Insect herbivores, chemical innovation, and the evolution of habitat specialization in Amazonian trees

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Abstract. Herbivores are often implicated in the generation of the extraordinarily diverse tropical flora. One hypothesis linking enemies to plant diversification posits that the evolution of novel defenses allows plants to escape their enemies and expand their ranges. When range expansion involves entering a new habitat type, this could accelerate defense evolution if habitats contain different assemblages of herbivores and/or divergent resource availabilities that affect plant defense allocation. We evaluated this hypothesis by investigating two sister habitat specialist ecotypes of *Protium subserratum* (Burseraceae), a common Amazonian tree that occurs in white-sand and terra firme forests. We collected insect herbivores feeding on the plants, assessed whether growth differences between habitats were genetically based using a reciprocal transplant experiment, and sampled multiple populations of both lineages for defense chemistry. *Protium subserratum* plants were attacked mainly by chrysomelid beetles and cicadellid hemipterans. Assemblages of insect herbivores were dissimilar between populations of ecotypes from different habitats, as well as from the same habitat 100 km distant. Populations from terra firme habitats grew significantly faster than white-sand populations; they were taller, produced more leaf area, and had more chlorophyll. White-sand populations expressed more dry mass of secondary compounds and accumulated more flavone glycosides and oxidized terpenes, whereas terra firme populations produced a coumaroyl-quinic acid that was absent from white-sand populations. We interpret these results as strong evidence that herbivores and resource availability select for divergent types and amounts of defense investment in white-sand and terra firme lineages of *Protium subserratum*, which may contribute to habitat-mediated speciation in these trees.

Key words: *Amazonia; ecological speciation; ecotypes; herbivory; natural enemies; plant defense; Protium subserratum; terra firme forests; tropical rain forests; white-sand forests.*

Introduction

Environmental gradients have been hypothesized to be engines of diversification in tropical ecosystems (Endler 1977, Smith et al. 1997, Moritz et al. 2000). Indeed, habitat specialization by plants across environmental gradients leads to high beta-diversity and greatly contributes to species diversity within regions, especially in tropical forests (Gentry 1986, Tuomisto et al. 2003). Herbivores play an important role in habitat specialization because they can magnify the differences in resource availability across habitats (Janzen 1974, Fine et al. 2004). But little is known about the mechanisms

underlying the evolution of habitat specialization and the extent to which herbivores contribute to phenotypic divergence during the speciation process, largely because comparative studies of host plants and their associated herbivores have not been attempted in recently diverged sister species (Futuyma and Agrawal 2009).

The evolutionary interaction between herbivores and their host plants is mediated by the suite of physical and chemical defenses employed by the host plant, as well as by adaptations by the herbivore to circumvent or detoxify defenses (Ehrlich and Raven 1964, Futuyma and Agrawal 2009). The type and amount of defense a plant allocates should represent an optimal strategy given the available resources and the abundance and identity of attackers (Herms and Matson 1992). Strong environmental gradients (i.e., contrasting habitats) can therefore influence plant–herbivore interactions in at

least two ways. First, the species composition and relative abundance of herbivore communities may turn over among habitats because herbivores are affected by habitat quality, structure, and interactions with predators (Novotny et al. 2005, Singer and Stireman 2005, Rodríguez-Castañeda et al. 2010). Second, the impact of herbivory on plant fitness may vary across habitats, especially across gradients in resource availability. For example, given trade-offs in allocation to growth and defense, the optimal defense allocation may be affected by differential costs of tissue replacement across habitats (Janzen 1974, Coley et al. 1985, Fine et al. 2006). Moreover, environmental heterogeneity may promote very different defense allocation strategies for different plant species depending on the type of defense employed (i.e., their elemental constituents and biosynthetic pathways), as well as the nature of resource limitation across habitats (i.e., light, nutrients, or water) (Bryant et al. 1983, Herms and Mattson 1992).

A long-standing hypothesis has linked escalation in plant defense that allows escape from insect herbivores to range expansion and speciation (Ehrlich and Raven 1964). Such escalation can include increases in the diversity of defense strategy (novel defense types), increases in the total amount of defense investment, or both (Agrawal et al. 2009). Range expansion, or merely an imperfect match between the distribution of plants and their natural enemies, may confront plants with different herbivore assemblages and/or variation in habitat resources across their range (Thompson 2005, Züst et al. 2012). This variation, in turn, may accelerate the evolution of differing defense strategies across habitats. Alternatively, natural enemies may not be major selective agents driving habitat specialization. In this case, we would predict that there would be few qualitative and quantitative defense differences between habitats, especially when diverging lineages of host plants occur in close proximity and also experience some gene flow across the habitat boundary.

White-sand forest habitat islands of the Western Amazon represent an excellent study system to investigate the role of natural enemies and plant defenses in the divergence of tree lineages across environmental gradients. White-sand patches occur immediately adjacent to other terra firme clay and brown-sand soils, providing a steep environmental gradient in soil fertility (Ruokolainen and Tuomisto 1998, Fine et al. 2005, 2006). Plant surveys of white-sand and neighboring forest types show strong patterns of specialization and changes in species composition, with many different genera containing habitat specialist species in both white-sand forests and the neighboring terra firme forests (Fine et al. 2010).

