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Differentiating temperate tree species and their organs using lipid biomarkers in leaves, roots and soil

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ABSTRACT

Although fatty acids and alcohols in plant polyesters can be useful indicators of organic matter provenance in soils and sediments, interpretation is limited by uncertainty in: (i) the distribution of lipids among plant species and their organs (e.g. leaves and roots) and (ii) the extent to which plant lipid composition is recorded in soils and sediments. In this study, we compare lipids in leaves, roots and soils from 11 temperate tree species. Base hydrolysis was used to release ester-bound lipids and solvent extraction was then used to recover both hydrolysable and "free" lipids. Leaf and root lipid composition varied substantially among the tree species and we highlight differences among evergreen conifers, deciduous broadleaved angiosperms and a deciduous conifer (*Larix decidua*). Some of the variation appears to be linked to the morphology and lifespan of leaves and roots. Soils preserved much of the variation in the leaf and root lipid composition of the overlying tree species. Yet, the concentration of some lipids in soil diverged from their concentration in tree leaves and roots, reflecting an undocumented input from understory plants and other plant organs (e.g. seeds) or variation in the extent of lipid preservation in soil. Finally, by comparing leaf and root lipid composition, our results help constrain the attribution of lipids to each of these plant organs. This allowed us to evaluate the utility of leaf-derived lipids as plant type biomarkers and to document a substantial contribution of root-derived lipids to soil beneath all 11 tree species.

1. Introduction

It is widely accepted that much of the organic matter (OM) in soils and some sediments is derived from plant litter, but more specific information on it is needed to understand the process of its stabilization and to reconstruct the contribution of different plant types and plant organs to the OM (Pancost and Boot, 2004; Medeiros and Simoneit, 2007; Amelung et al., 2008; Simpson et al., 2008). Plant lipids are well preserved in soil (Nierop, 1998; Rumpel et al., 2002; Nierop et al., 2003; Otto et al., 2005a) and sediments (Eglinton et al., 1968; Gough et al., 1993; Kastner and Goni, 2003; Ishiwatari et al., 2005) and some are exclusively or predominately associated with certain plant types, taxa or organs (e.g. leaves and roots; Hedges and Mann, 1979; Goni and Hedges, 1990b; Nierop, 1998). However, these biomarker associations were established with a relatively limited survey of plant species and organs. More extensive information on the distribution of lipid biomarkers among plant species and organs is therefore required for robust interpretation of their abundance in soils and sediments.

The lipid composition of plant roots is particularly understudied. This is a significant knowledge gap for two reasons. First, mortality of fine roots, i.e. those of <1–2 mm in diameter, results in a flux of plant carbon to soil that rivals the carbon flux through leaf litterfall (Fahey et al., 2005). Indeed, some authors have hypothesized that most soil OM (SOM) is derived from plant roots (Rasse et al., 2005). Second, plant type biomarkers established by analysis of above ground organs could yield faulty conclusions if these biomarkers are present in roots, but differently distributed among species. For example, Goni and Hedges (1990b) showed leaves of angiosperms and different gymnosperm plant families could be distinguished by the abundance of 14-hydroxytetradecanoic acid and positional isomers of dihydroxyhexadecanoic acid. Studies of a few temperate tree species (Matzke and Riederer, 1991; Nierop, 2001; Nierop and Verstraten, 2003) showed these potential biomarkers to be absent from roots. Yet, roots are known to contain similar compounds (Bernards, 2002; Graca and Santos, 2007), so analysis of a greater diversity of species is needed to confirm these results.

Lipids in plant leaves and roots are predominantely derived from wax, cutin and suberin. Plant wax is a mixture of 'free' lipids and relatively small, simple polyesters (Gulz, 1994). Cutin and

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suberin are larger, more complex polymers comprised of partially distinct monomers. Roots contain suberin but not cutin, which is found in above ground organs such as leaves, fruits and seeds (Kolattukudy, 2001). Thus, the abundance of cutin-specific and suberin-specific monomers in soil is a potential indicator of contributions from these above ground and below ground plant organs to soils and sediments (Nierop, 1998; Rumpel et al., 2002; Otto and Simpson, 2006). Monomers of wax esters, cutin and suberin also have potential as biomarkers for different plant types and taxa (Hunneman and Eglinton, 1972; Goni and Hedges, 1990b; Nierop and Verstraten, 2003; Blum et al., 2007).

The use of cutin and suberin monomers as biomarkers could be problematic because the attribution of certain biomolecules as being cutin- or suberin-specific is uncertain. Cutin and suberin are comprised of ω-hydroxyacids, α,ω diacids and di- or trihydroxyacids, but the compounds tend to have different chain length and relative abundance in each polymer (Kolattukudy, 2001; Bernards, 2002; Graca and Santos, 2007). Some authors attribute di- and trihydroxyacids to cutin alone (Kolattukudy, 2001; Kögel-Knabner, 2002; Amelung et al., 2008), while others suggest they are present in both polymers (Holloway, 1983; Matzke and Riederer, 1991; Bernards, 2002; Graca and Santos, 2007). Further, some authors agree that only abundant, long chain ω-hydroxyacids and α,ω diacids (e.g. $\geqslant C_{20}$) are diagnostic of suberin (Matzke and Riederer, 1991; Simpson et al., 2008), while others contend shorter chain α,ω diacids (e.g. C_{16} and C_{18}) are also characteristic of suberin (Kolattukudy, 2001; Kögel-Knabner, 2002; Amelung et al., 2008). These different views likely arise, in part, from insufficient knowledge of the differences in cutin and suberin composition among plant taxa. Finally, since much of the biochemical information about cutin and suberin comes from studies of horticultural crop tissues, bark and cork (Matzke and Riederer, 1991; Kolattukudy, 2001), previous findings are not representative of tree leaves or roots and likely have limited geochemical utility.

In order to assess the source specificity of plant lipids and evaluate their preservation in soil, we analyzed the lipids in leaves, roots and soil from monoculture plots of 11 temperate tree species. The plots are at the same site and were planted at the same time, ensuring that the lipid patterns of each tree species could be compared with minimal influence of climate, soil type or other external factors. For the plant and soil samples, we used base hydrolysis to release ester-bound constituents from cutin, suberin and wax esters. Solvent extraction was then used to recover lipids from the hydrolytic solution and sample residue, such that "free" and ester-bound lipids were combined.

2. Methods

2.1. Study site

Samples were from a common garden experiment in central Poland (51°14.87′N, 18°06.35E, altitude 150 m, mean annual temperature 8.2 °C). In 1970 and 1971, 14 tree species were planted in replicated monoculture plots (20 m × 20 m) after clear cutting an 80 yr old *Pinus sylvestris* plantation and plowing. The surface soil is derived from sandy glacial outwash. Following USDA soil taxonomy, soil in each plot was classified as a sandy, mixed, mesic Typic Ustipsamment or a fine-loamy, mixed, mesic Kanhaplic Haplustalf. The proportion of clay ranged from ca. 1% to 10% in the upper 20 cm. This variability in soil texture was likely introduced during plowing, which enriched the clay content of shallow soil layers when the underlying, clay-rich horizons were within plowing depth. For additional details on the common garden experiment see Reich et al. (2005) and Hobbie et al. (2007).

2.2. Sampling

We collected leaf, root and soil samples from two to six replicate plots of 11 species at the common garden, including six deciduous broadleaved angiosperms (*Acer platanoides*, *Acer pseudoplatanus*, *Fagus sylvatica*, *Quercus robur*, *Quercus rubra* and *Tilia cordata*), four evergreen conifers (*Abies alba*, *Pinus nigra*, *P. sylvestris* and *Pseudotsuga menziesii*) and one deciduous conifer (*Larix decidua*). All conifers belong to the family Pinaceae and have needle leaf morphology. In August 2008, the top 20 cm of mineral soil and roots of each plot were sampled by combining five soil cores collected within the inner 100 square m of the plot. Soil was sieved to 2 mm in the field and living fine roots that passed through the sieve were removed. Soil samples and roots were stored in coolers in the field and refrigerated in the lab prior to additional sample processing. Within 10 days of sampling, fine roots (<2 mm diameter) were separated manually from course roots. In August 2009, upper canopy leaves were collected from the same plots using shotguns. Leaf, root and soil samples were oven dried (60 °C) to constant wt. and homogenized using a ball mill.

