affect leaf palatability to herbivores, in actuality, herbivores attack the mature leaves of shade-tolerant tropical rain forest plants, such as *Inga*, at low rates. Consequently, we consider that LMA and leaf nitrogen of mature leaves are more important as adaptations for resource acquisition and habitat and not to herbivore pressure (Endara & Coley 2011).

Data were collected for young and mature leaves on 0.5–4-m tall saplings in the shaded understory from 2007 until 2011. More than 100 km of trails were walked regularly to search for plants, and collections were widely separated. Specifically, based on trail locations, we estimate that, for each ESU, our collections were made, on average, every 360 m. Leaf expansion rate was quantified for leaves between 20% and 80% of full size by measuring their area every 1–4 days until they were fully expanded. To quantify synchrony in leaf production for each ESU in the *I. capitata* complex, 30–70 individuals per ESU were marked and each plant was scored monthly for the presence of young leaves. *I.* cap1 was censused between June and

December of 2010. *I.* cap2, *I.* cap3 and *I.* het1 were censused between January and December 2007. Due to the low abundance of *I. heterophylla* het2 saplings, it was not possible to measure synchrony in leaf flushing or the following leaf traits. At each census, the number of ants visiting the extrafloral nectaries of expanding leaves was quantified (number of ants per nectary). The area of the nectary was estimated using a dial calliper. Leaf mass per unit area (LMA; g m^{-2}) was measured from discs of mature leaves of known area that were dried at approximately 70 °C for 3 days. Mature leaves were ground and analysed for leaf N content with a Costech 4010 Elemental Analyzer coupled to a Thermo Delta Plus XP IRMS (Costech Analytical Technologies, Valencia, CA, USA). The number and size of leaflets were calculated for at least three leaves per sampled individual, and the presence of wings on the rachis was recorded.

CHEMICAL ANALYSIS

Metabolites were extracted, separated, quantified gravimetrically and analysed using ultra-high-performance liquid chromatography (LC) or gas chromatography (GC) coupled to mass spectrometry (LC-MS and GC-MS, respectively). Expanding leaves from five individual understory saplings were collected for each ESU of *I. capitata* and *I. heterophylla*. For each individual sapling, we collected expanding leaves that were 80% of the average maximum size. Fresh leaves were dried in silica gel at room temperature and shipped to the University of Utah for chemical analysis. Only soluble metabolites were studied; thus, those covalently bound to cell walls were excluded (Lokvam & Kursar 2005).

Extract preparation

The protocol of Bixenmann, Coley & Kursar (2013) was followed with some modifications. For each sample, 300–500 g of vacuum-dried leaf material was homogenized using a ball mill (MM 200; Retsch, Haan, Germany) at 30 Hz for 30 s. Approximately 100 mg of each homogenized plant sample was weighed in an Eppendorf tube and mixed with 15 μL of a 1 mg mL^{-1} amino acid internal standard solution (a mixture of 20 amino acids labelled with 13C and 15N; Sigma-Aldrich, St. Louis, MO, USA) and 1.5 mL of 70% acetonitrile 30% water (v/v). We extracted with 70% acetonitrile (acetonitrile: water, 70:30, v/v) instead of ethanol, a typical solvent used for extraction, because we found that polygallate esters are unstable in ethanol (data not shown). After extraction for 10 min and centrifugation (13 793 × ***g***) for 5 min, the supernatant was transferred to a glass vial and the extraction repeated for a total of three times. The extraction was repeated two more times using 1 mL of 70% acetone (acetone:water, 70:30, v/v). The extracts were combined and dried under nitrogen gas until all organic solvents were evaporated. To remove lipids, 3 mL of water and 3 mL of hexane were added to the dried extract. After vortexing for 5 s, the extract was left to settle for a few minutes until two distinct layers formed. The non-polar fraction was then transferred to another pre-weighed glass vial and the extraction repeated with 3 mL of hexane. Both the non-polar and the polar organic fractions were dried under nitrogen gas and then under vacuum (0.8 torr) at ambient temperature.

The polar organic fraction was separated on an octadecylsilane (ODS; BAKERBOND@ 40 μm Prep LC Packing, AVANTOR, Center Valley, PA, USA) column. 2.9 g of ODS was dry-packed in a 10-mL syringe. The dried extract was suspended in 2 mL of water and transferred to the ODS column. Thirty mL of water was run through the column and collected in a pre-weighed glass vial (polar fraction). This process was repeated with 50% acetonitrile 50% water (v/v), followed by 100% acetonitrile to collect the phenolic and saponin fractions, respectively. After removal of solvents and vacuum drying (0.8 torr) at ambient temperature, each fraction was weighed. The weight for the saponin fraction was negligible and is not considered further.

GC-MS analysis. The water or polar fraction was analysed by GC-MS using a GCT Premier mass spectrometer (Waters, Milford, MA, USA) fitted with a GC6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) and a Gerstel MPS2 autosampler (Gerstel, Mülheiman der Ruhr, Germany). The dried polar fraction (0.25–0.47 mg) was suspended in 40 μL of 40 mg mL^{-1} *O*-methoxylamine hydrochloride in pyridine and incubated for 1 h at 30 °C. To this solution, 25 μL of *N*-methyl-*N*-trimethylsilyltrifluoracetamide was added using the autosampler and incubated for 30 min at 37 °C with shaking. One microlitre of the sample was injected in to the gas chromatograph at a 10:1 split ratio with the inlet temperature held at 250 °C. The gas chromatograph had an initial temperature of 95 °C for 1 min followed by a 40 °C min^{-1} ramp to 110 °C and a hold time of 2 min. This was followed by a second 5 °C min^{-1} ramp to 250 °C, a third ramp to 350 °C and then a final hold time of 3 min at 350 °C. A 30-m ZB-5MSi column (Phenomenex, Torrance, CA, USA) with a 5-m guard column was employed for chromatographic separation.

LC-MS analysis. Liquid chromatography was performed on an I-Class Acquity Ultra Performance Liquid Chromatography System (Waters). Dried phenolic extract (0.01–0.055 mg) was resuspended in 1 mL of 50% acetonitrile 50% water (v/v), centrifuged (13 793 × ***g***) for 5 min and the supernatant transferred to a HPLC vial. One microlitre of sample was injected on an Acquity UPLC BEH C18 column (50 mm × 2.1 mm × 1.7 μm) (Waters). Sample and column temperatures were held constant at 10 °C and 40 °C, respectively. Samples were eluted using a mobile phase of 0.3 mL min^{-1} with the gradient shown in Appendix S3. The mobile phases consisted of water with 0.1% formic acid (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B). The solvents are Fisher LC-MS grade.

Compounds were detected using a Xevo G2 QToF mass spectrometer (Waters) equipped with a lock spray and an electrospray ionization source (ESI). Spectra were collected in positive ionization mode (ES+) in the *m/z* range of 50–1200. The parameters of the ESI were as follows: capillary voltage of 2.3 kV, sampling cone voltage of 30V, extraction cone voltage of 4 V, source temperature of 120 °C, desolvation gas temperature of 400 °C, desolvation gas flow of 900 L h^{-1} and collision energy of 6 eV. The mass spectrometer was calibrated using a sodium formate standard (0.5 mM in 90% 2-propanol 10% water (v/v)), and leucine enkephalin (2 ng/μL) was used as a lock mass.

