Chemoecology
DOI 10.1007/s00049-015-0198-1

CHEMOECOLOGY

ORIGINAL ARTICLE

Habitat-specific divergence of procyanidins in *Protium subserratum* (Burseraceae)

John Lokvam[1] · Margaret R. Metz[2] · Gary R. Takeoka[3] · Lien Nguyen[1] · Paul V. A. Fine[1]

Received: 9 October 2014 / Accepted: 8 June 2015

Abstract In Amazonian Peru, the neotropical tree *Protium subserratum* Engl. (Burseraceae) occurs as distinct ecotypes on low nutrient white-sand (WS), intermediate fertility brown-sand (BS), and nutrient-rich clay (CS) soils. Genetic analysis indicates that these ecotypes are undergoing incipient speciation. Possible drivers of this divergence are habitat-specific herbivore faunas and differing resource availabilities. *Protium subserratum*, therefore, provides an ideal opportunity to investigate how defense chemistry evolves during lineage divergence. WS and BS races of *P. subserratum* are host to largely non-overlapping herbivore communities and they differ in chlorogenic acid, flavonoid, and oxidized terpene chemistry. Here, we investigate how another important class of anti-herbivore chemicals, procyanidins (PCs), varies among the ecotypes. We isolated the PCs from leaves of juvenile and adult trees from each ecotype and used spectroscopic and chemical techniques to characterize the chemical structures of their component monomers. We found that WS, BS, and CS ecotypes accumulate ca. 17 % of leaf dry weight as PCs. Within ecotypes, we found very little difference in PC type, neither by site nor by life stage. Among ecotypes, however, we observed a marked divergence in PC composition that arose at least in part from differences in their terminal and extension subunits. In addition, the average polymer length of BS and CS PCs was significantly greater than in WS ecotypes. We conclude that phenotypic differences in PCs in the WS versus BS and CS ecotypes of *P. subserratum* are consistent with selection by herbivores in different soil types that differ strongly in nutrient availability and may contribute to the evolution of habitat specialization.

Keywords *Protium subserratum* · Procyanidin · Defense chemistry · Herbivory · Habitat specialization · Speciation

Handling Editor: Marko Rohlfs.

✉ John Lokvam
lokvam@berkeley.edu

[1] Department of Integrative Biology, University of California, 1005 Valley Life Sciences Building 3140, Berkeley, CA 94720, USA

[2] Department of Biology, Lewis & Clark College, Portland, OR 97219, USA

[3] Agricultural Research Service, United States Department of Agriculture, Albany, CA 94710, USA

Introduction

In the Amazon basin, mosaics of contrasting soil types harbor completely different habitat-specialist tree communities (Gentry 1986; Ruokolainen and Tuomisto 1998; Fine et al. 2010). However, many of these habitat-specialist tree species have congeners that inhabit neighboring soil types. Because these soil types strongly differ in resource availability, resource allocation theory hypothesizes that tradeoffs in growth and defense may arise in plants that are under selection by herbivores and other natural enemies. For plants growing in high-resource versus low-resource habitats, theory predicts divergent strategies for growth and defense allocation (Janzen 1974; Coley et al. 1985). Indeed, a study of six genera that included white-sand (WS) and clay specialist trees found consistent differences in growth and defense strategies across soil types (Fine et al. 2006). However, to more closely link such habitat-specific strategies to the processes of lineage divergence

Published online: 25 June 2015

and speciation, one must investigate recently derived sister species or, ideally, lineages involved in incipient speciation and test the degree to which growth and defense strategies differ between such close relatives that occupy distinct habitats.

Recently, we investigated the chemical defenses and insect herbivore faunas of *P. subserratum* Engl. a species consisting of WS, brown-sand (BS), and clay soil (CS) habitat-specialists that appears to be undergoing incipient speciation (Fine et al. 2013a). Each of the three ecotypes occurs throughout the Peruvian Amazon. They are morphologically distinct and often occur within meters of each other in a parapatric distribution (Fine et al. 2013a; Misiewicz and Fine 2014). Phylogeographic analyses of the haplotypes of these three morphotypes as well as detailed population genetic study have revealed that they represent three evolutionary lineages although there is limited gene flow among them (Fine et al. 2013b; Misiewicz and Fine 2014). Thus, these ecotypes either represent recently derived species or lineages undergoing incipient speciation. In either case, this study system provides the opportunity to study phenotypic divergence in chemical defenses within the context of habitat specialization.

Earlier, we reported marked differences in defense chemistry among ecotypes, especially in flavones, oxidized terpenes, and quinic acid derivatives (Fine et al. 2013a). However, we also reported that both ecotypes accumulate large quantities of a class of phenolic metabolites that are known collectively as polymeric (epi)catechins or procyanidins (PCs). All three ecotypes accumulate PCs as 70–80 % of their total secondary metabolite investment. In this paper, we focus on the types of PCs that accumulate in the three *P. subserratum* ecotypes from different soil types. This group of compounds has well-documented anti-herbivore function (Feeny 1969; Swain 1979; Lokvam and Kursar 2005). However, within this broad category, there is an enormous array of structurally distinct molecules that likely differ in their deterrence mechanisms (Salminen and Karonen 2011) or are toxic to only certain types of natural enemies (Barbehenn and Constabel 2011). Among plant phenolics, two recognized modes of biological action are pro-oxidation and protein precipitation. Pro-oxidation generates reactive oxygen species that can damage herbivore guts; protein precipitation can lower food quality by reducing protein digestibility. PCs are not effective pro-oxidants (Barbehenn et al. 2006) yet within a certain pH range they can efficiently precipitate protein.

Plant resource allocation also differs with life stage such that PCs could also be expected to vary qualitatively and quantitatively across geographically separated populations and among ontogenetic stages. Boege and Marquis (2005) presented a model to explain how plant defense allocation should be optimized through ontogeny. They predicted that juveniles should allocate more energy toward chemical defenses than reproductive adults, because smaller individuals are more likely to experience herbivore-related mortality. Moreover, if different species or guilds of herbivores attack juvenile plants compared to those that attack reproductive adults, there may be qualitative differences in the chemical defenses expressed at different ontogenetic stages (Goodger et al. 2007).