2.3. Base hydrolysis and lipid extraction

Aliquots (500 mg) of soil samples from each plot ($n = 37$ total plots) were hydrolyzed and then extracted to account for the variability in soil texture. For leaf and root samples, single composite aliquots (10 mg) were hydrolyzed and extracted for each species ($n = 11$ for each plant organ). Composite aliquots were created by mixing equal proportions of material from each replicate plot. Hydrolysis was performed using sealed 50 ml glass centrifuge tubes. Sample aliquots were added along with 0.5 M KOH (5 ml; 5:1 MeOH/water, v/v). The headspace was flushed with N_2 before capping the tubes with Teflon lined lids. Tubes were submerged to the depth of the KOH solution in an 80 °C water bath (for 2 h). During heating, the tubes were removed and vigorously swirled every 30 min. Once cool, they were centrifuged and the KOH solution was transferred to a 60 ml glass vial. The sample pellet was rinsed 2× as follows: 2 ml fresh KOH solution was added to each tubes, which was shaken and centrifuged, and the supernatant was transferred to the 60 ml vial. Aqueous NaCl (25 ml; 5% w/v) was added to each vial and the resulting solution acidified to a pH slightly <2 using 6 M HCl. Lipids were extracted 5× from this solution: 3× using 1:1 hexane/EtOAc (v/v) and 2× using EtOAc. The first extraction was performed using 10 ml of 1:1 hexane/EtOAc which had been used to rinse the sample pellet (in two separate rinse steps of 5 ml each). The extracts were combined, concentrated under N_2, transferred to glass vials and evaporated just to dryness. Then, 500 μl of anhydrous pyridine was added and the vial stored at ca. 2 °C.

We assessed our method of hydrolysis and lipid extraction with recovery standards. Each batch of samples that were hydrolyzed and extracted included one centrifuge tube containing a mixture of 32 standards that was simultaneously subjected to the hydrolysis and extraction procedure. For a total of six of these batch standards, the average recovery of all 32 analytes was 91% (Supplementary Table 1).

2.4. Gas chromatography–mass spectrometry (GC–MS) and quantification

Within 2 weeks of hydrolysis, qualitative and quantitative analyses of the hydrolysis extracts were performed using GC–MS. Acids and alcohols were converted to the trimethylsilyl derivatives by combining aliquots of each lipid sample in pyridine (50 μl), and 10 μl internal standards in pyridine (3,4-dimethoxybenzoic acid and 12-hydroxyoctadecanoic acid) and 50 μl bistrimethylsilyltriflu-

oroacetamide (BSTFA; 99.9%, Sigma–Aldrich) in an autosampler vial and heating for 15–30 min at 60 °C. Within 24 h of derivatization, samples were analyzed using a Hewlett–Packard (HP) 6890 GC instrument coupled to a HP 5973 quadrupole mass spectrometer with electron impact ionization. Each sample was injected using pulsed splitless mode and an inlet temperature of 320 °C. Analytes were separated on a 30 m fused silica column (Agilent J&W DB-5; 0.25 mm, 25 μm) with He as carrier gas (1.5 ml/min). The temperature program was: 100 °C (hold 2 min) to 165 °C at 8°/min, to 260 °C at 3°/min, to 320 °C (hold 10 min) at 10°/min. Ionization energy was 70 eV and the scan range m/z 50–700.

Lipids were assigned by comparing their mass spectra with published spectra (Goni and Hedges, 1990a) and with spectra and retention times for standard compounds. Quantification was based on the most abundant ion in each spectrum that also had compound specificity. Peak areas of extracted target ions were normalized to an internal standard and the concentrations estimated by comparison with normalized peak areas of external standards run at seven different concentrations (from 0.001 μg/μl to 0.06 μg/μl). Following Filley et al. (2008), the majority of analytes were quantified by comparison with standards of the same identity (30 of 55 analytes), while others were quantified by comparison with lipids with similar structures (12 analytes). Finally, 13 analytes were quantified by extrapolation or interpolation using equations fit to the response factors of homologous compounds (Chaurasia et al., 1995). For details of target ions, qualifier ions and quantification approach used for each analyte, see Supplementary Table 1. The coefficient of variation (CV) for the estimated concentration of all 32 external standards ranged from 0.03 to 0.31 (i.e. the standard deviation was 3–31% of the mean; only two standards had CV > 0.1; Supplementary Table 1). The CV for each standard was based on data from an external standard mixture that was run every 10 samples (a total of 8×).

Target analytes included *n*-alcohols, *n*-alkanoic acids, α,ω-diacids, ω-hydroxyacids, and di- and trihydroxyacids. We did not quantify *n*-alkanes or branched lipids, such as *iso*- or *anteiso*-alkanoic acids. Other studies of forest soil have shown that these are generally a minor component of soil lipids (Naafs and van Bergen, 2002; Quénéa et al., 2004; Otto et al., 2005a) and qualitative assessment of the chromatograms suggested the same.

2.5. Composite vs. independent analysis of plant samples

For leaves of one tree species, we compared the lipid concentrations from one composite sample, made up of sub-samples from three different plots, to concentrations from separate analyses of sub-samples from the same three plots. The concentration of individual lipids in the composite sample correlated highly with the mean concentration of the separately analyzed sub-samples (i.e. separately analyzed plots; $P < 0.001$, R^2 0.95). For each lipid, its concentration in the composite sample diverged from the mean concentration of separately analyzed sub-samples by less than one standard deviation (Supplementary Fig. 1).

2.6. Statistical analysis

Statistics were calculated using JMP software (version 7.0.2, copyright SAS Institute Inc.). When necessary, variables were log transformed to improve normality and the transformed values were used. Analysis of variance (ANOVA) and analysis of covariance (ANCOVA) models were used to evaluate the effects of tree species and soil texture on lipid concentration. We used agglomerative hierarchical clustering to group tree species with similar lipid composition and lipids with similar distributions among species. We used the Euclidean distance metric to characterize similarity and Ward's method as the linkage algorithm. All descriptive and comparative statistics were performed on concentrations normalized to the mass of carbon in each sample.

3. Results and discussion

3.1. Methodological considerations

Because there are a variety of methods used to extract and analyze lipids from plants and soils, we begin our discussion with a brief review of methodologic factors that can influence measured lipid composition. Lipids are frequently distinguished on the basis of their "mode of occurrence" by first extracting the 'free' lipids with solvent and then releasing 'bound' lipids through base hydrolysis of the sample residue (Goossens et al., 1989; Otto and Simpson, 2007). In this study, we did not separate free and bound lipids because our primary aim was to assess how the lipid composition of leaves, roots and soils relating to different tree species were distinct (regardless of their mode of occurrence). *n*-Alkanoic acids (hereafter *n*-acids), *n*-alcohols and ω-hydroxyacids are commonly present in both 'free' and 'bound' lipid fractions (Otto and Simpson, 2007); thus we could not identify the mode of occurrence of these lipid groups in our samples. However, di- and tri-hydroxyacids and α,ω diacids with chain length $>C_9$ are generally not found in free lipid fractions (i.e. in solvent extracts; Goossens et al., 1989; Naafs et al., 2004; Otto and Simpson, 2007), unless the solvent extracts are further subjected to base hydrolysis (Quénéa et al., 2004); thus these lipid types were likely present as ester-bound moieties in our samples.

Some methods of OM depolymerization do not yield *n*-alcohols, such as the combined oxidation and hydrolysis that occurs in the presence of CuO and NaOH. This should be taken into consideration for samples where *n*-alcohols may be of interest. Goni and Hedges (1990a) observed substantial oxidation of the terminal OH in ω-hydroxyacid standards, producing α,ω diacids. Assuming terminal OH groups in *n*-alcohols are equally subject to oxidation, *n*-alcohols are likely to be converted to *n*-alkanoic acids during depolymerization with CuO and NaOH. Oxidation may occur due to the presence of oxidant (CuO) and the higher temperature of the reaction (150–170 °C vs. 70–100 °C for traditional base hydrolysis).

Some authors have suggested oxidation may be a source of short chain α,ω diacids and ω OH acids during traditional hydrolysis of plant and soil samples (Nierop et al., 2003). We cannot rule this out in our study, in particular for the C_9 α,ω diacid, which we observed to be abundant in leaves, roots and soil. However, it is unlikely that these compounds are artifacts of sample oxidation for the following reasons: (i) we recovered >90% of unsaturated acid standards that went through the full base hydrolysis procedure (e.g. octadecenoic acid, not shown), (ii) the temperature of our hydrolysis reaction was relatively mild (80 °C). It is more plausible that these compounds are actually present in plant and soil samples, although possibly as a result of natural oxidation (i.e. oxidation prior to sample hydrolysis). Goni and Hedges (1990a) also observed a high concentration of C_9 α,ω diacid in apple cuticles and provided similar evidence that its abundance was not likely attributable to oxidation during sample treatment.