In order to study the evolutionary processes involved in habitat specialization and the role of insect herbivores, an ideal study system would include recently derived sister species, or diverging lineages undergoing incipient speciation in different habitats. One common and diverse tropical tree genus, *Protium* (Burseraceae),

includes several species associated with white-sand forests, none of which are each other's closest relatives (Fine et al. 2005). Of these species, *Protium subserratum* Engl. (Engl.) comprises morphologically divergent ecotypes that correspond to white-sand and terra firme habitats, often occurring within meters of one another in a parapatric distribution (Daly and Fine 2011, Fine et al. 2013). Phylogenetic and phylogeographic analyses of 12 populations of *P. subserratum* ecotypes showed that the Peruvian terra firme (brown-sand and clay) ecotypes mostly share the exact same haplotypes, and that their common ancestor derives from a clade that includes almost all of the Peruvian white-sand populations (Fig. 1). Although there are consistent morphological differences that characterize the terra firme and white-sand ecotypes, there is also evidence of limited gene flow (or incomplete lineage sorting) between the two ecotypes (Fine et al. 2013). Thus, white-sand and terra firme ecotypes of *P. subserratum* are either a recently diverged sister pair or they represent two lineages that are undergoing incipient speciation. In either case, the system offers an excellent opportunity to compare herbivore communities and phenotypic divergence in the types and amounts of plant defense across gradients in resource availability. We collected insect herbivores, conducted a reciprocal transplant experiment, and analyzed plant defense chemistry from multiple populations of white-sand and terra firme lineages of this Amazonian tree to answer the following questions: (1) Are there differences in the number or diversity of insect herbivores between habitats? (2) Do white-sand and terra firme ecotypes differ in their amount of growth and defense investment? (3) Do white-sand and terra firme ecotypes express qualitatively different chemical defenses? (4) To what extent do differences in chemical defenses among populations correlate with differences in resource availability and herbivore community composition?

Methods

Insect herbivore sampling

We sampled insect herbivores from four populations of *Protium subserratum*: one population each was sampled from white-sand forest and terra firme forest (5 km apart) at the Centro de Investigación Jenaro Herrera (CIJH), and one population each was sampled from white-sand and terra firme forest (5 km apart) in the Allpahuayo-Mishana (AM) National Reserve near Iquitos, Peru (white and gray circles, white square and gray square without thick black border, Fig. 1). CIJH and AM are separated by ~100 km. Sampling in each location was undertaken for 12 months between March 2011 and March 2012. In each of the four sampling sites, 33–41 individual saplings (0.7–2.1 m in height) of *P. subserratum* were located and tagged within an area of ~1 ha, and initial height and leaf and leaflet number were recorded. Each plant was visited once every 4–7 d during the morning hours, and observed for 2–5 min,
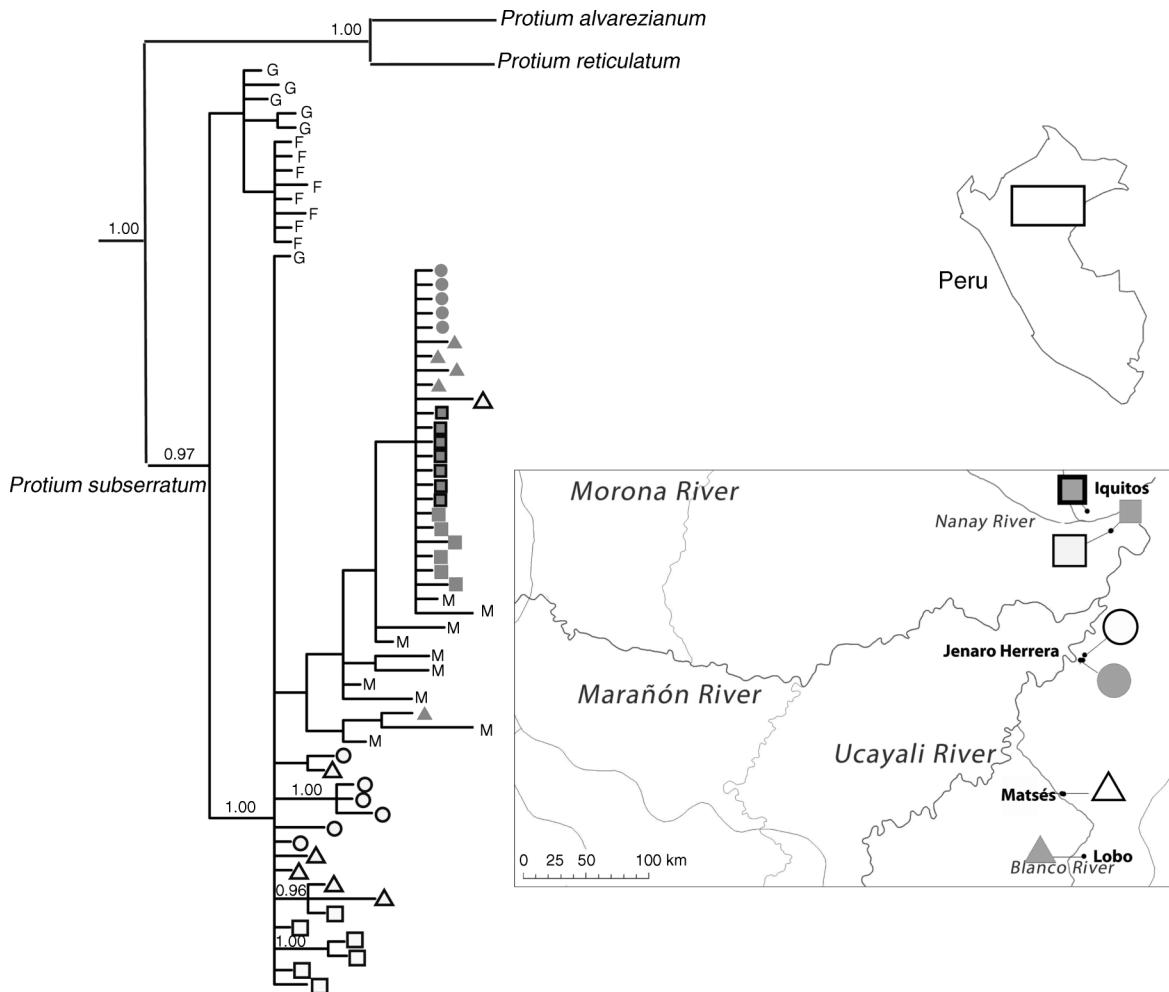
FIG. 1. Study system. (Left) A Bayesian phylogeny based on three nuclear genes of *Protium subserratum* populations from Fine et al. (2013) showing white-sand ecotypes from Peru (white circles, triangles, and squares), terra firme ecotypes from Peru (gray circles, triangles, and squares), and terra firme ecotypes from Manaus Brazil (indicated with "M"). Populations from Guyana (terra firme habitats) are indicated with "G," and those from French Guiana (terra firme habitats) are indicated with "F." Posterior probabilities >0.95 are shown. The sister taxa of *P. subserratum* included are from a phylogeny based on five chloroplast and nuclear genes (Daly and Fine 2011). (Right) The map highlights the area of study in Loreto, Peru, and the gray shaded and white shapes indicate sampling site locations, which also code the geographical locations of the tips on the population-level phylogeny.