For the present study, we examined the PC content of leaves from several populations of clay, BS, and WS ecotypes of *P. subserratum* at several sites across more than 100 km of Amazonian Peru. We ask the following questions: Has PC chemistry diverged among habitat-specialist ecotypes of *P. subserratum*? If so, what variations in chemical structure underlie these differences? Could PC structural variation have effects on biological activity, specifically, protein precipitation efficiency? And finally, to what extent does PC allocation (qualitatively or quantitatively) vary within each habitat-specialist ecotype with ontogenetic stage? Such a fine-scale characterization of defense metabolites that we present here is rarely performed but is crucial to understanding the relative importance of habitat, ontogeny, and geographic variation in natural selection for plant defense investment.

Materials and methods

Sample collection, preparation, and extraction

Leaf samples were collected from five populations of *P. subserratum* in the vicinity of Iquitos, Peru, in January–February, 2013. Samples were gathered from both juvenile trees (five from WS habitat, four from BS and three from CS) as well as adult trees (ten from WS, seven from BS and one from CS). In all cases, young, expansion-phase leaves were selected because this is the developmental stage that experiences the highest rates of herbivory (Coley and Barone 1996). Leaves were placed in envelopes and dried over repeatedly refreshed silica gel before transport to the University of California, Berkeley, in sealed plastic bags. In our laboratory, leaves were further dried at moderate vacuum (10^{-2} torr) at ambient temperature for 72 h. Dried leaves were then pulverized using a Mini Bead Beater (Biospec Products, Bartlesville, OK).

From each pulverized leaf sample, 100 mg (±1 mg to the nearest 0.1 mg) was placed in a 2.0-mL plastic centrifuge tube containing a micro stir bar and extracted 3× with 1.5 mL of 3:1 (v/v) hexane/acetone, 4× with 1.5 mL of 4:1 (v/v) ethanol/aq 0.5 % acetic acid, and finally 3× with 1.5 mL of 7:3 (v/v) acetone/aq 0.5 % acetic acid. Tubes were sealed and placed on a rapidly spinning stir

plate for 10 min before being centrifuged at 8000×*g* for two min. The resulting supernatant was removed by gas-tight syringe without disturbing the pellet. Like extractions were pooled into tared vials and dried first under a stream of nitrogen at 35 °C and then under moderate vacuum (10^{-2} torr) for 72 h at ambient temperature before being weighed. Preliminary chromatographic analysis showed that the aqueous acetone extraction comprised a subset of the aqueous ethanol extraction. In the following steps, these two extractions were combined into a single fraction (hereafter Phenolics).

Soluble procyanidin isolation

The Phenolics extraction from each sample was fractionated on Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO) to isolate PCs from all other extractable metabolites (Strumeyer and Malin 1975). Each Phenolics fraction was suspended in 2 mL of aq 0.1 % formic acid and applied to a 2.5-cm diameter chromatographic column containing 20 mL (in ethanol) of LH-20 that had been equilibrated with aq 0.1 % formic acid. Samples were eluted under low pressure with 40 mL of aq 0.1 % formic acid and 60 mL of ethanol to remove sugars, organic acids, and non-procyanidin phenolics. A final 40 mL elution of 7:3 (v/v) acetone/aq 0.1 % formic acid eluted the soluble PCs. Each acetone fraction was reduced to a small volume under vacuum and transferred to a tared vial. The remaining solvent was dried first under a stream of nitrogen at 35 °C and then under moderate vacuum (10^{-2} torr) at ambient temperature for 36 h before being weighed.

Characterization of procyanidin chemistry

Owing to the stereochemistry of the flavan-3-ol C ring (Fig. 1), the complexity of PCs can grow exponentially as polymer length increases. This class of metabolites, therefore, tends to be structurally complex and resistant to analysis by simple chromatographic methods. Nevertheless, using HPLC, we were able to separate several of the most mass-abundant PC oligomers (dimer through pentamer) found in each habitat type and to make comparisons among them. We then used mass spectrometric detection to build occurrence profiles for each oligomeric form for all study trees. Next, we used a chemical degradation to convert the chemically complex PCs to their component monomers and compared these among populations. Finally, we used HPLC with known standards and mass and nuclear magnetic resonance (NMR) spectroscopy to determine the chemical structures of the monomeric forms.

HPLC analyses

Analyses of soluble PCs and their degradation products were carried out by high-performance liquid chromatography (HPLC) using a Hitachi LaChrom Elite system (Hitachi High Technologies America, Pleasanton, CA) equipped with a photodiode array (PDA) detector (L-2455) configured in tandem with a SEDEX 75 evaporative light-scattering (ELS) detector (S.E.D.E.R.E, Alfortville, France). Electro-spray ionization mass spectrometry (ESIMS) was performed using an LCQ Fleet ion trap spectrometer (Thermo Scientific, Waltham, MA) with sample delivery effected through an Agilent 1100 HPLC