3.2. Abundant plant lipids

For leaves and roots, 20 lipids accounted for >80% of the mass of all 55 analytes. Polyhydroxyacids and ω-hydroxyacids with chain length $\geqslant C_{12}$ were the most abundant lipids in both plant organs (Fig. 1), reflecting a dominant contribution from cutin (for leaves) and suberin (for roots). Diacids (α,ω) with chain length $\geqslant C_{16}$ were also abundant in the hydrolysis extracts of leaves and roots. Several long chain *n*-acids and *n*-alcohols were present in high concentration in both leaves and roots, consistent with their

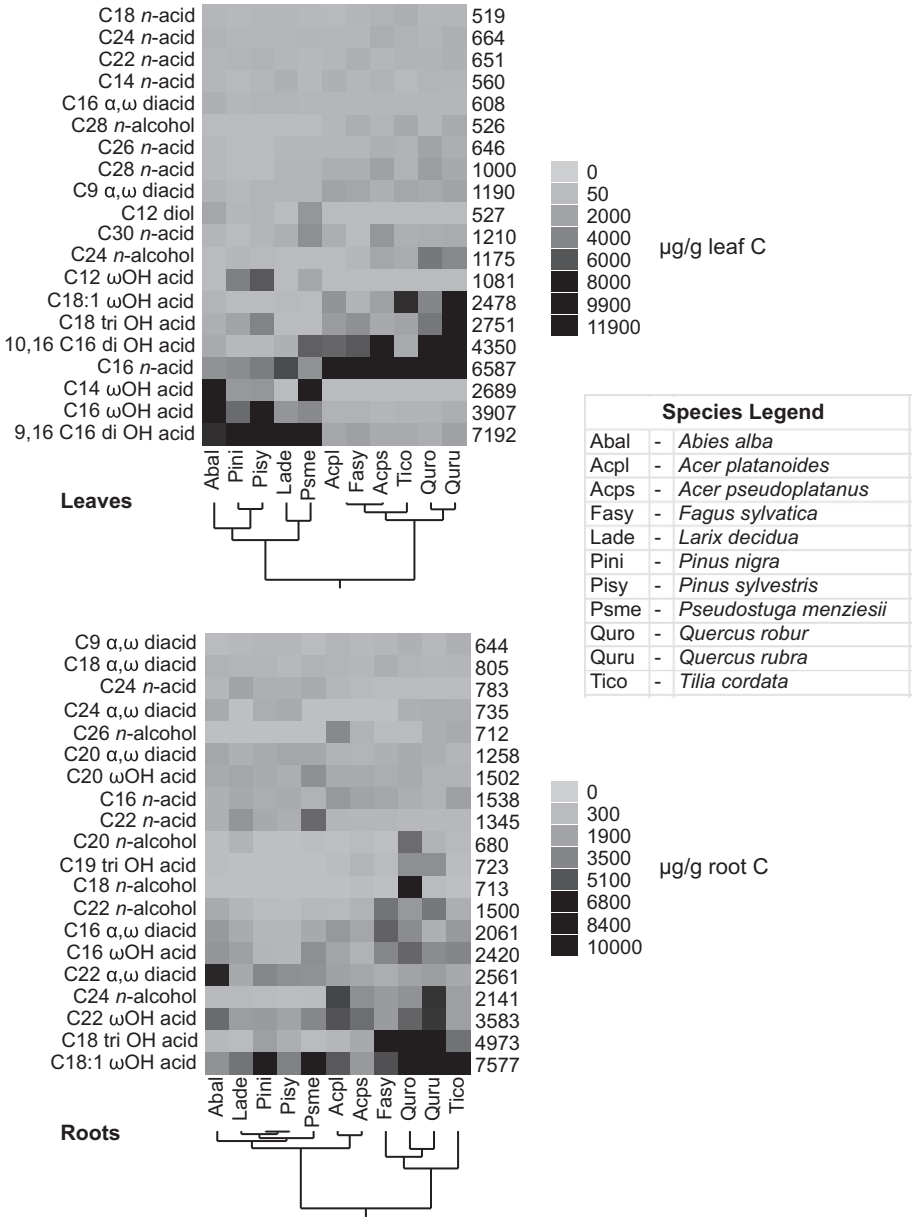

Fig. 1. Cluster diagrams of species variation ($n = 11$) in lipid concentration for the 20 most abundant lipids in each leaves and roots. The gray scale grid reflects the lipid concentration for each species (µg lipid/g tissue C). For each lipid, the average concentration of all 11 tree species is indicated to the right of the gray scale grid.

occurrence in plant waxes, where these lipids could occur freely or in wax esters, and in suberin (long chain *n*-acid and *n*-alcohols are thought to be rare, minor constituents of cutin; Kolattukudy, 2001). We highlight below the lipid patterns that most clearly distinguish plant types and organs. For a more complete depiction of the relative abundance of each analyte in leaves and roots, see Supplementary Figs. 2–5 in Supplementary Appendix 1.

3.3. Lipid distributions among tree species

There was a striking amount of variability in the lipid composition of leaves and roots of the 11 tree species. For close to half of the most abundant lipids in leaves and roots (21 of 40 lipids), concentration ranged at least tenfold between the species with the highest and lowest values. The concentration of all 40 of these abundant compounds showed greater than twofold variability among species. Cluster analysis revealed a strong phylogenetic pattern in lipid composition. For both leaves and roots, all five conifers were present in a cluster that excluded the deciduous broadleaved angiosperms (Fig. 1). Also, the lipid signatures of species from the same genus were consistently more similar to each other than to species from other genera.

Leaves and roots of deciduous broadleaved angiosperms contained substantially more *n*-alcohols (C_{20}–C_{30}, even chain length only) than leaves and roots of conifers (Fig. 2). A few other studies have documented similar taxonomic differences (e.g. Gulz, 1994), but our study is the first to show the patterns apparent in both leaves and roots. In a study of 48 temperate tree species, Diefendorf et al. (2011) reported that leaves of deciduous angiosperms had a concentration of *n*-alcohols 4–5× than in deciduous or evergreen gymnosperm leaves (based on solvent extracts that were subsequently hydrolyzed). In western Canada, Otto and Simpson (2005, 2006) showed *n*-alcohols were 10× more abundant in aspen (*Populus tremula*) leaves than in lodgepole pine (*Pinus contorta*) leaves. This was true for both solvent extractable and hydrolysable lipid fractions. Finally, using pyrolysis and