during which time all herbivores observed feeding on the leaves, stems, or meristems were collected by hand or with an aspirator. Multiple individuals from each morphospecies were often observed feeding on the same plants at the same time, and were counted but not collected. Larvae were collected and reared in the laboratory using fresh leaf material from unmarked *P. subserratum* individuals from the same habitat. In the laboratory, all insect specimens were classified to morphospecies by authors J. M. Ayarza. Zuñiga and M. Vásquez Pilco, and then assigned to family and subfamily by author G. P. A. Lamarre and to genus level by specialists associated with the amateur entomological society Société Entomologique Antilles-Guyane (SEAG) based in Cayenne, French Guiana (Appendix B).

Identification to species level is still in process, and, afterwards, all specimens will be deposited in museums in Peru, France, and the USA (Berkeley, California). Herbivores were most commonly found feeding on newly expanding leaves, which were much more prevalent in Jenaro Herrera compared to Iquitos (Appendix C).

We compared the abundance and composition of the herbivores feeding on plants in terra firme or white-sand habitats using regression and multivariate analyses. First, we used generalized linear models and a negative binomial error structure to compare the number of insects and morphospecies observed per plant and the number of host plants on which morphospecies were collected with crossed predictors of habitat type and

sampling site. These regressions account for overdispersion of the count data, which occurred due to insect counts being quite variable among plants. Second, we used a factorial analysis of variance (McArdle and Anderson 2001) to assess whether habitat type, sampling site, or the interaction of the two was a significant determinant of herbivore species composition. This is analogous to an ANOVA where the dependent variable is a multivariate matrix of species abundance. The herbivore community matrix was restricted to the 101 plants with ≥5 herbivore observations and the 31 insect morphospecies with ≥10 individuals observed across ≥5 unique plants. We then used a two-dimensional nonmetric multidimensional scaling (NMDS) ordination to illustrate the differences in the herbivore fauna at each site. Third, we performed an indicator species analysis (Dufrene and Legendre 1997) that examined the relative abundance of a morphospecies and its relative frequency among the four sampling areas. Finally, we summed insect observations across plants within a site (excluding the 51 morphospecies with single observations) to assess beta-diversity using pairwise Bray-Curtis dissimilarities.

Reciprocal transplant experiment

We located six populations of *Protium subserratum*, including three in white-sand habitats and three in clay and brown-sand habitats (all marked sites from the map in Fig. 1, except the gray circle). From each population, 60–100 *Protium subserratum* seedlings were collected from seedling carpets beneath 4–10 mother trees and placed in small plastic bags filled with soil. The bags were transported by boat to Iquitos and placed in a shaded area in the forest until all populations were ready to be planted. In September 2007, one seedling from each population was planted into 60 experimental plots, half of which were located in a white-sand forest in the AM Reserve near Iquitos, and half in a terra firme clay soil forest 10 km south on the Iquitos-Nauta highway in private land bordering the AM Reserve. Clay and white-sand soils differed significantly in nutrient availability and soil texture, with higher nitrogen, potassium, and percentage of clay content in clay soils and higher phosphorous and percentage of sand in white-sand soils (Appendix D; soil analyses conducted by the DANR lab, University of California, Davis, California, USA). Experimental exclosures were $2 \times 2 \times 2$ m wooden frames covered with 1-mm green mesh nylon netting to exclude herbivores. Controls were identical, except that they only had nylon netting covering the roof. Unfortunately, nylon netting was stolen from both white-sand and clay forest sites several times during the experiment, rendering the comparison of herbivore-excluded to control treatments invalid. Herbivore-exclusion treatment is thus not considered further.

We measured meristem height, number of leaves and leaflets, and average leaflet size until July 2009. In June 2010, we collected leaves from 62 seedlings to measure leaf toughness, leaf thickness, and chlorophyll content.

On three leaves per individual, leaf chlorophyll content was estimated using three values from a Minolta 210 SPAD 502DL meter (Spectrum Technologies, Plainfield, Illinois, USA). Leaf thickness was measured as the mean of three measurements with a digital micrometer (Mitutoyo Instruments, Singapore). Leaf toughness was measured as the average of three punch tests with a Chatillon penetrometer (Ametek, Largo, Florida, USA).

We assessed growth rates and defense allocation among white-sand and terra firme populations when grown in a common environment using a linear mixed-effects models with experimental soil type and origin soil type as crossed, fixed, independent variables, initial seedling height as a covariate, and maternal identity nested within population origin as random factors. We examined height growth (cm) through July 2009 using a standard starting point marked in September 2008, leaf area growth (cm^2) as the average size of the plant's leaflets multiplied by the difference between leaflet number in July 2009 and at transplant in September 2007, and chlorophyll content (μg/cm^2) derived from SPAD measurements using the calibration equation of Coste et al. (2010) derived for Neotropical trees. We examined differences in defense allocation with leaf toughness or leaf thickness as the dependent variables; predictor variables were the same, except that we did not include initial height as a covariate in these models.

Chemical methods

All chemical analyses reported here derive from field-collected individuals. Leaves were harvested from 5–9 individuals from each of four populations ($N = 25$ individuals) of *P. subserratum* growing near Iquitos and Jenaro Herrera, Peru (white and gray circles, white square and gray square with thick black border, Fig. 1). Leaves were kept in bags of silica-gel and dried at room temperature in ziplock bags before shipment to the University of California, Berkeley (California, USA) for immediate analysis of volatiles on GCMS (see Appendix A) and subsequently, to the University of Utah (Salt Lake City, Utah, USA) for nonvolatile chemistry.