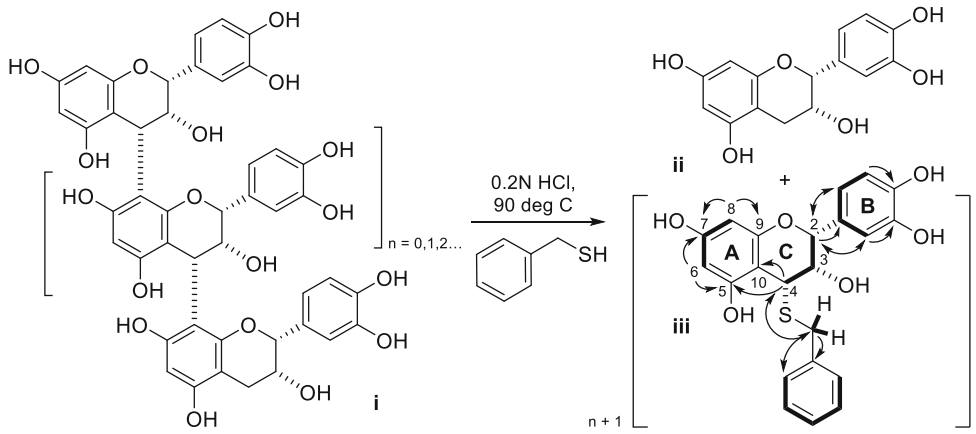

Fig. 1 (*i*) The structure of 4 → 8 linked poly-(epi)catechin (procyanidin). During the acid-catalyzed depolymerization of procyanidin in the presence of toluene-α-thiol, the lower (terminal) unit is released as an unmodified (epi)catechin (*ii*); the upper (extension) unit(s) are released as (epi)catechin-4-*S*-benzyl ethers. *Protium subserratum* thioethers (*iii*) were analyzed by nuclear magnetic resonance spectroscopy. Scalar-coupled ^{1}H–^{1}H linkage groups are shown by bold bonds; pertinent long-range ^{1}H–^{13}C correlations are shown with *arrows*. The stereochemistry at position **C**3 determines whether the monomeric subunit is catechin (3*S*) or epicatechin (3*R*). The stereochemistry at **C**3 is inferred from the magnitude of the coupling constant between H–**C**2 and H–**C**3. Refer to text for details

system (Agilent Technologies Inc., Santa Clara, CA). High-resolution mass spectra were recorded on Thermo Orbitrap Elite mass spectrometer (Thermo Scientific). NMR spectra were acquired using a Varian Inova 500 MHz spectrometer (Agilent) equipped with a z-gradient, indirect detection probe.

The PC-containing 70 % acetone LH-20 fractions were analyzed by HPLC using PDA/ELS and ESIMS detection. The solvent flow rate was 250 μL/min for all analyses. For the PC fraction, 1 mg of each sample was dissolved into 50 μL of a standard solution of 1:1 (v/v) methanol/aq 0.1 % formic acid containing 0.5 μg/μL (wt/v) each of chlorogenic and 2-OH-cinnamic acids as internal standards to correct for among-run variations in retention times. HPLC was carried out using an Atlantis T3 2 × 150 mm 3 μ ODS column (Waters Corp., Milford, MA). The injection volume for the PDA/ELS analyses was 5 μL; for ESIMS analyses, injection volumes were typically 2–4 μL. HPLC solvents were acetonitrile (**A**) and aq 0.1 % formic acid (**B**). HPLC run conditions were as follows: at 0 min, 5 % **A** in **B** followed by a linear gradient to 15 % **A** in **B** over 40 min and then an isocratic step to 63 min. ESIMS detection was in the positive-ion mode. These data were mass filtered using Excalibur software (Thermo Scientific) to provide a profile of each sample's oligomeric composition from dimers through pentamers.

For the thiolysis products analysis, a 2 × 250 mm 5 μ Pursuit XRs ODS column (Agilent) was used. Two μL injections were made directly from the sample vial reaction vessel. HPLC solvents were methanol (**A**) and aq 0.1 % formic acid (**B**). HPLC run conditions were as follows: at 0 min, 32 % **A** in **B** followed by an isocratic step to 1 min, a linear gradient to 45 % **A** in **B** at 2 min, and a linear gradient to 100 % **A** at 30 min. The relative mass abundances of given metabolites were determined by integration of ELS detector traces. ESIMS detection was in the negative-ion mode.

Thiolytic degradation of procyanidins

Acid-catalyzed degradation of PCs in the presence of toluene-α-thiol (Fig. 1) converts polymer-extension units to their respective flavan-4-*S*-toluene thioethers and frees terminal units as a simple monomers (Thompson et al. 1972). Stereochemically complex mixtures of polymers are thereby converted to a simple set of component parts. To carry this out, two mg of each sample's LH-20 acetone (PC) fraction was transferred to a 300-μL sample vial and dissolved into 125 μL of a 1:19 (v/v) toluene-α-thiol/methanol solution. Once dissolved, 125 μL of methanolic 0.2 N HCl was added, the solution was homogenized, and the vial was sealed. Each sample was immersed in a 95 °C water bath for three min and then rapidly cooled. Following centrifugation, samples were directly analyzed by HPLC.

Structural characterization of thiolysis products

Once ESIMS analysis of thiolysis products had provided the masses of the molecular ions of individual components, their chemical identities were determined in one of two ways. For the free monomers, comparisons based on molecular weight and retention time were made to authentic standards. For the thioethers, NMR spectrometric analysis of the purified compounds was required. Large-scale isolation of the thioethers was carried out as follows. Two gram of pulverized leaf material from a leaf sample known to contain the analytes of interest was extracted 3× with 100 mL of 4:1 (v/v) ethanol/aq 0.5 % acetic acid. After defatting, the aqueous extract was mixed with 400 mL of water-equilibrated LH-20 and washed with 1 L each of water and ethanol through a Büchner funnel. A final elution with 1 L of 7:3 (v/v) acetone/aq 0.5 % acetic acid isolated the PCs. This fraction was dried under a stream of nitrogen at 35 °C to yield 482 mg of a dark red powder. One hundred mg of this material was transferred to a 20-mL glass vial, dissolved in 5 mL each of the 1:19 toluene-α-thiol/methanol and methanolic 0.2 N HCl solutions described above. The vial was sealed under nitrogen, immersed in a 95 °C water bath for three min, and then rapidly cooled. The 10-mL reaction mixture was diluted to 100 mL with water. The resulting milky solution was applied to a water-equilibrated, 2 × 25 cm ODS column. The column was washed with sufficient water (150 mL) to raise the eluant pH to 5.5. The thioethers were eluted with 100 mL of methanol. This solution was dried at low pressure, redissolved in acetone, and then dried again. (Following the second drying, toluene-α-thiol was not detectable by HPLC.) Thioethers were transferred to a sample vial and dried under a stream of nitrogen at 35 °C to give 117 mg of a glassy solid. This material was redissolved in 500 μL of 1:1 methanol/aq 0.1 % formic acid. Purifications were carried out using an Atlantis T3 4.6 × 150 mm 3 μ ODS column (Waters) at a flow rate of 1 mL/min. The solvent system consisted of pre-mixed 32:68 (v/v) acetonitrile/aq 0.1 % formic acid. The injection volume was five μL. Separation was effected in a single 12 min isocratic step, with the two major thioethers eluting at 10.75 and 11.5 min, respectively. The eluate from multiple runs was collected by a fraction collector; like fractions were pooled and concentrated at low pressure. After residual solvent was evaporated under a stream of nitrogen, each fraction was dried at 10^{-2} torr for 24 h. This yielded 6 and 50 mg, respectively, of the two major thioethers. Each was redissolved in 700 μL of acetone-d_6 and submitted to NMR spectrometric analysis. The

following spectra were acquired for both samples: ^{1}H 1D; double-quantum-filtered, ^{1}H-^{1}H correlation (dqfCOSY); single-quantum-filtered, single-bond ^{1}H-^{13}C correlation (HSQC); and multiple-bond ^{1}H–^{13}C correlation (HMBC). NMR shifts were measured relative to tetra-methyl silane (0.0 ppm). Finally, each thioether was submitted for high-resolution mass spectrometric analysis.