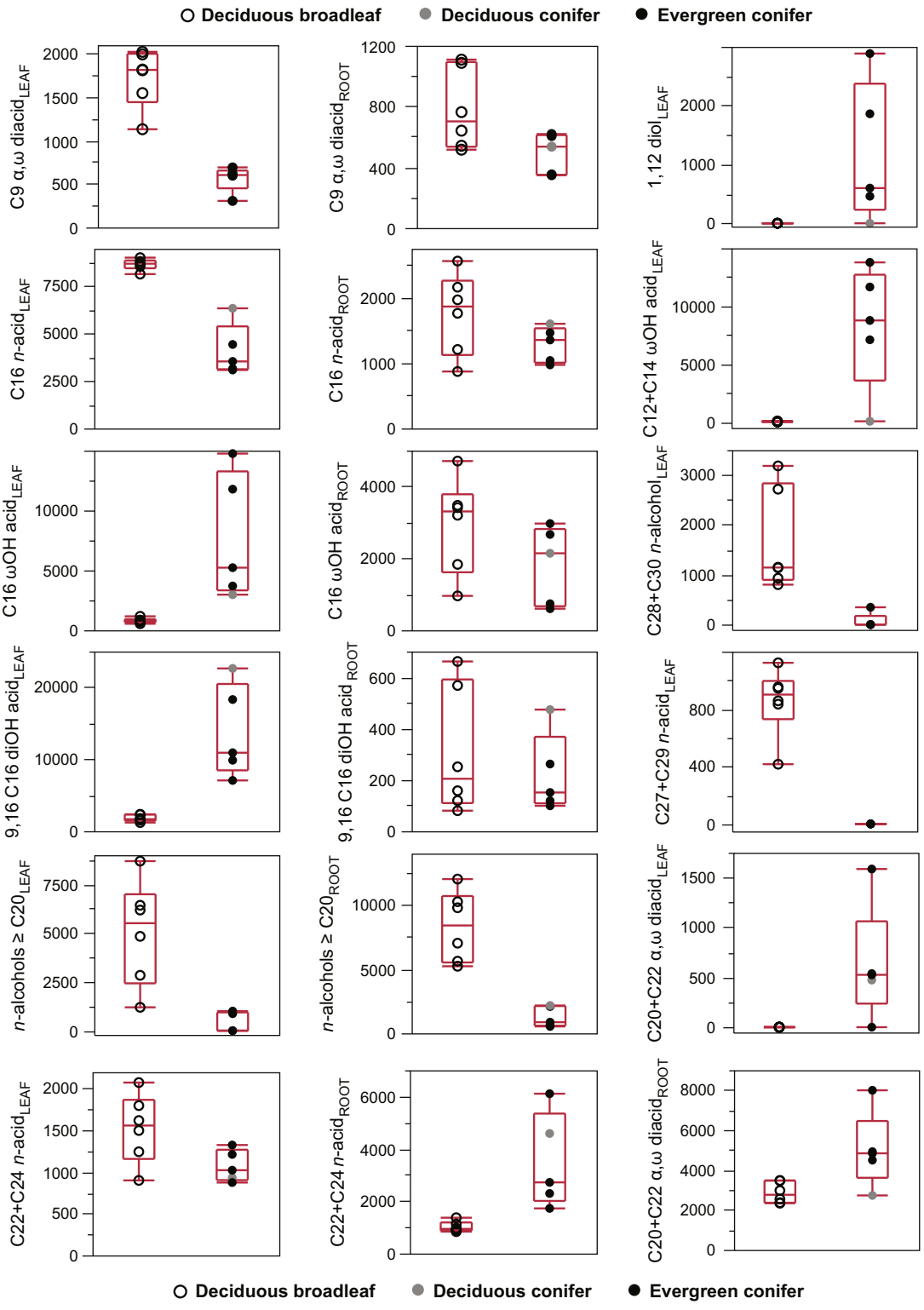

Fig. 2. Box plots of species variation in select leaf and fine root lipids (μg lipid/g tissue C). Each symbol represents the mean of one tree species (n = 11). Note legend at the top of the figure. For visual comparison, lipids below detection limit are represented as zero. Box plots show the median and 25% and 75% quartiles. The extent of the 'whiskers' indicates the range.

thermally assisted hydrolysis and methylation, Nierop and Verstraten (2003) observed that a series of *n*-alcohols was abundant in roots of oak (*Q. robur*) but not pine (*P. nigra* v. *maritime*).

Consistent with these published results, our work supports the use of *n*-alcohols as a tool for reconstructing the environmental distribution of plant types in the past (Jansen et al., 2008) and for discriminating sources of recalcitrant soil OM in modern soils. The extent of *n*-alcohol preservation in the environment is not well known, but *n*-alcohols have been found in a variety of soils (Naafs and van Bergen, 2002; Nierop and Verstraten, 2003; Quénéa et al., 2004; Otto and Simpson, 2005, 2006; Nierop et al., 2006; Jansen et al., 2008), sediments (Ishiwatari et al., 2005; White et al.,

2007) and plant fossils (Otto et al., 2005b). However, due to the paucity of data on plant roots, evaluation of additional plant species is necessary. It will also be necessary to compare the distribution of *n*-alcohols for trees with that of other plant types. Grass leaves and roots also produce *n*-alcohols, but input from trees and grasses can likely be distinguished by the dominance of C_{26} *n*-alcohols in grass tissue (Jansen et al., 2006).

The conifers and deciduous broadleaved angiosperms also differed substantially with respect to the abundance of dihydroxyhexadecanoic acid isomers (C_{16} diOH acid) in leaves (Fig. 2). Leaves of broadleaved species contained very little 9,16 C_{16} diOH acid isomer and substantial quantities of 10,16 C_{16} diOH acid, while the opposite was true for the five conifer species. This has been noted by others (Goni and Hedges, 1990b; Matzke and Riederer, 1991). Interestingly, the lipid composition of the deciduous conifer, *L. decidua*, was similar to evergreen conifers with respect to the relative abundance of C_{16} diOH acid isomers, but diverged for other lipids. For example, 1,12-dodecanediol (1,12 diol), 12-hydroxyhexadecanoic acid (C_{12} ωOH acid) and 14-hydroxyhexadecanoic acid (C_{14} ωOH acid) were found in evergreen conifer leaves (see also Goni and Hedges, 1990b; Matzke and Riederer, 1991), but not leaves of *L. decidua* or deciduous broadleaved angiosperms (Fig. 2). This distinction between *L. decidua* and evergreen conifers is novel and studies of other deciduous conifer species are needed to assess if it is a general pattern or not.

Our conifers all have needle leaves and are members of the family Pinaceae; their lipid signatures were not expected to be characteristic of conifers and gymnosperms with different leaf morphology. For example, Goni and Hedges (1990b) showed that gymnosperms from the Podocarpaceae, Cupressaceae and Araucariaceae families predominately produce 10,16 C_{16} diOH acid in their leaves (see also Hunneman and Eglinton, 1972). These gymnosperm families are thus indistinguishable from deciduous broadleaved angiosperms by way of C_{16} diOH acid isomers alone.

The roots of deciduous broadleaved angiosperms and conifers had a lipid composition less divergent than the lipid composition of their leaves. This is apparent in the clustering of *Acer* spp. more closely to conifers than to the other angiosperms (Fig. 1). Yet, the *Acer* spp., conifers, and other angiosperm trees still formed three distinct clusters. Moreover, some differences in root lipid composition were apparent at higher taxonomic levels, such as the difference in *n*-alcohol abundance of conifers and deciduous broadleaved angiosperms (Fig. 2). The five conifers had a higher concentration of C_{22} and C_{24} *n*-acids in the roots (but not in leaves), while the four evergreen conifers had roots with a higher concentration of C_{20} and C_{22} α,ω diacids (Fig. 2). Roots of *Quercus* spp., *F. sylvatica* and *T. cordata* were distinguished from all other species (Fig. 1) by a very high amount of 18-hydroxyoctadecenoic acid ($C_{18:1}$ ωOH acid), and/or 9,10,18 trihydroxyoctadecanoic acid (C_{18} triOH acid).

For some compounds, plant types or taxa were divergent for one organ but not the other. This is illustrated by the abundances of C_{16} *n*-acid, C_{22} and C_{24} *n*-acids, C_{16} ωOH acid and C_{16} diOH acid isomers (Fig. 2). Their utility as biomarkers for plant type or taxa requires knowledge of the relative contribution from leaves and roots to the preserved OM. For other lipids, including *n*-alcohols $\geqslant C_{20}$, the plant group patterns were similar for leaves and roots. Based on these results, we urge caution when interpreting patterns of biomarkers whose distribution in roots or other plant organs has not been widely evaluated.

In some instances, the similarities between plant taxa were notable. It has been suggested that C_{18} triOH acid may be diagnostic for angiosperm suberin (Nierop and Verstraten, 2003) and cutin (Goni and Hedges, 1990b). Yet, leaves and roots of *Pinus* spp. and *A. alba* contained it in substantial quantity (ca. 500–4000 μg/g C) and the angiosperm species displayed a wide range of concentration for C_{18} triOH acid (ca. 600–17,000 μg/g C; Fig. 1).

3.4. Comparing leaf and root lipid composition

Several compounds were very abundant in both organs. Three hydroxyacids were found to average >2000 μg/g C in both leaves and roots, specifically $C_{18:1}$ ωOH acid, C_{18} triOH acid and C_{16} ωOH acid (Fig. 1). These results and the results of separate surveys of plant tissues (Holloway, 1983; Matzke and Riederer, 1991) refute the suggestion that C_{18} triOH is primarily cutin derived (Kögel-Knabner, 2002; Amelung et al., 2008). Also abundant in both leaves and roots were *n*-acids (C_{16}, C_{22}, C_{24}), 1-tetracosanol (C_{24} *n*-alcohol), hexadecanedioic acid (C_{16} α,ω diacid) and nonanedioic acid (C_9 α,ω diacid).