In Utah, leaves were further dried at high vacuum (10^{-2} Torr; 1 Torr = 133.3 Pa) for 24 h, then pulverized using a Wig-L-Bug grinder (Dentsply Rinn, Elgin, Illinois, USA). Ground leaves (100 mg) were weighed into 2-mL centrifuge tubes and submitted to a polarity-gradient extraction protocol (Appendix A). This gave four extract fractions (lipids, medium polarity, high polarity, and hot-water soluble), as well as the marc (remaining leaf solids). Following removal of solvents and high-vacuum drying, each fraction was weighed. The medium- and high-polarity fractions, which together represented a majority of the extract mass, were combined and analyzed by high pressure liquid chromatography (HPLC) using diode array, evaporative light-scattering, and mass detection (see Appendix A for details). While flavans and flavones were readily

PLATE 1. White-sand ecotype of *Protium subserratum* seedling growing in the reciprocal transplant experiment. Note the hairs on the leaflet rachis, which are present whether this ecotype is planted in white-sand or clay soils. Terra firme ecotypes do not have any pubescence on their leaves. Photo credit: P. V. A. Fine.

identifiable by their chromatographic and UV/mass spectroscopic properties, two other classes of metabolites were not. One of these consisted of three isomeric quinic acid derivatives, the most abundant being *p*-coumaroylquinic acid (Appendix A). The second group consisted of a series of compounds having very similar HPLC retention times and UV absorption properties. The most abundant of these was a symmetrically substituted poly-acetyl, dicarboxylic acid derivative of squalene (Lokvam and Fine 2012; Appendix A). We consider each of these four classes of secondary metabolites to function in the deterrence of insect herbivores either directly or indirectly (see Appendix A)

We compared absolute and qualitative differences in leaf defense. First, we compared the dry mass of leaf defense chemicals in a linear model with soil type and study region as independent variables. Second, we tested whether habitat type, sampling site, or the interaction of the two, was a significant determinant of the relative allocation among flavan, flavone, quinic acid derivatives, and oxidized terpenes in each plant using a factorial analysis of variance. We used a two-dimensional NMDS ordination to illustrate the differences in allocation, overlaying vectors describing the relationship between the ordination space and defense chemical abundances or soil characteristics at each site.

Chemical variation as a function of soil, geography, genetic distance, and herbivore fauna

Finally, we examined how variation in leaf chemistry turned over across geographic, genetic, herbivore fauna, and soil type differences using multiple regression on distance matrices (MRM; Legendre et al. 1994, Lichstein 2007). The MRM analysis is analogous to partial Mantel tests that regress a response matrix against two or more distance matrices, and use permutations to determine the significance of the regression model and the predictor coefficients (Lichstein 2007). Leaf chemistry dissimilarities among 25 individual plants were regressed against four other distance matrices that represent potential drivers of plant chemical divergence. We asked whether similarities among plants in relative allocation to the four chemical classes were correlated to similarities in the herbivore fauna and soil resources experienced by the plant while controlling for the genetic and geographic distance among populations. The predictor matrices included (1) Bray-Curtis dissimilarities of summed insect observations across plants at a site and calculated for all site pairs; (2) site-level Bray-Curtis dissimilarities in soil characteristics using both nutrient availability (e.g., N, P, K) and the percentage content of clay, sand, and silt; (3) geographic and (4) genetic distances among populations reported in Fine et al. (2013); and (5) a block term to account for the nonindependence of insect fauna, soil, distance, and genetic dissimilarities among leaf chemical measurements at the same site.

All analyses were conducted in the statistical programming language R version 2.14.1 using the *lme4, languageR, multcomp, vegan, labdsv,* and *ecodist* packages (R Development Core Team 2011).
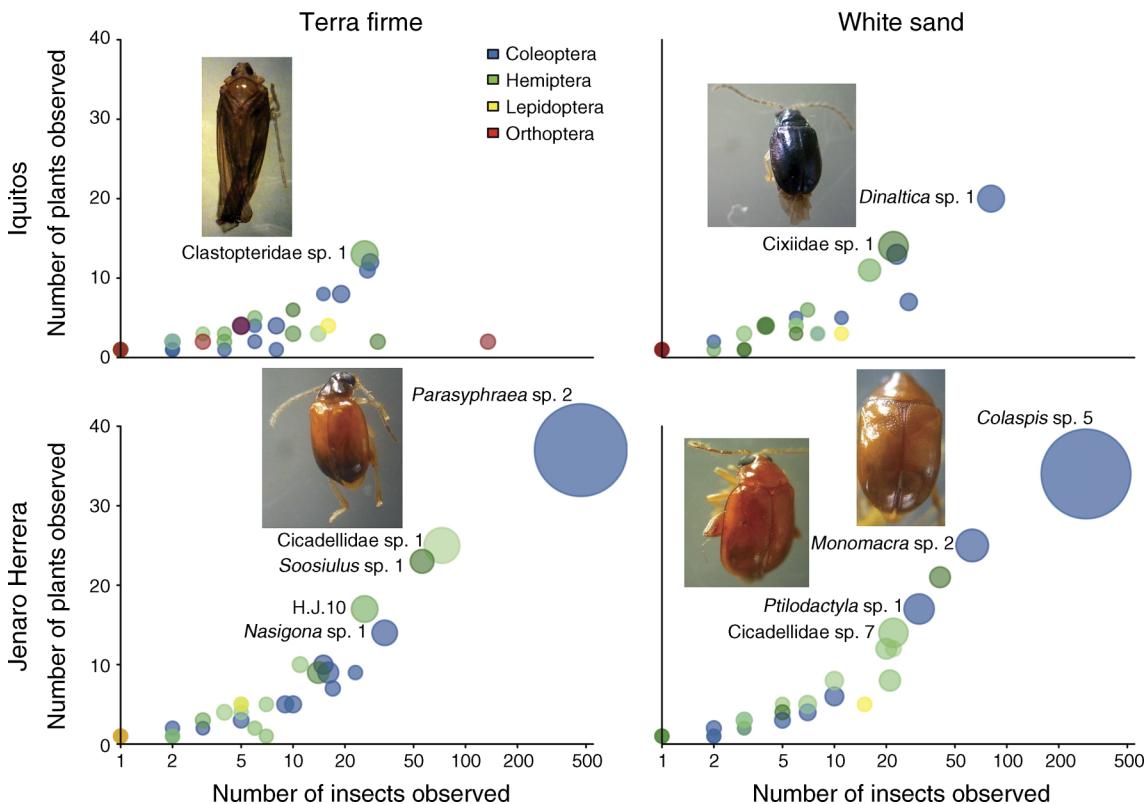
Fig. 2. Insect herbivore faunas: insect morphospecies abundances, distributions, and indicator species values for samples collected on *P. subserratum* plants in white-sand or terra firme habitats at Iquitos and Jenaro Herrera. Species are graphed according to the total number of insects observed (*x*-axis) and the number of unique plants at a site from which the morphospecies was collected (*y*-axis). Circles are scaled according to the indicator species value for the site, and morphospecies with an indicator species value ≥0.25 are listed by name. The color of the circle corresponds to the insect order. Photos of the most common insects per location are included in each panel.