Protein precipitation assay

The protein precipitation efficiency of PCs was estimated using a standard solution of bovine serum albumin (Hagerman and Butler 1978). Solutions ranging from 0.25 to 2 mg/mL were prepared using PCs from three juvenile and three adult individuals from WS and BS habitats. Five-point binding curves were generated following solubilization and conversion of bound PCs to iron-phenolate ions. Each solution's 510 nm absorbance served as a proxy for the relative quantity of PC-precipitated protein. The slopes of the linear portions of the binding curves provided a measure of the relative protein affinity of each sample. In all cases, binding curves had r^2 values >0.99.

Statistical analyses

We compared the relative mass percent of polymer terminal and extension units among habitat types (brown sand, CS, or white sand) using linear mixed effects models and Tukey post hoc comparisons. Habitat type was the independent fixed factor variable in each model, and source population was a random effect variable to account for potential non-independence of individuals at the same sampling site. The absolute mass investment in defense was compared between adults and juveniles within habitat type using similar models, where life stage was the independent predictor variable and source population was a random factor. All analyses were performed with the R statistical programming language (R_Development_Core_Team 2013) and the *lme4*, *lmerTest* (Bates et al. 2014), and *multcomp* (Hothorn et al. 2008) packages.

Results

Protium subserratum procyanidin content

In *P. subserratum*, the average mass investment in soluble PCs across all habitat types and plant age groups was 17.0 % (±1.2 SE) of leaf dry weight (LDW). Leaves from *P. subserratum* trees growing in BS, CS, and WS habitats all contain approximately the same mass investment in PCs (14.7 % ±1.5, 14.9 % ±3.5, and 19.6 ± 1.8, respectively). There was significantly greater investment in PCs in WS habitats compared to BS and CS habitats ($p = 0.04$). In addition to accumulating to higher concentrations, PCs from WS habitats showed markedly different chromatographic behavior compared to BS and CS habitats (Fig. 2).

PC oligomer composition

From HPLC–ESIMS analysis, a total of 33 oligomers were chromatographically resolvable, 12 from BS/CS and 21 from WS ecotypes, respectively (Fig. 3). Despite optimized source voltages, it is possible that the presence of some oligomers was the result of in-source fragmentation

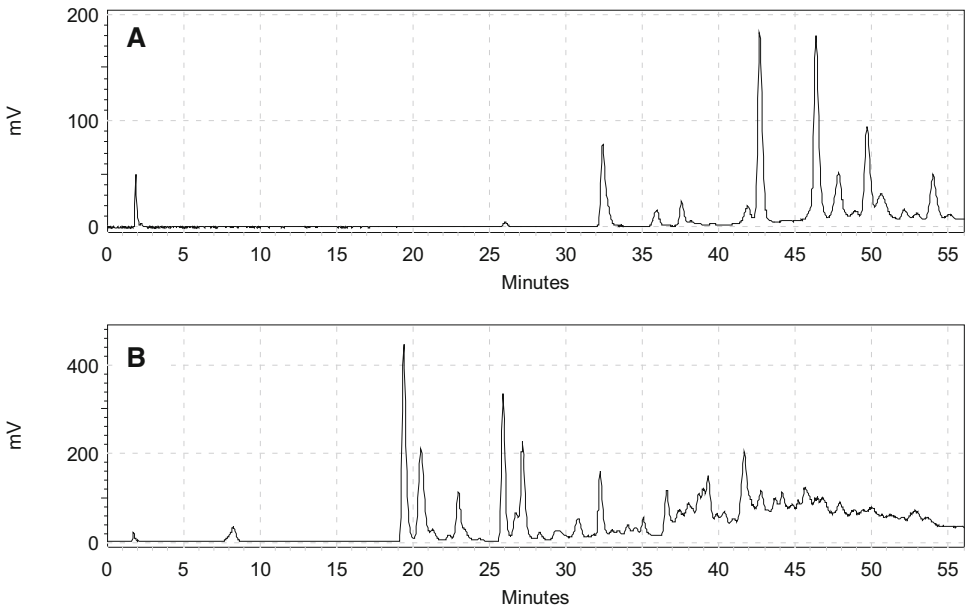

Fig. 2 Representative HPLC chromatograms of *P. subserratum* procyanidins (PCs) from an evaporative light-scattering detector. PCs from brown-sand/clay soil (**a**) and white-sand (**b**) ecotypes are shown in the *upper* and *lower panels*, respectively. Minutes from time of injection are shown on the *x*-axes. Detector response (instantaneous relative abundance) is shown in mV on the *y*-axes. Chromatographic conditions were identical for both analyses