Few lipids were exclusively or predominately associated with leaves or roots. Further, the distribution of lipids between leaves and roots was highly variable among tree species (Fig. 3). A good example is C_{18} triOH acid. For *A. platanoides* and *P. sylvestris*, leaves accounted for up to 80% of the total leaf and root production of this lipid (i.e. C_{18} triOH acid was predominately associated with leaves for these species). For other species, each organ had a similar concentration of C_{18} triOH acid (*A. pseudoplatanus*, *P. nigra*, *Q. rubra*). Finally, for several species, C_{18} triOH acid was produced exclusively or overwhelmingly in roots (*Fagus sylvestris*, *L. decidua*, *P. menziesii*, *Q. robur*). Such species-specific distributions between the leaves and fine roots were apparent for other lipids, including the C_{16} and C_{18} ωOH acids and α,ω diacids (Fig. 3). Some authors have suggested C_{16} or C_{18} α,ω diacids are derived primarily from suberin or roots (Quénéa et al., 2004), but our results and those of Matzke and Riederer (1991) contrast with this assertion. It is important to note, however, that some C_{16} and C_{18} hydroxyacids and diacids, while not diagnostic for leaves or roots of broad taxonomic groups (e.g. angiosperms or gymnosperms), could be diagnostic of certain families, genera, or species (e.g. high concentration of C_{18} triOH acid in *Fagus* and *Quercus* spp.).

Some lipids were more consistently associated with one or the other plant organs, regardless of tree species. Across all species, α,ω diacids and ωOH acids with chain length $\geqslant C_{20}$ were typically much more abundant in roots than leaves (Fig. 3). Matzke and Riederer (1991) also showed that these lipids were generally present in suberin but not cutin. However, their samples were dominated by suberin in cork and bark, with comparatively few root samples (6 out of 55 suberin sources). Thus, our study is novel because we specifically document that leaf-derived cutin and root-derived suberin of temperate trees generally differ according to the concentrations of α,ω diacids and ωOH acids $>C_{20}$. In addition, our results showed *n*-alcohols and *n*-acids with chain length $\geqslant C_{27}$ were primarily or exclusively present in leaves (Fig. 3), but this novel observation requires verification. Finally, our root samples did not contain C_{12} and C_{14} ωOH acids or 1,12 diol (Fig. 3), consistent with previous studies (Matzke and Riederer, 1991; Nierop, 2001; Nierop and Verstraten, 2003); these results substantiate the use of these lipids as cutin biomarkers with plant type specificity. In order to validate and identify lipid monomers that are consistently diagnostic for cutin or suberin, and for leaves or roots, we suggest that leaves, roots and other organs from a greater diversity of plant species should be characterized. Seeds and bark are of particular interest as these plant organs can contain cutin and suberin, respectively, but it is not known if the composition of cutin and suberin in different plant organs is unique.

3.5. Why does the lipid composition of leaves and roots vary with plant type?

We hypothesize that the lipid composition of leaves and roots is linked to the morphology and lifespan of leaves and roots, which varies more than tenfold among plant species (lifespan is the time between leaf or root development, i.e. birth, and senescence, i.e.

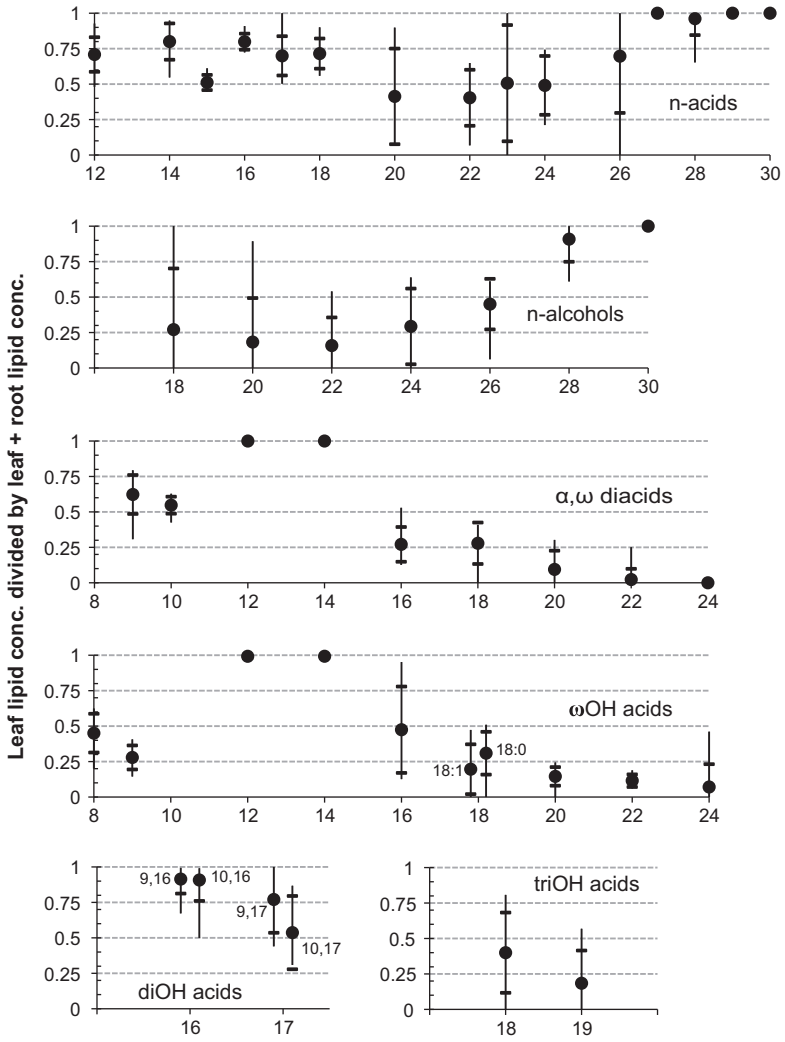

Fig. 3. Proportion of total leaf and root lipids that was leaf derived (*Y*-axis) for lipids of different chain length (*X*-axis) and functional group composition. Mean values calculated across all species (n = 4–11 for each lipid) are shown with black circles, the range is indicated by vertical lines and the SD is by horizontal bars. Lipids exclusively detected in leaves were assigned a value of 1. Lipids exclusively detected in roots were assigned a value of 0. Otherwise, the proportion was calculated as the concentration in leaves divided by the concentration in leaves + concentration in roots.

death). Leaves and roots that are long lived are thought to require proportionally more structural tissue and defense compounds to promote longevity. Likewise, leaves and roots that are thick or dense are expected to employ a more protective biochemistry because the loss of such tissues is more costly to plants than loss of thin, sparsely constructed tissue (Eissenstat, 1992; Poorter et al., 2009). It is unknown how, or if, leaf and root lipid composition varies with morphology and lifespan, but we expect such a relationship exists because lipid biopolymers protect against water loss and pathogens. Specifically, we expect investment in cutin, suberin, and plant wax should increase with lifespan and tissue thickness or density. Theoretically, plants could decrease investment in these polyesters, not only by reducing the total amount of a given polyester, but also by using polymer structures and monomers that require less energy to construct or maintain.

Since we did not exhaustively quantify all plant lipids, we were unable to estimate total plant investment in lipid polymers (as % biomass). Instead, we explored this hypothesis by evaluating correlations between the concentration of individual lipids, or lipid types, and data for the lifespan and morphology of roots and leaves. The investigation is exploratory because there is substantial uncertainty regarding how the structure, function and energetic cost of lipid polymers are dependent on any single lipid or lipid type. Nonetheless, several correlations support our hypothesis and merit further evaluation (Fig. 4). For example, the abundance of C_{16} *n*-acid in leaves declined as lifespan increased across all 11 tree species. For roots of all species, the abundance of *n*-alcohols $\geqslant C_{20}$ increased with specific root length (SRL, a morphological parameter that reflects root length per unit root mass; thick or dense roots have low SRL). In contrast, the relative abundance of α,ω diacids and *n*-acids $\geqslant C_{20}$ decreased as SRL increased. In suberin, *n*-alcohols that are ester-bound to ferulic acid could serve as a waxy water barrier or plasticizer that maintains flexibility (Kolattukudy, 2001; Bernards, 2002), perhaps indicating that high SRL roots (i.e. roots that are thin or sparsely constructed) utilize *n*-alcohol esters for these functions more than low SRL roots. The concentration of some lipids produced predominantly by different plant groups also varied with lifespan or morphology. The concentrations of C_{12} and C_{14} ωOH acid, lipids found only in evergreen conifer leaves, were correlated with leaf lifespan, but in the opposite direction. Within the deciduous broadleaved species, the concentration of 10,16 C_{16} diOH acid declined as specific leaf area (SLA) increased, while the relative abundance of C_9 α,ω diacid increased with SLA (SLA reflects leaf area per unit leaf mass; thick