INSECT HERBIVORES

To assess whether differences in defensive traits between the different ESUs relate to differences in herbivore choice, we performed the following: (i) a field survey of the abundance of leaf-chewing insects feeding on expanding leaves of the *I. capitata* species complex and (ii) a captive choice experiment with sawfly larvae (*Symphyta*, Argidae) that only fed on this species complex and was its most abundant herbivore. For the herbivore survey, all leaf-chewing insects that were

found feeding on the expanding leaves of saplings in the understory were recorded. Insects were collected by hand from the leaves between 2010 and 2011 for a period of 10 months, as part of a project that is examining the insect herbivore fauna feeding on the entire genus *Inga* in Los Amigos. Each plant was visited once every flush. All Coleoptera were classified to genus based on morphology by specialists associated with the Pontificia Universidad Catolica del Ecuador. All lepidopteran and sawfly insects were assigned to Molecular Operational Taxonomic Units (MOTUS) using COI sequences. PCR amplification and DNA sequencing were generated at the Canadian Center for Barcoding and in our laboratory using standard bar coding protocols (Ivanova, deWaard & Hebert 2006; deWaard *et al.* 2008). PCR amplification with either the LCO/HCO or LepF1/LepR1 primer pairs recovered a 658-bp region that was subsequently used to generate MOTUS.

For the feeding choice experiment, sawfly larvae and expanding leaves of the three *I. capitata* ESUs were collected in the field. In the laboratory, leaves were cut into square pieces of approximately 39 cm^2. Sawfly larvae were deprived of food overnight prior to the experiment, and then three pieces of leaf (one per ESU) were offered to an individual larva ($N = 9$). The experiment was carried out in Petri dishes lined with moistened filter paper. After 24 h, the area eaten on each square leaf piece was recorded using an acetate grid.

DATA ANALYSIS

Leaf traits

The censuses were analysed for synchronization in leaf production for each ESU using circular statistics (Zar 1999). Months were converted to angles between 0° and 360°. The vector length r was calculated for each population following Zar (1999). The length of the vector r varies between 0 and 1 and is a measure of seasonality. High values of r indicate aggregated phenological behaviour, and low values represent a uniform distribution of phenological activity throughout the year (Batschelet 1981). We determined whether ESUs differed in their season of leaf production using the Watson's test (U2) (Zar 1999).

Differences among ESUs in the number of ants visiting the extrafloral nectaries, nectary area, leaf expansion rate as per cent increase in area per day, LMA, number and size of leaflets, and phenolic contents were examined using analysis of variance (ANOVA) followed by Tukey's *post hoc* tests for multiple comparisons. Tests for normality of the data and appropriate data transformations were performed. These analyses were performed in the statistical programming language R version 3.0.1 (R Development Core Team 2011).

Chemical traits

Raw data from the GC-MS and the UPLC-MS were processed for peak detection and peak alignment using MarkerLynx (MassLynx v4.1; Waters, Manchester, UK) and XCMS (Smith *et al.* 2014). The output files from the UPLC-MS were further processed for data quality as follows: because no late eluting saponins were found, the retention time window of interest was delimited to 22 min (peaks at > 22 min to the end were discarded). Peaks (often referred to as 'features') that were not consistently detected were discarded. For this, all peaks that occurred in only one species and in three or fewer replicates of that species were discarded. Zero or missing values were replaced with half of the minimum positive value in the data set. Because our data set contained a large number of variables (> 490 peaks), multiple hypotheses were tested for each peak. Hence, we applied a filtering method in order to adjust for multiple testing (Hackstadt & Hess 2009). As recommended in Metaboanalyst (see below) for a sample size of about 500 peaks, we eliminated the 10% of peaks with the lowest intensities. For this, peak intensities were ranked based on the interquantile range. Peak intensities, or the total ion current, were normalized by the dry weight of the sample. The most important compounds for discriminating metabolic differences between these three ESUs, or 'biomarkers' were tentatively identified based on MS/MS or as unknowns that were classified based on retention time plus the mass to charge ratio (m/z, Appendix S6). Unknowns from the GC-MS analysis were compared to the NIST data base version 2.0 (2005) containing approximately 30 000 compounds.

To quantify metabolite-wide variation among *Inga* ESUs, multivariate statistical methods were used. First, normalized peak intensities were Pareto scaled. Subsequently, a PCA model, a PLS-DA model and hierarchical clustering were fitted on the scaled data in order to see grouping patterns. When the PCA model was non-significant (no clustering), the PLS-DA and hierarchical clustering analyses were not performed. The hierarchical clustering was performed using the Pearson's correlation similarity measure and the Ward's linkage clustering algorithm. All metabolomic data analyses were performed using the Metaboanalyst webserver (Xia *et al.* 2012).

Insect herbivores

COI sequences of sawflies and lepidopterans were assembled into contigs and manually edited using the program SEQUENCHER v5.1 (Gene Codes, Ann Arbor, MI, USA). The resulting sequences were subsequently aligned using the program MUSCLE (Edgar 2004) and clustered into MOTUS using the software package JMOTU (Jones, Ghoorah & Blaxter 2011). Then, the abundance and composition of these MOTUS were compared among the *I. capitata* ESUs using multivariate analyses. All the feeding records that were limited to a single individual in a particular host were not included in this analysis. For this reason, from 64 plants and 37 herbivore species that were originally sampled, only 38 plants and nine herbivore species were included in the analysis. Overlap in feeding records was estimated using the Bray–Curtis dissimilarity index with standardized raw data. The resulting matrix was then analysed for differences in herbivore communities between ESUs using a two-dimensional non-metric multidimensional scaling ordination and a permutational multivariate analysis of variance (PERMANOVA, Anderson 2001) with adjusted P values following the Holm–Bonferroni correction for multiple comparison testing. These analyses were performed using the R package vegan (Oksanen *et al.* 2013).

Host selection and feeding preferences for the sawfly larvae in the field and in the laboratory were estimated using a hierarchical Bayesian model designed for count data (Fordyce *et al.* 2011). This analysis was performed using the R package bayespref (Fordyce *et al.* 2011) in a Markov Chain Monte Carlo (MCMC) framework. Two models were run to estimate the strength of preference of the sawflies for a particular ESU. One model was run with individuals constrained to have equal preference for the different ESUs, and the other model was developed with variation in preference among ESUs (unconstrained). The deviance information criterion (DIC) value was used to compare the fit of each model. In both models, the MCMC chains were run for 5000 generations with the first 1000 generations discarded as a burn-in. Significant differences between ESUs were analysed using pair-wise comparisons of the proportion of times that a

sawfly had a greater preference parameter for a particular ESU at each step of the MCMC.

Results

INGA CAPITATA SPECIES COMPLEX

Leaf defence traits

Consistent with the colour and morphology of expanding and mature leaves, and with DNA sequence differences, each ESU had distinct defensive traits, with no intermediates observed. Total production of soluble phenolics in expanding leaves ranged from 32% to 39% of leaf dry weight across the three ESUs (Appendix S4), values that are typical for the genus as a whole. The fact that these metabolites had substantial detrimental effects in the laboratory at only 0.5–2% of diet (Coley *et al.* 2005; Lokvam & Kursar 2005; Lokvam *et al.* 2006) demonstrates that the *in vivo* level of circa 35% must be highly toxic. The profiles of defensive metabolites showed clear qualitative differences among ESUs (Appendix S5a). Quantitative analyses of the UPLC-MS metabolomics data (phenolics) for clustering showed complete separation among the three *I. capitata* ESUs, with cap3 being the most distinct (Fig. 1). Similarly, a PLS-DA analysis of the metabolites within the phenolic fractions in the UPLC-MS clearly separated cap1, cap2 and cap3 (Fig. 2a). Component 1 separates cap3 from cap1 and cap2, while the second component illustrates the clear contrast between cap1 and cap2 ($R^2 = 0.8$, $P < 0.05$, Fig. 2a). The most important biomarkers for discriminating metabolic differences between these three ESUs were a series of tyramine gallates and quinic acid gallates that are relatively more abundant in cap3. The chemistries of cap1 and cap2 were distinguished from each other and from cap3 by a series of unknowns and kaempferol-galloyl-hexose (Appendix S6).