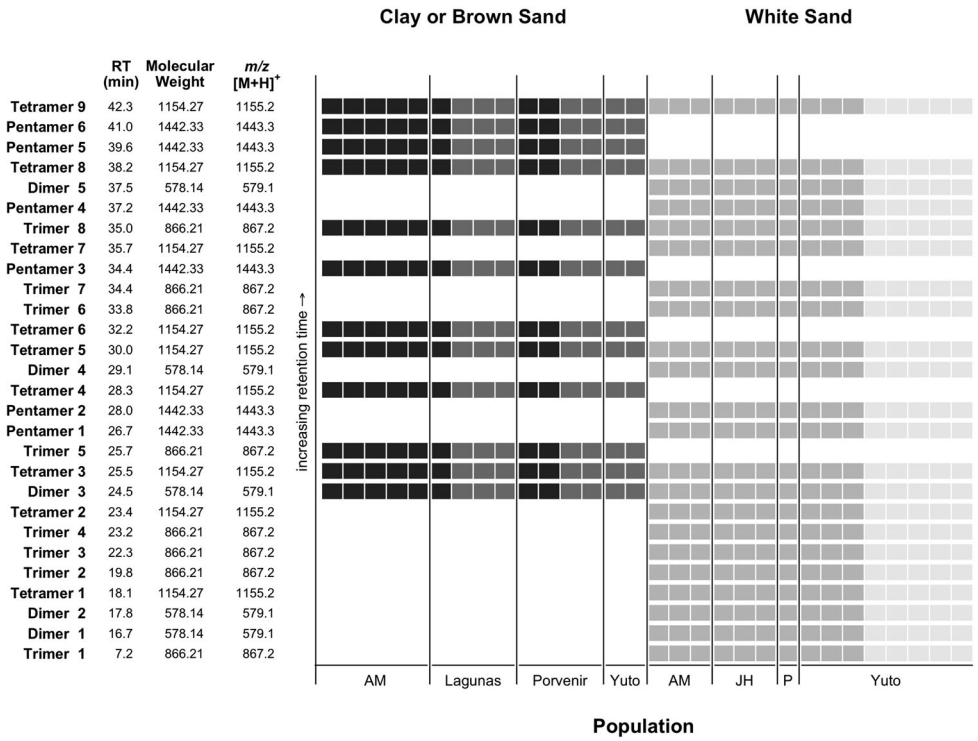

Fig. 3 *Protium subserratum* procyanidin oligomer occurrence profiles from HPLC–ESIMS analyses of leaves from each sampled tree, organized by ecotype (clay and brown sand on *left*; white sand on *right*), source population (*AM* Allpahuayo-Mishiani, *JH* Jenaro Herrera, *P* Porvenir), and life stage (adults in *dark shades*, juveniles in *light shades*). Oligomers on the *y*-axis are arranged in order of increasing chromatographic retention time

of higher order polymers. This analysis does not prove the presence of each oligomeric form in leaf tissue. Nevertheless, the oligomer occurrence profiles generated from this analysis were very useful for comparing *P. subserratum* PCs. Within habitat types, oligomer occurrence profiles were universal; between habitat types, overlap was limited to six oligomeric forms such that six were unique to BS/CS and 15 to WS ecotypes, respectively.

PC monomeric subunit composition

PC terminal units for all habitat types were overwhelmingly of a single monomeric type (Fig. 4). Using authentic standards (Sigma-Aldrich, St. Louis, MO), these were determined by HPLC–ESIMS to be epicatechin for BS and CS habitat samples, and catechin for WS habitat samples. ELS detection showed that a single type of extension unit comprised, on average, more than 98 % of the ELS-detected mass in samples from BS and CS habitats and 92 % in WS habitat samples (Fig. 4). However, a second and third type of extension unit (average 4.4 and 0.2 %) was observed in WS samples. Negative-ion ESIMS detection showed that the thioether derivatives of all three extension units gave nominal $[M–H]^-$ ions of *m/z* 411, consistent with (epi)catechin-type thioethers (Lokvam and Kursar 2005).The chemical structures of the two most abundant thioethers were determined after large-scale purification of each followed by high-resolution mass and NMR spectrometric analyses. The former gave $M + H^+$ ions of $m/z = 413.1053$ and 413.1059 μ, respectively, indicating a molecular formula of $C_{22}H_{21}O_6S$ (0.9874 and 1.2973 ppm error, respectively). NMR data from a COSY experiment (Fig. 1, iii) were interpreted with the help of 1D ^1H and phase-sensitive HSQC spectra. They showed five scalar-coupling networks, three of which were part of aromatic systems. One system included two meta-coupled protons and a second consisted of three protons, two meta-coupled, and two ortho-coupled. Integration of the 1D spectrum showed that the third aromatic system included a pair of double-intensity signals and one single-intensity signal, indicating a mono-substituted benzene ring. The remaining two spin systems comprised sp^3-hybridized centers, one with three protons, including two vicinal oxymethines; the other a methylene. An HMBC experiment provided the long-range $^1H–^{13}C$ correlations for the assembly of flavan-3-ol ring systems, both substituted at the C-4 position (Fig. 1, iii). This substitution pattern was consistent with linearly assembled procyanidin forms. To determine the stereochemistry at the critical C3 carbon, the coupling constants of the C2 protons were extracted from the 1D ^1H spectrum of both thioethers. For the sole thioether that was observed in samples from BS and CS habitats and for the dominant thioether from WS habitat samples, the C2 proton occurred as a broad singlet at 5.25 ppm. Its coupling to the C3 proton, apparent from the COSY spectrum, was not resolved in the 1D spectrum. This result indicated

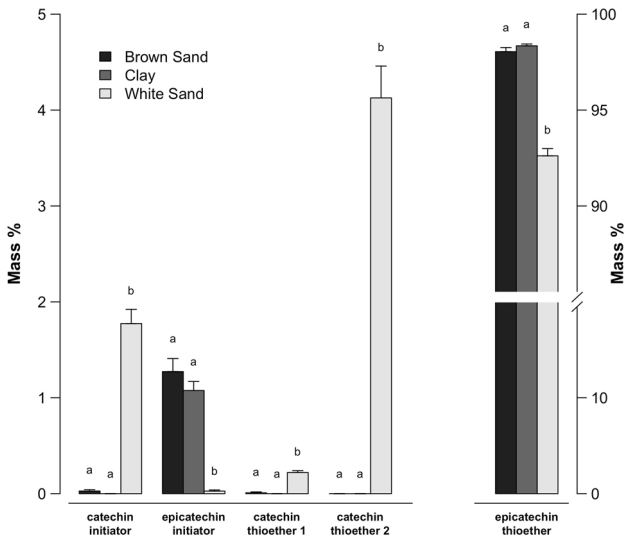

Fig. 4 Thiolytic degradation of PCs revealed that polymer initiator and extension units varied by ecotype of *P. subserratum.* Bins represent the mean mass percent across trees (with *standard error bars*). Brown sand/clay soil ecotypes had almost exclusively epicatechin initiators; white sand ecotypes almost exclusively catechin initiators. Epicatechin was the dominant extension unit in all ecotypes but catechin comprised approximately 4% of the extension units in white sand ecotypes. Models were run separately for each molecule; *small letters* above each group of bins indicate statistically significant differences from post hoc Tukey tests Fig. 5. Leaf-dry-weight mass percent of soluble procyanidins by habitat type and life stage. Significant differences between life stages within each habitat detected are indicated with an *asterisk*