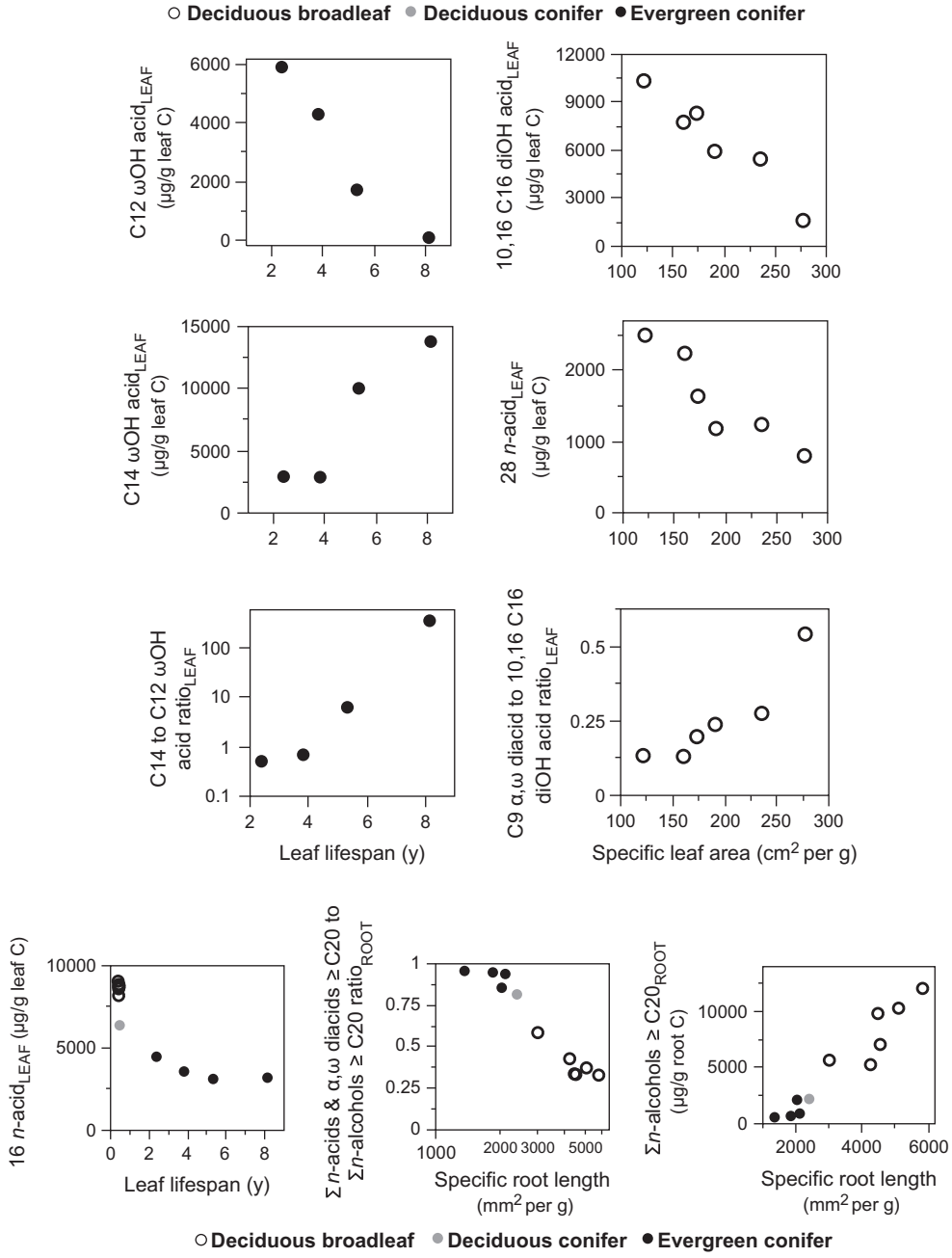

Fig. 4. Relationship between plant lipid concentration (µg lipid/g tissue C) and leaf lifespan, specific leaf area (SLA) or specific root length (SRL).

or dense leaves have low SLA). This could reflect, in part, that 10,16 C_{16} diOH acid contributes to the thickness of low SLA broadleaves by allowing cutin monomers to be cross-linked via the mid-chain OH group.

Our hypothesis can also be assessed, albeit indirectly, by comparing how extensively plant types differ with respect to the lipid composition of leaves and roots. Specifically, since leaf lifespan and morphology of evergreen and deciduous plants are typically more divergent than the lifespan and morphology of their roots (e.g. Withington et al., 2006), the lipid composition of evergreen and deciduous leaves should be more divergent than that of their roots. As noted above, this appears to be true in our study (Figs. 1 and 2).

Collectively, the results lend credence to the hypothesis that the lipid composition of leaves and roots is reflective of their morphology and lifespan, both across and within different plant groups. Yet, since the findings are based on a subset of lipids from temperate tree species, much work is needed to test if our results are representative of a wider range of plant lipids, plant taxa and biomes.

3.6. Abundant soil lipids

Cutin and suberin-derived compounds accounted for 8 of the 10 most abundant lipids recovered from soil (Table 1). This is consistent with several other studies of hydrolyzable lipids in forest soils (Naafs and van Bergen, 2002; Nierop et al., 2003; Quénéa et al., 2004; Otto et al., 2005a). The prominence of α,ω diacids and ωOH acids $\geqslant C_{20}$ indicates a dominant root contribution to soil lipids (see also Supplementary Fig. 5), which is emerging as a common feature of forest mineral soil (Nierop, 1998; Naafs and van Bergen, 2002; Rumpel et al., 2002; Nierop et al., 2003; Otto and

Table 1
Species and soil texture effects for the 25 most abundant soil lipids and other diagnostic lipid classes.

Lipid	Biomarker potential[a]	Mean conc. in soil (μg/g C)	Species effect[b]	Soil texture effect[b]	Combined effect[b]
C_{16} ωOH acid		2964	ns	ns	
9,16 C_{16} diOH acid	CL	2239	$P < 0.0001$, ${}_pR^2 = 0.74$	$P < 0.01$, ${}_pR^2 = 0.07$	$P < 0.0001$, $R^2 = 0.83$
C_{22} α,ω diacid	R	2111	ns	ns	
C_{22} ωOH acid	R	2044	$P < 0.05$, $R^2 = 0.31$	$P < 0.001$, ${}_pR^2 = 0.15$	$P < 0.0001$, $R^2 = 0.73$
C_{26} α,ω diacid	R	1900	ns	ns	
C_{18} triOH acid		1856	$P < 0.0001$, ${}_pR^2 = 0.70$	$P < 0.01$, ${}_pR^2 = 0.09$	$P < 0.0001$, $R^2 = 0.79$
C_{24} α,ω diacid	R	1583	ns	ns	
C_{16} *n*-acid		1188	$P < 0.01$, $R^2 = 0.60$	ns	
C_{24} ωOH acid	R	1131	ns	$P < 0.001$, $R^2 = 0.44$	
C_{22} *n*-acid		1003	$P < 0.1$, $R^2 = 0.42$	ns	
C_{20} α,ω diacid	R	996	ns	ns	
C_{16} α,ω diacid		976	$P < 0.0001$, ${}_pR^2 = 0.54$	$P < 0.05$, ${}_pR^2 = 0.04$	$P < 0.0001$, $R^2 = 0.81$
C_9 α,ω diacid		936	ns	$P < 0.01$, $R^2 = 0.22$	
10,16 C_{16} diOH acid	BL	885	$P < 0.0001$, ${}_pR^2 = 0.53$	$P < 0.001$, ${}_pR^2 = 0.12$	$P < 0.0001$, $R^2 = 0.83$
C_{30} *n*-acid	BL	866	$P < 0.1$, ${}_pR^2 = 0.23$	$P < 0.0001$, ${}_pR^2 = 0.22$	$P < 0.0001$, $R^2 = 0.73$
C_{20} ωOH acid	R	830	$P < 0.01$, ${}_pR^2 = 0.48$	$P < 0.05$, ${}_pR^2 = 0.06$	$P < 0.01$, $R^2 = 0.66$
$C_{18:1}$ ωOH acid		828	$P < 0.001$, $R^2 = 0.65$	ns	
C_{12} ωOH acid	ECL	817	$P < 0.01$, $R^2 = 0.56$	ns	
C_{24} *n*-acid		796	ns	ns	
C_{28} *n*-acid	BL	722	$P < 0.01$, ${}_pR^2 = 0.35$	$P < 0.01$, ${}_pR^2 = 0.10$	$P < 0.0001$, $R^2 = 0.79$
C14 ωOH acid	ECL	710	$P < 0.0001$, ${}_pR^2 = 0.77$	$P < 0.05$, ${}_pR^2 = 0.04$	$P < 0.0001$, $R^2 = 0.78$
C_{26} ωOH acid	R	651	ns	ns	
C_{26} *n*-acid		588	ns	$P < 0.001$, $R^2 = 0.30$	
C_{18} α,ω diacid		526	ns	ns	
C_{24} *n*-alcohol	BL + BR	516	$P < 0.0001$, ${}_pR^2 = 0.55$	$P < 0.01$, ${}_pR^2 = 0.06$	$P < 0.0001$, $R^2 = 0.88$
Other lipids					
Σα,ω diacids $\geqslant C_{20}$	R	6589	ns	ns	
ΣωOH acids $\geqslant C_{20}$	R	4655	ns	$P < 0.001$, $R^2 = 0.42$	
Σ*n*-alcohols $\geqslant C_{20}$	BL + BR	2257	$P < 0.0001$, ${}_pR^2 = 0.60$	$P < 0.001$, ${}_pR^2 = 0.08$	$P < 0.0001$, $R^2 = 0.90$
ΣC_{28} and C_{30} *n*-alcohol	BL	717	$P < 0.01$, ${}_pR^2 = 0.38$	$P < 0.0001$, ${}_pR^2 = 0.24$	$P < 0.0001$, $R^2 = 0.74$
ΣC_{27} and C_{29} *n*-acid	BL	694	$P < 0.05$, $R^2 = 0.29$	$P < 0.001$, ${}_pR^2 = 0.18$	$P < 0.001$, $R^2 = 0.71$
Σ*n*-acids $\geqslant C_{27}$	L	2263	$P < 0.05$, $R^2 = 0.20$	$P < 0.0001$, ${}_pR^2 = 0.21$	$P < 0.0001$, $R^2 = 0.79$