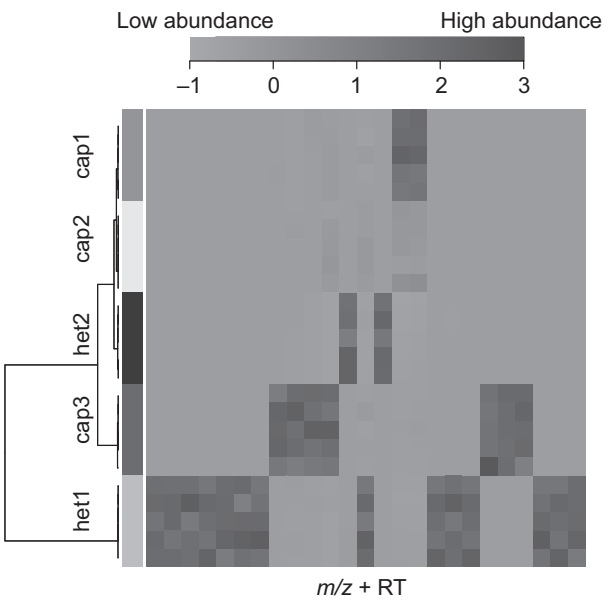

Fig. 1. Heatmap of a hierarchical clustering of *Inga capitata and Inga heterophylla* evolutionarily significant unit (ESUs) based on relative abundances of the most important 25 UPLC-MS phenolic metabolites. Each column represents a metabolite with a unique *m/z* and retention time; analyses are based on 5 individuals per ESU. Each row is one UPLC-MS analysis from one individual plant. Metabolites were identified as 'important' based on ANOVA. The colour scale for metabolite relative abundance is based on signal intensity (total ion current from the mass spectrometer).

Species of *Inga* also produce highly polar secondary metabolites such as toxic non-protein amino/imino acids that are isolated in the polar fraction and have been shown to have a toxic effect on herbivores (Coley *et al.* 2005; Lokvam *et al.* 2006). Metabolomic analysis of the secondary metabolites in the polar fraction included non-protein amino acids that were identified using standards (such as L-DOPA, β-alanine, homoserine, hydroxyproline) and uncharacterized compounds that are thought to be secondary metabolites because they were abundant and did not match any of the more than 30 000 known small molecules in the referenced data bases. This also showed a trend for differences among the ESUs ($R^2 = 0.8$, $P = 0.06$, Fig. 2b).

The timing and frequency of leaf production differed considerably between the ESUs within the *I. capitata* species complex (Fig. 3). Patterns of leaf flushing between cap2 and cap3 were significantly different ($P \leq 0.01$). ESU cap1 was not compared statistically as data were collected in a different year and for only 7 months. Low population vector lengths for cap2 and cap3 indicated low synchronization in leaf production (cap2 $r = 0.22$, cap3 $r = 0.26$), although relative peaks in leaf production were observed in July and October, respectively. In contrast, cap1 had a peak in September (of a different year) and, although several censuses were missed, a large population vector length ($r = 0.8$) for cap1 indicated a high degree of synchrony in leaf production.

The three ESUs differed also in the average area of extrafloral nectaries ($F_{2,140} = 47.75$, $P < 0.01$, Fig. 3). Ant visitation to extrafloral nectaries of *I. capitata* saplings differed among the three ESUs ($F_{2,84} = 5.71$, $P < 0.05$, Fig. 3). Ant abundance on cap1 and cap3, the terra firme ESUs, was two times higher than on cap2 ($P < 0.05$), the floodplain ESU. Another strategy to reduce the impact of herbivory is to expand leaves rapidly, which minimizes the period of greatest vulnerability to herbivores (Kursar & Coley 2003). We found that the three ESUs differ dramatically in the rate of leaf expansion, with cap1 showing a significantly higher percentage increase in leaf area per day than cap2 and cap3 ($F_{2,16} = 33.29$, $P < 0.01$, Fig. 3).

Insect herbivores

Consistent with the observed differences in defensive traits, the abundance and composition of the insect herbivore assemblages showed divergent patterns between the *I. capitata* ESUs (full model $F_{2,36} = 3.16$, $P < 0.01$; cap1 vs. cap2 $F_{1,22} = 2.37$, $P < 0.05$; cap1 vs. cap3 $F_{1,24} = 2.09$, $P < 0.05$; cap2 vs. cap3 $F_{1,26} = 1.77$, $P < 0.05$, Fig. 4). The ordination diagram showed separation among the three ESUs,

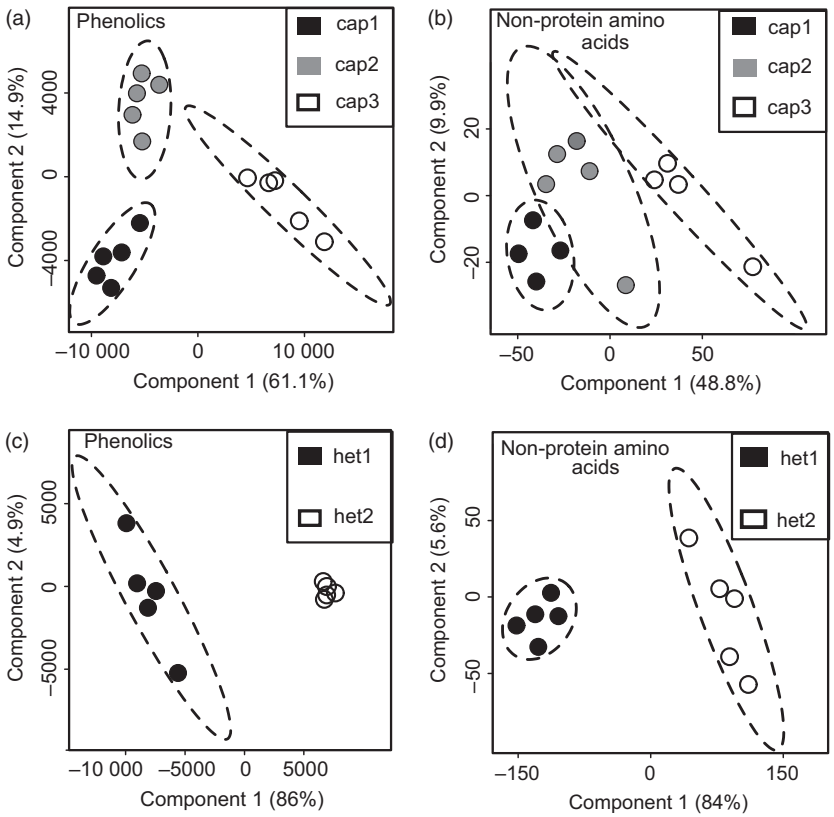

Fig. 2. Score scatter plots from a PLS-DA model fitted to the relative abundances of peaks obtained by metabolic fingerprinting. (a) and (b) *Inga capitata* species complex. (c) and (d) *Inga heterophylla* species complex. The percentage of the variation explained by each component is indicated on the axes. The ellipses delimited by the dotted lines represent the 95% confidence regions. *P* values are provided in the Results.