epicatechin (*cis*-2-3) stereochemistry. For the additional thioether that was observed in WS habitat samples, the C2 proton appeared as a doublet at 4.95 ppm with a coupling constant of 9.8 Hz, indicating catechin (*trans*-2-3) stereochemistry. The presence of the third, low-abundance thioether in WS samples is consistent with the kinetics of thioether formation from catechin extension units where two types of catechin thioether, one major and one minor, are observed (Thompson et al. 1972). In summary, BS and CS habitat PCs have almost entirely epicatechin as chain termini and epicatechin as chain extension units, while WS habitat PCs have almost entirely catechin as chain termini and both catechin and epicatechin as chain extension units (Fig. 4).

Estimates of relative PC polymer length were obtained using HPLC with ELS detection. ELS traces of the thiolysis products were integrated, and the ratio of extension units to termini for was calculated for each sample. Significant differences in relative polymer length were found between both BS and CS vs. WS habitat types ($p < 0.01$ in both cases, Fig. 4). Among all samples grouped by habitat type, this ratio was 52 for WS habitat samples ($N = 16$), 82 for BS habitat samples ($N = 10$), and 93 for CS habitat samples ($N = 4$). The difference between BS and CS samples was not significant.

Protein precipitation

The protein precipitation efficiency of leaf PCs from juvenile and adult trees from WS and BS habitats was measured using a standard bovine serum albumin solution. While all PCs had a marked ability to precipitate protein, no significant differences were observed in overall efficiency, either between age classes within habitat types or between habitat types.

Qualitative and quantitative differences within ecotypes: juveniles versus adults

Although WS and BS/CS ecotypes presented many qualitative differences (discussed above), we found almost no qualitative differences *within* ecotypes, either with respect to ontogenetic stage or geographic location (Fig. 5). However, adult trees of BS/CS ecotypes expressed significantly more mass investment of polymeric flavans than did juveniles in that habitat (Fig. 5).

Discussion

We found that although all *P. subserratum* ecotypes accumulate considerable quantities of PCs, the chemical structures of PCs being expressed by WS ecotypes differ markedly from those found in BS and CS ecotypes. These results significantly extend our understanding of *P. subserratum* secondary metabolite chemistry reported recently (Fine et al. 2013a, b). In our previous work, we described marked differences in flavones, quinic acid derivatives, and oxidized terpenes between WS and BS ecotypes but similar levels of soluble PC accumulation. Here, we demonstrate that across habitats, differences in PC chemistry are likewise pronounced. Using a chemical degradation, we found that the terminal units of WS PCs to be almost entirely catechin, while for BS and CS PCs, it was almost entirely epicatechin. Moreover, in BS/CS habitat types, there was a single type of extension unit. This type also predominated in WS habitats but approximately 5 % of the extension units consisted of a second type. Using NMR spectroscopic analyses, we found the predominant form in all habitats to be epicatechin and the minor form in WS habitats to be catechin. The fact that very small amounts of the minor termini were observed in all habitats, and that small amounts of the minor extension unit were observed in BS habitats indicates that genetic variation to express both of these forms is widespread and that the observed patterns of accumulation are likely a response to selection.

The chemical degradation we used also provided information on the chromatographically refractory high molecular weight polymeric forms. We found that in

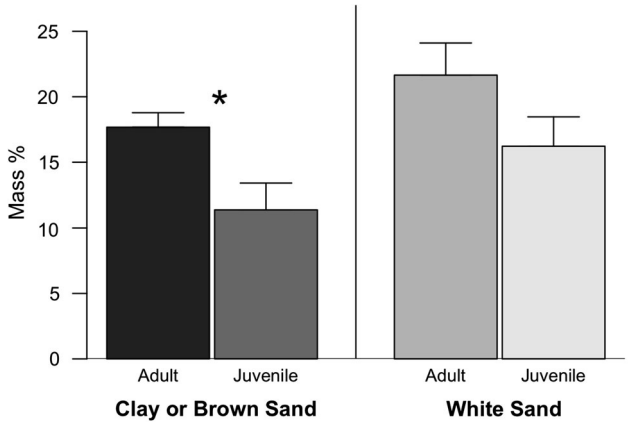

Fig. 5 Leaf-dry-weight mass percent of soluble procyanidins by habitat type and life stage. Significant differences between life stages within each habitat detected are indicated with an *asterisk*

addition to the contrast in monomeric composition between WS and BS/CS habitats, differences in the ratios of their extension unit-to-terminal units were highly significant. BS PC polymers are, on average, more than 50 % longer than those found in WS leaves. PC protein precipitation efficiency, however, did not differ among habitats. This is surprising in that precipitation efficiency has repeatedly been shown to correlate with polymer length (Kumar and Horigome 1986; Horigome and Okamoto 1988; Lokvam and Kursar 2005; Sugiyama et al. 2007). But these previous studies did not take into account the interaction between subunit chemical structure and polymer length with respect to protein binding. If the bioactivity of *P. subserratum* PCs does occur through protein precipitation, our data are consistent with a scenario where, in the BS lineage, selection for increased polymer length has acted in concert with selection for a shift in subunit composition such that protein precipitation efficiency is maintained.