[a] Assigned based on results in Figs. 2 and 3 (L, leaves; R, roots; B, broadleaved deciduous angiosperm; C, conifer; E, evergreen).
[b] Effects of tree species identity and soil texture were evaluated using ANCOVA tests; for instance, when both factors were significant, we report the total model statistics in the 'Combined effect' column; separate significance tests and partial R^2 values (${}_pR^2$) are given for the relevant effect in the 'Species' and 'Soil texture' effects columns; ${}_pR^2$ values were calculated by dividing sequential sum of squares for each factor by the total sum of squares for the dependent variable; in instances when only one factor was significant in the ANCOVA, we used a separate ANOVA or regression model to evaluate the effect of species or clay independent of the other factor (thus 'Combined' statistics are not given).

Simpson, 2006; Feng et al., 2010). Nonetheless, a substantial contribution from leaves is also apparent in the abundance of C_{16} diOH acid isomers and C_{14} and C_{12} ωOH acids.

The 25 most abundant soil lipids (Table 1) accounted for >80% of the total mass of analytes and were also dominated by cutin and suberin monomers (i.e. ωOH acids, α,ω diacids and di or trihydroxyacids). Other abundant soil lipids were C_{16} *n*-acid, several *n*-acids $\geqslant C_{20}$, and C_{24} *n*-alcohol. These lipids are not strictly associated with any single plant lipid polymer, but the *n*-acids and *n*-alcohols with chain length $\geqslant C_{20}$ are useful as generic plant biomarkers (Jansen et al., 2006; Simpson et al., 2008) and commonly occur in suberin and plant waxes.

3.7. Effect of tree species and soil properties on soil lipids

Variability in soil texture, summarized by the proportion of clay-size particles, correlated significantly and positively with the concentration of several soil lipids (Table 1). Clay likely facilitates preservation of plant lipids via increased protection in soil aggregates, which are more common in the clay rich plots at the site (personal observation), or through interaction with mineral surfaces that reduce lipid bioavailability (von Lutzow et al., 2006).

Tree species identity had a significant effect on 14 of the 25 most abundant lipids in the soil. Species effects were also apparent for individual lipids and lipid types diagnostic for plant taxa, functional types, or organs (Table 1). The explanatory power of tree species identity was generally greater than that of soil texture (evident in lower R^2 values). These differences among species are at least partially dependent on species-level variation in the lipid composition of leaves and roots, as evident from the similar species-level patterns observed for leaves and roots (Figs. 1 and 2) and soil (Fig. 5). Below, we briefly discuss the species-level variation in soil lipid composition in relation to plant lipid concentrations and other factors.

3.8. Comparing soil lipid composition among tree species

Cluster analysis revealed strong phylogenetic signals in the composition of soil lipids (Fig. 5). The four evergreen conifers are present in one cluster while the deciduous conifer (*L. decidua*) is positioned alone between two separate clusters of deciduous broadleaved angiosperms. Similar to the cluster diagram for root lipids, the two *Acer* spp. are quite divergent from the two *Quercus* spp. Much like the cluster diagrams for leaf and root lipids, species within a genus tended to have soil lipid compositions more similar to each other than to those of other species.

The concentration of some individual lipids and lipid types in soil (Fig. 6) was consistent with the differences we observed for the lipid composition of leaves and roots (Fig 2; see also Supplementary Fig. 4). For example, soil beneath deciduous broadleaved angiosperms had a higher concentration of *n*-alcohols $\geqslant C_{20}$, while evergreen conifer soil contained more 1,12 diol and C_{12} and C_{14} ωOH acids than did soil beneath deciduous species, including *L. decidua*. The results substantiate the use of these biomarkers for diagnosing input from these plant types in temperate

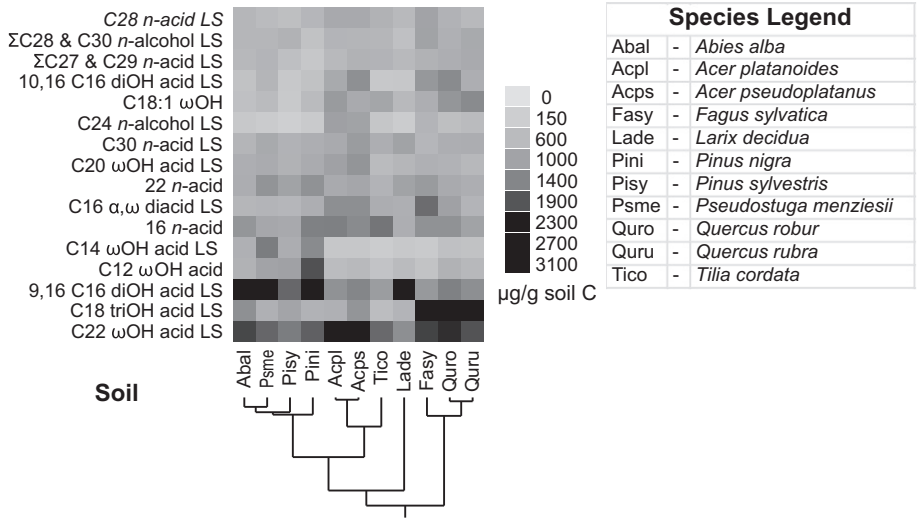

Fig. 5. Cluster diagram of soil lipid concentration (µg lipid/g soil C). In order to account for the confounding effect of soil texture and its variability among plots, the cluster analyses included only lipids for which soil lipid concentration differed significantly among the tree species (Table 1). For lipids with significant effects of species *and* soil texture, we used least squares adjusted mean value in the cluster analyses instead of the actual mean (i.e. the adjusted means are normalized to the mean % clay value using the coefficient for % clay calculated in the ANCOVA). Adjusted mean values are indicated by "LS" following the lipid name. The gray scale grid reflects the mean lipid concentration for each species.