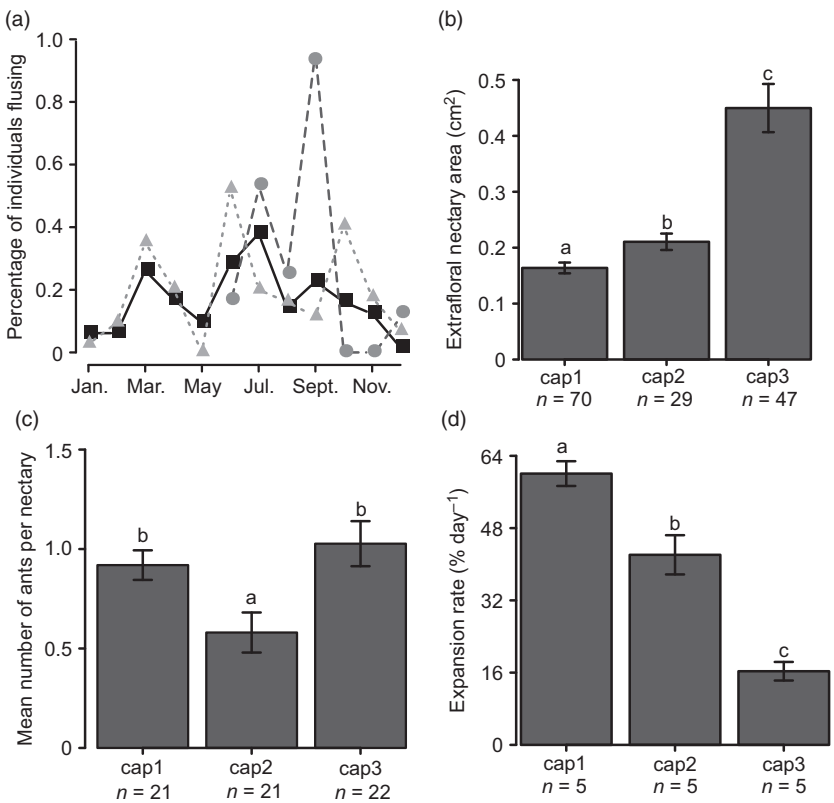

Fig. 3. Non-chemical defensive traits of leaves for the evolutionarily significant unit (ESUs) of the *Inga capitata* species complex. (a) phenological defences, (b) and (c) biotic defences and (d) developmental defences. In panel (a), circles represent cap1 ($n = 24$), squares represent cap2 ($n = 65$), and triangles represent cap3 ($n = 74$). Letters denote significant differences between ESUs. Bars are mean ± SE.

with cap1 supporting the most distinctive herbivore fauna. The permutational analysis of variance suggested that ESU is a more important factor than habitat in explaining the variation associated with the host selection by herbivores (ESU $R^2 = 0.2$, $P < 0.01$, habitat $R^2 = 0.007$, $P < 0.05$). Given that there are three ESUs, we also performed a more

restricted analysis that included only those herbivore species that were collected three or more times. No conclusions were affected, except that the differences between ESUs were more significant (full model: $P < 0.001$, ESU $R^2 = 0.25$, $P < 0.01$, habitat $R^2 = 0.005$, $P < 0.05$, Appendix S9).

Results from our choice experiment suggested that, even for a shared herbivore species, differences in chemical defences within the *I. capitata* complex are big enough to affect herbivore preference. While the sawflies were found on all *I. capitata* ESUs in the field, they showed a significantly higher preference for cap2 over cap1 and cap3, both in the field and in the choice experiment (pairwise post-burning comparisons, $P < 0.05$ for all comparisons between cap2 and the two other ESUs, Fig. 5, Appendix S8).

Leaf non-defence traits

Five non-defensive, functional traits were measured. LMA and leaf nitrogen content are widely used indicators of habitat specialization and photosynthetic ability. Both measures did not vary across ESUs (LMA: $F_{2,12} = 2.25$, $P = 0.15$, nitrogen: $F_{2,12} = 2.77$, $P = 0.1$, Fig. 6). The presence vs. the absence of wings on the rachis also did not show significant differences between ESUs (Fig. 6). Similarly, GC-MS analysis showed that primary metabolites in the polar fraction, such as protein amino acids, did not differ. The PCA model fitted to the primary metabolite data did not reveal separate clusters for any of the ESUs (Fig. 7a) neither for the first two principal components, nor for any other combination of components. However, saplings of cap1 had smaller leaflets ($P < 0.01$) and fewer leaflets per leaf ($P < 0.01$) than cap2 and cap3 (Fig. 6).

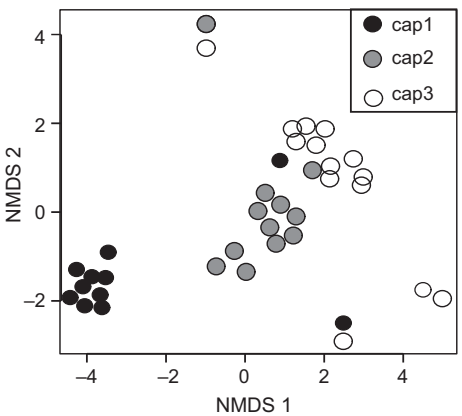

Fig. 4. Ordination diagram of 38 *Inga capitata* plants based on the similarities of their insect herbivore faunas (stress value = 0.05). Similarities in herbivore composition were calculated with the Bray–Curtis Index.

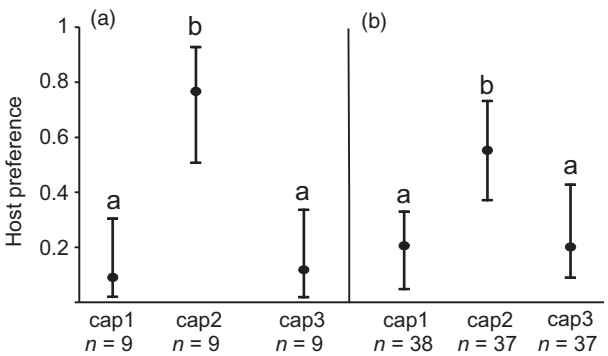

Fig. 5. Summary of sawfly host preferences in: (a) choice experiment and (b) field survey. In both cases, preference is plotted as the median and 95% confidence interval of the posterior probability distribution for population preference, estimated from a hierarchical Bayesian model (Fordyce *et al.* 2011). Lower-case letters denote posterior probabilities of > 0.95 for differences in preferences.

INGA HETEROPHYLLA SPECIES COMPLEX

Leaf defence traits

The two ESUs within the *I. heterophylla* complex are extremely different from each other with respect to their phenolic compounds (Appendix S5b), with het1 showing the greatest divergence (Fig. 1). In fact, *I. heterophylla* het2 more closely groups with *I. capitata* cap1 and cap2. For het1 and het2, total phenolic investment varied between 17% and 23% of leaf dry weight (Appendix S4). Compounds detected within the phenolic fraction in the UPLC-MS clearly separated het1 and het2 by the first axis ($R^2 = 0.9$, $P < 0.01$; Fig. 2c). Saplings of het1 are distinguished from het2 by the expression of relatively high abundant markers tentatively identified as tyrosine gallate, a class of compounds only known from *Inga* (Lokvam *et al.* 2007) and galloyl-L-DOPA (Appendix S6). Analyses of the non-protein amino/imino acid fraction also separated the two ESUs ($R^2 = 0.9$, $P < 0.05$; Fig. 2d), with the primary differences being high levels of free tyrosine and L-DOPA in het1. No marker phenolics or amino/imino acids were found in het2. An insufficient number of individuals of het2 did not enable us to perform statistical analyses for the other defensive traits.

Leaf non-defence traits

The two non-defensive traits for which we had sufficient data did not differ. Neither ESU had wings. The metabolic fingerprint of primary metabolites did not discriminate between het1 and het2 (Fig. 7).