But what factors drive the selection of such strong qualitative differences in PC subunit composition between WS and BS/CS ecotypes? We speculate that catechin- versus epicatechin-dominant PC assemblages have different (and as yet unknown) anti-herbivore function that may deter different species of herbivores. In a year of censusing insect herbivores on both WS and BS populations of *P. subserratum* juvenile plants, we found very little overlap in herbivore assemblages (Fine et al. 2013a, b). Chrysomelid beetles and cicadellid hemipterans dominated the assemblages on plants of both ecotypes, but different species were collected from the two different soil types. Chrysomelids and cicadellids are known to have neutral to acidic gut pH's (Knowles 1994; Chougule and Bonning 2012). In this pH range, PC-protein binding is kinetically favored and therefore could represent one possible defense mechanism against these herbivores. Notably, the neotropical tree genus *Inga* is also defended by PCs. At many sites, it is most heavily damaged by lepidopteran herbivores (Koptur 1985). Lepidopterans tend to have very high gut pH values as well as surfactant properties (Martin and Martin 1984; Dow 1992) that inhibit PC–protein complexation. This fact may explain why *Inga* species tend to have far higher leaf PC concentrations (Lokvam and Kursar 2005) than *P. subserratum.* Future work using feeding trials of captured herbivores could shed light on the particular effectiveness of different habitat-specific PC types against different species of herbivorous insects.

Our findings are largely congruent with those from a recent fine-scale microsatellite genetic study of several Peruvian *P. subserratum* populations that showed that WS and BS/CS ecotypes exhibit consistently different leaf morphologies and only experience gene flow extremely rarely, even when reproductive adults are growing in close proximity and flowering simultaneously (Misiewicz and Fine 2014). However, this study also showed some significant morphological and genetic differentiation between populations from BS vs. CS, while in our study, we found no qualitative or quantitative differences in PC chemistry between BS and CS individuals (Figs. 3, 4). Interestingly, a phylogeographic study of Peruvian WS, BS, and CS ecotypes showed no variation in haplotypes between BS and CS individuals (Fine et al. 2013b). Microsatellite studies are thought to reflect much more recent signals of population-level divergence than sequence data (Misiewicz and Fine 2014). If clay and BS ecotypes are experiencing incipient speciation, it does not appear to be related to their investment in PCs.

We found no evidence that within habitats, different life stages expressed qualitatively different PCs. This was surprising, as PCs are well known to deter insect herbivores, and most studies that have compared canopy and understory insect herbivores have reported very different assemblages (Basset et al. 1999). However, the canopy-level herbivores of *P. subserratum* have not been studied, and it is certainly possible that the same enemies attack these trees in juvenile and adult stages. Barone (2000) studied adults and seedling trees of two species in Panama and found very similar arthropod faunas, suggesting that for some trees, the same species of insect herbivores may attack both juvenile and adults. Future canopy fogging studies will be critical to understand the patterns of PC investment in these trees and how it relates to insect assemblages across different life stages. A second possibility is that PC expression is under selection by natural enemies at all life stages, but the plant experiences some sort of developmental constraint such that it cannot change its PC chemistry at different life stages. If this is the case, PC investment may represent an integrated and somewhat generalized defense that works across ontogenetic stages but is also very habitat-specific given the strong structural differences between WS and BS/clay ecotypes.

We did find significant differences in the amount of PCs between adult and juveniles in BS/clay ecotypes but not in WS ecotypes. For BS/clay ecotypes, adults expressed significantly more PCs than juveniles, which is opposite to the predictions of Boege and Marquis (2005) who proposed that juveniles should be under much stronger selection to produce defenses than adults given the relatively higher value of their leaves. Instead, our results are more consistent with the growth differentiation defense hypothesis (Herms and Mattson 1992) which posits that chemical defense investment is resource limited, and thus, adult plants with more access to light should have more resources to allocate to defenses. A recent meta-analysis showed that investment in constitutive chemical defenses, like PCs in woody plants, is often higher in adults than in juveniles, although there was large variation among sample studies and a wide variety of chemical defenses analyzed (Barton and Koricheva 2010). Future studies comparing metabolite investment across ontogeny in *P. subserratum* should study seedlings and also quantify investment in non-phenolic defense compounds.

Our study provides detailed characterization of PC investment in three ecotypes of *P. subserratum* that are undergoing lineage divergence. Among ecotypes, we found consistent differences in PC subunit composition between WS and BS/clay ecotypes. Within ecotypes, we found almost no variation across individuals, whether in different geographic locations or at different ontogenetic stages. For BS and clay ecotypes, adults accumulated significantly more PCs in their leaves than did juveniles, consistent with the growth differentiation defense hypothesis. The pronounced qualitative differences that we found among ecotypes in phenolics match our previous study that reported differences in flavones, oxidized terpenes, and quinic acid derivatives (Fine et al. 2013a). These chemical differences in PCs, the most mass-abundant group of anti-herbivore chemicals that the plants produce, are consistent with findings of the low levels of gene flow among populations from WS and BS/CS types as well as with non-overlapping insect herbivore faunas. We conclude that the phenotypic differences in phenolics in the WS versus BS and clay ecotypes of *P. subserratum* are consistent with a hypothesis of selection by herbivores in different soil types and may contribute to the evolution of habitat specialization by these trees.

Acknowledgments We thank the Ministerio del Ambiente of Peru for providing research and export permits. We thank Carlos Rivera of SERNANP-Allpahuayo-Mishana and the Instituto de Investigaciones de la Amazonia Peruana (IIAP) for institutional and logistical support. We would like to thank Italo Mesones, Magno Vásquez Pilco, J. Milagros Ayarza Zuñiga, Julio Sanchez for field assistance, and Leslie Harden for high-resolution mass spectrometry. We also thank Diego Salazar Amoretti for helpful suggestions to the manuscript. Funding was provided by NSF DEB 1254214 to PVAF.

Conflict of interest The authors declare they have no conflict of interest.