RESULTS

Insect herbivore assemblages collected from *P. subserratum* saplings varied significantly in both abundance and species composition across the four sampling locations (Appendix B). We observed 44% more insects on terra firme population plants than on white-sand plants (Appendix C). We collected 115 morphospecies, of which only 38 occurred at more than one site. Of the observed insects, 61% were chrysomelid beetles and 16% were cicadellid hemipterans (Appendix B). Each of the four locations had a unique group of 8–13 morphospecies that were considered indicator species (Fig. 2; sensu Dufrene and Legendre 1997). In addition to differences among the dominant herbivores, the species composition of the entire *P. subserratum* herbivore fauna exhibited high turnover among sites and habitats (Fig. 3; Appendix E). Differences between sites, habitat types, and their interaction explained 14%, 15%, and 11%, respectively, of the variation in herbivore species composition among the four sampling locations (Appendix F).

Growth strategies by terra firme and white-sand populations were significantly different (Fig. 4). All plants had more rapid growth in clay soils than in white-sand soils. However, these differences were not simply due to phenotypic plasticity. Terra firme populations exhibited significantly greater height and leaf growth and allocated more to chlorophyll production than white-sand populations in both soil types, demonstrating that different growth strategies have a genetic basis (Fig. 4; Appendix G). We also used the reciprocal transplant experiment to assess putative physical defense traits among populations. We found that leaf thickness and leaf toughness did not show a significant effect of lineage, but instead exhibited significant variation related to soil type, indicating that these traits were plastic at the seedling stage (Appendix H). By contrast, leaf pubescence was not plastic, and was observed only on white-sand populations, planted into both soil types (see Plate 1).

White-sand and terra firme populations exhibited strong differences in their amount and composition of leaf secondary metabolites. Total secondary metabolite production was significantly greater in field collections from white-sand populations (0.29 ± 0.04 g/g dry mass
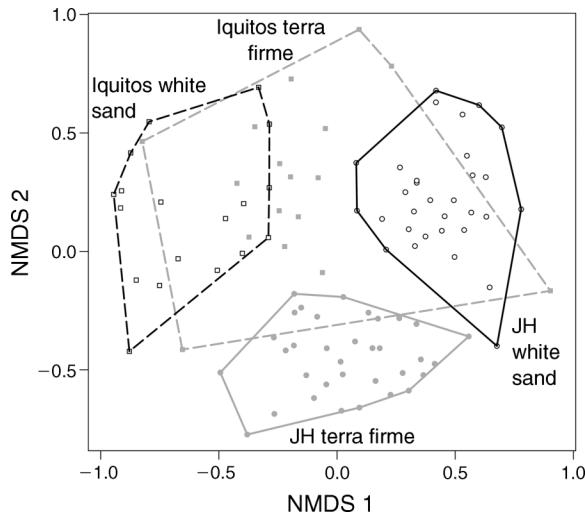
FIG. 3. Turnover in insect herbivore communities with habitat and distance. Results of a nonmetric multidimensional scaling (NMDS) ordination of herbivore assemblages from 101 plants in white-sand and terra firme habitats at the Iquitos (dashed lines and squares) and Jenaro Herrera (solid lines and circles) sites. Herbivore abundance data was restricted to the 31 insect morphospecies with ≥10 individuals observed across ≥5 unique plants. NMDS stress = 0.20.

[dm]) compared to terra firme populations (0.19 ± 0.01 g/g dm; Appendix H).

Gas chromatography analyses of leaves showed that, unlike most members of the family Burseraceae, *P. subserratum* does not yield measureable amounts of monoterpenes and only trace amounts of sesquiterpenes (Appendix A). Nevertheless, four classes of constitutive leaf defenses were identified in the populations of *P. subserratum*: flavans, flavones, quinic acid derivatives, and oxidized terpenes. The first consisted of flavan-3-ols, specifically (epi)catechin monomers and polymers (hereafter "flavan") and represented 80–90% of the mass of constitutive defenses in each of the four populations of *P. subserratum*. The other three metabolite classes were highly variable among populations and indicated differentiation between habitat types. These were flavone glycosides, primarily pentose and hexose-substituted quercetin (hereafter "flavone"); quinic acid derivatives, dominated by *p*-coumaroylquinic acid; and terpene acids, the most abundant being a poly-acetyl, dicarboxylic acid derivative of squalene (hereafter "oxidized terpene") (Appendix A). The relative abundance of these four classes of chemicals differed by habitat type, geographic location, and their interaction (accounting for 20%, 11%, and 10%, respectively, of variation in allocation; Appendix F). Quinic acid derivatives accumulated to relatively high concentration (~0.5–2% dm) in the two terra firme populations of *P. subserratum*, but these compounds are completely absent from the white-sand populations (Fig. 5). By contrast, terra firme plants expressed little to no flavone or oxidized terpene.