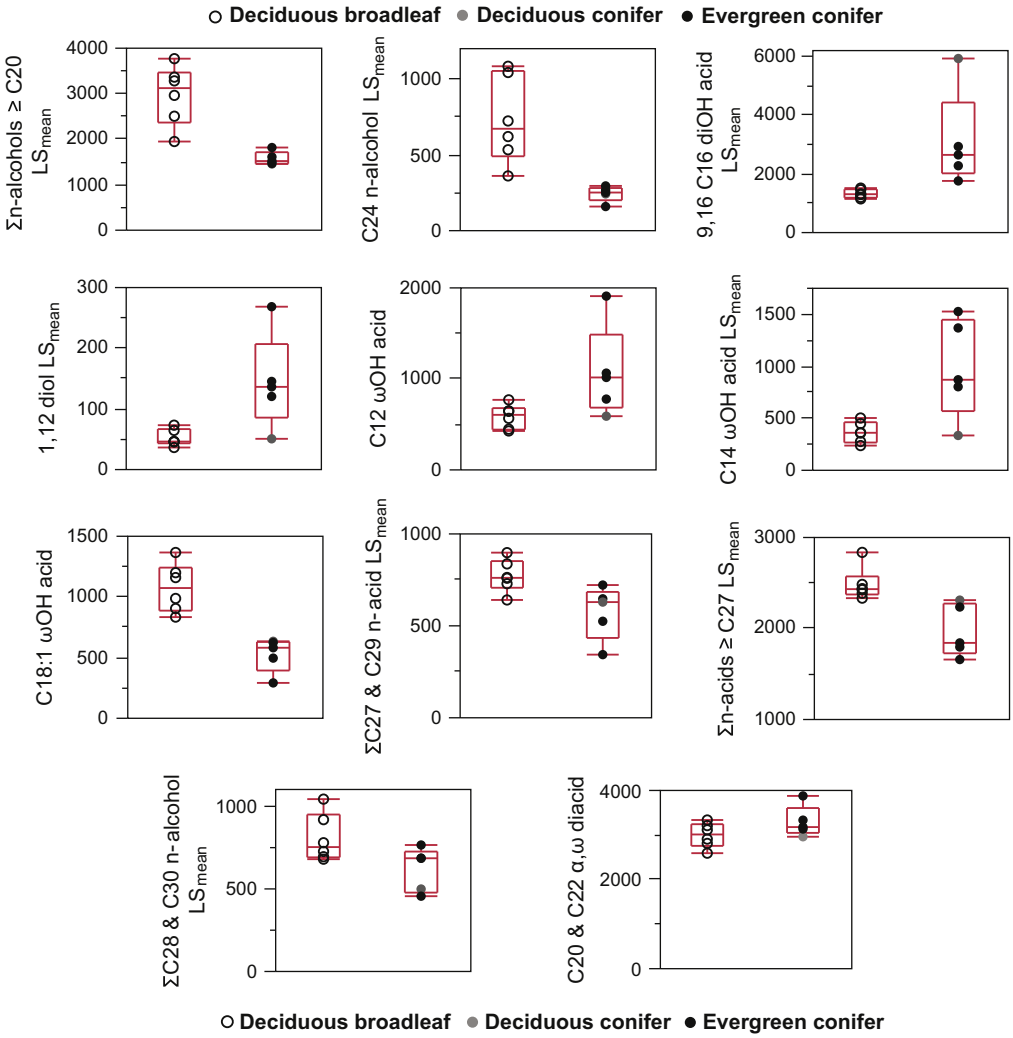

Fig. 6. Box plots of species variation in selected soil lipid concentration (µg lipid/g soil C). Each symbol represents the mean of one tree species (n = 11). Note legend at the top of the figure. For visual comparison, lipids below detection limit are represented as zero. Box plots show the median and 25% and 75% quartiles. The extent of the 'whiskers' indicates the range.

ecosystems. It is also notable that such differences in soil lipid composition among species can arise in less than 40 years.

Compared with the deciduous broadleaved angiosperms, conifers had a much higher concentration of the 9,16 C_{16} diOH acid isomer in soil and leaves (Figs. 2 and 6; Supplementary Fig. 4), but roots of conifer and broadleaved species contained a similar concentration of 9,16 C_{16} diOH acid (Fig. 2). In this case, the plant type difference in soil mirrors that of leaves because the lowest concentration of 9,16 C_{16} diOH acid in conifer leaves (ca. 7000 μg/g C) was 10× its highest concentration in roots of broadleaved species (ca. 700 μg/g C). Thus, any bias favoring preservation of root lipids was not able to mask the lipid composition of leaves. For soil samples with a strong bias toward root lipids, such as could occur in deep soil horizons, use of 9,16 C_{16} diOH acid as a Pinaceae biomarker could be problematic, especially if its concentration was relatively low.

For other lipids, their abundance in soils was not consistent with their abundance in leaves and roots of the overlying tree species, diminishing their utility as plant type indicators. Conifers had a higher concentration of C_{20} and C_{22} α,ω diacids in leaves than deciduous broadleaved angiosperms (Fig. 2), but both tree types had a similar concentration of these lipids in soil (Fig. 6). Likewise, although the angiosperms tended to have a much higher concentration of two *n*-acids (C_{27} and C_{29}) and two *n*-alcohols (C_{28} and C_{30}) than the conifers (Fig. 2), the two plant types had overlapping concentrations of these lipids in soil (Fig. 6; Supplementary Fig. 4). For one lipid, $C_{18:1}$ ωOH acid, its concentration in soil beneath the angiosperms was ca. 2× that in soil beneath the conifers (Fig. 6), despite the observation that these plant types had overlapping concentrations of it in both leaves and roots (Fig. 1; Supplementary Fig. 4).

A variety of factors are likely responsible for the disconnection between plant concentration and soil concentration for some lipids. For example, differences in the legacy of input from other plants, or in current input from understory species, can also distort the relationships between soil lipids and lipids of the overlying vegetation (Nierop et al., 2006). The presence of C_{12} and C_{14} ωOH acids in soil beneath deciduous trees is likely due to input from the *P. sylvestris* plantation that existed before the experiment was established. Input from sources other than roots and leaves (e.g. bark, seeds) could also distort soil lipid composition relative to that of roots and leaves. Finally, the process of OM decomposition and stabilization could be more distorting for some lipids.

3.9. Guidelines for interpreting plant lipid distributions in soils and sediments

Given these potentially distorting influences and the high variability among tree species with respect to leaf and root lipid composition (i.e. the rarity of lipids exclusively associated with a given plant taxa, plant functional type or plant organ), we have several suggestions for interpreting the abundance of plant lipids in soils and sediments. First, the origin of any lipid in soil or sediment can be confidently attributed to a specific plant taxa, functional type, or organ only if its distribution is known for the organs that contributed substantially to the preserved lipid pool. For example, when tracing the origin of a lipid in sediment to a specific plant taxa or functional type, knowing the distribution of lipids in leaves of different plants is insufficient for sediments where root-derived lipids are preserved. Second, published lipid data for different plant organs is not sufficient to represent organs of the many uncharacterized plant species. Thus, robust attribution of soil and sediment lipid provenance to a plant type, taxa, or organ requires analysis of many additional plant species. Due to these sources of uncertainty, more robust interpretations will be afforded when *multiple* lipid "biomarkers" are identified and used to collectively assess contributions of plant taxa, functional types, and organs to preserved lipids.

In our study, after accounting for the lipid composition of leaves and roots, the following lipids were the most consistently attributable to a certain plant taxon or functional type: C_{12} and C_{14} ωOH acids (diagnostic for evergreen conifers from the Pinaceae family), *n*-alcohols $\geqslant C_{20}$ (diagnostic for deciduous broadleaved angiosperms) and, conditionally, 9,16 diOH acid (diagnostic for Pinaceae in the absence of a strong root preservation bias). The most promising leaf "biomarkers" were C_{12} and C_{14} ωOH acids and *n*-acids and *n*-alcohols with chain length $>C_{26}$. The most consistent root biomarkers were α,ω diacids and ωOH acids $\geqslant C_{20}$.

4. Conclusions

We documented several novel patterns of lipid variation among tree species, including the following: (i) differences in the leaf lipid composition of evergreen conifers and a deciduous conifer, *L. decidua*, (ii) collectively, long chain *n*-alcohols ($\geqslant C_{20}$) were only abundant in leaves and roots of deciduous broadleaved angiosperms and (iii) several plant lipid concentrations correlated with the lifespan or morphology of leaves and roots. Many notable aspects of plant lipid composition were apparent in mineral soils beneath the 11 focal tree species, despite tremendous variation in factors that could distort those plant lipid signatures, including differences in plant litter input, soil chemistry, and the abundance and nature of decomposer organisms (Reich et al., 2005; Withington et al., 2006; Mueller et al., 2012). In addition, our comparison of leaf and root lipids from 11 tree species provided new information on the attribution of lipid origins to leaves and roots, which is required for robust interpretation of purported biomarkers of plant types, taxa and organs. We used these source constraints to document a substantial contribution of roots to soil lipids beneath all 11 tree species, providing evidence that this is probably a typical feature of mineral soils in temperate forests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.orggeochem.2012.08.014.

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