Discussion

DIVERGENCE IN DEFENCES

Insect herbivores are predicted to be major selective agents (Agrawal *et al.* 2012), and results from our analyses are consistent with this idea. First, we found a substantial investment in plant defences against herbivores. Total soluble phenolics accumulated to 32–39% of the dry weight of leaf tissue for the *I. capitata* species complex and to 17–23% for the *I. heterophylla* group. In addition, both groups invest in other costly

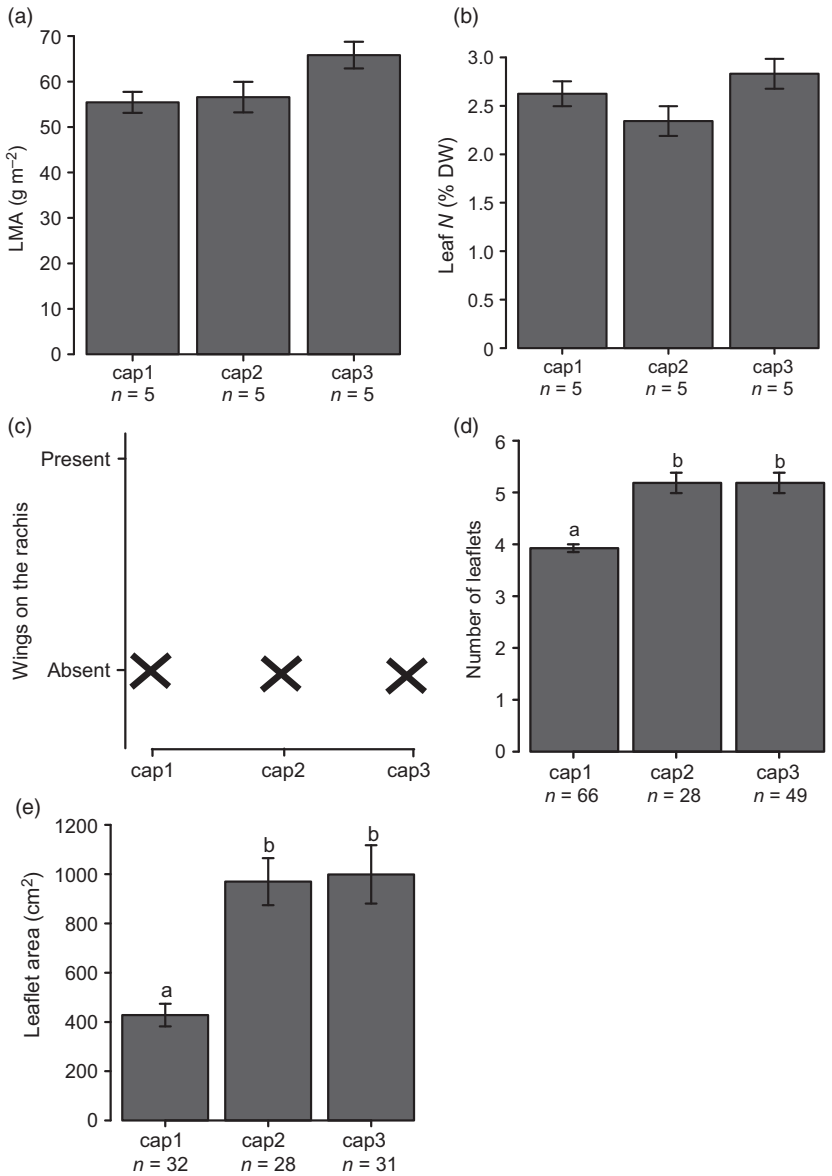

Fig. 6. Non-defensive traits of leaves for the evolutionarily significant unit (ESUs) of the *Inga capitata* species complex. Letters denote significant differences between ESUs. Bars are mean ± SE. LMA = Leaf mass per area and N = nitrogen.

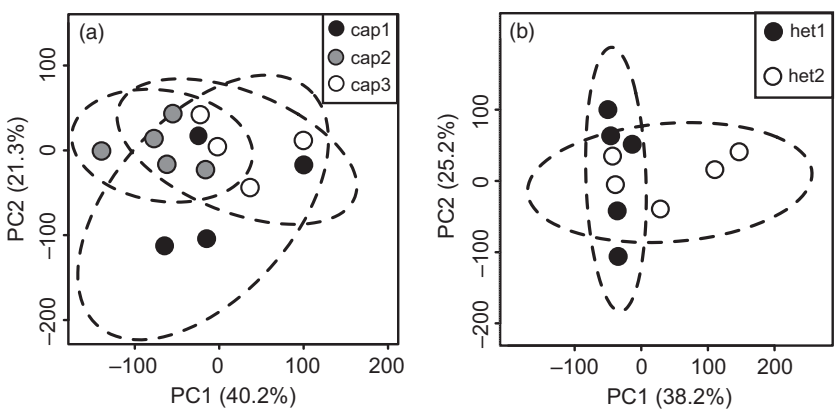

Fig. 7. Score scatter plots from a PCA model fitted to GC-MS data of primary metabolites. (a) *Inga capitata* species complex. (b) *Inga heterophylla* species complex. The percentage of the variation explained by each component is indicated on the axes. The ellipses delimited by the dotted lines represent the 95% confidence regions.

defences, such as non-protein amino acids, extrafloral nectar production, and phenological and developmental defences. Secondly, the factors with the highest divergence between closely related ESUs, for both species complexes, were the antiherbivore traits. And thirdly, for the *I. capitata* complex, close relatives were attacked by different insect herbivore assemblages. Taken together, these results are consistent with the hypothesis of strong selection on defences by herbivores

and suggest that these traits are evolutionarily labile (Agrawal *et al*. 2009, Kursar *et al.* 2009; Schemske 2009).

Among all defensive traits, the most contrasting and interesting differences were found in chemistry. Although all defences are important, clearly chemistry plays a central role in plant–herbivore interactions (Thompson 1988). The metabolomic analyses provided evidence for divergence in secondary metabolite expression (phenolics and non-protein amino acids) for each species complex by separating the different ESUs and identifying ESU-specific 'biomarkers' (Figs 1, 2 and 7; Appendix S6).

As with secondary metabolites, phenological defences diverged markedly between the *I. capitata* lineages (Fig. 3). Synchronous production of leaves is a strategy to satiate herbivores because, by flushing leaves simultaneously, leaf biomass production may exceed the capacity of insects to consume them; this is considered a phenological defence (Aide 1988, 1993). In addition, because in tropical forests, the temporal peak in leaf consumption by insect herbivores closely tracks leaf production (Murali & Sukumar 1993), especially for the most synchronous plant species (Lamarre *et al.* 2014), temporal separation of leaf production among species may be favoured as a strategy for partial escape from herbivores. Individuals of cap1 showed a greater synchrony in leaf production than cap2 and cap3 (Fig. 3). In addition, timing for leaf production was different between ESUs, that is, during the study period, the main peaks of leaf production were at different times of the year, with September for cap1 (data collected in a different year), July for cap2 and June for cap3. Young leaves are an ephemeral stage in the life of a leaf that lasts only a few weeks. Thus, time lags of only 2 weeks between ESUs would be biologically meaningful with different ESU's being available for oviposition at different times of the year (Aide & Londoño 1989).

Similarly, our analyses found differences among ESUs in the rate at which young leaves expand, with leaves from cap1 expanding significantly faster than leaves from cap2 and cap3 (Fig. 3). Shortening the window of vulnerability to herbivores provides a mechanism for temporal escape (Aide & Londoño 1989). It appears that the strategy of escaping herbivory by expanding leaves rapidly is fuelled by delaying the development of the chloroplast (delayed greening) until the leaf is fully expanded and defended by toughness (a defence syndrome termed 'escape species', see Kursar & Coley 2003). Although delayed greening reduces the photosynthetic capacity of young leaves, it also reduces the resources that are lost per gram of leaf eaten (Coley & Kursar 1996).