Pairwise dissimilarities among the defense compound composition of individual plants were well correlated with dissimilarities in the soil resources and the insect fauna associated with the plants in their respective
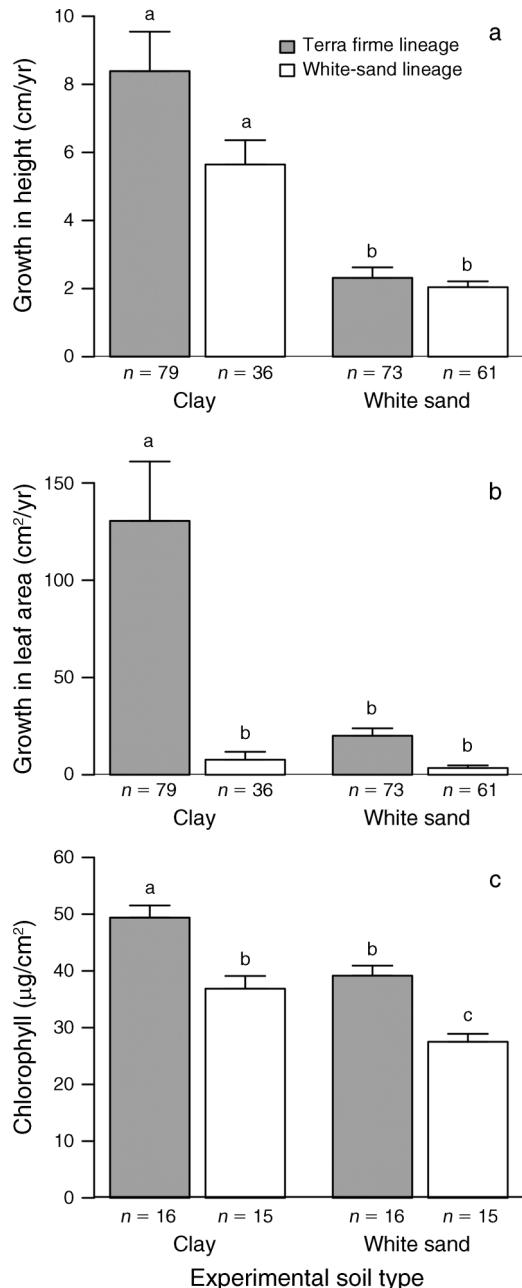

FIG. 4. Growth strategies differ between lineages. The figure shows allocation to growth by plants from populations of terra firme and white-sand ecotypes reciprocally transplanted into both soil types. Growth was measured by (a) growth in plant height, (b) growth in plant leaf area, and (c) SPAD measurements of chlorophyll content in the leaves. Bars are means ± 1 SE, averaged across populations and maternal identities. The number of plants in each treatment is given below the bars. Different lowercase letters indicate significant differences among groups using post hoc tests (*P* < 0.05).
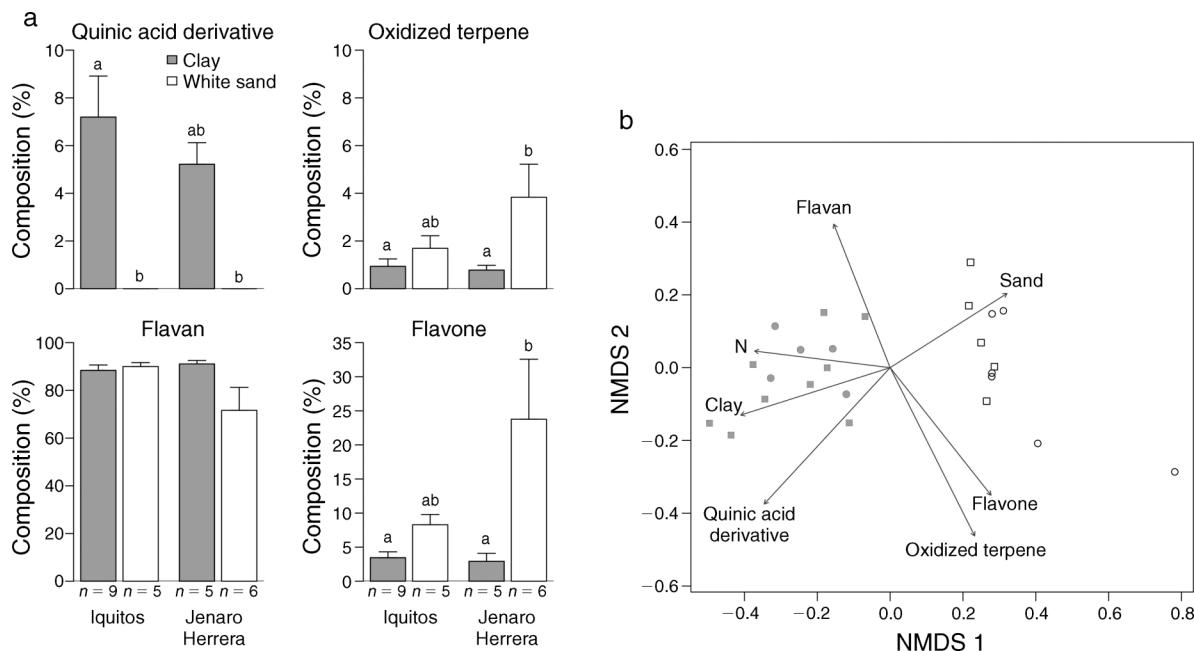
Fig. 5. Defense strategies differ between lineages from terra firme (gray bars and symbols) and white-sand (open bars and symbols) ecotypes at Iquitos (squares) and Jenaro Herrera (circles). (a) The relative abundance (percentage of total $60 + 100\%$ MeOH fractions) of four chemical defense compound classes from terra firme or white-sand ecotypes at Iquitos and Jenaro Herrera. Bars are means $\pm$ SE with sample sizes below. (b) An NMDS ordination of relative allocation of four defense classes in leaves from plants in both ecotypes, with vectors indicating the direction of maximal correlation with increasing abundance of chemical components. Site-level soil nutrient and texture characteristics are overlaid with arrows pointing in the direction of maximum correlation with available nitrogen and percentage clay or sand content. Different lowercase letters indicate significant differences among groups using post hoc tests ($P < 0.05$).

habitats, while also controlling for genetic and geographic distances and block effects among the populations ($R^2 = 0.53$, $P = 0.001$; Table 1).