References

Barbehenn RV, Constabel CP (2011) Tannins in plant-herbivore interactions. Phytochemistry 72:1551–1565. doi:10.1016/j.phytochem.2011.01.040

Barbehenn RV, Jones CP, Karonen M, Salminen JP (2006) Tannin composition affects the oxidative activities of tree leaves. J Chem Ecol 32:2235–2251. doi:10.1007/s10886-006-9142-8

Barone JA (2000) Comparison of herbivores and herbivory in the canopy and understory for two tropical tree species. Biotropica 32:307–317. doi:10.1111/j.1744-7429.2000.tb00474.x

Barton KE, Koricheva J (2010) The ontogeny of plant defense and herbivory: characterizing general patterns using meta-analysis. Am Nat 175:481–493. doi:10.1086/650722

Basset Y, Charles EC, Novotny V (1999) Insect herbivores on parent trees and conspecific seedlings in a rain forest in Guyana. Selbyana 20:146–158

Bates D, Maechler M, Bolker B, Walker S (2014) Ime4: Linear mixed-effects models using Eigen and S4. http://CRAN.R-project.org/package=Ime4

Boege K, Marquis RJ (2005) Facing herbivory as you grow up: the ontogeny of resistance in plants. Trends Ecol Evol 20:441–448. doi:10.1016/j.tree.2005.05.001

Chougule NP, Bonning BC (2012) Toxins for transgenic resistance to hemipteran pests. Toxins 4:405–429. doi:10.3390/toxins4060405

Coley PD, Barone JA (1996) Herbivory and plant defenses in tropical forests. Annu Rev Ecol Syst 27:305–335

Coley PD, Bryant JP, Chapin FS (1985) Resource availability and plant anti-herbivore defense. Science 230:895–899

Dow JAT (1992) pH gradients in lepidopteran midgut. J Exp Biol 172:355–375

Feeny PR (1969) Inhibitory effect of oak leaf tannins on the hydrolysis of proteins by trypsin. Phytochemistry 8:2119–2126

Fine PVA, Miller ZJ, Mesones I, Irazuzta S, Appel HM, Stevens MHH, Saaksjarvi I, Schultz LC, Coley PD (2006) The growth-defense trade-off and habitat specialization by plants in Amazonian forests. Ecology 87:S150–S162

Fine PVA, Garcia-Villacorta R, Pitman N, Mesones I, Kembrel SW (2010) Herbivores promote habitat specialization by trees in Amazonian forests. Ann MS Bot G 97:283–305

Fine PVA, Metz MR, Lokvam J, Mesones I, Milagros ZAJ, Lamarre GPA, Vásquez PM, Baraloto C (2013a) Insect herbivory, chemical innovation and the evolution of habitat specialization in Amazonian trees. Ecology 94:1764–1765

Fine PVA, Zapata F, Daly DC, Mesones I, Misiewicz TM, Cooper HF, Barbosa CEA (2013b) The importance of environmental heterogeneity and spatial distance in generating phylogeographic structure in edaphic specialist and generalist tree species of *Protium* (Burseraceae) across the Amazon Basin. J Biogeogr 40:646–661. doi:10.1111/j.1365-2699.2011.02645.x

Gentry AH (1986) Endemism in tropical versus temperate plant communities. In: Soulé ME (ed) Conservation biology: the science of scarcity and diversity. Sinauer, Sunderland, MA, pp 153–181

Goodger JQD, Choo TYS, Woodrow IE (2007) Ontogenetic and temporal trajectories of chemical defence in a cyanogenic eucalypt. Oecologia 153:799–808. doi:10.1007/s00442-007-0787-y

Hagerman AE, Butler LG (1978) Protein precipitation method for the quantitative determination of tannins. J Agric Food Chem 26:809–812

Herms DA, Mattson WJ (1992) The dilemma of plants: to grow or defend. Q Rev Biol 67:283–335. doi:10.1086/417659

Horigome T, Kumar R, Okamoto K (1988) Effects of condensed tannins prepared from fodder plant on digestive enzymes in vitro and in the intestine of rats. Br J Nutr 60:275–285

Hothorn T, Bretz F, Westfall P (2008) Simultaneous inference in general parametric models. Biom J 50:346–363

Janzen DH (1974) Tropical black water rivers, animals and mast fruiting by the Dipterocarpaceae. Biotropica 6:69–103. doi:10.2307/2989823

Knowles BH (1994) Mechanism of action of *Bacillus thuringiensis* insecticidal delta-endotoxins. Adv Ins Phys 24(24):275–308. doi:10.1016/s0065-2806(08)60085-5

Koptur S (1985) Alternative defenses against herbivores in *Inga* (Fabaceae: Mimosoideae) over an elevational gradient. Ecology 66:1639–1650

Kumar R, Horigome T (1986) Fractionation, characterization and protein-precipitating capacity of the condensed tannins from *Robinia pseudoacacia* L. leaves. J Agric Food Chem 34:487–489

Lokvam J, Kursar TA (2005) Divergence in structure and activity of phenolic defenses in two co-occurring *Inga* species. J Chem Ecol 31:2563–2580

Martin MM, Martin JS (1984) Surfactants—their role in preventing the precipitation of proteins by tannins in insect guts. Oecologia 61:342–345. doi:10.1007/bf00379632

Misiewicz TM, Fine PVA (2014) Evidence for ecological divergence across a mosaic of soil types in an Amazonian tropical tree: *Protium subserratum* (Burseraceae). Mol Ecol 23:2543–2558. doi:10.1111/mec.12746

R_Development_Core_Team (2013) R: A language and environment for statistical computing. Computing RFfS, Vienna, Austria

Ruokolainen K, Tuomisto H (1998) Tropical forests are not flat: how mountains affect herbivore diversity. Ecol Let 13:1348–1357

Salminen JP, Karonen M (2011) Chemical ecology of tannins and other phenolics: we need a change in approach. Funct Ecol 25:325–338. doi:10.1111/j.1365-2435.2010.01826.x

Strumeyer DH, Malin MJ (1975) Condensed tannins in grain-sorghum—isolation, fractionation, and characterization. J Agric Food Chem 23:909–914. doi:10.1021/jf60201a019

Sugiyama H, Akazome Y, Shoji T, Yamaguchi A, Yasue M, Kanda T, Ohtake Y (2007) Oligomeric procyanidins in apple polyphenol are main active components for inhibition of pancreatic lipase and triglyceride absorption. J Agric Food Chem 55:4604–4609. doi:10.1021/jf070569k

Swain T (1979) Tannins and lignins. In: Rosenthal GA, Janzen DH (eds) Herbivores: their interactions with secondary plant metabolites. Academic Press, New York, pp 657–682

Thompson RS, Jacques D, Haslam E, Tanner RJN (1972) Plant proanthocyanidins. Part 1. Introduction; the isolation, structure, and distribution in nature of plant procyanidins. J Chem Soc Perk Trans 1:1387–1399