Biotic defences also diverge between ESUs in the *I. capitata* group. Our field observations indicated that, although all the three lineages invest in active extrafloral nectaries, they differ in the area of the nectary (a proxy for the amount of nectar production, Rudger 2004; Díaz-Castelazo *et al.* 2005) and in the number of ants visiting each ESU (Fig. 3). Ant visitation to the two terra firme ESUs, cap1 and cap3, mirrors differences in extrafloral nectary size. Expanding leaves of cap1 received less ant visitation than leaves from cap3. This pattern could result from more nectar production in cap3, as ants respond positively to higher concentrations and volume of nectar (Bixenman *et al.* 2011). However, although cap2 has extrafloral nectaries that are intermediate in size, it received significantly lower ant visitation than the other two ESUs, presumably because it occurs in flooded forests where the abundances of ground-nesting ants might be lower (Pearson & Derr 1986).

In contrast to what we found with defensive traits, leaf functional traits that are unrelated to defence showed less variation between closely related ESUs. Neither species complex differed in the expression of primary metabolites (Fig. 7). Similarly, LMA and nitrogen content of mature leaves did not show significant differences between the ESUs of the *I. capitata* complex (Fig. 6). These findings suggest that adaptations to the abiotic environment, such as light and nutrients, may not have acted as drivers of divergence between closely related ESUs. However, cap1 did differ in the size and number of leaflets from cap2 and cap3 (Fig. 6), with cap1 having only four leaflets of consistently smaller size, and cap2 and cap3 having between four and six larger leaflets. Given that adults from these three ESUs show a reduction in leaflet size and mostly four leaflets per leaf (Pennington 1997; M. J. Endara pers. obs.), leaf morphologies may differ only at the sapling stage. The fact that cap2 occurs in the floodplain suggests that adaptations to seasonal flooding might be an important factor in divergence between this ESU and the terra firme ESUs, cap1 and cap2. Although we did not measure plant traits associated with flooding tolerance, quantitative trait-based studies have found that species adapted to flood-prone environments show higher LMA and greater leaf area than species from other habitats (Colmer & Voesenek 2009; Huber, Jacobs & Visser 2009). In our study, these traits did not differ between the terra firme and floodplain ESUs.

We also did not find distinct microhabitat preferences of the terra firme forms. ESUs cap1 and cap3, the shade-tolerant, terra firme ESUs coexist within metres of each other, similar to *I. heterophylla* ESUs het1 and het2. Moreover, all five ESUs occur in low-light microsites and show no preference for treefall light gaps.

Studies in other lineages have also suggested greater divergence among closely related species between defensive as compared to non-defensive traits. In the milkweeds, a significant correlation between variation in defensive traits and diversification has been found, while other, non-defensive traits did not show such a relationship (Agrawal *et al.* 2009). Likewise, close relatives of the genus *Psychotria* (Rubiaceae), an understory shrub of tropical forests, are more dissimilar in secondary metabolites than in traits associated with shade and drought tolerance (Sedio 2013). These findings suggest that defences are relatively more labile than other traits, and highlight the importance of insect herbivores in trait diversification.

DIVERGENCE IN INSECT HERBIVORE SELECTION

Consistent with the observed differences in defensive traits, the abundance and composition of insect herbivore communities

showed divergent host association between the *I. capitata* ESUs (Fig. 4). It is quite striking that these differences are consistent even when considering the whole *Inga* community in the study area (43 *Inga* species). Dissimilarities in Lepidoptera community between two of the three pairs of *I. capitata* ESUs are significantly greater than between more distantly related *Inga* species. The community mean dissimilarity index β_{RC} equals 0.69, whereas for cap1 vs. cap2, β_{RC} equals 0.74 and for cap2 vs. cap3, β_{RC} equals 0.92. But for cap1 vs. cap3, β_{RC} equals 0.10 (two-tailed test, alpha = 0.05, unpublished analyses of MJE; for β_{RC} analyses, see Chase *et al.* 2011). Similarly, Fine *et al.* (2013) found substantial differences in secondary metabolites and in the abundance and diversity of insect herbivores between two ecotypes of *Protium subserratum* that occur in white-sand and terra firme forests in the Peruvian Amazon. In our study, the two *I. capitata* lineages (cap1 and cap3) that occur within the same habitat, terra firme, showed the biggest difference in total herbivore assemblage (including Coleoptera, sawflies and Lepidoptera, Fig. 4). This may result from the fact that, in terms of phenolic composition, cap1 more closely groups with cap2 than with cap3 (Fig. 1). Taken together, these results suggest that herbivores might select for divergence in defences among coexisting lineages.

Our feeding choice experiment and field survey of sawfly larvae both showed a preference for cap2 over the other two ESUs. This suggests that differences in chemical defences within the *I. capitata* complex are big enough to affect herbivore preference, even for those herbivore species that are shared. Although many factors can influence host selection in the field, including habitat preferences, phenology and ant visitation to extrafloral nectaries, the primary factors assessed in our controlled choice experiment were leaf secondary metabolites and possibly nutrition. This consideration and the observation that, for sawfly larvae, host selection is related to plant phenolics (Opitz *et al.* 2012) are consistent with our bioassay results.

PATTERNS IN DEFENCE DIVERGENCE

Although both clades include closely related ESUs, two patterns of divergence in chemical defences are evident, with one species complex being more divergent than the other (Fig. 1). One trend is exemplified by the *I. heterophylla* complex, where the two ESUs express non-overlapping chemistry (Fig. 1 and Appendix S6). A switch in secondary compounds between sister species is often found across the genus *Inga* (Kursar *et al.* 2009). Similar results have been obtained for the Fabaceae in a recent study (Wink 2013), and for other groups, such as *Bursera* (Becerra 1997). These patterns diverge from the dominant paradigm of defence evolution, which predicts that closely related species have similar defences (Ehrlich & Raven 1964) and suggests that the production of novel defence mechanisms arise primarily through stepwise changes to structural genes coding for novel biosynthetic enzymes (Berenbaum & Feeny 1981; Berenbaum & Zangerl 1998; Berenbaum & Schuler 2010). We speculate that large shifts in defence chemicals between sister species can be better explained through changes in gene regulation rather than in structural genes for biosynthetic enzymes. In fact, most studies of systems at the genetic level report that chemical traits have diverged due to changes in regulation (Durbin *et al.* 2003; Tewari, Brown & Fristensky 2003; Windsor *et al.* 2005; Burow, Halkier & Kliebenstein 2010).

The second pattern in defence divergence is also consistent with regulatory changes. In the *I. capitata* complex, the three lineages within the group show related chemistry. The most common compounds expressed by the three ESUs are derived from the same pathways, with differences between close relatives found mainly at the level of expression of the different metabolites and/or structural complexity. For example, tyramine gallates, biomarkers for cap3, occur across the three ESUs. However, its relative abundance is much higher in cap3 than in cap1 and cap2. Similarly, in cap3 traces of an unknown with *m/z* of 144.08 is found, but this unknown is produced at high relative abundances only in cap1 (Appendix S6). These observations support the idea that structural genes are present in all ESUs, but ESUs differ in the extent to which they are down or upregulated.

Modifications in gene regulation may be a fast and simple mechanism for differential expression of metabolites between species. This would allow for rapid defence evolution and explain why close relatives are divergent in defences. Major shifts in defences would help to neutralize the advantages that short-lived herbivores have in an evolutionary arms race with long-lived trees.

Conclusions

The results from a number of recent studies suggest that herbivores play an important role in trait diversification and speciation in plants. Our functional trait approach provides evidence for enemy-related differentiation among closely related lineages. That marked phenotypic differences occur in defensive traits and not in other traits between sister lineages within a clade lead us to hypothesize that selection exerted by herbivores is one of the main ecological factors driving diversification. This interpretation is consistent with the proposal that the time scale for changes in abiotic selective pressures may be much longer than for natural selection due to biotic factors (Schemske 2002; Coley & Kursar 2014). Thus, plant traits that are adaptations to the arms race may evolve quickly in order to track counter-adaptations from their enemies. Simple and fast changes in defences through gene regulation are consistent with this hypothesis.