DISCUSSION

Insect herbivore diversity and abundance

We found that insect herbivores collected from *Protium subserratum* showed strong patterns of dissimilarity across different habitat types (Fig. 3). Moreover, we found significantly more insects feeding on terra firme plants than on white-sand plants, correlating with the large differences in resource availability between the habitat types. Taken together, these results suggest that there exists substantial variation in diversity and abundance of insect herbivores associated with *P. subserratum* across white-sand and terra firme habitats. In contrast, Novotny et al. (2007), using similar sampling methodology, found very little species turnover in four feeding guilds of herbivorous insects hand-collected from their host plants even across >500 km of Papua New Guinea lowland forest. However, when sampling across an elevational gradient, Novotny et al. (2005) found strong differences in herbivore assemblages in three widespread species of *Ficus*: Each tree species had a different dominant lepidopteran herbivore species in the lowlands compared to montane habitats. Similar results of high beta-diversity across elevational habitats

have been reported in lepidopteran herbivores specialized on *Piper* (Rodríguez-Castañeda et al. 2010).

Unlike Novotny et al. (2007), we found extremely high turnover with geographic distance (Appendix E). This may result from the fact that Jenaro Herrera and Iquitos are found on opposite sides of the Amazon River, which is known to be a major biogeographic

TABLE 1. Turnover in chemical-defense similarity with differences in habitat resources and insect assemblages after accounting for geographic and genetic distance.

Term	Estimate	P
Intercept	0.373	0.665
Insects	2.813	0.001
Soil	1.481	0.001
Distance	−0.007	0.001
Genetic distance	−0.399	0.001
Block	−1.203	0.001

Notes: Results of a multiple regression on matrices (MRM) analysis with the dependent-variable matrix being the pairwise differences among plants in the relative abundance of four leaf defense classes. These included 25 *Protium subserratum* plants from clay and white-sand soils at Iquitos and Jenaro Herrera, Peru (or 300 pairwise dissimilarities). Independent predictor variable matrices consisted of site-level pairwise dissimilarities in insect herbivore fauna and soil resource availability (Appendix E) and geographic or genetic distances among plant populations (Fine et al. 2013). A block-level term was included to account for the nonindependence of site-level predictors among plants at the same site. $R^2 = 0.52$, $P = 0.001$.

barrier for birds (Cracraft 1985; Álvarez et al., *in press*). Large differences in leaf production between Jenaro Herrera and Iquitos (Appendix C) could also play a role. Different assemblages of herbivores are known to feed on mature vs. expanding leaves (Novotny et al. 2003), which could explain the surprisingly high turnover in insect communities between locations. Increased sampling of plants producing new leaves in additional geographic locations on both sides of the Amazon River will be needed to understand the controls of the beta-diversity of the *P. subserratum* insect herbivore community. Finally, it is important to recognize that our sampling was limited to juvenile plants and lasted only 12 months. Insect herbivore populations can strongly vary in different years, and thus, it is possible that our sampling missed important herbivores that are associated with *P. subserratum*.

Divergence in plant defense strategies

Secondary compounds constitute 20–30% of the dry mass of leaf tissue in *P. subserratum*, suggesting that anti-herbivore chemical defense is an important energetic investment, especially for white-sand ecotypes. Consistent with the growth–defense trade-off hypothesis (Coley et al. 1985), we found higher growth rates and lower defense allocation in terra firme populations and lower growth rates and higher defense allocation in white-sand populations. The existence of this trade-off has been well supported by many different temperate and tropical studies looking at allocation to growth and defense in plants adapted to different light and nutrient availabilities, both within species (Herms and Mattson 1992, Agrawal et al. 2012, but see Woods et al. 2012), in interspecific comparisons (Herms and Mattson 1992, Fine et al. 2006, Endara and Coley 2011), and within clades (Agrawal et al. 2009).

Protium subserratum ecotypes also exhibited significant differences in the types of secondary metabolites they expressed, with terra firme plants containing quinic acids that were absent in white-sand plants, which in turn, produced significantly more flavones and oxidized terpenes (Fig. 5). All of the four defense compound classes that were present (flavans, flavones, oxidized terpenes, and quinic acids) have been associated with deterring insect herbivores and/or parasitoid signaling (see Appendix A). However, we acknowledge that none of the chemicals that we have characterized in this study have been subjected to feeding trials using herbivores collected on *P. subserratum* plants, and they may in fact function as deterrents to fungal pathogens or to different insects than those we have sampled. Investigating the function of these secondary metabolites will be an important direction of future research.

It is well known that plant defense exhibits substantial variation within populations of a single species (Whitham et al. 2006), as well as among closely related species (Becerra 1997, Kursar et al. 2009). However, while a few studies have investigated how variation within a species

correlates with geographic distance (Macedo and Langenheim 1989, Woods et al. 2012) or with differences in biotic interactions (Janzen 1975), we know of no other study that has compared populations of sister species' chemical defenses across an environmental or habitat gradient.

Similarities among individual plants in their allocation to different types of leaf defense chemicals were most strongly and positively correlated with differences in insect herbivores and soil resource availability among the locations those plants inhabited, even while accounting for the genetic and geographic distances among the plant populations (Table 1). This is an interesting result given the strong possibility of current (or recent) gene flow between ecotypes, and is consistent with strong selection by herbivores.

Alternatively, we cannot rule out that the qualitative and quantitative differences we report in defense chemistry from field-collected plants in the two habitats reflect phenotypic plasticity in secondary metabolite chemistry in the two ecotypes rather than genetically based traits under selection by natural enemies. If plasticity in defense allocation occurs, herbivores would be interpreted as reinforcing phenotypic differences between ecotypes rather than driving selection for different defense strategies.