Because four of the ESUs studied here co-occur as neighbours, our findings have significant implications for coexistence. In the tropics, growing evidence is showing dissimilarity in defences between close relatives occurring in sympatry. Thus, divergent selection on defensive traits by herbivores might be mandatory for coexistence of closely related neighbours in tropical forests and could potentially explain the astonishingly high local diversity of these forests (Coley & Kursar 2014).

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Data accessibility

Biomarker metabolites detected by LC-QTOF-MS and chromatograms: uploaded as Supporting Information. Insect herbivore species by host: uploaded as Supporting Information. Bar coding sequences: IBOL, project 'Geometrids of the world'.

References

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

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

Agrawal, A.A., Fishbeinc, M., Halitschke, R., Hastings, A.P., Rabosky, D.L. & Rasmann, S. (2009) Evidence for adaptive radiation from a phylogenetic study of plant defenses. *Proceedings of the National Academy of Sciences, USA*, **106**, 18067–18072.

Agrawal, A.A., Hastings, A.P., Johnson, M.T., Maron, J.L. & Salminen, J.P. (2012) Insect herbivores drive real-time ecological and evolutionary change in plant populations. *Science*, **338**, 113–117.

Aide, T.M. (1988) Herbivory as a selective agent on the timing of leaf production in a tropical understory community. *Nature*, **336**, 574–575.

Aide, T.M. (1993) Patterns of leaf development and herbivory in a tropical understory community. *Ecology*, **74**, 455–466.

Aide, T. & Londoño, E. (1989) The effects of rapid leaf expansion on the growth and survivorship of a lepidopteran herbivore. *Oikos*, **55**, 66–70.

Anacker, B.L. & Strauss, S.Y. (2014) The geography and ecology of plant speciation: range overlap and niche divergence in sister species. *Proceedings of the Royal Society B*, **281**, 20132980.

Anderson, M.J. (2001) A new method for a non-parametric multivariate analysis of variance. *Austral Ecology*, **26**, 32–46.

Batschelet, E. (1981) *Circular Statistics in Biology*. Academic Press, London.

Becerra, J.X. (1997) Insects on plants: macroevolutionary chemical trends in host use. *Science*, **27**, 253–256.

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, USA*, **106**, 18062–18066.

Berenbaum, M.R. & Feeny, P. (1981) Toxicity of angular furanocoumarins to swallowtail butterflies: escalation in a coevolutionary arms race? *Science*, **212**, 927–929.

Berenbaum, M.R. & Schuler, M.A. (2010) Elucidating evolutionary mechanisms in plant-insect interactions: key residues as key innovations. *Evolution Since Darwin: The First 150 Years* (eds M.A. Bell, D.J. Futuyma, W.F. Eanes & J.S. Levinton), pp. 269–290. Sinauer Associates, Sunderland, MA.

Berenbaum, M.R. & Zangerl, A.R. (1998) Chemical phenotype matching between a plant and its insect herbivore. *Proceedings of the National Academy of Science (USA)*, **95**, 13743–13748.

Bixenmann, R.J., Coley, P.D. & Kursar, T.A. (2011) Is extrafloral nectar production induced by herbivores or ants in a tropical facultative ant-plant mutualism? *Oecologia*, **165**, 417–425.

Bixenmann, R.J., Coley, P.D. & Kursar, T.A. (2013) Developmental changes in direct and indirect defenses in the young leaves of the neotropical tree genus *Inga* (Fabaceae). *Biotropica*, **45**, 175–184.

Brenes-Arguedas, T., Coley, P.D. & Kursar, T.A. (2008) Divergence in the chemical ecology of *Inga* between two Neotropical sites. *Journal of Ecology*, **96**, 127–135.

Brenes-Arguedas, T., Horton, M.W., Coley, P.D., Lokvam, J., Waddell, R.A., Meizoso-O'Meara, B.E. & Kursar, T.A. (2006) Contrasting mechanisms of secondary metabolite accumulation during leaf development in two tropical tree species with different leaf expansion strategies. *Oecologia*, **149**, 91–100.

Burow, M., Halkier, B.A. & Kliebenstein, D.J. (2010) Regulatory networks of glucosinolates shape *Arabidopsis thaliana* fitness. *Current Opinion in Plant Biology*, **13**, 348–353.

Chase, J.M., Kraft, N.J., Smith, K.G., Vellend, M. & Inouye, B.D. (2011) Using null models to disentangle variation in community dissimilarity from variation in α diversity. *Ecosphere*, **2**, 1–11.

Coley, P.D. & Aide, T.M. (1991) Comparison of herbivory and plant defenses in temperate and tropical broad-leaved forests. *Plant-Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions* (eds P.W. Price, T.M. Lewinsohn, W.W. Fernandes & W.W. Benson), pp. 25–49. John Wiley & Sons, New York, NY.

Coley, P.D. & Kursar, T.A. (1996) Anti-herbivore defenses of young tropical leaves: physiological constraints and ecological tradeoffs. *Tropical Forest Plant Ecophysiology* (eds S.S. Mulkey, R.L. Chazdon & A.P. Smith), pp. 305–336. Chapman and Hall, New York, NY.

Coley, P.D. & Kursar, T.A. (2014) Is the high diversity in tropical forests driven by the interactions between plants and their pests? *Science*, **343**, 35–36.

Coley, P.D., Lokvam, J., Rudolph, K., Bromberg, K., Sackett, T.E., Wright, L. *et al.* (2005) Divergent defensive strategies of young leaves in two Neotropical species of *Inga*. *Ecology*, **86**, 2633–2643.

Colmer, T.D. & Voesenek, L.A. (2009) Flooding tolerance: suites of plant traits in variable environments. *Functional Plant Biology*, **36**, 665–681.

Cornelissen, J.H., Lavorel, S., Garnier, E., Díaz, S., Buchmann, N., Gurvich, D.E., Reich, P.B., ter Steege, H., Morgan, H.D., van der Heijden, M.G., Pausas, J.G. & Poorter, H. (2003) A handbook of protocols for standardized and easy measurement of plant functional traits worldwide. *Australian Journal of Botany*, **51**, 335–380.

Díaz-Castelazo, C., Rico-Gray, V., Ortega, F. & Angeles, G. (2005) Morphological and secretory characterization of extrafloral nectaries in plants of coastal Veracruz, Mexico. *Annals of Botany*, **96**, 1175–1189.

Durbin, M.L., Lundy, K.E., Morrell, P.L., Torres-Martinez, C.L. & Clegg, M.T. (2003) Genes that determine flower color: the role of regulatory changes in the evolution of phenotypic adaptations. *Molecular Phylogenetics and Evolution*, **29**, 507–518.

Edgar, R. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, **32**, 1792–1797.

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

Endara, M.J. & Coley, P.D. (2011) The resource availability hypothesis revisited: a meta-analysis. *Functional Ecology*, **25**, 389–398.

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.

Fine, P.V.A., Metz, M.R., Lokvam, J., Mesones, I., Ayarza Zuñiga, J.M., Lamarre, G.P.A., Vásquez Pilco, M. & Baraloto, C. (2013) Insect herbivores, chemical innovation and the evolution of habitat specialization in Amazonian trees. *Ecology*, **94**, 1764–1775.

Fordyce, J.A., Gompert, Z., Forister, M.L. & Nice, C.C. (2011) A hierarchical Bayesian approach to ecological count data: a flexible tool for ecologists. *PLoS One*, **6**, e26785.