Plants' defense allocation to both C-based and N-based defenses can be affected by light, water, and nutrient availability (i.e., the carbon–nutrient balance hypothesis [Bryant et al. 1983], and the growth–differentiation balance hypothesis [Herms and Matson 1992]). For example, secondary metabolites such as amides, terpenes, and flavans can exhibit significant increases or decreases in concentration in response to experimental fertilization or natural differences in nutrient availability (Bryant et al. 1983, Dyer et al. 2004, Ormeño et al. 2008, Massad et al. 2012). Different classes of compounds derive from different biosynthetic pathways, which may make classes of secondary metabolites more or less likely to exhibit phenotypic plasticity under resource limitation (Massad et al. 2012).

However, Fine et al. (2006) measured physical and chemical defenses of seedlings of the white-sand ecotype of *P. subserratum* transplanted in clay and white sand and found no significant effect of soil type, consistent with the hypothesis that defense traits in this taxon are genetically based and not plastic responses to resource availability. In fact, none of the eight *Protium* species included in that reciprocal experiment demonstrated significant quantitative differences in phenolic or terpene allocation when transplanted in the contrasting soil type (see Appendix C from Fine et al. 2006: *Ecological Archives* E087-117-A3). Here, while we found some evidence in our reciprocal transplant for plasticity in leaf toughness (Appendix H), several other traits (growth rate, chlorophyll, and leaf pubescence) were not consistent with phenotypic plasticity.

Whether plastic or genetically based, chemical divergence in these two ecotypes represents important phenotypic differences that can influence host plant specialization by natural enemies. For example, host specialist insects appear to have undergone small-scale radiations within phenotypically plastic host plant species that occur in different environments (Wilson et al. 2012). Over time, variation in coevolutionary interactions among plants and their specialist enemies across the geographic mosaic should lead to diversification in both host plants and their enemies (Ehrlich and Raven 1964, Thompson 2005, Züst et al. 2012).

Historical biogeography and ecological speciation

Protium subserratum occurs in rain forests across northern South America. Phylogenetic and phylogeographic analyses of 12 populations from throughout its range found that western Amazonian terra firme populations contain very little haplotype diversity (consistent with recent population expansion) and are derived from a clade of white-sand populations (Fine et al. 2013). This is in agreement with the geological history of these two habitats. White-sand forests are older than the clays and brown sands of Andean origin that were laid down in Western Amazonia during the Miocene (Hoorn 1993). Integrating the results from our chemistry analyses into the historical and biogeographical context of this clade, we speculate that terra fime populations have increased their allocation to growth in the more nutrient-rich Andean-derived sediments, and, in the process, reduced allocation to leaf flavones and oxidized terpenes. In addition, terra firme populations appear to have shifted to initiate production of quinic acid derivatives, which may be giving them protection against terra firme-specific enemies, or may be more easily synthesized in high-resource soils.

There are currently no geographic barriers between white-sand forests and the terra firme forests that surround them, making it possible that these two ecotypes have undergone divergence without allopatry. Parapatric speciation across adjacent habitats is theoretically possible, provided that intermediate phenotypes are at a significant selective disadvantage in both habitats (Endler 1977). Several studies on tropical vertebrate species whose geographic distribution spans environmental gradients have found support for habitat-mediated selection and morphological divergence in the face of gene flow (Smith et al. 1997, Ogden and Thorpe 2002). Comparatively few studies of ecological speciation have been conducted on plants, and no studies have investigated the proximate mechanisms. For example, Savolainen et al. (2006) reported sister species of palms that have diverged parapatrically across an environmental gradient on a small tropical island, but they did not investigate the selective forces causing phenotypic divergence.

Leaving aside the question of whether divergence has occurred in these ecotypes in parapatry or allopatry, we believe that the differences in defense strategy we find agree with a growing consensus that plant defense traits are involved in diversification (Becerra et al. 2009, Kursar et al. 2009, Wilson et al. 2012). For example, Agrawal et al. (2009) found that defense traits showed significant correlations with diversification in the *Asclepias* clade, while several traits unrelated to defense did not. Moreover, defense traits, because of the allocation trade-off with growth, have been cited as an example of "two-dimensional incompatibility selection" that may accelerate the diversification process (Artzy-Randrup and Kondrashov 2006).

CONCLUSIONS

Populations of white-sand and terra firme ecotypes of *Protium subserratum* were attacked by herbivore assemblages differing in both abundance and species composition, exhibited significant differences in growth and defense allocation, and expressed qualitatively different secondary compounds. That these phenotypic differences occur in populations involved in incipient (or recent) speciation is consistent with the hypothesis that herbivores interact with environmental gradients to promote the evolution of habitat specialization in plants.

Environmental heterogeneity features prominently in terrestrial ecosystems throughout the globe, and sharp differences in resource availability and in insect herbivore assemblages likely occur across habitats in many regions. However, the tropics contain much higher herbivore diversity and abundance than temperate ecosystems (Erwin 1982, Coley and Barone 1996). The interaction between herbivores and environmental gradients thus may promote more habitat specialization at low latitudes, thereby increasing the rate of speciation (Schemske et al. 2009, Wilson et al. 2012) and contributing to the extraordinarily high species richness of tropical forests.

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SUPPLEMENTAL MATERIAL

Appendix A

Detailed chemistry methods and results (*Ecological Archives* E094-160-A1).

Appendix B

A table showing insect herbivore morphospecies by site (*Ecological Archives* E094-160-A2).

Appendix C

A table of the abundance and host plant distribution of herbivore morphospecies (*Ecological Archives* E094-160-A3).

Appendix D

A table showing soil variables from the reciprocal transplant experiment (*Ecological Archives* E094-160-A4).

Appendix E

A table showing Bray-Curtis dissimilarities in insect herbivore assemblages and host plant allocation to leaf secondary metabolites (*Ecological Archives* E094-160-A5).

Appendix F

A table showing variation in herbivore communities and relative abundance of leaf secondary metabolites (*Ecological Archives* E094-160-A6).

Appendix G

A table of growth allocation results from the reciprocal transplant experiment (*Ecological Archives* E094-160-A7).

Appendix H

A table of measurements of leaf size and physical and chemical defense traits for each ecotype (*Ecological Archives* E094-160-A8).