Fujita, K., van Bodegom, P. & Witte, J.P. (2013) Relationships between nutrient-related plant traits and combinations of soil N and P fertility measures. *PLoS One*, **8**, e83735.

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

Hackstadt, A.J. & Hess, A.M. (2009) Filtering for increased power for microarray data analysis. *BMC Bioinformatics*, **10**, 11.

Huber, H., Jacobs, E. & Visser, E.J. (2009) Variation in flooding-induced morphological traits in natural populations of white clover (*Trifolium repens*) and their effects on plant performance during soil flooding. *Annals of Botany*, **103**, 377–386.

Ivanova, N.V., deWaard, J.R. & Hebert, P.D.N. (2006) An inexpensive, automation-friendly protocol for recovering high-quality DNA. *Molecular Ecology Notes*, **6**, 998–1002.

Jones, M., Ghoorah, A. & Blaxter, M. (2011) jMOTU and Taxonerator: turning DNA barcode sequences into annotated operational taxonomic units. *PLoS One*, **6**, e19259.

Koptur, S. (1984) Experimental evidence for defense of *Inga* (Mimosoideae) saplings by ants. *Ecology*, **65**, 1787–1793.

Kursar, T.A. & Coley, P.D. (2003) Convergence in defense syndromes of young leaves in tropical rainforests. *Biochemical Systematics and Ecology*, **21**, 929–949.

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. (2009) The evolution of anti-herbivore defenses and their contribution to species coexistence in the tropical tree genus *Inga*. *Proceedings of the National Academy of Sciences, USA*, **106**, 18073–18078.

Lamarre, G.P.A., Mendoza, I., Fine, P.V.A. & Baraloto, C. (2014) Leaf synchrony and insect herbivory among tropical tree habitat specialists. *Plant Ecology*, **215**, 209–220.

Lokvam, J. & Kursar, T.A. (2005) Divergence in structure and activity of phenolic defenses of young leaves of co-occurring *Inga* species. *Journal of Chemical Ecology*, **11**, 2563–2580.

Lokvam, J., Brenes-Arguedes, T., Lee, J.S., Coley, P.D. & Kursar, T.A. (2006) Allelochemic function for a primary metabolites: the case of L-tryrosine hyper-production in Inga umbellifera (Fabaceae). *American Journal of Botany*, **93**, 1109–1115.

Lokvam, J., Clausen, T.P., Grapov, D., Coley, P.D. & Kursar, T.A. (2007) Galloyl depsides of tyrosine from young leaves of *Inga laurina*. *Journal of Natural Products*, **70**, 134–136.

Murali, K.S. & Sukumar, R. (1993) Leaf flushing phenology and herbivory in a tropical dry deciduous forest, southern India. *Oecologia*, **94**, 114–119.

Oksanen, J., Guillaume Blanchet, F., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H. & Wagner, H. (2013) *VEGAN: R package for community ecology*. Available at http://cran.r-project.org/web/packages/vegan/vegan.pdf.

Opitz, S.E.W., Boevé, J.-L., Nagy, Z.T., Sonet, G., Koch, F. & Müeller, C. (2012) Host shifts from Lamiales to Brassicaceae in the sawfly genus *Athalia*. *PLoS One*, **7**, e33649.

Pearson, D.L. & Derr, J.A. (1986) Seasonal patterns of lowland forest floor arthropod abundance in southeastern Peru. *Biotropica*, **18**, 244–256.

Pennington, T.D. (1997) *The Genus Inga*. The Royal Botanic Gardens, Kew, London, UK.

Pitman, N. (2007) *An overview of the Los Amigos watershed, Madre de Dios, southeastern Peru*. October 2007 version of an unpublished report available from the author at npitman@amazonconservation.org

R Development Core Team. 2011. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0. Available at http://www.R-project.org/.

Rudger, J. (2004) Enemies of herbivores can shape plant traits: selection in a facultative ant-plant mutualism. *Ecology*, **85**, 192–205.

Schemske, D.W. (2002) Ecological and evolutionary perspectives on the origins of tropical diversity. *Foundations of Tropical Forest Biology: Classic Papers with Commentaries* (eds R.L. Chazdon & T.C. Whitmore), pp. 163–173. University of Chicago Press, Chicago, IL.

Schemske, D.W. (2009) Biotic interactions and speciation in the tropics. *Speciation and Patterns of Diversity* (eds R. Butlin, J. Bridle & D. Schluter), pp. 219–239. Cambridge University Press, Cambridge.

Sedio, B. (2013) Trait evolution and species coexistence in the hyperdiverse tropical forest tree genus *Psychotria*. PhD Thesis, University of Michigan, Ann Arbor, MI.

Smith, C.A., Tautenhahn, R., Neumann, S., Benton, P. & Conley, C. (2014) *XCMS: R package for LC/MS and GC/MS data analysis*. Available at http://www.bioconductor.org/packages/release/bioc/manuals/xcms/man/xcms.pdf.

Tewari, S., Brown, S.M. & Fristensky, B. (2003) *Plant defense multigene families: I. Divergence of* Fusarium solani*-induced expression in* Pisum *and* Lathyrus. Available at http://arxiv.org/abs/q-bio/0310003.

Thompson, J. (1988) Coevolution and alternative hypotheses on insect/plant interactions. *Ecology*, **69**, 893–895.

Thompson, J. (2005) *The Geographic Mosaic of Coevolution*, 400 pp. University of Chicago Press, Chicago, IL.

deWaard, J.R., Ivanova, N.V., Hajibabaei, M. & Hebert, P.D.N. (2008) Assembling DNA barcodes: analytical protocols. *Methods in Molecular Biology: Environmental Genetics* (ed. C. Martin), pp. 275–293. Humana Press Inc., Totowa, NJ.

Windsor, A., Reichelt, M., Figuth, A., Svatos, A., Kroymann, J., Kliebenstein, D., Gershenzon, J. & Mitchell-Olds, T. (2005) Geographic and evolutionary diversification of glucosinolates among near relatives of *Arabidopsis thaliana* (Brassicaceae). *Phytochemistry*, **66**, 1321–1333.

Wink, M. (2013) Evolution of secondary metabolites in legumes (Fabaceae). *South African Journal of Botany*, **89**, 164–175.

Wright, I.J., Reich, P.B., Westoby, M., Ackerly, D.D., Baruch, Z. & Bongers, F. (2004) The world-wide leaf economics spectrum. *Nature*, **428**, 821–827.

Xia, J., Mandal, R., Sinelnikov, I., Broadhurst, D. & Wishart, D.S. (2012) MetaboAnalyst 2.0 – a comprehensive server for metabolomic data analysis. *Nucleic Acids Research*, **40**, W127–W133.

Zar, J.H. (1999) *Biostatistical Analysis*. Prentice-Hall, Upper Saddle River, NJ.

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Supporting Information

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

Appendix S1. Photographs of the *Inga* species.

Appendix S2. Maximum clade credibility tree of the species of the genus *Inga* in Los Amigos, Peru.

Appendix S3. Chromatographic gradient used for the LC-MS analyses.

Appendix S4. Phenolic content for *Inga* species.

Appendix S5. Total ion chromatograms showing relative intensities of peaks from the LC-QTOF-MS for the *Inga* species.

Appendix S6. Biomarker metabolites detected by LC-QTOF-MS.

Appendix S7. Insect herbivore species found on each *I. capitata* ESU.

Appendix S8. Deviance information criterion comparison of unconstrained vs. constrained models estimating the strength of preference of the sawflies for a particular ESU.

Appendix S9. Ordination diagram of 31 *Inga capitata* plants based on similarities of their herbivore faunas with a minimum number of 3 individuals per herbivore MOTU.